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Doctoral Thesis

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Abbreviations

5-LO: 5-Lipoxygenase
AAT: alpha 1 antitrypsin
Aβ: Amyloid β
ACN: Acetonitrile
AD: Alzheimer's Disease
ADAS-Cog: Alzheimer's Disease Assessment Scale-cognitive subscale
AGE: Advanced Glycation End-products
AIS: Axon Initial Segment
ALB: Albumin
APOA4: Apoliprotein A4
BCEA: Bivariate Contour Ellipse Area
BM: Basement Membrane
BMEC: Bone Marrow Endothelial Cells
BRB: Blood Retinal Barrier
BSA: Bovine Serum Albumin
C1NH: C1 Inhibitor
CFH: Complement Factor H
CHI3L1: Chitinase-3-like protein 1
CID: Collision-induced Dissociations
CLP: Coactosin-like-protein
CNS: Central Nervous System
CXCR4: Chemokine Receptor Type 4
DAG-PKC: Diacylglycerol-proteinkinase C
DAPK: Death-associated Protein Kinases
DCP: Deep Capillary Plexus
DR: Diabetic Retinopathy
DM: Diabetes Mellitus
DME: Diabetic Macular Edema
DPPIV: Dipeptidyl Peptidase IV
EC: Endothelial Cells
EBM: Endothelial Basal Medium
ECM: Extracellular Matrix
eNOS: endothelial Nitric Oxide Synthase
EPC: Endothelial Progenitor Cells
EPO: Eritropoietin
ERK: Extracellular signal-Regulated Kinase
ERM: Ezrin, Radixin and Moesin
ET-1: Endothelin 1
ETA: Endothelin Receptor A
ETB: Endothelin Receptor B
EUROCONDOR: European Consortium for the Early Treatment of Diabetic Retinopathy
FC: Fold change
FOXO1: Forkhead Transcription Factor 1
GA: Glial Activation
GAIT: Gamma Interferon-activated Inhibitor of Translation
GCL: Ganglionar Cell Layer
GFAP: Glial Fibrilary Acidic Protein
GLAST: Glutamate Aspartate Transporter
GLP-1: Glucagon-like Peptide 1
GLP-1R: Glucagon-like Peptide 1 Receptor

HPLC: High Performance Liquid Cromatography
HMGB1: High-Mobility Group Box-1
HMVEC: Human Dermal Microvascular Endothelial Cells
HREC: Human Retinal Endothelial Cell
iBRB: inner Blood Retinal Barrier
ICAM: Intercellular Adhesion Molecule
ICP: Intermediate Capillary Plexus
IL: Interleukin
INFγ: Interferon-γ
INL: Inner Neural Layer
IP-10: Interferon-inducible Protein 10
IPL: Inner Plexiform Layer
IRMA: Intraretinal Microvascular Abnormality
IRBP: Interphotoreceptor retinoid binding
KIF3A: Kinesin-like protein 3A
KKS: Kallilrein-kinin System
LTAA4: Leukotriene A4
LTA4H: Leukotriene A4 hydrolase
LTB4: Leukotriene B4
LTP: Long Term Potentiation
LTQ: Linear Trap Quadropole
MAC: Membrane Attack Complex
MAPK: Mitogen Activated Protein Kinase
MCI: Mild Cognitive Impairment
MCP: Monocyte Chemoattractant Protein
mfERG: mulifocal Electroretinogram
MMSE: Mini-Mental State Examination
MRI: Magnetic Resonance Imaging
MS: Mass Spectrometry
MSVI: Moderate and Severe Visual Impairment
NCAM: Neural Cell Adhexion Molecule
NeuN: Neuronal-Specific Nuclear Protein
NF-κβ: Nuclear transcription Factor-κβ
NMDA: N-Methyl-D-aspartate
NO: Nitric Oxide
NPDR: Non-Proliferative Diabetic Retinopathy
NRG: Neuroregulin
NVU: Neurovascular Unit
OCT: Optical Coherence Tomography
OCTA: Optical Coherence Tomography Angiography
ONL: Outer Nuclear Layer
OPL: Outer Plexiform Layer
oBRB: outer Blood Retinal Barrier
PBS: Phosphate Buffered Saline
PCR: Protein Chain Reaction
PEDF: Pigment Epithelium Derived Factor
PET: Positron Emission Tomography
PDGF: Platelet-Derived Growth Factor
PDR: Proliferative Diabetic Retinopathy
PKC: Protein Kinase C

RAGE: Receptor of the Advanced Glycation End-products
RhoGDI: Rho-GDP-dissociation Inhibitors
RIPA: Radio-immunoprecipitation Assay
RNFL: Retinal Neural Fiber Layer
ROS: Reactive Oxygen Species
RPE: Retinal Pigment Epithelium
SC: Superior Collicullus
SCP: Superficial Capillary Plexus
SD: Standard Deviation
SDF-1: Stromal Cell-Derived Factor
SDS-PAGE: Sodium Dodecyl Sufate Polyacrylamide Gel Electrophoresis
SST: Somatostatin
T2D: Type 2 Diabetes Mellitus
T1D: Type 1 Diabetes Mellitus
TF: Transferrin
TGFβ: Transforming Growth Factor β
THOP1: Thimet oligopeptidase
TNF: Tumor Necrosis Factor
TUNEL: Terminal Transferase dUTP Nick-End Labeling
UPR: Unfolded Protein Response
VCAM: Vascular Cell Adhesion Molecule
VEGF: Vascular Endothelial Growth Factor
ZIPK: Zipper Interacting Protein Kinase
ZO: Zonula Ocludens

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Abstract

Neurodegeneration is an early event in the pathogenesis of diabetic retinopathy (DR). In fact, the American Diabetes Association has recently defined DR as a highly tissue-specific neurovascular complication, thus emphasizing the importance of the neurovascular unit in the development of DR. The retina is a brain-derived tissue and there is growing evidence indicating that type 2 diabetic subjects are more prone to develop neurodegenerative processes such as Alzheimer's disease. Therefore, it seems reasonable to postulate that the mediators of the neurodegenerative process that occurs in the brain could also be present in the diabetic retina. On this basis, the general objective of this thesis is to investigate the relationship between retinal and brain neurodegeneration and its potential clinical and therapeutic implications.

The first part of this thesis focuses on experimental studies addressed to identifying mediators of impairment of the neurovascular unit. For this purpose a proteomic "hypothesis-free" study was performed comparing human retinas from non-diabetic and diabetic donors with and without glial activation (one of the hallmarks of retinal neurodegeneration). The Ingenuity Pathway Analysis revealed that several critical pathways related to brain neurodegenerative diseases were differently expressed in retinas from diabetic patients and in particular in those with glial activation. This observation supports the concept that a common soil exists in the neurodegenerative processes that occur in the retina and the brain. In addition, a downregulation of several proteins involved in axonal transport and the cytoskeleton was found in retinas from diabetic donors with glial activation. Furthermore, diabetes-induced glial activation was associated with a dysregulation of the complement system, as well as an upregulation of the proteins that govern the cytoskeleton changes. These results were confirmed by orthogonal methods. These novel findings contribute not only to our understanding of the mechanisms involved in the vascular leakage induced by neurovascular unit impairment, but could also have potential therapeutic implications.

The second part of this doctoral thesis is based on a "driven hypothesis" aimed at examining whether endothelin-1 plays a pivotal role in the neurovascular unit impairment that occurs in DR. This is based on the fact that by means of ETA receptors ET-1 leads to vascular damage and through ETB induces neurodegeneration. We first found an overexpression of both ET-1 and its receptors (ETA and ETB) in the retinas from diabetic donors in comparison with non-diabetic donors. Second, we found that topical administration of bosentan (a blocker of ETA and ETB receptors) prevented retinal neurodegeneration induced by diabetes in the db/db mouse model. In addition, the inhibition of diabetes-induced upregulation of PKC-β, TNF-α and VEGF plays an important role in the beneficial vascular action of bosentan. These dual beneficial effects of bosentan (neurotrophic and vasculotropic) and the pharmacokinetic results point to this drug as an excellent candidate to be tested in clinical trials.

The third part of the thesis is a clinical study addressed to reinforcing the concept that the assessment of retinal neurodegeneration could be a useful tool to identify those diabetic subjects at risk of developing dementia. Indeed, in a previous study we demonstrated that retinal sensitivity assessed by fundus-driven microperimetry was related to brain neurodegeneration and could be a useful biomarker for identifying patients with T2D who are at risk of developing Alzheimer's disease. In the present work, we assessed whether gaze fixation, a parameter that can also be

assessed by retinal microperimetry, is associated with cognitive impairment. This is based on previous evidence indicating that the capacity to maintain visual gaze on a single location (fixation) is hampered in Alzheimer's disease. Our results showed that gaze fixation is more unstable as cognitive impairment progresses. Moreover the assessment of fixational parameters significantly improves retinal sensitivity assessment in differentiating those subjects with mild cognitive impairment from normocognitive diabetic subjects. Therefore, the measurement of retinal sensitivity in combination with parameters of fixation by using microperimetry could be a reliable method for detecting prodromal stages of dementia in the T2D population. Hopefully, these findings will be the proof of concept of a large scale-clinical study.

Overall, this work contributes to the knowledge of the mediators of neurovascular unit impairment in the early stages of DR and increases our understanding of the link between retinal and brain neurodegeneration in the setting of the type 2 diabetic population. In addition, our findings suggest further candidates as new potential targets for the treatment of DR.

1. Introduction

1.1 Diabetic Retinopathy: concept and epidemiology

Diabetes mellitus affects more than 400 million people worldwide, and its prevalence is increasing. Most of the burden of this chronic disease comes from its complications, and given that diabetes is expected to increase from 388 million in 2013 to 592 million by 2030, the complications derived from diabetes will become even a more serious problem in the future (1).

Diabetic retinopathy (DR) is the most common complication of diabetes and remains the leading cause of preventable blindness among working-age individuals in most developed countries (1–3). Without treatment, DR progresses from mild, non-proliferative DR, to moderate and severe non-proliferative DR before the occurrence of proliferative DR (PDR), in which there is a growth of abnormal new retinal blood vessels. Concurrently, at any stage of retinopathy, due to exudation and edema in the macula (the central focal point of the retina), patients may also develop diabetic macular edema (DME) (4). In the diabetic population, the overall prevalence of DR is estimated to be 34.6% and 10% of them will present vision-threatening DR (defined by the presence of PDR and/or severe DME) (3). However, the individual lifetime risk of DR is 50-60% in a person with type 2 diabetes mellitus (T2D) and up to 90% in those with type 1 diabetes mellitus (T1D) (4).

Regarding the global causes of blindness and moderate and severe visual impairment (MSVI), DR is in the 5th position worldwide. It should be noted that from 1990 to 2010 the percentages of blindness caused by DR from all global blindness causes increased from 2.1% to 2.6% and regarding MSVI from 1.3% to 1.9% (2).

Healthcare costs for diabetic subjects are more than double the costs of those without diabetes (5,6), and the occurrence of major diabetes related complications in type 2 diabetic patients is associated with increased average medical costs annually (7,8). In this regard, it has been reported that the consumption of health care resources was almost double in type 2 diabetic patients with microvascular complications compared to patients without them (9). In addition it has been reported that average healthcare costs increase considerably with the severity of DR, which suggests that preventing the progression of DR may lower healthcare costs (10).

The main risk factors involved in DR development are diabetes duration, hyperglycemia and the presence of hypertension. T1D patients present a higher risk of DR (3,4). However, clinical studies have revealed that there is a substantial variation in the onset and severity of retinopathy that is not fully explained by the known risk factors (11–14). Indeed, in the ADVANCE Trial intensive glucose control to reduce glycosylated hemoglobin to 6.5% or lower had no effect on the 5-year incidence of DR (13). In the same study, the lowering of blood pressure to near normal levels did not achieve a further reduction in the progression of DR (13). In addition, some patients with poor control of glycemia or blood pressure do not develop DR (14). These data suggest that genetic factors play an essential role in accounting for the susceptibility to developing this late diabetic complication (15). In fact, heritability has been estimated to be as high as 25% for DR and 50% for PDR (16,17). A large number of putative genes and genetic variants have been reported in the literature and some of them exhibit consistent associations with DR (ALR2, VEGF and RAGE genes). However, these results have not been replicated in multiple populations, and therefore, no genes have achieved widespread acceptance as conferring a high risk of DR (15).

There are several studies demonstrating that tight control of blood glucose levels and blood pressure prevent and arrest the progression of DR (18–21). However, these measures are not easy to implement and even with a high degree of compliance are not always effective in preventing or arresting the development or progression of DR. In more advanced stages of DR, laser photocoagulation, intravitreal injections of corticosteroids or anti-VEGF agents and vitreoretinal surgery are recommended (22). All these treatments are expensive, require a vitreoretinal specialist and have a significant number of secondary affects (23,24).

One of the best strategies for arresting or preventing vision loss and reducing the associated economic-burden of this complication is the early diagnosis of DR based on screening programs. The early diagnosis of DR lead to physicians and patients to reinforce the diabetes care and the treatment of other risk factors and occasionally, when it reaches more advanced stages, may lead to the implementation of therapeutic procedures to prevent its progression (20,22). In addition, the presence of DR is an independent indicator of other diabetic complications such as diabetic nephropathy (25,26), cardiovascular disease (27,28) and stroke (29,30), thus increasing the risk of morbi-mortality of T2D patients. It should be noted that there is emerging evidence indicating that T2D subjects are more prone to develop dementia, which could be considered a “new” diabetic complication (31). Similar to the other diabetic complications, the ACCORD-mind and ACCORD-eye studies showed that DR was associated with predicted future cognitive decline in the T2D population (21,32). Therefore, the identification of those diabetic subjects with DR could help identify a subpopulation at risk of developing these devastating complications.

In the recent years, there has been considerable progress in understanding the key pathogenic factors involved in the development of DR, which represents an opportunity to find new therapeutic targets (23). Furthermore, the concept of DR as a microvascular disease has evolved into that of a more complex diabetic complication in which neurodegeneration plays a significant role (23,24,33,34). In fact, the American Diabetes Association has recently defined DR as a highly tissue-specific neurovascular complication involving progressive disruption of the inter-dependence between multiple cell-types in the retina (20). However, regardless these new insights, at present we are doing nothing directly addressed to the diabetic eye until advanced stages. Therefore, new treatments based on a better understanding of the pathogenesis of the early stages of DR are needed (23).

1.2 Pathogenesis of diabetic retinopathy

1.2.1 Overview of the pathogenic pathways involved in DR and the natural history of microvascular disease

Under hyperglycemic conditions, several pathways are triggered: polyol and the hexosamine pathways, the synthesis de novo of diacylglycerol-proteinkinase C (DAG-PKC), the formation of advanced glycation end-products (AGEs) and free radical production. All these pathways are crucial to the development of DR and other diabetic complications and are summarized in **Figure 1** (35). The activation of these pathways is associated with mitochondrial overproduction of reactive oxygen species (ROS) (36). The activation of PKC and the interaction between AGE and its receptor (RAGE) induce the activation of the NF-κβ nuclear transcription factor (37). In response to NF-κβ activation and ROS, pro-inflammatory cytokines are generated establishing a low-grade inflammatory state (38). Oxidative stress activates several transcription factors, among them FOXO1 (forkhead transcription factor) that impairs NO production (39). Moreover, the activation of all these pathways induces an imbalance between angiogenic and antiangiogenic factors (angiogenic >> antiangiogenic). Vascular endothelial-growth factor (VEGF) is the most important angiogenic factor involved in DR. When elevated it increases vascular permeability and it is implicated in generating DME. In addition, under hypoxic conditions it promotes neovascularization, which makes it an important factor in the development of PDR. On the other hand, several neuroprotectors factors like somatostatin (SST) or PEDF (Pigment Epithelium Derived Factor) are decreased in early diabetic retinopathy (40,41) (see section 1.2.2.2).

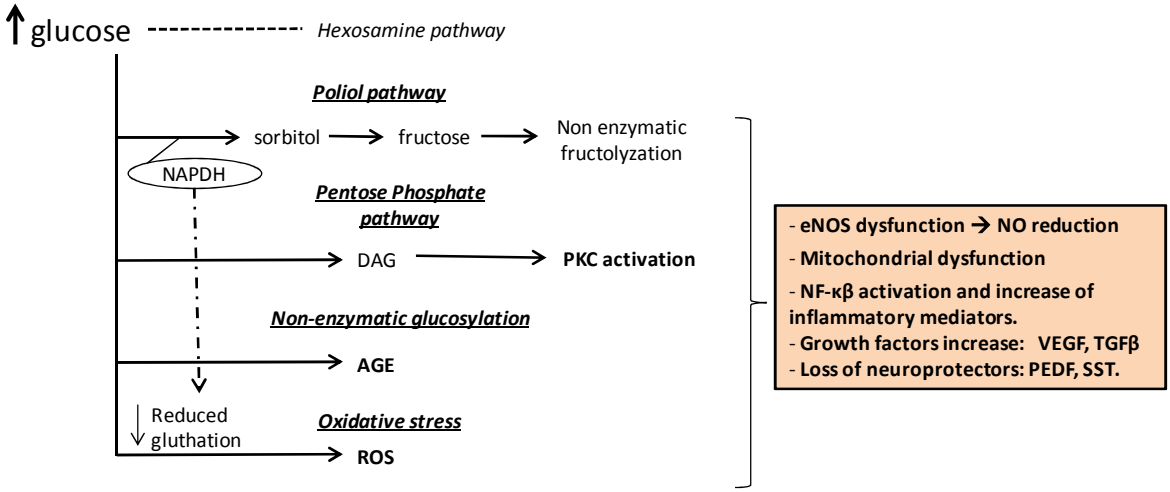


Figure 1. *Pathogenic pathways involved in diabetic complications*

The activation of all these pathways leads to abnormalities in both the neural retina (retinal neurodegeneration) and the capillary bed located in the inner retina (microangiopathic injury). The degenerative changes in the neuroretina include increased apoptosis, glial cell reactivity, microglial activation, and altered glutamate metabolism. When occurring together, these changes may explain some of the functional deficits in vision that first appear in diabetes even before vascular abnormalities can be appreciated (see section 1.2.2).

A summary of the natural history of DR is displayed in **Figure 2.**

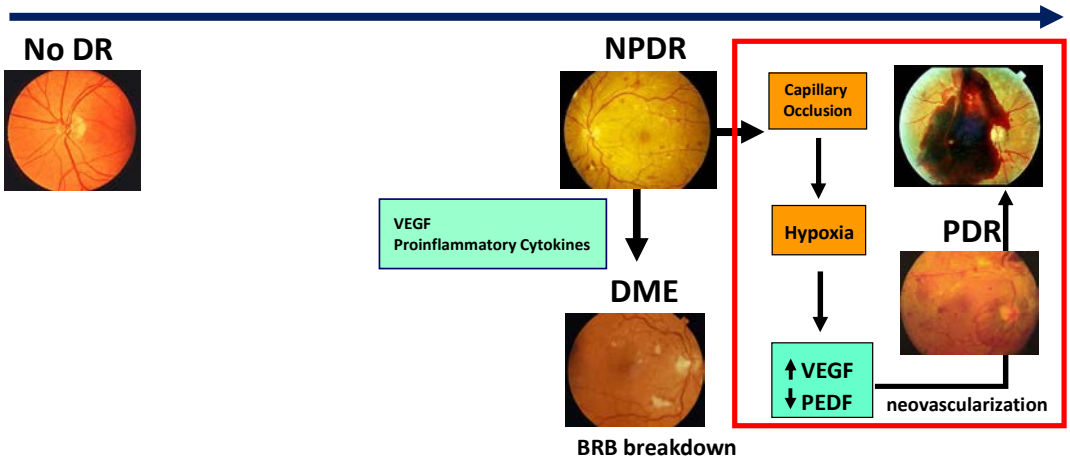


Figure 2. Natural evolution of DR. *The first stage that we can see by ophthalmologic examination is background retinopathy or non-proliferative diabetic retinopathy (NPDR). From this stage the natural history of the disease can follow two directions that do not exclude the other. One of them is the development of diabetic macular edema (DME) in which the most important pathogenic event is the breakdown of the BRB (this way is most frequent in type 2 diabetic patients). The other direction is towards PDR which is more frequent in type 1 diabetes and the primary event is hypoxia. In this setting capillary occlusion plays an essential role generating an imbalance between angiogenic and antiangiogenic factors, which finally stimulates neovascularization (the hallmark of PDR).*

The first vascular lesions that occur in the retina are the thickening of the basement membrane, the endothelial injury, the disruption of the tight junctions and pericyte loss (42). Pericyte dropout may have profound repercussion on capillary remodeling and are the main factor responsible for the first abnormalities detected in clinical examination by fundoscopy. Pericytes contain actin filaments and specific binding sites for endothelin and, in consequence, act as the smooth muscle cell of capillaries regulating the vascular tone and perfusion pressure (43). In addition, pericytes are in direct contact with endothelial cells through fenestrations in the basement membrane and may inhibit endothelial replication by locally releasing and activating transforming growth factor β1 (TGF-β1) (44). The thickening and the dysfunction of the basement membrane might disrupt the contact between pericyte and endothelial cells, thus impairing pericyte nutrition and contributing to its apoptotic death. The consequences of pericyte dropout are the loss of vascular tone regulation and, due to the deficit of TGF-β, the growth and proliferation of endothelial cells is also favored. These changes are crucial for the developing of microaneurysms (small outpouchings from retinal capillaries) and dot intraretinal hemorrhages, which constitute two of the earliest abnormalities that we can find in NPDR through ophthalmoscopic examination.

Optical Coherence Tomography Angiography (OCTA) allows the non-invasive evaluation of the retinal vasculature. Three layers are present in the macula: the superficial capillary plexus (SCP), found in the ganglion cell layer (GCL); the intermediate plexus (ICP), which is located between the IPL (Inner Plexiform Layer) and INL (Inner Neural Layer), and the deep plexus (DCP), between the INL and the OPL (Outer Plexiform Layer) (45). Nesper et al (46), quantified microvascular

abnormalities with increasing severity of DR by OCTA. They found that retinal capillary non-perfusion correlated with DR severity. Moreover, a significant decline in DCP blood flow was observed with increasing DR severity. Vujosevic et al (47) also examined by OCTA diabetic subjects without signs of diabetic retinopathy and healthy controls: in type 2 diabetic subjects they also found that DCP is more affected than SCP in pre-clinical DR. These findings suggest that capillaries in the DCP suffer an early loss of pericytes and subsequent reduction of blood flow, compared to the other plexuses.

The endothelial injury in this early state is not intense enough in most capillaries and, therefore, the endothelium's ability to synthesize vasodilator agents such as nitric oxide (NO) remains quite well preserved. The thickening of the basement membrane and the disruption of the tight junctions are determinant factors in the leakage of the retinal capillaries. Although thickened the basement membrane is dysfunctional and permits the passage of the intravascular content to the interstitial space, which is then amplified by the blood-retinal barrier breakdown due to the disruption of the tight junctions. The clinical consequence is the presence of hard exudates that represent the leakage of plasma constituents, especially lipids and proteins. DME is another consequence of these abnormalities and represents the leakage of plasma from the small blood vessels in the macula, the central portion of the retina that is responsible for the major part of visual function.

In advanced stages of NPDR, severe endothelial damage occurs and the profile of vasoactive factors synthesized by the endothelium becomes favorable to the vasoconstrictor agents (48). In addition, the NO is quenched by AGEs. Therefore, there is a clear predominance of vasoconstriction, thus favoring a hypoxic environment. As the disease progresses not only severe endothelial damage but also endothelial cell loss becomes a generalized phenomenon. In consequence, capillaries are constituted only by tubes of a thickened basement membrane, which are highly thrombogenic, and they could be occluded by classic thrombosis or even literally occluded by leucocytes. Obviously, these two pathways are crucial in worsening the ischemia and the consequence is preproliferative diabetic retinopathy. The clinical findings in fundoscopic examination are the presence of soft exudates which look like cotton-wool spots and which indicate a large area of retinal ischemia or infarction. Dilated capillaries and shunts between arteries and veins can also be identified in this largely avascular area and are known as intraretinal microvascular abnormalities (IRMAs). In addition, retinal veins may appear dilated, tortuous, and irregular in caliber. Arteries may appear white on inspection with an ophthalmoscope and are non-perfused when examined with fluorescein angiography. It should be emphasized that these sequential steps in the development of DR are not very clear and there is actually an overlap of all of them. In fact, capillary closures with areas of non-perfusion occur early in DR and they can be detected even earlier than microaneurysms by fluorescein angiography. However, capillary closures become widespread as the complication progresses and represent the predominant event in preproliferative DR. The end stage in the natural history of DR is PDR and its complications. As indicated above, PDR is the commonest sight-threatening lesion in type 1 diabetes. Severe hypoxia, acellular capillaries and leukostasis (the inappropriate adherence of leukocytes to the retinal capillaries) are the three main conditions for the initiation of PDR (41).

1.2.2 Pathogenesis of early stages of diabetic retinopathy

1.2.2.1 Neurovascular dysfunction

The term “neurovascular unit” (NVU), was first applied to the blood-brain barrier and refers to the functional coupling and interdependency of neurons, glial cells, and the highly specialized vasculature in the central nervous system (CNS) (49–52). In the context of the retina, the components of the NVU are diverse neural cell types (i.e. ganglion cells, amacrine cells, horizontal and bipolar cells), glia (Müller cells and astrocytes), professional immune cells (microglia and perivascular macrophages) and vascular cells (endothelial cells and pericytes) (49–52). All of these components are in intimate communication and maintain the integrity of the inner blood retinal barrier (iBRB). There is also another component of the BRB which is the outer BRB (oBRB). The oBRB is formed by the retinal pigment epithelium and endothelial cells from the choriocapillaries, and regulates the transport between choroid microcirculation and the neuroretina. Notably, in DR, the iBRB and the neurovascular unit are the main players and the impairment of the neurovascular unit is a primary event in the pathogenesis of DR (53–56). The intra-retinal vasculature lacks autonomic innervation and, therefore, a dynamic autoregulatory response of the NVU to complex circulatory and neural cues is essential to regulate blood flow through the inner retina (57,58). Neuronal and glial-mediated neurovascular coupling is the intrinsic physiological mechanism by which neural activity is coupled to blood flow and metabolism, thus enabling the retina to regulate blood flow in response to neural activity or metabolic demands (**Figure 3**).

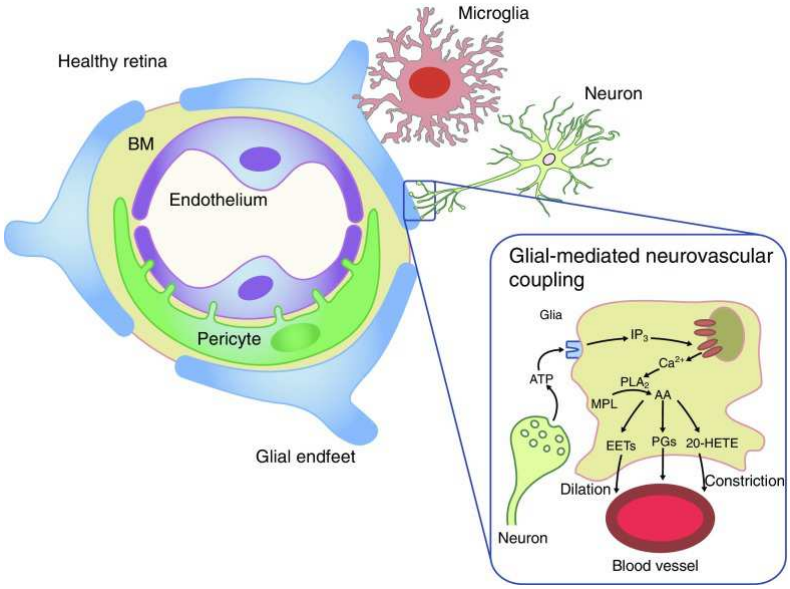


Figure 3. *Composition of the retinal NVU. Glial-mediated neurovascular coupling is schematically represented* (59).

Visual stimulation is a powerful modulator of retinal and optic nerve blood flow (60), and flicker light stimulation (intermittent flash) has been used to investigate this process because it increases neural activity. Flicker-induced retinal diameter change has been shown to deteriorate early in DR, and it has been reported in diabetic patients without structural microvascular abnormalities in the retina (51,52,57,58,61,62). These changes clearly demonstrate the relevance of neurovascular coupling or, in other words, interactions between the neurosensory retina and its blood vessels. The

progressive dysfunction of neurovascular coupling may be a key causative factor in the development of clinically evident DR, but longitudinal studies of retinal autoregulatory responses are needed.

The mechanisms underlying neurovascular coupling are complex and multifactorial and different types of cells are involved. Notably, glial cells have an essential role in the hemodynamic response by means of the production of vasoactive factors. Moreover, NO released from neural cells and endothelial cells acts as a modulator of the response. Interestingly, it has been reported that glial-induced vasodilating prostanoids are active at low NO concentrations, whereas vasoconstricting prostanoids are predominant at higher NO concentrations (34).

1.2.2.2 Neurodegeneration

A growing body of evidence clearly shows that neurodegeneration is an early event in the pathogenesis of DR and that it could be linked to the development of microvascular abnormalities (34). Therefore, the study of the underlying mechanisms leading to the early disruption of the NVU and neurodegeneration is essential for the development of new therapeutic strategies.

The hallmarks of diabetes-induced neurodegeneration are neural apoptosis and reactive gliosis. Both histological changes have been observed in experimental DR models and in the retina of diabetic donors before microangiopathy could be detected by fundus examination (63–65). Retinal ganglion cells and amacrine cells are the first neurons in which apoptosis is detected, but photoreceptors also have an increased apoptotic rate.

The most important mechanisms in the neurodegenerative process that occur in DR are:

- Extracellular glutamate accumulation. Glutamate is the major excitatory neurotransmitter in the retina and it is elevated in DR. The accumulation of glutamate leads to an overactivation of ionotropic glutamate receptors, mainly alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors, which results in an uncontrolled intracellular calcium response in postsynaptic neurons and cell death. This deleterious effect of glutamate on retinal neurons is known as “excitotoxicity”(34).

- Imbalance in the retinal production of neuroprotective factors. The retinal production of several neuroprotective factors such as PEDF, SST, interstitial retinol-binding protein (IRBP) and Glucagon-like peptide 1 (GLP-1) is downregulated in the retina of diabetic patients (34,66). On the other hand, an upregulation of neuroprotective but also pro-angiogenic factors such as VEGF and erythropoietin (EPO) also exists in the retinas from diabetic patients. Therefore, the overexpression of these factors could play a double role acting as neuroprotectors in the early stages but favoring vascular alterations in more advanced stages (40,41).

- Oxidative stress. As previously mentioned, the activation of the polyol and hexosamine pathways, AGE formation and the AGE-RAGE interaction, and PKC activation that occur under hyperglycemic conditions cause an overproduction of ROS (67). There is emerging evidence that

oxidative stress can damage neural (in particular retinal ganglionar cells) and microvascular retinal cells. One of the mechanisms might be through the impairment of high-affinity L-glutamate/L-aspartate transporter (GLAST) which is a transporter involved in glutamate clearance, thus favoring excitotoxicity. In addition, glutamate toxicity results in glutathione depletion thus contributing to oxidative stress (34).

- Neuroinflammation. The mechanism by which inflammatory cytokines contribute to neural apoptosis remains to be fully elucidated but excitotoxicity, oxidative stress and mitochondrial dysfunction are among the most important mediators (34).

- Axonal transport and neurotransmission: Diabetes alters the content of several synaptic proteins such as syntaxin-1, synapsin-1 and synaptophysin, thus leading to an impairment in neurotransmission (68). Moreover the impairment of axonal transport has been involved in neuronal cell death (69). In the retina of rats with streptozotocin-induced diabetes it has been shown that axonal transport is impaired in retinal ganglion cells (70). **The study of proteins involved in neurotransmission and axonal transport in early stages of DR will be one of the objectives of this thesis.**

1.2.2.3 Glial activation or reactive gliosis

At present it is unknown whether neural apoptosis or reactive gliosis is first in the neurodegenerative process occurring in the diabetic retina. However, reactive gliosis (or glial activation) may play a role in causing damage to retinal neurons and link the neurodegenerative process with microvascular disease. Indeed, the astrocytes and Müller cells of the NVU play a crucial homeostatic function by regulating retinal blood flow, water balance in the neural parenchyma, and maintaining barrier function (71). Specifically, Müller cells can undergo reactive gliosis, which is revealed by the aberrant expression of glial fibrillary acidic protein (GFAP) (**Figure 4**). Gliosis is associated with increased expression of VEGF and innate immune-related pathways, resulting in an overexpression of proinflammatory cytokines and BRB dysfunction (72,73). Apart from iBRB breakdown, Müller cells have also been implicated in neural apoptosis through glutamate excitotoxicity. Müller cells, in normal conditions, uptake glutamate through the GLAST and convert it to glutamine thanks to the specific enzyme glutamine synthetase. Both GLAST and glutamine synthetase are compromised in the diabetic retina (74).

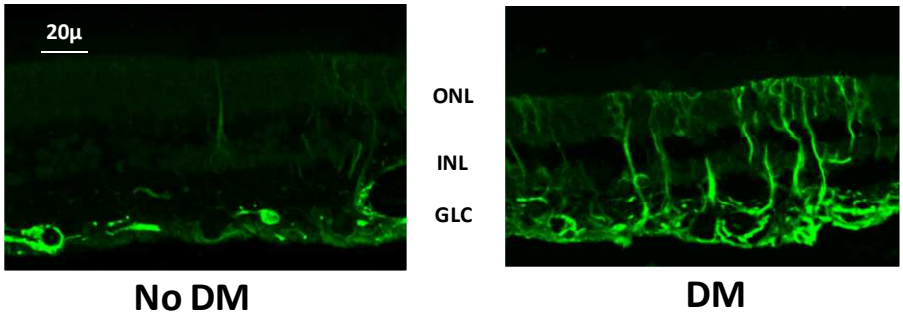


Figure 4. *Human retinal samples without glial activation (no DM) and with glial activation (DM), which is revealed by the aberrant expression of GFAP (in green).*

In addition to macroglial cells, activated microglia can also mediate diabetes-induced subclinical inflammation. Microglial activation is accompanied by a phenotype change toward an amoeboid shape and presents two opposite roles depending on the polarization of resident immune cells of the retina triggering proinflammatory (M1) or anti-inflammatory (M2) actions (72,73). In early stages of DR, the M2 response occurs concurrently with the M1 response ameliorating inflammation and delaying the progression of the disease. However, during the progression of DR, the M1 response is maintained whereas the M2 response declines and the classical pro-inflammatory signaling pathways are chronically activated (73). In fact, a shift from pro-survival to pro-neurotoxicity occurs and transcriptional changes in activated microglia, mediated via NF-κβ and extracellular signal-regulated kinase (ERK) signaling pathways, result in the release of various pro-inflammatory cytokines, chemokines, caspases and glutamate (75). These molecular mediators contribute to the disruption of the BRB and NVU impairment and to neuronal death.

1.2.2.4 Early vascular impairment

The early stages of DR include disruption of the iBRB, the thickening of the vascular basement membrane and the damage and subsequent loss of pericytes and endothelial cells.

1.2.2.4.1 Blood retinal barrier dysfunction

In both the iBRB and the oBRB the passage of proteins and many other macromolecules into the retina from the bloodstream is controlled by tight junctions between adjacent cells (i.e. occludin, claudins and ZO-1), which effectively block paracellular permeability. The increase in vascular permeability in diabetes occurs through two basic fluxes: a paracellular flux (between endothelial cells) and a transcellular flux (through the cells) (56,76). The paracellular flux consists in the formation of gaps between endothelial cells. The gaps are formed as a consequence of the endothelial contraction that follows actin and myosin contraction (77,78). In addition, transcellular flux could be mediated by membrane transporters or through the formation of endocytotic vesicle-like structures (56,76). The disruption of the BRB and in particular the iBRB is essential in the pathogenesis of DME (54).

The main molecular mechanisms causing vascular leakage in diabetes are the following:

- Advanced glycation end-products formation. There is emerging evidence indicating that AGEs increase vascular permeability both in vitro (79–81) and in vivo (82). One of the mechanisms involved in AGE-induced vascular leakage is the inflammatory pathway by means of the activation of NF- κβ. Another mechanism relies on the ability of glycosylated proteins to alter the properties of the glycocalyx, which surrounds the endothelial cells (76).

- Inflammatory mediators. Pro-inflammatory cytokines (i.e. IL-1β, TNF-α, IL-6, MCP-1) and complement components are secreted from RPE, glia and immune cells (83). In addition, it has recently been reported that photoreceptor cells release inflammatory products, which directly contribute to increased retinal endothelial permeability in mouse models of diabetes (84). Blood circulating leukocytes engage with adhesion molecules such as ICAM-1, VCAM and selectins on

the surface of the endothelial wall (leukostasis), and provoke the occlusion of capillaries. Such vascular-immune cell interactions also contribute to microvascular damage by releasing cytokines and superoxide via the respiratory burst, which alters the integrity of the NVU (83,85). In advanced stages of DR where immune privilege is compromised, circulating immune cells and serum proteins may infiltrate the retina and the vitreous, thus participating in chronic inflammation and retinal vascular and neuronal damage (85–88).

- Vascular Endothelial Growth Factor. VEGF, also known as vascular permeability factor (89), is an angiogenic factor that is upregulated by chronic hyperglycemia (90). It is synthesized locally in the retina by different cellular types, contributing to BRB breakdown and the development of DME (41,91). There is evidence suggesting that VEGF is able to increase permeability by incrementing not only paracellular flux, but also transcellular flux by inducing the formation of vesiculo-vacuolar organelles (92). In fact, anti-VEGF drugs are the first line therapeutic strategy for DME (4,22).

- PKC activation and downstream effectors. In chronic hyperglycemia, diacylglycerol (DAG) levels are elevated as a result of glucose catabolism. DAG enhances PKC activation, which has multiple effects: it alters enzyme activities in endothelial cells (altering the bioavailability of NO, increases ET-1 production and upregulating the expression of VEGF) and pericytes (upregulating PDGF, ROS, NF-κβ and MAPK). As a result, vascular permeability is increased, leukostasis is promoted and basement membrane thickens (93–95).

- Oxidative stress. In diabetic retinopathy ROS have a main role in inducing endothelial and pericyte apoptosis, contributing to BRB breakdown (67). Interestingly, Lu et al, observed that superoxide is able to increase pulmonary microcirculatory permeability in an animal model of type 1 diabetes (96). Moreover, ROS also contribute to endothelial dysfunction by means of a loss in NO bioactivity (97).

- ERM (ezrin, radixin and moesin) complex and cytoskeleton reorganization. Actin cytoskeleton organization is essential for endothelial barrier function (98). In fact, actin filaments are involved in tight junction (between endothelial cells) stabilization and focal adhesion plaque formation (between the endothelial cells and the extracellular matrix). When endothelial cells are activated in response to permeability-inducing agonists, two main changes in the cytoskeleton occur: a) Reorganization of the focal adhesions to sites where the stress actin fibers are anchored. b) Actomyosin contraction. These cytoskeleton changes induce the formation of gaps between endothelial cells due to the disassembly of cell-cell junctions and/or endothelial retraction (99–101). These structural rearrangements significantly contribute to paracellular flux, one of the aforementioned mechanisms of vascular leakage. ERM proteins activation mediates these cytoskeleton changes. **At present little is known regarding the role of ERM proteins in DR and, therefore, this will be one of the objectives of this thesis.**

1.2.2.4.2 Structural changes

As commented in section 1.2.1 the pericyte loss, the thickening of the basement membrane and the endothelial injury are the firsts structural changes detected in DR.

Pericyte are specialized contractile cells of neural crest, mesodermal and bone marrow origins, and regulate vascular tone and perfusion pressure (102). The loss of pericytes is one of the first histological alterations in DR. The loss of pericytes compromises capillary integrity leading to weakening of iBRB and vascular leakage. Following the loss of pericytes it is the loss of endothelial cells, and retinal capillaries become acellular (**Figure 5**). This so-called vasodegeneration or vasoregression is a central pathogenic response to chronic hyperglycemia and initiates the progressive ischemia which is characteristic of DR (103,104).

In parallel, as a result of the increased synthesis of vascular basement membrane components like collagen IV and laminin, in combination with a reduced degradation by catabolic enzymes, there is a thickening of the capillary basement membrane (BM). The vascular BM is a key component of the NVU and is essential for both structural integrity and cell-matrix interactions (24,105). These changes impair cell-cell communications and diminish the capacity of cell interactions that promote normal function and survivability of the NVU. In addition, the thickened BM has diminished its structural role as a barrier, thus favoring vascular leakage (106).

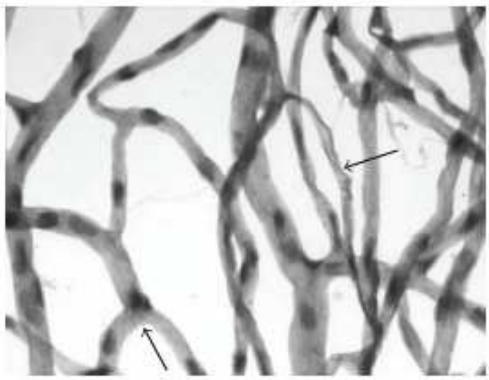


Figure 5. *Acellular capillaries. (from* (107)*)*

1.2.2.5 Mechanisms linking retinal neurodegeneration and early microvascular impairment

The potential mechanisms linking retinal neurodegeneration and early microvascular impairment are summarized in **Figure 6** (34). The most important are the following:

- VEGF and glutamate excitotoxicity. It has been demonstrated that hyperglycemia induces an increase in extracellular glutamate and the subsequent overactivation of NMDA receptors mediates VEGF production and BRB breakdown (108,109).

- Loss of neuroprotective factors. The loss of several neuroprotective factors such as PEDF or SST, which in normal conditions have a neuroprotective role, can contribute to the disruption of the BRB directly or through the upregulation of VEGF (110–113).

- Altered levels and dysfunctional EPC (endothelial progenitor cells). Conflicting results have been reported regarding EPC levels in diabetic subjects (114). Poor glycemic control appears to be associated with a reduction in the number of circulating EPCs, whereas an adequate control of glycemia seems to increase their numbers (115). In the setting of DR, an increase of EPCs have been reported in patients with PDR (116–119). However, some authors found a decreased level of

EPCs in NPDR (118,119). Apart from altered levels it has been observed that bone marrow derived EPCs from diabetic patients are dysfunctional, producing fewer endothelial cells with proliferative and migratory potential. It should be noted that EPC promotes vascular repair, preventing ischemic injury (120,121). Therefore, these dysfunctional EPCs could be one of the mechanisms involved in vasoregression and the neurodegenerative process that occur in the early stages of DR.

- Glial activation. The impairment of neurovascular coupling is an early event in DR. However little is known about the underlying mechanisms linking retinal neurodegeneration and the dysfunction of the neurovascular unit. As previously mentioned, glial cells have a key role in the hemodynamic response that governs the neurovascular coupling. In fact, activated glia can produce either dilatation or constriction, evoked by the same stimulus under different NO concentrations. In addition, glial cells, by means of increasing Ca^{2+}, can increase NO production, which in turn modulates the vascular response (58). Recent experimental studies have also found that an early activation of the microglia and complement system play a role in the damage of the retinal neurovascular unit (122). In addition, glutamate clearance by Müller cells and, in consequence, glutamate excitotoxicity is impaired in DR (74). To summarize, glial activation is implicated in both neurodegeneration induced by glutamate excitotoxicity (due to the impairment of glutamate removal) and in early microvascular impairment.

- The role of endothelin. Endothelin-1 (ET-1) is upregulated in the diabetic retina (123) and plays a dual deleterious action on microvessels and neurons. This is because of its capacity to bind to receptors A (ETA) which mainly mediates vasoconstriction and vasoregression (124), and receptors B (ETB) which are involved in retinal neurodegeneration (125,126). Therefore, ET-1 could be envisaged as a specific target for treating both early microvascular impairment and neurodegeneration. **However, there are no studies assessing ET-1 and ETA and ETB receptors in the early stages of DR in humans, and the potential effect of bosentan (a dual endothelin receptor antagonist) in preventing retinal neurodegeneration has not previously been explored. These two scientific gaps will be examined for the first time in this doctoral thesis.**

1.2.3 The role of inflammation and evidence from proteomic-based studies

Inflammation plays a crucial role in DR pathogenesis, being one of the main contributors of BRB breakdown, which is essential for DME development and carries important therapeutic implications (23). In fact, several proinflammatory cytokines, chemokines and adhesion molecules have been found elevated within the vitreous of diabetic patients with advanced stages of DR (127,128). The pro-inflammatory cytokines are generated as a response to oxidative stress and the activation of NF-κβ, generating a low-grade inflammatory state. The main cytokines and their connections are summarized in **Figure 7**. IL-1β is one of the main cytokines involved in diabetic complications. It is able to activate nuclear transcription factor-κβ (NF-κβ) and induce the expression of adhesion molecules and chemoattractant factors by the endothelium. NF-κβ can be also activated by RAGE interaction with HMGB1 (High-Mobility Group Box-1 Protein) which also functions as a pro-inflammatory cytokine (129).

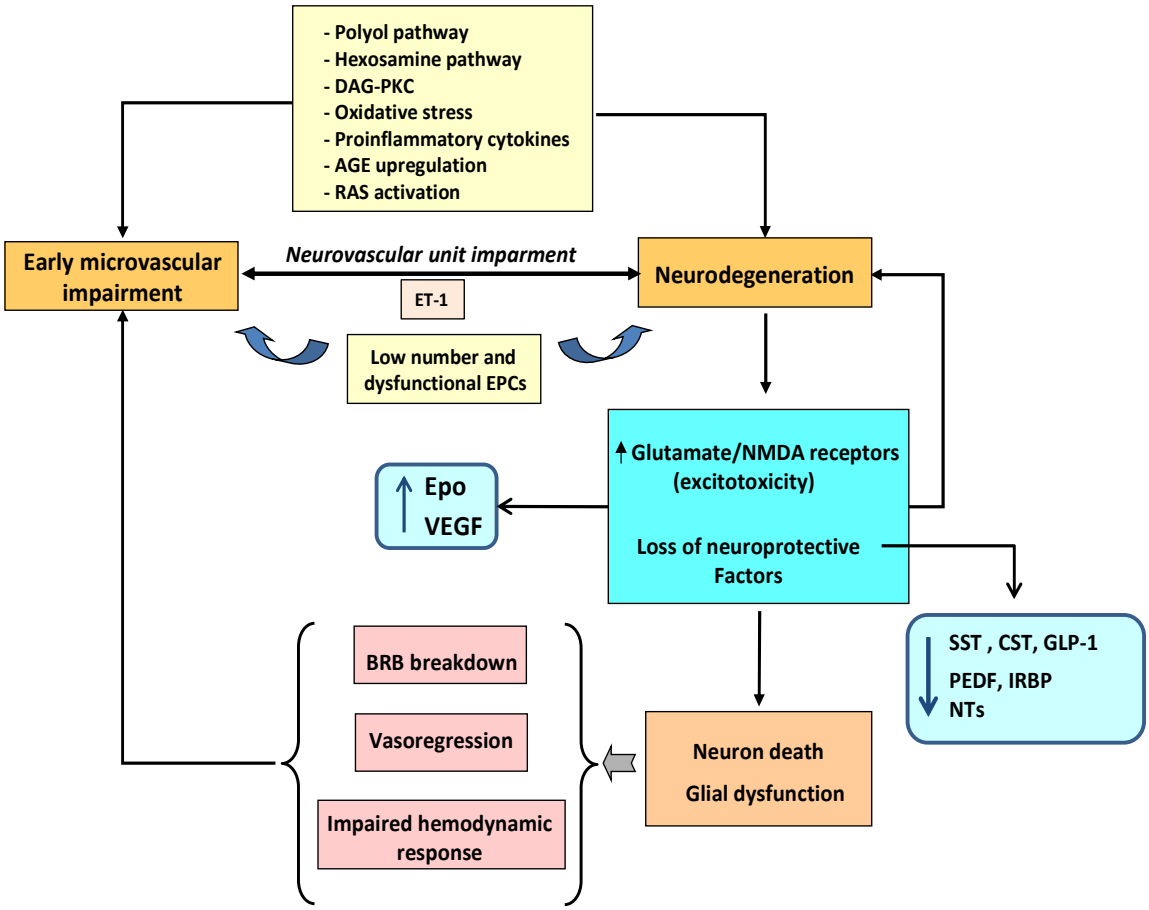


Figure 6. *Schematic representation of the main mechanisms leading to DR that link neurodegeneration and early microvascular impairment. ET-1 endothelin-1; EPC: endothelial progenitor cells; Epo: erythropoietin; VEGF: vascular endothelial growth factor; SST: somatostatin,; CST: cortistatin; PEDF: pigment epithelium derived factor; IRBP: interphotoreceptor retinoid-binding; NTs: neurotrophins. Adapted from* (34)

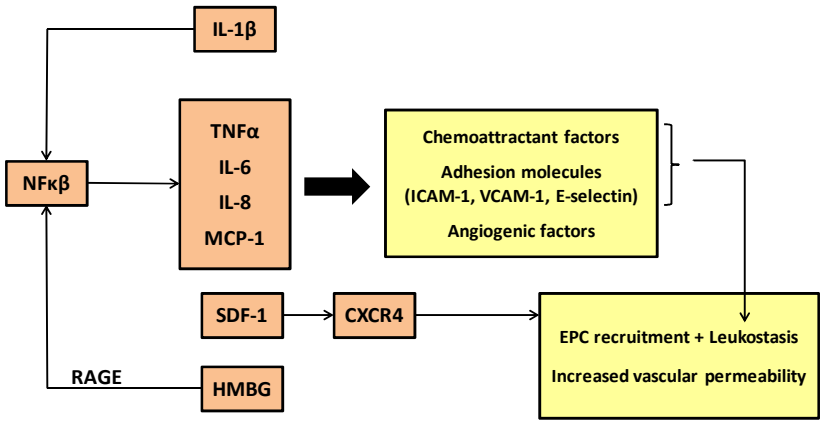


Figure 7. *Schematic representation of the mediators of inflammation involved in DR development. Adapted from* (38)

The activation of NF-κβ induces the expression of several pro-inflammatory cytokines. TNF-α is one of the most important and has been implicated in diabetes complications as it is able to amplify the inflammatory cascade. It should be mentioned that both IL-1β and TNF-α are able to increase vascular permeability and to induce the expression of adhesion molecules by endothelial cells (38,130,131). Both IL-8 and IL-6 have been found elevated in the vitreous of patients with PDR (38). IL-6 is able to increase endothelial cell permeability in vitro by rearranging actin filaments and by changing the shape of endothelial cells (132). IL-8 has been recognized as a potent chemoattractant and as an activator of neutrophils and T lymphocytes (133). MCP-1 (Monocyte Chemotactic Protein-1) is one of the main cytokines and it has been found in the vitreous from patients with PDR in the same range as that reported in pleural effusions of patients with pneumonia or tuberculosis, and its levels correlate with PDR activity (128). Under hyperglycemic conditions MCP-1 is produced by endothelial cells, RPE cells and Müller glial cells (134). All these cytokines increase the expression of adhesion molecules. The main adhesion molecule is ICAM-1 and an elevation of its soluble form has been found in DR (38). Adhesion molecules like ICAM-1 or VCAM-1 facilitate leukostasis, which is the irreversible adhesion of circulating leukocytes to the surface of endothelial cells (**Figure 8**). This phenomenon induces endothelial cells apoptosis, thus favoring vascular leakage (131). Finally, SDF-1 (Stromal Cell-Derived Factor I) is upregulated in damaged tissues and mobilizes stem/progenitor cells to promote their repair. SDF-1 acts through its receptor CXCR4 at several key steps in the process of ischemic repair, such as the recruitment of EPCs from the bone marrow (135). Moreover SDF-1 induces VEGF expression (136).

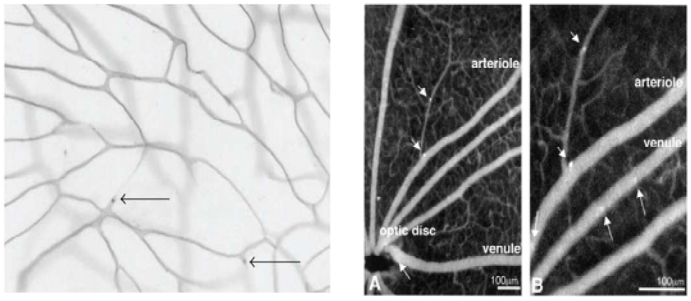


Figure 8. *Leukostasis phenomenon ex vivo (left panel)* (107) *and in vivo (right panel) in experimental models of diabetes* (137)

Proteomic analysis in the vitreous of the retinas from diabetic patients has contributed to the identification of the inflammatory mediators involved in DR. In this regard, two studies of the vitreous of diabetic subjects with PDR showed that several complement components were increased compared with control subjects (138,139). In addition, inflammation-associated proteins such as AAT, APOA4, ALB and TF have been found significantly elevated in the vitreous of PDR patients (140). Moreover, Gao et al (141) demonstrated that both extracellular carbonic anhydrase-I and kallikrein-mediated innate inflammation were involved in the pathogenesis of DME. Finally, two proteins related to inflammation were found differently expressed in the vitreous fluid of patients with DME in comparison with PDR and non-diabetic subjects: hemopexin (increased), which is an acute phase reactant that increases permeability; and clusterin (decreased), which limits the inflammatory response after injury (38,142).

The aforementioned studies focused on advanced stages of DR but inflammation is also an important player in early DR (34,83). In fact, one of the first signs of inflammation in DR, even before any microvascular abnormality is evident in the fundus exam, is the activation of glial cells which are one of the main sources of proinflammatory cytokines/chemokines (23,34). In addition, it has been demonstrated that hyperglycemia in retinal Müller cells triggers the expression of acute-phase response proteins and other inflammation-related genes (143). Therefore, glial activation is a primary event in the pathogenesis of DR that contributes both to neurodegeneration and early microvascular impairment (34). Vujosevic et al (88) performed a proteome analysis of the aqueous humour of diabetic subjects without DR in the fundus exam, with mild DR and controls, identifying several inflammatory cytokines that were differentially expressed among groups, including INF-γ, IL-1β, IL-3, IL-10, IP-10 and MCP-2. However, a proteomic approach in order to further identify the inflammatory candidates overexpressed in human retinas with early stages of DR has never been performed.

1.3 The assessment of retinal neurodegeneration

Retinal neurodegeneration can be examined morphologically or functionally. The structural consequence of the apoptotic death of retinal ganglionar cells is a reduced thickness of the inner layers and the nerve fiber layer. These early changes can be detected morphologically by optical coherence tomography (OCT) which is a non-invasive exam that is able to determine retinal layer thickness. The reduction of the ganglionar retinal cell layer and the nerve fiber layer can be detected in diabetic subjects even before any microvascular abnormality is revealed in the funduscopic exam (144).

The gold standard in assessing retinal function impairment is multifocal electroretinography (mfERG). One of the first alterations that can be detected in diabetic retinopathy is a delayed P1 implicit time and reduced amplitude of “a” and “b” waves (145). Impaired retinal function assessed by mfERG occurs in diabetic patients with no detectable evidence of microvascular abnormalities (146). In this regard, neuroretinal dysfunction assessed by mfERG has been reported in type 1 diabetic patients without BRB leakage measured by vitreous fluorometry (147). Furthermore, prospective studies, although performed in small numbers of patients, have shown that the increased implicit type in mfERG can predict the development of visible vascular abnormalities over a 1 to 3 year period (148–150). However, baseline fluorescein angiograms were not performed in these two studies, so it is possible that subclinical lesions existed at the study entry. All these findings raise the possibility that retinal neurodegeneration may precede the onset of diabetes-induced vascular changes. In fact in the baseline data of the EUROCONDOR study, which is the first clinical trial using neuroprotective drugs in the early stages of diabetic retinal disease, 58% of diabetic subjects without any microvascular abnormality in the 7 field funduscopic examination presented an abnormal mfERG (151).

mfERG is an expensive, cumbersome and time consuming examination which cannot be implemented in the general population and is restricted to clinical trials. Retinal sensitivity assessed by microperimetry might help overcome this inconvenience. Fundus-driven microperimetry evaluates the minimum light intensity that patients can perceive when spots of light stimulate specific areas of the retina. In some studies microperimetry has shown more sensitivity than mfERG in detecting early functional changes (152). In fact, diabetic subjects without signs of DR in the fundus exam show a reduced retinal sensitivity compared with non-diabetic subjects (153).

It should be noted that these structural and functional alterations have clinical implications in terms of deficiencies in sensory capacity that include decreased hue discrimination, contrast sensitivity, delayed dark adaptation and abnormal visual fields, thus resulting in an impairment of vision related quality of life (154–156).

1.4 Future perspectives in early stages of DR treatment based on pathogenic events

Current treatment strategies specifically directed to the eye target late stages of DR when vision has already been significantly affected. These strategies are invasive and consist in intravitreal injections of anti-VEGF or corticosteroids, laser photocoagulation and vitreo-retinal surgery (4). A better understanding of the pathogenesis of DR would permit the development of novel and more efficient preventive/interventional strategies against DR. The main strategies for treating DR in the early stages based on pathogenic events are summarized in **Figure 9** (adapted from (23)).

As previously mentioned, AGE formation and its interaction with RAGE induce vascular permeability and NF-κβ activation, incrementing the inflammatory cascade. In diabetic db/db mice, the administration of soluble RAGE fragments (a competitor of cellular RAGE for its ligands), prevented Müller cell dysfunction induced by diabetes and retinal capillary leukostasis in AGE-infused non-diabetic animals (81,157). Moreover, in other experimental approaches, targeting AGE accumulation or RAGE activation attenuated vascular lesions (158). Unfortunately, aminoguanidine, the first AGE inhibitor tested in diabetic patients was suspended due to its adverse effects (159). Antioxidant treatment has been also proposed in DR but despite the positive results in preventing retinal neurodegeneration and early microvascular impairment in rodents, clinical studies have been inconclusive (23).

Targeting inflammation is an emerging approach in DR treatment. In fact, corticosteroids are one of the main strategies (apart from anti-VEGF drugs) in the treatment of DME (4,22). In earlier stages of the disease it has been shown that the neutralization of IL-1β increases glutamine synthetase and GLAST expression in retinas from an experimental model of oxygen-induced retinopathy, thus reducing glutamate excitotoxicity (160). Another strategy that has been explored is the inhibition of the TNF-α receptor. The systemic administration of etanercept, a soluble TNF-α receptor that acts as a competitive inhibitor to block the effects of TNF-α binding to cells, reduces leukostasis, BRB breakdown and NF-κβ activation in the diabetic retina (161,162). Intravitreal injection of another TNFα-specific inhibitor, pegsunercept, led to a significant reduction in pericyte loss and capillary degeneration in diabetic rats (163,164). However, to the best of my knowledge no clinical trials using strategies addressed to block TNF-α in the diabetic eye are currently ongoing. The administration of aspirin and salicylates has proved useful in reducing retinal microvascular abnormalities induced by diabetes in experimental models (83). However, the clinical evidence that anti-inflammatory treatment could be beneficial for DR remains uncertain. The DAMAD Study Group reported that aspirin lowers the yearly increase in the number of definite microaneurysms (165). By contrast, the Early Treatment Diabetic Research Group did not find any benefit of aspirin treatment on DR. However, the dose used in this study was not high enough for anti-inflammatory purposes (166).

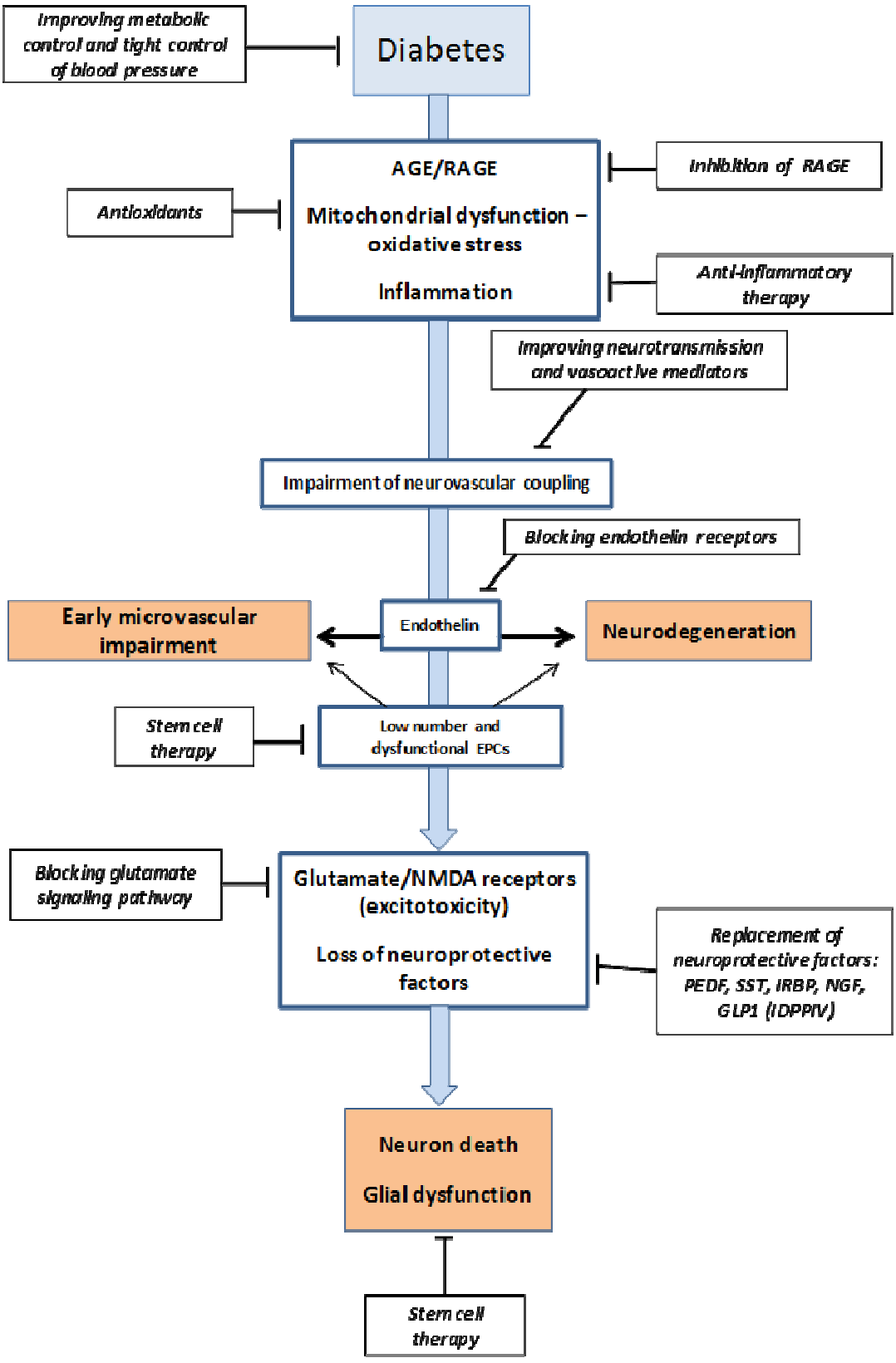


Figure 9. *Potential strategies for treating neurodegeneration. Adapted from* (23)

Recent evidence concerning the role of retinal neurodegeneration in early DR has aroused the possibility of using neuroprotective treatments to arrest the progression of DR. Several strategies have been proposed: blocking the glutamate signalling pathway, the replacement of downregulated neurotrophic factors (like PEDF or SST) or improving the neurovascular coupling function (23). As mentioned, SST is downregulated in the retina of diabetic patients without microvascular disease, and this downregulation is associated with retinal neurodegeneration (64,167). It has been shown that the topical administration of SST in streptozotozin-induced diabetic rats prevents retinal neurodegeneration. The main mechanisms involved in this benefitial effect were the prevention of the imbalance between proapoptotic and survival signalling caused by diabetes and the reduction of glutamate-induced excitotoxicity (167). The EUROCONDOR clinical trial, a multicentric, phase II-III, randomized controlled clinical trial to assess the efficacy of SST administered topically (eye drops) to prevent or arrest retinal neurodegeneration have demonstrated that topical administration of SST arrested the progression of neurodysfunction assessed by mfERG (implicit time) in those patients with some degree of neurodysfunction at baseline (168). This study has been the first clinical trial demonstrating that topical administration could be useful in treating DR and opens up the possibility of implementing non-invasive treatments in early stages of DR by using eye-drops.

The GLP-1 receptor is another target for preventing or arresting diabetes-induced retinal neurodegeneration. Apart from their impact on type 2 diabetes treatment, GLP-1R agonists have several pleiotropic actions, such as neuroprotection. Our group demonstrated that GLP-1 is downregulated in the human diabetic retina and that the topical administration of GLP-1R agonists prevented retinal neurodegeneration and vascular leakage in a spontaneous diabetic model (db/db mouse) (66). Similarly, topical treatment with DPP-IV inhibitors, by preventing GLP-1 degradation, was also effective in preventing the neurodegeneration and vascular leakage in db/db mouse model (169). However, no clinical trials aimed at demonstrating the effectiveness of either GLP-1R agonists or DPP-IV inhibitors have yet been conducted.

Stem cells have been also proposed for DR treatment. As has been mentioned, diabetic patients present less and dysfunctional EPC. There is evidence indicating that EPC could play a critical role in endothelial repair and maintenance. Therefore, the defective repair of injured retinal vessels by EPCs may contribute to the pathogenesis of DR (120,121). However, the usefulness and safety of EPC use as a therapeutic approach for DR remains to be determined (23).

The identification of new molecules implicated in DR pathogenesis could help in finding new targets for DR treatment. The results of the EUROCONDOR trial suggest that there are two differentiated phenotypes in early stages of DR: one in which neurodegeneration predominates and a second in which vascular damage is predominant (168). Therefore, in early stages of DR, targeting both neurodegeneration and microvascular impairment would appear the best option. In this regard, as previously commented, the inhibition of endothelin receptor A (which mediates vasoconstriction and vasoregression) and receptor B (which is involved in neurodegeneration) could be an optimal therapeutic strategy for the early stages of DR.

1.5 Retinal neurodegeneration as a tool for identifying type 2 diabetic subjects at risk of dementia.

1.5.1 Type 2 diabetes is a risk factor for the development of cognitive impairment and dementia

There is emerging evidence indicating that T2D is associated with cognitive impairment and dementia. Biessels et al (170), demonstrated in a meta-analysis that diabetic subjects presented at least a 2-fold higher risk of developing Alzheimer's disease (AD) than age-matched non-diabetic subjects. This increased risk was maintained even after adjusting for other vascular risk factors. This observation has been confirmed in other studies (171) and also in two large nationwide population-based studies performed in Taiwan with a follow-up of around 10 years (172,173). Diabetic subjects also present an increase of risk of mild cognitive impairment (MCI), which consists of cognitive impairment on standard tests but no impairment of activities of daily living and represents a transition state between normal cognitive function and dementia (174). The annual conversion rate from MCI to dementia ranges between 10-30% in the general population. In a case-control study, we found that diabetic patients converted to dementia at a higher rate and faster than age-matched control subjects independently of other risk factors of dementia (175). Therefore, diabetes is not only associated with cognitive impairment but it also accelerates the conversion from MCI to dementia. In fact, it has been demonstrated that T2D subjects presented brain structural abnormalities even before the diagnosis of dementia, consisting in global cerebral atrophy, loss of connectivity and small-vessel disease (176).

Among the diabetic population, the presence of microvascular complications is associated with even a higher risk of dementia. As has been mentioned, data from the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial and in particular from ACCORD-MIND and ACCORD-Eye sub studies showed that DR is associated with lower grey matter volume and predicted future cognitive decline in the T2D population (32). These results confirm the finding from the Edinburgh Type 2 Diabetes Study derived from cross-sectional data that lifetime cognitive decline is associated with DR (177). In addition, it has recently been reported that T2D patients with advanced retinopathy (i.e. PDR and DME) presented a 42% higher risk of incident dementia (178). Regarding diabetic nephropathy, there is some evidence that microalbuminuria is associated with accelerated cognitive decline in T2D (179,180). Altogether, these observations strongly suggest T2D patients with microangiopathy present a higher risk of cognitive decline and dementia than those patients without microangiopathy.

In addition, AD and diabetic complications share common pathogenic pathways. The impairment of insulin signaling, the presence of low-grade inflammation and the pathways directly related to chronic hyperglycemia, such as the accumulation of advanced glycation end-products (AGEs) and the increase in oxidative stress play an essential role in the pathogenesis of AD (23, 148,149). All these pathways have been implicated in diabetic complications and in particular with DR.

Apart from the underlying mechanisms shared between T2D and AD, hypoglycemia (in particular, severe hypoglycemic episodes) is a well-established independent risk factor for cognitive impairment and dementia in older people with T2D (183,184).

1.5.2 Strategies to identify diabetic subjects at risk of developing dementia

Both diabetes and dementia are expected to increase as both are age-related diseases. In the near future, it would be of interest to identify those diabetic subjects with a higher risk of dementia for several reasons:

- It could raise the possibility of implementing preventive treatments at early stages before dementia is established.

- Since it has been demonstrated that severe hypoglycemia is a risk factor for cognitive impairment and dementia, identifying those subjects at risk would help personalize diabetic treatment in order to reduce the hypoglycemic risk in this more vulnerable subpopulation.

- T2D patients with cognitive impairment are more prone to present an impaired self-management of diabetes, poor glycemic control, an increased incidence of diabetes-related complications and more hospital admissions. Therefore, in these patients the treatment of diabetes should be simplified in order to increase treatment adherence and improve the quality of life.

For all these reasons, and ultimately to implement a more personalized treatment, the early diagnosis of cognitive impairment and the identification of the subset of patients at a higher risk of developing AD is a challenge that needs special attention from healthcare providers and the development of specific patient-centered programs. Currently, in clinical practice, there are no reported phenotypic indicators or reliable examinations to identify type 2 diabetic patients with MCI. Moreover, the diagnosis of MCI is based on complex and time consuming neuropsychological tests which make their incorporation into current standards of care for the type 2 diabetic population unfeasible.

1.5.3 Could retinal neurodegeneration help in identifying the subjects at risk of developing dementia?

1.5.3.1 The common molecular soil in the neurodegenerative process of the brain and the retina

The retina is ontogenically a brain-derived tissue, and it has been suggested that it may provide an easily accessible and noninvasive way of examining the pathology of the brain. Therefore it could be postulated that in patients developing brain neurodegeneration there is a co-occurring neurodegenerative process in the retina (“the eye as a window of the brain”). Recently, in a collaborative study performed with the Penn State Hershey Eye Center (Pennsylvania, US) and University of Michigan Medical School (Michigan, US) we published the first results of a proteomic analysis using retinas from diabetic patients in very early stages of DR (185). This study revealed the presence in the neuroretina of typical connecting neurodegenerative and inflammatory processes which are likely to have strong commonality to the neurodegeneration of the brain. The PhD candidate has played a significant role in the analysis and interpretation of the results of this study which will be displayed in the results section of this doctoral thesis.

1.5.3.2 The usefulness of retinal assessments to identify subject with cognitive impairment

As previously mentioned multifocal electroretinography (mfERG), retinal microperimetry and spectral domain optical coherence tomography (SD-OCT) are among the current methods used to identify the presence of retinal neurodegeneration. These noninvasive methods permit us to detect functional and morphological abnormalities. Marziani et al (186) in a case-control study showed that both retinal nerve fiber layer (RNFL) and ganglionar cell layer (GCL) thickness measured by SD-OCT were reduced in AD patients compared with age-matched healthy subjects. Furthermore, it has been reported that around 40-50% of patients with AD presented abnormalities in electrophysiological studies of the retina (187). In addition, a decrease of retinal thickness of the RNFL has been reported in patients with AD and even in patients with MCI (188). However, in these studies the diagnosis of diabetes was not considered and, therefore, to investigate whether diabetes is the main factor accounting for these findings is a challenge to be met. In addition, some studies using a specialized camera with micron-level imaging resolution have shown that beta-amyloid levels can be detected in the retina and significantly correlate with beta-amyloid PET imaging (189,190). Therefore, it seems reasonable to postulate that detecting neurodegeneration in the retina of diabetic subjects could be a biomarker for the presence of neurodegeneration in the brain.

On this basis, we designed a prospective, nested case-control study (1:1:1) to evaluate whether retinal sensitivity assessed by fundus-driven microperimetry could be a useful and reliable screening test to identify those patients with T2D who are in the early stages of cognitive impairment. Our results suggested that retinal sensitivity assessed by microperimetry was related to brain neurodegeneration and could be a useful biomarker for identifying patients with T2D who are at risk of developing AD (191).

Microperimetry also permits us to examine the capacity to maintain visual gaze on a single location (fixation), which has been reported as being hampered in AD and other neurodegenerative diseases (192–196). However, little is known regarding the potential usefulness of the assessment of gaze fixation for identifying T2D patients at risk of AD. This is an important issue because the neural circuits involved in gaze fixation are not the same as those participating in retinal sensitivity and, therefore, complementary information could be drawn. **One of the objectives of this thesis will be to evaluate if gaze fixation abnormalities assessed by fundus-driven microperimetry could also be a useful tool to identify T2D at risk of dementia**.

2. Objectives

General objective:

To investigate the relationship between retinal and brain neurodegeneration and its potential clinical and therapeutic implications.

Specific objectives:

Experimental study

Free hypothesis:

1. To identify pathogenic mediators of neurodegeneration and vascular leakage induced by glial activation in human retinas by means of a proteomic approach.

Driven hypothesis

2. To assess the role of endothelin-1 (ET-1) in the pathogenesis of neurodegeneration and early microvascular impairment in an experimental model of diabetes.

Clinical Study

3. To explore whether retinal fixation assessed by retinal microperimetry could be useful for identifying those diabetic patients with cognitive impairment.

3. Methods

Experimental study: free hypothesis

3.1 Proteomic analysis in human retinas and validation of selected candidates

3.1.1 Proteomic analysis

3.1.1.1 Tissue acquisition and immunohistochemistry

Institutional review board approval was obtained for this study from Vall d'Hebron University Hospital. This study adheres to all the tenets of the Declaration of Helsinki. Human retinas were obtained from the Blood and Tissue Bank of Vall d'Hebron University Hospital. The study comprised a total of 10 donors with diabetes (5 with glial activation; 5 without glial activation) and 5 donors without diabetes, matched by age and sex. The clinical characteristics of the donors are described in **Table 1**.

Table 1. *Clinical characteristics of non-diabetic and diabetic donors included in the proteomic analysis.*

	Non diabetic donors	**Diabetic donors with GA**	**Diabetic donors without GA**	P
Age (years)	71.8 ± 7.1	68.6 ± 8.8	67.2 ± 7.2	n.s
Gender (M/F)	1/4	2/3	1/4	n.s
Diabetes treatment	-			
· Diet only		0	1	
· Oral agents		3	3	
· Insulin		1	0	
· Insulin + oral agents		1	1	
Cause of death				
· Coronary disease	1	2	1	
· Brain hemorrhage	2	1	1	
·Respiratory insufficiency	1	2	2	
· Other	1	-	1	
Death to harvest (hours)	3.8 ± 0.83	3.6 ± 0.8	4.0 ± 1.0	n.s

The diabetic donors had no history of DR or microcirculatory abnormalities in the ophthalmoscopic examinations performed during the two years before death. One eye cup was harvested, and both the retinal pigment epithelium (RPE) and the neural retina were soaked in paraffin. Retinal sections (5 μm) were deparaffinised in xyol and rehydrated in graded ethanol. To eliminate autofluorescence due to lipofucsin, slides were washed in potassium permanganate. The other eye cup was harvested in order to separate the neural retina from the RPE, and samples of both tissues were immediately frozen with liquid nitrogen and stored at -80ºC within 6 hours of death as previously described (185).

3.1.1.2 Neurodegeneration measurements

Measurements of glial activation: GA was evaluated by fluorescence microscopy using specific antibodies against glial fibrillary acidic protein (GFAP) following the methodology described elsewhere (197). To evaluate the degree of glial activation (GA), we used a scoring system based on the extent of GFAP staining (198) that had been used previously by our group (197). This scoring system was as follows: Müller cell endfeet region/ganglion cell layer (GCL) only (score 1); Müller cell endfeet region/GCL plus a few proximal processes (score 2); Müller cell endfeet plus many processes, but not extending to Outer nuclear layer (ONL) (score 3); Müller cell endfeet plus processes throughout with some in the ONL (score 4); Müller cell endfeet plus lots of dark processes from GCL to outer margin of ONL (score 5).

Immunohistochemical analysis for apoptosis assessment: Apoptosis was evaluated by TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) using the DeadEnd Fluorometric TUNEL System kit (PROMEGA, Madison, WI, USA). Cryosections of retina were permeabilized by incubation for 2 min on ice with 0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared. The secondary antibody was Alexa 488 goat-anti-rabbit (Invitrogen, San Diego, CA, USA).The number of TUNEL positive cells (green) was recorded in 3 fields of each retina that covered 212x212 μm. Images were acquired with a Lase Scanning Confocal (LSC) confocal laser scanning microscope (FV1000, Olympus. Hamburg, Germany). For evaluation by LSC the excitation wavelength was 488 nm and detection in the range of 515–565 nm (green) was used.

3.1.1.3 Proteomic analysis

Retinal lyses: Protein extracts from the whole neural retina were prepared by homogenization with radio-immunoprecipitation assay (RIPA) buffer (R0278; Sigma Aldrich Quimica S.A.) and 20mM NaF, 2mM Na3VO4, 2 mM PMSF. Extracts were cleared by microcentrifugation at 10000g for 10 minutes at 4ºC. The supernatant was aliquoted and stored at -80ºC.

Sample Preparation: A total of 60 μg protein (pooled from n=5 per group) was subjected to SDS-PAGE using 4% to 20% gel. Following electrophoresis, the gel was stained and each sample lane was cut into 16 fragments (1x3mm pieces). The fragments were placed in Eppendorf tubes and digested with trypsin using the following method. Briefly, fragments were distained with 8mg/mL (100mM) ammonium bicarbonate (NH_4HCO_3) in 50% acetonitrile (ACN) at pH=8.5. The samples were reduced with tris (2-carboxyethyl)phosphine (30mg/mL) in H_2O, incubated at 4°C for 2h, then alkylated with iodoacetamide (IAA; 18mg/ml (100mM) in 8mg/mL NH_4HCO_3, freshly made and kept in the dark) at room temperature for 30min. The alkylating buffer was removed and the samples were washed with distaining buffer. The gel fragments were dehydrated using ACN. Samples were digested with trypsin (10ng/μl in 8mg/mL NH_4HCO_3) at 37°C overnight. The tryptic peptides were transferred to fresh tubes. Additional peptides were extracted using 0.1% formic acid in 50% ACN and added to the original fractions. The samples were then filtered (NanoSep MF GHP 0.45μm) and concentrated prior to mass spectrometry (MS) analysis.

Nano-LC MS^2 was performed to detect peptides; 1 mL samples were loaded onto an Acclaim PepMap100 trapping column (100 um × 2 cm, C18, 5 μm, 100 Å, Thermo) at a flow rate of 20 μL/min using 4% aqueous ACN, and 0.1% formic acid as a mobile phase. The peptides were separated on an Acclaim PepMap RSLC column (75 μm × 15 cm, C18, 2 μm, 100 Å, Thermo) with a 90-min 4% - 40% linear gradient of acetonitrile in water containing 0.1% formic acid. The gradient was delivered by a Dionex Ultimate 3000 nano-LC system (Thermo) at 300nL/min. An LTQ OrbitrapVelos mass spectrometer (Thermo) was set to acquire data using the following data-dependent parameters: full FT MS scan at R 60,000 followed by 10 ion-trap MS^2 scans on most intense precursors with CID activation. Only the precursors with charge states +2 and higher were selected for MS^2; monoisotopic precursor selection was enabled, and the isolation window was 3 *m/z*. Dynamic exclusion duration was 15sec, repeated once over 30sec repeat duration.

Data analysis: The mass spectra were processed using Proteome Discoverer 1.3 (Thermo). The MS^2 data were analyzed with Sequest (Thermo, version 1.3.0.339) set up to search a database combining the current human_contam_fasta proteome, up1.4.0.288, Uniprot, and the common contaminants sequences, a total of 49,426 entries. The following search parameters were used: precursor tolerance 20ppm, fragment tolerance 0.8Da, dynamic modifications were Oxidation (+15.995Da, Met) and Deamidation (+0.984Da, Asn, Gln), static modification was Carbamidomethyl (+57.021Da, Cys). The resultant Sequest files were parsed into Scaffold (version 4.6.1) for validation filtering and the generation of non-redundant proteins. Uniprot was used to remove any obsolete protein identifications. A count value of one was added to each spectral count observation.

For each protein, 2-fold changes were considered differentially abundant (diabetic retinas without glial activation vs. non-diabetic control retinas, and diabetic retinas with glial activation vs. non-diabetic control retinas).

Pathway and Gene Ontology Analysis: Pathway enrichment analysis was performed using the Ingenuity Pathway Analysis (IPA) bioinformatics platform (provided in the public domain at www.Qiagen.com). For each of the three individual sample sets, identification of significantly represented pathways was performed using a −Log *P* value for each of the identified pathways. The significance value for each pathway was calculated using the right-tailed Fisher's exact test. The *P* values were corrected using the Benjamini-Hochberg (B-H) correction to control for the false discovery rate. A significance value cutoff of B-H *P* value ≤ 0.001 was selected to identify shared and unique pathways within each sample set. To evaluate the association of the identified proteins with neurological signaling, significant signaling pathways belonging to the category "Neurotransmitters and Other Nervous System Signaling" were selected for analysis.

Pathway activation analysis was performed using the comparison Analysis function on IPA. Briefly, a count value of one was added to each spectral count observation. For each protein, 2-fold changes (FCs) were computed (D/C and D+GFAP/C). The $Log_2(FC)$ was calculated for each protein within each of the two comparison groups. This dataset was uploaded to IPA, and only those proteins having a FC ≤ 0.5 or ≥ 1.5 were included in the final analysis.

3.1.2 Validation of selected candidates from the proteomic approach

3.1.2.1 Western blot analysis in human retinas

In order to validate the results obtained by proteomics, Western blot analyses in a second set of retinas was performed. For this purpose we also used human postmortem eyes obtained from non-diabetic (n=6) and diabetic donors (n=6) without visible microangiopathic lesions in the ophthalmological examinations performed during the preceding 2 years. Both groups were selected from our eye bank and were closely matched by age. The demographic characteristics are shown in **Table 2**. The eye cups were processed as previously described (185). In all cases glial activation was confirmed by GFAP immunofluorescence as reported elsewhere (198). The targets, dilution and primary antibodies used in western blot analyses are detailed in **Table 3.** For densitometric analysis of western blots, Image J software was used. Results were normalized to β-actin.

Table 2. *Clinical characteristics of non-diabetic and diabetic donors included in the validation study (inflammatory mediators).*

	Non diabetic donors	**Diabetic donors**	p
Age (years)	71.8 ± 8	72.3 ± 3	n.s
Gender (M/F)	3/3	3/3	n.s
Diabetes treatment	-		
· Diet only		0	
· Oral agents		3	
· Insulin		2	
· Insulin + oral agents		1	
Cause of death			
· Coronary disease	2	3	
· Respiratory insufficiency	2	2	
· Other	2	1	
Death to harvest (hours)	3.9 ± 0.9	4.1 ± 1.1	n.s

Table 3. *Targets, dilution, and sources of applied primary antibodies in western blot.*

Target Molecule	**Clone**	**Dilution**	**Source**
Complement C3	Goat	1/5000	Calbiochem#204869
Complement C9	Rabbit	1/3000	Serotec#AHC013
Clusterin α	Goat	1/1000	Santa Cruz Biotech#6420
Factor H	Goat	1/1000	R&D#AF4779
Complement C4b	Rabbit	1/2000	Abcam#181241
CD55	Rabbit	1/1000	Abcam#133684
CD59	Rabbit	1/200	Abcam#69084
CD46	Mouse	1/200	Santa Cruz Biotech#166159
C1NH	Mouse	1/1000	Santa Cruz Biotech#166159
Factor B	Rabbit	1/5000	QUIDEL#A-311
β-Actin	Mouse	1/60000	SIGMA#MA4792

3.1.2.2 In db/db mice

A total of 4 male db/db (BKS.Cg-Dock7m +/+ Leprdb/J) mice and 4 db/+ (Charles River Laboratories, Calco, Italy) aged 14 weeks were analyzed. This mouse carries a mutation in the leptin receptor gene and is a model for obesity-induced type 2 diabetes.

The mice were anesthetized (1 ml ketamine/0.3 ml xylazine) and transcardially perfused (p-formaldehyde 4%) before cervical dislocation. The eyes were immediately enucleated. This study was approved by the Animal Care and Use Committee of VHIR (Vall d'Hebron Research Institute).

Western blotting: A lysis buffer was prepared (RIPA buffer: phenylmethanesulfonylfluoride [PMSF], 1 mmol/l; Na3VO4, 2 mmol/l; NaF, 100 mmol/l) with a 1X protease inhibitor cocktail (Sigma, St Louis, MO, USA). Then, proteins were extracted from the neuroretinas in 80 µl of the buffer. A total of 20 µg protein was resolved by 8% and 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories). The primary antibodies mouse monoclonal anti-Ezrin (1:1000; ab4069; Abcam), rabbit monoclonal anti-Radixin (1:1000; ab52495; Abcam) and rabbit monoclonal anti-Moesin (1:1000; ab52490; Abcam) were incubated overnight at 4ºC, and on the following day, the suitable HRP secondary antibody anti-rabbit or anti-mouse (Dako Agilent, Santa Clara, CA, United States) was incubated for 1 hour at room temperature. Anti-cyclophilin A (1:10000; BML-SA296; Enzo) was used to normalize protein levels. Densitometric analysis of the autoradiographs was performed with Image J software.

Immunohistochemical analysis: Five sections of eyes were obtained per animal. Paraffined sections were deparaffinized in xylene and rehydrated in ethanol. Sections were fixed in acid methanol (-20 °C) for 1 minute and washed with 0.01M 4 phosphate buffered saline (PBS) at pH 7.4. Then, sections were incubated in blocking solution Protein Block Serum-Free Code X0909 (Dako North America Inc, CA, United States) for 1 h at room temperature and afterwards, they were incubated overnight at 4ºC with specific primary antibodies: mouse monoclonal to anti-Ezrin (1:50; ab4069; Abcam), rabbit monoclonal anti-Radixin (1:50; ab52495; Abcam), rabbit monoclonal anti-Moesin (1:50; ab52490; Abcam) and rabbit polyclonal anti- Anti-Collagen IV (1:400; ab6586; Abcam). The following day, after washing, sections were incubated with a fluorescent ALEXA 594 Donkey anti-Mouse (ab150108; Abcam) and 488 Goat anti-Rabbit preadsorbed (ab150081; Abcam) as a secondary antibody in blocking solution for 1 h, washed, nuclei were stained with Hoechst and mounted in Mounting Medium Fluorescence (Prolong, Invitrogen) with a coverslip. Images were acquired with a confocal laser scanning microscope (FV1000; Olympus, Hamburg, Germany). Five fields (three corresponding to the central and two to the peripheral retina) from each section were analyzed.

Experimental study: driven hypothesis

3.2 Assessing the role of endothelin-1 in early diabetic retinopathy

3.2.1 Human retinas

Human retinas from non-diabetic (n = 12) and diabetic (n = 12) were obtained selected from our Eye Bank (Vall d'Hebron Research Institute) as described in 3.1.1.1 section. The diabetic donors were free of funduscopic abnormalities or with mild DR in the ophthalmological examinations performed during the preceding 2 years, and were age-matched with the non-diabetic donors (68 ± 8 vs. 69 ± 7 years).

Reverse transcription polymerase chain reactions were carried out from the cDNA of each condition using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) using the 7,900 HT Sequence Detection System in 384-well optical plates with specific primers for ET-1, hETA, hETB and β-actin (**Table 4**). For each sample, qPCR was performed in duplicate and relative quantities were calculated using ABI SDS 2.0 RQ software (Applied Biosystems, Madrid, Spain) and the $2^{-\Delta\Delta Ct}$ analysis method with human β-actin as the endogenous control (primer in **Table 4**).

Table 4. *Primers used for PCR in human retinas.*

	Forward	**Reverse**
ET-1	5′-TCGTCCCTGATGGATAAAGA-3′	5'-GGCAAAAATTCCAGCACTTC-3'
hETA	5'-AAGGAATGGGAGCTTGAGAA-3'	5'-CAGAGGCATGACTGGAACA-3'
hETB	5'-TTTGCCTGGTCCTTGTCTTT-3'	5'-AAGCACGACTGCTTTTCCTC-3'
β-actin	5'-AGGCCAACCGCGAGAAGATGACC-3'	5'-GAAGTCCAGGGCGACGTAGCAC-3'

3.2.2 Animals

The neuroprotective effect of eye-drops containing bosentan was tested in the db/db mouse model. A total of 12 male db/db (BKS.Cg- + Lepr db/+ Lepr db/OlaHsd) mice aged 10 weeks were purchased from Charles River Laboratories. In addition, 6 non-diabetic (db/+) mice matched by age were used as the control group.

This study was approved by the Animal Care and Use Committee of VHIR (Vall d'Hebron Research Institute). All the experiments were performed in accordance with the tenets of the European Community (86/609/CEE) and ARVO (Association for Research in Vision and Ophthalmology).

3.2.3 Study design

The study design is summarized in **Fig. 10**. Bosentan or vehicle eye drops were administered directly onto the superior corneal surface of each eye using a syringe. One drop (5 µL) of bosentan

(0.5%) or vehicle was administered twice daily for 15 days. On day 15, mice were euthanized by cervical dislocation and the eyes would be immediately enucleated.

The pharmacokinetic profile of bosentan was studied in 35 pigmented HY79b female rabbits.

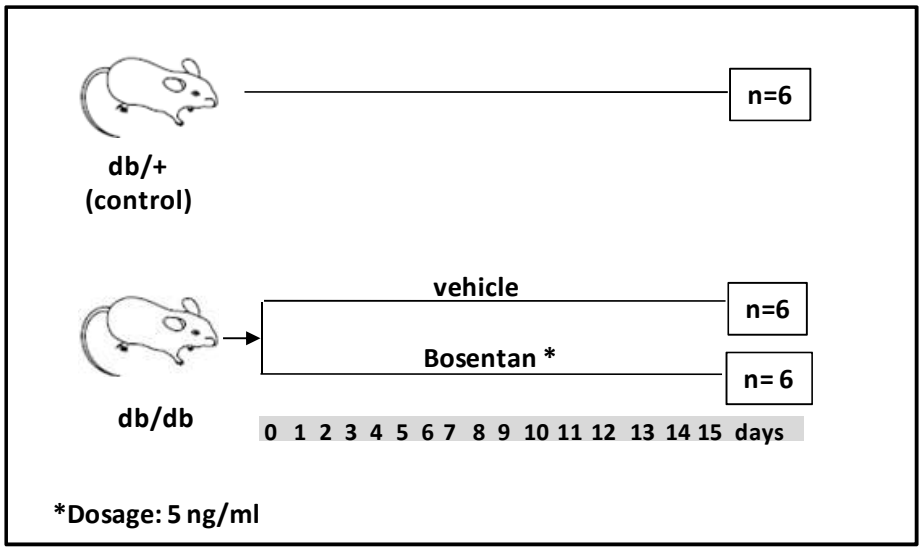


***Figure 10**. Study design of the experimental study using topical administration of bosentan*

3.2.4 Immunohistochemistry for endothelin (ET-1) and endothelin receptors (ETA-R and ETB-R)

All eyes were fixed in 4% paraformaldehyde after enucleation. Immunohistochemical studies were done on 4 µm-thick paraffined sections of the eyes thaw-mounted onto poly-L-Lysine treated slides and heated in an oven at 65 °C for 1 h to promote adherence to the slide. These sections were fixed in methanol/acid, rehydrated and washed in 0.01-M PBS and incubated in blocker (5% BSA, and 5% goat serum in PBS) for 1 h at room temperature. Then, they were incubated overnight at 4 °C with a rabbit anti-ET-1 (Ab 117757) diluted 1:1000 (prepared in 1% BSA in PBS); rabbit anti-ETA (Ab 117521) (Abcam Ltd., Cambridge, UK) and rabbit anti-ET-B (Millipore 3284) at 1:500 dilution (prepared in 1% BSA in PBS). After three washes in PBS, the sections were incubated with secondary antibody Alexa Fluor 594 conjugate for ET-1 or Alexa Fluor 488 conjugate for ETA and ETB (Molecular Probes-Life Technologies, Grand Island, USA). The sections were washed three times in PBS, counterstained with Hoestch and mounted with Mounting Medium Fluorescence (Prolong, Invitrogen) with a coverslip.

The immunofluorescence was quantified using laser confocal microscopy (Fluoview FV 1000 Laser Scanning Confocal Microscope Olympus; Olympus, Hamburg, Germany) and ImageJ software.

3.2.5 Neurodegeneration measurements and other immunohistochemical analyses

Measurements of glial activation: Glial activation was evaluated by Laser Scanning Confocal microscopy using specific antibodies against GFAP following the same methodology as in section 3.1.1.2 that has been described elsewhere (197). To evaluate the degree of glial activation, we used the same scoring system described in section 3.1.1.2.

Immunohistochemical analysis for apoptosis assessment: The same procedure described in section 3.1.1.2 was used.

Other immunohistochemical analysis: In order to clearly differentiate the ganglion cells from astrocytes within the GCL, an immunostaining for neuronal-specific nuclear protein (NeuN) was performed. Neuronal-specific nuclear protein was evaluated by immunofluorescence using a specific monoclonal antibody (anti-NeuN; ab104224; Abcam; Cambridge, UK).

We analyzed the PKC β protein, an important mediator of diabetic complications, which regulates ET-1 in the retinal vascular cells (ab189782; Abcam; Cambridge, UK). In addition, we evaluated the protein expression of TNF-α (ab8348; Abcam). Blood vessels were immunostained with collagen IV (ab6586; Abcam). For all the immunofluorescence analyses, after incubation with the secondary antibodies, the sections were counterstained with Hoetchst and mounted with Mounting Medium Fluorescence (Prolong, Invitrogen) with a coverslip. Comparative digital images from samples were recorded with a Fluoview FV 1000 Laser Scanning Confocal Microscope Olympus using identical brightness and contrast settings.

3.2.6 Pharmacokinetic analyses

A pharmacokinetic analysis after a single topical instillation of bosentan (0.5%) was performed. For this purpose, a single instillation in the right eye was administered to 35 female pigmented HY79b rabbits that were randomly distributed as summarized in **Table 5**. Bosentan concentration in plasma, aqueous humor and retina was measured using HPLC with MS/MS detection. This method was validated with respect to selectivity, linearity, precision, accuracy, matrix effect, extraction efficiency and dilution integrity. The pharmacokinetic parameters calculated included maximum observed concentration and time to maximum concentration.

Table 5. *Design of the pharmacokinetic study.*

Groups	Dose Regimen	Time-Points Post Dose	Animal Number
1	Single 50µL instillation in right eye	0.25 h	5
2		0.5 h	5
3		1 h	5
4		2 h	5
5		4 h	5
6		8 h	5
7		24 h	5

3.2.7 In vitro studies in Human Retinal Endothelial Cells

Human retinal endothelial cell (HREC) cultures were obtained from a vial of cryopreserved cells purchased from Innoprot (Biscay, Spain). Human retinal endothelial cells were thawed in the laboratory and cultured in endothelial basal medium (EBM) containing 5.5 mM D-glucose, 5% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin and ECGS supplement (Innoprot, Biscay, Spain). Human fibronectin (MerckMillipore, Madrid, Spain) was used at 5 µg/mL for cell attachment. The endothelial medium was changed every 3 days. Human retinal endothelial cells from passage 3 were used for the experiments and grown up to confluence. For cell stimulation, culture media were changed to a medium supplemented with 1% FBS. Cells were treated with TNF-α (10 ng/mL) (Miltenyi, Madrid, Spain) and bosentan (30 µM) for the final 24 h of the experiment.

3.2.8 Measurement of HREC Permeability

Permeability in HREC monolayers was obtained on permeable supports at 1,2 × 105 cells/well, PCF filters (MerkMillipore, Madrid, Spain). Inserts were incubated for 48 h at 37 °C in 5% CO_2-air to form the monolayer. Then, the medium was serum depleted on the upper side for 14 h before the treatments. Bosentan in PBS (30 µM) was include 1 h before TNF-α. Changes in the permeability were detected by changes in the fluorescence on the basal side of the insert. A total of 100 µg/mL of fluorescent FITC-Dextran (70 KDa) (Sigma, Madrid, Spain) was added to the upper side of the insert. Aliquots of 200 µL from the basal compartment were read in a SpectraMax Gemini (Molecular Devices, Sunnyvale, CA). Dextran data was obtained by extrapolation in a standard curve. Each condition was tested 3 times.

3.2.9 RNA extraction and quantitative real-time PCR

Human retinal endothelial cell RNA was extracted with Nzyol (Nzytech, Lisbon, Portugal). Reverse transcription polymerase chain reactions were carried out using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) using the 7,900 HT Sequence Detection System in 384-well optical plates with specific primers for human VEGF: 5'-TGCATTCACATTTGTTGTGCTGTAG-3' and 5'-GCAGATTATGCGGATCAAACC-3'. The primers for human ET-1 and human β-actin are detailed above. For each sample, qPCRs were performed in triplicate and relative quantities were calculated using ABI SDS 2.0 RQ software and the $2^{-\Delta\Delta Ct}$ analysis method with human β-actin as the endogenous control.

Clinical study

3.3 Exploring retinal fixation assessed by retinal microperimetry and its association with cognitive impairment

Data regarding fixation parameters obtained from the nested case-control study DIALRET (ClinicalTrial.gov: NCT02360527) were analyzed. A total of 33 patients with AD, 33 with MCI and 34 with normal cognition in whom a full report of fixation parameters were available were included in the study. These groups were well-matched by age, gender, BMI, cardiovascular risk factors and HbA1c. All these patients were selected from 214 consecutive type 2 diabetic patients attending a memory clinic (Fundació ACE, Barcelona, Spain). The study was conducted according to the Declaration of Helsinki and was approved by the local Ethics Committee.

The main inclusion criteria were: a) Age >65 years; b) Type 2 diabetes with a duration >5 years; c) Written informed consent which included accepting participation in brain MRI and PET, as well as a potential lumbar puncture; d) No apparent or mild non-proliferative DR according to the International Clinical Diabetic Retinopathy Disease Severity Scale (199). The exclusion of patients with more advanced DR was based on the fact that severe microvascular impairment could participate in neurodegeneration and the main aim of our study was to assess whether neurodegeneration of the brain and the retina runs in parallel, independently of the presence of overt microangiopathy.

The main exclusion criteria were: a) Patients with other neurodegenerative diseases of the brain or retina (i.e. glaucoma) or cerebrovascular diseases (Fazekas scale score $\leq$ 1) (200); b) A1c >10% (86 mmol/mol). The exclusion of patients with poor control was because very high blood glucose levels could affect retinal function (201). All patients underwent complete neuropsychological, neurological and psychiatric evaluations as previously described (202) including MMSE and ADAS-Cog. In addition, a biochemical analysis including HbA1c and a lipid profile was performed.

Retinal sensitivity was evaluated by fundus driven microperimetry (MAIA 3rd generation) as described elsewhere (191). For the evaluation of fixation, the MAIA microperimeter uses high-speed eye trackers (25 Hz) for the control of fixation losses and to register the fixation pattern. All fixation positions during the examination are used by the instrument to calculate the fixation indexes P1 and P2, which represent the percentage of fixation points inside a circle of 2 and 4 degrees of diameter, respectively. Subjects were classified as having stable fixation if P1 was more than 75%, relatively unstable fixation if P1 was less than 75% and P2 more than 75%, and unstable fixation if both P1 and P2 were below 75%. Microperimetry also provides a more accurate estimation of the fixation pattern using the bivariate contour ellipse area (BCEA). This area is calculated as an ellipse that covers fixation eye positions and takes into account 1 or 2 times the standard deviation, including consequently 63% (BCEA63) and 95% (BCEA95) of the fixation points. The area of this ellipse is calculated through major and minor axes, which are 2 orthogonal diameters, describing the extent of the fixation points (horizontal and vertical diameters) (203). For retinal sensitivity and fixation, data corresponding to the right eye was used, which was the first eye explored in all subjects.

3.4 Statistical analysis

For the proteomic study, the statistical analysis is described in section **3.1.1.3.**

Regarding the validation of selected candidates from the proteomic analysis, the assessment of the role of endothelin-1 and the effects of bosentan, data are presented as mean ± SD. Comparisons of continuous variables were performed using ANOVA and Student's T test. Comparisons of categorical variables were performed using the Fisher test. Levels of statistical significance were set at $p < 0.05$

In the clinical study, the Chi-square test for qualitative variables and ANOVA followed by DMS post-hoc tests for quantitative variables were used for assessing differences between groups. To evaluate the correlation between BCEA63 or BCEA95 and ADAS-Cog or MMSE, Spearman's correlation test and regression analysis were performed. Significance was accepted at the level of $p<0.05$. The Bonferroni correction was used for multiple comparisons. Logistic regression was used to predict the ROC curves and the Chi-squared test for ROC area comparison. Statistical analyses were performed with Stata statistical package.

4. Results

Experimental study: free hypothesis

4.1 Pathogenic mediators of neurodegeneration and vascular leakage induced by glial activation in human retinas by means of a proteomic approach

4.1.1 General overview of the proteome

The results of the general overview of the proteome have been already published elsewhere (185) and summarized in this section.

Similarity among groups was assessed by percent homology for each study group. **Figure 10** shows that 1414, 1487, and 1080 proteins were identified retinas in the C, D, and D+GFAP groups, respectively. Homology among all three sample groups encompassed 37% (*n* = 736) of the total assigned proteins. Between-group homology was greatest between groups C and D with an 18.1% similarity (*n* = 360), whereas the lowest was between groups C and D+GFAP, with only a 3.6% similarity (*n* = 72) between assigned protein IDs. The number of unique proteins expressed in each group was statistically similar (C = 246, D = 303, and D+GFAP = 184) as were the number of co-expressed proteins between any two groups (C and D = 360; C and D+GFAP = 72, D and D+GFAP = 88). The different profiles of the up and down-regulated proteins of the groups are represented in **Fig. 11**.

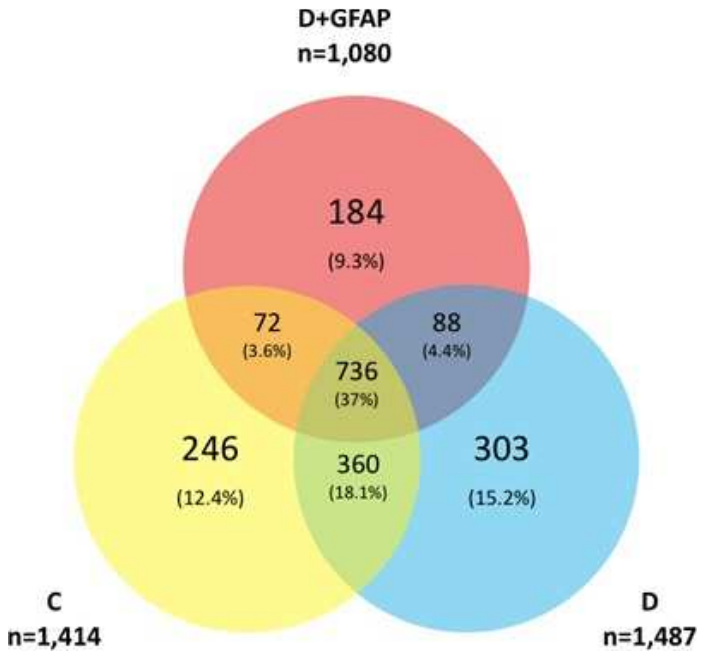


Figure 10. *Protein profile comparison. A total of 1414, 1487, and 1080 proteins were identified from retinas in the C, D, and D+GFAP groups, respectively. A common core of 736 proteins was identified. Number of unique proteins: C = 246, D = 303, and D+GFAP = 184. Groups C and D shared 360 expressed proteins, Groups C and D+GFAP shared 72, while groups D and D+GFAP shared 88.*

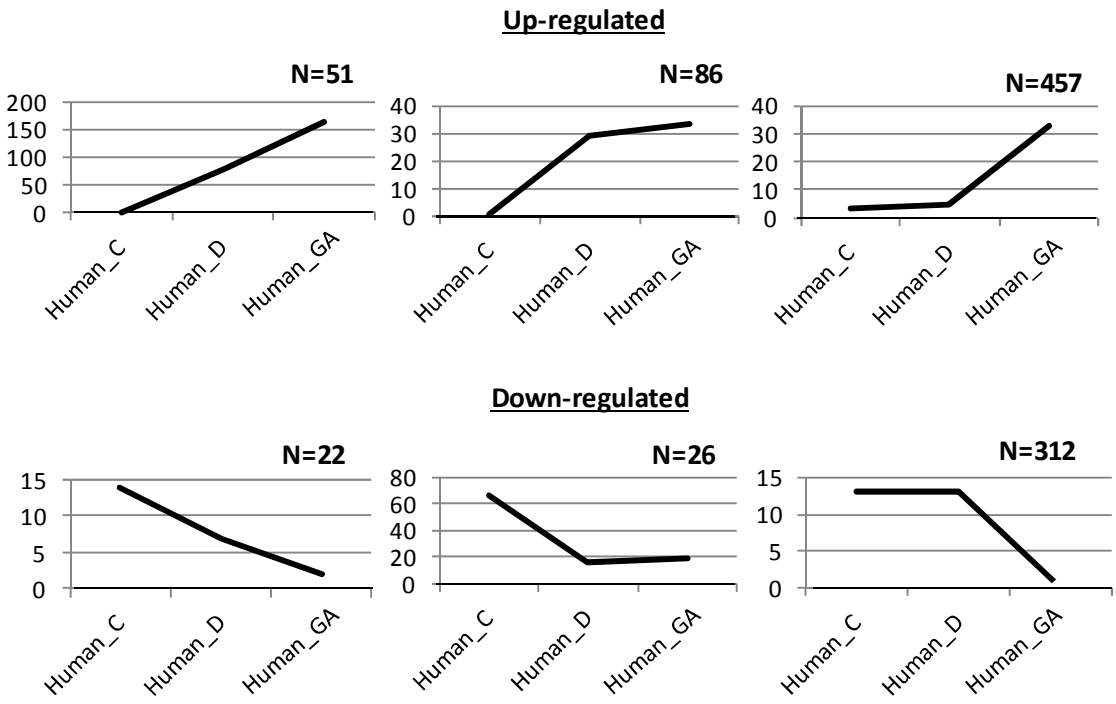


Figure 11. *There were 51 proteins that were upregulated in D+GFAP (or GA) > D > C, 86 that were upregulated in both diabetic samples compared to controls (C<D=GA) and 457 that were upregulated in D+GFAP compared with C and D (C=D<DA). On the other hand, 22 proteins were downregulated in the diabetic donors, especially the ones with glial activation (C>D>GA), 26 proteins that were downregulated in both diabetic samples (C>D=GA) and 312 proteins that were downregulated in GA compared with both D and C (C=D>GA).*

It should be noted, that the proteome profile revealed, not only the hyperexpression of GFAP (which was expected) but also an increase in the presence of albumin in the retinas with GA (**Table 5**). The increase in albumin indicates the presence of vascular leakage, one of the early signs of vascular impairment.

Table 5. *Spectral counts of GFAP and albumin in control retinas, diabetic retinas without glial activation and diabetic retinas with glial activation.*

	Controls	**Diabetes without GA**	**Diabetes with GA**	p=0.01
GFAP	123	240	528	C<D<DN
Albumin	83	89	231	C=D<DN

4.1.2 Identification of mediators of neurodegenerative brain diseases

A total of 35 signaling pathways related to "Neurotransmitters and Other Nervous System Signaling" are represented in a single group (non-diabetic donors [C], diabetic donors without GA [D], and diabetic donors with GA [D+GFAP]) were identified and they are summarized in **Fig. 13**.

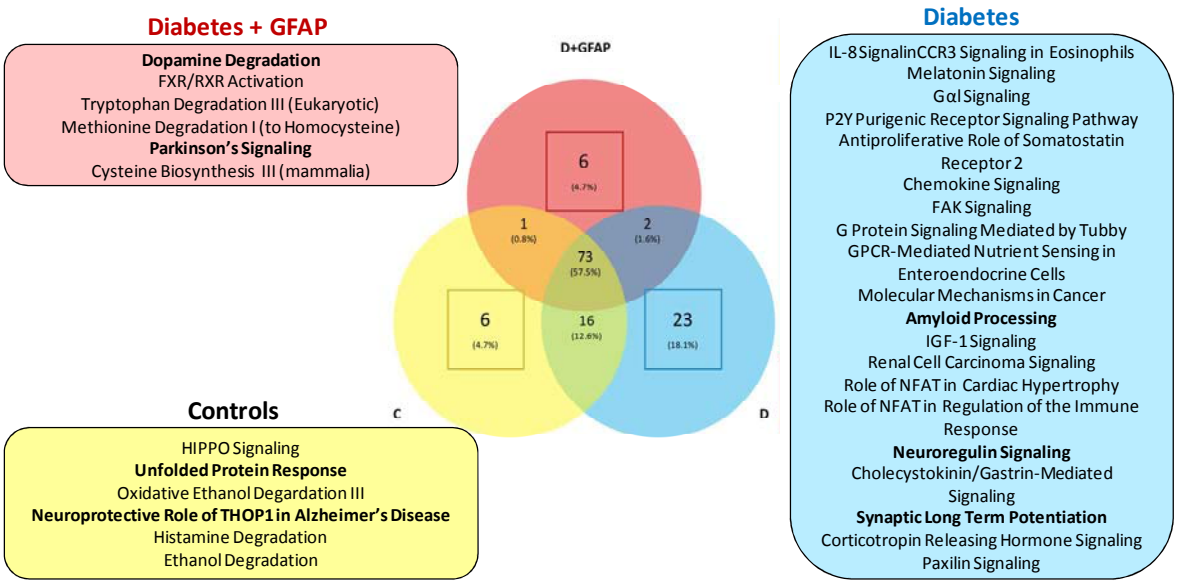


Figure 12. *Identified pathways belonging to the category "Neurotransmitters and Other Nervous System Signaling".*

The most relevant pathways were the following:

Samples from non-diabetic donors:

- Neuroprotective Role of THOP1 in Alzheimer's disease: THOP1 (Thimet oligopeptidase) mediates a compensatory neuronal response to increased Aβ in the brain, one of the central events in AD (204).

- Unfolded Protein Response (UPR): Impairment of the UPR has been involved not only in the accumulation of misfolded proteins in the brain of many neurodegenerative diseases (205), but also DR (206,207).

Samples from diabetic donors without glial activation

- Neuregulin signaling: The neuregulin family of ligands (NRGs) is important in synaptogenesis and neuronal survival. NRGs exhibit a neuroprotective role under inflammatory conditions by acting in concert with the cholinergic anti-inflammatory pathway (208).

- Synaptic long term potentiation: Synaptic long term potentiation (LTP) is a variation of synaptic transmission that functions in learning memory and has been involved in synaptic plasticity in the hippocampus and cerebellum (209–212).

- Amyloid processing: In AD, Aβ toxicity mediated by disruption of calcium homeostasis in neurons along with oxidative stress leading to increased MAP kinase activity, ultimately result in increased phosphorylation of Tau (213,214). Hyperphosphorylated Tau then is incorporated into the neurofibrillary tangles seen in AD. Amyloid processing incorporates these events, which are important precipitants of neurodegeneration in AD.

Samples from diabetic donors with glial activation

· Dopamine degradation and Parkinson's signaling: Dopamine is released by a subclass of amacrine cells in the retina and its involved in the diverse physiologic aspects of retinal neuromodulation, mediation of light responsiveness and clock gene regulation (215,216).

These findings will be commented on in the discussion section.

4.1.3 Proteins involved in the Axon Initial Segment (AIS) and axonal transport

The axon initial segment (AIS) is essential for neurotransmission (217). For this reason we wanted to analyze the proteins related to AIS and axonal transport. The results obtained in each group of samples are shown in **Table 6**.

Several proteins involved in axonal transport such as dynein-dynactin complex were downregulated in diabetic retinas with GA, whereas the kinesin group was upregulated in the retinas with glial activation. In addition, all cytoskeleton proteins belonging to axon initial segment (ankyrin, spectrin, neural cell adhesion molecule, neurofascin) were downregulated in the retinas from diabetic donors with GA.

4.1.4 Mediators of vascular leakage

Two main groups of proteins involved in vascular leakage have been identified: inflammatory mediators and proteins from the ERM complex.

4.1.4.1 Inflammatory mediators

The differential abundance of several inflammatory mediators among groups is displayed in **Table 7**.

ICAM-1, DDRGK domain-containing protein and chitinase-3-like protein 1 are incremented in retinas with glial activation. On the other hand, the bifunctional glutamate/proline tRNA ligase is decreased in retinas with glial activation. Regarding the complement system, an increase in C4b, as well as the complement inhibitors CD59 and isoform 4 of clusterin were found. In addition, a

downregulation of clusterin was observed. In addition, a downregulation of two essential inhibitory factors of the complement system, complement factor H and C1 inhibitor was detected.

4.1.4.1.1 Validation study by western blot analysis

Given the potential therapeutic implications and our previous background on study of the complement system in the vitreous fluid from patients with PDR (138), we first validated the proteins involved in the complement system. In order to facilitate interpretation of the results, the major findings are summarized in **Fig. 13**.

Table 6. *Spectral counts of proteins involved in axonal transport and structural proteins of the axon initial segment.*

			Controls	**Diabetes without GA**	**Diabetes with GA**	**p<0.01**
Proteins involved in the Axonal Transport	**Dynein**	Cytoplasmic dynein 1 heavy chain 1	10	14	5	**C=D>GA**
		Cytoplasmic dynein 1 light intermediate chain 1	13	14	5	**C=D>GA**
		Dynein light chain 2	33	39	19	**C=D>GA**
	Dynactin	Dynactin subunit 2	4	6	2	**C=D>GA**
		Isoform 6 of dynactin subunit 1	52	60	29	**C=D>GA**
	Kinesin	Kinesin light chain 1	1	2	14	**C=D<GA**
		Isoform 3 of kinesin light chain 4	5	2	22	**C=D<GA**
		Kinesin-like protein KIF3A	1	2	14	**C=D<GA**
Proteins from the Axon Initial Segment	**Ankyrin**	Isoform Br21 of Ankyrin 1	7	11	0	**C=D>GA**
		Ankyrin-2	117	122	45	**C=D>GA**
		Ankyrin-3	40	42	5	**C=D>GA**
	Spectrin	Isoform 2 of spectrin alpha chain	1064	1071	538	**C=D>GA**
		Spectrin β chain, non erythrocytic 2	108	128	33	**C=D>GA**
	Neural cell adhesion molecule	Neural adhesion molecule L1	16	21	2	**C=D>GA**
		Neural adhesion molecule 1	240	286	151	**C=D>GA**
	Neurofascin		11	7	5	**C=D>GA**

Table 7. *Spectral counts of proteins involved in inflammation.*

		Controls	Diabetes without GA	Diabetes with GA	p<0.01
ICAM-1		5	22	94	C<D<GA
Complement	C4-B	22	30	75	C=D<GA
	Complement factor H	8	2	0	C>D>GA
	CD59	6	7	43	C=D<GA
	C3	94	49	55	C>D=GA
	C1 inhibitor	15	2	7	C>D<GA
	Isoform 4 of clusterin	36	20	108	C=D<GA
Leukotriene biosynthesis	Leukotriene A-4 hydrolase	39	37	72	C=D<GA
	Coactosin-like protein 1	6	3	15	C=D<GA
	Prostaglandin reductase 1	10	18	2	C=D>GA
DDRGK domain-containing protein		3	5	10	C=D<GA
Chitinase-3-like protein 1		0	0	9	C=D<GA
Bifunctional glutamate/proline tRNA ligase		46	44	9	C=D>GA

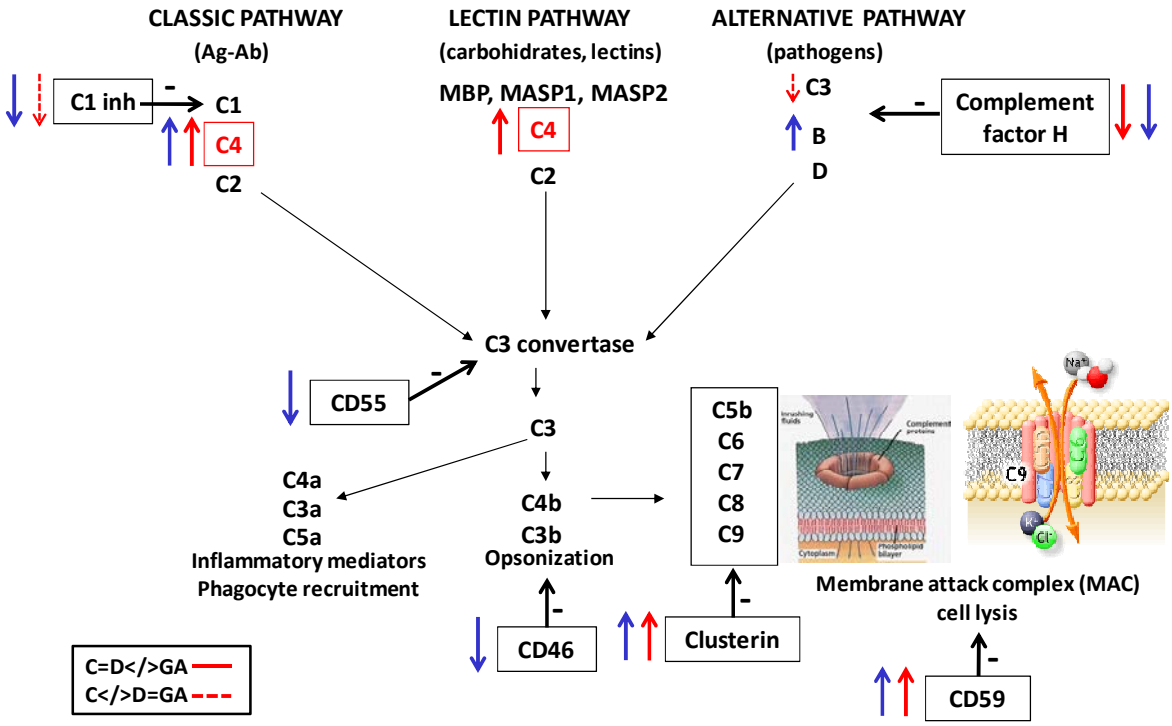


Figure 13. *Schematic description of the complement system. The complement cascade can be activated by three pathways (classic, lectin and alternative pathways). Activation leads to the activation of C3, which results in the formation of the membrane attack complex (MAC) and mediators of inflammation. In red, the changes observed in the proteomic analysis and in blue, the results from the western blot analysis are displayed.*

A significant downregulation of C1INH with an increase of C4 was observed in diabetic retinas with glial activation in comparison with non-diabetic control retinas (**Fig. 14**), thus confirming the results obtained by proteomic analysis. These findings suggest that activation of the classical pathway induced by the downregulation of C1INH exists in the early stages of DR. In addition, a significant downregulation of CFH along with an increase of Factor B was observed in diabetic retinas (**Fig. 15 A-B**), thus suggesting the activation of the alternative pathway.

As observed in the proteomic analysis, we found no significant difference in C3 expression between non-diabetic and diabetic retinas with glial activation. However, an increase, although non-significant, in several small bands corresponding to C3 fragments was detected in retinas from diabetic patients, suggesting C3 activation **(Fig. 16A)**. We also observed significant decreased levels of two additional complement inhibitors: CD55 and CD46 from the alternative pathway (**Fig. 16B-C**), which could contribute to complement activation.

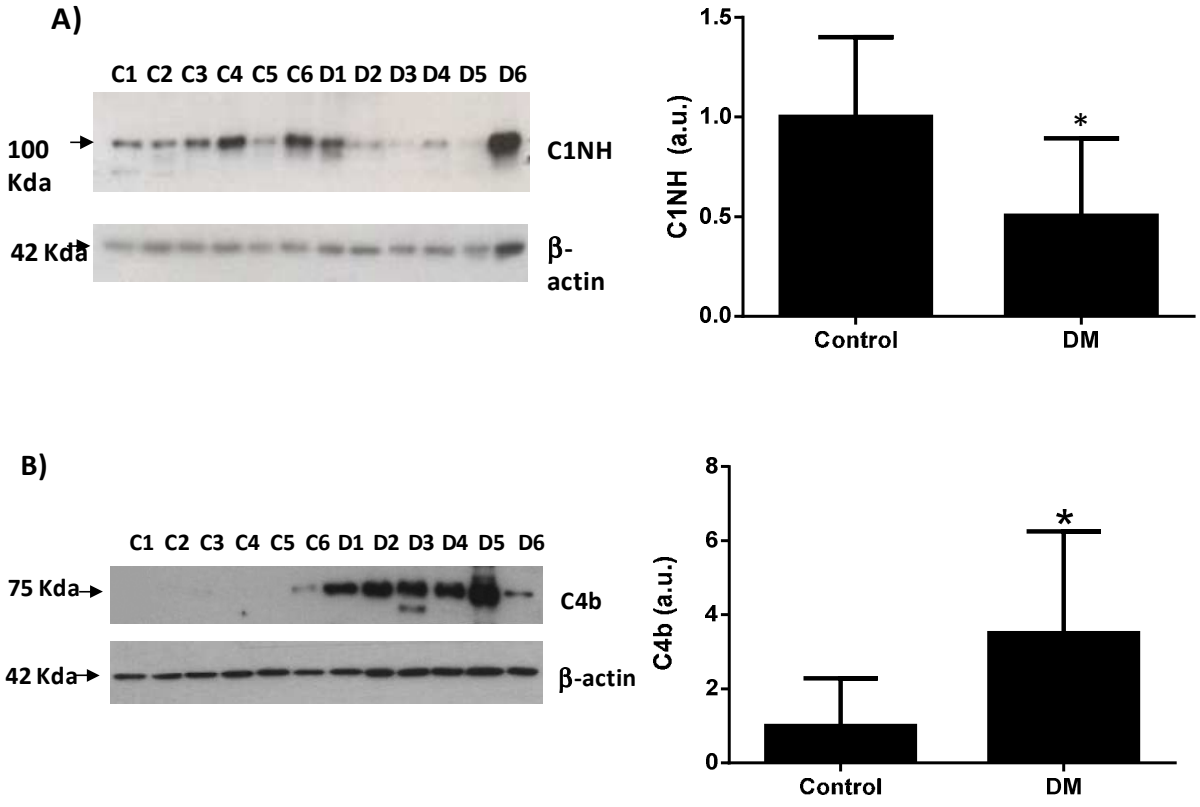


Figure 14. *Factors of the classical pathway of the complement system altered in diabetic retinas with glial activation (GA). A) Western blot analysis of C1INH in neuroretinas of non-diabetic donors (C1-C6) and diabetic donors with GA (D1-D6). Data are expressed as mean ± SD. *p<0.05. B) Western blot analysis of C4b in neuroretinas of non-diabetic donors (C1-C6) and diabetic donors with GA (D1-D6). *p<0.01.*

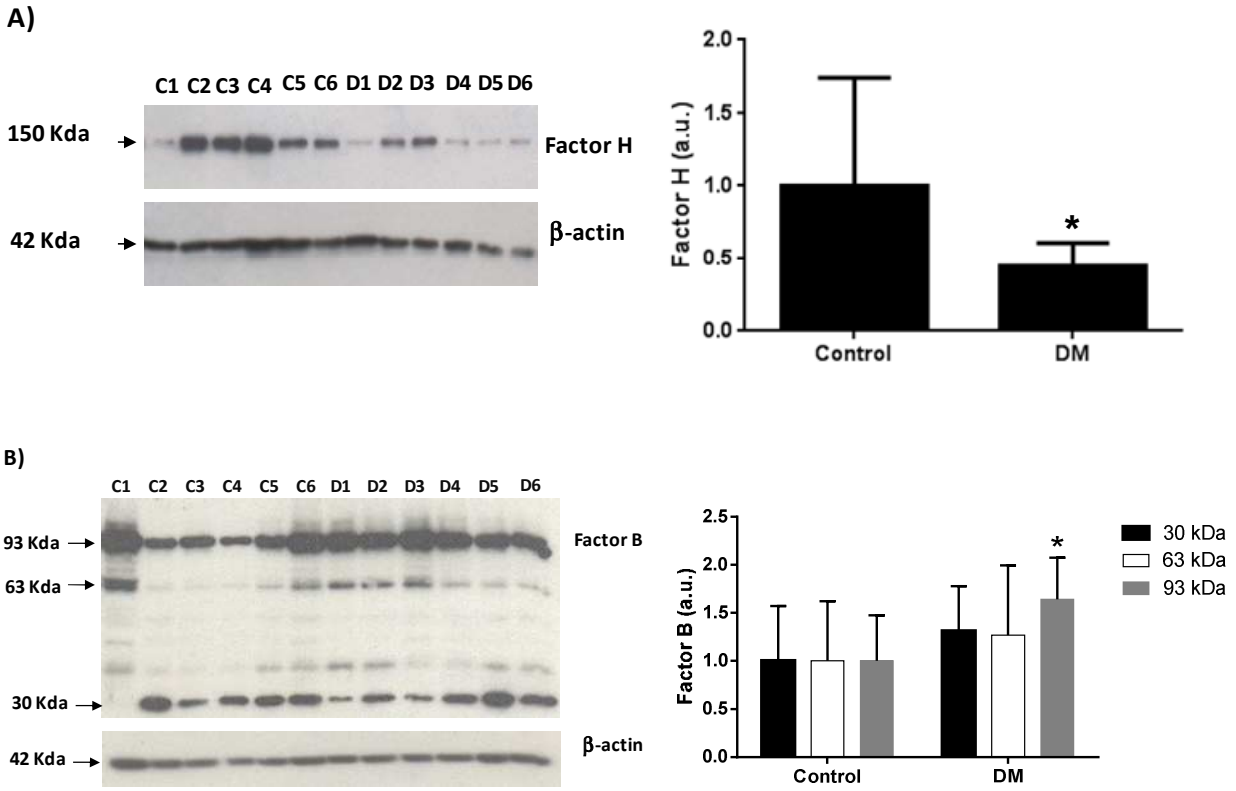
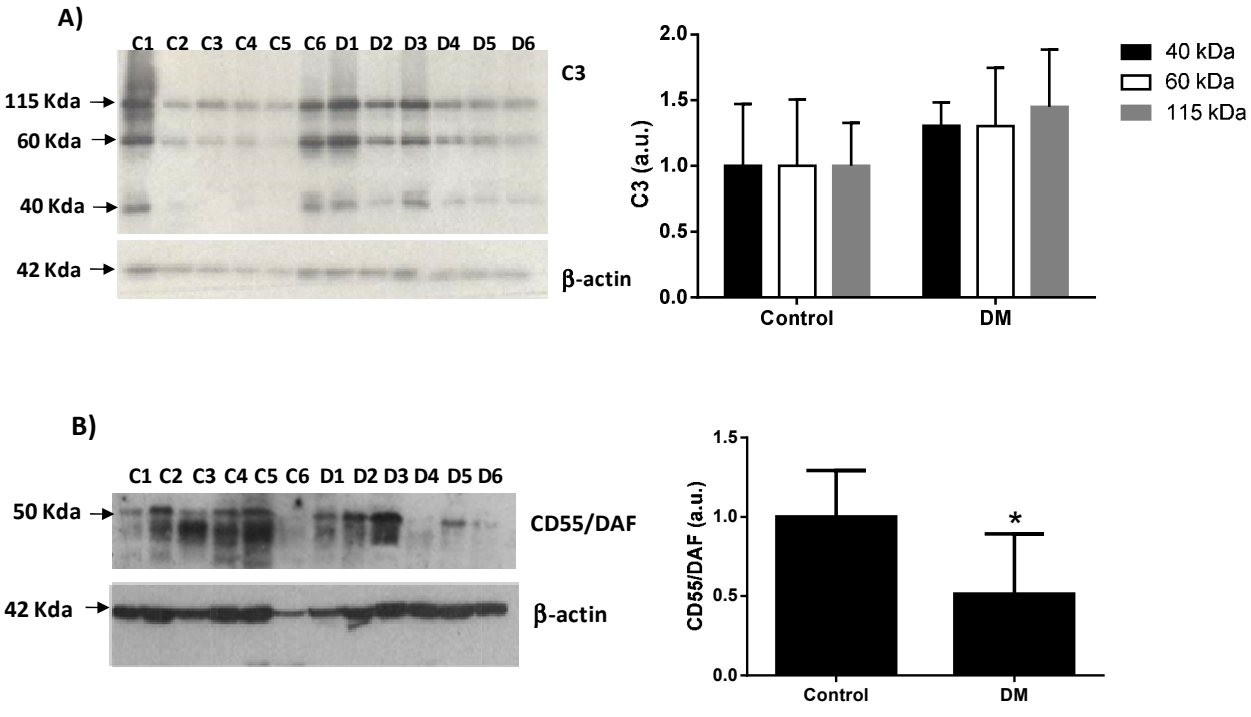


Figure 15. *Factors of the alternative pathway of the complement system altered in diabetic retinas with glial activation (GA). A) Western blot analysis of Complement Factor H in neuroretinas of non-diabetic donors (C1-C6) and diabetic donors with GA (D1-D6). Data are expressed as mean±SD. p=0.09. B) Western blot analysis of Factor B in neuroretinas of non-diabetic donors (C1-C6) and diabetic donors with GA (D1-D6). *p<0.05.*



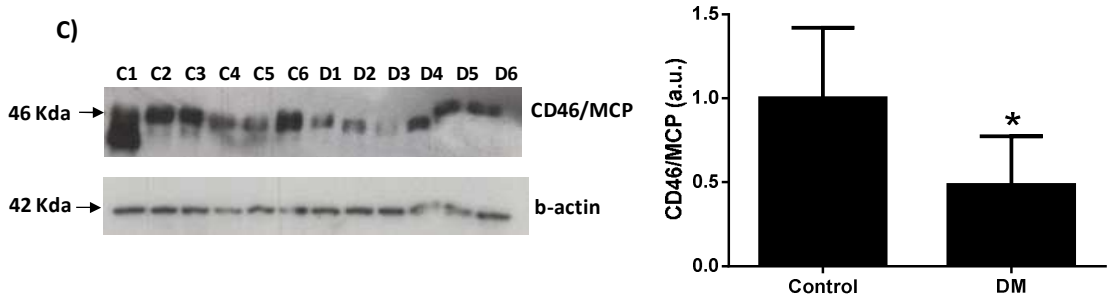
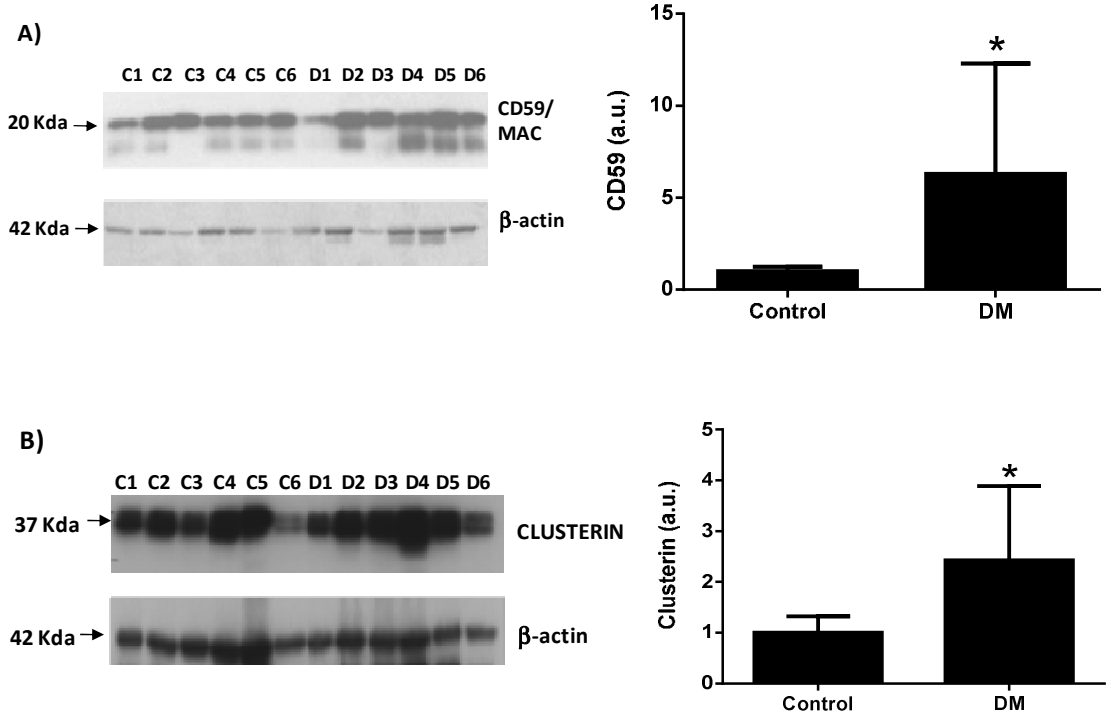


Figure 16. *Western blot analysis of C3 (A) in the human neuroretina. C1-C6: non-diabetic donors, D1-D6: diabetic donors with glial activation (GA). The presence of C3b fragments (60Kda and 42Kda) suggests C3 activation. The abundance of these fragments was higher in diabetic donors with GA (D1-D6) than in non-diabetic donors (C1-C6), but the differences were not statistically significant. Data are expressed as mean ± SD. B) and C) Western blot analysis C3 inhibitors: CD55 and CD46. *p <0.05.*

Finally, the increase in clusterin and CD59 **(Fig. 18A-B**) were confirmed, which could inhibit the enhancement of C9 (**Fig. 18C**) which, in turn, prevents the deleterious effect of MAC. Clusterin appeared as a thicker band because its high glycosylation (N-linked carbohydrates attached to clusterin) as previously reported (20, 21).



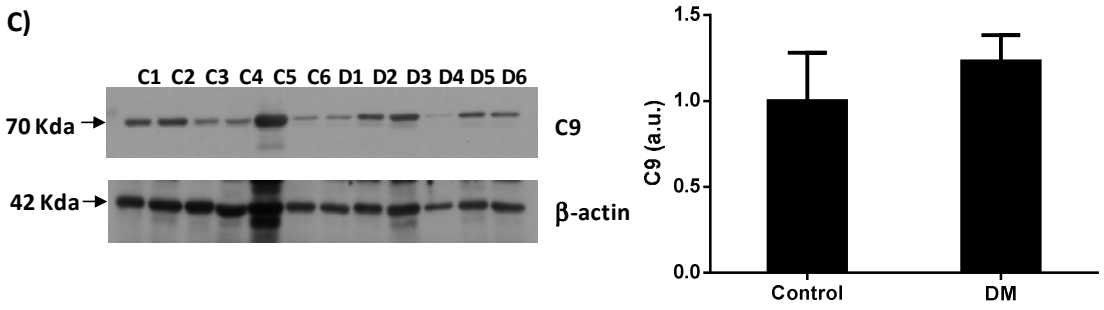


Figure 17. *Western blot analysis of C9 inhibitory proteins: CD59 and clusterin in retinas from non-diabetic donors and diabetic donors with glial activation (GA) (A and B). Data are expressed as mean ± SD. *p<0.05. C) Western blot analysis of C9 in human neuroretina of non-diabetic donors (C1-C6) and diabetic donors (D1-D6).*

4.1.4.2 ERM complex

4.1.4.2.1 Results in human retinas

ERM proteins when activated are able to act as downstream molecules of PKC and Rho GTPase pathways, acting as links between membrane molecules and actin filaments. Therefore, ERM proteins can be considered regulators of the cellular cortex – cytoskeleton that modulates plasmatic membrane shape (218). The changes in these proteins will have significant consequences in structural composition of cytoskeleton, thus favoring the vascular leakage. However, there is no information regarding effects of diabetes on these proteins in the setting of DR.

We have found among the ERM complex two patterns (**Table 8**):

- Proteins that are increased in the group of retinas with glial activation in comparison with both the control group or the retinas from diabetic patients without glial activation: Ezrin, Isoform 5 of Radixin, Moesin (ERM proteins) and Rho-GDP-dissociation inhibitors 1 and 2 (fragments).

- Proteins that are decreased when glial activation is present in comparison with both retinas from diabetic donors without glial activation or controls: Talin 1 and 2, and the Isoform A2 tight junction protein ZO-2.

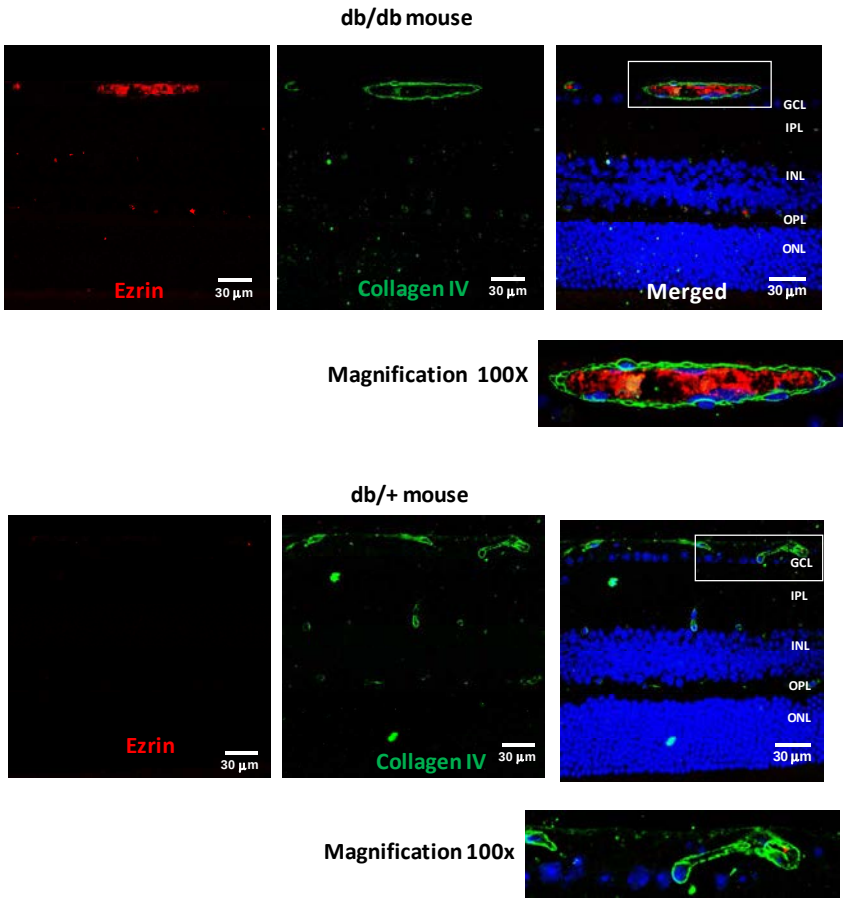
These findings represent a novel avenue in the underlying mechanisms involved in the BRB disruption that occurs in DR and will be commented in the discussion section.

Table 8. *Spectral counts of proteins of the ERM complex.*

	Controls	**Diabetes without glial activation**	**Diabetes with glial activation**	p<0.01
Ezrin	43	29	105	C=D<DN
Isoform 5 of Radixin	33	30	70	C=D<DN
Moesin	27	17	51	C=D<DN
Rho-GDP-dissociation inhibitor 1 (fragment)	15	13	46	C=D<DN
Rho-GDP-dissociation inhibitor 2 (fragment)	2	2	14	C=D<DN
Talin-1	81	108	58	C=D>DN
Talin-2	52	53	14	C=D>DN
Isoform A2 tight junction protein ZO-2	3	5	0	C=D>DN

4.1.4.2.2 Results in db/db mice

Western blot and immunohistochemistry assessments showed significant increase of Ezrin and Moesin in diabetic mice in comparison with non-diabetic mice (p<0.05) (**Fig. 18 and 20**). We also found an increase of Radixin in the immunofluorescence measurement but this was not confirmed by Western blot (**Fig. 19**).



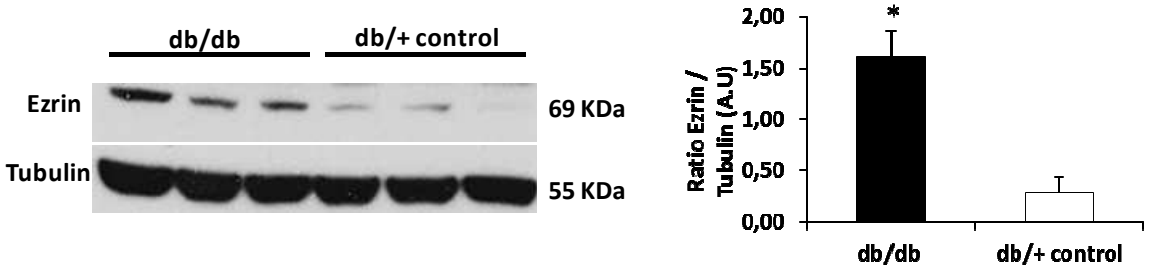


Figure 18. *Comparison of ezrin immunofluorescence (red) between representative samples from a diabetic mouse (db/db) (A) and non diabetic mouse (db/-) (B). Moreover collagen IV immunofluoresence is displayed in green. C) Western blot analysis of ezrin in diabetic mice (db/db) and non-diabetic mice (db/+). Data is expressed as mean ± SD. *p<0.05*

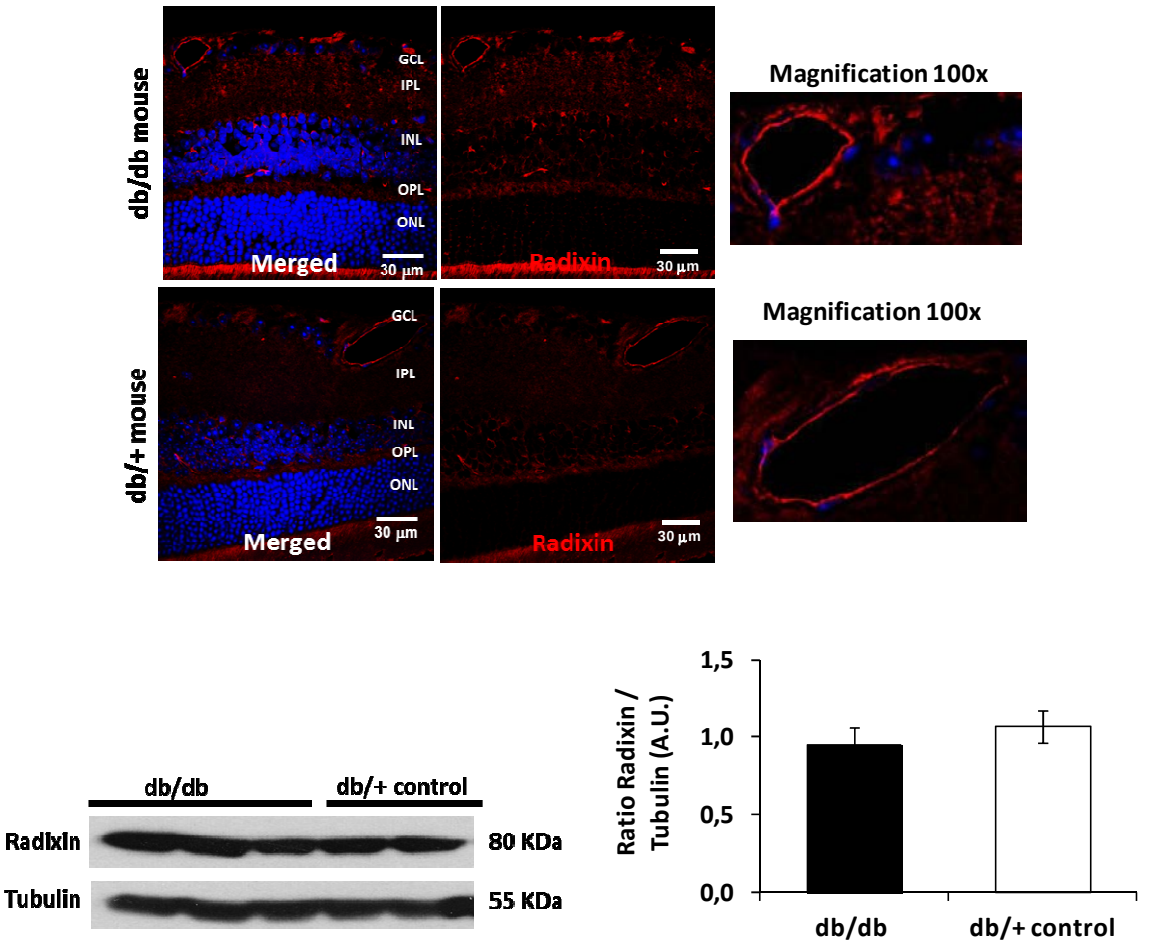


Figure 19. *Comparison of radixin immunofluorescence (red) between representative samples from a diabetic mouse (db/db) (A) and non-diabetic mouse (db/+) (B). (C) Western blot analysis of radixin in diabetic mice (db/db) and non-diabetic mice (db/+). Data is expressed as mean ±SD.*

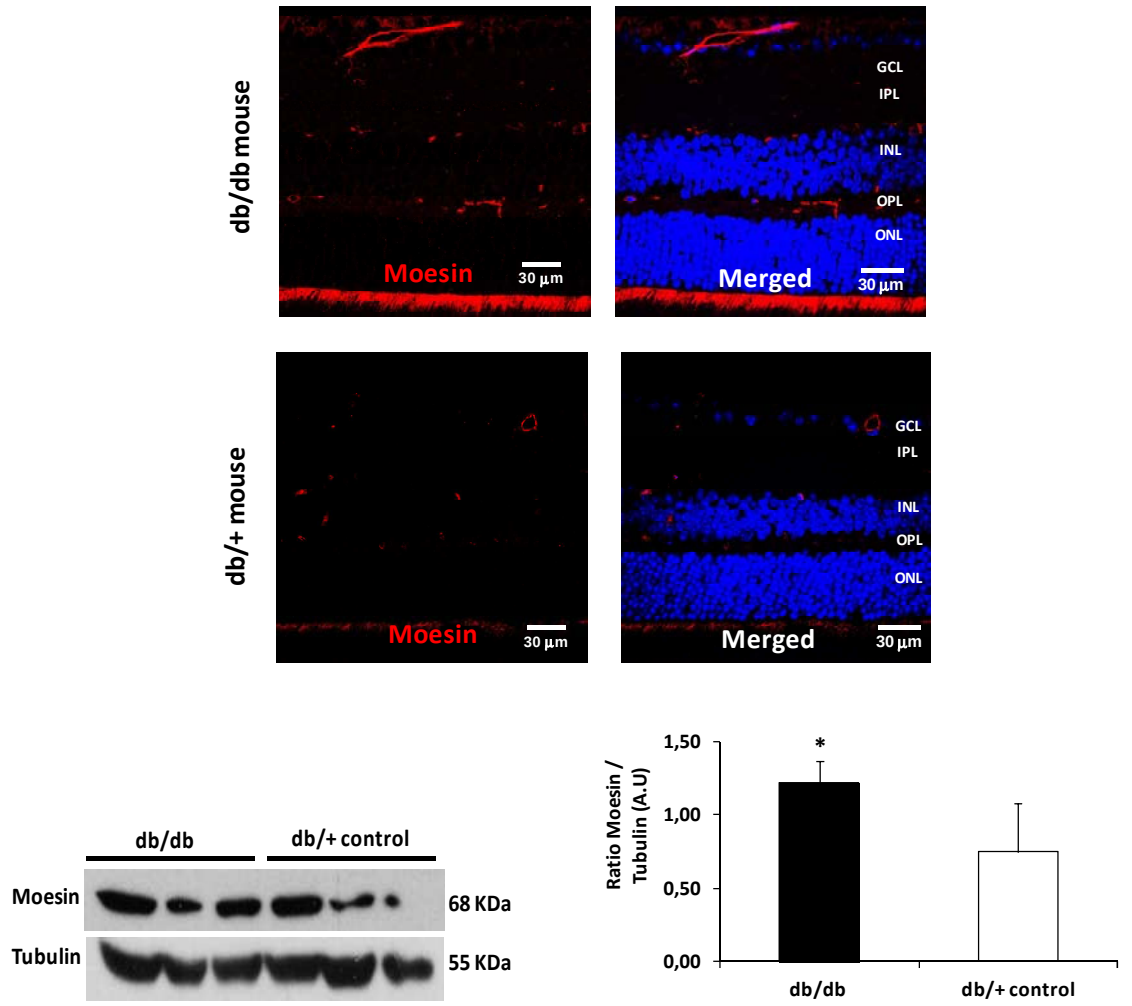


Figure 20. *Comparison of moesin immunofluorescence (red) between diabetic mouse (db/db) (A) and non-diabetic mouse (db/+) (B). (C) Western blot analysis of moesin in db/db mice and db/+ mice. Data is expressed as mean ±SD. *p<0.05*

Experimental study: driven hypothesis

4.2 Role of endothelin (ET-1) in the pathogenesis of neurodegeneration and early microvascular impairment in an experimental model of diabetes

4.2.1 Endothelin-1 and its receptors A (ETA) and B (ETB) were upregulated in the retina of diabetic donors

We found that ET-1 and ETB were upregulated in retinas from diabetic donors in comparison with retinas from non-diabetic donors (**Fig. 21**). Endothelin A-receptors (ETA) were also upregulated in the retinas from diabetic donors, but their increase did not achieve statistical significance.

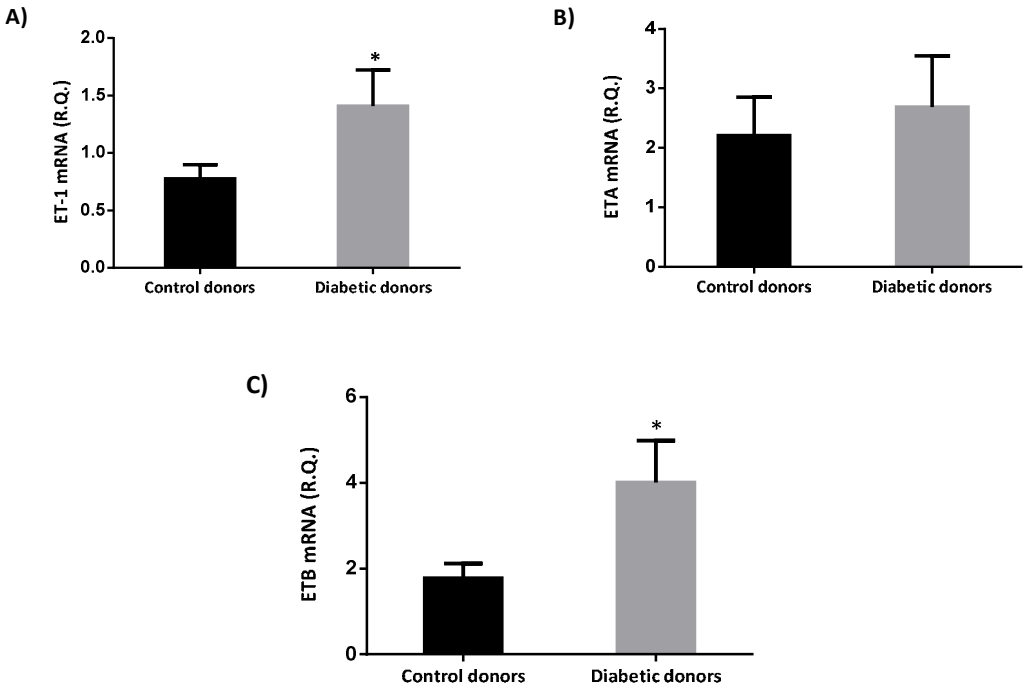


Figure 21. *Real-time quantitative RT-PCR analysis of Endothelin-1 (ET-1) mRNA (**A**) endothelin A-receptors (ETA) mRNA (**B**), and endothelin B-receptors (ETB) mRNA (**C**) in human retinas from diabetic and non-diabetic donors. The study was performed in 12 donors with diabetes and 12 donors without diabetes. R.Q.: Relative quantification. Data are mean ± standard error. The Student t test was used for comparisons. * $p < 0.05$.*

4.2.2 Bosentan ameliorated the upregulation of endothelin-1 and its receptors A (ETA) and B (ETB) in the retinas of diabetic mice

The expression of ET-1 was significantly higher in diabetic mice (db/db) in comparison with non-diabetic mice (**Fig. 22**). In addition, the content of both ETA and ETB were significantly higher in diabetic (db/db) mice than in non-diabetic control mice (**Fig. 22**). Bosentan was able to significantly reduce the upregulation of ET-1, ETA and ETB induced by diabetes.

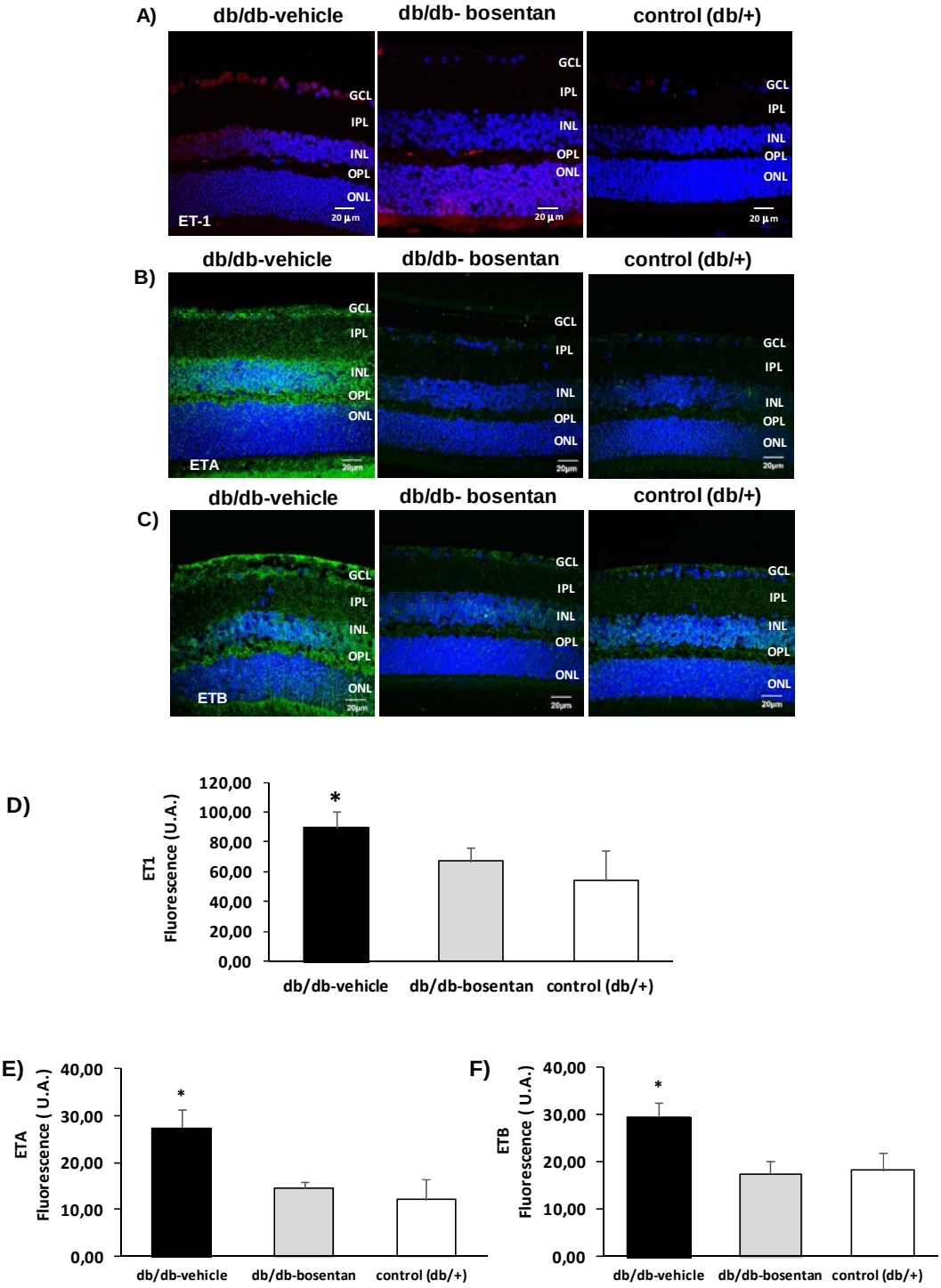


Figure 22. *(**A**) Comparison of ET-1 immunofluorescence (red) between representative samples from a diabetic mouse (db/db) mouse treated with vehicle, a diabetic mouse treated with bosentan, and a non-diabetic mouse (db/+). (**B**) Comparison of ETA immunofluorescence (green) between representative samples from a diabetic mouse (db/db) mouse treated with vehicle, a diabetic mouse treated with bosentan, and a non-diabetic mouse (db/+). (**C**) Comparison of ETB immunofluorescence (green) between representative samples from a diabetic mouse (db/db) treated with vehicle, a diabetic mouse treated with bosentan, and a non-diabetic mouse (db/+). Scale bar: 20 µm. (**D**–**F**) Quantification of immunofluorescence. AU: Arbitrary units. Data are expressed as mean ± SD. * $p < 0.05$ in comparison with the other groups.*

4.2.3 Bosentan prevented diabetes-induced neurodegeneration in diabetic mice

Blood glucose concentration and body weight at the end of treatment were similar in db/db mice treated with bosentan than in db/db mice treated with vehicle (**Fig 23**).

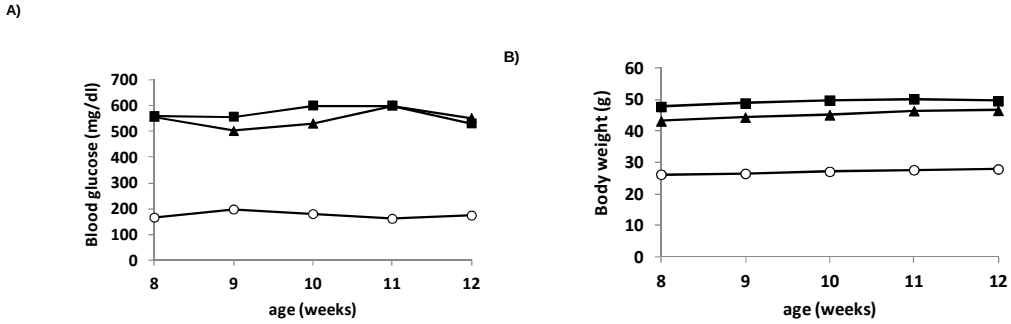
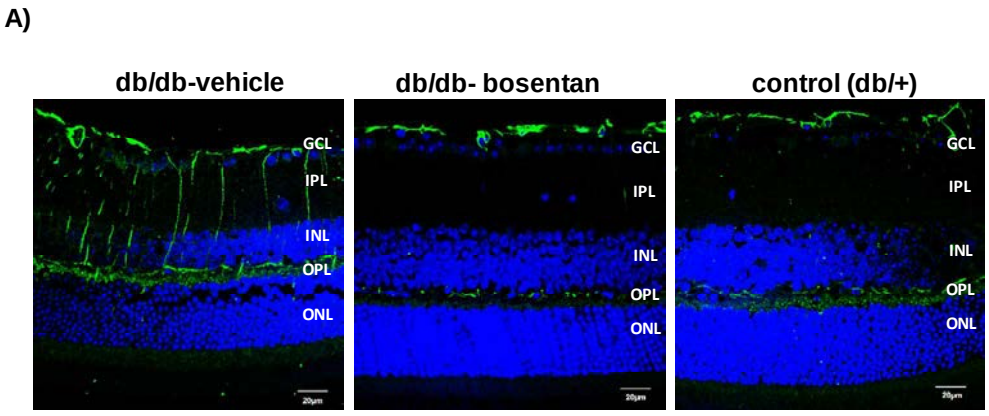


Figure 23. *Evolution of blood glucose (A) and body weight (B) in the experimental groups: db/db mouse treated with vehicle (black squares); a db/db mouse treated with bosentan (black triangles); and a non-diabetic mouse (db/+) (white circles).*

4.2.3.1 Glial activation

Glial fibrillary acidic protein (GFAP) expression was confined to the retinal ganglion cell layer in non-diabetic mice and therefore the GFAP score was ≤2 (**Fig. 24A,B**). The diabetic mice treated with vehicle presented significant higher GFAP expression than non-diabetic mice matched by age. Thus, 100% of diabetic mice presented a GFAP score ≥3. Bosentan administration for two weeks resulted in a significant decrease of reactive gliosis, and the GFAP score of the mice treated with bosentan was <3 in all cases (**Fig. 24A,B**).



B)

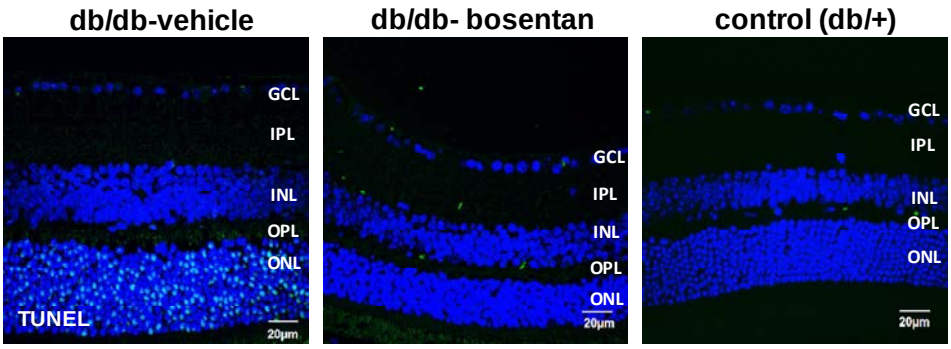
% positive GFAP labeling			
score	db/db-vehicle (%)	db/db- bosentan (%)	control (db/+) (%)
1	0.0	85.0	95.0
2	0.0	15.0	5.0
3	59.0	0.0	0.0
4	40.0	0.0	0.0
5	1.0	0.0	0.0

Figure 24. *Glial activation. (**A**) Comparison of glial fibrillary acidic protein (GFAP) immunofluorescence (green) between representative samples from a db/db mouse treated with vehicle, a db/db mouse treated with bosentan and a non-diabetic mouse. In the diabetic mouse treated with vehicle, the Müller cells' endfeet show abundant GFAP immunofluorescence and the radial processes stain intensely throughout both the inner and outer retina. Nuclei were labeled with DAPI (blue). GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner cell layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer. (**B**) Quantification of glial activation based on extent of GFAP staining. n = 6 mice per group (10 sections per retina).*

4.2.3.2 Retinal apoptosis

The apoptosis rate was significantly higher in diabetic mice treated with vehicle than in non-diabetic mice in all retinal layers (**Fig. 25A,B**). Bosentan administration resulted in a significant prevention of apoptosis in all retinal layers (**Fig. 25A, B**). The assessment of retinal ganglion cells in the GCL using NeuN immunofluorescence is showed in **Fig. 25 C, D**. The number of ganglion cells was significantly lower in diabetic mice treated with vehicle than in non-diabetic mice. Bosentan administration in diabetic mice significantly prevented the reduction of retinal ganglion cells observed in diabetic mice treated with vehicle.

A)



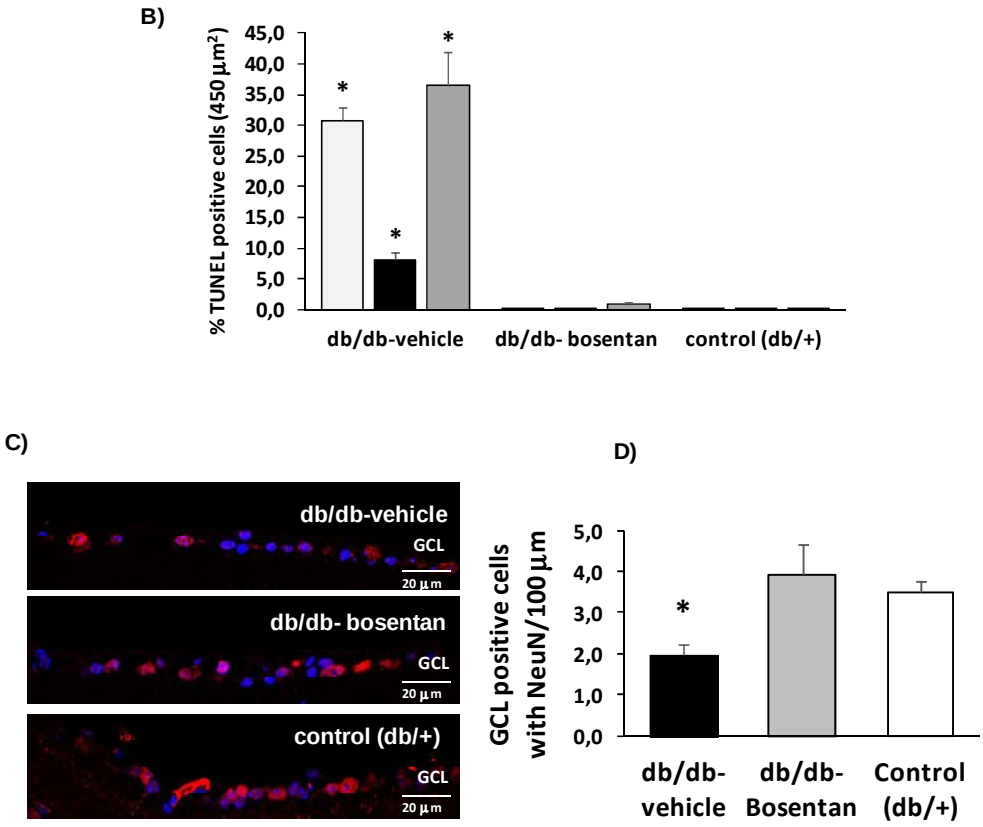


Figure 25. *Apoptosis. (**A**) Comparison of TUNEL immunofluorescence (green) between representative samples from a db/db mouse treated with vehicle, a db/db mouse treated with bosentan and a non-diabetic mouse. Scale bar: 20 µm. (**B**) Percentage of TUNEL positive cells in the retinal layers. * p < 0.001 in comparison with the other groups in all retinal layers (ONL, INL and GCL). n = 6 mice per group (10 sections per retina). (**C**) Comparison of NeuN positive cells (red) between representative samples from a db/db mouse treated with vehicle, a db/db mouse treated with bosentan and a non-diabetic mouse. Nuclei were labeled with DAPI (blue). (**D**) Quantification of NeuN positive cells.*

4.2.4 Bosentan decreased PKC-β, TNF-α and VEGF upregulation induced by diabetes

4.2.4.1 In vivo studies

We found that in db/db mice aged 12 weeks there was a significant increase of PKC-β in comparison with non-diabetic mice which was prevented by bosentan (**Figure 26 A,B**). Furthermore, we found that bosentan prevents the upregulation of TNF-α induced by diabetes in retinal vessels (**Figure 26C**).

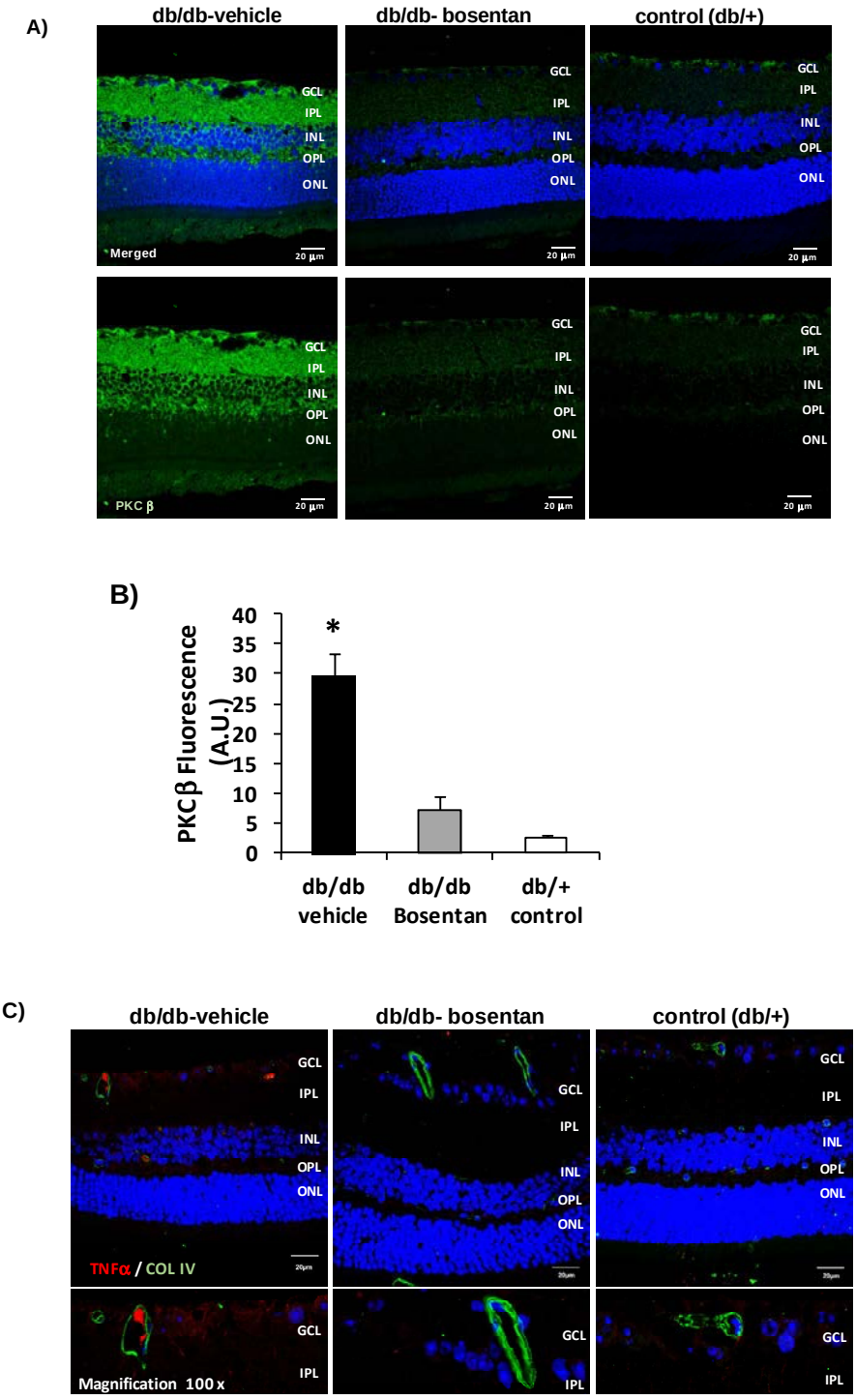


Figure 26. *(**A**) Comparison of PKC β immunofluorescence (green) between representative samples from a db/db mouse treated with vehicle, a db/db mouse treated with bosentan, and a non-diabetic mouse (db/+). Nuclei were labeled with Hoechst (blue). Scale bar: 20 µm. GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner cell layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer. (**B**) Quantification of PKC β immunofluorescence. A.U.: Arbitrary units. Data are expressed as mean ± SD. * p < 0.05 in comparison with the other groups. (**C**) TNF-α (red) immunofluorescence retinal images from a representative db/db mouse treated with vehicle, a db/db mouse treated with bosentan and a non-diabetic (db/+) mouse. Blood vessels were immunostained with collagen IV (green). Magnification at 100× shows the expression of TNF-α in retinal vessels in a representative diabetic mouse but not in a representative mouse from the other interventional groups.*

4.2.4.2 In vitro studies

Tumor necrosis factor alpha (TNF-α) induced an increase of mRNA levels of ET-1 in human retinal endothelial cells (HRECs). This upregulation was significantly reduced by bosentan (**Figure 27A**). The upregulation of ET-1 by TNF-α was associated with a significant increase of permeability that was prevented with bosentan (**Figure 27B**). Furthermore, bosentan prevented the upregulation of VEGF induced by high glucose in HRECs (**Figure 27C**).

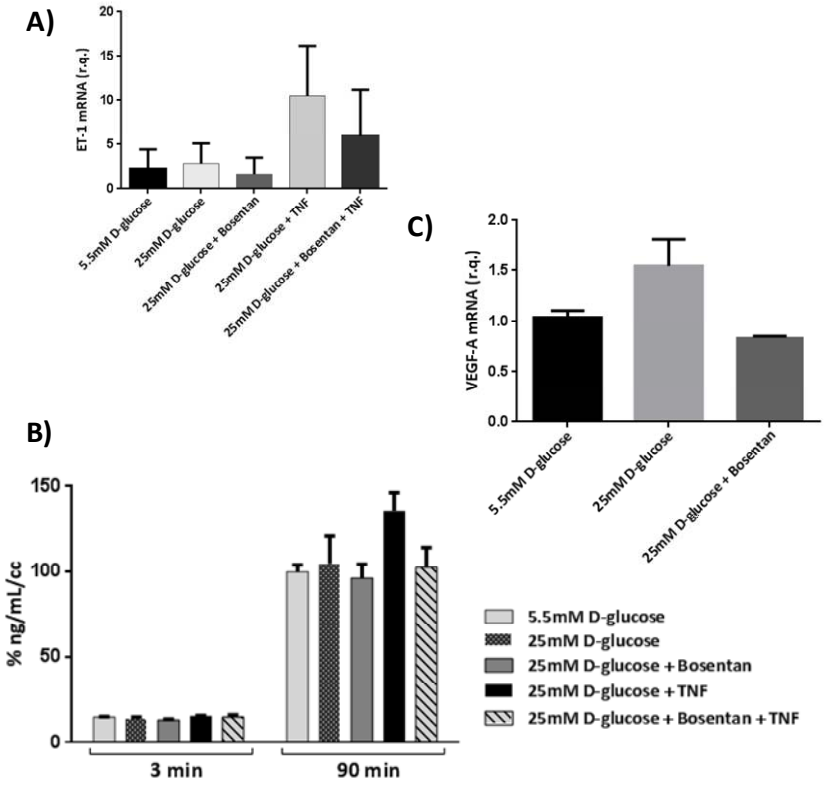


Figure 27. *(**A**) ET-1 mRNA expression in human retinal endothelial cells (HRECs) under different treatments including TNF-α (10 ng/mL) and bosentan (30 µM). R.Q.: Relative quantification. Data are expressed as mean ± SD. * p < 0.05 in comparison with the other conditions. (**B**) Results of 70 kDa dextran permeability in HRECs in the different conditions examined. The vertical axis is the concentration of dextran. Dextran permeability was measured at 3 and 90 min. * p < 0.05 in comparison with the other conditions. (**C**) Effect of bosentan on the upregulation of vascular endothelial growth factor (VEGF) induced by diabetes. * p < 0.05 in comparison with the other conditions.*

4.2.5 Pharmacokinetics

Bosentan content in ocular tissues and plasma after a single eye drop administration is shown in **Fig. 28**. Bosentan reached the peak level much earlier (0.5 h) before in retina than in the aqueous humor (2 h) and decreased rapidly afterwards. These results suggest that bosentan reaches the back of the eye through the trans-scleral route. Bosentan was detected in plasma only the first 30 min after administration and at very low concentration (0.50 ng/mL at 0.25 h and 0.48 ng/mL at 0.5 h).

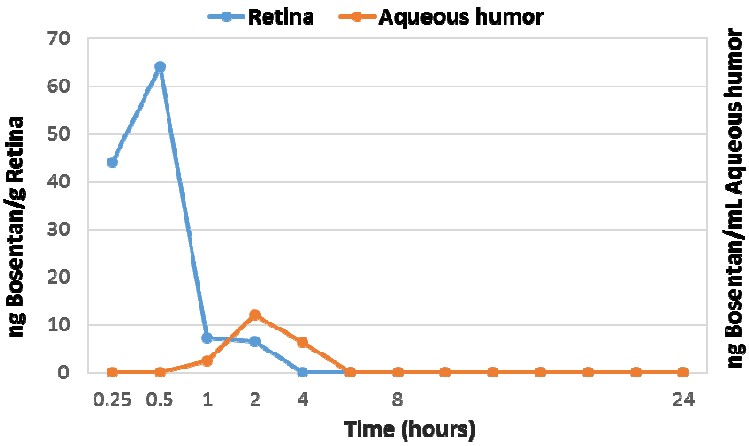


Figure 28. *Bosentan content in retina (blue line, ng/g) and aqueous humor (orange line, ng/mL) of rabbits treated with one instillation of bosentan (50 µL, 0.5%).*

Clinical study

4.3 Retinal fixation in normocognitive, MCI and AD diabetic subjects

The main clinical characteristics of subjects with type 2 diabetic included in the study are summarized in **Table 9**. No differences were found among groups in terms of age, sex, hypertension, dyslipidaemia, diabetes duration or HbA1c. The mean reliability index of the microperimetry test was above 90% in all groups.

Table 9. *General characteristics and parameters of retinal sensitivity and fixation*

	AD N= 33	**MCI N= 33**	**Normocognitive N= 34**	**p**
Age (years)	79±5.57	77.03±4.38	75.53±6.97	ns
Gender (males, %)	42.42	51.52	58.82	ns
IMC	27.72±3.79	28.28±4.48	30.28±4.45	ns
Hypertension (%)	78.79	75.76	73.53	ns
Dyslipidemia (%)	63.64	66.67	71.43	ns
Diabetes duration (years)	10.45±6.23	13.73±10.64	10.91±6.12	ns
A1c (% of Hb) DCCT	6.79±1.02	6.99±1.25	7.5±0.95	ns
Retinal sensitivity (dB)	16.95±6.01	21.05±4.82	23.39±2.47	<0.00001
Fixation P1 (%)	45.03±24.89	58.21±26.66	88.52±12.19	<0.00001
Fixation P2 (%)	70.2±20.61	81.03±15.60	96.09±3.66	<0.00001
Reliability	92.83±14.49	97.45±7.04	96.59±13.59	ns

Fixation was more unstable, in terms of P1 and P2 percentages, as cognitive impairment progressed. Therefore, the lowest fixation capacity was found in patients with AD and the highest in patients with normocognition, the differences between the groups being statistically significant ($p<0.00001$). To illustrate this point, a representative example of the fixation graph of each group (normocognition, MCI and AD) is shown in **Figure 29**. In addition, we also found a significant difference among groups on BCEA63 and BCEA95 ($p<0.00001$). The mean BCEA63 and BCEA95 for each group are represented in **Figure 30**.

A significant negative correlation was found between BCEA63 or BCEA95 and MMSE ($r=-0.4936$ and $r=-0.5301$ respectively; $p<0.00001$). In addition, BCEA63 and BCEA95 also significantly correlated with the Alzheimer's disease Assessment Scale-cognition sub-scale (ADAS-Cog) ($r= 0.428$ and $r=0.4716$, respectively; $p<0.00001$). In other words, a more unstable fixation correlated with a lower punctuation in the MMSE test and a higher score in the ADAS-Cog, indicating that fixation instability is associated with the degree of cognitive impairment (**Figure 31 a-d**).

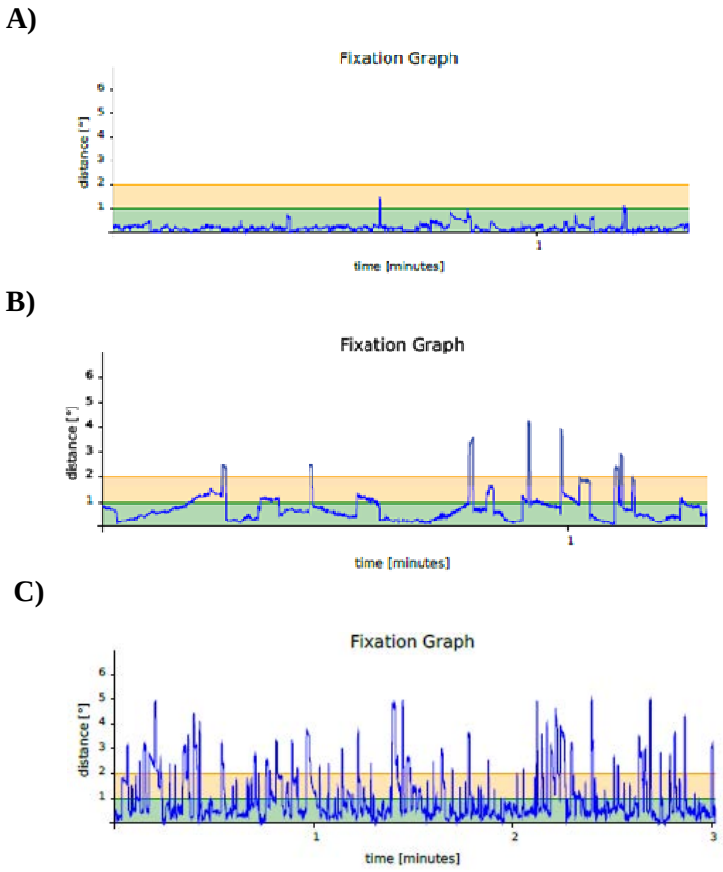


Figure 29. *Representative examples of the fixation graph of a normocognitive (**a**), MCI (**b**) and AD (**c**) subjects.*

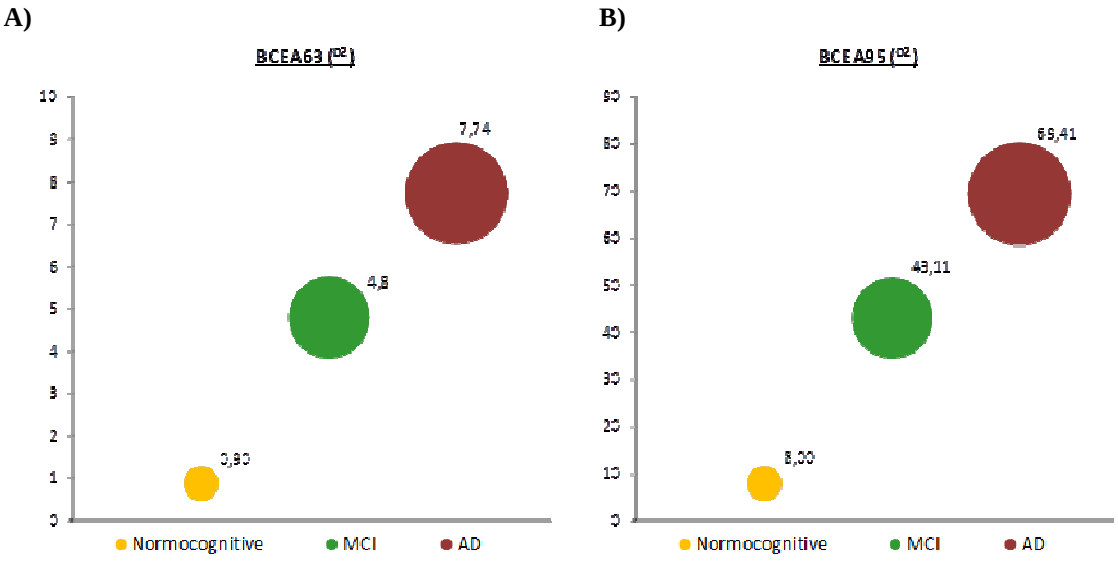


Figure 30. *Mean values of BCEA63 (**a**) and BCEA95 (**b**) (in ^{o2}) for normocognitive subjects (yellow), MCI (green) and AD (red).*

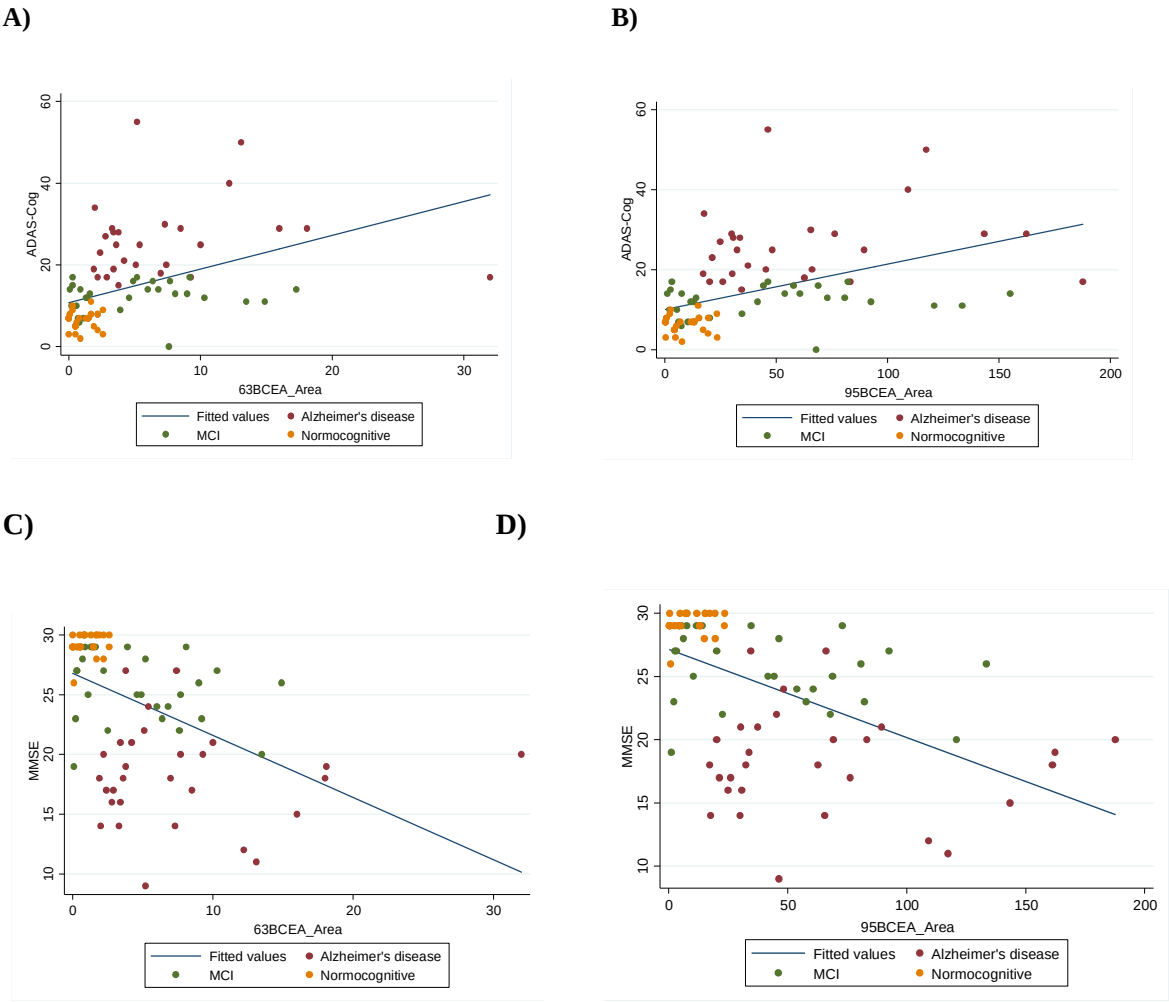


Figure 31. *Linear regression graphs between: (**a**) ADAS-Cog and BCEA63; (**b**) ADAS-Cog and BCEA95; (**c**) MMSE and BCEA63; (**d**) MMSE and BCEA95.*

Finally, a comparison regarding the capacity to discriminate MCI subjects from normocognitive subjects by using retinal sensitivity alone or by adding to the predictive model the parameters of fixation BCEA95 and P1 was performed. The results are summarized in **Table 8** and **Figure 32**. The addition of fixation assessment to the results obtained by measuring retinal sensitivity significantly increased the ROC area (p=0.0098), thus enhancing the capacity to discriminate between normocognitive and MCI diabetic subjects. The optimal cutoff based for maximum efficiency was 0.494191 with a sensitivity of 72.7% (95% CI: 55.8 to 84.9%), specificity of 87.9% (95% CI: 72.7 to 95.2%), positive predictive value of 85.7% and a negative predictive value of 76.3%. Sensitivity and specificity for every cutoff point is displayed in **Figure 33**.

Table 8. *ROC Area comparison between sensibility alone, and sensibility and fixation parameters (P1 and BCEA95).*

	ROC Area	Std. Err.	95% Conf interval	P
Sensibility	0.68	0.0662	0.55028 – 0.80969	0.0098
Sensibility + BCEA95 + Fixation P1	0.8604	0.0454	0.77145 – 0.94939	

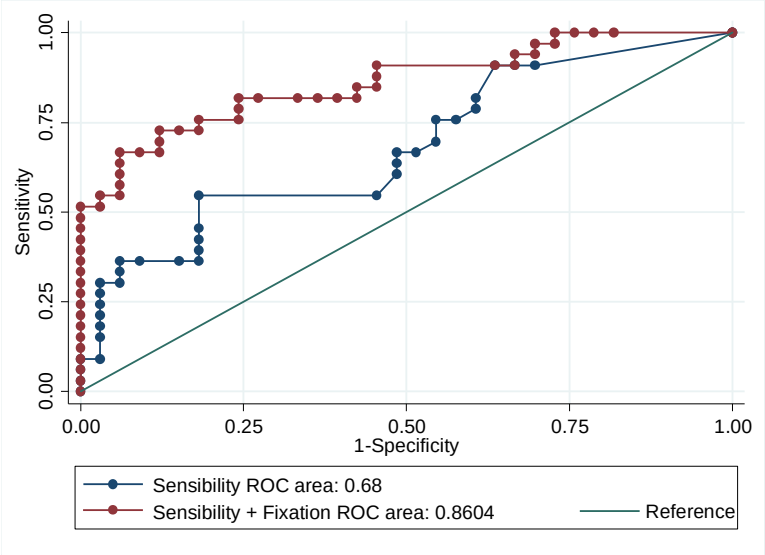


Figure 32. *ROC Area on sensitivity comparing retinal sensitivity alone assessed by fundus microperimetry vs. the combination of retinal sensitivity and fixation parameters (P1 and BCEA95).*

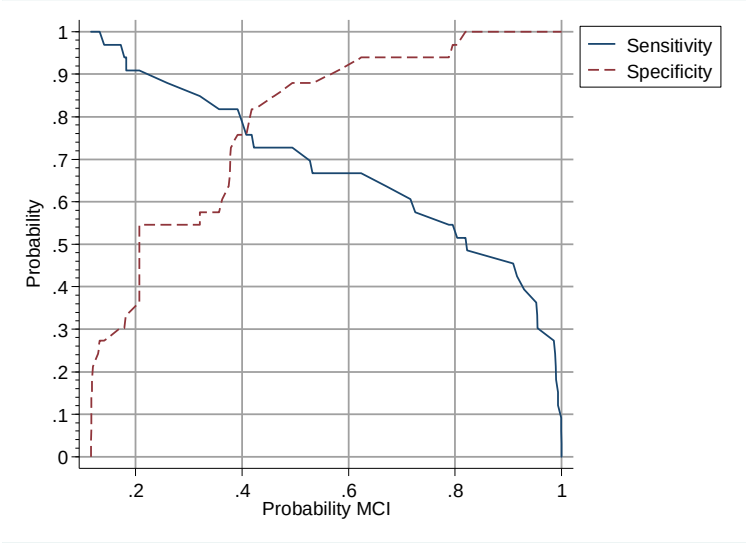


Figure 33. *Sensibility and sensitivity of retinal sensitivity, BCEA95 and fixation P1 for every cutoff point.*

5. Discussion

Experimental study: free hypothesis

5.1 Proteomic analysis of early diabetic retinopathy reveals mediators of neurodegeneration and vascular leakage

The present proteomic study allowed identification of proteome changes involved in the pathological processes that occur in the early stages of DR. We focused in proteins involving two crucial events: neurodegeneration and early vascular impairment. In this regard, it should be emphasized that retinas with glial activation also presented an increase in albumin, indicating the presence of vascular leakage.

5.1.1 Neurodegeneration

The Ingenuity Pathway Analysis revealed several pathways that are related to neurodegenerative diseases. This findings support the idea that the study of neurodegeneration in the diabetic retina could be useful to understand the neurodegenerative processes that occur in the brain in persons with diabetes. This concept has been further explored in the Clinical Study. On the other hand, we also identified a group of structural proteins of the axon initial segment and proteins involved in axonal transport, which could help to understand the pathogenic events involved in the neurodegeneration that occurs in diabetic retinas. In this section, both the pathways and the proteins identified will be discussed.

5.1.1.1 Pathways related to neurodegenerative brain diseases

A total of 35 pathways related to neurodegeneration or neuroregulation and specifically represented in a single group (non-diabetic donors [C], diabetic donors without GA [D], or diabetic donors with GA [D+GFAP]) have been identified. The most relevant pathways shared with brain neurodegenerative diseases will be discussed.

5.1.1.1.1 Samples from non-diabetic donors

In the non-diabetic control (C) group, the “Neuroprotective Role of THOP1 in Alzheimer's Disease” and “Unfolded Protein Response” pathways were uniquely present.

Neuroprotective Role of THOP1 in Alzheimer’s disease: THOP1 mediates a compensatory neuronal response to increased Aβ in the brain, one of the central events in AD (204). To the best of our knowledge, no data on the potential neuroprotective role of THOP1 in the retina have been reported. In a rabbit model of diabetes Aβ accumulation has been detected in multiple layers of the retina (219). Given the plausibility of retinal Aβ plaque formation and that the retina is ontogenically a brain-derived tissue, it could be hypothesized that THOP1 has neuroprotective functions in the retina similar to its role in the brain. Whether the loss of this signaling pathway

also occurs in the brains of type 2 diabetic patients remains to be elucidated, but could be a new mechanism to explain the growing evidence regarding the association between T2D and AD.

Unfolded Protein Response: The initial response to misfolded proteins in a healthy cell is to refold them back into their native shapes. Impairment of the UPR has been involved not only in the accumulation of misfolded proteins in the brain of many neurodegenerative diseases (205), but also DR (206,207). In our study, we observed a significant decrease in multiple Hsp70s (e.g., Hsp70Kda-4, Hsp70Kds-4L) in retinas of diabetic patients with GA, thus pointing to the accumulation of misfolded proteins as a potential pathway involved in the neurodegenerative process that occur in the early stages of DR. In contrast, we found an increase of isoform 2 of Hsp90-α and the isoform 2 of the stress-induced phosphoprotein 1 (STI1), an Hsp90 co-chaperone secreted by astrocytes, which protect neurons against Aβ oligomer-induced toxicity (220), suggesting a compensatory response.

5.1.1.1.2 Samples from diabetic donors without glial activation

In diabetic patients without GA (D group), the following neuroprotective pathways were upregulated: "Neuregulin Signaling," "Synaptic Long Term Potentiation," and "Amyloid Processing."

Neuregulin Signaling: The neuregulin family of ligands (NRGs) is important in synaptogenesis and neuronal survival. NRGs exhibit a neuroprotective role under inflammatory conditions by acting in concert with the cholinergic anti-inflammatory pathway (208). We found the "neuregulin signaling" pathway to be unique to the D group. Although few data exist on NRGs in the retina, numerous studies demonstrate neuroprotective effects of NRGs in the central nervous system (221). That the neuregulin pathway was genuine of D group suggests that its neuroprotective effects occur early in disease but are transient and are lost as the disease progresses.

Synaptic Long Term Potentiation: Synaptic long term potentiation (LTP) is a variation of synaptic transmission that functions in learning memory and has been involved in synaptic plasticity in the hippocampus and cerebellum (209–212). Several studies of the hippocampus in diabetic rat models have reported decreases in LTP relative to controls (210–212), which is inconsistent with our present finding. Further research is needed to elucidate the effects of diabetes on LTP and to determine whether changes in synaptic plasticity in the brain are generalizable to the retina.

Amyloid Processing: In AD, Aβ toxicity in neurons along with oxidative stress leading to increased MAP kinase activity, ultimately result in increased phosphorylation of Tau (213,214). Hyperphosphorylated Tau then is incorporated into the neurofibrillary tangles seen in Alzheimer's disease. Amyloid processing incorporates these events, which are important precipitants of neurodegeneration in AD. That the protective THOP1 pathway was present only in non-diabetic retinas while its counterpart, amyloid processing, was unique to diabetic retinas without glial activation may indicate a transitional role for this pathway in the progression of DR.

5.1.1.1.3 Samples from diabetic donors with glial activation

The “Dopamine Degradation” and “Parkinson's Signaling” pathways were unique to retinas with abundant GA (D+GFAP).

Dopamine degradation and **Parkinson’s signaling:** Dopamine is released by a subclass of amacrine cells in the retina (222) and its involved in the diverse physiologic aspects of retinal neuromodulation, mediation of light responsiveness and clock gene regulation (215,216). Disruption of the dopaminergic system is classically associated with Parkinson disease, but it also may occur in diabetes. It should be noted that, as occurs with AD, T2D patients have an increased prevalence of Parkinson disease (223).

5.1.1.2 Proteins from the axon initial segment

In retinas with glial activation we have found differential content in two groups of proteins: proteins involved in the axonal transport and proteins from the axon initial segment (AIS).

Axonal transport is essential for neuronal survival, and its impairment has been related to neurodegenerative processes (69). Among this group of proteins we found a downregulation of the dynein-dynactin complex in retinas with GA. This group of proteins is involved in the transport of cargos from the distal axon to the cell body (retrograde transport) and is also essential for many cellular functions including vesicle trafficking between Golgi and endoplasmic reticulum, endosome and lysosome motility, and spindle assembly. Furthermore, dynein-mediated transport has a key role in the removal of misfolded or degrade proteins (224). Fujiwara et al. (225) found in hypoccampal cultures that neurodegeneration induced by glutamate excitotoxicity resulted in profound alterations in components of the dynein-dynactin complex. In addition, the hyperexpression of dynein intermediate chain (a component of dynein that links dynein with dynectin) protected from glutamate-mediated neurodegeneration. It is worth mentioning that glutamate induced excitotoxicity is one of the main features in diabetic eye neurodegeneration (34). Overall our results suggest that the decrease in axonal transport proteins detected in the retinas with GA could participate in neural apoptosis observed in early stages of DR. By contrast we have found that members of the kinesin family, which are also involved in axonal transport (224), are overexpressed in retinas with GA. In fact, KIF3A is a protein for anterograde (from the cell body to the distal axon) fast axonal transport (226). Therefore, it could be hypothesized that in retinas with GA an imbalance between retrograde and anterograde transport occurs. It should be mentioned that our findings are different than those previously reported in rats with streptozotocin-induced diabetes (4). However, it should be mentioned that the retinas analyzed in the present study were from subjects with type 2 diabetes, whereas the retinas obtained from rats with streptozotocin-induced diabetes mimics type 1 or insulinopenic diabetes. In addition, it should be noted that streptozotocin is neurotoxic itself and, therefore, this deleterious effect should be taken into account when interpreting the results.

Regarding AIS proteins a generalized and marked downregulation was found. The AIS is localized at the proximal part of the axon and separates the somatodendritic domain from the axonal domain

(**Fig. 34**). It is essential for the initiation of the axon potential and axonal regeneration. Moreover, it plays a crucial role in maintaining axonal polarity by means of a specialized cytoskeleton organization that acts as a diffusion barrier between the two neuronal compartments. To the best of our knowledge, AIS proteins have not been previously investigated in diabetic retinopathy or human retinas. However, they but have been involved in the pathogenesis of neurodegenerative diseases like AD (217). We have found a lower content of spectrin, neural cell adhesion molecule, neurofascin and ankyrins in retinas with neurodegeneration in comparison with retinas without neurodegeneration and non-diabetic control retinas. Spectrin is a cytoskeleton protein essential in neuronal structure, and binds transmembrane proteins such as Neural Cell Adhesion Molecules (NCAM) or Neurofascin, through linkers like ankyrins (217, 226, 227). Interestingly, Schafer et al (229), demonstrated *in vitro* and *in vivo* that neuronal ischemic injury causes rapid and preferential disruption of the AIS by means of the proteolysis of Ankyrin G (also named Ankyrin 3) and Spectrin. Our results indicate that in diabetes-mediated neurodegeneration there is also a preferential disruption of the AIS. It should be mentioned that Ankyrin 2 (or Ankyrin B), participates in axonal transport by linking dynactin-dynein complex (230), which as mentioned above is also downregulate in diabetic retinas with GA. This connection reveals the intricate cross-talk that exists among these axonal proteins.

In summary, AIS structure and axonal transport are intimately related and both are essential for neuronal survival. Our findings indicate that glial activation in the setting of diabetes-induced neurodegeneration is associated with an impairment of axonal transport and a downregulation of the proteins that form the AIS which, in turn, could further increase neural retinal apoptosis. Since an increasing numbers of neurodegenerative diseases are being linked to defects in the axonal transport machinery, our result could contribute not only to fundamental knowledge about intracellular transport mechanisms but also to the development of new therapeutic strategies.

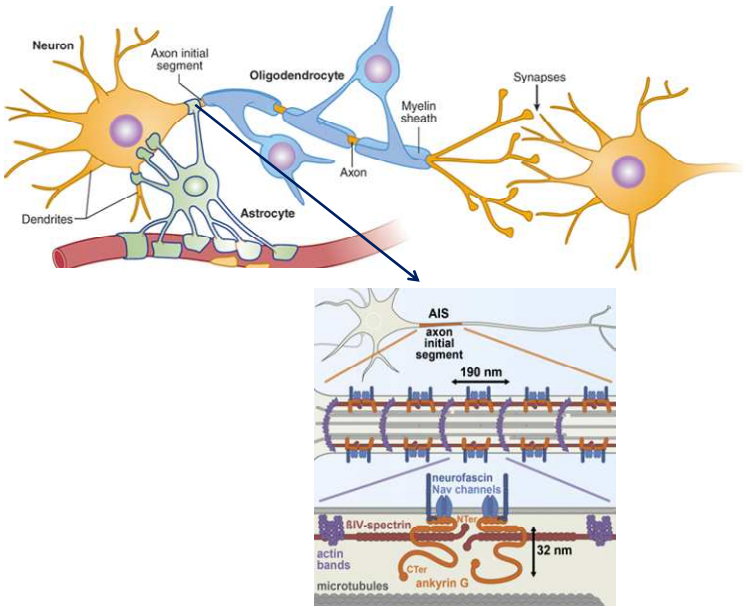


Figure 34. *Glial cells are in intimate contact with the AIS. Our findings indicate that glial activation is associated with a downregulation of the proteins that form the AIS.*

5.1.2 Proteins involved in vascular leakage

The increase in vascular permeability occurs early in DR pathogenesis can be detected by an increase in the presence of albumin in retinas with glial activation. The main molecular mechanisms involved in BRB dysfunction in diabetes are the formation of AGEs, inflammatory mediators, oxidative stress, PKC activation and its downstream effectors, and VEGF. By means of a proteomic approach we have identified several inflammatory mediators that could be involved in the early vascular impairment, which will be discussed in this section. Given the potential therapeutic implications and our previous background on study of the complement system in the vitreous fluid from patients with PDR (138), we first validated the proteins involved in the complement system. On the other hand, a differential abundance of ERM proteins have been found. This is a very interesting result because these proteins are essential in the cross-talk that exists between the cytoskeleton and endothelial cells that ultimately lead to vascular leakage. Since at present little is known regarding the role of ERM proteins in DR we decided to validate these candidates in the db/db mouse, a well-characterized model of DR (197).

5.1.2.1 Inflammatory mediators

5.1.2.1.1 Complement system

Complement activation is an important regulator and effector of the inflammatory process, and increased levels of several main components have been found in the vitreous fluid of diabetic patients with PDR (138,139). However, the role of the complement system in the early stages of DR remains to be elucidated. In the present study, we have found that glial cell activation is associated with a reset in the homeostasis of the complement system, consisting of a complex balance between the increase of several complement compounds and complement inhibitors that, although not leading to MAC activation, could participate in the pathogenesis of early stages of DR. Overall, this observation points to complement as a potential new therapeutic target of DR.

We previously reported the presence of activated complement products in the vitreous fluid of patients with PDR (138). Our findings have been further confirmed by other authors (139,231). We also detected a decrease of clusterin, an inhibitor of complement, in the vitreous fluid of patients with diabetic macular edema (142). The results from these two previous studies (138,142) in advanced DR, as well as the current results from the proteomic analysis are summarized in **Fig. 35**.

In addition, deposits of MAC have been reported within retinal blood vessels and choriocapillaries of human donors with diabetes (232,233), pointing to the role of the complement system in the vascular pathology of DR. Furthermore, a reduction of the retinal levels of the complement inhibitors CD55 and CD59 was detected in a study performed in retinas from diabetic subjects with NPDR (232).

Our findings from the proteomic analysis indicate that activation of the C4 component of the complement cascade occurs in the early stages of DR, but only when glial activation becomes evident. In our previous study an increase in C4 in vitreous from PDR patients was also detected

(138). Overall, these results indicate that the activation of the classical and/or the lectin pathways, leading to an increase of C4, may be implicated in the pathogenesis of both early and advanced stages of DR.

We have previously reported the activation of the alternative pathway of the complement system in patients with PDR, which results in an increase in C3 and factor B in the vitreous of subjects with PDR. However, in the present study, increased C3 was not observed in diabetic retinas. By contrast, factor B was increased in diabetic donors with glial activation in comparison with non-diabetic donors. Therefore, it seems that factor B rather than C3 may have a primary role in the activation of the alternative pathway during early stages of DR.

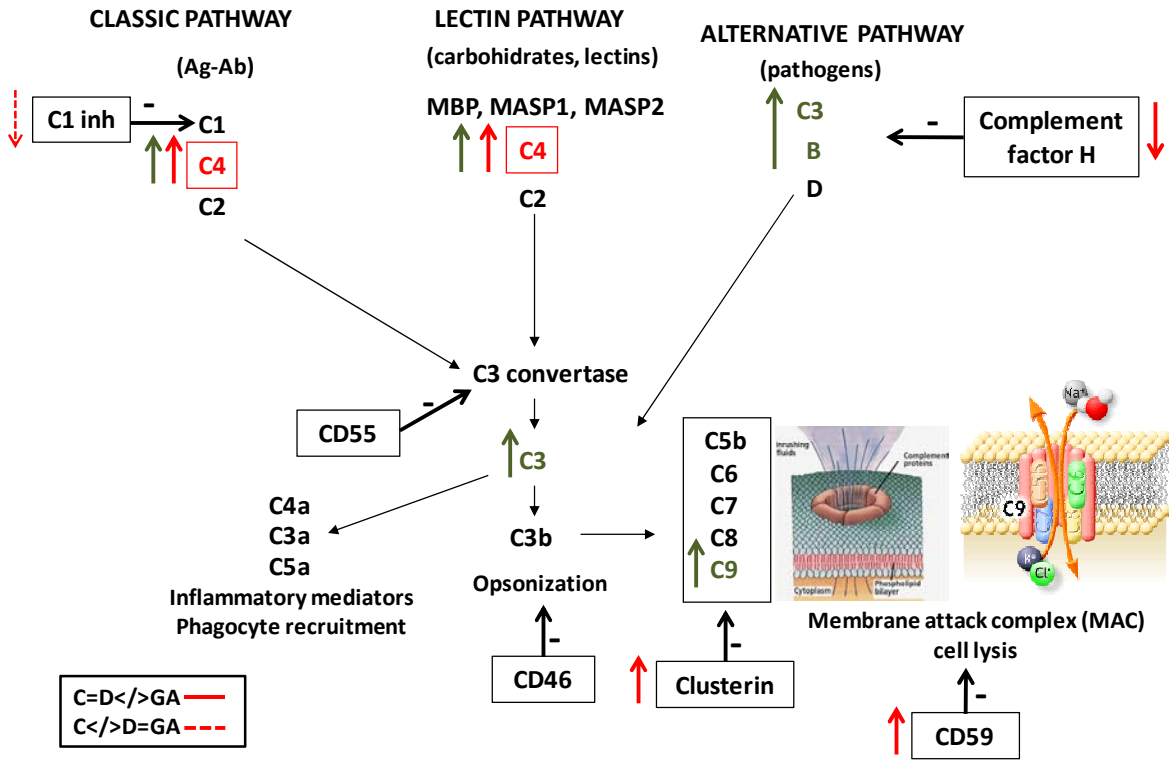


Figure 35. *Schematic description of the complement system. In red are represented the changes observed in the current proteomic analysis in human retinas with glial activation. In green are represented the changes observed in two previous proteomic analysis in human retinas from subjects affected with PDR (138) and diabetic macular edema (142).*

The dysregulation of the inhibitors of the complement system found in the present study merits special emphasis:

- First, the C1INH inhibitor was significantly reduced in diabetic retinas compared with non-diabetic retinas. Therefore, it is possible that the loss of this complement inhibitor could participate in activation of the classical pathway. Apart from C1INH, downregulation of CD55 was also detected in the set of retinas assessed by western blot analysis and, therefore, it might also be involved in classical pathway activation. It should be noted that a major role of C1INH is to prevent the development of excessive vascular permeability

and a partial deficiency of C1INH results in hereditary angioedema, a disease characterized by recurrent episodes of localized edema of the skin or of the mucosa of the gastrointestinal tract or upper airway. Although the anti-inflammatory effects of C1INH have been attributed to its inhibitory regulation of complement system, a direct effect cannot be ruled out (234). In addition, C1INH is also the primary regulator of the contact system, inhibiting factor XIIa and plasma kallikrein. Plasma kallikrein, a member of the kallikrein-kinin system (KKS), catalyzes the release of the bioactive peptide bradykinin, which induces inflammation, vasodilation and vessel permeability (234,235). Preclinical experiments on diabetic animals showed that inhibition of KKS components was effective in decreasing retinal vascular permeability, and KKS is an emerging therapeutic target for the treatment of diabetic macular edema DME (236,237). For these reasons the downregulation of C1INH observed in the diabetic retinas could be essential in favoring vascular leakage and can be considered as a new therapeutic target for treating the early stages of DR.

- Second, complement factor H (CFH) was also downregulated in retinas with glial activation compared with diabetic retinas without glial activation and controls. CFH regulates the complement system as a cofactor of complement factor I by inactivating C3b. CFH also accelerates the decay of the C3 convertase C3bBb of the alternative pathway, and competes with factor B for binding to C3b (238,239). The present study indicates that the decrease in CFH could be implicated in the activation of the alternative pathway of the complement system in early stages of DR. In this regard Lundh von Leithner et al (239) using a CFH-knockout mice demonstrated that the absence of CFH promoted the accumulation of C3 on the vascular endothelium in the neuroretina and the RPE-choroid interface, and it was associated with a reduced perfusion and leukostasis. In the same model, glial activation and thinning of the photoreceptor layer with a global increase in retinal thickness have been reported (240). This finding could be explained by the concurrence of neurodegeneration (glial activation and neural loss) and mild edema due to increased permeability of the BRB. Interestingly, in an animal model of Alzheimer's disease, a decrease in CFH was observed in both the retina and the brain, which correlated with beta-amyloid deposits (241). Moreover, the Y402H (1277T > C) polymorphism in CFH is associated with the development of Alzheimer's disease (242). All these findings are important because the activation of the alternative pathway secondary to CFH downregulation could be added to the recently identified common pathways related to neurodegeneration shared by the brain and the diabetic retina (185). It should be mentioned that a variation in the CFH gene has been associated with a significantly increased risk of age-related macular degeneration (238), and a polymorphism, rs800292 (I62V), in this gene has also recently been associated with DR (243).
- Third, in the western blot analysis we also observed a downregulation of two membrane-bound complement regulators: CD46 and CD55. CD46 is a cofactor for the factor-1 mediated cleavage of C3b and C4b, and CD55 accelerates the decay of already formed convertases. The downregulation of these complement inhibitors would contribute to the activation of the complement system
- Finally, complement inhibitors CD59 and clusterin were increased in retinas with glial activation. CD59 inhibits the final step in the MAC assembly. In fact, in an animal model of diabetes, human soluble CD59 (sCD59) transgene expressed from adeno-associated virus administered by an intravitreal injection attenuated the retinal vascular leakage, the

non-perfusion areas and ganglion cell apoptosis. Unexpectedly, sCD59 increased GFAP staining, suggesting that sCD59 is capable of activating Müller cells (244). Our findings suggest that the upregulation of CD59 observed in diabetic donors with glial activation could act as a compensatory mechanism for the aforementioned complement activation, thus preventing the deleterious effects of MAC activation. By contrast, Zhang et al. (232) found a significant reduction of CD59 in retinal samples from diabetic donors, thus suggesting that the loss of this regulatory mechanism may be the cause of increased complement activation. However, information regarding the fundus examination before death was unavailable in the study by Zhang et al. (232), and therefore, it is possible that diabetic donors had more advanced DR than in the current study. Regarding clusterin, we observed an upregulation in diabetic retinas with glial activation. Clusterin inhibits the inflammatory response by binding the C5B-9 complex, resulting in its inactivation (245). Moreover, clusterin is also capable of inhibiting VEGF-induced hyperpermeablility, thus abrogating BRB breakdown (246). In a previous study using vitreous fluid of patients with DME we found a decrease rather than an increase of clusterin in comparison with non-diabetic control (142). Overall, these finding suggest that in early stages of DR it is plausible that both CD59 and clusterin plays a protective role, while in more advanced stages of DR their loss may contribute to DME/DR development.

To the best of our knowledge this is the first study aimed at examining the relationship between glial activation and the complement system using human retinas in very early stages of DR, and the results of protein identification by mass spectrometry were validated by orthogonal methods. Our results suggest that, in early stages of DR, glial activation is associated with a dysregulation of the complement system, which could participate in the deleterious effects on both microvessels and neuroglia that occur in the diabetic retina. It should be noted that several treatment options targeting the complement system are being considered for its use in Age-related Macular Edema in Phase II and III clinical trials (247). Therefore, treatments aimed at modulating the complement system would appear to represent a promising option for treating DR.

5.1.2.1.2 Other inflammatory mediators identified

ICAM-1 (Intercellular Adhesion Molecule 1): ICAM- 1 is the most important adhesion molecule in DR. Indeed, ICAM-1 is found to be highly expressed in the blood vessels of the retina, choroid, and fibrovascular membrane in patients with diabetes, and its expression correlates with the number of migrated neutrophils in the retina and choroid of these patients, thus indicating that elevated ICAM-1 facilitates leukocyte recruitment (38). Accordingly, ICAM-1 blockade prevents diabetic retinal leukostasis and vascular leakage in a rat model of streptozotocin-induced diabetes (248).

DDRGK1 (DDRGK domain-containing protein): DDRGK1 expression is induced by endoplasmic reticulum stress (ERS) (249). Recently it has been demonstrated that DDRGK1 depletion decreases the expression of NF-κβ target genes (i.e. cytokines, chemokines and adhesion molecules), suggesting that DDRGK1 plays an important role in NF-κβ signaling pathway (249). There is increasing evidence indicating that NF-κβ plays an important role in the pathogenesis of early stages of DR, as it induces leukostasis by inducing ICAM-1 expression, and enhances the expression of pro-inflammatory cytokines and VEGF (83). In addition, DDRGK-1 depletion

induces ERSS-induced apoptosis (250). ERS has been involved in vascular abnormalities (loss of pericytes) and neuron apoptosis in DR (251). Thus, it could be hypothesized that under ERS, the overexpression of DDRGK-1 would have an anti-apoptotic and anti-inflammatory effect, protecting the retina from neurodegeneration. In our samples DDRGK1 was overexpressed in the retinas with glial activation, and we interpreted it as an attempt of protective response to ER stress.

Chitinase-3-like protein 1 (CHI3L1): CHI3L is a member of the protein family of chi-lectins and it is produced by a variety of cells, including neutrophils, monocytes/macrophages, monocyte-derived dendritic cells, osteoclasts, chondrocytes, fibroblasts, vascular smooth muscle cells and endothelial cells. CHI3L1 is regulated by various pro-inflammatory cytokines including IL-6, INF-γ, IL-1β and TNF-α, and stimulates the production of inflammatory mediators (MMP-9, CCL2, CXCL2, etc) and promotes proliferation and angiogenesis. However, no cellular receptors mediating its biological effects have been identified. CHI3L1 is elevated in individuals with obesity and/or T2DM, in association of other mediators of inflammation, and it has been implicated with endothelial dysfunction and atherosclerosis (252). It has been reported that plasma levels of CHI3L1 were higher in diabetic subjects with DR compared to controls. In addition, subjects affected with severe NPDR presented higher plasma levels of CHI3L1 than those with mild NPDR. Moreover, correlation analysis revealed that the outer diameter retinal blood vessels positively correlated with CHI3L1 levels, and negatively correlated with the number of retinal vessels (253). Additionally, a decreased DNA methylation of CHI3L1 was observed in a subject affected with PDR, which may contribute to the increased expression of this gene in diabetic patients (254). Similarly, CHI3L1 significantly correlated with diabetic nephropathy severity (albuminuria) in early stages of nephropathy in T2DM patients (255). To the best of my knowledge this is the first time that CHI3L1it is detected in the diabetic eye in humans. Our results showing an increase of this protein in those patients with glial activation suggest that CHI3L1could be a new pro-inflammatory player in the pathogenesis of DR.

Bifunctional glutamate/proline tRNA ligase: Bifunctional glutamate/proline tRNA ligase is a component of GAIT (gamma interferon-activated inhibitor of translation) complex which mediates INFγ-induced transcript-selective inhibition in inflammation processes. Among the targets that are suppressed by the GAIT complex are VEGF-A, ceruloplasmin, death-associated protein kinases DAPK and ZIPK, and several chemokines and their receptors (255, 256). We found it significantly downregulated in the retinas with glial activation. This finding could contribute to explain the results reported by Vujosevic et al, found a significant increase of INFγ in diabetic retinas compared with controls (88). INFγ upregulates a variety of pro-inflammatory parameters that have been implicated with diabetic retinopathy such as TNFα, interferon-inducible protein-10 (IP-10) and NF-κβ (38,257). Altogether, these results points to the downregulation of bifunctional glutamate/proline tRNA ligase as another mechanism accounting for the inflammatory milieu that exist in DR.

Leukotriene biosynthesis: Leukotrienes are a family of lipid mediators that play important roles in inflammatory reactions. Our findings are summarized in **Fig. 36** and suggest an increase in leukotriene biosynthesis in retinas with glial activation. Leukotrienes intensify the inflammatory response in the retina, upregulate the production of free radicals and subsequent oxidative stress and apoptosis of retinal neuronal and endothelial cells (258).

We have detected an increase in Coactosin-like-protein (CLP), which is a 5-Lipoxygenase (5LO) binding protein that up-regulates its activity both in vitro and in vivo (259). 5-LO is a key enzyme in leukotrienes biosynthesis: it catalyzes LTA4 production, which is the first step in leukotrienes biosynthesis. Diabetic 5LO knockout mice were protected from diabetes-induced increase in leukocyte adherence, didn't presented diabetes-induced increase in superoxide generation, and didn't express NF-κβ in the ganglion cell layer (260). Talahalli et al. demonstrated in a diabetic mouse model that retinal endothelial cells and glial cells can produce LTB4 from LTA4, and that the acquisition of LTA4 is by a transcellular passing from adhered leukocytes to endothelial cells (261). The presence of abundant coactosin-like protein 1 in the retinas of our diabetic donors with glial activation could be attributed to the local production by leukocytes infiltrating the retina or from leukocytes adhered to retinal microvasculature.

LTA4H (Leukotriene A-4 hydrolase), a key enzyme and rate-limiting step in the biosynthesis of leukotriene B4, was also increased. It is a potent chemoattractant that triggers adherence and aggregation of leukocytes to the endothelium (262). Interestingly, intravitreal injection of bestatin, an inhibitor of LTA4H, in a streptozotocin-induced NPDR animal model reversed BRD breakdown and inflammatory leukostasis (263).

Finally, prostaglandin reductase 1 (PTGR1) was reduced in retinas with glial activation. This enzyme catalyzes the conversion of LTB4 into its biologically less active metabolite (264). Our findings indicate that this enzyme is decreased in the retinas with glial activation. Consequently in these retinas leukotrienes will increase, thus intensifying the inflammatory response mentioned above. On the other hand, oxidative stress has been implicated in neurodegeneration and astrocytic-specific Nrf2 activation confers protection against oxidative stress. Dowell et al (265) found that PTGR1 is one of the critical enzyme implicated in Nrf2-mediated neuroprotection in astrocytes. Thus, it is possible that PTGR1 protects glial cells from oxidative stress and its downregulation participates in diabetes-induced neurodegeneration not only in the retina but also in the brain.

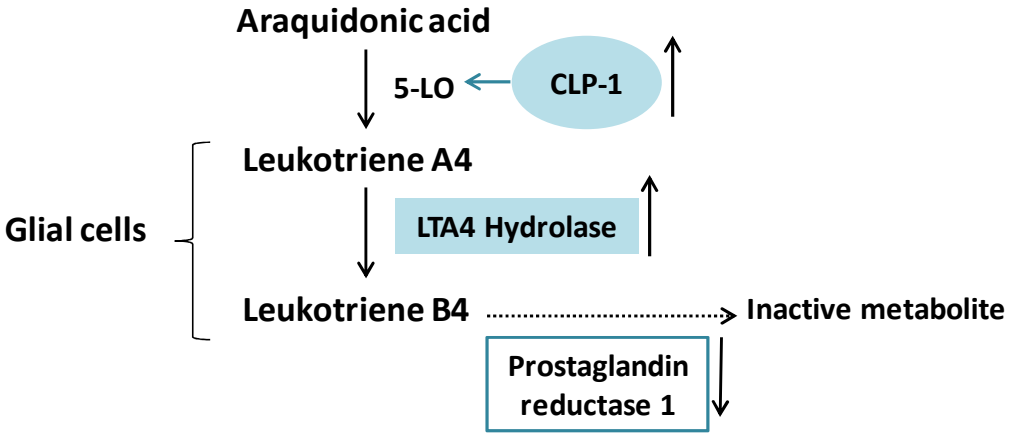


Figure 36. *A schematic representation of the findings obtained from the proteomic study in the setting of the main pathway involved in leukotriene biosynthesis.*

5.1.2.2 ERM proteins

Vascular permeability occurs by an increase of paracellular and transcellular flux. The paracellular flux consists in the formation of gaps between endothelial cells. The gaps are formed as a consequence of the endothelial contraction that follows actin and myosin contraction (77,78). Actin cytoskeleton organization is essential for endothelial barrier function. In fact, actin filaments associated with binding proteins form cytoplasmic focal adhesion plaques. Focal adhesion proteins bind to the transmembrane integrin receptor which, in turn, binds the extracellular matrix. Moreover, the actin cytoskeleton is invariably involved in the assembly and stabilization of tight junctions. When endothelial cells are activated in response to permeability-inducing agonists, two main changes in the cytoskeleton occur: a) Reorganization of the focal adhesions to sites where the stress actin fibers will be anchored. b) Actomyosin contraction. These cytoskeleton changes induce the formation of gaps between endothelial cells due to disassembly of cell-cell junctions and/or endothelial retraction (99–101).

In the proteomic analysis we have identified proteins involved in the cytoskeleton organization (ERM proteins and Rho-GDP-dissociation inhibitors 1 and 2) which were increased in retinas with glial activation whereas proteins that conform the focal adhesions (Talin 1 and 2) or tight junctions (Isoform A2 tight junction protein ZO-2) where decreased. Some o these findings have been also demonstrated in db/db mice.

5.1.2.2.1 ERM proteins and Rho-GDP-dissociation inhibitors

Ezrin, radixin and moesin (ERM proteins) are membrane-associated proteins and present a highly homologous primary structure. The three proteins, which were found increased in retinas with glial activation, share a common domain at the amino terminus: the 4.1-ezrin, radixin, moesin (FERM) domain, which makes them members of the band 4.1 superfamily. Through the carboxyl terminus domain they interact directly with membrane proteins (such as CD43, CD44, ICAM-1, -2 and -3) regulating their attachment to actin filaments. The regulation of membrane proteins and actin filament binding, in which ERM proteins are involved, is crucial in determining cell shape and surface structure, cell adhesion, motility, and the integration of membrane transport with signaling pathways (266–269).

ERM proteins present two conformations: an inactivated one, in which they are folded by an intramolecular interaction between the amino- and carboxy- terminal domains; and an activated conformation, where the two domains separate, thus unmasking their binding sites. To unfold the ERM proteins phosphorylation at a carboxy-terminal threonine residue is needed. Rho-kinases, Rho pathway, PKC-α and PKC-θ proteins are able to phosphorylate and subsequently activate the ERM proteins. In fact, ERM proteins seem to be effectors of the PKC and Rho pathways. Interestingly, there is evidence indicating that they could also be activated without C-terminal phosphorylation. Both phosphorylation-mediated activation and the non-phosphorylation dependent activation require the presence of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P) (267,270). Thus, ERM activation is regulated by a combination of phosphorylation and phospholipid binding.

Rho-GDP-dissociation inhibitors (RhoGDI) are also increased in retinas with glial activation. RhoGDI are a sequestering factor, and their interaction with the ERM proteins allow the RhoGTPases to become loaded with GTP and be subsequently activated. The Rho family of small GTPases acts as a molecular switch for the interaction of actin and myosin that induce endothelial contraction which is one of the mechanisms that participate in vascular permeability. At the same time, Rho-GTPases are able to activate ERM proteins by phosphorylation (**Fig. 37**).

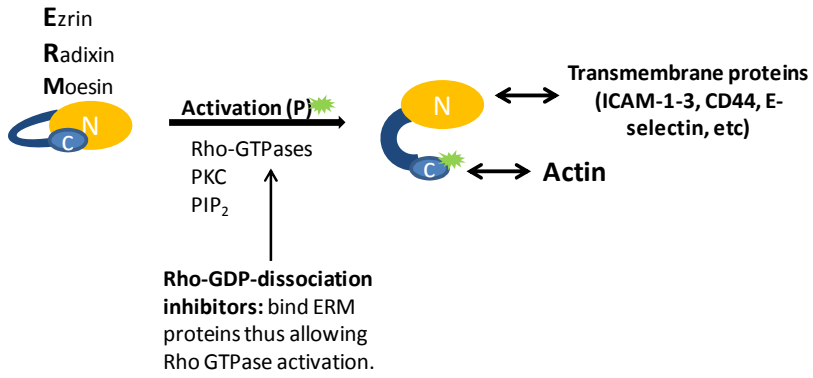


Figure 37. *Schematic representation of ERM proteins activation. When activated (phosphorylation = P), their binding sites are unfolded and are able to interact with transmembrane proteins and actin filaments.*

In summary, when ERM proteins are activated, they are able to bind both actin filaments and transmembrane proteins and lead to the reorganization of the cytoskeleton by forming stress fibers. Stress fiber formation and RhoGTPases activation, which are also activated by ERM phosphorylated proteins, induce actomyosin contraction which leads to vascular leakage (102.270-272). The changes that occur in endothelial cells following ERM activation are summarized in **Figure 38**.

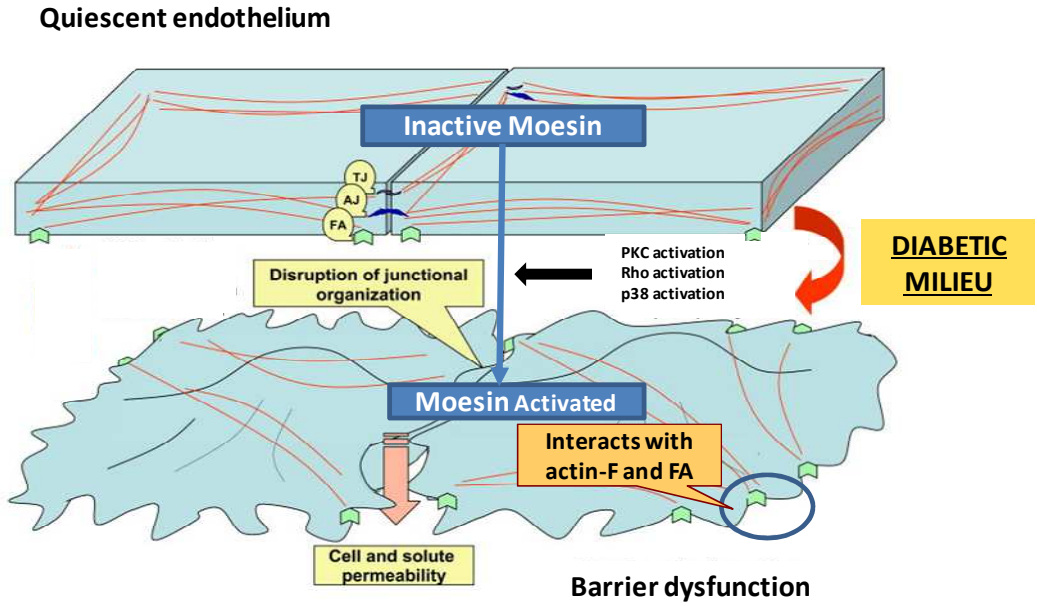


Figure 38. *Cytoskeleton changes in endothelial cells following moesin activation leading to increased permeability. Adapted from* (101)

We also showed an increase of ezrin and moesin proteins in the retinas of diabetic vs. non-diabetic db/db mice. Moreover, ezrin co-localized with collagen IV, thus suggesting that it is expressed in the retinal endothelial. However, we were not able to confirm the increase in radixin expression by western blot. In this regard it should mentioned that moesin has a more prominent effect than ezrin, and is the most expressed protein in several types of endothelial cells (271).

The implication of ERM proteins and their activation linked with the vascular leakage observed in early DR is also supported by the fact that several pathogenic pathways involved in diabetic complications are able to activate them. The pathways involved in ERM activation are summarized in **Fig. 39** and are the following:

*AGE mediated moesin phosphorylation induces vascular permeability***:** The accumulation of AGE in the diabetic milieu, by a direct effect and/or indirect effect through its receptor (RAGE), is one of the pathways involved in the increase of vascular permeability. But the exact mechanisms that lead to vascular leakage remain to be elucidated. Gup et al (272) demonstrated that the suppression of moesin expression (using a specific siRNA) prevented the cytoskeleton changes induced by AGE treatment in HMVEC (human dermal microvascular endothelial cells), which prevented albumin permeability. In addition, there is evidence that vascular leakage induced by AGE-mediated moesin phophorylation occurs in endothelial cells of brain (273) and retina (274) in murine models. AGEs activate ERM proteins through the activation of MAPK and RhoA kinase (266,268,269,275).

Inflammatory mediators that activate ERM proteins:

- High-mobility group box 1 (HMGB1): High-mobility group box 1 (HMGB1) is a non-histone DNA-binding protein that stabilizes nucleosome formation and facilitates transcription. Under inflammatory conditions, it migrates outside the nucleus to the extracellular space where it is able to interact with toll-like receptor (TLR)-2 or -4 and RAGE, activating NF-$\kappa\beta$ and inducing the expression of proinflammatory molecules such as TNF-α, MCP-1, and ICAM-1 (276,277). The levels of HMGB1 are increased in blood samples of type 2 diabetic subjects compared with controls (278) and it has been associated with vascular damage both in diabetic nephropathy (279) and DR (129,280). Lee et al (276) demonstrated that HMBG1 induced the reorganization of actin filaments and paracellular gap formation, and that these effects depended on moesin. Moreover, they found that the HMBG1 induced moesin-phosphorylation is mediated by the binding of RAGE, which activated the Rho- and p38 MAPK pathways.

- Tumor necrosis factor-α (TNF- α): TNF-α is a pro-inflammatory cytokine that has been associated with insulin-resistance, T2D and its microvascular complications (284, 285). TNF-α has been detected in the vitreous of diabetic patients and it is expressed in epiretinal membranes from PDR affected subjects (282). The main actions of TNF-α are the chemoattraction of inflammatory cells, the induction of apoptosis and the increase of vascular permeability. TNF-α action is mediated by the activation of PKC isoforms and p38 MAPK (130), which are able to phosphorylate ERM proteins. In fact, Koss et al (283) observed that Human Pulmonary Microvascular Endothelial Cells exposition to TNF-α induced the phosphorylation of ERM proteins localized in the cell periphery, thus leading to actin cytoskeleton reorganization and the formation of paracellular gaps.

Oxidative stress can also induce moesin phosphorylation: Mangialardi et al [79], studied bone marrow microangiopathy and in particular endothelial barrier dysfunction mediated by Rho-pathway. One interesting observation is that the exposure to N-acetyl-cysteine, which is a ROS scavenger, reduced the phosphorylation of moesin induced by diabetes in Bone Marrow Endothelial Cells (BMEC). This finding supports the concept that oxidative stress could also trigger moesin phosphorylation.

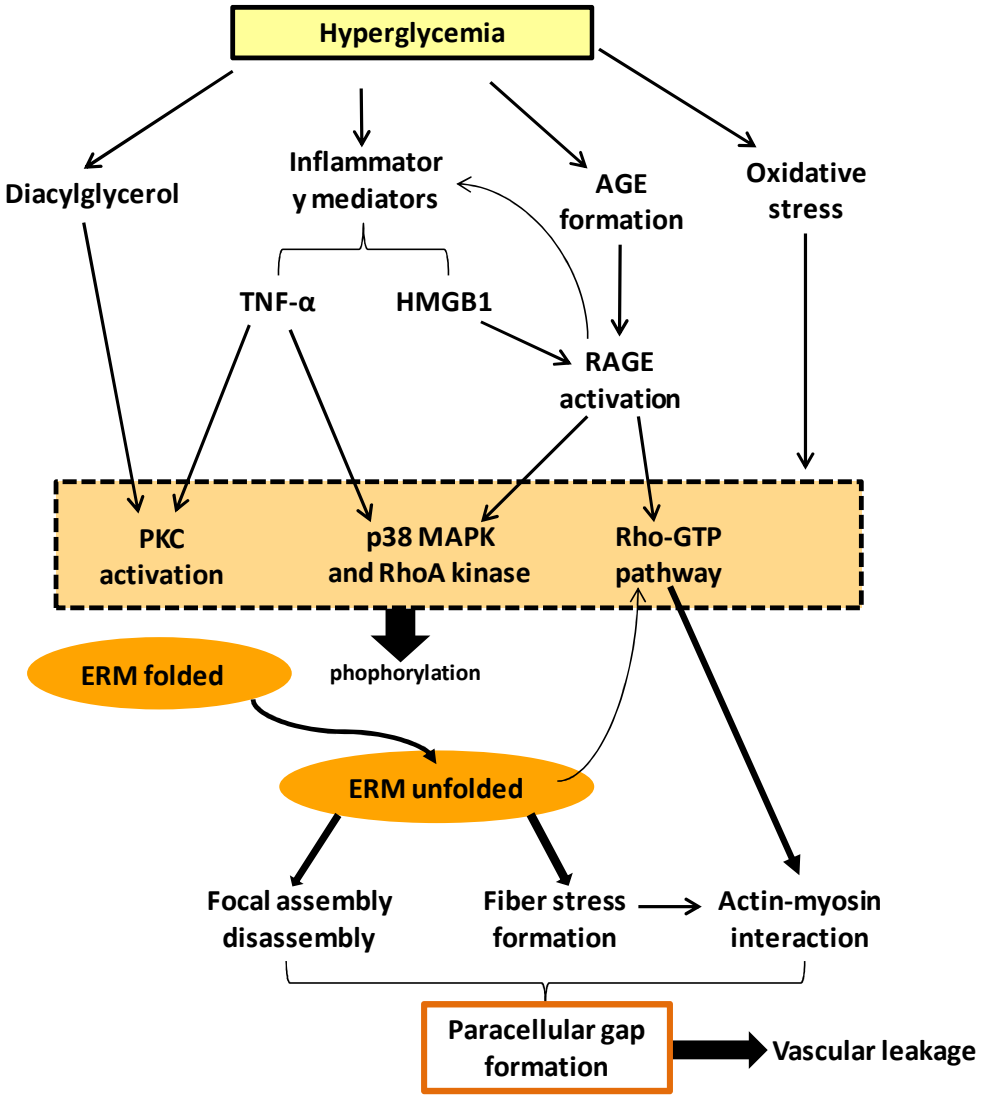


Figure 39. *Molecular pathways involved in ERM activation and vascular leakage in diabetes* (284).

5.1.2.2.2 Talin-1,2 and Isoform A2 tight junction protein ZO-2

The integrity of the BRB is maintained by adhesions between endothelial cells and the extracellular matrix (focal adhesions), and by adhesions between endothelial cells (tight junctions) (54). In the present study, Talin-1, Talin-2 and Isoform A3 tight junction protein ZO-2 were decreased in retinas with glial activation.

The isoform A3 tight junction protein ZO-2 is part of the zonula ocluddens (ZO) family, which is an essential component of tight junctions in retinal endothelial cells. Kim et al. (289) demonstrated in an animal model and cell cultures that an increased vascular permeability in diabetic retinas is accompanied by a decrease of ZO-1 and ZO-2. Our findings support that this concept is also true in humans. One of the proposed mechanisms is the downregulation of the expression of tight junction proteins by PKC activation mediated by TNF-α (130).

Talin is an important protein for cytoskeleton organization and focal adhesions. It connect integrin with the actin filaments, acting as a mechanical link between the ECM (Extracellular Matrix) and cytoskeleton (285–287) (**Fig. 40**). Integrins bind with proteins of the ECM in the cell surface, and can switch from an inactive (low affinity), primed (active but unengaged) and fully active (ligand-bound) states. The interaction between talin and integrin is a critical step for integrin activation (287–289). Once engaged, integrins cluster into nascent focal complexes and recruit additional proteins, maturing into long, stable focal adhesion (289). In endothelial cells, integrin activation is induced by growth factors, including VEGF, and mechanical stress (286). It could be hypothesized that in the retinal endothelium there is a constituent presence of talin that activates integrin promoting the formation of focal adhesions that would contribute in the maintenance of the BRB. Furthermore, in vitro assays have demonstrated that activated moesin can displace talin from integrin. Moesin-integrin interaction, inhibits integrin function, favoring membrane retraction and focal adhesion disassembly (288,289) (**Fig. 40**).

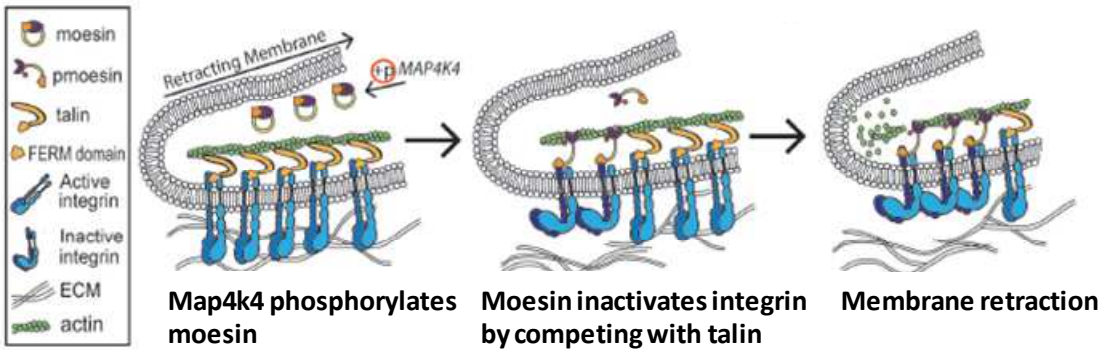


Figure 40. *Schematic representation of moesin-integrin interaction, following moesin activation and talin displacement. Adapted from* (289)

Based on all these results, we hypothesize that in diabetic retinas with glial activation, the loss of focal adhesions and tight junctions, and the endothelium contraction induced by ERM proteins (especially moesin) phosphorylation are one of the mechanisms implicated in the early microvascular impairment observed in DR (**Fig. 41**).

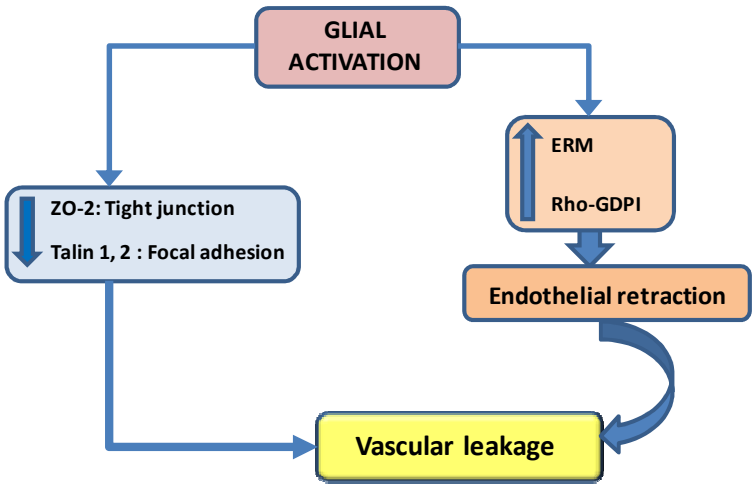


Figure 41. *Summary of the results from the proteomic analysis regarding proteins involved in the maintenance or disruption of the BRB, and its potential relationship with diabetes-induced vascular-leakage.*

5.1.3 Limitations of the Study

The "Free hypothesis" part of this thesis presents some limitations. The main limiting factors are the potential post-mortem artifacts in human retinas, which have been minimized due to the rapid collection of samples (less than 6 hours), and the small number of samples used in both human and db/db mouse experiments. Therefore, our findings need to be confirmed in larger number of samples. Secondly, the presence of microvascular abnormalities at death cannot be completely ruled out, but the absence of apparent microangiopathy in the ophthalmologic examinations performed 1 to 2 years before death and immediately before the euthanasia in mice makes this eventuality very unlikely. Third, which cell types within the complex cellular architecture of the retina contribute to the observed changes must be elucidated.

Experimental study: driven hypothesis

5.2 Topical administration of bosentan is effective preventing neurodegeneration in diabetic retina

Increased circulating levels of ET-1, a potent vasoconstrictor peptide, has been found in patients with diabetes, and a positive association with microangiopathy has been observed (290). In addition, ET-1 has been reported elevated in the vitreous (291) and aqueous humor (292) of diabetic patients with DR. Moreover, ETA and ETB have been found upregulated in rats with STZ-induced diabetes (123). In the present study ET-1 and its receptors ETA and ETB were early upregulated in the retina of db/db mice. In addition, we found for the first time that ET-1 and its receptors are upregulated in human retinas at very early stages of DR.

The mechanisms involved in the upregulation of ET receptors remain to be elucidated. The dual deleterious effect of ET-1 on both microvasculature and neurons point to ET-1/ETA-ETB system impairment as a crucial player in the neurovascular unit dysfunction (59).

In the present study we provided first evidence that topical ocular treatment with bosentan, a dual endothelin receptor antagonist, resulted in a significant decrease of both glial activation and the rate of apoptosis in comparison with diabetic mice treated with vehicle. These effects run in parallel with a decrease of ETA and ETB receptors. The beneficial effect of orally administered ET-1 receptor antagonist on microvascular abnormalities induced by diabetes such as decrease of retinal flow, basement membrane thickening, and capillary degeneration have been previously described (124,293–295). However, to our knowledge, no data regarding the beneficial effects of bosentan on retinal neurodegeneration caused by diabetes and its capacity of downregulate ETB has been previously reported. Chou et al. (124) reported that the orally administration of atrasentan, a selective ETA receptor antagonist, was able to ameliorate vascular regression in db/db mouse. However, the effect on neuroretinal apoptosis measured by TUNEL was only partial. This finding supports the idea that the blockage of the ETB receptor is essential to prevent retinal neurodegeneration induced by diabetes. In support of this notion it has been reported that ETB receptors are involved in retinal ganglion cell loss induced by glaucoma (125,296) and by optic nerve injury (126).

ET-1 expression induced by hyperglycemia in diabetes is partly due to activation of PKC-beta and -delta isoforms (297). It should be noted that the PKC β isoform is involved in diabetic complications and in particular in DR. In fact, it has been considered a therapeutic target (298). Notably, in the present study we found that bosentan prevented the upregulation of PKC β induced by diabetes in the neuroretina.

In addition, we found that bosentan inhibited the upregulation of ET-1 induced by TNF-α in HRECs. Moreover, bosentan significantly reduced the hyperpermeability induced by TNF-α in HRECs. Furthermore, bosentan topically administered was able to prevent the upregulation of TNF-α in retinas from diabetic mice. It should be noted that TNF-α participates in the breakdown of the blood-retinal barrier (BRB) by downregulating tight junction proteins of endothelial cells and favoring VEGF-induced permeability (130). Therefore, bosentan by inhibiting the upregulation

of both TNF- α and VEGF would also play a significant role in preventing the hyperpermeability induced by diabetes.

The results of the pharmacokinetic study performed on rabbits suggest that bosentan is mainly reaching the retina through the trans-scleral route. In addition, the pass to systemic circulation is negligible.

In conclusion, we found that upregulation of ET-1 and its receptors (ETA and ETB) is an early event in the diabetic retina, the topical (eye drops) administration of bosentan prevents retinal neurodegeneration induced by diabetes not only by blocking but also by downregulating ETB receptors. The inhibition of the diabetes-induced upregulation of PKC-β, TNF-α and VEGF induced by diabetes seems to play an important role in the beneficial vascular action of bosentan.

Clinical study

5.3 The assessment of retinal fixation by means of retinal microperimetry could be a useful tool to assess the risk of cognitive impairment in diabetic retinas

Currently, in clinical practice, there are no reported phenotypic indicators or reliable examinations for identifying type 2 diabetic patients with MCI. Moreover, the diagnosis of MCI is based neuropsychological tests whose complexity makes their incorporation into current standards of care for the type 2 diabetic population unfeasible. Fundus-driven microperimetry is a non-invasive examination that requires less than 5 minutes to evaluate macular function. In a previous study we demonstrated that the assessment of retinal sensitivity measured by fundus-driven microperimetry is related to brain neurodegeneration and could be a useful biomarker for identifying those patients at risk of developing AD (191). The anatomical pathways involved in the retinal sensitivity assessment are displayed in **Fig 42**. Our current findings indicate that fixational eye movement parameters assessed by microperimetry are useful for identifying T2D subjects at risk of dementia. Moreover, the addition these retinal fixation parameters to the assessment of retinal sensitivity significantly increase the capability to identify those subjects with MCI.

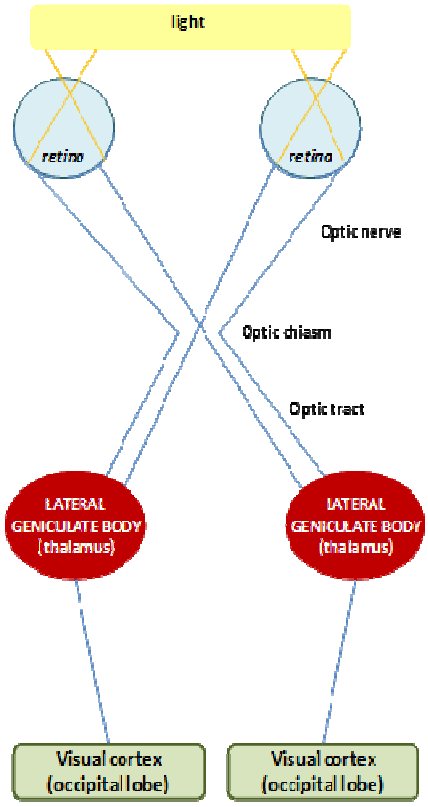


Figure 42. *Pathways involved in the assessment of retinal sensitivity.*

It could be argued that the benefits of the early diagnosis of dementia are questionable because treatment is still unavailable. However, unrecognized cognitive dysfunction can affect treatment adherence and diabetes self-management resulting in poor glycemic control, an increased

frequency of severe hypoglycemic episodes, diabetes-related complications and hospital admissions (31,299). In addition, the diagnosis of cognitive impairment permit us to offer a more personalized treatment (i.e. treatments with a very low capacity of provoking hypoglycemia), and to improve adherence by simplifying the prescribed treatments, thus resulting in an improvement of quality of life. Furthermore, retinal microperimetry is not influenced by non-cognitive functions such as mood or depressive disorders which could influence the results of neuropsychological tests. This is an important advantage given that the prevalence of depression is two-fold higher in type 2 diabetes compared with the general population worldwide (300), and has recently been reported as being 27,5% among type 2 diabetic patients in the Mediterranean population (301). This prevalence rate could be even higher in older adults with diabetes because depressive symptoms may be overlooked (302).

For all these reasons, the retinal functional assessment of ocular movements can be considered an objective method for identifying T2D patients with MCI. This method opens up new feasible procedure for testing cognitive status, which could easily be added to the routine ophthalmologic examination for the screening of DR.

Abnormalities in oculomotor function and viewing behavior have been previously reported in subjects affected with AD. In fact, several eye movements like prosaccades, antisaccades, and the microsaccades and saccadic intrusions that occur during fixation are altered in subjects with AD (192–194). In addition, during fixation, subjects affected with AD present a greater frequency of saccadic intrusions than normal subjects, causing instability gaze maintenance (193). Moreover, microsaccades that occur during normal fixation in AD are notably more oblique compared with normal adults (194).

Little is known about the usefulness of the study of movements to identify the prodromal stages of dementia. Kapoula et al (194) observed that MCI subjects present a major prevalence of oblique microsaccades during fixation compared with control subjects.

The techniques used to evaluate ocular movements were based on electrodes placed in the region around the eyes, and usually incorporated a support to restrict head movements (193,303). New eye-tracking systems have been developed and are usually video-based techniques, which has the advantage of being non-invasive (303–305). One of their main limitations is head movement, because even small movements may cause large errors in the estimation of a calibrated tracker (304). Fundus-driven microperimetry is a video-based technique with the advantage of incorporating a chin and forehead bars that restrict head movements. However, this device has never been used for the assessment of cognitive impairment.

Given that the T2D patients included in the present study did not present signs of DR or other retinal disease, the alterations in fixation detected in those patients with cognitive impairment could be attributed to a brain neurodegenerative disease rather than an intrinsic eye disease. Marseglia et al (306) showed that the presence of T2D affects perceptual speed, attention and primary memory in patients of 70-75 years. They postulate that these domains might be primarily affected by diabetes. It should be noted that fixational eye movements are affected by attention and working memory (192). For example, microsaccade rates transiently decrease during an attentional task (307). Therefore, the evaluation of fixation by fundus-driven microperimetry could be a biomarker

of the first neuropsychological abnormalities that occur in diabetes-related cognitive decline. On the other hand, it is also a possibility that neurons closest to the motor output that have been implicated in fixation, known as the omnipause neurons (located in the nucleus raphe interpositus of the paramedian pontine reticular formation) are affected by the neurodegenerative process. Other structures, like the superior colliculus could be also implicated. The superior colliculus (SC) contains an eye-centered map, and across this map neurons in the intermediate layers increase their firing rates when potential targets are present at particular eccentric locations triggering saccades to peripheral targets (308). The pathways involved in fixation and involuntary eye movements are summarized in **Fig. 41**. During microperimetry, the subjects are asked to maintain their gaze fixated on a central target, while stimuli with different intensities are presented at 37 points in 3 concentric circles of 2, 6, and 10 degrees of diameter (309). It could be hypothesized that in subjects with cognitive impairment are unable to inhibit the saccades triggered by the most eccentric stimuli and maintain their gaze fixated on a central target. Furthermore, certain areas of the cerebral cortex, such as the parietal and frontal cortex (frontal eye fields and the dorsomedial prefrontal cortex), show elevated firing rates during fixation. These areas could contribute to the control of fixation through descending projections to the SC and the omnipause region (308).

The significant increase in sensibility in discriminating T2D subjects with MCI from T2D normocognitive subjects using the combination of retinal sensitivity plus parameters of fixation (BCEA63 and BCEA95) could be attributed to the fact that the brain areas involved in retinal fixation are not the same as in retinal sensitivity. This would mean that this combined test is examining different neural circuits, thus improving the performance of the examination. In this regard, retinal sensitivity relies on the retina and the neural pathway of vision (**Fig. 43**). The first station of the optic tract is the lateral geniculate body of the thalamus, which is the first major visual processing region in the brain and plays a crucial role in relaying information from the retinal ganglion cells to the primary visual cortex (310). By contrast, the superior colliculus and the parietal and frontal cortex play an essential role in gaze fixation. There is evidence that both the geniculate body and the superior colliculus are affected by AD (311–313). Therefore, it could be hypothesized that the sensibility and sensitivity of microperimetry increases when fixation is taken into account because different pathways are being explored.

The main limiting factor of our study is that we have included patients without DR or only those patients with mild non-proliferative DR. Since the presence of more advanced stages of DR, and in particular DME, can alter fixation (314) we cannot extrapolate our results to the whole diabetic population.

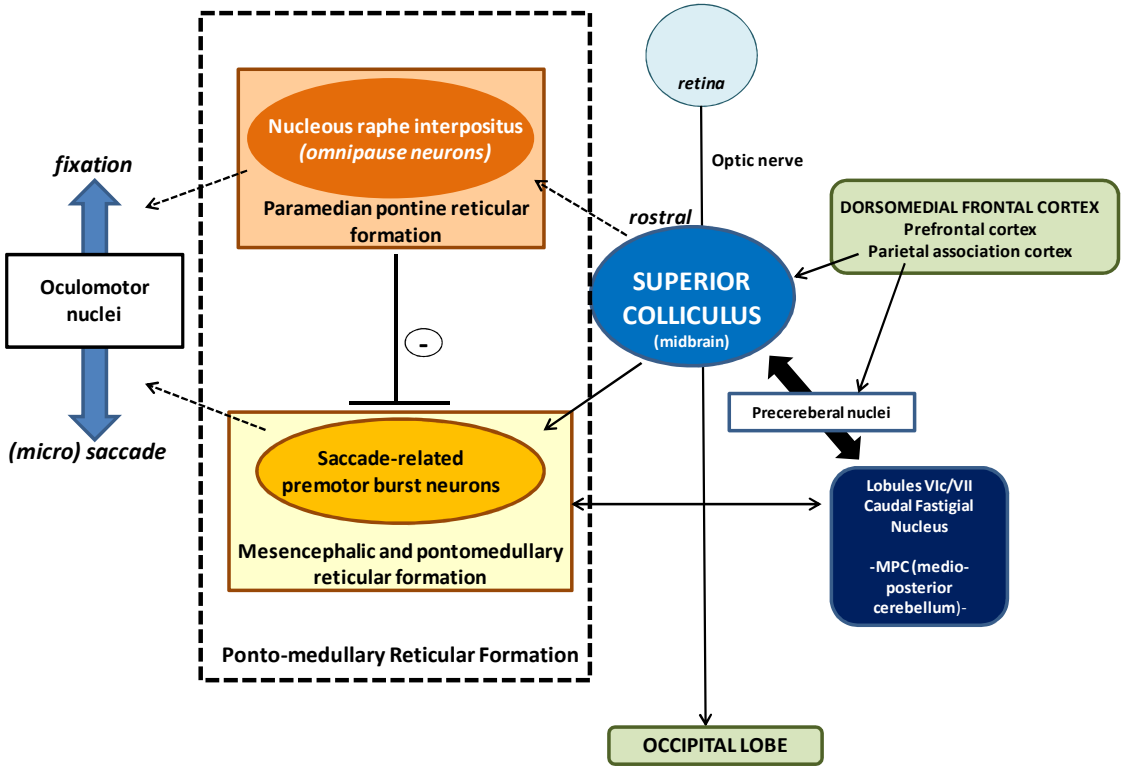


Figure 43. *Pathways involved in fixation and involuntary eye movements.*

6. Conclusions

1. Several pathways involved in the development of brain neurodegenerative disorders (i.e. Alzheimer's and Parkinson's disease) have been identified in the early stages of diabetic retinopathy. This results support the concept that a common soil exists in the neurodegenerative processes that occur in the retina and brain.

2. Proteins involved in axonal transport such as the dynein-dynactin complex and all the cytoskeleton proteins belonging to the axon initial segment (ankyrin, spectrin, neural cell adhesion molecule, neurofascin) are downregulated in the retinas from diabetic donors with glial activation.

3. Diabetes-induced glial activation is associated with a dysregulation of the complement system and other inflammatory mediators. This pro-inflammatory activity could participate in the deleterious effects on both microvessels and the neuroretina that occur in the diabetic retinas.

4. The upregulation of the ERM complex/ Rho-GDP dissociation inhibitors and the downregulation of talin participates in diabetes-induced vascular leakage.

5. The upregulation of ET-1 and its receptors (ETA and ETB) is an early event in the diabetic retina, and the topical administration of bosentan prevents retinal neurodegeneration induced by diabetes not only by blocking but also by downregulating ETB receptors. In addition, the inhibition of diabetes-induced upregulation of PKC-β, TNF-α and VEGF plays an important role in the beneficial vascular action of bosentan.

6. The assessment of gaze fixation abnormalities in T2D is related to cognitive status and could be a useful tool for identifying patients at risk of developing dementia. In addition, the assessment of retinal sensitivity in combination with parameters of fixation by using microperimetry could be a reliable method for detecting prodromal stages of dementia in the T2D population.

7. New Perspectives based on the provided results

The results of the present work support the concept that the study of neurodegeneration in the diabetic retina could help us to better understand the neurodegenerative processes that occur in the brain.

The identification of downregulated proteins (i.e. proteins involved in axonal transport) and the dysregulation of the complement system and proteins involved in the structural composition of cytoskeleton open up new avenues in basic research and provide new insights into the design of therapeutic approaches for treating early stages of DR.

The dual beneficial effects of bosentan (neurotrophic and vasculotropic) observed in our experimental model suggest that this drug is an excellent candidate for testing in clinical trials.

The results obtained by microperimetry combining the assessment of retinal sensitivity and gaze fixation, could be incorporated into the clinical practice. However, large-scale clinical trials are needed. In addition, specific research addressed to unraveling the molecular mediators involved in the impairment of the neurological circuits implicated in both retinal sensitivity and gaze fixation seems warranted.

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