TOWARDS A NEW THERAPY FOR DIABETIC RETINOPATHY:

RAS AND BERRIES

DANIELA FILIPA JORGE DA CRUZ SANTOS

Tese para obtenção do grau de Doutor em Mecanismos de Doença e Medicina Regenerativa

Doutoramento em associação entre:

Universidade NOVA de Lisboa (Faculdade de Ciências Médicas | NOVA Medical School - FCM|NMS/UNL)

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Orientador: Gabriela A. Silva, PhD, NMS-UNL

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Resumo

As doenças da retina são um conjunto de patologias que causam perdas significativas de visão ou, em estadios mais graves, cegueira. De entre este conjunto a Retinopatia Diabética (RD) é uma das mais comuns, sendo uma complicação associada à progressão da Diabetes *mellitus* (DM) que afeta maioritariamente adultos em idade ativa. A RD é classicamente descrita como uma complicação progressiva microvascular associada a inflamação crónica.

Embora o mecanismo exato de eventos associados à patofisiologia da RD não esteja completamente descrito, acredita-se que a exposição crónica a elevados níveis de glucose (hiperglicemia) desencandeia uma cascata de alterações bioquímicas e fisiológicas que inclui danos microvasculares e alterações na barreira hemato-retiniana que levam à perda da sua integridade. Estes eventos permitem a passagem de fluído e fatores com efeito prejudicial, como citoquinas, fatores de crescimento e fatores inflamatórios, assim como a promoção de alterações na expressão de fatores que podem causar danos celulares, desenvolvimento anormal de vasos sanguíneos na retina (neovascularização), inflamação crónica e isquémia.

Atualmente, existem terapias bastante eficazes para a RD, apenas aplicadas numa fase mais avançada da doença. No entanto, existe uma necessidade urgente de desenvolver alternativas que possam ser aplicadas na prevenção da progressão da RD, antes dos primeiros sinais de perda visual.

Neste trabalho propomos estudar a modulação do Sistema da Renina-Angiotensina e as propriedades bioativas dos metabolitos derivados de polifenóis como possíveis abordagens terapêuticas para melhorar as alterações moleculares que levam ao desenvolvimento de RD.

O Sistema da Renina-Angiotensina (SRA) é composto por um complexo número de vias, principalmente descrito pelo seu papel na regulação da pressão arterial. O SRA é expresso em diferentes tecidos, incluindo no olho, sendo responsável pelos efeitos locais e alterações a longo-prazo desses órgãos. Assim, tem vindo a ser sugerido como tendo um papel importante na patologia ocular. A literatura também descreve benefícios na ativação do eixo protetor do SRA para o tratamento de condições que resultam de inflamação crónica e fases proliferativas, como acontece na RD.

Por outro lado, os polifenóis e os seus derivados têm vindo a ser intensamente estudados pelas suas propriedades anti-oxidantes e anti-inflamatórias, com grande potencial terapêutico. Metabolitos sulfatados de Catecol e Pirogalol foram encontrados recentemente e pela primeira vez em circulação no plasma de voluntários após a ingestão de um puré de frutas rico em polifenóis. A identificação destes metabolitos fenólicos leva ao desenvolvimento de uma importante área de investigação no sentido de avaliar o seu potencial, não só em situação de tratamento de doença, mas também como retardadores do seu desenvolvimento.

Usando modelos celulares de retina e animais diabéticos, caracterizámos o SRA da retina e avaliámos como a sua modulação com um inibidor direto da renina (aliscireno) contribui para a melhoria da RD no que diz respeito a eventos de stress oxidativo, angiogénese e inflamação. Além disso, o efeito dos metabolitos de Catecol e Pirogalol na expressão de proteínas que estão descritas como estando envolvidas na patofisiologia da RD foi estudado, explorando o seu potencial terapêutico nestes modelos.

Neste trabalho foi possível mostrar que as células do epitélio pigmentar da retina (do inglês *retinal pigment epithelium*, RPE) expressam os principais componentes do SRA e que este sistema foi desregulado por concentrações elevadas de glucose. A exposição das RPE a angiotensina II diminui os níveis do fator de crescimento endotelial vascular (do inglês *vascular endotelial growth factor*, VEGF) e aumenta simultaneamente a expressão dos componentes do eixo protetor. Adicionalmente, a presença de angiotensina II estimula a produção de espécies reativas de oxigénio nas RPE. O tratamento das RPE com o inibidor direto da renina, aliscireno, diminui a atividade da renina e, consequentemente, diminui os níveis de angiotensina II. Os níveis de stress oxidativo e expressão do fator pro-angiogénico VEGF diminuem após ação do aliscireno e, simultaneamente, a expressão do fator derivado do epitélio pigmentar (do ingês pigment epithelium-derived factor, PEDF) aumenta. Finalmente, a ativação do eixo protetor através da sobre-expressão da enzima conversora da angiotensina 2 protege as células da RPE da inflamação e stress oxidativo.

Adicionalmente, a exposição das RPE ao metabolito sulfatado de pirogalol (Pyr-sulf) protege as células da inflamação em determinadas condições e efetivamente diminui a expressão de VEGF em células expostas a elevadas concentrações de glucose em hipoxia. Além disso, uma única injeção intraocular de Pyr-sulf diminuiu a expressão do marcador da microglia, Iba1, e do fator pró-angiogénico VEGF na retina de animais diabéticos numa fase precoce do desenvolvimento da RD.

Os nossos resultados do SRA da retina, juntamente com a demonstração de que o aliscireno efetivamente bloqueia o sistema em diferentes momentos da cascata, sugerem que este fármaco pode ser uma alternativa na prevenção dos efeitos prejudiciais que resultam da sobre-ativação do SRA na retina. Ao mesmo tempo, a ativação do eixo protetor do SRA (ACE2/Ang-(1-7)/Mas), descrito como tendo efeitos ao nível da célula que contrabalançam os observados pelo eixo ACE/AngII/AT1R, mostra-se como uma abordagem promissora. O efeito do metabolito sulfatado de pirogalol (Pyr-sulf), nas células RPE e na retina diabética abre uma oportunidade para continuar a explorar o efeito terapêutico destes metabolitos como anti-inflamatório na RD.

Palavras chave: Retinopatia Diabética, Sistema Renina-Angiotensina; metabolitos de polifenóis; epitélio pigmentar da retina.

Abstract

Among the group of acquired retinal diseases is Diabetic Retinopathy (DR), a Diabetes *mellitus* (DM) complication affecting mostly working-age adults approximately twenty years after the onset of diabetes, with a significant impact on people's quality of life and socioeconomic consequences. DR is classically regarded as complication with progressive microvascular features affecting the retina with a chronic inflammatory background.

Although the exact mechanism underlying DR pathophysiology is not yet fully understood, it is believed that chronic exposure to hyperglycemia triggers a cascade of biochemical and physiological changes that includes microvascular damage and inner brain-retinal barrier breakdown. These events allow extravasation of fluid and potentially toxic factors, including cytokines, growth factors and inflammatory factors, which might cause excitotoxic damage, vascular wall leakage, neovascularization, chronic inflammation and ischemia.

Nowadays, there are a few effective treatments for DR designed to act on later stages of the disease. However, there is an urgent need to target the progression of the disease, aiming a preventive therapeutic approach that can be useful before the first signs of blindness.

Here we propose to study the modulation of the Renin-Angiotensin System (RAS) and the bioactive properties of (poly)phenols-derived metabolites as possible therapeutic approaches towards DR.

The RAS is composed by an intricate system of pathways, mainly involved in the regulation of the blood pressure. It is known that tissue RAS is responsible for the localized effects and long-term changes in organs, suggesting a role in the pathogenesis of ocular conditions such as DR. Literature also shows evidence supporting the enhancement of the protective axis of RAS for treatment of conditions with chronic inflammation and proliferative stages, which are known DR hallmarks.

On the other hand, (poly)phenols and their derivatives have been intensively studied by their antioxidant and anti-inflammatory properties, with a putative therapeutic impact. The metabolites Catechol-*O*-sulfate (Cat-sulf) and Pyrogallol-*O*-sulfate (Pyr-sulf) were found for the first time by Santos and co-workers circulating in micromolar concentration in human plasma from volunteers after ingestion of a fruit purée. The

identification of these phenolic metabolites raises an emerging and very important research area towards their role in health and disease.

Using a cell model of retinal pigment epithelium cells and a diabetic mouse model, we characterized retinal RAS and evaluated how its modulation by a direct renin inhibitor, aliskiren, contributes to ameliorate the DR regarding oxidative stress, angiogenesis and inflammation. Moreover, we addressed the effect of both Catechol-*O*-sulfate and Pyrogallol-*O*-sulfate metabolites in the expression of several proteins known to have a role in DR pathophysiology, to explore their therapeutic potential.

Here we show that RPE cells express the main RAS constituents and this system was deregulated by hyperglycemic glucose concentrations. Exposure of RPE cells to angiotensin II increased the levels of the main pro-angiogenic factor VEGF in a concentration-dependent manner, while increasing the expression of the protective axis components. Additionally, angiotensin II also stimulated the production of ROS in RPE cells. Treatment of RPE cells with aliskiren inhibited the activity of renin and consequently decreased the levels of angiotensin II. The levels of oxidative stress decreased after treatment and the expression of anti-angiogenic factor PEDF was promoted along with the decrease in the expression of pro-angiogenic VEGF. Finally, the overexpression of ACE2 protects RPE cells from inflammation and oxidative stress.

Moreover, exposure of RPE cells to the (poly)phenol-derived metabolite Pyrogallol-*O*-sulfate, protect against inflammation and effectively decrease the expression of VEGF in cells under high glucose and hypoxia. Additionally, a single intraocular injection of Pyrogallol-*O*-sulfate was able to decrease the expression of the microglial marker Iba1 and pro-angiogenic VEGF in the diabetic retina earlier in the disease progression. Our findings of the RPE RAS, together with the demonstration that aliskiren effectively blocks this system at different steps of the cascade, suggest that aliskiren might be an alternative drug for preventing the deleterious effects derived from the overactivation of the RAS. At the same time, the activation of the ACE2/Ang-(1-7)/Mas axis, known to counterbalance the deleterious effects of ACE/AngII/AT1R axis, presents itself as a promising gene therapy approach. The effect of Pyrogallol-O-sulfate in RPE cells and diabetic retina opens an opportunity to further explore this metabolite as a therapeutic approach towards inflammation in DR.

Key words: Diabetic retinopathy, Renin-Angiotensin System; polyphenols' metabolites; retinal pigment epithelium.

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List of abbreviations

ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitor
AMD	Age-related Macular Degeneration
Ang	Angiotensin
ANOVA	Analysis of variance
ARB	Angiotensin Receptor Blocker
AT1R	Angiotensin II receptor 1
AT2R	Angiotensin II receptor 2
BRB	Blood-Retinal Barrier
Cat-sulf	Cathecol-O-Sulfate
cDNA	Complement deoxyribonucleic acid
CF	Cytosolic fraction
CMV	Citomegalovírus
DAPI	4', 6-Diamidino-2-Phenylindole
DFO	Desferrioxamine
DM	Diabetes mellitus
DME	Diabetic macular edema
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DR	Diabetic Retinopathy
DRI	Direct Renin Inhibitor
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLUT	Glucose transporter
HCl	Hydrochloric acid
HEK	Human embryonic kidney
HG	High Glucose
HRP	Horseradish peroxidase
Iba1	Ionized calcium-binding adapter molecule 1
IL-1β	Interleukin-1 beta
IL-8	Interleukin-8
IL-10	Interleukin-10
MAPK	Mitogen Activated Protein Kinase
Mas1	Mas receptor
MCP-1	Monocyte Chemotactic Protein-1
MF	membrane fraction
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NG	Normoglucose
PDR	Proliferative diabetic retinopathy

PEDF	Pigment epithelium-derived Factor
PBS	Phosphate-buffered saline
PI	propidium iodide
PR	Photoreceptor
Pyr-sulf	Pyrogallol-O-Sulfate
RAS	Renin-Angiotensin System
RES	Resveratrol
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
RT-qPCR	Quantitative Real-Time polymerase chain reaction
SD	Standard Deviation
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
TNF-α	Tumor necrosis factor alpha
TBS	Tris Buffered Saline
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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Simão S*, Santos DF*, Silva GA. Aliskiren inhibits the renin-angiotensin system in retinal pigment epithelium cells. Eur J Pharm Sci. 2016 Sep 20;92:22-7.
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doi: 10.1016/j.biochi.2018.06.019. Epub 2018 Jun 28. PMID: 29960032
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Santos DF, Cabrita B, Sequeira RL, Bitoque D, Simão S, Silva GA. Overexpression of ACE2 protects retinal pigment epithelium cells from inflammation and oxidative stress.
In preparation

Santos DF, Pais M, Silva GA. Polyphenol metabolite Pyrogallol-*O*-sulfate decreases microglial activation and pro-angiogenic VEGF in RPE cells and diabetic mouse model.
In preparation

Thesis Outline

The work presented in this document is a compilation of the research carried out between 2016 and 2020, supported by *Fundação para a Ciência e a Tecnologia* (Ref. PD/BD/114251/2016), com o apoio do FSE (*Fundo Social Europeu*) e do POCH (*Programa Operacional Capital Humano*).

These studies are integrated in the Ph.D. program in Mechanisms of Disease and Regenerative Medicine (ProRegeM), and were carried out at the Gene Therapy Lab, at Chronic Diseases Research Center (CEDOC), under the supervision of Gabriela A. Silva, PhD (NMS-UNL).

This thesis is constituted by six chapters. Chapter I consists of a **General Introduction** to eye anatomy and physiology, and ocular diseases, with particular emphasis on diabetic retinopathy. A brief overview on the molecules involved in diabetic retinopathy pathophysiology and new therapeutic approaches based in the modulation of the Renin-Angiotensin System and the potential of polyphenol-derived metabolites. In Chapter II is presented the **Methodology** used throughout the experimental work. Chapters III-V include the experimental work developed through the research project, which general aim was to investigate how modulation of the renin-angiotensin system and the bioactive properties of polyphenol-derived metabolites could present as theperaputic options for diabetic retinopathy. In chapter III, **"Towards a new therapy for diabetic retinopathy: Retinal renin-angiotensin system modulation"**, shows how direct renin inhibition and the activation of the protective axis of the renin-angiotensin system ameliorates diabetic retinopathy hallmarks. Chapter IV, **"Effect of polyphenol-derived metabolites in inflammatory-related markers"**, evaluates the anti-inflammatory potential of a polyphenol-derived metabolite towards diabetic retinopathy, in both cellular and a diabetic animal model. Chapter V, **"Effect of polyphenol-derived metabolites in angiogenesis-related proteins"**, shows that a polyphenol-derived metabolite efficiently decreases the expression of pro-inflammatory VEGF in retinal pigmented epithelium cells and in a diabetic mouse model. In the final chapter (Chapter VI), the main findings of this thesis are correlated and discussed considering the state of the art.

CHAPTER I
GENERAL INTRODUCTION

1. General Introduction

1.1. Diabetic Retinopathy

Diseases affecting the retina can be blinding disorders influenced by genetic and environmental factors altogether contributing to more than 25 % of blindness cases [1]. Most inherited retinal diseases are caused by mutations in genes expressed in the photoreceptors (PRs) and retinal pigmented epithelium (RPE) cells, being examples Lebers´ Congenital Amaurosis, Retinitis Pigmentosa and Choroideremia. Acquired retinal disorders include Age-related Macular Degeneration (AMD), glaucoma and Diabetic Retinopathy (DR), whose etiology is considered a combination of aging, environmental and genetic factors that damage the retina and RPE.

The focus of this study is DR, and thus a more detailed description will be given below.

Diabetes *mellitus* (DM) is a group of metabolic disorders characterized by abnormal glucose homeostasis in the blood resulting from defects in insulin secretion (clinically classified as type 1) and/or action (type 2) and leading to several associated complications, including DR.

DR remains one of the major causes of acquired visual impairment and/or blindness among diabetic population, affecting mostly working-age adults approximately twenty years after the onset of diabetes [2–4], with a significant impact on people's quality of life and socioeconomic consequences. With the increased prevalence of DM diagnosis worldwide in the last two decades (from 4.6 % of the global population in 2000 to 9.3 % in 2019) an increase in retinal complications as DR is expected to affect approximately one-third of all diabetics [5]. Moreover, an overall annual increase in the incidence of type 1 diabetes (T1DM) among children and adolescents is estimated around 3 % in many countries [6,7], particularly in those with less than 15 years of age. Therefore, an early diagnosis presents itself as a valuable tool to prevent vision impairment and further blindness.

DR is classically regarded as an inflammatory with progressive microvascular complication of diabetes that affects the retina. The retina (Figure 1.1) is a complex set of layered tissue that, along with vitreous humor, choroid and optic nerve forms the posterior segment of the eye. The vitreous humor acts as a shock absorber, supports the shape of the eye and is in contact with the retina, keeping it in place by pressing it into the choroid. The choroid is a vascular layer responsible for providing blood supply to retinal cells. The endothelial layer lining the inner retinal blood vessels acts as a

selective barrier, important for the maintenance of retinal function. The choriocapillaris is the second vascular bed that supplies the retina by diffusion of nutrients and oxygen, sustaining the high metabolic demands of the neural tissue.

The retina is the most metabolically active tissue of the human body, with a fast rate of glucose and oxygen consumption [8]. It is the sensory tissue that lines the inner surface of the posterior segment of the eye and is organized in seven major layers composed mainly by three cell types: neural cell (ganglion cells, müller ceels,bipolar neurons and amacrine cells), PRs (cones and rods) and RPE, that is located in-between the PRs and the choriod [9].

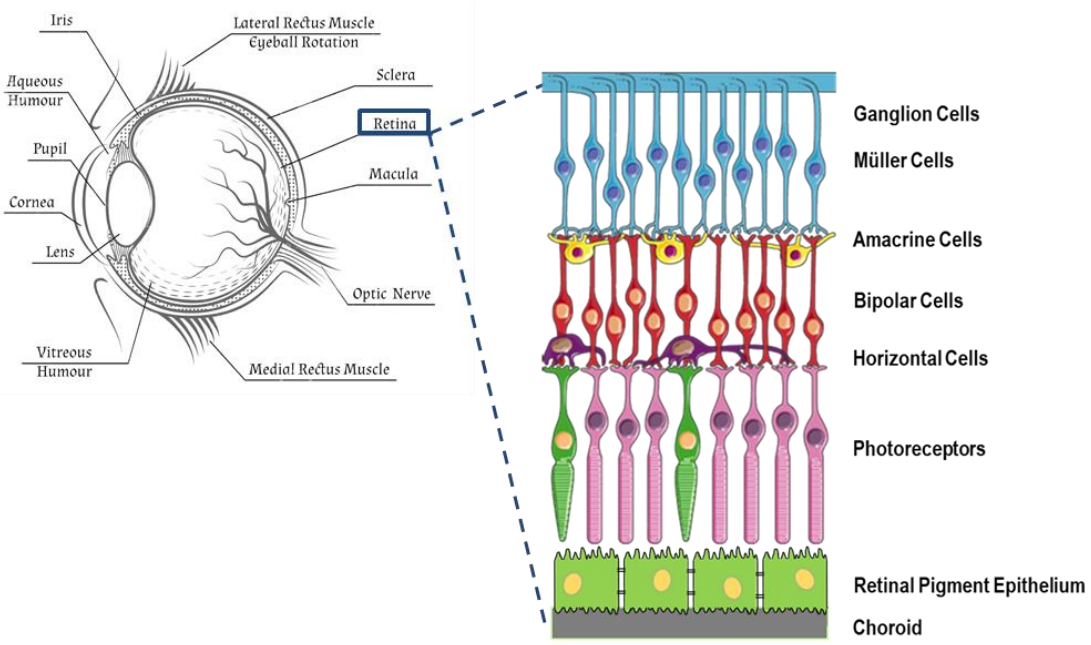


Figure 1.1 Schematic representation of the eye, highlighting the localization (left) and cell composition of the retina (right). The retina lines at the posterior segment of the eye, in close interaction with the choroid, forming the Blood-Retinal Barrier. It is composed by seven cell types, each with very important functions for the homeostasis of the retina and visual cycle, distributed in ten distinct layers (not represented). Adapted from Araújo *et al.*, 2018 [10] and 123RF digital stock agency.

RPE is a highly selective cell monolayer whose hexagonal cells are connected by tight-junctions, containing pigment granules [11]. The apical membrane faces the subretinal space, intereacting with the outer segment of PRs and the basolateral membrane is in contact with the Bruch membrane that is in direct contact with blood in fenestrated vessels of the choroid, forming the Blood-Retinal Barrier (BRB).

Among its functions are light absorption and transport of ions, water and metabolic end products from the subretinal space towards blood. RPE takes up nutrients,

such as glucose, retinol, and fatty acids, essential for the maintenance of PRs. Another important function of RPE cells is the re-isomerization of all-trans-retinal into 11-cis-retinal, a key element of the visual cycle. To ensure the excitability of PRs, the RPE phagocytes and digests the PR shedded outer segments. Furthermore, RPE produces and secretes a wide range of growth and neurotrophic factors that, because of its polarized nature, can either be secreted to the apical or basolateral membrane to support and maintain the structural basis of PRs and choriocapillaris endothelium [12]. The main functions of RPE are resumed in Figure 1.2.

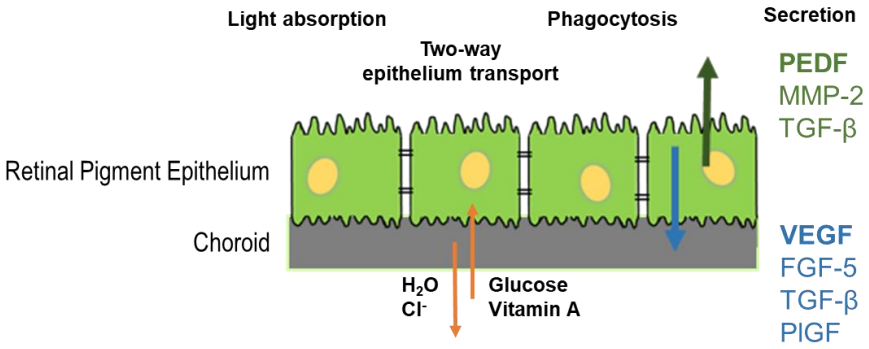


Figure 1.2 Schematic representation of the Retinal Pigment Epithelium and its main functions. Although it is a very thin layer of cells (~44 µm) it is responsible for many functions related to the visual cycle, nutrient and water exchanges, cell recycling and protein secretion. MMP-2 - matrix metalloproteinase-2; TGF-β - Transforming growth factor beta; FGF-5 - Fibroblast Growth Factor 5; PlGF – Placental Growth Factor. Adapted from Araújo *et al.*, 2018 [10].

1.1.1. Diabetic Retinopathy – pathophysiology

Although the exact mechanism underlying DR pathophysiology is not yet fully understood, it is believed that chronic exposure to hyperglycemia and other risk factors, such as hypertension [13], triggers a cascade of biochemical and physiological changes, including microvascular damage and retinal capillary basement membrane thickening that induces pericyte and endothelial cell death, causing inner BRB breakdown [14]. These events allow extravasation of fluid and potentially toxic factors, including chemokines, cytokines, growth factors and inflammatory factors, which might cause excitotoxic damage, vascular wall leakage, chronic inflammation and ischemia. Altogether, any of these factors might contribute to retinal neovascularization, formation of microaneurysms, edema and hemorrhages leading to visual impairment or irreversible blindness [2] (Figure 1.3).

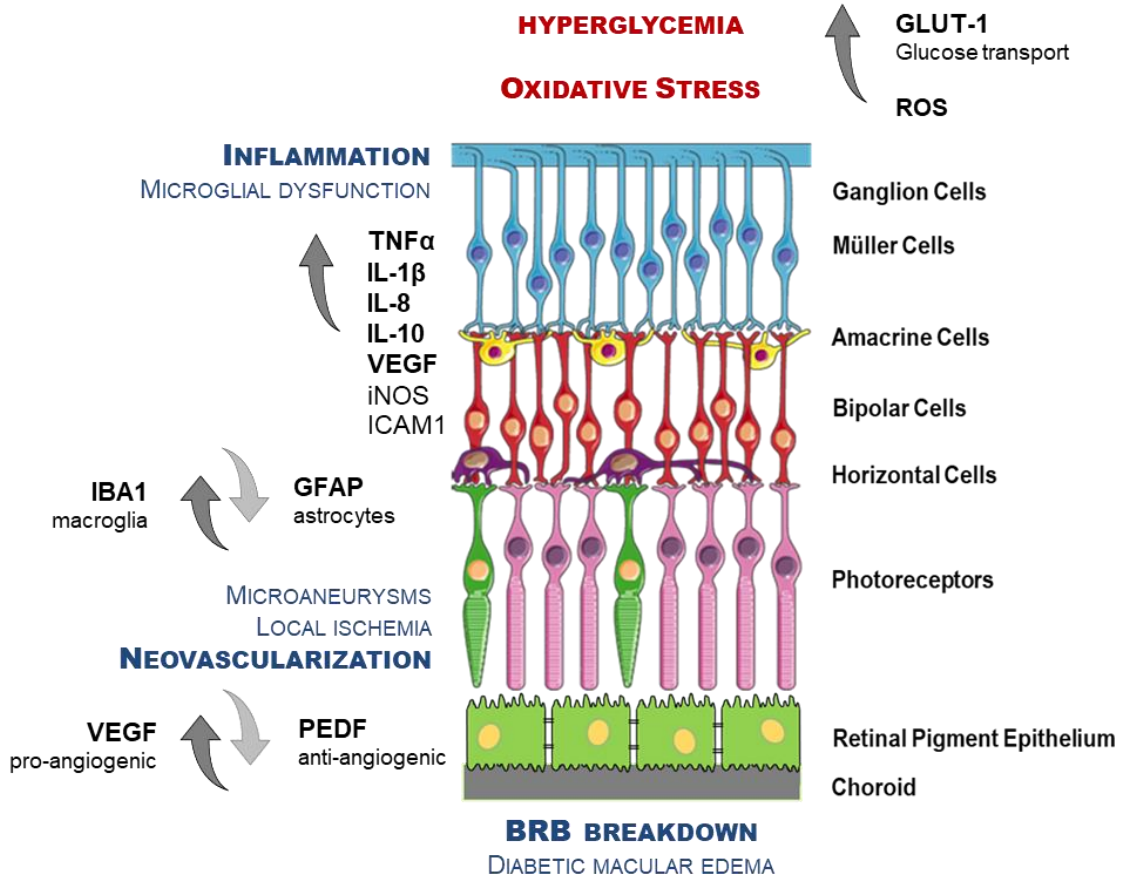


Figure 1.3 Schematic representation of the retina highlighting the pathophysiology of Diabetic Retinopathy and their hallmarks. Chronic hyperglycemia and oxidative stress have a great impact on cell metabolism and homeostatic functions, leading to microglial dysfunction and increase in the expression and secretion of pro-inflammatory molecules. Moreover, the BRB breakdown promotes a disruption in the RPE functions and the formation of Diabetic Macular Edema. The imbalance between pro- and anti-angiogenic proteins results in pathological neovascularization and vascular complications observed in advanced DR patients. iNOS – inducible Nitric Oxide Synthase; ICAM1 - Intercellular Adhesion Molecule 1. Adapted from Araújo *et al.*, 2018 [10].

1.1.1.1. Glucose

Chronic hyperglycaemia is the main cause of tissue damage, establishing the organ level complications that are clinically treated. In high glucose conditions most cell types reduce glucose cell uptake; however, endothelial, mesangial and neuronal cells cannot reduce this transportation effectively and are affected by excess of glucose [15].

Two landmark clinical trials have shown the close relation between blood glucose levels and the development and progression of DR: “The Diabetes Control and

Complications Trial" and "The UK Prospective Diabetes Study". In both studies, it was observed a reduction in DR progression upon hyperglycemia control [16,17].

The transport of glucose in the human retina was investigated at various ages and found to be exclusively mediated by one of the 12 isoforms of the sodium-independent glucose transporter (GLUT), the **GLUT1**, expressed in the endothelia of the retinal capillaries of the retina and RPE basal membrane, both part of the BRB [18].

Based on a gradient concentration, GLUT1 adapts the bidirectional transport of glucose to different situations, like hypoxia, growth factors and glucose levels itself [19]. Our group has shown that in a DR cell model, hyperglycemia and hypoxia directly affect GLUT1 expression and the secretory function of RPE cell [20]. It was found that not only the expression of GLUT1 is increased when RPE cells are in hypoxia under high glucose conditions, but also its membrane fraction increases under the same conditions. Moreover, the consumption of glucose is also affected pointing to an increase in the GLUT1 expression to achieve an effective glucose transport [20]. As a result of the loss of the barrier properties observed in the pathological vessels of proliferative DR (PDR), there is a concomitant absence of GLUT1 expression in these cells, as observed by Pardridge and colleagues [21]. Despite that, the intracellular concentration of glucose in diabetic retinas was found to increase, which highlights the crucial role of chronic hyperglycemia in the pathophysiology of DR [22].

The exact mechanisms by which high blood glucose levels produce diabetes complications are not clear. Nevertheless, it is known that hyperglycemia promotes alterations in biochemical pathways that leads to oxidative stress that notably contributes to induced inflammatory intermediate production, as previously reviewed [23]. Altogether, these events cause the disruption of BRB and the beginning of vascular complications associated with DR progression.

1.1.1.2. Oxidative Stress

Under physiological conditions, approximately 0.1 %–5 % of oxygen that enters the electron transport chain is reduced to superoxide; among outher products, the reactive oxygen species (ROS) are used in metabolic processes [24].

ROS are produced continuously in all cells to support normal cellular functions. However, an imbalance in ROS production and antioxidant defense system leads to oxidative stress, an important and common mechanism underlying most known types of chronic diseases such as diabetes [25,26]. In this context, its modulation rises a relevant

target for prevention or treatment DM. Generation of ROS is counteracted by effective enzymatic and non-enzymatic antioxidant systems, such as superoxide dismutase, glutathione peroxidase, catalase and glutathione which are diminished in the diabetic retina [27]. The possible sources of oxidative stress in diabetes might include auto-oxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants such as reduced glutathione and vitamin E, and impaired activities of antioxidant defense enzymes such as superoxide dismutase and catalase [27,28]. Hyperglycemic-derived ROS are crucial in the development of diabetic complications, with studies showing a positive correlation between both events [28]. However, the exact mechanism by which oxidative stress could contribute to the development of diabetic complications remains to be clarified.

The antioxidante defence system of the cell plays a crucial role in preventing the imbalance between excess formation and/or impaired removal of ROS that results in oxidative stress. Exogenous antioxidants are obtained from diet and include phytochemicals such as (poly)phenols. (Poly)phenols, such as flavonoids and phenolic acids, can act not only as direct antioxidants by preventing or scavenging ROS but also as metal chelators and modulators of cellular pathways [29–31]. Thus, a (poly)phenols'-enriched diet may affect human health, considering that it can have detrimental effects, or in contrast, being able to attenuate inflammation.

1.1.1.3. DR-related Inflammation

Another very important process to the progression of DR and that can be trigged by hyperglycemia and ROS stimulus, is inflammation**.** Inflammation is characterized by a biological response to harmful stimuli that protects cells of permanent damage. The duration of the inflammatory response could be beneficial when it is acute, however, chronic inflammation can have the opposite effect and, indeed, it is known to have a significant role in the development and progression of DR [32–34]. Understanding the mechanisms linking inflammation to DM and related complications is very important to develop new preventive strategies, by targeting inflammatory pathways. Several physiologic and molecular changes that are consistent with inflammation have been found in the retinas of diabetic animals and patients, which has been widely reviewed in the last decade [32,35,36].

Retinal microglia are the most important glial cell type in the retina because they act like macrophages, forming the first active immune defense in the neural retina. Microglia activation results from very sensitive calcium transport channels to small

alterations in extracellular calcium. This activation promotes a cytokine-induced activation cascade to help microglia in inflammation process. However, the permanent activation of microglia can lead to retinal degeneration and chronic inflammation.

Ionized calcium-binding adapter molecule 1 (**Iba1**) is a specific protein up-regulated after microglia activation [37], promoting the migration and proliferation of cells involved in the vascular process and the deregulation of the cell cycle. Recently, we have found a significant increase in the expression of Iba1 in the retina of a spontaneous type I diabetic mouse, the C57BL/-Ins2^{Akita}-6J (Ins2^{Akita}), at an early stage of DR progression, with a tendency to increase in older retinas, compared with non-diabetic mice [38]. These results point to the importance of microglia activation in DR progression, preceding the vascular alterations observed in later stages, and are in accordance with previous work where the activation of microglial has been described in both early and late DR patients [39].

The retinal macroglia, comprising Müller cells and astrocytes, are also part of the retinal glia cells. Particularly, Müller cells have an intimate anatomical association with retinal neurons and blood vessels that translates into a rapid response to retinal homeostatic challenges. Thus, the role of these cells in the pathogenesis of retinal microangiopathy deserves to be investigated.

The glial fibrillary acidic protein (**GFAP**), a well-known sensitive marker for retinal gliosis, was found to be increased in the diabetic retinas [40,41]. The expression of GFAP itself changes with diabetes, which was found limited to astrocytes in normal retinas but predominantly in Müller cells after induction of diabetes [40]. These changes are consistent with the concept that altered glial-endothelial cell interactions at the BRB contribute to DR, as the individual glial cell types react differentially to the diabetic state. Whereas the number of Müller cells increases, the activation of astrocytes decreases, shown by the decrease in the expression of GFAP [41]. As so, glial behavior may contribute decisively to the onset and development of neuropathy in the diabetic retina.

The inflammatory response also involves specific mediators like cytokines, interleukins and chemokines. In diabetic patients, particularly those with non-proliferative DR [42], a significant increase in the levels of pro-inflammatory cytokines, such as monocyte chemoattractant protein (MCP)-1 [43,44], tumor necrosis factor α (TNF-α) [34,42], interleukine-1 beta (IL-1β) [34,42,45], interleukine-6 (IL-6) [42,46] and interleukin-8 (IL-8) [42,44] is described, which induces the expression of other pro-inflammatory proteins and neuronal degeneration.

IL-1β, besides being produced by caspase-1, it is associated with local inflammatory response enhanced under hyperglycemic conditions [47]. In fact, Busik and co-workers suggested that high levels of IL-1β induced by glucose stimulation lead to vascular dysfunction and cell death via a paracrine mechanism [48]. IL-1β associated with other cytokines is responsible for collagen and fibroblasts synthesis, resulting in proliferation and contraction, influencing angiogenic activity. Therefore, targeting IL-1β as a therapeutic approach has been studied for most chronic diseases, including DR. Previous studies demonstrated that the use of IL-1β inhibitors ameliorate inflammation and photoreceptor degeneration in aged rats [49] and can prevent degeneration of retinal vasculature in diabetic mice [50]. Also, the use of antioxidants reduces the IL-1β expression promoted by diabetes in Streptozotocin (STZ)-induced DR rats [51], representing a possible therapeutic approach for DR.

In addition, **TNF-α** is a pro-inflammatory molecule secreted by Müller cells, which have been associated with promotion of vascular dysfunction and BRB disruption [52,53], being promising targets to treat or prevent ocular vascular pathologies. Several studies show the link between the levels of TNF-α and disease progression in diabetic patients [54,55]. Moreover, a relationship between Vascular Endothelial Growth Factor (VEGF), TNF-α and IL-6 was observed in the development of the DR in children with T1DM [56].

1.1.1.4. Growth factors

As mentioned before, one of the major characteristics of DR are vascular changes along the progression of the disease, trigged by chronic and sustained hyperglycemia. **Neovascularization**, a dynamic process of formation of new blood vessels from pre-existing ones, is critical to the development of every tissue in the body. In the initial stages of angiogenesis, a feedback loop with VEGF, a potent pro-angiogenic factor, and its receptor (VEGFR) is well characterized as being the central mechanism by which migratory tip cells are selected to undergo the cellular mechanism of angiogenesis [57].

Retinal ischemia is an important component of the pathogenesis of retinal neovascularization. Ischemia induced by cellular hypoxia is responsible for a broad range of signaling pathways to overexpress angiogenic stimulators such as VEGF [58]. In a normal retina, RPE expresses a small amount of VEGF and high levels of pigment epithelium-derived factor (PEDF), a potent anti-angiogenic molecule, with antioxidant and anti-inflammatory properties as well. The balance between pro- and anti-angiogenic

molecules is disrupted in DM due to hypoxia, leading to up-regulation of VEGF and down-regulation of PEDF [38].

VEGF, particularly VEGF-A, is a protein from a large family of angiogenic proteins, that is secreted by different cells in retina, mostly by the basolateral side of RPE. The main functions of these proteins are the regulation of angiogenesis and vascular permeability, with several studies correlating the imbalance in its expression and of its receptors with the severity of DR and AMD [58,59]. The VEGF signal involves the binding of this factor to specific tyrosine-kinase receptors called VEGFR, promoting proliferation and migration of endothelial cells, and the formation of new blood vessels [60]. VEGF is also involved in the inflammatory response since it is strongly expressed in wound healing, where microvascular permeability and angiogenesis are increased [61].

Splice variants of VEGF such as the b isoforms, and in particular $VEGF_{165b}$, was shown to be a potent anti-angiogenic isoform of VEGF with positive outcome in treating induced choroidal neovascularization [62]. We have recently shown that $VEGF_{165b}$ is expressed by RPE cells and is decreased in the diabetic retina, opening a window to explore its therapeutic potential using gene therapy [63].

On the other side there is one of the most relevant molecules of the RPE secretome, the pigment epithelial-derived factor (PEDF). **PEDF** is an apically secreted protein from the serine protease inhibitor (serpin) super family. In 1999, PEDF was identified as an anti-angiogenic factor which prompt it immediately to be considered as a potential therapeutic target for DM [64]. In fact, PEDF has been administered with positive effects in photoreceptor survival, morphology, and function as demonstrated recently in a rat model of choroidal neovascularization [64–66]. Alternatively, a prolonged therapeutic effect can be achieved with gene therapy. In a phase I trial of a single intravitreal injection of a PEDF-expressing adenovirus in 28 advanced AMD patients, the high-dose were more likely to maintain or reduce CNV size up to 12 months, but the reasons why it failed to move to phase II are unknown [67].

Shortly after its discovery, low levels of PEDF were correlated with Retinitis Pigmentosa and Lebers´ Congenital Amaurosis [64], and also found to be decreased in the retinas from patients with proliferative DR and Retinopathy of Prematurity [68,69]. On the other hand, overexpression of PEDF prevents the formation of new blood vessels, conferring protection to the retina [70]. Our group has shown that sustained expression of PEDF up to 3 months by an episomal vector, both in cultures of human RPE cells and in the retina of a DR mice model after a single subretinal injection, decreases VEGF, GLUT1, and inflammation markers [71].

As a consequence of the imbalance between angiogenic factors, neovascularization is promoted but the new blood vessels are usually weak, fragile and leaky which allows proteins, fluids and debris to enter the retina [2,72].

1.1.2. Current therapies for Diabetic Retinopathy

Clinically, DR is classified in two distinct phases based on the presence of visible ophthalmological changes and the manifestation of retinal neovascularization [73]. An early non-proliferative phase, characterized by increased vascular permeability, microaneurysms and intraretinal hemorrhages; and a late and advanced proliferative phase (PDR), characterized by retinal neovascularization in the retina and posterior surface of the vitreous, ischemia and hemorrhages [2,74]. Patients with early PDR are at a 75 % risk of developing high-risk PDR in 5 years, which highlights the rapid progression of PDR [75].

Nowadays, there are a few effective treatments for DR. Tight glycemic control is the most efficient therapy to slow the progression of DR. Blood pressure should also be taken in consideration since hypertension aggravates the disease by increasing blood flow, and causing mechanical damage to the vascular endothelial cells, which in turns stimulates the production of VEGF [2,72].

Current treatments for DR are designed to act on later stages of this pathology, targeting ocular lesions resulting from the disease progression and that play a major role in visual impairment, such as neovascularization and Diabetic Macular Edema (DME). DME is a condition in which fluid accumulates underneath the central macula due to a breakdown of the BRB resulting in vison loss [76,77].

In pathologies associated with abnormal angiogenesis, like DR or wet AMD, intravitreal administration of monoclonal antibodies - mainly ranibizumab (Lucentis®), bevacizumab (Avastin®), or fusion proteins that block VEGF activity – such as aflibercept (Eylea®, Zaltrap®) - is considered as first line therapeutic approach. Although this treatment can improve patients' visual acuity, it depends on the baseline visual acuity and requires a tight follow-up regime and repetition of injections [78]. In both cases, this procedure is extremely invasive and might have deleterious side-effects towards macular edema and increasing ocular pressure as well as on other retinal cells, such as RPE and PRs [79–81]. Other reported side effects include pain, subconjunctival hemorrhage, uveitis, and it has also been described that the viability of PRs, choriocapillaris and Müller cell signaling may also be negatively affected [78]. In addition to anti-VEGF injections there are

treatments with corticosteroids as alternative to be used on DR patients with DME that do not respond to anti-VEGF treatment [82,83].

Laser photocoagulation is also a treatment approach for neovascularization to slow down vision loss caused by PDR and DME [84]. This technique aims to cause laser burns in specific spots within the retina to help regression and slow progression of retinal neovascularization and reduction of ischemia induced by VEGF production. However, despite its efficiency preventing visual loss, the damaging nature of laser carries several ocular side-effects, including poor light-dark adaptation and decrease in visual acuity. Moreover, if the therapy is not started timely, halting of vision loss cannot be guaranteed [2].

Vitrectomy is the most effective surgical treatment for complications of advanced retinopathy, such as vitreous hemorrhage and retinal detachment. However, despite its efficiency reducing the risk of retinal neovascularization development, it markedly increases the risk of iris neovascularization and cataract formation [2].

Considering the growing numbers of DM incidence and prevalence and that these forms of treatment only uphold the progression of DR temporarily, there is an urgent need to seek therapeutic alternatives that either have longer lasting effects or can target DR in earlier stages of progression.

1.2. Renin-Angiotensin System

As mentioned, hypertension is a risk factor in DR development. Hypertension is a result of an imbalance in blood pressure, which is regulated by the classical hormonal cascade, the Renin-Angiotensin System (RAS), described in detail in the next section.

Cumulating evidence shows that RAS is an intricate system of pathways, involving a variety of enzymes and peptides. From all the pathways known to date, our interest falls over two main axes. The first is the classically known Angiotensin (Ang)-converting enzyme (ACE)/Angiotensin II (Ang II)/Angiotensin II type1 receptor (AT1R) axis, also designated as the detrimental axis and the second is the ACE2/Angiotensin (1-7) (Ang (1-7))/Mas receptor (Mas1) axis, also known as the protective axis, which will be explained in the next section (Figure 1.4).

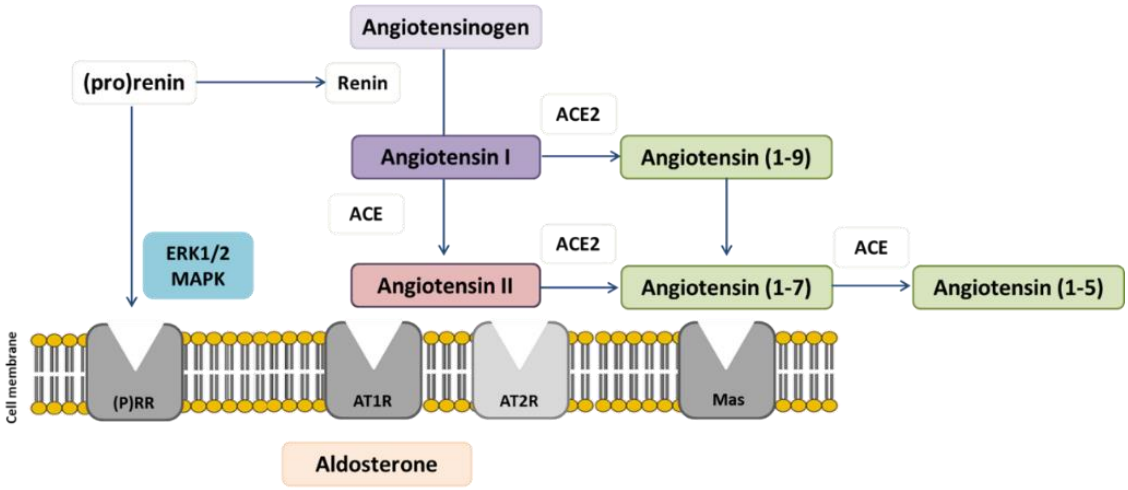


Figure 1.4 Simplified schematic representation of the Renin-Angiotensin-Aldosterone System. The RAAS is a very complex hormonal system with several intervenients. Here is represented an Angiotensin II-independent axis, where the interaction of the renin with its receptor promotes the activation of the extracellular signal-regulated kinases (ERK)-1/2-mitogen activated protein kinase (MAPK) cascade, with consequences on cell proliferation and survival. Regarding the Angiotensin II-dependent axes it is described a prejudicial axis (that results in the formation of Angiotensin II) with an impact on vasoconstriction, angiogenesis and inflammation; and a beneficial axis [that results in the formation of Angiotensin (1-7)]. Adapted from Choudhary *et al*., 2017 [85].

The classical RAS pathway begins with the peptide Angiotensinogen, produced by the liver, that is cleaved by the enzyme Renin, produced by the kidney. The product of this reaction is Angiotensin I (Ang I), a biologically inactive peptide. However, it is the substrate of the ACE, transforming it in Angiotensin II (Ang II), the main effector of this pathway. This peptide has two known receptors: AT1R and Angiotensin II type 2 receptor (AT2R). Each receptor mediates opposing effects. When Ang II interacts with AT1R vasoconstriction is induced, expression of pro-inflammatory and angiogenic factors is promoted as well as cellular proliferation, and the production of ROS is stimulated [86,87]. In the literature, it is indicated that when Ang II interacts with the AT2R, opposing effects to the ones mediated by AT1R are promoted [88]. However, these are not fully understood. It is thought that, in general, the interaction of Ang II with AT2R lead to vasodilation and inhibit cell proliferation [89,90]. Some reports have shown that this can happen in certain cell types or tissues, while in others the interaction with AT2R can lead to similar effects to the ones promoted by AT1R such as cellular proliferation and growth [85,89,91]. In addition, these two angiotensin receptors are known to homo- or heterodimerize, similarly to other membrane receptors. It has been shown that these receptors are more

feasible to homodimerize and that AT2R can induce a negative crosstalk, counteracting the AT1R mediated effects [92].

Although this is the commonly known prejudicial axis, there are other pathways that exert the same prejudicial effects. As highlighted in Figure 1.4, there is a pathway involving renin but not the formation of Ang II capable to induce effects such as vasoconstriction. In the kidney, renin is produced mainly in its inactive form, (pro)renin. (Pro)renin presents an extra peptide chain that covers the catalytic site of the enzyme. To be in its active form, proteolytic activation occurs, the pro-segment is cleaved, originating renin in its mature form. However, both forms, (pro)renin and renin, can bind the renin receptor and induce vasoconstriction [93,94].

A second axis of this system, ACE2/Ang (1-7)/Mas1, has been designated as a protective axis (Figure 1.4). The protective arm of RAS has different components from the ones belonging to the previous axis. Firstly, a biologically active peptide different from Ang II was discovered, Ang (1-7) [93,94]. In 2000, ACE2 was identified and reported by two independent research groups as an homologous to ACE and described as a RAS effector [95,96]. In addition, a functional receptor for the active heptapeptide was described: the Mas1 receptor [97]. The interaction between Ang (1-7) and Mas1 not only inhibits vasodilation, cell growth and proliferation, but also decreases ROS production [98,99]. This ACE2/Ang (1-7)/Mas1 axis is then able to counterbalance the deleterious effects of the ACE/Ang II/AT1R axis, thus becoming a valuable therapeutic target.

Of utmost importance for its therapeutic value is the formation of Ang (1-7). This peptide can induce beneficial effects by itself, but its formation is also at the expense of Ang II, since the latter is the substrate used by ACE2. In general, the benefits arise from eliminating the harmful effector. ACE2 has high affinity for Ang II, converting it quite efficiently into Ang (1-7), but their enzymatic activity also includes de degradation of Ang I, leaving ACE2 to be considered as a regulator of the effects of both axes of RAS. Despite, the rate of this conversion is slower than the rate of interaction between Ang II and the AT1R [100]. In Figure 1.4 it is also represented the alternative pathways to obtain Ang (1-7). One of the well-known is through the action of ACE2 over Ang I. Although not as efficiently, ACE2 can cleave Ang I, forming Angiotensin (1-9), which is subsequently cleaved by ACE, producing Ang (1-7) [101].

1.2.1. Tissue RAS

When RAS was firstly described it was thought that it only exerted systemic effects. Currently it is known that components of RAS are expressed in tissues and organs such as brain, heart, kidney and the eye, being directly involved in the function of these organs. From all these organs, the eye has been studied to evaluate the expression of RAS components and their potential involvement in ocular physiology and/or pathophysiology. In early studies, RAS components were found in the eye [102,103] but there was a lack in identifying the origin of the ocular RAS by either local production or selective uptake by ocular tissues from the circulatory RAS. This question has been clarified after findings of Danser *et al*. that showed that RAS' components were expressed by the neural retina, RPE and choroid, among other ocular structures [102,104]. The presence of renin, angiotensinogen as well as ACE was detected, showing a localized synthesis of these components, and supporting the existence of an ocular RAS independent from the systemic RAS. Also from Danser's work, it was shown that the eye has its own Ang II production system, later identified in Müller cells [90], due to the higher concentration of RAS components in the retina when compared to plasma concentration, and that angiotensin peptides are unable to cross the BRB, suggesting that RAS components in the ocular tissues are locally synthesized [104]. These observations were further confirmed by Brandt *et al*. after finding the renin mRNA (stands for messenger Ribonucleic Acid) in the eye [105].

Further work has shown that, although independent, ocular or other localized RAS can be modulated by the systemic components. A model proposed by Milenkovic *et al*., indicates that the circulating Ang II reaches the RPE layer by diffusion from the choroidal vessels and stimulate the AT1 receptors present and its effects [106]. One of the main findings of this study was that this interaction with circulating high levels of Ang II lead to a subsequent decrease of the renin levels in this layer of the retina, giving an apparent regulating role of ocular RAS to RPE. In a more recent work, White *et al*. furthered the knowledge of ocular RAS, demonstrating the expression pattern of RAS' classical components across all structures of the human eye [87]. There is thus evidence that retinal tissues strongly express these components, and those being a hallmark for some ocular diseases since these structures are then able to strongly produce angiotensin peptides.

With the cumulating evidence of the existence of the protective arm of RAS, a characterization of the same axis at an organ and tissue level was also pressing, with the Mas receptor and Ang (1-7) detected across the ocular tissues. Additionally, the

enzyme ACE2 is also expressed in ocular tissues. Being that the classical components can also be found in these layers, the presence of ACE2, Ang (1-7) and Mas1 shows a physiological role in the balance between deleterious and beneficial axes. However, further studies on the involvement of RAS on the eye physiology are necessary.

In terms of pathway, there are no known changes between systemic and tissue RAS. Angiotensinogen is the initial peptide and renin is responsible for its conversion in Ang I. However, according to the tissue or organ different enzymes can be responsible for the conversion of Angiotensin I to Ang II, as in the heart, where chymase is the major enzyme responsible for this conversion [107]. Nevertheless, the conversion to Ang II happens and its effects are mediated by the angiotensin receptors.

Ang II elicits effects over the retinal vasculature, inducing vasoconstriction, reducing blood flow in the retina, as well as potentiating inflammation and oxidative stress. Additionally, Ang II is known for having a role in pericyte uncoupling, which is a substantial hallmark of vasculature changes, since the loss of interaction between pericyte and endothelial cells leads to an enhance response to growth and angiogenic factors, such as VEGF, by endothelial cells [108,109].

Several studies have been performed to better characterize ocular and other local RAS systems. The balance between both axes plays a role in maintaining the function of several organs and the imbalance is responsible for the long-term injuries, such as DR.

1.2.2. RAS and DR

It is acknowledged that tissue RAS is responsible for the localized effects and long-term changes in organs suggesting a possible role in the pathogenesis of ocular conditions such as AMD, glaucoma and DR.

To better understand the role of tissue RAS in physiology and pathophysiology (Figure 1.5), several studies aimed to clarify RAS alterations in the scope of the organs level.

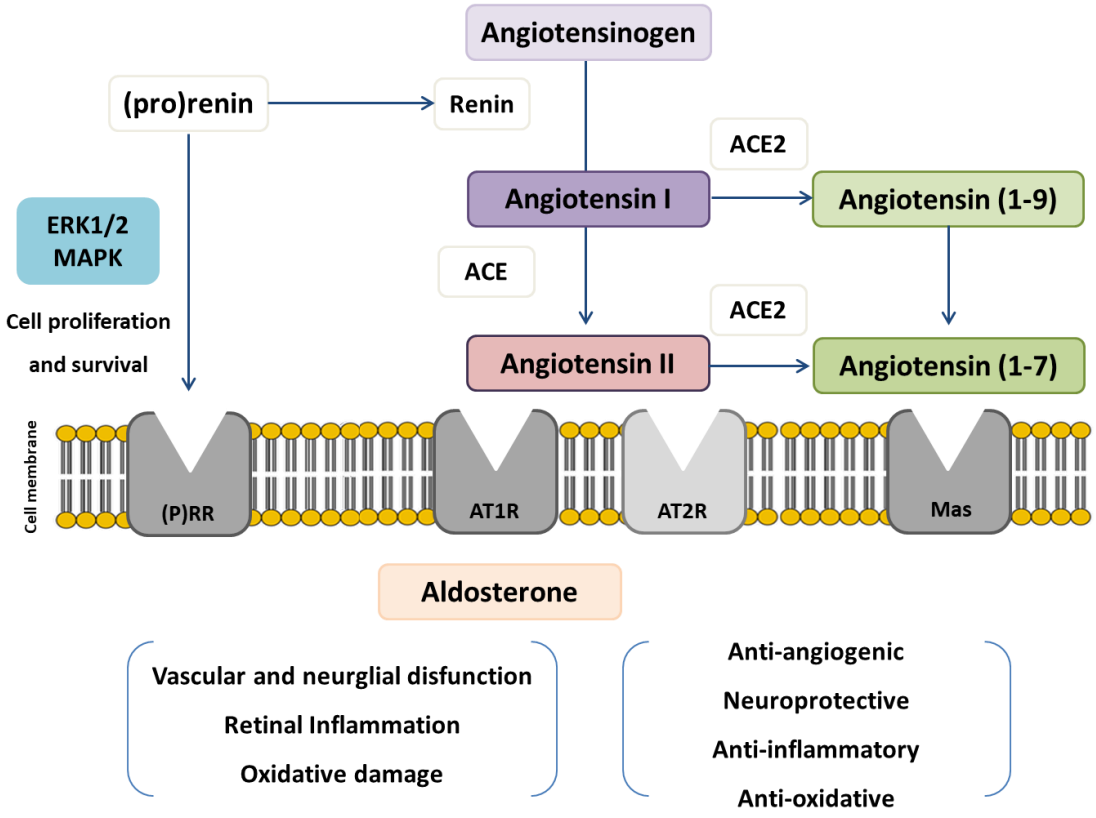


Figure 1.5 Renin-Angiotensin System involvement in Diabetic Retinopathy hallmarks. The production of Angiotensin II, the active peptide of RAS, can lead to different effects on cells, depending on which receptor is activated. The binding of Angiotensin II to AT1R promotes vascular and neuroglial disfunction, retinal inflammation and production of reactive oxygen species. On the other hand, when Angiotensin II binds to AT2R or Mas receptor it stimulates protective effects on cells. Moreover, several signaling pathways, such as the ERK1/2 MAPK, can be active by the binding of (pro)renin to its receptor, which is involved in cell proliferation and survival mechanisms. Adapted from Choudhary *et al*., 2017 [85].

In the previous section it was possible to realize that most studies of RAS alterations target the affected organs. However, for ocular alterations of RAS it is assumed that the same alterations are observed. In diabetic or hypertensive conditions, alterations of ACE2 expression is observed in the heart and kidney, normally a decrease in its expression [108]. By association, it is assumed that in the diabetic retina there is also a decrease in the expression of ACE2. In support to this decrease there is evidence that the deletion of the ACE2 encoding gene leads to an increased production of Ang II in tissue [110,111]. Studies have been made in ocular tissues, namely in samples of diabetic patients, where classical RAS components such as AT1R and renin were found to be elevated [104].

Further supporting RAS alterations in ocular pathologies, namely in patients with DR, the levels of renin, renin and Ang II are elevated in vitreous samples along with elevated levels of VEGF (angiogenic factor) [112]. In addition, the Mas receptor has been described to be strongly expressed in retinal blood vessels, mediating the vasodilatory effects of Ang (1-7) [97].

In animal models for diabetes, upregulation of ACE2 and Mas receptor is linked to reno-protective effects, suggesting a therapeutic effect of this axis for renal damage [113]. Also, in animal models for Type 2 DM it has been shown that Ang (1-7) can reverse hyperglycemia and its consequences [113,114]. In ACE2 knockout animal models, Ang (1-7) was shown to improve insulin resistance and oxidative stress [113,115]. Widening the scope of existing evidence, in diabetic patients, altered expression of ACE2 or changes in its activity play a role in the progression of diabetic nephropathy, where the levels of ACE2 were found to decrease [116].

Literature also shows evidence supporting the enhancement of this RAS protective arm for treatment of conditions with chronic inflammation and proliferative stages, which are known DR hallmarks. Experimental disease models have shown that Ang (1-7)/Mas receptor signaling is able to suppress Ang II induced effects. In brain endothelial cells Ang II-promoted ROS production was suppressed by the activation of Mas receptor by Ang (1-7) [113]. As mentioned above, it is thought that crosstalk between receptors of RAS may have a role in the mediation of their effects, inducing vasorelaxation of microvessels in mice [113].

To sum up, the ACE2/Ang (1-7)/Mas receptor axis is of therapeutic value for counterbalancing the detrimental effects. Although some benefits of RAS inhibition may be due to the increased expression of protective components, as detailed in the latter section, the modulation of RAS by overexpressing protective components like ACE2 or Ang (1-7) is an approach to be considered. Studies where lentiviral delivery of ACE2 was performed have shown beneficial effects and that the manipulation of ACE2 has therapeutic value. Additionally, the Mas receptor may play a key role in some retinal pathologies, since it has been shown that its expression in retinal tissue is higher than the expression of the angiotensin receptors. Therefore, an overexpression of the Ang (1-7) receptor should also be considered and further evaluated.

Alterations of this hormonal system have also been implicated in ocular diseases other than DR, such as age-related macular degeneration and glaucoma. For both pathologies, RAS overactivation has also been tightly linked to the development or progression of the diseases. In AMD, RPE is also compromised and as progression

occurs, neovascularization in the choroidal vessel can arise. In this progression Ang II, when signaling is mediated by AT1R, and (pro)renin have been identified as key players [108]. In glaucoma, a crucial pathological player is intraocular pressure, which is increased in disease. From studies performed, RAS has been attributed a role in the regulation of intraocular pressure through the mediation of aqueous humor formation and clearance [108].

It has been shown that RAS can be modulated by glucose levels, with diabetic patients showing elevated levels of renin and renin [108,117]. Comparatively to DR hallmarks, it is known that Ang II is an angiogenic growth factor and can also induce VEGF expression and enhance its effects, promoting neovascularization; has a role in BRB breakdown, inducing RPE damage; and can increase vascular permeability by inducing the expression of chemokines and recruiting inflammatory cells.

Studies aiming to elucidate the role of RAS in the retina are recurrent. However, studies involving RAS and RPE are scarce and the RPE, as part of the BRB, is known to be affected in DR. Conducting more studies to the role of RAS in RPE may lead to a better understanding of the underlying mechanisms of BRB dysfunction and may help prevent one of DR's hallmarks, BRB breakdown.

1.2.3. Inhibition of the Renin-Angiotensin System as a therapeutic strategy

The major risk factors in DR establishment are hyperglycemia and hypertension, which can be modifiable. Several clinical trials, such as the "The UK Prospective Diabetes Study" and the "The Diabetes Control and Complications Trial", have shown that a tight blood glucose control can lead to a reduced risk of developing DR and delay the progression of established retinopathy. Additional clinical trials linked to "The UK Prospective Diabetes Study" such as the ACCORD and ADVANCE studies, where a tighter blood glucose is aimed combined with a tight blood pressure control, showed that a tight glycemic control tends to be beneficial in retinopathy and that a threshold for benefits of the blood pressure control may exist, since in the ACCORD clinical trial the effects of blood pressure control were less evident. Still, lowering blood pressure in hypertensive diabetic patient leads to a reduced risk of developing macro- and microvascular complications. These modifiable risk factors are usually part of a medical approach to manage DR establishment or progression. However, these tight patterns are hard to achieve in practice and associated complications like hypoglycemia may

arise. Nevertheless, since there is evidence that RAS is overactive in DR, there is an interest in the effects of RAS blockade in DR development (Figure 1.6).

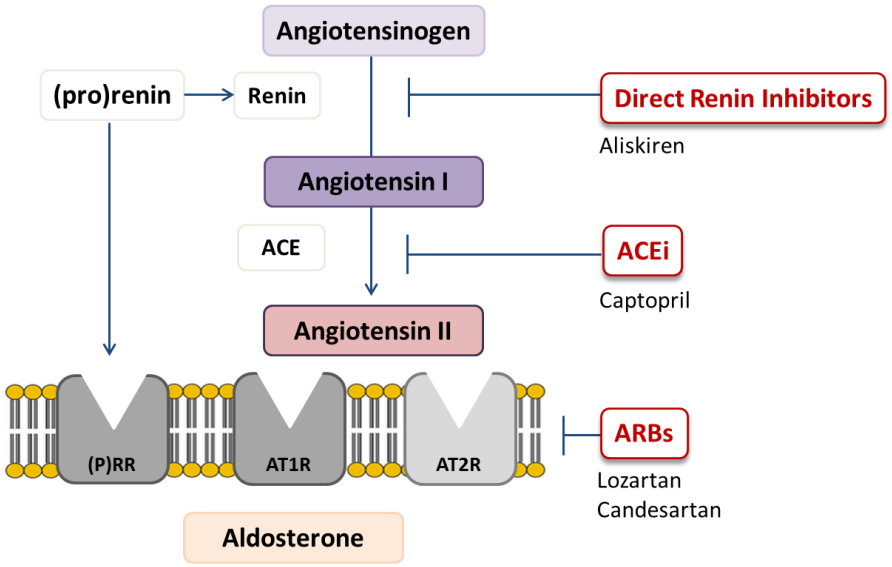


Figure 1.6 Schematic representation of RAS inhibitors aiming the Angiotensin II-dependent deleterious axis. The Angiotensin Converting Enzyme inhibitors (ACEi) and the Angiotensin Receptor Blockers (ARBs) are currently the first line of treatment for hypertension. The new Direct Renin Inhibitors (DRI) were intensively studied in the last decades and the first and only drug of this class, aliskiren, was approved in 2007. Adapted from Choudhary *et al*., 2017 [85].

Research on the physiological role of RAS in regulation of blood pressure and fluid homeostasis lead to the development of the first antihypertensive drugs. When the role of Ang II was discovered, drugs able to block its formation by inhibiting the activity of ACE (ACEi) were developed. Later, the continued research lead to the development of another type of RAS inhibitor. Angiotensin II type 1 receptor blockers (ARBs) function as antagonists for Ang II by inhibiting its interaction with the AT1R. These drugs have long been used as therapy, although not always effective in decreasing the levels of Ang II. By blocking the receptor, the local and circulating levels of Ang II increase. With the knowledge of the protective arm, it is hypothesized that the therapeutic effects of these drugs may come from the metabolite of Ang II, Ang (1-7). Research data indicates that with anti-hypertensive drug administration, levels of ACE2 expression are increased, along with the increase of Ang (1-7) levels.

RAS inhibitors are mainly used as hypertension treatment, but it is known that ACEi and ARB can reduce the risk of incidence and of DR progression in diabetic patients, conferring a degree of protection in DR models, and showing that they can also

modulate ocular RAS and are a valuable treatment option in reducing DR development and progression. A compilation of evidence by Pernomian *et al.* shows that the use of common RAS blockers can improve endothelial function by their blockade mechanism and by anti-inflammatory and antioxidant mechanisms, usually thought to be a consequence of the increase in Ang (1-7) levels [99]. Additionally, studies on animal models of diabetes show a prevention of early VEGF increased expression with RAS blockade. When withholding this increase in VEGF expression, there should be no increase in vascular permeability and dysregulation of endothelial cell growth, preventing some DR hallmarks. These types of RAS inhibitors act on later steps of the cascade and incomplete suppression of Ang II can occur. It is known that this system presents a compensatory mechanism when ARBs are used that leads to an increase of renin levels. This way, the Ang II independent cascade can be favored, and prejudicial effects induced. Since this treatment approach does not fully upholds DR development or progression, a new method of inhibition of RAS becomes an interesting approach.

Currently, there is a new type of RAS inhibitors, the direct renin inhibitors, that have a different spectrum of action. Only one of the developed direct renin inhibitors is clinically approved and commercially available. Aliskiren, commercially available as Rasilez® or Tekturna® (in the USA), acts on the catalytic center of renin, inhibiting its activity and blocking RAS at its initial and rate limiting step. Experimental studies on reperfusion injury, with tissue damage induced by the return of blood supply, demonstrated that aliskiren prevents this type of injury. Alhough common RAS blockers can also prevent this type of injury, the expression of AT1R, the receptor that has been shown to mediate the effects in this injury, was decreased upon administration of aliskiren. With this type of blockade retinal perfusion injury was almost completely prevented.

In summary, the use of ACEi or ARBs have been shown to improve dysfunction from diabetes. However, these drugs do not confer a full degree of protection against DR. Diabetic patients still show disease progression or development, since only a small percentage of these drugs can cross the BRB or because of RAS' compensatory mechanisms. Knowing that aliskiren inhibits RAS at its rate limiting step raises interest on its possible effects in preventing DR development and progression.

1.3. Natural compounds as therapeutic strategy

In the last decades, studies on the impact of (poly)phenols properties in health and cellular metabolism have increased the interest on these molecules. In fact, some of them are already well recognized by general population, as well as their antioxidant and anti-inflammatory effects. Also, there is accumulating evidence that (poly)phenols in natural products are beneficial against human diseases, with great impact on angiogenesis. Particularly in the context of DR, the beneficial effects of resveratrol (3,4′,5-trihydroxy-stilbene, RES) on retinal vascular complications [118] and oxidative stress [119] were first observed in the beginning of the decade. However, our interest falls on two specific phenolic metabolites, as described in the following section.

1.3.1. (Poly)phenol-derived metabolites

(Poly)phenols are members of a large family of compounds, chemically characterized by the presence of one or more hydroxyl groups attached to an aromatic ring. They can be classified according to the number of phenol rings in their structure and on the basis of structural elements that bind these rings to one another, which confer different chemical behavior and physicochemical properties. As pleiotropic compounds it may act against several disease-relevant biological pathways [120]. (Poly)phenol-derived metabolites are plant secondary metabolites with potential health-promoting effects that have been studied in experimental cell and animal models [121–125], as well as human studies [126,127].

(Poly)phenols, such as phenolic acids and flavonoids, can be found in high amounts in dietary products. Many authors have identified new phenolic metabolites in nutritional intervention studies after the ingestion of fruits and/or vegetables. Raspberries are fruits rich in these bioactive compounds and some studies with raspberry extracts have shown anti-inflammatory capacity. Also, these metabolites have been studied for their neuroprotective effects showing to decrease intracellular ROS levels, modulation of glutathione and activation of caspases without compromising the cell viability and functionality. The consumption of cranberry juice was also recently correlated with an improvement in vascular function in healthy males, due to the presence of specific metabolites identified in the plasma [128].

In Portugal, a consumption of about 300 grams of fresh fruit per day was estimated, with berries accounting for 9 % of total fresh fruit intake, which still represents a relative low consumption. Continuous and prolonged intake of (polyp)phenols have

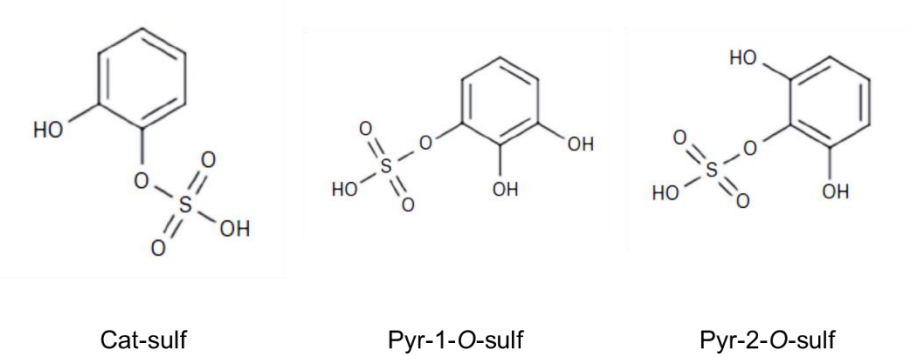
been associated with lower risk of several chronic non-communicable diseases, such as diabetes and low-grade inflammation.

However, the preventive or therapeutic action of these compounds are dependent on the amount consumed and their bioavailability, meaning the proportion of nutrient that is digested, absorbed and metabolized, which is generally low because the absorbed forms are more likely to be metabolites of the native forms of the compound found in food. In the first step and before absorption, the compounds are hydrolyzed by intestinal enzymes/colonic microflora and during absorption the (poly)phenols suffers methylation, sulfation and/or glucuronidation and reaches the blood and tissues in a different form from those present in food. Then, metabolites of (poly)phenols may follow different pathways of excretion, via the biliary or the urinary route, depending on their size.

1.3.1.1. Cathecol-*O*-sulfate and Pyrogallol-*O*-sulate metabolites

The intestinal absorption levels and the nature of the metabolite circulating in the plasma is directly related with the structure of the (poly)phenols. The metabolism of (poly)phenols starts as soon as the compounds enter the body, although some are rapidly absorbed, others are not. In particular, the presence of sulfated metabolites of Catechol and Pyrogallol was confirmed for the first time by Santos and co-workers in human plasma from volunteers after ingestion of a fruit purée [126]. The metabolites Catechol-*O*-sulfate (Cat-sulf) and Pyrogallol-*O*-sulfate (Pyr-sulf) were found in several volunteers at baseline, reaching concentrations up to 20 µM in some volunteers that can exert effects in vivo (Figure 1.7). Moreover, phase II metabolites of pyrogallol and catechol were relatively abundant in urine samples of volunteers [127].

In this study, two sulfated metabolites of pyrogallol were identified in similar proportions: Pyrogallol-1-*O*-sulfate is a conjugate of Pyrogallol and sulphate, member of isoflavones and Pyrogallol-2-*O*-sulphate is a member of resorcinol's, shown to be the main form transported.



Cat-sulf Pyr-1-*O*-sulf Pyr-2-*O*-sulf

Figure 1.7 Structure of (poly)phenol-derived metabolites, Cathecol-O-sulfate (A) and Pyrogallol-O-sulphate isomers 1 (B) and 2 (C). Both metabolites were found in high amounts in plasma and urine of human volunteers after ingestion of a (poly)phenol-rich fruit puree. Cathecol-*O*-sulfate is a member of phenols, Pyr-1-O-sulphate is a member of isoflavones and Pyr-2-O-sulphate a member of resorcinols.

The identification of these phenolic metabolites, that were found to circulate in micromolar concentrations, raises an emerging and very important research area towards their role in health and disease. Indeed, these two metabolites (Cat-sulf and Pyr-sulf) have been recently studied in different models of neurodegenerative and cardiovascular disorders, showing the ability of these small molecules to cross the BBB, modulate the brain cell towards neuroprotection and have an effect on cardiomyocyte beating following prolonged stimulation. Also, it was shown for the first time that the most abundant metabolite in circulation, Pyr-sulf, was also the most effective in preventing oxidative damage in an endothelial cells' model.

1.3.2. Therapeutic potential of (poly)phenol-derived metabolites for retinal diseases

There are several studies on the anti-diabetic effects of (poly)phenols. These compounds influence the glycemia through different mechanisms like inhibition of glucose absorption in the gut or in peripheral tissues. Some studies show the inhibition of glucose transporters by (poly)phenols [129,130]. For example, onion (poly)phenols have the capacity to protect diabetic patients from oxidative stress and (poly)phenols from vegetables act as potent anti-diabetic agents because they lowered the levels of blood glucose and increase plasma insulin [131,132].

Studies specifically related to the effect of (poly)phenols or their metabolites on retinal diseases are scarce. However, the interest in this topic increased in the last decade, particularly in studies on the effect of RES [119,133,134]. Ola *et al*. reviewed evidence

pointing out the potential metabolic sources and pathways related to the increase of oxidative stress in DR and the role of dietary flavonoids, particularly flavanones, flavanols, flavanols, isoflavones, flavones and anthocyanins, in the modulation of redox homeostasis in the diabetic retina [135]. More recently, the benefic effect of different carotenoids and (poly)phenols in the prevention and treatment of age-related ocular disorders was reviewed, highlighting the role of these metabolites in oxidative stress and inflammation [136].

Specifically, epigalloccatechin-3-gallate, the main (poly)phenol component of green tea (leaves of *Camellia sinensis*), was identified as new inhibitor of ocular angiogenesis with influence in ocular vascular permeability [137]. Therefore, treatment with nutraceuticals (foods providing medical or health benefits) at early stages of DR may represent a reasonable alternative to act upstream of the disease, preventing its progression. In particular, *in vitro* and *in vivo* studies have revealed that a variety of nutraceuticals have significant antioxidant and anti-inflammatory properties that may inhibit the early diabetes-driven molecular mechanisms that induce DR, reducing both the neural and vascular damage typical of DR.

1.4. Specific aims

Knowing that targeting DR remains a challenge, is important to understand how we can develop new therapeutic strategies towards the prevention of its progression.

Thus, our main goal is to investigate novel therapeutic approaches towards Diabetic Retinopathy, based either on the Renin-Angiotensin System modulation or on the biological proprieties of (poly)phenol-derived metabolites. Using cell and animal models of DR, we aimed to:

- investigate the presence of RAS components in retinal cells and a diabetic mouse model;

- explore the therapeutic potential of the inhibition of the RAS with aliskiren, a direct renin inhibitor, by evaluation of some of the hallmarkrs of DR, such as angiogenesis and oxidative stress;

- evaluate the therapeutic potential of modulation of the ACE2-Ang(1-7)-Mas receptor axis of RAS, by ACE2 overexpression in retinal cells;

- explore the potential of (poly)phenols-derived metabolites, specifically Catechol-O-sulfate and Pyrogallol-O-sulfate, in the expression of proteins involved in angiogenesis and inflammation, both in retinal cells and a diabetic mouse model.

CHAPTER II
METHODOLOGY

2. Methodology

2.1. Cell culture

The use of cell models that mimic diabetic conditions and diabetic animal models are crucial to study the underlying pathophysiology of DR and further development of new therapies.

In vitro cell models can be either primary cultures or continuous cell lines of human origin. In the beginning, *in vitro* studies of human RPE cells has been done with primary cultures, but these short-term cultures and tend to lose some epithelium characteristics. Thus, the need to create a continuous cell line was imperative. In 1995 a group of investigators extracted cells from a globe of the eye of a dead 12-year-old boy, and they were able to maintain the cells in culture for more than 200 passages [138]. This cell line, named D407, is now considered a spontaneously transformed RPE cell line that, although not capable of polarizing in culture nor synthesizing pigment, retain nevertheless the RPE unique epithelial morphology and characteristics, which makes it a great model to study alterations in different conditions.

D407 cells, a human spontaneously transformed RPE cell line [138], were kindly provided by Dr. Jean Bennett (University of Pennsylvania, USA). A human embryonic kidney cell line (HEK), HEK293T, kindly provided by Dr. Guilherme Ferreira (University of Algarve, Portugal), were used as control for the expression of RAS components. Cells were maintained in 5 % CO_2–95 % air at 37 °C and grown in Dulbecco's Modified Eagle Medium (DMEM) with 25 mM D-glucose (Sigma-Aldrich, USA or GE Healthcare Life Sciences, USA) supplemented with 1 % penicillin/streptomycin (Sigma-Aldrich, USA), 1 % glutamine (Sigma-Aldrich, USA) and fetal bovine serum (FBS, Sigma-Aldrich, USA) (5 % for D407 and 10 % for HEK293T). For sub-culturing, cells were dissociated with a trypsin-Ethylenediaminetetraacetic Acid (EDTA) solution (Sigma-Aldrich, USA), split 1:5 and cultured in 21 cm^2 culture plates (Orange Scientific, Belgium). Culture medium was changed every 2 days.

Standard culture protocols suggest growing D407 cells in DMEM with 25 mM D-glucose, considered a high glucose concentration; however, for certain experiments we have cultured the cells in DMEM with 5.5 mM D-glucose (non-diabetic conditions) with no deleterious effects on cell viability or morphology (Figure 2.1).

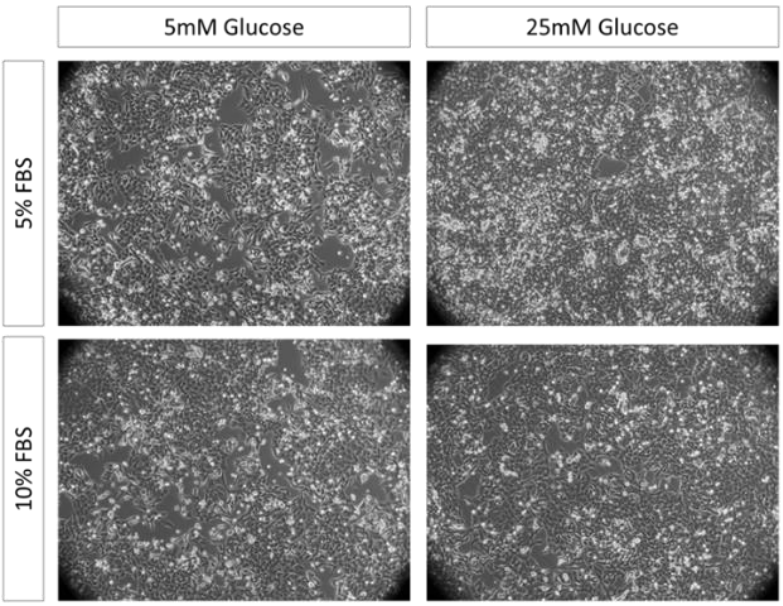


Figure 2.1 Representative microscopy images of RPE cells in culture in different concentrations of glucose and FBS. Cells were kept in culture in standard conditions under normal glucose levels (5.5 mM) until exposure to high glucose medium (25 mM) and 5 % or 10 % FBS. At least two independent experiments were performed.

2.1.1. Glucose and Hypoxia

For the experiments regarding the effect of glucose, RPE were seeded at a density of $3x10^5$ cells/well in a 6-well plate (Orange Scientific, Belgium). For each independent experiment cells were cultured either in DMEM with 5.5 mM glucose, to mimic a physiological concentration, or in DMEM with 25 mM glucose, to mimic a hyperglycemic condition, or in DMEM with 5.5 mM of D-glucose containing D-Mannitol (Applichem, Germany) with a final concentration of 25 mM, as an osmolarity control, as it is known to be metabolically inert in mammalian cells as energy source [139].

Desferrioxamine (DFO, Sigma-Aldrich, USA), an iron chelating agent, was used as a hypoxia-mimetic agent, at a final concentration of 100 µM [140,141]. Under normoxic conditions and in the presence of iron, hypoxia-inducible factor-1α is hydroxylated and subsequently degraded by the proteasome. In the presence of DFO, the iron required for the enzymatic activity of prolyl hydroxylases is removed by its chelating capacity, allowing hypoxia-inducible factor-1α to be stabilized and dimerize with its β-subunit, originating a functional complex that is translocated to the nucleus [140].

2.1.2. Angiotensin II and Aliskiren

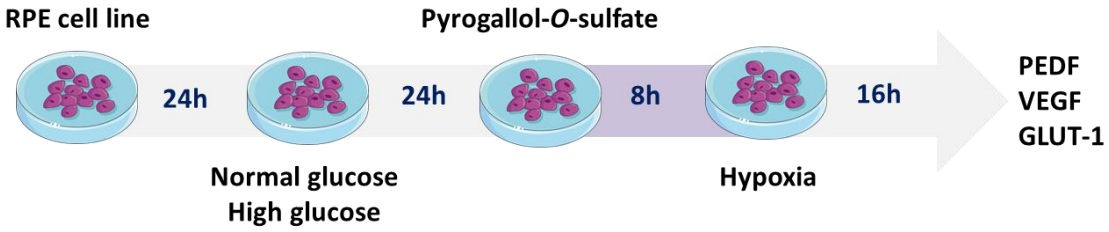
RPE cells were exposed to Angiotensin II (Santa Cruz Biotechnology, USA) at different concentrations (100 and 200 µM) and collected 12 or 24 hours after for evaluation of the expression of VEGF, ACE2, Mas receptor and levels of ROS and Ang (1-7). Similarly, aliskiren (Selleckchem, USA) was tested for 2 h, 4 h or 24 h and cells collected afterwards for detection of renin and Ang (1-7) levels and renin receptor, ACE2 and Mas receptor expression.

2.1.3. Treatment with (poly)phenol-derived metabolites

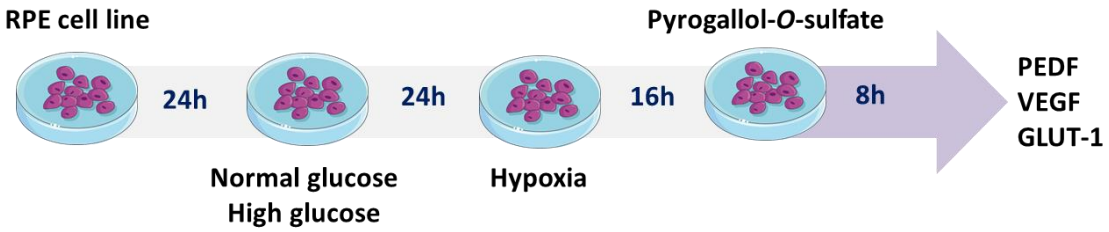
(Poly)phenol-derived metabolites were kindly provided by Dr Cláudia Nunes Santos (CEDOC, Portugal), synthetized as explained by Pimpão *et al.* [126]. The final residues were kept at -20 ºC until reconstitution in Dimethyl sulfoxide (DMSO) to a final working concentration of 100 mM.

To evaluate the effect of the polyphenolic metabolite in RPE cells, cells were plated with DMEM with 5.5 mM glucose, mimic a physiological concentration. The next day, the medium was changed to DMEM with 5.5. mM glucose or DMEM with 25 mM glucose, to mimic a hyperglycemic condition. Depending on the therapeutic approach to test, Cathecol-*O*-sulfate (Cat-sulf, 5 µM) or Pyrogallol-*O*-sulfate (Pyr-sulf, 6.5 or 11 µM) were incubated in media without FBS in three different protocols represented below (Figure 2.2). Hypoxia was chemically induced with DFO for 16 h.

(A) One single dose as pre-treatment up to 8 h before hypoxia. The metabolite was removed from the cell culture after the incubation period.



(B) One single dose after induction of hypoxia. Cells were exposed to the metabolite for 8 h.



(C) One single dose, 24 h after the increase of the glucose in the culture medium, prior to induction of hypoxia. The metabolite was left for 24 h in contact with the cell culture.

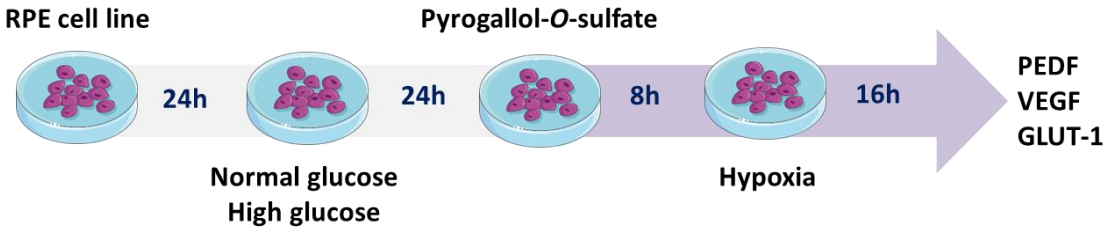
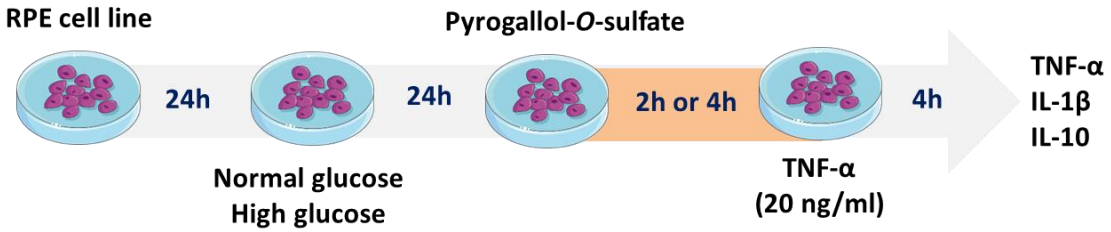


Figure 2.2 Schematic representation of the different protocols designed to evaluate the effect of Pyrogallol-O-sulfate in the expression of proteins of interest in RPE cells. Cells were kept in culture in standard conditions under normal glucose levels (5.5 mM) until exposure to high glucose medium (25 mM). Treatment with Pyr-sulf was performed before **(A)** or after **(B)** induction of hypoxia up to 24 hours **(C)** of total incubation period. The expression of PEDF, VEGF and GLUT-1 was analyzed in whole cells lysates by Western blot.

2.1.4. TNF-α-based inflammatory cell model

For the experiments regarding the effect of Pyr-sulf in the expression of pro- and anti-inflammatory cytokines, RPE were seeded at a density of $3.0x10^5$ cells/well in a 6-well plate (Orange Scientific, Belgium) in DMEM with 25 mM glucose. Recombinant human TNF-α protein (Peprotech, UK) was reconstituted in water with 0.1 % BSA and added to the cell medium at a final concentration of 20 ng/ml for 4 hours. The expression of TNF-α, IL-1β and IL-10 was analyzed by Western blot upon treatment with Pyrogallol-O-sulfate before or after the inflammatory stimulus. The protocol used for both experimental setups are schematized below (Figure 2.3).

(A) One single dose as pre-treatment for 2 or 4 h before inflammatory stimulus of TNF-α. The metabolite was removed from the cell culture after the incubation period.



(B) One single dose after exposure to TNF-α. Cells were exposed to the metabolite for 2 or 4 h.

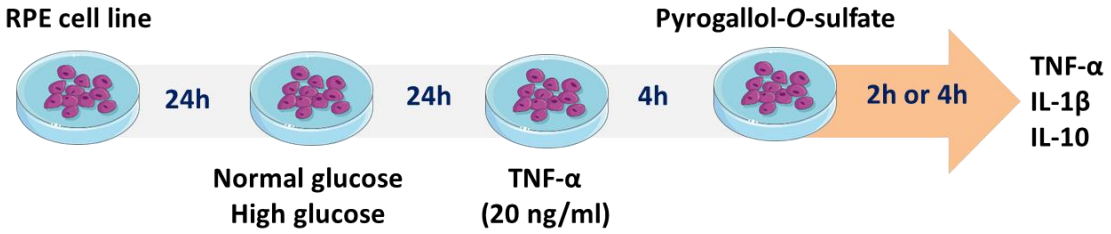


Figure 2.3 Schematic representation of the different protocols used to evaluate the effect of Pyrogallol-O-sulfate in the expression of TNF-α, IL-1β and IL-10 in RPE cells. Cells were kept in culture in standard conditions under normal glucose levels (5.5 mM) until exposure to high glucose medium (25 mM). Treatment with Pyr-sulf was performed before **(A)** or after **(B)** stimulus with TNF-α (20 ng/ml) up to 4 hours of total incubation period. The expression of TNF-α, IL-1β and IL-10 was analyzed in whole cells lysates by Western blot.

2.2. Immunocytochemistry

Cells at a density of $2x10^4$ or $6x10^4$ cells/well were grown overnight in glass coverslips. Then were fixed with 4 % paraformaldehyde for 15 min at room temperature, permeabilized and blocked in 10 % normal serum in phosphate-buffered saline (PBS) with 0.2 % TritonX-100 for 30 min at room temperature. Primary antibodies for renin, renin receptor and ACE2 were diluted in PBS with 0.2 % TritonX-100 and incubated for 1 h at room temperature. After three washes cells were incubated with the secondary antibody Alexa Fluor® 488 (1:2000, A-11008, Thermo Fisher Scientific, USA) for 1 h at room temperature. 4', 6-Diamidino-2-Phenylindole (DAPI) was included in the secondary antibody solution to mark the nuclei. Cells were visualized using a fluorescence microscope at a 630 X magnification (Axio Observer Z2, Zeiss).

2.3. Protein extraction and quantification

2.3.1. Whole cell lysates and supernatants

Confluent cells were grown in FBS-free culture medium for 48 h, with the appropriate glucose concentration (5.5 or 25 mM D-glucose). The conditioned medium of each cell line was collected and concentrated using Amicon Ultra-2 filters (Merck-Millipore, Germany). To obtain cellular lysates, cells were washed with cold PBS 1X and homogenized in radioimmunoprecipitation assay buffer (RIPA) [50 mM Tris-Hydrochloric acid (HCl, Sigma-Aldrich, USA) pH 7.4, 1 % NP-40 (Merck Millipore, Germany), 0.25 % Na-deoxycholate (Sigma-Aldrich, USA), 150 mM Sodium chloride (Sigma-Aldrich, USA) and 1 mM EDTA (Sigma-Aldrich, USA)] supplemented with protease inhibitors cocktail 1x (Roche, Germany). The cells were then scrapped from the bottom of the well, kept on ice for 20 min, centrifuged (16 200 g, 20 min, 4 °C) and the supernatant collected and stored at -20 ^{o}C until further analysis.

2.3.2. Citosolic and Membranar Fractions isolation

To obtain cytosolic and membrane fractions, cells were homogenized in lysis buffer [20 mM Hepes pH 7.4, 1 mM EDTA, 250 mM sucrose (Sigma-Aldrich, USA)] supplemented with protease inhibitors cocktail 1 x. Lysates were centrifuged to pellet nuclei and cellular debris. Supernatants were centrifuged (100 000 g, 1 h at 4 °C) and the obtained supernatants correspond to the cytosolic fraction. The remaining pellet was

resuspended in 10 mM Hepes pH 7.4, 250 mM sucrose and protease inhibitors to obtain the membrane fraction.

2.3.3. Cell lysates for Ang 1-7 levels measurement

Protein extraction for the detection of Angiotensin 1-7 in cell lysates was performed according to recommended protocol. The medium from the 6-well plate was discarded, and cells were washed with cold PBS 1X. Cells were dissociated with trypsin-EDTA for about 2 minutes at 37 ºC. After adding DMEM supplemented with 25 mM D-glucose and 5 % FBS, the suspension was centrifuged for 5 minutes at 1000 g. Cells were resuspended with PBS and counted. For each $1x10^6$ cells, 200 µl of PBS 1X were added to wash the cells thrice. Freeze-thaw cycles using liquid nitrogen were performed to lyse the cells with a final centrifugation step at 1 500 g, for 10 minutes at 4 ºC. The supernatant was collected and stored at -80 °C until further use.

2.3.4. Protein quantification

Protein concentration was determined by the Bradford Method[142], using the Bio-Rad protein assay dye reagent concentrate (BioRad, USA) in cellular lysates, retinal tissue, and concentrated conditioned media. v (NZYTech, Portugal or Thermo Fisher Scientific, USA) was used as standard (1 mg/mL; 0.5 mg/mL; 0.25 mg/mL; 0.125 mg/mL and 0.0625 mg/mL) prepared in miliQ water. Samples were also diluted in miliQ water in 1:5 or 1:10 ratios for cell lysates and retinas, respectively. The cell and standard samples were loaded into a 96-well plate, and the dye reagent added to each well. The absorbance was read in a Biotrak II Plate reader (Amersham Biosciences, Germany) at 595 nm and concentration of protein was determined by linear regression.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

2.4.1. Renin activity

Renin activity was measured in RPE cells grown in DMEM culture medium with 5.5 or 25 mM glucose incubated in the presence or absence of aliskiren [50 and 100 µM (30 and 60 µg/ml in absolute dose)] for 2 h and 4 h by means of a fluorometric assay kit (MAK157, Sigma-Aldrich, USA). Cellular lysates were collected and processed according with the manufacturers' instructions. Fluorescence was recorded every 5 min during 60 min (540/590 nm (Ex/Em); InfiniteM200, TECAN).

2.4.2. Angiotensin II levels (Sigma)

Angiotensin II levels were measured in RPE cells upon treatment with aliskiren [100 µM (60 µg/ml in absolute dose); 4 h] by ELISA kit (RAB0010, Sigma-Aldrich, USA). Cellular lysates were collected and processed according with the manufacturers' instructions, and the absorbance measured at 450 nm (InfiniteM200, TECAN). Values were normalized to protein concentration in each condition and expressed as percentage of control, with the control being to be the condition without aliskiren.

2.4.3. Angiotensin (1-7) levels

The levels of Angiotensin (1-7) were measured in RPE cells grown in DMEM culture medium with 5.5 or 25 mM glucose, and in the presence of Angiotensin II or aliskiren by ELISA kit (NBP2-69078, Novus Biologicals, USA). Assay procedure was performed according to the supplied manufacturer's instructions and absorbance read at 450 nm on a plate reader (Biotrak II Plate Reader, Amersham Biosciences, Germany). The concentration of Ang (1-7) in the tested samples was obtained from a four-parameter logistics curve generated with the OD450 nm values after normalization to the zero OD, in Graph Pad/PRISM 6 software, from which the values of the concentration were interpolated from.

2.5. Measurement of ROS levels

Intracellular ROS levels were measured in human RPE using a cell-based fluorometric assay based on deacetylation and oxidation of non-fluorescent reduced CM-H_2DCFDA (C6827, Thermo Fisher Scientific, USA). Cells were grown at 10.000 cells/well in 96-well plates and were exposed to either aliskiren or angiotensin II. Cells were washed with PBS 1X and loaded with 10 µM CM-H_2DCFDA for 30 min at 37 °C. Fluorescence intensity was measured in a microplate reader (InfiniteM200, TECAN) at 494/522 nm (Ex/Em) during 60 min.

2.6. Cell viability

2.6.1. Calcein

RPE cells were seeded at $1x10^4$ cells/well in 96-multiwell plates and incubated with aliskiren [50 and 100 µM (30 and 60 µg/ml in absolute dose)] for 2 h and 4 h. Cells

were also incubated with ethanol (Sigma-Aldrich, USA, or Merck Millipore, Germany), as the positive control for cell death and DMSO (Sigma-Aldrich, USA) as the vehicle for aliskiren. Cells were incubated with calcein-AM (C3100MP, 5 µM, Thermo Fisher Scientific, USA) for 30 min at 37 °C and the fluorescence was measured (Ex. 493/Em. 530 nm; InfiniteM200, TECAN).

2.6.2. MTT assay

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma-Aldrich, USA) is a yellow substrate that, when metabolized by the cells yields a dark blue color, denoting the formation of formazan. So, the metabolic activity of D407 cells is directly proportional to the quantity of formazan formed [143].

D407 cells were seeded at a density of $2x10^4$ cell/well in a 48-well plate, with a volume of DMEM of 500 µl/well with 5.5 mM glucose (supplemented with 1 % penicillin/streptomicin and 5 % FBS), hence considered the physiological condition. After 24 h the medium was changed, and cells were exposed to different conditions as previously described in 2.1.3. Treatment with the polyphenolic metabolites Cathecol-*O*-sulfate or Pyrogallol-*O*-sulfate was performed according to the protocol described in Figure 2.2 C.

In each well 25 µL of MTT at a final concentration of 0.25 mg/ml was incubated at 37 ºC for 3 hours. At the end of the incubation period, the formazan was dissolved with 250 µl of 0.04 N HCl in isopropanol 100 % and the absorbance was measured in a Biotrak II Plate reader (Amersham Biosciences, UK) at 540/620 nm.

2.6.3. Trypan Blue Exclusion Test of Cell Viability

Cells were seeded at a density of $3x10^5$ cell/well in a 6-well plate and cell density determined 72 h after. A 0.4 % solution of Trypan Blue (K940, VWR, USA) in PBS1X was added in a proportion of 1:1. Cell suspension with trypan blue was loaded into an hemocytometer and examine immediately under a microscope at low magnification. The number of blue staining cells and the number of total cells was counted, and the percentage of cell viability determined by the equation 2.1.

$$\% \text{ viable cells} = 1 - \frac{\text{number of blue cells}}{\text{number of total cells}} \times 100$$

Equation 2.1 Representation of the mathematical equation for the percentage of viable cells evaluated by Trypan blue exclusion assay

2.6.4. Flow Cytometry

D407 cells were seeded at a density of $3x10^4$ cell/well in a 6-well plate, for a final volume of 2000 µl/well of DMEM with 5.5 mM medium (supplemented with 1 % penicillin/streptomicin and 5 % FBS), the standard condition. After 24 h the media was changed, and cells were exposed to different media conditions either 5.5 mM glucose or 25 mM glucose. Treatment with the Cat-sulf (C_f=5 µM) was maintained for 24 h. After, the cells were collected with trypsin and the cell suspension centrifuged 5 min at 300 g. The pellet was resuspended in PBS 1X, transferred to an identified tube and centrifuged 5 min at 300 g. The supernatant was discarded, the pellet resuspended in 500-1000 µl of PBS 1X and centrifuged twice 5 min at 300 g. The pellet was finally resuspended in PBS 1X and incubated with PI at a final concentration of 5 µM for 5 minutes in the dark. The samples were analyzed on a FACS Scan apparatus (Becton Dickinson FACScan, USA) and data were processed using FlowJo™ Software (Ashland, USA). Data were collected for 10.000 events.

2.7. Immunoblotting

The samples were diluted in RIPA byffer with 4x Laemmli Sample Buffer (BioRad, USA) and heated at 95 ºC for 5 minutes for deoxyribonucleic acid (DNA) denaturation. A total of 30 µg of protein were loaded in a 10 % to 15 % SDS acrylamide gel, as suitable, along with 3 µl of a PageRuler Plus Prestained Protein Ladder 2x (Thermo Fisher Scientific, USA). Electrophoresis was performed at 100-120 V in electrophoresis buffer [25 mM Tris-HCl, 192 mM Glycine (Enzymatic, Portugal) and 0.1 % SDS (AppliChem, Germany)] until the dye reaches the bottom of the gel.

Proteins were electrotransferred to polyvinylidene difluoride (GE Healthcare Life Sciences, USA) previously activated for 20 seconds with methanol, followed by deionized H_2O for 20 seconds and cold transfer buffer (0.192 M glycine, 0.025 M Tris pH 8.3, 10 % methanol) for 5 minutes, or nitrocellulose (NC, GE Healthcare Life Sciences (USA) membrane through a wet transfer or semi-dry transfer method for 20 minutes and 20 V (Trans-Blot Turbo®, BioRad, USA). The membranes were then blocked with 5 %

non-fat dry milk in Tris buffered saline (TBS) with 0.1 % Tween-20 (Sigma-Aldrich, USA) (TBS-T) for 1 h at room temperature, with gently agitation. Primary antibodies, listed in Table 2.1, diluted in 5 % non-fat dry milk or 5 % bovine albumin serum in TBS-T were incubated overnight at 4 ºC with gentle agitation. The membranes were subsequently washed with TBS-T and incubated with the correspondent secondary antibodies IgG conjugated with HRP (Santa Cruz Biotechnology, USA) for 1 h at room temperature. After washing three times for 5 minutes each with TBS-T, the enhanced chemiluminescence Prime or Select Western Blotting Detection Reagent (1:1, GE Healthcare Life Sciences, USA) was used for visualization in a GelDoc or ChemiDoc™ imaging System (BioRad, USA). To ensure that samples were evenly loaded the membranes were stripped and re-probed for β-tubulin or β-actin.

For stripping, the membrane was washed three times with TBS-T for 5 minutes, incubated for 5 minutes with 0.2 N Sodium Hidroxyde (NaOH, Sigma-Aldrich, USA) or mild stripping solution (Glycine, SDS, Tween-20 pH=2.2), followed by a triple TBS-T wash for 5 minutes and incubated with the new primary antibody.

Table 2.1 List of primary and secondary antibodies, with the respective technical information, used for detection of proteins of interest by Western blot.

Primary antibody	Dilution	Reference	Brand
Renin	1:500	sc-22752	Santa Cruz Biotechnology, USA
Renin receptor	1:1000	ab40790	Abcam, UK
Mas receptor	1:500	sc-390453	Santa Cruz Biotechnology, USA
ACE2	1:2000	NBP1-76614	Novus Biologicals, USA
VEGF-A	1:500-1:1000	ab46154	Abcam, UK
$VEGF_{165b}$	1:500	ab14994	Abcam, UK
PEDF	1:500-1:1000	07-280	Merck Millipore, Germany
Iba1	1:500	SAB2500041	Sigma-Aldrich, USA
GFAP	1:10000	ab7260	Abcam, UK
GLUT-1	1:2000	ab32551	Abcam, UK
TNF-α	1:500	ab9635	Abcam, UK
IL-1β	1:500	ab9722	Abcam, UK
IL-10	1:500	ab133575	Abcam, UK
β-actin	1:5000	A5441	Sigma-Aldrich, USA
tubulin	1:10000	sc-5274	Santa Cruz Biotechnology, USA
Goat anti-mouse igG-HRP	1:5000	sc-516162	Santa Cruz Biotechnology, USA
Goat anti-rabbit igG-HRP	1:5000	sc-2004	Santa Cruz Biotechnology, USA
Donkey anti-goat igG-HRP	1:5000	sc-2020	Santa Cruz Biotechnology, USA

2.8. Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

2.8.1. RNA Extraction

To promote the lysis of the cultured cells, TRIzol™ Reagent (Sigma-Aldrich, USA) was added (300 µl) to each well. After homogenization, all the volume was transferred to a previously identified tube and incubated 2-3 minutes with chloroform (VWR, USA) to precipitate the DNA. After centrifugation (12 000 g, 4 ºC, 15 min), the aqueous part containing the ribonucleic acid (RNA) was carefully transferred to a new tube and the pellet resuspended with isopropanol for 10 minutes. The samples were centrifugated (12 000 g, 4 ºC, 10 min) and the supernatant removed. A solution of 75 % ethanol was used to wash the RNA and the sample centrifugated again (7 500 g, 4 ºC, 5 min) and the pellet resuspended in 20 µl of RNAse-free water (Sigma-Aldrich, USA). To evaluate the quality and quantity of the RNA, samples were analyzed in a NanoDrop™ 2000 (Thermo Scientific, USA) spectrophotometer.

2.8.2. cDNA synthesis

The complement deoxyribonucleic acid (cDNA) was synthetized with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The RNA extract was diluted to a ratio of 1000 ng of protein per 10 µl of RNAse-free water. To perform the reaction, each tube had a final volume of 20 µl (10 µl of the sample and 10 µl of the master mix). The reactions were done in a MyCycler™ Thermocycler (BioRad, USA).

2.8.3. Quantitative Real-Time PCR

This technique was used to analyze de expression of PEDF, VEGF, IL-8 and IL-1β genes and β-Actin gene as a control. To perform the RT-qPCR, we used the cDNA obtained, prepared a mix with RNase-free water, containing the correspondent primers, forward and reverse, and Ssofast Evagreen Supermix (BioRad, USA). In a 96 well-plate, 13 µl of the Mix and 2 µl of the diluted cDNA sample were added, for a final volume of 15 µl. The reactions were done in a 7300 real Time PCR System (Applied Biosystems).

The primers sequence and annealing temperature are listed in Table 2.2. For the PCR reactions, the conditions were as followed: denaturation at 95 ºC for 1 minute, followed by 40 cycles of denaturation at 95ºC for 15 seconds and annealing for 15 seconds at the gene-specific temperature and a final extension step at 65 ºC for 30

seconds. The β-actin was used has housekeeping gene, to normalize the mRNA levels of PEDF, VEGF, IL-8 and IL-1β. For mRNA quantification we used the comparative method represented in equation 2.2 [144].

Table 2.2 List of primers sequence and respective annealing temperature used for quantification of human genes for IL-8 and IL-1β by RT-qPCR.

Gene	Primer sequence	Annealing temperature (ºC)
hIL-8	Fw: 5' ATAAAGACATACTCCAAACCTTTCCAC 3'	62.3
	Rv: 5' AAGCTTTACAATAATTTCTGTGTTGGC 3'	
hIL-1β	Fw: 5' AAATACCTGTGGCCTTGGGC3'	62.3
	Rv: 5' TTTGGGATCTACACTCTCCAGCT 3'	
hβ-Actin	Fw: 5' GCAAAGACCTGTACGCCAAC 3'	59
	Rv: 5'AGTACTTGCGCTCAGGAGGA 3'	

Fw: forward; Rv: reverse

$$relative\ expression = 2^{-\Delta\Delta CT}$$

$$\Delta\Delta CT = [C_{T\,(gene\ of\ interest\ )} - C_{T\,(\beta-actin\ )}]_{Treated\ \ sample\ } - [C_{T\,(gene\ of\ interest\ )} - C_{T\,(\beta-actin\ )}]_{Untreated\ \ sample}$$

Equation 2.2 Representation of the mathematical equation of the comparative method used for mRNA quantification of IL-8 and IL-1β in RPE cells

2.9. pEPito-hCMV-hACE2 vector construction

pEPito-hCMV-eGFP-IRES-IB was used as backbone, as described before [145,146]. The plasmid pBluescript IISK containing the human ACE2 flanked by NhE I (5') and BspE I (3') restriction sites were synthetized (GeneCust, France). The amplified DNA fragments of the human ACE2 gene and pEPito-hCMV-eGFP vector were purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany), according to the manufacturer's instructions, and the digested fragments (Figure 2.4 A) were mixed with DNA ligase, resulting in a recombinant vector carrying the gene of interest (Figure 2.4 B) (Figure 2.4).

Chemically competent *Escherichia coli* GT115 bacteria (Invitrogen, USA) were transformed using 30 ng of plasmid. Transformed bacteria were selected on Luria broth plates containing ampicillin (Sigma-Aldrich, USA). After bacterial propagation, plasmid DNA from three clones were isolated using the QIAGEN® Plasmid Mini Kit (QIAGEN,

Germany), according to the manufacturer's instructions, and digested with NhE I (5') (Figure 2.4 C) and EcoR I (Figure 2.4 D).

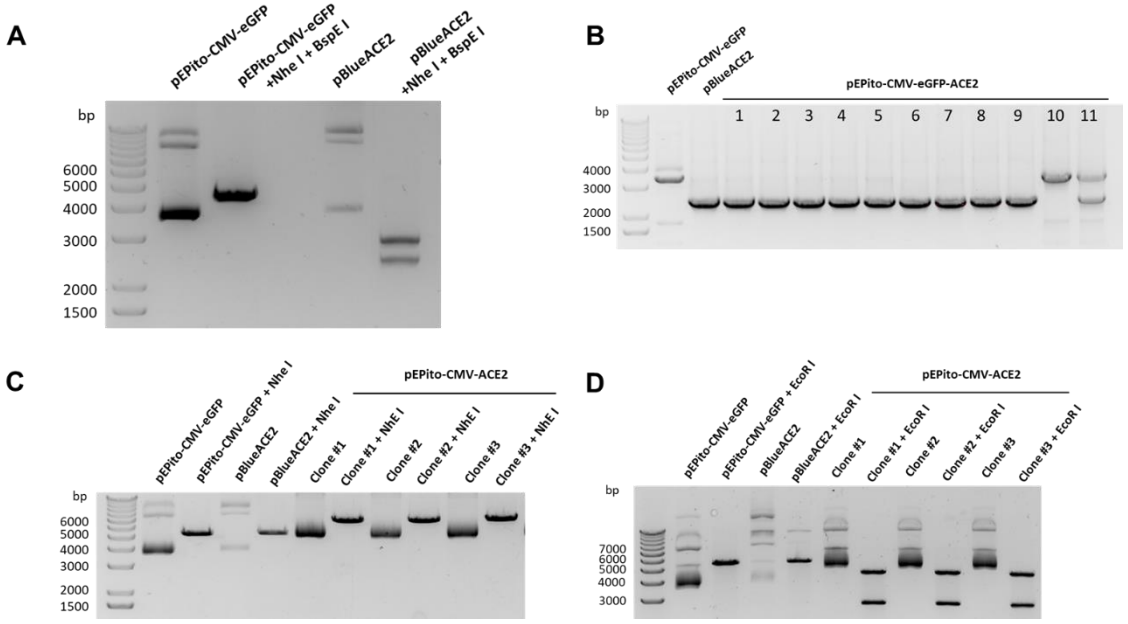


Figure 2.4 pEPito-hCMV-hACE2 vector construction. (A) Representative image of pEPito-CMV-eGFP and pBluescript SK II ACE2 purification, with and without digestion with NhE I and BspE I enzymes**. (B)** Representative image of products of ligation of pBlueACE2 inserted in the backbone of pEPito-CMV-eGFP. Representative image of three different clones of pEPito-CMV-ACE2 with and without digestions with NhE I **(C)** and EcoR I **(D)** enzymes.

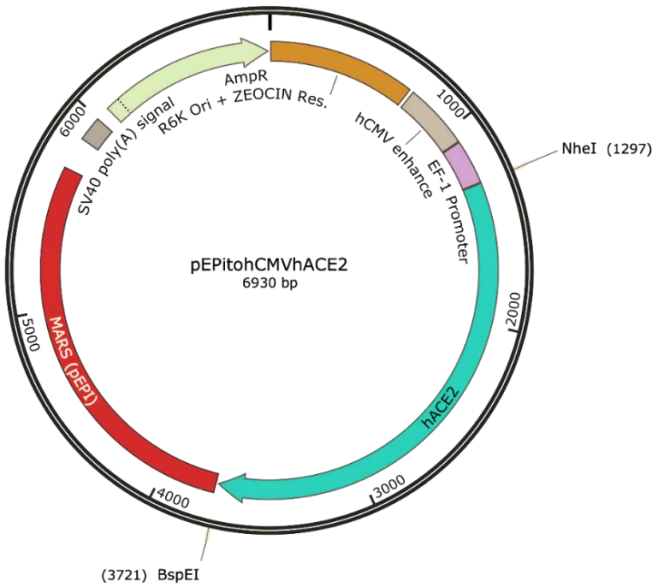


Figure 2.5 Schematic representation of the pEPito-hCMV-hACE2 vector. The human gene of ACE2 was inserted into the non-viral episomal expression vector pEPito under a humanized CMV promoter containing an eGFP-BSD cassette, in which an enhanced green fluorescent protein (eGFP) is expressed.

The new plasmid pEPito-hCMV-hACE2 with a 6930 bp size (Figure 2.5) was confirmed by sequencing with specific primers: forward primer 5'-GATCGCTAGCAATGTCAAGCTCTTCCTGG-3' and reverse primer 5'-TAGTCCGGAGCTAAAAGGAGGTCTGAAC-3'.

2.10. Evaluation of ACE2 expression driven by the pEPito vector in vitro

Transfection was performed using FuGENE® HD (Promega, USA) with a 3:1 ration (µL of FuGENE® HD:µg of DNA), according to the manufacturer's instructions. Briefly, $1.5x10^5$ cells were seeded in a 6-well tissue culture plate (Orange Scientific) and 24 h after seeding, cells were transfected with 1 µg of DNA. Cells were collected at 24 h, 48 h and 72 h after transfection for protein extraction.

2.11. In vivo model: $Ins2^{Akita}$ mice

Since DR is a complex disease with both genetic and environmental influences, diabetes in animal models can be pharmacologically induced or by genetic modification, as reviewed in [147].

These genetic models of diabetes have been gaining special attention in DR research, since they are highly reproducible and enable longitudinal studies of complications, providing a good background to test new therapies and for drug screening. There are at least five different genetic mouse models of DR, vary in progression and pathology of disease, that include strain-specific, spontaneous, and genetically edited mutations.

The C57BL/-$Ins2^{Akita}$-6J ($Ins2^{Akita}$) mice model is a spontaneous monogenic model for phenotypes associated with T1DM. This model carries a single base-pair substitution in the insulin 2 gene that leads to incorrect folding of the insulin protein. Therefore, the promoted toxicity in pancreatic β cells reduces the insulin secretion. Heterozygous $Ins2^{Akita}$ mice are fertile and develop several symptoms of T1DM, including hyperglycemia, hypo-insulinemia, polydipsia and polyuria by 3 to 4 weeks old without insulitis or obesity. The phenotype is more severe and progressive in males and untreated homozygotes rarely survive beyond 12 weeks of age. Progressive retinal abnormalities begin as early as 12 weeks after the onset of hyperglycemia. Retinal complications include increased vascular permeability, alterations in the morphology of astrocytes and microglia, increased apoptosis and thinning of the inner layers of the

retina. Also, the frequency of apoptotic retinal neurons increases and retinal blood flow decreases.

The Ins2^{Akita} mice develop both early signs of DR (microaneurysms, vascular damage, and increased vascular leakage) and some signs of proliferative DR including neovascularization (NV) and new capillary bed formation. In previous studies it was shown in these mice a loss of the BRB function, an increase in vascular permeability, and a thickening of the vascular basement membrane. Furthermore, the number of leukocyte adherent to the vascular wall was significantly elevated and microglia is activated, which confirm that the inflammation component of DR is present in these mice. Recently, we demonstrated that the balance between pro- and antiangiogenic molecules is disrupted in Ins2^{Akita} mice either in neural retina or RPE, in early and late stages of the disease. Moreover, increased levels of Iba1 were restricted to the early stages of the disease, as well as decreased levels of GFAP, suggesting that Ins2^{Akita} mice develop an early inflammatory response [38].

2.11.1. Animals

Male C57BL/-Ins2^{Akita}-6J heterozygote and C57BL/6J age-matched (wild-type) mice from The Jackson Laboratory, USA, were used. These mice present a dominant point mutation in the insulin 2 gene, that spontaneously induces diabetes, developing hyperglycaemia as early as 4 weeks of age [148]. With the progression of hyperglycaemia, diabetic mice can develop retinal complications similar to what is observed in DR [148], making them a good model to study early alterations of DR.

2.11.2. Housing

Animals were maintained and handled in accordance with the Portuguese and European Laboratory Animal Science Association (FELASA) Guide for the Care and Use of Laboratory Animals, the European Union Council Directive 2010/63/EU for the use of animals in research and the Association for Research in Vision and Ophthalmology (ARVO) for the use of animals in ophthalmic and vision research. Animal experiments have been performed under the approval of the Portuguese national body DGAV (*Direção Geral de Alimentação e Veterinária*) and the institutional Ethics Review Board (CEFCM).

Mice were housed in individually ventilated cages, under controlled temperature and with continuous access to food and water, on a 12 h dark/light cycle. To confirm the diabetic phenotype, blood glucose levels were measured 2 months after birth, with a drop of blood obtained by a tail cut on a reactive glucose strip (Contour Next, Ascencia Diabetes Care, Portugal). The mice presenting blood glucose levels equal or higher than 250 mg/dl were considered diabetic [148].

Male diabetic C57BL/-Ins2^{Akita}-6J and non-diabetic C57BL/-Ins2^{Akita}-6J age-match mice (The Jackson Laboratory, USA) were used as DR model. The main goal of the following experimental design is to assess the effect of Pyr-sulf on the expression of proteins involved in inflammation and angiogenesis in the neural retina and RPE of animals at different ages (4, 6, 8 and 9 months of age). The estimated number of subjects in each group, in order to demonstrate a statistically significant difference ($p < 0.05$), with a difference in means of 50 %, was at least 5 animals per group. For this study, a total of 70 animals were used.

2.11.3. Intraocular injections of Pyrogallol-O-sulfate in C57BL/Ins2^{Akita} mice

The animals were randomly assigned for different experimental groups: C57Bl/6 (non-diabetic) sham; C57Bl/6 (non-diabetic) Pyr-sulf injection; C57BL/-Ins2^{Akita}-6J (diabetic) sham and C57BL/-Ins2^{Akita}-6J (diabetic) Pyr-sulf injection. The experimental protocol for the intraocular injections is schematized below (Figure 2.5).

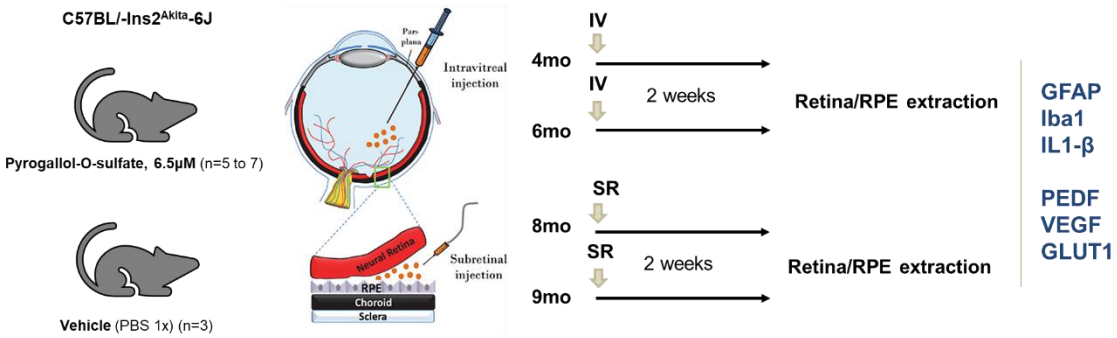


Figure 2.6 Schematic representation of the intraocular injections of Pyrogallol-O-sulfate in diabetic and non-diabetic mice, targeting different retinal cells at different stages of DR progression. The expression of proteins having a role in inflammation and angiogenesis were assessed by Western blot, two weeks after a single injection.

Intraocular injections were performed in anesthetized animals with Avertin® ((2,2,2-tribromoethyl alcohol (Sigma-Aldrich, USA) with 2-methyl-2-butanol (Sigma-Aldrich, USA); intraperitoneal injection of 0.5 ml/25 g) and pupils were dilated using 1 % tropicamide (Edol, Portugal). Cornea was superficially anesthetized with Anestocil (Edol, Portugal) as an eye drop. A 30-gauge needle was used to puncture a hole approximately 1 mm below the limbus, in the immobilized animals. The left eye was injected intravitreal or subretinally, with 2 µl of Pyr-sulf, at a final concentration of 6.5 µM, or saline solution in one eye using a Hamilton syringe 10 µl 701 RN injector at 50 nl/sec. The non-injected contralateral eye was used as control. After the injection gentamicin and dexamethasone were applied in the injected eye to reduce the risk of infection.

No changes in grooming, behavior, food or water consumption were observed after injection. Additionally, visual inspection of the eyes of animals within the experimental groups revealed no signs of ocular inflammation nor changes in overall health status.

2.11.4. Retina and RPE protein extraction

Two weeks after Pyr-sulf injection, the animals are humanely sacrificed by cervical dislocation and the eyes removed. The neural retina and RPE, as previously described [149]. Samples were mechanically homogenized in ice-cold RIPA buffer containing a protease inhibitor cocktail. and kept on ice for 20 minutes. The extracts were then centrifuged (20 minutes, 4 ºC, 16 100 g). The supernatant was collected and quantified by Braford method, as previously described in section 2.3.4, with a dilution of 1:10, for Western blot. The samples were stored at -80 ºC until use.

2.12. Statistical analysis

Arithmetic means are given with standard error of the mean (SEM) or standard deviation (SD), when more suitable. Statistical analysis was performed using an unpaired t-test with or without Welch's correction, One-way Analysis of Variance (ANOVA) with Dunnet's multiple comparison test or Two-way ANOVA followed by the Sidak's or Bonferroni´s Multiple Comparison test for multiple comparisons. A value of $p < 0.05$ was considered to be statistically significant.

CHAPTER III

TOWARDS A NEW THERAPY FOR DIABETIC RETINOPATHY:

RETINAL RENIN-ANGIOTENSIN SYSTEM MODULATION

This chapter is based on the following scientific articles:

Simão S, Santos DF, Silva GA. **Aliskiren inhibits the renin-angiotensin system in retinal pigment epithelium cells**. European Journal of Pharmaceutical Sciences. 2016 Sep 20;92:22-7. doi: 10.1016/j.ejps.2016.06.019. Epub 2016 Jun 23. PMID: 27343695.

Simão S, Santos DF, Silva GA. **Aliskiren decreases oxidative stress and angiogenic markers in retinal pigment epithelium cells**. Angiogenesis. 2017 Feb;20(1):175-181. doi: 10.1007/s10456-016-9526-5. Epub 2016 Oct 1. PMID: 27695972.

Santos DF, Cabrita B, Sequeira RL, Bitoque DB, Simão S, Silva GA. **Overexpression of ACE2 protects retinal pigment epithelium cells from inflammation and oxidative stress.** in preparation

3.1. Abstract

There is growing evidence on the role of ocular renin–angiotensin system in the development of diabetic retinopathy, particularly due to the trigger of oxidative stress and angiogenesis. While targeting RAS might present a significant therapeutic potential, current RAS-based therapies are mainly focus on hypertension and still ineffective in halting the progression of diseases.

The direct renin inhibitors, such as aliskiren, are a potential therapeutic alternative. However, it is unclear how aliskiren acts in the retina, particularly in the retinal pigment epithelium, the cellular monolayer responsible for the maintenance of retinal homeostasis whose role is deeply compromised in several retinal diseases. We firstly analyzed the expression and activity of the main RAS components from both the deleterious and protective axes in RPE cells. Time- and concentration-dependent treatments with aliskiren were performed to modulate different pathways of the RAS in RPE cells. Also, we proposed to investigate the role of glucose on the expression of retinal RAS, the effect of activating the protective RAS axis and whether aliskiren protects RPE cells from angiogenesis and oxidative stress.

Our data demonstrate that RPE cells express the main RAS constituents and this system was deregulated by hyperglycemic glucose concentrations. Exposure of RPE cells to angiotensin II increased the levels of the main pro-angiogenic factor, VEGF in a concentration-dependent manner, while tends to increase the expression of the protective axis components. Additionally, angiotensin II also stimulated the production of reactive oxygen species in RPE cells. Treatment of RPE cells with aliskiren inhibited the activity of renin and consequently decreased the levels of angiotensin II. The levels of oxidative stress decreased after treatment and the expression of anti-angiogenic factor PEDF was promoted. Finally, the overexpression of ACE2 protects RPE cells from inflammation and oxidative stress.

Our findings of the RPE RAS, together with the demonstration that aliskiren effectively blocks this system at different steps of the cascade, suggest that aliskiren might be an alternative drug for preventing the deleterious effects derived from the overactivation of the RAS. At the same time, the activation of the ACE2/Ang-(1-7)/Mas receptor axis, known to counterbalance the deleterious effects of ACE/AngII/AT1R axis, presents itself as a promising gene therapy approach.

3.2. Introduction

Retinal diseases are serious visual threatening disorders and persist as the leading cause of acquired blindness worldwide. DR is considered a microvascular complication derived from sustained hyperglycemia. How hyperglycemia induces changes in the retina in DR is not entirely defined, but it is known that glucose triggers angiogenesis and oxidative stress in retinal cells contributing to cellular damage [150,151]. Recently, we demonstrated that the equilibrium between angiogenic and anti-angiogenic factors was disrupted by both hyperglycemia and ROS in the retina of a diabetic mouse model [63]. We also demonstrated that the RPE cells, which constitute the outer BRB were particularly susceptible to both glucose and ROS [63], highlighting the importance of studying the RPE in the context of DR.

The RAS is a set of intricate pathways with a well-defined function in the regulation of blood pressure and body fluid homeostasis. The RAS was initially identified in the kidney but currently it is well established that it is also present in other tissues such as the retina [87,108,152]. The finding of a specific RAS within the retina, namely in RPE cells, and the fact that deregulation of this system triggers hallmarks of DR such as angiogenesis and oxidative stress [112,152], support a role for RAS in this pathology. Therefore, the use of RAS blockers (ACEi or ARBs) has been tested in experimental models of DR and AMD [153,154]. Clinical trials using ARBs or ACE inhibitors to prevent or decrease the progression of DR [155,156] have shown these to be mildly protective. This limited success may be related with the fact that these conventional RAS inhibitors act in downstream steps of the RAS, promoting a compensation feedback mechanism that increases renin[157] and compromises the effectiveness of the treatment.

A new generation of RAS inhibitors are the direct renin inhibitors (DRI). Renin is the rate-limiting step of the RAS and its direct inhibition promotes high and sustained RAS blockage. Presently the only DRI available for medical use is aliskiren, an antihypertensive drug [158]. Aliskiren was shown to be effective in decreasing retinal ischemic damage in an experimental model [159]. Wilkinson-Berka *et al.* compared the effectiveness of an ACE inhibitor with aliskiren in preventing pathological features in models of retinopathy [160] and concluded that aliskiren conferred a similar or greater level of protection than the ACE inhibitor, suggesting that aliskiren has potential for the treatment of retinopathies. Despite that, studies on the effect of aliskiren upon retinal cells are scarce and the mechanism of action of this drug upon the retinal RAS has not been described so far. Batenburg *et al.* showed that aliskiren has anti-inflammatory properties in Müller cells [161], but, to date, no studies were conducted on RPE. The RPE

has a fundamental role in the homeostasis of the retina, in maintaining the integrity of the outer blood retinal barrier, and its function is deeply compromised in DR and AMD [11,162]. How RAS might contribute to the impairment of the role of the RPE is unknown.

Therefore, the aim of the present study is to better characterize the RAS, explore how glucose modulates the RAS in these cells and to determine how a direct renin inhibitor (aliskiren) influences the different components of this system and expression of DR markers in RPE cells.

The obtained data will contribute to elucidate the interplay of the RAS and RPE cells and to assess if aliskiren or overexpression of ACE2 are potential drugs for the treatment of retinopathies.

3.3. Results and Discussion

3.3.1. Renin and (pro)renin receptor are expressed in RPE cells

Expression and cellular localization of renin and renin receptor were evaluated in RPE and HEK293 cells (Figure 3.1). HEK293 cells were used as control in this first set of experiments since both renin and renin receptor were previously described in the kidney [163,164]. Both proteins localize in the cytoplasm and in the perinuclear space of RPE and HEK293 cells (Figure 3.1 A). Figure 3.1 B shows that similar to HEK293, RPE cells synthesize and secret renin and express the renin receptor. The expression of the receptor was higher in the cytosolic fraction (CF) when compared with the membrane fraction (MF) in both cell lines (Figure 3.1 C).

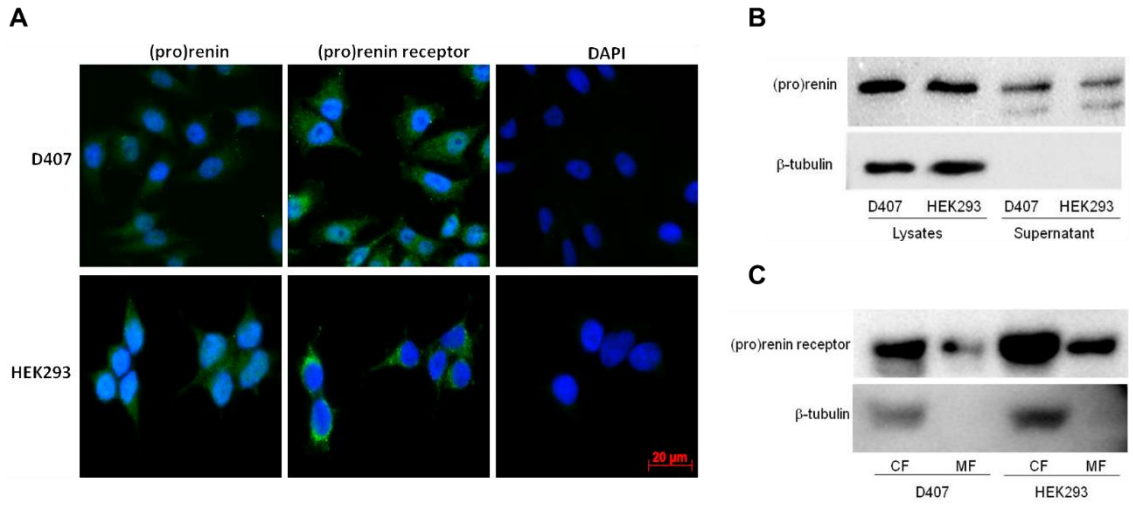


Figure 3.1 Expression of renin and renin receptor in RPE cells. (A) Cellular localization of renin and renin receptor in D407 RPE and HEK293 cells assessed by immunocytochemistry. HEK293 cells were used as the positive control for the expression of RAS components. Cells were stained with anti-renin (green) or anti-renin receptor (green) antibodies and counter-stained with DAPI to visualize the nuclei (blue). Images were obtained in a fluorescence microscope with a 630x magnification. Scale bar = 20 µm. Images are representative of three independent experiments. **(B)** Representative immunoblots of renin in cellular lysates and supernatants of D407 RPE and HEK293 cells. β-Tubulin was used as the loading control, N=3. **(C)** Representative immunoblots of renin receptor in cytosolic (CF) and membrane fractions (MF) collected from D407 RPE and HEK293 cells. β-Tubulin was used as the loading control, N=3.

Interestingly, we have found that the secreted renin presented two different bands, probably corresponding to the two independent proteins: renin (41 kDa) and (pro)renin (46 kDa), suggesting that cleavage of the prosegment might occur in the extracellular space. The expression of renin at the mRNA and protein levels was previously demonstrated by others in RPE cells, namely in freshly isolated mouse RPE

and retinal tissue [106]. In the present study we also demonstrated that D407 RPE cells express the renin receptor, with higher levels in the cytoplasm compared with the membrane. Considering the cellular distribution of the renin receptor, our results are in accordance with the finding that this receptor has a predominant subcellular localization, such as the endoplasmic reticulum and Golgi [165]. The translocation of the receptor to the cellular membrane, where binding to renin occurs, might take place only following a certain stimulus. The presence of this receptor in the cellular membrane was previously found in primary cultures of human RPE cells and in the ARPE-19 RPE cell line [166].

3.3.2. Glucose modulates the expression of both deleterious and protective RAS axis components in RPE cells

To further characterize the retinal RAS and its role in the pathogenesis of DR, we investigated the effect of glucose in the expression of some of RAS components in RPE cells.

Renin and its receptor are key players in the RAS due to its actions at the rate-limiting step of the system. Expression of renin was evaluated in cellular lysates and supernatants of RPE cells exposed to 5.5 mM (normoglucose) or 25 mM of glucose (high glucose) (Figure 3.2). The levels of the (pro)renin receptor were higher when RPE cells were grown in the high glucose than in the normoglucose medium, as shown in Figure 3.2 A. The levels of (pro)renin expressed in lysates of RPE cells were more elevated in high glucose than in normal glucose conditions (Figure 3.2 B), while the levels of (pro)renin secreted to the extracellular space decrease in high glucose medium (Figure 3.2 B). Renin activity followed an increasing trend when RPE cells are exposed to 25 mM of glucose, although statistical significance was not reached (Figure 3.2 C).

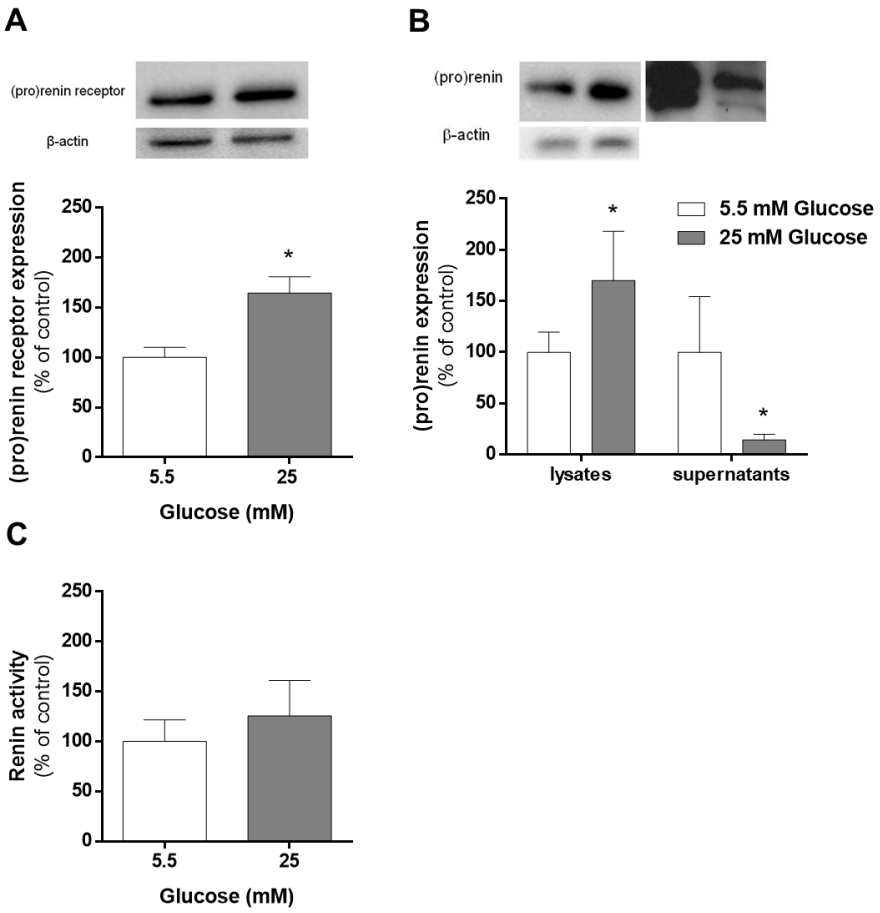


Figure 3.2 Effect of glucose in the expression of RAS in RPE cells. (A) Representative immunoblots and densitometric analysis of renin receptor expression in the presence of 5.5 and 25 mM of glucose in cell lysates of RPE cells. β-actin is used as loading control. Values are expressed as percentage of control, with control being the condition with 5.5 mM of glucose. * $p<0.05$ compared with control values, determined by unpaired t test, N=3. **(B)** Representative immunoblots and densitometric analysis of renin expression in the presence of 5.5 and 25 mM of glucose in cell lysates and supernatants of RPE cells. β-actin is used as loading control. Values are expressed as percentage of control, with control being the condition with 5.5 mM of glucose. * $p<0.05$ compared with control values, determined by multiple unpaired t test, N=6. **(C)** Renin activity in the presence of 5.5 and 25 mM of glucose in cellular lysates of RPE cells. Values are normalized to protein concentration in each condition and expressed as percentage of control, with control being the condition with 5.5 mM of glucose, N=5.

Our results show that high glucose increases the levels of renin in the intracellular milieu of RPE cells, but the levels of renin secreted to the extracellular medium were significantly decreased. These findings demonstrate that hyperglycemia promotes the overexpression and retention of renin in RPE cells. Similar results were obtained with rat mesangial cells where high glucose decreased the levels of renin in the culture medium, resulting in its intracellular accumulation [167]. Although statistical significance was not

reached, a trend was observed for the activity of renin in the presence of high glucose in cellular lysates of RPE cells. Nevertheless, the retention of renin in the intracellular compartment induced by excess of glucose will activate the angiotensin II-dependent pathway, ensuing angiotensin II synthesis.

The renin receptor was also modulated by high glucose in RPE cells. This receptor has a predominant subcellular localization [165], and its translocation to the cellular membrane takes place only upon stimulation. In the present experiment, the levels of the renin receptor were measured in total lysates and not in the membrane and cytosolic fractions. Since high glucose promoted the overexpression and retention of renin in the intracellular space, we hypothesize that this condition might also favor the retention of the renin receptor within the RPE cells.

As part of the protective axis of RAS, the expression of ACE2, Mas receptor and the levels of Ang (1-7) were evaluated in RPE cell lysates exposed to normoglucose or high glucose conditions. The three components of the protective axis of RAS were detected in our experimental setup, as shown in Figure 3.3.

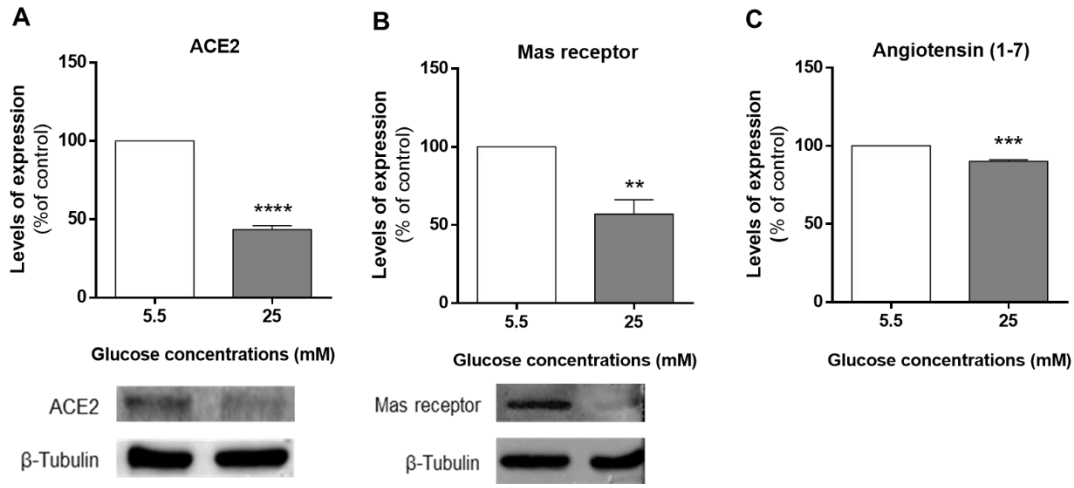


Figure 3.3 Effect of glucose in the expression of RAS protective axis components in RPE cells. Representative immunoblots and densitometric analysis of ACE2 **(A)** and Mas receptor **(B)** in the presence of 5.5 or 25 mM of glucose in cell lysates of RPE cells. β-tubulin is used as loading control. Values are expressed as percentage of control, with control being the condition with 5.5 mM of glucose. ** $p<0.01$, **** $p<0.0001$ compared with control values, determined by unpaired t test, N=3. **(C)** Densiometric analysis of Angiotensin (1-7) levels determined by ELISA. Values are expressed as percentage of control, with control being the condition with 5.5 mM of glucose. **** $p<0.0001$ compared with control values, determined by unpaired t test, N=3.

Here, the opposite is observed where the levels of ACE2 and Angiotensin (1-7), as well as the expression of its biological receptor, Mas1, decrease significantly in high glucose compared to normal glucose conditions (Figure 3.3 A-C). Interestingly, when inner medullary collecting duct cells were exposed to high glucose (30 mM D-glucose), the activity of ACE2 increased in the intracellular compartment, resulting in high levels of Ang (1-7) in the same cellular compartment [168]. Also, it was observed an increase in the ACE2 enzymatic activity in induced-diabetic mouse retina [169]. However, the same group had previously reported a decrease in ACE2 expression in the diabetic kidney [170], as observed in the RPE cells from our study. This implies that different organs respond differently to the diabetic environment and could explain the contradictory effect of glucose in cells and organs. Moreover, the specific pathways that modulate ACE2 expression are unknown. Crackower *et al*. had addressed that question, showing that hypoxia may be responsible to stimulate the expression of this enzyme [110]. Our study lacks the expression of RAS components under hypoxia, which could lead to results in line with those observed in other previously mentioned cell models.

These results indicate that glucose modulates RAS, leading to an imbalance of the protective axis. With decreased expression of the protective components and increased expression of renin, its receptor and Ang II, the prejudicial effects overrule the beneficial effects of the protective axis of RAS. Considering that ACE2 is a key regulator of Ang II and Ang (1-7) levels and their contribution to the disease, this modulation/effect in expression can determine the development and progression of the pathology towards retinal damage.

3.3.3. Activation of the renin–angiotensin system promotes angiogenesis and oxidative stress in RPE cells

VEGF is a potent inducer of angiogenesis, but oxidative stress also contributes to this process. To assess if activation of the RAS contributes to deleterious effects in RPE cells via VEGF and oxidative stress activation, these were exposed to angiotensin II. Following, it was observed that the levels of VEGF increased in a concentration-dependent manner (Figure 3.4 A). Additionally, exposure of RPE cells to angiotensin II increased the production of ROS in a time-dependent manner (Figure 3.4 B).

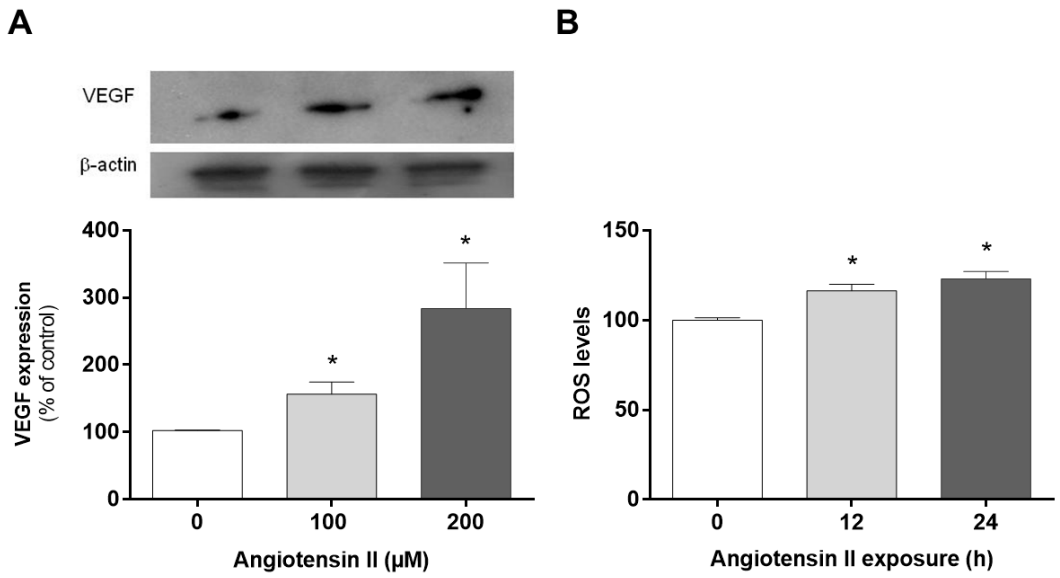


Figure 3.4 Effect of activation of RAS on angiogenesis and oxidative stress in RPE cells. (A) Representative immunoblots and densitometric analysis of VEGF expression in the presence of angiotensin II (0, 100, 200 μM; 24 h) in RPE cells. β-actin is used as the loading control. Values are expressed as percentage of control with control being the condition without angiotensin II. * $p < 0.05$ compared with control values, determined by multiple t test, N=5. **(B)** Levels of ROS in the presence of angiotensin II (0, 12, 24 h; 200 μM) in RPE cells. Values are expressed as percentage of control with control being the condition without angiotensin II. * $p < 0.05$ compared with control values, determined by multiple t test, N=4.

This result, which contributes to the harmful effects associated with the ACE/Ang II/AT1R axis, leads to the need to target RAS pathways. Several inhibitors have emerged to block this system at different steps, showing to effectively decreased the production of ROS in the retinas of ischemic rats [171], as well as the levels of VEGF in the retinas of streptozotocin-induced diabetic rats [172,173]. Despite these results, the RAS was not effectively blocked, since these drugs promote a compensation feedback mechanism that increases renin [174,175].

3.3.4. Activation of the renin-angiotensin system increases the expression of the protective components in a time-dependent manner

Knowing that Ang II is the substrate of ACE2 to form Ang (1-7), we evaluated the expression of the protective components in RPE cells previously exposed to Ang II at 200 μM concentration for 12 h and 24 h. The levels of expression of the three components tend to increase in a time-dependent manner upon RAS activation with Ang II (Figure 3.5).

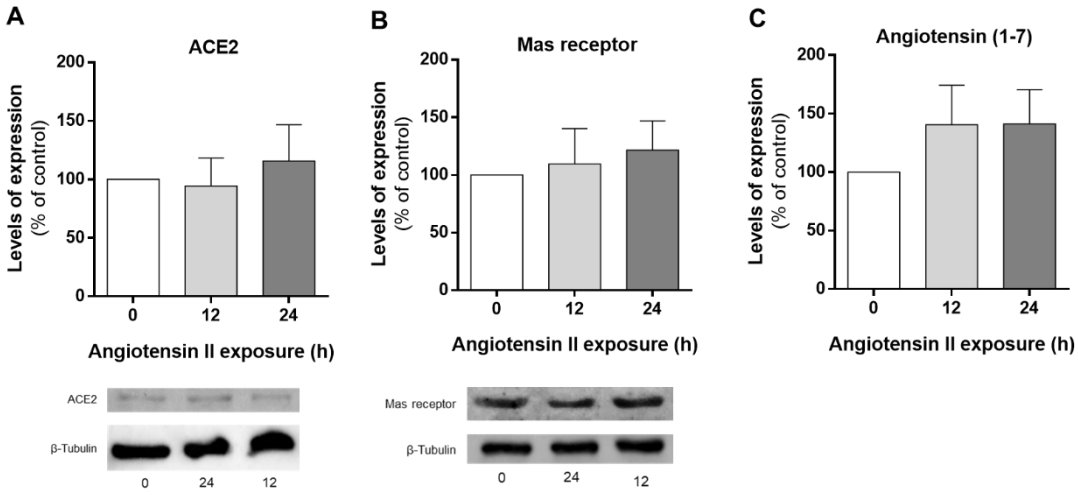


Figure 3.5 Effects of RAS activation in the expression of RAS' protective components. Representative immunoblots and densitometric analysis of ACE2 **(A)** and Mas receptor **(B)** expression in RPE cells after stimulation with Ang II (200 μM) for 12 h and 24 h. α-Tubulin is used as loading control. Values are expressed as percentage of control with control being the condition without angiotensin II (0 h), N=3. **(C)** Densiometric analysis of Angiotensin (1-7) levels determined by ELISA. Values are expressed as percentage of control with control being the condition without angiotensin II. (0 h), N=2.

Since the studies performed in RPE cells are scarce, we need to interpolate these results based on other cell types or organs. It is acknowledged that ACE2 is important in heart function but there are conflicting results on its regulation by angiotensin peptides, namely Ang II. Reports from animal models of myocardial infarction and hypertension show an increase in ACE2 expression associated with cardiovascular diseases, parallel to elevated plasma levels of Ang II [176–178]. Also Lin *et al.*, reported that an Ang II stimulus induced ACE2 expression in human cardiac fibroblasts [178]. However, when studying the regulation of ACE2 by angiotensin peptides, Gallagher *et al*. showed that Ang II lead to a decrease in the expression of ACE2 in both rat cardiac myocytes and fibroblasts [176]. While emphasizing that ACE2 regulation by angiotensin peptides can be dependent on the physiological or pathological processes involved, it is suggested that upregulation of ACE2 plays a compensatory role to the increased Ang II levels [178].

When hypertension is associated with diabetic nephropathy, another common diabetic and kidney damaging complication, is has been reported an increase in the expression of ACE, associated with high Ang II levels [177,179]. Thus, in hypertension, is suggested that the alteration in the balance between the two ACE enzymes shifts towards favoring Ang II formation, down-regulating ACE2 expression in the kidney, accompanied by an increase in ACE levels [177]. This imbalance could lead to more renal damage and progression of renal disease since excess Ang II production is favored

instead of its degradation through ACE2 catalysis. However, a report indicates that, in spontaneously hypertensive rats, ACE2 is highly expressed in the kidney early on and only after the establishment of hypertension is seen a decrease in the expression of ACE2 [180,181]. Even in initial stages of diabetes, ACE2 is highly expressed and with the development of retinopathy its expression decreases [182]. In line with these reports, in diabetic rodent models are observed early increases in the expression of ACE2 [182,183], which has been found to be decreased in older diabetic mice [183]. This indication leads back to the possibly compensatory and early protective role of ACE2, in several diseases, associated with RAS activation.

In this study, the activation of RAS through an Ang II stimulus increases the expression of pro-angiogenic VEGF while increasing ROS production. At the same time, the expression of the protective components of RAS tends to increase. We hypothesize that there is an attempt to compensate the increase of VEGF and ROS in the cell through the increase in the expression of the components of the protector axis of RAS. However, the molecular mechanisms of this increase are unknown. We have observed that hyperglycemic conditions imbalance the expression of the RAS' protective components. Yet, an activation of RAS tends to increase the expression of the same components. Further research should be carried in order to better understand the molecular mechanisms involved and the apparent trigger that reverts the initial protective trend of this hormonal system.

3.3.5. Aliskiren does not affect the viability of RPE cells

Due to the complexity of RAS pathways, several inhibitors have emerged to block this system at different steps. Therapies targeting RAS inhibition are based in ACEi and ARBs acting on later steps of this cascade, by inhibiting Ang II formation or the mediation of its prejudicial effects by the AT1R receptor. These drugs have been proven to confer protection in DR development and progression [13,156] but being an intricate system of pathways there are ways to subvert the action of these inhibitors, bringing limited success to these therapies. Direct renin inhibitors raise interest for blocking RAS at its initial step. The only commercially available direct renin inhibitor is aliskiren.

In order to test the effects of aliskiren in the RPE cells, we performed a viability assay to determine the concentrations and times of exposure that would be safe for retinal cells. RPE cells were exposed to 50 and 100 μM of aliskiren (30 and 60 μg/ml in absolute dose) during 2 h and 4 h and none of these conditions altered the viability of

the cells. DMSO had no effect upon the cellular viability, in opposition to ethanol, which decreased cell viability, as expected (Figure 3.6).

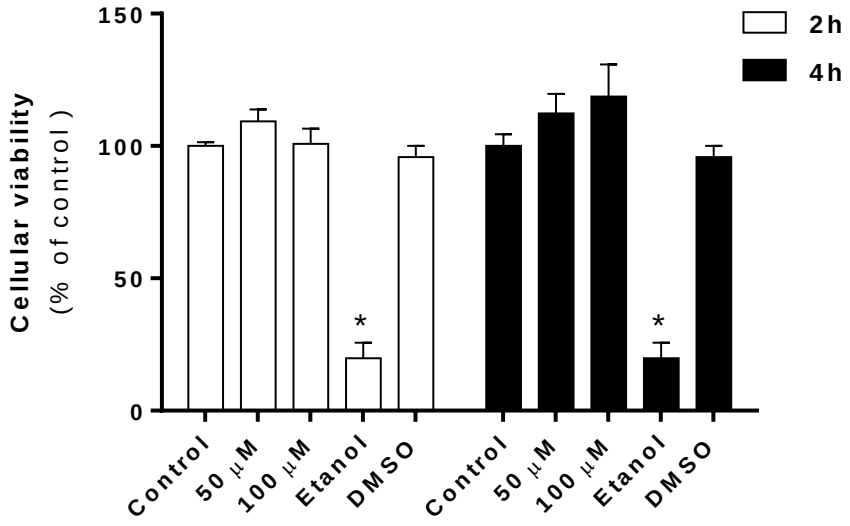


Figure 3.6 Impact of aliskiren in the viability of RPE cells. Graph representing the viability of RPE cells in the presence of aliskiren [50 and 100 μM (30 and 60 μg/ml in absolute dose)] incubated during 2 h and 4 h measured by means of the Calcein-AM assay. Ethanol was used as the negative control for viability. DMSO was used as vehicle for aliskiren. Values are expressed as percentage of control, with control assumed to be the condition of 0 μM aliskiren. * $p < 0.05$ compared with aliskiren treatment, determined by Two-way ANOVA with Dunnett's Multiple Comparison test, N=4.

Because the effects of aliskiren on in vitro retinal cells are scarce in the literature, the range of concentrations of aliskiren used in the present study was chosen based on other cell types [161,184–186]. In the present study concentrations of aliskiren in the micromolar range were found to be safe for RPE cells and therefore were used to modulate the RAS.

3.3.6. Aliskiren alters the distribution of the (pro)renin receptor in RPE cells

The effect of aliskiren on the expression of the (pro)renin receptor was evaluated in both cytosolic (CF) and membrane fractions (MF) of RPE cells (Figure 3.7). In the presence of aliskiren [100 μM (60 μg/ml in absolute dose); 4 h] the expression of the receptor increased in the CF and decreased in the MF of RPE cells.

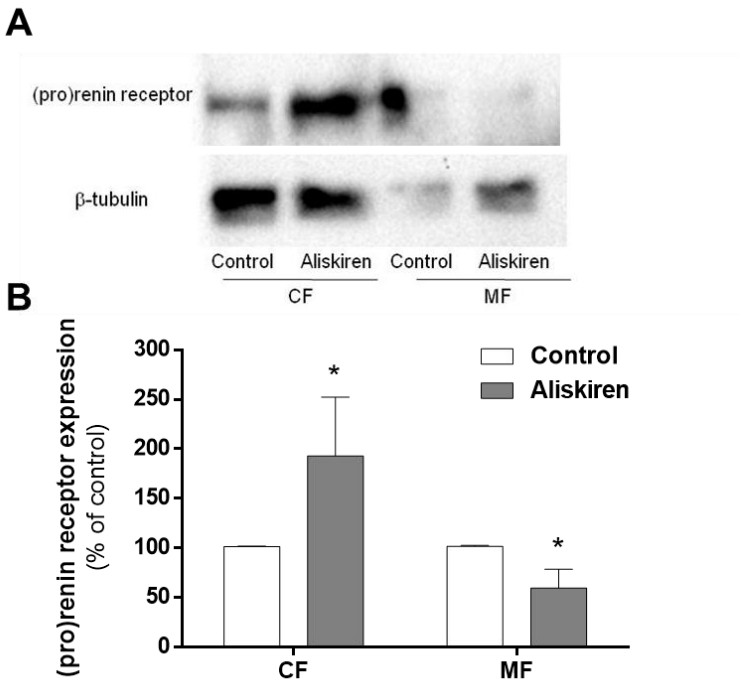


Figure 3.7 Effect of aliskiren in the renin receptor expression in the membrane of RPE cells. (A) Representative immunoblots of renin receptor expression in the cytosolic (CF) and membrane fractions (MF) in the presence of aliskiren [100 µM (60 µg/ml in absolute dose); 4 h] in D407 RPE cells. **(B)** Densitometric analysis of renin receptor expression in the cytosolic (CF) and membrane fractions (MF) in the presence of aliskiren [100 µM (60 µg/ml in absolute dose); 4 h] in D407 RPE cells. β-Tubulin was used as the loading control. Values were expressed as percentage of control, with control assumed to be the condition without aliskiren. * $p < 0.05$ compared with control values, determined by multiple unpaired t test, N=5.

3.3.7. Aliskiren decreases the activity of renin, levels of angiotensin II, ACE2 and Mas receptor in RPE cells

RPE cells were exposed to aliskiren and the activity and expression of renin was assessed. When RPE cells were exposed to 100 µM of aliskiren (60 µg/ml in absolute dose) during 4 h the activity of renin was significantly inhibited (Figure 3.8 A), but no changes were observed in its expression levels (Figure 3.8 B and C). Angiotensin II acts downstream renin and its levels were significantly decreased by aliskiren [100 µM (60 µg/ml in absolute dose); 4 h] in RPE cells (Figure 3.8 D).

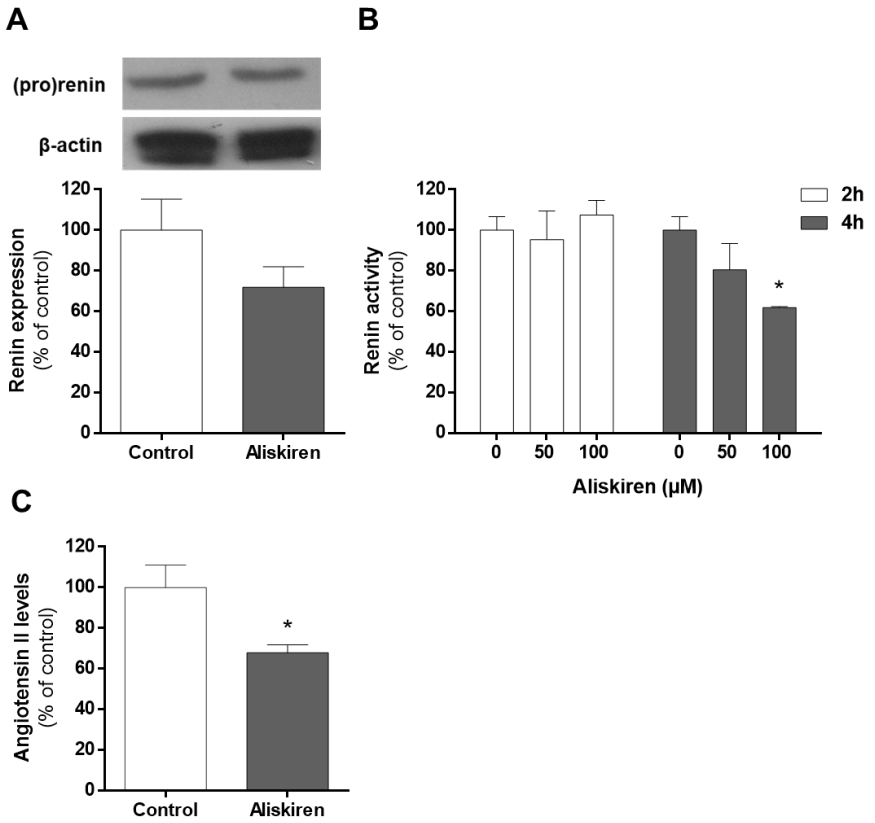


Figure 3.8 Effect of aliskiren in renin expression and activity and angiotensin II levels in RPE cells. **(A)** Representative immunoblots and densiometric analysis of renin expression in the presence of aliskiren [100 μM (60 μg/ml in absolute dose); 4 h] in D407 RPE cells. β-Tubulin was used as the loading control. Values are expressed as percentage of control, with control assumed to be the condition without aliskiren, N=6. **(B)** Renin activity in the presence of aliskiren [50 and 100 μM (30 and 60 μg/ml in absolute dose)] incubated during 2 h and 4 h in D407 RPE cells, measured by a fluorimetric assay. Values were normalized to protein concentration in each condition and were expressed as percentage of control, with control assumed to be the condition of 0 μM aliskiren. * $p < 0.05$ compared with control values, determined by Multiple t test, N=3. **(C)** Levels of angiotensin II measured in cellular lysates from D407 RPE cells in the presence of aliskiren [100 μM (60 μg/ml in absolute dose); 4 h] by means of an enzyme immunoassay. Values were normalized to protein concentration in each condition and were expressed as percentage of control, with control assumed to be the condition without aliskiren. * $p < 0.05$ compared with control values, determined by an unpaired t-test with Welch's correction, N=5.

Moreover, the expression levels of ACE2, Mas receptor and Angiotensin (1-7) were measured in RPE cells exposed to aliskiren for 4 and 24 h. It was observed that the expression of ACE2 tends to decrease 4 h after renin inhibition (p=0.1695), although without reaching statistical significance (Figure 3.9 A), with no alterations in the expression of Mas receptor (Figure 3.9 B). However, the levels of the beneficial effector, Ang (1-7) (Figure 3.9 C), seem to have the opposite tendency, increasing its expression upon 4 h of exposure to aliskiren.

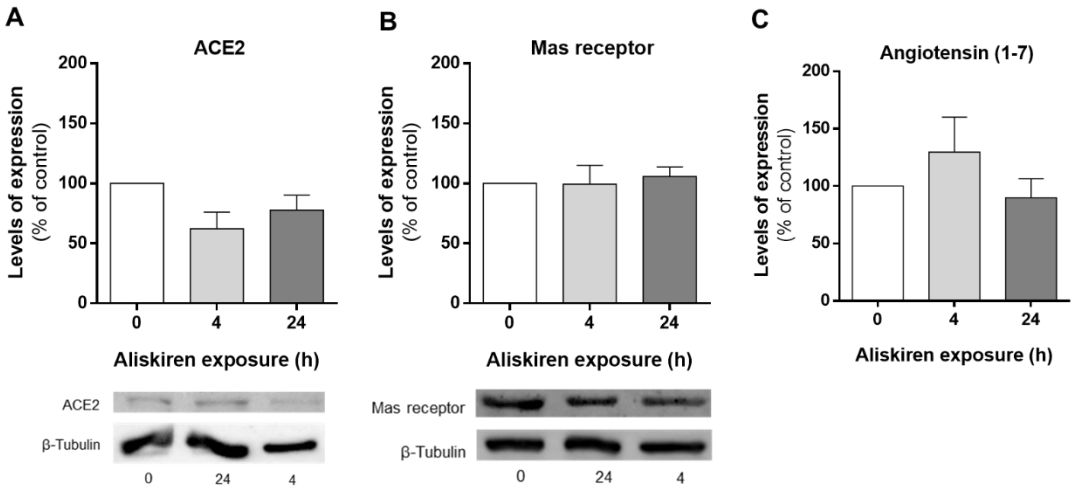


Figure 3.9 Effect of aliskiren on the expression of RAS' protective components. Representative immunoblots and densitometric analysis of ACE2 **(A)** and Mas receptor **(B)** expression in RPE cells after stimulation with aliskiren (100 μM) for 4 h and 24 h. α-Tubulin is used as loading control, N=3. **(C)** Densiometric analysis of Angiotensin (1-7) levels determined by ELISA. Values are expressed as percentage of control, with control being no exposure to aliskiren (0 h), N=3.

Aliskiren blocked renin activity in RPE cells but had no effect on the levels of the protein, suggesting that the exposure times chosen for this study enable aliskiren to modulate the activity of renin but not its levels. This is in accordance with the mechanism of action of aliskiren, which is based on the binding of the drug to the catalytic center of renin [158], a process much faster than the time needed for the expression of proteins. As a result of the decrease in renin activity, there was a significant reduction in angiotensin II. The latter peptide induces vasoconstriction in several tissues including the retina and promotes angiogenesis, inflammation and oxidative stress [108,153]. Also, inhibition of RAS by aliskiren seems to have effect on the expression of ACE2 and Angiotensin (1-7) (Figure 3.9 A, C). By blocking the formation of the angiotensin peptides, it seems that the expression of some key players of the protective arm of RAS decreases. Since there is less Ang II, the main substrate for Ang (1-7), there is less need of ACE2 in the cell. Unexpectedly, the same tendency was not observed for Ang (1-7) (Figure 3.9 C). Aliskiren acts on the catalytic center of renin [187], thus, the tendency to increase Ang (1-7) levels cannot be explained by the favorable pathway of formation, where Ang II is degraded. Other sources of formation of Ang (1-7) could explain this result and should be further explored.

3.3.8. Aliskiren prevents oxidative stress and angiogenesis in RPE cells

RPE cells were exposed to aliskiren (100 µM) up to 24 h followed by the measurement of ROS levels, angiogenic and anti-angiogenic factors (Figures 3.10 and 3.11). Aliskiren significantly decreased the production of ROS (Figure 3.10) and the levels of VEGF in a time-dependent manner (Figure 3.11 A and B), an effect that reached statistical significance after a 24 h of exposure.

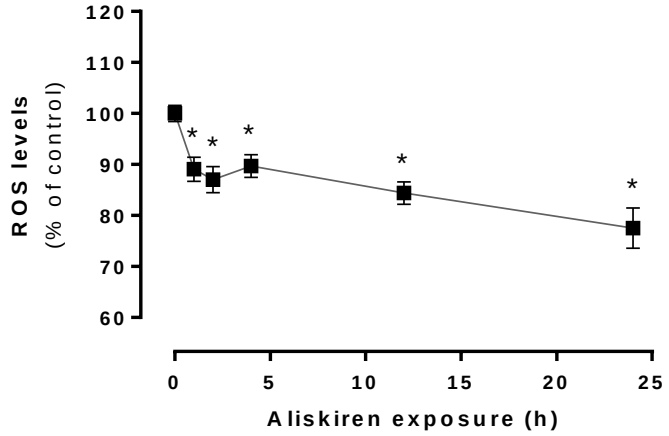


Figure 3.10 Effect of aliskiren in oxidative stress levels in RPE cells. Levels of ROS in the absence (0) and presence of aliskiren (1, 2, 4, 12, 24 h; 100 µM) in RPE cells. Values are expressed as percentage of control with control being the condition without aliskiren (0). * $p<0.05$ compared with control values, determined by Dunnett's multiple comparison test, N=6.

On the other hand, the levels of anti-angiogenic PEDF increased in RPE cells in a time-dependent manner following aliskiren treatment, reaching statistical significance after 24 h of exposure (Figure 3.11 B).

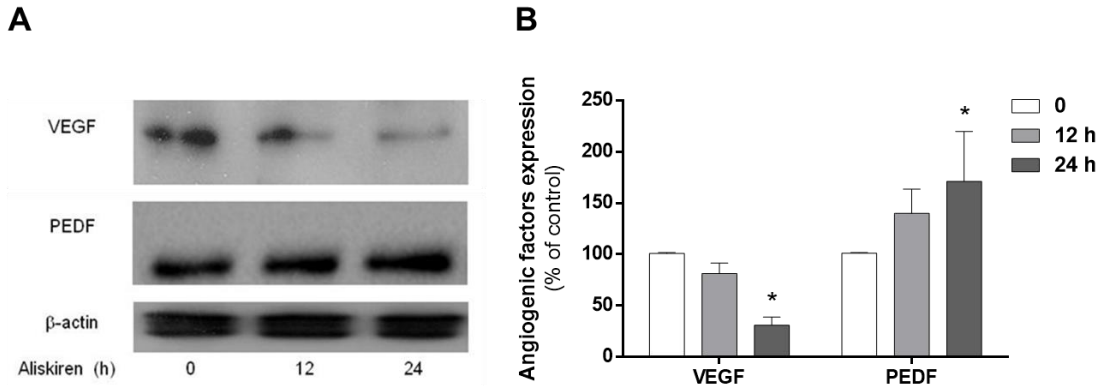


Figure 3.11 *(continued on the following page)*

Figure 3.11 Effect of aliskiren in the expression of proteins involved in angiogenesis in RPE cells. (A) Representative immunoblots and **(B)** densitometric analysis of VEGF and PEDF expression in the presence of aliskiren (0, 12, 24 h; 100 μM) in RPE cells. β-actin is used as the loading control. Values are expressed as percentage of control with control being the condition without aliskiren. * $p<0.05$ compared with control values, determined by by multiple t test, N=6.

It was previously described that patients with retinopathies have increased levels of angiotensin II [108] and was also shown that angiotensin II might induce breakdown of the RPE basement membrane [188]. Our results demonstrate that aliskiren is effective in decreasing angiotensin II and, consequently, the production of ROS in RPE cells in a sustained and time-dependent manner, suggesting that this drug has antioxidant capacity in RPE cells and therefore might be a candidate drug to protect these cells from the deleterious effects derived from the overproduction of this peptide.

It is well established that in patients with retinal diseases the balance between angiogenic and anti-angiogenic factors is shifted towards the former, promoting angiogenesis and neovascularization, and this is crucial for disease progression [68]. In the retina, RPE cells are one of the sources of VEGF [189,190] and inhibition of this factor constitutes the main therapeutic approach to reduce ocular neovascularization. PEDF, synthesized by RPE cells, is known to suppress angiogenesis and is also down-regulated in retinal diseases [68]. Our findings demonstrate that aliskiren decreases the levels of VEGF while up-regulating PEDF, successfully contributing to restore the balance between angiogenic and anti-angiogenic factors in RPE cells.

Overall, our findings suggest that aliskiren combines antioxidant and anti-angiogenic functions over RPE cells. To the best of our knowledge, none of the classical RAS blockers used in the treatment of retinopathies gather these properties and for that reason aliskiren might offer advantages over those RAS inhibitors for the treatment of these diseases. Interestingly, in a recent systematic review aimed to evaluate the effect of RAS inhibitors on diabetic retinopathy it was found that ACE inhibitors were better than ARBs for the treatment of this disease [191]. We hypothesized that this finding might be related with the fact that the ACE is a preceding component in the RAS cascade than the angiotensin II receptor, supporting our theory that upstream components of the RAS might be more adequate targets to promote effective RAS inhibition.

Despite our promising findings regarding upstream RAS inhibition with aliskiren, and considering our previous findings, in which we observed dysregulation in the expression of both RAS axis by glucose, along with the evidence of the important role of the ACE/AngII/AT1R axis in DR hallmarks, such as angiogenesis and oxidative stress,

we hypothesized that targeting the protective axis of RAS can be also a valid therapeutic approach.

The concept that shifting the balance of the RAS towards the vasodilatory axis is beneficial has been supported by studies at the organ level, such as in the kidney [192]. Particularly, the overexpression of ACE2 was studied in two different animal models of DM, namely STZ-induced diabetic eNOS$^{-/-}$ mouse model, which has been recently shown to develop accelerated retinopathy [193] and the STZ-induced diabetic SD rat model, conferring protection against development of DR in animal models [194] or resulting in an improvement in the inflammatory response by activating the ACE2/Ang-(1-7)/Mas axis in human RPE cells [195].

We hypothesize that a gene therapy approach towards the activation of the protective axis could present as a therapeutic solution for DR. To test this hypothesis, we have chosen to overexpress the ACE2 in RPE cells and evaluate the expression and/or levels of the protective axis components, as well as its effects on inflammation, oxidative stress and angiogenesis. The human gene of ACE2 was inserted into the backbone of the pEPIto episomal vector under a humanized citomegalovírus (CMV) promoter, as described previously in Section 2.9.

3.3.9. ACE2 is effectively overexpressed in RPE cells

The plasmid containing the human gene of ACE2 was transfected into RPE cells, and efficiency of transfection was confirmed by microscopy by green flurorescent protein (GFP) autofluorescence of the empty plasmid transfected in a control group of cells. (Figure 3.12 A) and the expression of ACE2 was evaluated by Western blot at 24, 48 or 72 hours post transfection (Figure 3.12 B). The cellular localization and the expression of ACE2 in RPE cells was confirmed by immunocytochemistry (Figure 3.12 C).

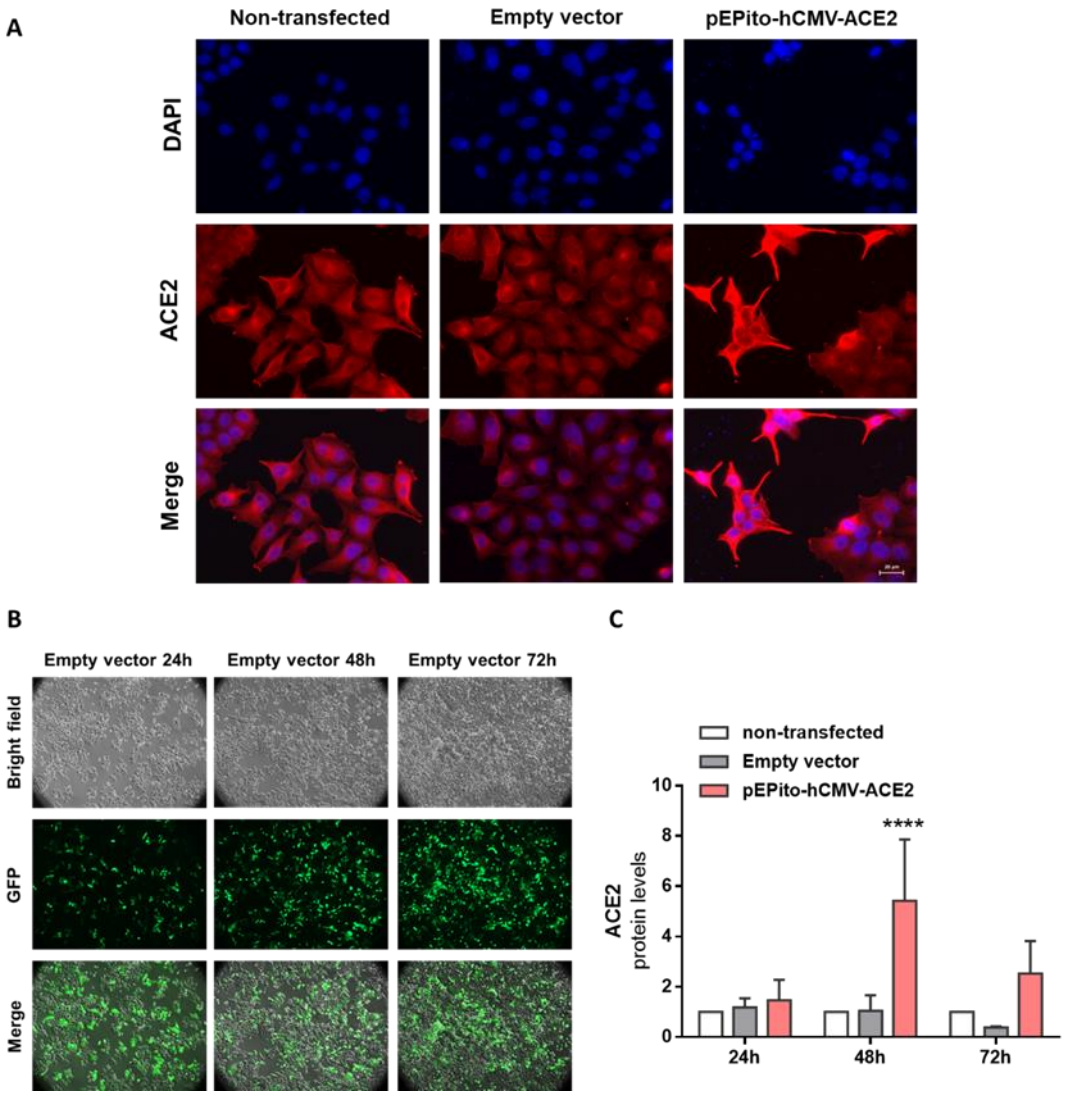


Figure 3.12 Overexpression of ACE2 driven by pEPito-hCMV-ACE2 in human RPE cells up to 72 h. (A) Immunostaining of RPE cels with anti-ACE2 (red) antibody, counter-stained with DAPI (blue) to visualize the nuclei. Images were obtained in a fluorescence microscope with a 630x magnification. Scale bar = 20 µm. Images are representative of two independent experiments. (**B)** Representative images and **(C)** densiometric analysis of protein levels of ACE2 after transfection determined by Western blot. Values are expressed as mean ± SD, **** $p < 0.0001$ compared with non-transfected cells (NT), determined by Dunnett's multiple comparisons test, N=3.

As observed in figure 3.12 A and B, the ACE2 plasmid significantly upregulated the expression of ACE2 in RPE cells after 48 h of transfection. Also, by immunocytochemistry, it is possible to observe perinuclear and cytoplasmatic expression of ACE2 within the RPE cells, which is not altered by transfection. Overexpression of ACE2 driven by pEPito-hCMV-ACE2 is once again confirmed by the intensification in the staining of ACE2. Most components of RAS, including ACE2/Ang-(1-7)/Mas axis, have been confirmed in various tissues and organs, including the eye[152]. The overexpression

of components of the protective axis of RAS was recently achieved and studied in RPE cells by Fu *et al.*[195] having in mind their putative role in the improvement of Aβ-induced inflammatory response in human RPE cells. Indeed, the activation of the ACE2/Ang-(1-7)/Mas axis, particularly by overexpressing ACE2 in RPE cells, shown as a promising therapeutic approach for inflammatory response in AMD [195].

Here we show that overexpression of ACE2 is successfully achieved in RPE cell line, D407, 48 hours after transfection, as there is a significant increase in the expression of ACE2 in cells transfected with pEPito-hCMV-ACE2 compared with non-transfected cells. Longer exposure to ACE2 plasmid does not result in upregulation of the ACE2 expression, possibly because there is an increase in cell death as shown by figure 3.12 B. Also, we show the perinuclear and cytoplasmatic expression of ACE2 in RPE cells by immunostaining, which is enhanced by pEPito-hCMV-ACE2 transfection.

3.3.10. The levels Mas receptor are increased by ACE2-overexpression in RPE cells

The protective axis of RAS is also composed of a small peptide Ang-(1-7) and its receptor, Mas. The expression of both elements was analyzed by ELISA or Western blot upon ACE2-overexpression in RPE cells (Figures 3.13 A, B).

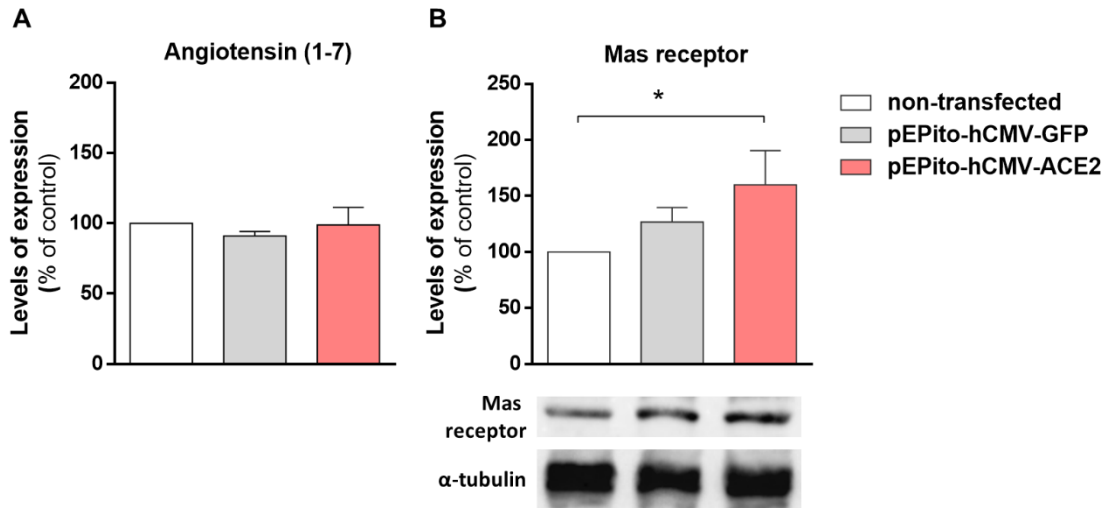


Figure 3.13 Levels of Angiotensin (1-7) and expression of Mas receptor in ACE2-overexpressed RPE cells. (A) Densiometric analysis of Angiotensin (1-7) levels determined by ELISA. Values are expressed as percentage of control with control being the non-transfected cells, N=3. **(B)** Densiometric analysis of the Mas receptor and representative immunoblots. α-tubulin is used as the loading control. Values are expressed as percentage of control with control being the non-transfected cells. * $p < 0.05$ determined by Dunnett's multiple comparisons test, N=3.

Figure 3.13 shows no differences at the levels of Ang-(1-7) in RPE cells transfected with pEPito-hCMV-ACE2, compared with the non-transfected cells. However, there is a significant increase in the expression of Mas receptor.

Although there is an increase in the expression of ACE2 promoted by pEPito-hCMV-ACE2 transfection, it is not reflected in the levels of Ang-(1-7). Moreover, the increase in the expression of the receptor without affecting the levels of Ang-(1-7) may have to due with the fact that we are looking to protein levels which takes longer to be affected by certain stimuli. An analysis of the mRNA levels or the secreted form of the peptide could leds us to important information regarding the effect of ACE2 overexpression in this context.

3.3.11. ACE2-overexpression does not alter the levels of proteins involved in angiogenesis but protects RPE cells from inflammation and oxidative stress

As previously described, inflammation and pathological angiogenesis are great contributors for DR progression. The expression of anti-angiogenic PEDF and pro-angiogenic VEGF were evaluated by Western blot in ACE2-overexpressed RPE cells (Figure 3.14 A). Also, pro-inflammatory markers, TNF-α and IL1-β, and the levels of ROS were assessed in ACE2 driven by pEPito-hCMV-ACE2 RPE cell (Figure 3.14 B, C).

We found no differences in the expression of PEDF or VEGF when ACE2 is overexpressed in RPE cells were compared with non-transfected cells (Figure 3.14 A). On the other hand, the expression levels of TNF-α in RPE cells transfected with pEPito-hCMV-ACE2 are similar to the levels found in non-transfected cells, protecting them from the increase observed by pEPito-hCMV-GFP (Figure 3.14 B). The same is observed for oxidative stress, with a significantly decrease in the levels of ROS in cells overexpressing ACE2, compared to empty plasmid (Figure 3.14 C).

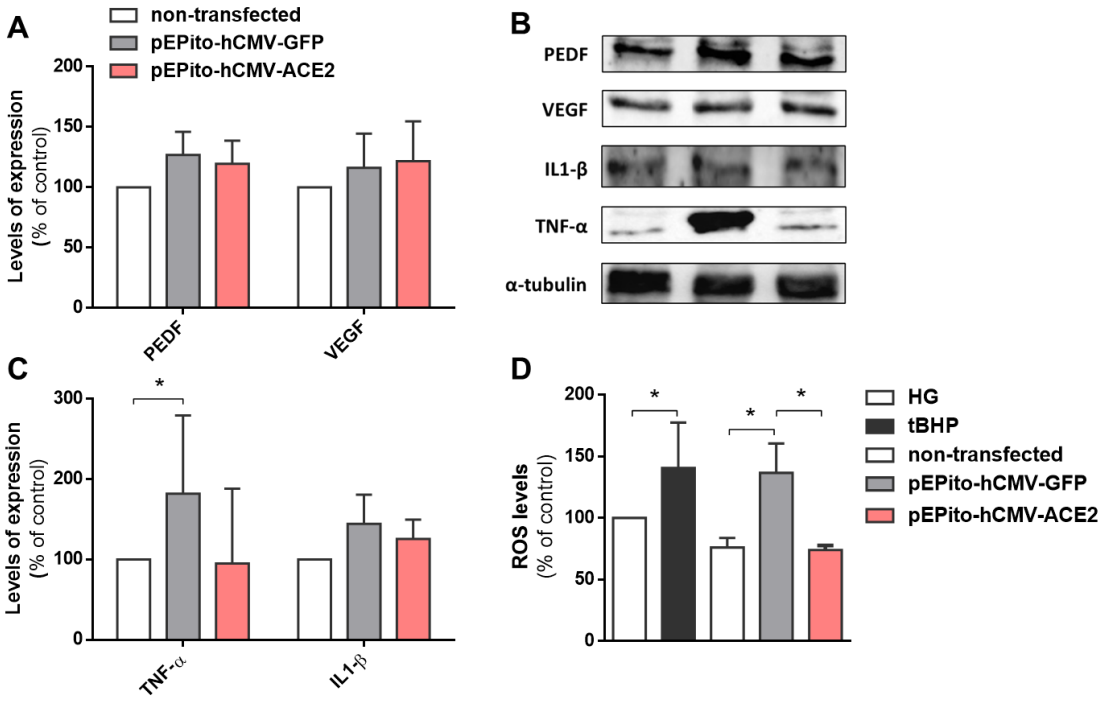


Figure 3.14 Expression of several markers relevant for the DR phenotype in ACE2-overexpressed RPE cells. (A) Densiometric analysis of PEDF and VEGF and **(B)** representative immunoblots. Values are expressed as percentage of control with control being the non-transfected cells. α-tubulin is used as the loading control, N=4. **(C)** Densiometric analysis of TNF-α and IL1-β. Values are expressed as percentage of control with control being the non-transfected cells. * $p < 0.05$, determined by Sidak's multiple comparisons test, N=3. **(D)** Densiometric analysis of the levels of ROS. Values are expressed as percentage of control with control being the non-transfected cells. * $p < 0.05$ compared to control cells under high glucose, non-trasnfected cells or empty vector, determined by Tukey's multiple comparisons test, N=3.

3.3.12. Characterization of RAS in a diabetic animal model

The present work has also aimed to characterize the expression of RAS components in the retina of diabetic mice (Figure 3.15). Studies performed with induced diabetic animal models have shown the retinal expression of the protective components decreased with the duration of diabetes, while the expression of the classical components increased [194].

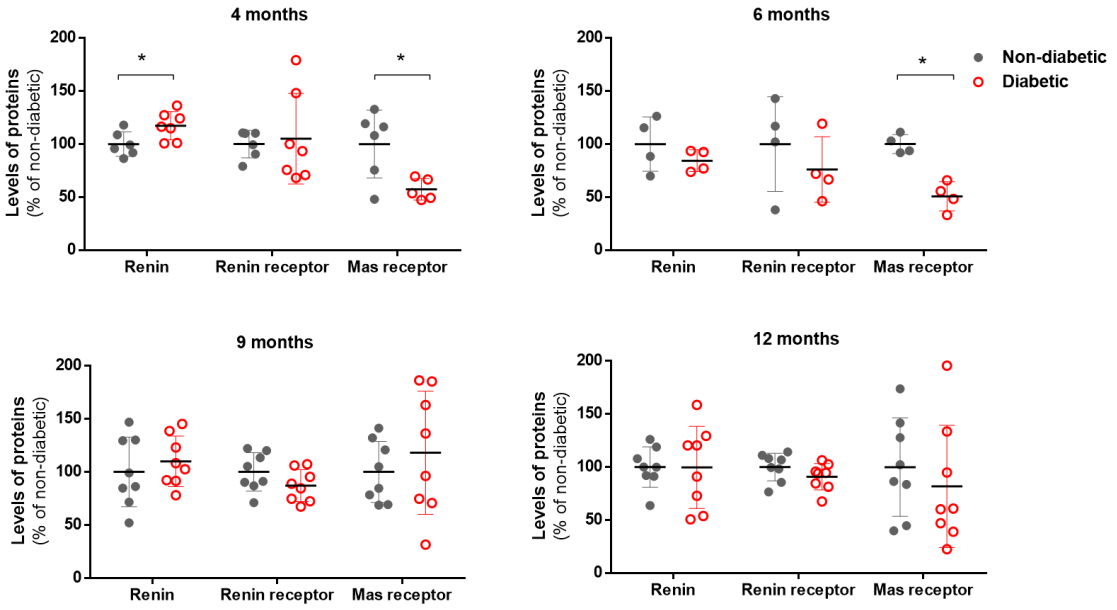


Figure 3.15 Expression of classical and protective RAS components in the mouse eye. Densiometric analysis of the expression of renin, renin receptor and Mas receptor in the retinas of wild-type and diabetic mice at different ages (4, 6, 9, and 12 month-old). Results are shown as a percentage of control non-diabetic mice. α-Tubulin was used as loading control. * $p < 0.05$ compared to non-diabetic retina, determined by Multiple t test, N=4 to 8.

Expression of components from both prejudicial and protective axis were evaluated in the retina of diabetic C57BL/6-Ins2^{Akita}/J and C57BL/6J age-matched (non-diabetic) mice. In the retina of diabetic 4 month-old animals the expression of renin increases while the expression of the Mas receptor decreases, compared with non-diabetic retinas. The expression of the renin receptor is maintained at similar levels in both diabetic and non-diabetic retinas (Figure 3.15 A). In 6 month-old diabetic retinas, the expression of all components evaluated tends to decrease, being observed a higher decrease on the expression levels of Mas receptor (Figure 3.15 B). The expression of renin, renin receptor and Mas receptor remains unchanged in the diabetic retinas of older mice (9- and 12-months, Figure 3.15 C and D), compared with non-diabetic animals.

While for the Ins2^{Akita} mice this is the first time this analysis is performed, for wild-type mice the expression of the Mas receptor has been described in the literature as being higher than the expression of the angiotensin receptors [97].

It has also been shown that the retina produces (pro)renin and its receptor [166,196]. In addition, diabetic patients show increased levels of (pro)renin [102] and the binding of (pro)renin to (pro)renin receptor has been associated with the development of ocular disease [108,152], with proven association with angiogenic activity in PDR [197]. The interaction

of (pro)renin to its receptor mediates similar effects to the ones mediated by AT1R but independently of this receptor [94].

In the present work, we also observed a lower level of expression of renin and (pro)renin receptor in the diabetic retinal tissue when compared to non-diabetic retinas. Nevertheless, the expression levels are still higher than the Mas receptor levels (Figure 3.15 B). From these results we hypothesize that this initial RAS imbalance, observed by the Mas receptor decreased expression, may be one of the triggers to develop DR. As the diabetic mice age, the diabetic complications tend to aggravate. However, it seems that the RAS expression reaches again an equilibrium between the classical and protective components. This may be explained also by the characterization of $Ins2^{Akita}$ mice by Barber *et al*., which indicates that this animal model can only develop the earlier stages of DR [148]. Since the expression of the RAS components seem to reach this equilibrium, there is no longer an overruling of the prejudicial effects of RAS over the beneficial effects, resulting in a delay in progressing to the severe stages of DR.

CHAPTER IV

EFFECT OF (POLY)PHENOL-DERIVED METABOLITES IN INFLAMMATION-RELATED MARKERS

This chapter is based on the following scientific article:

Santos DF, Pais M, Silva GA. Polyphenol metabolite Pyrogallol-*O*-sulfate decreases microglial activation and pro-angiogenic VEGF in RPE cells and diabetic mouse model. *In preparation*

4.1. Abstract

(Poly)phenols-derived metabolites are small molecules resulting from (poly)phenols degradation that can be found in circulation following (poly)phenols-rich diet. The described impact of (poly)phenols in health and cellular metabolism have increased the interest on these molecules. Particularly, in the context of DR, the beneficial effects on inflammation models raise some interest. The presence of sulfated metabolites of Catechol and Pyrogallol was confirmed in human plasma from volunteers after ingestion of a fruit purée.

We aimed to study both Catechol-*O*-sulfate and Pyrogallol-*O*-sulfate by addressing their effect in the expression of cytokines involved in inflammation and proteins involved in retinal glial activation in RPE cells and the diabetic retina. The expression of TNF-α, IL-1β and IL-10 was measured upon treatment of RPE cells with Pyrogallol-O-sulfate, exposed to the cells before or after the inflammatory stimulus. Experiments on diabetic mouse model, the $Ins2^{Akita}$, were performed by a single intraocular injection of the metabolite, targeting the posterior and anterior layers of the retina. The expression of Iba1 and GFAP was measured from neural retina and the expression of IL-1β from RPE of diabetic and non-diabetic mice, two weeks after treatment.

We were able to observe a protective role of Pyr-sulf from TNF-α-induced pro-inflammatory expression in RPE cells, upon pre-treatment of RPE cells. Moreover, a single intraocular injection of Pyr-sulf significantly decrease the expression of microglial marker Iba1 in the diabetic retina at an early stage of DR progression in a diabetic mice model. The expression of GFAP significantly increase in the diabetic retina of older mice upon treatment.

These first set of results highlights Pyrogallol-O-sulfate metabolite as a promise treatment for inflammatory deleterious effects related to DR progression and deserves further investigation.

4.2. Introduction

In the last decades, studies on the impact of (poly)phenols in health and cellular metabolism have increased the interest on these molecules. Particularly in the context of DR, the beneficial effects of resveratrol (RES) on neovascularization and oxidative stress were first observed in the beginning of the decade [118,119].

(Poly)phenol-derived metabolites are plant secondary metabolites with potential health-promoting effects that have been studied in experimental cell cultures and animal models, as well as human clinical studies [121,122,124,126,127]. The presence of sulfated metabolites of Catechol and Pyrogallol was confirmed for the first time by Santos and colleagues in human plasma from volunteers after ingestion of a fruit purée[126], which were found to reach concentrations up to 20 µM in some volunteers. Moreover, phase II metabolites of pyrogallol and catechol were relatively abundant in urine samples of volunteers [127].

However, the preventive or therapeutic effects of these compounds are dependent on the amount consumed and their bioavailability, meaning the proportion of nutrient that is digested, absorbed and metabolized; this is generally low, because the absorbed forms are more likely to be metabolites of the native forms of the compound found in food.

Studies specifically related to the effect of (poly)phenols or their metabolites on retinal diseases are scarce. Recently, the beneficial effect of different carotenoids and (poly)phenols in the prevention and treatment of age-related ocular disorders was reviewed, highlighting the role of these metabolites in reducing oxidative stress and inflammation [137]. DR development and progression are highly affected by inflammation and the presence of increased levels of pro-inflammatory molecules in retinal cells creates an opportunity to study the role of (poly)phenol-derived metabolites in both retinal cells and diabetic mice models for their anti-inflammatory properties.

Here we aim to study both Catechol-*O*-sulfate and Pyrogallol-*O*-sulfate in RPE cells and diabetic mice retina, addressing their effect in the expression of molecules involved in inflammation and retinal glial activation.

4.3. Results and Discussion

4.3.1. Metabolic activity, but not cell viability, of RPE cells is affected by treatment with Cathecol-O-sulfate

As far as we know, this is the first time that Cathecol-*O*-sulfate and Pyrogallol-*O*-sulfate are studied in RPE cells. As a first approach, the metabolic activity of these cells upon treatment with these (poly)phenol-derived metabolites was investigated.

The mean concentration of metabolites found in the plasm of human volunteers 4 h after the ingestion of the fruit purée was chosen to perform the assay [126]. In the same study, it is reported the presence of two sulfated metabolites of pyrogallol, being the isomer 2 the more abundant. For that reason, a metabolite for Pyrogallol-*O*-2-sulfate was synthetized and used in all the experiments. To measure the metabolic activity in RPE cells exposed to Cat-sulf or Pyr-sulf for 24 h upon exposure to hypoxia for 16 or 24 h, an MTT assay was performed. DMSO was used as negative control, as it is reported that high concentrations of this reagent exert toxic effects on cells [198].

As observed in Figure 4.1, the metabolic activity of RPE is compromised upon treatment with Cat-s in both timepoints of exposure to hypoxia, with percentages below 80 %, which is the threshold for most cells in culture [199].

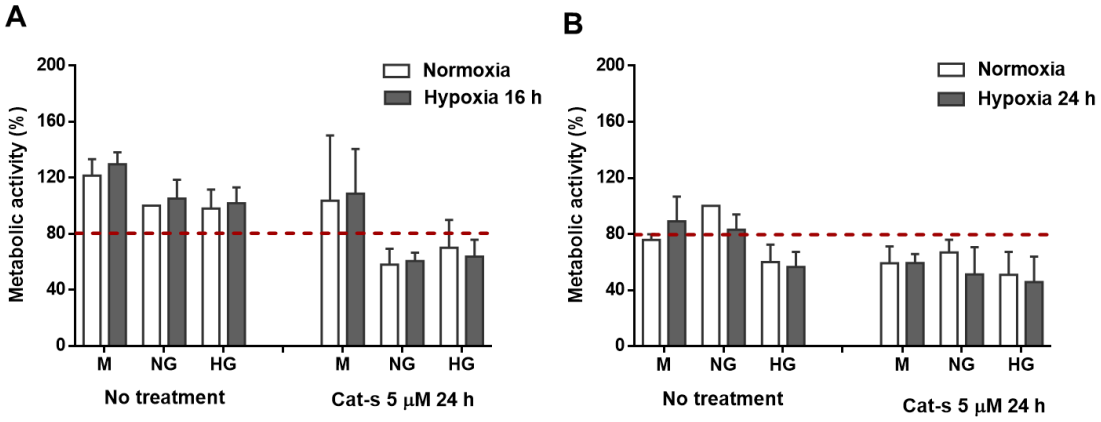


Figure 4.1 Effect of Catechol-O-sulfate (5 μM, 24 h) on RPE cell metabolic activity under high glucose and hypoxia. (A) RPE cells under normal (NG, 5.5 mM glucose) or high glucose (HG, 25 mM glucose) with chemically induced hypoxia for 16 h (DFO 100 μM), assessed by MTT assay. **(B)** RPE cells under normal (NG, 5.5 mM glucose) or high glucose (HG, 25 mM glucose) with chemically induced hypoxia for 24 h (DFO 100 μM), assessed by MTT assay. Values are expressed as percentage of control (NG, normal glucose under normoxia). Mannitol (M) was used as osmolarity control. N=4.

Because the MTT assay is an indirect measure of cell viability, we performed further assays to confirm this effect. Therefore, the number of propidium iodide (PI) positive cells was assessed by Flow Cytometry, as PI is used as a DNA marker to evaluate cell viability. In the same line, the Trypan blue assay allows to determine the number of viable cells present in a cell suspension, based on the principle that live cells possess intact cell membranes that exclude certain dyes. The observations from both experiments (Figure 4.2 A and B), as well as the microscopy imagens from cultured cells after treatment (Figure 4.2 C) reveals that the cell viability of RPE is not disturbed by Cat-sulf.

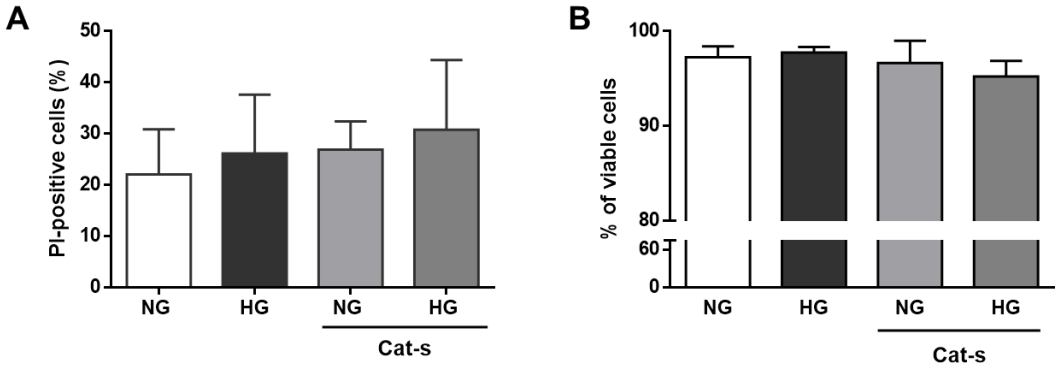


Figure 4.2 Effect of Catechol-O-sulfate (5 µM, 24 h) on RPE cell viability under high glucose and hypoxia. (A) Cell viability of RPE cells under normal (NG, 5.5 mM glucose) or high glucose (HG, 25 mM glucose) assessed by PI-positive cells, N=4. **(B)** Cell viability of RPE cells under normal (NG, 5.5 mM glucose) or high glucose (HG, 25 mM glucose) assessed by Trypan blue assay, N=3. Values are presented as percentage of mean ± SD.

The opposite effect was observed when RPE cells were treated with Pyr-sulf under the same conditions (Figure 4.3).

Although cells under hypoxia for 24 h show a decrease in the percentage of metabolic activity, reaching values very close to the threshold (dashed red line, Figure 4.3 B), the RPE cells under hypoxia for 16 h are not metabolically affected by the treatment with Pyr-sulf (Figure 4.3 A).

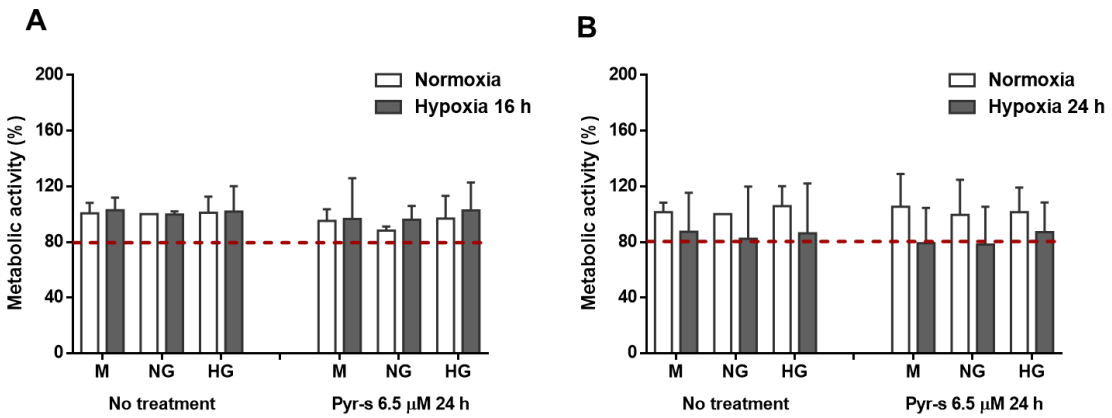


Figure 4.3 Effect of Pyrogallol-O-sulfate (6.5 µM, 24 h) on RPE cell metabolic activity under high glucose and hypoxia. (A) RPE cells under normal (NG, 5.5 mM glucose) or high glucose (HG, 25 mM glucose) with chemically induced hypoxia for 16 h (DFO 100 µM), assessed by MTT assay. N=4. **(B)** RPE cells under normal (NG, 5.5 mM glucose) or high glucose (HG, 25 mM glucose) with chemically induced hypoxia for 24 h (DFO 100 µM), assessed by MTT assay. N=5. Values are expressed as percentage of control (NG, normal glucose under normoxia). Mannitol (M) was used as osmolarity control. Two-way ANOVA with Dunnett's multiple comparisons test.

Having these results in mind, the following experiments were performed only with Pyr-s treatment under 16 h of hypoxia.

4.3.2. Treatment of RPE cells with higher concentrations of Pyrogallol-O-sulfate negatively affects their metabolic activity

In previous studies, namely the one that describes the presence of this conjugated phenolic metabolite in human plasma, a peak concentration of Pyr-sulf of about 11 µM was confirmed in 8 volunteers [126]. To investigate if this concentration had a deleterious effect on RPE cells, an MTT assay in RPE cells exposed to 11 µM Pyr-sulf up to 8 h was performed. DMSO was used as negative control.

We have observed that higher concentrations of Pyr-sulf decrease the metabolic activity of RPE in all time-points evaluated, independently of the levels of glucose in the medium and hypoxia (Figure 4.4 A and B).

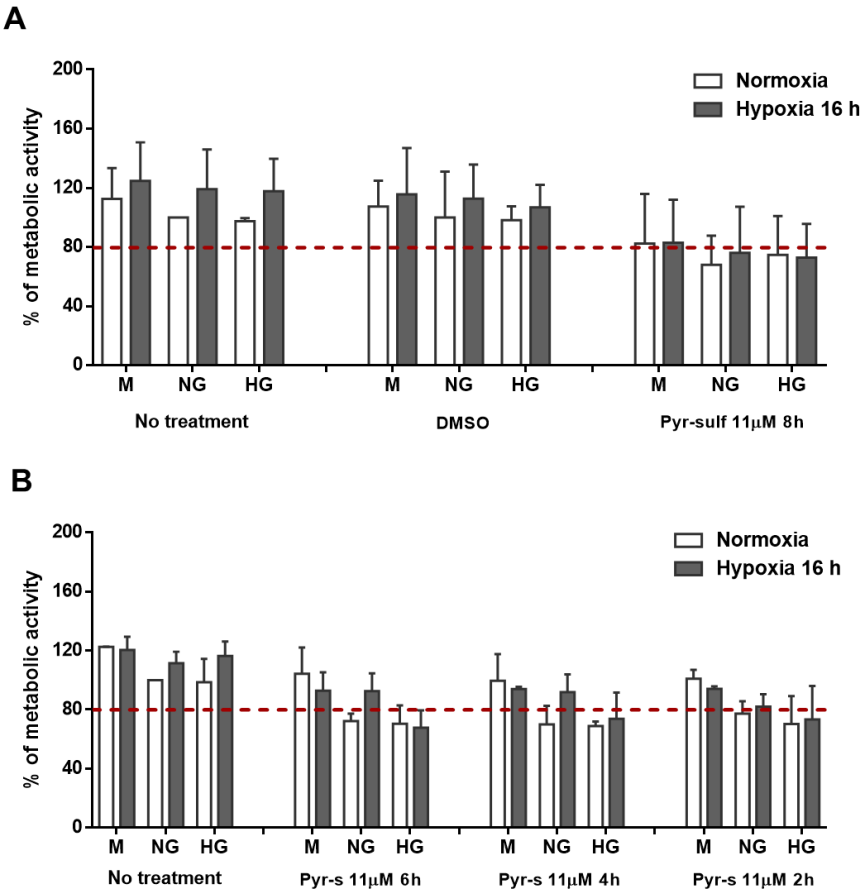


Figure 4.4 Effect of Pyrogallol-O-sulfate (11 µM, 24 h) on RPE cell metabolic activity under high glucose and hypoxia. (A) RPE cells under normal (NG, 5.5 mM glucose) or high glucose (HG, 25 mM glucose) with chemically induced hypoxia for 16 h (DFO 100 µM), exposed to Pyr-sulf treatment for 8 h, N=4. **(B)** RPE cells under normal (NG, 5.5 mM glucose) or high glucose (HG, 25 mM glucose) with chemically induced hypoxia for 16 h (DFO 100 µM), exposed to Pyr-sulf treatment from 2 to 6 h, N=3. Values are expressed as percentage of control (NG, normal glucose under normoxia). Mannitol (M) was used as osmolarity control and DMSO as vehicle, N=2. Two-way ANOVA with Dunnett's multiple comparisons test.

Taking this into account, the experimental protocol was designed to address the effect of Pyr-sulf treatment up to 24 h, at a final concentration of 6.5 µM, in the expression of proteins known to be involved in DR pathophysiology and progression.

Having in mind a therapeutic approach that could benefit DR patients earlier in the progression of the disease, we started by testing the hypothesis that (poly)phenol-derived metabolites, in particular the Pyrogallol-O-sulfate, have biological significance as anti-inflammatory molecules.

4.3.3. Gene and protein expression of pro-inflammatory cytokines increase in TNFα-treated RPE cells.

Chronic inflammation is an important hallmark of disease progression, and DR, being a disease with a significant inflammatory component, is no exception. The (poly)phenols'-derived metabolites have been studied for their anti-inflammatory properties in several cell models [122,200]. Our hypothesis is that treatment of RPE cells with Pyr-sulf decrease the expression of inflammatory-related cytokines having an impact in the inflammatory environment and progression of DR.

To test our hypothesis, the mRNA expression of pro-inflammatory IL-1β and IL-8 were evaluated in RPE cells under high glucose and hypoxia by RT-qPCR.

It is observed a slight increase in the gene expression of both cytokines evaluated, with a more pronounced increase of IL-1β in RPE cells under hypoxia (Figure 4.5 A), compared to cells in normoglucose.

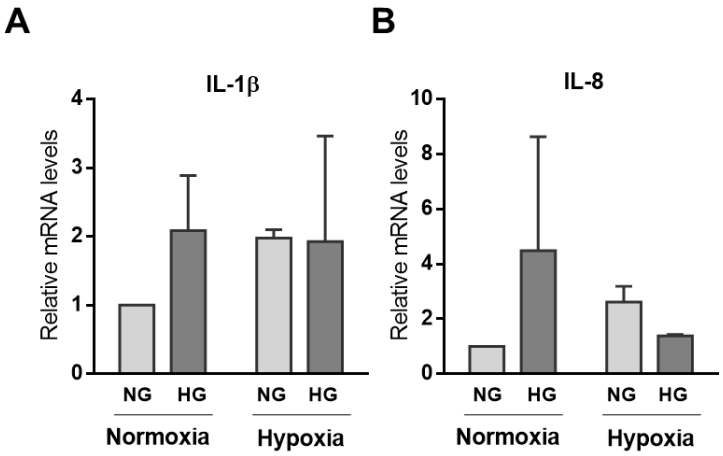


Figure 4.5 RT-qPCR analysis of pro-inflammatory genes in RPE cells. (A) Gene expression of IL-1β in RPE cells in normoglucose (NG) or high glucose (HG), under normoxia or hypoxia. β-Actin was used as housekeeping control, N=3. **(B)** Gene expression of IL-8 in RPE cells in normoglucose (NG) or high glucose (HG), under normoxia or hypoxia. β-Actin was used as housekeeping control, N=2.

Because the basal expression levels observed for IL-1β and IL-8 were relatively low, we designed an RPE-inflammatory cell model upon a pro-inflammatory stimulus. Cells were challenged with different concentrations of TNF-α up to 24 h and the protein expression of TNF-α and IL-1β were assessed by Western blot.

Human RPE cells incubated with TNF-α (10 or 20 ng/ml) for 4 hours expressed significant levels of TNF-α (Figure 4.6 A) and IL-1β (Figure 4.6 B), while the expression

of both cytokines remains unaltered after 8 or 24 hours of exposure to the inflammatory stimulus.

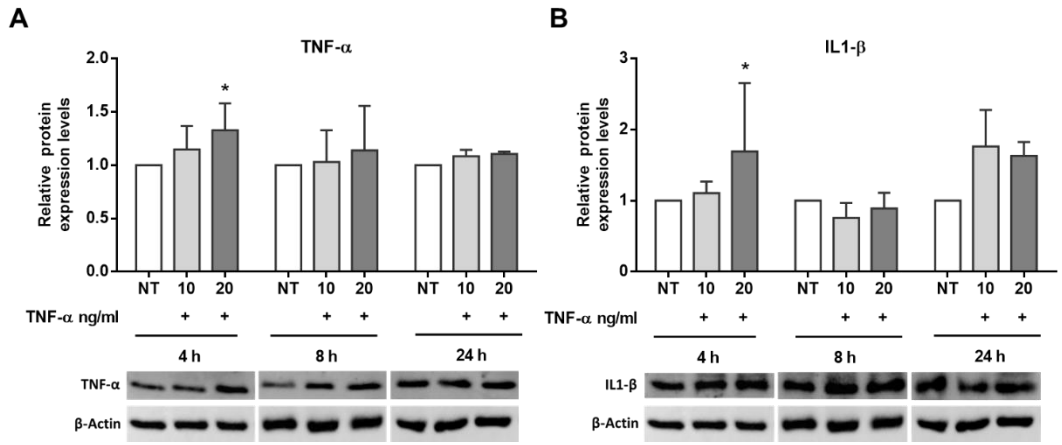


Figure 4.6 Expression of TNF-α and IL-1β in RPE cells upon stimulus with TNF-α at different concentrations and exposure times. (A) Relative protein expression levels of TNF-α in cells with or without (NT = non-treated) TNF-α (10 or 20 ng/ml) up to 24 hours, assessed by Western blot. **(B)** Relative protein expression levels of IL-1β in cells with or without (NT = non-treated) TNF-α (10 or 20 ng/ml) up to 24 hours, assessed by Western blot. Protein levels were normalized for β-Actin. Data are expressed as mean ± SD, Two-way ANOVA with Dunnett's multiple comparisons test, * $p<0.05$, n=4.

4.3.4. Pre-treatment of RPE cells with Pyrogallol-O-sulfate affects the expression of pro-inflammatory cytokines.

Pro-inflammatory cytokines such as TNF-α and IL-1β are secreted by infiltrating lymphocytes or macrophages and may play a role in triggering RPE dysfunction associated with DR [52,201].

To evaluate the anti-inflammatory potential of treatment with Pyr-sulf, RPE cells were challenged with TNF-α for 4 h and the expression of pro-inflammatory cytokines IL-1β and IL-8, together with the anti-inflammatory cytokine IL-10 were evaluated after 2 and 4 h of treatment with Pyr-sulf. Having in mind a potential preventive effect of treatment with Pyr-sulf preceding the inflammatory stimulus cells were exposed to Pyr-sulf before the inflammatory stimulus as represented below (Figure 4.7).

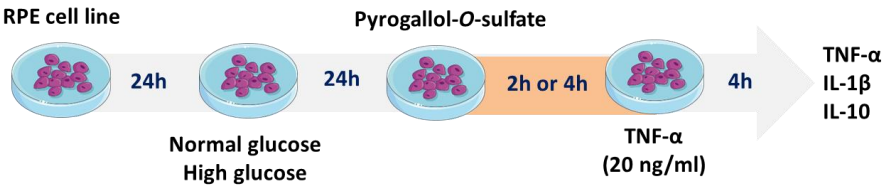


Figure 4.7 Schematic representation of treatment with Pyrogallol-O-sulfate before stimulus with TNF-α (20 ng/ml) up to 4 hours of total incubation period. The expression of TNF-α, IL-1β and IL-10 was analyzed in whole cells lysates by Western blot.

In Figure 4.8, pre-treatment of RPE cells with Pyr-sulf for 2 h tends to decrease the expression of TNF-α (Figure 4.8 B) and IL-1β (Figure 4.8 C) to levels similar to non-treated (NT) cells, protecting the cells from the increse that is observed when exposed to the inflammatory stimulus or Pyr-sulf alone. The same profile is observed for the expression of anti-inflammatory IL-10 (Figure 4.8 D). Longer exposure to Pyr-sulf (4 h) prevents the increase in the expression levels of IL1-β (Figure 4.8 F) and IL-10 (Figure 4.8 G), but not of TNF-α (Figure 4.8 E).

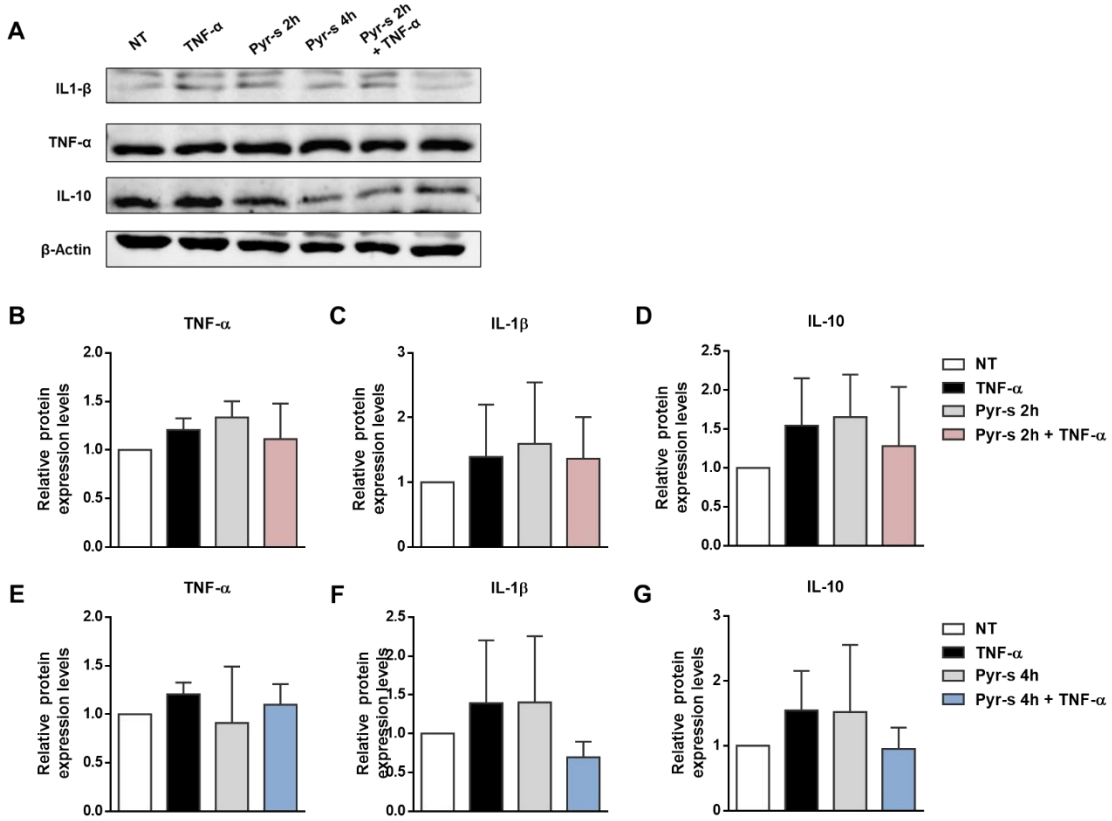


Figure 4.8 *(continued on the following page)*

Figure 4.8 Expression of pro- and anti-inflammatory cytokines upon pre-treatment of RPE cells with Pyrogallol-O-sulfate followed by TNF-α stimulus. **(A)** Representative imagens of the expression of IL1-β, TNF-α and IL-10 in RPE cells treated with Pyr-sulf. **(B-D)** Quantitative data of the expression of pro-inflammatory cytokines TNF-α and IL1-β and anti-inflammatory IL-10 upon treatment with Pyr-sulf for 2 hours. **(E-G)** Quantitative data of the expression of pro-inflammatory cytokines TNF-α and IL1-β and anti-inflammatory IL-10 upon treatment with Pyr-sulf for 4 hours. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD, N=4.

4.3.5. Treatment of RPE cells with Pyrogallol-O-sulfate has no effect on TNF-α-challenged cells.

Testing the effect of treatment with Pyr-sulf, RPE cells were first exposed to TNF-α and then treated with Pyr-sulf (Figure 4.9).

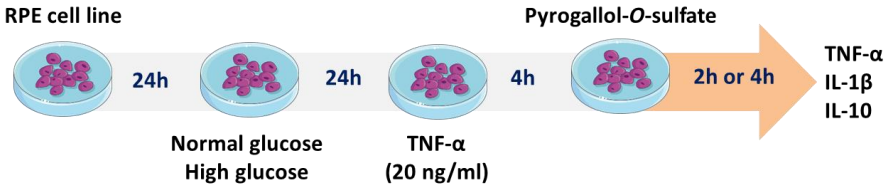


Figure 4.9 Schematic representation of treatment with Pyrogallol-O-sulfate after stimulus with TNF-α (20 ng/ml) up to 4 hours of total incubation period. The expression of TNF-α, IL-1β and IL-10 was analyzed in whole cells lysates by Western blot.

No significant effect in the expression of pro-inflammatory cytokines upon 2 hours of treatment (Figure 4.10 B and C) is observed. However, there is a tendency to increase the expression of anti-inflammatory IL-10 in cells inflammatory cells treated with Pyr-sulf for 2 hours (Figure 4.10 D). Treatment of RPE cells with Pyr-sulf alone for 4 hours shows a trend to decrease the expression of TNF-α, compared to the inflammatory stimulus alone (Figure 4.10 E). No differences were observed in the expression of IL1-β in this experimental condition (Figure 4.10 F). Pyr-sulf alone also tends to increase the expression of IL-10 (Figure 4.10 G), while inflammatory cells treated with Pyr-sulf for 4 hours shows the opposite tendency, compared to non-treated cells of Pyr-sulf alone.

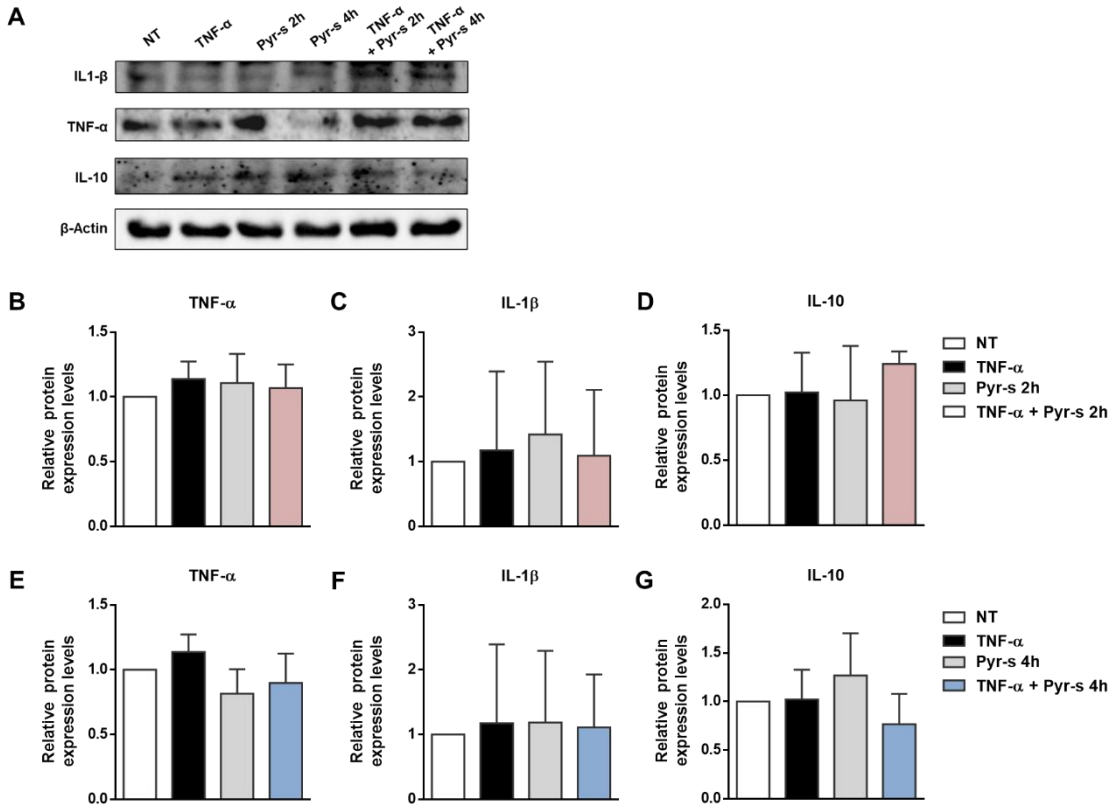


Figure 4.10 Expression of pro- and anti-inflammatory cytokines treated with Pyrogallol-O-sulfate in cytokine-treated RPE cells. **(A)** Representative imagens of the expression of IL1-β, TNF-α and IL-10 in RPE cells treated or not with Pyr-sulf. **(B-D)** Quantitative data of the expression of pro-inflammatory cytokines TNF-α and IL1-β and anti-inflammatory IL-10 upon treatment with Pyr-sulf for 2 hours. **(E-G)** Quantitative data of the expression of pro-inflammatory cytokines TNF-α and IL1-β and anti-inflammatory IL-10 upon treatment with Pyr-sulf for 4 hours. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD, n=4.

It is known that, in response to specific cytokine stimulation, RPE produces several cytokines that play important roles in ocular inflammation [202–204]. Also, studies show that TNF-α and IL1-β are deeply involved in BRB breakdown, inflammation and pathological angiogenesis, leading to the development of retinal degenerative diseases and DR [201,205].

(Poly)phenols-derived metabolites have been studied for their anti-inflammatory properties in several cell and animal models of disease. Here we studied the effect of Pyrogallol-O-sulfate, a (poly)phenol-derived metabolite present in human plasma and urine upon berries ingestion [126,127], in the expression of several cytokines in a TNF-α-based inflammatory cell model. RPE cells were exposed to Pyr-sulf before or after a TNF-α stimulus, and the expression of TNF-α, IL1-β and IL-10 were assessed by Western blot.

The results reveal a trend for pre-treatment of Pyr-sulf 2 hours prior to the inflammatory stimulus to protect the RPE cells from the increase in TNF-α and IL1-β expressions. This points to the anti-inflammatory potential of Pyr-sulf when before the onset of an inflammatory profile in the cell. Longer exposure of cells to Pyr-sulf before the inflammatory TNF-α stimulus can benefit the overall inflammatory status by decreasing the expression of IL1-β, but not TNF-α or IL-10. When RPE cells are exposed to Pyr-sulf for 2 hours following the TNF-α stimulus, it is only observed a mild increase in the expression of anti-inflammatory IL-10.

Additionally, our results reveal that we were able to establish a mild inflammation cell model, as the expression of the pro-inflammatory cytokines analyzed does not increase significantly with TNF-α alone. Nevertheless, we believe that (poly)phenol metabolites may have a role in RPE cell protection against inflammation, by decreasing microglia activation. This hypothesis is supported by studies where (poly)phenol-derived metabolites, particularly Pyr-sulf, shown to be neuroprotective [121] or having a role in reducing neuroinflammation [206–208]. In our study we have analyzed cellular expression of cytokines, which we know to be secreted proteins. A more detailed evaluation of the secreted levels of pro- and anti-inflammatory cytokines could lead us to more accurate results regarding the effect of Pyr-sulf in the inflammatory prolife of RPE cells.

As far as we known, this is the first time that this particular (poly)phenol metabolite is studied in retinal cells at their physiological concentration. As next step towards the investigation of the anti-inflammatory effects of Pyrogallol-*O*-sulfate towards DR, an experimental setup using a diabetic mouse model was designed.

4.3.6. One-single intravitreal injection of Pyrogallol-O-sulfate decreases the expression of microglial marker Iba1 in a diabetic mice model

Chronic subclinical inflammation has been associated with DR progression, characterized by increased cytokine production and activation of microglial and macroglial cells [32,209]. We previously characterized the expression of several markers of DR progression in a spontaneous T1DM model, the $Ins2^{Akita}$, and found an increase in the expression of microglia marker Iba1 in the neural retina of 6 month-old diabetic mice [38]. At the same time point, the expression of GFAP decreases in diabetic retina, compared to the non-diabetic retina [38].

In this context, we have analyzed the protein levels of Iba1 and IL-1β, both markers of microglia activation, and GFAP, a glial intermediate filament expressed by astrocytes, in the retina and RPE of 4, 6, 8, and 9 month-old male heterozygote $Ins2^{Akita}$

mice two weeks after one-single intravitreal injection of Pyr-sulf, targeting the neural retina, as schematized below (Figure 4.11).

The experimental design is described in detail in Chapter II, Section 2.11.3. Briefly, diabetic and age-matched non-diabetic animals were randomly divided into two groups, one receiving a single intraocular injection of Pyr-sulf and the other receiving the vehicle solution. After two weeks, the retina and RPE of the mice were isolated and protein extracted for Western blot analysis of the expression of proteins of interest.

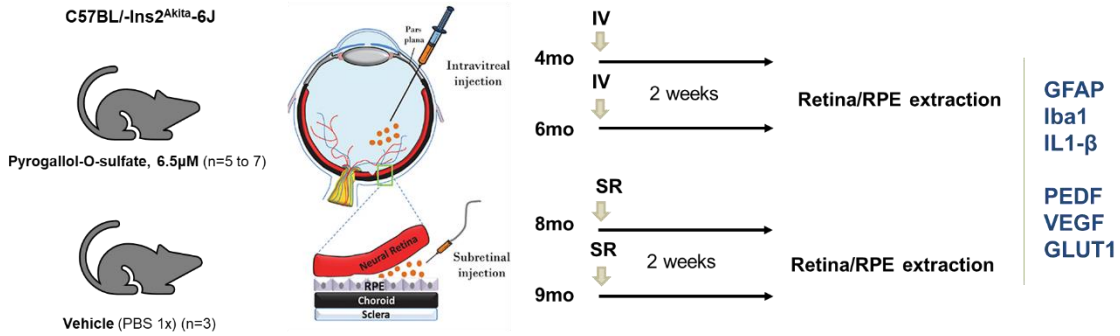


Figure 4.11 Schematic representation of the intraocular injections of Pyrogallol-O-sulfate in diabetic and non-diabetic mice, targeting different retinal cells at different stages of DR progression. The expression of proteins having a role in inflammation and angiogenesis were assessed by Western blot, two weeks after a single injection.

Blood glucose levels and body weight were measured at the time of injection (Figure 4.12). Ins2^{Akita} mice weighed significantly less in all time points evaluated compared with control mice (Figure 4.12 A). Additionally, Ins2^{Akita} mice displayed significantly increased concentrations of blood glucose compared with non-diabetic mice at all analyzed time points (Figure 4.12 B). These data are consistent with previous reports in the literature [148,210].

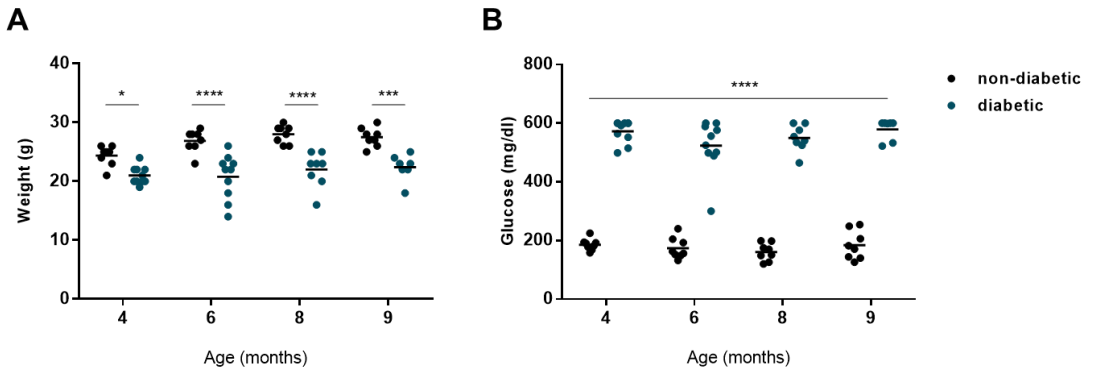


Figure 4.12 Characterization of non-diabetic and diabetic mice according to their body weight and blood glucose levels. **(A)** Body weight of non-diabetic and diabetic mice with 4-, 6-, 8- and 9-months of age. Values are represented as mean ± SD. * p<0.05, *** p<0.001, **** p<0.0001 is significantly different compared to non-diabetic mice, determined by Sidak's multiple comparisons test. **(B)** Blood glucose levels of non-diabetic and diabetic mice with 4-, 6-, 8- and 9-months of age. Values are represented as mean ± SD. **** p<0.0001 is significantly different compared to non-diabetic mice, determined by Sidak's multiple comparisons test. N=7 to 10.

The results, plotted in Figure 4.13, show the expression of Iba1 and GFAP normalized for levels of expression of the contralateral non-injected eye of animals either injected with a vehicle (PBS 1x) or Pyrogallol-*O*-sulfate at a final concentration of 6.5 µM.

The time-points chosen are related to early DR progression in this mice model, where inflammation has a very important role [38,148]. Here we corroborate the previous observations in 6 month-old mice, where expression of Iba1 increases (Figure 4.13 B), a tendency that is also observed for 4 month-old mice (Figure 4.13 A), when comparing the retinas injected with the PBS vehicle from non-diabetic and diabetic mice. Regarding the expression of GFAP, a decrease in its expression is observed in 6 month-old mice (Figure 4.13 D) similar to what was previously described [38].

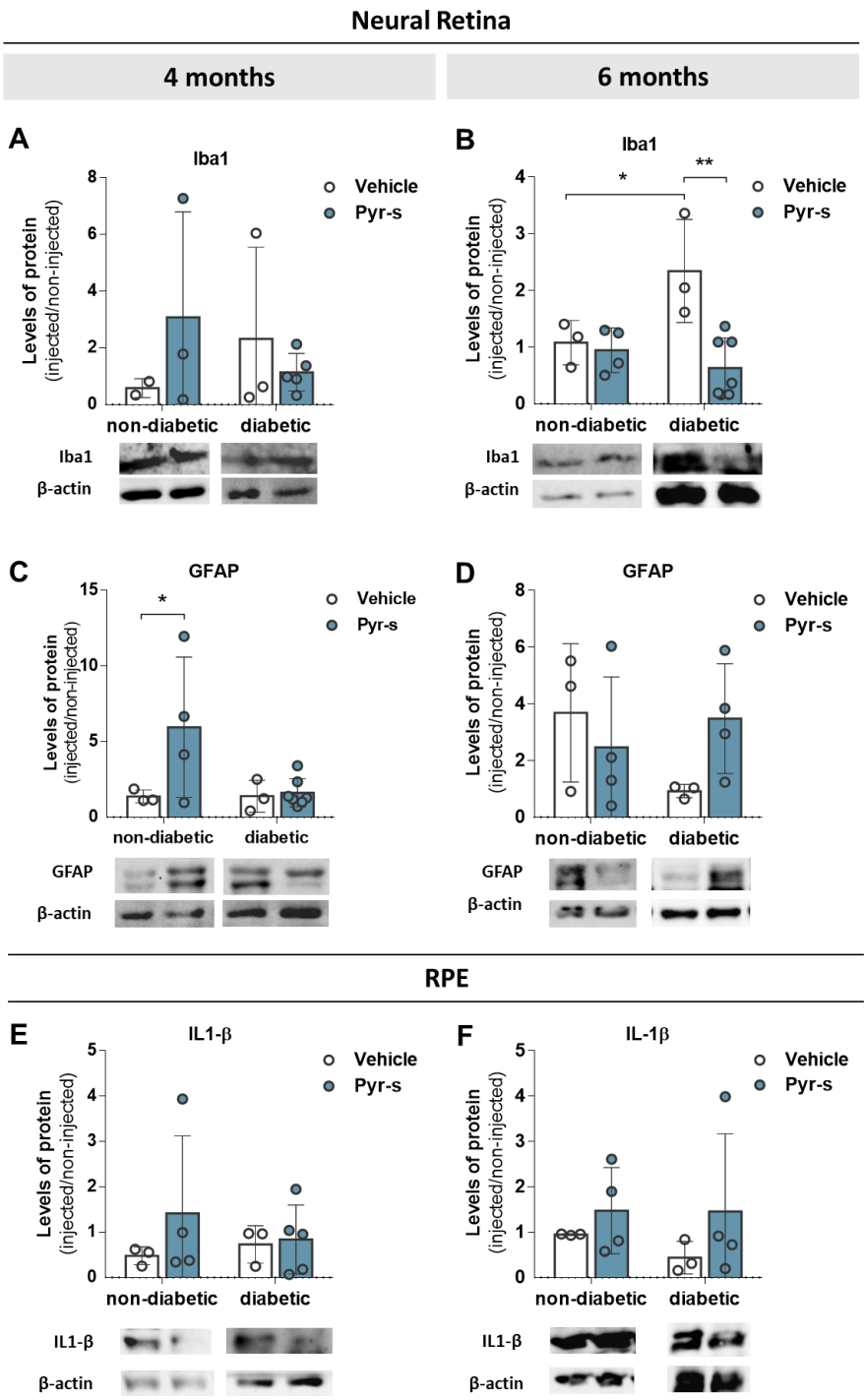


Figure 4.13 Inflammatory response in Ins2^{Akita} mice after one-single intravitreal injection of Pyrogallol-O-sulfate. **(A-B)** Protein expression levels and quantitative data of Iba1 in the retina of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice, with 4 and 6 month-old, respectively. **(C-D)** Protein expression levels and quantitative data of GFAP in retina of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice. **(E-F**) Protein expression levels and quantitative data of IL1-β in RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice, with 4 and 6 month-old, respectively. Protein levels were normalized to β-Acin. Data are expressed as mean ± SD (N=3 to 6 mice/group), * $p<0.05$, ** $p<0.01$ by two-way ANOVA (age and genotype) with Sidak's multiple comparison test.

Treatment of diabetic retinas with the (poly)phenol-derived metabolite Pyr-sulf, effectively decreases the expression of Iba1, contrary to the tendency observed in the retina of these animals starting at 4 months of age, as observed previously [38] (Figure 4.13 B). However, the intravitreal injection of Pyr-sulf in non-diabetic mice increases the expression of GFAP with 4 months old (Figure 4.13 C) with a clear tendency to have the same effect in diabetic retinas later at 6-month (Figure 4.13 D). The expression of IL-1β was also measured in the RPE in both time-points (Figure 4.13 E and F). The injection with Pyr-sulf had no effect in the expression of this cytokine.

4.3.7. One-single subretinal injection of Pyrogallol-O-sulfate increases the expression of GFAP in a diabetic mice model

Although RPE is not the main source of cytokines and other proteins involved in inflammation, the inflammatory environment in these cells have a very important role in DR progression [211]. In response to the cellular environment, the microglia may become overactivated, up-regulating pro-inflammatory factors that induce oxidative stress, neuronal degeneration and neovascularization [209,212–214].

The expression of the same cell markers was measured in the retinas of 8 and 9-month-old $Ins2^{Akita}$ mice two weeks after one-single subretinal injection of Pyr-sulf, targeting RPE cells (Figure 4.14).

In our previous study, the expression of Iba1 tends to increase in the 9 month-old diabetic retina but is not altered in older mice [38]. Here we analyzed the expression of Iba1, GFAP and IL1-β in 8- and 9 month-old mice to understand if the administration of Pyr-sulf before an advanced stage of DR (as previously established to be at 9 months) has a positive effect on retinal health.

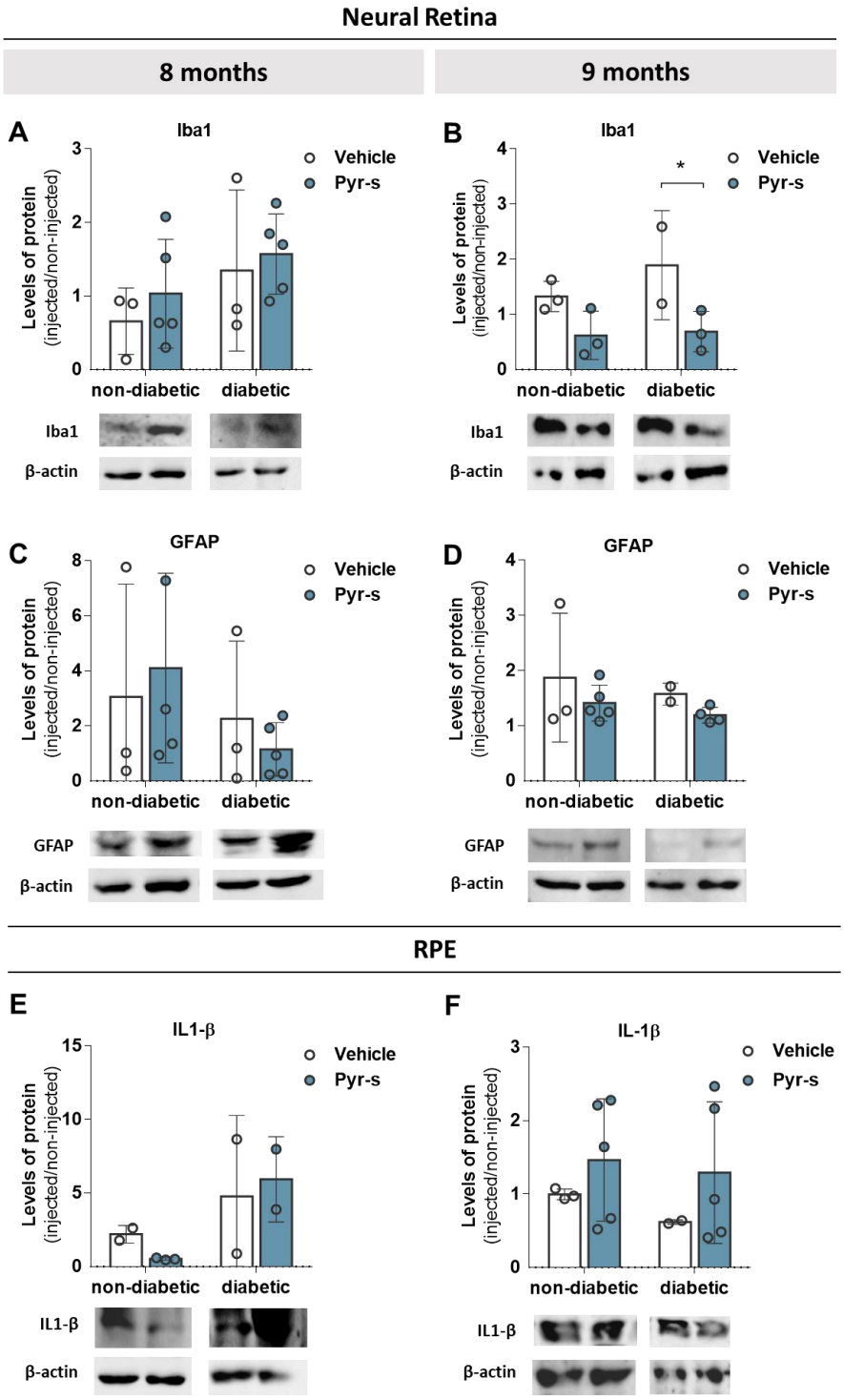


Figure 4.14 Inflammatory response in Ins2^{Akita} mice after one-single subretinal injection of Pyrogallol-O-sulfate. **(A-B)** Protein expression levels and quantitative data of Iba1 in retina of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice, with 8 and 9 month-old, respectively. **(C-D)** Protein expression levels and quantitative data of GFAP in retina of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice, with 8 and 9 month-old, respectively. **(E-F**) Protein expression levels and quantitative data of IL1-β in RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice, with 8 and 9 month-old, respectively. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD (N=2 to 5 mice/group) * $p<0.05$ by two-way ANOVA (genotype and treatment) with Sidak's multiple comparison test.

Treatment of diabetic 9 month-old mice with one-single subretinal injection effectively decreases the expression of Iba1 in the neural retina (Figure 4.14 B), but not in 8 month-old mice (Figure 4.14 A). Also, the expression of GFAP was not affected by Pyr-sulf neither in animals with 8- (Figure 4.14 C) or 9 month-old (Figure 4.14 D). Similar to what was previously described in our study in younger mice, the expression of IL1-β was not altered by treatment with both vehicle or Pyr-sul (Figure 4.14 E and F).

Consistent with our results in the $Ins2^{Akita}$ mouse, the activation of microglia has been described in both early DR patients and other animal models, as reflected by the increased expression of Iba1 [148,215]. This seems to be thwarted by treatment with pyrogallol-*O*-sulfate in animals with early (6 month-old) or advanced (9 month-old) DR features, highlighting the anti-inflammatory potential of Pyr-sulf as a bioactive metabolite in diabetic retina. In accordance to our results, digested raspberry metabolites used at physiological levels has previously exhibited anti-inflammatory activity, not only by the reduction of Iba1 expression, but also by inhibiting the release of nitric oxide and TNF-α [207].

Additionally, macroglia undergoes prominent changes in early DR, that includes a decrease in the astrocytic population and reduced coverage of retinal blood vessels by glial cell processes [41,216]. Moreover, it is described an increased permeability of BRB in the early stages of DR in $Ins2^{Akita}$ mice [148,210], in line with the decreased levels of the astrocyte marker GFAP observed in all tested ages [38]. Here we observed a tendency to the expression of GFAP to increase upon treatment with Pyr-sulf in diabetic 6 month-old retina. As it is described that the number of astrocytes decreases as DR progresses, these results need further confirmation regarding the astrocytic population, in order to evaluate whether Pyr-sulf has a role in maintaining the number of astrocytes, protecting the retinal blood vessels, or if this tendency to increase the expression of GFAP enhances macroglia activation promoting the progression of DR. Because the injection of the vehicle does not increase the expression of GFAP, when the diabetic retina is compared to non-diabetic, our hypothesis relies in a non-pathological role of Pyr-sulf driven by GFAP expression.

CHAPTER V

EFFECT OF (POLY)PHENOL-DERIVED METABOLITE IN ANGIOGENESIS-RELATED PROTEINS

This chapter is based on the following scientific article:

Santos DF, Pais M, Silva GA. Polyphenol metabolite Pyrogallol-*O*-sulfate decreases microglial activation and pro-angiogenic VEGF in RPE cells and diabetic mouse model. *In preparation*

5.1. Abstract

It is widely accepted that diabetic retinopathy is a microvascular complication of diabetes, with a progressive pathophysiology towards neovascularization. Current therapies for DR are designed to act on later stages of this pathology, targeting ocular lesions resulting from the disease progression and that play a major role in visual impairment. As these treatments have a limited efficiency, there is an urgent need to alternative therapeutic strategies targeting earlier stages of the disease progression and that are as less invasive possible.

In the last decades, studies on the impact of (poly)phenols properties in health and cellular metabolism, accumulates evidence that (poly)phenols in natural products are beneficial against human diseases, with great impact on angiogenesis.

As Diabetic Retinopathy progresses towards pathological angiogenesis, where the imbalance in the expression of pro- and anti-angiogenic proteins play a crucial role, we aim to study Pyrogallol-*O*-sulfate in RPE cells and diabetic mice retina, addressing their effect in the expression of proteins involved in angiogenesis and glucose transport. The expression of PEDF, VEGF and GLUT1 were analyzed upon treatment in RPE cells under high glucose and hypoxia. Intraocular injections of Pyrogallol-*O*-sulfate were executed in a diabetic mouse model and the expression of the same proteins measured in neural retinal and RPE two weeks after treatment.

Here we observe a significant decrease in the expression of pro-angiogenic VEGF, both in RPE cells and in the diabetic retina, upon treatment with Pyrogallol-*O*-sulfate.

These observations open an opportunity to further explore the effect of (poly)phenol-derived metabolite in DR-related angiogenesis.

5.2. Introduction

In the last decades, studies on the impact of (poly)phenols properties in health and cellular metabolism have increased the interest on these molecules, accumulating evidence that (poly)phenols in natural products are beneficial against human diseases, with great impact on angiogenesis. Particularly in the context of DR, the beneficial effects of resveratrol (RES) on retinal vascular complications [118] and oxidative stress [119] were first observed in the beginning of the decade.

(Poly)phenols are members of a large family of compounds, chemically characterized by the presence of one or more hydroxyl groups attached to an aromatic ring. (Poly)phenol-derived metabolites are plant secondary metabolites with potential health-promoting effects that have been studied in experimental cell and animal models, as well as human clinical studies.

Many authors have identified new phenolic metabolites in nutritional intervention studies after the ingestion of fruits and/or vegetables. In particular, the presence of sulfated metabolites of Catechol and Pyrogallol was confirmed for the first time by Santos and co-workers in human plasma from volunteers after ingestion of a fruit purée [126] . The metabolites Catechol-*O*-sulfate (Cat-sulf) and Pyrogallol-*O*-sulfate (Pyr-sulf) were found in several volunteers at baseline to be relatively abundant not only in plasma but also in urine samples of volunteers [127].

The identification of these phenolic metabolites, obtained from exact mass identification in human samples, raises an emerging and very important research area of synthesis of new (poly)phenol metabolites to be used as standards. In the same way, these new synthetic metabolites can be further used for research in disease models.

These two metabolites (Cat-sulf and Pyr-sulf) have been recently studied in different models of neurodegenerative and cardiovascular disorders, showing the ability of these small molecules to cross the BBB, modulate the brain cell towards neuroprotection and have an effect on cardiomyocyte beating following prolonged stimulation. Also, it was shown for the first time that the most abundant metabolite in circulation, Pyr-sulf, was also the most effective in preventing oxidative damage in an endothelial cells' model.

There are several studies on the anti-diabetic effects of (poly)phenols. These compounds influence the glycemia through different mechanisms like inhibition of glucose absorption in the gut or in peripheral tissues. Studies specifically related to the

effect of (poly)phenols or their metabolites on retinal diseases are scarce. However, the interest in this topic increased in the last decade, particularly in studies on the effect of RES [119,133,134]. Ola *et al*. reviewed evidence pointing out the potential metabolic sources and pathways related to the increase of oxidative stress in DR and the role of dietary flavonoids, particularly flavanones, flavanols, flavanols, isoflavones, flavones and anthocyanins, in the modulation of redox homeostasis in the diabetic retina [135]. More recently, the benefic effect of different carotenoids and (poly)phenols in the prevention and treatment of age-related ocular disorders was reviewed, highlighting the role of these metabolites in oxidative stress and inflammation [137].

Specifically, epigalloccatechin-3-gallate, the main (poly)phenol component of green tea (leaves of *Camellia sinensis*), was identified as new inhibitor of ocular angiogenesis with influence in ocular vascular permeability [137]. Therefore, treatment with nutraceuticals (foods providing medical or health benefits) at early stages of DR may represent a reasonable alternative to act upstream of the disease, preventing its progression.

Our goal is to investigate the biological properties of (poly)phenol-derived metabolites, namely Pyrogallol-*O*-sulfate, addressing their effect in the expression of molecules involved in DR-related angiogenesis using RPE cells and diabetic mice retina.

5.3. Results and Discussion

5.3.1. Pre-treatment of RPE cells with Pyrogallol-O-sulfate before hypoxia tends to decrease the expression of pro-angiogenic VEGF

(Poly)phenols-derived metabolites have been studied for their potential to prevent neuroinflammation. Recent studies show that pre-incubation of microglial cells with Pyrogallol-*O*-sulfate before LPS insult is able to decrease the expression of several markers of inflammation [217]. As described before, DR is characterized as an inflammatory and microvascular disease of the retina. It is well-known the role of hypoxia in neovascularization, but also the imbalance between pro-and anti-angiogenic proteins is one of the main contributors to disease progression [38].

Our hypothesis is that in RPE cells under high glucose, pre-treatment with Pyr-sulf before inducing hypoxia prevents the imbalance in the expression of anti-angiogenic PEDF and pro-angiogenic VEGF. Also, the expression of glucose transporter GLUT-1, known to increase in cells under high glucose and hypoxia [20], is expected to be downregulated by pre-treatment with Pyr-sulf.

RPE cells were exposed to the (poly)phenol-derived metabolite up to 8 h before induction of hypoxia. The expression of two of the proteins involved in the angiogenic process, PEDF and VEGF, and of the glucose transporter in the retina, GLUT1, was evaluated 16 h after hypoxia by Western blot (Figure 5.1).

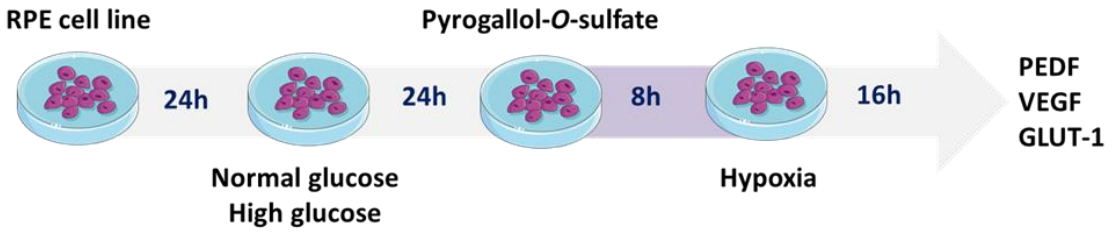


Figure 5.1 Schematic representation of pre-treatment of RPE cells with Pyrogallol-O-sulfate for 8 h before induction of hypoxia. The expression of PEDF, VEGF and GLUT1 were analyzed in whole cell lysates by Western blot.

Figure 5.2 shows the relative expression of PEDF, VEGF and GLUT-1 in cells in normal glucose (NG) and high glucose (HG) under normoxia and hypoxia with and without treatment with Pyr-sulf (Pyr-s) up to 8 hours before induction of hypoxia. Cells in normal glucose under normoxia were considered the control condition, as representative of the physiological situation.

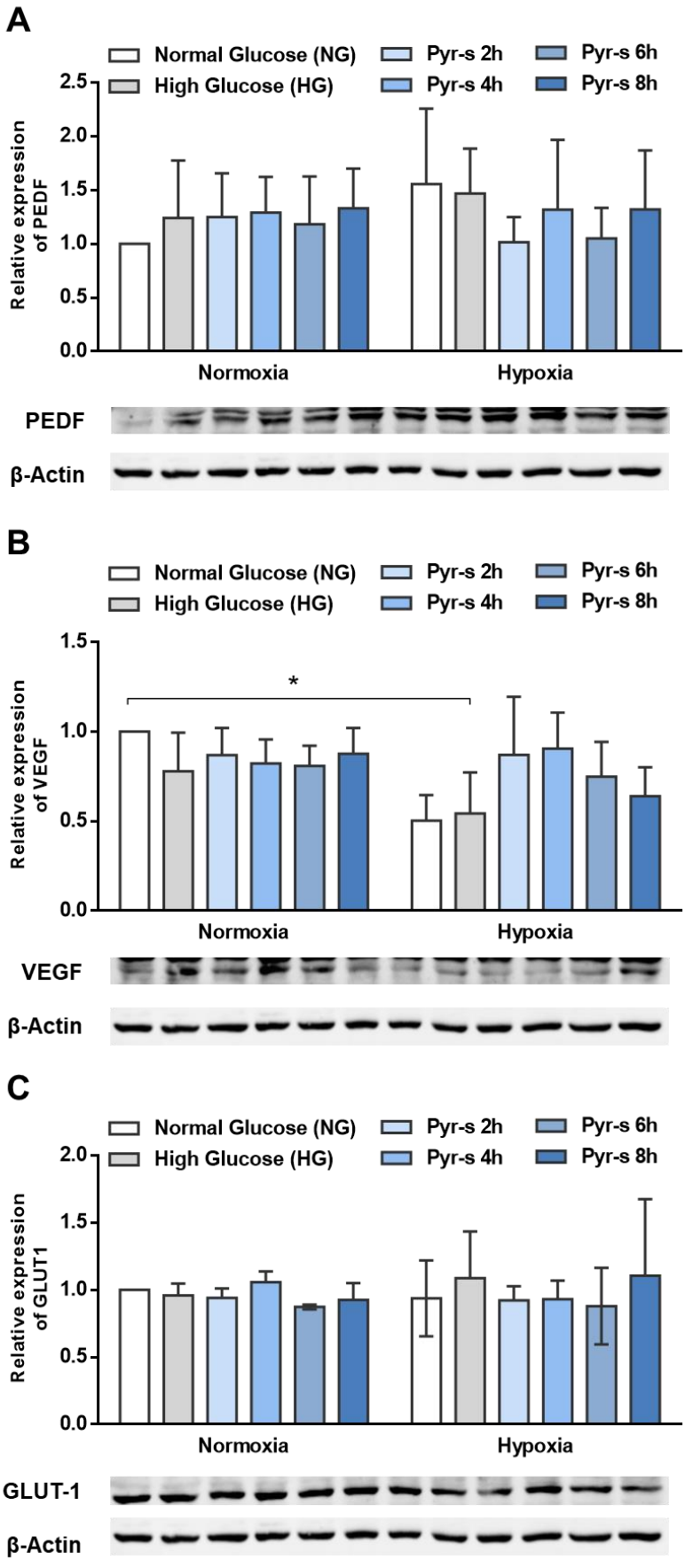


Figure 5.2 (continued in the following page)

Figure 5.2 Effect of pre-treatment of RPE cells with Pyrogallol-O-sulfate for 8 h before chemically induced hypoxia. (A) Levels of PEDF in RPE cells normal (NG) and High (HG) glucose, quantitative data normalized by the intensity of β-Actin, N=3. **(B)** Western blot analysis of VEGF protein levels in RPE cells after treatment under hypoxia, quantitative data normalized by the intensity of β-Actin. * $p<0.05$ by Two-way ANOVA with Dunnett's multiple comparisons test, N=3. **(C)** Western blot analysis of GLUT-1 protein levels in RPE cells after treatment under hypoxia, quantitative data normalized by the intensity of β-Actin, N=3.

Here we show that the expression of PEDF (Figure 5.2A) or GLUT-1 (Figure 5.2 C) are not altered by pre-treatment with Pyr-sulf before the hypoxic insult, at any of the timepoints evaluated. However, despite a significant decrease in the expression of VEGF in cells under high glucose and hypoxia compared to the experimental control cells that is opposite to what is described in the literature, pre-treatment of RPE cells with Pyr-sulf for 8 before hypoxia is able to decrease the expression of VEGF, compared to the experimental condition mimicking the physiological condition (Figure 5.2 B). This result shows that pre-treatment of RPE cells with Pyr-sulf does not prevent the increase in VEGF levels in cells under high glucose and hypoxia. Although, it is able to decrease the expression of VEGF in hypoxic cells compared to the physiological condition.

In these cells, we expected to observe the decrease in the expression of PEDF and increase in the expression of VEGF and GLUT-1 in cells under high glucose and hypoxia; however, that was not the case, which may be explained by the experimental setup. The standard cell culture protocol for this cell line is to keep the cells in high glucose medium. Here we designed the experiments based on a hypothesis that cells under normal glucose medium will be more representative of the physiological conditions and hyperglycemic conditions will be induced by increasing the levels of glucose in the cell media. However, when cells are under high glucose the expression levels of the proteins of interest are not altered, maybe because cells are adapted with the levels of glucose present in high glucose media. Increasing the time of exposure or the levels of glucose may be critical to improve this experimental setup. Also, before inducing hypoxia in these cells, the medium containing Pyr-sulf is removed, as well as for the conditions without pre-treatment. As so, the measured protein levels refer to those produced by the cell in the 16 hours prior to the cell lysis for protein extraction, which may not be sufficient to produce the known differences described for cells under high glucose and hypoxia at the protein level. An evaluation of the mRNA and protein levels should be carried out comparing cells maintained in high or low glucose and exposed to the opposite glucose concentrations, followed by the same experimental conditions as performed here.

5.3.2. Treatment of RPE cells with Pyrygallol-O-sulfate after hypoxia tends to increase the expression of PEDF without compromise the expression of VEGF or GLUT1

Most of the DR cases are diagnosed at later stages, where ischemia is already established and is the main cause of pathological angiogenesis promoted by the increase in the levels of VEGF in the retina. Here we address the hypothesis that RPE cells benefit from treatment with Pyrogallol-O-sulfate after being exposed to high glucose and chemically-induced hypoxia, decreasing the expression of pro-angiogenic VEGF and glucose transporter GLUT-1 and increasing the expression of anti-angiogenic PEDF.

The expression of pro- and antiangiogenic factors, as well as GLUT-1, was evaluated in RPE cells treated with Pyrogallol-O-sulfate for 8 h after induction of hypoxia (Figure 5.3).

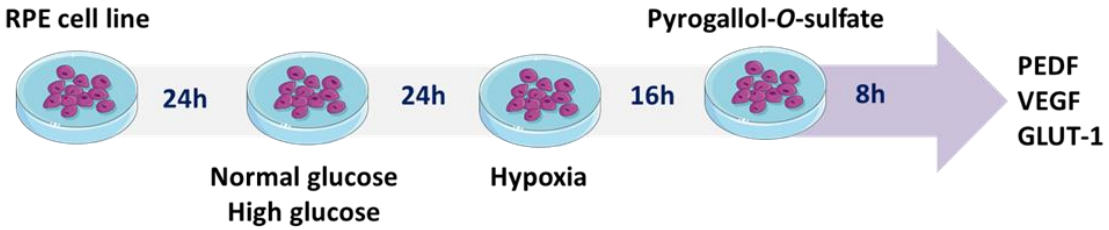


Figure 5.3 Schematic representation of treatment of RPE cells with Pyrogallol-O-sulfate for 8 h after stress induced by hypoxia. The expression of PEDF, VEGF and GLUT1 were analyzed in whole cell lysates by Western blot.

Figure 5.4 shows the relative expression of PEDF, VEGF and GLUT-1 in cells without (NT) or with Pyr-sulf treatment for 8 hours after induction of hypoxia. Values measured in cells under hypoxia were normalized for the respective control in normoxia. Cells in normal glucose without treatment were considered the control experimental condition, as representative of the physiological situation.

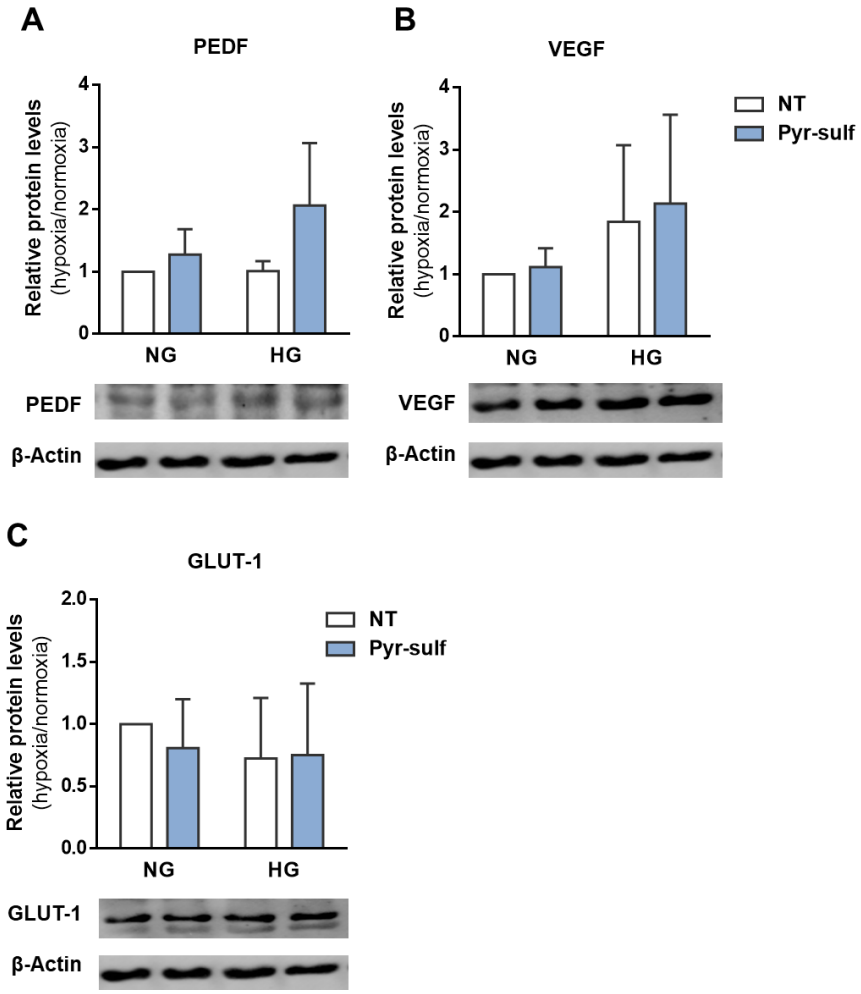


Figure 5.4 Effect of treatment of RPE cells with Pyrogallol-O-sulfate for 8 h after chemically induced hypoxia. (A) Levels of PEDF in RPE cells normal (NG) and High (HG) glucose, quantitative data normalized by the intensity of β-Actin, Two-way ANOVA with Sidak's multiple comparisons test, N=3. **(B)** Western blot analysis of VEGF protein levels in RPE cells after treatment under hypoxia, quantitative data normalized by the intensity of β-Actin, N=3. **(C)** Western blot analysis of GLUT-1 protein levels in RPE cells after Pyr-sulf treatment under hypoxia, quantitative data normalized by the intensity of β-Actin, N=3.

Treatment of RPE cells under high glucose (HG) with Pyr-sulf for 8 hours after hypoxia tend to increase the expression levels of anti-angiogenic PEDF (Figure5.4 A). The levels of pro-angiogenic VEGF (Figure 5.4 B) and glucose transporter GLUT-1 (Figure 5.4 C) are not affected by treatment.

5.3.3. Treatment of RPE cells with Pyrogallol-O-sulfate for 24 h decreases the expression of pro-angiogenic VEGF

In previous experimental approaches, we analyzed the effect of Pyr-sulf as protector of the deleterious effects induced by hypoxia on RPE cells and its potential role in reverting those effects after hypoxia is already established. However, we were also interested in the hypothesis of a beneficial effect of prolonged and continuous exposure of RPE cells to Pyr-sulf.

To test this hypothesis, Pyr-sulf was added to the cell medium 8 hours before induction of hypoxia and maintained up to 24 hours, as described in Figure 5.5. The expression levels of PEDF, VEGF and GLUT-1 were measured in whole cell lysates by Western blot.

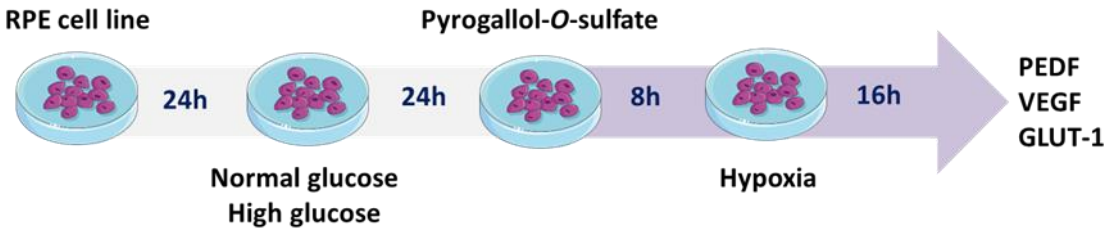


Figure 5.5 Schematic representation of treatment of RPE cells with Pyrogallol-O-sulfate for 24 h. The expression of PEDF, VEGF and GLUT1 were analyzed in whole cell lysates by Western blot.

Figure 5.6 shows the relative expression of PEDF, VEGF and GLUT-1 in cells in normal glucose (NG) and high glucose (HG) under normoxia and hypoxia with and without treatment with Pyr-sulf (Pyr-s) up to 24 hours. Cells in normal glucose under normoxia were considered the control experimental condition.

When cells are exposed to Pyr-sulf before induction of hypoxia, with Pyr-sulf remaining in the cell medium for 24 hours, the expression of VEGF significantly decreases, compared to normoglucose cells in normoxia. Moreover, cells in high glucose and hypoxia show an increase in VEGF, as discribed before, which is prevented by treatment with Pyr-sulf for 24 hours (Figure 5.6 B). The expression of both PEDF or GLUT-1 are not altered by treatment with Pyr-sulf in these conditions (Figure 5.6 A and C).

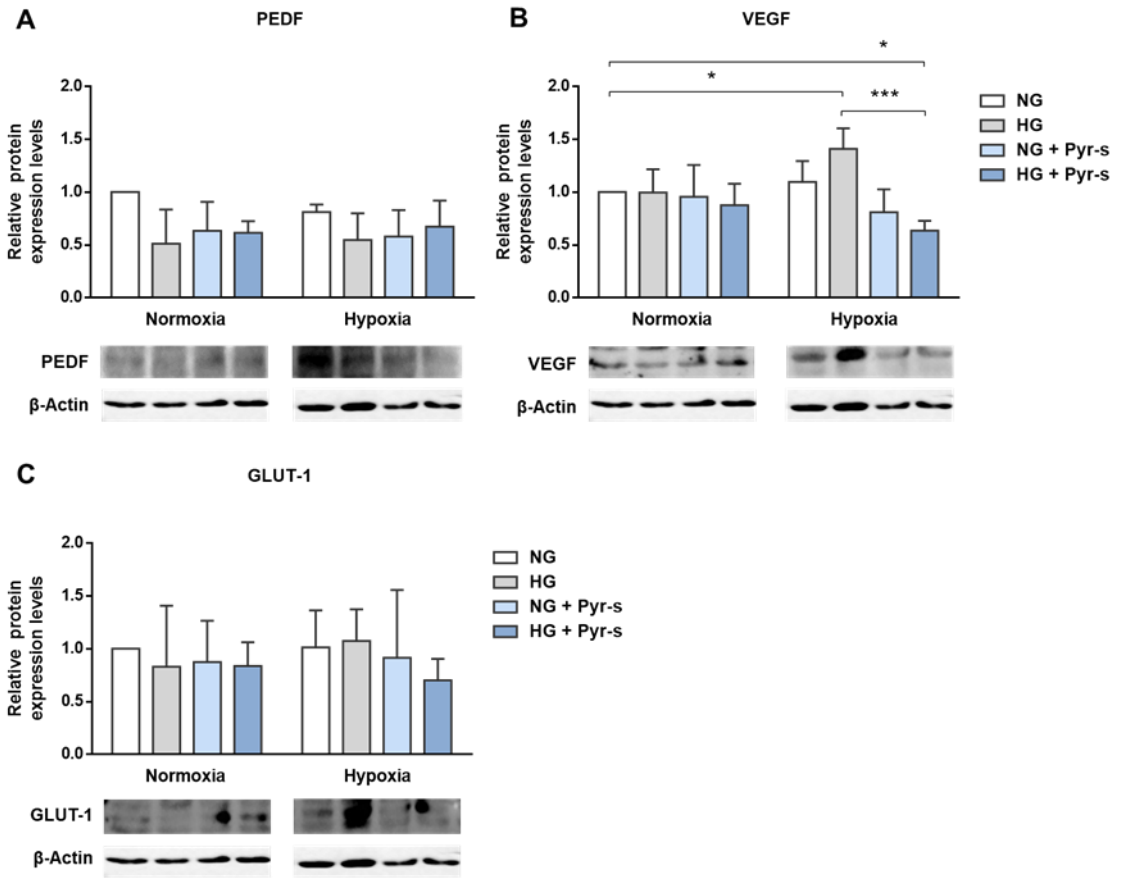


Figure 5.6 Effect of treatment of RPE cells with Pyrogallol-O-sulfate for 24 h under high glucose and hypoxia. (A) Levels of PEDF in RPE cells normal (NG) and High (HG) glucose, quantitative data normalized by the intensity of β-Actin, N=5. **(B)** Western blot analysis of VEGF protein levels in RPE cells after treatment under hypoxia, quantitative data normalized by the intensity of β-Actin. * $p<0.05$, *** $p<0.001$ by Two-way ANOVA with Dunnett's multiple comparisons test, N=5. **(C)** Western blot analysis of GLUT-1 protein levels in RPE cells after treatment under hypoxia, quantitative data normalized by the intensity of β-Actin, N=5.

Taking together the results from the three experimental approaches, regarding the effect of Pyr-sulf on the expression of PEDF, VEGF and GLUT-1 in RPE cells, we can observe that the time of exposure causes different outcomes.

When cells are pre-treated with Pyr-sulf for 8 hours before being exposed to hypoxia a decrease is observed in the expression of VEGF (Figure 5.2 B). However, the effect is lost if the treatment with Pyr-sulf is applied after the hypoxia is fully established (Figure 5.4 B). On the other hand, the expression of PEDF is promoted if cells in high glucose are treated with Pyr-sulf after hypoxia (Figure 5.4 A). The prolonged exposure of RPE cells under high glucose and hypoxia to Pyr-sulf significantly decreases the expression of pro-angiogenic VEGF, compared to both physiological conditions

(normoglycemia and normoxia) and non-treated cells under high glucose and hypoxia (Figure 5.6 B).

We must take into consideration the fact that Pyrogallol-O-sulfate is a secondary metabolite derived from (poly)phenols that, once uptaken by the cell, can be further metabolized into new products. If those products exert biological activities, it could explain the differences in the expression of the proteins analyzed according to the time of exposure of different cells to Pyr-sulf.

Also, both PEDF and VEGF are secreted proteins whose secreted levels were not analyzed in this study. Secreted proteins' levels should be measured in the cell culture medium to better understand if treatment with Pyr-sulf has an impact in the secretory function of RPE cells.

Several studies correlate bioactivity of (poly)phenols-derived metabolites with an improvement in vascular function in healthy humans, linked to the presence of newly identified plasma metabolites [128,218]. Also, a study shows that Epigalloccatechin-3-gallate, the main (poly)phenol component of green tea, effectively protected RPE cells from cell death and attenuated mRNA expressions of key angiogenic factors (MMP-9, VEGF, VEGFR2) by inhibiting the generation of ROS, with further evidence on the inhibition of ocular neovascularization and vascular permeability [137]. In the same line, RES, present in red wine, as well as other diet-derived (poly)phenols, was shown to have antiangiogenic properties, as reviewd in [219]. Our results point to a potential anti-angiogenic effect of Pyr-sulf on RPE cells that is dependent on the time of exposure of the cells to the bioactive metabolite. Also, further studies focused on the vascular features performed in a more robust hyperglycemia retinal model would help to understand the extension of the potential of treatment with Pyr-sulf in DR prevention and progression.

Regarding the levels of GLUT-1, which were not affected by Pyr-sulf in any of the experimental setups, an evaluation of the glucose consumption by the cells may be useful to determine if Pyr-sulf affects glucose uptake without significantly changing the expression of TNF-α in the cell membrane.

To extend our studies to a diabetic animal model, a proof-of-concept protocol was designed, based on the least invasive strategy and best approach for a therapeutic effect using the Pyrogallol-O-sulfate.

5.3.4. A single intravitreal injection of Pyrogallol-O-sulfate does not affect the expression of pro-angiogenic VEGF in the RPE of a diabetic mouse model

Unpublished data from our group has confirmed the impairment in the balance between pro- and anti-angiogenic factors in the vitreous of patients with DR, favoring pro-angiogenic molecules, more pronounced in the PDR stage.

Our group has also documented the above findings in a mouse model of type I diabetes, the Ins2^{Akita}, where it is observed a significant increase in the expression of VEGF in the neural retina of 9 month-old mice, while the expression of PEDF decreases. The same tendency is observed for VEGF in these animals [38].

To further evaluate the effect of Pyrogallol-O-sulfate in the expression profile of pro- and anti-angiogenic factors with disease progression in Ins2^{Akita} mouse model, we have assessed the protein levels of VEGF-A and PEDF in neural retina and RPE cells of 4, 6, 8 and 9 months-old mice two weeks after one-single intravitreal (Figures 5.8) or subretinal (Figures 5.9) injection of Pyr-sulf.

The experimental design is described in detail in Chapter II, Section 2.11.3. Briefly, diabetic and age-matched non-diabetic animals were randomly divided into two groups, one receiving a single intraocular injection of Pyr-sulf and the other receiving the vehicle solution. After two weeks, the retina and RPE of the mice were isolated and protein extracted for Western blot analysis of the expression of proteins of interest. The number of animals per group varies depending on the total number of animals available, without compromising the power of the experimental design.

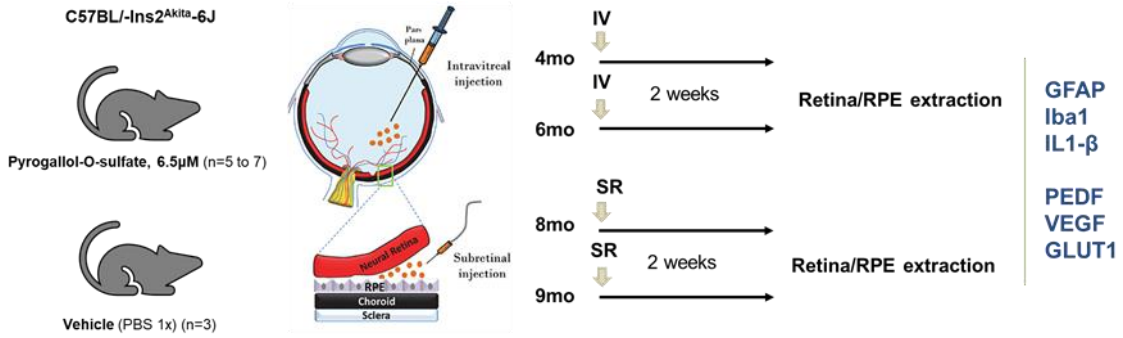


Figure 5.7 Schematic representation of the intraocular injections of Pyrogallol-O-sulfate in diabetic and non-diabetic mice, targeting different retinal cells at different stages of DR progression. The expression of proteins having a role in inflammation and angiogenesis were assessed by Western blot, two weeks after a single injection.

Treatment with Pyr-sulf does not affect the expression of PEDF, VEGF or GLUT in the neural retina of both non-diabetic or diabetic animals with 4 months of age (Figure 5.8 A, C and E). Despite no significant results were observed, the intravitreal injection of Pyr-sulf tends to increase the expression of PEDF (Figure 5.8 B) and decrease the expression of GLUT-1 (Figure 5.8 F) in the RPE of diabetic animals with 4 months of age.

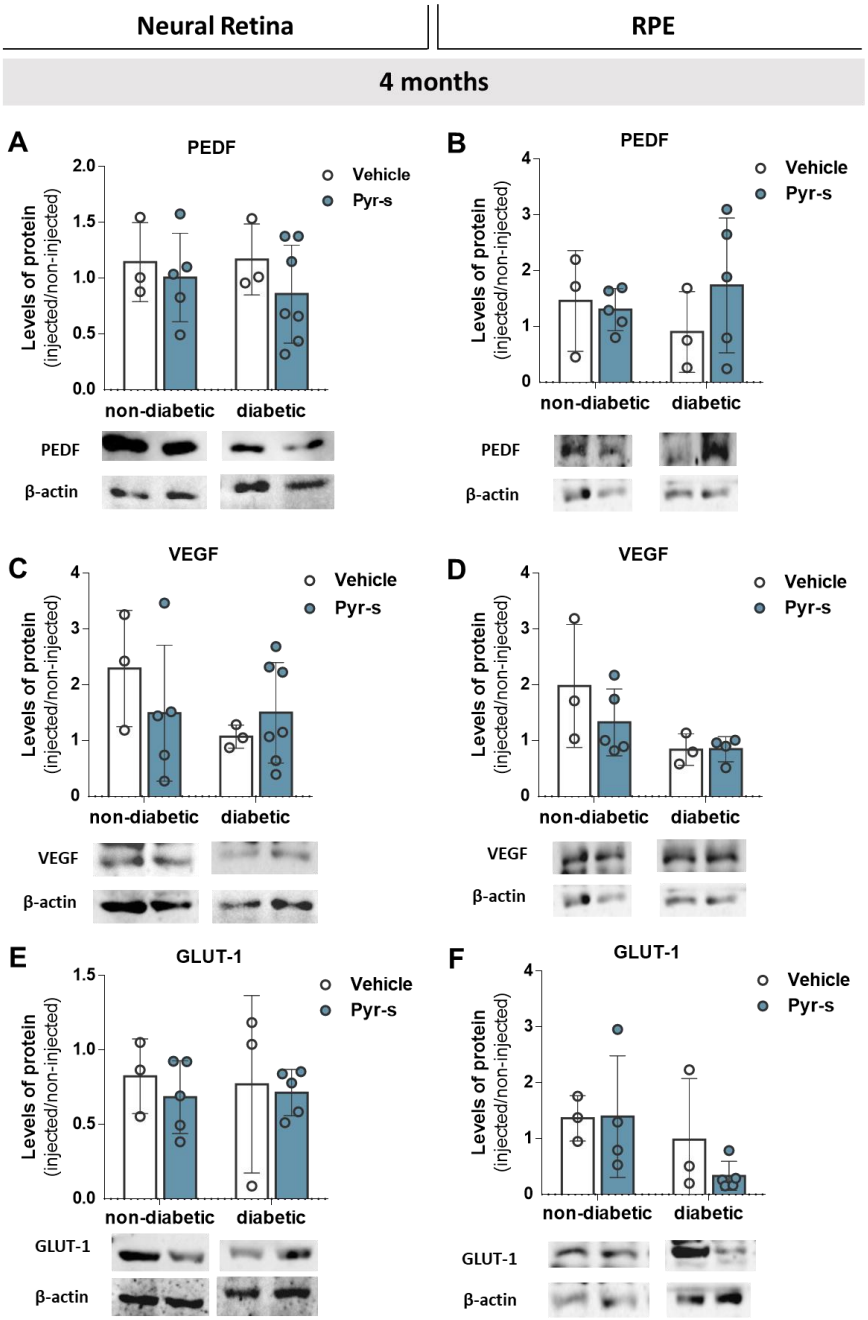


Figure 5.8 *(continued in the following page)*

Figure 5.8 Expression of angiogenic and glucose transporter proteins in 4 month-old Ins2^{Akita} mice after a single intravitreal injection of Pyrogallol-O-sulfate. **(A-B)** Protein expression levels and quantitative data of PEDF in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 4 month-old. **(C-D)** Protein expression levels and quantitative data of VEGF in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 4 month-old. **(E-F**) Protein expression levels and quantitative data of GLUT-1 in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 4 month-old. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD (N=3 to 7 mice/group), Two-way ANOVA (age and genotype) with Sidak's multiple comparison test.

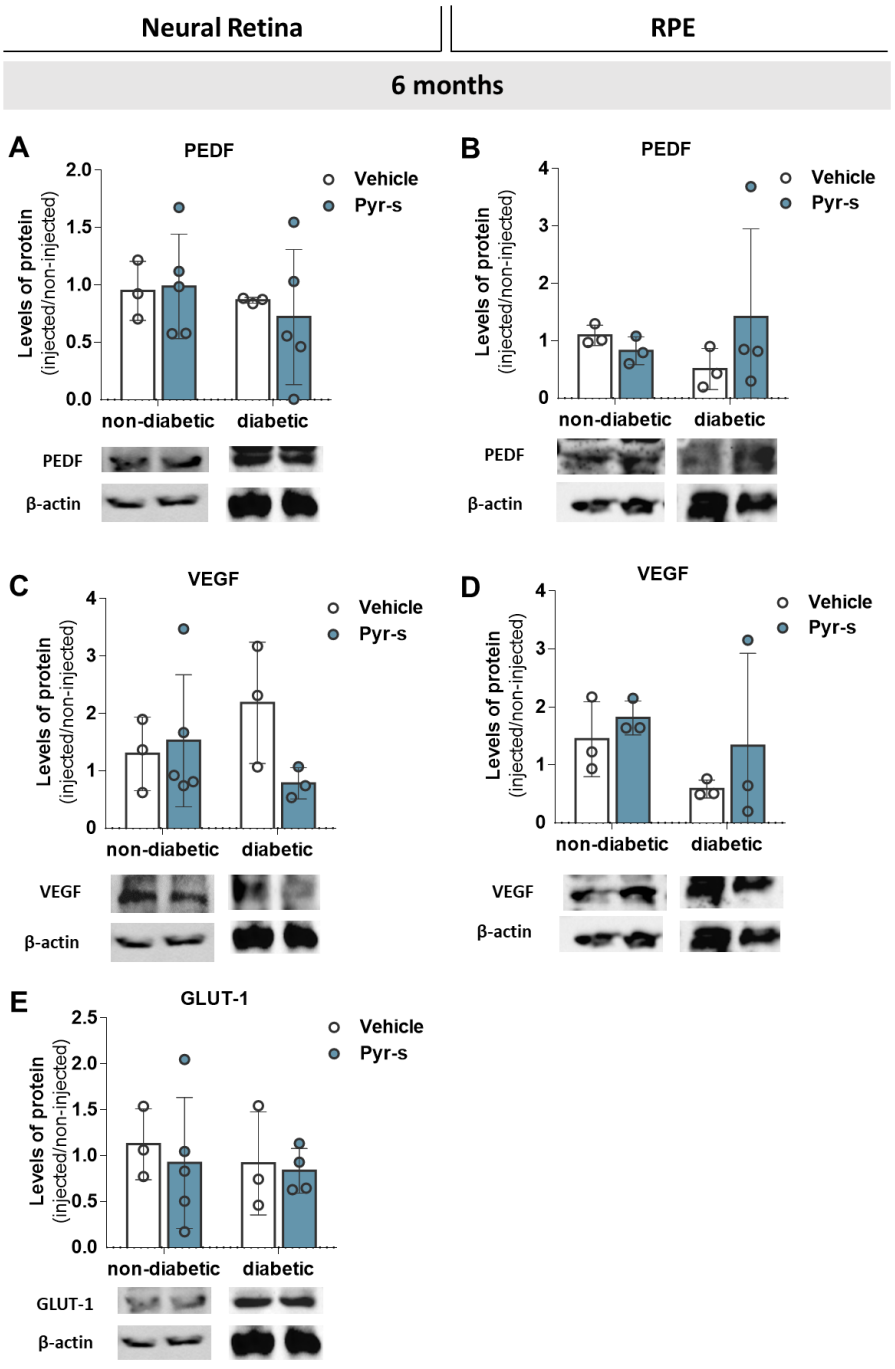


Figure 5.9 *(continued in the following page)*

Figure 5.9 Expression of angiogenic and glucose transporter proteins in 6 month-old Ins2^{Akita} mice after a single intravitreal injection of Pyrogallol-O-sulfate. **(A-B)** Protein expression levels and quantitative data of PEDF in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 6 month-old. **(C-D)** Protein expression levels and quantitative data of VEGF in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 6 month-old. **(E-F**) Protein expression levels and quantitative data of GLUT-1 in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 6 month-old. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD (N=3 to 5 mice/group), Two-way ANOVA (age and genotype) with Sidak's multiple comparison test.

When observing the effect of one-single intravitreal injection of Pyr-sulf in animals with 6 months of age, the expression of VEGF tends to decrease in the diabetic retina (Figure 5.9 C), compared to vehicle, with no effect in the expression of PEDF (Figure 5.9 A) or GLUT-1 (Figure 5.9 D). No alterations in the expression of both PEDF or VEGF were observed in diabetic and non-diabetic retina of the animals injected with vehicle, as previously observed in our study [38]. Regarding the RPE, treatment with Pyr-sulf does not have effect in the expression of PEDF (Figure 5.9 B) or VEGF (Figure 5.9 D). We were unable to detect the expression of GLUT-1 in these samples (Figure 5.9 E).

5.3.5. A single subretinal injection of Pyrogallol-O-sulfate decrease the expression of pro-angiogenic VEGF in the RPE of a diabetic mouse model

In the neural retina of animals with 8 months of age, treatment with Pyr-sulf does not have significant effect on the expression of PEDF (Figure 5.10 A), VEGF (Figure 5.10 C) or GLUT-1 (Figure 5.10 E), regardless of the genotype. However, there is a tendency for the single subretinal injection of Pyr-sulf to increase the expression of PEDF in RPE of both non-diabetic and diabetic mice (Figure 5.10 B). Also, a significant decrease in the expression of VEGF is observed in diabetic RPE upon treatment with Pyr-s (Figure 5.10 D). Treatment with Pyr-sulf additionally decreased the expression of GLUT-1 in the RPE of non-diabetic animals (Figure 5.10 F).

Likewise, the expression of PEDF, VEGF or GLUT-1 are not affected by a subretinal injection of Pyr-sulf in mice with 9 months old, both in the retina or RPE, with exception for a tendency to decrease the expression of VEGF in non-diabetic retina (Figure 5.11 C) and increase the expression of GLUT-1 in diabetic retina (Figure 5.11 E), compared to vehicle.

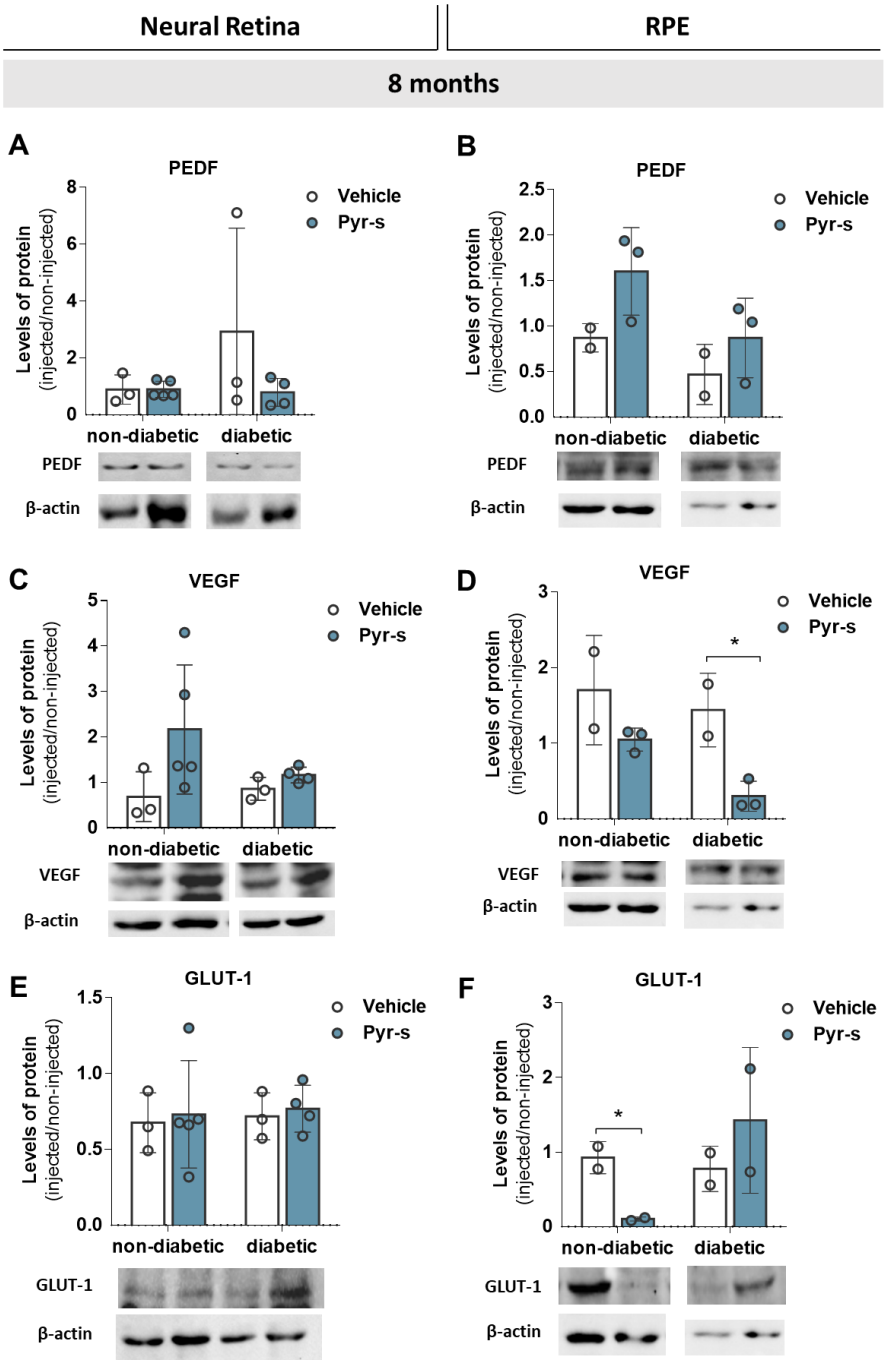


Figure 5.10 Expression of angiogenic and glucose transporter proteins in 8 month-old Ins2^{Akita} mice after a single subretinal injection of Pyrogallol-O-sulfate. **(A-B)** Protein expression levels and quantitative data of PEDF in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 8 month-old. **(C-D)** Protein expression levels and quantitative data of VEGF in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 8 month-old. **(E-F**) Protein expression levels and quantitative data of GLUT-1 in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 8 month-old. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD (N=2 to 5 mice/group), * $p<0.05$ by Two-way ANOVA (age and genotype) with Sidak's multiple comparison test.

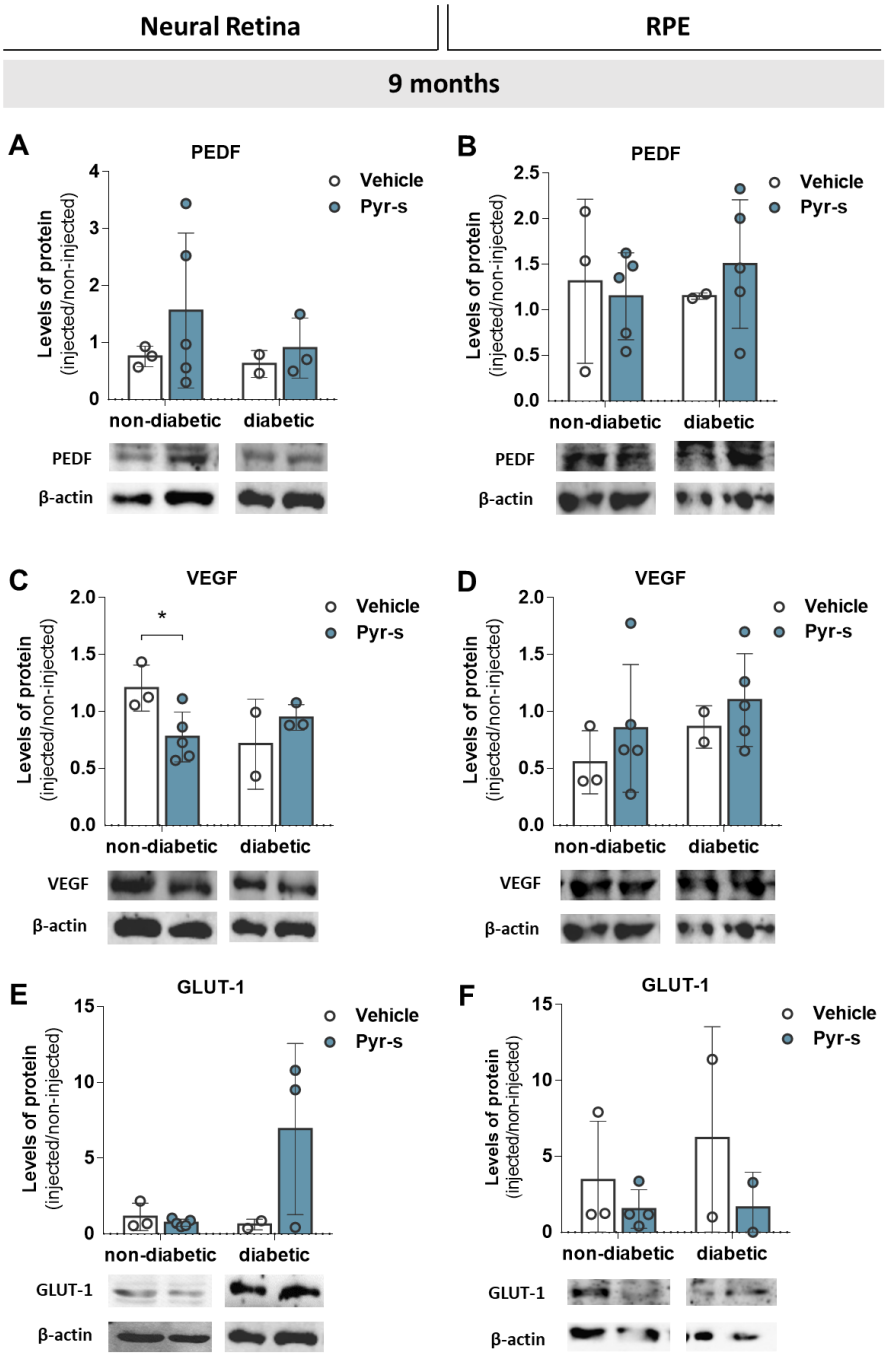


Figure 5.11 Expression of angiogenic and glucose transporter proteins in 9 month-old Ins2^{Akita} mice after a single subretinal injection of Pyrogallol-O-sulfate. **(A-B)** Protein expression levels and quantitative data of PEDF in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 9 month-old. **(C-D)** Protein expression levels and quantitative data of VEGF in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 9 month-old. **(E-F**) Protein expression levels and quantitative data of GLUT1 in the retina and RPE of Ins2^{Akita} (diabetic) mice and age-matched non-diabetic mice with 9 month-old. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD (N=2 to 5 mice/group), * $p<0.05$ by Two-way ANOVA (age and genotype) with Sidak's multiple comparison test.

A tendency to increase the expression of VEGF was previously reported by our group in the RPE of diabetic Ins2^{Akita} mice at 9 months old [38]. Here we show that a single subretinal injection of Pyr-sulf was able to prevent that increase, as shown by the decrease in the expression of VEGF in the RPE of diabetic 8 month-old mice. Although Pyr-sulf treatment does not have an effect in the expression of VEGF in the RPE of diabetic animals with 9-months of age, it also does not increase its expression, revealing that it is not a promoter of imbalance in the expression of proteins involved in pathological angiogenesis involved in the progression of DR.

The analysis of the expression of PEDF, VEGF and GLUT-1 that follows intravitreal injection, thus targeting the neural retina, in younger mice, shows a tendency to decrease the expression of VEGF in the diabetic retina upon treatment in animals with 6 months of age. This is a very exciting outcome, as it points to a beneficial effect of Pyr-sulf in the prevention of neovascularization by decreasing the expression of a very important pro-angiogenic agent earlier in DR progression. The expression of GLUT-1 also tends to decrease in the RPE of 4 month-old mice treated with Pyr-sulf by intravitreal injection, showing that it is possible to have an effect promoted by Pyr-sulf in the outer layer of the retina after intravitreal administration.

Altogether, intraocular injection of Pyrogallol-O-sulfate decreases the expression of pro-angiogenic VEGF in diabetic retina and RPE, at different stages of DR progression, showing how it can be explored for a therapeutic approach towards DR.

CHAPTER VI
GENERAL DISCUSSION AND CONCLUSION

6.1. General Discussion and Conclusion

Classically regarded as a progressive microvascular complication of diabetes with an inflammatory background, DR remains one of the major cause of acquired visual impairment and/or blindness among diabetic population [2–4].

The retina, the most metabolically active tissue of the human body, is the sensory tissue that lines the inner surface of the posterior segment of the eye and is composed mainly by three cell types including the RPE, that is located in-between the PRs and the choriocapillaris [9].

RPE is a highly selective cell monolayer, with the apical membrane facing the subretinal space hat interacts with the outer segment of PRs, and the basolateral membrane contacting with the Bruch membrane, that is in direct contact with blood in fenestrated vessels of the choroid, forming the BRB. Among other functions, RPE takes up nutrients, such as glucose, and produces and secretes a wide range of growth and neurotrophic factors that, because of its polarized nature, can either be secreted to the apical or basolateral membrane to support and maintain the structural basis of PRs and choriocapillaris endothelium [12].

Although the exact mechanism underlying DR pathophysiology is not yet fully understood, it is believed that the chronic exposure to hyperglycemia triggers a cascade of biochemical and physiological changes, causing inner BRB breakdown [14]. These events are potentiated by oxidative stress and inflammation, which promote increase in the production of ROS, cytokines and growth factors, altogether contributing for visual impairment or irreversible blindness [2].

As mentioned before, one of the major characteristics of DR is vascular changes along the progression of the disease, trigged by chronic and sustained hyperglycemia. Due to retinal Ischemia during diabetes, the balance between pro- and anti-angiogenic molecules is disrupted, leading to up-regulation of pro-angiogenic VEGF and down-regulation of anti-angiogenic PEDF. As a consequence of the imbalance mostly between these two growth factors, neovascularization is promoted but the new blood vessels are usually weak, fragile and leaky, which allows proteins, fluids and debris to enter the retina [2,72].

At present, there are few effective treatments for DR. Tight glycemic control is the most efficient therapy to slow the progression of DR. Blood pressure should also be taken in consideration since hypertension aggravates the disease by increasing blood

flow, and causing mechanical damage to the vascular endothelial cells, thus stimulating the production of VEGF [2,72]. However, current treatments for DR are designed to act on later stages of this pathology, targeting ocular lesions resulting from the disease progression. In pathologies associated with abnormal angiogenesis, like DR or wet AMD, intravitreal administration of monoclonal antibodies or fusion proteins that block VEGF activity is considered as first line therapeutic approach. Considering the growing numbers of diabetes incidence and prevalence and that these forms of treatment only uphold the progression of DR temporarily, there is a need to seek therapeutic alternatives that either have longer lasting effects or can target DR in earlier stages of progression.

Knowing that targeting DR remains a challenge, it is important to understand how we can prevent its progression. Our main goal is to investigate different and novel therapeutic approaches towards DR, based on the RAS modulation and biological proprieties of (poly)phenol-derived metabolites.

The RAS is an intricate system of pathways, involving a variety of enzymes and peptides. Of all the pathways known to date, our interest falls over two main axes. The first is the classically known ACE/Ang II/AT1R axis, also designated as the prejudicial axis and the second is the ACE2/Ang (1-7)/Mas1 axis, also known as the protective axis. It is now acknowledge that components of RAS are not only systemically expressed but also expressed at the organ level, including the eye [102,103,68,105]. Several studies have been performed to better characterize ocular and other local RAS systems. The balance between both axes plays a role in maintaining the function of several organs and the imbalance is responsible for the long-term injuries, such as DR.

Studies aiming to elucidate the role of RAS in the retina are recurrent. However, studies involving RAS and RPE are scarce and the RPE, as part of the BRB, is known to be affected in DR. The precise mechanisms contributing to RPE dysfunction in DR are not completely clarified, but it is known that angiogenesis and oxidative stress are two major contributors [11,220]. Here we aimed to further characterize the RAS in a cell model of RPE and how RAS modulation could contribute to ameliorate DR outcome.

The RAS has been described as being strongly influenced by glucose [221], and evidences show a correlation between the overactivation of RAS and DR [108,167,222]. In fact, we were able to detect components from both axis in RPE cells and that they are modulated by glucose levels, overall promoting an imbalance between the deleterious and protective arm. The RAS can be activated by two different pathways: an angiotensin II-dependent and an angiotensin II-independent pathway. The angiotensin II-dependent pathway takes place in the intracellular compartment and involves the conversion of

angiotensinogen to angiotensin I by renin. Angiotensin I is then converted to angiotensin II by the action of ACE. When RPE cells were exposed to Angiotensin II, it is observed a significant increase in the expression of pro-angiogenic VEGF and the production of ROS. Also, there is a tendency to increase the presence of the components from the ACE2/Ang(1-7)/Mas axis in a time-dependent manner, as Ang II is the substrate of ACE2.

RAS-based therapies have been focused on ACE inhibitors or ARBs but with limited success, since disease progression could not be prevented. The main limitation lies on the very downstream targets within the RAS cascade where these blockers act, highlight the need of alternative drugs to target the RAS in a sustained manner for the treatment of retinal diseases.

The DRIs are a recent class of RAS inhibitors of great interest since they act exactly at the rate-limiting step of the system – renin – with Aliskiren being the only DRI approved for medical use. Comparing with the traditional RAS inhibitors, the DRIs have the advantage of acting directly on renin, the rate-limiting step of the cascade, and for that reason these drugs might be more effective candidates to block the RAS. Aliskiren is the only DRI clinically approved and because their direct effects on RPE cells are unknown, namely in pathological conditions, we tested RAS inhibition with aliskiren in RPE cells at different levels of the cascade.

In order to bind renin, the receptor must be translocated from the cytoplasm to the membrane [165]. Our data demonstrated for the first time that aliskiren decreases the amount of the renin receptor in the cellular membrane, favoring its retention in the intracellular space of RPE cells. Blocking the translocation of this receptor to the membrane is imperative to prevent the downstream effects derived from its stimulation, such as ERK1/2-MAPK activation [165]. We have tested this hypothesis, and subjected RPE cells to a time-course with aliskiren [100 μM (60 μg/ml in absolute dose), during 10, 30, 60, 120 and 240 minutes] and the activation of ERK1/2 was measured (Figure 6.1). Following an initial phosphorylation (up to 30 minutes), ERK1/2-MAPK was inhibited by aliskiren in a sustained manner in RPE cells.

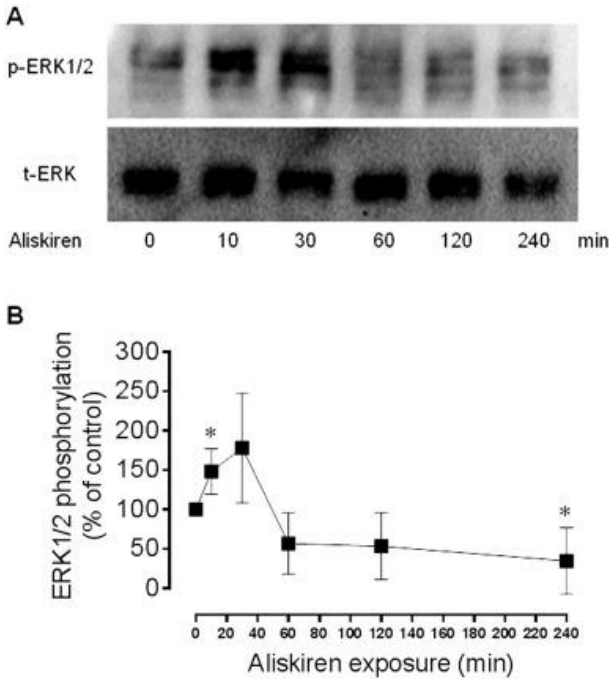


Figure 6.1 Effect of aliskiren in the phosphorylation of ERK1/2 in RPE cells. (A) Representative immunoblots of p-ERK1/2 time-course in the presence of aliskiren [100 µM (60 µg/ml in absolute dose)] in D407 RPE cells and **(B)** Densitometric analysis of p-ERK1/2 time-course in the presence of aliskiren [100 µM (60 µg/ml in absolute dose)] in D407 RPE cells. Values were normalized to t-ERK and expressed as percentage of control, with control assumed to be the condition without aliskiren. * $p<0.05$ compared with control values, determined by Dunnett's Multiple Comparison test, N=4.

Moreover, the expression and activity of renin is decreased by RAS inhibition with aliskiren, as the levels of Ang II. Similarly to what we have observed, a paper has described in aortic HSMC cells that aliskiren decreased the RAS activity by binding to the catalytic center of renin and by changing the expression of the renin receptor, which reduced the activation of downstream signaling pathways [184]. At the same timepoint as these observations, the expression of ACE2 tends to decrease, which may be explained by the fact that there is less Ang II. However, the levels of Ang (1-7) tend to increase, pointing to an alternative pathway to produce this peptide.

In our study, we demonstrate for the first time that aliskiren effectively reduced the production of ROS in RPE cells in a sustained and time-dependent manner, suggesting that this drug has antioxidant capacity. Also, the expression of pro-angiogenic VEGF decreases, while increase the expression of anti-angiogenic PEDF, highlighting its potential as a therapeutic approach for DR. Indeed, the retinal protection of aliskiren

was proven to be similar or greater than ACE inhibition in a non-proliferative DR animal model [160].

Despite our promising findings regarding upstream RAS inhibition with aliskiren, we considered our previous findings, in which we observed dysregulation in the expression of both RAS axis by glucose. As so, we hypothesized that targeting the protective axis of RAS can be also a valid therapeutic approach by using a gene therapy approach towards the activation of the protective axis. To test this hypothesis, we have chosen to overexpress the ACE2 in RPE cells and evaluate the expression and/or levels of the protective axis components, as well as its effects on inflammation, oxidative stress and angiogenesis.

ACE2 overexpression significantly increases the expression of Mas receptor in RPE cells, with no effect on the levels of Ang (1-7), raising some questions. Here we measure the levels of protein but not the gene levels, which could address an earlier effect of the ACE overexpression over the Ang (1-7) levels. Regardless, the levels of inflammatory marker TNF-α decreased in RPE cells overexpressing ACE2 as well the levels of ROS.

The activation of ACE2/Ang (1-7)/Mas axis was proven to be relevant to the anti-inflammatory effects of the ARBs, such as telmisartan and olmesartan. As a consequence of the increase in the expression of ACE2/Ang (1-7)/Mas1 by ARBs there is a reduction in the expression of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6; and increase in the expression of anti-inflammatory cytokine IL-10 [223,224]. Apart from the direct effects induced by ligation of Ang (1-7) to Mas1, it is postulated that the Mas1 receptor can interact with the angiotensin receptors in a similar way to the dimerization observed between AT1R and AT2R. Reports show that hetero-oligomerization of Mas1 with AT1R resulted in altered signaling of the AT1 receptor with a direct impact in its response to Ang II [225]. It is also reported that the interaction of Mas1 with other receptors may be necessary in order for Ang (1-7) to induce significant physiological effects. For example, Mas1 can form a heterodimer with B2 receptor, a bradykinin receptor, leading to a potentiation of the effects of its ligand [226].

A preliminary characterization of the expression of some RAS components was performed in the retina of a diabetic mouse model, the $Ins2^{Akita}$, at different ages. Our observations point to an imbalance in the deleterious and protective axis components, as the expression of renin increases, while the expression of Mas receptor decreases in the diabetic retina, compared to age-match non-diabetic tissue. However, this imbalance is not observed in aged mice.

A more complete characterization of the RAS elements in the retina (including the RPE) of these animals would help to better target DR progression through RAS modulation. The levels Angiotensin II and Angiotensin (1-7) were evaluated by confocal imaging and quantitative immunohistochemistry during the development of streptozotocin-induced diabetes in rats, showing a higher intensity and extent of Ang II staining compared with nondiabetic eyes, but lower intensity with a reduced distribution of Ang (1-7) immunoreactivity [227]. Moreover, angiotensin II was found in Müller cells and its receptors localized in the blood vessels and neural cells in the human retina, pointing to a role on the onset and severity of retinovascular disease [90]. Understanding how diabetes influence the expression of these elements in the $Ins2^{Akita}$ retinas will be of a great value to design an experimental setup that evaluates the effect of RAS modulation, either by RAS inhibition with aliskiren or activation of the protective axis, in DR-related hallmarks.

There are several studies on the anti-diabetic effects of (poly)phenols. These compounds influence the glycemia through different mechanisms like inhibition of glucose absorption in the gut or in peripheral tissues. Some studies show the inhibition of glucose transporters by (poly)phenols. For example, onion (poly)phenols have the capacity of protect diabetic patients from oxidative stress and (poly)phenols from vegetables act as potent anti-diabetic agents by decreasing the levels of blood glucose and increase plasma insulin.

Studies specifically related to the effect of (poly)phenols or their metabolites on retinal diseases are scarce. However, the interest in this topic increased in the last decade, particularly in studies on the effect of RES, as reviewed in [228]. More recently, the benefic effect of different carotenoids and (poly)phenols in the prevention and treatment of age-related ocular disorders was reviewed, highlighting the role of these metabolites in oxidative stress and inflammation [137]. In particular, *in vitro* and *in vivo* studies have revealed that a variety of nutraceuticals have significant antioxidant and anti-inflammatory properties that may inhibit the early diabetes-driven molecular mechanisms that induce DR, reducing both the neural and vascular damage typical of DR.

The presence of sulfated metabolites of Catechol and Pyrogallol was confirmed for the first time by Santos and co-workers in human plasma from volunteers after ingestion of a fruit purée[126] . The metabolites Cat-sulf and Pyr-sulf were found in several volunteers at baseline reaching, in some volunteers, concentrations up to 20 µM that can exert effects *in vivo*. Moreover, phase II metabolites of pyrogallol and catechol were relatively

abundant in urine samples of volunteers [127]. These two metabolites (Cat-sulf and Pyr-sulf) have been recently studied in different models of neurodegenerative and cardiovascular disorders, showing the ability of these small molecules to cross the BBB, modulate the brain cell towards neuroprotection and have an effect on cardiomyocyte beating following prolonged stimulation. Also, it was shown for the first time that the most abundant metabolite in circulation, Pyr-sulf, was also the most effective in prevent oxidative damage in an endothelial cells' model. Here we aim to address the effect of both Cat-sulf and Pyr-sulf in the expression of proteins involved in inflammation and angiogenesis, using RPE cells and a diabetic mouse model.

To the best of our knowledge, this is the first time these (poly)phenol-derived metabolites are tested in RPE cells, so a first approach was to evaluate how RPE are metabolic affected by the presence of Cat-sulf or Pyr-sulf. RPE cells, under high glucose and hypoxia, are metabolically affected by treatment with Cat-sulf (5 µM, 24 h) but not by Pyr-sulf (6.5 µM, 24 h). However, the cell viability is not affected by Cat-sulf, as observed by two different assays, leaving an opportunity to explore the metabolic pathway(s) that may be disturbed by this metabolite.

As previously highlighted, inflammation plays a prominent role in DR pathophysiology. In line with this, we aimed to contribute with new insights on the potential of Pyr-sulf in preventing inflammation and neovascularisation in the retina, thus showing itself as a therapeutic approach for early-stage DR patients. Our first hypothesis was that RPE cells pre-treated with Pyr-sulf before the inflammatory stimulus will be protected from the exacerbated increase in the expression of pro-inflammatory cytokines. We were able to establish a model of acute inflammation, by challenged RPE cells with a pro-inflammatory cytokine, TNF-α, and measured the levels of expression of pro- and anti-inflammatory proteins that are trigged by inflammation. In fact, there is a tendency for pre-treatment with Pyr-sulf to protect RPE cells from increasing the expression of TNF-α and IL-1β, but only in specific conditions. However, this experimental cell model deserves further investigation as the levels of cytokines selected were only measured in whole cell lysates lacking the levels of secretion.

The levels of PEDF and VEGF, whose expression is imbalanced in DR patients, as well as the glucose transporter GLUT1 were analyzed in RPE cells treated with Pyr-sulf in three different protocols. When Pyr-sulf was added before induction of hypoxia, the expression of pro-angiogenic VEGF tends to decrease, suggesting a role in protecting RPE cells from the deleterious effects that are promoted by the increase in the expression of VEGF. On the other hand, it is observed when Pyr-sulf is added after

full establishment of hypoxia, where a tendency to increase the expression of anti-angiogenic PEDF is observed. The expression of VEGF is effectively decreased when RPE cells are exposed to Pyr-sulf for 24 hours, starting before induction of hypoxia.

One phenomenon that may explain the different outcomes of Pyr-sulf treatment, depending on the protocol, is their metabolism within the cells. The proposed cellular pathways of Pyr-sulf metabolism was previously designed and quantified in a blood-brain barrier cell model [121]. The same characterization in RPE cells upon exposure to Pyr-sulf could help to understand the novel cellular metabolites present at the time-points previously evaluated and their relative amounts in the cell, to further study their physiological relevance. Also, it is known that PEDF, VEGF and the cytokines evaluated are mainly secreted by cells, including RPE, whose levels were not evaluated in this study. Assessing the secreted levels of these proteins could help to understand the secretory profile of RPE cell upon treatment with Pyr-sulf and, therefore, highlight the biological outcome towards inflammation and angiogenesis.

To further study the therapeutic potential of Pyr-sulf we evaluated the expression of several proteins known to be involved either in DR-inflammatory process or in neoangiogenesis after intraocular injection of the metabolite in $Ins2^{Akita}$ mice. Diabetic and age match non-diabetic animals were divided in groups at different ages, covering different stages of DR progression, and the eye injected with vehicle or Pyr-sulf at the same concentration used *in vitro*. A single injection was performed, intravitreously or subretinally, and the expression of several proteins of interest were measured in the neural retina and RPE.

The expression of the microglia marker, Iba1, significantly decreased in diabetic retina after a single intravitreous injection of Pyr-sulf, in 6 month-old animals. Indeed, the injection of Pyr-sulf in 4 month-old animals tends to have the same effect, although without reaching statistical significance. The decrease in the expression of Iba1 is maintained when Pyr-sulf is subretinally injected in 9 month-old diabetic mice.

Regarding the expression of GFAP, it is observed a significant increase in its expression in non-diabetic mice with 4-month of age upon intrevitreous injection of Pyr-sulf, compared to vehicle. The tendency is maintained in diabetic retinas from 6 month-old animals, but Pyr-sulf was able to maintain the expression of GFAP very close to the levels observed in the animals receiving the vehicle. For all the results, the expression of any of the proteins analyzed was not altered by the injection of vehicle, as represented in supplementary data.

In the normal retina, astrocytes label more heavily with anti-GFAP than the Müller cell endfeet (which predominately express the type-III intermediate filament, vimentin), but after injury there is a rapid up-regulation of GFAP by Müller cells [41,215]. In our studies, elevated GFAP expression happens early and becomes close to the control levels in later stages of DM upon injection of Pyr-sulf. As to date no study has been able to connect increased levels of GFAP to any functional outcome, immunohistochemistry of the expression of GFAP, comparing diabetic with non-diabetic retina upon injection with vehicle or Pyrogallol-*O*-sulfate will better complete these studies, in order to evaluate the cell population expressing GFAP and their functional role in this model.

Concerning the levels of proteins contributing to angiogenesis, it is observed a tendency to decrease the expression of pro-angiogenic VEGF in the diabetic retina of the 6 month-old animals upon injection with Pyr-sulf. The decrease in the expression of VEGF is also observed in the diabetic RPE of animals with 8-months of age, and non-diabetic retina of 9 month-old mice, subretinal injected with Pyr-sulf.

In agreement with what is described for PDR patients, we previously described an increase of VEGF in late stages of the disease in $Ins2^{Akita}$ mice both in the neural retina and the RPE [38,210]. However, at earlier stages, we have observed significantly decreased levels of VEGF in RPE cells and a slight decrease in the neural retina [38]. Here, a single intraocular injection of Pyr-sulf, at the physiological concentration found in human plasma, significantly decrease the expression of VEGF at earlier stages, that is maintained in older animals, assessed two weeks after injection. These studies, together with those reviewed by Cao *et al.* [219], increases interest in (poly)phenols as an important group of therapeutic natural compounds in the treatment of neovascular-dependent disorders.

Pyr-sulf injection also had beneficial effects in non-diabetic retinas by decreasing the expression of the glucose transporter, GLUT1, in 8 month-old mice, a tendency that is maintained not only in non-diabetic, but also in diabetic retina in 9 month-old animals.

Also, evaluation of the retina by optical coherence tomography and electroretinogram upon treatment at different ages will address the role of Pyrogallol-O-sulfate in the maintenance of retinal function.

Altogether, this thesis contributed with new insights about:

- Retinal RAS components
- Effect of glucose in the expression of the main RAS components in RPE cells
- Mechanism of action of RAS inhibition by aliskiren in the RPE cells
- Potential of the activation of ACE2/Ang (1-7)/Mas receptor towards inflammation and oxidative stress in RPE cells
- Effect of a single intraocular injection of the (poly)phenol-derived metabolite Pyrogallol-*O*-sulfate in microglial activation and pro-angiogenic protein expression in the diabetic retina

In short, our data demonstrate that RPE cells express the main RAS constituents and this system was deregulated by hyperglycemic glucose concentrations. Exposure of RPE cells to angiotensin II increased the levels of the main pro-angiogenic factor VEGF in a concentration-dependent manner, while tends to increase the expression of the protective axis components. Additionally, angiotensin II also stimulated the production of ROS in RPE cells. Treatment of RPE cells with aliskiren inhibited the activity of renin and consequently decreased the levels of angiotensin II. The levels of oxidative stress decreased after treatment and the expression of anti-angiogenic factor PEDF was promoted. Finally, the overexpression of ACE2 protects RPE cells from inflammation and oxidative stress.

Moreover, exposure of RPE cells to the (poly)phenol-derived metabolite Pyrogallol-*O*-sulfate, protect against inflammation and effectively decrease the expression of VEGF in cells under high glucose and hypoxia. Additionally, a single intraocular injection of Pyrogallol-*O*-sulfate was able to decrease the expression of the microglial marker Iba1 and pro-angiogenic VEGF in the diabetic retina earlier in the disease progression.

Our findings of the RPE RAS, together with the demonstration that aliskiren effectively blocks this system at different steps of the cascade, suggest that aliskiren might be an alternative drug for preventing the deleterious effects derived from the overactivation of the RAS. At the same time, the activation of the ACE2/Ang-(1-7)/Mas axis, known to counterbalance the deleterious effects of ACE/AngII/AT1R axis, presents itself as a promising gene therapy approach. The effect of Pyrogallol-O-sulfate in RPE cells and diabetic retina opens an opportunity to further explore this metabolite as a therapeutic approach towards inflammation in DR.

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Supplementary data

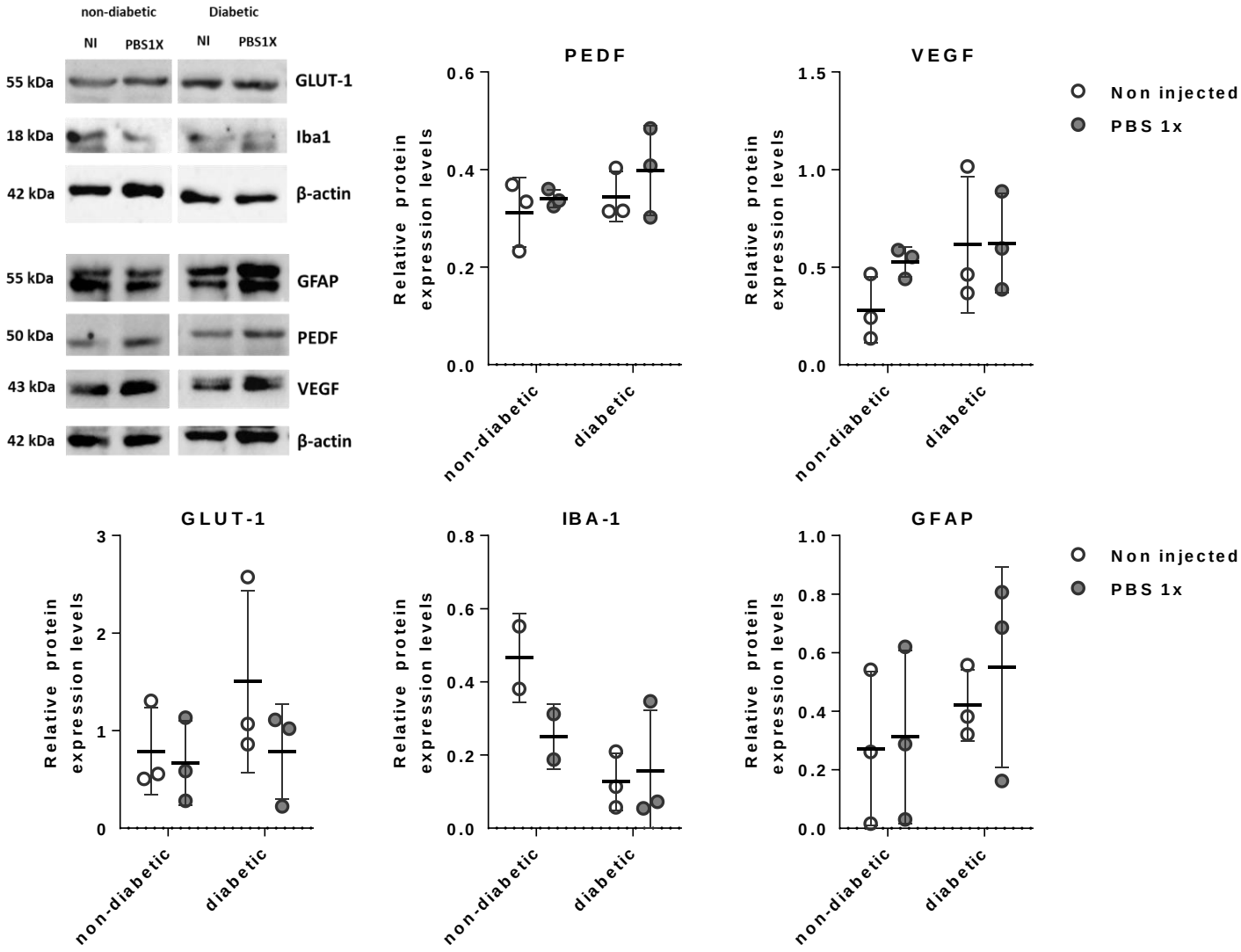
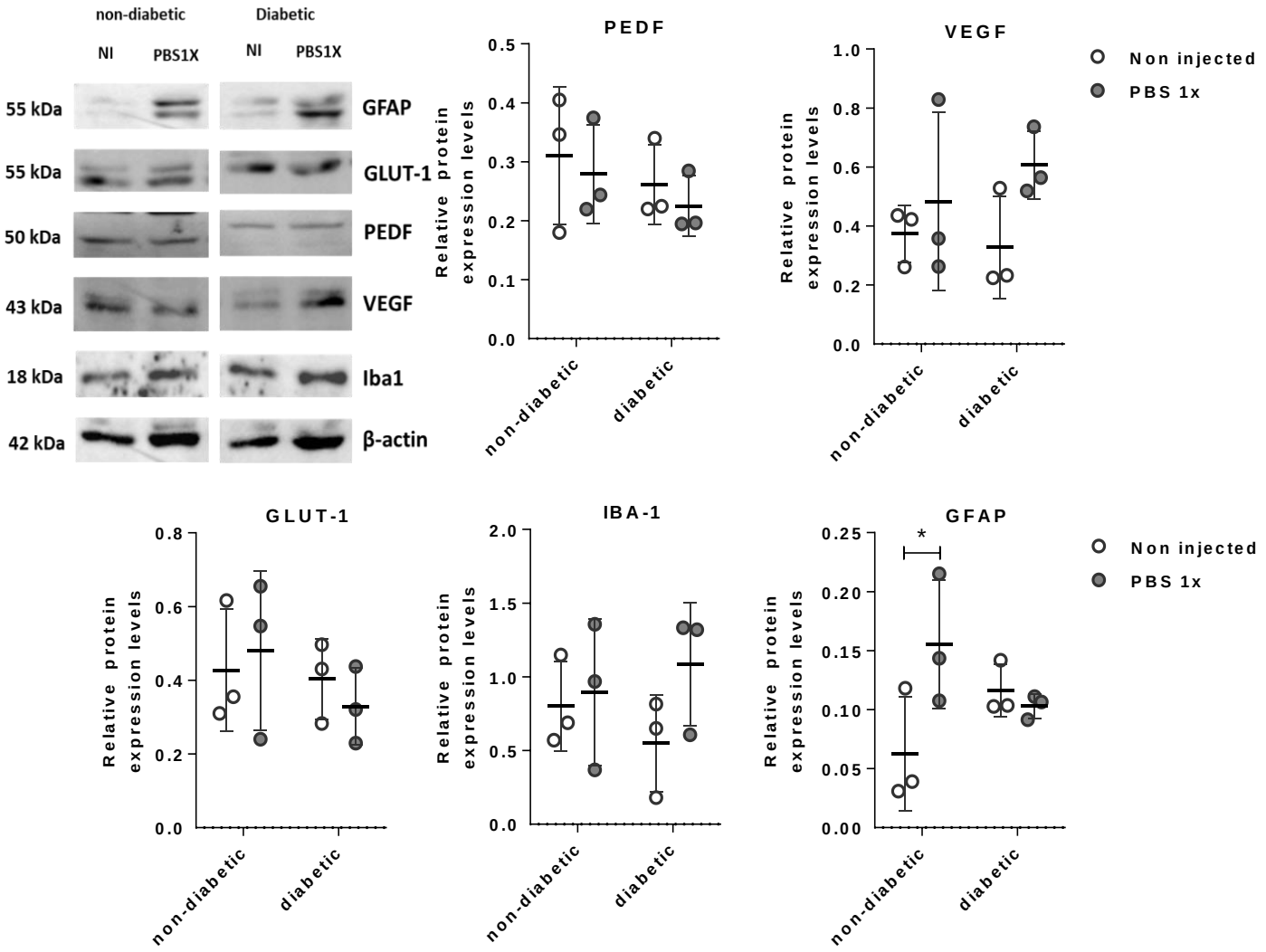
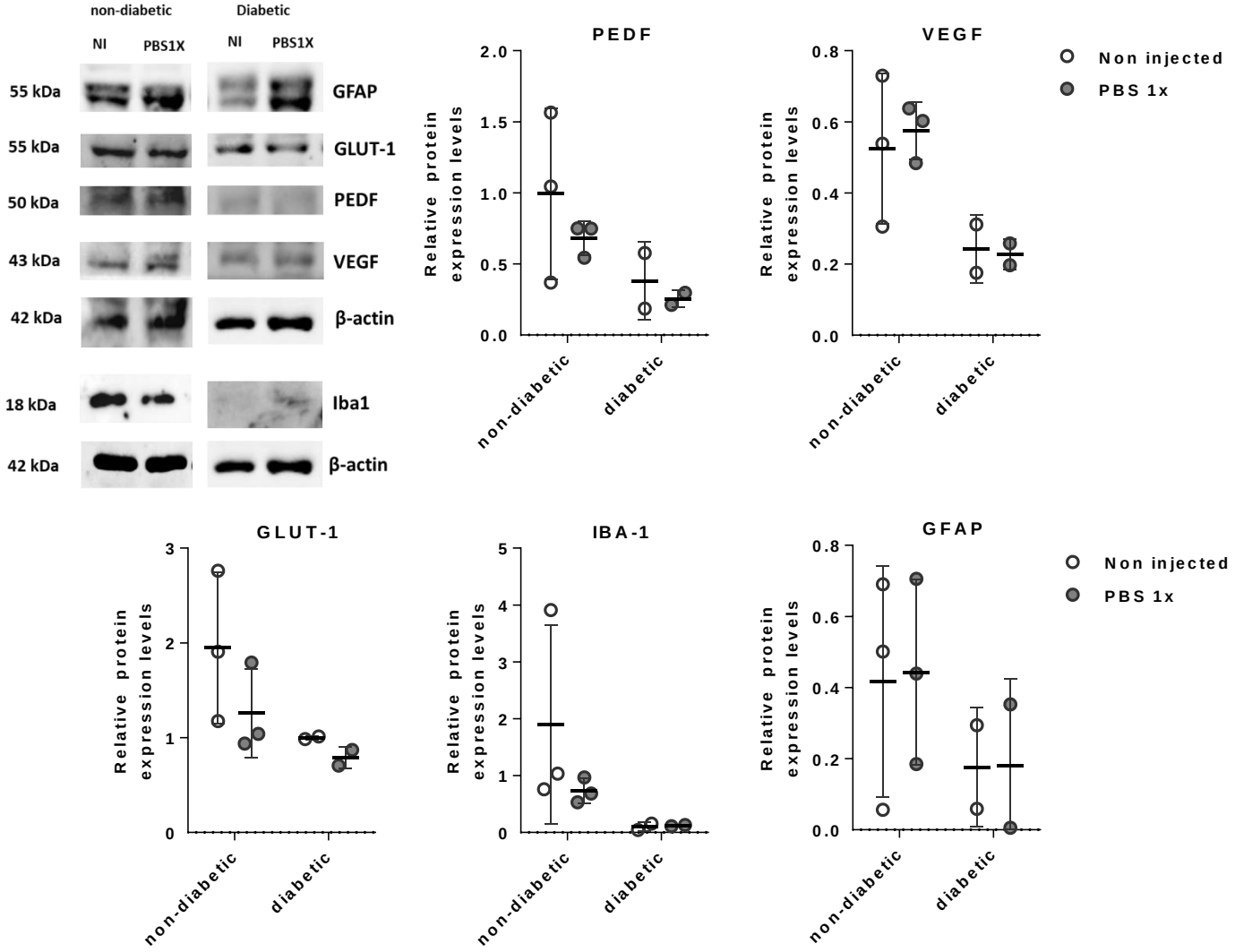


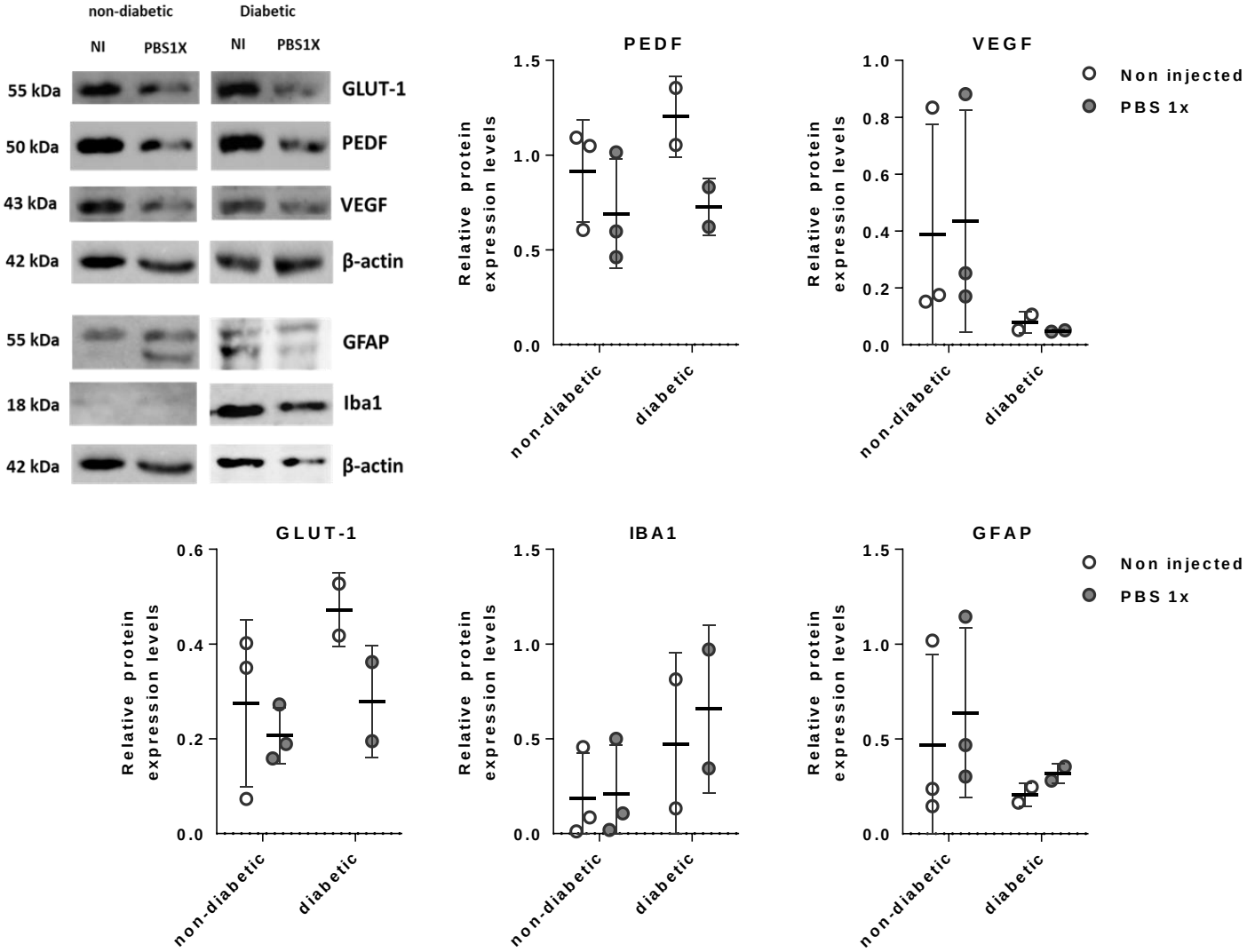
Figure Expression of proteins of interest followed by intraocular injections of vehicle solution (PBS 1x) in diabetic and age-match non-diabetic animals with 4 month-old. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD (N=2 to 3 mice/group).



Expression of proteins of interest followed by intraocular injections of vehicle solution (PBS 1x) in diabetic and age-match non-diabetic animals with 6 month-old. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD (N=2 to 3 mice/group). * $p<0.05$ is significantly different compared to contralateral non-injected eye, determined by Two-way ANOVA (age and genotype) with Sidak's multiple comparison test.



Expression of proteins of interest followed by intraocular injections of vehicle solution (PBS 1x) in diabetic and age-match non-diabetic animals with 8 month-old. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD (N=2 to 3 mice/group).



Expression of proteins of interest followed by intraocular injections of vehicle solution (PBS 1x) in diabetic and age-match non-diabetic animals with 9 month-old. Protein levels were normalized to β-Actin. Data are expressed as mean ± SD (N=2 to 3 mice/group).

TOWARDS A NEW THERAPY FOR DIABETIC RETINOPATHY: RAS AND BERRIES

ERRATA

Página	Onde se lê	Leia-se
v	A exposição das RPE a angiotensina II diminui (…)	A exposição das RPE a angiotensina II aumenta (…)
Xxiii, 97, 117	Santos DF, Pais M, Silva GA.	Santos DF, Pais M, Santos CN, Silva GA.
64	mRNA levels of PEDF, VEGF, IL-8 and IL-1β	mRNA levels of IL-8 and IL-1β
68; Figure 2.6	Pyrogallol-*O*-sulfate (n=5 to 7)	Pyrogallol-*O*-sulfate (n=2 to 7)
75	Figure 3.1 B shows (…)	Figure 3.1 B and C shows (…)
84	(…) activity of renin was significantly inhibited (Figure 3.8 A) (…)	(…) activity of renin was significantly inhibited (Figure 3.8 B) (…)
84	(…) but no changes were observed in its expression levels (Figure 3.8 B and C).	(…) but no changes were observed in its expression levels (Figure 3.8 A).
84	(…) in RPE cells (Figure 3.8 D).	(…) in RPE cells (Figure 3.8 C).
90; Figure 3.12	Empty vector	pEPito-hCMV-GFP
92	(…) increase observed by pEPito-hCMV-GFP (Figure 3.14 B).	(…) increase observed by pEPito-hCMV-GFP (Figure 3.14 C).
92	(…) overexpressing ACE2, compared to empty plasmid (Figure 3.14 C).	(…) overexpressing ACE2, compared to empty plasmid (Figure 3.14 D).
106, Figure 4.8 A		Falta Pyr-sulf 4h+TNFa
126	(…) Pyr-sulf in these conditions (Figure 5.6 A and C).	(…) Pyr-sulf in these conditions (Figure 5.6 A and C), although there is a tendencey for treatment with Pyr-sulf decrease the expression of GLUT-1 in cells under high glucose and hypoxia compared to control cells.
128	(…) without significantly changing the expression of TNF-α in the cell membrane.	(…) without significantly changing the expression of GLUT-1 in the cell membrane.
129	(…) levels of VEGF-A and PEDF in neural retina and RPE cells (…)	(…) levels of VEGF-A and PEDF in neural retina and RPE (…)