Brigite Ladeira Cabrita

Degree in Biology

Characterization and modulation of the Renin-Angiotensin System in Diabetic Retinopathy

Dissertation for obtention of the Master's degree

in Molecular Genetics and Biomedicine

Supervisor: Professor Gabriela A. Silva, PhD, CEDOC, Nova Medical School

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Abstract

Diabetes *mellitus* is a chronic disease whose numbers of affected individuals are substantially increasing. Diabetes-associated complications include Diabetic Retinopathy (DR), the main cause of visual impairment and blindness. DR is a progressive pathology affecting the retina, caused by chronic hyperglycaemia. From the several associated risk factors, hypertension has been considered a key player in DR. Renin-Angiotensin System (RAS) is the hormonal cascade responsible for the control of blood pressure. This system can be divided in two main axes: a deleterious one that promotes vasoconstriction, inflammation, angiogenesis and increased oxidative stress and a parallel axis known to counterbalance these effects. Both axes' components have been identified in the eye and we have shown that the prejudicial axis is modulated by glucose.

In this work we aimed to characterize the expression of the RAS protective axis in retinal pigment epithelium (RPE) cells and evaluate the effects of its modulation by glucose and direct renin inhibition. The expression of Angiotensin-converting enzyme 2, Angiotensin (1-7) and Mas1 were detected in RPE cells. A decrease in the expression of these components was observed in high glucose conditions, showing a RAS imbalance. These results are supported by the results obtained in the retina of a type 1 diabetic animal, the $Ins2^{Akita}$ mouse.

RAS dysregulation triggers DR hallmarks such as oxidative stress and angiogenesis. Thus, RAS blockade can affect DR development and progression. When simulating RAS overactivation, expression of the protective components increased over time, pointing to an initial protective role that needs further investigation. When treating cells with a renin-blocker, aliskiren, decreased levels of Angiotensin-converting enzyme 2 and Mas1 were observed, probably caused by the non-formation of angiotensin peptides. Overall our results are indicative of an early effect of the protective arm of RAS in the glucose-induced RAS dysregulation.

Keywords: Diabetic Retinopathy, Renin-Angiotensin System, Angiotensin-converting enzyme 2, Angiotensin (1-7), Mas1, Aliskiren

Resumo

A Diabetes *mellitus* (diabetes) é uma doença crónica cujo número de indivíduos afetados tem aumentado substancialmente. As complicações associadas à diabetes incluem a Retinopatia Diabética (RD), que é a principal causa de deficiência visual e perda de visão. A RD é uma patologia progressiva que afeta a retina, sendo causada por hiperglicemia crónica. Dos vários fatores de risco associados, a hipertensão têm-se destacado como um fator crucial no desenvolvimento da RD. O sistema da Renina-Angiotensina é o sistema hormonal responsável pelo controlo da pressão arterial. Podem destacar-se dois eixos principais: um, prejudicial, que promove vasoconstrição, inflamação, angiogénese e aumento de stress oxidativo e outro eixo paralelo conhecido por contrabalançar estes efeitos. Os componentes de ambos os eixos já foram identificados no olho e já demonstramos que os componentes do eixo prejudicial são modulados pela glucose.

O objetivo deste estudo consiste em caracterizar a expressão do eixo protetor do sistema Renina-Angiotensina em células do epitélio pigmentado da retina (EPR) e avaliar os efeitos da sua modulação pela glucose e pela inibição direta da renina. A expressão da enzima conversora da angiotensina 2, angiotensina (1-7) e Mas1 foi detetada nas células EPR. Foi observada uma diminuição destes componentes em condições de elevada glucose, indicando um desequilíbrio do sistema da renina-angiotensina. Estes resultados são corroborados pelos resultados obtidos de retinas do modelo animal de diabetes do tipo 1, o murganho $Ins2^{Akita}$.

A desregulação deste sistema desencadeia processos de stress oxidativo e angiogénese, característicos de RD. Assim, o bloqueio do sistema da renina-angiotensina poderá afetar o desenvolvimento e progressão da RD. Ao simular a sobre ativação do sistema da renina-angiotensina, a expressão dos componentes protetores tendeu a aumentar com o tempo, possivelmente devido a um papel inicial de proteção. Ao tratar as células com um inibidor da renina, Aliscireno, verificou-se uma tendência de diminuição da expressão da enzima conversora da angiotensina e Mas1 em fases iniciais, possivelmente devido à ausência de formação dos péptidos da angiotensina. Os nossos resultados são indicativos de um desequilíbrio deste sistema induzido pela glucose, apesar do aparente papel protetor inicial do eixo protetor.

Palavras-chave: Retinopatia Diabética, Sistema Renina-Angiotensina, Enzima conversora da angiotensina 2, Angiotensina (1-7), Mas1, Aliscireno

Contents

List of Figures

Abbreviations

ACE – Angiotensin-converting enzyme

ACE2 – Angiotensin-converting enzyme 2

ACEi – Angiotensin-converting enzyme Inhibitors

AMD – Age-related Macular Degeneration

Ang II – Angiotensin II

Ang (1-7) – Angiotensin 1-7

ARBs – Angiotensin II type 1 receptor blockers

AT1R – Angiotensin II type 1 receptor

AT2R – Angiotensin II type 2 receptor

BRB – Blood-Retinal Barrier

BSA – Bovine Serum Albumin

CT – Control Reaction

DME – Diabetic Macular Oedema

DMEM – Dulbecco's Modified Eagle's Medium

DR - Diabetic Retinopathy

EDTA - Ethylenediamine tetra acetic acid

ELISA - Enzyme-linked Immunosorbent Assay

FBS – Foetal Bovine Serum

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDR – Proliferative Diabetic Retinopathy

PRR – (Pro)Renin Receptor

RAS – Renin-Angiotensin System

RIPA – Radioimmunoprecipitation Assay

ROS – Reactive Oxygen Species

RPE – Retinal Pigment Epithelium

SEM – Standart Error of the Mean

SD – Standart Deviation

TBS-T – Tris-buffered Saline with Tween

VEGF – Vascular Endothelial Growth Factor

Chapter 1. Introduction

1.1. Diabetes and Diabetic Retinopathy

Diabetes *mellitus* (diabetes) is a condition of constant elevated blood glucose levels and it presents two typologies: Diabetes type 1, where people affected are insulin dependent and diabetes type 2 where people affected have an ineffective use of the insulin the body produces. This chronic disease was estimated to affect a total of 422 million people globally in 2014 (WHO, 2016), and is expected to affect 429 million people by 2030 (Wild, Roglic, Green, Sicree, & King, 2004). The prevalence of this disease has substantially increased (WHO, 2016), along with the life expectancy of the people affected by diabetes, raising concerns over the diabetes-associated complication (Santiago, Boia, Aires, Ambrósio, & Fernandes, 2018).

Uncontrolled diabetes can lead to several long-term complications. The most common microvascular complication of diabetes is diabetic retinopathy (DR) (Hammes, 2013; Nentwich, 2015), being the main cause of the visual impairment and/or blindness of the people affected and in a working age range (Fletcher, Phipps, Ward, Vessey, & Wilkinson-Berka, 2010; Klein, Klein, Moss, Davis, & Demets, 1984a, 1984b). DR is characterized as a progressive pathology affecting the retina, established due to chronic hyperglycaemia, though the exact mechanism by which it develops is not yet fully understood (Sjølie, Dodson, & Hobbs, 2011). With the increasing incidence and life expectancy of diabetic patients, the higher is the risk of developing DR (Fletcher et al., 2010; Garcia, 2014; Nentwich, 2015).

There are several risk factors associated with DR development, being most related to metabolic alterations. An easily associated risk factor is hyperglycaemia. It has been shown and will be approached in more detail in the following sections, that a tight control of blood glucose levels can delay the establishment or even reduce the progression of DR and other diabetes complications (Clinical Trial DCCT, 1993). However, complications persist and a considerable percentage of diabetic patients still develop and progress through the disease stages (Antonetti, Klein, & Gardner, 2012). Dyslipidaemia, hypertension and chronic inflammation are other associated risk factors. (Choudhary, Kapoor, Singh, & Bodakhe, 2017; Duh, Sun, & Stitt, 2017; Fletcher et al., 2010; Lee, Wong, & Sabanayagam, 2015; Santiago et al., 2018; Sjølie et al., 2011; van Leiden et al., 2002). All have their contribution to the establishment and progression of DR. Yet, hypertension has been recently highlighted in research as a key player (Duh et al., 2017; Fletcher et al., 2010; Turner, Matthews, Neil, & Mcelroy, 1998; Yau et al., 2012), and its regulation system will be approached in a following section.

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 transportation to the inside of the cell. However, endothelial, mesangial and neuronal cells cannot reduce this transportation effectively and are affected by the excess of glucose (Brownlee, 2005). Though hyperglycaemia is systemic, cellular mechanisms are involved in the development of diabetic complications.

DR affects the retina, a complex set of layered tissue, that presents its own set of blood vessels, responsible for oxygen and nutrient supply. The endothelial layer lining the inner retinal blood vessels acts as a selective barrier, important for the maintenance of retinal function. Also, as part of this selective barrier is the Retinal Pigment Epithelium (RPE), that is located in-between the photoreceptors and the choriocapillaris (Garcia, 2014) (Figure 1.1). 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 (Antonetti et al., 2012; Lechner, O'Leary, & Stitt, 2017).

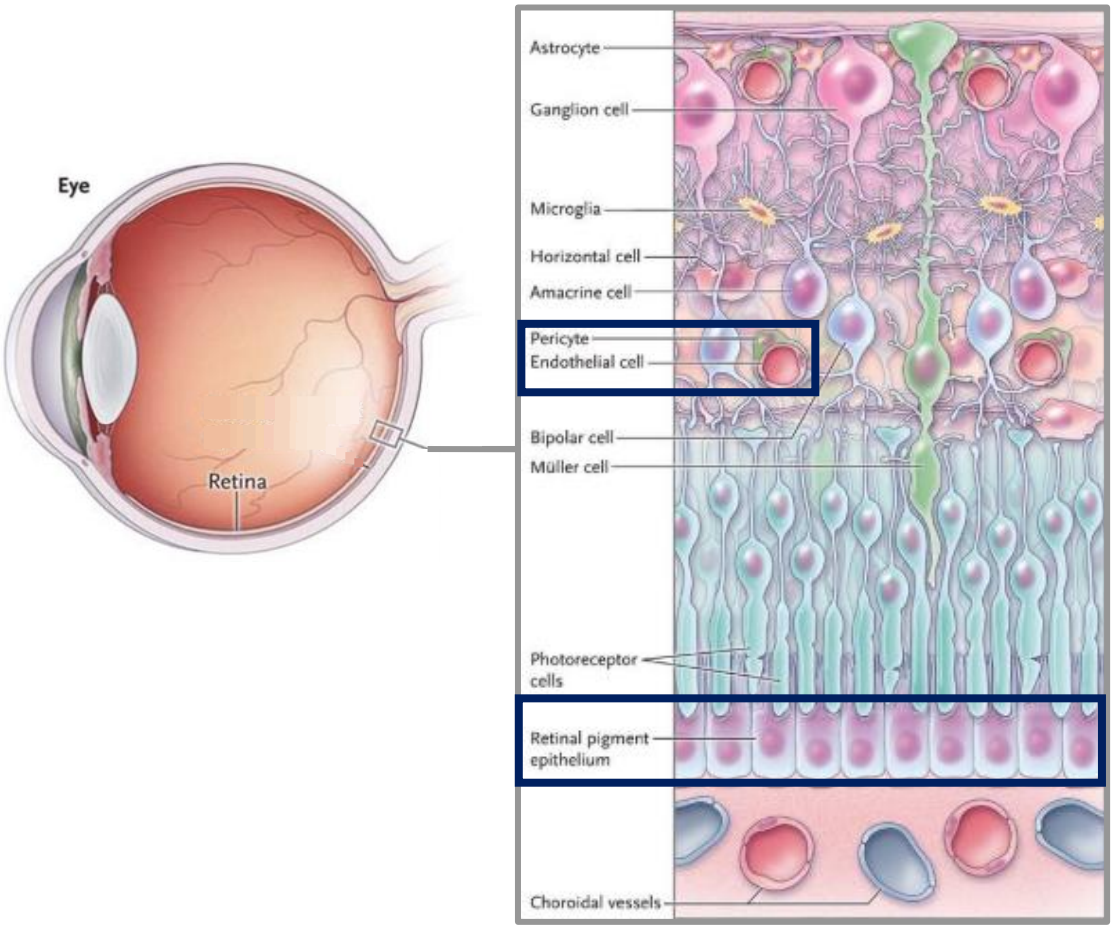

Figure 1.1 – Schematic representation of the retina and vascular beds. The retina is a complex set of layered tissue. It presents two vascular beds that constantly nourish the tissue and serve as a selective barrier. The inner blood-retinal barrier (BRB) is maintained by the tight interaction between pericytes and vascular endothelial cells while the outer BRB e assured by the Retinal Pigment Epithelium (RPE). Both these BRB elements are highlighted by blue boxes. Adapted from Antonetti et al., 2012.

The high glucose levels in the endothelial cells lead to several metabolic alterations, responsible for an increase in the production of reactive oxygen species (ROS) (Brownlee, 2005). This increase in oxidative stress induces a low and continuous inflammatory response. The prolonged altered ROS production and chronic inflammation lead to cellular dysfunction and altered cell-to-cell communication between endothelial cells and pericytes as well as to pericyte loss, a DR feature, followed by endothelial cell death (Antonetti et al., 2012; Frank, 2004; Santiago et al., 2018). Consequently, areas of non-perfusion and tissue hypoxia are developed (Fletcher et al., 2010) and the blood-retinal barrier (BRB) starts losing its integrity, resulting in fluid leakage into the retina (Santiago et al., 2018). RPE is also

affected by diabetic conditions. Just like in the inner retinal vessels, metabolic alterations lead to RPE dysfunction and BRB breakdown, occurring fluid leakage from the choriocapillaris, originating oedema (Lechner et al., 2017; Simó, Villarroel, Corraliza, Hernández, & Garcia-Ramírez, 2010). Still, the underlying mechanism of RPE dysfunction is not entirely known but oxidative stress, inflammation and angiogenesis seem to be involved (Simão, Santos, & Silva, 2017; Simó et al., 2010).

The prolonged ischemia in the retina added to the excess oxidative stress and chronic inflammation induce the expression of several cytokines and growth factors in order to try and restore the supply of oxygen and nutrients to the retinal tissue. Moreover, these factors will increase vascular and BRB permeability, establishing a favourable environment for neovascularization (Fletcher et al., 2010; Frank, 2004; Patz, 1982; Santiago et al., 2018). Among these growth factors, one is particularly known to have its expression increased in DR, being attributed a pivotal role in the development of this vascular pathology. The vascular endothelial growth factor (VEGF) promotes mitogenic effects on endothelial cells (Frank, 2004), that are enhanced due to the loss of pericytes, since this loss leads to dysregulation of endothelial cells, allowing uncontrolled proliferation (Orlidge, 1987; Santiago et al., 2018). Neovascularization is one of the most severe hallmarks of DR. In the most severe cases, the newly formed blood vessels can reach the vitreous cavity and generate vitreal haemorrhage leading to vision loss (Frank, 2004).

The diagnostic of DR is performed based on retinal microvascular alterations. However, by analysing what is known so far from the underlying mechanism in DR development, there are no vascular alterations that can be detected visibly in the initial stages of DR development. Thus, DR is defined as asymptomatic in its earlier stages. However, patients can show reduced blood flow, indicative of retinal vessel alterations, but still are considered retinopathy-free until the appearance of the first visible clinical features (Lechner et al., 2017).

By evaluation of microvascular alterations present and detectable on the retina, DR is broadly categorized in non-proliferative diabetic retinopathy and proliferative diabetic retinopathy (PDR). In non-proliferative diabetic retinopathy, the weakened blood vessels form microaneurysms, venous beading (dilation of blood vessels) and intra-retinal haemorrhages (Figure 1.2). PDR is defined by the presence of neovascularization (Duh et al., 2017; Garcia, 2014; Santiago et al., 2018; Wu, Fernandez-Loaiza, Sauma, Hernandez-Bogantes, & Masis, 2013). Additionally, lesions related with BRB damage can be detected by the presence of hard exudates (leakage of lipoproteins) and cotton-wool spots (resulting from the ischemic changes) in the retina (Antonetti et al., 2012; Duh et al., 2017; Nentwich, 2015) (Figure 1.2).

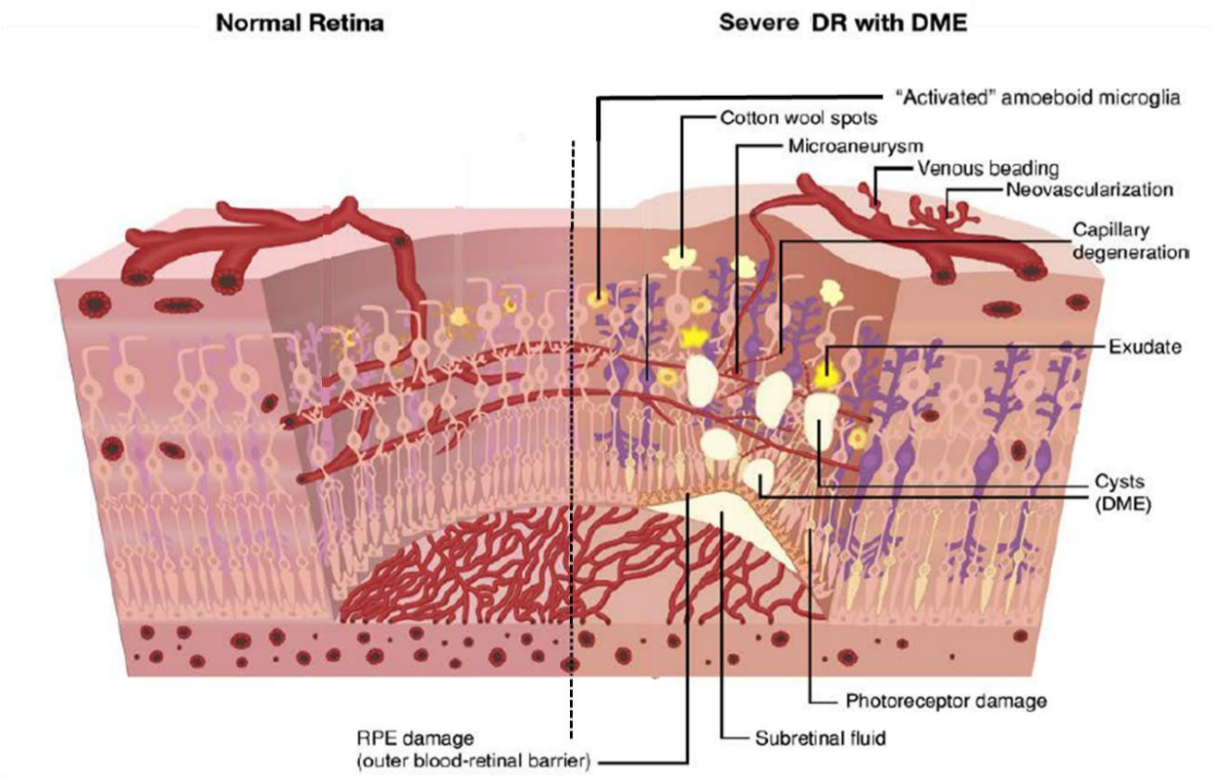

Figure 1.2 – Retinal and vascular alterations of Diabetic Retinopathy. Multiple vascular abnormalities are present in the diabetic retina, from venous beading to neovascularization. The first clinical sing of DR are microaneurysms and venous beading. As the disease progresses the BRB loses its integrity and fluid leaks into the retina. After reabsorption the lipoproteins in the leaked fluid form hard exudates. In the more advanced stages of DR, neovascularization is the diagnosing feature. Adapted from Duh et al., 2017.

It is acknowledged that the aggravated neovascularization is one of the reasons individuals with DR lose or have visual impairment. However, one other lesion plays a major role in visual impairment: Diabetic Macular Oedema (DMO or DME). This is a consequence of the BRB breakdown and accumulation in the retina of the leaked fluids, leading to retinal thickening and in more severe cases retinal detachment. The reabsorption of these leaked fluids generates the hard exudates. DME is commonly associated with later stages of DR but can be developed at any stage of DR (Antonetti et al., 2012; Duh et al., 2017; Frank, 2004; Klaassen, Van Noorden, & Schlingemann, 2013; Santiago et al., 2018).

DR treatment is designed to act on later stages of this pathology. The main treatment options are laser photocoagulation, intravitreous injections and vitrectomy (Duh et al., 2017). Photocoagulation is the most common form of treatment for neovascularization in order to slow vision loss caused by PDR and DME (Antonetti et al., 2012; Fletcher et al., 2010). Though it can significantly reduce the severe visual loss, sometimes patient's vision is not restored or improved and is itself a destructive technique (Duh et al., 2017; Fletcher et al., 2010; Frank, 2004; Santiago et al., 2018). Additionally, this treatment is often ineffective on patient with DME (Schmidt-Erfurth et al., 2017). Since VEGF is a key player in BRB breakdown and neovascularization, anti-VEGF injections have been used and proven to be quite

effective to treat neovascularization, even when used in patients presenting DME (Duh et al., 2017). Although this treatment can improve patients' visual acuity, it requires a tight follow-up regime and repetition of injections (Duh et al., 2017). In addition to anti-VEGF injections there are treatments with corticosteroids as an alternative to be used on DR patients with DME that do not respond to anti-VEGF treatment (Santiago et al., 2018; Schmidt-Erfurth et al., 2017). Lastly, vitrectomy is performed in severe cases of neovascularization, mainly in patients with PDR (Duh et al., 2017; Nentwich, 2015; Santiago et al., 2018).

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 a treatment that either has 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). Cumulating evidence shows that RAS is an intricate system of pathways, involving a variety of enzymes and peptides (White et al., 2015). Of all the pathways known to date, our interest falls over two main axes. The first is the classically known Angiotensin-converting enzyme (ACE)/Angiotensin II (Ang II)/Angiotensin II type1 receptor (AT1R) axis, also designated as the prejudicial axis and the second is the Angiotensin-converting enzyme 2 (ACE2)/Angiotensin (1-7) (Ang (1-7))/Mas1 axis, also known as the protective axis, which will be explained in the next section.

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 (Keidar, Kaplan, & Gamliel-Lazarovich, 2007; White et al., 2015). However, it is the substrate of the ACE, transforming it in Ang II, the main effector. This peptide has two known receptors: AT1R and Angiotensin II type 2 receptor (AT2R) (Simões e Silva, Silveira, Ferreira, & Teixeira, 2013; Touyz & Berry, 2002) (Figure 1.3). Each receptor mediates opposing effects of each other. 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 (Mehta & Griendling, 2007; Simões e Silva et al., 2013; Verma et al., 2012; White et al., 2015). In literature, it is indicated that when Ang II interacts with the AT2R, opposing effects to the ones mediated by AT1R are promoted. 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, (Chung, Kühl, Stoll, & Unger, 1998; Senanayake et al., 2007). Some reports have showed 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 (Choudhary et al., 2017; Chung et al., 1998; Keidar et al., 2007; Levy, 2005). 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 (Miura, Matsuo, Kyia, Karnik, & Saku, 2010; Prasad, Verma, & Li, 2014).

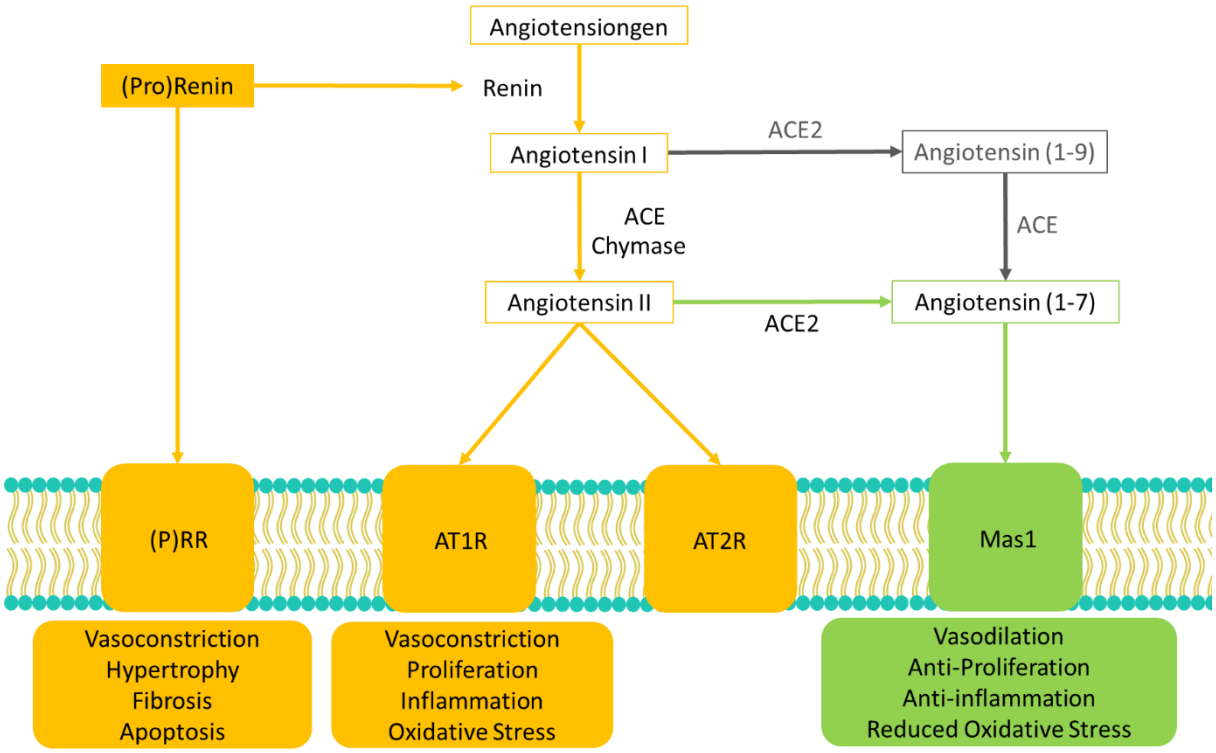

Figure 1.3 – Simplified scheme of RAS. Currently, there more known enzymes and peptides associated with this cascade, being a more complete schematic found Holappa et al., 2017 with the known components up to the date of publication. As it is represented, the same enzymes can act on different peptides and different enzymes can on the same substrate, as the case of Chymase and ACE. Nevertheless, the preferred followed pathway is the one where enzymes have more affinity towards their substrates. In this scheme are highlighted in colour the main axis. The prejudicial ACE/Ang II/AT1R axis in yellow and the protective ACE2/Ang (1-7)/Mas1 axis in green. In yellow is also the axis that induces prejudicial effects in an Ang II independent manner.

Although this is the commonly known prejudicial axis, being an intricate system there are other pathways that exert the same prejudicial effects. As highlighted in Figure 1.3, there is a pathway involving renin but not involving the formation of Ang II to induce effects such as vasoconstriction. In the kidney, renin is produced mainly in its inactive form, prorenin (Nguyen et al., 2002; Ribeiro-Oliveira et al., 2008). Prorenin presents an extra peptide chain that covers the catalytic site of the enzyme. To be in its active form, proteolytic activation occurs and the prosegment is cleaved, originating renin in its mature form (Alcazar, Cousins, Striker, & Marin-Castano, 2009; Guang, Phillips, Jiang, & Milani, 2012; Holappa, Vapaatalo, & Vaajanen, 2017). However, both forms, prorenin and renin, can bind the (pro)renin receptor (PRR) and induce vasoconstriction (Holappa et al., 2017; Nguyen et al., 2002).

Hypertension is the most associated pathology to alterations of RAS, namely its overactivation and mediation by the AT1 receptor. Still, RAS overactivation can be associated with other pathologies. In cardiovascular alterations such as heart failure, Ang II levels are elevated (Keidar et al., 2007; Serneri et al., 2001), along with increased expression of ACE (Danser et al., 1997; Serneri et al., 2001). In addition, levels of prorenin and renin are also elevated in heart failure (Danser et al., 1997; Serneri et

al., 2001). Dysregulation of RAS can also lead to kidney injuries. In hypertensive nephropathy, there is a strong increase in the expression of ACE in kidney structures (Koka et al., 2008). Alterations of this hormonal system have also been implicated in ocular diseases other than DR, such as age-related macular degeneration (AMD) and glaucoma. For both pathologies, RAS overactivation has also been tightly linked to the development or progression of the diseases (Alcazar et al., 2009; Holappa et al., 2017). In AMD, RPE is also compromised and as progression occurs, neovascularization in the choroidal vessel can arise (Garcia, 2014). In this progression Ang II, when signalling is mediated by AT1R, and prorenin have been identified as key players (Alcazar et al., 2009; Choudhary et al., 2017). In glaucoma, a crucial pathological player is intraocular pressure, which is increased in disease (Giese & Speth, 2014; Holappa et al., 2017). From studies performed, RAS has been attributed a role in the regulation of intraocular pressure through the mediation of aqueous humour formation and clearance (Holappa et al., 2017).

It has been shown that RAS can be modulated by glucose levels. Diabetic patients show elevated levels of (pro)renin and renin (Fletcher et al., 2010; Luetscher, Kraemer, Wilson, Schwartz, & Bryer-Ash, 1985). 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 (Fletcher et al., 2010; Otani, Takagi, Suzuma, & Honda, 1998), has a role in BRB breakdown, inducing RPE damage (Pons, Cousins, Alcazar, Striker, & Marin-Castaño, 2011; Simão, Santos, & Silva, 2016), and can increase vascular permeability by inducing the expression of chemokines and recruiting inflammatory cells (White et al., 2015).

Knowing that hypertension is a risk factor in DR development and seeing an apparent involvement of this system in developing DR hallmarks such as BRB breakdown and increased vascular permeability, one of the first approaches of treatment is the inhibition of RAS, which will be explored in detail in the last section of this chapter.

1.3. Protective Axis

In the previous section, we described that Ang II is commonly considered as the main prejudicial effector of the RAS and that AT1R mediates its effects. A second axis of this system, ACE2/Ang (1-7)/Mas1, was mentioned and designated as the protective axis (Figure 1.3).

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) (Campagnole-Santos et al., 1989; Schiavone, Santos, Brosnihan, Khosla, & Ferrario, 1988; Simões e Silva et al., 2013). In helping establishing this heptapeptide as a RAS effector, an enzyme homologous to ACE was identified and described: the ACE2 (Donoghue et al., 2000; Simões e Silva et al., 2013; Tipnis et al., 2000). In addition, a functional receptor for the active heptapeptide was discovered: the Mas1 receptor (R. A. S. Santos et al., 2003; Simões e Silva et al., 2013). Upon the interaction between Ang (1-7) and Mas1 effects such as vasodilation, cell growth and proliferation are inhibited and ROS production is decreased (Moon et al., 2011; Pernomian, Pernomian, & Restini, 2014; Simões e Silva et al., 2013). This ACE2/Ang

(1-7)/Mas1 axis is then able to counterbalance the deleterious effects of the ACE/Ang II/AT1R axis, becoming of valuable therapeutical target. Of upmost importance in its therapeutical value is the formation of Ang (1-7). This peptide can induce beneficial effects itself, but also its formation is at the expense of Ang II, since this latter peptide is the substrate used by ACE2. In general, the benefits come from eliminating the prejudicial effector (Hernandez Prada et al., 2008; Simões e Silva et al., 2013). ACE2 has a high affinity for Ang II, converting it quite efficiently to Ang (1-7) (Ferrario, Ahmad, Joyner, & Varagic, 2010; Simões e Silva et al., 2013; Vickers et al., 2002), leaving ACE2 to be considered as the Ang (1-7) forming enzyme and a regulator of the effects of both axes of RAS (Donoghue et al., 2000; Gallagher, Ferrario, & Tallant, 2008a; Karnik, Singh, Tirupula, & Unal, 2017; Simões e Silva et al., 2013; Tipnis et al., 2000). Despite this pathway being efficient and some Ang II may be always converted in Ang (1-7), the rate of this conversion is slower than the rate of interaction between Ang II and the AT1 receptor (Keidar et al., 2007; Reudelhuber, 2006). In Figure 1.3 is also represented alternative pathways to obtain Ang (1-7). One of the most known is through the action of ACE2 over Ang I. Though not as efficiently, ACE2 can cleave Ang I, forming Angiotensin (1-9), which is subsequently cleaved by ACE, producing Ang (1-7) (Simões e Silva et al., 2013; Vickers et al., 2002).

Apart from the direct effects induced by ligation of Ang (1-7) to Mas1, it is suspected that the Mas1 receptor can interact with the angiotensin receptors in a similar way to the dimerization between AT1R and AT2R (Keidar et al., 2007; Kostenis et al., 2005; Miura et al., 2010; Prasad et al., 2014). Reports show that hetero-oligomerization of Mas1 with AT1R resulted in altered signalling of the AT1 receptor, with impact in its response to Ang II (Ferrario, 2011; Karnik et al., 2017; Kostenis et al., 2005). It is even reported that the interaction of Mas1 with other receptors may be necessary in order of Ang (1-7) 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 (Cerrato, Carretero, Janic, Grecco, & Gironacci, 2016; Karnik et al., 2017).

To show how this axis has a protective role, studies have been made in order to see effects of the altered expression of the protective components. The kidney is the target of several of these studies, just as it happens for the effects of the ACE/Ang II/AT1R axis. In the kidney Ang(1-7) is able to induce its known vasodilation effects on renal arterioles, having Mas1 mediating these effects (Dharmani, Mustafa, Achike, & Sim, 2007; Ferrario et al., 2010). In turn, inhibition of the ACE2 enzyme leads to an increase in Ang II in the kidney tissue while avoiding the formation and beneficial effects of Ang (1-7) (Burgelova et al., 2009; Ferrario et al., 2010). It has been shown that in chronic kidney disease, the higher expression of ACE2 has protective effects (Dilauro, Zimpelmann, Robertson, Genest, & Burns, 2010; Ferrario et al., 2010). In acute kidney injury the activity of ACE2 was shown to be decreased while the inhibition of ACE lead to an improvement in ACE2 activity (Ferrario et al., 2010; Velskoska, Dean, Burchill, Levidiotis, & Burrell, 2010)

When it comes to cardiovascular pathologies or complications such as heart failure, it has been shown that after the injury, there is an increase on the expression of ACE2, consequently increasing Ang (1-7) and its credibility as a protective effector (Gallagher, Ferrario, & Tallant, 2008b). In atherosclerosis, the enhancement of the systemic expression of the protective components ACE2 and

Ang (1-7) is thought to decreases the pathological progress, possibly due to the antioxidative and anti-inflammatory effects of the heptapeptide (Karnik et al., 2017). Additionally, it has been shown in detail as well that ACE2 enzyme is quite important in cardiac function. In animal models, disruption or ablation of ACE2 severely impairs cardiac function as on the other hand overexpression of the same enzyme improves cardiac function and protects from Ang II dependent cardiac hypertrophy and fibrosis and prevented hypertensive pathology in the heart. The complete deletion of the *ace2* gene has been shown to completely abolish cardiac function (Crackower, Sarao, Oliveira-dos-Santos, Da Costa, & Zhang, 2002; Gallagher et al., 2008a, 2008b; Huentelman et al., 2005; Keidar et al., 2007). The loss of ACE2 was also associated to increased circulating Ang II levels and in tissue (Gallagher et al., 2008b).

Considering the diabetic condition, in animal models for diabetes, an upregulation of ACE2 and Mas1 is linked to renoprotective effects, suggesting that, in diabetes, this axis can be used as a therapeutical target for renal damage (Karnik et al., 2017). Furthermore, Ang (1-7) administration prevented cardiac dysfunction in *db/db* mice (Karnik et al., 2017; Papinska, Soto, Meeks, & Rodgers, 2016). Also, in animal models for type 2 diabetes it has been shown that Ang (1-7) can reverse hyperglycaemia and its consequences (Karnik et al., 2017; S. H. S. Santos et al., 2014). In ACE2 knockout animal models, Ang (1-7) was shown to improve insulin resistance and oxidative stress (Cao, Yang, Xin, Xie, & Yang, 2014; Karnik et al., 2017). Widening the scope of existing evidence, in people affected by diabetes, 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 (Ferrario, 2011).

Literature also shows evidence supporting the enhancement of this RAS protective “arm” for treatment of conditions with chronic inflammation and proliferative stages (Simões e Silva et al., 2013), which are known DR hallmarks. Experimental disease models have shown that Ang (1-7)/Mas1 signalling is able to supress Ang II induced effects. In endothelial cells in the brain, Ang II promoted ROS production was supressed by the activation of Mas1 receptor by Ang (1-7) (Karnik et al., 2017; Xiao et al., 2015). As mentioned above, it is thought that crosstalk between receptors of RAS may have a role in the mediation of their effects. Heterodimerization of Mas1 with B2 receptor, a bradykinin receptor , leads to a potentiation of the effects of the agonistic ligand (Cerrato et al., 2016), inducing vasorelaxation of microvessels in mice (Karnik et al., 2017).

To sum up, the ACE2/Ang (1-7)/Mas1 axis is of therapeutical value for counterbalancing the prejudicial effects. Though some benefits of RAS inhibition may be due to the increased expression of protective components, elaborated in the last section, the modulation of RAS by overexpressing protective components like ACE2 or Ang (1-7) is an approach to consider. Studies where lentiviral delivery of ACE2 is performed have shown beneficial effects and that the manipulation of ACE2 is of therapeutical value (Huentelman et al., 2005; Verma et al., 2012). Additionally, the Mas1 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 (Prasad et al., 2014). Therefore, an overexpression of Ang (1-7)’s receptor should also be considered and further evaluated.

1.4. Tissue RAS

When RAS was firstly described it was thought that it only exerted systemic effects but currently it is known that components of RAS are expressed in tissues like endothelial and organs such as brain (White et al., 2015), heart (Ribeiro-Oliveira et al., 2008), kidney (Ribeiro-Oliveira et al., 2008) and the eye (Danser et al., 1989; Giese & Speth, 2014; Wagner et al., 1996; White et al., 2015), being directly involved in the function of these organs (Giese & Speth, 2014). From all these organs, the eye has been studied to find the expression of RAS components and their involvement in ocular physiology and/or pathophysiology. Ocular RAS has been characterized across a variety of species, including human. In the human eye, Wagner et al. showed that RAS' components were expressed by the neural retina, RPE and choroid, among other ocular structures. The presence of renin, angiotensinogen as well as ACE was detected, showing a localised synthesis of these components and supporting the existence of an ocular RAS independent from the systemic RAS (Wagner et al., 1996). Also, from Danser's work, it was highlighted that the eye has its own Ang II production system, 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 (Danser, Derkx, Jaap, Paulus, & Schalekamp, 1994). Additional work is indicative that, although being independent, ocular or other localized RAS can be modulated by the systemic components (Choudhary et al., 2017). 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. One of the changes shown by 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 (Milenkovic et al., 2010), giving an apparent regulating role of ocular RAS to RPE. In a more recent work, White et al. reinforced the knowledge of ocular RAS, demonstrating the expression pattern of RAS' classical components across all structures of the human eye. There is evidence that retinal tissues strongly express these components, being a hallmark for some ocular diseases, since these structures are then able to strongly produce angiotensin peptides (White et al., 2015).

With the cumulating evidence of the existence of the protective arm of RAS, a characterization of the same arm at an organ and tissue level was also pressing, with the Mas1 receptor and Ang (1-7) detected across the ocular tissues (Vaajanen, Kalesnykas, Vapaatalo, & Uusitalo, 2015). Additionally, the enzyme ACE2 is also expressed in ocular tissues (Holappa, Valjakka, & Vaajanen, 2015). 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. Yet, it is acknowledged that tissue RAS is responsible for the localised effects and long-term changes in organs suggesting a possible role in the pathogenesis of ocular conditions such as (AMD), glaucoma and DR (Danser et al., 1989; Holappa et al., 2017; Kurihara, Ozawa, Ishida, Okano, & Tsubota, 2012).

In terms of cascade 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. For example, in the heart, Chymase is the major enzyme responsible for this conversion

(Figure 1.3) (Keidar et al., 2007; Urata, Kinoshita, Misono, Bumpus, & Husain, 1990). 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 (Garcia, 2014), as well as potentiating inflammation and oxidative stress (Choudhary et al., 2017; Fletcher et al., 2010). Additionally, Ang II is known for having a role in pericyte uncoupling (Fletcher et al., 2010; Garcia, 2014), which is a crucial hallmark, 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 (Fletcher et al., 2010; Giese & Speth, 2014; Nadal, Scicli, Carbini, Nussbaum, & Scicli, 1999). With this uncoupling, the BRB loses its integrity and fluid leaks into the retina, reaching another DR hallmark and one of the main causes of visual impairment of DR as mentioned in the first section.

To help understand the role of tissue RAS in physiology and pathophysiology, several studies have aimed to RAS alterations in the scope of the organs abovementioned. In previous sections it was possible to realise that most studies of RAS alterations are already performed targeting the affected organs. For example, in hypertensive and diabetic nephropathy, the kidney is the study target. Likewise, in cardiovascular diseases, cardiac cell types are the study object. However, for ocular alterations of RAS it is assumed that the same alterations are observed in other organs. In diabetic or hypertensive conditions, in the heart and kidney are observed alterations of ACE2 expression, normally a decrease in its expression. By association it is assumed that in the diabetic retina, there is also a decrease in the expression of ACE2 (Fletcher et al., 2010). Supporting this decrease, there is evidence that the deletion of the ACE2 encoding gene leads to an increased production of Ang II in tissue (Crackower et al., 2002; Wong et al., 2007). In cardiac cells, when exposed to Ang II, there is a reduced expression of ACE2 (Gallagher et al., 2008a). Anyhow, studies have been made involving ocular tissues. In samples of diabetic patients, classical RAS components such as AT1R and renin are elevated in ocular tissues (Danser et al., 1994, 1989; Dominguez et al., 2016). Further supporting RAS alterations in ocular pathologies, in patients with DR, levels of prorenin, renin and Ang II are elevated in vitreous samples along with elevated levels of VEGF (angiogenic factor) (Choudhary et al., 2017; Danser et al., 1989; Fletcher et al., 2010). In addition, Mas1 receptor has been described to be strongly expressed in retinal blood vessels, mediating the vasodilatory effects of Ang (1-7) (Prasad et al., 2014). In studies using RPE cells it has been shown that hyperglycaemia lead to a higher expression of PRR and renin, and activation of RAS lead to an increase of the expression levels of VEGF and an increased production of ROS (Simão et al., 2017).

It is possible to see that several studies have been performed in order to better characterize ocular and other local RAS systems. The balance between both axes under analysis here plays a role in maintaining the function of several organs and the imbalance is responsible for the long-term injuries. Studies aiming for 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.

1.5. Inhibition of the Renin-Angiotensin System

The major risk factors in DR establishment are hyperglycaemia and hypertension, which can be modifiable. Several clinical trials, such as the UKPS and the DCCT(DCCT, 1993; UKPDS, 1998), show 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 to UKPDS , where a tighter blood glucose is aimed combined with a tight blood pressure control, like the ACCORD and ADVANCE studies, showed that a tight glycaemic 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 (Beulens et al., 2009; The ACCORD Study Group and ACCORD Eye Study Group, 2010). Still, lowering blood pressure in hypertensive diabetic patient leads to a reduced risk of developing macro and microvascular complications (Antonetti et al., 2012; Sjølie et al., 2011). 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 hypoglycaemia may arise. Notwithstanding, since there is evidence that RAS is overactive in DR, there is an interest in the effects of RAS blockade in DR development.

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 an antagonist for Ang II, and inhibit its interaction with the AT1R, inhibiting the mediation of its prejudicial effects (Holappa et al., 2017). These drugs have long been used as therapy, although these drugs are not always effective in decreasing the levels of Ang II, which is the case of ARBs. By blocking the receptor, the local and circulating levels of Ang II increase. With the knowledge of the protective arm, it is acknowledged that the therapeutical effects of these drugs may come also from the metabolite of Ang II, Ang (1-7) (Gallagher et al., 2008a; Iyer, Ferrario, & Chappell, 1998; Karnik et al., 2017; Keidar et al., 2007). Research data indicates that with anti-hypertensive drug administration, levels of ACE2 expression are increased, along with the increase of Ang (1-7) levels (Iyer et al., 1998; Keidar et al., 2007; Simões e Silva et al., 2013; Sukumaran et al., 2011).

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 and confer a degree of protection in DR models (Antonetti et al., 2012; Tenkumo et al., 2014), and showing that they can also modulate ocular RAS and are a valuable treatment option in reducing DR development and progression (Choudhary et al., 2017; Fletcher et al., 2010). 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 also by anti-inflammatory and anti-oxidant mechanisms, usually thought be a consequence of the increase in Ang (1-7) levels (Pernomian et al., 2014). Additionally, studies on animal models of diabetes show a prevention of early VEGF increased expression with RAS blockade (Fletcher et al., 2010; Zheng et al., 2009; Zheng, Chen, Xu, Li, & Gu, 2007). 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 favoured, and prejudicial effects induced (Fletcher et al., 2010; Tenkumo et al., 2014). 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 acts on the catalytic center of renin, inhibiting its activity and blocking RAS at its initial and rate limiting step (Rahuel et al., 2000). Experimental studies of reperfusion injury, tissue damage induced by the return of blood supply, demonstrate that aliskiren prevents this type of injury. Though 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 (Tenkumo et al., 2014).

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, due to the fact that only a small percentage of these drugs can cross the BRB (Verma et al., 2012) 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.

1.6. Objective

It is recognised that the dysregulation of ocular RAS is one of the cause of DR hallmarks such as oxidative stress and angiogenesis. As above mentioned, common RAS inhibitors confer some attenuation of these hallmarks. Work performed by our group (Simão et al., 2017) has shown the effects of aliskiren on RAS and DR hallmarks, in a human RPE cell line. Though showing a similar protection to commonly used RAS inhibitors, aliskiren was able to effectively decrease renin activity and consequently, Ang II levels, and alter the distribution of the (pro)renin receptor in RPE cells. Additionally, aliskiren decreased VEGF levels and ROS production, suggesting a role in angiogenesis and oxidative stress in these cell model (Simão et al., 2016, 2017). Despite RAS blockade having shown mild protection on DR development and progression, as mentioned, an interest is raising in modulating the expression of the components of the ACE2/Ang (1-7)/Mas1 axis. This therapeutical approach may be used as complementary to RAS inhibition or as a completely alternative therapy.

In this study we will analyse the expression of ACE2, Ang (1-7) and Mas1 receptor in a human RPE cell line (D407 cells) and their modulation by glucose. In addition, we will evaluate the effects of RAS activation on these protective components in RPE cells as well as the effects of the direct renin inhibition, using aliskiren, on the same components. These results will lead to a better understanding of RAS in

RPE cells and evaluated the potential of aliskiren as a RAS blocker therapy. In addition, the expression of some components of RAS will be evaluated in retinas from a type 1 diabetic animal, the $Ins2^{Akita}$ mouse.

To evaluate the potential therapeutical value of the modulation of RAS by the expression of RAS protective components, we will perform the delivery of a vector expressing the human form of ACE2. This approach may be able to give some insight on the use this method as a DR therapy.

Chapter 2. Material and Methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM) with 25 mM D-glucose and ECL Western Blotting Detection reagents were purchased from GE Healthcare Life Sciences (USA). Culture medium supplements (Penicillin/Streptomycin (PenStrep)), L-glutamine and Foetal Bovine Serum (FBS)), trypsin-EDTA, molecular grade water and TRIzol reagent were purchased from Sigma (USA). Isopropanol, ethanol and chloroform were purchased from Merck Millipore (USA). Protease inhibitor cocktail was purchased from Roche (Germany). Bovine Serum Albumin was purchased from Nzytech (USA). Protein assay dye reagent concentrate and 4x Laemmli sample buffer were purchased from BioRad (USA). RNeasy® Mini kit and QIAquick Gel Extraction kit were purchased from QIAGEN (Germany). cDNA synthesis kit was purchased from Applied Biosystems (USA). Dream Taq enzyme was purchased from Thermo Fisher Scientifics and the 1kb ladder plus from Invitrogen (USA).

Antibodies against Renin (sc-22752), Mas1 (sc-390453), Goat anti-Rabbit (sc-2004) and Goat anti-Mouse (sc-516162), conjugated with Horse Radish Peroxidase were purchased from Santa Cruz Biotechnology (USA). Antibody against (pro)renin receptor (ab40790) was purchased form Abcam (UK). α-Tubulin (T5168, Sigma Aldrich, USA) was used as loading control.

Aliskiren was purchased from Selleckchem (USA) and resuspended in Dimethyl Sulfoxide (Sigma, USA) (working concentration of 6.6 mM). Angiotensin II was purchased from Santa Cruz Biotechnology (USA) and resuspended in deionized water (working concentration of 19.12 mM).

Human Angiotensin (1-7) detection kit (NBP2-69078) and antibody against ACE2 (NBP1-76614) were purchased from Novus Biologicals (USA).

2.2. Cell Culture

In this work we have used a spontaneously transformed human Retinal Pigment Epithelium cell line, D407 cells (Davis et al., 1995), kindly provided by Dr. Jean Bennett (University of Pennsylvania, USA). Cells were cultured in in 25 cm^2 t-flasks (Orange Scientific, Belgium) containing Dulbecco's Modified Eagle's Medium (DMEM) with 25 mM D-glucose, supplemented with 1% Penicillin/Streptomycin, 1% L-Glutamine and 5% foetal bovine serum, and kept in a humidified chamber at 37ºC with 5% CO_2. The medium was changed every 2 days. After reaching confluence (70%-90%), cells were washed with PBS 1x and detached with trypsin-EDTA at 37ºC. After 5 minutes DMEM with 25 mM D-glucose was added to the flask and the final volume centrifugated (1 min, 0.3xg). The pellet was resuspended in the fresh medium and the cells were seeded or maintained in culture.

For the experiments regarding the glucose effects, RPE cells were seeded at a density of $3{,}0x10^5$ cells/well in a 6-well plate (Orange Scientific, Belgium). For each independent experiment, cells were

kept in DMEM with 25 mM D-glucose or in DMEM with 5.5 mM D-glucose (supplemented with 5% FBS and 1% PenStrep) (Figure 2.1). After 24h, protein was extracted as described in the next section.

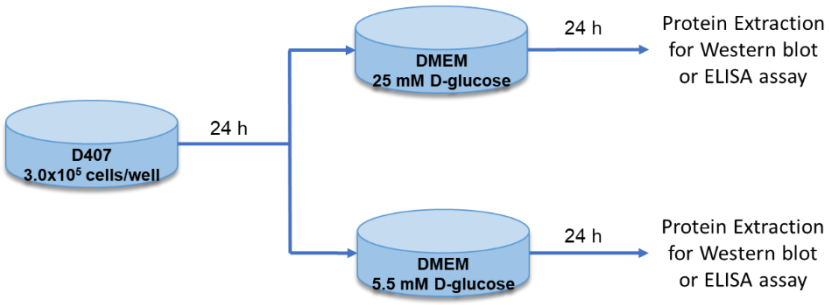

Figure 2.1 – Schematic representation of the experiments regarding glucose effects on RAS.

On the RAS modulation study, D407 cells were cultured in 6-well plates in a density of 3,0x10^5 cells/well. For each experiment, cells were stimulated with Ang II 200 µM (24h and 12h) or Aliskiren 100 µM (24h and 4h) in FBS-free culture medium (Figure 2.2). These time points were chosen based on previous published work from our group (Simão et al., 2016, 2017) where significant alterations were observed on the prejudicial axis of RAS upon the same type of stimuli and incubation periods. After incubation with Ang II or Aliskiren was complete, protein was extracted as described in the next section.

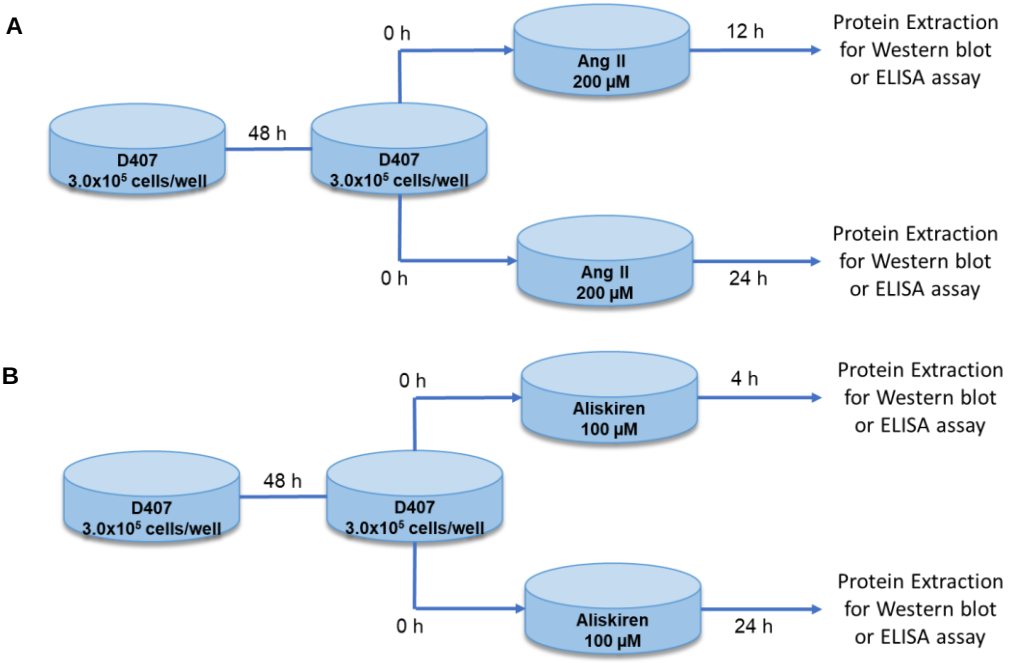

Figure 2.2 – Schematic representation of the experiments of RAS modulation with Ang II (A) or with Aliskiren (B).

2.3. Protein Extraction and Quantification

To obtain cell lysates, the medium from the 6-well plate was discarded, and the cells were washed with cooled PBS 1x. After, 100 µl of lysis solution of radioimmunoprecipitation assay (RIPA) buffer (Tris-HCl 50 mM pH 7.4, 1% NP-40, 0,25% Na-deoxycholate, NaCl 150 mM, EDTA 1 mM) with protease inhibitor cocktail was added. The cells were then scrapped from the well bottom. From here, the whole volume of the remainder suspension from each well was collected to an identified tube and left on ice

for 20 min. At last, the tubes were centrifuge at 16800xg at 4ºC, for 20 minutes, collecting the supernatant and storing it at -20°C.

For the samples used for the detection of Angiotensin 1-7 (Novus Biologicals, USA, NBP2-69078), protein extraction was performed according to recommended protocol. Briefly, the medium from the 6-well plate was discarded, and the cells were washed with cooled PBS 1x. Cells were dissociated with trypsin for about 2 minutes at 37ºC. After adding DMEM with 25 mM D-glucose supplemented with 5% FBS, the suspension was centrifuged for 5 minutes at 1000xg. Cells were resuspended with PBS and counted. For each $1x10^6$ cells, 200µl of PBS 1x were added to wash the cells 3 times. Freeze-Thaw cycles using liquid nitrogen were performed to lyse the cells and a final centrifuging step at 1500xg, for 10 minutes at 4ºC was performed. The supernatant was collected as well but stored at -80°C.

The total protein present in the samples collected was quantified using the Bradford Method (Bradford, 1976). In summary, the Bradford Method is based on the colour shift upon ligation of the Coomassie Brilliant Blue G-250 to the proteins in solution. Upon interaction, the dye colour shifts from red to blue in a very quick process (Bradford, 1976). Our samples were diluted in 1:5 or 1:10 ratios for cell lysates and for retinas, respectively, in deionized water. A Bovine Serum Albumin solution of 1 mg/ml was used to prepare a calibration curve, with sequential 1:2 dilutions. After preparation in a 96-well plate (Orange Scientific, Belgium), the respective volume Protein Assay dye reagent was added and the plate read at 590nm (Biotrak II Plate reader, Amersham Biosciences, Germany).

2.4. Western blot/Immunoblotting

For the characterization of RAS' components, protein samples were diluted with RIPA buffer in 1:5 ratio, and denatured at 95°C for 5 minutes, after adding 1x Laemmli sample buffer, prepared as recommended. Electrophoresis was performed with Sodium Dodecyl Sulphate (SDS) gels, where the separating gel contains 10% or 12% acrylamide. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Sciences, USA) through wet transfer or semi-dry transfer (Trans-Blot Turbo®, BioRad, USA) methods. Blocking was performed as standard protocol with skim milk 5% in TBS-T for 2 hours. The membranes were incubated with primary antibody against Renin (1:250 dilution) and ACE2 (1:2000 dilution) enzymes and for PRR (1:1000 dilution) and Mas1 (1:500 dilution) receptors, diluted in skim milk 5% or BSA 5% in Tris-buffered saline with 0.1% Tween (TBS-T) overnight at 4°C and with the secondary antibody, Goat anti-Rabbit or Goat anti-Mouse HRP conjugated (1:5000 dilutions in skim milk 5% or BSA 5% in TBS-T), for 1h at room temperature, For the loading control, incubations of the primary (α-Tubulin) and secondary antibodies (Goat anti-Mouse HRP conjugated) were performed for 1 hour at room temperature. After each incubation, the membranes were washed 3 times for 5 minutes with TBS-T. The membranes were incubated with ECL Western Blotting Detection reagent or ECL Select Western Blotting Detection reagent and the chemiluminescence was detected using a Chemidoc system (BioRad, USA).

2.5. RNA Extraction

Total RNA from SH-SY5Y cells, a clone subline of the human neuroblastoma cell line SK-N-SH (Biedler, Roffler-tarlov, Schachner, & Freedman, 1978), grown and differentiated in a monolayer in a 6-well plate was isolated using TRIzol reagent or RNeasy® Mini kit, both according to manufacturer's instructions, briefly described below.

2.5.1. TRIzol isolation

The cell culture medium is discarded and 300µl TRIzol reagent were directly added to each well. After homogenization, the volume of the well was collected to clean and previously identified tube. 60µl of chloroform were added and gently mixed. After a 2-3-minute incubation, samples were centrifuged at 12000xg at 4°C, for 15 minutes. The clear aqueous phase was carefully transferred to new tubes, without interfering with the interphase. To this phase, 150µl of isopropanol were added and incubated for 10 minutes, followed by a centrifuging step at 12000xg at 4°C, for 10 minutes. The supernatant is gently discarded, and the pellet resuspended with 300µl of 75% ethanol. The sample is briefly put on the vortex and then centrifuged at 7500xg at 4°C, for 5 minutes. The supernatant is discarded, and the tubes left open for 10 minutes to air dry the pellet. At last, 20µl of RNase-free water were added to resuspend the pellet. After an incubation at 55°C for 10 minutes the samples were quantified using Nanodrop (Thermo Fisher Scientifics, USA) and stored at -80°C.

2.5.2. RNeasy® isolation

After discarding the cell culture medium, 350µl of the RLT buffer were added to each well. After homogenization, the lysates were collected to designated tubes. 350µl of 70% ethanol were added to the lysates and homogenized by pipetting up and down. The volume was then transferred to RNeasy spin columns and centrifuged at 9000xg for 15 seconds. After, 700µl of RW1 buffer were added and the column was again centrifuged at 9000xg for 15 seconds. Next, 500µl of RPE buffer were added and the column was centrifuged at the same settings. After repeating this latter step, the collector tube is substituted with a new one and the column centrifuged at full speed for 1 minute. After switching to a new collector tube, 40µl of RNase-free water was added to the column and centrifuged. This last step was repeated using the eluted RNA. After quantification using Nanodrop (Thermo Fisher Scientifics, USA), samples were stored at -80°C.

2.6. cDNA Synthesis

RNA extracted from differentiated SH-SY5Y cells was converted in cDNA using a cDNA synthesis kit. After quantification, dilutions of the RNA were prepared in order to achieve a working amount of 1000 ng of RNA in a 10 ul volume. The dilutions were prepared with molecular grade water. A master mix was prepared with the supplied reaction buffer, dNTPS, random primers, RNase inhibitor and the enzyme transcriptase. Molecular grade water was added to make up a 10ul mix volume. In PCR tubes, the volume of the RNA dilutions was added to 10ul of the master mix. The cDNA synthesis was performed according to the following steps: initial step at 25°C for 10 minutes, second step at 37°C for 120 minutes and a third step at 85°C for 5 minutes.

2.7. ace2 Amplification

The cDNA obtained was used in a polymerase chain reaction (PCR) in order to amplify the human gene of ACE2. For that, specific primers for further cloning processes were designed. The final primers used were as followed: forward primer 5'-GATCGCTAGCAATGTCAAGCTCTTCCTGG-3' and the reverse primer 5'-TAGTCCGGAGCTAAAAGGAGGTCTGAAC-3'. After preparing the PCR mixtures according to manufactures conditions for Dream Taq, the samples were initially denatured at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, gradient for primer optimization from 68°C-78°C or from 77°C-81°C for 30 seconds and 72°C for 2 minutes and 30 seconds. A final extension step was performed at 72°C for 10 minutes. After amplification, the samples were separated in a 1% agarose gel, using the molecular marker 1kb ladder plus and running the electrophoresis at 90V.

2.8. Gel Extraction

When amplification band was obtained, the band was carefully excised from the agarose gel using a clean scalpel and saved in a tube. The DNA fragment was than purified form the gel using the QIAquick Gel Extraction Kit. Briefly, after weighing the excised fragment 3 volumes of Buffer QG were added to the tube and incubated at 55°C until the gel was completely dissolved. After 1 volume of isopropanol was added to the tube and the complete volume transferred to a QIAquick column. The column was centrifuged for a minute at 17.900xg. The flow-throw was discarded and 500µl were added to the column. The column was centrifuged for one minute at 17.900xg. 750µl of Buffer PE were added to the QIAquick column and centrifuged again at the same settings. The flow-throw was discarded, and the centrifuging step repeated. 50µl of Buffer EB were added to the column and the column centrifuged for one minute at 17.900xg. The eluted DNA was quantified using Nanodrop (Thermo Fisher Scientifics, USA).

2.9. Angiotensin (1-7) Detection

To detect Ang (1-7) in our cell lysates an Enzyme-linked Immunosorbent Assay (ELISA) kit was used. According to manufacturer's instructions the standard solution and dilutions, biotinylated antibody dilution and the HRP conjugate dilution were prepared. Assay procedure was performed according to the supplied manufacturer's instructions. Briefly, 50µl of standard solution and correspondent dilutions and of the samples were added to the wells. Quickly after, 50µl of the biotinylated antibody were added to each well and incubated for 45 minutes at 37°C. The wells were aspirated and washed 3 times with the wash buffer. Next, 100µl of the HRP conjugate were added to each well and incubated for 30 minutes at 37°C. The wells were then aspirated and washed 5 times, following the addition of 90µl of the substrate reagent and incubated for 15min at 37°C. After the incubation, 50µl of the stop solution were added to each well and the plate immediately read at 450nm 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 OD450nm values, after normalization to the zero OD, in Graph Pad/PRISM 6 software, from which the values of the concentration were interpolated from.

2.10. Animals

Male C57BL/6J Ins2^{Akita} (diabetic) 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 (Barber et al., 2005). With the progression of hyperglycaemia, diabetic mice can develop diabetic complications such as diabetic retinopathy (Barber et al., 2005). In addition, these animals are able to show a similar develop of retinal complications similar to streptozotocin-induced diabetic rats (Barber et al., 2005), making them a good model to study early alterations of DR. All experimental procedures were carried out according to the Portuguese and European Union FELASA regulations 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. This work was carried out under a project approved of DGAV and by the Ethics Committee of the host institution (CEFCM, NOVA Medical School).

2.10.1. Housing

Mice were housed in individually ventilated cages, under controlled temperature and with continuous access to food and water, on a 12h dark/light cycle. In order 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 (Barber et al., 2005).

2.10.2. Retina extraction

6-month-old mice were humanly sacrificed by cervical dislocation and the eyes were removed. The eyes were dissected, and the neural retina was extracted. Samples were mechanically homogenized in RIPA buffer with protease inhibitors, using a motor pestle, and kept on ice for 20 minutes. The extracts were then centrifuged at 16800xg at 4°C, for 20 minutes. The supernatant was collected and stored at -80°C. Total protein quantification was performed by the Bradford Method, as briefly described in the section 2.3.

2.11. Statistical Analysis

The data are presented as the mean ± SEM or mean ± SD, when more suitable. The obtained data were analysed using unpaired t-test with Welch's correction, One-way ANOVA with Dunnet's multiple comparison test or a 2-way ANOVA with Fisher's LSD test as appropriate to each data set. P-value <0.05 was considered statistically significant.

Chapter 3. Results

3.1. Characterization of ACE2/Ang (1-7)/Mas1 axis of RAS in RPE cells

ACE2, Ang (1-7) and Mas1 are now long acknowledged components of the protective arm of RAS. The expression of these proteins was evaluated in cell lysates of D407, a human RPE cell line, by exposing them to culture media with low and high glucose concentrations. The low glucose medium contains 5.5 mM and the high glucose medium 25 mM of D-glucose. The three protective components of the RAS were detected in our samples in all independent experiments carried out (Figure 3.1.1 and 3.1.2; Annexes I, Table 1). The levels of expression of these proteins are decreased when RPE cells are exposed to high glucose, compared to the levels of expression in low glucose (Figure 3.1.1) and a more accentuated decrease is observed on the expression of Mas1, the biological receptor for Ang (1-7) (Figure 3.1.1B). The levels of Ang (1-7) seem unaltered (Figure 3.1.2).

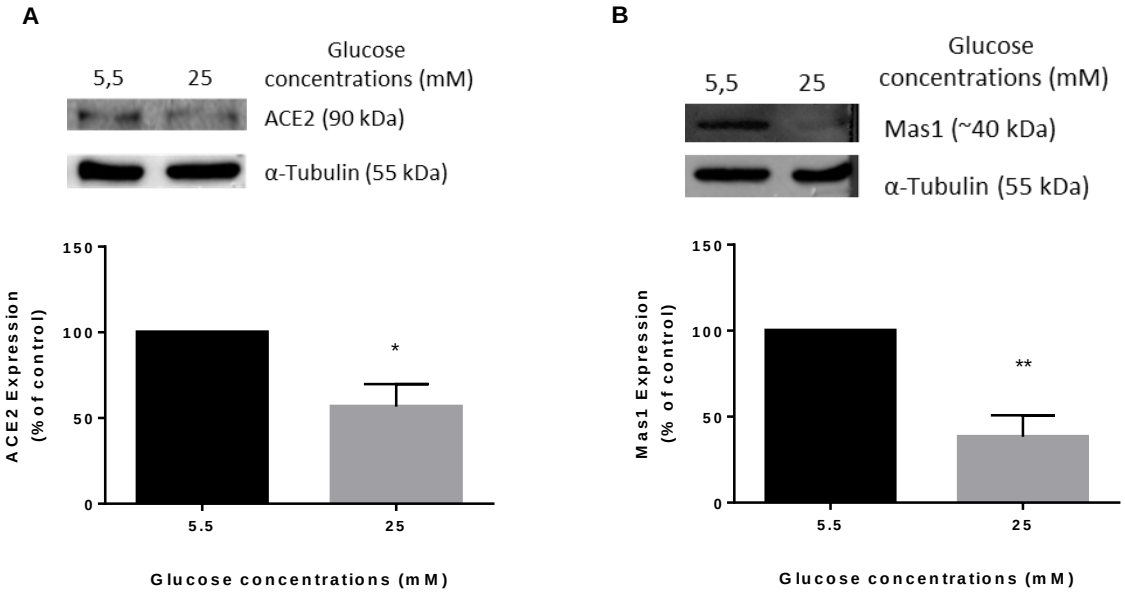

Figure 3.1.1 – Expression of the protective components of the RAS in RPE cells. Representative immunoblots and densiometric analysis of ACE2 (**A**) and Mas1 (**B**) expression in the presence of 5.5mM or 25mM of glucose. As loading control, α-Tubulin was used. The results of panels **A** and **B** are representative of 4 and 5 independent experiments, respectively. *P<0.05 and **P<0.01 determined by unpaired t-test with Welch's correction. The results are presented as mean ± SEM.

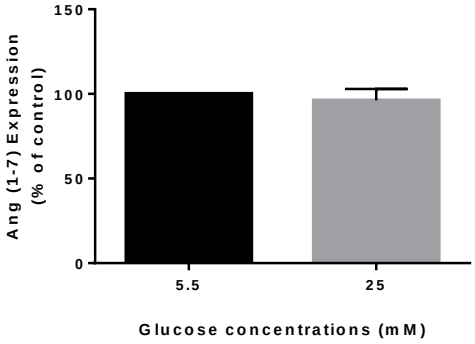

Figure 3.1.2 - Levels of Ang (1-7) detected in RPE cells. These expression levels were detected by an ELISA assay in the presence of 5.5 mM or 25mM of glucose. The results are shown as a percentage of control, being the control condition the 5.5mM of glucose. The results are representative of 3 independent experiments and presented as mean ± SEM, applying an unpaired t-test with Welch's correction.

3.2. Characterization of RAS in a diabetic animal model

Expression of components from both prejudicial and protective axis were evaluated in C57BL/6-Ins2^{Akita}/J (Ins2^{Akita}, diabetic) heterozygote and C57BL/6J age-matched (wild-type) mice retinas and RPE layer. In 6-month old retinas, the expression of all components tends to decrease, being observed a higher decrease on the expression levels of Mas1, although without reaching statistical significance (Figure 3.2).

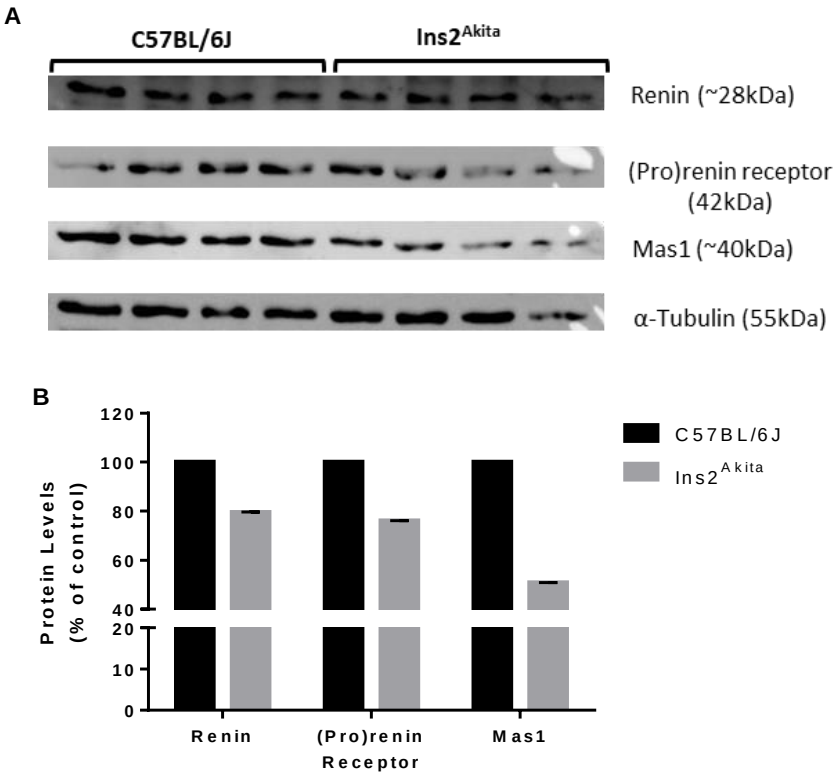

Figure 3.2 – Expression of classical and protective RAS in the mouse eye. **A** Representative immunoblots of renin, (pro)renin receptor and Mas1 detection and **B** densiometric analysis of their expression in the retinas of wild-type and diabetic 6-month old mice (n=4 for each strain). As loading control, α-Tubulin was used. The results are shown as a percentage of control, being the control condition, the retinas extracted from wild-type mice. The results are presented as mean ± SD, applying a 2-way ANOVA with Fischer's LSD test.

3.3. Modulation of RAS by Ang II

Ang II is the prejudicial effector of this hormonal cascade. Its interaction with the AT1 receptor is known to trigger DR hallmarks such as oxidative stress and inflammation. RPE cells were exposed to Ang II at 200μM concentration for 12h and 24h and the expression levels of the protective components evaluated in cellular lysates. The expression levels of all three components tend to increase in a time-dependent manner (Figure 3.3.1 and 3.3.2).

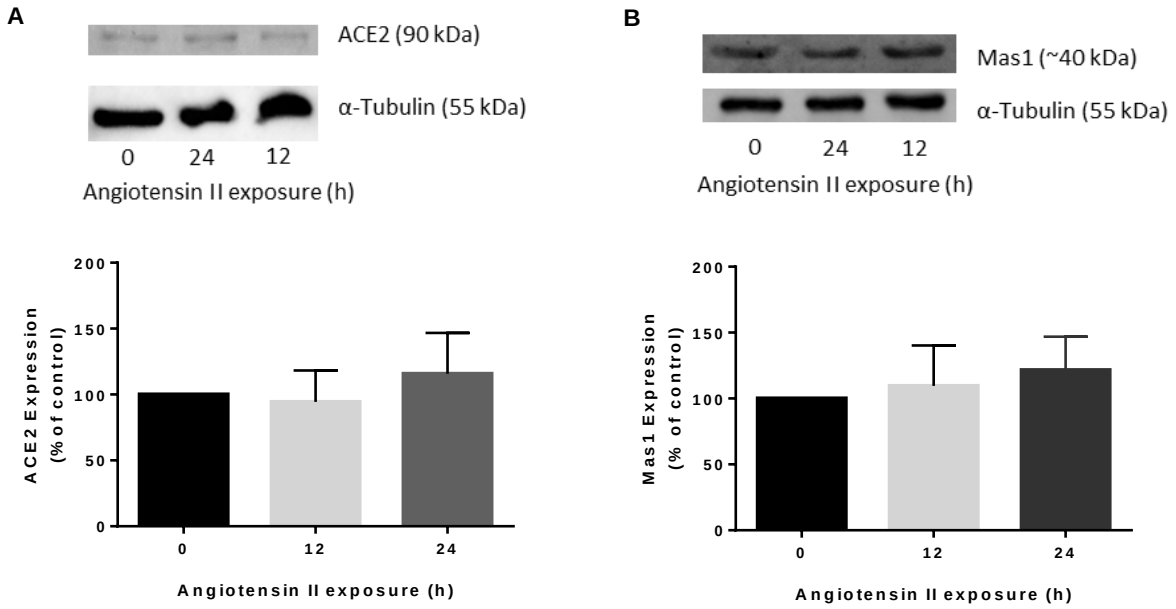

Figure 3.3.1 – Effects of Ang II on the expression of RAS' protective components. Representative immunoblots and densiometric analysis of ACE2 (**A**) and Mas1 (**B**) expression after stimulation with Ang II (200µM) for 12 and 24h. As loading control, α-Tubulin was used. For both proteins 3 independent experiments were carried. The results are presented as mean ± SEM, applying a One-way ANOVA with Dunnet's multiple comparison test.

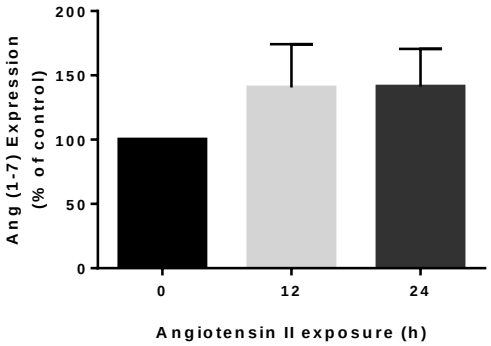

Figure 3.3.2 – Effect of Ang II on Ang (1-7) in RPE cells. Levels of expression were analysed after stimulation with Ang II (200µM) for 12 and 24h. The results are shown as a percentage of control, being the control condition no exposure to Ang II (0h). The results are representative of 2 independent experiments performed by ELISA assay and presented as mean ± SEM, applying a One-way ANOVA with Dunnet's multiple comparison test.

3.4. Modulation of RAS by Aliskiren

Aliskiren is the only commercially available direct renin inhibitor, blocking the RAS cascade in its initial step. The same type of exposure assay was performed on RPE cells. Cells were exposed to Aliskiren (100µM) and cellular lysates were obtained after 4h and 24h of exposure. The levels of expression of ACE2 and Mas1 tend to show a higher decrease at 4h of exposure to aliskiren, although without reaching statistical significance (Figure 3.4.1). However, the levels of the beneficial effector, Ang (1-7), seem to have the opposite tendency, increasing its expression at 4h of exposure to aliskiren (Figure 3.4.2).

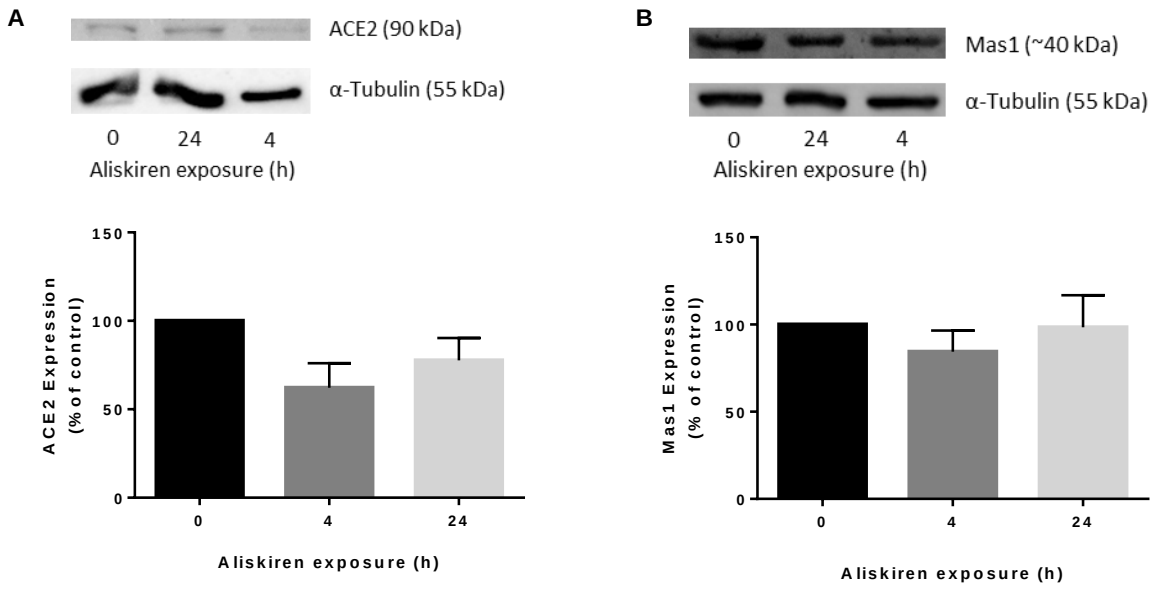

Figure 3.4.1 – Effects of Aliskiren on the expression of RAS' protective components. Representative immunoblots and densiometric analysis of ACE2 (**A**) and Mas1 (**B**) expression after stimulation with Aliskiren (100µM) for 4 and 24h. As loading control, α-Tubulin was used. 3 independent experiments were performed for ACE2 and 4 for Mas1. The results are presented as mean ± SEM, applying a One-way ANOVA with Dunnet's multiple comparison test.

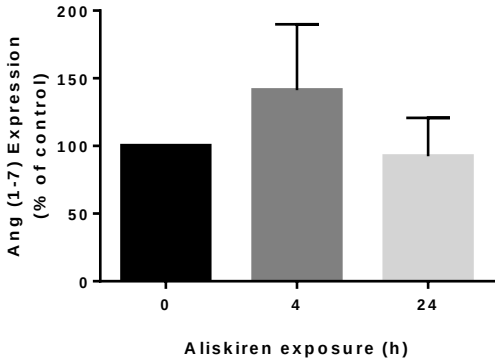

Figure 3.4.2 - Effect of aliskiren on Ang (1-7) in RPE cells. Levels of expression were detected after stimulation with Aliskiren (100µM) for 4 and 24h. The results are shown as a percentage of control, being the control condition 0h of exposure to the direct renin inhibitor. The results are representative of 2 independent experiments by ELISA assay and are presented as mean ± SEM, applying a One-way ANOVA WITH Dunnet's multiple comparison test.

3.5. Modulation towards the protective axis

In this work, it was attempted to obtain the cDNA of the human ACE2 enzyme to clone it in a plasmid vector for expression in RPE cells. To first obtain the cDNA, we chose the SH-SY5Y cell line due to the documented high levels of expression of ACE2 (Thul et al., 2017). cDNA from RNA extracted from differentiated SH-SY5Y cells was used to amplify the human form of ACE2. A gradient of temperatures from 68°C to 78°C was firstly used to optimize the amplification protocol. A faint band around 1.6kb was obtained at 78.2°C (Figure 3.A). The temperature gradient was then shifted to higher temperatures (77°C-81°C), obtaining an amplification product of approximately 1.6kb at 79.2°C (Figure 3.5B). This latter band was then cut out of the agarose gel and the DNA band purified. After DNA quantification a

new amplification was carried at the two temperatures where the ~1.6kb amplicon was obtain. No band of the same molecular weight was obtained (Figure 3.5).

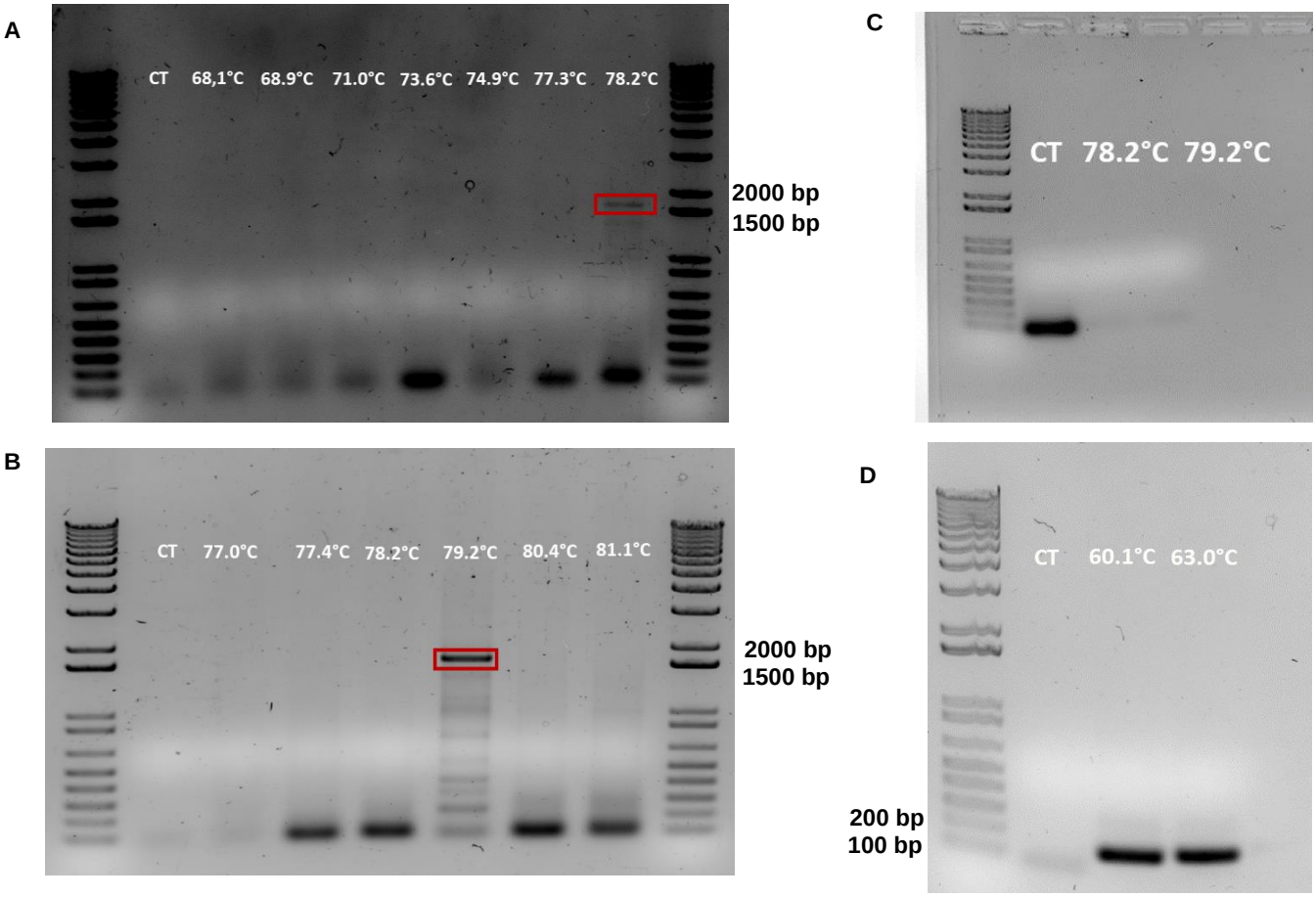

Figure 3.5 – Amplification of *ace2* gene from cDNA of differentiated SH-SY5Y. **A** amplification at 78.2°C with the temperature gradient from 68°C to 78°C and **B** amplification at 79.2°C with the temperature gradient from 77°C to 81°C. In red squares is highlighted the obtained amplicons of approximately 1.6 kb. CT corresponds to the PCR reaction control which reaction was held at the lowest temperature of each gradient (68.1°C for **A** and 77.0°C for **B**). The red squares highlight the resulting amplification band. **C** Amplification of the purified DNA from the amplicon obtained in **B**. CT corresponds to the PCR reaction control which was held ate the lowest of the chosen temperature. No amplification band was obtained at the used annealing temperatures of the PCR. **D** amplification of β-actin as control for cDNA integrity. CT corresponds to the PCR reaction control which was held at the lowest of the chosen temperatures.

Chapter 4. Discussion

With the high prevalence of diabetes, concern over its long-term complications grows bigger. DR is the most common microvascular complication of diabetes and one of the major vision loss threatening causes. Although the molecular mechanisms that lead to DR establishment are not well known, a combination of factors has been tightly associated with this pathology. RAS has been linked to DR, playing a key role in the pathology development. In summary, diabetic patients with diabetic retinopathy have elevated vitreal levels of prorenin, renin and Ang II (Danser et al., 1989; Fletcher et al., 2010). In work published by our group (Simão et al., 2016), it is demonstrated that RPE cells express prorenin and its receptor. Additionally, the expression of these components was shown to be modulated by glucose. When exposed to higher levels of glucose, these components have elevated expression, occurring an increasing trend in renin activity and, consequently, Ang II levels. With higher Ang II levels, angiogenesis and oxidative stress are promoted (Simão et al., 2016, 2017).

In this work, the expression of the protective components of RAS was evaluated. We found that D407 cells, a human RPE cell line, expresses the three components of the protective arm of RAS: ACE2, Ang (1-7) and Mas1 (Figure 3.1.1, Annexes I, table1). Previous studies have already characterized in depth the expression pattern of the Mas1 receptor in the human eye and indicated the local expression of ACE2 and Ang (1-7) (Holappa et al., 2015; Vaajanen et al., 2015). Acknowledging that RPE expresses these components, the deleterious effects of Ang II can be counterbalanced by the ACE2/Ang (1-7)/Mas1 axis in this retinal layer. When exposing RPE cells to high glucose levels, the expression of ACE2 and Mas1 is affected, showing a significant decrease of its levels. When analysing the expression levels of Ang (1-7), only a slight trend of decrease is observed in its expression levels. One of the reasons for this may be the need of more replicates since the detection method used for Ang (1-7) expression (ELISA) is more sensitive to errors, such as inaccurate pipetting or improper preparation of working solutions. 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 prorenin, PRR (Simão et al., 2016) and Ang II (Danser et al., 1994), 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 in disease (Ferrario, 2011), this modulation/effect in expression can determine the development and progression of the pathology maybe accelerating retinal damage (Wong et al., 2007).

The present work has also aimed to characterize the expression of RAS components in the retina of diabetic mice. 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 (Verma et al., 2012). In this work we have used the $Ins2^{Akita}$ mouse, which is a type 1 diabetes model that rapidly develops diabetes and can develop diabetic complications such as diabetic retinopathy (Barber et al., 2005). Our results show that at 6 months of age, $Ins2^{Akita}$ mice's retinas have a lower expression of Mas1 when compared with age-matched wild-type mice, also showing a RAS imbalance of a protective component (Figure 3.2B). While for the $Ins2^{Akita}$ mice this is the first time this analysis is performed, for wild-type mice the Mas1 expression is higher than the

expression of the angiotensin receptors, consistent to what has been described in the literature (Prasad et al., 2014).

It has also been shown that the retina produces prorenin and its receptor, PRR (Alcazar et al., 2009; Danser et al., 1997). In addition, diabetic patients show increased levels of prorenin (Danser et al., 1989) and the binding of prorenin to PRR has been associated with the development of ocular disease (Fletcher et al., 2010; Giese & Speth, 2014). The interaction of prorenin to PRR mediates similar effects to the ones mediated by AT1R but independently of this receptor (Nguyen et al., 2002). In the present work, we also observe a lower level of expression of renin and PRR in the retinal tissue, when compared to wild-type retinas. Nevertheless, the expression levels are still higher than Mas1 levels (Figure 3.2B). In previous work performed by our group, the expression of renin, PRR and Mas1 was evaluated in the retinas of mice from different ages (4, 9 and 12 months of age). When viewing these unpublished results, it was possible to see an elevated decrease in the expression of the Mas1 receptor in the retinas of 4-month old diabetic mice, accompanied by an increased expression of renin and PRR when comparing with wild-type mice's retinas. For the retinas of 9 and 12-months old mice, there was a tendency for a achieving an equilibrium in the expression of these RAS components. From these results we can suggest that this initial RAS imbalance, observed by the Mas1 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 (Barber et al., 2005). Since the expression of RAS' components seem to reach this equilibrium there is no longer an overruling of the prejudicial effects of RAS over the beneficial effects, there is a delay in developing the severe stages of DR.

Previous results of studies on RPE cells demonstrate that activation of RAS in these cells leads to the increase of ROS production, indicative of oxidative stress, and the increased expression of VEGF, a known major angiogenic factor involved in DR pathogenesis (Simão et al., 2017). In our study, based on the concentration and time of exposure to Ang II at which these results were observed (Simão et al., 2017), we observed that the expression of the protective components tends to increase in a time-dependent manner (Figure 3.3.1 and 3.3.2).

Since the studies performed in RPE cells are uncommon, 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 piling conflicting results on its regulation by angiotensin peptides, namely Ang II. Reports from animal models are conflicting with clinical findings, where in animal models of myocardial infarction and hypertension Ang II can reduce the expression of ACE2, the clinical findings show an increase in ACE2 expression associated with cardiovascular diseases, parallel to elevated plasma levels of Ang II (Gallagher et al., 2008b; Koka et al., 2008; Lin, Pan, Wen, Yang, & Kuan, 2010). In a report by Lin et al., it was indicated that an Ang II stimulus induced ACE2 expression in human cardiac fibroblasts. 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 (Lin et al., 2010). 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 (Gallagher et al., 2008b). This study helps show the conflicting results on ACE2 regulation by angiotensin peptides and that its fine regulation may have to be considered within the scope of the physiological or pathological roles.

During hypertension it is known that, associated with elevated Ang II levels, there is a decrease in the expression of ACE2 (Koka et al., 2008). 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 (Huang, Chen, Truong, & Lan, 2003; Koka et al., 2008). Thus, in hypertension, is suggested that the alteration in the balance between the two ACE enzymes shifts towards favouring Ang II formation (Koka et al., 2008). Additionally, it has been noted that in hypertensive nephropathy, the same alteration in ACE and ACE2 balance occurs. There is a down-regulation of ACE2 expression in the kidney, accompanied by an increase in ACE levels (Koka et al., 2008). This imbalance could lead to more renal damage and progression of renal disease, since excess Ang II production is favoured instead of its degradation, which can be beneficial through ACE2 catalysis of Ang II. 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 (Keidar et al., 2007; C Tikellis et al., 2006). Even in initial stages of diabetes, ACE2 is highly expressed and with the development of retinopathy its expression decreases (Keidar et al., 2007; Ye et al., 2004). In line with these reports, in diabetic rodent models are observed early increases in the expression of ACE2 (Wong et al., 2007; Ye et al., 2004), which has been found to be decreased in older diabetic mice (Christos Tikellis et al., 2003; Wong et al., 2007). This indication leads back to the possibly compensatory and early protective role of ACE2, in several diseases, associated with RAS activation.

In this study, activation of RAS, through an Ang II stimulus, seems to lead to an increase in the expression of the protective components of RAS. This goes in accordance with several reports on ACE2 expression mentioned, mainly in diabetic models, and shows a trend in the protective role of this protective arm of the RAS cascade. However, the molecular mechanisms of this increase are unknown. We have seen that hyperglycaemic 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.

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. These drugs have been proven to confer protection in DR development and progression but being an intricate system of pathways, in section 1.5 we saw that 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 our study, the concentration used was based on the study carried out by Simão et al. (Simão et al., 2016). When exposing RPE cells to

100µM of Aliskiren we see a tendency for a decrease at 4h of exposure on the expression of ACE2 and Mas1 (Figure 3.4.1A and B). In previous work from Simão et al., Aliskiren effectively blocked renin activity, leading to a decrease in Ang II levels, at 4h of exposure (Simão et al., 2016). 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 and Mas1 components of protective RAS. Unexpectedly, the same tendency was not observed for Ang (1-7) (Figure 3.4.2). Aliskiren acts on the catalytic center of renin (Rahuel et al., 2000). Thus, this raise in Ang (1-7) cannot be explained by the favourable pathway of formation, where Ang II is degraded. However, since in literature it is possible to see interaction between RAS and other pathways, such as bradykinin one, there may be an yet unknow pathway of formation of Ang (1-7). Still, we also believe that there may have occurred a loss of efficacy of the working aliskiren solution. Without an efficient RAS blockade, the expression of RAS components can be normalized, and the system can follow its regular and favourable pathways.

Since aliskiren has been shown to efficiently inhibit RAS, studies have been performed aiming to discover any additional benefits of the direct renin inhibition. Some of these have shown that inhibition of RAS with aliskiren confers a similar protection to common RAS inhibitors in hypertension and nephropathy (Fletcher et al., 2010; Persson et al., 2009; Whaley-Connell et al., 2010). From the clinical trials performed, the ALTITUDE trial stands out. In this trial, type 2 diabetic patients were given aliskiren, on top of common treatments, to see if there was a reduction on renal and cardiovascular complications, since RAS compensates the common blockade by an increase in circulating renin levels. Due to the adverse effects on patients' condition this study had to be halted. It was assumed the adverse effects emerged from the combination of aliskiren and common RAS blockers, ACEi or ARBs. New trials on aliskiren effects have taken course, however, when targeting combination of drugs, none has a combination of ACEi or ARBs with aliskiren (Guha, Mookerjee, & Dastidar, 2012). It is still not known if aliskiren can confer a similar or greater protection in DR. Clinical trials could perhaps aim at the effects of aliskiren alone in diabetic complications.

Summarizing, in this study it is shown that hyperglycaemia leads to an imbalance of the protective arm of RAS, albeit its apparent initial protective role when this system is overactivated. RAS blockade with aliskiren seems to lead to a decrease in the expression of the protective components as well as of the prejudicial effector, Ang II. Albeit the lack of Ang II appearing beneficial, the expression of the protective components is also affected, being unable to induce beneficial effects efficiently. In addition, the amount of aliskiren that crosses the BRB may be the same as the other commonly used RAS blockers not conferring more protection than ACEi or ARBs. With this, the manipulation of RAS towards the protective axis becomes a more interesting therapeutical approach.

In line with this hypothesis, there are studies where this modulation is performed delivering ACE2 or Ang (1-7) in viral vectors. Verma et al. showed that the delivery of either vector reduced the vascular permeability in diabetic mice's retinas, reduced the number of acellular capillaries and prevented the oxidative stress induced retinal damage. Nevertheless, the delivery of the ACE2 vector decreases the high levels of Ang II while increasing the levels of Ang (1-7) (Verma et al., 2012). Supporting this

protective role, the delivery of a lentiviral ACE2 expressing vector reversed cardiac hypertrophy in rats (Huentelman et al., 2005; Koka et al., 2008). The local delivery of ACE2 expressing vector seems to provide a good strategy to counterbalance the classical RAS signalling and prejudicial effects.

To test the potential of this therapeutical approach, it was attempted to obtain the *ace2* gene to further clone it on a vector and deliver this ACE2 expressing vector to RPE cells. Since we have seen that the human RPE cell line used in this work expressed the ACE2 enzyme, we firstly tried to obtain the *ace2* gene from cDNA samples of RNA extracted from D407 cells. When trying to amplify this gene by PCR no amplification was observed. The following option was to amplify this gene from another cell line that expressed in higher levels the *ace2* gene. The human neuroblastoma subclone SH-SY5Y cell line was chosen (Thul et al., 2017). As seen in Figure 3.5A and B, an amplicon of approximately 1.6kb was obtained. However, the expected size of this gene is approximately 2.5kb (Nucleotide – NCBI; Komatsu et al. 2000). To understand what this amplicon is the band seen in Figure 3.5B was isolated and the DNA purified. However, the yield of this process was very low. In order to augment the amount of DNA a subsequent PCR was performed with the product of the purification product. The result of this amplification attempt seen in Figure 3.5C shows that no amplification occurred on the used annealing temperatures. This leaves us still unable to clearly understand what the amplification product obtained and seen in Figure 3.A and B is. One hypothesis that we have not been able to test the circularization of the amplified copies, since the primers used contain restriction sites on the extremities. If there is any matching occurring between these sequences, the amplicon circularizes and can migrate further during electrophoresis. Another alternative would be to sequence the obtained product. However, this was not possible due to the low yield of the purification process. An additional alternative to obtain an *ace2* expressing plasmid was via Doctor Chandana Herath (University of Melbourne, Australia), which has worked with an *ace2*-expressing plasmid. Although a positive response was provided, no plasmid vector was sent to us and therefore an *ace2*-plasmid has been ordered from a commercial vendor.

Chapter 5. Conclusion

With this present work we show that RPE expresses ACE2, Ang (1-7) and Mas1, supporting the presence of the protective axis in ocular RAS. Additionally, we were also able to show that this axis is modulate by glucose, lowering the expression levels of its components. This indicates an imbalance of the protective axis, enhancing the deleterious effects of RAS and maybe accelerating retinal damage. In the retinas of diabetic animals, we have shown that at 6 months of age the expression a low expression of the Mas1 receptor, supporting the early imbalance of the ACE2/Ang (1-7)/Mas1 axis. Yet, when RAS is overactive our results show a potentially earlier protective role of this axis, since the expression of the three components tends to increase.

Aliskiren, previously shown by our group to effectively inhibit renin in RPE cells (Simão et al., 2016), has shown to also have an impact on the expression of mainly ACE2 and Mas1, reducing their expression. Although no Ang II dependent effects seem to be induced when aliskiren is used to inhibit RAS, we are also impairing the expression of these protective components and preventing the beneficial effects of this axis.

With the results presented in this work, a higher interest rises over the potential of the modulation of RAS by expressing the protective components as therapeutical approach. Work by Verma et al. and Dominguez et al. (Dominguez et al., 2016; Verma et al., 2012) has shown that this approach is promising as a therapy for DR. At the time of submission of this dissertation, an *ace2*-plasmid has been ordered from a commercial vendor so this modulation of RAS can be accessed in future work. The aim will be the delivery of this expression vector to RPE cells and evaluate the effects of this delivery in the expression of RAS components and DR hallmarks such as oxidative stress, inflammation and angiogenesis.

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Annexes

I. Human Ang (1-7) ELISA data

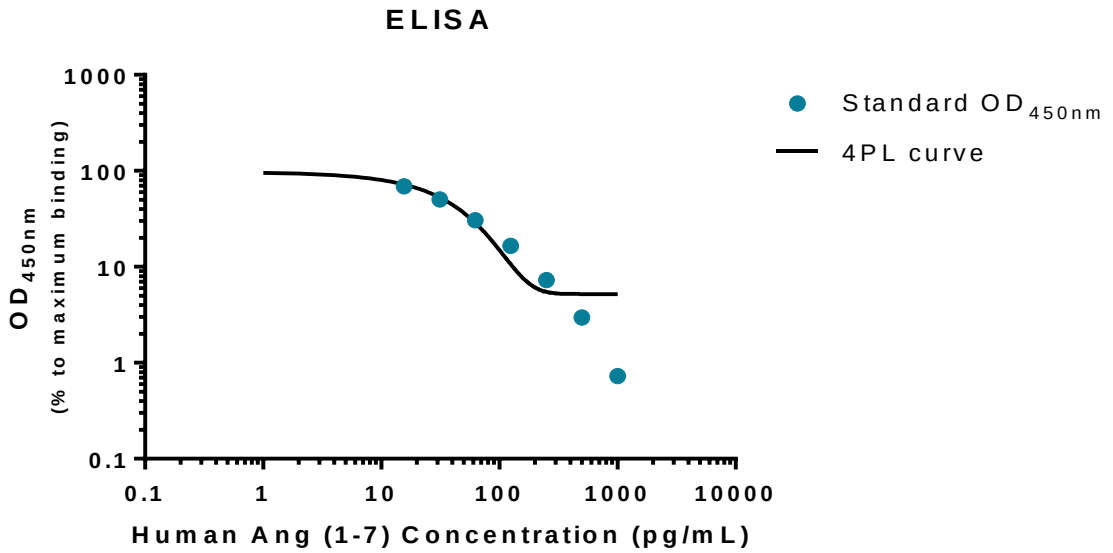

Figure I.1 – Four-parameter logistics curve standart curve. The concentrations of the standart solution vary from 0 to 1000 pg/mL, using dilution in a 1:2 ratio. The OD values are represented as a percentage of the maximum binding since this ELISA uses a competitive principle and, for the maximum binding to be considered in the data processing, a ratio B/B0 is applied to all OD values. In this ratio B is the absorbance of each well and B0 is the absorbance of the well where the standart sample with no concentration of Ang (1-7), which, in this case, will be the well with the highest OD value.

Table I.1 – Concentration of Ang (1-7). The samples used were tested in duplicates. After the obtained OD_{450nm} values were averaged and the ratio B/B0 applied. The concentration of Ang (1-7) present in the tested samples was interpolated from the four-parameter logistics standart curve represented in Figure I.1.

Sample	Average OD_{450nm}	Interpolated Concentration (pg/mL)	N
5.5 mM glucose	1,479	8,278604	1
25 mM glucose	1,456	9,087541	
5.5 mM glucose	1,345	13,16074	2
25 mM glucose	1,381	11,8074	
5.5 mM glucose	1,320	14,12017	3
25 mM glucose	1,360	12,59289	
Control	1,4995	7,566954	1
Ang II 12h	1,3445	13,17977	
Ang II 24h	1,3515	12,91396	

Aliskiren 4h	1,3135	14,37236	
Aliskiren 24h	1,4545	9,140687	
Control	1,450	9,300438	2
Ang II 12h	1,4315	9,961882	
Ang II 24h	1,4195	10,39505	
Aliskiren 4h	1,4695	8,611364	
Aliskiren 24h	1,547	5,950462	