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Characterization of pro- and anti-angiogenic factors in models of Diabetic Retinopathy

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This MSc Thesis has been carried out under the supervision of Professor Gabriela Silva, PhD and Sofia Calado, MSc, at the Biomedicine Research Center (CBMR) of the University of Algarve.

Liliana Teresa da Silva Alves

Faro, 2015

Citation

"Man may be the captain of his fate, but he is also the victim of his blood sugar."

- Wilfrid G. Oakley
Transactions of Medical Society of London (1962),
78:16

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Abstract

Diabetic retinopathy (DR) is a microvascular complication associated with *Diabetes mellitus* (DM) and the leading cause of blindness in developed countries. Hyperglycemia and hypoxia are suggested to play essential pathophysiological role in the onset, progression and prognosis of DR. Moreover, hypoxia inducible factor-1 (HIF-1), which is stabilized under hypoxic conditions, acts as a transcription factor of several genes such as vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT1). It was also described that an imbalance between pigment epithelium-derived factor (PEDF), a potent angiogenic inhibitor, and VEGF, the major angiogenic stimulator, is present in patients that suffer of DR. However little is known about the role of this imbalance in DR progression.

Therefore the goal of this study was to characterize the expression of GLUT1, pro-, and anti-angiogenic factors involved in the development and progression of DR, using *in vitro* and *in vivo* models of the disease.

The present study demonstrates that in D407 cells, an immortalized retinal pigmented epithelium (RPE), the levels of the glucose transporter GLUT1 are increased in the response to hypoxia and hyperglycemia and this increase is more pronounced in the cell membrane fraction. Additional support to our in vitro results is the fact that GLUT1 expression levels are increased in $Ins2^{Akita}$ mice, a model of type 1 DM.

Regarding PEDF expression *in vitro*, there was no significant difference between tested conditions, but we observed the tendency to decrease in hypoxic conditions. *In vivo,* we could see a significant decrease of PEDF expression in the retina of 6 month-old mice; but the key result is the marked decrease detected in the RPE cell layer. VEGF mRNA levels were increased *in vitro* and *in vivo* at 6 month-old animals, but this was not observed for the protein levels. At 12 months of age, mRNA levels were similar in WT and diabetic mice, but the protein levels are significantly decreased in diabetic mice, a potential consequence of the overall senescence observed for these animals.

Taken together, these results suggest that the increase in GLUT1 and the decrease in PEDF expression levels are associated with the development of diabetic retinopathy.

Keywords: Diabetic retinopathy (DR); hypoxia; hyperglycemia; vascular endothelial growth factor (VEGF); glucose transporter 1 (GLUT1); pigment epithelium-derived factor (PEDF);

Resumo

A retinopatia diabética (DR) é uma complicação microvascular da retina que causa cegueira a mais de 10 000 pessoas diabéticas anualmente. Esta doença resulta da exposição prolongada da retina a elevados níveis de glucose resultantes do estado diabético.

Os processos envolvidos no desenvolvimento das complicações associadas à diabetes são ainda desconhecidos, no entanto pensa-se que estas poderão estar associadas a uma maior captação da glucose por parte da retina e a uma exposição prolongada à hipóxia que leva à neovascularização da retina.

A glucose é transportada na barreira hemato-retiniana através do transportador GLUT1, que é regulado pela concentração sanguínea de glucose e pelo fator indutor de hipóxia, HIF-1. O HIF-1 tem um papel importante em condições de hipoxia pois funciona como factor de transcrição de vários genes importantes como o VEGF, que é o principal potenciador da neovascularização. Também foi descrito que pacientes que sofrem DR possuem um desequilíbrio entre os fatores pro-angiogénicos e anti-angiogénicos como o VEGF e o PEDF, respetivamente.

Com base nestes dados, este trabalho teve como objetivo avaliar os níveis de expressão do transportador GLUT1 e dos fatores VEGF e PEDF em sistemas *in vitro* de células da retina D407 em condições de hipóxia e normóxia sujeitas a diferentes concentrações de glucose. Foi ainda avaliada a expressão do GLUT1 e dos fatores VEGF e PEDF *in vivo*, em amostras de retina de ratinhos $Ins2^{Akita}$, um modelo animal de DM tipo I, que apresenta algumas características da DR, e em amostras de animais "*wild type*" não diabéticos. Esta avaliação foi feita ainda em dois momentos na vida dos ratinhos: aos 6 meses de idade, que representa o início do desenvolvimento da doença em que começa a mostrar alguns sintomas característicos de DR, como microaneurismas e vazamento vascular, e aos 12 meses de idade, que representa um estádio crónico da doença.

Os resultados mostram *in vitro,* nas células RPE imortalizadas D407 um aumento do transportador GLUT1 quando estas são sujeitas a condições de hiperglicemia e hipóxia. Mas o que se destacou foi o facto de este aumento ser mais predominante nas membranas das células o que sugere que há um aumento não só da concentração como também da sua translocação para a membrana celular.

Em relação aos níveis de expressão do PEDF *in vitro* apesar de esta diminuição não ser significativa, apresenta tendência para diminuir em condições de hipóxia. Contudo, nos

níveis de expressão do VEGF *in vitro* não se verificou qualquer alteração nas condições testadas.

Nos resultados *in vivo* podemos verificar que houve um aumento da expressão do transportador GLUT1 na retina de ratinhos $Ins2^{Akita}$ em comparação com os ratinhos "*wild type*" de 6 meses de idade, sugerindo que este aumento dos transportadores poderá estar associado ao desenvolvimento de DR em estádios precoces da doença. Já aos 12 meses este aumento não é significativo o que poderá estar associado à senescência que a retina apresenta nesta idade.

Em relação ao PEDF *in vivo*, podemos constatar que em amostras de retina e de epitélio pigmentar da retina (RPE) de ratinhos de 6 meses houve uma diminuição significativa na sua expressão aos 6 meses de idade. Já aos 12 meses essa expressão foi abolida na RPE dos ratinhos diabéticos.

Por último, os níveis de expressão do VEGF *in vivo* são controversos pois podemos verificar um aumento da expressão do mRNA nas retinas dos ratinhos $Ins2^{Akita}$, aos 6 meses de idade, mas não da proteína. Porém, aos 12 meses não se verificou alteração dos níveis de mRNA entre estirpes mas sim uma diminuição da expressão proteica que pode estar associado ao estádio avançado da doença nos ratinhos $Ins2^{Akita}$.

O nosso estudo sugere que o GLUT1 e o PEDF podem ser componentes importantes para o desenvolvimento de DR, sendo este último mais importante no estádio inicial da doença.

Palavras-chave: Retinopatia diabética (RD); Hipóxia; Hiperglicemia; GLUT1; VEGF; PEDF; células D407; $Ins2^{Akita}$.

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Abbreviations

AGEs	Advanced glycation end products
AR	Aldose reductase
BM	Basement membrane
BRB	Blood retinal barrier
CO_2	Carbon Dioxide
DAG	Diacylglycerol
DFO	Desferrioxamine;
DM	Diabetes Mellitus
DMEM	Dulbecco’s Modified Eagle's Medium
DR	Diabetic retinopathy
ECs	Endothelial Cells
EPO	Erythropoietin
FBS	Fetal bovine serum;
Fw	Forward Primer
GCL	Ganglion cell layer
GDM	Gestational diabetes
GLUT1	Glucose transporter 1
GR	Glutathione reductase
$H_2C_2O_4$	Oxalic acid
HCl	Hydrochlorid acid
HIF-1	Hypoxia-inducible factor-1
HPO_4	Hydrogen Phosphate
ID	International Dollars
IDF	International Diabetes Federation
IGF2	Insulin-like growth factor-2
ILL	Inner limiting layer
INL	Inner nuclear layer
IPL	Inner plexiform layer
KCl	Potassium chloride
$KMnO_4$	Potassium Permanganate
KOH	Potassium hydroxide
NaCl	Sodium chloride

NCDs	Non-communicable diseases
NFL	Nerve fiber layer
NOS	Nitric oxide synthase
NPDR	Non proliferative diabetic retinopathy
O_2	Oxygen
OCT	Optimal cutting temperature
OLL	Outer limiting layer
ON	Over night
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PBS	Phosphate-buffered saline
PCA	Perchloric acid
PDR	Proliferative diabetic retinopathy
PEDF	Pigment Epithelium–Derived Factor
PFA	Paraformaldehyde
PHD	Prolyl hydroxylase
PKC	Protein kinase C
POS	Photoreceptor outer segments
RCL	Rod and cone layer
Rev	Reverse Primer
ROS	Reactive oxygen species
RPE	Retina Pigmented Epithelium
rpm	Rotation per minute
RT	Room Temperature
SDH	Sorbitol dehydrogenase
T1DM	Diabetes type 1
T2DM	Diabetes type 2
TBS.T	Tris-Buffered Saline, 0.1% Tween 20
TGFα	transforming growth factor- α
VEGF	Vascular endothelial growth factor
WT	Wild Type

1. Introduction

1.1 Diabetes

In the last decades, scientists have been searching for a cure or an efficient treatment for diabetes, an epidemic disease that is growing in an alarming rate. [1] Although some treatments like drug therapy combined with advanced technology and prevention measures seem to be effective, the battle to halt this growing is being lost.

International diabetes federation (IDF) announced that in 2014, 382 million people have diabetes and are expected to rise more 205 million by 2035. [2] More concerning is the fact that Portugal is the country with the highest prevalence of diabetes (13.09%) from all Europe besides Turkey (14.71%). [3] Associated with the increase of diabetic population is the increase of health expenditures. In 2013, the global health spending to treat diabetes complications was about 581 billion of International Dollars (ID) and is expected to increase to almost ID 678 billion in 2035. In other words, it was spent on average ID 1,522 per person in 2013. [2]

The main problem in this century is the increase of risk factors associated with diabetes like obesity, sedentary lifestyle, smoking, alcohol and unhealthy diet. [4] Since diabetes is one of the most non-communicable diseases (NCDs) and one of the leading cause of death, the associated complications are of paramount importance for the population. [2] [5]

Diabetes Mellitus (DM) is a metabolic disease characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action or both. The majority of cases of DM fall into two broad categories designated type 1 (T1DM) and type 2 (T2DM) but there are other types of DM like gestational diabetes (GDM) or DM derived from genetic defects. Both T1DM and T2DM are preceded by a phase of abnormal glucose homeostasis as the disease progresses. While T1DM is a result of an absolute or near-total insulin deficiency, T2DM is a group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion and increased glucose production. [6] [7]

T1DM also known as insulin-dependent diabetes results from a cellular mediated autoimmune destruction of the pancreatic β-cells and corresponds only to 5-10% of those with diabetes.

On the other hand, T2DM or non-insulin dependent diabetes includes individuals who have insulin resistance and relative insulin deficiency. It is estimated that 90-95% of those with diabetes have this type and they do not need insulin treatment to survive. Most of the

T2DM diabetic patients are obese which can cause some degree of insulin resistance itself. [6] [7]

Long term complications of diabetes are associated with chronic hyperglycemia exposure. Generally, hyperglycemia effects on the body can be separated into macrovascular (injury in large blood vessels) and microvascular (injury in small blood vessels) complications. The macrovascular complications include coronary artery disease, peripheral arterial disease, and stroke, while microvascular complications include diabetic nephropathy, neuropathy, and retinopathy. [8]

Among the diabetic complications, this work will focus in the diabetic retinopathy, one of the most common microvascular complications. [8]

1.2 Retina and BRB

The retina is a tissue that allows us to see the world. The function of the retina is to convert light signal into neural signal and send it to the brain through the optic nerve. To understand pathological changes in diabetic retinopathy it is important to know about its morphology and physiology. The retina is the innermost layer of three layers of the eye and is composed by two sub-layers, the neural retina and retinal pigmented epithelium (RPE), which are surrounded by the choroid and the humor vitreous (fig. 1.1). [9] The two sub-layers have distinct functions and physiologies.

The RPE layer consists of cuboidal cells that surround the neural retina and acts as a barrier that absorbs scattered light, regulates the ion transport between the retina and the choroid, removes the free radicals and is essential to the visual cycle. [9]

The neural retina is composed by five neural cells (photoreceptors, bipolar cells, ganglion cells, horizontal cells and amacrine cells) that are disposed by nine layers (fig. 1.1). [10] The nine layers (from choroid to vitreous body) are:

1. Rod and cone layer (RCL) – photoreceptors with different properties. While rods have low spatial resolution and are extremely sensitive to light, cones has high spatial resolution and are relatively insensitive to light. Therefore rods are specialized for sensitivity and cones are specialized for acuity and also responsible for color perception. [11]
2. Outer limiting layer (OLL) – a natural barrier between the sub retinal space and outer nuclear layer, localized between the photoreceptors and Müller cell processes. [9]

3. Outer nuclear layer (ONL) - contains cell bodies of the photoreceptors. [9]
4. Outer plexiform layer (OPL) - includes axons of the photoreceptors and dendrites of association neurons in the inner nuclear layer. [9]
5. Inner nuclear layer (INL) - contains the nuclei of various neurons, specially the bipolar cells, amacrine cells, and horizontal cells. [9]
6. Inner plexiform layer (IPL) - consists of axons and dendrites that connect neurons of the INL with the ganglion cells. [9]
7. Ganglion cell layer (GCL) - contains cell bodies of ganglion cells. [9]
8. Nerve fiber layer (NFL) – formed by axons of the ganglion cells, that composes the optic nerve. [9]
9. Inner limiting layer (ILL) - consists of terminal expansions of other Müller cell processes that cover the collagenous membrane of the vitreous body. [9]

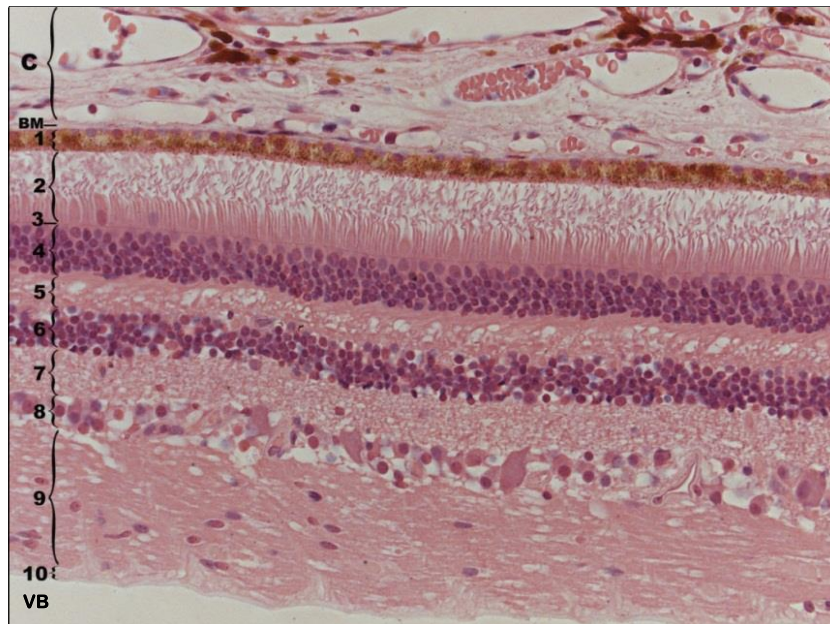

Figure 1.1: Histological section of the human retina showing the retinal layers with hematoxylin/eosin staining. 1: RPE; 2: RCL; 3: OLL; 4: ONL; 5: OPL; 6: INL; 7: IPL; 8: GL; 9: NFL; 10: ILL; C - choroidal vascular layers; BM: Bruch's membrane; VB – Vitreous body. Adapted from [12].

1.3 Diabetic Retinopathy

1.3.1 Clinical Aspects

Diabetic retinopathy (DR) is considered the leading cause of blindness in working-age adults. [2] The prevalence of DR after 20 years of DM diagnosis is higher 90% for patients with T1DM and > 60% of those with T2DM. [13]

DR is a retinal microvascular disorder that occurs as a complication of DM and is characterized by signs of retinal ischemia like microaneurysms, hemorrhages, cotton-woolspots, intraretinal microvascular abnormalities, venous caliber abnormalities, neovascularization and increased retinal vascular permeability. These pathological changes in the retinal microvasculature are the result of chronic exposure to elevated blood glucose. [14] [15] In addition, some studies showed that after the blood glucose levels have returned to normal, the retinal lesions persisted meaning that an early detection is crucial to an effective treatment. [16]

There are two types of DR: non-proliferative DR (NPDR) and proliferative DR (PDR). NPDR occurs when there are only intraretinal microvascular changes, such as altered retinal vascular permeability and eventual retinal vessel and capillary closure. [17] In the non-proliferative phase, retinopathy is categorized further into four levels of severity: mild, moderate, severe, and very severe (described in Table 1.1).

Neovascularization is not an element of this phase however, in advanced NPDR, nonperfusion of the retina and retinal ischemia may be developed and lead to the proliferative phase. [17]

PDR is characterized by neovascularization responding to hypoxic mechanisms. These new vessels, which grow up near the optic disc, break easily leading often to vitreous hemorrhage, fibrous bands and retinal detachment leading to blindness. [18]

Table 1.1: Classification of the severity of non-proliferative diabetic retinopathy. Adapted from [17].

Levels of Severity	Lesions Present
No retinopathy	No retinal lesions
Mild	Mild levels of microaneurysms and intraretinal hemorrhage
Moderate	Moderate levels of microaneurysms and intraretinal hemorrhage
Severe	Presence of one of the following features: 1. Severe intraretinal hemorrhage in all 4 quadrants; 2. Venous beading in 2 or more quadrants; 3. Moderate intraretinal microvascular abnormalities in at least 1 quadrant.
Very severe	Presence of two or more of the above-mentioned features described in severe non-proliferative diabetic retinopathy

1.3.2 Pathways of hyperglycemia-induced damage

It has been proposed several biochemical mechanisms relating hyperglycemia and retinal microvascular complications such as polyol pathway, advanced glycation end products (AGEs) accumulation, protein kinase C (PKC) activation and oxidative stress (Fig. 1.2). [19]

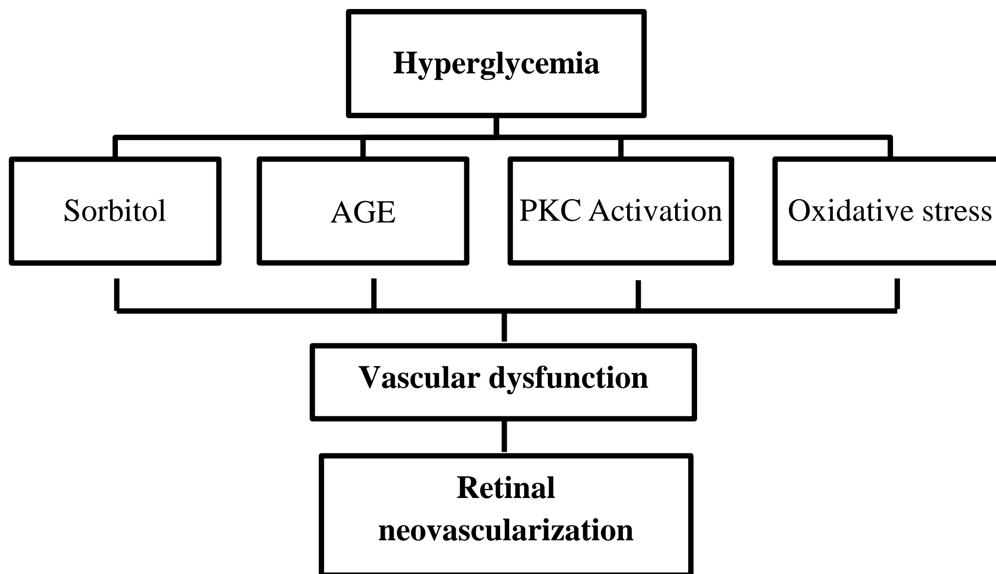

Figure 1.2: Pathophysiology of DR. Hyperglycemia instigates a cascade of events leading to retinal vascular endothelial dysfunction. Adapted from [20].

1.3.2.1 Polyol Pathway

Several studies show that polyol pathway may be a plausible contributor to DR and other complications. This pathway is highly active under hyperglycemia conditions and consists in reducing glucose to sorbitol which is then converted to fructose (fig. 1.3). Aldose reductase (AR), the first and rate-limiting enzyme of the pathway, is present in all cells types (neurons, Müller glia, pericytes, and endothelial cells) that are affected by diabetes. [21]

The effects of this pathway are variable. Sorbitol is an alcohol, polyhydroxylated and strongly hydrophilic contributing to its intracellular accumulation in the cells which consequently increase the osmotic stress/pressure. [22]

Oxidative stress, PKC activation and AGEs production are other effects of polyol pathway.

The fructose produced by the polyol pathway can enter in the formation of fructose-3-phosphate and 3-deoxyglucosone which are powerful glycating agents and AGE precursors. [22]

It is also known that reactive oxygen species (ROS) can work both as signaling and damaging molecules which triggers pro-inflammatory responses as well as apoptosis. So it may be expected that chronic activity of polyol pathway in diabetes is sufficient to initiate the oxidative stress that is observed in the diabetic retina and should be partly responsible for cellular changes that have the potential to shape the development and progression of DR (fig. 1.2). [22]

Figure 1.3: Effects of polyol pathway activation. Increased of cytosolic glucose causes accelerated transformation of glucose to sorbitol by aldose reductase (AR). In the second step of the polyol pathway, sorbitol is oxidized to fructose by sorbitol dehydrogenase (SDH). Finally, fructose produced in the second step of the polyol pathway is a precursor of AGEs. Adapted from [22].

1.3.2.2 AGEs Accumulation

Some patient-based studies have demonstrated that the levels of AGEs in serum are correlated with the clinical progression of DR. [23]

Non-enzymatic glycation reactions between reducing sugars and the free amino groups on proteins, lipids, and DNA are inevitable but in the presence of high levels of glucose these reactions are enhanced. [24]

Thus, over time, even middle hyperglycemia can lead to a significant adduct accumulation of AGEs on long-lived macromolecules. [24] [23]

Some studies have demonstrated that AGEs induce toxic effects on retinal pericytes by causing oxidative stress and subsequent apoptosis. In addition, other studies have indicated that AGEs cause osteoblast differentiation and calcification in retinal pericytes, which eventually also leads to apoptosis. Thus, both events may contribute to pericyte loss in the retinal capillaries that are present in DR (fig. 1.2). [24]

1.3.2.3 PKC activation

In DR, PKC mediates changes in endothelial permeability, blood flow, and both the formation and response to angiogenic growth factors contributing to retinal leakage, ischemia, and neovascularization. PKC activation also contributes to loss of capillary pericytes, an early feature of DR (fig. 1.4). [25]

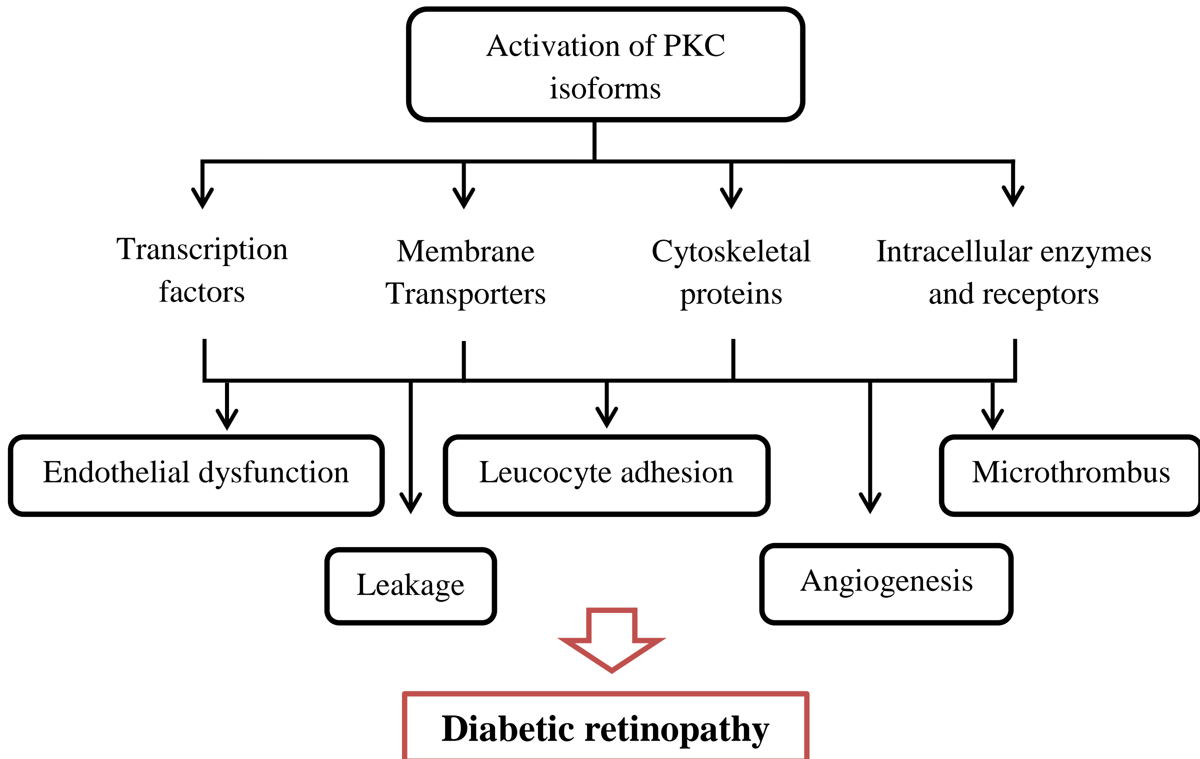

Figure 1.4: PKC, especially PKCβ, mediated phosphorylation of transcription factors, cytoskeletal proteins, enzymes and transporters leads to many of the pathophysiological features of DR. Adapted from [25].

The PKC molecule is part of a serine/threonine kinase family that catalyzes phosphorylation of key proteins involved in signal transduction. It has individual isozymes that are activated by diacylglycerol (DAG), a molecule that is upregulated in vascular tissues by hyperglycemia. Differences in structure and substrate requirements allowed to identify approximately 12 different PKC isoforms in which PKCβ is the one that demonstrates the most significant increase in hyperglycemia. [26]

PKC has also been implicated in the stimulation of vascular endothelial growth factor (VEGF) expression, an important angiogenic factor that will be further discussed in this work. [27]

1.3.2.4 Oxidative Stress

Oxidative stress is the cytopathic consequence of the generation of excess ROS beyond the capacity of a cell to defend against them. It represents an imbalance between the production of oxidants and antioxidant defense leading to potential tissue damage.[28] [29]

Formation of ROS is increased in hyperglycemia by several events and sources of ROS such as disruption of the mitochondrial electron transport chain, formation of AGEs products, auto-oxidation of glucose, flux through the polyol pathway, uncoupling of nitric oxide synthase (NOS), and activation of PKC. [29]

Moreover, chronic exposure to oxidative stress can induce damage to biological macromolecules such as DNA, lipids, proteins, carbohydrates, disruption in cellular homeostasis and generation of other ROS which creates further damage in the retina. [28]

The retina has several defense mechanisms to minimize oxidative stress such as glutathione, vitamins A, C, E, and enzyme systems like superoxide dismutase, catalase, glutathione reductase (GR) and glutathione peroxidase.[29]

High activity is required to protect the retinal neurons and particularly rod outer segments from oxidative stress due to their high metabolism, which leads to a substantially less antioxidant activity in the retinal microvascular endothelial cells. Then, this imbalance in the endothelial cells may be the explanation for the oxidative stress in retinal capillaries under diabetic conditions (fig. 1.2). [29]

1.3.3 Pathophysiological Process

All the biochemical mechanisms described above may induce clinical changes in the retina that characterizes DR namely pericyte loss, blood retinal barrier (BRB) breakdown, inflammation, apoptotic response and neovascularization.

1.3.3.1 Pericyte loss

Pericytes are the supporting cells of the microvasculature and regular components of capillaries in almost all human tissues and organs associated mostly with stabilization and hemodynamic processes of blood vessels. Pericytes in the retina are completely embedded within the capillary basement membrane (BM), surrounding the endothelial cells (ECs) originating the capillary tube. The density of pericyte coverage in the capillary varies from organ to organ but the highest density is found in the retinal microvasculature. One explanation for the high pericyte coverage of capillaries of the retina is the function of blood vessels in this specialized organ. The blood supply to the retina is the highest among

all organs of the body and the need for vessel tightness is particularly high in the retina for proper visual function. Therefore, the greater the number of pericytes covering the EC tube, the better the barrier functions. [30]

Some studies show that pericyte possesses contractile properties and control capillary blood flow in response to local modulation by vasoactive mechanisms, but the most prominent function is their role in vascular growth and vessel stabilization. [30]

Moreover, pericytes loss in DR consequently alters the blood flow in the retinal capillaries which can lead to capillary leakage, vessel occlusion and ultimately microaneurysms formation. [31] Furthermore, pericyte loss destabilizes the BRB integrity, making it more permeable. Due to these consequences, pericyte loss is one of the earliest changes in DR. [30]

1.3.3.2 Inflammation in DR

Most of the metabolic and physiologic abnormalities found in DR are consistent with the presence of a chronic inflammation which may play a critical role in the development of the early and late stages of the disease. [32]

Inflammation is a signal-mediated response to cellular insult that embraces a variety of functional and molecular mediators, including recruitment and/or activation of leukocytes. While acute inflammation has beneficial effects against those insults, chronic inflammation can cause undesirable effects like tissue destruction. [33]

Some studies have shown that DR is associated with increase of inflammatory markers like leukocytes, cytokines, interleukins, platelets, intercellular adhesion molecules and growth factors. Whether the presence of this inflammatory markers in DR is due to the vasculopathy or whether they are directly involved in the damage is not yet elucidated. [34] Nevertheless, leukostasis, a major component of inflammatory process, contribute to capillary non-perfusion and has been postulated to be a factor in endothelial cell deaths and breakdown of the BRB. Diabetic retinal vascular leakage, capillary non-perfusion, and endothelial cell damage are associated with leukocyte recruitment and adhesion to the retinal vasculature which correlate with increased expression of inflammatory markers. [35]

Therefore, these inflammatory markers are important in the development of early stages of DR.

1.3.3.3 BRB breakdown

Another important structure present in the retina is BRB. It is an important barrier that is essential to maintain the function and structure of the retina. It controls fluid and molecular movement between the ocular vascular beds and the retinal tissues and prevents leakage into the retina of macromolecules and other potentially harmful agents.

It consists of two layers, the inner BRB (iBRB) and outer BRB (oBRB) (Fig. 1.5). The iBRB is formed by tight junctions between ECs from neighboring capillaries and the oBRB is composed by tight junctions between RPE cells. [36] The BRB maintenance is covered by the foot processes of astrocytes, Müller cells and pericytes that contribute to a proper BRB functioning and integrity.

The BRB breakdown present in DR is characterized by an increased vascular permeability, which comes from molecular changes in proteins of the tight junctions complex and loss of pericytes. Ultimately, BRB breakdown leads to microaneurysms and neovascularization. [37]

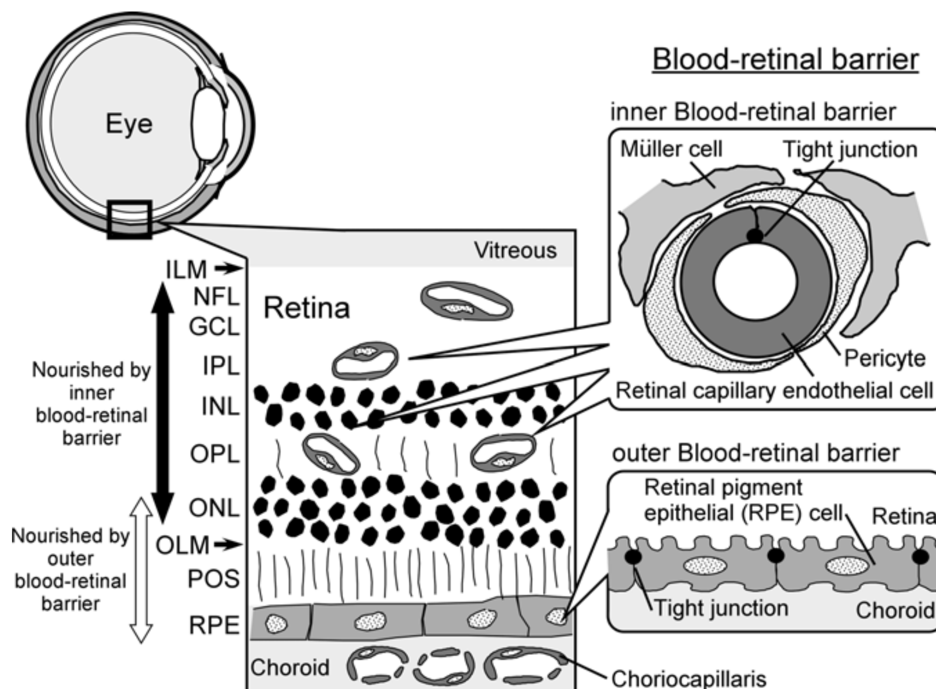

Figure 1.5: Schematic diagram of the inner and outer blood–retinal barrier and its location in the retina. The retinal cell layers seen histologically consist of RPE cells, photoreceptor outer segments (POS=RCL); outer limiting "membrane" (OLM); outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL); ganglion cell layer (GCL); nerve fiber layer (NFL); inner limiting "membrane" (ILM). Adapted from [38].

1.3.3.4 Capillary Dropout and Neovascularization

Capillary dropout is characterized by the loss of capillary components like pericytes and ECs that causes degeneration, obliteration of capillaries, ischemia, release of angiogenic growth factors and eventually retinal neovascularization. [39]

Capillaries provide nutrients and oxygen to the retina and remove its waste products in order to maintain the retinal homeostasis. In general, the high caliber blood vessels are localized in the innermost portion of the retina while capillaries are found between the nerve fiber and inner nuclear layers. [39]

In the disease there is loss of pericytes during the initial stages, which is followed by the loss of ECs. The small vessels start to bleed and leak to the surrounding tissue and the retina become ischemic/ hypoxic. In response, there are release of growth factors leading to the growth of new vessels (neovascularization) and occlusion of the pre-existing ones. The new blood vessels are abnormal and leaky which may cause microaneurysms. They can be intraretinal or extend into the vitreous cavity leading to vitreous hemorrhage and ultimately blindness. [39]

1.4 Hypoxic Response

From all the tissues in the human body, retina shows the highest oxygen (O_2) consumption and metabolic activity. This high consumption is due to the energetic requirement of the photoreceptors for the signal transduction. [40] So, an adequate and continuous O_2 supply is essential for a good retinal function. [41] [42]

One interesting fact is when the retina is under a systemic hypoxia condition (a non-ischemic form of O_2 deprivation), retinal function is impaired. Therefore, O_2 is a limited metabolite essential for a normal retinal function. [43]

Although the retina receives supplies from two separate circulations (retinal and choroidal circulation), the photoreceptor layer is avascular and the O_2 supply is barely enough. [44] Consequently, when rods operate at maximum activity, hypoxia or, in extreme cases, anoxia may develop in the inner portions of the retina in diseases with less than optimal O_2 supply. [40]

In DR, the presence of hyperglycemia and capillary dropout may expose retina to a pseudo-hypoxia and lately to hypoxia condition that can activate adaptive cell responses. One of the responses is the stabilization of hypoxia-inducible factor-1 complex (HIF-1). [41] [45] [46]

HIF-1 is a heterodimeric nuclear transcription factor composed of two units: an α and a β subunit. Both subunits are constitutively expressed in cells but HIF-1α is usually degraded under normoxia conditions by specific prolyl hydroxylases (PHDs) that targets HIF-1α for proteasome degradation.[41] [45]

Under hypoxia conditions, HIF-1α is stabilized, dimerises with HIF-1β, and translocates to the nucleus, where acts as a transcription factor of a series of genes involved in angiogenesis, glycolytic energy metabolism, cell proliferation and survival (Fig. 1.6), such as: [41] [45] [47]

- VEGF, involved in angiogenesis,
- Glucose transporter 1(GLUT1)/ glycolytic enzymes that increases the glucose uptake;
- Erythropoietin (EPO) that is essential for blood cells formation;
- Insulin-like growth factor-2 (IGF2) and transforming growth factor- α (TGF-α) that contributes to cell proliferation.

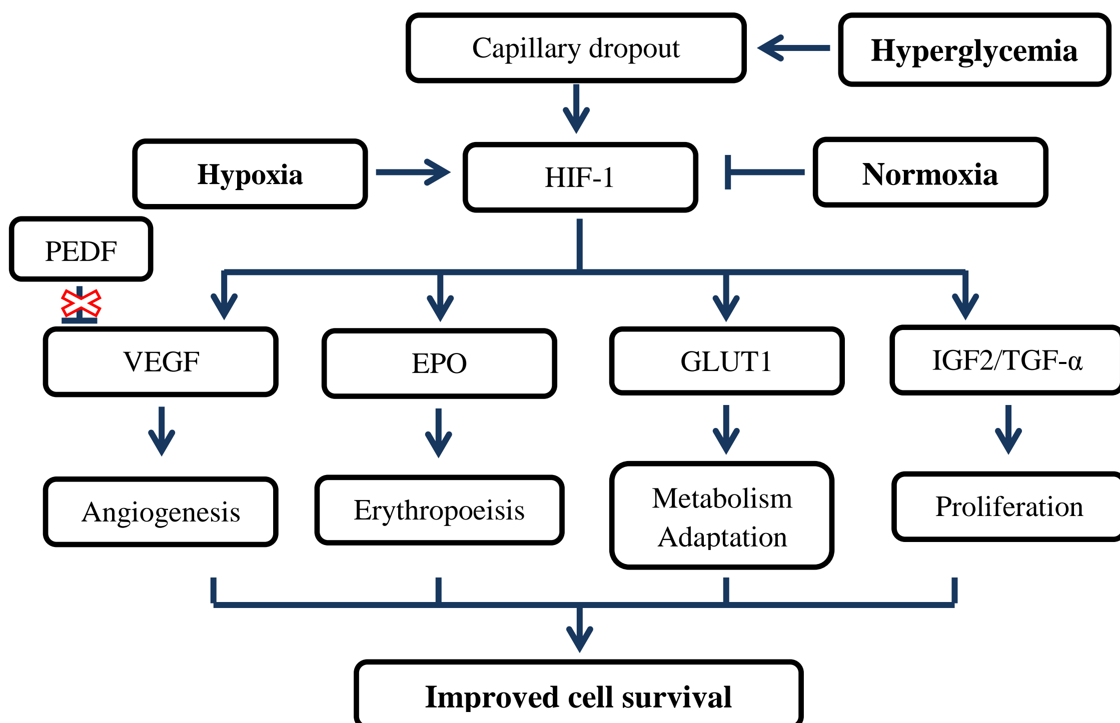

Figure 1.6: HIF-1 activity under hypoxia and normoxia conditions. Adapted from [47].

The activation of those genes by hypoxia may be the cause of the characteristic symptoms visible in DR like abnormal growth of blood vessels and neovascularization and aneurysms. Therefore, both hypoxia and hyperglycemia conditions can activate responses that drive to DR symptoms.

1.5 Factors modified in DR

To date, several possible mechanisms have been implicated in the development of DR. These mechanisms are mostly dependent on excessive transport of glucose into retinal cells, which is done by GLUT1. It has been described that this transporter is upregulated by hyperglycemia. In hypoxic conditions, as was previously described, HIF-1α is stabilized and leads to the transcription of several genes like *glut1* and *vegf*, a potent angiogenic factor that is overexpressed in DR and is mostly responsible for the vascular alterations. Also, it is known that in DR there is an imbalance between VEGF and PEDF counteracting action. [16]

Therefore, these three factors have an important role in the development of DR.

1.5.1 Glucose transporter 1 (GLUT1)

Glucose is essential for normal metabolic activity in the neural tissue. Glucose demand by the retina is enormous, which means that it is necessary an adequate glucose delivery from the circulation to supply all retinal needs. Glucose uptake to the retina is exclusively performed through the BRB by sodium-independent glucose transporter GLUT1. Therefore, changes in the GLUT1 abundance in the BRB may have profound consequences in the glucose delivery to the retina. [48]

GLUT1 is expressed in high density in erythrocytes and blood-tissue barriers (such as BRB) while the remaining GLUTs have other tissue-specific localization. Comparison studies among *glut1* of various species reveal high levels of conservation (>95%) which highlight the critical and universal importance of this transporter in cellular metabolism. Other proof of its importance is the fact that GLUT1 expression is detected in various cell types of the retina during development as early as 8 weeks of gestation. [48]

The expression of GLUT1 is regulated/ modulated by several factors such as hypoxia, growth factors and glycemia.

In cases of DR, the retina is submitted both to hypoxia and hyperglycemia. Under hypoxic conditions it is expected that the levels of GLUT1 arises by HIF-1 activation. [41] However, conflicting studies have been published regarding GLUT1 contribution for the development and progression of the disease. While some studies have reported reduction of the GLUT1 protein levels in the retina with chronic hyperglycemia [49] [50], other studies demonstrated an upregulation of the GLUT1 in the early development of DR. [14]

Moreover, it has been shown that VEGF may play indirectly a role in the GLUT1 expression on DR conditions. Specifically, a study showed that VEGF upregulates the translocation of cytosolic GLUT1 to the plasma membrane surface which consequently cause an intracellular increase of glucose in retinal cells. [51]

Therefore, the levels of GLUT1 may vary with the course of the disease.

1.5.2 Vascular endothelial growth factor (VEGF)

Recent evidence shows that new vessels growth and maturation are highly complex and coordinated processes, requiring a sequential activation of a series of receptors by numerous ligands. [52]

VEGF is not only a potent angiogenic factor and plays a role in normal physiological functions such as bone formation, hematopoiesis, wound healing and development, but also is a strong vascular permeability enhancer. [53] VEGF signaling represents a critical rate-limiting step in physiological and pathological angiogenesis. [52]

Its presence is so important that even heterozygous knockout of the *vegf* gene results in embryonic lethality. Also, VEGF is known as a vascular permeability factor based on its ability to promote vascular leakage which enhances its importance in other mechanisms like inflammation.[52]

Nowadays, the VEGF family comprises seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PLGF (placental growth factor). [52]

VEGF (also referred to as VEGF-A) has high affinity for two receptors, VEGFR-1 and VEGFR-2 both of which are express on vascular ECs. [53]

Regulation of VEGF expression is made by several factors but the most important are the oxygen tension and growth factors. [52] In DR, due to hypoxia exposure and HIF-1 activation VEGF levels are increased, as described in several publications. [54] [55] [56]

1.5.3 Pigment Epithelium–Derived Factor (PEDF)

In order to have a normal angiogenic growth, the balance between pro and anti-angiogenic factors should be tightly controlled. The disruption of this balance plays an essential role in the development of diseases. [57] PEDF was first identified as a 50 kDa secreted glycoprotein in conditioned medium from cultured fetal human RPE cells that belongs to the serine proteinase inhibitor superfamily. [58] It displays several biological activities such as neurotrophic and neuroprotective properties, anti-vasopermeability, anti-inflammatory, anti-fibrosis, and anti-cancer activities and function as an endogenous inhibitor of angiogenesis. [58] [59]

PEDF was described to inhibit angiogenesis in a number of *in vivo* assays and the decreased levels of PEDF in the vitreous and ocular tissues were associated with PRD and choroidal neovascularization. More specifically, it has been shown that PEDF and VEGF have a reciprocal interaction and PEDF counteract the angiogenic potential of VEGF which means that the increased of one of them leads to a decreased of the other. In other words, the VEGF overproduction in DR provokes a decreased production of PEDF which subsequently leads to BRB breakdown and neovascularization. [59] [60]

Thus, PEDF may play important roles in preventing the development of RD. [59]

1.6 RD Models

In vivo or *in vitro* models are important tools for understanding the biological mechanisms that are involved in the development of diseases. The *in vitro* models display several advantages over *in vivo* models like the ability to control the environment, test a specific hypothesis, no need of animal sacrifice, faster results and the amount of reagents spent are less than *in vivo*.

However, there are some disadvantages regarding this system. The environment is not precisely equal to the *in vivo* and due to that, the *in vitro* models do not behave exactly like *in vivo*.

So, the *in vivo* models have the advantage that the tissues are under similar conditions to those detected in human. Nevertheless is more expensive and takes more time to obtain the results.

1.6.1 *In vitro* models: D407 cell line

The RPE cells present in the human eye are part of the oBRB, which controls the entrance and exit of substances like nutrients, growth factors and hormones. Extensive investigations of human RPE have been limited by the need of using primary cultures. Moreover, they rarely survive more than 8 to 10 passages and they often lose their initial morphology. [61] Due to these, investigators seek out for a continuous cell line that would keep both the morphology and the function of the RPE. There are several commercially available RPE cell lines and the cell line used in this work is D407 cell line.

D407 cell line retains most of the RPE characteristics which make it possible to study potential alterations in the RPE in different conditions. This cell line came from human RPE primary culture that was spontaneously transformed and immortalized. The D407

cells retain epithelial morphology and possess a keratin-containing cytoskeleton however they lose some enzymatic activity and their polarization. [61]

1.6.2 *In vivo* models: Ins2^{Akita} mouse

Animal models are being used by numerous groups to study the pathogenesis of DR. They remain an essential part to understand the pathogenesis process and to identify promising ways to inhibit the retinal disease. There are several RD animal models like rodents, primates, cats, dogs which develop the disease by three pathways: born with the preposition to develop diabetes, induced by chemicals or develop T2DM by turning them obese. [62]

The common drugs used to induce diabetes are alloxan and streptozotocin (fig. 1.7). Although both drugs incite Langerhans isles inflammation and pancreatic β-cells destruction, they act differently. While streptozotocin, an alkaline compound, will alkylate DNA and consequently fragment it, alloxan will lead to an increase of ROS within the cells and its accumulation leads to cell death (Fig. 1.7). [63] Nevertheless, diabetes induced by toxins is becoming less successful because the animal models are becoming more resistant to drugs.

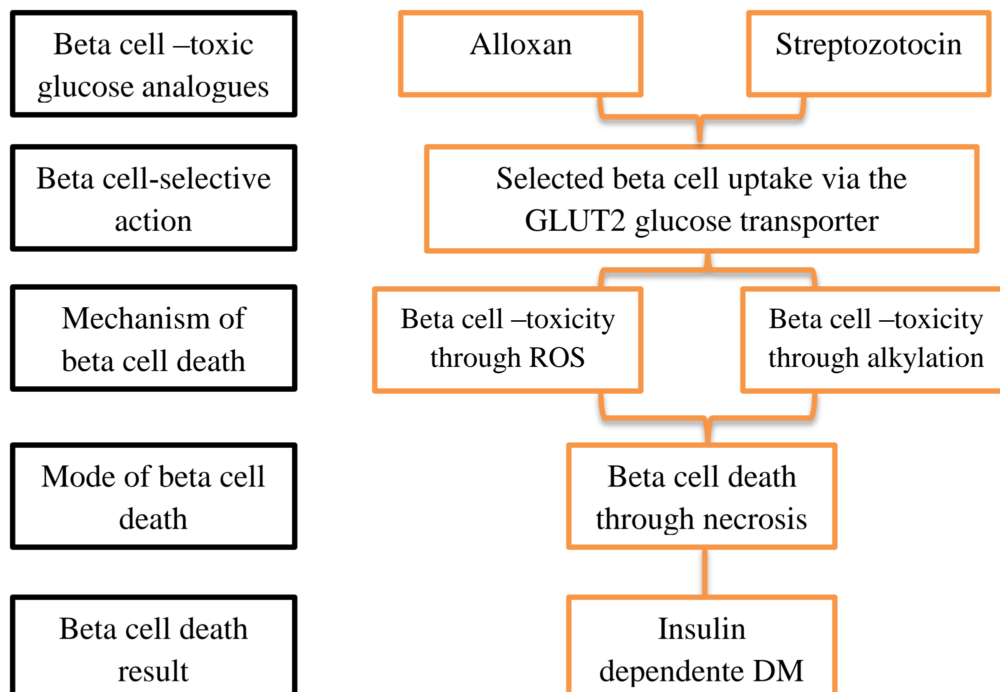

Figure 1.7: Schematic representation of the toxic effects of the glucose analogues alloxan and streptozotocin in pancreatic β cells, which produce chemical diabetes. Adapted from [63].

So, the study of the Ins2^{Akita} mouse, a spontaneous model for T1DM, is of great potential for studying diabetes' complications including DR. C57BL/6J Ins2^{Akita} mouse descend from heterozygotes male mouse for the *Ins2* gene crossed with C57BL/6J *wild-type* (WT) females. These mice possess a point mutation in the insulin gene 2 that causes the misfolding of insulin protein. This misfolded protein will accumulate in the β cells' endoplasmic reticulum and activate several responses that lead to cell death. From β-cell death results hyperglycemia and hipoinsulinemia. These symptoms start to appear as early as 4 weeks which leads to an early death. They show an increase of vascular permeability, loss of pericytes, loss of BRB function and a decrease of retinal layer thickness, characteristics of RD development. [64]

2. Objective

It has been previously described that glucose is exclusively transported into the retina by GLUT1 and hyperglycemia present in DR causes an increase of this transporter in the retina. It is also known that HIF-1 plays an important role under hypoxic conditions by acting as a transcription factor of several genes, such as GLUT-1 and VEGF. It has also been described that there is an imbalance between angiogenic and anti-angiogenic factors like VEGF and PEDF in DR. Our working hypothesis is that the increase of GLUT1 and the imbalance between VEGF and PEDF are the main cause of DR development.

Therefore, using DR models, the general aim of this work was to characterize the expression of GLUT1, VEGF and PEDF and it was divided in two parts:

2.1. Characterization of pro- and anti-angiogenic factors in *in vitro* models.

2.2. Characterization of pro- and anti-angiogenic factors in *in vivo* models.

3. Materials and Methods

3.1 MTT assay

Cells were seeded at a density of 5.0×10^4 cells/ well in 24-well flat-bottom tissue culture plates and were maintained for 72h in culture medium containing 5 mM of D-Glucose, 25 mM of D-Glucose or 25 mM of mannitol. DFO was added 16h prior the MTT assay in order to induce hypoxia. At 72 h, 50 µL of MTT (5 mg/mL, Sigma-Aldrich) were added to each well and incubated for 4 hours, at 37 ºC. To dissolve the formed formazan crystals, the medium was replaced with 500 µL of 0.04N HCl/ Isopropanol. Absorbance was measured using a micro-plate reader (Tecan Infinite 2000, USA), at 570 nm (for cell viability/formazan formation) and 630 nm (background/cell debris). After subtracting the background (optical density (OD) = OD570 nm – OD630 nm), cell viability was calculated as follows: Cell viability (%) = (OD sample)/ (OD control) × 100. Cells incubated with a latex extract in culture medium were used as control for cell death.

3.2 Cell culture and Treatment

The *in vitro* studies were performed in the human RPE cell line, D407. This cell line was derived from the eyeball of a 12 year-old child [61] and have been extensively used as a model of the RPE. [61] D407 cells were maintained in humidified atmosphere with 5% CO_2 at 37ºC in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma) containing 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (Sigma) and 1% L-Glutamin (Sigma).

D407 cells were exposed to two oxygen tension conditions, normoxia or hypoxia, and treated with 2 concentrations of glucose, 5.5mM (corresponding to a normoglycemic condition) or 25mM (corresponding to a hyperglycemic condition) (Table 3.1). [65] As a control, the cells were also treated with DMEM containing mannitol, at a final concentration of 25mM.

Mannitol is a sugar alcohol that can be used as an osmotic control substance and a glucose metabolism control because does not influence significantly the blood sugar levels. [66] [67]

Normoxia condition represents retina under normal circumstances, which worked as a control, and hypoxia condition represents retina under pathological conditions like in DR.

Table 3.1: The conditions tested *in vitro* models.

Normoxia	Hipoxia
DMEM 5mM D-Glucose	DMEM 5mM D-Glucose
DMEM 25mM D-Glucose	DMEM 25mM D-Glucose
DMEM 25 mM mannitol	DMEM 25 mM mannitol

For induction of hypoxia, it was used desferrioxamine (DFO, Sigma) at a final concentration of 100 μM, previously described in other study. [68]

DFO is an iron chelating agent often used as a hypoxia-mimetic agent to stabilize HIF-1α. [69] Under normoxic conditions and in the presence of iron, HIF-1α is hidroxylated by prolyl hydroxylases (PHDs) and subsequently degraded by the proteasome. On the other hand, under hypoxia or in the presence of DFO, due to the chelation of iron (which is required for enzymatic activity of PHD), HIF-1α is stabilized and dimerizes with its β-subunit, originating a functional complex (fig. 3.1). [70] [69]

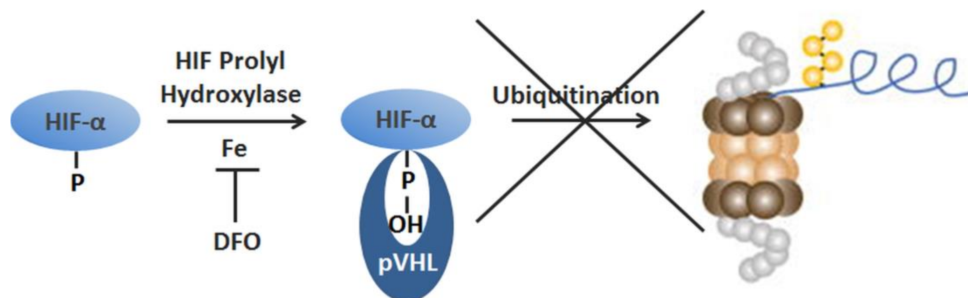

Figure 3.1: Inhibition of the HIF hydroxylation by DFO. Adapted from [71].

3.3 Animals

C57BL/6J Ins2^{Akita} heterozygote male mice (Jackson Laboratory, Bar Harbor, ME) were used as a DR model. The females were not used in this study because the disease progression is slower and less uniform. [64]

C57BL/6J age-matched (WT; Jackson Laboratory, Bar Harbor, ME) mice were used as a control group.

Mice were housed in plastic cages in a pathogen-free environment, with continuous access to food and water on a 12 hour light/ dark schedule.

The diabetic phenotype was confirmed 2 months after birth by blood glucose > 250 mg/dL (One Touch UltraEasy, Lifescan) in a drop of blood from a tail's cut.

C57BL/6J Ins2^{Akita} heterozygote mice and WT mice were sacrificed at the age of 6 and 12 months, that corresponds to the onset and chronic stage of the disease .

3.4 RT-PCR and qRT-PCR

RNA Extraction and Reverse Transcription

Total RNA from cell samples were extracted using the RNA extraction kit “Quick-RNA™ MiniPrep” (Zymo Research) according to the manufacter’s instructions.

Total RNA from neural retina were extracted using “RNeasy mini kit” (Qiagen) according to the manufacter’s instructions.

After spectrophotometric quantification of RNA using *NanoDrop 2000c* (Thermo scientific), reverse transcription was carried out using “The High Capacity cDNA Reverse Transcription Kits” (Applied Biosystems) according to the manufacter’s instructions.

Polymerase chain reaction (PCR)

cDNA amplification was performed by using 1µg of RNA as template, PEDF, VEGF, GLUT1 or β-ACTIN specific primers (10µM; see Appendix 1), dNTPs, cDNA, taq (Invitrogen), taq buffer, $MgCl_2$ (50mM) and sterile water. The amplification parameters set for PEDF and VEGF were 95ºC for 5 min, 95ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min (total 35 cycles) and for GLUT1 and β-ACTIN were 95ºC for 5 min, 95ºC for 30 s, 62ºC for 30 s, 72ºC for 1 min (total 35 cycles).

The amplification products were loaded in a 2% electrophoresis gel and stained with GreenSafe Premium (NzyTech).

Real Time PCR (qRT-PCR)

Specific primers for PEDF, VEGF, GLUT1 or β-ACTIN (5µM) were used for qRT-PCR (Appendix 1). The qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) according to the manufacter’s instructions.

The average CT (threshold cycle) of fluorescence units was used to analyze the mRNA levels. PEDF, VEGF and GLUT1 mRNA levels were normalized by β-ACTIN RNA levels. Quantification was calculated as: mRNA levels (percent of control) = $2^{\Delta(CT)}$ with $\Delta(CT) = C_{T\,(PEDF,\,VEGF,\,GLUT1)} - C_{T\,\beta\text{-Actin}}$.

3.5 Western Blot analysis

Primary Antibodies: Anti-PEDF antibody ab180711 (rabbit, Abcam); Anti-VEGF antibody ab46154 (rabbit, Abcam); Anti-Glucose Transporter GLUT1 antibody ab32551 (rabbit, Abcam). Dilutions described in table 3.2.

Secondary Antibodies: Goat anti-mouse IgG-HRP: sc-2005 (Santa Cruz Biotechnology); goat anti-rabbit IgG-HRP: sc-2004 (Santa Cruz Biotechnology).

Table 3.2: Antibodies dilutions for Western Blot.

Western Blot				
	1°Antibody dilution	Time	2° Antibody Dilution	Time
GLUT1	1:3000	ON/4ºC	1:5000	1h/RT
VEGF	1:1000	ON/4ºC	1:5000	1h/RT
PEDF	1:1000	ON/4ºC	1:10000	1h/RT
β-Actin	1:10000	1h/RT	1:5000	1h/RT

Protein Extraction

For protein extraction from cells culture samples, the D407 cells were plated in 60 mm dishes until reach 90% of confluence. The whole extract proteins were collected in cold RIPA buffer (50 mM Tris-HCl pH 7.4, 0.25% Na-deoxycholate, 1% NP-40, 150 mM NaCl, 1mM EDTA) containing protease inhibitor cocktails.

The lysate was cleared by centrifugation at 13200 rpm for 20 min at 4ºC and the supernatant stored at – 20ºC, for further quantification using the Bradford assay.

Eyes from animals of 6 and 12 month-old were dissected after cervical dislocation. For neural retina isolation, the anterior chamber, containing the cornea, lens, vitreous and aqueous humor was removed, and the retina was peeled from RPE. Retinas and RPE were dissected, homogenized in 100µl of cold RIPA buffer and incubated at 4ºC for 20 min. Homogenates were cleared by centrifugation at 13200 rpm for 20 minutes at 4ºC and stored at -80ºC. The protein concentration was determined using the Bradford assay.

Western blot

A total of 30 µg of protein were resolved by 12% SDS-PAGE gel, transferred to a PVDF membrane (GE HealthCare) and blocked in Superblock Blocking solution (Thermo Scientific), containing 0.1% of Tween-20 for 1h at RT. Then, the membrane was incubated

ON, at 4ºC with the primary antibody diluted in Superblock Blocking solution containing 0,1% Tween 20.

The membrane was washed with Tris-Buffer Saline (20 mM Tris base, 150 mM NaCl pH 7,6) with 0,1% tween 20 (TBS.T; 3 x 10 min), and was probed with an HRP-conjugated secondary antibody for 1h at RT diluted in Superblock Blocking solution 0,1% Tween 20.

As a loading control, we used β-Actin. For β-Actin detection, the membrane was blocked for 1h at RT with Superblock Blocking solution 0,1% Tween 20 and incubated for 1h at RT with β-Actin antibody diluted in Superblock Blocking solution 0,1% Tween 20. After it was washed with TBS.T (3 x 10 min), it was probed with HRP-conjugated secondary antibody diluted in Superblock Blocking solution 0,1% tween for 1h at RT.

Immunoreactive bands from the membrane were detected through *ChemiDoc MP System* (Bio-Rad) by its chemiluminescence, using an ECL plus kit (GE Healthcare).

3.6 Immunocytochemistry and Immunohistochemistry

Primary Antibodies: Anti-PEDF antibody Sc-25594 (rabbit, Santa Cruz); Anti-VEGF antibody ab46154 (rabbit, Abcam); Anti-Glucose Transporter GLUT1 antibody ab32551 (rabbit, Abcam). Dilutions are in table 3.3 and 3.4.

Secondary antibodies: Alexa fluor Goat anti-rabbit 594 (Molecular Probes).

Immunocytochemical Staining of PEDF, VEGF and GLUT1

Table 3.3: Antibodies dilutions for Immunocytochemistry.

Immunocytochemistry				
	1ª Antibody dilution	Incubation Time	2º Antibody dilution	Incubation Time
GLUT1	1:250	1h/RT	1:500	1h/RT
VEGF	1:100	1h/RT	1:500	1h/RT
PEDF	1:100	1h/RT	1:500	1h/RT

D407 cells were seeded at a concentration of 7 x 10^4 cell/well and grown on sterile glass coverslips in 12-well plates with culture media containing different concentrations of D-Glucose, for 3 days. To mimic hypoxic conditions, the cells were then incubated with DFO at concentration of 100 μM, for 16h and fixed for 10 min in ice-cold methanol, for 10

minutes. Methanol is an organic solvent that preserve the cells by removing its lipids, then dehydrate them and precipitate the proteins present in the cells. [72]

Following fixation, the cells were washed in 0,2% Triton X-100 in Phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7,4) (3 x 5 min) and incubated with a blocking solution (1% goat serum in PBS 0,2% Triton X-100) for 30 min, at room temperature (RT) before incubating with the primary antibody diluted in PBS 0.2% Triton X-100 for 1h at RT.

After 3 washes with PBS-T, the coverslips were incubated with the secondary antibody, for 1h at RT, protected from light. The cells were then washed in PBS 0,2% Triton X-100 (3 x 5 min) and mounted with Fluoromount G (Electron Microscopy Sciences) containing DAPI, for following light microscopy analysis.

Images were obtained using an AxioVision microscope with a 63× objective, using the appropriate filter sets (Axio Observer Z2, Zeiss).

Immunohistochemical staining of PEDF, VEGF and GLUT1 in the mouse retinas

Table 3.4: Antibodies dilutions for Immunohistochemistry.

Immunohistochemistry				
	1ª Antibody dilution	Incubation Time	2º Antibody dilution	Incubation Time
GLUT1	1:250	ON/4ºC	1:500	1h/RT
VEGF	1:100	ON/4ºC	1:500	1h/RT
PEDF	1:100	ON/4ºC	1:500	1h/RT

The eyes were enucleated and immediately fixed in ice-cold 4% paraformaldehyde (PFA) in PBS, for 16 hours, cryoprotected in 30% sucrose (Sigma) in PBS and embedded in optimal cutting temperature (OCT; VWR) mounting medium, for cryosection. 10 µm thick serial sections were performed, placed on glass slides and stored at -20ºC. After drying for 1h30, the frozen sections were permeabilized with PBS 0.2% Triton X-100 for 10 min.

The retina is rich in endogenous fluorescence components such as melanin, lipofuscin and carotenoids which difficult the visualization of normal fluorescence. [73] To reduce the autofluorescence of the retina, the slices were incubated with 0.25% of potassium permanganate ($KMnO_4$) in PBS for 15 min at RT, followed by a wash step with PBS 0.2%

Triton X-100 (2 x 5 min) and treated with 5% of oxalic acid ($H_2C_2O_4$) in PBS. Both $KMnO_4$ and $H_2C_2O_4$ have been described as bleachers to the retina autofluorescence. [74]

After reducing the autofluorescence, the sections were washed with PBS 0.2% Triton X-100 (3 x 10 min), blocked with 1% of goat serum in PBS 0.2% Triton X-100 for 1h at RT, followed by incubation with the primary antibody diluted in PBS 0.2% Triton X100.

After an ON incubation at 4ºC, , the cells were washed with PBS 0.2% Triton X-100 (4 x 10 min) and then incubated with the secondary antibody diluted in PBS 0.2% Triton X-100 for 1h at RT, in a humid chamber protected from light.

The samples were then washed with PBS 0.2% Triton X-100 (4 x 10 min), mounted on glass slides with Fluoromount G containing DAPI and the images were obtained using an AxioVision microscope with a 40× objective, using the appropriate filter sets (Axio Observer Z2, Zeiss).

3.7 Cellular protein membrane extraction

Cells were grown on 100 mm dishes to reach confluence and were washed with ice-cold PBS (2x). The cells were homogenized in 1 ml of homogenization buffer (20 mM HEPES pH 7.4; 1mM); EDTA; 250 mM Sucrose; 1x protease inhibitors cocktail).

Homogenates were cleared by centrifugation at 3500 rpm for 10 minutes at 4ºC and the supernatant transferred to a fresh centrifuge tube. The lysate was then centrifuged at 100 000 g for 1h at 4ºC.

The resulting supernatant, corresponding to the cytosolic fraction, and the pellet, corresponding to the membrane fraction, was resuspended in a resuspension buffer (10 mM HEPES pH 7.4; 250 mM Sucrose; 1x protease inhibitor cocktail).

The protein concentration was determined using the Bradford assay.

4. Results and Discussion

As previously reported, DR is a microvascular complication associated with DM and the leading cause of blindness in developed countries. [75] Some studies have shown that mechanisms involving hyperglycemia and hypoxia are key factors in the onset, progression and prognosis of the disease. [42] [76] The increase of glucose by GLUT1 activates several biochemical pathways that culminate with DR appearance. [14] It was also described that in patients suffering DR there is an imbalance between pro- and anti-angiogenic molecules, such as VEGF and PEDF, respectively, that are also responsible for the onset of the disease. [59] [60]

So, with this study, we intended to evaluate the expression of GLUT1, VEGF and PEDF in DR models under DR conditions.

4.1 Characterization of pro- and anti-angiogenic factors in *in vitro* models

To evaluate the expression of pro- and anti-angiogenic factors we developed an *in vitro* system DR, using human RPE cells. D407 cells were cultured either with 5 mM of D-Glucose (corresponding to normoglycemia) or 25 mM of D-Glucose (simulating hyperglycemia). In addition, cells were subjected to hypoxia by the addition of DFO, a chelating agent that induces hypoxia by inhibiting HIF-1α degradation at the proteasome.

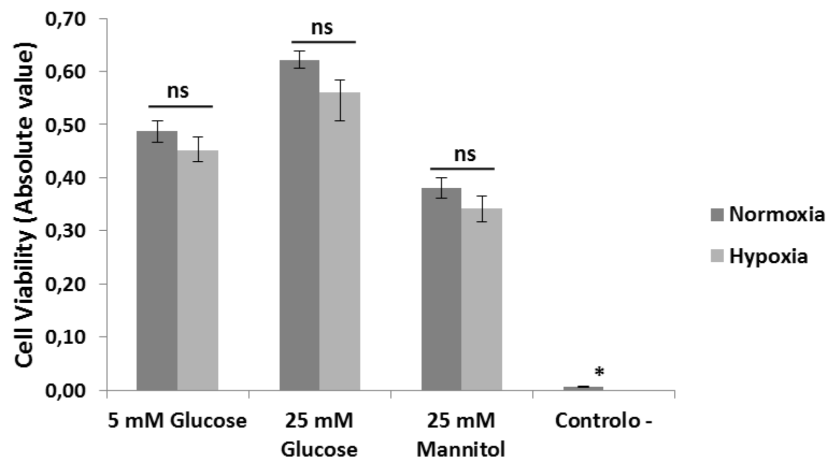

Figure 4.1: Effect of glucose and DFO upon cellular viability of RPE cells. Cellular viability, measured by MTT assay, of D407 RPE cells in the presence of different concentrations of glucose (5.5 mM and 25 mM of D-Glucose) and mannitol upon exposure to DFO during 16h. Latex extract was used as the negative control for viability. *P<0.05 indicates that latex extract values are significantly different from glucose values determined by Sidak's multiple comparisons test.

To exclude possible toxicity induced by the addition of DFO to the cells, we decided to perform an MTT assay. The MTT assay is a colorimetric assay that assesses the

cytotoxicity and the cell viability under defined conditions in which viable cells convert MTT into a purple colored formazan product which it is measurable. [77] Our results demonstrate that DFO does not induce significant cell death in all the concentrations tested (fig. 4.1).

4.1.1 Characterization of GLUT1 in D407 cells

In order to assess how glucose concentration and hypoxia influences GLUT1 expression, we decided to analyze GLUT1 mRNA levels by real-time qPCR (fig. 4.2). Our results show that hypoxia induces an increase (fig. 4.2A) of 20-fold in GLUT1 mRNA (fig. 4.2B). According to literature, the increase detected in hypoxia is expected due to the HIF-1 cascade activation. [78] Moreover we found no significant differences in GLUT1 mRNA levels among the various glucose concentrations in which the cells were cultured (Fig. 4.2). This result is also in line with literature that state that there is no significant change in the levels of GLUT1 mRNA between cells incubated in medium with low and high glucose. [50]

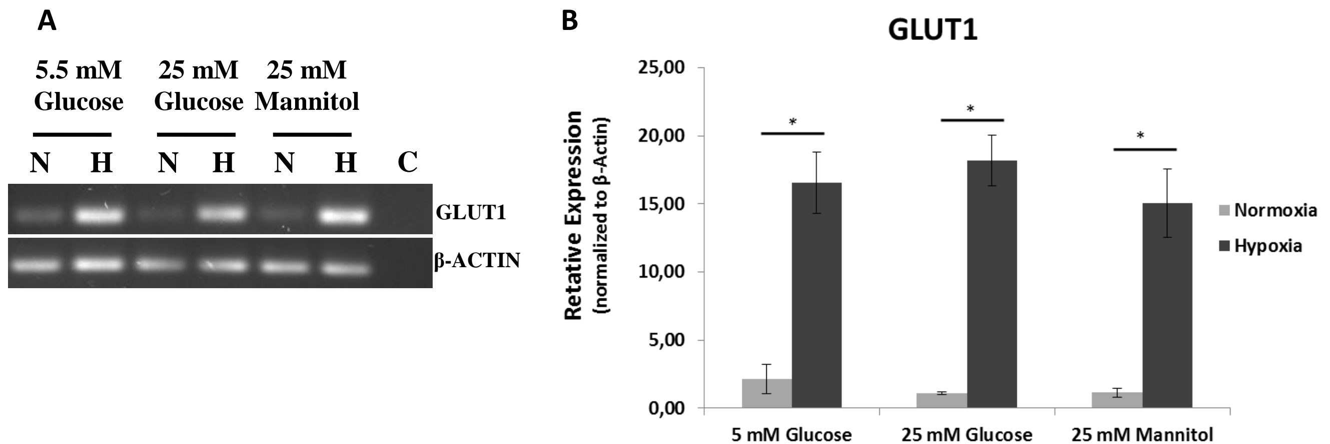

Figure 4.2: GLUT1 mRNA expression in RPE cells cultured under diabetic environment. PCR (A) and qPCR (B) analysis of GLUT1 in D407 cells cultured under normoxia (N) and hypoxia (H) conditions and different concentrations of glucose in the culture medium: 5 mM of D-Glucose (corresponding to normoglycemia), 25 mM of D-Glucose (simulating hyperglycemia) and 25 mM of Mannitol (osmolarity control). N=3, *$P<0.05$ indicates significant differences in the mRNA levels of GLUT1 in hypoxia compared with the cells cultured in normoxia, determined by Tukey's multiple comparisons test.

We also evaluated GLUT1 expression at the protein level. We found a marked increase in GLUT1 protein expression induced by hypoxia in the cells cultured under diabetic conditions (fig. 4.3). However, figure 4.3 shows no difference, at the protein level, in the normoglycemic conditions, in normoxia compared with hypoxia. This means that despite the increase of GLUT1 mRNA levels due to hypoxia (fig. 4.2B), there is no translation into

protein. One hypothesis to explain this result is the translation machinery is diminished in hypoxic conditions which may affect the translation of GLUT1. [79] On the other hand, we verify an increase of about 1.5 times in GLUT1 protein (fig. 4.3B) in the cells cultured with 25 mM of D-Glucose subjected to hypoxia when compared with the cells cultured with the same glucose concentration under normoxia conditions, showing a positive effect of glucose concentration in the translation of GLUT1 mRNA into protein. These results are complementary with the previous results and support by the literature. [80]

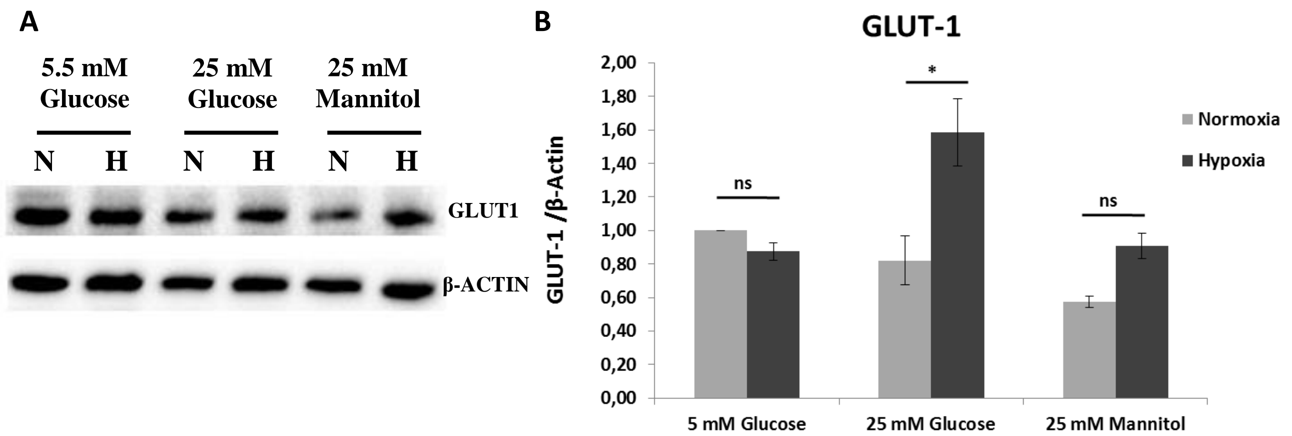

Figure 4.3: Effects of glucose and hypoxia in GLUT1 protein expression. (A) corresponds to Western Blot analysis of GLUT1 in the different treatments performed and (B) represents the quantitative data normalized by the intensity of β-ACTIN bands. N=4, *P<0.05 represents significant difference and ns indicates no significant difference in GLUT1 protein level in the cells cultured under hypoxia with high glucose concentration medium, determined by Tukey's multiple comparisons test.

In order to better understand the mechanism underlying GLUT1 overexpression in the cells cultured in a diabetic condition, we performed an immunocytochemistry to trace GLUT1 in human RPE cells (fig. 4.4). There is a clear overexpression of GLUT1 in the membrane of the hypoxic cells compared with the normoxic cells (fig. 4.4). These results are in accordance with the previous hypothesis and the literature which says that in cells and tissues such as cardiac and skeletal muscle there is a major translocation of Glut4 and Glut1 induced by hypoxia. [81]

Also, it is possible to observe in figure 4.4 that GLUT1 staining was stronger in the hypoxic cells cultured under a hyperglycemic condition compared with the cells cultured under a normoglycemic condition which is in accordance with the previous results from Western blot.

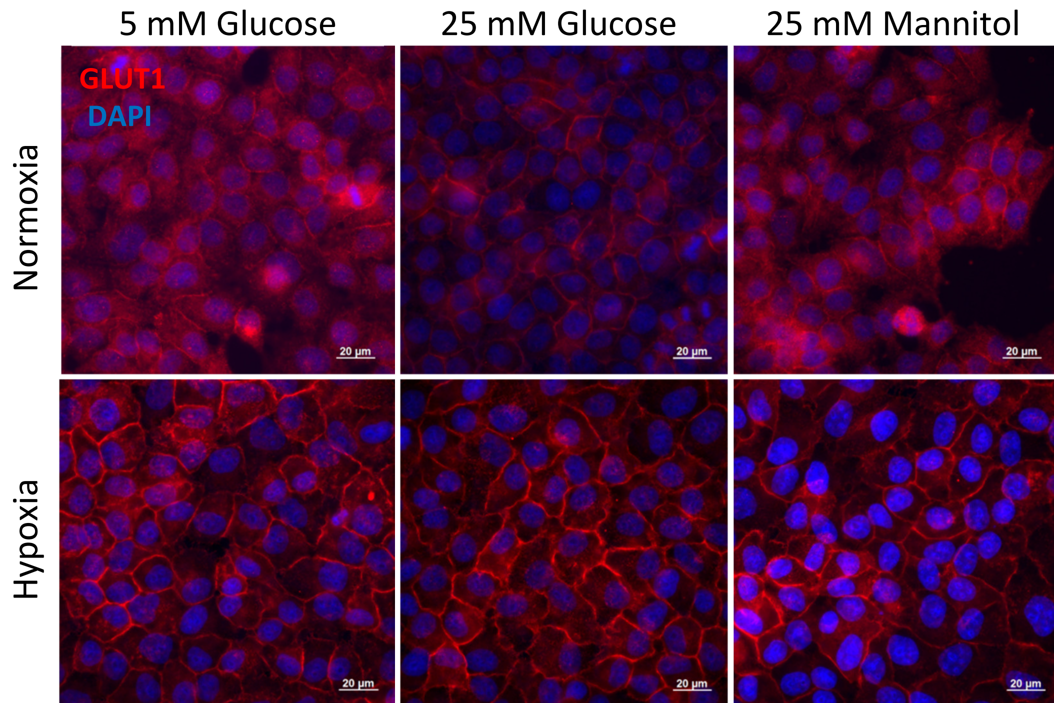

Figure 4.4: Immunocytochemistry staining of GLUT1 (red) in human RPE cells. D407 cells were cultured with different concentrations of glucose and were subjected to hypoxia and normoxia conditions. DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 20 μM.

To prove our previous results we decided to isolate cytosolic and membrane proteins and we evaluated GLUT1 expression in both fractions by Western blot (fig. 4.5). As we can see illustrated in figure 4.5, there is a marked increase of GLUT1 expression in the membrane fraction of the hypoxic cells compared with the normoxic cells which is in accordance with the hypothesis that there is translocation of the GLUT1 to the membrane in response to hypoxia (fig. 4.5).

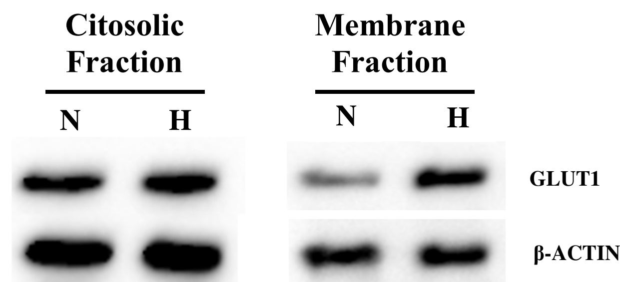

Figure 4.5: GLUT1 stabilization in the cell membrane in response to hypoxia. Western Blot analysis of GLUT1 in the cytosolic and membrane fraction of D407 cells cultured with 25 mM of D-Glucose, subjected to normoxia (N) and hypoxia (H).

Moreover, we could not observe a significant increase of GLUT1 in the cytosolic fraction of the hypoxic cells, supporting the idea that the increase that the overall increase in GLUT1 that we observed in the Western blot analysis is due to an increase of GLUT1 expression in the those cells. This increase can be due to the fact that hypoxic cells need to increase glucose uptake due to enhanced anaerobic glycolytic pathway. [82]

4.1.2 Characterization of PEDF in D407 cells

After the evaluation the GLUT1 expression, we decided to assess the PEDF expression in hypoxic conditions and cultured underlow glucose and high glucose concentrations. First, we decided to analyze the mRNA levels of PEDF by real time qPCR. Despite our efforts and after changing several times the primers used for this experiment (appendix 1), we were not able to access PEDF mRNA expression due to unspecific amplification of the negative control. Due to this, we decided to move on and evaluate the protein level of PEDF by Western blot on those conditions (fig. 4.6).

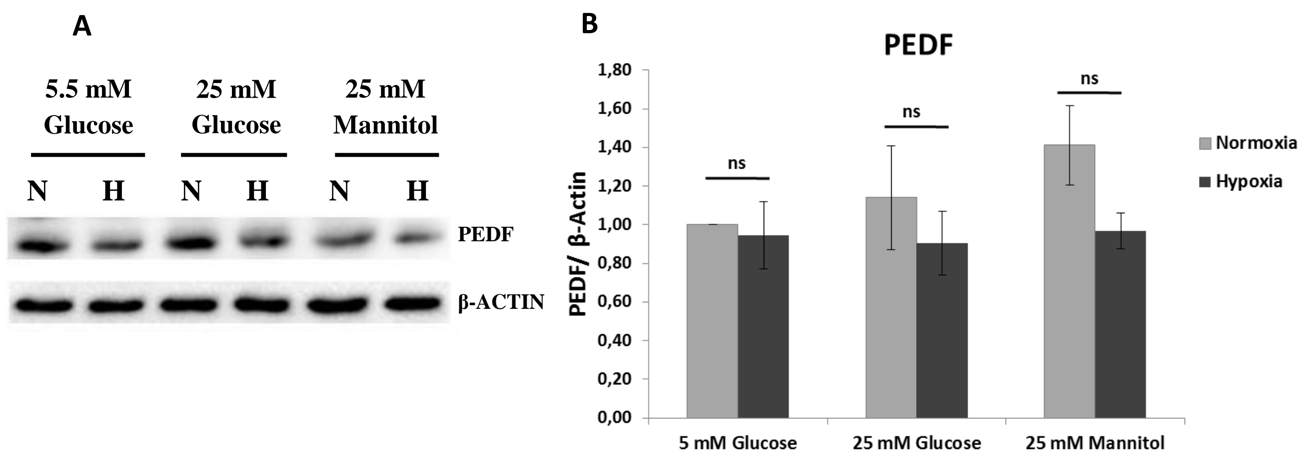

Figure 4.6: Effects of glucose and hypoxia in PEDF protein expression. (A) Corresponds to Western Blot analysis of PEDF in the different treatments performed and (B) represents the quantitative data normalized by the intensity of β-Actin bands. N=4, ns represents no significant difference ($P>0.05$) in PEDF protein level in the cells cultured under hypoxia with high glucose concentration medium, determined by Tukey's multiple comparisons test.

As we can see represented in figure 4.6B there is no significant difference between hypoxic and normoxic conditions in the cells cultured under normoglycemia and hyperglycemia conditions. Nevertheless we can see in figure 4.6 (A and B) there is a slight tendency for decrease in PEDF expression in the cells cultured under hypoxia conditions when compared with the cells cultured under normoxia conditions. This result is in

accordance with the literature. [83] The slight decrease of the PEDF expression may be explained by the fact that this factor is produced and secreted by RPE cells. This can be the reason why we cannot detect a significant decrease in the intracellular protein. [84] Since PEDF is a secreted protein, it would be interesting to evaluate the protein levels in the supernatant of the cells and verify if its expression is altered.

Subsequently, we evaluate the expression levels of PEDF in D407 cells by immunocytochemistry (fig. 4.7).

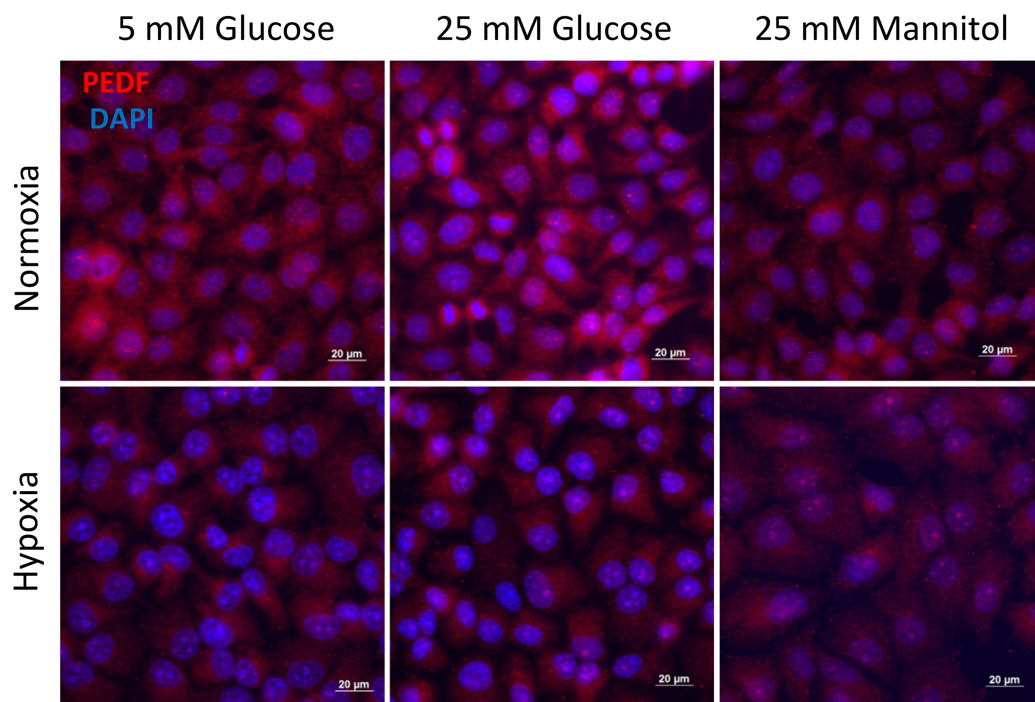

Figure 4.7: Immunocytochemistry staining of PEDF (red) in human RPE cells. D407 cells were cultured with different concentrations of glucose and were subjected to hypoxia and normoxia conditions. DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 20 µM.

As we can see in figure 4.7 there is a slight decrease in PEDF expression in the cells cultured under hypoxic condition. This result is in accordance to our Western blot results in which we found a tendency to decrease in PEDF protein.

4.1.3 Characterization of VEGF in D407 cells

Knowing that VEGF expression levels are imbalanced in DR, we also decided to evaluate the VEGF expression in hypoxic conditions and under normoglycemia and hyperglycemia conditions. To start, we analyzed the mRNA expression levels of VEGF by real time qPCR (fig. 4.8).

In our PCR (fig. 4.8A) result it seems that there is a slight increase between normoxia and hypoxia. This result was confirmed by qPCR analysis (fig. 4.8B) which shows that hypoxia induces a significant increase (of about 3 times) in VEGF mRNA than in normoxia. These results are in accordance with the literature which says that HIF-1α, that it is stabilized in hypoxia, induces VEGF expression. [56]

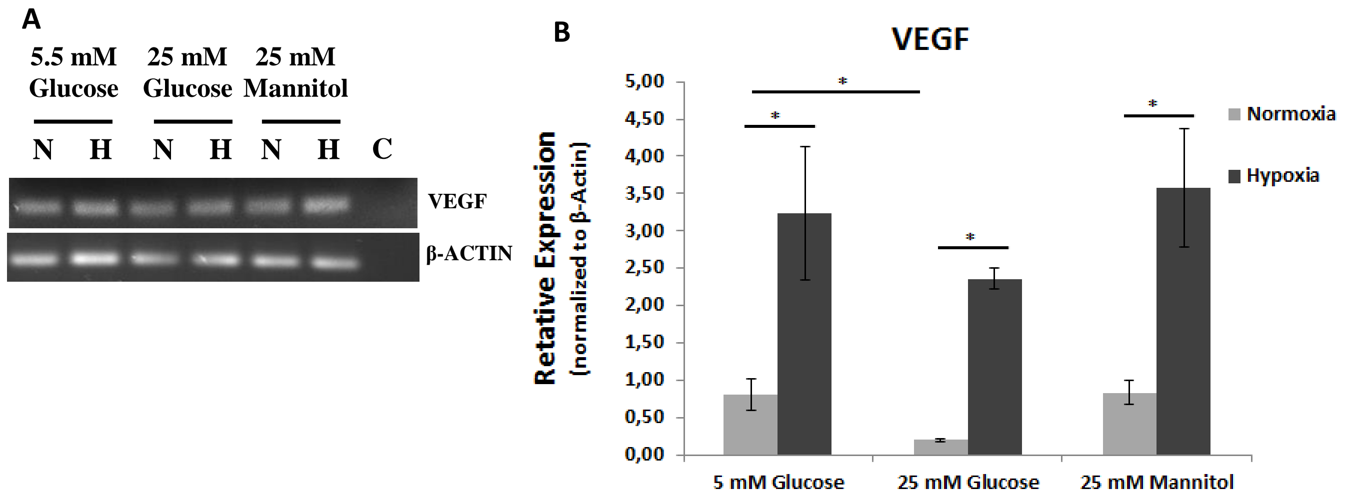

Figure 4.8: VEGF mRNA expression in RPE cells cultured under diabetic environment. PCR (A) and qPCR (B) analysis of VEGF in D407 cells cultured under normoxia (N) and hypoxia (H) conditions and different concentrations of glucose in the culture medium: 5 mM of D-Glucose, 25 mM of D-Glucose and 25 mM of Mannitol. N=3, *P<0.05 indicates significant differences and ns indicates non-significant differences in the mRNA levels of VEGF in hypoxia compared with the cells cultured in normoxia, determined by Tukey's multiple comparisons test.

However, when we compared different glucose concentrations under normoxia conditions, we can see that there is a slight decrease of the VEGF mRNA expression in hyperglycemia (fig. 4.8B). This result was opposite to what we were expecting. We were expecting to have a stronger increase in VEGF mRNA expression in the cells cultured with high glucose medium. One hypothesis to explain this result is the fact that the VEGF is a factor that regulates glucose passage across blood barriers so, before the typical increase of VEGF mRNA expression, maybe there is a decrease as a defense mechanism of the cell to prevent more glucose entrance. [85] In addiction, other study demonstrates that hyperglycemia has been shown to reduce the levels of VEGF RNA whose our results seems to be in accordance.[86] So, we can say that hypoxia maybe is the main promoter of the VEGF mRNA increased expression.

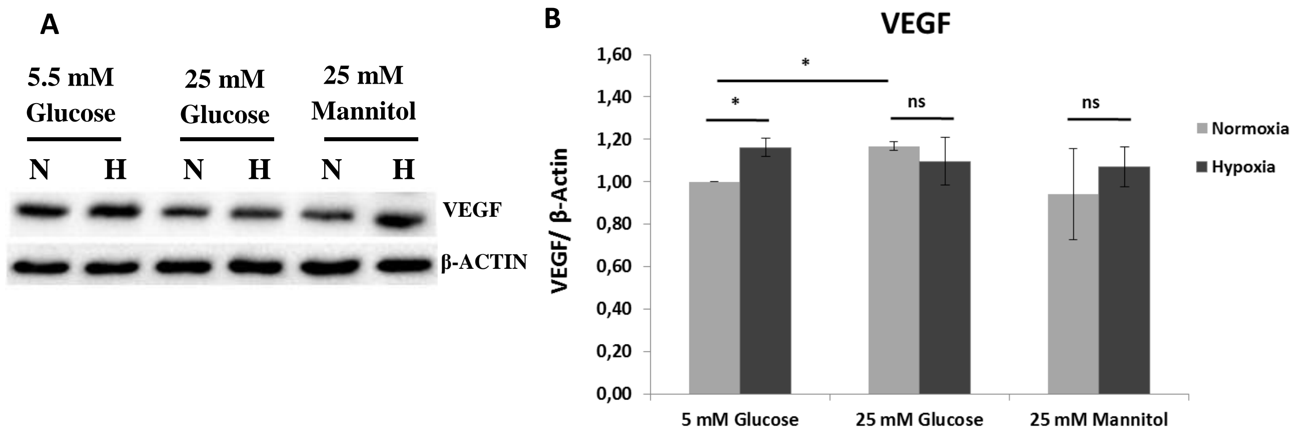

Figure 4.9: Effects of glucose and hypoxia in VEGF protein expression. (A) Corresponds to Western Blot analysis of VEGF in the different treatments and (B) represents the quantitative data normalized by the intensity of β-Actin bands; N=4 ,ns indicates no significant difference in VEGF protein level in the cells cultured under hypoxia with high glucose concentration medium, determined by Tukey's multiple comparisons test.

Next, we decided to analyze the protein expression of VEGF (fig. 4.9). In figure 4.9A we can see that in normoglycemia there is a slight increase of VEGF protein comparing normoxia with hypoxia which is in accordance with the qPCR results and the literature (Fig. 4.9B). [87] Similarly, despite the slight decrease at mRNA level, there is a significant increased at protein level in the cells cultured with high glucose medium compared with the cells cultured with normoglycemic medium. This result suggests that the hyperglycemia effect on VEGF expression is induced at translational level. [88] However, when we evaluated the protein expression in high glucose condition, we can see that there is not a significant difference between normoxia and hypoxia conditions. This result is not in accordance with the literature that shows an increase in VEGF expression levels due to hyperglycemia and hypoxia. One possible explanation is the counteraction of PEDF in mRNA translation into protein. As we described earlier, PEDF expression levels are not changed in the presence of hypoxia and hyperglycemia, so we hypothesized that PEDF may lead to the blockage of VEGF production, as it was previously described by Takenaka et al. [89] Moreover, similarly to PEDF, VEGF is also a secreted protein and in critical conditions such as hypoxia, the produced protein is secreted to the supernatant decreasing the intracellular content of VEGF, explaining the slight decrease we observed. [90]

In order to confirm the previous results, we also performed an immunocytochemistry to trace VEGF in human RPE cells (fig. 4.10). However, our results seem to show that there is no significant alteration in the expression of VEGF which is partially in accordance with the Western blot analysis (fig. 4.9).

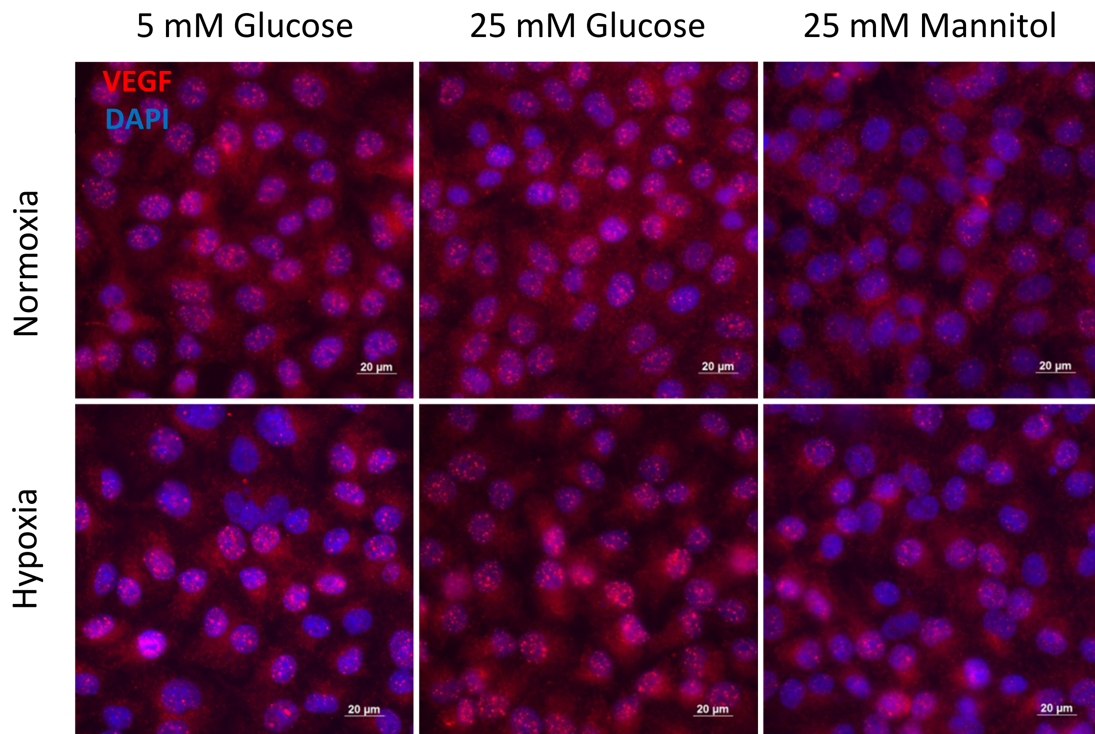

Figure 4.10: Immunocytochemistry staining of VEGF (red) in human RPE cells. D407 cells were cultured with different concentrations of glucose and were subjected to hypoxia and normoxia conditions. DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 20 µM.

4.2 Characterization of pro- and anti-angiogenic factors in *in vivo* models

To better understand the role of these factors in DR, we evaluated their expression *in vivo*. In this study we used retina and RPE samples of C57BL/6 WT and Ins2^{Akita} mice with 6 and 12 months of age.

Previous studies in Ins2^{Akita} mice indicated that clinical symptoms of DR start at 6 month of age, which represents the initial phase of the disease. [91] [92] Other study also showed that at 9 months of age the Ins2^{Akita} start to develop PDR. [92] Therefore, the evaluation of angiogenic factors in mice with 12 months already represents severe state of the disease.

4.2.1 Characterization of GLUT1 in mouse models

Similarly to what was performed for the *in vitro* models, we started by analyzing GLUT1 mRNA expression levels by real time qPCR in retinas of 6 and 12 month-old mice (fig. 4.11).

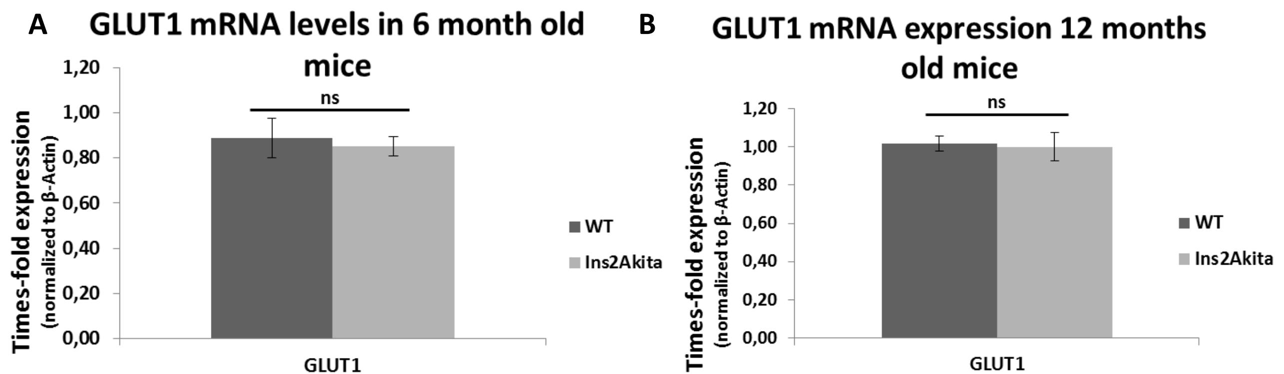

Figure 4.11: qPCR analysis of GLUT1 in C57BL/6 WT and Ins2^{Akita} with 6 (A) and 12 months (B) of age. N=6; non-significant (ns) indicates no significant differences in the mRNA levels of GLUT1 in WT compared and Ins2^{Akita}, determined by Tukey's multiple comparisons test.

In figure 4.11, in contrast to what we expected, there is no significant difference in GLUT1 expression in the WT retinas, when compared with the retinas of Ins2^{Akita} mice, for both the samples of animals with 6 and 12 months-old (fig. 4.11B). This can be due to the fact that at 6 months of age mice are at the onset of the disease, which may not yet affect the mRNA expression of GLUT1. [91] Likewise, we found no significant differences in the GLUT1 mRNA expression levels at 12 months of age. This can be related with the severe state of the disease where the retina is senescent. [93]

The expression of GLUT1 was also evaluated by Western blot and as we can see in figure 4.12, there is a significant increase of GLUT1 protein in the retina of 6 month-old Ins2^{Akita} when compared with the WT animals.

This increase is in accordance to what we were expecting and may indicate that the mice are at the beginning of the disease when hyperglycemia is already installed and, consequently, there is an overexpression of GLUT1 protein. However this is not in agreement with our qPCR results in which we see no increase in the mRNA expression of GLUT1 in the retinas of the diabetic mice, when compared with WT (non-diabetic). This could be due to the fact that the mice may be in the early phase of the disease where the increase of the expression may be mediated at translational level leading to a difference at the protein level but not at the mRNA level. [81]

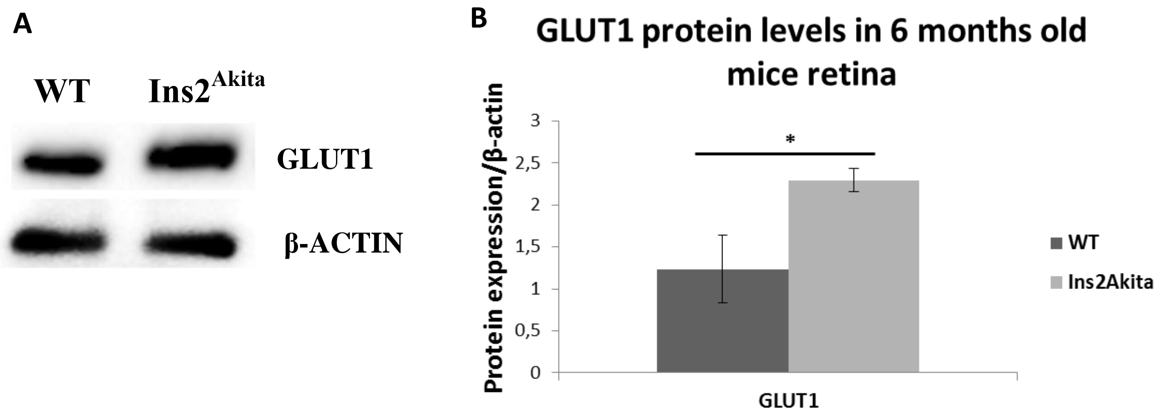

Figure 4.12: GLUT1 expression in C57BL/6 WT and Ins2^{Akita} with 6 months of age in the retina. (A) corresponds to Western blot analysis of GLUT1 expression levels and (B) corresponds to the quantitative data normalized by the intensity of β-Actin bands. N=4; *P<0.05 represents significant difference in glut1 protein level GLUT1 between C57BL/6 WT and Ins2^{Akita}, determined by Tukey's multiple comparisons test.

Nevertheless, there is no significant difference in the expression of GLUT1 at 12 months of age in both strains (fig. 4.13A and 4.13B). This result is in accordance with the mRNA levels at this age which may also be associated with the presence of a severe state of the disease that is installed at that age. [93] However, it seems that GLUT1 protein has tendency to be higher in Ins2^{Akita} than in WT. This result is in accordance with the literature that says that in the presence of chronic hyperglycemia there is an increase of Glut1 expression. [14]

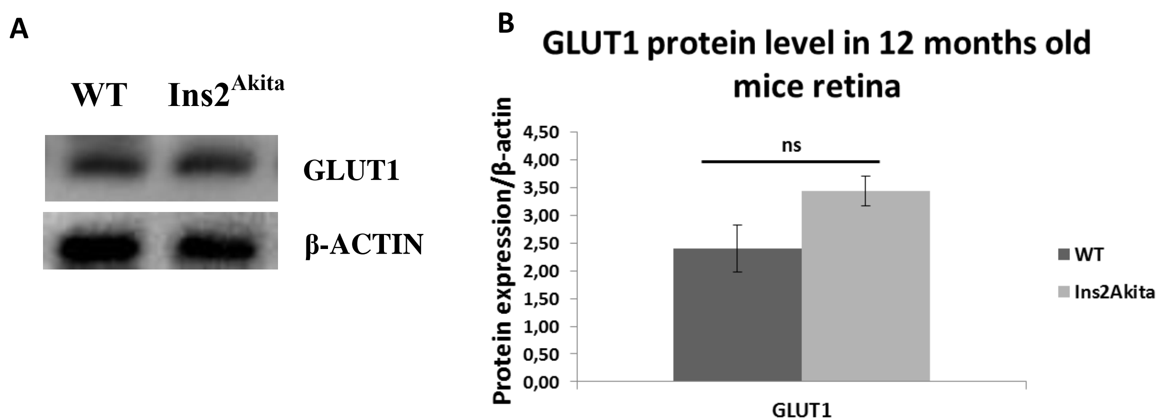

Figure 4.13: GLUT1 expression in C57BL/6 WT and Ins2^{Akita} with 12 months of age in the retina. (A) corresponds to Western blot analysis of GLUT1 expression levels and (B) corresponds to the quantitative data normalized by the intensity of β-Actin bands. N=3; ns indicates no significant differences in the protein expression of GLUT1 in WT compared with Ins2Akita, which was determined by Tukey's multiple comparisons test.

Figure 4.14 represents an immunohistochemistry of Glut1 in the retina of animals with 6 months. Interestingly there is a marked increase in Glut1 staining in the membrane of the retinal cells of Ins2^{Akita} mice. This result is in accordance with our previous results in D407 cells (fig. 4.5) that shows a predominant expression in the cellular membranes.

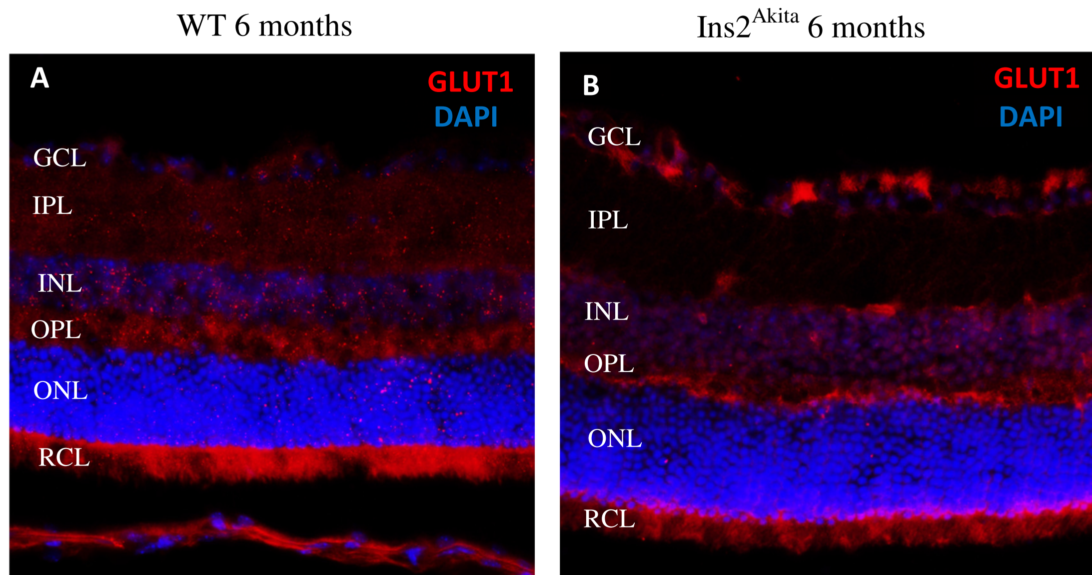

Figure 4.14: Immunohistochemistry staining of GLUT1 in retina sections of C57BL/6 WT (A) and Ins2^{Akita} (B) with 6 months of age. GCL - Ganglionar cell layer; IPL- inner plexiform layer; INL – Inner nuclear layer; OPL – outer plexiform layer; ONL – outer nuclear layer; RCL – Rod and cones layer. Red staining corresponds to GLUT1expression and DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 20 µM.

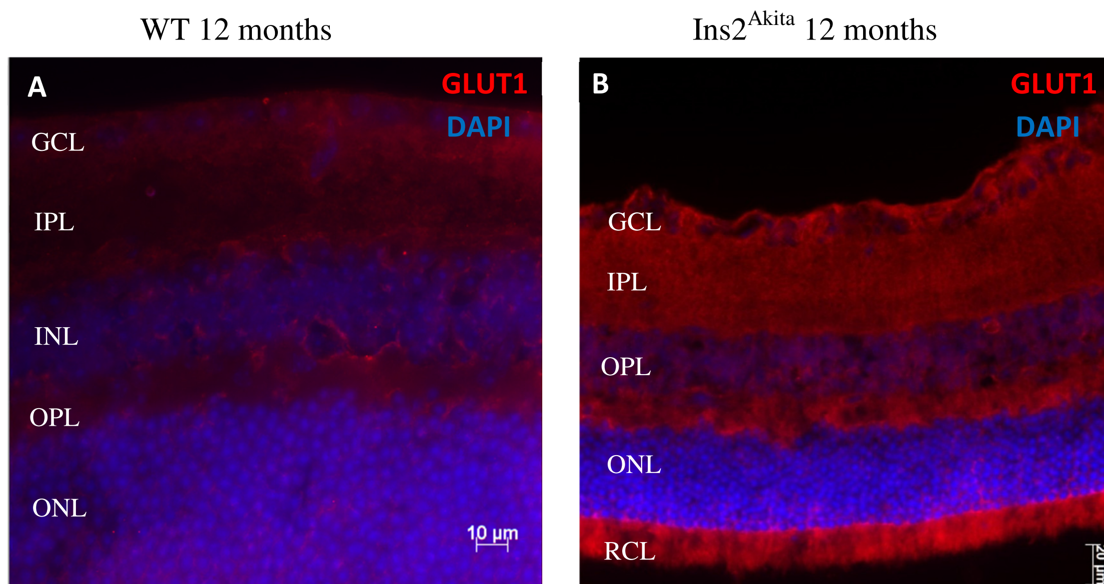

Figure 4.15: Immunohistochemistry staining of GLUT1 in retina sections of C57BL/6 WT (A) and Ins2^{Akita} (B) with 12 months of age. GCL - Ganglionar cell layer; IPL- inner plexiform layer; INL – Inner nuclear layer; OPL – outer plexiform layer; ONL – outer

nuclear layer; RCL – Rod and cones layer. Red staining corresponds to Glut1expression and DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 10 and 20 μM.

For the immunohistochemistry of Glut1 transporter at 12 months of age, there seems to be an increase of intensity in the expression of GLUT1 in Ins^{2Akita} comparing with WT (fig. 4.15). This result is in accordance to what it was expected, since from the literature in the presence of chronic hyperglycemia there is an increase of this transporter. [14] Also, this result confirms the tendency of GLUT1 expression to be higher in Ins2^{Akita} that we described earlier, which possibly would be significant if we had increased the sample number. However, we did not see the same marked increase of this transporter localized in the membranes that we saw at 6 months of age. Instead, the expression was spread throughout the entire cell. This may indicate that GLUT1 was not being translocated to the membranes and if cytosolic GLUT1 is elevated, the mRNA production may not vary, which explains the unchanged mRNA expression at 12 months of age.

Summarizing, the GLUT1 expression in Ins2^{Akita} start to increase by the presence of hyperglycemia but in the early phase of the disease this increase is mainly at the translational level leading to an increase of GLUT1 in the membrane of the retinal cells. With the progression of the disease we can see that this increase is maintained.

4.2.2 Characterization of PEDF in mouse models

After analyzing GLUT1 expression, we decided to analyze the expression levels of PEDF. We started to analyze the expression of PEDF mRNA levels by real-time qPCR (fig. 4.16). Our result shows that there is not a significant difference in the expression of PEDF mRNA between WT and Ins2^{Akita} in both time-points that we analyzed. It is known that the mice start developing some features of DR at 6 months of age, meaning that they are at the beginning of the disease which may not yet influence the PEDF mRNA production. However as we can see in figure 4.16A there is a tendency to increase the PEDF mRNA expression in Ins2^{Akita} mice. Knowing that hyperglycemia elevates the levels of angiogenic factors, such as VEGF, and at 6 months of age the hyperglycemia is already installed, this increase of PEDF expression may be to counteract the increase of this angiogenic factors. [94] However, as we can see in figure 4.16B, that represents 12 months of age and the most severe state of the disease, there is no significant difference between both strains. One possible explanation to these unchanged levels could be the senescence that it is present in the retina at this age even for the WT mice.

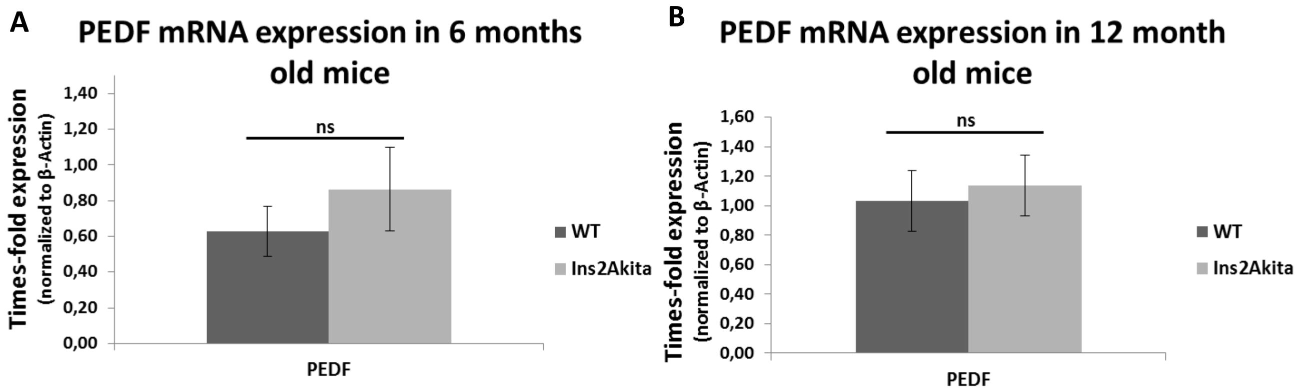

Figure 4.16: qPCR analysis of PEDF in C57BL/6 WT and $Ins2^{Akita}$ with 6 months (A) and 12 months of age (B); N= 6; ns indicates no significant differences in the mRNA levels of PEDF in WT compared and $Ins2^{Akita}$, determined by Tukey's multiple comparisons test.

We decided to next analyze the protein levels of PEDF by Western Blot in the retina and RPE layer. We can see in figure 4.17 and 4.18 a decreased expression of PEDF both in the retina (fig. 4.17) and RPE layer (fig. 4.18) of the diabetic mice. Although the PEDF expression in the retina is not in accordance with the mRNA levels from figure 4.16, this was expected based on the literature, which shows that PEDF is decreased in cases of DR. [59] So analyzing both mRNA and protein expression, we can say that mRNA levels have a tendency to increase because it is trying to compensate the decrease of PEDF protein expression. In addiction it seems that the decrease of PEDF expression is much more pronounced in the RPE layer than in the retina which is also expected because the RPE layer is one of the major production sources of PEDF (fig.4.18). [83]

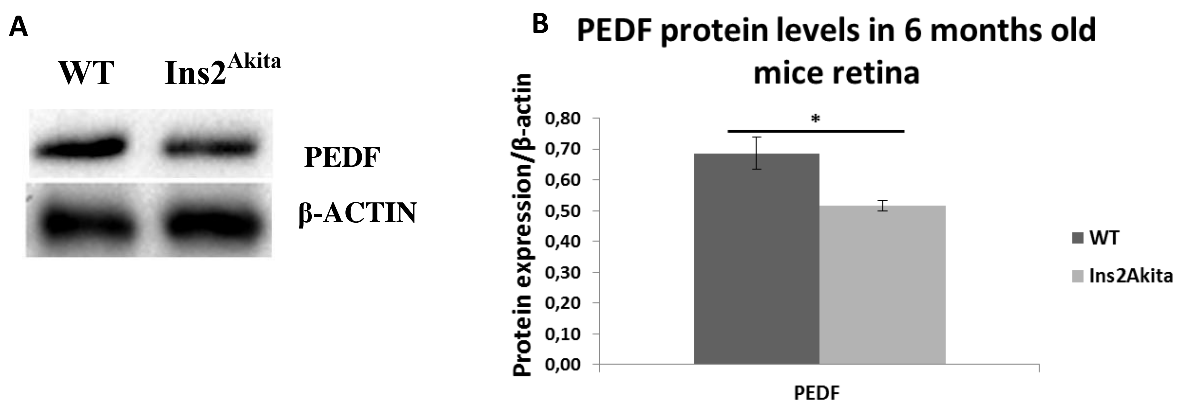

Figure 4.17: Western Blot analysis of PEDF expression in C57BL/6 WT and $Ins2^{Akita}$ with 6 months of age in the retina. (A) corresponds to Western blot analysis of PEDF expression levels and (B) corresponds to the quantitative data normalized by the intensity of β-Actin bands. N=3; *P<0.05 indicates significant differences in the protein levels of PEDF in WT compared and $Ins2^{Akita}$, which was determined by Tukey's multiple comparisons test.

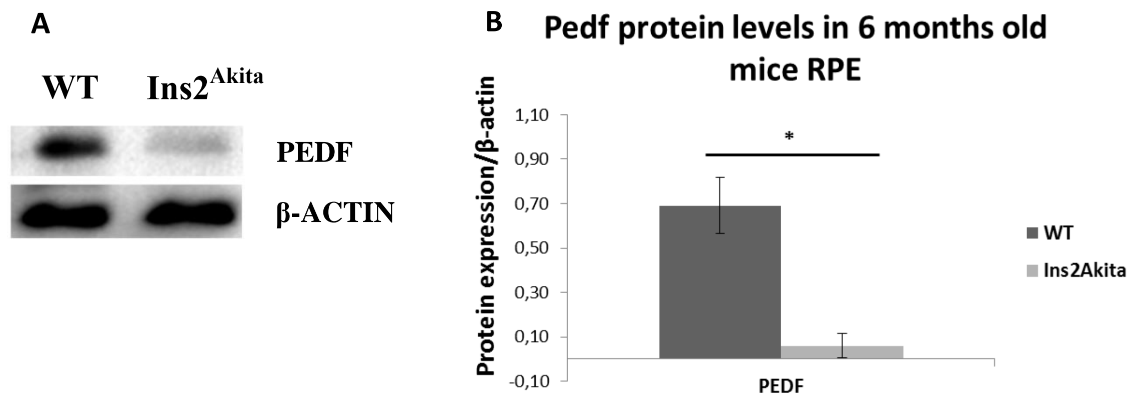

Figure 4.18: Western Blot analysis of PEDF expression in C57BL/6 WT and Ins2^{Akita} with 6 months of age in the RPE layer. (A) corresponds to Western blot analysis of PEDF expression levels and (B) corresponds to the quantitative data normalized by the intensity of β-ACTIN bands. N=3; *P<0.05 indicates significant differences in the protein levels of PEDF in WT compared and Ins2^{Akita}, which was determined by Tukey's multiple comparisons test.

Afterwards we analyzed the protein levels of PEDF in mice with 12 months old (fig. 4.19 and 4.20). In the retina of 12 months old mice there is non-significant difference in the PEDF expression between WT and Ins2^{Akita} (fig. 4.19). This is in accordance with the mRNA levels at 12 months of age (fig. 4.16) which also show a non-significant increase of its expression. However we can see that Ins2^{Akita} mice have a tendency to increase the PEDF expression when compared with WT. This increase of PEDF protein maybe is an attempt to counteract the angiogenic factors that are described to be elevated at this stage. [95]

When we evaluated the expression in the RPE layer, the protein expression of PEDF in Ins2^{Akita} with 12 months of age is completely abolished comparing with the PEDF expression in the RPE of WT mice (fig. 4.20). One possible explanation is that in conditions of chronic hypoxia there is a necessity of increasing the vascularity and angiogenesis. [96] To do so, it is necessary to increase angiogenic factors and subsequently decrease the anti-angiogenic factors. So the abolishment of PEDF proteins in the RPE of 12 months Ins2^{Akita} mice may be derived from this decrease of the anti-angiogenic factors in response to chronic hypoxia. [97]

However this result is not in accordance with the tendency of the PEDF expression to increase in the retina. This means that despite the abolition of PEDF in the RPE, PEDF levels in the retina in general are slightly increased. This increase maybe is associated with the production of PEDF by other retinal cells like Müller cells in which it was described by

Yang et al. that under hypoxia conditions, anti-angiogenic activity is secondary to the neuroprotective function inherent to PEDF. [98] Other possibility is that the RPE layer is dysfunctional at this age which may lead to the non-production and consequently the degradation of its principal factor. [99]

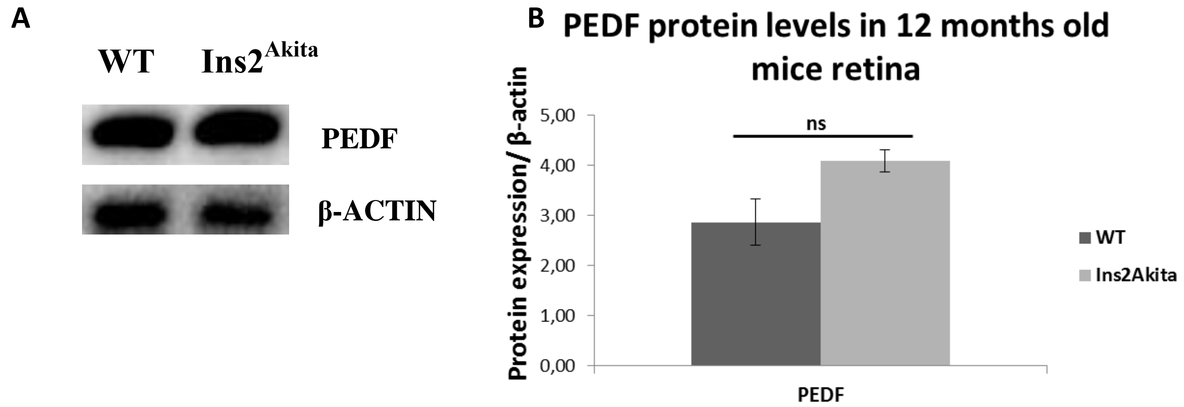

Figure 4.19: Western Blot analysis of PEDF expression in C57BL/6 WT and Ins2^{Akita} with 12 months of age in the retina. (A) corresponds to Western blot analysis of PEDF expression levels and (B) corresponds to the quantitative data normalized by the intensity of β-ACTIN bands. N=3; *P<0.05 indicates significant differences and ns indicates non-significant differences in the protein levels of PEDF in WT compared with Ins2^{Akita}, which was determined by Tukey's multiple comparisons test.

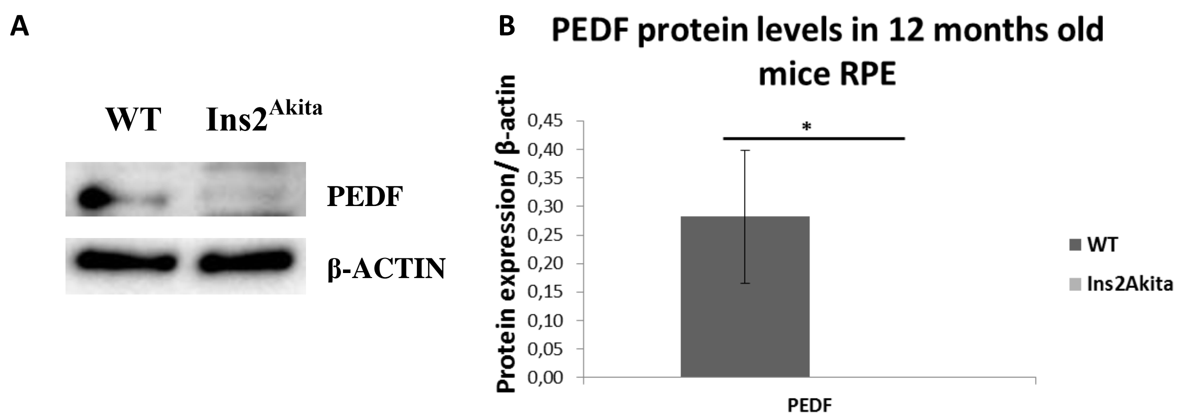

Figure 4.20: Western Blot analysis of PEDF expression in C57BL/6 WT and Ins2^{Akita} with 12 months of age in the RPE layer. (A) corresponds to Western blot analysis of PEDF expression levels and (B) corresponds to the quantitative data normalized by the intensity of β-ACTIN bands. N=3; *P<0.05 indicates significant differences and ns indicates non-significant differences in the protein levels of PEDF in WT compared with Ins2^{Akita}, which was determined by Tukey's multiple comparisons test.

Subsequently, we also performed an immunohistochemistry to evaluate PEDF expression in the retina and in the RPE layer. As we can see in figure 4.21, it seems to be a slight decrease of the PEDF expression in the Ins2^{Akita} at 6 months of age compare with

WT with the same age in the retina. This result is in accordance with our Western blot results (fig. 4.17) and with the literature. Likewise, we can see that there is a significant decrease of the Pedf expression in Ins2^{Akita} mice compared with the 6 months-old WT mice in the RPE layer (fig. 4.22) supporting our previous results.

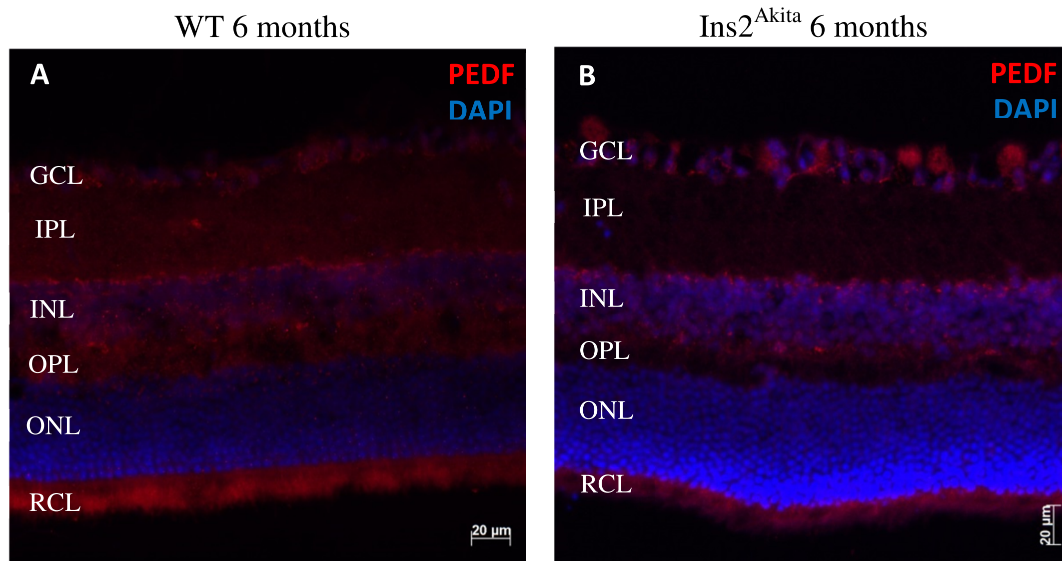

Figure 4.21: Immunocytochemistry staining of PEDF in retina sections of C57BL/6 WT (A) and Ins2^{Akita} (B) with 6 months of age. GCL - Ganglionar cell layer; IPL- inner plexiform layer; INL – Inner nuclear layer; OPL – outer plexiform layer; ONL – outer nuclear layer; RCL – Rod and cones layer. Red staining corresponds to PEDF expression and DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 20 µM.

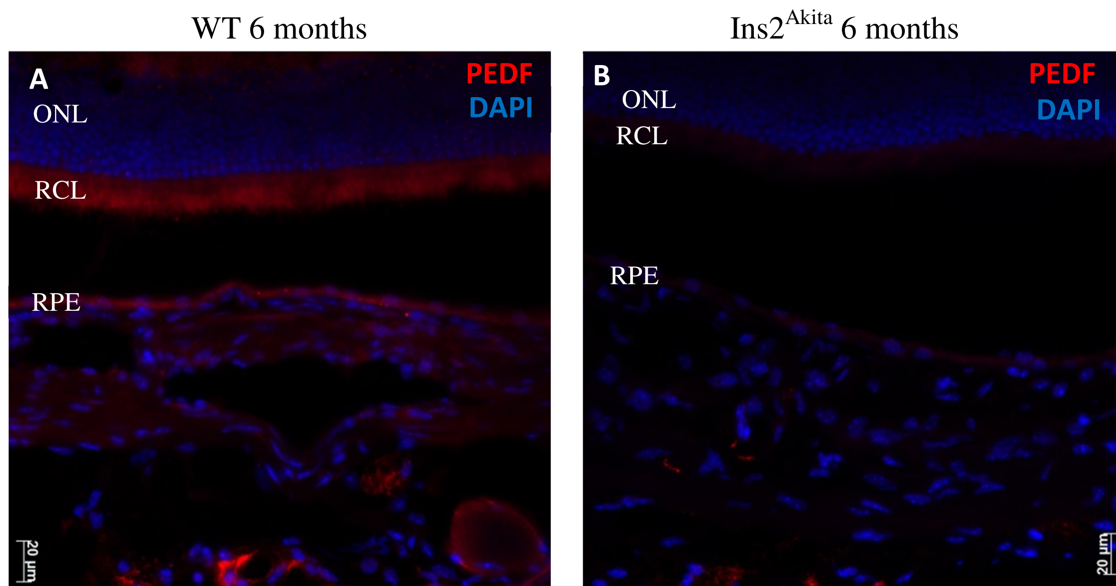

Figure 4.22: Immunocytochemistry staining of PEDF in RPE sections of C57BL/6 WT (A) and Ins2^{Akita} (B) with 6 months of age. ONL – outer nuclear layer; RCL – Rod and cones layer; RPE - retina pigmented epithelium. Red staining corresponds to PEDF expression and DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 20 µM.

Afterwards, through figure 4.23 we can see that there is no significant difference in the PEDF expression between Ins2^{Akita} and WT at 12 months of age which also corroborates with the Western Blot analysis. We also tried to perform an immunohistochemistry against PEDF to stain the RPE layer of the animals with 12 months of age but the samples at this age were more damaged than usual. This can be due to RPE impairment along the progression of the disease. RPE composes the oBRB and is one of the first structures affected by the DR is the BRB. [99] [100]

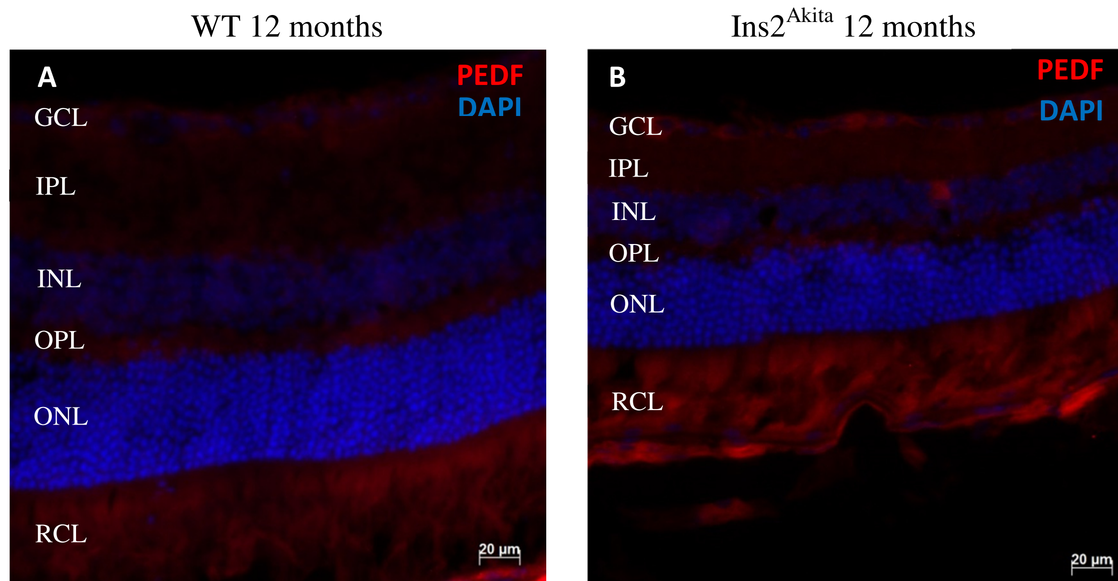

Figure 4.23: Immunocytochemistry staining of PEDF in retina sections of C57BL/6 WT (A) and Ins2^{Akita} (B) with12 months of age. GCL - Ganglionar cell layer; IPL- inner plexiform layer; INL – Inner nuclear layer; OPL – outer plexiform layer; ONL – outer nuclear layer; RCL – Rod and cones layer. Red staining corresponds to PEDF expression and DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 20 µM.

Summarizing, the PEDF expression levels in Ins2^{Akita} mice at 6 months of age are decreased in both retina and RPE layer when compared with WT mice. In addition, this decrease is more pronounced in the latest. For mice with 12 months of age this decrease is only detectable in the RPE layer.

4.2.3 Characterization of VEGF in mouse models

Afterwards we decided to analyze the VEGF expression in WT and Ins2^{Akita} mice with 6 and 12 month-old. We first analyzed the VEGF mRNA expression by real-time qPCR. As we can see in figure 4.24A, at 6 months of age there is a significant increase of the VEGF mRNA expression in Ins2^{Akita} compare with WT. This result is in accordance with

the literature that associated an increase of VEGF mRNA expression with the appearance of the disease.[19] However, at 12 months of age despite it seems to have a tendency to an increase in VEGF expression, it is not significant (fig.4.24B).

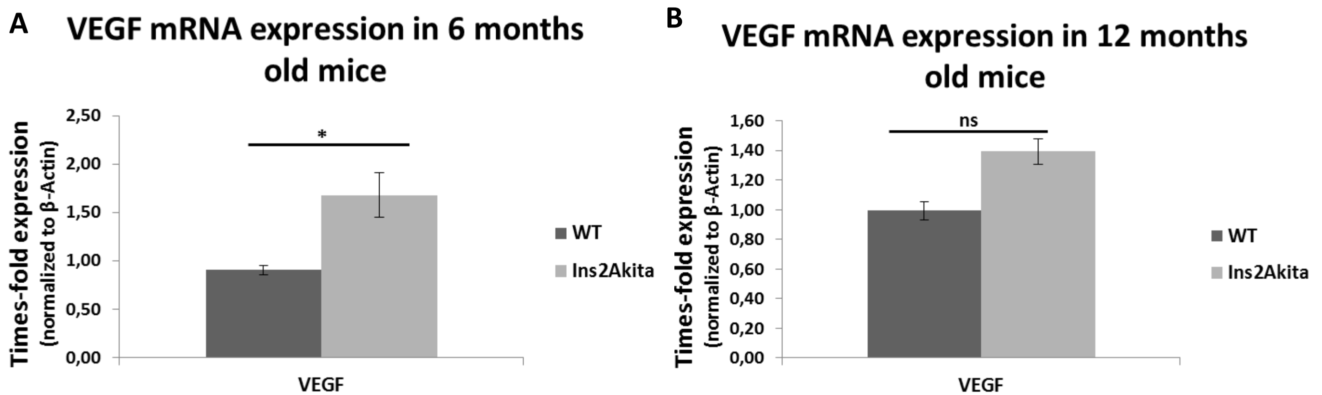

Figure 4.24: qPCR analysis of VEGF mRNA expression in C57BL/6 WT and $Ins2^{Akita}$ with 6 months of age (A) and 12 months of age (B). N=6; *$P<0.05$ indicates significant differences in the VEGF mRNA expression and ns indicates non-significant differences between WT and $Ins2^{Akita}$, which was determined by Tukey's multiple comparisons test.

Next, we analyzed the protein levels of VEGF in WT and $Ins2^{Akita}$ mice with 6 months and 12 months of age by Western blot (fig. 4.25 and 4.26). Our results show that there are no significant differences between WT and $Ins2^{Akita}$ mice at 6 months of age (fig. 4.25). This is not what we were expecting, taking into account the previous result from the qPCR and the literature. One hypothesis is that there might be degradation of the protein or other phenomenon that interferes with the translation of the mRNA. In fact, knowing that the translational machinery is diminished in hypoxic conditions, maybe the VEGF translation at the onset of the disease is also affected. [79]

Our result at 12 months of age shows that there is a significant decrease in VEGF protein expression (fig. 4.26) comparing WT with $Ins2^{Akita}$ mice. This is the opposite of what we were expecting but this result can be explained by aging retinal cell death in the retina of the 12 month-old $Ins2^{Akita}$ mice. [101] [93]

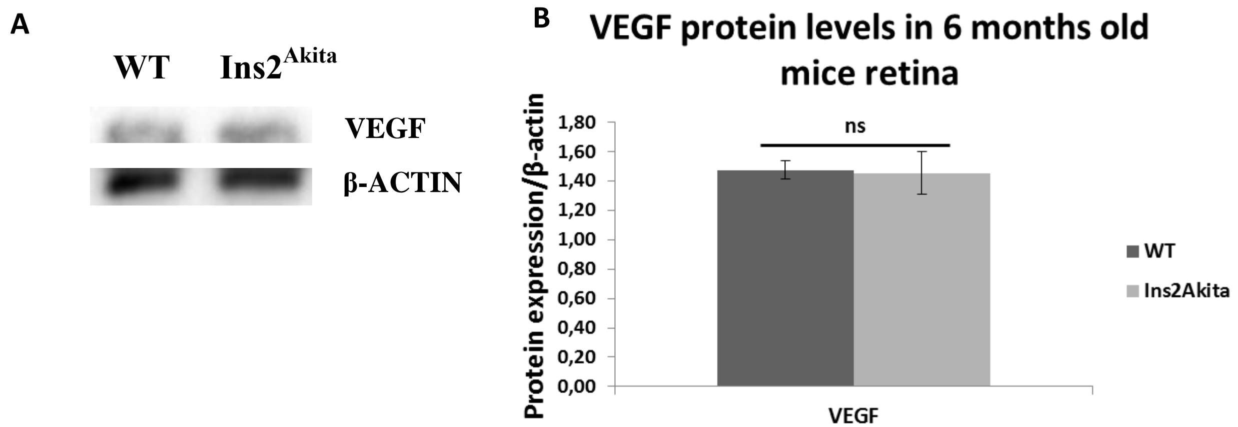

Figure 4.25: Western Blot analysis of VEGF expression in C57BL/6 WT and Ins2^{Akita} with 6 months of age in the retina. (A) corresponds to Western blot analysis of VEGF expression levels and (B) corresponds to the quantitative data normalized by the intensity of β-Actin bands. N=3; *P<0.05 indicates significant differences in the VEGF mRNA expression and ns indicates non-significant differences between WT and Ins2^{Akita}, which was determined by Tukey's multiple comparisons test.

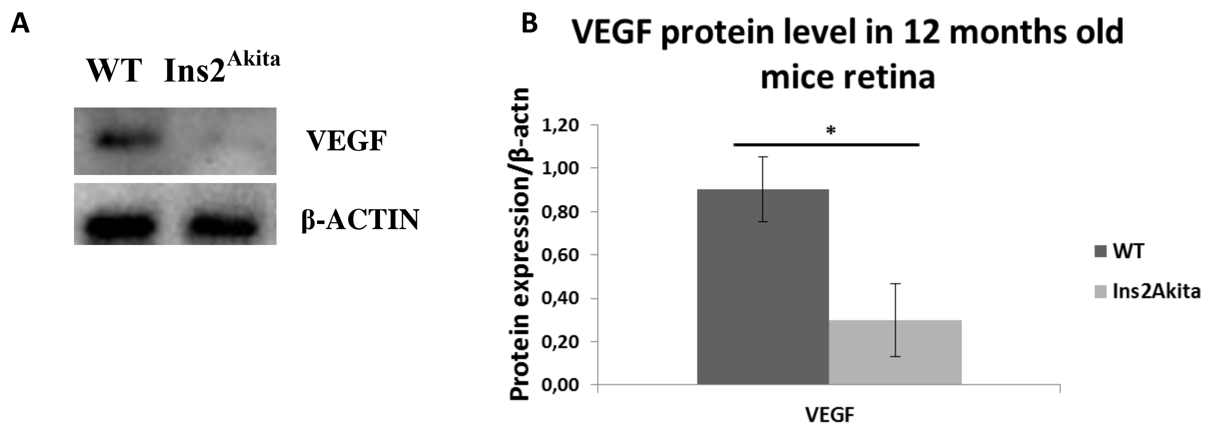

Figure 4.26: Western Blot analysis of VEGF expression in C57BL/6 WT and Ins2^{Akita} with 12 months of age in the retina. (A) corresponds to Western blot analysis of VEGF expression levels and (B) corresponds to the quantitative data normalized by the intensity of β-Actin bands. N=3; *P<0.05 indicates significant differences in the VEGF mRNA expression and ns indicates non-significant differences between WT and Ins2^{Akita}, which was determined by Tukey's multiple comparisons test.

Lastly, we also analyzed the VEGF expression in retinal samples by immunohistochemistry. As we can see in 6 month-old mice (fig. 4.27), it seems to be an increase of the VEGF expression in Ins2^{Akita} mice compared with WT. When we analyzed the expression of VEGF in mice with 12 months of age (fig. 4.28), it seems that there is no difference between WT and Ins2^{Akita}. These results are not what we expected based on the Western blot results but the differences may be due to the fact that both

immunohistochemistry and Western blot are a qualitative method which may not detect the small differences between tested conditions.

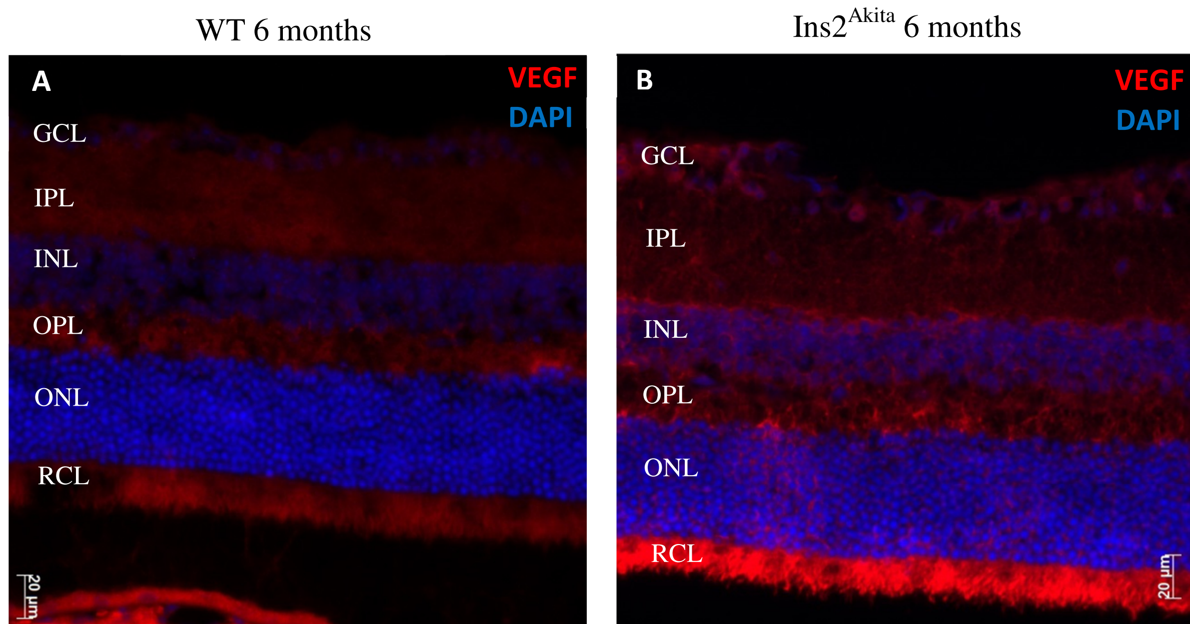

Figure 4.27: Immunocytochemistry staining of VEGF in retina sections of C57BL/6 WT (A) and Ins2^{Akita} (B) with 6 months of age. GCL - Ganglionar cell layer; IPL- inner plexiform layer; INL – Inner nuclear layer; OPL – outer plexiform layer; ONL – outer nuclear layer; RCL – Rod and cones layer. Red staining corresponds to VEGF expression and DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 20 µM.

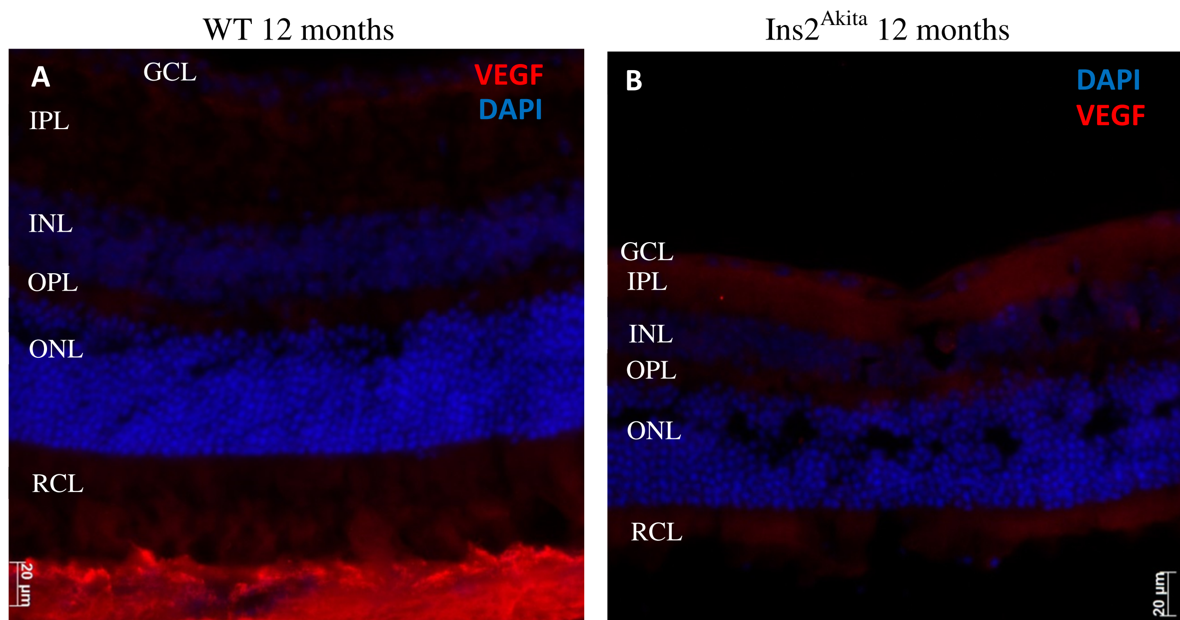

Figure 4.28: Immunocytochemistry staining of VEGF in retina sections of C57BL/6 WT (A) and Ins2^{Akita} (B) with 12 months of age. GCL - Ganglionar cell layer; IPL- inner plexiform layer; INL – Inner nuclear layer; OPL – outer plexiform layer; ONL – outer

nuclear layer; RCL – Rod and cones layer. Red staining corresponds to VEGF expression and DAPI (blue) represents the nuclei. Magnification: 63X, scale bar: 20 μM.

5. Conclusion

RD is a microvascular complication associated to diabetes and the major cause of blindness among adults in developed countries. With the increase of T2DM, diabetic complications like RD are expected to rise, which makes it even more necessary to find a cure or a treatment for this pathology.

The DR *in vitro* and *in vivo* models, like D407 cells and Ins2^{Akita} mice, respectively, are essential for the understanding of the biochemical mechanisms that leads to DR onset and progression. While D407 cells show a big similarity with the RPE cells that are present in the oBRB, the Ins2^{Akita} mouse is a spontaneous mouse model of T1DM that shows to be a suitable model for the study of diabetic complications like RD.

The typical chronic hyperglycemia present in diabetic patients to which the retina is subjected leads to hypoxic conditions that activate a cascade of biochemical pathways that alter the levels of GLUT1 and pro and anti angiogenic factors such VEGF and PEDF. [16]

It is known that glucose transport from blood to retina is almost exclusively made by GLUT1, so alterations on its abundance and action may be associated with the DR symptoms. [14] Also, it is known that chronic hypoxia conditions lead to retinal neovascularization, [42] thus angiogenic factors are also associated with the development of DR pathology. The most important angiogenic inducer that it is activated by HIF-1 (a factor stabilized by hypoxia) is VEGF; a potent angiogenic inhibitor produced by RPE cells, that it is positively correlated with oxygen concentrations, is PEDF. [57]

Therefore, the aim of this study was to evaluate and characterize the expression levels of GLUT1 and angiogenic factors such VEGF and PEDF in models of DR both *in vivo* and *in vivo* models.

In our *in vitro* studies we were able to demonstrate that the expression levels of GLUT1 were increased in the cells cultured with medium containing glucose concentration simulating diabetes and subject to hypoxia. This increase was associated to an increase in GLUT1 in in the membrane of the cells. This means that hypoxia induces a translocation of the transporter to the membranes which was also described in early studies of the GLUT4 in skeletal muscle. [81]

Regarding PEDF, we can say that *in vitro* there is no significant alteration in the expression levels comparing normoxia with hypoxia and normoglycemia with hyperglycemia. However it seems to have a tendency to decrease when D407 cells are exposed to hypoxia which is what we expected. One possible explanation is that D407

cells are a model of RPE and the RPE layer is the one of the major producer of PEDF that is secreted to act as neurotrophic and anti-angiogenic factor in the inner retina. This means that changes in PEDF levels may be more significant in the medium where they are released. [102]

Regarding VEGF, the *in vitro* results show a significant increase at the mRNA levels. However when we analyzed the protein levels we only saw an increase of VEGF expression in normoglycemic conditions when we compare normoxia with hypoxia and in normoxia conditions when we compare normoglycemia with hyperglycemia. This may result from the fact that immunochemistry and Western blot are qualitative methods and not quantitative methods like qPCR which may not detect small differences among tested conditions.

Since VEGF is also a secreted factor that acts through VEGF receptors that are present in the membrane of the endothelial cells, it would be more accurate to analyze VEGF expression in the culture medium of the cells. For this reason the intracellular levels of VEGF may not alter significantly.

In our *in vivo* models, our study shows some contradictory data. At 6 months of age, GLUT1 mRNA did not alter when comparing both strains but there is an increase of its protein in the retina of $Ins2^{Akita}$ mice. This could result from the fact that at 6 months of age they are starting developing the disease which means that the increased of GLUT1 expression is maybe due to an increase in translation and not in transduction. In contrast we found no differences in GLUT1 expression in the retina of 12 month-old $Ins2^{Akita}$ mice, when compared with the age-matched non-diabetic control. In fact, some reports showed that at this age the retinal cells become senescent and there are more cell death events, which can explain our results. [93]

Similar results were verified for PEDF *in vivo*, where the mRNA levels did not show a significant difference between WT and $Ins2^{Akita}$ mice at 6 and 12 months of age; however when we analyzed at the protein level there was a significant decrease of PEDF protein both retina and RPE of $Ins2^{Akita}$ mice compared with WT at 6 months of age. These results are in agreement with Han et al. in which they also showed diminished PEDF protein expression in $Ins2^{Akita}$ mice. [95]

For the older mice we can see by Western blot and immunohistochemistry analysis that there is no significant difference in PEDF protein levels in the retina of WT and $Ins2^{Akita}$ mice. However we found that there is no production of PEDF in the RPE of the 12 month-

old Ins2^{Akita} mice, showing a marked influence of the time exposure to hyperglycemia in the maintenance of RPE function and PEDF production.

Regarding VEGF, in this study we found a significant increase in the mRNA levels of VEGF between both strains at 6 months of age, which is in line with the literature. [95] However there were no significant differences at the protein level. As it was proposed before, this may result from the diminished function of the translational machinery in hypoxic conditions. [79] At 12 months of age the only significant difference detected between strains was a decrease of its protein expression. This may be associated with the retinal ageing and senescence that is present at this age, which may be impaired the VEGF expression. [93] [101]

In conclusion, we could not see a clear correlation between PEDF and VEGF but we demonstrated a decrease of PEDF and an increase of GLUT1 that could be associated with the onset of the DR.

6. Future Perspectives

DR remains the leading cause of blindness and visual impairment in the working age population. Research over the past few decades has identified many biochemical pathways that are associated with the pathophysiology of DR, but not a single one emerged as the sole responsible. Since a cure is the ultimate goal, these studies are important to better understand the disease and help develop a novel therapeutic that can ease the DR symptoms.

Our work was focused on angiogenic factors, VEGF and PEDF, and Glut1, which may collectively be responsible for the onset of DR.

In contrast to what we were expecting when we started to develop this work, we could not see a clear correlation between PEDF and VEGF in D407 cells or in *in vivo* models. Therefore, in the future we should try to use another in *vitro* model, such as human endothelial cells (HUVEC) that are the cells responsible for the majority of VEGF production. It would also be interesting to analyze the expression of both VEGF and PEDF in the supernatant of the cells in all the conditions tested before to see if there is any alteration on these secreted factors. Ideally we should also evaluate the expression of VEGF, PEDF and GLUT1 in Ins2^{Akita} with 9 months of age, since it was previously described that at this stage DR is already installed but not as severe as at 12 months, where we had most of the contradictory results. [95]

In the future we would like to analyze the expression of HIF-1α factor in those same conditions because HIF-1α is directly correlated with VEGF and GLUT1 and would be interesting to see their levels and correlate with these results. And if possible, increase the sample numbers for increased statistical significance.

Taking into account some of the data like the increase in GLUT1 and the decrease of PEDF expression, we could in the future investigate therapies like the blockage/inhibition of GLUT1 or overexpression of PEDF and see if the phenotype is reversed on these mice.

7. References

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8. Appendix

Appendix 1: Table of real time qPCR Primers.

Name	Sequence	Fragment
hGLUT1_FW_RT	TCACTGTGCTCCTGGTTCTG	233 bp
hGLUT1_Rev_RT	CCTGTGCTCCTGAGAGATCC	
hPEDF_FW	TAGACCGAGAACTGAAGACCG	308 bp
hPEDF_Rev	TGTGTCTGTGTCCCTCAGTAC	
hPEDF_FW(2)	TATCACCTTAACCAGCCTTTCATC	83 bp
hPEDF_Rev(2)	GGGTCCAGAATCTTGCCAATG	
hPEDF_RT_FW(3)	TATGACCTGTACCGGGTGCGA	70 bp
hPEDF_RT_Rev(3)	CCACACTGAGAGGAGACAGGAGC	
hVEGF_FW	ACTTCTGGGCTGTTCTCG	141 bp
hVEGF_Rev	TCCTCTTCCTTCTCTTCTTCC	
hGAPDH_FW	ACATCATCCCTGCCTCTACTG	232 bp
hGAPDH_Rev	ACCACCTGGTGCTCAGTGTA	
hACTINβ_FW	CATGTACGTTGCTATCCAGGC	250 bp
hACTINβ_Rev	CTCCTTAATGTCACGCACGAT	
mGlut1_FW	GCTGTGCTTATGGGCTTCTC	114 bp
mGlut1_Rev	CACATACATGGGCACAAAGC	
mPedf_FW	AATCACCCGACTTCAGCAAGA	73 bp
mPedf_Rev	TCGAAAGCAGCCCTGTGTT	
mGapdh_FW	GGTGAAGGTCGGTGTGAACG	123 bp
mGapdh_Rev	ACCATGTAGTTGAGGTCAATGAAGG	
mActinβ_FW	AGAGGGAAATCGTGCGTGAC	138 bp
mActinβ_Rev	CAATAGTGATGACCTGGCCGT	