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Dipartimento di Medicina Sperimentale

PhD School in Life Sciences

PhD Course in Translational Medicine

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Modulation of diabetic retinopathy by melanocortin MC1-MC5 receptor agonists "*in vivo and in vitro* studies "

PhD Coordinator
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Chapther 5

Acknowledgements

These 3 years of PhD has been rich in experience from both a professional and personal point of view. Firstly I would like to thank all those who have allowed this, that is my thesis director Prof. D'Amico, who despite the many dark moments, together, we have always managed to find the light. These 13 years lived together have allowed us to share a lot not only at a professional level.

I thank Dr. Clara Di Filippo that despite "some divergence" during the journey deep down she has always been even working behind the scenes.

I thank all my laboratory colleagues both those present, and those who have now taken different ways. The friendship and respect that binds us will never divide us.

The PhD has allowed me to live a year in Valencia, “my second home” where I met wonderful people. I start thanking my professors

Francisco Javier Romero and Jorge Miquel Barcia who, from the very first day, made me feel a RETOS. I'd like to thank the RETOS especially Lorena, Maria, Javier, Natalia, Beatriz e Daniel for the help and the friendship shown.

How not to say thanks to "my Valencian family", who shared everything with me.

I thank Sandra my sister, not of blood but of heart, for her there are no words, too many things lived together in a year, lots of laughter, there have been even tears but in the end we have always managed to keep going. Thanks for all. You already know that in that "all" there is the heart you have given me.

I owe my family a thank and in particular my mother for always being there for me even when I was far away, taking over many things.

My thanks go even to Alessandro's family, especially to my mother-in-law, who until her last day has always supported me on this journey and has always been proud of me and what I was doing.

Last but not least I say thanks to my husband Alessandro, the person who allowed me to live this path at 360°, the man who, even while remaining alone most of the time, has renounced my presence but never my cultural, professional and especially my personal growth. He also encouraged me when I was about to give up even though he was the one who needed me, so that my doctorate and life project continues to take shape.

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Abstract

For this doctoral purpose were used ed three different experimental models: I) human retinal pigment epithelial cells ARPE-19, II) primary retinal cell culture, III) a mouse model of streptozotocin (STZ)-induced Diabetic retinopathy.

Diabetic retinopathy is a leading cause of adult blindness and is the most common complication of diabetes. It affects morethan 90% of people with diabetes, ultimately leading to retinal edema, neovascularization, and, in some patients, vision loss [1, 2]. Systemic control of blood glucose can slow down the progression of diabetic retinopathy but fails to stop or reverse clinical signs of it [3, 4]. Hence understanding the molecular pathways governing the pathophysiology of DR and targeting them is essential to the prevention of catastrophic visual loss arising from vision-threatening complications of diabetic retinopathy such as macular edema, vitreous hemorrhage, and

tractional retinal detachment. Melanocortins are endogenous peptides that possess a wide range of biological activities, including inhibition of leukocyte activation, promotion of inflammation resolution, and the ensuing tissue protection [5–12]. These effects on the immune response are brought about by five distinct melanocortin receptors, termed from MC1 to MC5, ubiquitously expressed except for the MC2 which is localised to the adrenal glands [13]. Within the eye, MC3, MC4, and MC5 are expressed in the inner neural retinal layers [14, 15], with MC3 and MC4 expression being reported also in the layer of retinal ganglion cells [14, 15]. MC5 alone has been detected in the neural outer plexiform layer, whilst MC1 and MC5 are detected in retinal pigment epithelial cells [16, 17]. There is scant knowledge on the biology associated with these receptors in the eye. Work is limited to the most common melanocortin peptide, α-melanocyte stimulating hormone (α-MSH), which activates all MC receptors (except MC2), controls the development and neurotrophism of the ocular tissues [18–20], and exerts protective effects on the retinal vascular endothelial cells [21, 22]. The present study aimed at establishing the efficacy of melanocortin peptides in the prevention of DR and

characterizing the MC subtypes involved. Was to investigate the role of the melanocortin receptor (MCR) 5 in the high glucose induced release of VEGF-containing exosomes from human retinal pigment epithelial cells ARPE-19 and in the resulting neovascularization. Here I show that retinal pigment epithelial cells ARPE-19 cultured in a medium containing 35mM of D-glucose have augmented ROS formation and augmented release of vascular endothelial factor (VEGF)-containing exosomes, compared to ARPE-19 cells cultured in a medium containing 5mM of D-glucose (standard medium). The exposure of these cells for 9 days to 10-10M of PG-901, a melanocortin receptor (MCR) 5 agonist, reduces this pro-oxidant challenge by reducing the ROS generation, the number of exosomes released and their VEGF content. ARPE-19 derived VEGF-containing exosomes promoted neovascularization in HUVEC cells, an effect that was markedly reduced by 10-10M PG-901 but not by the MCR3/4 agonist MTII (0.30 nmol) or the MCR1 agonist BMS-470539 (10-5 M). The MCR5-related action in ARPE-19 cells was accompanied by an increased expression of two coupled factors, the cytochrome p4502E1 (CYP2E1) and the nuclear factor kappa b (Nf- kB). These are both involved in the high glucose

signaling, in the ROS generation and interestingly, reduced by the MCR5 agonist in ARPE-19 cells. Altogether, these data suggest MCR5 as modulator of the responses stimulated by glucose in ARPE-19 cells, possibly translated into a modulation of the retinal pigment epithelium response to diabetes in vivo.

In primary retinal cells culture I analyzed retinal photoreceptors are particularly vulnerable to local high-glucose concentrations. Oxidative stress is a risk factor for diabetic retinopathy development, I focused on their role in primary retinal cell cultures in high-glucose concentrations. After eye enucleation from wild-type male C57BL/6 mice, retinal cells were isolated, plated in high-glucose concentration and treated with melanocortin receptors 1 and 5 agonists and antagonists. Immunocytochemical and biochemical analysis showed that treatment with melanocortin receptors 1 and 5 agonists reduced anti-inflammatory cytokines and chemokines and enhanced manganese superoxide dismutase and glutathione peroxidase levels, preserving photoreceptor integrity. According with these evidences, we propose a major

role of melanocortin receptors 1 and 5 on primary retinal cell response against high glucose or oxidative insults. I used of a mouse model of STZ-induced DR, an experimental system suitable for replicating the early signs of non proliferative DR, such as loss of retinal pericytes and capillaries, thickening of the vascular basement membrane and increased vascular permeability [23] .

CHAPTER 1

Introduction

1.1 General Eye Anatomy

The eye is the most fundamental part of the visual system. It is irregularly spherical, and its size varies from one individual to another depending on their ethnicity and gender, although the differences are minimal [24].

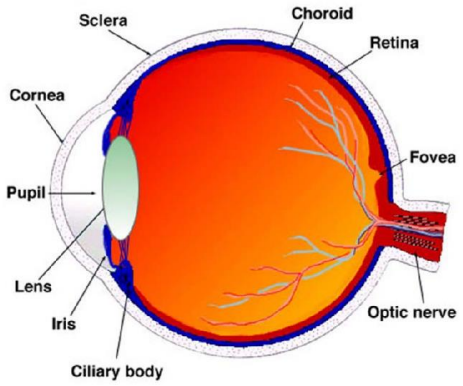

Figure 1. General anatomy of the eye. *A drawing of a section through the human eye with a schematic enlargement of the retina [24].*

The most important area in the eye is the image-processing organ known as the

retina. The retina is a thin, multilayered, highly organized structure responsible for receiving light stimuli from the outside and turn them into electrical impulses, which are send towards the occipital cortex, where images are finally processed.

1.2 Retina

The retina is placed in the back of the ocular globe, in contact with the vitreous humor and the retinal pigment epithelium (RPE). Anteriorly, it limits with the ciliary epithelium of the pars plana, in this area the retina forms a circular toothed structure known as the ora serrata. The retina contains many cell types. Among them, there are two types of photoreceptors: the cones and the rods, which nuclei form the outer nuclear layer (ONL) and their prolongations form the outer plexiform layer (OPL). Cones detect the fine shape, the colors and movements of visual objects. They require high light intensity and work in photopic conditions (day light). The rods do not discriminate colors and are very sensitive to low light intensities. Thus, rod photoreceptor cells work in scotopic conditions (night vision).

The optic nerve (ON), located at the center of the retina, is composed of retinal

ganglion cell axons on their way to the brain and incoming blood vessels that ramified into the retina to nurture the numerous neurons and glia cells [24].

The *macula lutea* is a region of the central retina required for high acuity vision. Cones are predominant in this area, whereas a majority of rods are

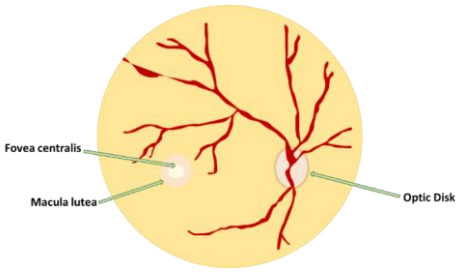

Figure 2. *Human eye fundus, with macula and fovea near center.*

located at the periphery. Although, the macula corresponds to less than 10% of the retinal surface, half of the retinal ganglion cells (RGCs) – neurons that form the optic nerve – are located in this region. The *fovea centralis* – located at the center of the *macula lutea* – is a depression in the inner retinal surface specialized in maximal visual acuity (Fig.2).

1.3 Histology of retina

As aforementioned, the retina is formed of several layers of cells (Fig.3). From the outer area to inner side we find: the retinal pigment epithelium (RPE), the photoreceptor outer segments (POS), the photoreceptor inner segments (IS), the external limiting membrane (EML), the outer nuclear layer (ONL), the outer plexiform layer (OPL) (formed by synapses between photoreceptors, bipolar, and horizontal cells), the inner nuclear layer (INL) (containing bipolar, amacrine and horizontal cells), the inner plexiform layer (IPL) (making synaptic contact with the ganglion cells), the nerve fiber layer (NFL), and the inner limiting membrane (ILM).

The inner layers of the retina, from the POS to the ILM, are often known as the neural retina and consist of seven main types of cells: photoreceptors (rod and cones), bipolar cells, horizontal cells, amacrine cells, retinal ganglion cells, and Müller cells[25] (Fig.3).

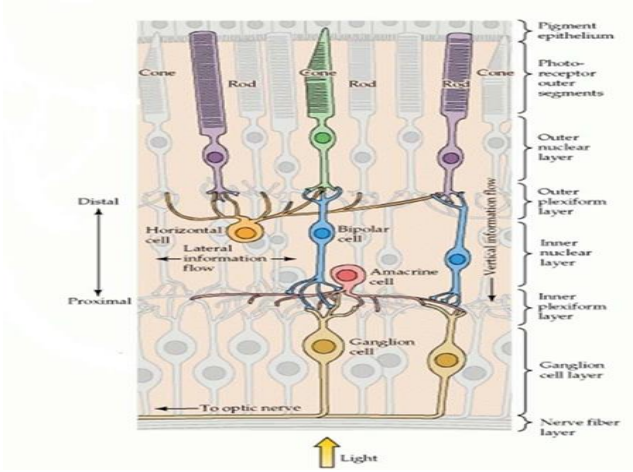

Figure 3: Structure of the retina. *A three-neuron chain—photoreceptor, bipolar cell, and ganglion cell—provides the most direct route for transmitting visual information to the brain. Horizontal cells and amacrine cells mediate lateral interactions in the outer and inner plexiform layers, respectively. The terms inner and outer designate relative distances from the center of the eye (Levine et al., 2014).*

The retinal pigment epithelium (RPE) is a single cell layer firmly anchored to the choroid plexus between the Bruch's membrane and the photoreceptor cell layer [25-27]. Choroid plexus is the vascular network that supplies with nutrients and oxygen the outer parts of the eye.

1.4 Retinal Pigment Epithelium

The RPE is one of the tissues of the body with higher metabolic activity. The apical side of RPE the cell extends forming microvilli that make direct contact with the POS. On their lateral walls, RPE cells form tight,

adherens gap junctions, while on their basal side, the RPE contact the underlying Bruch's membrane, that separates the retina from the choroids [28-30]. As the name suggests, the RPE contains pigment, particularly melanin, which is located in granules in the apical side of the cells. In humans, the pigment is observed after 5 weeks of gestation. (Fig. 4)

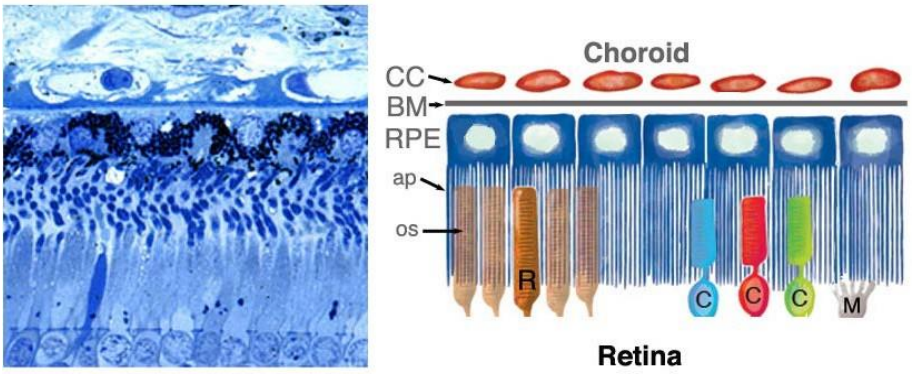

Figure 4: Retinal pigment epithelium*: CC, Choriocapillaris, BM, Bruch´s membrane, ap, apical proceses of the RPE enwrapping POS, photoreceptor outer segments, R rods, C, cones [31].*

Embryologically, it derives from neurosensory retina During embryogenesis [31], RPE cells are involved in the formation of the ciliary body and the iris, they control the termination of the optic fissure and influence retinal neurogenesis and ganglion cells. Thus, the RPE plays an important role in maintaining the function and the integrity of the retina and choroid [32].

1.5 Physiology of the Retinal Pigment Epithelium

The functions of the RPE are diverse and all of them are important. It absorbs light and protects against photooxidation [33]. RPE is part of the blood- retinal barrier (BRB), and it regulates the entrance of nutrients into the retina. In fact, RPE transports nutrients, ions, water, and metabolic-end products from one side to the other [34]. RPE is critical for phototransduction – the process of transforming a photon into electrical impulses – since it is in charge of the reisomerization of all-trans-retinal into 11-cis-retinal, a key element of the visual cycle

[25].

1.6 Retinal vascularization

Nutrition and oxygenation of the retina comes from two sources [35]:

- The outer retina, which corresponds to photoreceptors and RPE cells, is nourished by nutrients that arrive through the choriocapillaris layer.
- Two thirds of the inner retina (from the INL to MLI) receive vascularization of retinal capillaries derived from the central retinal artery (CRA, a branch of the ophthalmic artery) (Fig. 6). Such vascularization is located in two separate planes: 1) The surface capillary network is located in the nerve fiber layer and ganglion cells. 2) The deep capillary, denser and more complex than the surface layer, is located in the inner nuclear layer.

To understand the vascularization of the retina, it is important to review the histology of the retinal capillaries (Fig.6). Capillaries contain three layers:

- Endothelial cells (EC): Connected to each other by tight junctions. EC, together with the pericytes and astrocytes, secrete cytokines that promote binding between these cell types to form the BRB [36].

- Basal membrane (BM): Layer formed by a matrix of collagen type IV, laminin, fibronectin, and proteoglycans (mainly heparan sulfate). It covers the outer surface of the EC.

- Pericytes: They are practically cells embedded in glycoproteins of the extracellular matrix and placed in the outer side of the BM. They posses multiple pseudopodal processes that form gap junctions with EC through fenestrations of the BM. Moreover, pericytes possess actin filaments and endothelin receptors, which allow them to act as smooth

muscle fibers, thus regulating the caliber of the vessels and participating in the self-regulation of microvascular circulation (ability of the vessels to maintain an adequate flow independently of changes in pressure and metabolic needs).

- Pericytes produce and maintain the glycoprotein matrix and help preserve the BRB. They also secrete factors that inhibit the growth of endothelial cells (e.g. TGFb1), increase prostaglandin (PGI2) secretion, and protect the retina from oxidative stress (OS) [317].

- The cellular component (EC and pericytes) and BM should be considered an anatomical-functional unit, where modifications that occur in the cellular component significantly influence the BM and vice versa.

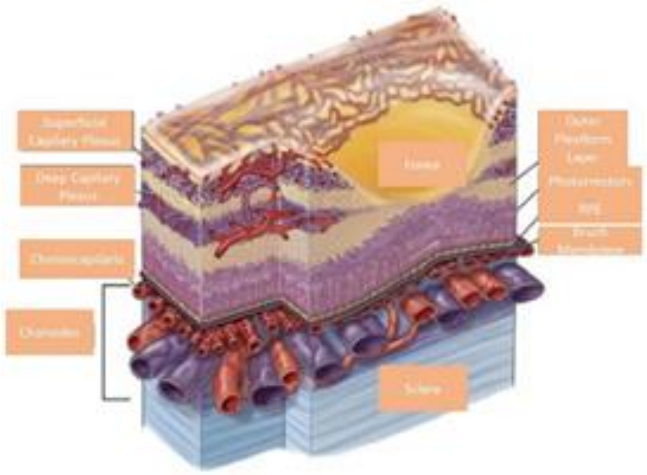

Figure 5: Retinal vascularization. *Drawing showing vasculature of the retina and choroid [37].*

1.7 Retinal diseases

Because the visual sense is so important for the reception of information, blindness creates social and personal damage. There are several diseases that can lead to loss of vision: age-related macular degeneration (AMD), glaucoma, diabetic retinopathy (DR), retinitis pigmentosa (RP), and Stargadt disease (SGTD). Among them, AMD is the leading cause of irreversible blindness in Western civilization [38].

1.8 Age-related Macular Degeneration

AMD affects the macula, affecting central vision and the ability to see fine details. A 2014 meta-analysis predicts that 196 million people will have AMD by 2020 increasing to 288 million by 2040 [40]. There are two principal forms of the disease: dry and wet AMD. Dry AMD, is a degenerative phase, which is associated with the development of drusen, subretinal deposits composed primarily of lipids and proteins that enlarge with the development of the disease and age [40].

Wet, exudative or neovascular AMD occurs when unstable blood vessels grow and extend from the choroid in a process called choroidal neovascularization (CNV). These new blood vessels are fragile and leaky and therefore their content can extravasate an affect vision [41] .

Excess of VEGF induces the progression of AMD towards its neovascular form. It represents a contributory factor in the initiation of angiogenesis as it directs the migration

of the “endothelial tip cells”. Tip cells are specialized endothelial cells that guide vessels to growth towards hypoxic or inflamed tissue [42]. The “tip” phenotype is controlled by the expression of the Notch ligand DLL4 in endothelial cells exposed to a VEGF gradient, triggering Notch activation in adjacent cells [43].

These signaling events further ensure the selection of the initial Notch-inactive cell as a single tip cell leading the new blood vesssels, as opposed to the Notch-active tip cells forming the base of the sprout [43-44]. Ultimately, the inhibition of VEGF-mediated sprouting by Notch promotes the maturation and quiescence of the capillary network [45].

Blood-retinal barrier (BRB) breakdown and vascular leakage is the leading cause of blindness of diabetic retinopathy (DR). Hyperglycemia-induced oxidative stress and inflammation are primary pathogenic factors of this severe DR complication. An effective interventional modality against the pathogenic factors during DR is needed to curb BRB breakdown and vascular leakage. This study sought to examine the protective effects melanocortin stimulating on diabetic retinopathy of a mouse model, in primary cells culture and ARPE-19 cells treated with high glucose 35mM , studying the mechanisms

the MCR. Melanocortin receptors represent a family of G-protein-coupled receptors classified in five subtypes and are expressed in retina. Our previous data indicate that subtypes 1 and 5 receptor agonists exert a protective role on experimental diabetic retinopathy.

CHAPTER 2

2.1 Blindness and Diabetic Retinopathy

As the worldwide prevalence of diabetes mellitus continues to increase, diabetic retinopathy remains the leading cause of visual impairment and blindness in many developed countries. Between 32 to 40 percent of about 246 million people with diabetes develop diabetic retinopathy. Approximately 4.1 million American adults 40 years and older are affected by diabetic retinopathy. This glucose-induced microvascular disease progressively damages the tiny blood vessels that nourish the retina, the light-sensitive tissue at the back of the eye, leading to retinal ischemia (i.e., inadequate blood flow),

retinal hypoxia (i.e., oxygen deprivation), and retinal nerve cell degeneration or death. It is a most serious sight-threatening complication of diabetes, resulting in significant irreversible vision loss, and even total blindness [46].

The leading causes of chronic blindness include cataract, glaucoma, age-related macular degeneration, corneal opacities, diabetic retinopathy, trachoma, and eye conditions in children (e.g., caused by vitamin A deficiency) [46]. Age-related blindness is increasing throughout the world, as is blindness due to uncontrolled diabetes.

The estimated number of people visually impaired in the world is 285 million, with 39 million blind and 246 million having low vision; 65% of people visually impaired and 82% of all blind are aged 50 and above (Table 1-1) [47].

Age (in years)	Population (millions)	Blind (millions)	Low Vision (millions)	Visually Impaired (millions)
0 – 14	1848.50	1.421	17.518	18.939
15 – 49	3548.2	5.784	74.463	80.248
50 and older	1340.80	32.16	154.043	186.203
all ages	6737.50	39.365 (0.58)	246.024 (3.65)	285.389 (4.24)

Table 1-1: *Global estimate of the number of people visually impaired by age, 2010; for all ages in parenthesis the corresponding prevalence (%) [47].*

The distribution of people visually impaired in the six WHO Regions is shown in Table 1-2 with the percentage of the global impairment shown in parentheses [47]. About 90% of the world's visually impaired live in low-income settings [48].

Globally, the major causes of blindness are cataract, glaucoma, age-related macular degeneration, and diabetic retinopathy (Figure 6) [49]. Diabetic retinopathy is a serious eye problem in the world, and also the most common diabetic eye disease. It is the leading cause of blindness in working-aged adults in the United States and around the word [50- 51].

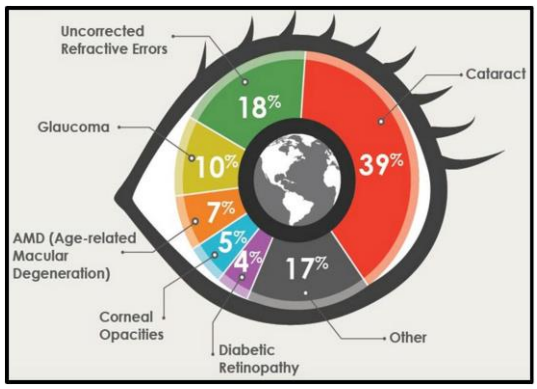

Figure 6: *Global causes of blindness due to eye diseases and uncorrected refractive errors [49].*

Diabetic eye disease refers to a group of eye problems that people with diabetes may face as a complication of diabetes, and all can cause severe vision loss or even blindness [52]. Diabetic eye disease may include diabetic retinopathy, cataract, and glaucoma. Cataract is a clouding of the eye's lens and develops at an earlier age in people with diabetes. Glaucoma results from rise in fluid pressure inside the eye that leads to optic nerve damage and loss of vision. A person with diabetes is nearly twice as likely to get glaucoma as other adults. Diabetic retinopathy is caused by diabetes. It affects the retina, the light-sensitive tissue at the back of the eye, and causes the most blindness in U.S. adults [5]. It affects the vision of more than half of the 18 million people diagnosed with diabetes age 18 or older. People with diabetes should have a complete eye exam through dilated pupils at least once a year.

2.2 Stages of Diabetic Retinopathy

The retina is the light-sensitive tissue at the back of the eye. A healthy retina is necessary for good vision. Diabetic retinopathy is a gluocose-induced microvasuclar disease, leading to progressive damage to the tiny blood vessels that nourish the retina [53]. The damaged blood vessels may swell and leak blood and other fluids, causing clouding of vision [54]. This circulation problems cause retinal tissue to become oxygen deprived, resulting in retinal hypoxia (i.e., lack of oxygen) and irreversible retinal nerve cell degeneration and death. It is a serious sight-threatening complication of diabetes. Diabetic retinopathy usually affects both eyes, leading to significant vision loss if left untreated. Normal vision and the same scene viewed by a person with diabetic retinopathy are compared in Figure 6 (A,B) [52].

Figure 6. *(A) Normal vision. (B) The same scene viewed by a person with diabetic retinopathy [52].*

Diabetic retinopathy has four stages or severity levels [52, 54, 55], which are summarized in Table 2 [10]:

Proposed disease severity level	Findings observable upon dilated ophthalmoscopy
Mild NPDR	Microaneurysms only
Moderate NPDR	More than just microaneurysms but less than severe NPDR
Severe N*β*I/PDR	No signs of PDR, with any of the following: 1. More than 20 intraretinal hemorrhages in each of four quadrants 2. Definite venous beading in two or more quadrants 3. Prominent intraretinal microvascular abnormalities in one or more quadrants
PDR	Neovascularization and/or vitreous/preretinal hemorrhage

Table 2. *International Clinical Diabetic Retinopathy Disease Severity Scale [55].*

Mild Nonproliferative Diabetic Retinopathy (NPDR). At this earliest stage, small areas of balloon-like bulges called microaneurysms may protrude from the walls of the retina's tiny blood vessels.

Moderate NPDR. As the disease progresses, some blood vessels that nourish the retina are blocked.

Severe NPDR. Many more blood vessels are blocked, depriving several areas of the retina with their blood supply.

Proliferative Diabetic Retinopathy (PDR). At this advanced stage, the retina sends signals for nourishment, triggering the growth of new blood vessels that are abnormal and fragile. The new blood vessels grow along the retina and toward the vitreous, the gel-like fluid that fills the inside of the eye. They may leak blood into the vitreous, causing severe vision loss and blindness.

The classic retinal microvascular signs of non proliferative diabetic retinopathy are microaneurysms, hemorrhages, hard exudates (lipid deposits), cotton wool spots (accumulations of axoplasmic debris within adjacent bundles of ganglion cell

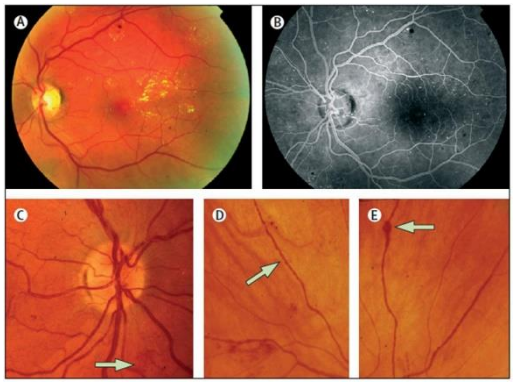

Figure 7. *Non-proliferative diabetic retinopathy. Cardinal signs are retinal microaneurysms, hemorrhages, and hard exudates (A and B); and intraretinal microvascular abnormalities (C, arrow); venous beading (D, arrow); and venous loop formation (E, arrow).*

axons), venous dilation and beading, and intraretinal microvascular abnormalities (dilated pre-existing capillaries; Figure 7).

The pathogenesis of PDR:

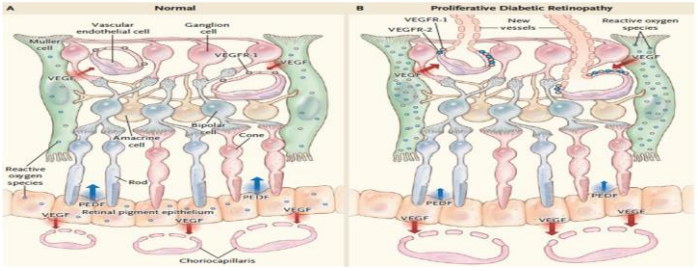

Figure 8: *Retinal anatomy and mechanisms of proliferative diabetic retinopathy [11]. A normal retina is shown in Panel A, and a retina from a patient with proliferative diabetic retinopathy is shown in Panel B are schematically shown in Figure 8 [47]*

Several polypeptide growth factors and their cell-membrane receptors have possible relevance to the pathogenesis of diabetic retinopathy, but vascular endothelial growth factor (VEGF) and its receptors, VEGFR-1 and VEGFR-2, and pigment-epithelium–derived factor (PEDF), for which no receptor has yet been identified, are currently undergoing the most intensive investigation [56]. These two growth factors are both produced in the retinal pigment epithelium, where their constitutive secretion appears to be highly polarized. Retinal neovascularization in diabetic retinopathy nearly. Always occurs away from the retinal pigment epithelium and toward the vitreous space. There is evidence that both VEGF and PEDF are produced in retinal neurons and in glial cells, such as in Müller cells [56]. In the normal retina, VEGFR-1 is the predominant VEGF receptor on the surface of retinal vascular endothelial cells, but in diabetes, VEGFR-2 appears on the endothelial-cell plasma membrane (Figure 1-4) [56].The appearance of retinal neovascularization is a hallmark of PDR (Figure1-5) [57]. Fibrovascular proliferation is a characteristic of advanced proliferative disease

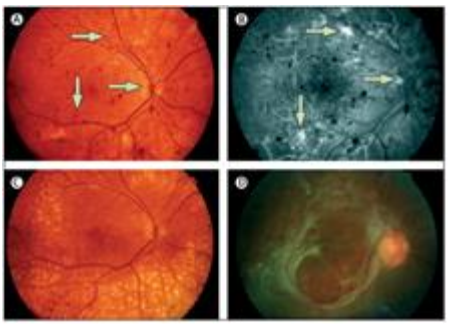

Figure 9. *Proliferative diabetic retinopathy [57]. Neovascularization, a hallmark of proliferative diabetic retinopathy (A, arrows), which can be identified on fluorescein retinal angiogram (B, arrows); resolution of retinopathy with panretinal photocoagulation (C);progression of retinopathy without treatment to fibroproliferative disease (D).*

2.3 Causes of Vision Loss and Risk Factors

Blood vessels damaged from diabetic retinopathy can cause vision loss in two ways [52, 54]:

Fluid can leak into the macula, the area of the retina that is responsible for sharp, clear, central vision and allows us to see colors and fine detail. The fluid makes the macula swell, blurring vision. This condition is called macular edema [58]. It can occur at any stage of diabetic retinopathy, although it is more likely to occur as the disease progresses. About half of the people with proliferative diabetic retinopathy also have macular edema.Fragile, abnormal blood vessels can develop and leak blood into the center of the eye, blocking vision. This is proliferative diabetic

retinopathy and is the fourth and most advanced stage of the disease. Other complications include detachment of the retina due to scar tissue formation and the development of glaucoma, an eye disease causing progressive damage to the optic nerve. In cases of proliferative diabetic retinopathy, the cause of this nerve damage is extremely high pressure in the eye. If left untreated, proliferative diabetic retinopathy can cause severe vision loss and even blindness.All people with diabetes–both type 1 and type 2–are at risk for the development of diabetic retinopathy. The longer the patients have diabetes, the more likely they will develop diabetic retinopathy. Between 40 to 45 percent of Americans diagnosed with diabetes have some stage of diabetic retinopathy [52;59].

People with other medical conditions such as high blood pressure and high cholesterol are at greater risk [54]. Pregnant women face a higher risk for developing diabetes and diabetic retinopathy [59]. If gestational diabetes develops, the patients are at much higher risk of developing diabetes as they age. Everyone with diabetes is recommended to get a comprehensive dilated eye examination at least once a year, since macula edema and proliferative diabetic retinopathy can develop without symptoms, such that patients are at high risk for vision loss.

2.4 Symptoms

Often there are no visual symptoms in the early stages of diabetic retinopathy. In the later stages, symptoms of the disease may include [54]:

1. **Blurred vision.** Prolonged periods of high blood sugar can lead to the accumulation of fluid at the lens inside the eye that controls eye focusing. This changes the curvature of the lens and results in the development of symptoms of blurred vision. Blurred vision may also occur when the macula (i.e., the part of the retina that provides sharp central vision) swells from leaking fluid.

2. **Seeing spots or floaters in the field of vision.** If new blood vessels grow on the surface of the retina, they can bleed into the eye and block vision. When the bleeding occurs, patients will see a few specks of blood, or spots, “floating” in the field of vision. If spots occur, patients should see an eye care professional as soon as possible. They may

need treatment before more serious bleeding occurs. Hemorrhages tend to happen more than once, often during sleep. If left untreated, proliferative diabetic retinopathy can cause severe vision loss and even blindness.

2.5 Diagnosis and Detection

Diabetic retinopathy and macular edema can be detected through a comprehensive eye examination that includes [52, 54]:

- **Visual acuity test.** This eye chart test determines the extent to which central vision has been affected.
- **Dilated eye exam.** Drops are placed in eyes to widen, or dilate, the pupils. This allows the eye care professional to see more of the inside of eyes to check for signs of the disease. The eye care professional uses a special magnifying lens to examine the retina and optic nerve for signs of damage and other eye problems.

❖ **Tonometry.** An instrument measures the pressure within the eye. Numbing drops may be applied to eyes for this test.

The eye care professional checks the retina for early signs of the disease, including [52]:

1. Leaking blood vessels.

2. Retinal swelling (macular edema).

3. Pale, fatty deposits on the retina (signs of leaking blood vessels).

4. Damaged nerve tissue.

5. Any changes to the blood vessels.

Supplemental testing may include fluorescein angiography [52, 54, 59]. In this test, a special dye is injected into the arm, and pictures are taken as the dye passes through the blood vessels in the retina.The test allows the evaluation and identification of any abnormal blood vessel growth and leaking blood vessels.

2.6 Current Treatments of Diabetic Retinopathy

Treatment of diabetic retinopathy depends on the stage of the disease. During the first three stages of diabetic retinopathy, no treatment is needed, unless macular edema occurs.

2.7 Laser Surgery

If the disease advances, leakage of fluid from blood vessels can lead to macular edema, which is treated with laser surgery. This procedure is called focal laser treatment (photocoagulation) [52, 54]. Up to several hundred small laser burns are created in areas of the retina with abnormal blood vessels to try to seal the leaks and reduce the amount of fluid in the retina. A patient may need focal laser surgery more than once to control the leaking fluid.When blood vessel growth is more widespread throughout the retina, as in proliferative diabetic retinopathy, a laser surgery called scatter laser treatment is needed [52, 60]. 1,000 to 2,000 scattered laser burns are created in the areas of the retina away from the macula, causing the abnormal blood vessels to shrink and disappear. Because a high number of laser burns are necessary, two or more sessions usually are required to complete treatment. With this procedure, peripheral vision may be partially lost in order to

preserve central vision. Scatter laser treatment may slightly reduce color vision and night vision .

2.8 Vitrectomy

In more advanced cases such as severe bleeding, a surgical procedure called a vitrectomy may be needed to restore sight by removing significant amount of blood from the center of the eye (vitreous gel) [52, 61]. Retinal detachment, defined as separation of the light-receiving lining in the back of the eye, resulting from diabetic retinopathy, may also require surgical repair [9].

A vitrectomy is performed under either local or general anesthesia. A doctor makes a tiny incision in the eye of a patient. Next, a small instrument is used to remove the vitreous gel that is clouded with blood and replace it with a salt solution to maintain the normal shape and health of the eye. Since the vitreous gel is mostly water, the patient will notice no change between the salt solution and the original vitreous gel. The patient's eye will be red

and sensitive. The patient will need to wear an eye patch for a few days or weeks to protect the eye, and also need to use medicated eyedrops to protect against infection.

2.9 Anti-VEGF Therapy

Anti-VEGF therapies are important in the treatment of diabetic retinopathy. They can involve monoclonal antibodies such as bevacizumab, antibody derivatives such as ranibizumab, or orally-available small molecules that inhibit the tyrosine kinases stimulated by VEGF [62]. Both antibody-based compounds are commercialized.

The efficacy of treatment with the anti-VEGF agents ranibizumab and bevacizumab indicates that VEGF contributes to the pathogenesis of diabetic macular edema and reflects successful translational research.

2.10 Severe Drawbacks of Current Treatments

Although current treatments of diabetic retinopathy (i.e., laser treatment, vitrectomy surgery and anti-VEGF therapy) can reduce vision loss, they only slow down but cannot stop the degradation of the retina. Patients require repeated treatment to protect their sight. Once some people develop proliferative diabetic retinopathy, they will always be at risk for new bleeding.

The current treatments also have significant drawbacks. Laser therapy is focused on preserving the macula, the area of the retina that is responsible for sharp, clear central vision, by sacrificing the peripheral retina since there is only limited oxygen supply. Therefore, laser therapy results in a constricted peripheral visual field, reduced color vision, delayed dark adaptation, and weakened night vision [63]. Vitrectomy surgery increases the risk of neovascular glaucoma, another devastating ocular disease, characterized by the proliferation of fibrovascular tissue in the

anterior chamber angle [64]. Anti-VEGF agents have potential adverse effects, and currently there is insufficient evidence to recommend their routine use.

CHAPTER 3

3.1 Family of Vascular endothelial growht factor (VEGF)

VEGF is a peptide discovered by Napoleone Ferrara[65], who described it as a potent mitogen for follicular cells from bovine pituitary [65]. Harold Dvorak had already isolated VEGF baptizing it as vascular permeability factor (VPF), because of its great capacity to induce plasma extravasation [66]. The VEGF family is essential for proliferation and differentiation in vasculogenesis, angiogenesis, and lymphangiogenesis [66].

The VEGF family consists of seven genes that give rise to dimeric glycoproteins structurally related (Fig. 10).

- VEGF-A (VEGF)
- VEGF-B
- VEGF-C
- VEGF-D

- VEGF-E (orphan virus parapox)
- VEGF-F (snake venom)
- PIDF (Placental growth factor)

The gene VEGF-A generates 5 isoforms from alternative splicing resulting in peptides with 121, 145, 165, 189, and 206 amino acids. Isoforms 121, 165, and 189 are found most frequently [67]. VEGF synthesis can be stimulated by various growth factors (EGF, IGF-1, TNF, TNFa, TNFß, and interleukins such as IL-6, IL-10, and IL-33) and hormones like TSH, ACTH, estradiol, and progestins [68].

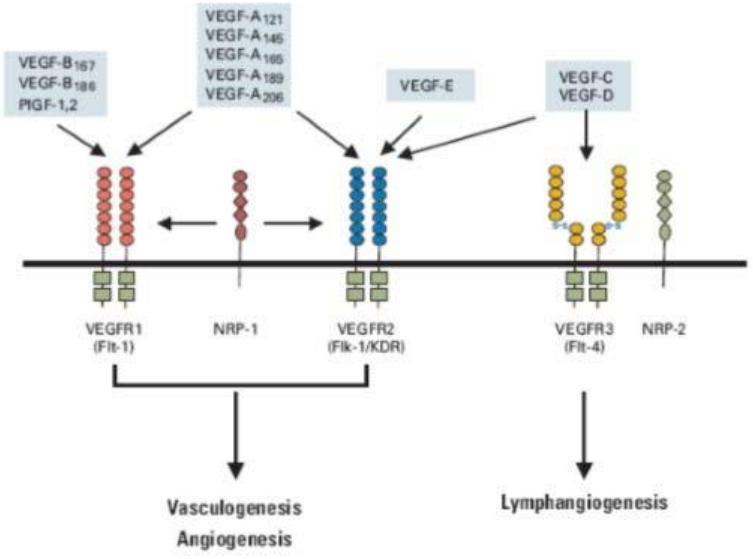

Figure 10: Ligands and receptors of the VEGF family. *The mammalian family of VEGF ligands consists in VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PlGF). VEGFA binds to both VEGF receptor 1 (VEGFR1) and VEGFR2. VEGFB and PlGF bind exclusively to VEGFR1. Heterodimers of VEGFA and PlGF have been identified that can bind to and activate VEGFR2. VEGFR3 is a specific receptor for VEGFC and VEGFD [69].*

3.2 VEGF Receptors and functions

The VEGF family activates three tyrosine kinase transmembrane receptors (Fig. 11):

- VEGFR1 or Flt -1. It has affinity for VEGF-A, VEGF-B, and PlGF-1 and -2 factors. This receptor is expressed in ECs, smooth muscle cells, and monocytes, among others.

- VEGFR2, KDR or Flk-1. It has affinity for VEGF-A, VEGF-C VEGF-D,

and VEGF-E factors. This receptor is expressed predominantly in ECs.

- VEGF3 or Flt -4. It has high affinity for VEGF-C and VEGF-D factors. This receptor is located in the lymph ECs.

In addition, there are three receivers without tyrosine kinase:

- SVEGFR1. A soluble form of VEGFR1 might work as a natural scavenger for VEGF as it competes by polypeptide binding without an intracellular effect.

- NP1. It is capable of binding PIGF, VEGF-B, and VEGF-A165 but not VEGF-A121. It functions as a synergistic co-receptor in the interaction of VEGF with VEGFR2 and VEGFR1, increasing tumor angiogenesis.

- NP2. It binds to VEGF-A145 and VEGF-A165, PlGF, and BEGF-C and is related to the formation of lymphatic vessels [70].

Most angiogenic promoting signals produced by the VEGF-A are mediated by VEGFR2. This receptor consists of an extracellular region of seven domains tyrosine kinase of 70 amino acids. Besides being expressed in ECs, VEGFR2 is also expressed in neurons, osteoblasts [71] and RPE [72].

Exposure to VEGF in ECs allows flow of substances of different sizes through different mechanisms: (1) the formation of fenestrations, allowing a flow of solutes with small molecular weight [73] ; (2) the induction of invaginations of the cell membrane, allowing the flow of small proteins through the cytoplasm; (3) the construction of endothelial junctions, allowing transport of high molecular weight proteins [74]. The size and number of cell junctions and pores are affected by VEGF in a dose-dependent manner. It was suggested that very specific concentrations of exogenous VEGF might manipulate the vascular barrier [75]. Blocking the communication between adjacent ECs occurs by binding of VEGF with receptor 2 through activation of Src kinases and Yes7 and phosphorylation of connexin-43 (Fig. 11).

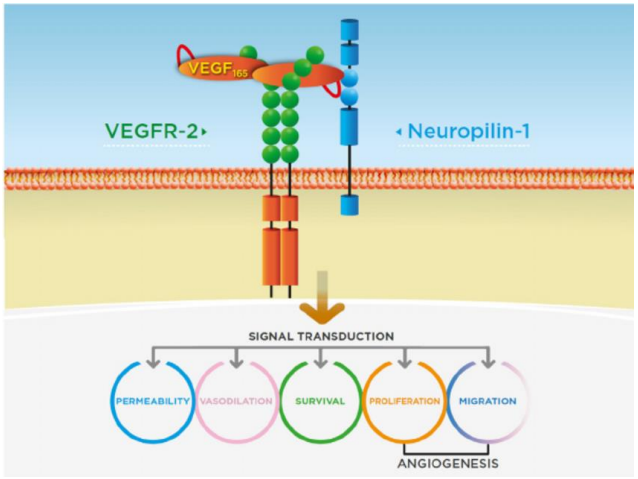

Figure 11: *Signaling pathways and interactions between VEGF and VEGFR2 [76].*

Many of the proteins of adherens junctions (VE- cadherin, β-catenin, among others) are also phosphorylated by the tyrosine kinase domain of VEGFR2, after activation thereof, leading to a weakening of cell-cell junction between ECs in vivo [77]. In the absence of VEGF, VEGFR2 activation due to stress is sufficient to induce a vascular permeability mediated by integrins causing the reorganization of the proteins of adherens junctions [78;79].

VEGFR1 is formed by an extracellular domain with seven immunoglobulin-like domains, a transmembrane region, and an intracellular tyrosine kinase domain. The expression of this receptor is stimulated by different injuries, such as

hypoxia or EtOH exposure, among others [80]. Although the domain has a very low activity, some scientists suggest that VEGFR1 is critical in angiogenesis [81]. This is evident because VEGFR1 deficient mice die by vascular disorganization, so this receptor would function as captor of VEGF-A during embryogenesis, regulating VEGFR2 activity by competition with substratum [82].

3.3 Angiogenesis and Exosomes

The influence of exosomes on developmental tissue has been suggested. They have an important role in osteogenesis, both *in vitro and in vivo*, since they seem to be implicated in bone regeneration (Qin et al., 2016). Besides, exosomes from Human- Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cells (hiPSC-MSC) can stimulate bone regeneration and angiogenesis in critical-sized calvarial defects in ovariectomized rats [83]. Exosomes have a strong implication in diseases such as cancer. Recent studies demonstrate that the anti-angiogenic effect of the exosome is cell-specific, since exosomes derived from bone marrow MSCs showed an opposite effect on the expression

of VEGF and bFGF in tumor cells [84]. Moreover, platelet-derived microvesicles (PMV) – circular fragments from the surface membranes of activated platelets – and exosomes released from platelets, could contribute to metastatic spread, transferring integrin CD41. Further, a recent report showed that the increased level of circulating PMV is a strong predictor of metastasis in patients with gastric cancer [85]. In addition, the theory that exosomes modulate cancer angiogenic processes has been strengthened [86]. Actually, exosomes obtained from plasma of glioma patients display pro-angiogenic features, indicating that glioma derived exosomes play a role in initiating angiogenesis [87]. Additionally, exosomes from LAMA84 chronic myeloid leukemia (CML) cells affect vascular remodeling in vitro through an IL-8 mediated activation of VCAM-1[88]. Moreover, exosomes induce migration and tube formation, but did not affect EC proliferation [89].

CHAPTER 4

4.1 Cellular Communication

In multicellular organisms, cells communicate by extracellular signaling molecules such as nucleotides, lipids, and proteins. These molecules, after being released into the extracellular medium, bind to receptors of their neighboring cells, inducing intracellular signaling and modifying the physiological state of the recipient cell. In addition to the release of these signaling molecules, eukaryotic cells are able to release membrane-bound vesicles to the extracellular medium. These vesicles contain numerous proteins, lipids, and nucleic acids that might affect the fate of the target cells. These vesicles are known as extracellular vesicles (EVs) [90].

It has been observed that EVs are involved in cell communication, transporting information (proteins, mRNA, miRNAs, and even DNA fragments) between cells [91]. Thus, a donor cell produces EVs and

another cell is able to incorporate and interpret the information that is inside the EV. The information in the form of nucleic acids and proteins contained in these vesicles can trigger processes such as cell proliferation, differentiation, migration, and apoptosis in the recipient cells. EVs are involved in processes such as immune suppression of tumor metastasis and angiogenesis [92-94].

Since it is a relatively new issue, it appears necessary to standardize the nomenclature for the different types of EVs. A recent article by Maria Mittelbrunn [95] makes a very convenient classification of the EVs, while collecting information on the mechanisms of formation of these vesicles. Based on their origin and size, EVs can be classified into three different groups: exosomes, ectosomes (microvesicles), and apoptotic bodies (AB) (Fig.12).

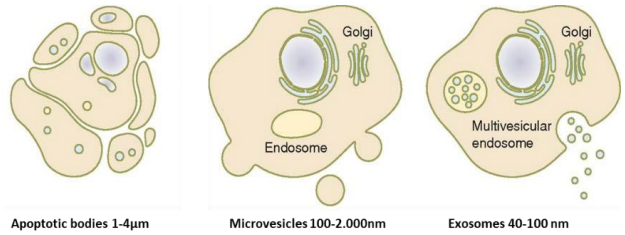

Figure 12. Types of Extracellular vesicles. *Extracellular membrane vesicles as a mechanism of cell-to-cell communication [96].*

4.2 Exosomes

The term "exosome" was proposed for the first time in the early 1980s to designate membranous vesicles secreted during maturation of chicken reticulocytes [97]. In this study, it was observed that the absent transferrin receptor in mature erythrocytes was previously released in small vesicles of endocytic origin. Thus, initially it was thought the main function of exosomes was the elimination of unnecessary molecules for the mature cell. After that conclusion, the study of exosomes was interrupted for several years.

Lately, interest in exosomes was renewed thanks partially to the research of Raposo and collaborators, who revealed for the first time the relevance of exosomes in adaptive immune response [98].

Exosomes have a size between 40 and 150 nm in diameter and a density of 1.13-1.19 g/ml in sacarose [99]. They are rich in proteins derived from the plasma membrane of the endocytic pathway and cytosol, but may also contain small amounts of proteins from the Golgi, ER, and the nucleus (Fig. 13).

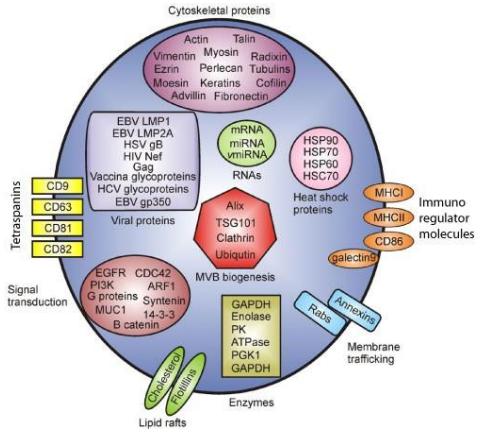

Figure 13: Typical structure and content of exosomes. *Exosomes are surrounded by a phospholipid bilayer and contain proteins, such as annexins, that are important for transport; tetraspanins for cell targeting; and other proteins, such as Alix and TSG101, that are involved in exosomal biogenesis from endosomes [99].*

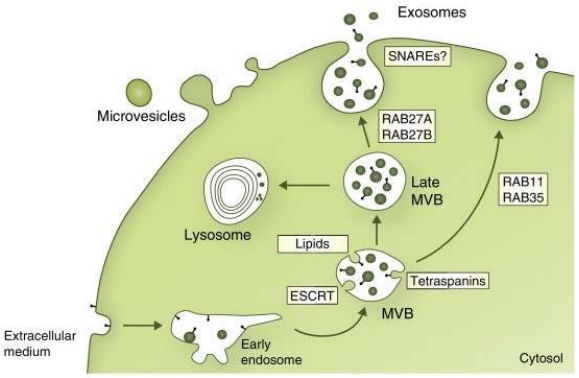

Figure 14: Exosomes biogenesis. *ESCRT machinery, lipids (such as ceramide), and tetraspanins are involved in ILVs formation. The MVBs can fuse with lysosome or with Plasmatic Membrane (PM). Several RAB proteins (RAB11, RAB27 and RAB35) have been shown to be involved in the transport of MVBs to the PM and in exosome secretion [100].*

The MVBs can follow two routes: the degradative and the exocytic. In the degradative pathway, the fusion of MVBs with lysosomes (forming the amphisome) induces degradation of proteins and lipids present in them, allowing also the removal of excessive membrane[101;102]. In the exocytic route, the MVBs fuse with plasma membrane in a process where a number of proteins are involved: Rab11, RAB35, and RAB27, just to name a few [103]. Figure 7 compares biogenesis of exosomes, ectosomes, and

apoptotic bodies. Exosomes biogenesis determines the orientation of the membrane, so the proteins have the same orientation as in the plasma membrane [98,100,104].

Moreover, comparison of exosomes membrane with the plasma membrane of the cell from which they derive allow the conclusion that they are not simple fragments of it. Unlike other types of microvesicles, exosomes lack certain highly abundant proteins of the plasma membrane. For example, it has been observed that dendritic cells (DC)-derived exosomes lack FcyRII/III receptor [105], exosomes from T cells lack CD28 and CD45 [106], and exosomes from B cells lack the transferrin receptor [98].

Today, the complete cellular machinery of the merging mechanism between MVBs and plasma membrane is not completely understood. Several studies have shown that some proteins, such as soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors (SNAREs) or some types of Rab (Rab11 and Rab27), may play an important role in the transport and merge with the plasma membrane [107-110]. Moreover, it

has been demostated that diacylglycerol kinase in T cells, and Big2 protein in endothelial cells are involved in these processes [111;112].

Exosomes are secreted into the extracellular medium of established cell lines, including: intestinal [99] and traqueobronquial [113] epithelial cells, mesenchymal stem cells [114], fibroblasts [115], reticulocytes [116], mastocytes [117], platelets [118], DCs [119-121], T cells [122], B cells [123], keratinocytes [124], glial cells [125], and microglia [127],neurons [128], astrocytes [129], and numerous tumoral cell lines [132-133]. Exosomes have also been described in various biological fluids such as serum[134;135], bronchoalveolar fluid [136], urine [137], tumor effusions [138], sperm [139], amniotic fluid [140] saliva [141], and breast milk [143] and colostrum [144].

4.3 Secretion

Exosome release can be either constitutive or regulated by different external stimuli, including increased levels of intracellular Ca2+ [145]. Exosomes secretion can also be stimulated exogenously by cellular stress promoters (heat shock or UV radiation) [146]; and drugs, such as calcium ionophores [147;148], sequestering agents , and inhibitors of [149], cholesterol synthesis [150]. Once formed the MVB, it should fuse with the plasma membrane in order to release its content and secrete the exosomes. A family of proteins involved in the intracellular transport is the Rab family. Depending on the cell type, there are different molecules involved in the process of EV secretion. In the K562 cell line, it has been shown that Rab11 is necessary for releasing calcium-induced exosomes. In oligodendrocytes, Ra[151], b35 plays an important role in the secretion of exosomes[152], Rab27b and Rab27a control various steps of the exosome secretion pathway in HeLa cells [153]. The final step of the merge between MVBs and the plasma membrane is probably mediated by SNARE

proteins, although the cellular machinery is not completely understood yet. As aforementioned, exosome release can be enhanced by any damage applied to the cell. Few studies have focused on the interaction of exosomes with the recipient cell. The results obtained suggest four possible mechanisms of interaction: (I) receptor- mediated endocytosis[154], (II) cell surface adhesion ([155-159], (III) fusion with the plasma membrane[160], and (IV) phagocytosis [161].

4.4 Molecular Composition

The protein composition of exosomes varies depending of the cell type as well as the [164] and are enriched with heat shock proteins after heat stress [165]. Moreover, exosomes secreted by DCs express CD11c [166] and, upon activation, are enriched with MHC-II, CD86, and the intercellular adhesion molecule 1 (ICAM- 1) [167].

Regardless of the cell of origin, exosomes share a set of proteins that are considered "exosomal markers" [168].These markers include: proteins

involved in the biogenesis of MVBs (Alix, clathrin, and Tsg101), tetraspanins (CD9, CD63, and CD81), which is one of the most abundant families in exosomes; and heat shock proteins (hsp) that are involved in antigen presentation, such as hsp60, hsc70, hsp70, and hsp90.

Other proteins found in exosomes are: ICAM-1; lipid rafts associated proteins, as flotillin-1; proteins involved in cellular traffic (annexins, syntaxin, dynamin); metabolic enzymes (enolase, aldolase, ATP citrate lyase, fatty acid synthase); proteins involved in signal transduction, such as kinases and phosphatases; and cytoskeletal proteins, such as actin, tubulin, and vimentin. Other proteins detected in exosomes are ATPases, albumin, histones, and transcription factors (TF) [166-173].The machinery that make proteins enter the MVBs is not exactly known. Several mechanisms appear to be involved, including: the endosomal sorting complex required for transport (ESCRT), which recognizes ubiquitination signals, protein aggregation into oligomers, and the presence of proteins in lipid rafts [174-176]. Exosomes present lipid rafts enriched in sphingomyelin, ceramide

and cholesterol, and exposed to the outer face of the lipid bilayer that is normally found in the cytoplasmic face of the plasma membrane,[176-179]. These lipids can change depending of the origin cell or the proteins expressed in the plasma membrane of these cells [180].

Some studies have shown the presence of mRNAs and miRNAs in exosomes released by mast cells of mouse and human [181]. These RNAs were biologically active, thus conferring new functions to the recipient cells after their capture. Later, Skog and collaborators confirmed their presence in exosomes secreted by a glioblastoma human cell line [182]. This discovery has enhanced interest in exosomes in recent years.

To date, the transcriptome of exosomes from different types of cells , for example, pancreatic cells [183], neurons [184], monocytes [186] , B cells [187], T cells and DCs [188] and from body fluids like serum [189], and saliva [190]. The data obtained suggest a mechanism for targeting exosomes that are enriched with certain RNAs, depending on their cellular origin.

The necessity to collect information on the composition of proteins, lipids, and genetic material, in exosomes from different sources has led to the creation of the database ExoCarta (www.exocarta.org). To date, they have been included in the database: 135 articles published in peer-reviewed journals, a total of 4,520 protein entries, 194 lipid entries, 1,639 mRNAs entries and 764 miRNAs entries.

The fact that the composition of exosomes (proteins, lipids, mRNAs and miRNAs) is a reflection of the type of cell and the physiological state of such cell has led to propose exosomes as biomarkers for diagnosis of various human diseases [190].

4.5 Exosome functions

The first function attributed to exosomes was the ecto-enzymes transport of the membrane [183]. Later this exosomes function was expanded and it was proposed that exosomes are sweeping obsolete proteins and excessive cell membrane [190]. Today, besides being considered as vehicles of different types of molecules, it is known that exosomes can be captured by recipient cells through specific nonrandom mechanisms [191]. The content of exosomes induces a response in the host cell comparable to conventional systems triggered by cell signaling [192], so they can be considered regulators of cell-cell communication. Exosomes are involved in the survival and metastasis of tumors by modifying the microenvironment [192]. It has been described, the role of exosomes in communication between neurons in neurodegenerative diseases such as Alzheimer, Parkinson and prion-induced neurodegeneration [187]. They have also a role in cardiovascular diseases, such as atherosclerosis [193], heart

hyperplasia, and cardiomyocyte dysfunction[195]. Despite the limited studies in the liver, it has been found that hepatocytes release and receive exosomes[196]. Moreover, exosomes have been linked to the pathogenesis of hepatocellular carcinoma[197], viral hepatitis [198] and hepatic inflammation [199]. Furthermore, the exosomes isolated from mouse hepatocytes contain cytochrome P450, a family of enzymes implicated in drug detoxification [188].

4.6 Exosomes and the RPE

It has been recently proved the presence of exosomes in extracellular medium of ARPE-19 cells, a commercial RPE cell line. Unlike other tissues, there are only a few studies about exosomes from RPE [165]. RPE-released exosomes were altered when cells were under OS. Biasutto et al.; [169] described that exosomes from ARPE-19 cells varied their protein cargo when they were subjected to low levels of stress [190].Other studies showed that mitochondrial DNA damage enhanced exosome release when cells were under stress

[191].Furthermore, exosomes released after stimulation with ciytokines, inhibit T cellactivation and induce monocyte death, which does not occur if exosomes are derived from unstimulated cells [192]. Exosomes also play an important role in diseases such as AMD. Recently, it was found that the complement regulatory protein (CD59) was increased in regions of the RPE of early AMD patients, but decreased in advanced form of AMD [153]. The study showed that this protein was released within exosomes to the subretinal space [198].

4.7 Stress Stimulates Exosomes Production

Exosomes production is stimulated by OS generated by the presence of ETOH Atienzatr et al., 2016 showed that was able to stimulate exosome production in ARPE-19. They showed that ETOH-induced exosomes appeared to have an increased protein content. So we were stimulated the ARPE-19 cells with different stimuli I used high glucose for nine days and after nine days of exposure, it was observed a dramatical increase of reactive oxygen species (ROS) and descreased of viability cells (XTT). Interestingly, after treatment with melanocortin agonist receptors MCR 1 and 5 to reduced ROS and

increased viability cells production, it was observed a decreased exosomes formation, also found that exosomes are quite stable under physiological and pathological conditions. Increase of exosomes and their protein content, after a brief high glucose treatment, leads to denatured proteins and cellular damage.

CHAPTHER 5

Melanocortin

Melanocortins are endogenous peptides that possess a wide range of biological activities, including inhibition of leukocyte activation, promotion of inflammation resolution, and the ensuing tissue protection [200-205]. These effects on the immune response are brought about by five distinct melanocortin receptors, termed from MC_1 to MC_5, which are ubiquitously expressed except for the MC_2 which is localised to the adrenal glands [206]. Within the eye, MC_3, MC_4, and MC_5 are expressed in the inner neural retinal layers [206; 207], with MC_3 and MC_4 expression being reported also in the layer of retinal ganglion cells [206; 207]. MC_5 alone has been detected in the neural outer plexiform layer, whilst MC_1 and MC_5 are detected in retinal pigment epithelial cells [208; 209]. There is scant knowledge on the biology associated with these receptors in the eye.

The melanocortin system is composed by the agonists adrenocorticotropic hormone and , α,β and γ melanocyte stimulating hormone, and two naturally

occurring antagonists, agouti and agouti-related protein. These ligands act by interaction with a family of five melanocortin receptors (MCRs), assisted by MCRs accessory proteins (MRAPs). MCRs stimulation activates different signaling pathways that mediate a diverse array of physiological processes, including pigmentation, energy metabolism, inflammation and exocrine secretion. This study focuses on the regulatory mechanisms of MCRs signaling, highlighting the differences among the five receptors. MCRs signal through G-dependent and independent mechanisms and their functional coupling to agonists at the cell surface is regulated by interacting proteins, namely MRAPs and b-arrestins. The knowledge of the distinct modulation pattern of MCRs signaling and function may be helpful for the future design of noveldrugs able to combine specificity, safety and effectiveness in the course of their therapeutic use.

The melanocortin system has an important role in the regulation of a variety of biological functions like pigmentation, inflammation, stress response, sexual behavior and energy homeostasis [200]. This multiplicity of actions of the melanocortin system is possible due to a highly regulated interaction of

different biological molecules, namely different agonists, two naturally occurring antagonists, five different receptors and specific accessory proteins, which all integrate the melanocortin system.

5.**1. Agonists: the melanocortins**

The melanocortin peptides include the adrenocorticotropic hormone (ACTH) and a, b and c-melanocyte-stimulating hormones (α,β,γ -MSH) (Table 4). They are generated upon successive cleavages of the polyprotein proopiomelanocortin (POMC), mainly expressed in the pituitary and arcuate nucleus of the hypothalamus. Melanocortins are then released into the blood stream or move through the sympathetic nervous system to the whole organism. POMC was also found in other regions of the central nervous system and in a variety of peripheral tissues like skin, lung, kidney, adrenal glands, gastrointestinal and genitourinary tract [201]. The ubiquitous expression and the tissue-specific proteolytic cleavage of POMC surely contribute to the extraordinary complexity of functions associated to melanocortins.

5.2 Antagonists: agouti and agouti-related protein (AgRP)

One of the most interesting aspects of the melanocortin system is the presence of two naturally occurring antagonists, the agouti and the agouti-related protein (AgRP) (Table 4). A great interest in agouti emerged when it was discovered that dominant mutations in the mice gene induce obesity, insulin resistance, yellow coat and predisposition for tumors [203]. In mice, the agouti protein is mainly expressed in the skin, but in those mutants, agouti is ectopically expressed in other tissues. Due to agouti affinity for different MCRs, pleiotropic phenotypes associated with the ectopic expression of agouti were indeed predictable(Table 4). In fact, the coat pigmentation arises from an antagonic effect on MC1R whereas the other effects may result from the antagonism of other MCRs in different tissues. For instance, the obesity syndrome may derive from the blockade of MC4R signaling in the central nervous system. The agouti-signaling protein (ASIP) is the human homolog of the mouse agouti gene. It was detected in the skin, heart, ovary, testis, foreskin, adipose tissue, liver and kidney [204] and, although not so well characterized,

it also appears to regulate human pigmentation [205]. The AgRP was later discovered based on its sequence homology to agouti. It is mainly expressed in the adrenal gland and hypothalamus, where it acts as a potent orexigenic factor by antagonizing MC3R and MC4R (Table 4).

In fact, the effect of AgRP overexpression is similar to that described for agouti, excluding the pigmentation changes, because it does not antagonize MC1R [206]. Its function in adrenal glands remains to be elucidated since it has a lower antagonist effect on MC5R and no binding affinity for MC2R, the MCRs with major expression in this tissue [207].

MCR	Agonist affinity	Antagonists	Tissue expression	Main function
MC1R	α-MSH ≥ ACTH = β-MSH > γ-MSH	Agouti	Skin; immune cells; brain; pituitary; corpus luteum; placenta; testis; adipocytes	Pigmentation; anti-inflammatory
MC2R	ACTH	Agouti	Adrenal gland; skin; adipocytes	Adrenal steroidogenesis
MC3R	γ-MSH = α-MSH = ACTH > β-MSH	Agouti; AgRP	Brain; placenta; stomach; pancreas; duodenum; heart; testis; mammary gland; muscle cells; kidney; immune cells	Energy homeostasis; anti-inflammatory
MC4R	α-MSH ≥ ACTH > β-MSH > γ-MSH	Agouti; AgRP	Brain; sympathetic nervous system; muscles; adipocytes	Energy homeostasis
MC5R	α-MSH ≥ ACTH > β-MSH > γ-MSH	Agouti; AgRP	Exocrine glands; adrenal gland; pituitary; kidney; liver; lung; lymphatic system; testis; ovary; uterus; gastrointestinal tract; skin; skeletal muscle cells; adipocytes; B-lymphocytes; brain	Exocrine gland secretion; fatty acid β-oxidation; adipocyte lipid metabolism

Table 4 *Melanocortin receptor family : agonist affinity antagonist, tissue distribution and main function*

5.3 The melanocortin receptors (MCRs): physiological role

The research in the melanocortin field has gained new insights with the identification of high levels of binding sites for a-MSH in human melanoma cells by Tatro and colleagues [208]. Later on, a cDNA library from these cells allowed cloning the first MCR, initially named a-MSHreceptor [209; 210]. Concomitantly, a second receptor was cloned from human adrenal cells and termed ACTH receptor due to its high affinity for ACTH binding [211]. Afterwards, three more MCRs were discovered [211–215]and then all were numbered from 1 to 5 according to the order of their cloning (MC1R–MC5R). Structurally, all MCRs belong to the class of the GPCRs, which have and N-terminal extracellular domain, seven transmembrane regions and a C-terminal cytoplasmic domain [216].

The most exciting characteristics of MCRs regard the different binding affinities for the diverse melanocortins and antagonists and the absence of functional uniformity (Table 4). In fact, no other receptors have their activity regulated by both agonists and antagonists, making the MCRs unique among

the superfamily of GPCRs. This type of modulation allows the increase on specificity and accuracy of melanocortin's biological functions. The MC1R is recognized as the classic receptor of melanocytes because it is particularly involved in the regulation of skin and hair pigmentation. The activation of MC1R promotes the synthesis of eumelanin (dark pigment) and decreases the production of pheomelanin (yellow pigment) which results in the darkness of skin and hair [217]. MC1R was also described in a large number of different immune cells where it exerts an anti-inflammatory effect [218; 219]. The main function of MC2R is the control of steroidogenesis in the adrenal gland and, consequently,mutations in MC2R gene accounts for 25 % of all cases of the familial glucocorticoid deficiency (FGD), a rare autosomal recessive disorder characterized by a severe glucocorticoid deficiency, associated with failure of adrenal responsiveness to ACTH [220–222]. This deficiency is also observed in the MC2R knockout mice [223].

Moreover, MC2R is present in human skin cells [224] and in mouse adipocytes [225–228], where it was suggested to regulate lipolysis [229]. MC3R and MC4R are expressed at the nervous system and are intimately related with the

central regulation of energy homeostasis. Human mutations on MC3R gene are frequently related to obesity and type 2 diabetes [230] and some reports indicate that MC3R polymorphisms are associated with increased risk of childhood obesity [231–234]. Genetic disruption of the MC4R has been found tocause obesity in mice and mutations on MC4R human gene are responsible for up to 6 % of morbid obese individuals [235]. MC4R is also involved in the regulation of the sexual behavior and erectile function [236, 237] and pain [238, 239]. The MC5R was the last receptor of this family to becloned and was found to be expressed in a variety of organs and tissues. The MC5R knockout mice revealed a deficient function of the sebaceous and other exocrine glands. Additional roles to MC5R have been attributed, namely the activation of regulatory T-lymphocytes during ocular immunity [240–243], immunomodulatory functions in B lymphocytes [244] and stimulation of cytokine secretion in adipocytes [245]. In skeletal muscle cells, MC5R was specifically implicated on the regulation of fatty acid oxidation [246], while in adipocytes MC5R promotes lipolysis and impairs fatty acid re-esterification [246,248].

5.4 Accessory proteins for MCRs cell surface targeting

Two melanocortin receptor accessory proteins (MRAP1 and MRAP2) regulate the transport of the MCRs towards the cell membrane and also the ligand-induced signaling [248] (Table 5). The presence of interacting factors for MCRs function was first suggested by Noon et al. [49] who failed to obtain a correct trafficking of MC2R to cell surface when the receptor was expressed in cells that lack endogenous expression of the melanocortin system interveners. MRAP1 was further established as essential for MC2R trafficking and signaling [140, 250–253], a reason why 20 % of FGD type 2 patients have mutations in MRAP gene [200]. Later on, a closely related protein, named MRAP2, was found in adrenal gland and hypothalamus [248, 252]. Whereas MRAP1 is essential for MC2R-signaling activation, MRAP2 seems to be a competitive inhibitor of MRAP1, decreasing its ability to bind MC2R [252; 254] (Table 5). MRAPs assist MC2R targeting to cell membrane by facilitating ER export and further post-translational processing at Golgi apparatus, most

probably glycosylation[255]. Although MRAPs are only essential for MC2R targeting to cell surface [248, 256], they are able to interact with all five MCRs regulating their trafficking and cell surface expression (Table 5) [257; 258].

	MRAP1		MRAP2	
	Cell surface expression	Cell signaling	Cell surface expression	Cell signaling
MC1R	= [48, 55]	= [48]	= [48]	= [48]
MC2R	↑ [48, 51–55]	↑ [48, 51–55]	↑ [48, 52, 54]	↑ [48, 52, 54]
MC3R	= [48, 55]	= [48]	= [48]	↓ [48] ↑ [59]
MC4R	↓ [48, 54] = [55]	↓ [48] = [54]	↓ [48, 54]	↓ [48] = [54] ↑ [59]
MC5R	↓ [48, 51, 55]	↓ [48]	↓ [48, 51]	↓ [48]

Table 5 *The role of MRAPs on MCRs cell surface expression and signaling capacity*

When MRAP1 is present, it impairs the MC4R and MC5R trafficking to plasma membrane while having no effect on cell surface expression of MC1R and MC3R [248; 251]. Intriguingly, Chan et al. [248] demonstrated that both MRAPs act as negative regulators of MC1R, MC3R, MC4R and MC5R signaling, but recently, Asai et al. [259] showed that MRAP2 expression

increases MC3R and MC4R signaling. The use of different agonists can explain the conflicting results: Chan et al. used NDPMSH, a synthetic analogue of a-MSH, whereas Asai et al. used a-MSH [248; 259]. MRAP2 deletion causes obesity in mice and heterozygous variants of MRAP2 gene are associated with early onset obesity in humans [259]. Although not yet clear, MRAP2 seems to regulate body weight by facilitating MC4R function but may also act through other receptors expressed in peripheral tissues since MRAP2-null mice develop the obesity syndrome without hyperphagia [259, 260]. Besides MRAPs, other proteins were identified as potential accessory proteins for MCRs, namely attractin, attractin-like protein (ALP) and mahogunin ring finger 1 (MGRN1) 200, 261, 262]. MGRN1 contains a ‘‘really interesting new gene’’ (RING) finger domain characteristic of the E3 ubiquitin ligases. In fact, ubiquitination is a frequent modification of GPCRs important for their targeting for proteasome degradation, but also regulates GPCRs signaling,internalization and lysosomal degradation [263–265]. It was recently demonstrated that MGRN1 impairs MC1R and MC4R function by inhibiting receptor functional coupling to the cyclic adenosine monophosphate (cAMP)

pathway [266]. These authors also suggested that signaling inhibition by MGRN1 occurs independently of receptor ubiquitination or internalization and might be specific for the MCR subfamily of GPCRs since it is not observed for the b2-adrenergic receptor (b2-AR) [66]. However, the role of MGRN1 on MC2R function was postulated to be related with trafficking and/or degradation rather than with signaling since MC2R became ubiquitinated in the presenceof MGRN1 but did not exhibit differences in cAMP production [261]. Endoplasmic reticulum-resident chaperones, such as calnexin and calreticulin, the 70 kDa heat-shock protein (Hsp70) family, the receptor activity modifying proteins (RAMPs) and the GTPases family of Rab and Sar1/ARF all function broadly to facilitate folding and ER export of numerous GPCRs [257; 267]. It was recently shown that Hsc70, the cognate cytosolic Hsp70 protein, promotes cell surface expression and signaling of intracellular retained obesity-related MC4R mutants [268].

5.5. MCRs intracellular signaling

At the plasma membrane, MCRs are activated by ligand binding and undergo conformational changes, which triggera complex intracellular network that translates extracellular signals into biological responses. Since MCRs belong to the GPCRs family, much of the research and progress on the field of MCRs signaling was inspired on GPCRs data.

5.6 G protein-dependent signaling

Classically, GPCRs signaling is primary mediated by the interaction with the heterotrimeric G-proteins, which consistof an a-subunit that binds GDP and a non-dissociable complex composed by b and c-subunits. Ligand binding facilitates the exchange of GDP to GTP on a-subunit promoting the dissociation of G-protein from the receptor and the dissociation of the bc heterodimer from GTP bound a-subunit. Induction of signaling is terminated with the hydrolysis of GTP and subsequent re-association of inactive GDP-bound a-subunit with bc complex, waiting for a new cycle of receptor activation.

According to the sequence homology and functional similarities, G-protein a-subunits are divided into fourclasses: Gs, Gi/o, Gq/11 and G12/13 (Fig. 15) [269].

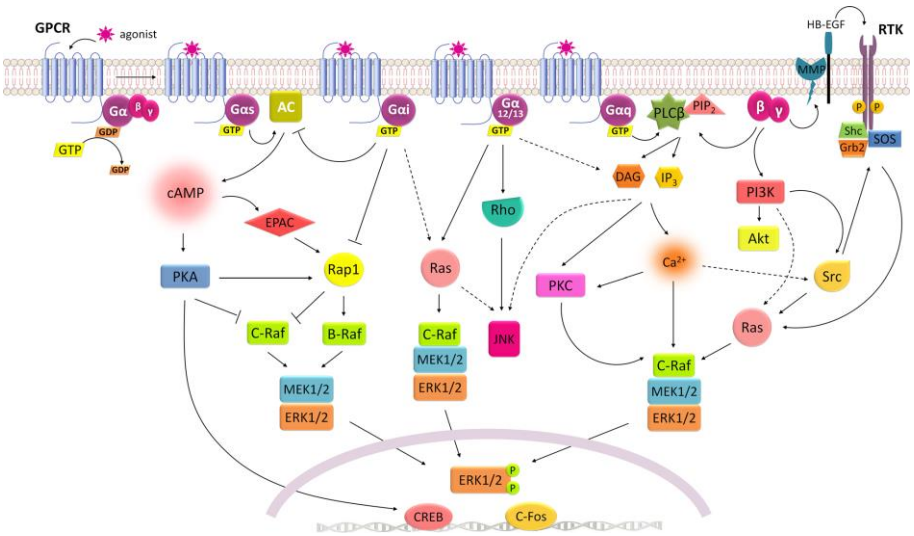

Figure 15. *Integrated mechanisms for G-protein-mediated signaling. Gs signaling is mainly conveyed through activation of AC/cAMP/PKA pathway, which inhibits C-Raf, but also leads to the activation ofRap1 that relays in B-Raf stimulation of ERK1/2 signaling. Giinhibits AC activity blocking its inhibitory effect on C-Raf and concomitantly stimulates ERK1/2 signaling by a Ras-dependentmechanism. G12/13 strongly stimulates JNK activity but can also activate ERK1/2 through Ras or by DAG/PKC pathway. Gq can stimulate ERK1/2 via PLCb/DAG/PKC as well as PLCb/IP3/Ca^{2+}signaling mechanisms, either by direct phosphorylation of C-Raf byPKC or by a Ras-dependent manner, which may involve the recruitment of Src. Besides a-subunit, the complex bc released froma subunit during GPCR activation is also able to promote ERK1/2 signaling through stimulation of PLCb or PI3K. PI3K usually relays in Akt phosphorylation, but also leads to Src and/or Ras stimulation of ERK1/2. In addition, G-proteins can mediate the transactivation of RTK through its bc-subunits. Activation of PI3K leads to Srcmediated receptor tyrosine kinase phosphorylation and subsequentrecruitment of Shc, Grb2 proteins and SOS to stimulate Ras activity and ERK1/2 phosphorylation. RTK transactivation mediated by bcsubunits may also occur through an inside-out model. This mechanism is well described for the epidermal growth factor (EGF) receptor, in which bc activates matrix metalloprotease (MMP) proteins that cleave the ectodomains of membrane-bound growthfactors (HB-EGF) to generate soluble EGF ligands that are released from the cell to activate its RTK. Whatever the mechanism of ERK1/ 2 activation is, these kinases are then able to phosphorylate a wide variety of cytoplasmic and nuclear targets. When translocating to thenucleus, ERK1/2 initiate gene transcription by phosphorylating several transcription factors like CREB and c-Fo*

The signaling cascades generated by GPCRs activation areparticularly dependent on the subtype of G-protein that couples to the receptor. For

example, the protein Gs interacts with adenylyl cyclase (AC) thus mediating the increase in intracellular cAMP levels and subsequent activation of protein kinase A (PKA), whereas Gi protein inactivates AC and blocks PKA activation (Fig. 16) [269; 270].

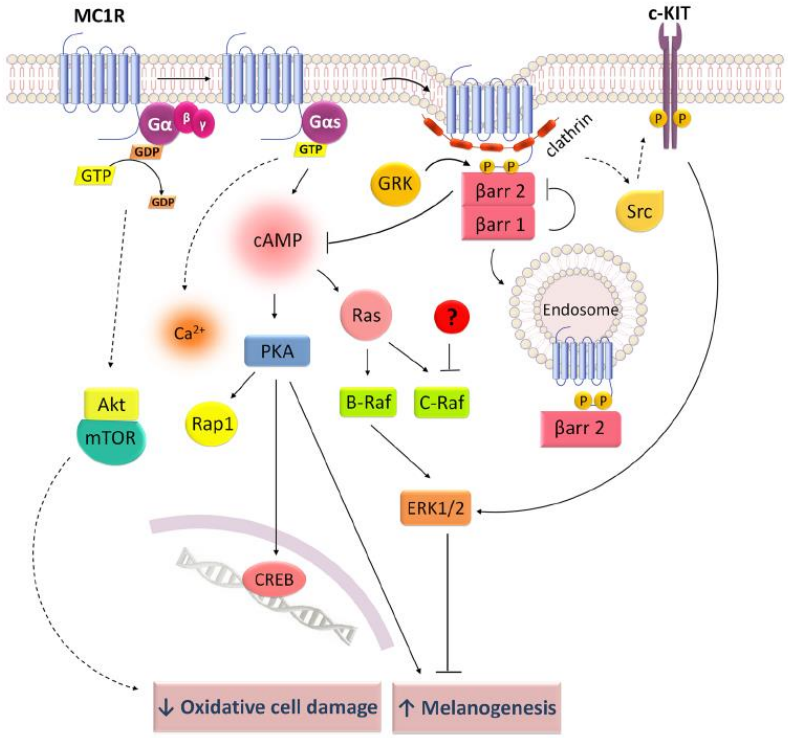

Figure 16 . Signaling mechanisms of melanocortin 1 receptor (MC1R).
MC1R couples to Gs stimulating cAMP/PKA and increasing intracellularCa^{2+} levels. Ligand binding to MC1R promotes a cAMPdependentactivation of Rap1 and Ras, which leads to ERK1/2 phosphorylation through B-Raf signaling. By c-KIT transactivation, MC1R is also able to activate ERK1/2. b-arrestin 2 mediates MC1R internalization and desensitization, decreasing cAMP production but not affecting ERK1/2 activation. b-arrestin 1 competes with b-arrestin 2 for MC1R binding, which blocks b-arrestin 2-mediated cAMP inhibition. cAMP/PKA signaling increases synthesis of melanogenic pigments whereas ERK1/2 seems to have an inhibitory effect on thisprocess. Akt/mTOR apparently increases cell survival during oxidative stress.

Conversely, downstream effectors for Gq/11 comprise inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG),both originated from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C-b (PLCb), and their signaling is conveyed mainly through the activation of protein kinase C (PKC) and $Ca2^{++}$ pathways (Fig. 1) [269].While G12/13 signaling is thought to be mediated by Rho- GTPases, it also modulates PKA and PKC activity [269; 271; 272]. Notwithstanding, GPCRs frequently couple to more than one G-protein subtype, thereby initiating a complex intracellular signaling network rather than a simple cascade sequence [269; 272; 273]. For instance, b2-AR is able to couple to both Gs and Gi [274], orexin-2 receptor is functionally coupled to Gs, Gi and Gq [275] and the thrombin protease activated receptor 1 couples to Gi/o, Gq/11 and G12/13 [276; 277]. For this, the quantification of specific early messengers like intracellular cAMP for Gs, or $Ca2^{++}$ for Gq, is no longer sufficient to measure receptor activity, which requires the evaluation of multiple signaling pathways. In addition to the a-subunits, the bc dimer also has regulatory functions, although initially thought

to have no relevance on GPCR signaling. It has been demonstrated that bc subunits modulate several intracellular pathways like phosphoinositide 3-kinase (PI3K), extracellular signalregulated kinase 1/2 (ERK1/2), PKA and PKC (Fig. 15) [272, 278–279]. As part of the GPCRs family, all MCRs are functionally coupled to Gs and stimulate the cAMP/PKA pathway [281]. Additionally, MC3R, MC4R and MC5R were found to signal through Gi/o [272–274] and the Gq was also associated with MC4R activation [275]. MCRs also induce $Ca2^{++}$ levels and promote ERK1/2 activation both in native and in overexpressing cell systems, although conveyed by different mechanisms [272, 276–274]. Indeed, many recent studies have highlighted a wide diversity of signaling partners downstream of MCRs. MC1R activation triggers the cAMP pathway, induces $Ca2^{+}$ levels and stimulates ERK1/2 but it seems to have no effect on PKC pathway [276, 277, 278, 279] (Fig. 15). In HEK293 cells expressing MC1R and also in human melanomacells, a-MSH-mediated PKA activation induces the phosphorylation of the transcription factor CREB [26]. In rodent melanocytes, the interaction of different pathways and specific interveners was described: cAMP activates RAP-1 through PKA and

additionally induces RAS phosphorylation, which activates B-RAF, MEK and ERK1/2[284] (Fig. 16). a-MSH-dependent cAMP activation mediates biosynthesis of photoprotective melanin pigments whereas ERK1/2 pathway seems to downregulate melanogenesis [273]. Human MC1R mutants expressed in heterologoussystems present a reduced cAMP signaling in response to a-MSH but activate ERK1/2 with the same efficiency as MC1R wild type, suggesting no dependence between ERK1/2 and cAMP pathways [274; 276]. This combined signaling might assume a protective role in skin cancers, where MC1R mutations are highly frequent and compromisecAMP signaling, but ERK1/2 pathway is still able to regulate cell survival by decreasing melanogenic activity.

In retinal pigment epithelium cells, a-MSH activates ERK1/2 and AKT/mTOR signaling pathways, reducing the hydrogen peroxide induced cell damage [279] (Fig. 16), a function that might be crucial to promote cell survivalunder oxidative stress conditions. Besides Gs/cAMP/PKA pathway, MC2R is also able to signal through $Ca^{2+,}$ PKC and ERK1/2 [276], activating several transcriptional factors like CREB, c-Fos and c-Jun [278–279] (Fig. 3). In

HEK293/FRT cells overexpressing MC2R and in human fasciculata cells, ACTH-induced ERK1/2 activation is PKA dependent [286]. By contrast, in Y1 and H295R adrenocortical cells, ERK1/ 2 signaling revealed to be independent from PKA [286]. Additionally, PKC is not involved in ERK1/2 phosphorylation in both H295R and HEK293/FRT cells expressing MC2R [279, 260]. In H295R cells, Janes et al. [289] showed no interference of Ca2? on ERK1/2 activation and did not observe any differences in p38 pathway after ACTH stimuli, however, Roy et al. [279] have demonstrated sustained p38 activation in HEK293/FRT cells expressing MC2R. The key factors that define which mechanisms might be activated under different situations seem to be the potency and duration of stimuli that may depend not only on the cell type and agonist concentration, but also on MC2R surface expression and occupancy that can be temporally regulated by the synchronization of different signaling pathways. In Y1 cells, for example, ACTHstimulated ERK1/2 activation leads to a cyclic phosphorylation of SF-1, a transcription factor important for the transcriptional regulation of several steroidogenic enzymes and the MC2R itself, but, in parallel, ACTH activation of PKA is able to decrease ERK1/2

phosphorylation thereby reducing SF-1 function and MC2R expression [101]. ERK1/2 signaling has been closely associated with proliferation and differentiation processes, important to maintain adrenal structure and function. Using the Y1 cell line, Lotfi et al. demonstrated that acute stimulations of ACTH induce cell cycle progression independently of cAMP, possibly mediated by ERK1/2, however, chronic exposure to ACTH had a cAMP-dependent inhibitory effect on cell proliferation. Together, the data demonstrate a dual effect of MC2R in adrenal function, coordinating both cAMP/PKA and ERK1/2 pathways through a compensatorymechanism to counterbalance the hypertrophic and steroidogenic capacity of adrenal glands (Fig. 17).

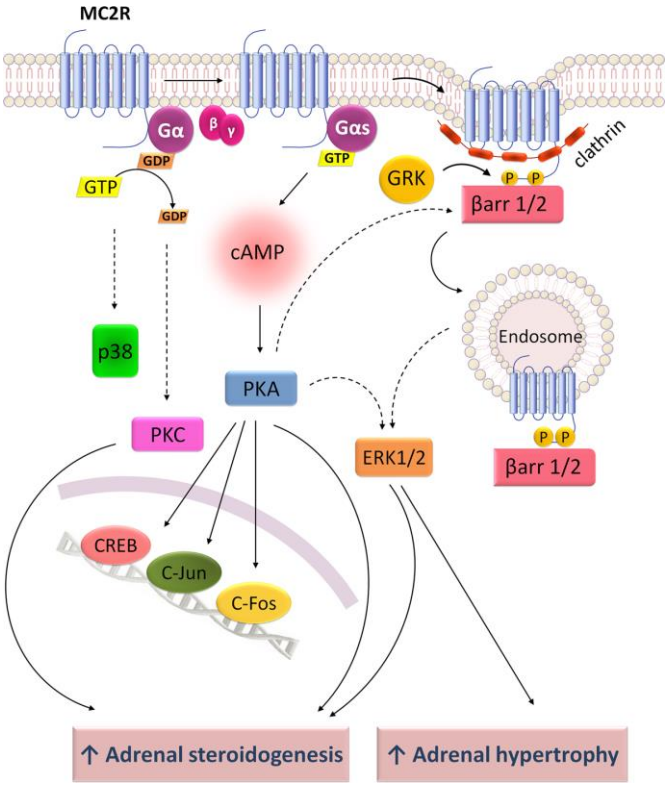

Figure 17 *Signaling mechanisms of melanocortin 2 receptor (MC2R). MC2R couples to Gsand stimulates cAMP/PKA.Activation of ERK1/2 isdependent on PKA and MC2R internalization. MC2R also leads to the phosphorylation of p38 and PKC. MC2R promotes biphasic ERK1/2 activation, with a first transient wave, dependent from receptor internalization, but independent of b-arrestins. ERK1/2 seems to be related with proliferation and differentiation processes regulating the trophic andsteroidogenic properties of ACTH in adrenal glands. PKA and PKC are also mediators of adrenal steroidogenesi.*

MC3R is also able to induce cell proliferation [272, 276] and to signal through both cAMP/PKA and ERK1/2 [272,277].In HEK293 cells, NDP-MSH binding tooverexpressed MC3R increases cell growth rate uponactivation of ERK1/2 signaling by a PI3K- and Gi/o dependent mechanism, but independently from PKA, $Ca^{2+}$and PKC [272] (Fig. 18).

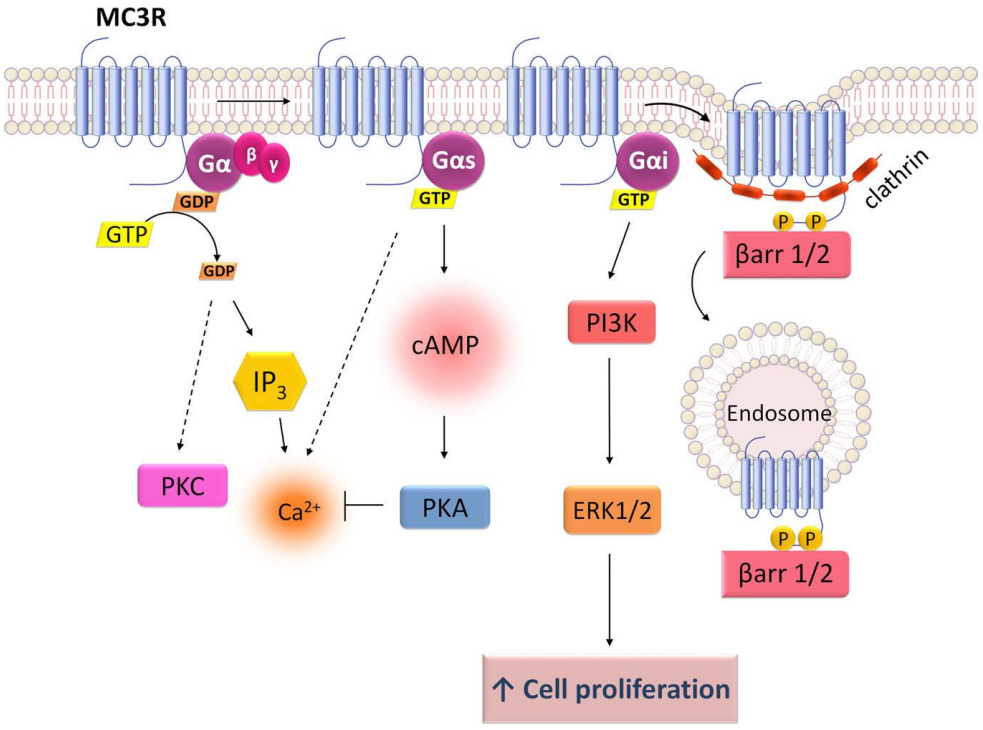

Figure 18 *Signaling mechanisms of melanocortin 3 receptor (MC3R). MC3R couples to Gs stimulating cAMP/PKA but is also able to couple to Gi driving to ERK1/2 pathway through activation of PI3K. MC3R receptor is also known to increase intracellular Ca^{2+}levels and to interact with PKC and IP3. MC3R activation induces cell proliferation through a PI3K/ERK1/2- mediated mechanism that may be related to neuronal regeneration.*

Similarly, in a neuronal cell line, the presence of MC3R is associated with an increase in cellular proliferation regulated by AKT [276]. In fact, an important neurotrophic role has been attributed to the melanocortins [272] and although the physiological significance of MC3R-mediated proliferation it still

unsolved, it is possible to be related with neuronal regeneration. MC3R can also increase the intracellular Ca^{++} levels [276] and interact with PKC pathway [279] (Fig. 18). Some authors report that the increased Ca^{2+} influx is dependent from IP3 [277] whereas others do not indicate the involvement of IP3 during calcium mobilization [279]. MC4R can couple to Gi/o and Gq, besides Gs, and switches from second messengers such as cAMP and $Ca^{2+}$to trigger other downstream pathways than PKA, like PKC,PI3K and ERK1/2 [278; 279] (Fig. 19).MC4R promotes Ca^{2+}formation through a mechanismmediated by PLCb and IP3 [279]. NDP-a-MSH stimulation of MC4R endogenously expressed in hypothalamic cells promotes cAMP activation and transient ERK1/2 phosphorylation through Ca^{2+}/PKC pathway but independently of PKA, PI3K and Gi protein [271] (Fig. 19). By contrast, in CHO cells stably transfected with MC4R, NDP-a-MSH induces a PKA-independent but PI3K-dependent phosphorylation of ERK1/2 [272] and in HEK293 cells expressing MC4R, Gi protein mediates NDP-a-MSH induced ERK1/2 phosphorylation [271]. Structurally, theMC4R cytoplasmic tail was found to be necessary for the activation of ERK1/2 signaling [272]. Regarding the functional consequences

of the different signaling mechanisms, a PKA-dependent increase of ERK1/2 phosphorylation was reported in solitary nucleus neurons after central administration of MTII in rats as a required mechanism for MC4R suppression of appetite, possibly mediated by CREB phosphorylation [281]. Notwithstanding, Chai et al. [221] showed a PKA-independent ERK1/2 signaling important to mediate the anti-apoptotic effects of melanocortins in hypothalamic cells. In fact, the neurotrophic role of melanocortin peptides has been widely associated to a neuroprotective effect, which reduces cell damage induced by neuronal injuries and increases recovery from nerve lesions [188]. Additionally, NDP-a-MSH inhibits c-Jun NH2-terminal kinases (JNK) activity and insulin receptor substrate 1 phosphorylation in HEK293 cells expressing human MC4R. This interaction of themelanocortin ergic system with the insulin signaling involves AKT phosphorylation and increases insulin stimulated glucose uptake [113]. In an heterologous cell system stably expressing MC5R, the receptor activation promotes cAMP and Ca^{2+} increase, independently of IP3 [114]. In a similar cellular system, MC5R was found to

elicit two parallel signals when activated by a-MSH: cAMP/PKA and ERK1/2 pathways (Fig. 19).

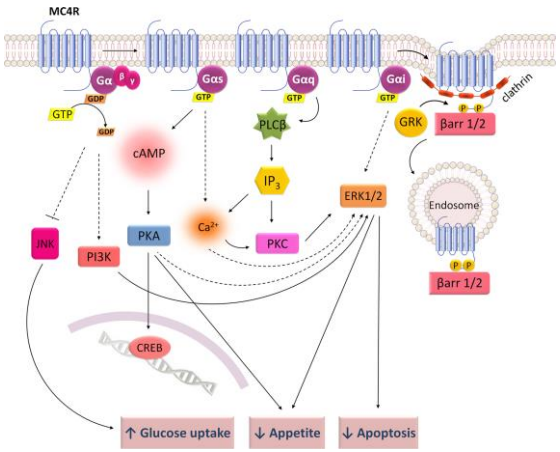

Figure 19 *Signaling mechanisms of melanocortin 4 receptor (MC4R). MC4R couples to Gs stimulating cAMP/PKA and to Gq activatingPKC dependently from PLCb and IP3. It also activates ERK1/2 via Gi or through mechanisms dependent from* $Ca^{2+,}$ *PKA, PKC or PI3K. A PKA-dependent ERK1/2 signaling is associated with food intake suppression, whereas a PKA-independent ERK1/2 seems to decreasecell apoptosis upon neuronal damage. MC4R also inhibits JNK activity, possibly to increase insulin-stimulated glucose uptake.*

In adipocytes, cAMP/PKA revealed to be important for lipolysis activation by a-MSH/MC5R whereas ERK1/2 pathway seems to be crucial for decreasing adipocyte fatty acid re-esterification [247]. ERK1/2 activation occurs independently of PKA, PKC and AKT but requires PI3K and leads to a downstream phosphorylation of 90-kDa ribosomal S6 kinases (p90RSK) and mitogen- andstress-activated protein kinase 1 (MSK1) [274] (Fig. 20).

Moreover, only a small fraction (10 %) of activatedp90RSK and ERK1/2 translocates to the cell nucleus inducing c-Fos expression. The cAMP/PKA activation by MC5R is dependent of Gs protein and induces nuclear CREB phosphorylation whereas ERK1/2 pathway is regulated through a biphasic mechanism with an early transient and a late sustained activation, both mediated by Gi protein [273] (Fig. 20).

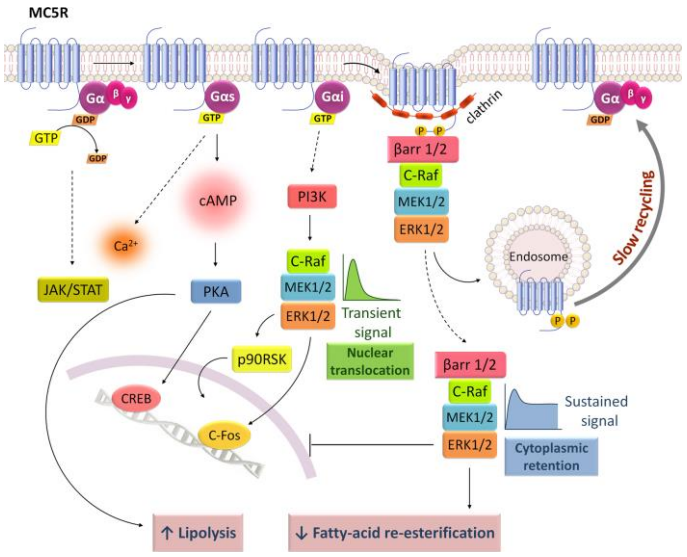

Figure 20 *Signaling mechanisms of melanocortin 5 receptor (MC5R). MC5R activates two parallel signaling pathways, Gs/cAMP/PKA andGi/PI3K/ERK1/2 that promote CREB phosphorylation and c-fos expression, respectively. MC5R is also known to increase intracellular* Ca^{2+} *levels and promote JAK/STAT activation. ERK1/2 signaling occurs through a biphasic fashion with an early transient peak dependent from Gi protein and a second one, more sustained in time, regulated by Gi and b-arrestins. MC5R internalizes independently from b-arrestins but these scaffold molecules seem to retain ERK1/2 signaling in the cytoplasm to mediate MC5R-dependent decrease of fatty acid re-esterification in adipocytes. MC5R-dependent increase of lipolysis rate is regulated by PKA activation.*

Additionally to the known role of b-arrestins in GPCR internalization, they are also recognized as central scaffold proteins for signal transduction of many GPCRs even after receptor internalization and desensitization. b-arrestins act as adapters for Src-family tyrosine kinases and have a major role as scaffolds for ERK1/2 signaling. In some cases, internalized GPCRs form complexes with barrestins and all the components of the ERK1/2 cascade that anchor signaling to intracellular endocytic vesicles for long periods of time [116, 117, 129, 130]. In this situation, b-arrestins interaction induces a slow ERK1/2 activation, more sustained in time and restricted to the cytoplasm [131]. Otherwise, other GPCRs dissociate from b-arrestins shortly after movement of the receptor into CCPs and do not colocalize with b-arrestin complexes in endosomes [130, 132, 133]. In this case, duration and distribution of ERK1/2 pathway activated by b-arrestins is quite similar to the G-protein-dependent mechanisms, which lead to a rapid and transient phosphorylation of ERK1/2 that, in general, translocate to nuclear compartments. The b-arrestins-dependent ERK1/2 activation was firstly demonstrated for b2-AR by [134;135]. b2-AR as shown to activate

ERK1/2 through a biphasic mechanism, comprising a rapid and transient phase dependent from G-protein and a second one more sustained in time and driven by b-arrestins [132]. Intriguingly, the b2-AR rapidly dissociates from b-arrestins upon internalization and does not form stable receptor-b-arrestin complexes on endosomes, even though it is able to promote a sustained ERK1/2 activation [133]. The MC5R seems to behave similar to b2-AR [273]. Although b-arrestins are not involved in MC5R internalization, they appear to function as scaffolds to prolong ERK1/2 signaling in the cytoplasmic compartment and prevent their nuclear translocation [273]. A difference between MC5R and b2-AR arises in the type of G-protein involved: b2-AR activation induces an early Gs/Gi proteins.Dependent before the last b-arrestins-dependent ERK1/2 signaling [232], whereas MC5R promotes a transient and a sustained ERK1/2 activity, both dependent on Gi but not on Gs protein [273]. The earlier phase of ERK1/2 activation occurs independently of b-arrestins and seems to be important for nuclear translocation and c-Fos expression whereas the late sustained ERK1/2 activation, Gi and barrestins driven, may be associated with cytoplasmic functions [273] (Fig. 20). In fact,

in adipocytes, a decrease in the cytoplasmic activity of phosphoenolpyruvate carboxykinase (PEPCK), important to glyceroneogenesis, was linked to the a-MSH-mediated ERK1/2 phosphorylation [47]. Similarly to MC5R, MC2R also promotes a biphasic ERK1/2 signaling [101] with the first transient activation being independent from b-arrestins [89]. It is still unknown whether the second wave of ERK1/2 phosphorylation requires b-arrestins, however, we can speculate a similar mechanism between MC2R and MC5R. Indeed, MC2R internalization is needed to promote a transient ERK1/2 activation [270] which occurs independently from b-arrestins [271], suggesting that b-arrestins recruitment might be a later event occurring after ERK1/2 activation. In this regard, it is still possible that b-arrestins binding to MC2R might form an intracellular complex retaining receptor signaling in cytoplasmic endosomes and thus being responsible for the second period of ERK1/2 phosphorylation. This mechanism could be an explanation for the lower nuclear translocation of ERK1/2 observed by Roy et al.; [273]. Even though b-arrestin 1 and 2 have an apparent redundant function in MC5R signaling, recent data implicated b-arrestins in MC1R internalization and signaling in an isoform specific way: b-

arrestin 2 inhibits MC1R agonist- dependent cAMP production, but not ERK activation, and colocalizes with the receptor in endocytic vesicles promoting internalization. b-arrestin 1, by contrast, functionally competes with b-arrestin 2 for binding to MC1R and consequently increases signaling upon displacement of b-arrestin 2 [279]. Given the physiological significance of the melanocortins, they are now recognized as a target for the pharmacological treatment of different disorders like obesity, diabetes and erectile dysfunction [277, 279]. Their therapeutic potential was, so far, only attained by designing analogues of MCRs agonists. However, MCRs present high homology between each other and cross-reactions of their agonists, causing unwanted side-effects, are frequently observed. For instance, AstraZeneca in collaboration with Palatin technologies developed a melanocortin analogue for the treatment of obesity, AZD2820, but it was discontinued following serious adverse effects observed in phase I clinical trials. Moreover, bremelatonide (PT-141), initially developed for the treatment of obesity, promises efficacy for the treatment of sexual dysfunction and the phase III clinical trials are expected soon [237, 238]. MC4-NN2-0453 developed by Novo Nordisk, also failed to demonstrate

effects in weight loss causing several skin-related adverse effects, headache and sexual disturbances [278]. Considering all these data, a promising approach for generating more effective and safer drugs may involve the management of a specific pathway step. Indeed, many GPCRs have been shown to elicit different responses when activated by distinct ligands, a phenomenon that was recently named as ‘‘biased agonism’’ [280, 281]. This concept arises from the discovery that the same receptor is able to activate both G-protein and b-arrestins-dependentsignaling pathways. Compared to a ‘‘normal ligand’’ that signals with similar efficacy through all the pathways available for the receptor, a ‘‘biased ligand’’ favors one pathway over another [276, 277]. This property has highlighted the therapeutic potential of ‘‘biased ligands’’ and the opportunity to design and produce selective drugs with improved efficacy and less unwanted effects [278]. A diversity of biased ligands for GPCRs has been identified so far [278]. In the setting of MCRs, biased agonism may constitute an encouraging challenge in drug development.

An alternative approach considers the specific intracellular, instead of extracellular, delivery of melanocortin peptides.

Innovative data revealed that a-MSH complexes with MC4R at the endoplasmic reticulum, a condition that induces maximal amplitude and constant signaling of cAMP production at the cell surface. In that complex, MC4R acquires a stable active conformation that does not become desensitized [279].

Development of agonists for intracellular targeting, instead of binding to MCRs at cell surface, is thus an attractive challenge aiming drug specificity. All the data discussed in this review stresses out the strategical importance to study the molecular mechanisms and signaling pathways activated by receptors and agonists in the different tissues and cells. In fact, the intervention in a particular signaling cascade activated by a specific MCR seems to be the rational way to improve the efficacy and long-term safety of the pharmacological therapies.

AIM OF THE STUDY

The objective of this study was to understand whether, the melanocortin pathway is involved in the development of diabetic retinopathy and have a modulation of its receptors subtypes by means of nealy drugs can have potential protective effect, since the 5 receptor subtypes of melanocortin are expressed on the retina. This studies have been conducted on 3 different experimental models two in vitro (ARPE-19 and primary and retinal cell cultures) and one on an animal model ofdiabetes- induced retinopathy.

The first aim of this study was to investigate the role of the melanocortin receptor (MCR) 5 in the high glucose induced release of VEGF-containing exosomes from human retinal pigment epithelial cells ARPE-19 and in the resulting neovascularization.

The second aim of the this study focused on their role in primary retinal cell cultures in high-glucose concentrations and oxidative stress are a risk factor for diabetic retinopathy development. Retinal photoreceptors are particularly

vulnerable to local high-glucose concentrations, so see what modal receptors of melanocortin could preserve the photoreceptor.

The third aim have been was to evaluate whether melanocortin receptors (MC) could be protective tissue protective circuit in a model of streptozotocin-(STZ) induced diabetic retinopathy (DR) in mice.

CHAPTER 6

MATERIAL AND METHODS: In vitro models

6.1 ARPE-19 Cell Culture and Treatments

Human retinal pigment epithelial cell line ARPE-19 was obtained from American Type Culture Collection (ATCC). ARPE-19 cells were cultured in Dulbecco's modified Eagle's medium/Nutrient mixture F12 (DMEM/F12, Invitrogen) supplemented with 5 mM HEPES buffer, 7.5% NaHCO3, 10% fetal bovine serum (for exosome: 5% fetal bovine serum depleted exosome), 1% penicillin/streptomycin and were maintained at 37ºC and 5% CO2. Depending on the technique, cells were cultivated in six-cell culture well plates or P-100 at a starting seeding density of 2 x 105 cells/well and 1 x 106 cells/wells respectively. For cell viability assays, ARPE-19 cells were also seeded at 6 × 103 per well in a 96-cell culture well plate and grew to confluence for 9 Days with high glucose 35 mM and 24 h with H_2O_2. After 9 days, cells were incubated for 24 h with MC1R / MC5R agonist and antagonist melanocortins

receptor. MTII from Bachem Ltd. (Saffron Walden, Essex, UK), and SHU9119 from Phoenix Pharmaceuticals (Karlsruhe, Germany). Other compounds were supplied (BMS- 470539,AGRP) or synthetized (PG-901, PG20N) by Professor Grieco (University of Naples Federico II). All compounds B were stored at −20°C before use and dissolved in sterile PBS,pH 7.4.

6.1.1 Passage and Maintenance of ARPE-19 Cells

ARPE-19 cells are adherent cells. To subculture them for growth, medium was aspirated, cells were rinsed with PBS. There after, trypsin-EDTA was added and cells were incubated at 37 ºC for 5 to 10 minutes for cell detachment. Following trypsinisation complete medium was added and transferred into T75 flasks. Cells were maintained at 37ºC and 5% CO2 and medium was changed every two days. Cells were used from 18 to 20 passages.

6.1.2 Heat Inactivation of Fetal Bovine Serum

FBS was heated to 56ºC for 45 min in a water bath and then aliquoted into 50 mL aliquots and stored at -20ºC.

6.1.3 Counting Cells

Cells were counted using a Neubauer haemacytometer (Fisher). 20 μL of cells was mixed with 20 μL of 0.4% Trypan blue solution to make a 1/2 dilution. The cells were counted using the grid on the haemocytometer. The mean number of cells was calculated and this was multiplied by the dilution factor and then by 104 to scale the volume of the haemocytometer (0.1 mm3) to cells/mL.

6.1.4 Primary retinal cell cultures

Retinal cell cultures were obtained according to Santiago et al. [2] with some modifications. Briefly, mice (n = 10) were anesthetized by intraperitoneal injection of ketamine/medetomidine (ketamine 100 mg/kg and medetomidine 0.25 mg/kg). After eye enucleation, retina was dissected under sterile conditions using the enzymes trypsin and collagenase A [22]. After dissociation, the cells were collected by centrifugation and resuspended in

Eagle's minimum essential medium (MEM) supplemented with 26 mM NaHCO3, 25 mM HEPES, 10% heat-inactivated foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were maintained in humidified atmosphere of 5% CO2 air at 37°C. The cells were plated at a density of 2.0 x 10^6 cells per cm2 on 24-well plates or 35 mm Petri dishes, coated with poly-D-lysine (0.1 mg/ml; Sigma-Aldrich, St Louis, MO, USA). Two days after, cells were incubated for 20 days with high-glucose concentration 25 mM D-glucose (high glucose) or 5 mM D-glucose (control) [11]. After this, retinal cell cultures were treated for 24 hrs with MCR agonists PG-901 (MC5 agonists, 10^{-10} M); (BMS-470539, 10-^{5} M) [23]. Each treatment was repeated three times.

6.1.5 HUVEC CELLS

Human umbilical vein endothelial cells (HUVEC) were isolated as described previously (Sobrino et al., 2010) Briefly, umbilical veins were perfused with 1% collagenase solution and incubated at 37°C for 15 min. Endothelial cells were

recovered in specific endothelial growing medium (EGM)-2 (Lonza, Cultek, Barcelona, Spain) and incubated at 37°C and 5% CO2.

6.1.6 Cryogenic Storage of ARPE-19 Cells

To freeze for future use, cells were detached using as mentioned above trypsinEDTA. The detached cells were washed with 9ml of complete medium and pelleted by centrifugation at 1200g for 3 min. The supernatant was aspirated and cells were resuspended in cryogenic solution (90% (v/v) FCS, 10% (v/v) dimethyl sulfoxide). 1ml of the cell stock was transferred into each cryogenic vial (Nunc). They were stored at -80ºC for 48hrs, then transferred to liquid nitrogen (-196 ºC) for storage. Cells were thawed by defrosting the cells, which were then transferred to eppendorfs and centrifuged into a pellet at 400 rpm for 5 min. The freezing medium was removed and the cells were resuspended in 10 mL of complete medium, placed into a T75 flask and maintained at 37°C, 5% CO2. For adherent cells, medium was changed the next day once cells had attached.

6.1.7XTT assay

Cell viability as metabolic activity were measured using 3´-[1-phenylaminocarbonyl-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate, (XTT, Cell Proliferation Kit II; Roche 11465015001). RPE cells were seeded at 6 x10^3 cell/well in a 96-well cell culture plate for 24 hrs. Cells were rinsed with PBS (Aurogene AU-L0615) twice and then were incubated with 0.3mg/ml of XTT final solution for 6 nhrs at 37°C in 5% CO_2. Mitochondrial activity was measured by fluorescence multiple reader (Victor X5; Perkin Elmer) at 550nm.

6.1.8 Determination of ROS levels

Levels of ROS were measured using 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Santa Cruz Biotechnology CAS 4091-99-0), which is converted to a nonfluorescent derivate (H_2DCF) by intracellular esterases. This molecule can be oxidized by ROS producing intracellular

dichlorofluorescein (DCF). ARPE-19 cells were seeded at a $6x10^3$cells/well in a 96 well plate. Cells were rinsed with PBS twice and then were incubated with 15 µM of H_2DCFDA for 15 min at 37°C in 5% CO_2. Total intracellular ROS production was measured by fluorescence multiple reader (Victor X5; Perkin Elmer) excited at 485 nm and emission at 530nm.

6.2 In vivo models : Diabetic Retinopathy

This study was performed according to the guidelines of the Ethic Committee for animal experiments at the University of Studies of Campania "Luigi Vanvitelli". C57BL/6 mice (Envigo^{++}, Italy) aged 7 to 10 weeks were rendered diabetic with one intraperitoneal injection of STZ (65 mg/kg, Sigma-Aldrich, St. Louis,MO, USA) freshly dissolved in 10mM citrate buffer (pH 4.5). Development of diabetes (defined by blood glucose greater than 250mg/dL) was verified 1 week after the STZ injection (Glucometer Elite XL; Bayer Corp., Elkhart, IN). Blood glucose levels were checked intermittently throughout the study in order to confirm the maintenance of the diabetic condition. C57BL/6 mice were divided into 8 groups (n = 10 animals per

group), labelled consecutively from 1 to 10 to repeat the fluorescein angiography (FAG) to the same animal at each time point considered. Mice were randomised into the following experimental groups: (1) non diabetic mice; (2) diabetic mice; (3) diabetic mice treated with intravitreal injection of the MC1 receptor agonist BMS-470539 [24]; (4) diabetic mice treated with intravitreal injection of the mixed MC3-MC4 receptor agonist MTII [25]; (5) diabetic mice treated with MC1 receptor antagonist agouti related protein (AGRP; [26]); (6) diabetic mice treated with intravitreal, MC5 agonist PG-901 [286]; (7) diabetic mice treated with intravitreal MC3-MC4 receptor antagonist SHU9119, [285]; (8) diabetic mice treated with intravitreal MC5 receptor antagonist PG20N, [285]. In all cases, animals were monitored over a 16-week period for the development of diabetes, with specific analyses at weeks 8, 12, and 16 when fluorescein angiography was conducted. At the end of each time course the animals were sacrificed and the eye ball was displaced forward by placing curved forceps around the posterior part and cut in two halves. On one half of each eye the cornea was cut using a sharp blade or scalpel, and the retina was squeezed through the cut together with residual pigment epithelium

and lens by applying gentle pressure with the forceps. Dissected retina was placed in cooled PBS, freed from nonretinal tissue using the forceps, and immediately frozen in liquid nitrogen and stored at −80°C for subsequent biochemical analysis.The other half of each eye was fixed by immersion in 10% neutral buffered formalin and paraffin-embedded for immunohistochemistry.

6.2.3 Intravitreal Injections.

Seven days after the development of diabetes the mice were anesthetized by Ketamine 0.01 ml/10 gr + Medetomidine 0.01ml/10gr for mice. Tropicamide (5%) was instilled into the right eye of each animal, in order to induce dilatation of pupils, and tetracaine (1%) was injected for local anaesthesia. Physiological saline or MC receptor ligand preparations (5 μL volume) were administered via intravitreal injection into the right eye using sterile syringes fitted with a 30-gauge needle (Microfine; Becton Dickinson AG, Meylan, France), as previously described [283]. The following MC receptor ligands were used, at the indicated dose as selected from the reported publications: BMS-470539, 33 μmol [284]; MTII, 9.3 nmol [285]; SHU 9119, 9 nmol [286];

PG-901, 7.32 nM [285]; PG20N, 130 nM [286]; agouti related protein or AGRP, 1 μg [286]. Each compound was injected every 4 weeks from the development of diabetes.

6.2.4 Fluorescein Angiography (FAG).

FAG was performed by using a Topcon TRC-50DX apparatus (Topcon, Tokyo, Japan) following intraperitoneal injection of 10% fluorescein sterile solution (1 mL/kg body weight, AK-Fluor; Akorn, Inc.). Fundus photographs were captured in order to display the retinal vasculature and to evaluate the early typical alterationsof diabetic microangiopathy.

RNA Isolation and Quantization

Total RNA was extracted from the heart tissue (~50mg) using the Rneasy Plus Mini Kit (Qiagen) according the manufacturer's protocol from Animal Cells. Then RNA was quantified using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

mRNA Reverse-transcription and Real Time PCR Reaction

❖ Retinal primary cells culture

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, WestSussex, UK), according to the manufacturer's instructions. Contaminating DNA was removed from RNA preparations performed with theAmbion_ Thurbo DNA-free system (Life Technologies, Waltham, MA,United States) using manufacturer's instructions. The concentration andpurity of the RNA were then analysed using the Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was obtained by reverse transcription (RT) of 1 lg of totalDNase-treated RNA, with the Superscript III reverse transcriptase system (Invitrogen, Carlsbad, CA, USA) and oligo(dT)15 as a primer following manufacturer's protocol. Real-time PCR was performed with Read Mix PCR Master Mix (ThermoScientific, Waltham, MA, United States)and the following amplification profile: 95°C for 2 min.; 35 cycles -

94°C for 30 sec., 55°C for 35 sec. and 72°C for 65 sec., followed by final elongation step at 72°C for 5 min. Each 25 reaction consisted of 1 of diluted cDNA (150 ng/ll RNA), 22.5 of 1.19 ReddyMix PCR MasterMix, 1 of ddH2O and 1 of commercially available primer for amplification of mouse MC1R and MCR5 (Qiagen). mRNA data were normalized relative to GAPDH and then used to calculate expression levels. Negative controls were either RT without enzyme or PCR without cDNA template. The protocol for the RT-PCR .

❖ Retinal tissues

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, West Sussex, UK), according to the manufacturer's instructions. Contaminating DNA was removed from RNA preparations using the Ambion Turbo DNA-free system (Life Technologies, Paisley, UK) using manufacturer's instructions.The concentration and purity of the RNA were then analysed using the Nandrop ND-1000 (NanoDrop Technologies, Wilmington, DE). ComplementaryDNA(cDNA)was obtained by reverse transcription (RT) of 1 μg of total DNase-treated RNA, using the Superscript III Reverse Transcriptase System

(Invitrogen, Carlsbad, CA, USA) and oligo(dT) primers following manufacturer's protocol.

Conventional PCR was performed for detecting the expression of murine *MCR1, MCR3,* and *MCR5* genes using cDNA (150 ng/reaction), specific primers (Quantitect Primer Assays, Qiagen, West Sussex, UK), and Thermo Scientific 1.1x ReddyMix PCR Master Mix (Life Technologies, Paisley, UK). The following amplification profile was applied: 95°C for 2 min; 35 cycles 94°C for 30s, 55°C for 35s, and 72°C for 65 s, followed by final elongation step at 72°C for 5min. Melanocortin receptor expression was quantified using the predesigned Quantitect Primers (ABI Prism 7900 Sequence Detection System; Applied Biosystems Inc.) and 2x Power SYBRGreenMastermix (Applied Biosystems,Thermo Fisher Scientific Inc., Paisley, UK). The absence of unspecific products was confirmed by analyzing the included dissociation end step. Cycle threshold (Ct) values were measured and calculated by the Sequence Detector software. Relative amounts of mRNA in diabetic retinas were normalized to endogenous control (*GAPDH*) and to the healthy controls. Relative Mrna contents were calculated using the$x = 2^{-\Delta\Delta Ct}$ equation.

Western blotting analysis

- ARPE-19 Cells

ARPE-19 cells treated or not were collected in RIPA Buffer (Sigma-Aldrich R0278) and protease inhibitor cocktail (Roche 11873580001). Equal amount of protein (20µg) were loaded and analyzed by SDS-PAGE on 4-12% SDS-Polyacrylamide gel electrophoresis and electroblotted onto polyvinylidenedifloride membranes (PVDF; Millipore IPFL10100) by wet transfer. Membranes were incubated overnight at 4°C with antibodies against MCR1 (abcam ab180776), MCR2 (abcam ab180793), MCR3 (abcam ab203671), MCR4 (abcam ab24233), MCR5 (abcam ab133656), VEGF (Santa Cruz sc57496), NF-kb (ab32536), Cyp2E1 (Abcam, ab28146) and β-actin (Santa Cruz Biotechnology sc-8432) as loading control. Finally, membranes were incubated for 2 hours at room temperature with anti-mouse (sc-2005) and anti-rabbit IgG-HRP (Santa Cruz Biotechnoloy sc-2004). Bands were visualized with ECL (Pierce, Thermo Scientific, 32132) and detected with Image Quant LAS-400 mini (GE Healthcare, Uppsala, Swedem). Protein levels

were quantified by densitometry using ImageJ software (NIH, Bethesda, MD,USA).

❖ Primary retinal cell lysates

Retinal cell lysates obtained following the protocol described by Baptista et al., [10]. Briefly, cells were washedwith ice-cold phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10 Na2HPO4, 1.8 KH2PO4, pH 7.4, at 4°C) and lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 1 mM DTT) supplemented with complete miniprotease inhibitor cocktail tablets and phosphatase inhibitors (10 mM NaF and 1 mM Na3VO4). Lysates were incubated on ice for 30 min. and centrifuged at 16,000 9 g for 10 min. at 4C°. The protein concentrations were determinate as described by Bradford (1976). The primary polyclonal antibodies used are anti-manganese superoxide dismutase MnSOD (dilution 1:200; Millipore, Merck, Milan, Italy) and anti-glutathione peroxidase (GPx) (dilution 1:200; Abcam, UK). Anti-β-actin was used as loading control,

with an enhanced chemiluminescence detection reagent (ECL). Protein bands were quantified by densitometry performed with a Bio-Rad ChemiDoc MP Imaging system. Secondary antibodies used were antimouse and anti-rabbit (dilution 1:1000; Santa Cruz Biotech, CA, USA).

- Retinal tissues

Western blot was performed on the retinal tissues 16 weeks after the onset of diabetes, monitoring the macrophage M2 marker, the mannose receptor CD206, and the macrophage M1 marker, the integrin αX/CD11c. Retinal samples were homogenized on ice using RIPA buffer (Santa Cruz Biotechnology, Milan, Italy), containing a protease inhibitor cocktail tablet (Roche Diagnostics,Mannheim,Germany). Lysates were centrifuged at 12,000 g and the supernatant was collected. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Milan, Italy). A total of 100 μg of proteins were separated on denaturing 8% SDS-PAGE and transferred to PVDF membrane. The following primary antibodies were used: anti-M2 mannose receptor (CD206) (1 : 400, Abcam, Cambridge, UK), anti-M1 Integrin Alpha X/CD11c (1 : 200, Bioss, USA). Donkey anti-rabbit

polyclonal IgG (Abcam, Cambridge, UK) and goat anti-mousepolyclonal IgG (Santa Cruz Biotechnology, USA) secondary antibodies were used at concentration of 1 : 1000 and 1 : 2000, respectively.

Immunocytochemistry

Cells cultured in glass coverslip were fixed with 4% paraformaldehydein PBS pH 7.4 for 10 min. at room temperature. After fixation, the cultureswere washed with PBS and incubated 1 hr with blocking solution5% BSA serum (Sigma-Aldrich) 0.05% Tween in PBS, and then incubated overnight with monoclonal anti-opsin (1:1000; Sigma-Aldrich) and anti-recoverin (1:1000; Abcam, Cambridge, UK) antibodies. Alexa Fluor_488 (Jackson Laboratory, West Baltimore Pike, West Grove, PA, USA)- conjugated goat polyclonal antibody (1:1000) was used as secondary for opsin detection. Cy3-conjugated goat polyclonal anti-rabbit (Jackson Laboratories; 1:400) was used as secondary for recoverin detection. Nuclei were counterstained by DAPI. Quantification of fluorescence intensity was determined by LEICA software

(Milan, Italy). The method used by Alessio et al. [24] was applied to calculate the percentage of positive cells in each microscope field. This was calculated by the number of green or red (opsin or recoverin) positive cells of 400 cells in six different microscope fields according to the previous method [24].

Exosomes isolation and size distribution

For exosomes isolation, ARPE-19 cells were cultured as previously described using 1% Exosome-depleted FBS (Thermo Fisher 4478359). After 9 days, 10 mL of culture media from ARPE-19 cells treated or not were processed using Total Exosomes Isolation Reagent from cell culture media (Thermo Fisher 4478359) for exosomes isolation. The exosome pellet was stored at 4°C in PBS 1x. Exosomes identity was confirmed by the nanoparticle tracking

SystemNanoSight N S300 following manufactures protocols (Malvern Instruments, Malvern, UK).

Enzyme–Linked Immunoabsorbent Assay (ELISA)

The quantitative determination of human vascular endothelial growth factor (VEGF) was assessed using Human VEGFA ELISA Kit (Abcam ab100662), according to the manufacturer's protocol, on ARPE-19 cells, and I was Assay for Endogenous α-MSH within the Retina. A commercial kit (Phoenix Pharmaceuticals, Inc., Karlsruhe, Germany) was used following the manufacturer protocol in order to assess the levels of the protein within the retina of non diabetic and diabetic mice with retinopathy.

Cytokines Array

A specific kit (ARY006, R&D Systems, Abingdon, UK) was used for the simultaneous measurementof the production of a number of pro- and anti-inflammatory cytokines and chemokines from mouse retinas.

Electron microscopy

Cells were seeded in a Lab-Tek chamber slide with eight wells (Nalge Nunc International,Naperville,IL, USA) and were fixed in 3% glutaraldehyde for 2 hrs at 37°C. Cells were post-fixed in 2% OsO4 for 1 hr at room temperature and stained in 2% uranyl acetate in the dark for 2 hrs at 4°C. Finally, cells were rinsed in distilled water, dehydrated in ethanol and infiltrated overnight in Durcupan resin (Fluka, SigmaAldrich, St. Louis, MO, USA). Following polymerization, embedded cultures were detached from the chamber slide and glued to araldite blocks. Ultrathin sections (0.06–0.08 lm) were prepared with the Ultracut and stained with lead citrate. Finally, grids were covered with handmade Formvar and photomicrographs were obtained under a transmission electron microscope FEI Tecnai G2 Spirit (FEI Europe, Eindhoven, Netherlands)using a digital camera Morada (Olympus Soft Image Solutions GmbH, M€unster, Germany). Exosome pellets were

resuspended in PBS and ultracentrifuged at 120,000 9 g for 70 min. at 4°C. After that, approximately 10 lg of the sample was resuspended in PBS on parafilm. The sample was fixed by depositing a drop of 2% PFA on the parafilm and placing the grid (Mesh with Formvar) on top of the drop. Negative staining was performed with 2% uranyl acetate. Photomicrographs were obtained using the transmission electron microscope previously described. Exosomes were identified under the microscope solely based on size and morphology.

Flow cytometry

Exosomes were scrutinized by applying anti-CD9,CD81,VEGF and VEGFR2 (Abcam), a well-established exosome marker [39–41], using a FACScan flow cytometer (Beckman Coulter, Alcobendas, Spain).. Five hundred thousand events were collected for each sample. Results were analysed with BD FAC Suite software (Fullerton, CA, USA).

Vasculogenesis assay

Vasculogenesis was analysed in Matrigel (Becton Dickinson, Bedford, MA, USA) as previously described [44]. After treating the cells in the absence or presence of RPE- derived exosomes for 24 hrs, HUVEC (6,9 x 104 cells/ well) were recovered and seeded on Matrigel-coated wells for 5 hrs. Matrigel was previously diluted with EGM-2 media, FBS free and allowed to solidify for 30 min. at 37°C. Then, pictures were taken with a Nikon Eclipse-Ti inverted microscope (Nikon, Tokyo, Japan).

Immunohistochemistry

Ocular tissue sections (5 μm) were serially cut from paraffin-embedded tissue and labeled for the detection of Ki-67 by immunohistochemistry, according to previous published protocol [31]. Briefly, sections wereincubated with primary mouse monoclonal anti-ki67 (PP-67) antibody (dilution 1 : 250, Abcam, Cambridge, UK) for 30 min at roomtemperature. Sections were then washed

with PBS and incubated with biotin-conjugated goat anti-mouse IgG secondary antibodies and avidin-biotin peroxidase complex (DBA, Milan, Italy).

Compounds

BMS-470539 and PG-901 were used as MCR1 and MCR5 agonist, respectively, although PG901 also binds with antagonistic activity MC3Rand MC4R [285, 286]. Compounds were supplied by Professor Grieco (Pharmacy Department, University of Naples Federico II).

Animals

All the experimental procedures were performed according to University of studies of Campania "L.vanvitelli"guidelines of the Ethic Committee for animalexperiments. Three-week-old male C57BL/6 mice (18–22 g) (Harlan, Milan, Italy) were housed in standard cages (n = 10 per cage) with acycle of 12 hrs light (7 a.m. to 7 p.m.) and 12 hrs dark, humidity and temperature automatically controlled to 60% and 21 _ 1°C, respectively.

Statistical Analysis

ARPE-19 cell and primary cell results of each experiment are presented as mean ± S.E.M. of the three treatments since experiments were repeated three times to insure consistency of results. Statistical significance was determined using ANOVA followed by Bonferroni's test. For the immunocytochemistry, the mean ± S.E.M. of the percentages was calculated and expressed in graph. Differences were considered significant when *P < 0.05 versus high glucose, **P < 0.01 versus high glucose and °P < 0.01 versus control. For the *in vivo* experiments, all values expressed as mean ± SEM of n = 10 mice. Statistical analyses were assessed either by Student's t-test (when only two groups were compared) or one-way analyses of variance (ANOVA), followed by Dunnett's *post hoc* test (more than two experimental groups). A probability of $p < 0.05$ was considered sufficient to reject the null hypothesis.

Chapther 7

7.1 Results

7.1.1 ARPE-19 Results

ARPE-19 cell culture: ARPE-19 cells presented a typical fusiform shap with little processes,in contrast after glucose and H2O2 exposure challenge ARPE-19 were transformed lose their fusiform shape and become round.

7.1.2 Expression of MC receptors in ARPE-19 cells

Figure 21 showed the marked expression of melanocortin receptors 5 (MCR5) in ARPE-19 cells, in contrast to the poor expression of the other melanocortin receptor subtypes. Particularly, the MCR1 was more expressed than the MCR2-MCR4.

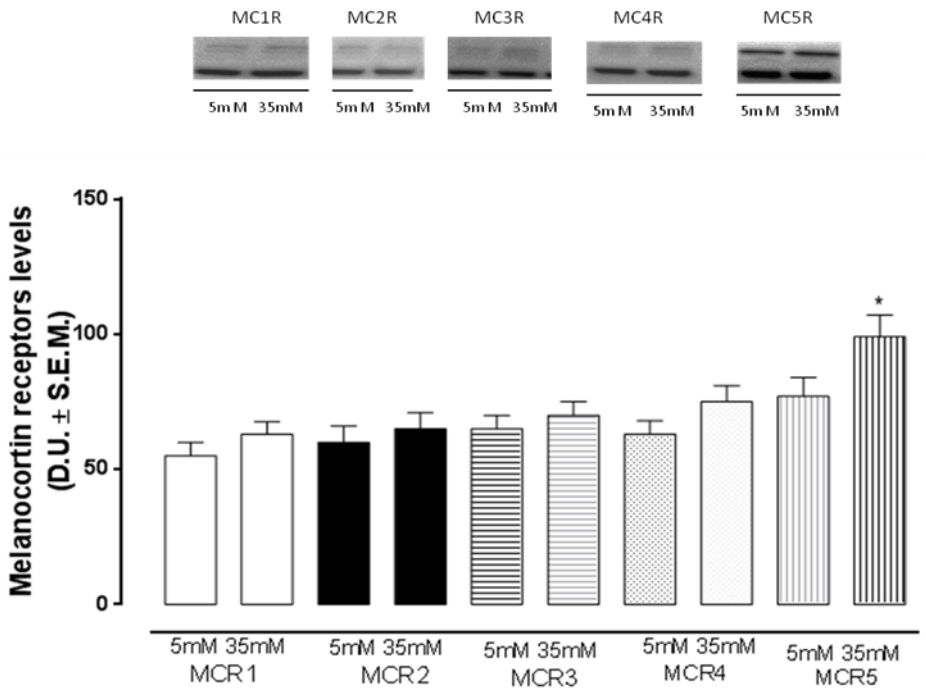

Figure 21. Melanocortin receptors expression protein.*Western blotting analysis showing that MCR5 are the most expressed receptors compared to MCR1, MCR2, MCR3 and MCR4 in ARPE-19 cells cultured in high glucose conditions (D-glucose, 35 mM). D.U. = Densitometric Units.*

7.1.3 ARPE-19 cells increased ROS production under pro-oxidant challenges and MCR5 agonist normalized this increase.

As expected, control ARPE-19 cells did not produce significant ROS levels. The addition of 35 mM D-glucose (HG) resulted in a significant increase in ROS production. This phenomenon was significantly reduced when the

compound PG-901 (10^{-10}M), MCR5 agonist, was added. In contrast, the incubation of cells with the melanocortin receptor MCR1 agonist BMS-470539 (10^{-5} M) or with the mixed MCR3/4 agonist MTII (0.30 nmol) does not produce any significant decrease of ROS levels ($P<0.01$) (Figure 22), thus excluding an active participation of these receptors in the ARPE-glucose-ROS circuit. Interestingly, the stimulation of the MCR1 decreased the ROS production after H_2O_2 challenge. Based on the contention that a glucose-ROS-dependent mechanism may drive other downstream melanocortin actions within the ARPE cells, the sequent experiments were performed by using MCR5 agonists only against the HG stimulus.

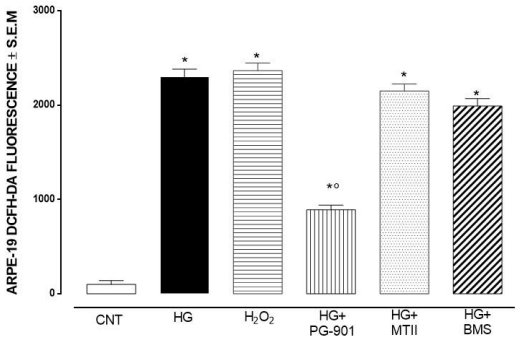

Figure 22. ARPE-19 ROS production. *Total intracellular ROS from ARPE-19 cells exposed to standard medium (CNT); High Glucose (HG, 35mM); H_2O_2 (100μM); HG+PG-901 (10^{-10}M); HG+MTII (0.30 nmol); HG+BMS-470539 (10^{-5}M) were analyzed by H_2DCFH. Values are expressed as mean ± S.E.M. Experiments were repeated three times to insure consistency of results. Significance levels expressed as P<0.01 (*) versus CNT, P<0.01 (°) versus HG.*

7.1.4 PG-901 treatment increases the ARPE-19 cells survival following high glucose exposure.

XTT assay showed that treatment of ARPE-19 cells with 35 mM D-glucose medium significantly reduced cell viability compared to the standard medium (CNT) (P<0.05 *vs* CNT). ARPE-19 cells exposed to HG and incubated for 24 hours with 10^{-10}M PG-901 showed a significant increase of survival compared

to cells exposed to high glucose alone (P<0.01 vs HG), hence positively interfering with metabolic activity (Figure 23).

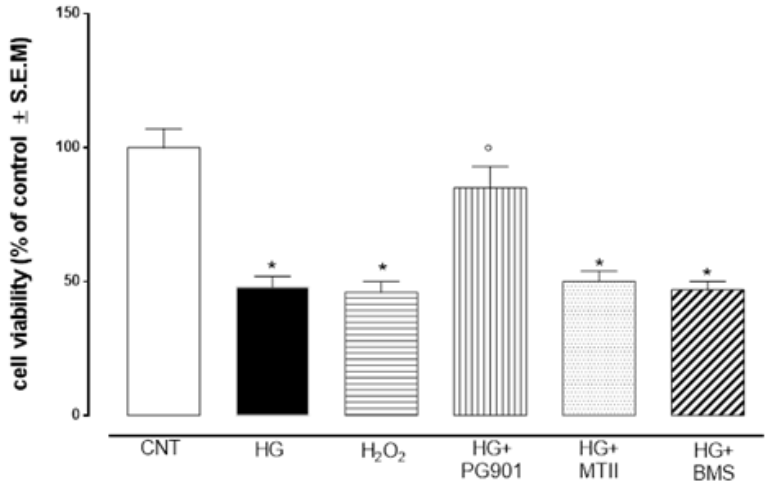

Figure 23. *XTT assay showing the cell viability as percentage of the standard medium (CNT). Compared to the control, 35 mM glucose (HG) led to a significant decrease of the cell viability. PG-901 (10^{-10}M) increased cell survival in ARPE-19 exposed to 35mM glucose.* ***(B)*** *Cell viability after: HG+MTII (MCr3/4 agonist, 0.30 nmol); H_2O_2+MTII; HG+BMS (MCr1 agonist, 10^{-5}M). The results are reported as the mean ± S.E.M. Experiments were repeated three times to insure consistency of results. P<0.01 (*) versus CNT; P< 0.01 (°) versus HG.*

7.1.5 Oxidative Challenges increased VEGF exosome release and MCR5 agonist.

Nine days after HG or H_2O_2 exposure, ARPE-19 cells release extracellular vesicles (Figure 24) as measured with the nanosight techinique.

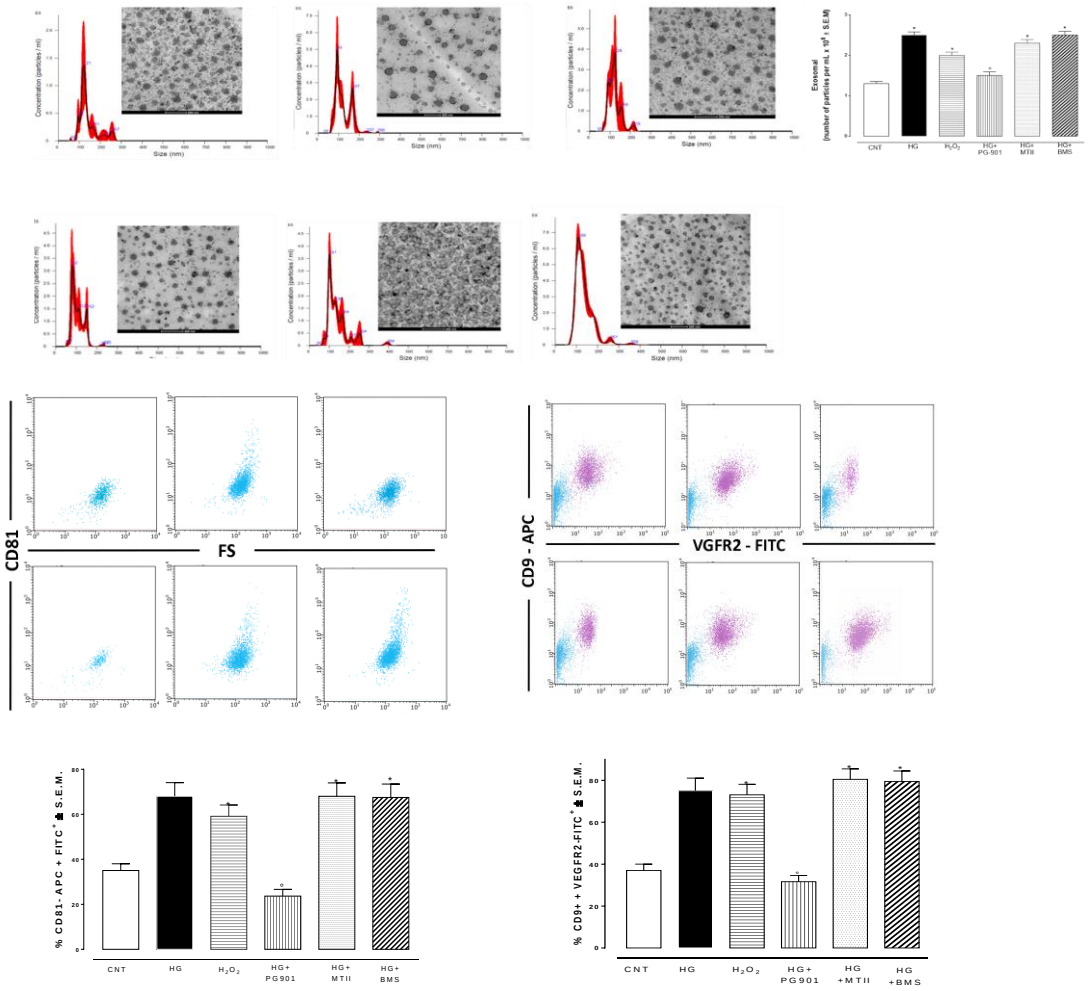

Figure 24. Characterization of exosomal vesicles released by ARPE-19 cells treated or not. (A) *Size-distribution analysis and exosomes number was performed by Nanoparticle Tracking Analysis,exosomes released into the extracellular medium from HG (35mM) treated cells were detected by electron microscopy. (**B** Exosomes detection was performed by Flow citometry targeting CD81, with a relative quantification expressed in the bar graph)**(C)** Exosomes detection was performed by Flow citometry targeting CD9, with a relative quantification expressed in the bar graph. Scale bar 200nm. Experiments were repeated three times to insure consistency of results. Significance levels expressed as P<0.01 (*) versus control (CNT) and P<0.01 (°) versus HG. CNT= standard medium; HG=35 mM D-glucose.*

Extracellular medium containing 35 mM D-glucose presented an 80% significant increased levels of exosomes labeling for VEGF (CD9-VEGF) and VEGFR2 (CD9-VEGFR2) (Figure 25) with respect to the medium containing 5 mM D-glucose.

Fitting with the previous finding, the addition of MCR5 agonist reduced the number of CD9-VEGF and CD9-VEGFR2 exosomes (Figure 25). ELISA (VEGF-A) and western blot analyses indicated that VEGF protein levels were increased after both pro-oxidant challenges HG and H_2O_2 and reduced after MCR5 agonist exposure in ARPE-19 cell homogenates and in the exosomes released from them (Figure 26A, 26D).

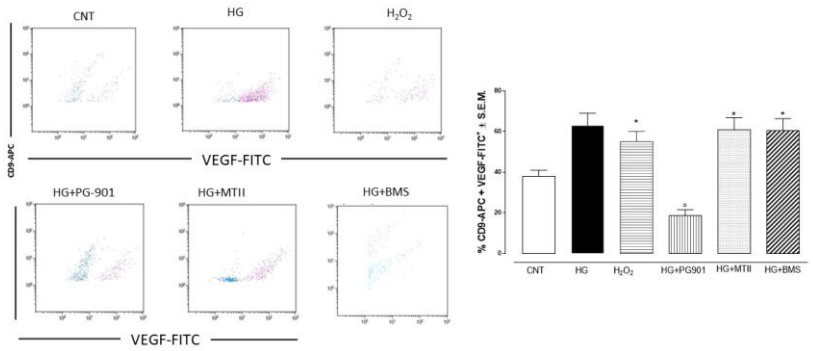

Figure 25. Characterization of the exosomal cargo. *ARPE-19 released exosomes were isolated and scrutinized by flow cytometry by anti-CD9, anti-VEGFR2 (A) and anti-VEGF (B). Treatments as in figure 4. Experiments were repeated three times to insure consistency of results. Significance levels expressed as P<0.01 (*) versus control (CNT) and P<0.01 (°) versus HG. CNT = standard medium; HG= 35 mM D-glucose.*

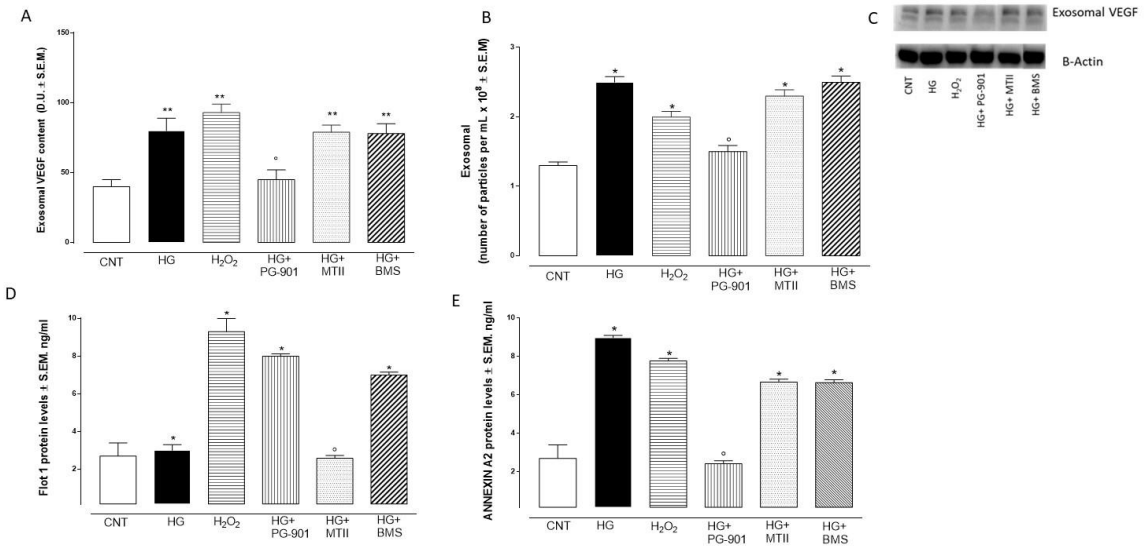

Figure 26. VEGF expression in ARPE-19 cells and ARPE-19 isolated exosomes. (A) *VEGF protein levels measured by ELISA assay in ARPE-19 cells.* ***(B, C)*** *VEGF protein levels measured by western blot in exosomes. (D,E) Flot 1 and ANX2 protein levels measured by ELISA assay in ARPE-19 cells. Experiments were repeated three times to insure consistency of results. Values are expressed as (pg/ml) mean ± S.E.M. (N=3 repeats). Significance levels expressed as P<0.05 (*) versus CNT, P<0.01 (**) versus CNT P<0.01 (°) versus HG. CNT=standard medium; HG=35 mM D-glucose; D.U. = Densitometric Units.*

7.1.6 ARPE-19 Released Exosomes promoted Vasculogenesis

In the absence of exosomes HUVEC cells produced little vascular processes theirselves. Similarly, exosomes derived from vehicle (control) treated ARPE-19 cells promoted few nodes and tubes (Figure 27). In contrast, HG (35 mM) and H_2O_2 (100 μM) induced exosomes resulted in a significant increase in HUVEC tube formation with a significant increase on nodes and tubes. Interestingly, exosomes induced from

HG/H_2O_2+MCR5 agonist PG-901 treated cells resulted in a significant decrease in tube and node formation (Figure 27).

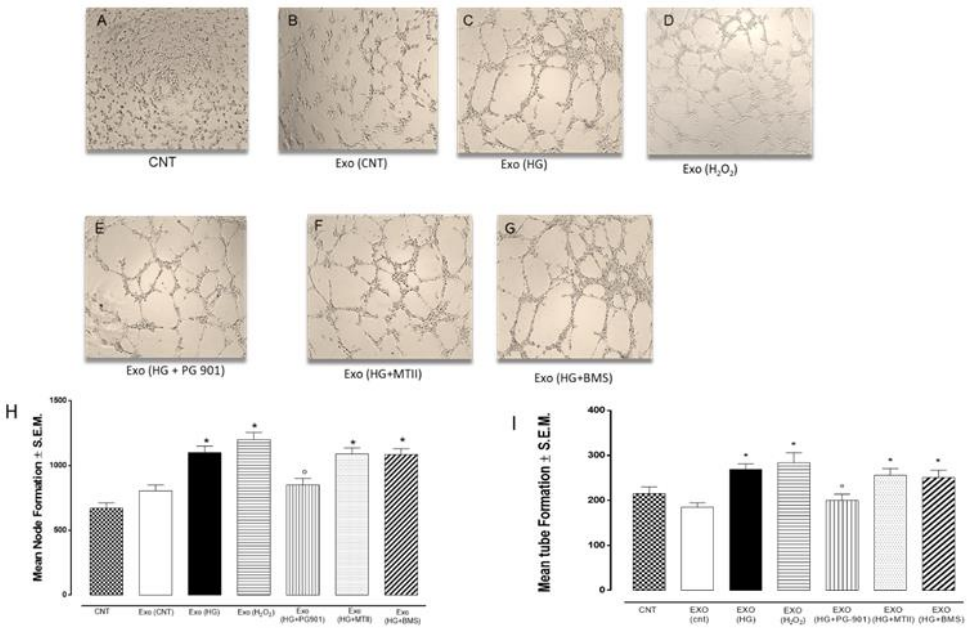

Figure 27. Exosome-induced Vasculogenesis in HUVEC cells. *ARPE-19 released exosomes from all the experimental conditions were seeded within HUVEC cells on Matrigel. Specific tube formation was shown 5 hours later. Representative images of tubular structures from:* ***(A)*** *HUVEC cells seeded with exosomes free medium;* ***(B)*** *HUVEC cells seeded with exosome-containing medium;* ***(C)*** *HUVEC cells seeded with HG (35 mM) induced exosomes;****(D)*** *HUVEC cells seeded with H_2O_2(100 µM) induced exosomes;* ***(E)*** *HUVEC cells seeded with HG+MCr5 agonist PG-901 (10^{-10}M);* ***(F)*** *HG+MCr3/MCr4 agonist MTII 0.30 nmol;* ***(G)*** *HG+BMS (10^{-5}M) ;* ***(H)*** *Node formation and* ***(I)*** *Tube Formation. Scale bar 500µm. Experiments were repeated three times to insure consistency of results****.*** *Values are expressed as mean ± SEM. Significance levels expressed as P<0.01 (*) versus EXO (CNT) and P<0.01 (°) versus HG. CNT=ARPE -19 cells in standard exosome free medium.*

7.1.7 Oxidative-induced NF-kB and Cyp2E1 expression are modulated by MCR5 agonist.

Western blot analysis indicated (Figure 28) how both pro-oxidant challenges (HG and H_2O_2) led to a significant NF-kB and Cyp2E1 protein expression compared to control levels. More interesting, the exposure to MCR5 resulted in a significant reduction of NF-kB and Cyp2E1 protein levels when compared to HG/H_2O_2-treated cells.

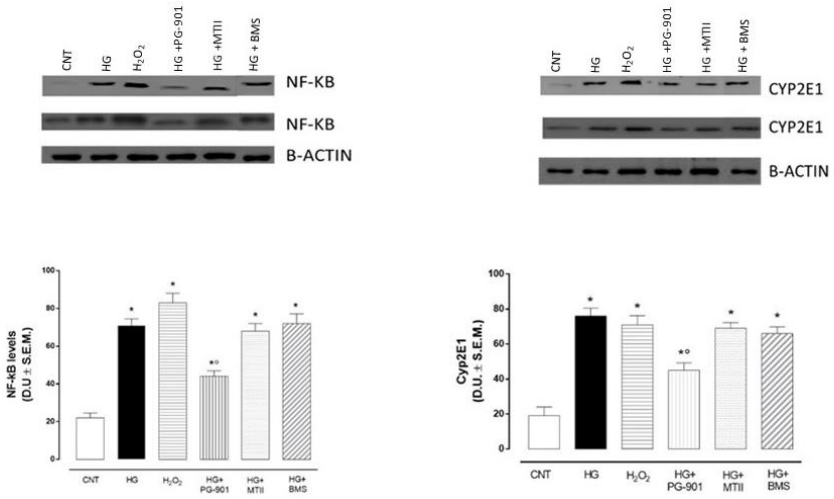

Figure 28. NF-kB and Cyp2E1 protein expression. *Protein extracts on the different gropus were analyzed by Western blot against NF-kB and Cyp2E1. Representative images and relative levels assesed by densitomerty of bands of 65 kD (NF-kB) and 57 kDa (Cyp2E1) are presented. β-Actin is used as loading control. Data are expressed as densitometric units (D.U.) and presented as mean ± S.E.M. Experiments were repeated three times to insure consistency of results. Significance levels expressed as P<0.01 (*) versus control and P<0.01 (°) versus HG*

7.2. Primary retinal cells culture results

In order to study the presence effect of MCR in other retinal cell types primary retinal cell cultures were perfomerd an analyzed when their were incubated for 20 days with high glucose.

7.2.1. MCR1 and MCR5 gene expression and protein in retinal cells cultured in high glucose

RT-PCR showed a significant increase of MCR1,5 gene expressions in retinal cells after high-glucose exposure compared to control cells ($P < 0.01$ versus control). In contrast, both MCR1,5 genes were significantly reduced ($P < 0.01$ versus control) in the presence of the MCR1,5 agonists (PG901 and BMS-470539,respectively) (Fig. 29A and B).To confirm gene expression data, MCR1,5 protein levels were measured by ELISA assay, and fitting with RT-PCR results, protein levels show the same expression profile. MCR1,5 protein levels were significantly increased under high-glucose conditions. Consistently

with RT-PCR, MCR1,5 agonists were able to reduce the high-glucoseincreased MCR1,5 protein levels (Fig. 30A and B).

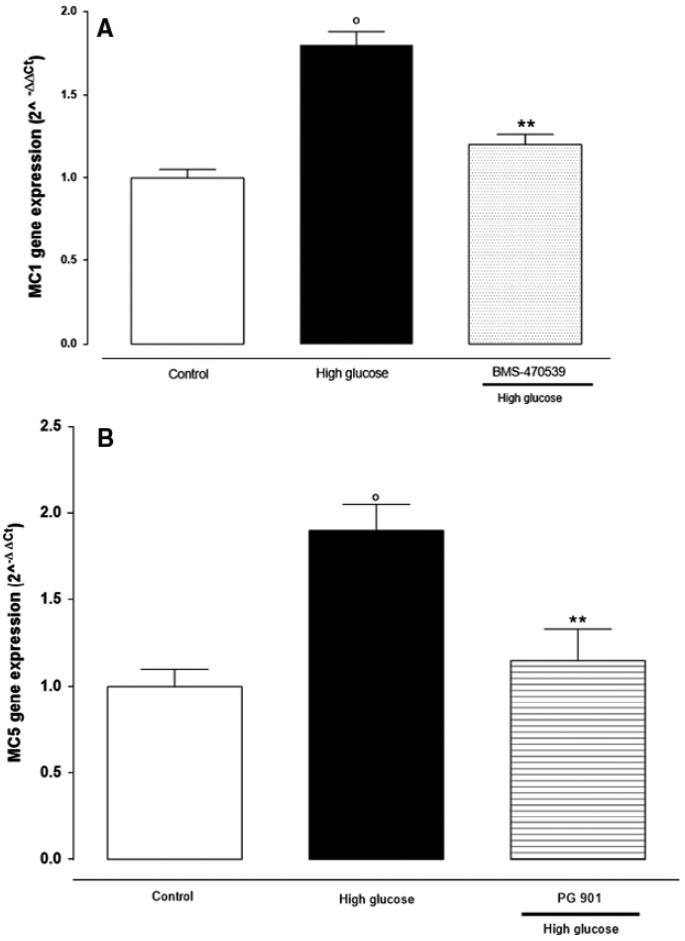

Figure 29. *RT-PCR analysis showed (A) MCR1 (melanocortin receptors 1)and (B) MCR5 (melanocortin receptor 5) gene expression in retinal cellscultured in high-glucose (25 mM) concentration, and in the presence or absence of MCR1 agonist BMS-470539 and MCR5 agonist PG- 901. The results are reported as the mean _ S.E.M. of n = 3 treatments and the significant results expressed as °P < 0.01 versus control and **P < 0.01 versus high glucose.*

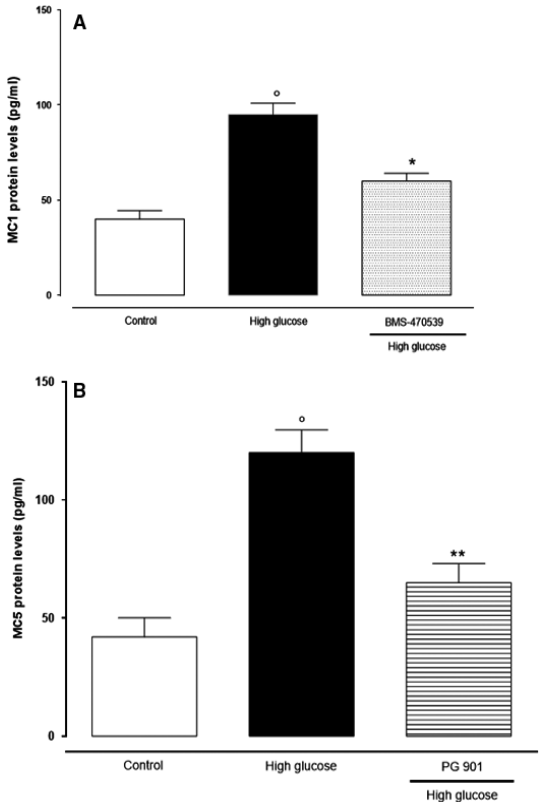

Figure 30. *ELISA assay showed high levels of MCR1,5 protein in retinal cells cultured in high-glucose concentration. These were significantly decreased by the treatment with BMS-470539 and PG- 901 (MCR1 and MCR5 agonists). The results are reported as the mean _ S.E.M. of n = 3 treatments and the significant results expressed as °P < 0.01 versus control and **P < 0.0 versus high glucose.*

7.2.2. Decreased MnSOD and GPx enzyme levels are restored by MCR1,5 agonists

MnSOD and GPx antioxidant enzymes were significantly decreased after high-glucose exposure compared to normal glucose (control) cultured cells (Fig. 31A and B). Conversely, MnSOD and GPx levels were significantlyincreased after MCR1,5 agonist treatment (Fig. 31A and B).Able to reduce the high-glucose increased MCR1,5 protein levels (Fig. 32A and B).

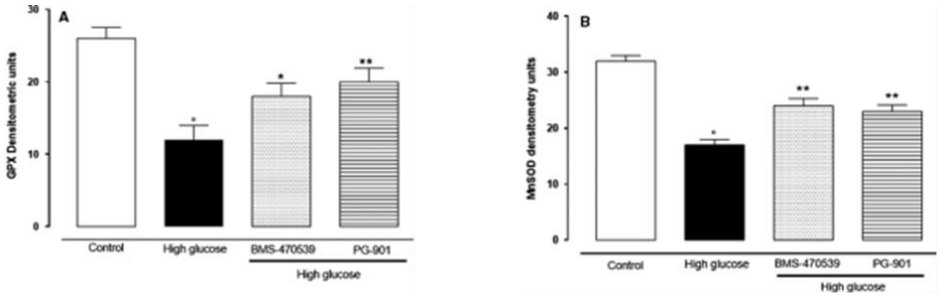

Figure 31. *Western blotting analysis showed that high glucose (25 mM) decreases MnSOD and GPx enzyme levels; (A, B). Treatment with the compounds BMS-470539 (MCR1 agonist) and P-901 (MCR5 agonist) restored the MnSOD and GPx enzyme levels. (A, B). MnSOD: manganese superoxide dismutase; GPx: glutathione peroxidase. The results are reported as the mean _ S.E.M. of n = 3 treatments and the significant results expressed as *P < 0.05 versus high glucose, **P < 0.01versus high glucose and °P < 0.01 versus control.*

7.2.3. Opsin and recoverin labelling

Among the different cell types included in the retinal cell culture, the presence of photoreceptors can be recognized by the presence of recoverin and opsin. Under control conditions (5 mM Glucose), photoreceptors exhibit large cytoplasm expansions, and opsin is sparsely distributed along the cytoplasm membrane. In contrast, photoreceptors exposed to high-glucose concentration (25 mM) present less opsin labelling (Fig. 32), as evidenced by the percentage of opsinpositive cell on the total of cells counted. However, the addition of the MC receptor agonists, PG901 and BMS-470539, and melanocortin receptors 1 and 5 to high-glucose-treated photoreceptors presented a pattern of opsin labelling more similar to that shown after control conditions. Control recoverin-positive cells present a red dye with cytoplasm location (Fig. 32). However, high-glucose-treated cells present almost null recoverin reactivity .In contrast, the addition of PG901, or BMS-470539, resulted in evident pattern of labelling similar to that observed under control conditions.

Structurally, high-glucose-(25 mM) cultured cells appear with abnormal morphology of photoreceptors characterized by stringy, swelled and

compressed size, with respect to the control (5 mM). In contrast to this, treatment of high-glucose-cultured cells with the compounds BMS-470539 and PG-901 improved photoreceptors morphology that indeed appear less distorted, more regular and more similar to the control cells.

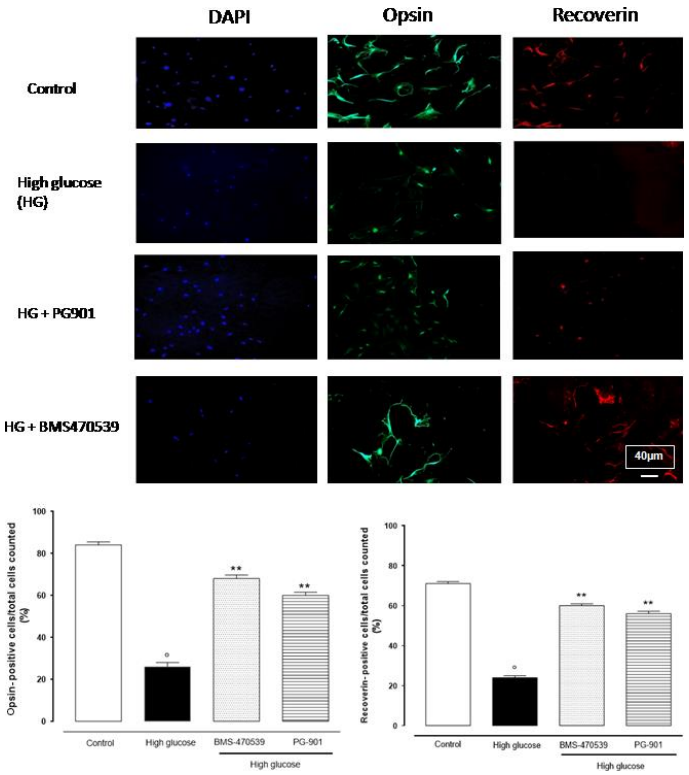

Figure 32. *Depicted are representative immunocytochemistries of retinal cells cultured in 5 mM or 25 mM glucose and labelled with opsin, recoverinand 40,6-diamidino-2-phenylindole (DAPI) antibodies. Cells were treated with BMS-470539 and PG-901, and representative microscopic fields foreach treatment are shown. Accordingly, the percentage of recoverin and opsin-positive cells is represented in the graph. The results are expressedas mean _ S.E.M. of the percentages of positive cell/total cell counted in each analysed field for each treatment. The statistical significance wasreported as °P < 0.01 versus control; **P < 0.01 versus high glucose. 409 magnification.*

7.3 In vivo studies results

In order to continue the previus results , mice were induced diabetic by intraperitoneal injection of STZ (65 mg/kg) to C57BL/6mice significantly increased glycemia levels, which remained almost constant throughout the duration of the 16-week observation (Table 1).These levels were not affected by drug treatment at any of the time points under investigation. In contrast, the endogenous levels of α-MSH within the retina were significantly reduced after 16 weeks of diabetes(nondiabetic, 72 ng/mL ± 7; diabetic, 27 ng/mL ± 11).

7.3 .1 STZ-Induced Diabetes Causes Structural and Microvascular Changes in Mouse Retinas

Fluorescein angiography showed structural changes in the retinal vessels with an increased vascular tortuosity and microvascular changes in 8 of the 10 mice analyzed (data at week 12). These alterations became more evident at week 16 after STZ. Indeed, an irregular retinal vessel caliber and microaneurysmswere

seen at both time points (Figure 33). No signs of deviation from the normal retina vascularization were seen after 8 weeks of diabetes (Figure 33).

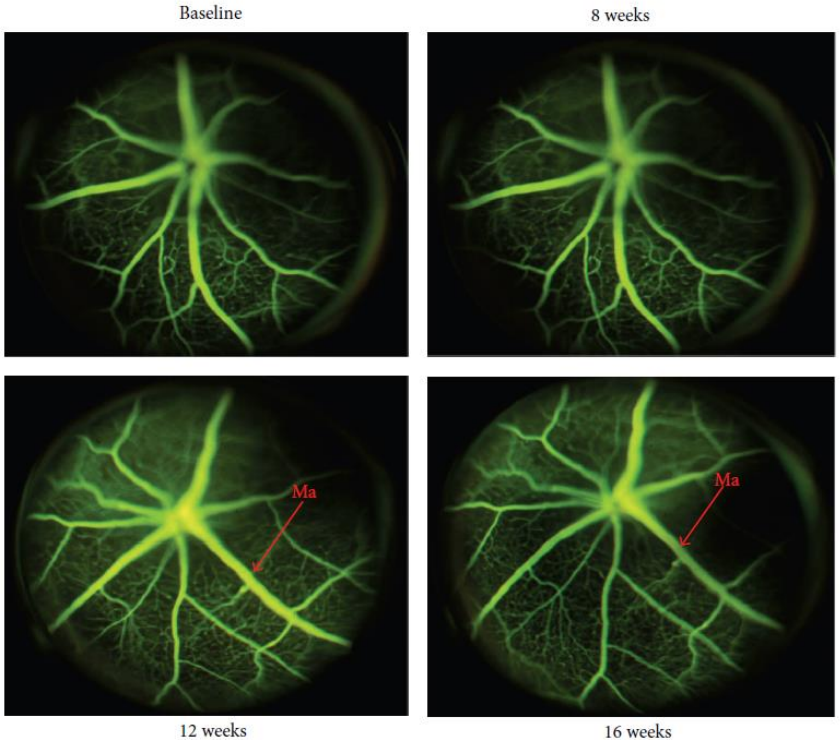

Figure 33. *Representative images of FAG performed at baseline and after 8, 12, and 16 weeks fromdiabetes induction. There were no alterations worthy of note at 8 weeks. At 12 weeks from diabetic induction there was an increase in vascular tortuosity with some microvascular changes such as microaneurysms that were also evident after 16 weeks of diabetes. Each group consisted of 10 mice in which 8 developed clear signs of retinopathy. Ma = microaneurysms.*

7. 3.2. Melanocortin Receptors Are Expressed in the Retina of Mice Suffering from Diabetic Retinopathy.

Next we determined the expression patterns of selected MC receptor expression in the retina of diabetic mice that had developed retinopathy, with a focus on MC1, MC3, andMC5 receptors since implicated in the process of inflammation and tissue protection. By conventional PCR we could detect the MC1 and MC5 signals, but not the MC3 (Figure 34). Using qPCR, MC1 and MC5 displayed a plastic response to diabetes, with elevated expression being quantified by week 16 (Figure 34).

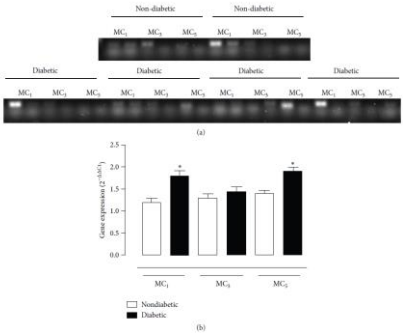

Figure 34. *Real Time PCR for the expression of melanocortin receptor subtypes within the retina. (a) Representative traces of the RT-PCRand (b) relative 2−ΔΔCt gene expressions forMC1, MC3, andMC5 receptors assayed after 16-week follow-up in nondiabetic mice, and diabetic mice with retinopathy. Total RNA was extracted using RNeasy Plus Mini Kit and commercially available primer for amplification of mouse MC1, MC3, andMC5 receptors. Negative controls were either RT without enzyme or PCR without cDNA template.*

7.3.3. Melanocortin Receptor Activation Modulates the Development of Diabetic Retinopathy.

Intravitreal injections of the MC1 receptor agonist BMS-470539 (33 mmol) or MC5 receptor agonist PG-901 (7.32 nM) decreased retinal damage,as demonstrated by FAG. Indeed, regular course and caliber of retinal vessels without microvascular changes or vessel leakage were present at each time point considered afterintravitreal injection of MC1 receptor agonist BMS-470539 and MC5 receptors agonists PG-901, as compared to theuntreated diabetic mice with retinopathy (Figure 35).

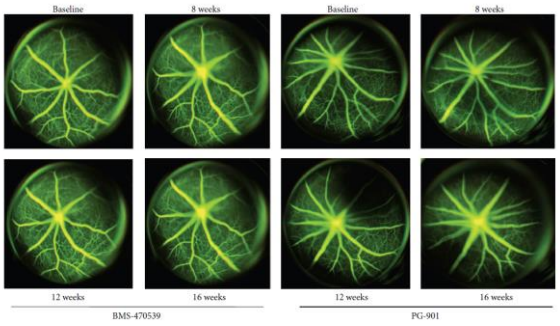

Figure 35. *Representative pictures of FAG showed a regular course and caliber of retinal vessels without microvascular changes or vesselleakage at every time point following intravitreal injection of the MC1 melanocortin receptor agonist BMS-470539 and of the MC5 agonistPG-901.The number of mice for each group was n = 10 nondiabetic mice (baseline) and 8 diabetic mice with retinopathy.*

To investigate a potential involvement of the MCpathway by endogenous peptides, we tested the effect of receptor antagonists. Intravitreal injection of PG20N (MC5antagonist) and AGRP(MC1 antagonist) worsened the retinal injury with evident changes already after 8 weeks afterinduction of diabetes. Due to the presence of a venousloop and an extensive retinal vessel leakage with progressive dye diffusion (Figure 36) at 16 weeks after induction,hyperfluorescent areas were observed after treatmentwith either of the two compounds. Of interest, intravitreal injection of molecules that activate MC3, like the agonist MTII (dual MC3-MC4 agonist) or the antagonist SHU9119 (dual MC3-MC4 antagonist), did not produce changes of the microvascular bed into the retina of diabetic mice (Figure 37).

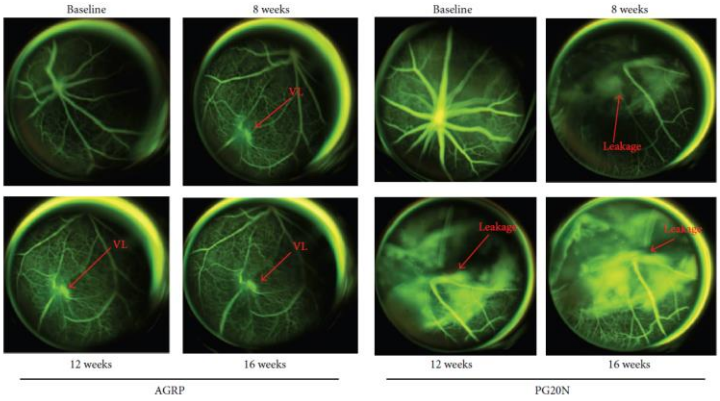

Figure 36. *FAG performed at baseline (nondiabetic) and after 8, 12, and 16 weeks from diabetes induction after the intravitreal injection of the MC1 receptor antagonist AGRP, and the MC5 receptor antagonist PG20N. At baseline no vascular alterations were present 8 weeks following diabetic induction, FAG depicted an increased vascular tortuosity with hyperfluorescent area due to the presence of a venous loop in the retinal inferior nasal area.The first retina damage appears 8 weeks following diabetic induction and was characterized by an extensive hyperfluorescent area of vascular leakage. At 12 and 16 weeks this hyperfluorescent area was extended with progressive dye diffusion. The number of mice for each group was n = 10 nondiabetic mice and 8 diabetic mice with retinopathy. VL = venous loop.*

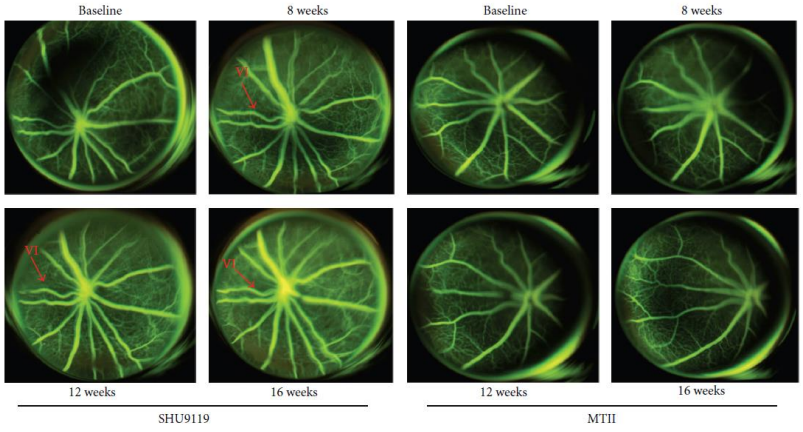

Figure 37. *Representative FAGs after intravitreal MC3-MC4 receptor antagonist SHU9119, and MTII, MC3-MC4 receptor agonist in diabeticmice with retinopathy. Evident was a progressive increase of the vessel irregularity during the follow-up without microvascular abnormalities or vessel leakage. The number of mice for each group was n = 10 nondiabetic mice (baseline) and 8 diabetic mice with retinopathy. VI =vascular irregularity.*

7.3.4. Melanocortin Receptor Activation Modulates Levels of Retinal Occludin.

In diabetic mice suffering from retinopathy, cellular tight junctions were damaged as demonstrated by the low levels of occludin detected in retinal homogenates(Figure 38A). Of interest, levels of this cell junction marker progressively decreased with the development of retinopathy, reaching the lowest level detected at week 16 after STZ (Figure 38A). Such a nadir was further reduced following intravitreal injection of the MCR1 and MCR5 receptor antagonist AGRP and PG20N, respectively, over the different time points (Figure 38A). Conversely, administration of BMS-470539 or PG-901 increased the retinal occludin levels at 8 weeks, 12 weeks (data not shown), and 16 weeks, compared to untreated diabetic mice ($p < 0.01$, Figure 38A), demonstrating a protective effect downstream activation of MCR1 or MCR5, respectively. Modulation of MCR3 signals was without effects (Figure 38A).

7.3.5 Melanocortin Receptor Activation Modulates Levels of VEGF in the Retina of Diabetic Mice.

As we observed profound alterations in the microcirculation, visual images complemented by the loss of tight junction proteins (of which occludin was selected as faithful marker), we then measured expression of a fundamental angiogenic factor. Retinal levels of VEGF were increased (+6< 0.01 versus diabetic; Figure 38B) or MC1 antagonist AGRP (+36.2 ± 1.8%, p < 0.01 versus diabetic; Figure 36).Agonism at MC1 or MC5 decreased levels of VEGF in the retina of diabetic mice back to levels detected in retinas of untreated diabetic mice (p < 0.01; Figure 38 B). MC3-MC4 appear not be involved in this protective effect, as the compounds MTII and SHU9119 failed to modulate VEGF levels assayed during the development of retinopathy in diabetic mice (p > 0.05; Figure 38B).

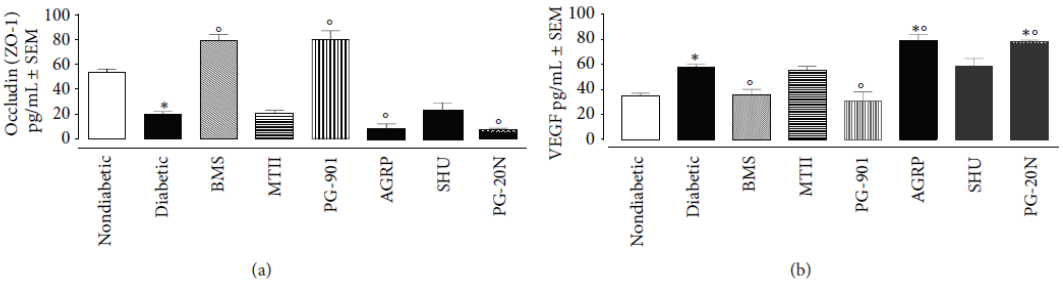

***Figure 38 A and B**. Occludin and vascular endothelial growth factor levels into the retina of STZ-diabetic mice. A Quantikine ELISA kit was usedin order to assay after 16 weeks of diabetes the levels of occludin into the retina of nondiabetic and diabetic mice following intravitreal administration of selectivemelanocortin receptor agonists/antagonists:MC1 receptor agonist BMS-470539;MC3-MC4 melanocortin receptor agonist MTII;MC1 receptor antagonist agouti related protein (AGRP); MC5 melanocortin receptor agonist PG-901;MC3-MC4 melanocortin receptor antagonist SHU9119; MC5 melanocortin receptor antagonist PG20N. The values represent the mean ± SEM of 8–10 observations.Significant differences against nondiabetic mice are expressed as $*p < 0.01$. Significant differences versus diabetic are expressed as $\circ p < 0.01$.*

In line with this trend, immunohistochemistry for ki-67 showed a decrease in the percentages of positive stained area/total stained area following intravitreal injection of BMS-470539 (MCRF1 agonist, −64.2 ± 6% versus diabetic) or PG-901 (MCR5 agonist, −68.6 ± 7%) as calculated against the values quantified in week 16 diabetic mice (Figure 39).

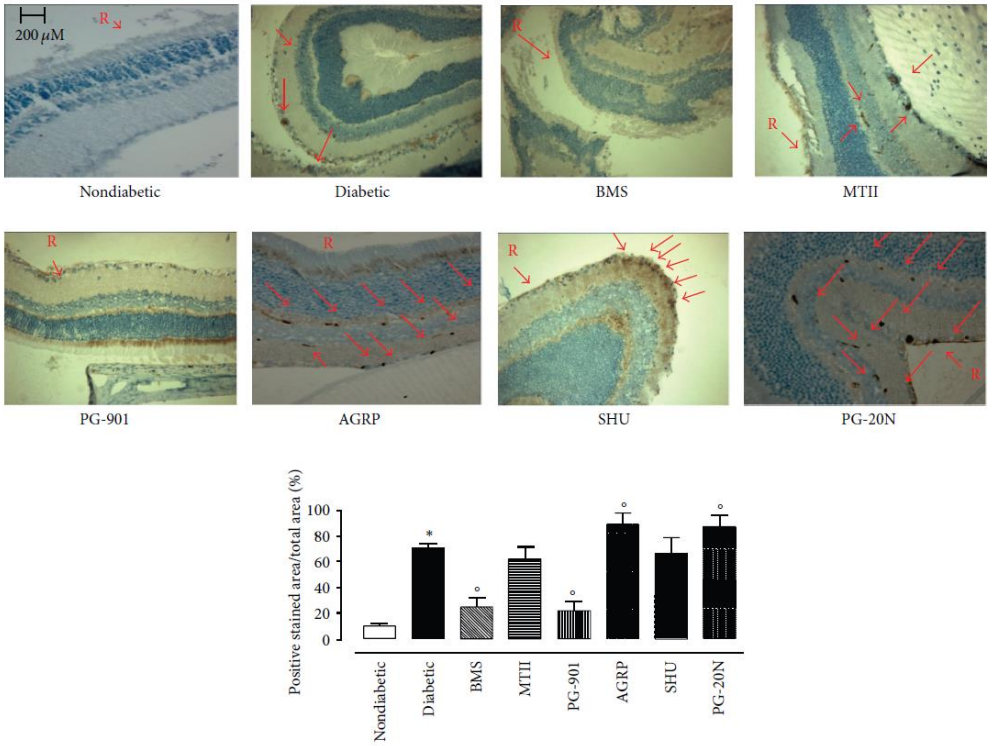

Figure 39. *Representative immunohistochemistry after 16 weeks of diabetes for ki-67 in the retina of nondiabetic mice (nondiabetic), STZdiabetic mice with retinopathy (diabetic) after intravitreal treatment or not withMC1 receptor agonist BMS-470539;MC3-MC4 melanocortin receptor agonist MTII; MC1 receptor antagonist agouti related protein (AGRP); MC5 melanocortin receptor agonist PG-901; MC3-MC4 melanocortin receptor antagonist SHU9119;MC5 melanocortin receptor antagonist PG20N. Percentage of positive stained area/total areawith significant differences against nondiabetic mice is expressed as* $*p < 0.01$*. Significant differences versus diabetic are expressed as* $\circ p < 0.01$*.R = retina; arrows indicate the positive immunostaining.*

7.3.6 Melanocortin Receptor Activation Changes retinal Macrophage Phenotype.

Western blotting of retina homogenates showed that the presence of the CD11c marker for M1 macrophages was increased in diabeticmice at all time points considered, compared with healthy nondiabetic mice (Figure 40). There was a good correlation between the number of M1 macrophages and the levels of occludin in the retina with an $r2$= 0.9732. The number of M1 macrophages was further increased by intravitreal injection of the MC1 or MC5 antagonist (Figure 40). In contrast, intravitreal injection of the MC1 or MC5 agonist in diabetic mice affected by retinopathy decreased the binding for CD11c (Figure 40).To complement these analyses, we then also assessed expression of an M2 marker, the mannose receptor CD206. Intravitreal treatment with BMS-470539 or PG-901 increased CD206 detection, when compared to diabetic mice (Figure 40), in line with the improvement of the ocular signs recorded with the FAG. This suggests an elevatedpresence of M2 subtype macrophages.

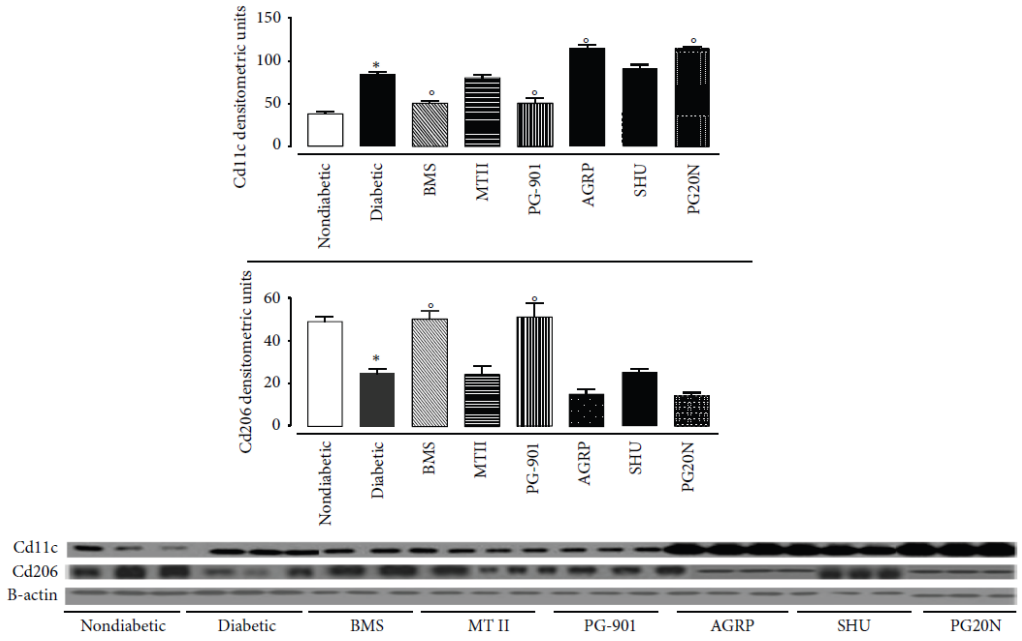

Figure 40. *Expression of retinal Cd11c and Cd206 in eyes of mice with retinopathy. 16-week time point western blotting traces and relative densitometric units of binding to anti-Cd211 and Cd206 antibodies in the retina of nondiabetic mice (nondiabetic), STZ-diabetic mice with retinopathy (diabetic) after intravitreal treatment or not with MC1 receptor agonist BMS-470539; MC3-MC4 melanocortin receptor agonist MTII;MC1 receptor antagonist agouti related protein (AGRP);MC5 melanocortin receptor agonist PG-901;MC3-MC4 melanocortin receptor antagonist SHU9119; MC5 melanocortin receptor antagonist PG20N. Significant differences against nondiabetic mice are expressed as* $*p < 0.01$*; significant differences versus diabetic are expressed as* $\circ p < 0.01$.

7.3.7 Melanocortin Receptor Activation Modulates Cytokine and Chemokine Expression Profiles

Around 40 cytokines and chemokines were analysed using the retinal extracts. The proinflammatory cytokine IL-1β was increased by ~61% in response to the development of retinopathy (p < 0.01 versus nondiabetic mice without retinopathy; Figure 41).The melanocortin receptor antagonistsAGRP and PG20N further increased expression levels for IL-1α, IL-1β, IL-6, MIP-1α, MIP-2α, and MIP-3α (Figure 41). For example, IL-1β was increased by 26 ± 2% and 28 ± 1.8%, respectively, for AGRP and PG20N (p < 0.01 versus diabetic). Administration of BMS-470539 or PG-901 significantly diminished IL-1α, IL- 1β, IL-6, MIP-1α, MIP-2α, and MIP-3α in the retina (p <0.01 versus diabetic; Figure 41). MTII and SHU9119 did not significantly affect the expression of these proinflammatory mediators (Figure 41). A similar trend was observed for the proinflammatory chemokines MIP-1α,MIP-2α, andMIP-3α, where the highest levels were reached in diabetic mice 16 weeks after diabetes induction (Figure 41). Modulation of MC1 and MC5 receptor

signaling by means of agonists and antagonists significantly modified MIP 1α, MIP-2α, andMIP-3α levels (Figure 41). Finally, and of further interest, while IL-10 levels were low in the retina of diabetic mice with retinopathy, the expression of this anti-inflammatory cytokine was significantly increased after the injection of either MCR1 or MCR5 agonist, for example, +64 ± 7% and +56 ± 4.2% for BMS-470539 and PG- 901, respectively (p < 0.01 versus diabetic mice) (Figure 41).

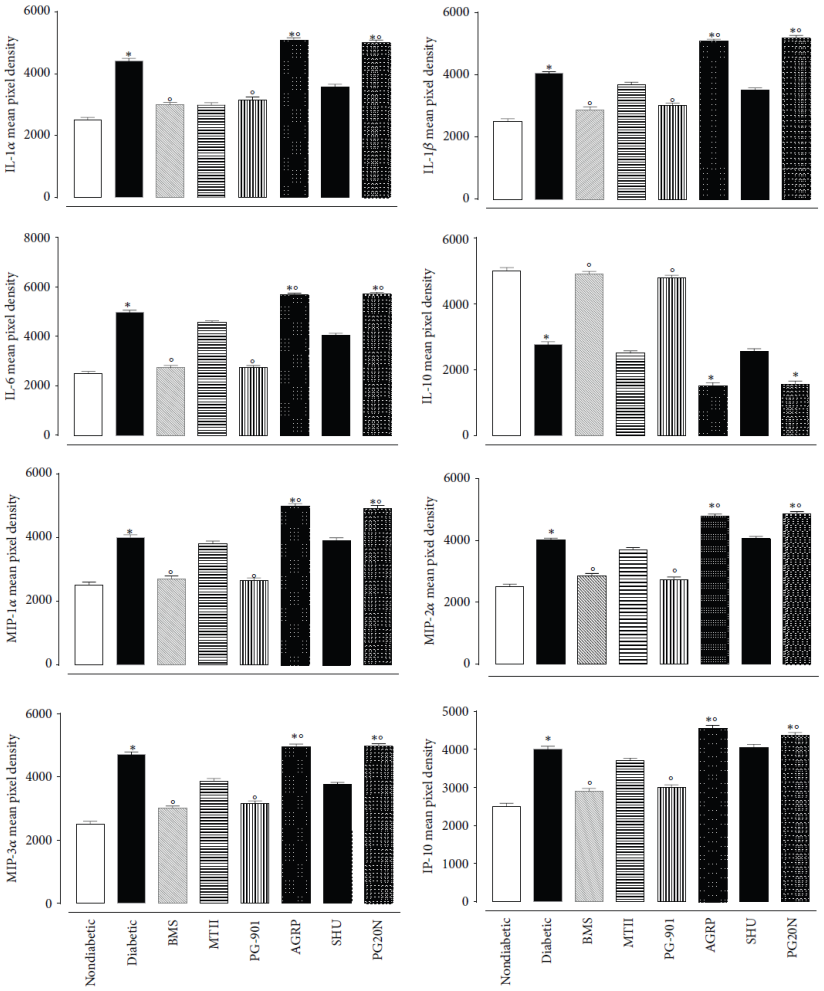

Figure 41 *Array of cytokines and chemokines released in the retina of diabetic mice. A specific ELISA kit was used in order to quantify the levels of cytokines and chemokines after 16 weeks of diabetes in mice treated or not with the melanocortin receptor agonists/antagonists asin Figure 40.The values represent the mean ± SEM of 8–10 mice per group. Significant differences against nondiabetic mice are expressed as* $*p < 0.01$*; significant differences versus diabetic are expressed as* $\circ p < 0.01$.

Chapther 8

DISCUSSION

Diabetic retinopathy is a leading cause of adult blindness and is the most common complication of diabetes. It affects morethan 90% of people with diabetes, ultimately leading to retinal edema, neovascularization, and, in some patients, vision loss [1, 2]. Systemic control of blood glucose can slow down the progression of diabetic retinopathy but fails to stop or reverse clinical signs of it [3, 4]. Hence understanding the molecular pathways governing the pathophysiology of DR and targeting them is essential to the prevention of catastrophic visual loss arising from vision-threatening complications of diabetic retinopathy such as macular edema, vitreous hemorrhage, and tractional retinal detachment. One critical factor in diabetes is the glucose over drive that finally results in reactive oxygen species (ROS) production [18] affecting the cell membranes and proteins and leading cell miss function or even cell death [7]. These ROS are detrimental for cells in several organs, including the eye where they determine alterations of the structure and

functionality of retinal cells [19]. These latter include amacrine cells, Muller cells, ganglion cells and the photoreceptors [11]. Of special interest are the cells of the retinal pigment epithelium (RPE) because of their physiological role in retinal homeostasis. These cells are sensibles to various oxidant injury stimuli such as those induced by RPE exposure to visible light or those derived from endogenous metabolism [20,21]. Following these stimuli RPE cells lead mobilization of a number of ROS and mediators that ultimately control the integrity and the function of RPE itself and of other cells and tissues into the retina [22]. In this context, the present study shows that a culture of human retinal pigment epithelial cells ARPE-19 exposed to 35 mM D-glucose, a condition that mimics the diabetic hyperglycemia in vivo [11], generates a high glucose driven over expression of ROS that conditions the survival of the cells themselves. Indeed, this over ROS production is accompanied by a reduction of cells viability with respect to the cells cultured in standard medium containing 5 mM D-glucose. Consequence of the exaggerated glucose-induced ROS production in the eye is often the initiation of a process of vascular alterations, damage, leakage and cell proliferation [10] leading the

neovascularization of the retina [16,23]. This neovascularization involving vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), and the angiopoietins among others [23]. Previous studies have hypothesized that neovascularization of the retina is initiated by a ROS-dependent release of VEGF-containing exosome vesicles from retinal cells leading the formation of the new vessels [9,24,25]. These exosomes are small vesicles, between 50 and 150 nm in diameter [9], released by several cell types and stimuli [26-29] and contain genetic material, proteins and inflammatory factors essentials for cell-cell communication [9]. In the present setting a high concentration of glucose into the medium represents a strong stimulus to induce increase of ROS and exosomes release from ARPE-19 after nine days of exposure. These exosomes mostly characterized by high quantity of VEGF protein into them, aimed at stimulate neoangiogenesis in other cells. Indeed, the VEGF-containing exosomes increase the nodes and tubes formation when they are transfered to HUVEC cells. This is the first time that is described. However, the most important and novel finding is another as it identifies the MCR5 one of the

receptor important for this neoagiogenesis. Melanocortins are endogenous peptides that possess a wide range of biological activities, including inhibition of leukocyte activation, promotion of inflammation resolution, and the ensuing tissue protection [5–12]. These effects on the immune response are brought about by five distinct melanocortin receptors, termed from MC1 to MC5, ubiquitously expressed except for the MC2 which is localised to the adrenal glands [13]. Within the eye, MC3, MC4, and MC5 are expressed in the in Melanocortins are endogenous peptides that possess a wide range of biological activities, including inhibition of leukocyte activation, promotion of inflammation resolution, and the ensuing tissue protection [5–12]. These effects on the immune response are brought about by five distinct melanocortin receptors, termed from MC1 to MC5, ubiquitously expressed except for the MC2 which is localised to the adrenal glands [13]. Within the eye, MC3, MC4, and MC5 are expressed in the inner neural retinal layers [14, 15], with MC3 and MC4 expression being reported also in the layer of retinal ganglion cells [14, 15]. MC5 alone has been detected in the neural outer plexiform layer, whilst MC1 and MC5 are detected in retinal pigment epithelial cells [16, 17].

There is scant knowledge on the biology associated with these receptors in the eye. Work is limited to the most common melanocortin peptide, α-melanocyte stimulating hormone (α-MSH), which activates all MC receptors (except MC2), controls the development and neurotrophism of the ocular tissues [18–20], and exerts protective effects on the retinal vascular endothelial cells [21, 22]. The present ner neural retinal layers [14, 15], with MC3 and MC4 expression being reported also in the layer of retinal ganglion cells [14, 15]. MC5 alone has been detected in the neural outer plexiform layer, whilst MC1 and MC5 are detected in retinal pigment epithelial cells [16, 17]. There is scant knowledge on the biology associated with these receptors in the eye. Work is limited to the most common melanocortin peptide, α-melanocyte stimulating hormone (α-MSH), which activates all MC receptors (except MC2), controls the development and neurotrophism of the ocular tissues [18–20], and exerts protective effects on the retinal vascular endothelial cells [21, 22]. At the present, the exosomal release from these cells and the consequent exosomes-induced node and tube formation in HUVEC cells was significantly reduced by the incubation of ARPE-19 with PG-901, agonist at the melanocortin receptor

MCR5. This receptor is the only one of the 5 melanocortin receptors, called MCR1-MCR5, expressed into the retinal pigment epithelial cells [11,12]. So MCR5 it is cautiously proposed a receptor having a major role in the antioxidative action and defensive response against the high glucose stimulation of human pigment epithelial cells ARPE-19. The MCR5 role is not limited, however, to the tagets earlier mentioned. Indeed, the data presented here show that the reduced pro-vascular drive of MCR5 in ARPE-19 is accompanied by reduced expression of two coupled factors [30], the cytochrome p4502E1 (CYP2E1) and the nuclear factor kappa b (Nf- kB) involved in high glucose signaling and in the ROS generation. Notewhorthy, the activation of Nf-Kb by high glucose has been demonstrated in several in vivo and in vitro studies [30] as well as the subsequent increase of CyP2E1 caused by this transcription factor [30]**.** CyP2E1 already been involved in ARPE-19 oxidative stress and exosomes release [9]. To our knowledge, this is the first time that it is described, and may pave the way to an action of the MCR5 against the high glucose damage that also involves a modulation of the Nf-kB and CyP2E1 pathways.

The retina receives light, converts it into neural signal, and sends thesxe signals on to the brain for visual recognition (6).It has a very complex structure with 10 layers, and has multiple neurons including photoreceptors and ganglion cells. The present study shows that murine primary retinal cells exposed to a high-glucose medium express a damaged photoreceptors phenotype. This demonstrated by a morphological assessment and by a decrease of two markers of cell membranes and photoreceptors integrity, the opsin and recoverin onto the cell surface.

It is well known that high glucose in diabetes is an independent risk factor for several vascular and non-vascular diseases [26], and promotes direct cellular alterations by inducing a stress response independently of the diabetic condition [26–28]. At level of the retina, a persistent hyperglycaemia leads derangement of retinal vessels and retinal structure causing retinopathy [13]. Several previous studies indicated different pathways and pattern of mediators as responsible of this damage, including oxidative stress and inhibition of antioxidant enzyme gene expression [4, 8, 29]. They do not describe, however, the role of melanocortin peptides and their receptors in this mechanism.

Endogenous melanocortins are peptides that control many physiological and pathological processes through the activity of different 7-transmembrane G-protein-coupled receptors called MCR1-5 [13]. These MCR, probably due to their role on skin cancer, skinrelated diseases or even obesity [30], MCR, have attracted attention of many researchers on the last two decades, from 75 results in 1998 to 270 results in 2015 (PubMed). Indeed, beyond melanocyte regulation, MCR are related to other cell-signalling pathways such as the leucocytes activation, the promotion of inflammation resolution and the consequent tissue protection [13]. Moreover, it has been shown that a-MSH or other MCR agonists has immunosuppressive activity in experimental uveitis [25, 31] and also protects retinal endothelial cells from oxidative-induced damage [32]. Fitting with this knowledge, in an initial study, we described for the first time that MCR1,5 agonists help diabetic retinopathy by concretely protecting retinal vascular network [13, 32] in a murine model, through the inhibition of the local inflammatory and immune responses [13]. To these pioneering results have been added now the new data of an antioxidant and defensive response of the retinal cells following activation of MCR1,5.

Particularly, here we show that MCR1,5 agonists promote a protective response on photoreceptors of high-glucose-cultured primary retinal cells by preserving their structure from the abnormal morphology and cytoplasm swelling induced by high glucose. High glucose also promotes an evident MCR1,5 overexpression in these cells, and MCR1,5 agonists normalize this increase. From the biochemical point of view, the activation of the MCR1,5 was accompanied by restoring of the levels of both GPx and MnSOD enzymes, impaired by high-glucose exposure [13]. Noteworthy, impaired antioxidant enzymes by high-glucose results in a high accumulation of hydrogen peroxide (H2O2) and superoxide (O2 •_), and a reduced nitric oxide (NO) bioavailability [33], thus driving retinal cells towards derangement. Photoreceptors are case sensitive to high-glucose conditions [10] playing a pivotal role on diabetic retinopathy [12]. In fact, photoreceptor cell membranes are particularly rich in polyunsaturated fatty acids and extremely vulnerable to oxidative damage being the major site of superoxide generation in diabetes [34]. As MCR1,5 modulate the nuclear transcription of the cAMP response element-binding protein (CREB) [17, 35] and CREB as a redox-regulated

pathway modulating MnSOD transcription [36], a possible theoretical frame, supporting this proposal, is that high-glucose exposure overexpresses MCR1,5 and the addition of MCR1,5 agonists lead to cAMP-PKA-CREB, increasing MnSOD transcription. Beyond this and in view of the present results, we cautiously propose a major role of MCR1,5 on cell response against high glucose or other oxidative insults. In diabetic retinopathy, oxidative stressis increased in the retina and its capillary cells, and icreased oxidative stress is considered one of the major metabolic abnormaalities associated with the development of diabetic retinopathy [3,12]. As mentioned above, inaddition to autoxidation of glucose, the major biochemical abnormalities altered by hyperglycemia also fuel into increased ROS production, and to make the bad situation worse, biochemical abnormalities diated by hyperglycemia milieu themselves are also trigger by free radicals [12].

Conclusion

Although clinically DR can be managed by controlling the metabolic pathway of glucose, we conclude this study by proposing that endogenous melanocortin system stimulation in the eye through local activation of MC1 and MC5 can reduce the damage to the retina caused from diabetes. This could be the beginning of a melanocortin therapy for DR, especially considering that different natural and synthetic melanocortin receptor agonists are under clinical trial [52, 53].

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