UNIVERSITAT ROVIRA i VIRGILI

MOLECULAR BASES OF EYE INFLAMMATION

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TREBALL FINAL DE GRAU DE BIOQUÍMICA I BIOLOGIA MOLECULAR.

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Abstract

Diabetic macular edema (DME) is a disease caused by diabetic retinopathy (DR). It represents the main cause of visual impairment in people suffering from diabetes mellitus, which has a prevalence in the population between 6 and 8%. It occurs due to a series of microvascular changes in diabetes that lead to a vessel incompetence and is associated with blood-retinal-barrier (BRB) dysfunction and the consequent accumulation of extracellular fluid within the central retina. C-reactive protein (CRP) is an acute phase reactant that is increased in patients with diabetic retinopathy. Thus, we sought to investigate whether the different forms of CRP (pentameric and monomeric) could affect to the integrity of the inner BRB consequently contributing to the development of DME.

Key Words: C reactive protein; macular edema; blood-retinal barrier.

Resum

L’edema macular diabètic (EMD) és una malaltia causada per la retinopatia diabètica (DR). Representa la principal causa de discapacitat visual de persones que pateixen diabetis mellitus, la qual té una prevalença en la població entre un 6 i 8%. Sorgeix a causa d’una sèrie de canvis microvasculars en la diabetis que condueixen a una incompetència del vasos i s’associa amb la disfunció de la Barrera hemato-retiniana (BRB) amb la conseqüent acumulació de líquid extracel·lular a l’interior de la retina central. La proteïna C reactiva (CRP) és un reactant de fase aguda que augmenta en pacients amb retinopatia diabètica. Amb aquest motiu, es va buscar investigar si les diferents formes de la CRP (pentamèrica i monòmerica) podrien afectar a la integritat de la BRB , que com a conseqüència contribueix al desenvolupament de EMD.

Paraules clau: proteïna C reactiva; edema macular; barrera hemato-retiniana.

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List of Abbreviations

AJ, Adherent Junction.

BBB, Blood-Brain Barrier.

BRB, Blood-Retinal Barrier.

DME, Diabetic Macular Edema.

CD-31, Cluster of Differentiation 31

CRP, C Reactive Protein

DR, Diabetic Retinopathy.

EC, Endothelial Cell.

GUK, Guanylate Kinase

HRMEC, Human Retinal Microvascular Endothelial Cell.

iBRB, inner Blood-Retinal Barrier.

IL-1β, Interleukin 1β.

IL-8, Interleukin 8.

JAM, Junctional Adhesion Molecules.

MAGUK, Membrane-associated Guanylate Kinase

mCRP, monomeric C Reactive Protein.

RPE, Retinal Pigment Epithelium.

oBRB, outer Blood-Retinal Barrier.

pCRP, pentameric C Reactive Protein.

RPE, Retinal Pigment Epithelium.

TJ, Tight Junction.

VEGF, Vascular Endothelial Grow Factor.

ZO, Zonula Occludens

Introduction

The retina

The retina is an essential neural tissue which is highly differentiated, sensitive to light. It is located in the posterior segment of the eyeball, between the vitreous body and the choroid *(Fig. 1)*. The main function is to convert the light received into a nerve impulse that travels to the brain through the optic nerve and is finally transformed into an image, which we are able to perceive.

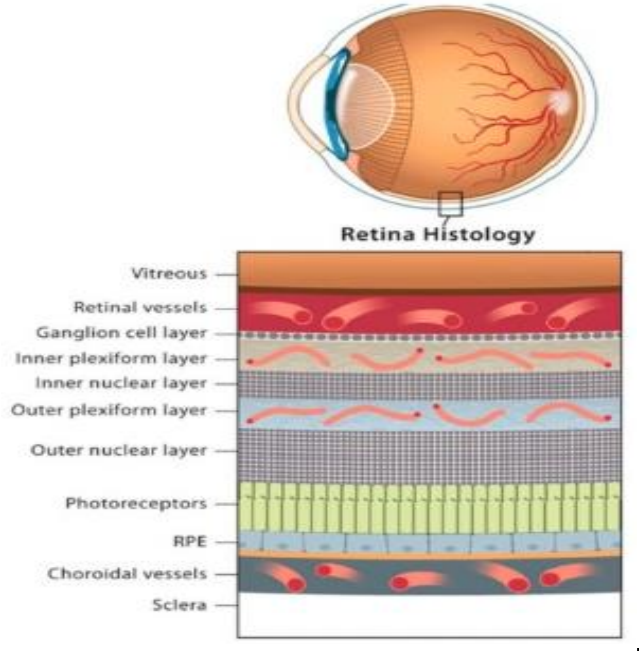


Figure 1. *Anatomical structure of the human eye.*[1]

The retina is irrigated by two vascular systems, one is the central retinal artery and the other are the choriocapillaris. It is formed by different cell types that are organized into eight distinct layers, as follows vascular cells (pericytes and endothelial cells (ECs)) and microglia (Muller cells and astrocytes). Also, for different groups of neurons made up of photoreceptors, bipolar cells, amacrine cells, horizontal cells and ganglion cells) and pigment epithelium.

The retinal structure makes it extremely vulnerable to internal and external insults. In addition, has a poor renewal and repair capacity, alongside a low functional autonomy[2]. It has the Blood-Retinal Barrier (BRB), that prevents potentially toxic molecules from entering the inner retina and mediates the supply of different nutrients due to the hight metabolic rate of the retina.

The Blood Retinal Barrier

The blood–retinal barrier (BRB) is a restrictive physiological barrier that protects the retina from the regulation of water and plasma permeability, i.e. the water flux into and out of the retina, also regulates flow of nutrients and the delivery of amino acids and sugars.

The structure of this barrier is composed of the outer BRB (oBRB) and the inner BRB (iBRB). The main component of the oBRB is the Retinal Pigment Epithelium (RPE), maintained by Tight Junctions (TJs). Where RPE is a single polarized monolayer of cells that controls the transport between the retina and the choriocapillaris of the choroid. The iBRB is composed of ECs surrounded by Müller cells, which nourish the inner two thirds of the retina and regulates transport across retinal capillaries. *(Fig. 2)*

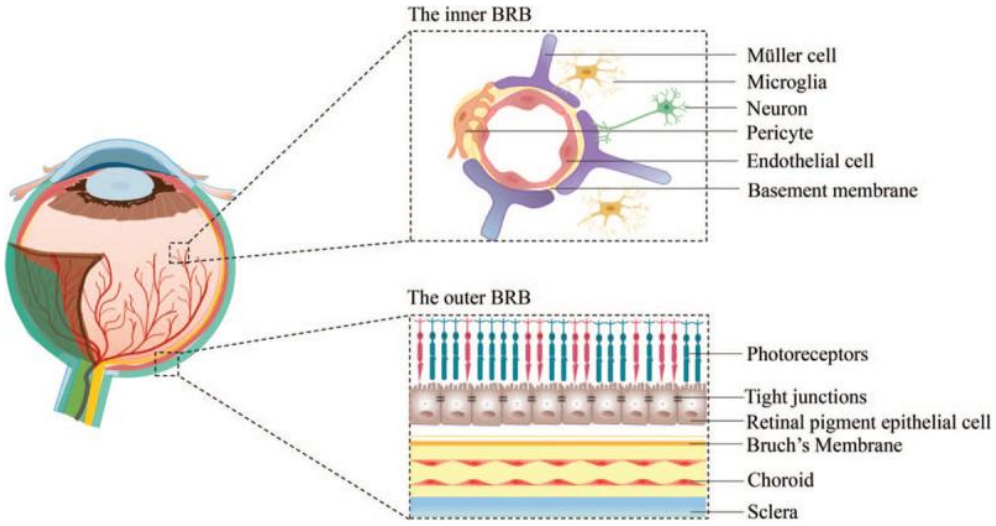


Figure 2. *Structure of the eye with the different barriers.*[3]

Among its many functions, the BRB regulates fluids and molecular movement between the ocular vascular beds and retinal tissues and keeps the microenvironment of the retina and retinal neurons [4]. The BRB also maintains proper neural homeostasis and protects the neural tissue from potential blood borne toxicity by preventing leakage of macromolecules and other potentially harmful agents into the retina. Which would cause a loss of the integrity of BRB. As a consequence, a loss of vision and the development of diseases such as Diabetic Macular Edema (DME) or Diabetic Retinopathy (DR).

The inner blood-retinal barrier:

The iBRB is a selectively permeable regulator of the input of molecules between the circulating blood and the retina and is located in the inner retinal microvasculature.

This barrier is composed of ECs united by TJs and Adherents Junctions (AJs), surrounded by pericytes and glial cells, involved in paracrine interactions but they do not form a continuous layer. In turn are also covered by Müller cells, astrocytes and neurons. In this way they form a monolayer that separates two domains, on the one hand the abluminal domain and the luminal, that is, the retina of the circulation blood.

Among its main functions is to prevent the free diffusion between the circulating blood and the neural retina, from a regulation of the paracellular movement of fluids and molecules through the retinal capillaries. Both of paracellular and transcellular movement are one of the main characteristics of movement between the membrane. Therefore, if a decrease in transcellular movement or transcytosis is observed it will mean a decrease also of receptors, transporters and mediators within the endothelial cells, which play a fundamental role in the supply of nutrients and are responsible for the efflux of neurotransmitters and their metabolites.[3]

In relation to the paracellular movement, this has been restricted between adjacent vascular ECs due to binding complexes, exactly TJs, which is composed of more than 40 proteins. Therefore, an increased permeability in both transporters is indicative of a dysfunction in iBRB, which in turn would lead to an abnormal and distribution of these TJs.

Other functions include providing nutrients to the retina and removing endogenous organisms and foreign objects from the retina, such as inflammatory lymphocytes, blood-borne pathogens, excessive enzymes, and other toxic compounds.

The iBRB shares several features with the blood-brain-barrier (BBB). These similarities are that these are constituted by the same narrow union of ECs, that is, TJs, AJs... And therefore, also for the same cytoplasmic proteins, such as

Claudins, Occludins, ZO, among others. Another similarity is that they share the same transport mechanisms between barriers, since they have the same characteristic of having a selective permeability. Such transports are both those that are mediated by transporters and those that are mediated by receivers, caveolae's...

Molecular constituents of iBRB:

iBRB is composed of narrow junctions, TJs and AJs, between retinal ECs. These endothelial cells are surrounded by pericytes, which are closely associated with the adjacent basement membrane surrounded by astrocytes, and Müller cells as a structural scaffolding for iBRB. The retinal endothelial layer functions as an 'epithelium' and in this way is directly associated with its differentiation and with the polarisation of BRB function. (Fig.3)

ECs provide enough oxygen and glucose for neuronal function while restricting the flux of other molecules and cells in order to protect the neuronal environment. When bound by TJs, they act as tangential points between the plasma membranes of adjacent cells or at the point of contact between a cell and its respective microvessel. In addition, TJs have the function of acting both as a selective physical barrier and as active complexes. To be able to restrict paracellular diffusion of solutes from active transport through the BBB / iBRB and the prevention of the entering and damaging of harmful materials (toxins, infections, endobiotics...).

TJs are made up of more than 40 transmembrane proteins, such as junctional adhesion molecules (JAMs), Claudins, Occludins... These proteins are bound to other cytoskeleton and cytoplasmic scaffold proteins expressed in ECs, such as ZO-1, ZO-2 and ZO-3.

The glial cells play an important role in maintaining the normal structure, metabolism, and function of the retina. Where Müller cells are distributed radially across all retinal layers, and astrocytes are found only on the vitreal side of the retina, within the nerve fiber and ganglion cell layers. They are considered to influence the activity of retinal endothelial cells and of the iBRB by transmitting regulatory signals to endothelial cells indicating changes in the microenvironment of the retinal neuronal circuitry.[5],[6]

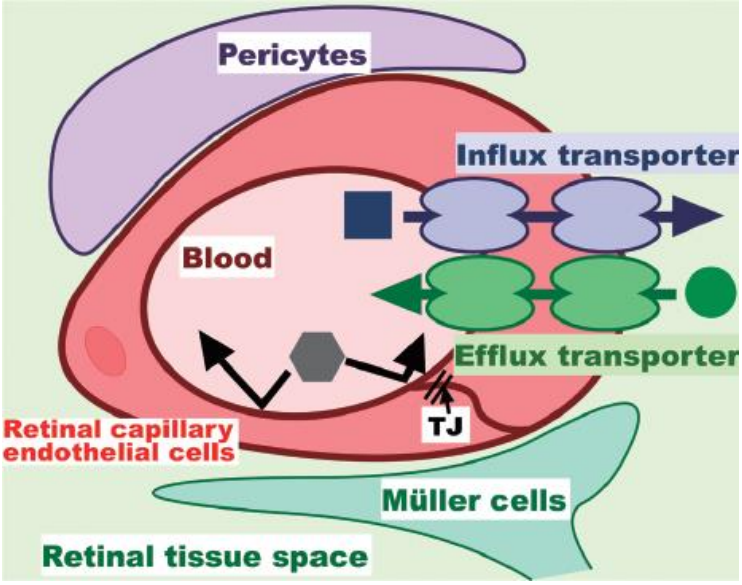


Figure 3. *iBRB structure.*[7]

Zonula Occludens (ZO)

ZO is a scaffolding protein, which plays a crucial role in the assembly of functional TJs and AJs and anchors macromolecular binding complexes to cytoplasmic actin. Which is involved in intercellular unions that is expressed in all ECs and also is a component of both BRB and BBB. Its expression is related to the degree of confluence and increases with barrier tightness, for example, when the permeability of iBRB increases, it causes a decrease in the content of the ZO-1 protein.

There are three different types, first is ZO-1, which belongs to the membrane-associated guanylate kinase (MAGUK) family of proteins and incorporates three PDZ domains: an SH3 domain, a GUK domain and a proline-rich domain at the C-terminal end. ZO-1 disruption in a functional epithelial monolayer result in a loss of barrier function and a reorganization of apical actin and myosin.

Decreased expression of ZO-1 is associated with severe plasma leakage observed in multiple sclerosis and diabetes.

Claudin (Cldn)

Claudins are integral membrane proteins with four transmembrane domains and two extracellular and one intracellular domain. They have cytoplasmic,

intracellular NH2– and COOH– ends and two extracellular loops. Their carboxy-terminal end contains a PDZ-binding motif, which is required for pairing with ZO (ZO-1, ZO-2, ZO-3…), a TJ scaffolding protein. This C-terminal tail is the region that confers stability to claudins, and it has been found that the exchange of the C-terminal tails coincides with a reversal of the half-life of the proteins.[8]

Claudins are distinguished from other tight-binding proteins, such as Occludin, in that their extracellular domains have a certain degree of homology and mediate size selectivity and ion flux across the various barriers. Occludin appears to have a more regulatory role in tight junction.[8]

Claudin-5 is abundantly expressed at the iBRB, expressed over 500-fold compared to the next highly expressed claudin subtype[9]. It is ubiquitously expressed in all vascular beds whereas Claudin 1, 3, and 12 are specific to the brain microvasculature. In addition, they have shown lower but inducible mRNA expression levels.

Deletion of barrier-forming claudins (e.g., claudin-1 or claudin-5) dramatically increases paracellular permeability. In contrast, an abundance of TJ plaque was found after overexpression of these claudins. And most of they share a common motif, -YV in the c-terminal region. There are 27 claudins discovered to date that can be classified as closed, selectively permeable based on their functions

Among some of its functions is that of mediating in the selective transport of ions paracellularly, from the formation of pores and barriers. As well as determine the maximum size of molecules that diffuse through a layer of ECs.

Breakdown of the iBRB:

iBRB dysfunction can be caused by a variety of factors, including loss of barrier bonds between cells, vascular endothelial growth factor, damage induced by end products of advanced glycation, and oxidative stress. This vascular dysfunction, in turn, generates a greater permeability of the barrier and a degeneration of the vessels of the retinal capillaries.

The iBRB provides the first layer of protection to the retina, if the barrier is broken, it will be protected by the cells of the retina. These cells of the retina provide protection by suppressing the response of local inflammation. This will prevent

the onset of diseases, such as DR. In addition, there is a third layer of protection formed by the systemic immune system by creating an antigen-specific tolerance or suppressive immune response upon detection of antigenic signals.

When the three layers are not sufficient and there is trauma or direct injury, external infections, neuronal or glial injury, the iBRB is finally altered. This disruption is caused by exogenous antigens and leukocytes that have entered the retina and activated the innate immune response, leading to the initiation of other adaptive immune responses and causing excessive inflammatory injury to the retina.

Breakdown of iBRB is a serious lesion in diabetic patients, since it maintains vascular and tissue homeostasis and modulates many physiological processes. If it left untreated can lead to DME, which will probably lead to severe visual impairment in the absence of rapid intervention. Therefore, DR and DME are the consequences of the advance of these diseases due to an alteration that affects the integrity of the iBRB.[10]

Diabetic Retinopathy:

Diabetic retinopathy is a debilitating microvascular complication of diabetes mellitus. And is one of the most common microvascular complications of diabetes mellitus, which often induces ophthalmologic complications. Its incidence increases with the duration of the disease, so that after 20 years of diabetic evolution, all type 1 diabetics and 60% of type 2 diabetics present some degree of retinopathy.

In inflammatory retinal disorders like DR, retinal circulation is impaired and eventually stops. Where the vascular endothelium responds to blood flow and changes function to maintain tissue homeostasis and adapt to injury under can contribute to the development of DME, pathologic neovascularization, and bleeding, contributing to severe vision loss.

Clinically DR begins with a decrease in night vision, but after being exposed to light, it can be recovered a little. There are also alterations in the colours. There are two types of DR, the first is proliferative, which manifests early. In contrast, no proliferative causes vascular dilation of the retina, in addition to capillary

occlusion, increased permeability of the blood vessels of the retina, retinal exudation, bleeding, among other characteristics.

Therefore, DR, by causing a blockage of the blood vessels in the retina, leads to a decrease in blood supply. Which produces ischemia and with less irrigation, the new vessels that will form will be weaker and will tend to break more easily. This breakdown will cause haemorrhages and finally the development of DME.

The edema produced by a loss in the capacity of renewal and vascular recovery of the retina, will cause a decrease in its functional autonomy and therefore, will not be able to function alone. EMD is the leading cause of blindness in diabetic patients and can develop at any stage of RD.

Growth factors, such as VEGF, and cytokines, such as IL-1β and IL-8 are related to the degradation of the iBRB and are elevated in DR, promoted by angiogenesis. VEGF modulates loss of tight junction integrity or enhanced transport mechanisms in endothelial cells in the early stages of DR. Therefore, it induces phosphorylation of cell-cell binding molecules such as VE-cadherin, Occludin and ZO-1 and finally causes iBRB breakdown. A treatment against the liberation of VEGF or other factors like proinflammatories cytokines, IL-8 or IL-1β, would be those one which cause an inhibition. As is the case of anti-VEFG, small interfering RNA, minocycline... [10]

IL-1β and IL-8 are pro-inflammatory cytokines, whose maturation and secretion into the extracellular environment and is associated with the progression of retinal pathologies [11]. Therefore, they are present when there is an inflammation in ECs in order to promote the development of certain immune cells[12].

Treatment of diabetic rodents with a range of agents that either modulate protein kinase C activation, prevent formation of reactive oxygen species (ROS), or regulate aldose reductase activity can prevent diabetes-mediated rises in VEGF expression and prevent iBRB dysfunction. [13]

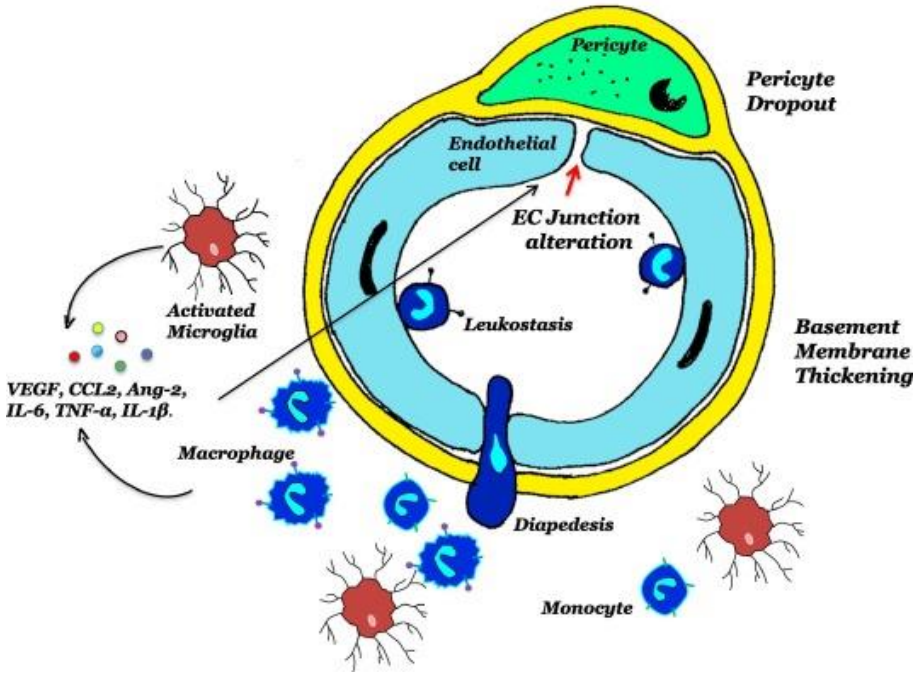


Figure 4. *BRB's alteration.*[14]

C Reactive Protein (CRP)

CRP is a nonspecific acute phase reactant that is a member of the pentraxin proteins (*Fig. 5*), which are pattern recognition proteins that are an integral part of the innate immune system and is responsible of the host-defense [15]. Is to say that consists of five identical and noncovalently 23kDa protomers arranged symmetrically around a central pore and circulates in blood as a pentamer (pCRP). pCRP can dissociate under particular pathogenic conditions in absence of $Ca^{2+,}$ low pH and high urea concentrations, into a pro-inflammatory monomer (mCRP) upon binding to certain surfaces or specific ligands.

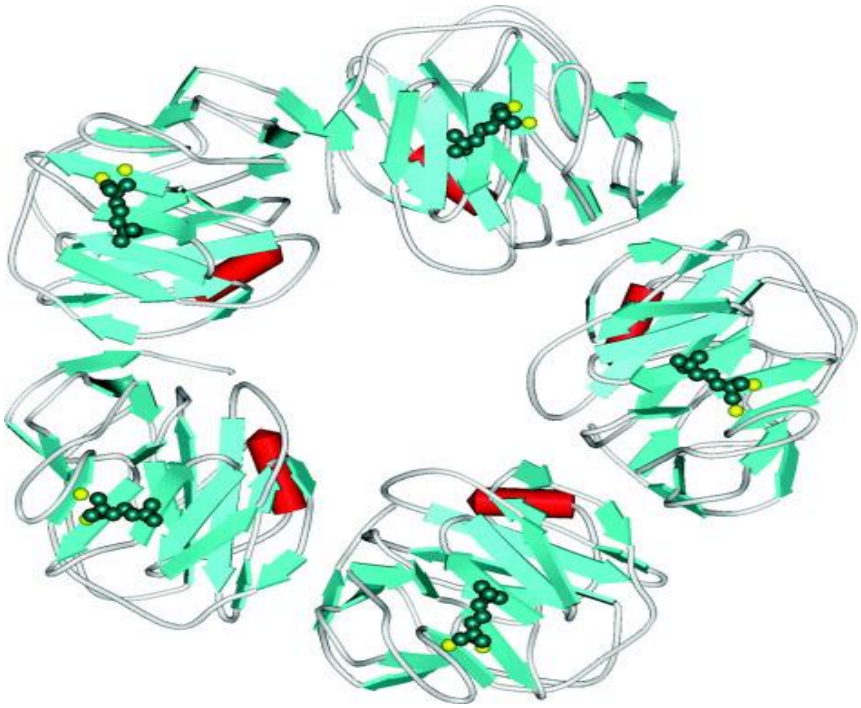


Figure 5. *C Reactive Protein (CRP) Structure.*[16]

CRP is produced and synthesized in the liver in response to inflammatory cytokines and assists in complement binding and phagocytosis by macrophages. Where mCRP shows a proinflammatory phenotype in several cell types and it has been shown that the mCRP, but not the pCRP, contributes to the alteration of the oBRB in vitro. Although the origin of this protein in retinal tissue is not known. [14,15,16]

pCRP rapidly dissociates into a monomeric form (mCRP) with a solubility, antigenicity, tissue localization, binding ligands, and functions distinct from those of pCRP. It is one of many acute phase reactants that are elaborated in response to inflammation and/or tissue injury, and its upregulation is in line with inflammatory mediators (cytokines) produced by cells actively involved in the tissue injury environment, such as IL-6, IL-1β, TGFβ and TNF-α. Which induce diseases such as DR, which can induce DME.

Thus, CRP levels tend to be proportional to the intensity of the inflammatory process and, therefore, the levels of this marker are sensitive to subtle changes in the acute phase response. And once inflammation decreases, CRP will consequently begin to decrease.

Under conditions of acute inflammation, pCRP may increase to 50 to 100 mg/L within 4 to 6 hours following a mild or moderately noxious stimulus. pCRP levels double every 8 hours and peak 36 to 50 hours after the onset of inflammation or injury. Mild increases in pCRP between 2 mg/L and 10 mg/L are considered metabolic inflammation. In contrast, markedly elevated pCRP levels (>100-500 mg/L) are strongly associated with bacterial infections.

Therefore, the increased presence of the monomeric form of an acute inflammatory protein, CRP, is significance of an infection. Apart from a large amount of mCRP, there will also be other increased factors, such as proinflammatory cytokines, IL-8 or IL-1β. Low-grade persistent inflammation and oxidative stress, where mCRP is involved, lead to the interruption of oBRB. [17]

Hypothesis and Objectives

Hypothesis

The initial hypothesis of the present work was that pCRP and mCRP may affect the integrity and functionality of the iBRB.

Objectives

The main objective of the present work was to evaluate the effect of CRP isoforms on barrier function and integrity

For this purpose, the following sub-objectives were addressed:

- To optimize culture conditions (growth media and substrate coating) of Human Retinal Microvascular Endothelial Cells (HRMECs) to study its barrier properties
- To study the effect of CRP isoforms on the barrier properties of HRMEC by determining the effect on:
    - Paracellular permeability
    - TJ distribution
    - Proinflammatory cytokine production

Materials and methods

Cell culture

The cells used in the experiments were Human Retinal Microvascular Endothelial Cells (HRMEC), which were cultivated in different media and coatings to see where they grew better. Where they formed the best barrier and created better joints, such as TJ, like Zonula Occludens 1 (ZO-1). Cluster of differentiation 31 (CD-31), an EC marker was also observed as it is the endothelial cell adhesion molecule, that is, an indicator that we are dealing with endothelial cells such as HRMEC, among other.

Cells were cultured in 12 well plates with polycarbonate membrane Transwell™ filters (refereed as Inserts from now on) cryopreserved of HRMEC passage 6. They were on different mediums and coatings, as seen below in the *Table 1.* The medium was supplemented by 1% of penicillin/streptomycin (P/S) and they were placed in an incubator at 37ºC with 5% of CO_2.

***Table 1**. Medium and Coatings conditions in HRMEC.*

EGM2 Control	EGM2 at 10% FBS Control	MV2 Control	MV2 at 10% FBS Control
EGM2 Gelatin 0,2 %	EGM2 at 10% FBS Gelatin 0,2 %	MV2 Gelatin 0,2%	MV2 at 10% FBS Gelatin 0,2%
EGM2 50 µg/ml Fibronectin	EGM2 at 10% FBS 50 µg/ml Fibronectin	MV2 50 µg/ml Fibronectin	MV2 at 10% FBS 50 µg/ml Fibronectin

Cells were passed from one flask of T25 to another of T75 by trypsinization before reaching 90-100% confluence. We didn't let them reach 100 percent confluence because otherwise they could start dying for lack of space and nutrients. The cells used in this assay ranged between passage 6 and 9 depending on the experiment.

Different type of culture was done. First, the effect of CRP, both mCRP and pCRP, on iBRB was observed. Secondly, various cytokines, such as IL-8 and IL-1B, were cultivated in addition to VEGF.

Passage and maintenance

To passage HRMECs, when they are confluent, from T25 to T75 or from T75 to the inserts, medium from the culture surface was removed and cells were rinsed with PBS. Then PBS was removed and 0,05% (w/v) trypsin (Trp) to detach the cells from the plate and it was incubated for 1 minute. The volume needed of trypsin varies depending on the culture surface. 2ml for T25 cm2 flasks and 5ml T25 cm2 flasks. After 1 min, the enzymatic digestion was stopped by adding a treble volume neutralization medium (any cell culture medium containing FBS which have trypsin inhibitors).

Following, cells were seeded in the Transwells™ containing MV-2 medium at 10% FBS and placed in the incubator for 30 minutes before placing the insert with medium in both sides of the Transwells™.

The amount of cells that the insert contains depends on how many cells have been obtained in the previous culture, counted in the Neubauer Chamber. And when an insert was seeded, 100 µl were added to the volume of the apical compartment of the insert (0,5 ml). The culture was placed in the incubator.

Transwell™ filters

To cultivate HRMEC, it was used Transwell™ filters, there are for polycarbonate membrane cell culture and the growth area is of 1.12cm^2 and 0.4 µm pore size, as we can see in the *Figure 1*.

This type of inserts has a high pore density that allows a greater exchange through the membrane for transport studies and offers versatility by allowing the surface of cells to be exposed simultaneously to the growth medium and to the variable condition of the experiments, in this case, the treatments with different CRPs at different concentrations. Also have better cell fixation properties and are adequate for barrier assays, as it provides independent access to the basolateral and apical compartments, with the aim of been studied on HRMEC.

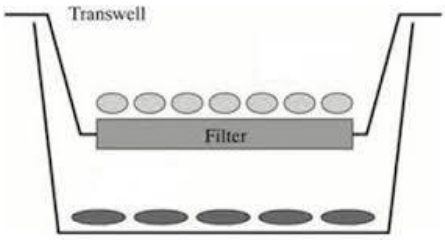


***Figure 6**. Schematic drawing of a Transwell™ filter.*

Cell Counting

The cell count was carried out to know approximately how many cells were in the 75T to calculate how many were seeded in each Transwell™.

Therefore, the Neubauer chamber was used, it consists of a thick crystal slide, (30 x 70 mm and 4 mm thickness) and it has a central area, where the cells count is performed. As well, the chamber has three parts, on one hand, is the central part, where the counting grid has been set on the glass and on the other hand, it has a double chamber, where are two counting areas that can be loaded independently.

The sample loaded to the chamber, that were 10 µl for each area to obtain an accurate cell counting, this volume was prepared by diluting 10 µl of 1/10 cell suspension with 20 µl of Trypan Blue and 70 µl of MV2 at 10% FBS. Trypan blue is used as a vital stain to selectively colour dead tissues or cells blue.

Once the dilution is prepared, introduced 10 µl in one of the chambers, ensuring that the entire chamber is covered by the dilution without bubbles. Eventually, place the Neubauer chamber on the microscope and observed. It started counting the 4-grid square of each chamber and when the 8-grid square were counted, it was determined the cell number by counting these 8 areas through *Equation 1.*

$$Total\ number\ of\ cells = \frac{X_1 + X_2 + \cdots + X_8}{8}\ x \frac{1}{10^{-4}}\ x\ Total\ volume\ of\ cells$$

***Equation 1.** Total number of cells.*

After, the tube was centrifugated at 270 RCF for 5 minutes, with the objective that the cells precipitate and thus obtain a cell pellet. The medium where the cells were found was discarded to just keep the pellet and thus resuspended the pellet in the desired volume.

Cryopreservation

For cryopreservation, cells were trypsinized to detach the cells from the flask culture. Which were passed into a tube containing MV2 at 10% FBS. Later, 1ml was relieved in a cryovial and immediately placed in the freezer (-80ºC) in Thermo Scientific™ Mr. Frosty Freezing Container overnight. Finally, cryovials were transferred to a liquid nitrogen container.

FITC-dextran paracellular permeability

The objective of doing this assay is that it will express the paracellular permeability, from the µg of FITC- DEXTRAN 40 KDa that transfer to the basolateral compartment from the apical one, in addition to giving information to the pore size of TJ, that is, inform about the barrier integrity of the cultures. The results were expressed in ratio, that is, dividing the OD of the basal compartment of each time by the OD of the apical compartment at 180 minutes and multiplying by 100. Finally expressing it as% of FITC-Dextran diffusion rate.

From Pierce™ FITC Antibody Labeling Kit, it started by adding 10 µl of FITC-dextran in the apical compartment of every Transwell™ to finally obtain a concentration of 500 µg/ml. Mixed and incubated the plate at 37ºC. Consequently 250 µl of supernatant was collected from the basolateral compartment at time 20 and 80. In each collection, 250 µl of fresh medium was added to always have 1,500 µl in the basolateral compartment and 500 µl in the apical compartment. When it reached 180 minutes, 250 µl was collected from the apical area and 250 µl from the basal area.

The standard curve was prepared and in a 96 well plate, 10 µl / well of each sample was pipetted and to analyse the samples, the plate was read in a fluorescence plate reader at 490 nm excitation and 520 nm emission with a normal gain.

The % of diffusion with which the graphs of tests of coatings and means have been made, % FITC, was generated from the fraction between the different times, 20, 80, 120 of the basolateral compartments between 120 minutes of the apical compartment.

Transendothelial Electrical Resistance Measurements (TEER)

Trans endothelial electrical resistance (TEER) is a quantitative technique with the aim of measuring the integrity and permeability of TJs dynamics in cell culture of HRMEC and of the cell monolayer. The values of TEER obtained are an indicator of the ohmic resistance, notwithstanding these values may be modified according to the temperature, the passage number of the cells…

TEER consists of a cellular monolayer of HRMEC cultured on a Transwell™, in other words, a semipermeable filter inserts which have a separation of compartments, one basolateral (lower) and the other apical (upper). For the measurement is used two electrodes, one of them is placed in the apical and the other in the basal and both electrodes are separated by the cellular monolayer. Finally, it is obtained the ohmic resistance, which the ratio is calculated from dividing this resistance by the Transwell™ area, which measures 1.12 cm^2.

In the Figures observed in Results and Discussion of the media and coatings tests, given that there were negative TEER values, to normalize correctly, +30 was added to each value and then normalized with respect to the EGM2 Control.

C-Reactive Protein (CRP) treatments

All the processes that have been carried out were based on the pentameric form of the pCRP, which is introduced into a membrane. In such a way that when the dialysis is carried out, only the sodium azide (NaN_3) comes out and the rest of the protein remains in the inside. It is dialyzed in a buffer with which the protein itself is made, thus ensuring that nothing else enters or leaves the membrane. The volume of the pCRP dialization buffer is five hundred times more than the volume of protein, which is composed about 20mM Trizma, 280mM NaCl and 5mM $CaCl_2$ on pH 8.

Therefore, once the protein has been introduced into the membrane and into the buffer, it is maintained in agitation with two changes of buffers, for example, one of 3 hours and another ON at RT.

Endotoxin Elimination

Endotoxin Removal Kit (Rapid)(ab239707) can quickly and effectively eliminate endotoxins to < 0.05 EU/ml in solutions containing proteins, in this case of the CRP.

Firstly, regenerate Rapid Endotoxin Removal Spin Column and equilibrate Rapid Endotoxin Removal Spin Column in Endotoxin Removal Equilibration Buffer. Firstly, snapped off the bottom plug, removed the cap and placed the column in an Endotoxin-free Collection Tube. Spined and discarded the solution. Later, it was added 0.5 ml Endotoxin Removal Equilibration Buffer and again quick Spin to removed and discarded the solution. Next, regenerated the column by adding 0.5 ml Rapid Endotoxin Removal Regeneration Buffer and again quick Spin to removed and discarded the solution. Repeated the last step and added 0.5 ml Rapid Removal Wash Buffer Quick Spin to removed and discarded the solution and repeated twice the last step. It was equilibrated agarose by adding 0.5 ml Endotoxin Removal Equilibration Buffer Quick Spin to removed and discarded the solution and repeat the last step. After, placed the column in a new Endotoxin-free Collection Tube.

Applied sample of pCRP (0.1 - 0.5 ml) to the column. Let the sample pass through the column by gravity and repeat load flow-through several times (3-5 times). Quick Spin to collect the sample and determined the endotoxin concentration of the processed sample. If the final endotoxin concentration is above the desired endotoxin concentration. Placed plug and cap back and store the column in 200 µl Endotoxin Removal Equilibration buffer with 20% ethanol at 4ºC.

Finally, the pCRP is stored in one of the endotoxin-free vials.

Monomerization of pCRP

Afterwards, half of pentamer is monomerized by mixing the same amount of pCRP as the mixture of 16M Urea and 20mM EDTA that has been done previously with free-endotoxin water. And it is incubated for 3 hours at 37 ° C to go from pCRP to mCRP, in such a way that the concentration of the monomer decreases by half with respect to that of pCRP.

Once the monomer is obtained, it is re-dialyzed in the same way as with the pentamer but with a different buffer, in this case with TBS, which is composed by

10mM Trizma and 50mM NaCl and with 3 changes of buffer, i.e., starting with 2 hours, then ON and finally 3 hours. After, the mCRP is stored in one of the endotoxin-free vials.

Endotoxin Quantification

The Thermo Scientific™ Pierce™ LAL Chromogenic Endotoxin Quantification Kit is a quantitative endpoint assay for the detection of gram-negative bacterial endotoxins, in this case of the CRP. The developed colour intensity is proportional to the amount of endotoxin present in the sample and can be calculated using a standard curve. Therefore, in this kit three different substrates were used, the standard stock solution, LAL and chromogenic substrate. The aim of this assay is to obtain an amount equal to or less than 0,125 EU/ml, both pCRP and mCRP.

On the one hand, the Endotoxin Standard Stock Solution begins to be prepared, which according to the Endotoxin Units (EU) that comes in the vial. As in our case there were 22 EU / ml (X), applying [(X - 1) / 20] ml the amount of endotoxin-free water that must be added to the freeze-dried amount of Stock Solution is obtained. Once prepared, the solution must be vortex for 15 minutes in a high-speed vortex mixer before use.

Afterwards dilutions are made according to the table below.

Table 2. *Dilutions of Endotoxin Standard Stock Solution.*

Vial	Volume of Endotoxin Standard Stock (ml)	Volume of Vial A (ml)	Endotoxin-free Water (ml)	Final endotoxin concentration (EU/ml)
A	0,05	-	1,05 (X-1)/20	1
B	-	0,25	0,25	0,50
C	-	0,25	0,75	0,25
D	-	0,10	0,90	0,10

On the other hand, the Chromogenic Substrate is prepared, like each vial contains approximately 7 mg of lyophilized substrate, added 6,5 ml of endotoxin-free water to yield a final concentration of 2mM approx. Save at 37ºC before use.

Once the necessary substrates have been prepared, finally the Limulus Amebocyte Lysate (LAL) is prepared, since once prepared it must be used immediately. The LAL reagent contains lyophilized lysate prepared from the circulating amebocytes of the crab *Limulus polyphemus*. It's prepared with 1,4 ml of endotoxin-free water, swirled and protected from the light.

To start with the 96 wells plate previously reheated to 37ºC, it was dispensed 50 µl of each standard, blank, pCRP or mCRP, depending. Then, when it was added 50 µl of LAL reagent to each well, beginning timing as the LAL is added and mixed. Then the plated was covered. After 10 minutes, exactly, it was added 100 µl of Chromogenic Substrate solution to each well. Mixed and covered he plate. Next, after exactly 6 minutes, it was added 100 µl of Stop Reagent, it contains 25% acetic acid and measured the absorbance at 405-410 nm on a plate reader and obtained the standard curve with linear regression to determine the endotoxin concentration of each sample, i.e., pCRP and mCRP.

BCA protein quantification assay

Bicinchoninic acid (BCA) was used to create an intense purple complex with ions Cu^{1+} in alkaline medium. From BCA Protein Assay Kit (K001), where the reagent forms the basis of an analytical method capable of monitoring the cuprous ion produced in a reaction between proteins with Cu^{2+} in an alkaline medium. So as to our objective was to quantify how much pCRP or mCRP protein was left.

Firstly, prepare the standards with pure distilled water and followed the volumes of the table for two standards, that is, for pCRP and mCRP, following the *Table 3.*

Table 3*. Dilutions to make the standard BSA pattern.*

Vial	Diluent Volume (µl)	BSA Volume (µl)	Final Concentration BSA (µg/ml)
A	0	25 of **Stock**	2.000

B	9,5	28,5 of **Stock**	1.500
C	24	24 of **Stock**	1.000
D	12,5	12,5 of **B**	750
E	22,5	22,5 of **C**	500
F	20	20 of **E**	250
G	15	15 of **F**	125
H	20	5 of **G**	25
I (Blank)	25	0	0

As the standard line is useful for both CRP, for this reason we look at both the concentration of pCRP which is approximately a volume of 1,000 µl and for the monomer that is 500 µl or 250 µl. Once the vials with which we will make our standard line are prepared, in a 96-well plate, we put 10 µl of each sample per well with their respective duplicates. Next, 200 µl of Working Reagent is pipetted per well, which has an A: B dilution of 50:1, and the plate is left at 37°C for 30 minutes, protected from light. Eventually, it is read at 562nm using a four-parameter curve.

As a conclusion to this test, it was observed that the amount of mCRP protein was slightly diluted in the process, whereas pCRP did not.

Electrophoresis

The aim of the electrophoresis is separate the fragments of the pCRP and the mCRP according to the molecular weight (MW), in which the pentameric form has to be approximately on the 115 KDa and the monomeric form on the 23 KDa.

Firstly, the samples, pCRP and mCRP, were prepared with Loading buffer, it was composed of dH_2O, 0,5M Tris-HCl at pH 6,8, Glycerol, 10% SDS and 0,5% Bromophenol blue. It was calculated a quantity of 2 µg of protein to be used and charged in the gel 0,10 µl of the mixture that was done before.

The gel was prepared starting from the mixture of two gels, the first is Separating, which is composed of 1,5M Tris-HCl pH 8,8, Acrylamide, dH_2O, 10% SDS 1/20, 15% PA and TEMED. The aim of the separating gel is to separate the proteins based on their molecular weight (MW), instead the aim of the Stacking gel is to

line up all the protein samples loaded on the gel, so that they can enter the separating gel at the same time. Hence the composition of stacking is 0,5M Tris-HCl at pH 6,8, Acrylamide, dH_2O, 10% SDS 1/20, 15% PA and TEMED.

Once the gel is made, it is introduced into the electrophoresis tray, which was filled with Running buffer, made up of Trizma base, Glycine and 1/20 SDS. The aim of this running buffer is to contain ions that conduct current through the gel. Once the time has passed and the protein has run correctly a 30mCA constant and approximately 50 minutes, the gel is carefully extracted, and it is stained with Coomassie Blue to be able to observe the results easily.

Immunostaining procedure – Immunofluorescence

The immunostaining procedure was used to determine two different proteins of TJs. On one hand, in the 12 different conditions was determined the protein ZO-1, indicator of the presence of TJs, and on the other, in two wells was also determined the protein CD-31, indicator of ECs.

Firstly, the cell plate with the 12 conditions was washed twice with PBS until it was covered for 5 minutes. Next, they were fixed with 0.5 µl of 3.8% Paraformaldehyde (PFA) at room temperature for 20 minutes. Secondly, cells were washed again with PBS for three times, if the If immunofluorescence is not done immediately, cells are left in NaN_3 0.02% until realize.

Therefore, if it is done immediately, they were permeabilized with 400 µl 0.2% Triton for 15 minutes at room temperature (RT). In the case of the wells where both ZO-1 and CD-31 are determined, it is permeabilized with the Triton with the highest concentration, in our case it was the same concentration in both. Re-wash twice with PBS for 5 minutes. Later, block nonspecific binding sites with 500 µl BSA at 1% PBS at RT for 15 minutes, twice.

After washing with PBS twice, incubate them with the primary antibody. In the case of only wanting to determine the ZO-1, use Mouse anti-ZO1 (1: 100) and in the case of determining CD-31, Rabbit anti-CD31 (1:50) is used. Before blocking, while washing occurs, the diluted antibody is centrifuged to 10.000 rcf for 10 minutes. Afterward, put a piece of parafilm on the plate, where the primary antibody will go and deposit our cells on top and stored ON at 4ºC.

The next day, wash cells with PBS twice and incubate with 50 µl of a mixture of the secondary antibody, DAPI and 1% BSA-PBS. This mixture contains the secondary antibody of ZO-1, Mouse AF488 (1: 200) and where determine CD-31, Rabbit AF568 (1:100). It also contains DAPI, which is diluted 1:100 in 1% BSA-PBS and then has a 1:50 dilution with the secondary Ab, in total, diluted 1:5000. The secondary antibody was centrifuged in the same way as the primary, and were incubated with the mixture during 1 hour at RT in the dark.

Finally, they were washed three times with PBS and mount the sample and observe them under the high-speed spectral confocal microscope Leica TCS-SP5 was carried out using the appropriate filters.

ZO-1 and Cldn-5 intensity quantification

The signal intensity of the previously marked proteins, both ZO-1 and Cldn-5, was quantified from Image J. The program opened the desired image from the high-speed spectral confocal microscope Leica TCS-SP5. The channels were split and select the desired channel.

Once the ZO-1 or Cldn-5 channel is selected, the Threshold is adjusted and the following images are recorded with the same Threshold. Once adjusted, 5 lines of approximately the same size of the TJs are made and one of the cytoplasm. The higher the cytoplasm intensity, the lower the ratio, as there will be more background, and therefore worse.

When the data collected by the Region of Interest (ROI) manager is available, it is exported and an Excel is created with the results.

Enzyme-Linked Immunosorbent Assays (ELISA)

In all HRMECs, before immunofluorescence, supernatants were collected, that is, the 500 µl from the apical compartment and the 1500 µl from the basolateral compartment, which were centrifuged at 1200 RPM for 5 minutes and collected by freezing them at -80ºC. ELISA was performed in order to observe the effect of different factors in HRMEC.

VEGF is a vascular endothelial growth factor, which has antiapoptotic effects on endothelial cells, increasing their vascular permeability and promoting cell migration. In addition to participating in angiogenesis that induces diabetic retinopathy, due to its effects on vascular permeability and neovascularization.

We started growing 100 µl/well on 96-well plates (Corning™ Costar™ 9018) from Mouse anti-human VEGF Capture Antibody (1:1000 in PBS) at RT. The next morning, the liquid was discarded and washed three times with Washing Buffer, i.e., with PBS-Tween 0.05% at a pH between 7.2 and 7.4. Subsequently, non-specific binding sites were blocked with 300 µl/well of 1% BSA-PBS during 1h to RT. After time, the dilutions were prepared to obtain the straight pattern.

Table 4. *Preparation straight pattern of VEGF.*

Point nº		1% BSA-PBS (µl)	VEGF standard line (pg/ml)
1	500 µl of VEGF 2000 pg/ml.	0	2000
2	250 µl of 1	250	1000
3	250 µl of 2	250	500
4	250 µl of 3	250	250
5	250 µl of 4	250	125
6	250 µl of 5	250	62,5
7	250 µl of 6	250	31,3

8	0	250	0

Once the dilutions of the straight pattern have been made, the liquid is discarded from the plate and washed again, three times, with the Wash Buffer. And 100 µl/well of each standard and sample dilution was added in duplicate. Pass 2h RT and discard the liquid from the plate and wash with Wash buffer 3 times. Then 100 µl/well of Biotinylated Goat anti-Human VEGF Detection Antibody was added (1:2000 in 1% BSA-PBS). When she had already been 2 hours incubating at RT, she discarded the liquid and washed 3 times with Wash Buffer.

Then it was incubated for 20 min at RT with 100 µl/well of avidin-HRP, where over time the liquid was dissolved and washed 3 times. To incubate for 20 min at RT with 100 µl/well of TMB 1x and finally added 50 µl/well Stop Solution (1M H_2PO_4). And the plate was read at 540/570 nm and 450nm, with the OD value expressed as OD450-OD540/570 nm. And indicating that it is a pattern curve 4 parameters.

IL-8 and IL-1β are proinflammatory chemokines, therefore, a presence of these in the iBRB means that is surely inflamed because of the mCRP. Where the CRP High Sensitive ELISA kit was used, ELISA procedure is the same as in VEGF, except that in the case of IL-1. Where the sample was diluted 1/15 and the dilution of the capture antibody is 1:250 in Coating Buffer. And blocking with 200 µl/well of Diluent (1X).

Table 5. *Preparation straight pattern of IL-1β.*

Point nº		Recombinant human IL-1 beta (pg/ml)	ELISA/ELISASPORT Diluent (1X) (µl)
1	500 µl of recombinant human IL-1 beta,	150	0
2	250 µl of 1	75	250
3	250 µl of 2	35	250
4	250 µl of 3	17,5	250

5	250 µl of 4	8,75	250
6	250 µl of 5	4,375	250
7	250 µl of 6	2,1875	250
8	0	0	250

The detection antibody in this case was pre-titrated, biotin-conjugated anti-human IL-1 beta. And the diluted avidin also 1:250 in diluent (1X) and reading as standard curve: log-log.

In the case of IL-8, the Human IL8 Uncoated ELISA Kit was used. Comparing it with the ELISA made in VEGF, this every time it is washed it has to be 3 to 5 times, instead of only 3. When preparing the standard dilutions and our samples with 1x diluent. And the other changes as in the ELISA of IL-1β.

Table 6. *Preparation straight pattern of IL-8.*

Point nº		IL-8 (pg/ml)	Diluent (5X) (µl)
1	500 µl of recombinant human IL-1 beta,	250	0
2	250 µl of 1	125	250
3	250 µl of 2	62,5	250
4	250 µl of 3	31,25	250
5	250 µl of 4	15,62	250
6	250 µl of 5	7,81	250
7	250 µl of 6	3,9	250
8	0	0	250

Results and Discussion

Medium tests and coatings

In the first set of experiments obtained were the type of medium and coatings that made the HRMEC cells more confluent, with stronger TJs... from the results obtained in the TEER, FITC and even with immunofluorescence. In this way, we recreate the iBRB with the HRMECs and thus be able to observe if the CRP causes alterations in the membrane.

A higher value in TEER means a higher resistance across the cellular monolayer. Therefore, it also confirms the integrity of the TJs and of the cell monolayer. Also reflects the ionic conductance of the paracellular pathway in the epithelial monolayer, whereas the flux of non-electrolyte tracers indicates the paracellular water flow, as well as the pore size of the tight junction.

Starting with the TEER results, the effect of different media on cells can be observed in *Figures 6*, *7*, *8* and *9*. In *Figure 6*, it could be observed that the best coating that made the cells more confluent was that of Fibronectin, even though on the last day of TEER measurement that of Gelatin increased above. Continuing with *Figure 7*, Gelatin's coating is ahead, although it is very close to that of Fibronectin. Both EGM2 and EGM2 at 10% in FBS with respect to MV2, their electrical resistance is lower.

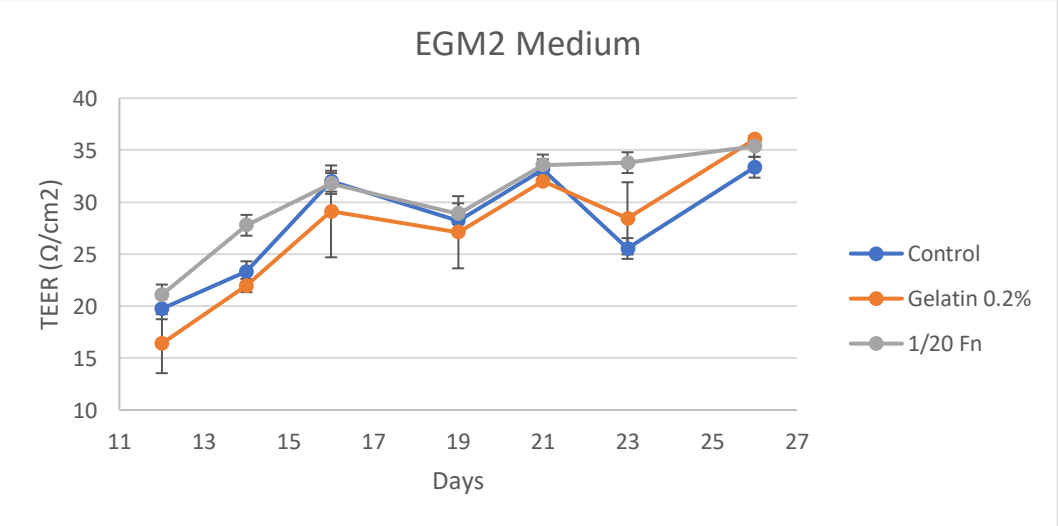


Figure 7. *TEER measurements results according to EGM2.*

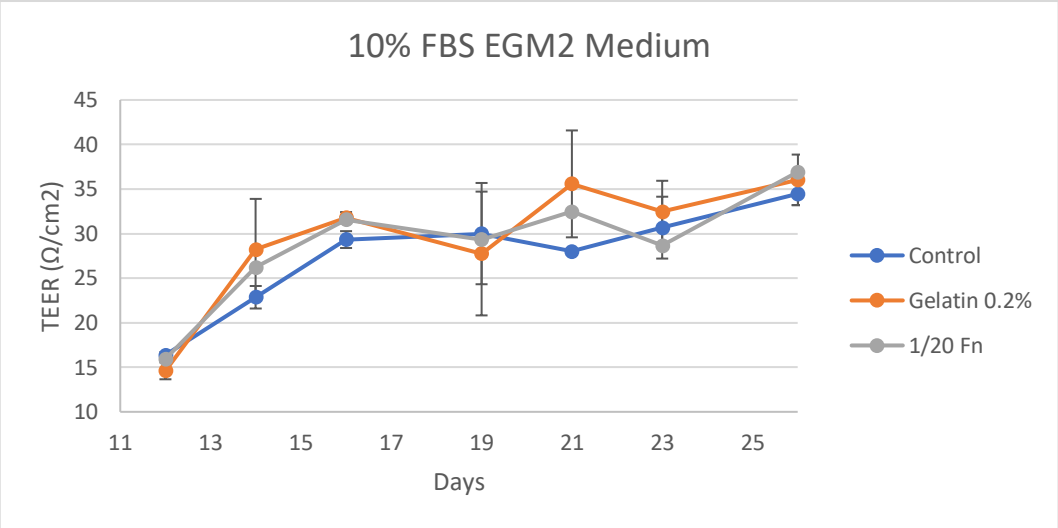


Figure 8. *TEER measurements results according to EGM2 at 10% FBS.*

Continuing with *Figure 8*, Fibronectin is the one that had the highest values in TEER. And finally, *Figure 9*, this medium had the highest TEER values compared to the others, where Fibronectin was the best coating followed by Gelatin. In conclusion, the best coating that made the HRMEC cells have stronger TJ was 50 µg/ml Fibronectin followed by Gelatin.

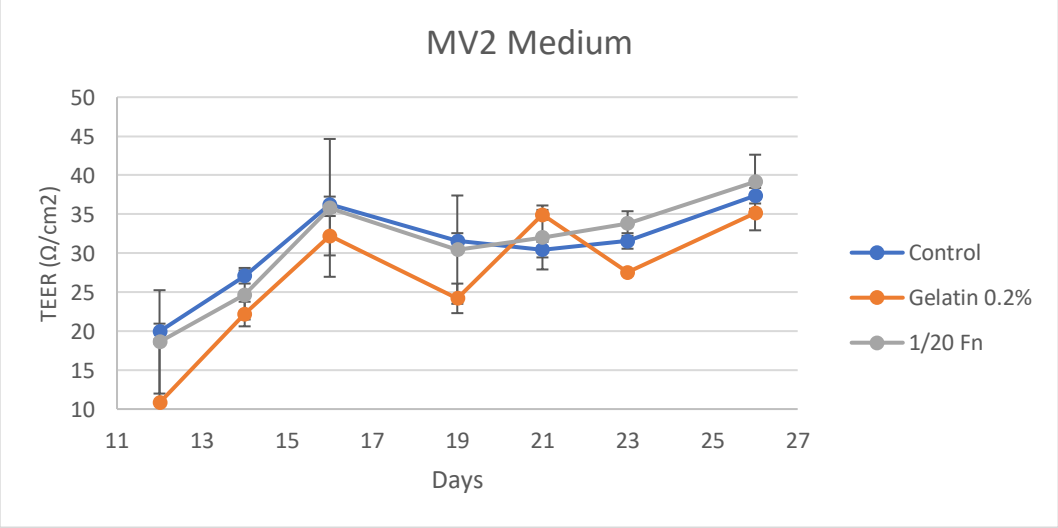


Figure 9*. TEER measurements results according to MV2.*

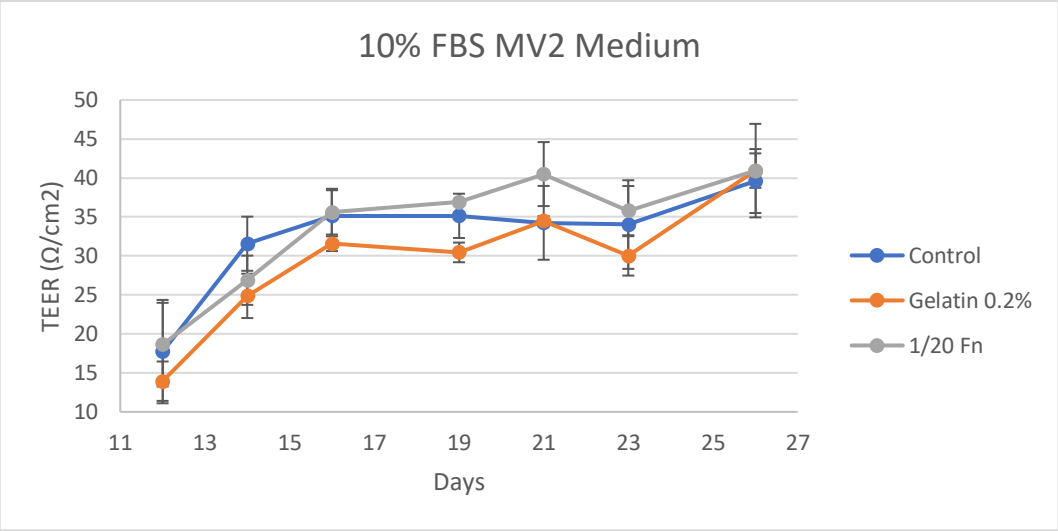


Figure 10. *TEER measurements results according to MV2 at 10% FBS.*

Then we looked at the effect of culture media. Where in *Figure 10*, it was observed that the best medium was that of MV2 at 10% FBS followed by MV2 and that of EGM2 being the worst. In *Figure 11*, the same results were observed as in *Figure 10*, as in *Figure 12*.

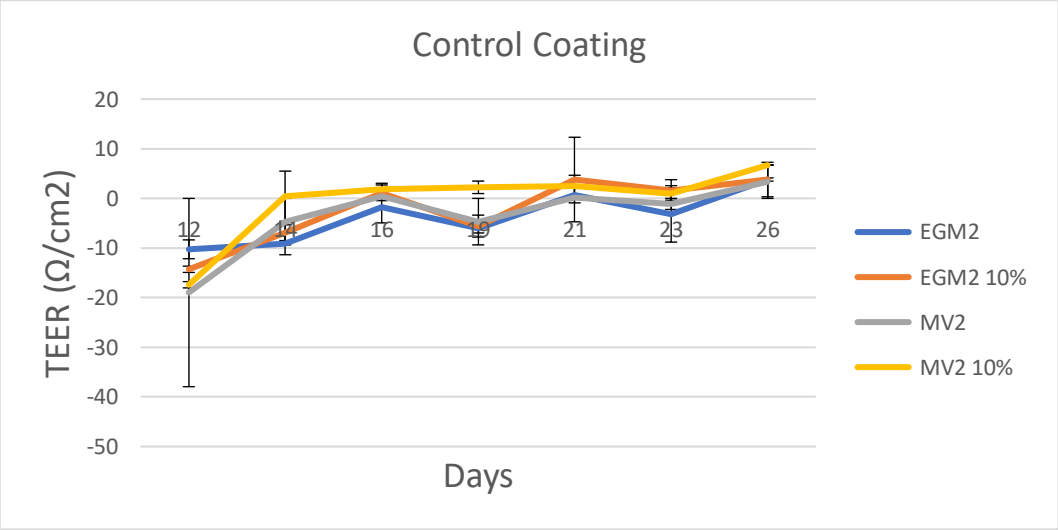


Figure 11. *TEER measurements results according to Control.*

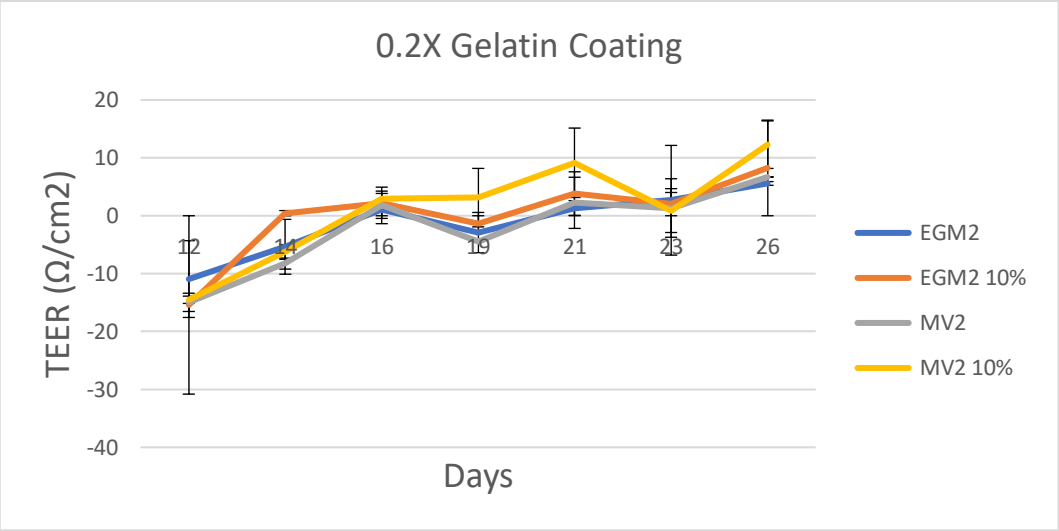


***Figure 12**. TEER measurements results according to 0,2 % Gelatin.*

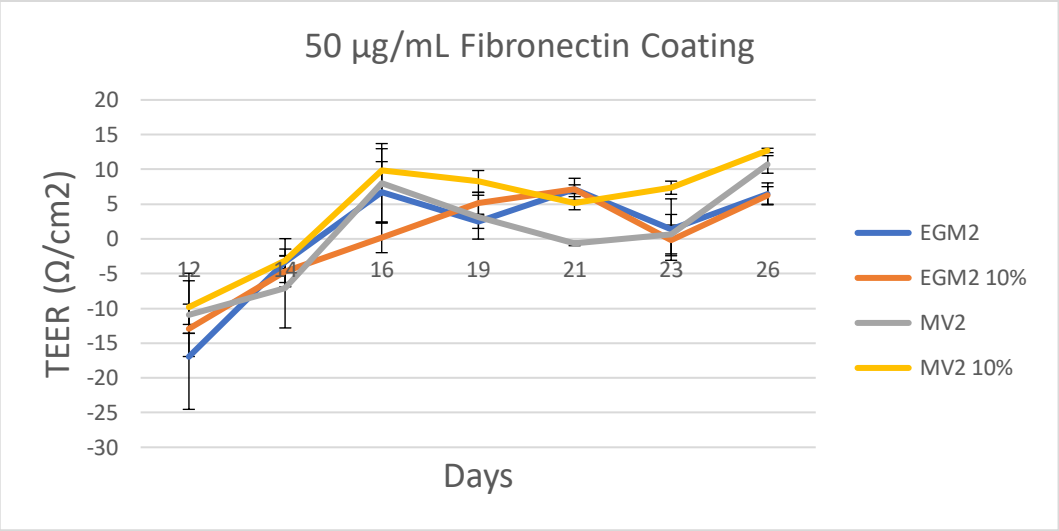


***Figure 13.** TEER measurements results according to 50 µg/ml Fibronectin.*

For this reason, since the values of MV2 at 10% FBS in both 0,2% Gelatin coating and 50 µg/ml Fibronectin were very similar, it was decided first to be cultured HRMEC cells in T25 or T75 with 0,2 % Gel. coating and then passed to inserts with Fibronectin coating. The worst medium was EGM2 and without coating, i.e., Control.

According to FITC diffusion experiments, it was confirmed that the MV2 medium at 10% FBS with 50 µg/ml Fibronectin coating was the best for HRMEC barrier function.

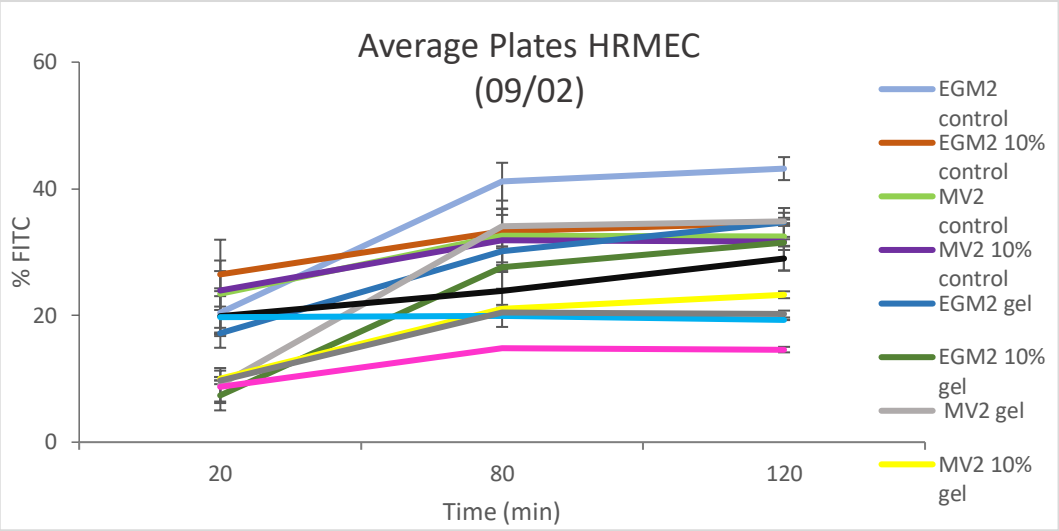


Figure 14. *FITC measurement.*

From another point of view, only by selecting the values of the averages of the ratios at 120 minutes, that is, the last time, the difference in effects caused by all the media such as coatings was observed. Since EGM2 Control was the one that obtained the highest values, that is, it did not create enough barrier and it is not the most favourable for our objectives. On the other hand, those with the lowest values were those of the MV2 medium at 10% FBS, with a difference that of the Fibronectin medium.

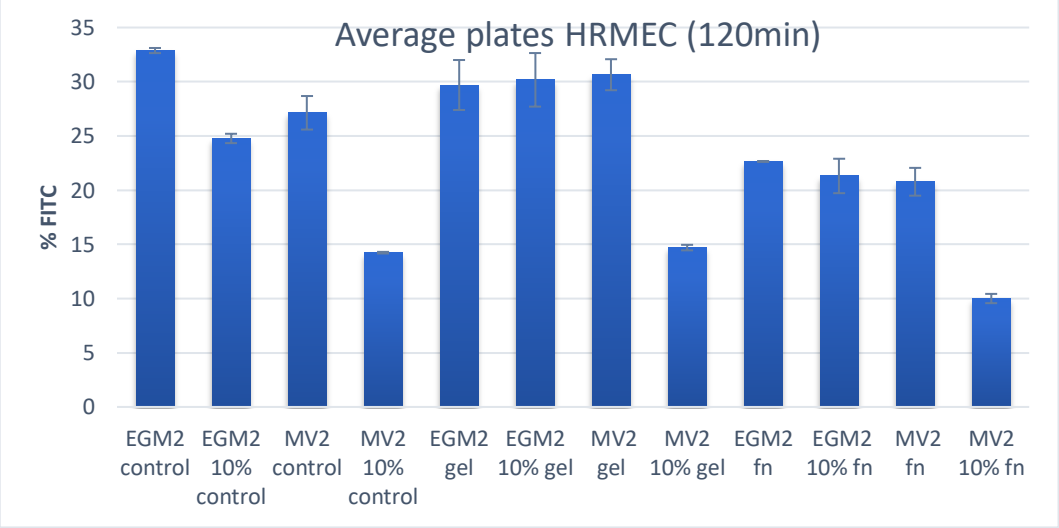


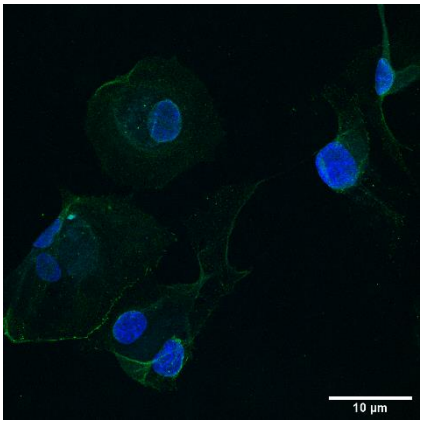
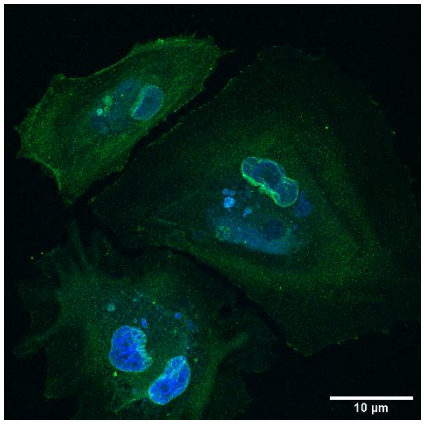
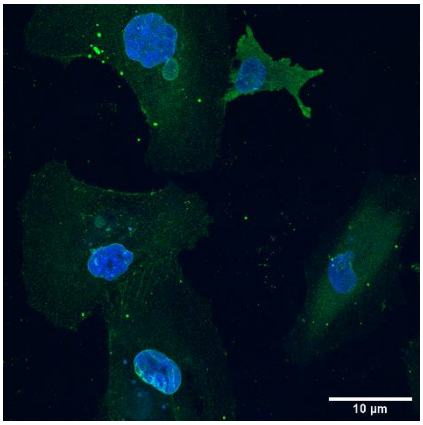
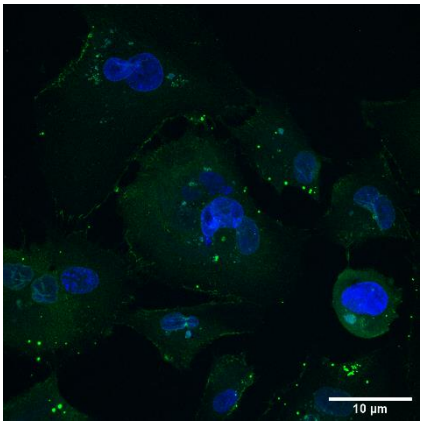
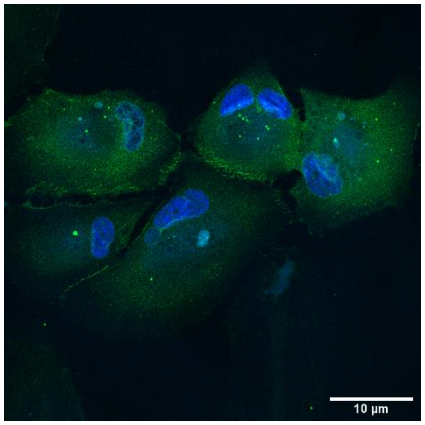
Figure 15. *FITC measurements at 120 minutes.*

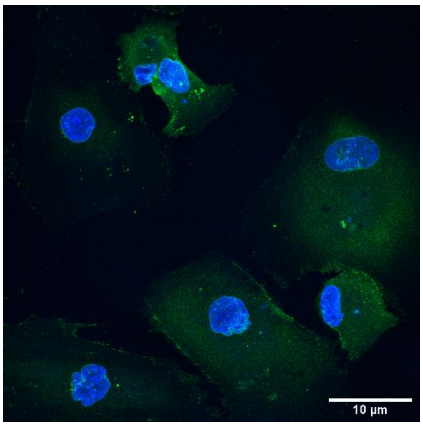
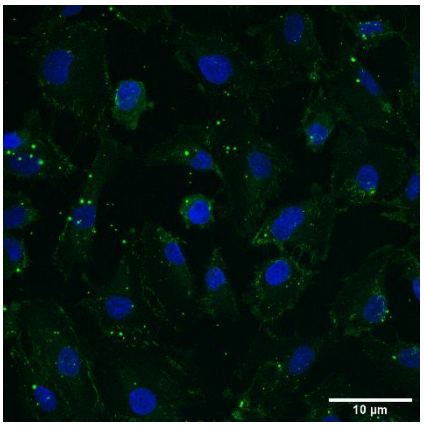
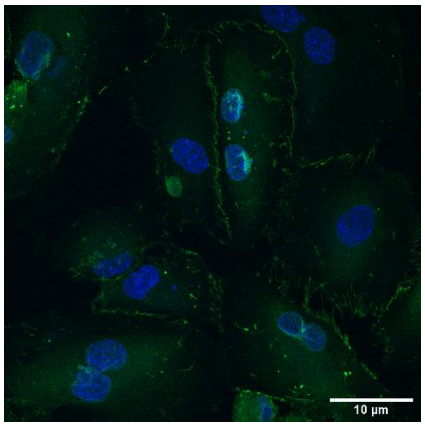
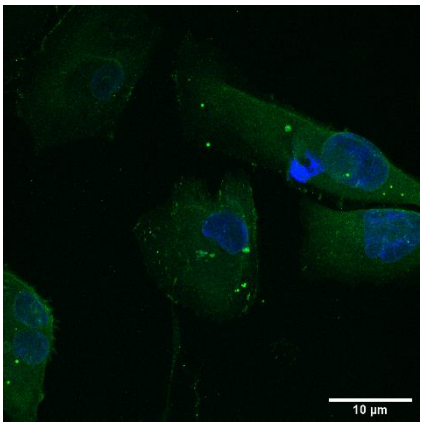
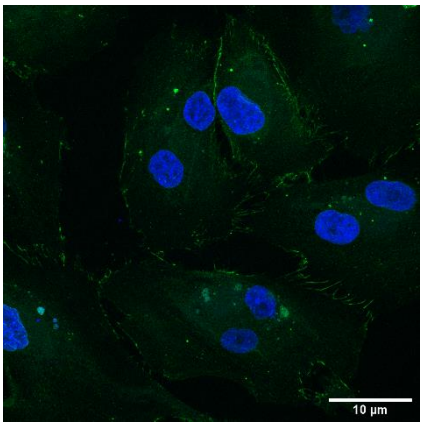
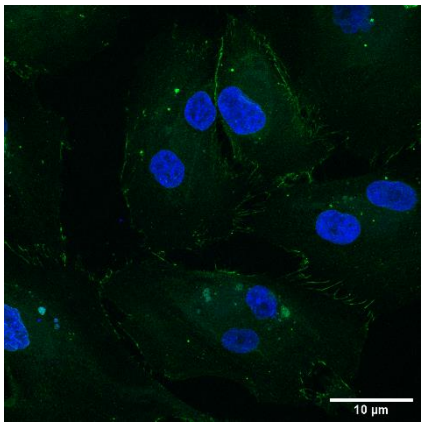
The endothelial phenotypic morphology and the presence of a monolayer was confirmed by the immunostaining procedure,

The immunofluorescence images showed, firstly, a marker for endothelial cells in red colour that belongs to the CD-31 protein, which, as its name indicates, is

found in endothelial cells, exactly at the intercellular junctions. Second, and in the other Figures, the ZO-1 protein is found in green colour, indicative of the morphology of TJs, that is, it is expressed more strongly when it is attached to more endothelial cells. Therefore, the more endothelial cells there are, the more intense the expression of ZO-1 will be in the membrane of these cells. Finally, in blue colour is DAPI, which is a DNA fluorescent marker, which has the nucleus.

Table 7. *Comparative table of the effect of different media and coatings on HRMEC culture.*

	Control	**0,2% Gelatin**	**50 µg/ml Fibronectin**
EGM2			
EGM2 at 10% FBS			

MV2			
MV2 at 10% FBS			

Effect of CRP isoforms on HRMEC barrier function

Once obtained the optimal medium and coating for HRMEC's growth, i.e., Fibronectin coating and MV2 at 10% FBS, they were added in the apical and basolateral compartments. Henceforth to know if mCRP affected the barrier of these cells, they were cultured with different concentrations of CRP. Where CRP was added in the apical compartment of the Insert (*Figure 6*) and later observed if there was CRP in the basolateral compartment. This fact would show the permeability of iBRB from the TEER and FITC- dextran diffusion rate and thus, determine barrier's integrity.

It was cultured with the monomer at 5 µg/ml and also at 10 µg/ml, to be able to observe if with a lower concentration it affected in the same way. It was also cultured with pCRP, with different concentrations as well as mCRP, in order to observe that pCRP did not cause effects on the barrier. And finally, they were also cultivated without CRP to be able to compare it with the others and verify that the barrier was not altered by not being treated.

In the following Figures it was observed that when cells were treated with mCRP, there was an increased in paracellular permeability with respect of untreated cells or cells treated with pCRP. This fact suggests that mCRP induced a barrier disruption in HRMEC.

The result was (*Figure 15*) according to FITC that the permeability of mCRP was higher respect the other, so it created a lower barrier resistance, therefore had a lower barrier permeability, and therefore the presence of ZO-1 is disorganized, due to the disruption. In contrast, the Control or pCRP had more difficulty in crossing the barrier, characteristics contrary to the previous one, that is, it presents a greater permeability in the barrier and a better organization of ZO-1.

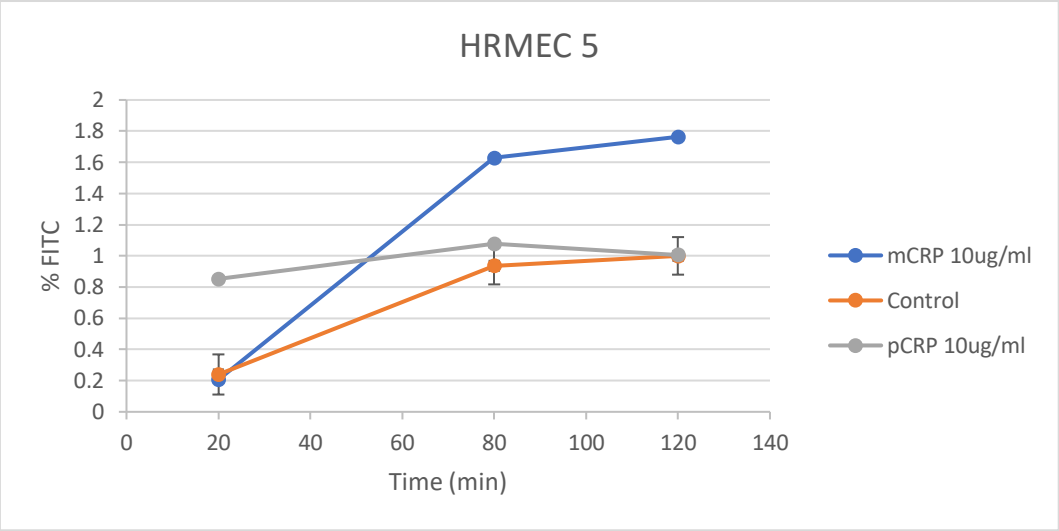


Figure 16. *HRMEC 5's FITC.*

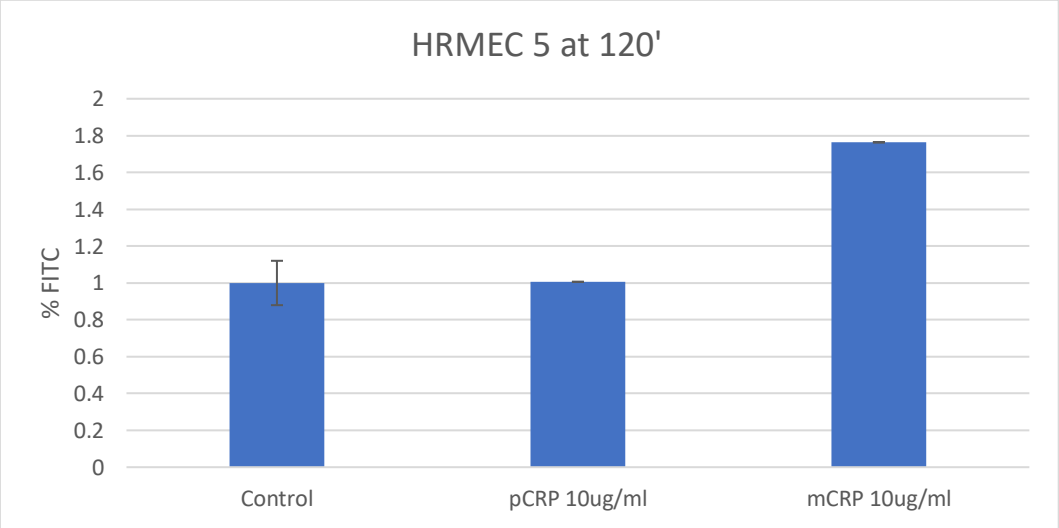


Figure 17. *HRMEC's FITC at 120 min.*

In the next set of experiments, HRMEC 6, conditions were evaluated in duplicate, both Control and mCRP or PCRP at 5 µg/ml and at 10 µg/ml. As seen in *Figure 17*, in this experiment we were not able to validate our first results as mCRP did not increase the paracellular permeability as measured by FITC-dextran diffusion rate.

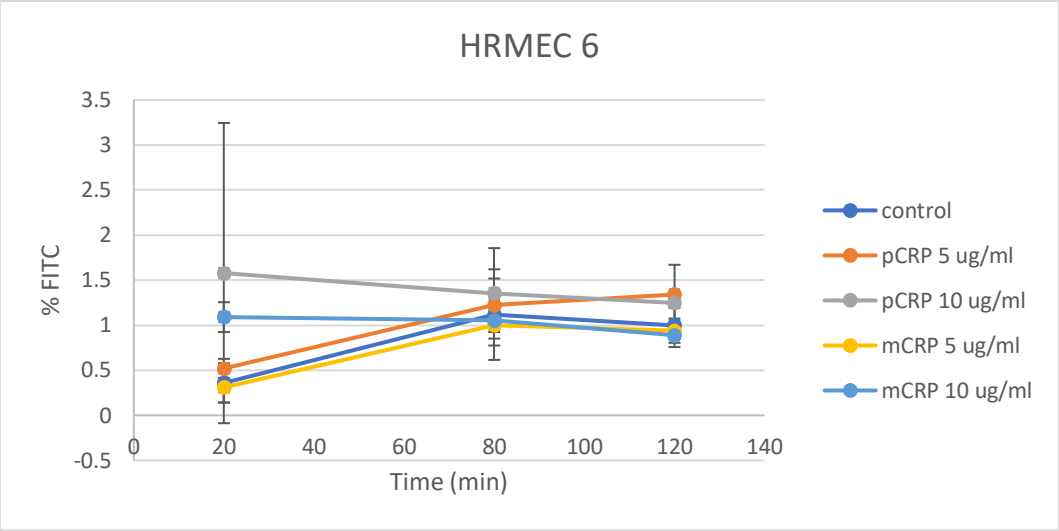


Figure 18. *HRMEC 6's FITC.*

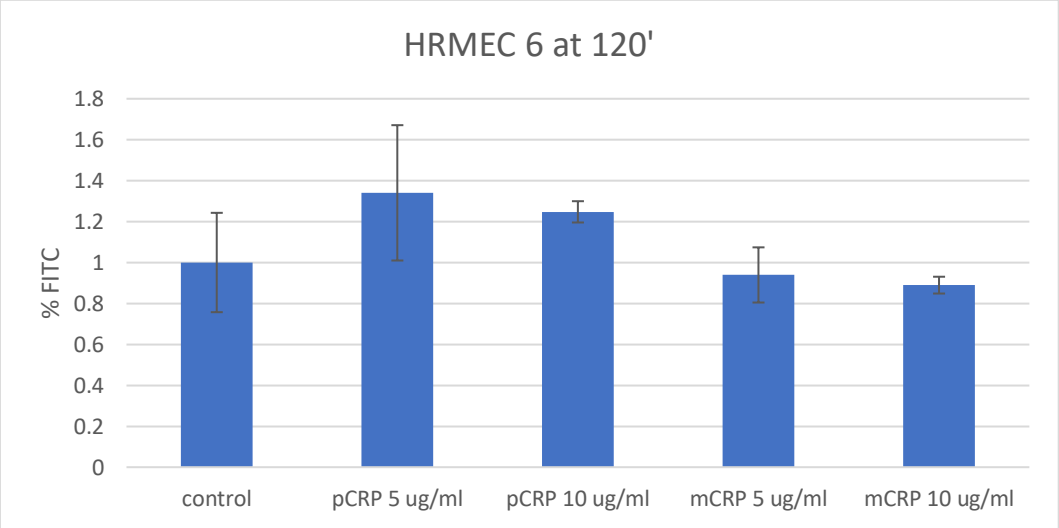


Figure 19. *HRMEC 6's FITC at 120 min.*

The conditions of HMREC 7 were evaluated, where the result should have been that mCRP5 and mCRP10 presented a higher FITC and both pCRP and Control with lower and similar values. But the result has been that Control has presented quite high values, that is, that the membrane has presented great permeability. On the other hand, at 120 min, the results have been better, since the highest value belongs to mCRP5, which is our target, indicating the disruption of iBRB, followed by mCRP10 and pCRP5.

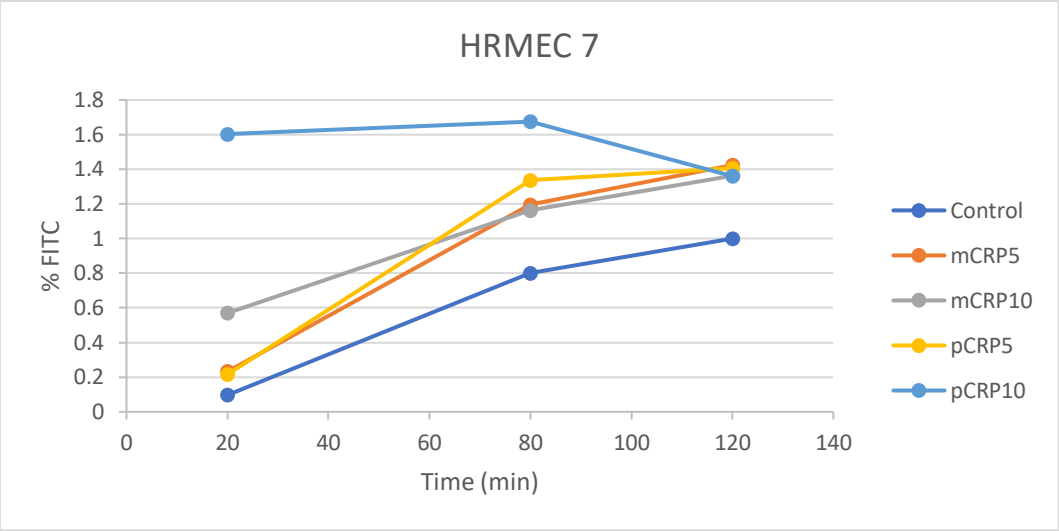


Figure 20. *HRMEC 7's FITC.*

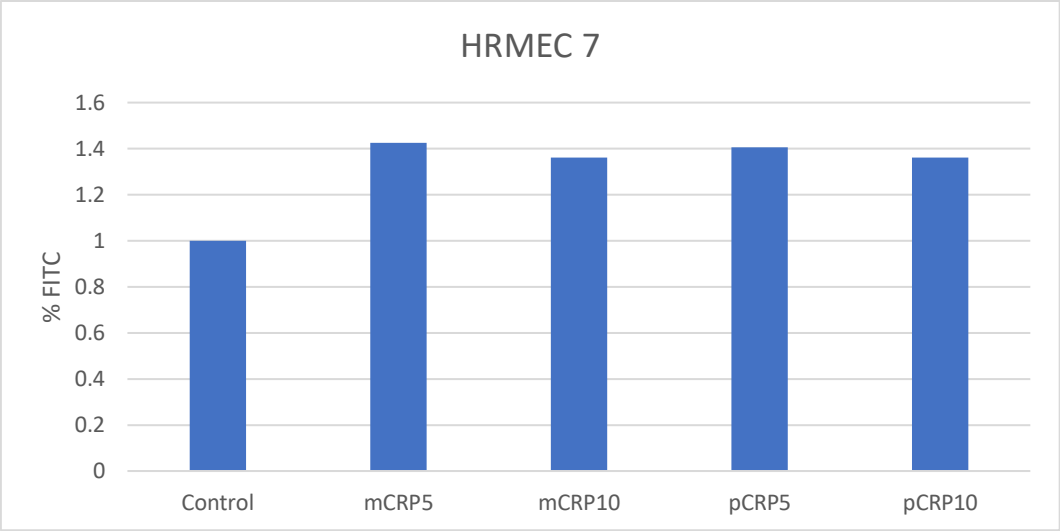


Figure 21. *HRMEC 7's FITC at 120'.*

In the HMREC 8 values we observed the expected results, where mCRP10, in the 120', is the highest value indicating a high disruption in iBRB because it has caused a lower permeability.

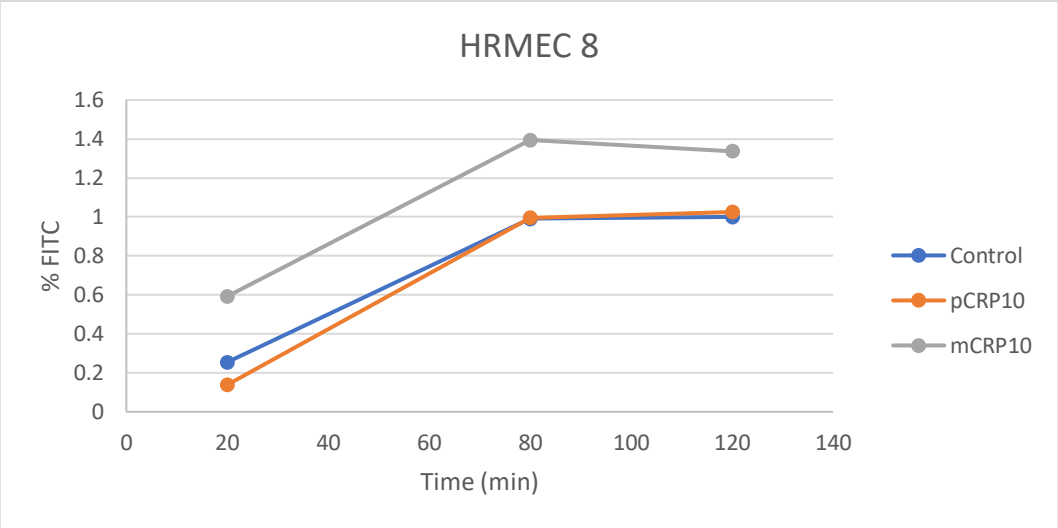


Figure 22. *HRMEC 8's FITC.*

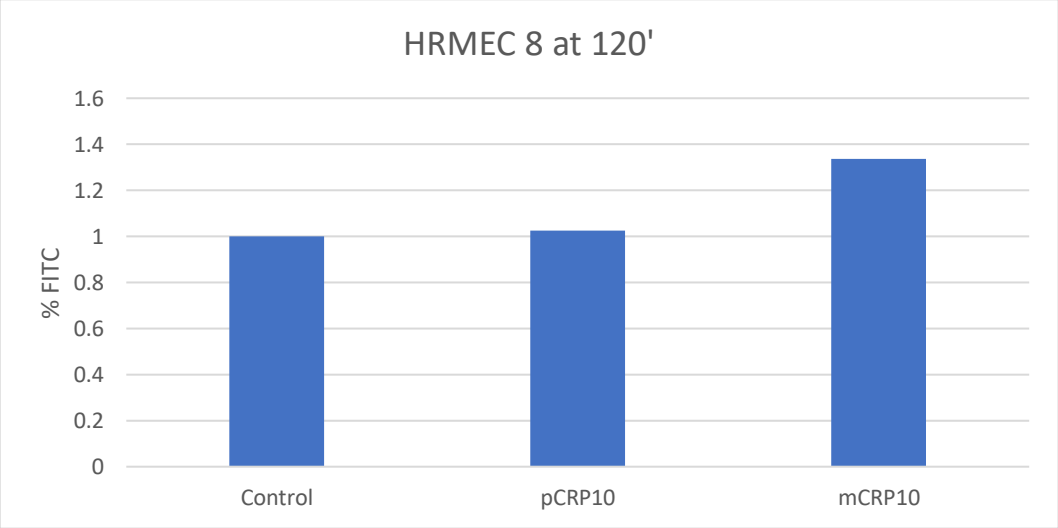


Figure 23. *HRMEC 8's FITC at 120'.*

In *Figure 24*, the combined results of HMEC and normalised results can be observed. pCRP5 is the highest value followed by mCRP5 and mCRP10, meaning that pCRP5 has affected iBRB in a way that has made it less resistant to permeability and has had greater disruption in the barrier. As has been said throughout the work, who should have caused more disruption is the monomer and who should not have caused modifications in said membrane is Control and pCRP. Therefore, these observed results indicate that pCRP at lower concentrations could cross iBRB causing membrane alterations, followed by mCRP.

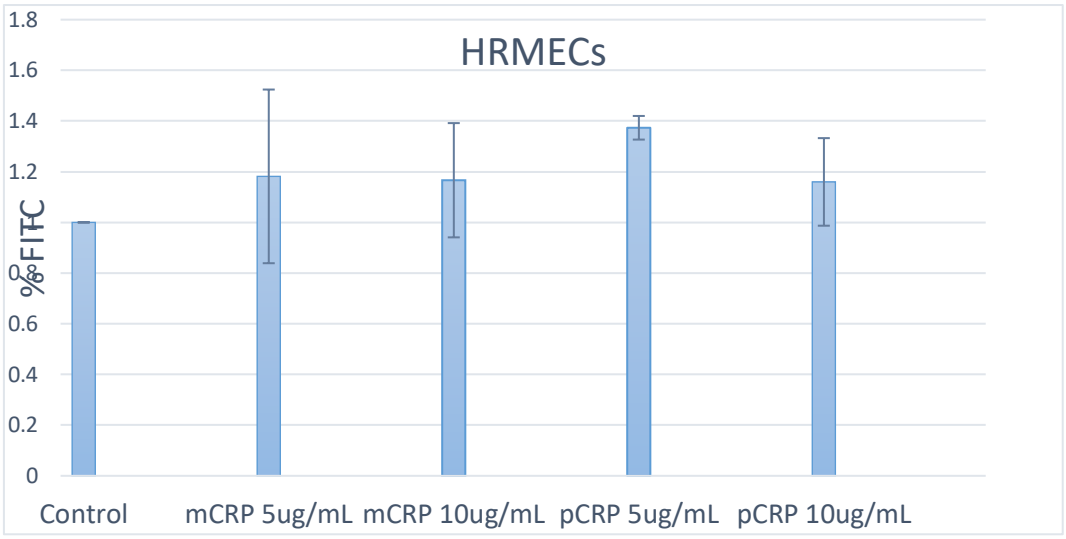


Figure 24. *Normalized HRMEC joint results.*

Enzyme-Linked Immunoabsorbent Assay (ELISA):

First of all, when VEGF was measured an effect on HMREC5 was observed but in the rest, there was almost no effect. This effect of VEGF on HRMEC implies a certain degradation in iBRB, achieving the objective of the research. Since the effect that there has been of VEGF in HRMEC5 but it has only caused the desired effect in one of four crops, so it is not conclusive.

Secondly, when IL-1β was measured, no conclusive results were observed, since the cytokine concentration was either very low or almost non-occurring. And regarding IL-8 neither.

Conclusions

A proper integrity and function of the iBRB is vital for the retina and iBRB disruption is involved in the development of sight-threatening diseases such as DME.

In the present work we were able to find appropriate cell culture conditions of HRMEC to study barrier function. HRMEC cultured in fibronectin surfaces and grown in MV2 media were able to form a proper barrier function. In addition, our results indicated that the mCRP effects of 5µg/ml may compromise iBRB function increasing the paracellular permeability. However, tight junctions are very complex structures and the relative contribution of the various proteins to the barrier function remains unclear [20]. In addition, the ELISA results also did not confirm our hypothesis, since almost no proinflammatory cytokine was observed in the culture.

Our findings have not been conclusive because they were not able to perform enough replicates It would be necessary to carry out a greater number of cultures with duplicates in the amounts of CRP that are introduced to be able to see if mCRP actually causes a disruption in iBRB, which leads to the development of diseases such as DME.

Bibliography

[1] A. Naylor, A. Hopkins, N. Hudson, y M. Campbell, «Molecular Sciences Tight Junctions of the Outer Blood Retina Barrier», doi: 10.3390/ijms21010211.

[2] M. Chen, C. Luo, J. Zhao, G. Devarajan, y H. Xu, «Immune regulation in the aging retina», *Progress in Retinal and Eye Research*, vol. 69. Elsevier Ltd, pp. 159-172, 01-mar-2019, doi: 10.1016/j.preteyeres.2018.10.003.

[3] X. Yang, X. W. Yu, D. D. Zhang, y Z. G. Fan, «Blood-retinal barrier as a converging pivot in understanding the initiation and development of retinal diseases», *Chin. Med. J. (Engl).*, vol. 133, n.º 21, pp. 2586-2594, nov. 2020, doi: 10.1097/CM9.0000000000001015.

[4] L. Liu, X. Liu, L. Liu, y · X Liu, «Roles of Drug Transporters in Blood-Retinal Barrier», doi: 10.1007/978-981-13-7647-4_10.

[5] Y. A. Komarova, K. Kruse, D. Mehta, y A. B. Malik, «Protein Interactions at Endothelial Junctions and Signaling Mechanisms Regulating Endothelial Permeability», *Circ. Res.*, vol. 120, n.º 1, pp. 179-206, ene. 2017, doi: 10.1161/CIRCRESAHA.116.306534.

[6] C. G. Fresta *et al.*, «A new human blood–retinal barrier model based on endothelial cells, pericytes, and astrocytes», *Int. J. Mol. Sci.*, vol. 21, n.º 5, mar. 2020, doi: 10.3390/ijms21051636.

[7] Y. Kubo y K. Hosoy, «Inner Blood-Retinal Barrier Transporters: Relevance to Diabetic Retinopathy», en *Diabetic Retinopathy*, InTech, 2012.

[8] M. Campbell, P. S. Cassidy, J. O'Callaghan, D. E. Crosbie, y P. Humphries, «Manipulating ocular endothelial tight junctions: Applications in treatment of retinal disease pathology and ocular hypertension», *Progress in Retinal and Eye Research*, vol. 62. Elsevier Ltd, pp. 120-133, 01-ene-2018, doi: 10.1016/j.preteyeres.2017.09.003.

[9] M. Díaz-Coránguez, C. Ramos, y D. A. Antonetti, «The inner blood-retinal barrier: Cellular basis and development», *Vision Res.*, vol. 139, pp. 123-137, oct. 2017, doi: 10.1016/j.visres.2017.05.009.

[10] M. Rudraraju, S. P. Narayanan, y P. R. Somanath, «Regulation of blood-retinal barrier cell-junctions in diabetic retinopathy», *Pharmacological Research*, vol. 161. Academic Press, p. 105115, 01-nov-2020, doi: 10.1016/j.phrs.2020.105115.

[11] R. Natoli *et al.*, «Microglia-derived IL-1β promotes chemokine expression by Müller cells and RPE in focal retinal degeneration», *Mol. Neurodegener.*, vol. 12, n.º 1, abr. 2017, doi: 10.1186/s13024-017-0175-y.

[12] R. C. Russo, C. C. Garcia, M. M. Teixeira, y F. A. Amaral, «The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases», *Expert Review of Clinical Immunology*, vol. 10, n.º 5. Expert

Reviews Ltd., pp. 593-619, 2014, doi: 10.1586/1744666X.2014.894886.

[13] P. Canning, J. V Glenn, D. K. Hsu, F.-T. Liu, T. A. Gardiner, y A. W. Stitt, «Inhibition of Advanced Glycation and Absence of Galectin-3 Prevent Blood-Retinal Barrier Dysfunction during Short-Term Diabetes», *Exp. Diabetes Res.*, vol. 10, 2007, doi: 10.1155/2007/51837.

[14] E. A. Urias, G. A. Urias, F. Monickaraj, P. McGuire, y A. Das, «Novel therapeutic targets in diabetic macular edema: Beyond VEGF», *Vision Res.*, vol. 139, pp. 221-227, oct. 2017, doi: 10.1016/j.visres.2017.06.015.

[15] J. M. Seddon, S. George, B. Rosner, y N. Rifai, «Progression of age-related macular degeneration: Prospective assessment of C-reactive protein, interleukin 6, and other cardiovascular biomarkers», *Arch. Ophthalmol.*, vol. 123, n.º 6, pp. 774-782, jun. 2005, doi: 10.1001/archopht.123.6.774.

[16] S. Black, I. Kushner, y D. Samols, «C-reactive protein», *Journal of Biological Chemistry*, vol. 279, n.º 47. Elsevier, pp. 48487-48490, 19-nov-2004, doi: 10.1074/jbc.R400025200.

[17] S. Romero-Vázquez *et al.*, «Activation of C-reactive protein proinflammatory phenotype in the blood retinal barrier in vitro: Implications for age-related macular degeneration», *Aging (Albany. NY).*, vol. 12, n.º 14, pp. 13905-13923, jul. 2020, doi: 10.18632/aging.103655.

[18] B. Molins *et al.*, «Complement factor H binding of monomeric C-reactive protein downregulates proinflammatory activity and is impaired with at risk polymorphic CFH variants», *Sci. Rep.*, vol. 6, mar. 2016, doi: 10.1038/srep22889.

[19] B. Molins *et al.*, «C-reactive protein isoforms differentially affect outer blood-retinal barrier integrity and function», *Am. J. Physiol. - Cell Physiol.*, vol. 312, n.º 3, pp. C244-C253, mar. 2017, doi: 10.1152/ajpcell.00057.2016.

[20] M. Mesquida *et al.*, «Modelling macular edema: The effect of IL-6 and IL-6r blockade on human blood-retinal barrier integrity in vitro», *Transl. Vis. Sci. Technol.*, vol. 8, n.º 5, 2019, doi: 10.1167/tvst.8.5.32.