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Innovative therapeutic approaches for the atrophic Age-related Macular Degeneration (“dry” AMD)

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Abstract

Age related macular degeneration (AMD) is a major concern regarding blindness in the world. In western countries, where visual alterations due to minor pathologies as cataract and uncorrected refractive errors are easily resolved, AMD represent the main cause of blindness. Of the two existing forms of the disease, while the neovascular is more aggressive and progress quickly, geographic atrophy is the one still lacking an appropriate therapy. My PhD program was focused on investigating AMD features, trying to understand if some approaches I tested could be able to provide some suggestion about potential future therapies on “dry” AMD.

In my research I developed three main projects. The most important part of the work regards the study of integrins and their fundamental role in cell adhesion in a context of interaction between retinal pigmented epithelium (RPE) and immune cells. I investigated how co-culture of these different cell lines can lead to simulate an inflammatory state inducing cell signaling, cytokine production and cell death. The use of integrin antagonists developed in our laboratory, showed how these effects can be reverted.

A secondary approach regards the use of antioxidants and their role in epigenetic modifications in ARPE-19 cells to investigate how these compounds might exert their well-known protective role on AMD. Commonly used antioxidants as Lutein and Quercetin do not induce clear epigenetic modifications through histone H3 acetylation indicating only a limited involvement.

Finally, during the period abroad of my PhD program, I studied through an RNA-sequencing strategy how statins might exert a protective role in AMD. Following previous findings from my host laboratory, I investigated if treating ARPE-19 cells and primary culture of RPE cells with different concentrations of Atorvastatin could induce modification in expression of genes related to fundamental pathways as phagocytosis, debris removal and lipid metabolism. I was able to detect any genes involved in these modifications, without being able to prove a clear trend indicating a massive pathways involvement.

Summary

Introduction

1. Human eye anatomy

The eye is the organ dedicated to receive and to process incoming light from the World around us into impulses suitable with the central nervous system (CNS), sending them to the brain through the optic nerve to perceive images (Fernald 1997). Among our five senses, is also the one we rely on the most and the one we mainly use to analyze information and detect what happens in the environment.

Eyes are spheroidal organs located into the orbits, where six extraocular muscles are responsible for their movements, connected to the sclera, the most external layer of the eye bulb, coating nearly the entire surface. Indeed, to permit light to pass, the anterior part of the eye is covered with a clear surface called cornea. Regarding the parts that compose the eyeball, it is possible to distinguish two main sections: the anterior segment and the posterior segment (**fig. 1**). The anterior segment is filled with aqueous humor and comprise about one third of the eye, including every part ahead of the vitreous: cornea, iris, ciliary bodies and lens. Moreover, it is possible to divide the anterior segment in the anterior and the posterior chambers: the anterior chamber is located between the posterior surface of the cornea (corneal endothelium) and the iris, while the posterior chamber is located between the iris and the frontal face of the vitreous. The posterior segment comprises the remaining two thirds of the globe, from the vitreous membrane to the optic nerve. In between there are, in anterior to posterior order, vitreous humor, retina and choroid. It is common for ophthalmologists to specialize in specific parts and pathologies of the eye and usually the main distinction is between anterior and posterior segment specialists (Boyd and Turbert 2018).

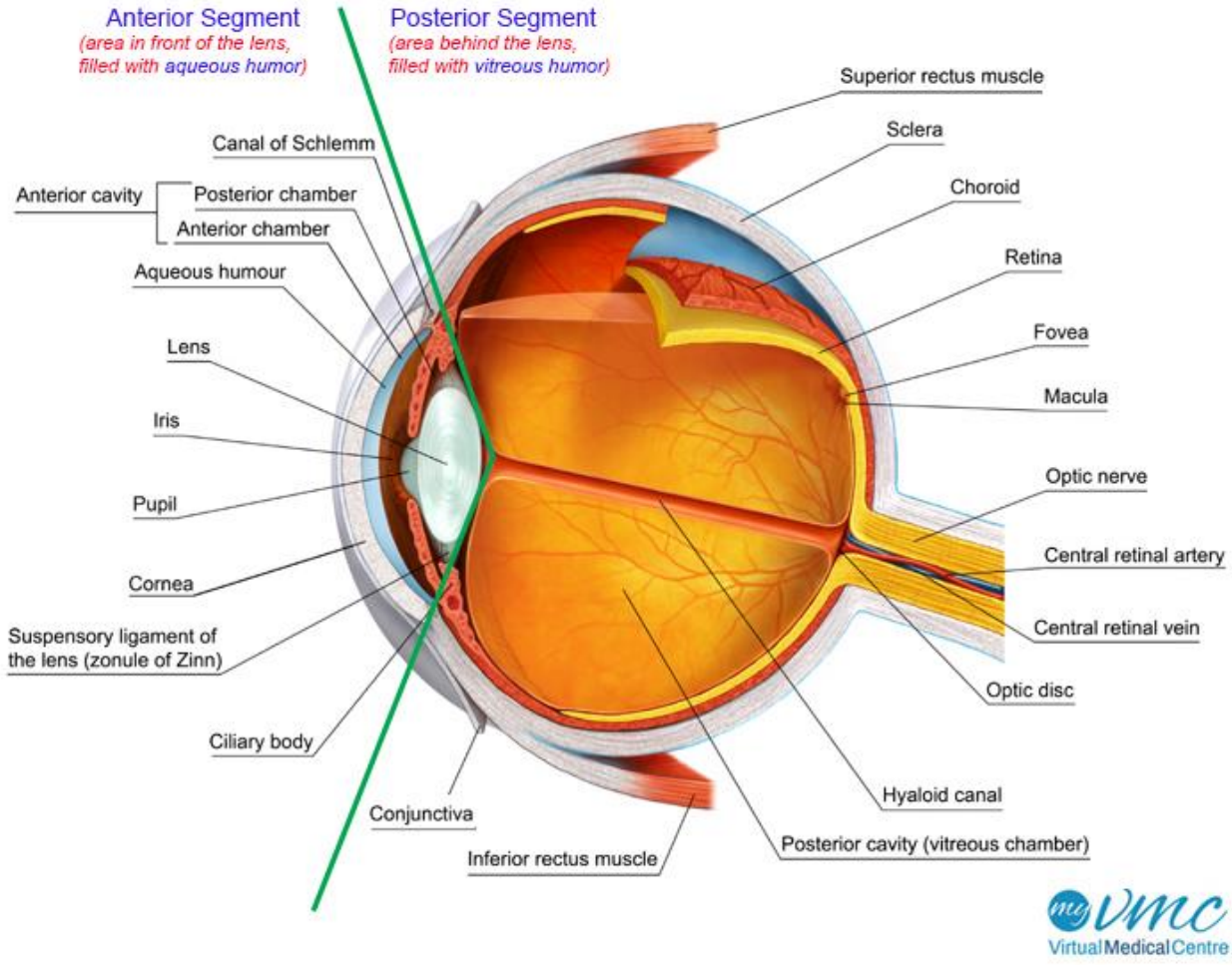


Fig. 1. Human eye segments. *Detailed section of the components of anterior and posterior segments of the human eye (Virtual medical center 2018).*

Usually, to classify the different parts of the eye is common also to speak about layers (**fig. 2**). Indeed, the bulb has a laminar organization where it is possible to distinguish three main sections depending on their functions and depth (Tortora and Grabowski 1993):

- The most external layer is the fibrous tunic, consisting of the sclera in the posterior part and the cornea on the anterior part. Indeed, as discussed before, sclera is an opaque tissue covering the eye for about the 80%, while cornea is only on the remaining frontal part, allowing light to enter and reaching the retina.
- Vascular tunic, also known as uvea, is the middle layer and contains vessels carrying blood throughout the eye. Is composed by the iris, the ciliary body and the choroid. The choroid invests the posterior 80% of the bulb and extends front until the limit between retina and ciliary body, called *ora serrata*. The ciliary body

connects the choroid to the circumference of the iris, which is a circular diaphragm presenting at its center a rounded aperture, the pupil, that allows light to pass through.

- The most internal layer is the nervous tunic, also known as retina, deputed to catching light signals from outside, converting them into impulses and send them to the brain (Purves et al. 2001).

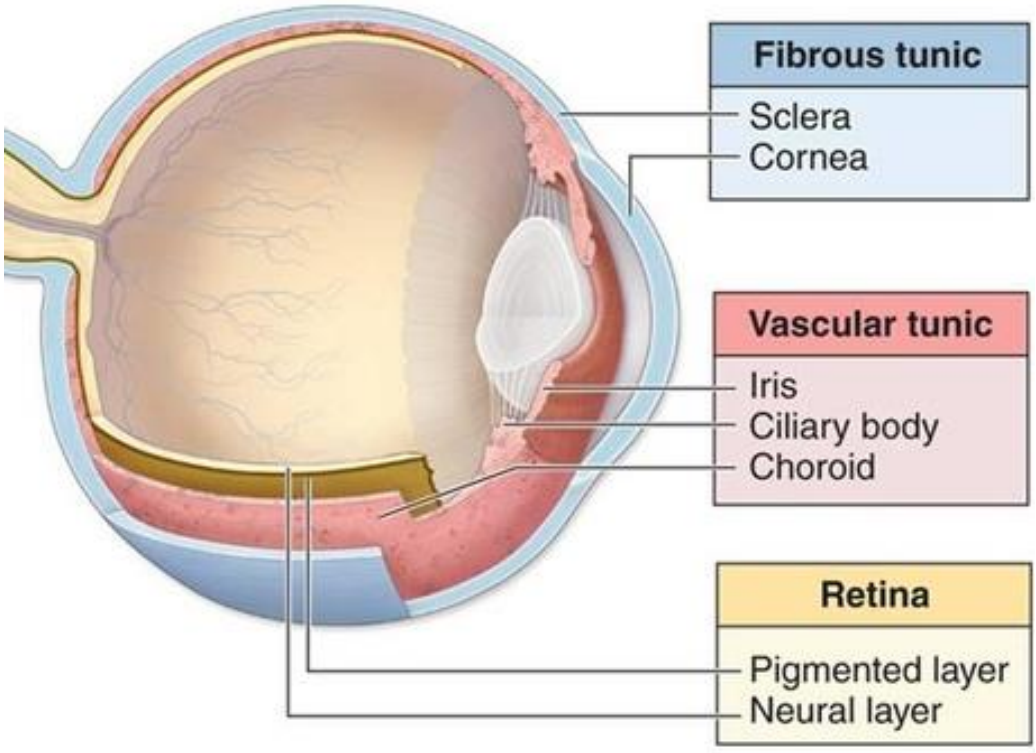


Fig. 2. Human eye tunics. *Detailed section of the different layers of the human eye (Quizlet Inc. 2016).*

1.1 Blood-ocular barriers

The eye is one of the few parts of the body with a so-called "immune-privilege", making it very special. In this group of organs, including CNS, placenta and testicles, normal immune response is limited, probably to protect them from damage that may occur as a consequence of the activation of immunity, leading to swelling, increasing of temperature and other potentially detrimental reactions (Boyd 2018). In the eye, this peculiar condition seems to be due mainly to two factors: the anterior chamber associated immune deviation (ACAID), a particular behavior of the immune system, that maintains inflammations and antigens reactions (Stein-Streilein and Streilein 2002) and the protection and isolation of this organ

given by a peculiar lymphatic system and the blood-ocular barriers (Chen 2009; Taylor 2009; Yücel et al. 2018).

Two main parts characterize the blood-ocular barrier system: the blood-aqueous barrier (BAB) in the anterior segment and the blood-retinal barrier (BRB) in the posterior segment. These barriers protect the eye from circulating cells diffusion and spreading of inflammation as would occur in other tissues and sites, conferring to it, as said, the status of "immune-privileged" organ (Cunha-Vaz 2009).

BAB is an epithelial barrier, made by the non-pigmented epithelium of the ciliary body, the posterior pigmented epithelium of the iris and the endothelium of the iridial vessels (Freddo 2013). These layers have tight junctions, gatekeepers of the paracellular transport, limiting the selective diffusion of ions and small solutes through the space between neighboring cells. BAB contributes to maintain the immune-privileged conditions of the eye, selectively controlling leukocytes access to the aqueous humor. The epithelial and endothelial cells of the BAB express many immunoregulatory factors and its breakdown, independently of the etiology, is manifested by an increase in the aqueous humor protein concentration (Coca-Prados 2014).

BRB (**fig. 3**) is one of the most important barriers of the body, with interesting similarities to the blood brain barrier (BBB) (Díaz-Coránguez, Ramos, and Antonetti 2017; Steuer et al. 2005). It is important to understand that the retina is nourished through two different vascular systems: retinal vasculature supplies oxygen and nutrients only to the inner retinal layers, while the external part of the retina is under the responsibility of the choroidal vasculature (Choi et al. 2013). Considering this distinction, it is simple to comprehend why the BRB consists in two parts, the inner and the outer component. The inner component is made by the retinal vascular endothelium with its intercellular tight junctions and selectively protects the retina from foreign substances coming from the retinal blood circulation. The outer component is made by the retinal pigmented epithelium (RPE), a monolayer of polarized cells supporting the photoreceptors and located above the fenestrated choriocapillaris layer of the choroid. RPE exerts a fundamental transport processes along with its critical barrier characteristics, strictly controlling the diffusion of molecules and substances coming from the circulation (Rupenthal, Huang, and Chen 2018). RPE, moreover, is an important contributor in maintaining the immune privileged status of the eye, not only protecting retina from external threats, but also regulating leukocytes activity (Stein-Streilein 2008).

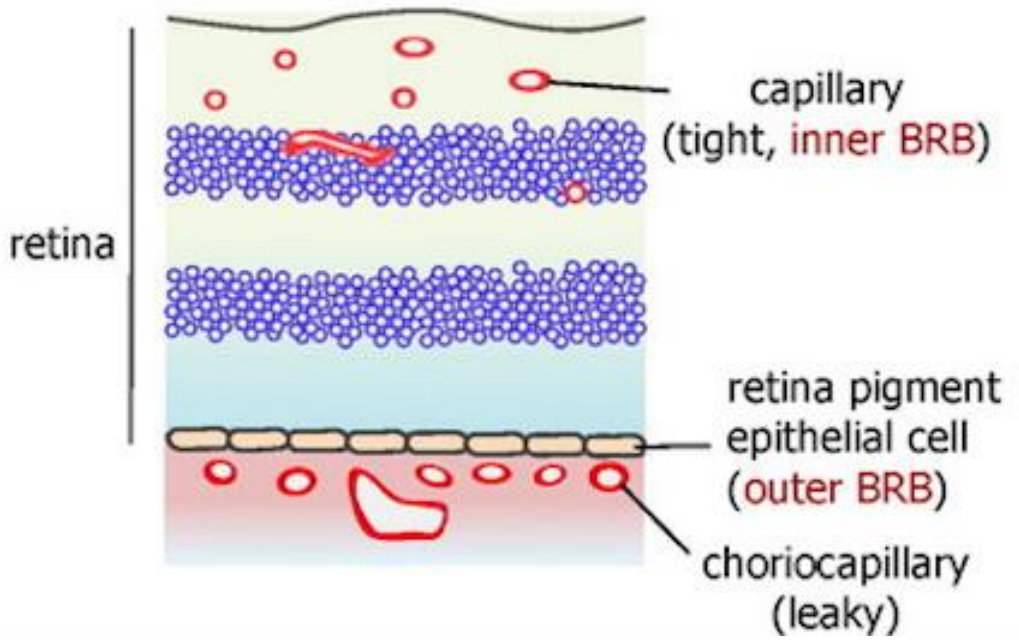


Fig. 3. Blood-retinal barrier. *Blood-retinal barrier (BRB) components in detail, comprehending the inner and the outer BRB.*

1.2 Sclera

Sclera is also generally known as the white part of the eye, because it is the outer layer of the bulb and externally in humans it comprises a white coloration. Together with the cornea, they compose the external layer of the bulb, covering the eye for about the 80% of its surface. It is made mainly by connective tissue containing collagen and elastic fibers intersected and bundled together, conferring to the eyeball strength and flexibility. Sclera main roles are protection from impacts and damages, maintenance of the shape of the eye and to serve as solid attachment for the 6 extrinsic eye muscles, responsible for the movement of the bulb (Forrester et al. 2016). It can be divided in three layers:

- Episclera, the external part, that is a highly vascularized membrane laying beneath the conjunctiva in the anterior part of the eye and attached to Tenon's capsule, a thin sheath that surrounds the eyeball;
- Scleral stroma, the intermediate layer, made of irregularly arranged collagen fibers, scattering light and conferring the white color to the tissue (Annear and Petersen-Jones 2012);
- Lamina fusca, the innermost layer, in contact with the choroid (Maggs 2008).

1.3 Cornea

Together with the sclera, cornea constitutes the external layer of the eye, the fibrous tunic, in the frontal part of the eye. It is located in front of the iris, pupil and anterior chamber and acts as the eye's outermost lens. Indeed, cornea provides 65-75% of human eye's focusing power contributing together with the lens in properly addressing light to the retina: when light strikes the cornea, it refracts the light onto the lens. Cornea also serves as a filter that screens off UV light from the sun. Without this protective action, the lens and the retina would be exposed and damaged by UV rays. Furthermore, cornea acts as a barrier against dirt, germs and other particles that can harm the eye, sharing this protective task with the eyelids, eye sockets, tears, and sclera (National Eye Institute 2016).

Cornea is a highly organized tissue, even if appears and is supposed to be completely clear to allow light transition. For this reason, it does not present blood vessels in normal healthy conditions but receives nourishment from tears and aqueous humor. Indeed, through the blinking of the eyes, tears produced by the lacrimal glands distribute across the cornea, contributing to protect and clean eye surface from dirt and pathogens, lubricating the eye and supplying ions, lipids, oxygen and other substances to the cornea. It is possible to distinguish five different layers of the cornea (Ludwig and Sevensma 2019):

- Epithelium, the outermost layer, acting as a shield against external substances and germs and intercepting oxygen and nutrients from tears before redistributing them to the other parts of the cornea.
- Bowman’s layer made of layered collagen fibers.
- Stroma, the thickest part of the tissue. It is composed mainly by water and collagen, provides to the tissue its shape, resistance and elasticity. The organization of the fibers in this layer is essential for the light-conducting properties of the cornea.
- Descemet's membrane, a thin but resistant acellular layer constituted by collagen fibers, different from the ones in the stroma.
- Endothelium, the thin, innermost cellular monolayer of the cornea. Cells composing this layer are responsible to regulate fluids and solute transport between the stroma and the aqueous humor (Blackburn et al. 2019; W. M. Bourne 2003).

1.4 Conjunctiva

It is a tissue that coats the anterior, non-corneal part of the eye and the interior part of the eyelids. It provides protection and lubrication to the eye surface by producing and spreading mucus and tears. The conjunctiva of the eye consists of an epithelial layer composed of stratified squamous and stratified columnar epithelium. It is a non-keratinized tissue with interspersed goblet cells, where are present also blood vessels, lymphatic channels, immune cells and accessory lacrimal glands. It has a role in preventing microbial entrance into the eye and playing a part in immune surveillance (Shumway and Wade 2019). It can be considered as maid by three parts:

- Bulbar conjunctiva covers the anterior surface of the sclera and stopping at the junction with the cornea;
- Palpebral conjunctiva covers the inner surface of both upper and lower eyelids.
- Conjunctival fornices forms the junction between palpebral and bulbar conjunctivas. These parts are loose and flexible, allowing movements of the globe and the eyelids.

In its entirety, conjunctiva is continuous, making impossible for any kind of object (dust, particles, contact lens, etc.) to get lost behind the eye bulb (Perkins and Davson 2019).

1.5 Iris

Iris is a flat, thin, circular membrane of the eye, that have the role of modifying the amount of light passing through the pupil and reaching the retina, controlling its size and diameter. Iris is situated in the anterior segment of the eye and physically separates anterior and posterior chamber. Looking at the iris from outside is possible to notice two distinct areas: the pupillary zone (central) and the ciliary zone (peripheral), separated through a wavy border, the collarette. The pupillary zone extends from pupillary margin to collarette, while ciliary zone extends from collarette to iris root, the outer edge of the iris, attached to the ciliary body (Alward and Longmiur 2017).

Iris is composed mostly of connective tissue and smooth muscle fibers and consists in an anterior fibrovascular layer and a posterior epithelial layer:

- The anterior fibrovascular layer, also known as stromal layer, is a vascular connective tissue having no anterior epithelial covering and is the part of the iris where musculature lies. A sphincter muscle rings the pupil, causing its

contraction, while dilator muscles spreads radially, causing pupil dilatation. Blood vessels of the iris are also mostly located in the stromal layer with a radial disposition. Too much or too little light can hamper vision. The muscular iris moves to shrink the pupil if there is too much light and widen it if there is not enough. This is an involuntary function, controlled by the brain (Bloom and Czyz 2019).

- The posterior epithelial layer is composed itself by two different epithelial layers: the anterior layer has little pigmentation and is continuous with the outer (pigmented) layer of the ciliary body. The posterior layer is densely pigmented and faces the posterior chamber. This layer is continuous with the nonpigmented layer of ciliary epithelium. When we speak about the color of our eyes, we refer to iris color. Despite the wide range of colors, the only pigment that contributes substantially to normal human iris color is melanin. The coloring of the iris is due to variable amounts of total melanin in general and in the relative amount of eumelanin (brown/black melanin) and pheomelanin (red/yellow melanin) produced by melanocytes, visible in brown-eyed people and in blue and green-eyed people, respectively (Sturm 2004).

1.6 Ciliary body and lens

Ciliary body, together with the iris, constitutes the anterior part of the vascular tunic, also known as uvea, and connects the iris and the other component of this layer, the choroid, which lies in the posterior part of the eye. It separates the posterior chamber from the vitreous humor and has the essential roles of producing the aqueous humor and regulating its flow other than controlling the change of shape of the lens.

Lens, or crystalline lens, is an ellipsoid transparent and biconvex structure that, together with the cornea, helps refracting light in order to have a proper focus on the retina. The outermost part of the lens is called capsule and is an elastic membrane composed of collagen that completely surrounds it. The lens must be transparent and for this reason it lacks of blood vessels and receives its nourishment through the aqueous humor, helped by the lens epithelium, a monolayer of cuboidal epithelial cells regulating most of the homeostatic function of the lens, that lies in the anterior part of it (Andley 2008). The bulk of the lens is made by fibers organized in concentric layers, mostly constituted by water-soluble proteins called crystallins (Andley 2007).

Ciliary body is composed of muscle, vessels and epithelium. The anterior part of the ciliary body is called *pars plicata* and is characterized by the ciliary processes, inward folding arranged in circle, responsible for the production of the aqueous humor and its secretion in the posterior chamber. From there, the liquid can then flow through the pupil and reach the anterior chamber, where is collected through the trabecular meshwork in the Schlemm's canal (Borges- Giampani and Giampani 2013).

The ciliary muscle consists in different separate muscle fibers. The contraction of the longitudinal fibers opens the trabecular meshwork and Schlemm`s canal, allowing the liquid to being removed. The circular ciliary fibers affect the zonular fibers (zonula of Zinn), a system of fibrous strands that connects the ciliary body with the lens, enabling changes in lens shape for light focusing. When the muscle contracts, it moves forward releasing the tension on the lens caused by the zonular fibers and leading the lens itself to assume a more spherical shape, adapting the view to short-range focus. On the other side, when there is muscle relaxation, fibers become tensed and stretched, flattering the lens and increasing the focal distance (Riordan-Eva and Whitcher 2008) (**fig. 4**).

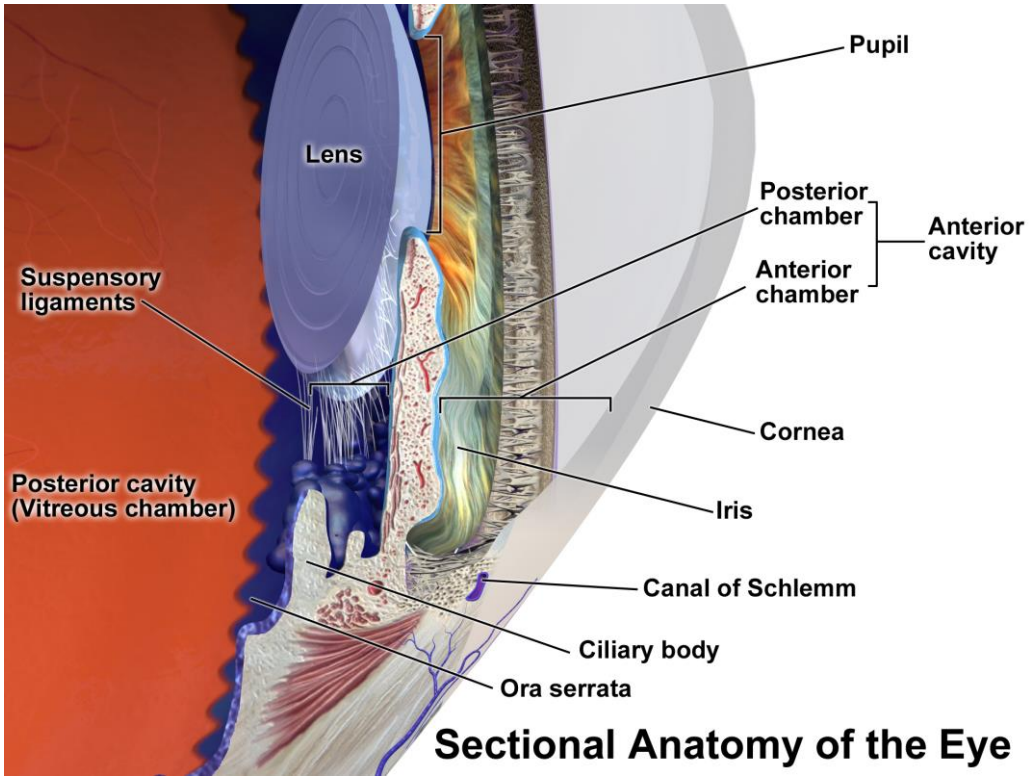


Fig. 4. Anterior segment. *Sectional anatomy of the anterior segment of the eye (Blausen Medical Communications 2014)*

1.7 Vitreous humor

The vitreous humor, also known as vitreous body or just vitreous, is an inert, transparent hydrophilic gel constituting four-fifths of the globe volume and spanning from the posterior limit of lens and ciliary body (anteriorly), to the retina (posteriorly). It is a single block made for the 99% of water beside hyaluronic acid, sugars, ions, collagen and other proteins. It is coated by a layer of collagen called vitreous of hyaloid membrane and its shape follows the spheroidal profile of the eye with an anterior depression called *patella fossa* corresponding to the area of contact with the lens (Bowling and Kanski 2015).

Despite the low number of solid particles, vitreous has a consistency that supports and maintains the shape and internal pressure of the eye, protecting the globe from damages and alterations and avoiding retinal detachments. It also has an important role as a barrier in diffusion of substances between the anterior and the posterior segments and as a metabolic buffer, acting as a reservoir for the metabolism of ciliary body and retina, with several substances accessing the vitreous rather than the bloodstream, due to the tight and selective blood barriers of the eye. Least but not last, obviously, its most important role is to allow light passage to the retina, guaranteed by vitreous high transparency given by the low concentration of macromolecules (Khurana and Khurana 2006).

1.8 Choroid and eye vascularization

Along with ciliary body and iris, choroid forms the vascular layer of the eye, known as uvea. It provides nourishment and oxygen to outer retina and lies between retina and sclera, accounting for the 85% of total blood flow of the eye (Delaey and Van De Voorde 2000).

Indeed, there are two main circulations in the eye: uveal and retinal (**fig. 5**). They both origins from a main vessel deputed to carry blood into the eye: the ophthalmic artery, a branch of the internal carotid artery. This main artery separates in several vessels nourishing the orbit and the eye bulb. Therefore, it is possible to distinguish different branches addressed to different areas and layers of the eye (Ehrlich et al. 2017):

- Central retinal artery pierces the sheath of the optic nerve behind the globe and for its last tract travels inside the nerve. After entering in the eye, it divides to supply the inner retina.
- Ciliary arteries can be further divided into anterior, long posterior and short posterior. Anterior ciliary arteries travel with the extraocular muscles, penetrating

the sclera near to the limbus to then reach the major arterial circle of the iris, nourishing sclera, conjunctiva and iris. There are two long posterior ciliary arteries in each eye, penetrating the sclera around the optic nerve and travelling into the suprachoroidal space, between sclera and choroid, all the way up to the ciliary body, reaching the arterial circle of the iris. Short posterior ciliary arteries pierce the sclera closer to the optic nerve, supplying the choroid and the optic disc (optic nerve head) through the circle of Zinn-Haller, an anastomotic ring of vessels (Kiel 2010).

The venous system outflow exiting the eye happens primarily through the central retinal vein (running in parallel with the central retinal artery) and the vorticose veins, collecting blood from the choroid (Cheung and McNab 2003; Kutoglu et al. 2005).

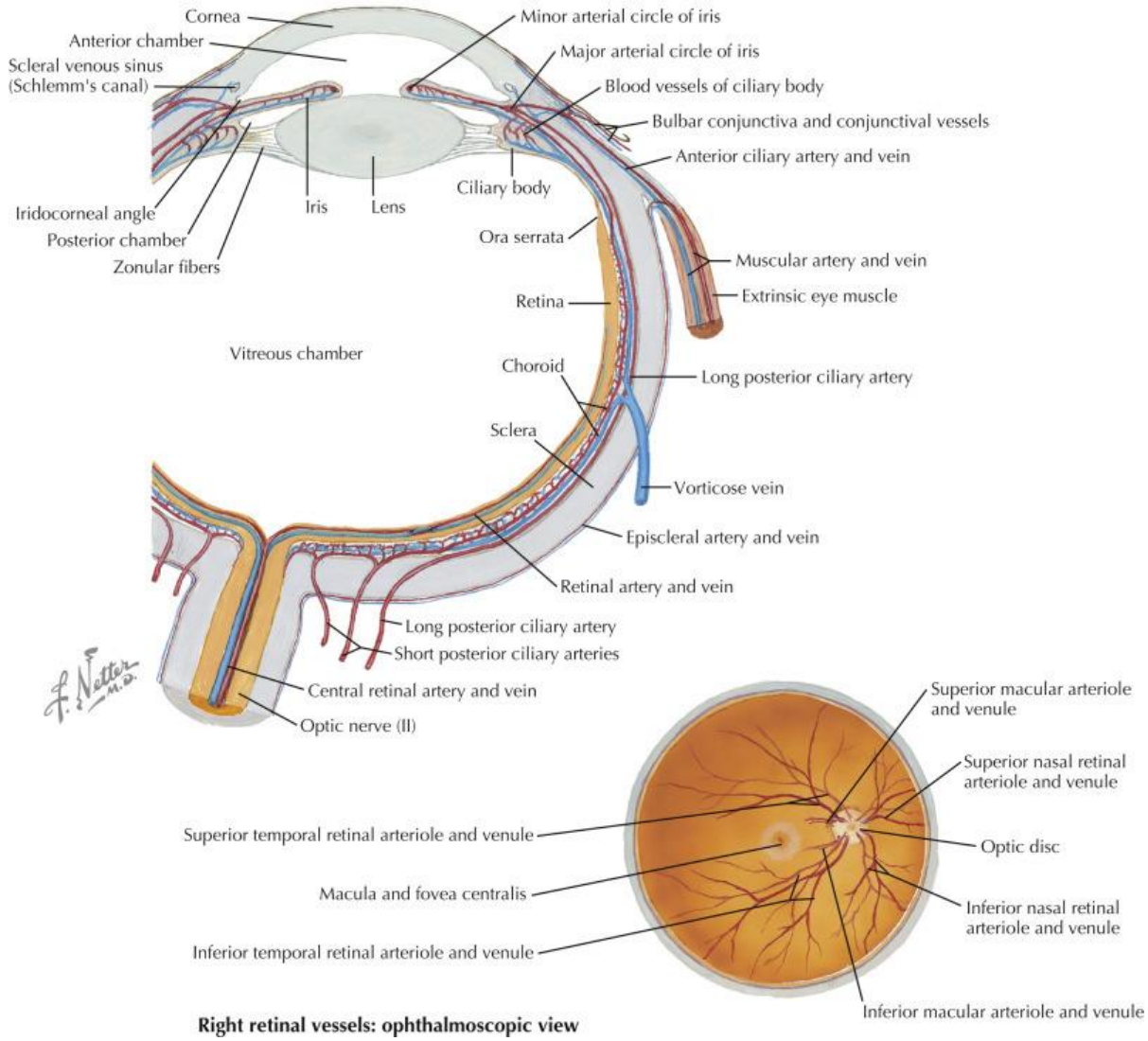


Fig. 5. Eye blood system. *Eye arterial and venous vascular systems in detail (Felten et al. 2016).*

1.9 Retina

The retina is probably the most important component of the eye, having the role of capturing light focused by cornea and lens from the outside, converting it into a nervous signal and sending it to the brain. It extends from the *ora serrata*, a serrated line representing the junction between retina and ciliary body, to the posterior part of the eye. It comprises a central part called macula, where photoreceptor concentration is maximum (especially in its central part, a depression of the tissue called *fovea centralis*) and the optic disc (also known as optic nerve head or blind spot) where optic nerve exits the eye and there is no presence of photoreceptors (Anastasi, Balboni, and Motta 2007).

Retina is a complicate and deeply organized tissue with the so-called neurosensory retina as the inner main actor in visual process, together with the outer part of the retina, the RPE. Retina is a full-fledged part of the CNS deriving, during development, from the diencephalon and composes the nervous tunic, the innermost layer of the eye (Forrester et al. 2016).

Opposite to what would be the most intuitive feature, retina is inverted (**fig. 6**). Visual information is encoded as nervous signals by photoreceptors, processed by retinal neurons, and then sent to the brain via the optic nerve, but photoreceptors are not the first exposed cells to light. On the contrary, they are the most external part of the neuroretina and light has to pass through all the other layers before reaching them (Kröger and Biehlmaier 2009).

As said, several different layers compose neurosensory retina:

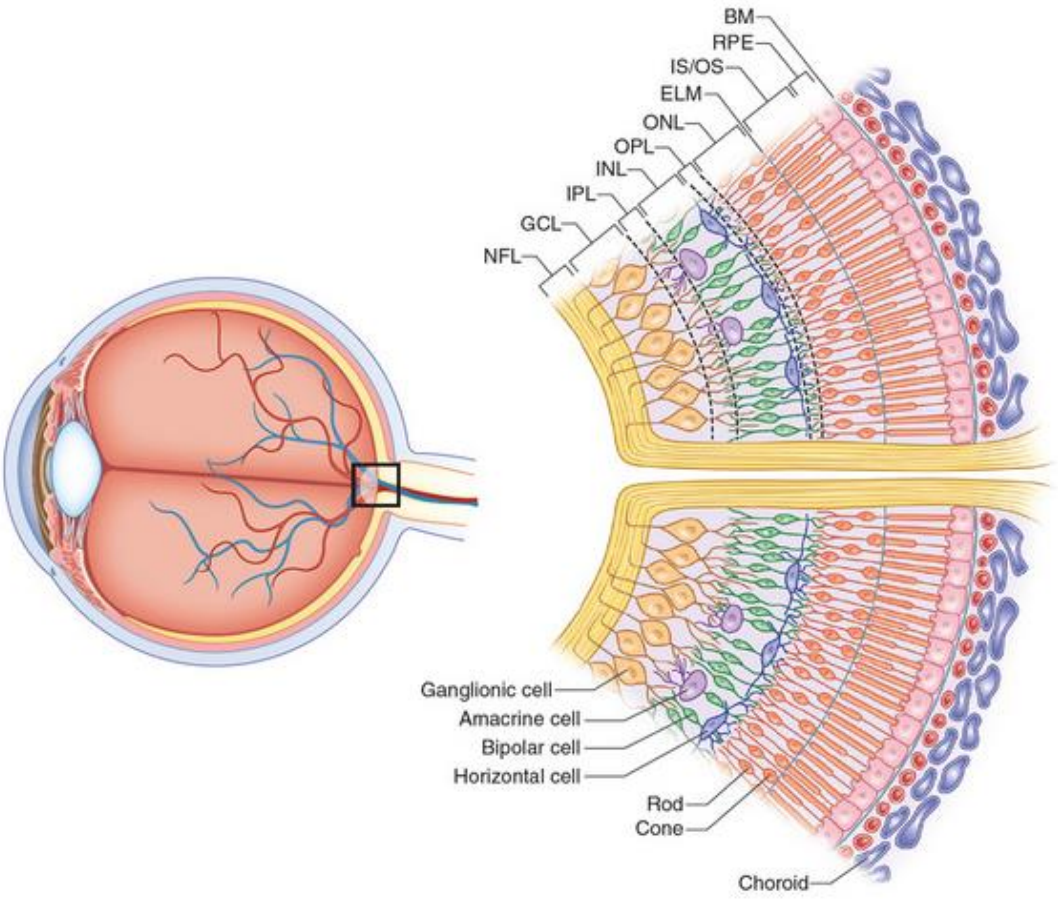


Fig. 6. Retina. *Retinal layers and cellular components in detail (The Retina Reference 2019).*

Proceeding from the vitreous to the choroid there are:

- Internal limiting membrane (ILM), the boundary between retina and vitreous body. It is composed by the expanded terminations of Müller cells except for the optic disc, where they are replaced by astrocytes (Remington 2012).
- Nerve fiber layer (NFL), where axons of the ganglion cells gather to reach the optic nerve. Indeed, this layer is thicker in the area close to the optic disc, where the nerve exits the eye, while is thinner in the periphery of the retina.
- Ganglion cell layer (GCL), where lie their nuclei.
- Inner plexiform layer (IPL) contains synapses between bipolar cell axons and dendrites of ganglion and amacrine cells.

- Inner nuclear layer (INL) contains nuclei and surrounding cell bodies of amacrine cells, bipolar cells, and horizontal cells.
- Outer plexiform layer (OPL) is where the projections of photoreceptors create a complex network of synapses with dendrites of bipolar and horizontal cells.
- Outer nuclear layer (ONL) is the layer where lies the bodies of photoreceptors.
- External or outer limiting membrane (ELM/OLM), layer that separates the external portions of the photoreceptors from their cell nuclei.
- Photoreceptors inner segment (IS) and outer segments (OS). As seen above, nuclei and synaptic bodies of photoreceptors are in ONL and OPL, respectively. Over the OLM, in the outermost pert of the retina, are IS and OS (Kolb 2005; The Retina Reference 2019).

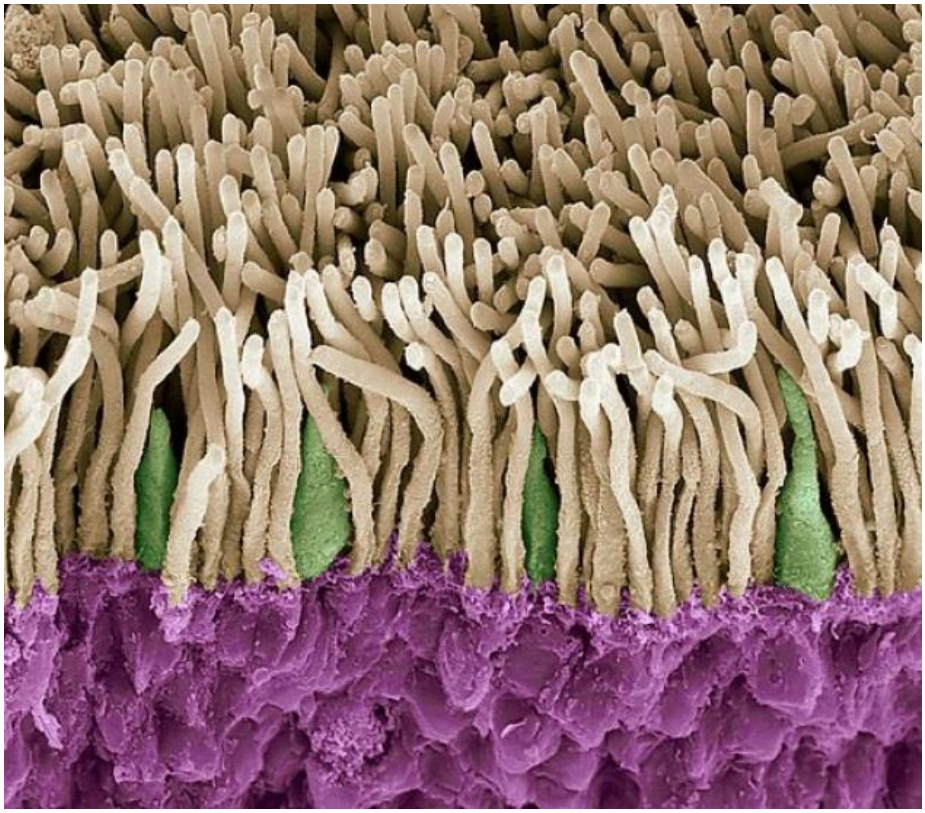


Fig. 7. Cone and rods. *Scanning electron micrograph of human rod (gray) and cone cells (green) adjacent to the outer nuclear layer (purple) (Gschmeissner 2015).*

1.9.1 Photoreceptors

Humans have two different kinds of photoreceptors: cones and rods (**fig. 7**). There are approximately 115 million rods and 6.5 million cones in the human eye. Rods are responsible for sensing contrast, brightness and motion, while cones are responsible for fine resolution, spatial resolution and color vision. As names suggest, cones have a conical shape and are short and large, while rods are longer and thin. The relative density of photoreceptors varies depending on different regions of the retina: in the periphery, rods are predominant (30000/mm2), while cones density increases nearer to the macula, with the *fovea* having exclusively cones presence (150000/mm2) (Forrester et al. 2016).

Both have the same general structure, with a synaptic termination (OPL), their bodies containing the nuclei (ONL) and the most external part composed by IS and OS. IS is the part of the cell specialized in metabolic activity, with high number of mitochondria for ATP production, ribosomes and having the role of assembling opsin for the OS. OS is the part deputed to light absorption through specialized disks filled with the visual pigments, molecules that absorb light at certain wavelength (Kolb 2012) (**fig. 8**).

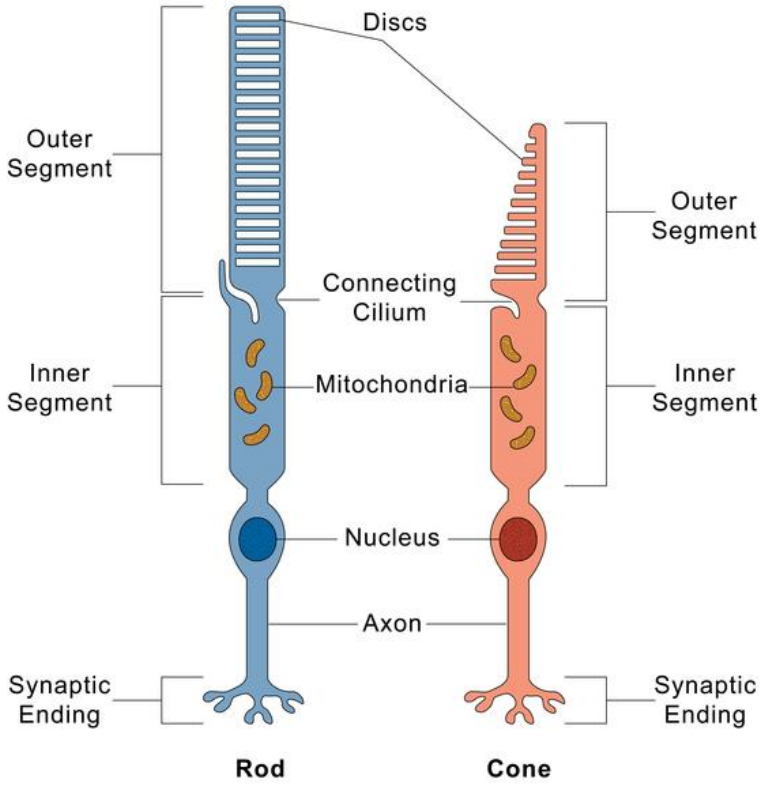


Fig. 8. Cone and rods. *Schematic representation of cones and rods cellular components (NIH 2019).*

In human, usually, there are three types of cones and a single type of rods. Rods contain the visual pigment rhodopsin and are sensitive to blue-green light with a peak sensitivity of 496 nanometers (nm) wavelength. Rods are highly sensitive and are used for vision in the dark. Cones contain cone opsins as their visual pigments and, depending on the exact structure of the opsin molecule, are maximally sensitive to either long (L-cones for red light, peak at 560 nm), medium (M-cones for green light, peak at 530 nm) or short wavelengths (S-cones for blue light, peak at 420 nm) (Forrester et al. 2016) (**fig. 9**).

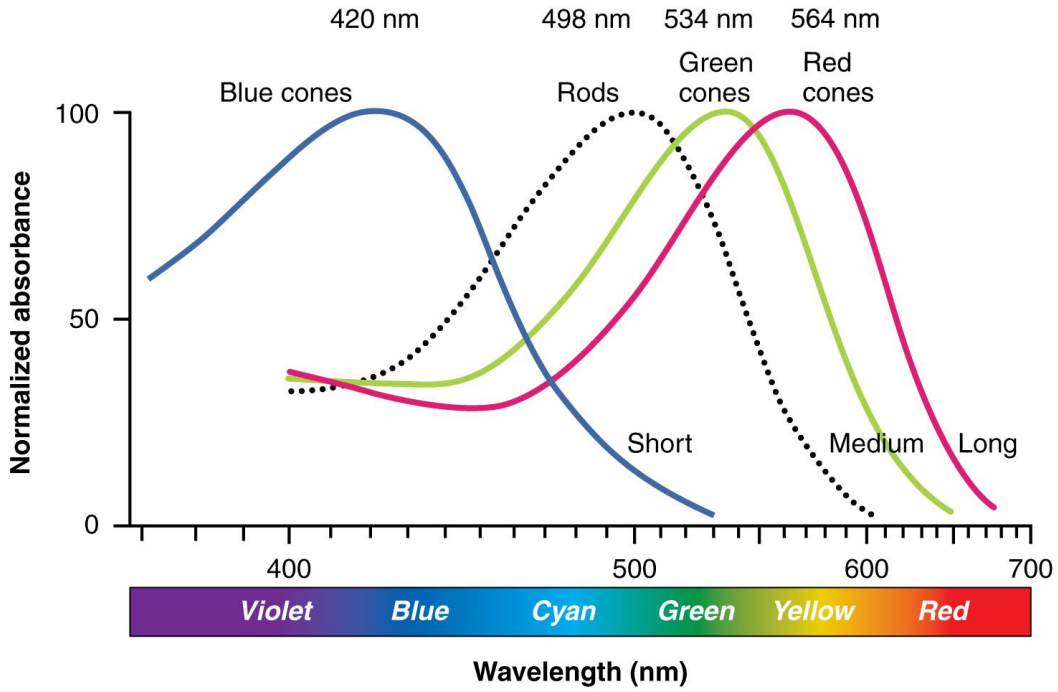


Fig. 9. Cones and rods pigments wavelengths. *Human rod cells and the different types of cone cells each have an optimal wavelength. However, there is considerable overlap in the wavelengths of light detected. The color we perceive is a result of the ratio of activity of our three types of cones (Openstax College 2013).*

1.9.2 Retinal pigmented epithelium (RPE)

RPE is a monolayer of highly polarized hexagonal cells having several fundamental roles for the visual process (**fig. 10**). It is located between the neuroretina and the Bruch's membrane, the internal part of the choroid: the apical microvilli of the RPE cells interdigitate with the photoreceptor outer segments (POS), while the RPE basal side is attached firmLy to the its basement membrane that is the innermost layer of Bruch’s membrane (Thumann et al. 2013).

There are about 3.5 million RPE cells rich in melanin that confers to the tissue its characteristic brown color. Melanin role is to absorb light, to minimize the excess of signals that could create alterations in visual perception, and to act as an antioxidant against ROS production (Hu, Simon, and Sarna 2008).

RPE exerts a tremendous amount of essential functions for vision. Of course, as said, is involved in capturing scattered light, but has also a principal role, for example, in the constant renewal of POS. In the eye, light is focused by cornea and lens onto the macula, resulting in a strong concentration of photo-oxidative energy added to a high perfusion and saturation of oxygen derived from choroidal blood other than, of course, oxidation due to metabolic

activity (Kruk, Kubasik-Kladna, and Aboul-Enein 2015). Retina put in place mechanisms of prevention of oxidative stress as, for example, the presence of ROS scavenger and light absorbent pigments such as lutein and zeaxanthin, yellow carotenoids from which derives the name *macula lutea* (in Latin, yellow spot) (Koushan et al. 2013). Despite of that, the continuous oxidative stress leads to damages to the POS, needing a constant repair and renew through the RPE, one of the most actively phagocytic cells in our body (Mao and Finnemann 2013).

Another essential function of the RPE is participating in the visual phototransduction. Everything starts from 11-*cis*-retinal, a photosensitive retinoid produced by RPE and deriving from vitamin A or retinol. This molecule migrates to the POS where it binds opsin, the pigment present in photoreceptors. When a light photon strikes 11-*cis*-retinal, its configuration changes in all-*trans*-retinal and the opsin changes its conformation and binds another protein that is called transducin. This molecule activates a phosphodiesterase, leading to the breakdown of cGMP, lowering its concentration in POS and reducing the cGMP available for binding to membrane channels that in this way close, which results in hyperpolarization and generation of a nervous impulse. To restore the initial conditions, there is the contribution of another protein called arrestin that attenuates the activation of transducin (Gurevich and Benovic 1995; Kiser, Golczak, and Palczewski 2014; Purves et al. 2001).

RPE is also responsible for secretion of several factors needed for regulates neighboring cells and is also able to communicate with the immune system, controlling and reducing its activity in healthy conditions. As seen above, this behavior, along with RPE barrier properties, confers to the eye "immune-privileged" conditions in comparison to the majority of other tissues of the body (Strauss 2010).

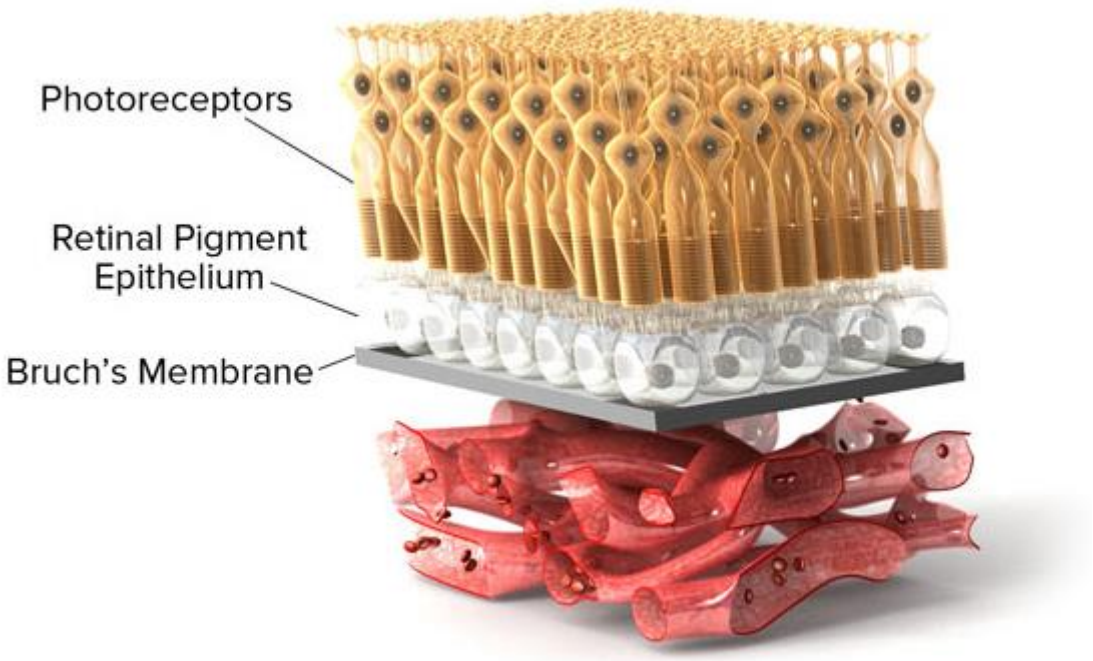


Fig. 10. RPE. *Schematic representation of the healthy RPE with the related elements: choroid, Bruch’s membrane and photoreceptors (Eye Care Fundamentals 2014).*

2. Blindness in the World

Beside the improvements in research and medical science in general, blindness is still a relevant concern not only in developing countries, but also in the so-called "western World".

The International Classification of Diseases classifies vision impairment in two groups:

Distance vision impairment

Mild – presenting visual acuity worse than 6/12 m (20/40 ft)

Moderate – presenting visual acuity worse than 6/18 m (20/60 ft)

Severe – presenting visual acuity worse than 6/60 m (20/200 ft)

Legal blindness – presenting visual acuity worse than 3/60 m (20/400 ft)

Near vision impairment

Presenting near visual acuity worse than 6/12 m (20/40 ft)

These values mean that, for example, a subject with mild visual impairment, sees at 6 meters of distance what a normal sighted subject sees at 12 meters, while 6/6 is considered as normal vision (Keeffe et al. 2013).

Need to be highlighted that visual impairment has also an important subjective component depending on how the individual experience his/her condition. Factors as the country of residence and widely different health care systems, unfortunately affect the chances to access to different treatments and supports in order to prevent, delay, or treat pathologies as well as ameliorate life conditions when is too late or there are no options to intervene (World Health Organization 2018).

Considering all the existing conditions, from the less impacting and easily resolvable to the legal blindness, in the whole world nearly 1.3 billion people suffer for some kind of visual impairment. In details 188.5 million have mild vision impairment, 217 million have moderate to severe vision impairment, and 36 million people are blind (R. R. A. Bourne et al. 2017). In the last years, population growth and expanded life span augmented the risk to acquire vision impairment and this problem is going to rise considerably in the next decades. We will need to face with acceleration in the increasing numbers of people affected by visual issues (**fig. 11**). Prevalence is not only age-related but, unfortunately, also dependent on the level of development of the countries, with a prevalence of moderate to severe blindness in regions like North Africa, Middle East and South Asia.

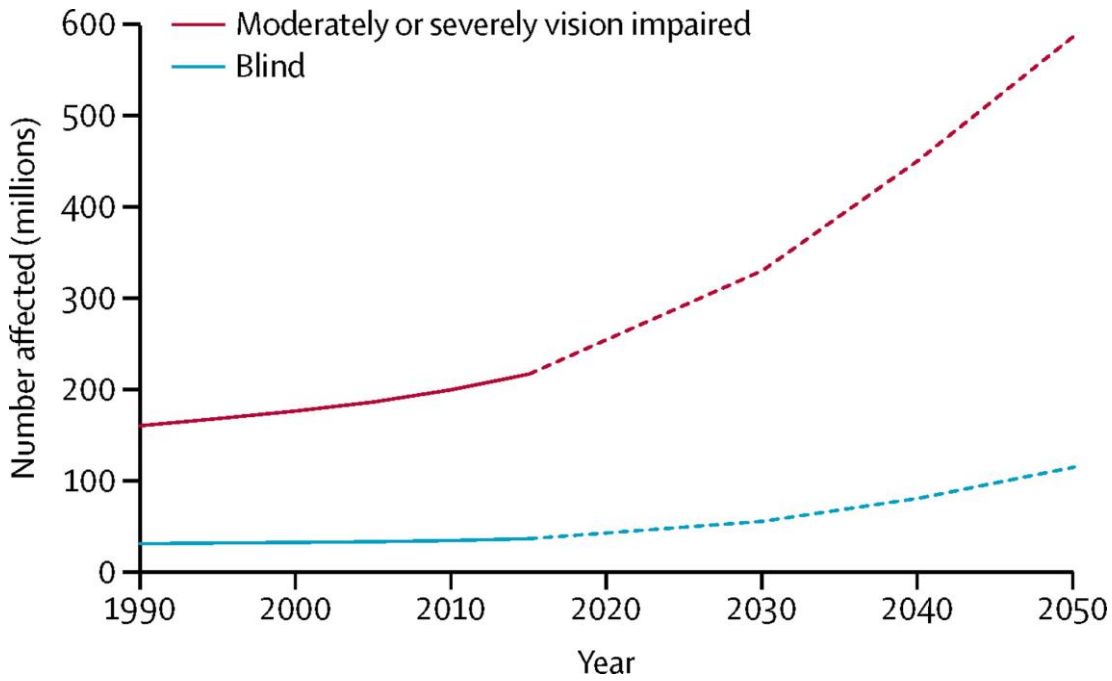


Fig. 11. Blindness in the World. *Global trends and predictions of numbers of people who are blind or moderately and severely vision impaired, form 1990-2050 (R. R. A. Bourne et al. 2017).*

2.1 Main causes

2.1.1 Uncorrected refractive errors

There are several pathologies and conditions related to visual impairment and among them, uncorrected refractive errors are the most common. Considering all different types and gravity of affection, in some situations they can deeply affect quality of life. Uncorrected presbyopia is considered the most diffuse of these refractive errors, given that 1 billion people are estimated to be affected (Fricke et al. 2018). Presbyopia is an age-related condition in which the ability to focus up close becomes more difficult. As the eye ages, the lens can no longer change shape enough to allow the eye to focus close objects clearly. Other common forms of refractive errors are:

-Myopia (nearsightedness), a condition where close objects appear clearly, while objects far away appear blurry. With myopia, light comes to focus in front of the retina instead of on the retina.

-Hyperopia (farsightedness), a common type of refractive error where distant objects may be seen more clearly than near ones. However, people experience hyperopia differently. Some people may not notice any problems with their vision, especially when they are young.

For people with significant hyperopia, vision can be blurry for objects at any distance, near or far.

-Astigmatism, a condition in which the eye does not focus light evenly onto the retina, the light-sensitive tissue at the back of the eye. This can cause images to appear blurry and stretched out (World Health Organization 2010).

Refractive errors cannot be prevented, but they can be diagnosed by an eye examination and treated with corrective glasses, contact lenses or refractive surgery. If corrected in time and by eye-care professionals, they do not impede the full development of good visual function. Correction is provided in different forms according to factors as gravity of the defect and age of the subject (Kandel et al. 2017).

2.1.2 Cataract

Cataract is globally the leading cause of reversible blindness and visual impairment. The only available treatment for cataract is surgery, but outcomes are usually good and complications can be easily prevented or managed (Lam et al. 2015). This condition is more common in populations with low socioeconomic status and in developing countries. For this reason, the greatest challenge remained to fight is the growing amount of patients with cataract blindness in the developing World due only to lack of access to affordable surgery (Perruccio, Badley, and Trope 2010; Nair, Chou, and Geiss 2014). Efforts aimed at training additional cataract surgeons in these countries do not keep pace with the increasing demand associated with ageing population demographics. In the absence of strategies that can prevent or delay cataract formation, it is important to focus efforts and resources on developing models for efficient delivery of cataract surgical services in underserved regions (Mundy, Nichols, and Lindsey 2016).

Usually cataracts develop because of ageing of the crystalline lens. Degeneration and accumulation of altered crystalline fibers can depend by different reasons like oxidation, diabetes, inherited genetic disorder, inflammations, etc. and this leads to lens opacity (Sinha, Kumar, and Titiyal 2009; Allen and Vasavada 2006). Moreover, different kinds of trauma can bring to cataract. This conditions may also be developed due to smoking, diabetes, use of corticosteroids or other drugs (Kelly et al. 2005) manly in developed countries. In third World regions, unfortunately, malnutrition, dehydration and excessive

exposure to sunlight are still some of the cause of the pathology progression (Zodpey et al. 1999; Taylor et al. 1988).

2.1.3 Glaucoma

Glaucoma is a group of slowly progressive eye disorders resulting from increased intraocular pressure (IOP) (Yadav, Rajpurohit, and Sharma 2019). This increase causes alterations to the optic nerve, situated on the posterior part of the eye. If the high pressure in the eye is not controlled and reduced promptly, long term exposure can lead to permanent damage of the optic nerve and consequent vision loss (Hertzog et al. 2013). The IOP of a healthy human eye lays between 10 to 21 mm Hg (Timothy and Nneli 2007). In glaucoma, the major risk factor, although not the only one, is an IOP maintained above the usual range. Normally the aqueous humor, the fluid present in the anterior part of the eye, flows out through the trabecular meshwork and drain into the Schlemm's canal, in the posterior chamber. If this system gets blocked, the liquid accumulates (Swarup S. Swaminathan, Dong-Jin Oh 2014).

Less common causes of this pathology include physical or chemical injury to the eye, severe eye infection, blocked blood vessels inside the eye bulb and inflammatory conditions. Even if not often, sometimes eye surgery to correct other conditions can lead to glaucoma. It usually affects both eyes, but it may be worse in one than the other (Schwartz and Budenz 2004).

Among all the different forms of glaucoma, it is possible to distinguish two main variants:

-"Open or wide angle" glaucoma is the most common type of the pathology and develops slowly. “Open-angle” means that the angle where the iris meets the cornea is as wide and open as it should be and the trabecular meshwork looks normal, but the fluid doesn't flow as it would be supposed to. Unfortunately, this form of glaucoma is mostly asymptomatic and without regular eye exam there is a risk of late diagnose, when peripheral vision starts to be affected (Saccà et al. 2019).

-"Angle-closure" glaucoma is less common and can develop both in an acute and chronic form. This form is due to a reduction in the space between iris and cornea, obstructing the flow-through of the aqueous humor. The chronic type can develop slowly without early symptoms, as the open "open angle" glaucoma, while the acute form can lead to severe pain, nausea, visual disturbance and need to be treated immediately (Wright et al. 2016).

2.1.4 Diabetic retinopathy (DR)

This pathology of the retina is a consequence of diabetes mellitus, where chronic high blood sugar levels leads to alterations and damages to the small blood vessels of retina (Kusuhara et al. 2018). Indeed, atherosclerosis and vascular lesion are the main reason for impaired life expectancy in diabetic patients, leading to the well-known mechanism of production of glycated products and ROS, alteration of the vascular endothelium, accumulations of lipids and other metabolites with finally thrombosis and vessel damage (Rask-Madsen and King 2013; Aouiss et al. 2019).

In the earlier stages, no symptoms usually appear, and the disease progresses unnoticed until it affects vision. In this phase, we can speak about NPDR (non-proliferative diabetic retinopathy). When edema and/or bleeding occur, blurred or distorting vision may arise, and floating spots can start to appear. With time, if not properly threated, these symptoms can worsen and develop permanently, leading to vision loss. The advanced status of DR, when retina starts growing new blood vessels is called PDR (proliferative diabetic retinopathy). This neovascularization cause additional damage to the retina, bleeding that can affect also the vitreous and even retinal detachment (NEI 2015).

2.1.5 Corneal opacity

Cornea is the anterior transparent layer on the front of the eyeball. When cornea get damaged or altered, can become opaque leading to blurry and/or reduced vision. Corneal opacity is a consequence of certain types of insult to this usually clear tissue, modifying passage of light through it to the retina and this may cause cornea to appear white or clouded over (Robaei and Watson 2014). Principal causes of this dysfunction are to be addressed to both physical and chemical eye injuries or to infections like measles, herpes simplex and conjunctivitis. Other reasons may be vitamin A deficiency, dry eye, keratoconus and several others syndromes and congenital pathologies (Burton 2009). After minor injuries or scratches, cornea usually heals on its own. Deeper injuries and infections can cause corneal scarring, pain, sensitivity to light, headache, etc. If not prevented or treated appropriately, corneal opacity can lead to severe to total visual loss. Options of treatment include, depending on the source of the issue and on the stage of the damage: antibiotics, antivirals, steroids up to laser surgery through phototherapeutic keratectomy (PTK) and corneal transplant (Rathi, Vyas, and Sangwan 2012).

2.1.6 Trachoma

Trachoma is the leading cause of infectious blindness Worldwide (Resnikoff et al. 2004). This condition is caused by a gram-negative intracellular bacterium named Chlamydia trachomatis (Mohseni and Takov 2019). The pathogen can be contracted by direct contact with someone affected, using common tools, bedding, clothes, etc. Nevertheless, the most frequent way of spread is through particular species of flies that have been in contact with discharge from the eyes or nose of an infected person. Where is diffused, trachoma usually affects especially 3-5 aged children and become less frequent and shorter in duration in older people (Mariotti, Pascolini, and Rose-Nussbaumer 2009). Given that living in proximity of others with active disease is the major cause of contagion, re-acquisition and chronic infections are common in endemic areas even if our immune system can be able to clear autonomously a single episode. In these situations, after repeated and continued infections, damage to the eye can evolve from redness, photosensitivity, burning sensation, lacrimation and palpebral swelling in the first stages up to scarring in the inside of the eyelid that can even turns inwards for the lesions and leading to eyelashes rubbing against the eye (trichiasis) leading to serious damages to the corneal surface. If not properly threated (in the last stages surgical intervention can be necessary), this can result in visual impairment, opacities and blindness. As said, in endemic communities, this can occur in childhood, but onset of visual impairment is more common in adults around 30-40 years old and women are usually more affected, probably for their more frequent contact with children. Just improving hygiene conditions and access to clear water can make a considerable difference together with antibiotics and proper treatments (World Health Organization 2019).

Must be highlighted that there is certain variability among the described causes across different areas of the World, highly related to economics and lifestyle. Indeed, cataract has a higher expression in underdeveloped and developing countries compared to industrialized ones, due to lack of access to proper treatments and surgery. On the other side, in western World, most common diseases are diabetic retinopathy, glaucoma and age-related macular degeneration, related to social habits and greater lifespan.

World Health Organization estimates that, globally, approximately 80% of vision impairment can be avoided, especially in poor countries. There are effective interventions available to prevent and treat several diseases that need only to be addressed somehow to those who are excluded from the treatments. For example, uncorrected refractive error can

be corrected with glasses while cataract surgery can easily restore vision. Vision rehabilitation is also effective in improving functioning for people with irreversible vision impairment. More has to be done to help areas in difficulty with tools that for us are taken for granted (World Health Organization 2018).

3. Age related macular degeneration

Age related macular degeneration (AMD) is a pathology that represents one of the biggest concerns regarding eye care nowadays, being the third cause of blindness worldwide, but the first if considering only developed countries (WHO 2018). Several studies indicate how AMD is foreseen to be a serious issue in the future with an expected rise of its prevalence in the next decades, due to the increase of human lifespan and a consequent larger elderly population (Miller, Bagheri, and Vavvas 2017). Indeed, as indicated by the name itself, aging is the main feature of the disease, being clinically apparent usually in people over 50 years of age and with a much higher prevalence in older people, especially after 75 years of age (BrightFocus Foundation 2016; Jonas, Cheung, and Panda-Jonas 2017).

3.1 Pathogenesis

AMD affects the macula, the central part of the retina, responsible for color and high-resolution vision. The latter phenomenon is due to the largest number of cones of the whole tissue, especially in the fovea, a small depressed zone where only cone cells are present (van Lookeren Campagne et al. 2014). As the pathology proceeds and worsens, central vision can start to appear blurry and distorted leading, in the latest phase, to a complete and permanent loss of central sight. At this point, the only option left is to learn how to better use the residual peripherical vision (Seiple et al. 2005).

The most characteristic clinical sign of AMD is the presence of small yellowish spot in the macula, called drusen, together with alteration in tissue pigmentation. Drusen are deposits of protein, lipids and cellular debris accumulating in the subretinal space, between the RPE and the Bruch’s membrane (Thoreson and Margalit 2014). They can be classified based on their characteristics and dimensions, but the main distinction is between hard and soft drusen. Hard drusen are usually smaller and with defined edges. They are related to a lower progression risk of the pathology. Soft drusen are instead bigger and with irregular and not well-defined shape. They tend to cluster together and are highly related with the risk of

developing AMD (Klein et al. 2004). Even if drusen are a hallmark of AMD, their presence is highly common in elderly population. In the first phases of the disease, vision is usually not affected. During years or decades, growth of deposits and consequent tissue alteration, lead to sight symptoms and AMD development (Ambati and Fowler 2012).

In the last phase of the pathology it is possible to distinguish between two forms, generally called “wet” and “dry” AMD. In the exudative or “wet” neovascular AMD (NVAMD), the progress of the disease comprises new vessels formation in the choroid invading the RPE. These new vessels are weak and leak fluids and blood, leading to hemorrhages and worsening of lipid deposits. With time, macula can lift up from its normally flat position and alteration of the tissue can result scarring and losing of function (AMDF 2019). This is not present in the non-exudative or “dry” form that is also usually recognized as geographic atrophy (GA) (van Lookeren Campagne et al. 2014). Moreover, a certain degree of overlap must be considered, giving that patients with NVAMD experienced the “dry” form. In the “dry” form, increase of deposits and drusen lead to tissue alterations and losing of functions due to thinning and drying of the macula with death of RPE and photoreceptors (AMDF 2019). NVAMD account approximately for 10-15% of AMD cases, but is responsible for about 90% of blindness related to the pathology (Kolar 2013). This is due to its more rapid and severe progression compared to GA that, conversely, develops slowly (Morris et al. 2007).

3.2 Risk factors

Despite several studies on causes and risk factors have been made, AMD pathogenesis is still not fully elucidated. Indeed, being a multifactorial and chronic condition, several elements can be related to it and understanding the global mechanism of the disease is still a complicated target to reach. As other pathologies progressing during elderly, AMD develops due to gradual impaired functions and reduced restoration mechanisms: processes as autophagy and deposit removal start decreasing their efficiency (Nowak 2014). Moreover, as seen above, RPE is a tissue with a high metabolic activity having the role, for example, of renewing POS and participate in visual phototransduction. In this conditions, deposit of debris is a common event facilitated by autophagy and lysosome activity failure during aging, leading to accumulation of metabolites (Golestaneh et al. 2018). Several kinds of protein, lipids and cellular components constitute deposits and drusen such as albumin, clusterin, Apoliprotein E (ApoE), complement factors, immunoglobulins,

amyloid-β and many others (Wang et al. 2010; Wolf 2003) including lipofuscin, a yellowish pigment made of accumulated and damaged proteins, fats and derivates of the visual phototransduction process (Biesemeier, Schraermeyer, and Eibl 2011). RPE is also constantly exposed to light and, even if protected by the anterior structures of the eye as cornea and lens, UV rays can strike retina and concur to the oxidative damage characteristic in AMD (Balaiya et al. 2010; Glickman 2011). Smoking is well known for having a strong correlation with AMD (Seddon et al. 1996) as well as obesity, hypertension and diabetes mellitus, due to their role in vascular alteration and oxidative stress (Chakravarthy et al. 2010).

As said previously, retina is characterized by the presence of the blood-retinal barrier, conferring an immune privilege if compared to other tissues. However, in this complex environment, accumulation of debris and oxidation of macromolecules can lead to immune system activation (Ambati and Fowler 2012). Activated complement system is one of the best-known components of this mechanism, concurring to initiate the inflammation. Complement is constituted by plasma proteins that in presence of certain signals enhances the ability of antibodies and phagocytic cells to restore normal conditions (Janeway et al. 2001). Moreover, cytokines as tumor necrosis factor alpha (TNF-α), interferon-γ (IFN- γ) and several interleukins contribute to stimulate inflammation and activate immune cells (Sato et al. 2018): resident glial cells, macrophages, monocytes and cells from the blood circulation are then recruited and stimulated, contributing to the AMD multifactorial damage (Frederick and Kleinman 2014).

Genetics is another important contributing element of the disease. One of the strongest correlations found between AMD and genetic alterations, regards complement factor H (CFH), widely recognized as a major risk factor in the inflammatory component of AMD. Indeed, CFH inhibits a key activation step in complement activation, thereby reducing complement-induced host cell damage and inflammation (Ambati and Fowler 2012). Another prominent genetic association with AMD concerns the region on chromosome 10 harboring two genes: high temperature required factor A1 (HTRA1) and age-related maculopathy susceptibility protein 2 (ARMS2) genes (Grassmann et al. 2017). While the functional pathway involving the ARMS2/HTRA1 genes is unknown, the alternative complement pathway where CFH participates is well described and annotated and further

investigations on the entire complement system, revealed associations between AMD risk and other complement components (Deangelis et al. 2011). Genetic association was found also in elements involved in cholesterol metabolism as ApoE, a protein involved in cholesterol membrane transportation. Moreover, ATP-binding cassette transporter A1 (ABCA1), hepatic lipase (HL, coding genes named LIPC) and cholesteryl ester transfer protein (CETP) are other genes involved in cholesterol metabolism and transport shown to have some degrees of connection with AMD. Metalloproteinase inhibitor 3 (TIMP3) is another gene that has been related to AMD, due to its role in degradation of extracellular matrix (ECM). In situations as inflammation and oxidative stress, it can be induced and its genetic variants have been associated to AMD (Kolar 2013; Shaw et al. 2016).

3.3 Therapies

To date, there is no treatment available for GA, while there have been developed therapeutic solutions for NVAMD. The first approach for the “wet” form of the pathology was laser photocoagulation, the use of a laser directly on retinal leaky vessels to close them and reduce edema and tissue damage. The problem was that this treatment produced burns and lesions and many clinicians objected to this approach causing immediate destruction of central vision in patients who could have various degrees of residual macular function (Virgili and Bini 2007).

After laser photocoagulation, the photodynamic therapy (PDT) was developed. It consists in the use of a light treatment after administering verteporfin, a drug working as a photosensitizer. When the drug is exposed to a specific wavelength of light, it produces free radicals that kill nearby cells. This treatment results in a selective destruction and sealing of leaking vessels while leaving healthy ones intact, but without excluding risks of retinal scars and additional vision loss. Moreover, even the most successful treatments do not preclude reoccurrence, making multiple treatments likely. Rate of vision loss may be slowed down and some sight may be preserved, but this method is not a cure and even in the best case scenario it preserves the status quo not restoring vision that is already lost (AMDF 2019). A breakthrough in NVAMD has been the anti-VEGF therapies, currently the most common and effective clinical treatment. Ranibizumab, bevacizumab and, with time, aflibercept and pegaptanib are all molecules that with different mechanism antagonize vascular endothelial growth factor (VEGF), a protein that stimulates the formation of blood vessels. These drugs

are administered monthly through intravitreal injections, blocking new vessels formation and even receding blood vessels already present. Anti-VEGFs slow down the progression of the disease and, in some cases, lead to moderate gains in vision (AMDF 2019).

As said above, to date, GA still doesn’t have any cure, leaving with no options to restore vision loss and macular damages to RPE and photoreceptors. During years were studied several nutrients linked with decreased likability of AMD progression and was demonstrated that people constantly consuming vegetables rich in antioxidants in their diet, have less risks to develop the pathology (Merle et al. 2019; Pratt 1999). The National Eye Institute’s Age-Related Eye Disease Study (AREDS) found that the progression of AMD could be delayed or prevented by taking nutritional supplements with a specific high-dose formulation of antioxidants (vitamin C, vitamin E, beta-carotene and zinc). A follow-up trial (AREDS2) was completed in 2013. In that study researchers found that the addition of omega-3 fatty acids to the supplements did not improve the formula’s success, but the antioxidants lutein and zeaxanthin proved to be safer than beta-carotene, which increases the risk of lung cancer for smokers or ex-smokers (Tisdale et al. 2019). Patients at risk of developing AMD are recommended to take AREDS-type supplements, as this formulation has been proven to reduce the risk of progression to advanced AMD (Krishnadev, Meleth, and Chew 2010).

Aims

1. Integrins as a potential target for “dry” AMD

1.1 Integrins

Integrins are cell surface heterodimeric receptors that mediate attachment of cells to extracellular matrix (ECM) and take part into cell-to-cell interactions. The integrin family comprises several glycoprotein in mammals, formed by the combination of 18 α and 8 β subunits that can assemble into 24 different heterodimers (Barczyk, Carracedo, and Gullberg 2010).

Integrins have a globular head and two leg regions (one from an α-subunit and one from a β-subunit) inserted within the plasma membrane and, therefore, each subunit comprises an extracellular domain, a transmembrane domain, and a cytoplasmic tail (Srichai and Zent 2010). Integrin activity is regulated by conformational changes and it is possible to distinguish between three states: bent, extended and extended with an open headpiece (**fig. 12**). In the two extended states, the ligand-binding site faces away from the cell surface, while is oriented toward the plasma membrane in the bent conformation, impeding ligand engagement. Activation requires the extended-open state, which binds ligand with much higher affinity than the bent-closed and extended-closed states (Askari et al. 2009; Campbell and Humphries 2011; Li and Springer 2017). This model is currently the most widely accepted, but there is also evidence that integrins can bind ligand in a bent (or partially unbent) conformation (Arnaout, Goodman, and Xiong 2007).

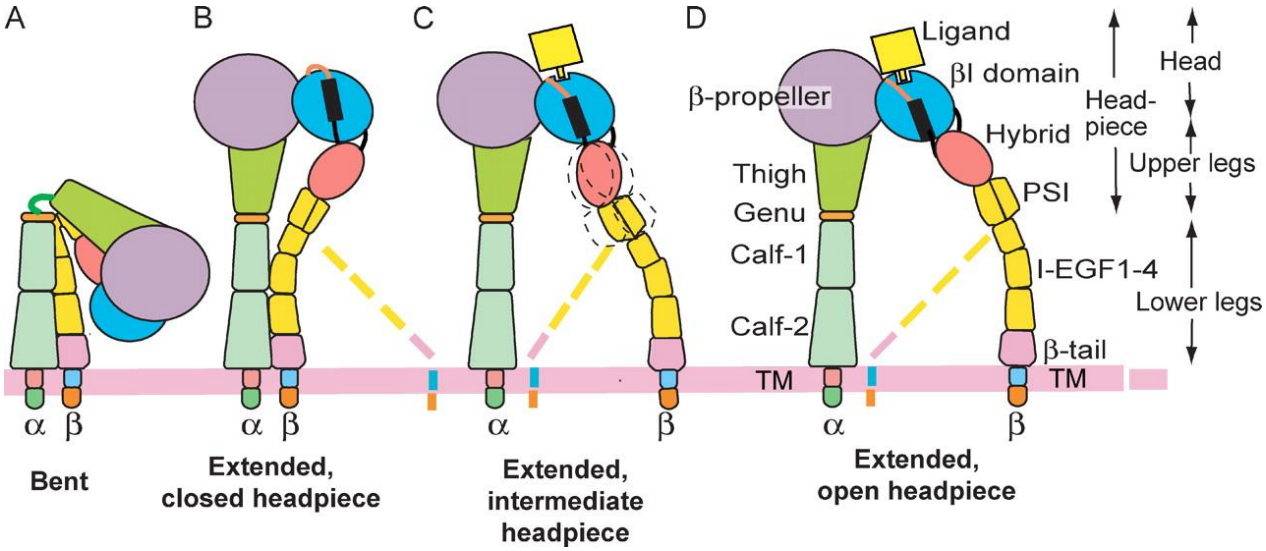


Fig. 12. Integrin conformations. *Different conformations of integrin heterodimers dependent on the activation state (Yu et al. 2012).*

Other than having roles in cell adhesion, integrins mediate also signal transduction. Extracellular domains can bind ECM proteins such as collagen, laminin, fibronectin, vitronectin, and some other cellular receptors, while the cytoplasmic domains can associate directly with several cytoskeletal proteins and intracellular signaling molecules. These interactions modulate fundamental cell processes and functions as cell adhesion, proliferation, migration, and apoptosis (Pan et al. 2016). One of the pathways most involved in integrins transduction, is the MAPK/ERK pathway. The signaling pathway culminates in ERK activation and is activated by extracellular stimuli such as adhesion to the extracellular matrix or growth factors. Cell adhesion activates ERK by binding of integrins at the cell surface to extracellular matrix proteins such as fibronectin (Juliano et al. 2004; Yee, Weaver, and Hammer 2008).

As seen above, integrins can undergo to conformational changes in their extracellular domains and this is due to signaling events inside cells. This process, often referred as inside-out signaling, is initiated by adaptor molecules that affect the position of the cytoplasmic tails of α and β subunits relative to each other and to the plasma membrane. The best-known positive regulators of integrin activation are the adaptor molecules talin 1 and the kindlins. Upon activation, integrins initiate ligand-dependent intracellular signaling, a process that is called outside-in signaling because it is initiated by the binding of extracellular ligands (Ley et al. 2016).

1.1.1 Leukocyte integrins

Inflammation leads to leukocyte recruitment in the site of injury, a process involving adhesion molecules, chemokines, cytokines and other regulatory molecules. Leukocyte recruitment into inflamed tissue follows a specific cascade of events, beginning with capturing of circulating leukocytes to the endothelium, followed by rolling, adhesion to endothelial cells, strengthening, crawling, and finally transmigration. During this process, integrins play an essential role (Herter and Zarbock 2013) (**fig. 13**).

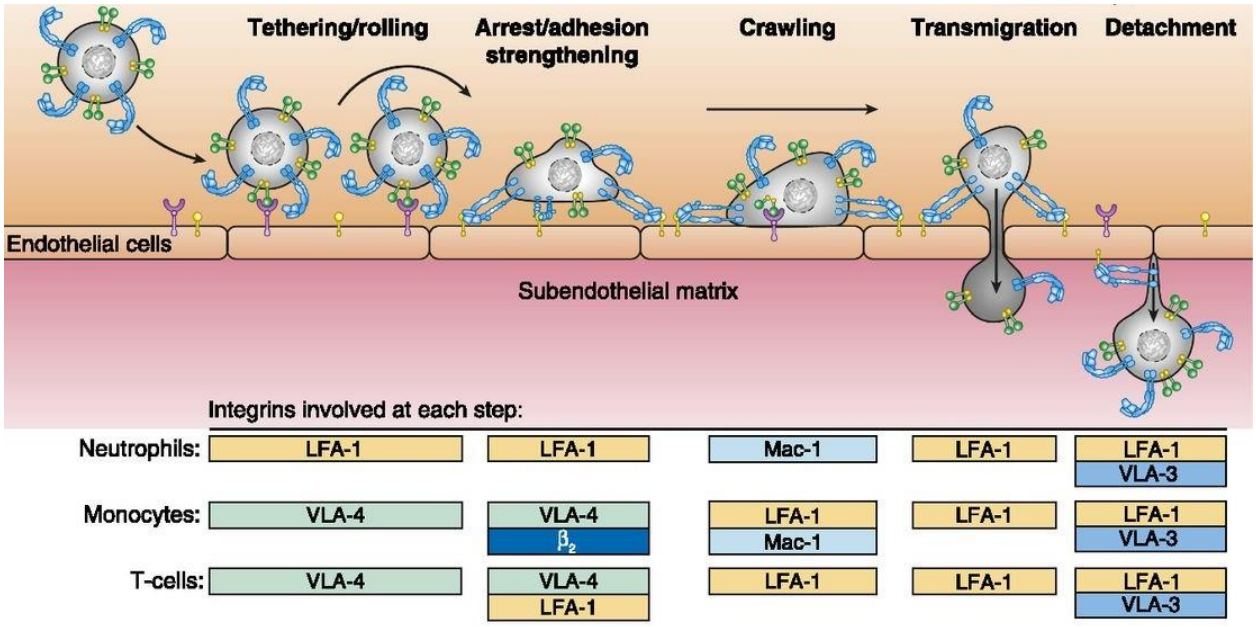


Fig. 13. Diapedesis. *Detailed diapedesis mechanism of immune cells and fundamental involvement of integrins (Herter and Zarbock 2013)*.

Among all the different family components, integrins expressed predominantly by leukocytes consist of a β2 subunit coupled with one of several α subunit counterparts (αLβ2, αMβ2, αXβ2, and αDβ2) or an α4 subunit with its β subunit counterparts (α4β1 and α4β7). Specifically, a prominent role in controlling extravasation and inflammation is leaded by αLβ2, or lymphocyte function-associated antigen-1 (LFA-1) and α4β1 or very late antigen-4 (VLA-4) (Hyun, Lefort, and Kim 2009).

αLβ2 is involved in various cell-cell interactions, such as T cells-antigen presenting cells, B cells-T cells and natural killer (NK) cells-target cells and in the formation of the immunological synapse (Grakoui et al. 1999; Shimaoka and Springer 2003) and its

deficiency is related to defects in immune responses and pathologies as leukocyte adhesion deficiency (LAD) (Ghosh et al. 2006; Springer 1994). αLβ2 binds to intercellular adhesion molecules (ICAMs) and among them ICAM-1 is its principal ligand (Marlin and Springer 1987).

Integrin α4β1 is expressed by most resting lymphocytes, eosinophils, and monocytes (Luo, Carman, and Springer 2007) and is an important component in immune function, playing a role in lymphocyte differentiation and homing, as well as in tissue-specific migration during inflammation. Vascular cell adhesion protein 1 (VCAM-1) and fibronectin are their main ligand (Butcher et al. 1999). Although the αLβ2/ICAM-1 interaction is crucial in the structure of the immunological synapse, the α4β1/VCAM-1 interaction also contributes to its formation (Mittelbrunn et al. 2004).

1.2 New antagonist molecules of leukocyte integrins

As said in the dedicated chapter, RPE is a monolayer in the retina having several fundamental functions including regulation of nutrients, transport to the photoreceptors, POS phagocytosis and renewal, scattered light absorption and, being part of the BRB, protection of the retina and contribution to prevent spread of substances from the blood circulation (Rupenthal, Huang, and Chen 2018). RPE cells play also an important role in immune responses, expressing major histocompatibility complex (MHC) molecules, adhesion molecules, and cytokines. Cytokines secreted by RPE cells contribute in different ways to immune and inflammatory responses (Gregerson et al. 2007). Among these molecules, ICAM-1 is a cell surface protein that mediates adherence of leukocytes through the interaction with the integrin LFA-1 (or αLβ2) and Mac-1 (or $\alpha_M\beta2$) (Devine, Lightman, and Greenwood 1996; De Fougerolles, Qin, and Timothy 1994). Moreover, ICAM-1 expression on vascular endothelial cells regulates binding and cross-migration of leukocytes and extravascular leukocyte trafficking (Frank and Lisanti 2008; Wee et al. 2009). ICAM-1 was also found on human RPE cells as well as VCAM-1. Both molecules are involved in extravasation and inflammation and their expression is upregulated in response to inflammatory cytokines such as TNF- α, interleukin-1β (IL-1β) and IFN-γ and related to AMD pathology (Benhar et al. 2016; Chirco 2017; Dorecka et al. 2013; Nagineni et al. 1996; Thichanpiang et al. 2014; Uddin et al. 2018). Indeed, during AMD, RPE cells contribute to cytokine production, intensifying inflammatory state and immune cells recruitment (Fukuoka,

Strainic, and Medof 2003; Planck et al. 1993; Sato et al. 2018). Together with the immune cells, they are reported to produce several different cytokines as IL-1β, IFN-γ, TNF-α among the others and this is reported to increase adhesion molecule production as ICAM-1 and VCAM-1 (Ardeljan et al. 2014; Crane and Liversidge 2008; Platts et al. 1995; Whitcup et al. 2013). These inflammatory conditions can affect and impair RPE function, leading to their dysfunction and, eventually, death through different mechanism as apoptosis, autophagy and necrosis (Dunaief et al. 2002; Kaarniranta et al. 2017; Telegina, Kozhevnikova, and Kolosova 2017).

Leukocyte recruitment to inflamed tissues forms the basis for any type of local immune response. Targeting this process remains an attractive chance to support immune system and to suppress inflammation-induced tissue damage. Progress in drug development has been limited, possibly due also to integrin redundancy in their roles and interactions (Srichai and Zent 2010).

As a part of my PhD program, I investigated the role of small molecules, antagonists of leukocyte integrin to fight “dry” AMD. In my laboratory have already been synthesized and studied a series of integrin antagonists that may block α4β1 and/or αLβ2 integrins (Baiula et al. 2016; Dattoli et al. 2014; Tolomelli et al. 2015). Furthermore, were discovered small molecules that block both α4β1 and αLβ2 integrins (Baiula et al. 2016). Differently from monoclonal antibodies, binding selectively only one integrin, small molecules may be directed towards two different leukocyte integrins and could possess a safer profile. αLβ2 and α4β1 integrin antagonists may target different immune cells such as monocytes, macrophages and lymphocytes, blocking their adhesion to endothelial and RPE cells mediated by interactions with, respectively, ICAM-1 and VCAM-1.

Targeting leukocyte integrins has proven applications in diseases such as multiple sclerosis (MS), Crohn disease (CD) and ulcerative colitis (UC) (Hahn et al. 2015). The αL integrin subunit is the target of efalizumab, which was on the market for psoriasis, even if was withdrawn in 2009 because of an association with a fatal brain infection, the progressive multifocal leukoencephalopathy (PML) (Kuehn 2009). A topical αLβ2 integrin inhibitor, lifitegrast, was approved by the US Food and Drug Administration (FDA) in July 2016 for the treatment of dry eye disease (DED). Lifitegrast is a small-molecule integrin antagonist designed to reduce inflammation by binding αLβ2 integrin and blocking the interaction with its cognate ligand ICAM-1, that is overexpressed in corneal and conjunctival tissues in

patients with DED (Abidi, Shukla, and Ahmad 2016). Natalizumab is a humanized monoclonal antibody that prevents α4β1 integrin on leukocytes from binding VCAM-1 on vascular endothelial cells in the CNS as well as in the intestine. Has been approved for therapeutic use in MS (Hutchinson 2007) and later, even if was reported to affect cerebral antiviral immunity and in some cases cause PML, was approved for Crohn disease in patients who did not respond to or were intolerant of conventional treatment (Park and Jeen 2018). Vedolizumab is a humanized monoclonal antibody against α4β7-integrin that inhibits the adhesion of leukocytes to the endothelium by blocking the interaction between α4β7 integrin and MAdCAM-1 expressed on blood vessels and lymph nodes associated with the gastro-intestinal (GI) tract. Has been approved for the treatment of CD, but while natalizumab inhibits leukocyte trafficking in multiple organs, including the brain, vedolizumab acts only on gut-trophic α4β7 heterodimers and, therefore, inhibits lymphocyte trafficking selectively in the intestine. Moreover, even if MAdCAM-1 exists rarely at the blood-brain barrier, vedolizumab is known to have no effect on CNS immunity (Döring et al. 2011).

Beyond drugs targeting leukocyte integrins, among the approved drugs available on the market, there are several addressing other integrins as therapeutic target. For example, abciximab, tirofiban and eptifibatide are, respectively, a monoclonal antibody and two small molecules antagonizing αIIbβ3 integrin against platelet aggregation (Ley et al. 2016). Integrins often bind their ligands through recognition of short amino-acid sequences and arginine-glycine-aspartic acid (RGD) is a frequent recognition motif present in ECM molecules such as fibronectin, vitronectin, and laminin. Based on these findings, synthetic peptides and peptidomimetics displaying the RGD motif have been developed as integrin ligands and antagonists, inhibiting the adhesion of cells to ECM proteins, thereby controlling integrin-mediated biological functions (Kapp et al. 2017; Nieberler et al. 2017).

2. Analyzing antioxidants effects on RPE

2.1 Oxidation and AMD

As reported in the paragraph about AMD, oxidation is one of the main features of every age-related pathology, contributing to detrimental effects during years. Even if there are several different mechanisms and theories proposed to elucidate the aging process, it is generally agreed that there is a correlation between aging and the accumulation of oxidative

damaged proteins, lipids, and nucleic acids that have been shown to increase as a function of age (Stadtman 2006). Conversely, factors acting in the way of decelerating protein oxidation have been shown to increase lifespan, while their reduction has been reported to shorten it. Several age-related diseases are associated with an elevated amount of oxidized molecules that can reflect deficiencies in one of the functions deputed to maintain the balance between oxidants, antioxidant and repair or discard of damaged elements (Krisko and Radman 2019).

As said, in the retina and especially in the macula, progression of aging process leads to characteristic features as formation of deposits, usually under the RPE, in the space between retina and Bruch’s membrane. These deposits constitute the hallmark of AMD, the drusen, and are composed by several different proteins and lipids (Knudtson et al. 2004). With time AMD develops, causing alteration in structure and function of retina and choroid and leading in the last phases to atrophy of the RPE and, consequently, to photoreceptor death due to inadequate support by the RPE. RPE and photoreceptor death in GA, bring to central vision loss as final result of the pathology (Khandhadia and Lotery 2010).

Reactive oxygen species (ROS) are formed as a natural byproduct of body metabolism and are highly reactive atoms, ions or molecules that contain oxygen, either free radicals or peroxides. ROS can be produced during metabolic activities as glycolysis, Krebs cycle, mitochondrial cellular respiration, but also by the immune system as a defense against pathogens and by environmental factors such as smoking, pollution and radiations. Uncontrolled ROS can cause severe damage to cells and the body has therefore evolved to provide an efficient system of antioxidants enzymic and nonenzymic activity to neutralize their detrimental potentials (Muller et al. 2007). However, if ROS levels exceed antioxidating capacity, as a result of either excess of ROS production or reduced antioxidant capability, then oxidative damage may develop. Unfortunately, this is exactly what happens during the aging process, where repair mechanisms activity decrease. This can lead to progressive dysfunction due to abnormal production of proteins and lipids that are essential for cellular activity (Harman 1956).

Retina is a source of high metabolic activity and is exposed to a constant source of oxidative stress from incoming light, leading to photo-oxidation, which in turn generates ROS and this happens especially in its central part, the macula. As seen above, with age, the balance between production of ROS and local antioxidant levels is altered, allowing

molecular and cellular damage. Systemic ROS and antioxidant levels in AMD reflect this process (Khandhadia and Lotery 2010).

With time, RPE cells also accumulate granules containing a fluorescent pigment called lipofuscin, composed by undigested products from the phagocytic process of POS and also autophagy results in accumulation of lipofuscin granules (Ryan 2006). Lipofuscin granules contain toxic substances, including retinoids (products of the visual cycle), and modified protein and lipid products (Ng et al. 2008). In vitro, RPE cells loaded with lipofuscin exhibit a significant reduction in phagocytic capacity and, when exposed to light, is possible to detect increase of ROS formation damaging the RPE (Nilsson et al. 2003; Wassell et al. 1999).

Given that retina is highly susceptible to oxidative damage, it has developed a range of protective mechanisms that include carotenoids, antioxidant enzymes and other substances. Lutein and zeaxanthin are found in high concentration in the retina and might be protective for AMD (Packer 1993). These carotenoids are not synthesized in mammal but need to be obtained from the diet and has been reposted that an insufficient intake of these antioxidant can contribute to AMD pathogenesis. For this reason, carotenoids and many other dietary compounds and supplements have been studied in several surveys and trials to assess their protective role in AMD (Ozawa et al. 2012; Pawlowska et al. 2019).

2.2 Histone acetylation and antioxidants

Histones are a family of small, alkaline and positively charged proteins termed H1, H2A, H2B, H3, and H4 located in the nucleus of eukaryotic cells. Their main function is to allow DNA to package into basic structural units, called nucleosomes, ensuring appropriate access to it. DNA is negatively charged, due to the phosphate groups in its phosphate-sugar backbone, so histones bind with it very tightly. This enfolding in several sequentially higher order structures, lead eventually chromatin to chromosome formation. Indeed, chromatin is composed by DNA and proteins, and histones are the main proteins present in chromatin. Two copies of each of histones H2A, H2B, H3, and H4 come together to form a histone octamer, which binds and wraps approximately 1.7 turns of DNA, or about 146 base pairs. The addition of one H1 protein wraps another 20 base pairs, resulting in two full turns around the octamer, forming a structure called chromatosome (**fig. 14**).

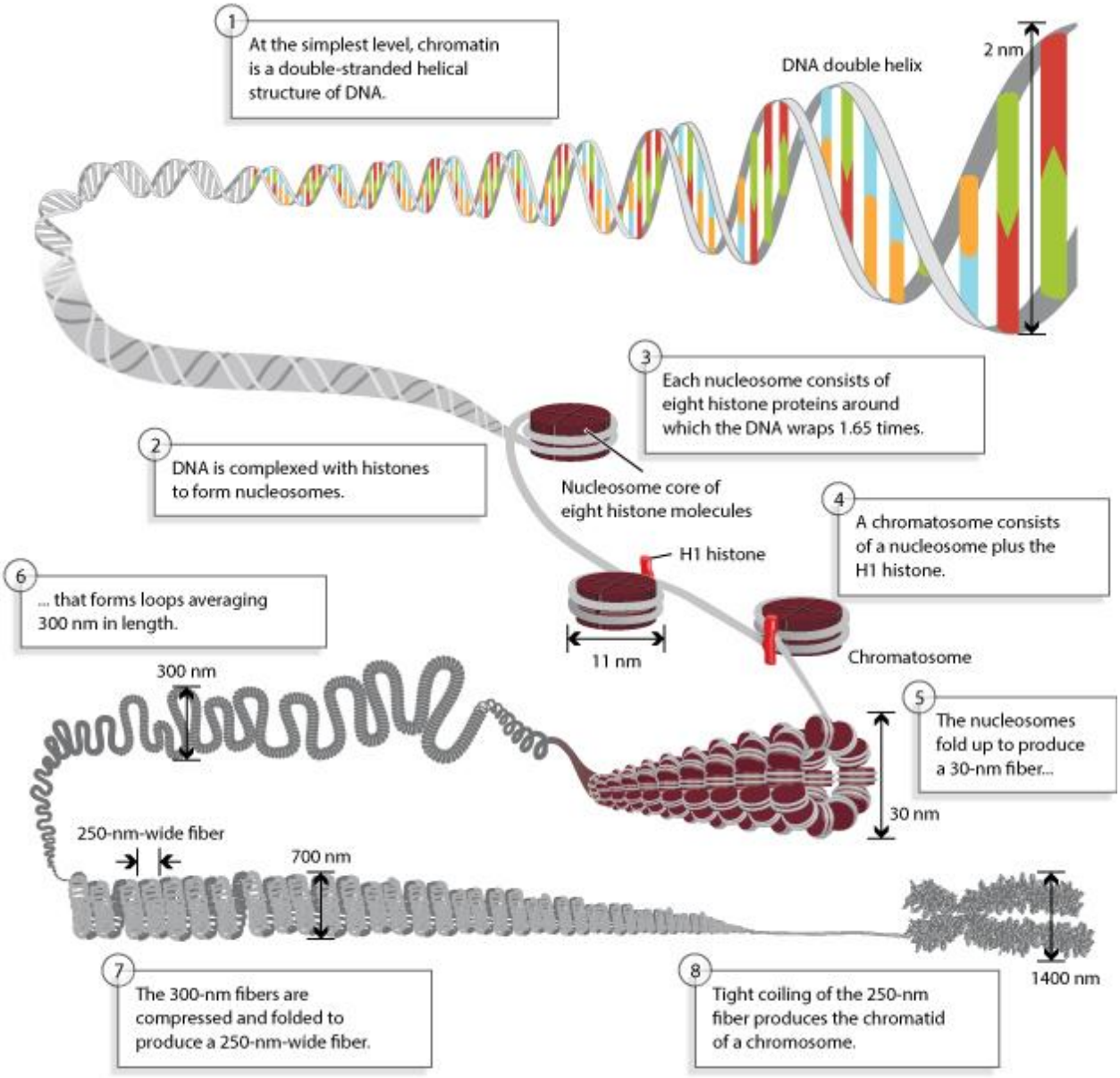


Fig. 14. Histones. *Detailed successive step of DNA folding and histone involvement (Annunziato 2008).*

Given that DNA wrap around histones, these proteins are recognized as having roles in gene regulation and expression. This behavior and the study of the different modifications happening to DNA and reflecting gene expression, without involving direct modification of the nucleic acid chain sequence, is called epigenetic (van Holde 1989). Processes such as transcription and replication require the two strands of DNA to separate temporarily, allowing polymerases to access to the DNA template. Nucleosomes and more complexed folded structures create barriers to the enzymes that unwind and copy DNA. For this reason, it is

important for cells to have a way for open chromatin fibers, allowing transcription and replication. This can happen through some histones modification exerted by specific enzymes producing acetylation, methylation or phosphate groups addition on histones (Fischle, Wang, and Allis 2003) or directly adding and modifying groups bounded to the DNA chain (Bird 2007). These processes are reversible allowing modified or remodeled chromatin to return to its compact state when transcription or replication is completed (John and Rougeulle 2018).

Several kind of epigenetic studies in multiple different fields are trying to investigate roles of nutrients, behaviors, conditions, chemicals, contaminants and other elements on gene expression modification, made through alteration of histones or directly on groups attached on the DNA chain (Bai et al. 2018; Csoka and Szyf 2009; Heinbockel and Csoka 2018; Mason 2018; Seo et al. 2018). Epigenetic is one of the fields in biomedical science that is having most of the attention now, due to the incredible number of functions and elements that can be involved. The study of chromatin alterations is changing our view of how nature and genome express themselves, leading us to understand how lifestyle and environment influence our gene expression over time and how these changes can be passed through generations (Ledford 2012).

Speaking about antioxidants and their effect on the epigenome (the overall multitude of DNA and chromatin modifications), hundreds of molecules have been studied for their play in the most diverse pathologies and conditions. (Eryılmaz Pehlivan 2019; Guillaumet-Adkins et al. 2017; Rodriguez and El-Osta 2018; Rong et al. 2016). Moreover, have been conducted several studies investigating the potential benefits of antioxidants also on RPE, with different compounds reported to exert epigenetic modification resulting in beneficial effects related to AMD pathology (Gemenetzi and Lotery 2014; Kwa and Thrimawithana 2014; Tokarz, Kaarniranta, and Blasiak 2016).

One of the most important epigenetic modification is histone acetylation, a process in which lysine residues of histones are added with an acetyl group (Khan and Khan 2010). This mechanism is reversibly controlled by two families of enzymes: histone acetyltransferases (HAT) and deacetylases (HDAC), respectively adding and removing the acetyl group and thus influencing gene expression. Because of its amino group, lysine is a positively charged amino acid, which binds strongly to the negatively charged DNA molecule. The addition of the acetyl group neutralizes this positive charge reducing the

interaction between histones and DNA, leading to a more open structure that is more accessible to the transcriptional machinery. Histone acetylation therefore leads to transcriptional activation (Ellenbroek and Youn 2016; Fan, Baeza, and Denu 2016).

In AMD, some studies are already directed on investigating histone acetylation. Clusterin, an important component of drusen, has been reported to be an inhibitor of protein aggregation, a complement inhibitor and acting against angiogenesis. Drugs as Valproic acid (VPA), an inhibitor of histone deacetylase (HDAC) leading to histone hyperacetylation, have been reported to notably increase clusterin production (Suuronen et al. 2007). VPA has been found also to have beneficial effects on RPE and photoreceptors, but with controversial outcomes depending on conditions and patients’ genotypes (Berner and Kleinman 2016; Mitton et al. 2014; Tzekov et al. 2010; Vent-Schmidt et al. 2017).

Epigenetic field is undergoing a constant development and need to be deeply studied to understand the exact mechanisms related to chromatin modification. Purpose of a part of my PhD studies, was to investigate the role of some antioxidants frequently related with beneficial effects on RPE and AMD aging features. I tried to understand if these results could be reached through epigenetic variations and, specifically, histone acetylation. Among the histones presents in chromatin, histone H3 is a core histone and is well reported as a fundamental regulator of gene expression other than one of the most modified histones (Lee, Smith, and Shilatifard 2010; Oliver and Denu 2011). I studied the modification in histone H3 acetylation after treatment with several antioxidants already reported as having beneficial effects on RPE or even in use for prevention and treatment of AMD pathology.

3. Possible beneficial effects of statins in AMD

Statins are a class of therapeutic agents well known and used for lowering lipid levels in blood, especially regarding cholesterol. Cholesterol is a lipidic molecule both synthetized by our organism and taken through food. It is important for the biosynthesis of steroid hormones, bile acids and vitamin D but, most of all, it is a fundamental component of cellular membranes (Maxfield and Tabas 2005). Together with other lipids, cholesterol is carried in the blood by different lipoproteins as chylomicrons, very low-density lipoprotein (VLDL), high-density lipoprotein (HDL) and low-density lipoprotein (LDL). These plasma particles have distinct roles in lipids transportations and different relative amounts of cholesterol. For

example, HDL carries cholesterol from tissues to the liver to be eliminated and for this reason is generally recognized as “good cholesterol” (Lund-Katz and Phillips 2010). On the other side, LDL is generally recognized as “bad cholesterol” and is reported to be a high-risk factor for atherosclerosis and cardiovascular diseases, carrying circulating cholesterol in the blood to where it is needed, but being responsible also for its deposit into the vessel walls in case of excess (Pirahanchi and Huecker 2019).

Statins exert their roles through inhibiting a specific step of cholesterol synthesis, the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) into mevalonate. This effect is mediate by blocking the enzyme HMG-CoA reductase and doing so, they exert a potent lipid-lowering effect that reduces cardiovascular risk and decreases mortality. Since the mevalonate pathway also influences endothelial function, inflammatory response and coagulation, statins have a whole set of beneficial effects on cardiovascular system (Pinal-Fernandez, Casal-Dominguez, and Mammen 2018).

As widely reported, lipids and cholesterol are a determinant component of drusen, the hallmark of AMD and they have been related with the pathology progression (Pikuleva and Curcio 2014; Wang et al. 2010). Moreover, AMD shares several similarities with cardiovascular diseases both in risk factors and pathogenic features, as the composition of drusen and arteriosclerotic deposits (Miller 2013; Mullins et al. 2000; Tomany et al. 2004; Yip et al. 2015).

Last part of my research was conducted during the period abroad of my PhD program. I spent one year at the Angiogenesis Laboratory of Dr. Demetrios Vavvas, Department of Ophthalmology, Massachusetts Eye and Ear Infirmary (MEEI), Harvard Medical School, Boston, Massachussetts, USA. Researches in the lab pointed out how trying to contrast cholesterol levels, might have positive effects in AMD progress and improve patients’ life conditions. In Dr. Demetrios Vavvas laboratory, was reported that atorvastatin could improve membrane fluidity and cellular phagocytic function in human RPE cells and attenuate inflammation (Tian et al. 2017). Moreover, in a clinical study conducted by Dr. Vavvas, was reported that patients receiving intensive treatment with statins resulted in regression of drusen deposits. This was associated with vision gain and no subjects in the study progressed to advanced neovascular AMD (Vavvas et al. 2016).

Starting from these assumptions, my research focused on the investigation of the mechanisms that may be responsible for a protective role of statins in AMD but focusing on common doses clinically used in routine therapy.

Experimental methods

1. Cell cultures

All the following cells were cultured in 75 cm^2 flasks and maintained in incubators at 37°C, in a humidified atmosphere of 5% CO_2.

1.1 ARPE-19 cells

ARPE-19 (ATCC® CRL2302™) cells are a spontaneously arising retinal pigment epithelia cell line derived in 1986 by Amy Aotaki-Keen from normal eyes of a 19-year-old male who died from head trauma in a motor vehicle accident. These cells form stable monolayers, which exhibit morphological and functional polarity. In addition, ARPE-19 expresses the RPE-specific markers CRALBP and RPE-65 (Dunn et al., 1996).

These cells were grown in Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (DMEM/F12; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), antibiotic-antimycotic (Gibco) 100x (100 units of penicillin, 100 µg of streptomycin, 0.25 µg of amphotericin B/mL medium plus 0.85% of Fungizone®).

1.2 Jurkat Cells

Jurkat, Clone E6-1 (ATCC® TIB-152™), were established from the peripheral blood of a 14-year old boy with T cell leukemia by Schneider et al., and was originally designated as JM. The Clone E6-1 cells produce large amounts of IL-2 after stimulation with phorbol esters and either lectins or monoclonal antibodies against the T3 antigen (both types of stimulants are needed to induce IL-2 production).

The base medium for this cell line is RPMI-1640 Medium (Gibco). To prepare the complete growth medium, FBS is added to the base medium to a final concentration of 10%.

1.3 Co-culture

ARPE-19 cells were seeded in 6-well plates and cultured until monolayers were formed. Later, 1 million of Jurkat cells were added for different incubation periods (1, 16, 24 and 48 hours) and cultivated with ARPE medium. At the end of the co-culture, immune cells were removed by doing three washes with PBS (phosphate buffered saline), and ARPE-19 cells

were detached with Trypsin/EDTA 1% solution (Lonza). Finally, cells were centrifugated and pelleted to be stored at -80° for future uses.

2. Treatments

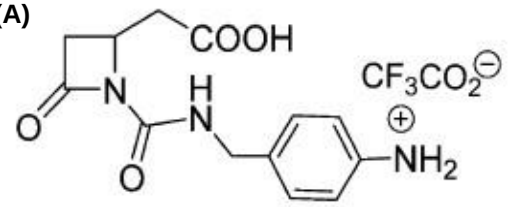
2.1 Integrin antagonists

Integrin antagonists employed in this study were provided by chemist research groups headed by Prof. Daria Giacomini and Prof. Luca Gentilucci. These compounds had demonstrated a strong antagonist activity against αLβ2 (MN27) and α4β1 (DS-70) integrins as shown in previous papers from Spampinato laboratory (Baiula et al., 2016; Dattoli et al., 2018) (**Table 1**). DS-70 and MN27 were used on Jurkat cells that express both α4β1 and αLβ2 integrins. Cells were pre-incubated with different concentrations (10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M diluted from stock 10^{-2} M) of antagonists for 30 minutes at 37°C before adding Jurkat cells to ARPE-19 cells for 24 or 48 hours. Finally, the cells were collected separately and stored as described above.

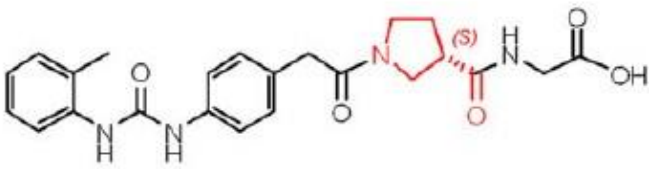
Integrin antagonists	**Solid phase binding/SPA IC50 (nM)[a]**	**Jurkat cell adhesion α4β1/VCAM-1 IC50 (nM)[b]**	**Jurkat cell adhesion αLβ2/ICAM-1 IC50 (nM)[b]**	**K562 cell adhesion α5β1/FN IC50 (nM)[b]**
MN27 (A) (Baiula et al., 2016)	6.7 ± 2.5 (αLβ2)	574.0 ± 1.7	0.39 ± 0.02	44.5 ± 2.6 (agonist)
DS-70 (B) (Dattoli et al., 2018)	8.3 ± 3.2 (α4β1)	5.04 ± 0.51	>5000	>5000

Table 1. MN27 and DS-70 structures and properties. *Characterization of integrin antagonists MN27 and DS-70 carried out on previous studies (Baiula et al. 2016; Dattoli et al. 2018). [a]IC_{50} values of considered antagonists evaluated through competitive solid-phase binding assay to specific ligands (ICAM-1 for $\alpha_L\beta_2$) or through scintillation proximity assay (SPA, FN for $\alpha_4\beta_1$). [b]IC_{50} values of considered antagonists evaluated through cellular adhesion assays (Baiula et al., 2016; Dattoli et al., 2018). Data obtained from six independent experiments carried out in quadruplicate. Data are expressed as mean ± SD.*

(A)



(B)



2.2 Integrin antibodies

Neutralizing antibodies anti-VCAM-1 (BD Pharmingen™), anti-ICAM-1 (BD Pharmingen™) anti-alpha 4 (abcam) were added to ARPE-19 cells at saturation concentration (10 µg/mL) for one hour before adding Jurkat cells; thereafter, the co-culture was extended for 24 hours. Neutralizing antibody anti-α4β1 integrin (Abcam) was pre-incubated with Jurkat cells and after one hour of incubation, immune cells were added to ARPE-19 cells for 24 hours. Cells were collected separately and stored as previously described.

2.3 Antioxidants

Flavanon (K & K laboratories), Quercitrin (K & K laboratories), Quercetin (Sigma) and Lutein (Sigma) were administered after solving powders in 100% ethanol. Stock solutions of Flavanon, Quercetin and Lutein were prepared at 1 mg/mL, while Quercitrin stock solution was prepared at 2 mg/mL.

3. Extractions and quantifications

3.1 Protein extraction

Proteins were extracted by cell homogenization in MAPK buffer lysis (50mM Tris-Cl, 300mM NaCl, 1mM EDTA, 1mM Na3VO4, 1mM NaF, and 10% glycerol) with protease and phosphatase inhibitors mix (2 µg/mL aprotinin, 2 µg/mL leupeptin, 0,5 mg/mL benzamidine, 2mM PMSF and 2mM PPI). The homogenates were sonicated for 10 seconds and then centrifuged at 17000 g for 25 minutes at 4°C. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's instructions.

Total histones were extracted using Histone Extraction Kit (abcam) according to manufacturer’s instructions. Histones concentration was determined through Lowry method using Jasco V-530 spectrophotometer (Jasco) and RADLIG data analysis software (Biosoft Corp.).

3.2 RNA extraction

Cells pellets were resuspended in 350 μL TRIzol™ Reagent (Sigma) to obtain a homogeneous suspension. After three minutes of incubation, 90 μL chloroform was added, the suspension was inverted vigorously and incubated on ice for 2-4 minutes. Samples were centrifuged at 16000 g for 15 minutes at 4°C. The upper aqueous phase of each sample, containing RNA, was transferred in a new tube and added 400 µl of cold isopropanol. Each sample was inverted and stored at -80 °C overnight, in order to precipitate RNA which is insoluble in isopropanol. The following day, samples were centrifuged at 16000 g for 15 minutes at 4°C. Thereafter, pellets were washed with 200µl of ice cold 75% ethanol containing dithyl-pyrocarbonate (DEPC, RNAase inhibitor, which preserves RNA integrity). Samples were centrifuged at 16000 g for 5 minutes at 4 °C and pellets were dried for 3-4 minutes under vacuum conditions. Hence, pellets were dissolved in 10 µl of water supplemented with DEPC and nucleic acid concentrations were determined at Nanodrop spectrophotometer (Millipore).

4. Western blot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed according to standard procedures.

Polyacrylamide gel were formed after polymerization of acrylamide monomers and small amounts of N,N'-Methylenebisacrylamide in presence of ammonium persulphate (UltraPURE® Life Technologies) 10% p/V and TEMED (Sigma). Once the polyacrylamide gel was ready, extracted protein samples were thawed on ice, diluted with nuclease- and protease-free water added of loading dye and denatured at 95°C for three minutes.

Total protein lysates were used to analyze p42/44 MAPK expression (extracellular signal-regulated kinase, ERK 1 and 2). Electrophoresis was carried out on a 12% polyacrylamide gel with at 20 mA and transferred to nitrocellulose membranes (0,2 μm, Bio-Rad). The

membranes were blocked for an hour with 5% non-fat milk in 0.1% TBS-T (0,1% Tween-20, 10mM Tris-HCl, 150mM NaCl, pH = 8). Dilutions of primary antibodies were made in 5% BSA 0,1% TBS-T. The membranes were incubated overnight in agitation at 4 °C with either rabbit polyclonal antibody anti-phospho-ERK 1/2 (Cell Signaling Technology) or anti-total ERK1/2 (Cell Signaling Technology) in 5% BSA in 0,1% TBS-T.

Total histones were used to analyze acetylation levels of Histone H3. Electrophoresis was carried out on a 15% polyacrylamide gel with at 20 mA and transferred to nitrocellulose membranes (0,2 μm, Bio-Rad). The membranes were blocked for an hour with 5% non-fat milk in 0.05% TBS-T (0,05% Tween-20, 10mM Tris-HCl, 150mM NaCl, pH = 8). Dilutions of primary antibodies were made in 5% BSA 0,05% TBS-T. The membranes were incubated overnight in agitation at 4°C with either rabbit polyclonal antibody anti-acetyl histone H3 (Millipore) or anti-histone H3 (Millipore) in 5% BSA 0,05% TBS-T.

The following day, after proper washes membranes were incubated with the secondary antibody (goat anti-rabbit HRP-conjugated IgG; Santa Cruz) for 1.5 hours in agitation at room temperature. After washes, membranes were exposed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) to the LAS3000 instrument (Fujifilm). The signal density of the bands was measured using software Aida Image Analyzer v.3.45 and normalized to the associated control band signal density (100%).

5. Reverse transcription and quantitative PCR

Ribonucleic acid (RNA) was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit™ (Applied Biosystems. 20 μL reaction. 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C and ∞ at 4°C) on the same day of the extraction according to manufacturer's instructions. To perform reverse-transcription PCR, was used Applied Biosystems 2720 Thermal Cycler® (Applied Biosystems) instrument. Obtained cDNA samples were stored at -20 °C until further usage.

Quantitative PCR (qPCR) was performed using the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) and StepOne® instrument (Applied Biosystems). The gene expressions of IL-1β (primer sequence forward: 5'-CATGAGCTTCGTACAAGGAGAAAG-3'; primer sequence reverse: 5'-CAGGTACAGATTCTTCCCCTTGA-3') were normalized to the house-keeping gene L19

(primer sequence forward: 5’-CTAGTGTCCTCCGCTGTGG-3’; primer sequence reverse: 5’-AAGGTGTTTTTCCGGCATC-3’) and calculated according to the ΔΔCt method. All reactions regarding RT or PCR were performed with RNAse free supplies (Euroclone).

6. Flow cytometry

Collected cells were labelled to detect cellular molecules of interest through cytofluorometry. Anti-ICAM-1 monoclonal antibody (BD Pharmingen), anti-VCAM-1 (BD Pharmingen) and anti-alpha4 monoclonal antybody (abcam), were used in 1% BSA/HBSS (Hanks’ Balanced Salt Solution, LifeTechnologies) for 45 min at 4°C. After two washes in 1% BSA/HBSS cells were incubated with Goat anti-Rabbit IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor 488 in 1% BSA/HBSS (Invitrogen) for 45 min at 4°C. After two washes with 1% BSA/HBSS, cells were resuspended in PBS and analyzed at Guava® easyCyte™ flow cytometer (Millipore) and 10000 cells/sample were analyzed.

Cell death was rate by measuring flow cytometry analysis. Cell pellets were analyzed on the harvesting day with a 1:1 dilution of DMEM/F12 supplemented with 10% FBS and Guava Nexin® reagent (Millipore). Cells were incubated for twenty minutes at room temperature in the dark. After, early apoptosis and late apoptosis/necrosis were detected with Guava® easyCyte™ flow cytometer (Millipore) and data were analyzed with software guava InCyte™.

7. RNA-sequencing

Total RNA was extracted using RNeasy® Mini kit (QIAGEN) according to manufacturer’s instructors. A brief RNA spot quality check was carried out through Nanodrop spectrophotometer (Millipore) and agarose gel, using SYBR® Safe as stain. Samples were shipped to ©Arraystar Inc. for RNA-sequencing procedure.

The company ran and guaranteed quantity and quality of total RNA samples through RNA quantity and purity test by NanoDrop ND-1000 and RNA integrity and gDNA contamination test by denaturing agarose gel electrophoresis. 1-2 µg of total RNA was used to prepare the sequencing library in following steps including total RNA enrichment by oligo (dT) magnetic beads (rRNA removed), RNA-seq library preparation using KAPA Stranded RNA-Seq Library Prep Kit (Illumina), which incorporates dUTP into the second cDNA strand and

renders the RNA-seq library strand-specific. The completed libraries were qualified with Agilent 2100 Bioanalyzer and quantified by absolute quantification qPCR method. To sequence the libraries on the Illumina HiSeq 4000 instrument, the barcoded libraries were mixed, denatured to single stranded DNA in NaOH, captured on Illumina flow cell, amplified in situ, and subsequently sequenced for 150 cycles for both ends on Illumina Hiseq 4000 instrument.

Image analysis and base calling were performed using Solexa pipeline v1.8 (Off-Line Base Caller software, v1.8). Sequence quality was examined using the FastQC software. The trimmed reads (trimmed 5’, 3’-adaptor bases using cutadapt) were aligned to reference genome using Hisat2 software. The transcript abundances for each sample was estimated with StringTie and the FPKM value for gene and transcript level were calculated with R package Ballgown. The differentially expressed genes and transcripts were filtered using R package Ballgown. The novel genes and transcripts were predicted from assembled results by comparing to the reference annotation using StringTie and Ballgown, then use CPAT to assess the coding potential of those sequences. Then use rMATS to detect alternative splicing events and plots. Principle Component Analysis (PCA) and correlation analysis were based on gene expression level, Hierarchical Clustering, Gene Ontology, Pathway analysis, Gene Ontology, Pathway analysis, scatter plots and volcano plots were performed with the differentially expressed genes in R, Python or shell environment for statistical computing and graphics.

8. Statistical methods

The data obtained in this study are expressed as mean and standard deviation from at least three independent experiments. Statistical comparisons were evaluated through one-way ANOVA and post hoc Newman–Keuls test or t-test repeated measures, using Prism© GraphPad 8 (GraphPad Software Inc.) Statistical significance is stated as $p < 0.05$.

Results

1. Integrins as a potential target for “dry” AMD

1.1 Expression of adhesion molecules

Main part of my PhD program regards the study of integrins. Mainly, my research is focused on the role of integrins in cell adhesion analyzed in a contest of ARPE-19 and immune cells interactions and their possible involvement in AMD development.

First, I tried to detect integrins and adhesion molecules in ARPE-19 through cytofluorimetry, to evaluate if they are expressed and their quantification.

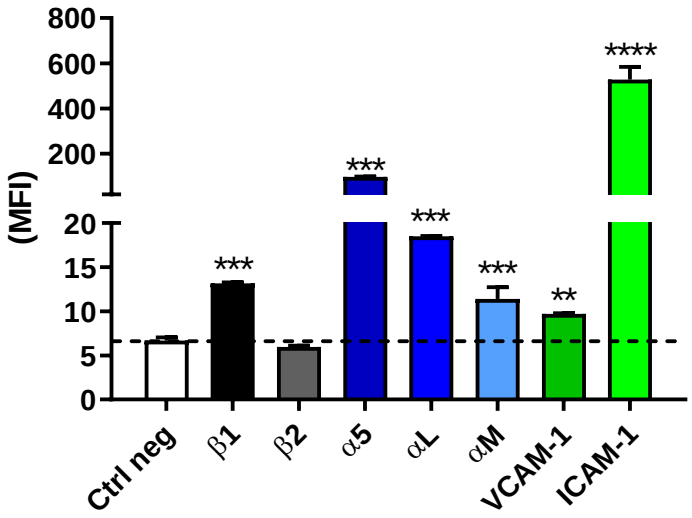


Fig. 15. Integrin expression in ARPE-19. *Analysis of the expression of integrins and molecules of adhesion in ARPE-19 cells. Analysis was made through cytofluorimetry. (**p<0.01 vs ctrl neg, ***p<0.001 vs ctrl neg, ****p<0.0001 vs ctrl neg).*

As previously reported (see p. 40), ICAM-1 and VCAM-1 are expressed in RPE cells, especially during inflammation. I was able to confirm this data in ARPE-19 together with many integrin subunits essential to mediate cell adhesion (**fig. 15**).

I tried then to evaluate how the expression of adhesion molecules would have been affected by using cytokines to simulate an inflammatory condition.

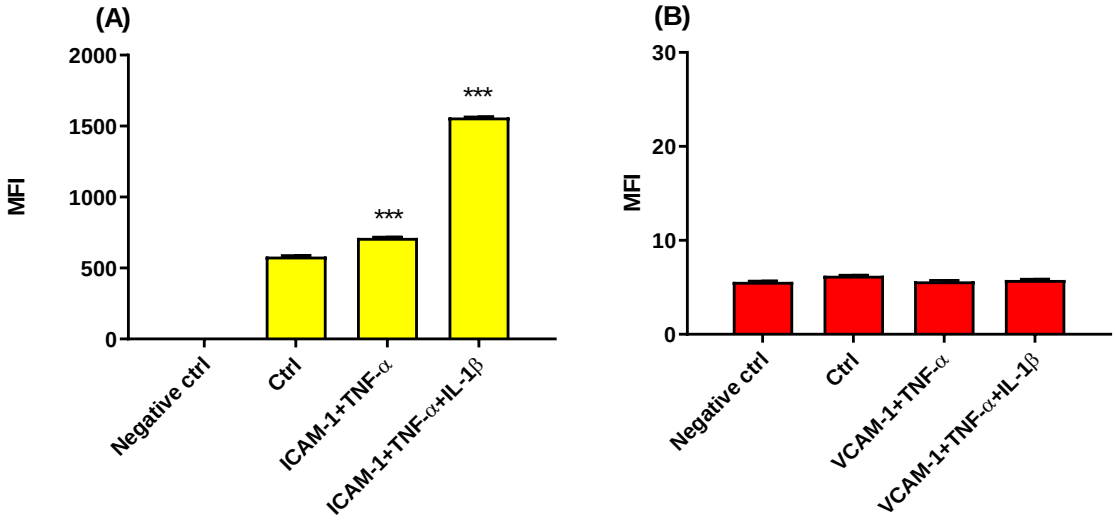


Fig. 16. Expression of ICAM-1 (A) and VCAM-1 (B) simulating inflammation. *Analysis of the expression of adhesion molecules VCAM-1 and ICAM-1 in ARPE-19 cells after administration of either TNF-α (10 ng/mL) or TNF-α (10 ng/mL) and IL-1β (10 ng/mL) for 24 hours. Analysis was made through cytofluorometry (***p<0.001 vs ctrl neg).*

After treating ARPE-19 cell with either TNF-α (10 ng/mL) or TNF-α (10 ng/mL) and IL-1β (10 ng/mL), it is possible to notice a null variation in VCAM-1 expression, while ICAM-1 shows a marked increase (**fig. 16**).

1.2 Simulating cell recruitment

After confirming that ICAM-1 and VCAM-1 are expressed in ARPE-19 cells in basal conditions and after pro-inflammatory cytokines administration, I was interested in what happens in presence of immune cells. As I said previously (p. 40), integrins might play a fundamental role in AMD, contributing to infiltration and adhesion of immune cells to the RPE. To determine what happens during their activation, I conducted experiments plating immune cells (Jurkat cells) on a monolayer of ARPE-19 and analyzing their response.

1.2.1 Cell signaling

First, I focused on cell signaling. Indeed, as described at page 38, integrins bind with their substrates and activate signaling cascades, that ultimately lead to cellular responses as proliferation, migration, and apoptosis. I focused on MAPK/ERK pathway, reported to be a fundamental pathway in integrin cellular transduction.

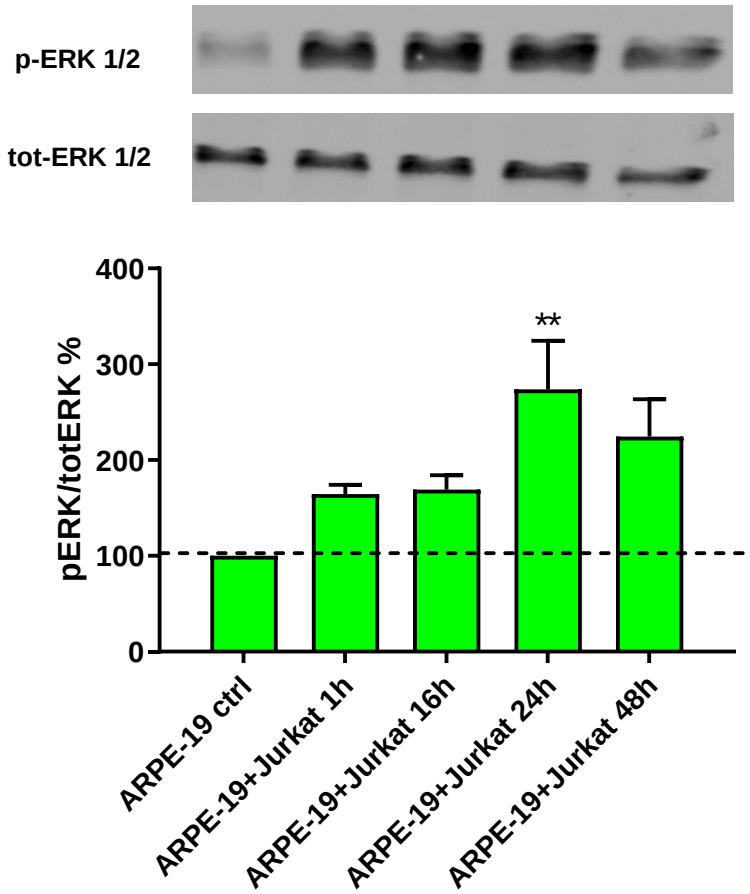


Fig. 17. Cell signaling in co-culture. *Detection of cell signaling activation in ARPE-19 cells plated in co-culture with Jurkat cells. Analysis was made through western blots, detecting p-ERK/tot-ERK ratio in ARPE-19 cells after 1, 16, 24 and 48 hours of co-culture (**p<0.01 vs ctrl).*

As shown in **fig. 17**, after plating Jurkat cells on ARPE-19 cells, it is possible to notice a time-dependent increase in cell signaling activation. Detecting p-ERK/tot-ERK ratio through western blot, I was able to detect an increase in cell signaling with a peak at 24 hours and a decrease at 48 hours, probably due to excessive cell death after a long interaction of ARPE-19 cells with Jurkat cells.

1.2.2 Expression of ICAM-1 and VCAM-1

After analyzing cell signaling, I was interested in studying adhesion molecules expressed in ARPE-19. In the same condition adopted for the study of MAPK/ERK activation, I evaluated any change of ICAM-1 and VCAM-1 expression after co-culture (**fig. 18**).

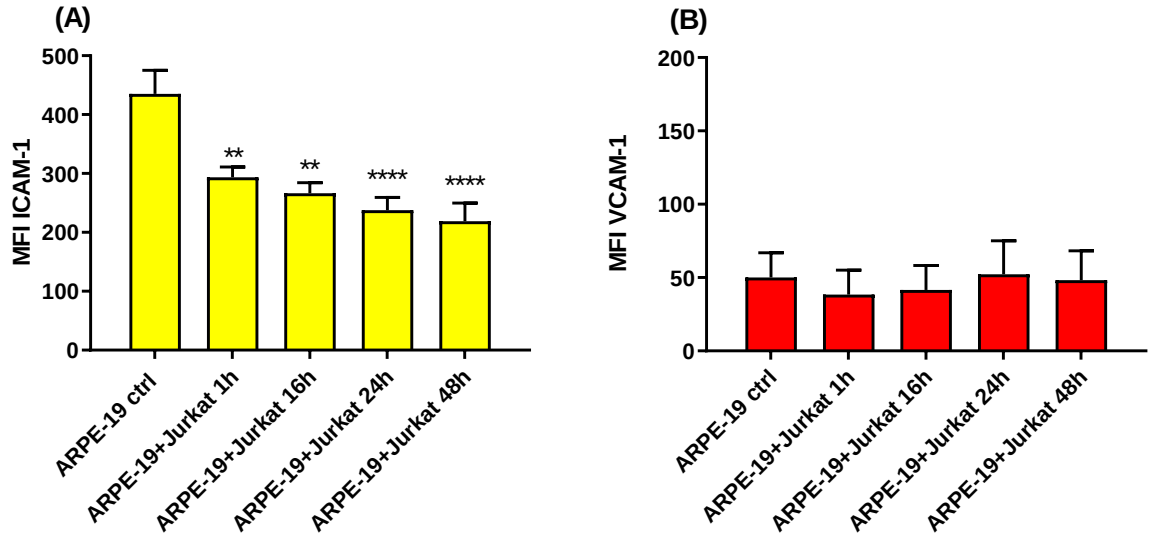


Fig. 18. ICAM-1 (A) and VCAM-1 (B) expression in co-culture. *Detection of ICAM-1 and VCAM-1 expression in ARPE-19 cells plated in co-culture with Jurkat cells. Analysis was made through cytofluorimeter in ARPE-19 cells after 1, 16, 24 and 48 hours of co-culture (**p<0.01 vs ctrl, ****p<0.0001 vs ctrl).*

While VCAM-1 shows no variation at any of the different time points, surprisingly ICAM-1 seems to decrease its expression in ARPE-19 cells.

1.2.3 IL-1β expression

Since I simulate an inflammatory state by reproducing conditions of inflammation in the presence of immune cells, I am also interested in understanding how ARPE-19 respond to inflammation in cytokines production. During AMD, in fact, several different cytokines are produced both by RPE and immune cells.

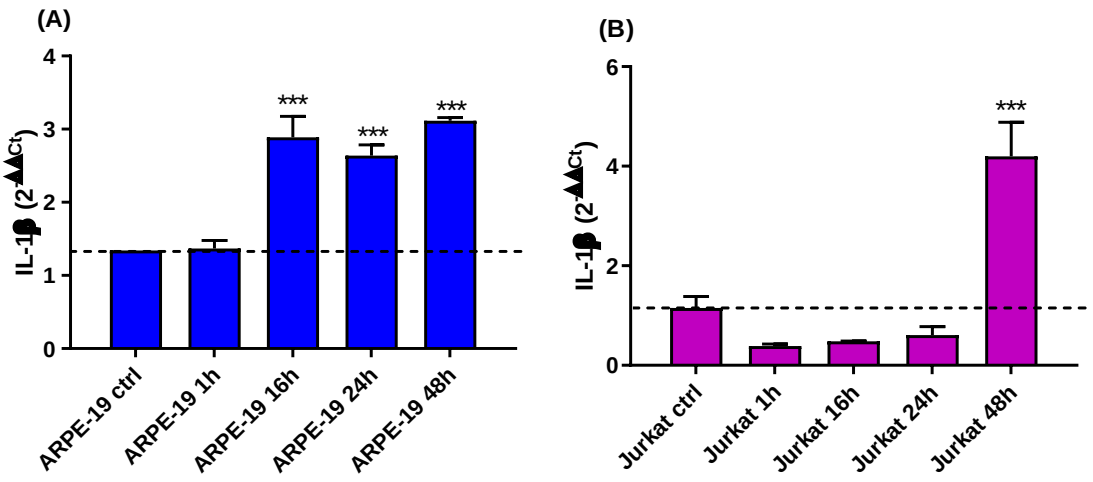


Fig. 19. IL-1β expression in co-culture. *Detection of IL-1β expression in ARPE-19 cells* **(A)** *and Jurkat cells* **(B)** *plated in co-culture. Analysis was made through qPCR after 1, 16, 24 and 48 hours of co-culture (***p<0.001 vs ctrl).*

After co-culture, IL-1β expression on both ARPE-19 cells and Jurkat cells results in a marked production increase (**fig. 19**).

1.2.4 Apoptosis and late apoptosis/necrosis

In the same conditions adopted to evaluate signaling pathways, including adhesion molecule expression and cytokine production, I wanted to analyze if cell death of ARPE-19 was affected by the interaction with Jurkat cells.

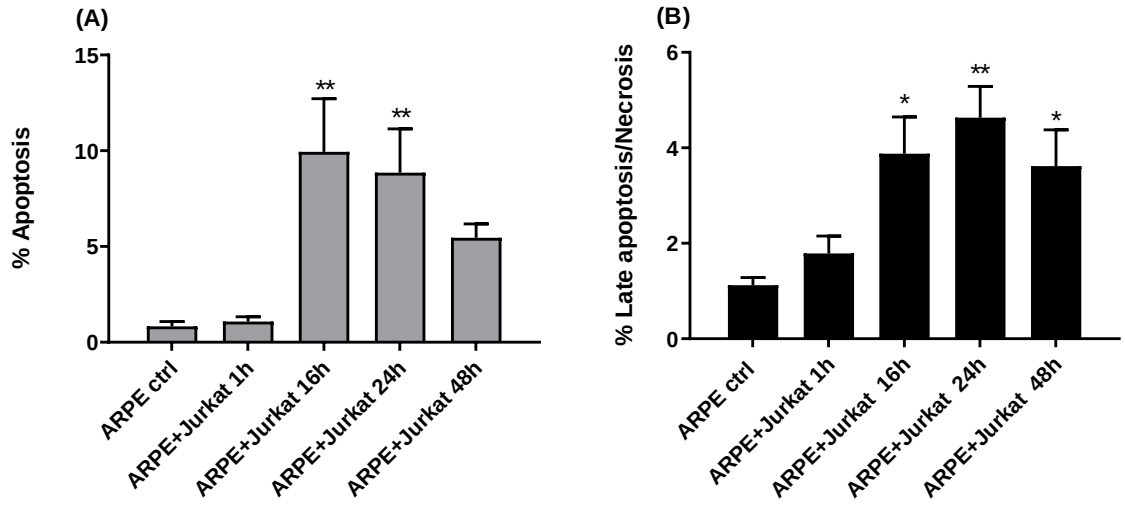


Fig. 20. Apoptosis and late apoptosis/necrosis in co-culture. *Detection of apoptosis* **(A)** *and late apoptosis/necrosis* **(B)** *in ARPE-19 cells plated in co-culture with Jurkat cells. Analysis was made through cytofluorimetry after 1, 16, 24 and 48 hours of co-culture (*p<0.05 vs ctrl, **p<0.01 vs ctrl).*

As shown in **fig. 20**, there is a marked increase of in apoptosis and late apoptosis/necrosis in ARPE-19 cells after co-culture with Jurkat cells. As for cell signaling (**fig. 17**), it is possible to notice a peak between 16 and 24 hours with a decrease at 48 hours.

1.3 Treatment with integrin antagonists

As reported in the dedicated chapter (p. 40), I have assayed different molecules with both integrin agonist and antagonist activity. In my research, I decided to use two compounds in order to evaluate if they are able to block the inflammatory features related to ARPE-19 cells and Jurkat cells in co-culture.

DS-70 was used as antagonist of integrin α4β1 while MN27 was used as integrin αLβ2 antagonist.

1.3.1 Signaling

First, I pre-incubated the cells with the compounds before repeating the adhesion assay in ARPE-19 cells/Jurkat cells in the same condition tested before (**fig. 17**).

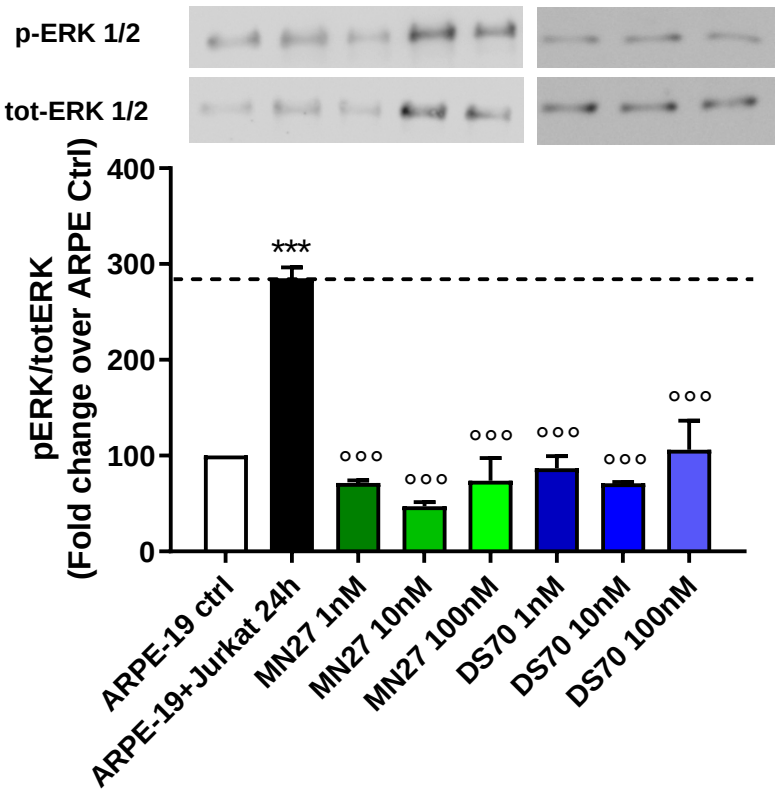


Fig. 21. Cell signaling after treatment with antagonists. *Detection of cell signaling activation in ARPE-19 cells plated in co-culture with Jurkat cells after treatment with integrin antagonists MN27 and DS-70. Analysis was made through western blots, detecting p-ERK/tot-ERK ratio in ARPE-19 cells after 24 hours of co-culture. MN27 and DS-70 were administered at 1, 10 and 100 nM concentration (***p<0.001 vs ARPE ctrl, °°°p<0.001 vs ARPE-19+Jurkat 24h).*

As reported in **fig. 21**, compared to the control, both MN27 and DS-70 were able to reduce p-ERK/tot-ERK ratio in ARPE-19 cells, after 24 hours of co-culture with Jurkat cells.

1.3.2 IL-1b

As shown in signaling experiments, integrin antagonists were tested also before analyzing cytokine production in both ARPE-19 cells and Jurkat cells.

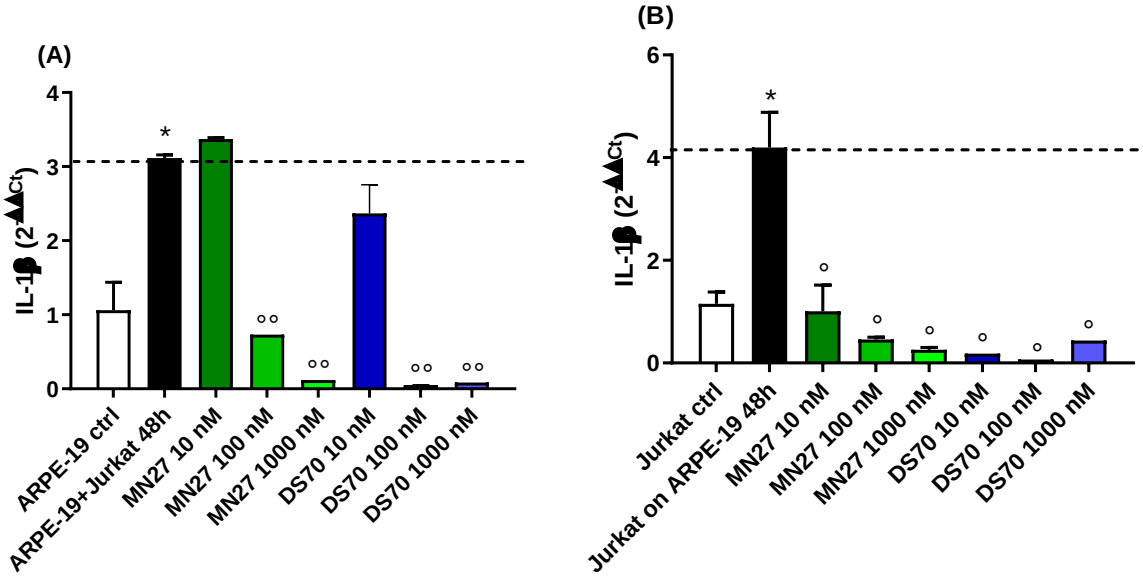


Fig. 22. IL-1β expression after treatment with antagonists. *Detection IL-1β expression in ARPE-19 cells* **(A)** *plated in co-culture with Jurkat cells* **(B)** *after treatment with integrin antagonists MN27 and DS-70. Analysis was made through qPCR in ARPE-19 cells after 48 hours of co-culture. MN27 and DS-70 were administered at 10, 100 and 1000 nM concentration (*p<0,05 vs ctr, °p<0.05 vs ARPE-19+Jurkat 48 h, °°p<0,01 vs ARPE-19+Jurkat 48 h).*

Both MN27 and DS-70 can markedly reduce IL-1β production in ARPE-19 cells, after co-culture with Jurkat cells for 24 hours (**fig. 22**). At 10 nM concentration, neither of the two compounds was able to induce a statistically significant modification in IL-1β expression. Instead, at 100 nM and 1000 nM concentration, both compounds were able to induce a notable reduction.

1.3.3 Apoptosis and late apoptosis/necrosis

As made for signaling and IL-1β production, I tested MN27 and DS-70 by evaluating their effects in counteracting cell death in ARPE-19 cells co-cultured with Jurkat cells for 24 hours.

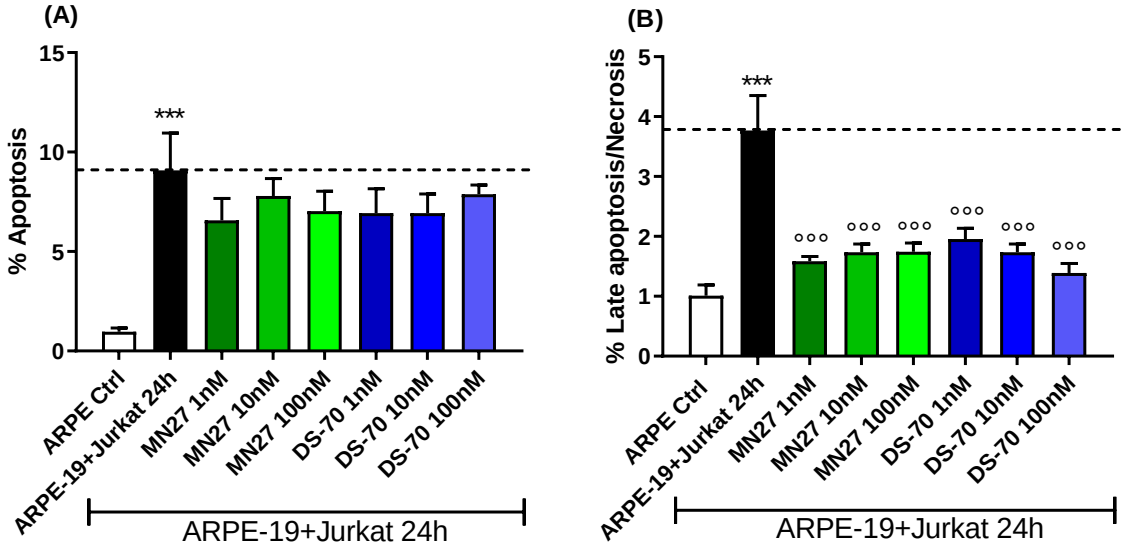


Fig. 23. Apoptosis and late apoptosis/necrosis after treatment with antagonists. *Detection of apoptosis* **(A)** *and late apoptosis/necrosis)* **(B)** *in ARPE-19 cells plated in co-culture with Jurkat cells after treatment with integrin antagonists MN27 and DS-70. Analysis was made through cytofluorimetry in ARPE-19 cells after 24 hours of co-culture. MN27 and DS-70 were administered at 1, 10 and 100 nM concentration (***p<0.001 vs ARPE-19 ctrl, °°°p<0.05 vs ARPE-19+Jurkat 24h).*

As expected from previous results, both MN27 and DS-70 are able to reduce cell death in ARPE-19 cells after co-culture with Jurkat cells for 24 hours (**fig. 23**).

1.4 Confirmation with monoclonal antibodies

As final confirmation of our analysis, I demonstrated that our results in reducing intracellular signaling in ARPE-19 cells was dependent on the integrin antagonists administered in co-culture. According to this hypothesis, intracellular signaling was increased in a time-dependent way after Jurkat cells adhesion to ARPE-19 cells in a context that, in a simplified procedure, simulated an inflammatory condition. I speculate that this adhesion is dependent on the interaction between integrins and their correspondent adhesion molecules. I focused specifically on αLβ2/ICAM-1 and α4β1/VCAM-1. To confirm these studies, I administered to ARPE-19/Jurkat co-culture different antibodies to block the adhesion molecules of interest, and then I analyzed cellular signaling evaluating the MAPK/ERK pathway modification.

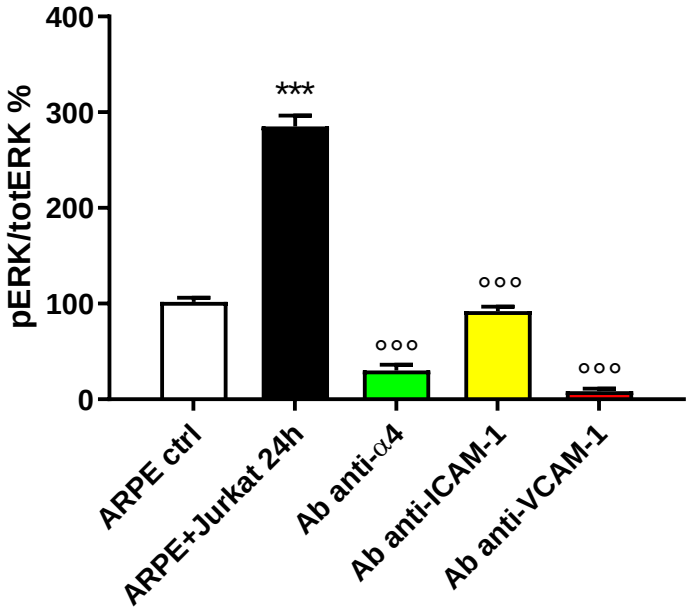


Fig. 24. Cell signaling after treatment with antibodies. *Detection of cell signaling activation in ARPE-19 cells plated in co-culture with Jurkat cells after treatment with monoclonal antibodies specific for integrin subunit α4, ICAM-1 and VCAM-1. Analysis was made through western blots, detecting p-ERK/tot-ERK ratio in ARPE-19 cells after 24 hours of co-culture. α4, ICAM-1 and VCAM-1 monoclonal antibodies were administered at 10 µg/mL for 1 hour (***p<0,001 vs ctrl, °°°p<0,001 vs ARPE-19+Jurkat 24h).*

As we can see (**fig. 24**), using antibodies for integrin subunit α4 and for ICAM-1 and VCAM-1, was possible to markedly reduce intracellular signaling in ARPE-19 cells in co-culture with Jurkat cells for 24 hours.

2. Studying antioxidants effects on RPE

In this part of my PhD research, I decided to investigate different antioxidants (**fig. 25**) commonly considered to be beneficial for AMD prevention and development. Indeed, I analyzed the effects of some flavonoids and carotenoids commonly reported to protect from AMD progress and part of supplements prescribed to patients in the early stages of this pathology (Cao et al. 2010; Domalpally et al. 2019; Kook et al. 2008; Liu et al. 2017; Pawlowska et al. 2019; Xu et al. 2016). Specifically, I am interested in understanding which is the mechanism behind their action and if this includes epigenetic modification in RPE cells.

I started from Flavanon, a molecule that has the basic rigid structure of flavonoids, a well-known class of antioxidants, but without any functional group. I used it as a reference. Later, I analyzed Quercetin, a flavonoid well known for its great antioxidant and anti-inflammatory properties and its glycoside, Quercitrin, widely present in fruits and vegetables (Anand David, Arulmoli, and Parasuraman 2016). Moreover, I analyzed Lutein, a carotenoid used in AMD prevention as part of the AREDS 2 study (Chew et al. 2012).

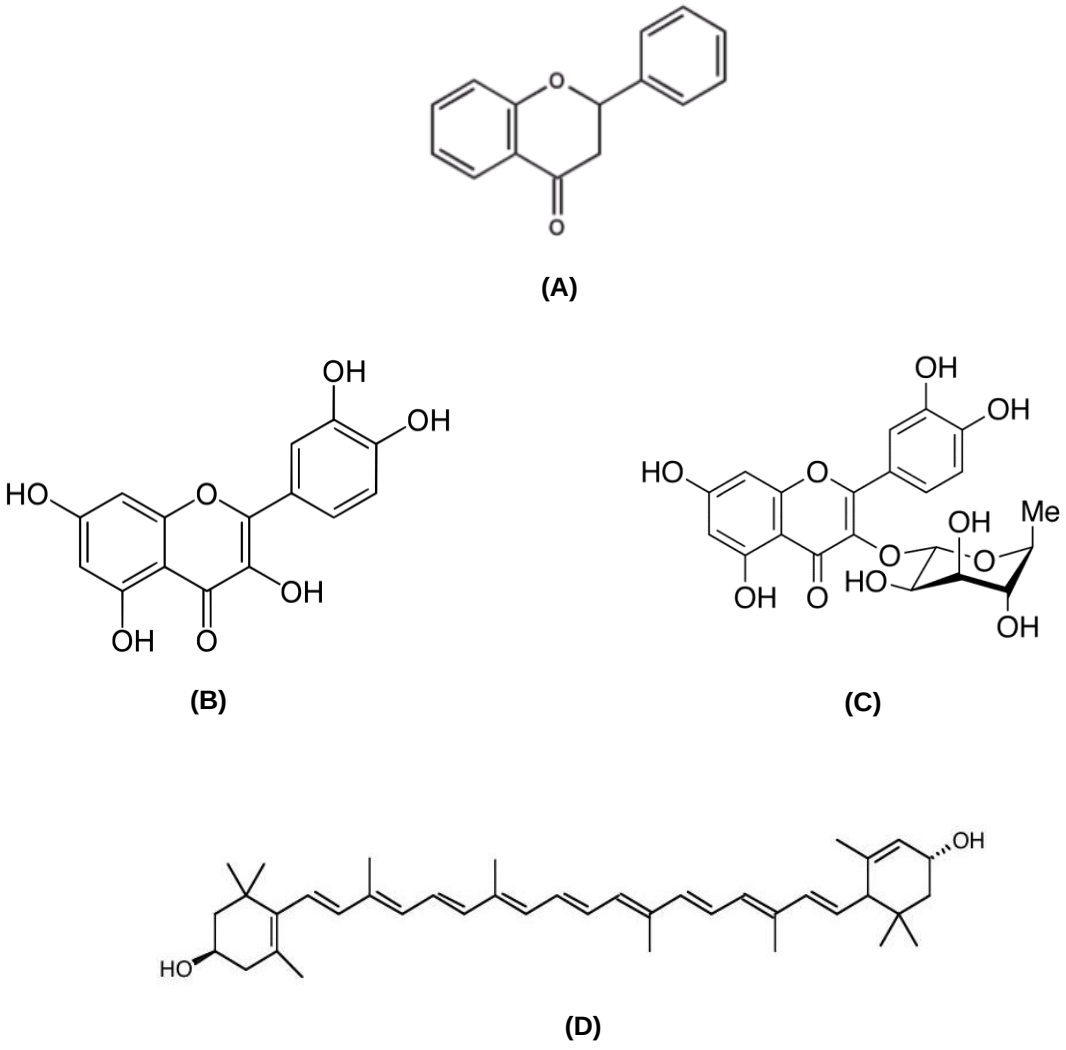


Fig. 25. Tested antioxidants. *Flavanon* **(A)***, Quercetin* **(B)***, Quercitrin* **(C)** *and Lutein* **(D)** *molecular structures.*

2.1 Role of antioxidants in cell death

First, I checked if the compounds could be responsible for increasing cell death, checking variation in early and late apoptosis/necrosis through cytofluorimetry assay.

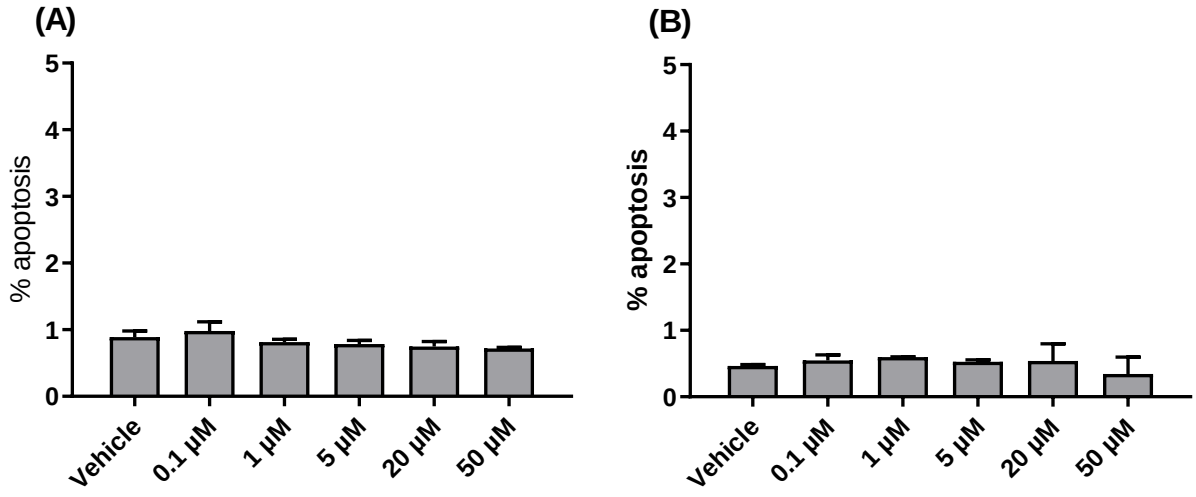


Fig. 26. Effects of Flavanon on apoptosis. *Early apoptotic fractions after administration of Flavanon (0.1, 1, 5, 20 and 50 µM) for 24* **(A)** *and 48 hours* **(B)** *in ARPE-19 cells. Analysis was made by cytofluorometry.*

As shown in **fig. 26**, administration of Flavanon for 24 and 48 hours in ARPE-19 cells did not induce any modification in early apoptosis. The same result was reported for late apoptosis/necrosis (**fig. 27**)

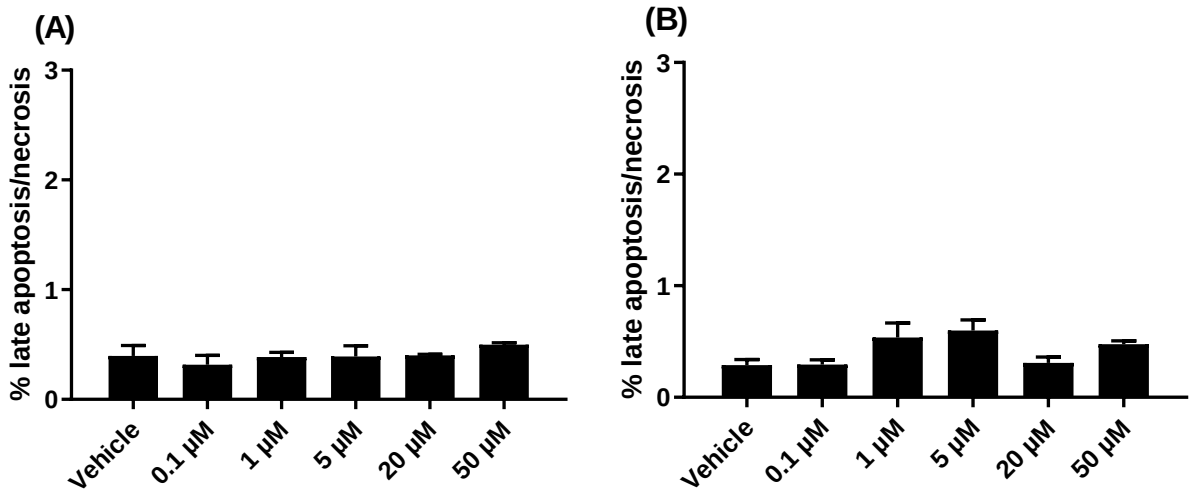


Fig. 27. Effects of Flavanon on late apoptosis/necrosis. *Late apoptotic/necrotic fractions after administration of Flavanon (0.1, 1, 5, 20 and 50 µM) for 24* **(A)** *and 48 hours* **(B)** *in ARPE-19 cells. Analysis was made by cytofluorometry.*

I have also repeated the analysis on cell death administering Quercitrin in the same conditions.

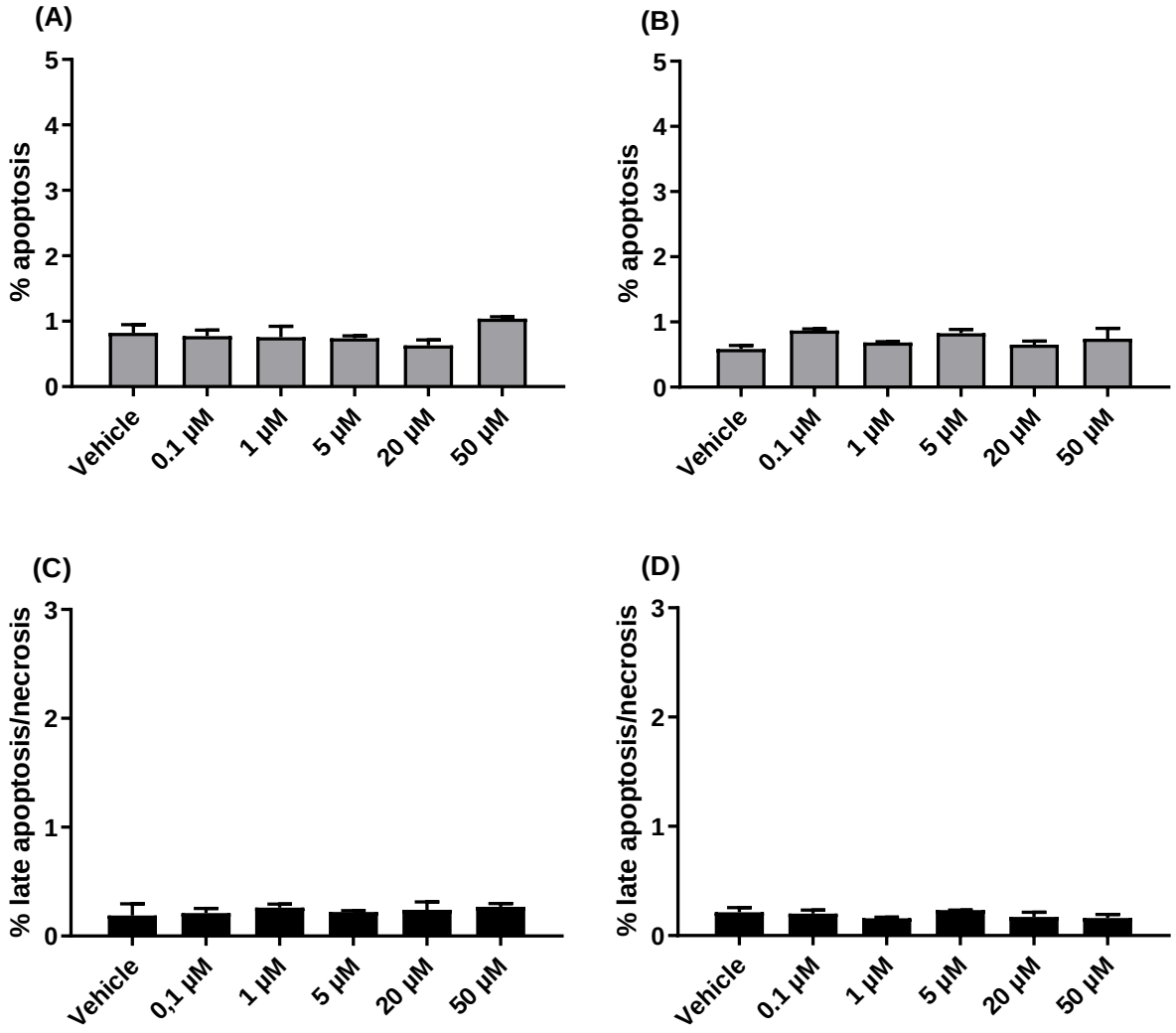


Fig. 28. Effects of Quercitrin on apoptosis and late apoptosis/necrosis. *Early apoptotic fractions after administration of Quercitrin (0.1, 1, 5, 20 and 50 µM) for 24* **(A)** *and 48 hours* **(B)** *in ARPE-19 cells. Late apoptotic/necrotic fractions after administration of Quercitrin (0.1, 1, 5, 20 and 50 µM) for 24* **(C)** *and 48 hours* **(D)** *in ARPE-19 cells. Analysis was made by cytofluorometry.*

As for Flavanon, Quercitrin did not induce any modification in cell apoptosis and late apoptosis/necrosis (**fig. 28**), confirming to not be responsible for increasing cell death.

2.2 Effects of Antioxidants on histone H3 acetylation

I evaluated if there is any variation in histone H3 acetylation, after treatment of ARPE-19 with the antioxidant compounds Lutein and Quercetin. This could lead help to understand if

antioxidants may have a role in epigenetic modification and if that is one of the ways through which they exert their protective role on RPE cells.

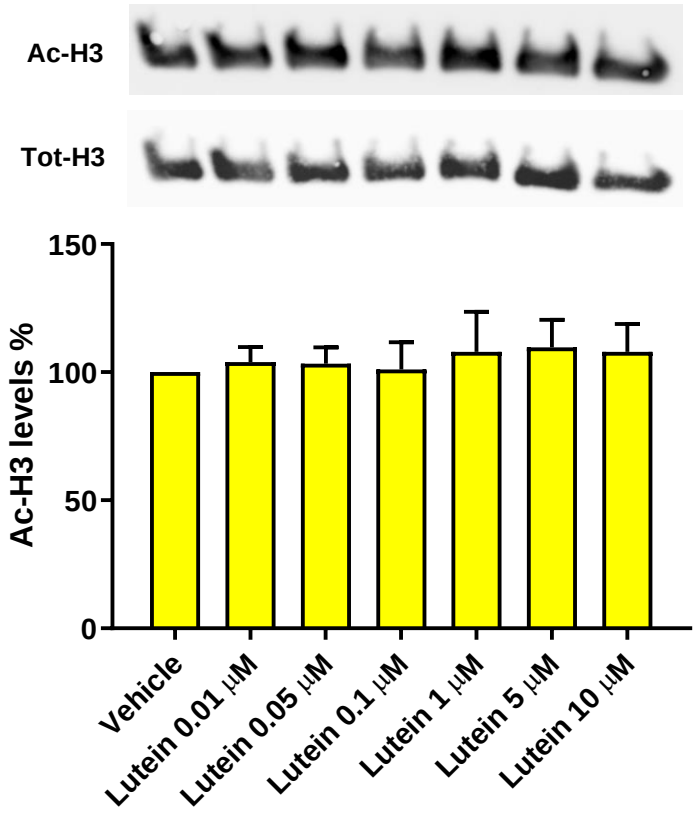


Fig. 29. Effects of Lutein on histone H3 acetylation. *Histone H3 acetylation reported to total histone H3 after administration of Lutein (0.01, 0.05, 0.1, 1, 5 and 10 μM) for 48 hours in ARPE-19 cells.*

Lutein administered for 48 hours to ARPE-19 does not modify histone H3 acetylation (**fig. 29).** I assayed in the same conditions the flavonoid Quercetin and, even with a reductive trend, it did not show any statistically significant modification of histone H3 acetylation (**fig. 30**).

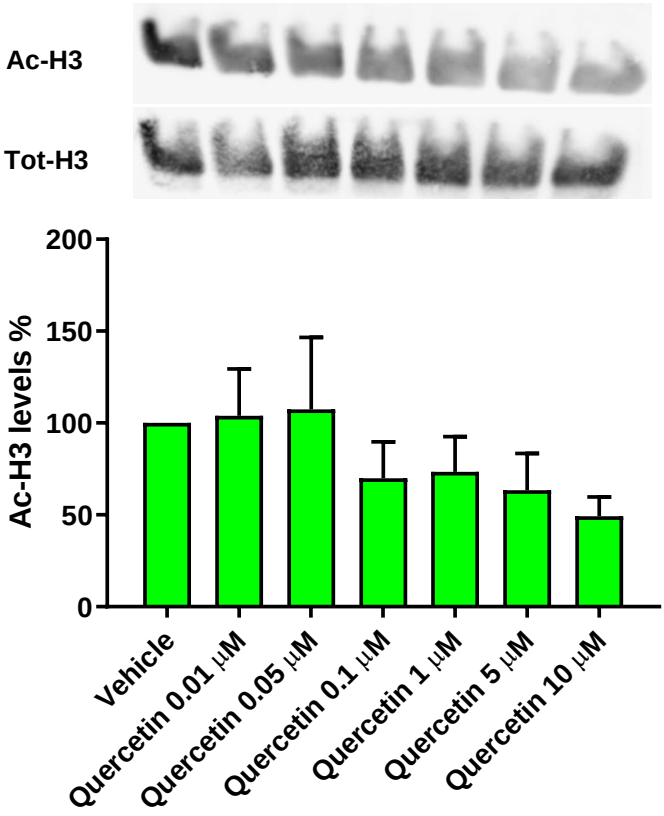


Fig. 30. Effects of Quercetin on histone H3 acetylation. *Histone H3 acetylation reported to total histone H3 after administration of Quercetin (0.01, 0.05, 0.1, 1, 5 and 10 μM) for 48 hours in ARPE-19 cells.*

3. Possible beneficial effects of statins in AMD

As described in the aims (p. 47), this research was carried out during the period that I spent at the Angiogenesis Laboratory with Dr. Vavvas and his team who reported a potential protective effect of statins in ARPE-19 cell exerted through an increase in the phagocytic capacities and a reduction of inflammatory cytokines production (Tian et al. 2017). Moreover, a clinical study conducted on AMD patients, reported that high doses of statins may cause regression of AMD features as drusen and in some cases even an improvement in visual acuity (Vavvas et al. 2016). Following these outcomes, I started a project to investigate which cellular mechanisms could be involved in statins potential effects on RPE cells. Therefore, I carried out a study to assay gene expression modifications induced by atorvastatin in ARPE-19 cells through RNA-sequencing.

3.1 ARPE-19

The first part of the research was conducted in ARPE-19 cells and among the statin drug family was selected atorvastatin, considering the promising previous results obtained by Dr. Vavvas and coworkers (Tian et al. 2017; Vavvas et al. 2016).

The choice of the proper dose to use in the study was made considering reports in the scientific community underlying how drug concentrations used in *in vitro* experiments are often too high if compared to the real doses reached in human plasma (Burgos-Morón et al. 2010; Liston and Davis 2017; Salehi et al. 2018). Therefore, I followed a study reporting human serum concentration of statins (Björkhem-Bergman, Lindh, and Bergman 2011) and picked 15 nM as the basal dose. Indeed, 15 nM is the mean concentration in human serum after administration of the most common dose of administration of atorvastatin in humans (20 mg). Moreover, I picked another concentration of 60 nM to treat ARPE-19, correspondent to the higher dose of atorvastatin commonly used in human beings (80 mg). The administration of the two doses were made at two different time-points: 3 hours and 3 days. Among all the RNA-sequencing results, I focused especially on pathway reports, allowing to determine whether the differentially expressed mRNAs are enriched in certain biological pathways, based on KEGG (Kyoto Encyclopedia of Genes and Genomes) databases.

Only the higher dose (60 nM) administered for 3 days, seemed to be able to affect mechanisms related to debris degradation, phagocytosis, membrane fluidity or other elements reported to have protective or improving effects on AMD features.

Indeed, two genes belonging to endocytosis pathway (hsa04144) (**fig. 31**) were reported to be upregulated in these conditions.

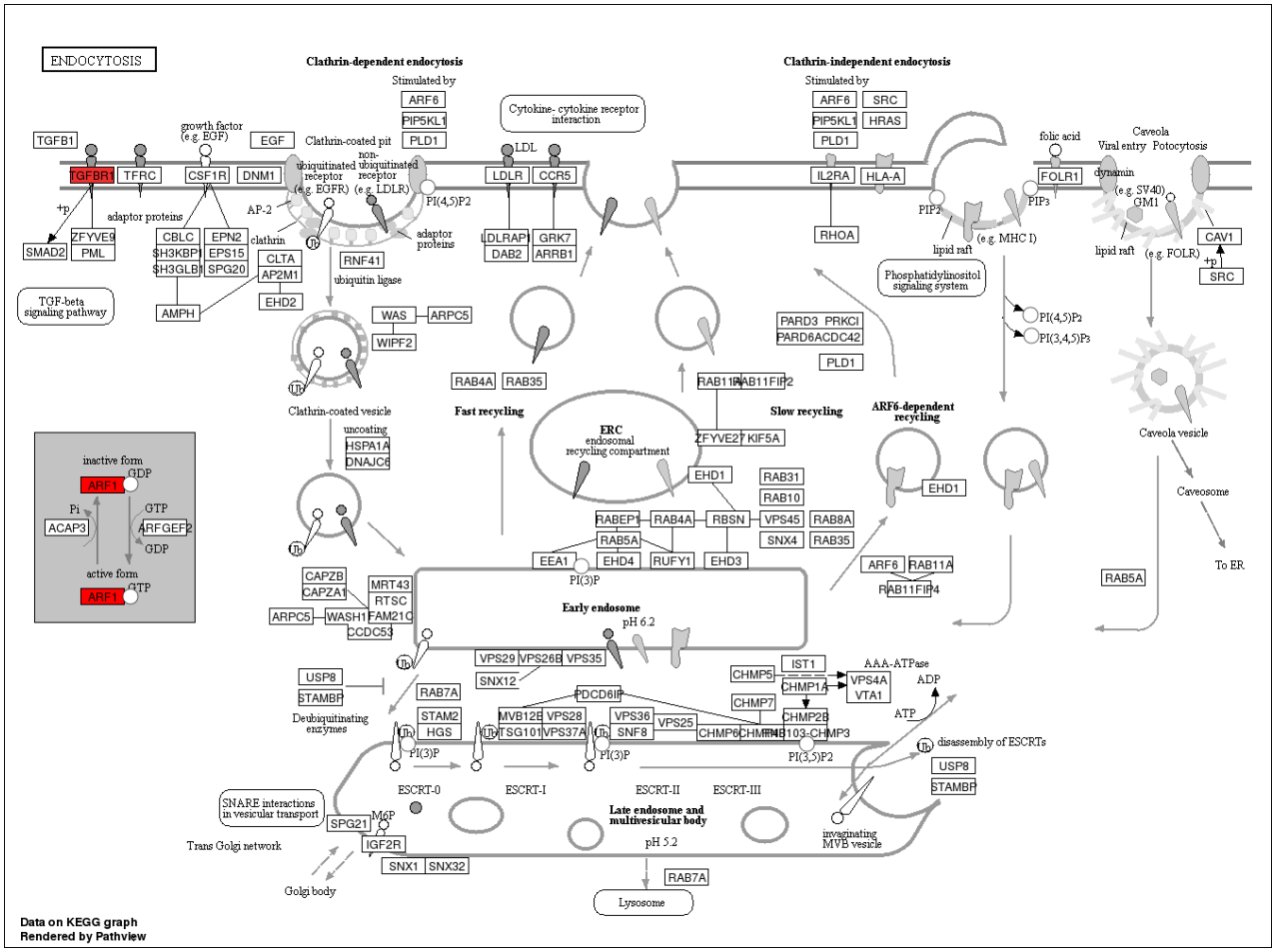


Fig. 31. KEGG pathway hsa04144, endocytosis (Homo sapiens): *Green marked nodes are associated with down-regulated genes, red marked nodes are associated with up-regulated or only whole dataset genes, white nodes have no significance.*

Nevertheless, considering the number of genes belonging to this pathway compared to the only two statistically upregulated, does not allow to draw any conclusion.

3.2 Primary RPE cells

Therefore, I decided to carry out another experiment, using primary RPE human cells (hRPE). Again, I used two different doses (15 nM and 60 nM), but this time administered for seven days.

Interestingly, the analysis reported how the drug administration at both doses, compared to the vehicle, was able to upregulate several pathways, while either no pathways were reported to be downregulated and no effects were reported to be reached by comparing the effect of the lower dose to the higher.

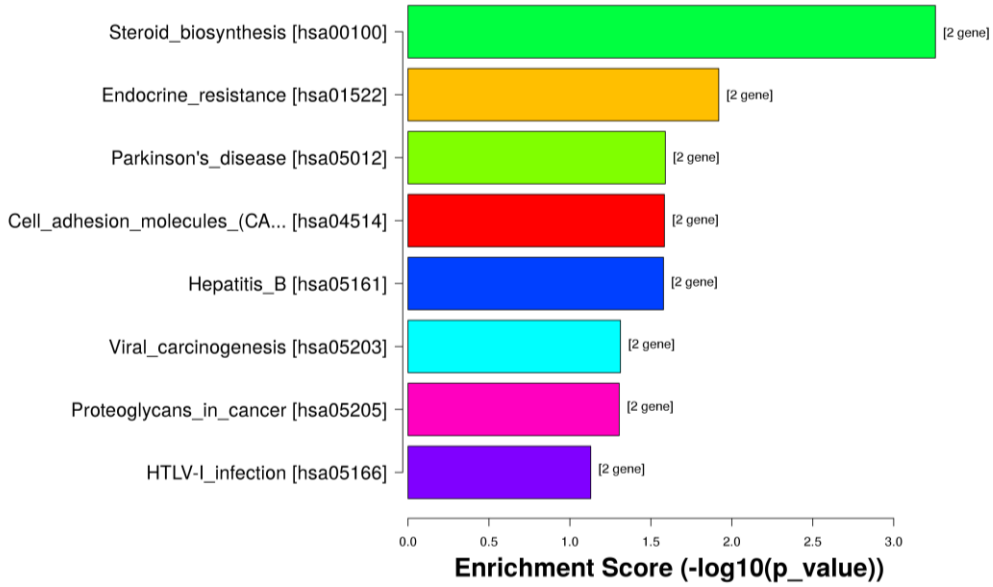


Fig. 32. Pathway analysis Atorvastatin 15 nM vs vehicle, up-regulation: *Top significant pathways ordered from top to bottom by p-value (-log10 scaled), with the most significant pathway on the top. Pathway analysis based on the latest KEGG database for the differentially expressed genes. Determination of differentially expressed mRNAs enriched in certain biological pathways. Up-regulation comparing low dose (15 nM) vs vehicle in primary RPE cells. The p-values calculated by Fisher’s exact test are used to estimate the statistical significance of the enrichment of the pathways between the two groups.*

Interestingly, for the 15 nM dose, it is possible to notice from the enrichment score report (**fig. 32**) that among all the pathways upregulated, none of them could be related to any protective or beneficial effects on AMD.

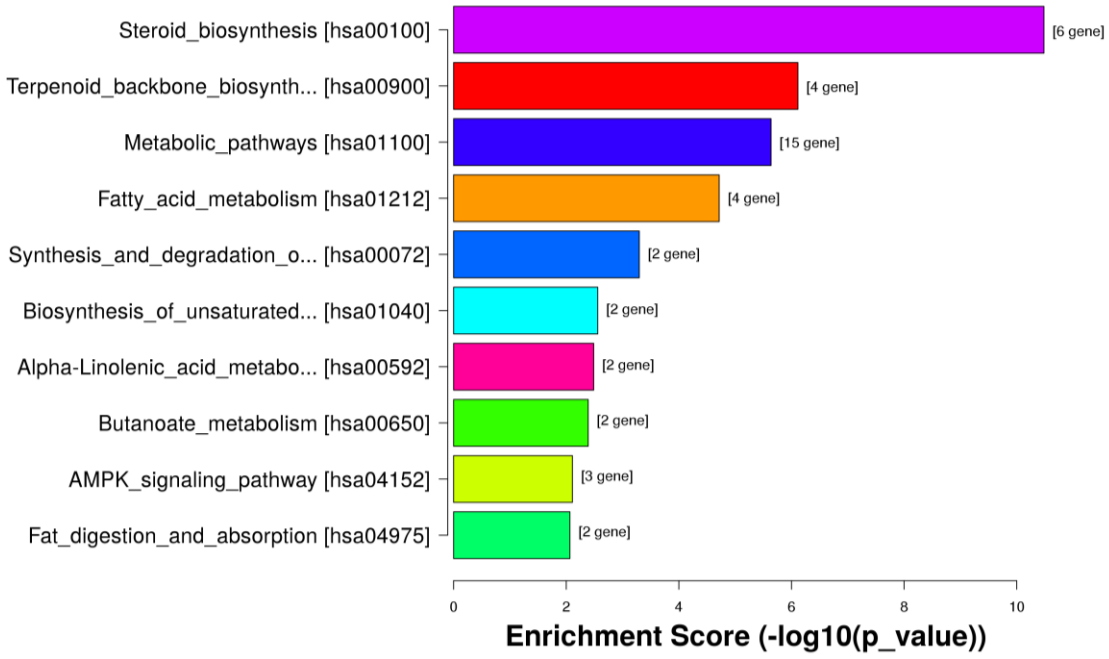


Fig. 33. Pathway analysis Atorvastatin 60 nM vs vehicle, up-regulation: *Top significant pathways ordered from top to bottom by p-value (-log10 scaled), with the most significant pathway on the top. Pathway analysis based on the latest KEGG database for the differentially expressed genes. Determination of differentially expressed mRNAs enriched in certain biological pathways. Up-regulation comparing high dose (60 nM) vs vehicle in primary RPE cells. The p-values calculated by Fisher’s exact test are used to estimate the statistical significance of the enrichment of the pathways between the two groups.*

Instead, at 60 nM dose treatment, among the different pathways upregulated, it is possible to notice an increase in fat digestion and adsorption pathway (hsa04975), with two genes involved (**fig. 33**).

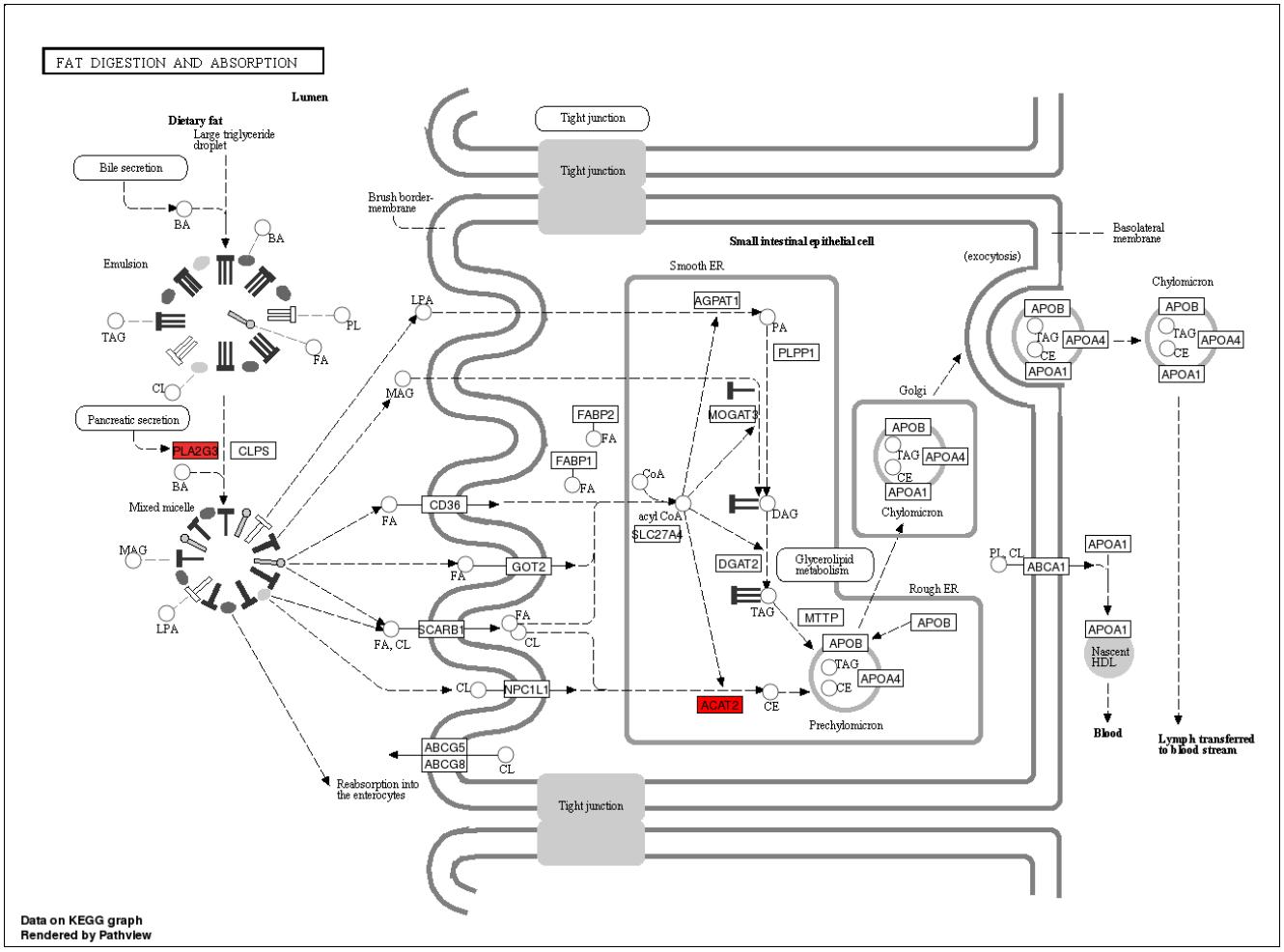


Fig. 34 KEGG pathway hsa04975, fat digestion and absorption (Homo sapiens): *Green marked nodes are associated with down-regulated genes, red marked nodes are associated with up-regulated or only whole dataset genes, white nodes have no significance.*

As showed in the study carried out in ARPE-19 (**fig. 31**), also for this research (**fig. 34**) it is possible to notice how only two genes are upregulated in the pathway of interest. Again, this result is unfortunately not sufficient if compared to the overall pathway analysis and to the elevated number of genes potentially involved. It is possible to assume a certain degree of correlation between our drug and the effects, but for claiming a definitive effect of atorvastatin in modifying fat digestion and absorption or other mechanism related to AMD improvements, deeper studies need to be made.

Conclusions

AMD is still a huge burden in our society with enormous prevision of growth in the future due also to the stable increase in human lifespan, leading the average population age to raise continuously in next decades. As other age-related diseases such as Alzheimer disease, many efforts are made aiming to find any effective therapy but, being a multifactorial pathology with a considerable number of different factors composing it, it is difficult even just to understand its basic mechanisms. Finding new approaches and therapies is turning out to be a hard challenge, with different failed attempt on the way.

My PhD program focused precisely on studying and understanding better the role of retinal pigmented epithelial cells in AMD, in order to obtain a deeper view of what relies in cellular mechanisms of age-related macular degeneration. My work was mainly carried out in vitro and, even if it allows a simplified context, I have tried to obtain numerous information from my research aiming to traduce my findings in possible useful outcome.

Main part of my work regarded the study of the role of integrins in the AMD frame. I focused on understanding how react ARPE-19 cells in the presence of immune cells as Jurkat and in a context of simulated inflammation in order to reproduce what may happen in AMD. After confirming literature data reporting integrins and adhesion molecule expression in ARPE-19 cells, I have tried to induce inflammation by using inflammatory cytokines as TNF-α and IL-1β and measuring what would have been the response to ICAM-1 and VCAM-1 production in ARPE-19. My findings show that while VCAM-1 expression does not change, ICAM-1 increases markedly in ARPE-19 cells treated with TNF-α and IL-1β 10 ng/ml for 24 hours. Therefore, I focused on cellular interactions between ARPE-19 cells and Jurkat cells, trying to evaluate cell signals possibly related to inflammation that could be also mediated by integrins. After 48 hours in co-culture, indeed, Jurkat cells presence induces a considerable increase in the MAPK/ERK pathway, a cell signaling pathway related to integrin activation. Jurkat cells induce in ARPE-19 also a decrease of ICAM-1 expression and I assume this could be a possible inflammatory reaction by ARPE-19 in order to reduce or prevent any cellular damage. Moreover, Jurkat cells may increase apoptosis and late apoptosis/necrosis other than leading to an increase in the inflammatory cytokine IL-1β production, detected both in Jurkat cells and ARPE-19 cells.

After this first part of research, I was interested in understanding if it was possible to stop the interaction mediated by integrins and if this could represent a possible treatment option for AMD, reducing inflammation and cell death in vitro. For this reason, in the same conditions tested for Jurkat and ARPE-19 cells in co-culture, I administered two integrin antagonists synthetized and analyzed previously in our laboratory. These molecules, MN27 and DS-70, have been reported to be able to reduce all the previously evaluated parameters. Indeed, MAPK/ERK pathway signaling, IL-1β production and cell death are reduced after the administration of the two compounds.

Finally, I tried to confirm if these results are due to integrin antagonism and the effects detected were related on a partial counteract of integrin receptors. I have administered monoclonal antibodies directed against ICAM-1, VCAM-1 and integrin alpha-4 in the co-culture of ARPE-19 and Jurkat, at saturation concentrations. Adopting this procedure, it is possible to evaluate a reduction in cell signaling through the MAPR/ERK pathway, indicating that blocking integrins are able to induce a reduction in cell signaling due to ARPE-19/Jurkat cell interaction.

In conclusion, this part of my research reports results indicating that integrin antagonists can modulate cell to cell interactions between ARPE-19 cells and cells of the immune system such as Jurkat cells. Administering MN27 and DS-70 antagonists, respectively for αLβ2 and α4β1 integrins, the interaction αLβ2/ICAM-1 and α4β1/VCAM-1 is counteracted. These compounds are able to reduce immune cells adhesion to ARPE-19 cells in vitro, an effect mediated by leucocyte integrins. This data still need further investigations and I already carried out different other experiment on other immune cell type as THP-1 and monocytes to give more evidence to these data. Future works will be pointed to increase the number of the experiment replicates, repeating the same experiments already concluded but on differ immune cells and investigating different inflammatory conditions to evaluate all the different features of the complex multifactorial environment of AMD pathology.

The second part of my research was focused on studying antioxidants molecules, widely used in the prevention of “dry” AMD, but still their mechanism of action is not well understood. Aim of my approach was to understand if these compounds are able to modify epigenetic expression through histone acetylation modification on ARPE-19 as a possible explanation of their well-known protective effects on cell death and damage. I administered

flavonoids as Flavanon, Quercetin and its glycoside, Quercitrin other than a carotenoid widely used in AMD prevention, Lutein.

Unfortunately, none of the molecules showed to be clearly able to induce epigenetic modifications through histone acetylation. Only Quercetin indicated a pretty consistent trend even if not statistically significant. These compounds are reported to be effective in AMD prevention and are used in this pathology to slow down the disease progression. Future studies deeply investigating how these compounds act, are necessary to elucidate and finally clear out any doubt on their efficacy.

In the last part of my research I focused in studying statins and their potential role in “dry” AMD prevention and protection. Starting from previous findings from Dr. Demetrios Vavvas and coworkers, I investigated through RNA-sequencing the gene expression modification on ARPE-19 cells and human primary RPE cells. After collecting the data, I noticed that Atorvastatin administrated to RPE cells is able to partially induce an increased expression of genes related to augmented metabolism and phagocytosis that can be helpful in AMD features. Nevertheless, the genes involved, if compared to the whole number of genes belonging to the pathways of interest are unfortunately not enough to support the hypothesis that the treatment is able to exert a massive protective action. More efforts and experiments are needed to keep investigating if and how statins may turn out to be potential allied against “dry” AMD. Unfortunately, I was not able to conclude my work before the end of my period abroad. In the last part of my period there, I focused on preparing a further experiment to administer atorvastatin to C57BL/6 mice. I decided to run the analysis both in male and female mice by gavage that could be similar to human route of administration. I conducted blood tests on mice treated with 2.5, 5, 20, 25, 50, 100 mg/kg of atorvastatin for seven days to check the serum levels reached. My purpose was to establish the appropriate dose and the proper formulation of the drug in the suspension used for the gavage.

References

Abidi, Afroz, Pooja Shukla, and Ali Ahmad. 2016. “Lifitegrast: A Novel Drug for Treatment of Dry Eye Disease.” *Journal of Pharmacology & Pharmacotherapeutics* 7(4): 194.

Allen, David, and Abhay Vasavada. 2006. “Cataract and Surgery for Cataract - Clinical Review.” *Bmj* 333(July): 128–32.

Alward, Wallace, and Reid Longmiur. 2017. “Anatomy of the Angle - American Academy of Ophthalmology.” *American Academy of Ophthalmology*.

Ambati, Jayakrishna, and Benjamin J. Fowler. 2012. “Mechanisms of Age-Related Macular Degeneration.” *Neuron* 75(1): 26.

AMDF. 2019. “Dry vs Wet Age-Related Macular Degeneration.” *American Macular Degeneration Foundation*.

Anand David, Alexander Victor, Radhakrishnan Arulmoli, and Subramani Parasuraman. 2016. “Overviews of Biological Importance of Quercetin: A Bioactive Flavonoid.” *Pharmacognosy reviews* 10(20): 84–89.

Anastasi, Giuseppe, Giuseppe Balboni, and Pietro Motta. 2007. *Trattato Di Anatomia Umana, Vol. 3*. Edi. Ermes.

Andley, Usha P. 2007. “Crystallins in the Eye: Function and Pathology.” *Progress in Retinal and Eye Research* 26(1): 78–98.

Andley, Usha P. 2008. “The Lens Epithelium: Focus on the Expression and Function of the Alpha-Crystallin Chaperones.” *The international journal of biochemistry & cell biology* 40(3): 317–23.

Annear, Matthew J., and Simon M. Petersen-Jones. 2012. “Surgery of the Ocular Surface.” *Equine Surgery*: 770–92.

Annunziato, A. 2008. “DNA Packaging: Nucleosomes and Chromatin.” *Nature Education*.

Aouiss, A., D. Anka Idrissi, M. Kabine, and Y. Zaid. 2019. “Update of Inflammatory

Proliferative Retinopathy: Ischemia, Hypoxia and Angiogenesis.” *Current Research in Translational Medicine*.

Ardeljan, Christopher P, Daniel Ardeljan, Mones Abu-Asab, and Chi-Chao Chan. 2014. “Inflammation and Cell Death in Age-Related Macular Degeneration: An Immunopathological and Ultrastructural Model.” *Journal of clinical medicine* 3(4): 1542–60.

Arnaout, M Amin, Simon L Goodman, and Jian-Ping Xiong. 2007. “Structure and Mechanics of Integrin-Based Cell Adhesion.” *Current opinion in cell biology* 19(5): 495–507.

Askari, Janet A, Patrick A Buckley, A Paul Mould, and Martin J Humphries. 2009. “Linking Integrin Conformation to Function.” *Journal of cell science* 122(Pt 2): 165–70.

Bai, Zhong-Tian et al. 2018. “Epigenetic Actions of Environmental Factors and Promising Drugs for Cancer Therapy.” *Oncology letters* 15(2): 2049–56.

Baiula, Monica et al. 2016. “New β-Lactam Derivatives Modulate Cell Adhesion and Signaling Mediated by RGD-Binding and Leukocyte Integrins.” *Journal of Medicinal Chemistry* 59(21): 9721–42.

Balaiya et al. 2010. Clinical Ophthalmology *Clinical Ophthalmology Evaluation of Ultraviolet Light Toxicity on Cultured Retinal Pigment Epithelial and Retinal Ganglion Cells*.

Barczyk, Malgorzata, Sergio Carracedo, and Donald Gullberg. 2010. “Integrins.” *Cell and Tissue Research* 339(1): 269–80.

Benhar, Inbal, Kitty Reemst, Vyacheslav Kalchenko, and Michal Schwartz. 2016. “The Retinal Pigment Epithelium as a Gateway for Monocyte Trafficking into the Eye.” *The EMBO Journal* 35(11): 1219–35.

Berner, Andre K., and Mark E. Kleinman. 2016. “Therapeutic Approaches to Histone Reprogramming in Retinal Degeneration.” In *Advances in Experimental Medicine and Biology*, , 39–44.

Biesemeier, Antje, Ulrich Schraermeyer, and Oliver Eibl. 2011. "Chemical Composition of Melanosomes, Lipofuscin and Melanolipofuscin Granules of Human RPE Tissues." *Experimental Eye Research* 93(1): 29–39.

Bird, Adrian. 2007. "Perceptions of Epigenetics." *Nature* 447(7143): 396–98.

Björkhem-Bergman, Linda, Jonatan D. Lindh, and Peter Bergman. 2011. "What Is a Relevant Statin Concentration in Cell Experiments Claiming Pleiotropic Effects?" *British Journal of Clinical Pharmacology* 72(1): 164–65.

Blackburn, Brecken J, Michael W Jenkins, Andrew M Rollins, and William J Dupps. 2019. "A Review of Structural and Biomechanical Changes in the Cornea in Aging, Disease, and Photochemical Crosslinking." *Frontiers in bioengineering and biotechnology* 7: 66.

Blausen Medical Communications. 2014. "Medical Gallery of Blausen Medical." *WikiJournal of Medicine* 1(2): 10.

Bloom, Jeffrey, and Craig N. Czyz. 2019. StatPearls *Anatomy, Head and Neck, Eye Iris Sphincter Muscle*. StatPearls Publishing.

Borges- Giampani, Adriana Silva, and Jair Giampani. 2013. "Anatomy of Ciliary Body, Ciliary Processes, Anterior Chamber Angle and Collector Vessels." In *Glaucoma - Basic and Clinical Aspects*, InTech.

Bourne, Rupert R.A. et al. 2017. "Magnitude, Temporal Trends, and Projections of the Global Prevalence of Blindness and Distance and near Vision Impairment: A Systematic Review and Meta-Analysis." *The Lancet Global Health*.

Bourne, W M. 2003. "Biology of the Corneal Endothelium in Health and Disease." *Eye* 17(8): 912–18.

Bowling, Brad (Bradley), and Jack J. Kanski. 2015. *Kanski's Clinical Ophthalmology : A Systematic Approach*.

Boyd, Kierstan. 2018. "The Eye and Immune Privilege." *American Academy of Ophthalmology*.

Boyd, Kierstan, and David Turbert. 2018. “Parts of the Eye.” *American Academy of Ophthalmology*.

BrightFocus Foundation. 2016. “Age-Related Macular Degeneration: Facts & Figures.” *BrightFocus*.

Burgos-Morón, Estefanía et al. 2010. “The Dark Side of Curcumin.” *International Journal of Cancer* 126(7): NA-NA.

Burton, Matthew J. 2009. “Prevention, Treatment and Rehabilitation.” *Community eye health* 22(71): 33–35.

Butcher, Eugene C. et al. 1999. “Lymphocyte Trafficking and Regional Immunity.” *Advances in Immunology* 72: 209–53.

Campbell, Iain D, and Martin J Humphries. 2011. “Integrin Structure, Activation, and Interactions.” *Cold Spring Harbor perspectives in biology* 3(3): a004994.

Cao, Xiaoguang et al. 2010. “The Effects of Quercetin in Cultured Human RPE Cells under Oxidative Stress and in Ccl2/Cx3cr1 Double Deficient Mice.” *Experimental Eye Research* 91(1): 15–25.

Chakravarthy, Usha et al. 2010. “Clinical Risk Factors for Age-Related Macular Degeneration: A Systematic Review and Meta-Analysis.” *BMC ophthalmology* 10: 31.

Chen, L. 2009. “Ocular Lymphatics: State-of-the-Art Review.” *Lymphology* 42(2): 66–76.

Cheung, Ning, and Alan A. McNab. 2003. “Venous Anatomy of the Orbit.” *Investigative Opthalmology & Visual Science* 44(3): 988.

Chew, Emily Y et al. 2012. “The Age-Related Eye Disease Study 2 (AREDS2): Study Design and Baseline Characteristics (AREDS2 Report Number 1).” *Ophthalmology* 119(11): 2282–89.

Chirco, Kathleen Rose. 2017. “Mechanisms of Pathophysiology and Methods for Regeneration of the Choriocapillaris in Age-Related Macular Degeneration.” University of Iowa.

Choi, WooJhon et al. 2013. "Choriocapillaris and Choroidal Microvasculature Imaging with Ultrahigh Speed OCT Angiography." *PloS one* 8(12): e81499.

Coca-Prados, Miguel. 2014. "The Blood-Aqueous Barrier in Health and Disease." *Journal of Glaucoma* 23(8 Suppl 1): S36-8.

Crane, Isabel J., and Janet Liversidge. 2008. "Mechanisms of Leukocyte Migration across the Blood–Retina Barrier." *Seminars in Immunopathology* 30(2): 165.

Csoka, Antonei B., and Moshe Szyf. 2009. "Epigenetic Side-Effects of Common Pharmaceuticals: A Potential New Field in Medicine and Pharmacology." *Medical Hypotheses* 73(5): 770–80.

Cunha-Vaz, José. 2009. "The Blood–Retinal Barrier in Retinal Disease." *European Ophthalmic Review* 03(02): 105.

Dattoli et al. 2018. "DS-70, a Novel and Potent A4 Integrin Antagonist, Is an Effective Treatment for Experimental Allergic Conjunctivitis in Guinea Pigs." *British journal of pharmacology* 175(20): 3891–3910.

Dattoli, Samantha D. et al. 2014. "Synthesis and Assay of Retro-A4β1 Integrin-Targeting Motifs." *European Journal of Medicinal Chemistry* 73: 225–32.

Deangelis, Margaret M, Alexandra C Silveira, Elizabeth A Carr, and Ivana K Kim. 2011. "Genetics of Age-Related Macular Degeneration: Current Concepts, Future Directions." *Seminars in ophthalmology* 26(3): 77–93.

Delaey, Christophe, and Johan Van De Voorde. 2000. 32 Ophthalmic Res *Regulatory Mechanisms in the Retinal and Choroidal Circulation*.

Devine, L., S. L. Lightman, and J. Greenwood. 1996. "Role of LFA-1, ICAM-1, VLA-4 and VCAM-1 in Lymphocyte Migration across Retinal Pigment Epithelial Monolayers in Vitro." *Immunology* 88(3): 456–62.

Díaz-Coránguez, Mónica, Carla Ramos, and David A Antonetti. 2017. "The Inner Blood-Retinal Barrier: Cellular Basis and Development." *Vision research* 139: 123–37.

Domalpally, Amitha et al. 2019. “Prevalence, Risk, and Genetic Association of Reticular Pseudodrusen in Age-Related Macular Degeneration: Age-Related Eye Disease Study 2 Report 21.” *Ophthalmology*.

Dorecka, Mariola et al. 2013. “Exendin-4 and GLP-1 Decreases Induced Expression of ICAM-1, VCAM-1 and RAGE in Human Retinal Pigment Epithelial Cells.” *Pharmacological Reports* 65(4): 884–90.

Döring, Axinia et al. 2011. “TET Inducible Expression of the A4β7-Integrin Ligand MAdCAM-1 on the Blood-Brain Barrier Does Not Influence the Immunopathogenesis of Experimental Autoimmune Encephalomyelitis.” *European Journal of Immunology* 41(3): 813–21.

Dunaief, Joshua L., Tzvete Dentchev, Gui-Shuang Ying, and Ann H Milam. 2002. “The Role of Apoptosis in Age-Related Macular Degeneration.” *Archives of Ophthalmology* 120(11): 1435.

Ehrlich, Rita et al. 2017. “Anatomy and Regulation of the Optic Nerve Blood Flow.” *Reference Module in Neuroscience and Biobehavioral Psychology*.

Ellenbroek, Bart, and Jiun Youn. 2016. “Environment Challenges and the Brain.” *Gene-Environment Interactions in Psychiatry*: 107–39.

Eryılmaz Pehlivan, Fadime. 2019. “Vitamin C: An Epigenetic Regulator.” In *Vitamin C - an Update on Current Uses and Functions*, IntechOpen.

Eye Care Fundamentals. 2014. “Retinal Pigment Epithelium & Bruchs Membrane (RPE & BM).” *Youtube*.

Fan, J., J. Baeza, and J.M. Denu. 2016. “Investigating Histone Acetylation Stoichiometry and Turnover Rate.” *Methods in Enzymology* 574: 125–48.

Felten, David L. et al. 2016. “Sensory Systems.” *Netter’s Atlas of Neuroscience*: 353–89.

Fernald, Russell D. 1997. “The Evolution of Eyes.” *Brain, Behavior and Evolution* 50(4): 253–59.

Fischle, Wolfgang, Yanming Wang, and C David Allis. 2003. "Histone and Chromatin Cross-Talk." *Current opinion in cell biology* 15(2): 172–83.

Forrester, John V. et al. 2016. "Anatomy of the Eye and Orbit." *The Eye*: 1-102.e2.

De Fougerolles, AR, Xi Qin, and A Timothy. 1994. "Characterization of the Function of Intercellular Adhesion Molecule (ICAM)-3 and Comparison with ICAM-1 and ICAM-2 in Immune Responses." *The Journal of Experimental Medicine* 179(2): 619.

Frank, Philippe G, and Michael P Lisanti. 2008. "ICAM-1: Role in Inflammation and in the Regulation of Vascular Permeability." *American journal of physiology. Heart and circulatory physiology* 295(3): H926–27.

Freddo, Thomas F. 2013. "A Contemporary Concept of the Blood-Aqueous Barrier." *Progress in retinal and eye research* 32: 181–95.

Frederick, Paul A, and Mark E Kleinman. 2014. "The Immune System and AMD." *Current ophthalmology reports* 2(1): 14–19.

Fricke, Timothy R. et al. 2018. "Global Prevalence of Presbyopia and Vision Impairment from Uncorrected Presbyopia: Systematic Review, Meta-Analysis, and Modelling." *Ophthalmology*.

Fukuoka, Y, M Strainic, and M E Medof. 2003. "Differential Cytokine Expression of Human Retinal Pigment Epithelial Cells in Response to Stimulation by C5a." *Clinical and Experimental Immunology* 131(2): 248.

Gemenetzi, M, and A J Lotery. 2014. "The Role of Epigenetics in Age-Related Macular Degeneration." *Eye (London, England)* 28(12): 1407–17.

Ghosh, Shamik et al. 2006. "The LFA-1 Adhesion Molecule Is Required for Protective Immunity during Pulmonary Mycobacterium Tuberculosis Infection." *The Journal of Immunology* 176(8): 4914–22.

Glickman, Randolph D. 2011. "Ultraviolet Phototoxicity to the Retina." *Eye & Contact Lens: Science & Clinical Practice* 37(4): 196–205.

Golestaneh, Nady et al. 2018. "Dysfunctional Autophagy in RPE, a Contributing Factor in Age-Related Macular Degeneration." *Cell Death & Disease* 8(1): e2537–e2537.

Grakoui, A. et al. 1999. "The Immunological Synapse: A Molecular Machine Controlling T Cell Activation." *Science* 285(5425): 221–27.

Grassmann, Felix, Iris M Heid, Bernhard H F Weber, and International AMD Genomics Consortium International AMD Genomics Consortium (IAMDGC). 2017. "Recombinant Haplotypes Narrow the ARMS2/HTRA1 Association Signal for Age-Related Macular Degeneration." *Genetics* 205(2): 919–24.

Gregerson, Dale S. et al. 2007. "Interaction of Retinal Pigmented Epithelial Cells and CD4 T Cells Leads to T-Cell Anergy." *Investigative Opthalmology & Visual Science* 48(10): 4654.

Gschmeissner, Steve. 2015. "Gene Therapy Cures Blindness by Replacing Vision Cells in Eyes." *Newscientist*.

Guillaumet-Adkins, Amy et al. 2017. "Epigenetics and Oxidative Stress in Aging." *Oxidative medicine and cellular longevity* 2017: 9175806.

Gurevich, V V, and J L Benovic. 1995. "Visual Arrestin Binding to Rhodopsin. Diverse Functional Roles of Positively Charged Residues within the Phosphorylation-Recognition Region of Arrestin." *The Journal of biological chemistry* 270(11): 6010–16.

Hahn, Lindsay et al. 2015. "Vedolizumab: An Integrin-Receptor Antagonist for Treatment of Crohn's Disease and Ulcerative Colitis." *American Journal of Health-System Pharmacy* 72(15): 1271–78.

Harman, D. 1956. "Aging: A Theory Based on Free Radical and Radiation Chemistry." *Journal of Gerontology* 11(3): 298–300.

Heinbockel, Thomas, and Antonei B Csoka. 2018. "Epigenetic Effects of Drugs of Abuse." *International journal of environmental research and public health* 15(10).

Herter, Jan, and Alexander Zarbock. 2013. "Integrin Regulation during Leukocyte

Recruitment." *The Journal of Immunology* 190(9): 4451–57.

Hertzog, Lars H., Kathleen G. Albrecht, Lori LaBree, and Paul P. Lee. 2013. "Glaucoma Care and Conformance with Preferred Practice Patterns." *Ophthalmology* 103(7): 1009–13.

van Holde, Kensal E. 1989. "The Proteins of Chromatin. I. Histones." In *Springer Series in Molecular and Cell Biology*, , 69–180.

Hu, Dan-Ning, John D. Simon, and Tadeusz Sarna. 2008. "Role of Ocular Melanin in Ophthalmic Physiology and Pathology." *Photochemistry and Photobiology* 84(3): 639–44.

Hutchinson, Michael. 2007. "Natalizumab: A New Treatment for Relapsing Remitting Multiple Sclerosis." *Therapeutics and clinical risk management* 3(2): 259–68.

Hyun, Young-Min, Craig T Lefort, and Minsoo Kim. 2009. "Leukocyte Integrins and Their Ligand Interactions." *Immunologic research* 45(2–3): 195–208.

Janeway, Charles, Paul Travers, Mark Walport, and Mark J Shlomchik. 2001. "The Complement System and Innate Immunity."

John, Rosalind M, and Claire Rougeulle. 2018. "Developmental Epigenetics: Phenotype and the Flexible Epigenome." *Frontiers in cell and developmental biology* 6: 130.

Jonas, Jost B, Chui Ming Gemmy Cheung, and Songhomitra Panda-Jonas. 2017. "Updates on the Epidemiology of Age-Related Macular Degeneration." *Asia-Pacific Journal of Ophthalmology* 6(6): 493–97.

Juliano, R L et al. 2004. "Integrin Regulation of Cell Signalling and Motility." *Biochemical Society transactions* 32(Pt3): 443–46.

Kaarniranta, Kai et al. 2017. "Autophagy Regulates Death of Retinal Pigment Epithelium Cells in Age-Related Macular Degeneration." *Cell biology and toxicology* 33(2): 113–28.

Kandel, Himal, Jyoti Khadka, Michael Goggin, and Konrad Pesudovs. 2017. "Impact of

Refractive Error on Quality of Life: A Qualitative Study." *Clinical and Experimental Ophthalmology*.

Kapp, Tobias G. et al. 2017. "A Comprehensive Evaluation of the Activity and Selectivity Profile of Ligands for RGD-Binding Integrins." *Scientific Reports* 7(1): 39805.

Keeffe, Jill et al. 2013. "New Systematic Review Methodology for Visual Impairment and Blindness for the 2010 Global Burden of Disease Study." *Ophthalmic Epidemiology*.

Kelly, Simon P. et al. 2005. "Smoking and Cataract: Review of Causal Association." *Journal of Cataract & Refractive Surgery* 31(12): 2395–2404.

Khan, Shahper N., and Asad U. Khan. 2010. "Role of Histone Acetylation in Cell Physiology and Diseases: An Update." *Clinica Chimica Acta* 411(19–20): 1401–11.

Khandhadia, Sam, and Andrew Lotery. 2010. "Oxidation and Age-Related Macular Degeneration: Insights from Molecular Biology."

Khurana, A. K., and Indu. Khurana. 2006. *Anatomy and Physiology of Eye*. CBS Publishers.

Kiel, Jeffrey W. 2010. The Ocular Circulation *Anatomy*. Morgan & Claypool Life Sciences.

Kiser, Philip D, Marcin Golczak, and Krzysztof Palczewski. 2014. "Chemistry of the Retinoid (Visual) Cycle." *Chemical reviews* 114(1): 194–232.

Klein, Ronald, Tunde Peto, Alan Bird, and Mylan R. Vannewkirk. 2004. "The Epidemiology of Age-Related Macular Degeneration." *American Journal of Ophthalmology* 137(3): 486–95.

Knudtson, Michael D. et al. 2004. "Location of Lesions Associated with Age-Related Maculopathy Over a 10-Year Period: The Beaver Dam Eye Study." *Investigative Opthalmology & Visual Science* 45(7): 2135.

Kolar, Petr. 2013. "Classification and Clinical Features of AMD." In *Age-Related Macular Degeneration - Etiology, Diagnosis and Management - A Glance at the Future*, InTech.

Kolb, Helga. 2005. Webvision: The Organization of the Retina and Visual System *Simple Anatomy of the Retina*. University of Utah Health Sciences Center.

———. 2012. Webvision: The Organization of the Retina and Visual System *Photoreceptors*. University of Utah Health Sciences Center.

Kook, Daniel et al. 2008. “The Protective Effect of Quercetin against Oxidative Stress in the Human RPE In Vitro.” *Investigative Opthalmology & Visual Science* 49(4): 1712.

Koushan, Keyvan et al. 2013. “The Role of Lutein in Eye-Related Disease.” *Nutrients* 5(5): 1823–39.

Krishnadev, Nupura, Annal D Meleth, and Emily Y Chew. 2010. “Nutritional Supplements for Age-Related Macular Degeneration.” *Current opinion in ophthalmology* 21(3): 184–89.

Krisko, Anita, and Miroslav Radman. 2019. “Protein Damage, Ageing and Age-Related Diseases.” *Open Biology* 9(3): 180249.

Kröger, Ronald H.H., and Oliver Biehlmaier. 2009. “Space-Saving Advantage of an Inverted Retina.” *Vision Research* 49(18): 2318–21.

Kruk, Joanna, Katarzyna Kubasik-Kladna, and Hassan Y Aboul-Enein. 2015. “The Role Oxidative Stress in the Pathogenesis of Eye Diseases: Current Status and a Dual Role of Physical Activity.” *Mini reviews in medicinal chemistry* 16(3): 241–57.

Kuehn, Bridget M. 2009. “Efalizumab Withdrawn.” *JAMA* 301(20): 2085.

Kusuhara, Sentaro et al. 2018. “Pathophysiology of Diabetic Retinopathy: The Old and the New.” *Diabetes and Metabolism Journal* 42(5): 364–76.

Kutoglu, Tunc, Bulent Yalcin, Necdet Kocabiyik, and Hasan Ozan. 2005. “Vortex Veins: Anatomic Investigations on Human Eyes.” *Clinical Anatomy* 18(4): 269–73.

Kwa, Faith A A, and Thilini R Thrimawithana. 2014. “Epigenetic Modifications as Potential Therapeutic Targets in Age-Related Macular Degeneration and Diabetic Retinopathy.” *Drug Discovery Today* 19(9).

Lam, Dennis et al. 2015. “Cataract.” *Nature Reviews Disease Primers* 1: 15014.

Ledford, Heidi. 2012. “Epigenetics: Marked for Success.” *Nature* 483(7391): 638–39.

Lee, Jung-Shin, Edwin Smith, and Ali Shilatifard. 2010. “The Language of Histone Crosstalk.” *Cell* 142(5): 682–85.

Ley, Klaus, Jesus Rivera-Nieves, William J. Sandborn, and Sanford Shattil. 2016. “Integrin-Based Therapeutics: Biological Basis, Clinical Use and New Drugs.” *Nature Reviews Drug Discovery* 15(3): 173–83.

Li, Jing, and Timothy A Springer. 2017. “Integrin Extension Enables Ultrasensitive Regulation by Cytoskeletal Force.” *Proceedings of the National Academy of Sciences of the United States of America* 114(18): 4685–90.

Liston, Dane R, and Myrtle Davis. 2017. “Clinically Relevant Concentrations of Anticancer Drugs: A Guide for Nonclinical Studies.” *Clinical cancer research : an official journal of the American Association for Cancer Research* 23(14): 3489–98.

Liu, Huijun et al. 2017. “Protective Effect of Lutein on ARPE-19 Cells upon H2O2-Induced G2/M Arrest.” *Molecular Medicine Reports* 16(2): 2069–74.

van Lookeren Campagne, Menno, Jennifer LeCouter, Brian L Yaspan, and Weilan Ye. 2014. “Mechanisms of Age-Related Macular Degeneration and Therapeutic Opportunities.” *The Journal of Pathology* 232(2): 151–64.

Ludwig, Parker E., and Karlin Sevensma. 2019. “Anatomy, Head and Neck, Eye Cornea.”

Lund-Katz, Sissel, and Michael C Phillips. 2010. “High Density Lipoprotein Structure-Function and Role in Reverse Cholesterol Transport.” *Sub-cellular biochemistry* 51: 183–227.

Luo, Bing-Hao, Christopher V. Carman, and Timothy A. Springer. 2007. “Structural Basis of Integrin Regulation and Signaling.” *Annual Review of Immunology* 25(1): 619–47.

Maggs, David. 2008. “Cornea and Sclera.” *Slatter’s Fundamentals of Veterinary Ophthalmology*: 175–202.

Mao, Yingyu, and Silvia C Finnemann. 2013. “Analysis of Photoreceptor Outer Segment Phagocytosis by RPE Cells in Culture.” *Methods in molecular biology (Clifton, N.J.)* 935: 285–95.

Mariotti, S P, D Pascolini, and J Rose-Nussbaumer. 2009. “Trachoma: Global Magnitude of a Preventable Cause of Blindness.” *The British journal of ophthalmology* 93(5): 563–68.

Marlin, Steven D., and Timothy A. Springer. 1987. “Purified Intercellular Adhesion Molecule-1 (ICAM-1) Is a Ligand for Lymphocyte Function-Associated Antigen 1 (LFA-1).” *Cell* 51(5): 813–19.

Mason, Laura Elizabeth. 2018. “Epigenetics and Drug Discovery.” *Technology Networks*.

Maxfield, Frederick R., and Ira Tabas. 2005. “Role of Cholesterol and Lipid Organization in Disease.” *Nature* 438(7068): 612–21.

Merle, Bénédicte M.J. et al. 2019. “Mediterranean Diet and Incidence of Advanced Age-Related Macular Degeneration: The EYE-RISK Consortium.” *Ophthalmology* 126(3): 381–90.

Miller, Joan W. 2013. “Age-Related Macular Degeneration Revisited – Piecing the Puzzle: The LXIX Edward Jackson Memorial Lecture.” *American Journal of Ophthalmology* 155(1): 1-35.e13.

Miller, Joan W, Saghar Bagheri, and Demetrios G Vavvas. 2017. “Advances in Age-Related Macular Degeneration Understanding and Therapy.” *US ophthalmic review* 10(2): 119–30.

Mittelbrunn, M. et al. 2004. “VLA-4 Integrin Concentrates at the Peripheral Supramolecular Activation Complex of the Immune Synapse and Drives T Helper 1 Responses.” *Proceedings of the National Academy of Sciences* 101(30): 11058–63.

Mitton, Kenneth P et al. 2014. “Different Effects of Valproic Acid on Photoreceptor Loss in Rd1 and Rd10 Retinal Degeneration Mice.” *Molecular vision* 20: 1527–44.

Mohseni, Michael, and Veronica Takov. 2019. StatPearls *Chlamydia*. StatPearls

Publishing.

Morris, Brid, Fraser Imrie, Ana-Maria Armbrecht, and Baljean Dhillon. 2007. “Age-Related Macular Degeneration and Recent Developments: New Hope for Old Eyes?” *Postgraduate medical journal* 83(979): 301–7.

Muller, Florian L. et al. 2007. “Trends in Oxidative Aging Theories.” *Free Radical Biology and Medicine* 43(4): 477–503.

Mullins, Robert F., Stephen R. Russel, Don H. Anderson, and Gregory S. Hageman. 2000. “Drusen Associated with Aging and Age-Related Macular Degeneration Contain Proteins Common to Extracellular Deposits Associated with Atherosclerosis, Elastosis, Amyloidosis, and Dense Deposit Disease.” *The FASEB Journal* 14(7): 835–46.

Mundy, Kevin M., Erin Nichols, and Jennifer Lindsey. 2016. “Socioeconomic Disparities in Cataract Prevalence, Characteristics, and Management.” *Seminars in Ophthalmology* 31(4): 358–63.

Nagineni, Chandrasekharam N., R.Krishnan Kutty, Barbara Detrick, and John J. Hooks. 1996. “Inflammatory Cytokines Induce Intercellular Adhesion Molecule-1 (ICAM-1) MRNA Synthesis and Protein Secretion by Human Retinal Pigment Epithelial Cell Cultures.” *Cytokine* 8(8): 622–30.

Nair, Parvathy, Chiu-fang Chou, and Linda S Geiss. 2014. “NIH Public Access.” 154(6 0): 1–17.

National Eye Institute. 2016. “Facts About the Cornea and Corneal Disease.” *NEI*.

NEI. 2015. “Facts About Diabetic Eye Disease.” *National Eye Institute (NEI)*.

Ng, Kwok-Peng et al. 2008. “Retinal Pigment Epithelium Lipofuscin Proteomics.” *Molecular & cellular proteomics : MCP* 7(7): 1397–1405.

Nieberler, Markus et al. 2017. “Exploring the Role of RGD-Recognizing Integrins in Cancer.” *Cancers* 9(9).

NIH. 2019. “Cone and Rod Distrophy.” *Genetics Home Reference*.

Nilsson, Sven Erik G., Staffan P. Sundelin, Ulf Wihlmark, and Ulf T. Brunk. 2003. “Aging of Cultured Retinal Pigment Epithelial Cells: Oxidative Reactions, Lipofuscin Formation and Blue Light Damage.” *Documenta Ophthalmologica* 106(1): 13–16.

Nowak, Jerzy Z. 2014. “AMD-the Retinal Disease with an Unprecised Etiopathogenesis: In Search of Effective Therapeutics.” *Acta Poloniae Pharmaceutica ñ Drug Research* 71(6): 900–916.

Oliver, Samuel S, and John M Denu. 2011. “Dynamic Interplay between Histone H3 Modifications and Protein Interpreters: Emerging Evidence for a ‘Histone Language.’” *Chembiochem : a European journal of chemical biology* 12(2): 299–307.

Openstax College. 2013. *Anatomy & Physiology*.

Ozawa, Yoko et al. 2012. “Neuroprotective Effects of Lutein in the Retina.” *Current Pharmaceutical Design* 18(1): 51.

Packer, Lester. 1993. “Antioxidant Action of Carotenoids in Vitro and in Vivo and Protection against Oxidation of Human Low-Density Lipoproteins,B.” *Annals of the New York Academy of Sciences* 691(1 Carotenoids i): 48–60.

Pan, Li, Yuan Zhao, Zhijie Yuan, and Guixin Qin. 2016. “Research Advances on Structure and Biological Functions of Integrins.” *SpringerPlus* 5(1): 1094.

Park, Sung Chul, and Yoon Tae Jeen. 2018. “Anti-Integrin Therapy for Inflammatory Bowel Disease.” *World journal of gastroenterology* 24(17): 1868–80.

Pawlowska, Elzbieta et al. 2019. “Dietary Polyphenols in Age-Related Macular Degeneration: Protection against Oxidative Stress and Beyond.” *Oxidative Medicine and Cellular Longevity* 2019: 1–13.

Perkins, Edward, and Hugh Davson. 2019. “Human Eye: Definition, Structure & Function.” *Encyclopaedia Britannica*.

Perruccio, Anthony V., Elizabeth M. Badley, and Graham E. Trope. 2010. “A Canadian

Population-Based Study of Vision Problems: Assessing the Significance of Socioeconomic Status.” *Canadian Journal of Ophthalmology* 45(5): 477–83.

Pikuleva, Irina A., and Christine A. Curcio. 2014. “Cholesterol in the Retina: The Best Is yet to Come.” *Progress in Retinal and Eye Research* 41: 64–89.

Pinal-Fernandez, Iago, Maria Casal-Dominguez, and Andrew L Mammen. 2018. “Statins: Pros and Cons.” *Medicina clinica* 150(10): 398–402.

Pirahanchi, Yasaman, and Martin R. Huecker. 2019. StatPearls *Biochemistry, LDL Cholesterol*. StatPearls Publishing.

Planck, Stephen R., Xiao-Na Huang, Joseph E. Robertson, and James T. Rosenbaum. 1993. “Retinal Pigment Epithelial Cells Produce Interleukin-1 β and Granulocyte-Macrophage Colony-Stimulating Factor in Response to Interleukin-1α.” *Current Eye Research* 12(3): 205–12.

Platts, K E et al. 1995. “Cytokine Modulation of Adhesion Molecule Expression on Human Retinal Pigment Epithelial Cells.” *Investigative ophthalmology & visual science* 36(11): 2262–69.

Pratt, S. 1999. “Dietary Prevention of Age-Related Macular Degeneration.” *Journal of the American Optometric Association* 70(1): 39–47.

Purves, Dale et al. 2001. *Anatomy of the Eye*. Sinauer Associates.

Quizlet Inc. 2016. “Flashcard Eye Anatomy | Quizlet.”

Rask-Madsen, Christian, and George L King. 2013. “Vascular Complications of Diabetes: Mechanisms of Injury and Protective Factors.” *Cell metabolism* 17(1): 20–33.

Rathi, Varsha M, Sharadini P Vyas, and Virender S Sangwan. 2012. “Phototherapeutic Keratectomy.” *Indian journal of ophthalmology* 60(1): 5–14.

Remington, Lee Ann. 2012. *Clinical Anatomy and Physiology of the Visual System*. Elsevier/Butterworth-Heinemann.

Resnikoff, Serge et al. 2004. 82 Bulletin of the World Health Organization *Global Data on*

Visual Impairment in the Year 2002.

Riordan-Eva, Paul, and John P. Whitcher. 2008. "Anatomy & Embryology of the Eye." *Vaughan & Asbury's General Ophthalmology (17th ed.)*.

Robaei, Dana, and Stephanie Watson. 2014. "Corneal Blindness: A Global Problem." *Clinical & Experimental Ophthalmology* 42(3): 213–14.

Rodriguez, Hanah, and Assam El-Osta. 2018. "Epigenetic Contribution to the Development and Progression of Vascular Diabetic Complications." *Antioxidants & Redox Signaling* 29(11): 1074–91.

Rong, Xianfang et al. 2016. "Effects of Histone Acetylation on Superoxide Dismutase 1 Gene Expression in the Pathogenesis of Senile Cataract." *Scientific Reports* 6(1): 34704.

Rupenthal, Ilva, Di Huang, and Ying-Shan Chen. 2018. "Overcoming Ocular Drug Delivery Barriers through the Use of Physical Forces." *Advanced Drug Delivery Reviews* 126: 96–112.

Ryan, Stephen J. 2006. *Retina*. Elsevier/Mosby.

Saccà, Sergio Claudio et al. 2019. "Substances of Interest That Support Glaucoma Therapy." *Nutrients* 11(2): 1–23.

Salehi, Bahare et al. 2018. "Resveratrol: A Double-Edged Sword in Health Benefits." *Biomedicines* 6(3).

Sato, Tomohito et al. 2018. "Intraocular Inflammatory Cytokines in Patients with Neovascular Age-Related Macular Degeneration before and after Initiation of Intravitreal Injection of Anti-VEGF Inhibitor." *Scientific Reports* 8(1): 1098.

Schwartz, Kenneth, and Donald Budenz. 2004. "Current Management of Glaucoma." *Current Opinion in Ophthalmology* 15(2): 119–26.

Seddon, Johanna M., Walter C. Willett, Frank E. Speizer, and Susan E. Hankinson. 1996. "A Prospective Study of Cigarette Smoking and Age-Related Macular Degeneration in

Women." *JAMA: The Journal of the American Medical Association* 276(14): 1141.

Seiple, William et al. 2005. "Eye-Movement Training for Reading in Patients with Age-Related Macular Degeneration." *Investigative Opthalmology & Visual Science* 46(8): 2886.

Seo, Mi Kyoung et al. 2018. "Effects of Antipsychotic Drugs on the Epigenetic Modification of Brain-Derived Neurotrophic Factor Gene Expression in the Hippocampi of Chronic Restraint Stress Rats." *Neural Plasticity* 2018: 1–10.

Shaw, Peter X et al. 2016. "Oxidative Stress, Innate Immunity, and Age-Related Macular Degeneration." *AIMS molecular science* 3(2): 196–221.

Shimaoka, Motomu, and Timothy A. Springer. 2003. "Therapeutic Antagonists and Conformational Regulation of Integrin Function." *Nature Reviews Drug Discovery* 2(9): 703–16.

Shumway, Caleb L., and Matthew Wade. 2019. StatPearls *Anatomy, Head and Neck, Eye Conjunctiva*. StatPearls Publishing.

Sinha, Rajesh, Chandrashekhar Kumar, and Jeewan S Titiyal. 2009. "Etiopathogenesis of Cataract: Journal Review." *Indian Journal of Ophthalmology* 57(3): 245.

Springer, Timothy A. 1994. "Traffic Signals for Lymphocyte Recirculation and Leukocyte Emigration: The Multistep Paradigm." *Cell* 76(2): 301–14.

Srichai, Manakan Betsy, and Roy Zent. 2010. "Integrin Structure and Function." In *Cell-Extracellular Matrix Interactions in Cancer*, Springer New York, 19–41.

Stadtman, Earl R. 2006. "Protein Oxidation in Aging and Age-Related Diseases." *Annals of the New York Academy of Sciences* 928(1): 22–38.

Stein-Streilein, Joan. 2008. "Immune Regulation and the Eye." *Trends in Immunology* 29(11): 548–54.

Stein-Streilein, Joan, and J Wayne Streilein. 2002. "Anterior Chamber Associated Immune Deviation (ACAID): Regulation, Biological Relevance, and Implications for Therapy."

International reviews of immunology 21(2–3): 123–52.

Steuer, Heiko et al. 2005. "Functional Characterization and Comparison of the Outer Blood–Retina Barrier and the Blood–Brain Barrier." *Investigative Opthalmology & Visual Science* 46(3): 1047.

Strauss, Olaf. 2010. "The Retinal Pigment Epithelium." *Webvision: The Organization of the Retina and Visual System*.

Sturm, R. 2004. "Eye Colour: Portals into Pigmentation Genes and Ancestry." *Trends in Genetics* 20(8): 327–32.

Suuronen, Tiina et al. 2007. "Epigenetic Regulation of Clusterin/Apolipoprotein J Expression in Retinal Pigment Epithelial Cells." *Biochemical and Biophysical Research Communications* 357(2): 397–401.

Swarup S. Swaminathan, Dong-Jin Oh, Min Hyung Kang. 2014. "Aqueous Outflow: Segmental and Distal Flow." *J Cataract Refract Surg.* 40(8): 1263–1272.

Taylor. 2009. "Ocular Immune Privilege." *Eye* 23(10): 1885–89.

Taylor, Hugh R. et al. 1988. "Effect of Ultraviolet Radiation on Cataract Formation." *New England Journal of Medicine* 319(22): 1429–33.

Telegina, D. V., O. S. Kozhevnikova, and N. G. Kolosova. 2017. "Molecular Mechanisms of Cell Death in Retina during Development of Age-Related Macular Degeneration." *Advances in Gerontology* 7(1): 17–24.

The Retina Reference. 2019. "Normal Retinal Anatomy." *The Retina Reference*.

Thichanpiang, Peeradech, Steven J Harper, Kanokpan Wongprasert, and David O Bates. 2014. "TNF-α-Induced ICAM-1 Expression and Monocyte Adhesion in Human RPE Cells Is Mediated in Part through Autocrine VEGF Stimulation." *Molecular vision* 20: 781–89.

Thoreson, W.B., and E. Margalit. 2014. "Macular Degeneration." *Reference Module in Biomedical Sciences*.

Thumann, Gabriele, Guorui Dou, Yusheng Wang, and David R. Hinton. 2013. "Cell Biology of the Retinal Pigment Epithelium." In *Retina*, Elsevier, 401–14.

Tian, Bo et al. 2017. "Atorvastatin Promotes Phagocytosis and Attenuates Pro-Inflammatory Response in Human Retinal Pigment Epithelial Cells." *Scientific reports* 7(1): 2329.

Timothy, C. O., and R. O. Nneli. 2007. "The Effects of Cigarette Smoking on Intraocular Pressure and Arterial Blood Pressure of Normotensive Young Nigerian Male Adults." *Nigerian journal of physiological sciences : official publication of the Physiological Society of Nigeria* 22(1–2): 33–36.

Tisdale, Alanna K. et al. 2019. "Association of Dietary and Supplementary Calcium Intake With Age-Related Macular Degeneration." *JAMA Ophthalmology* 137(5): 543.

Tokarz, Paulina, Kai Kaarniranta, and Janusz Blasiak. 2016. "Inhibition of DNA Methyltransferase or Histone Deacetylase Protects Retinal Pigment Epithelial Cells from DNA Damage Induced by Oxidative Stress by the Stimulation of Antioxidant Enzymes." *European Journal of Pharmacology* 776: 167–75.

Tolomelli, Alessandra et al. 2015. "Dehydro-β-Proline Containing α 4 β 1 Integrin Antagonists: Stereochemical Recognition in Ligand–Receptor Interplay." *ACS Medicinal Chemistry Letters* 6(6): 701–6.

Tomany, Sandra C. et al. 2004. "Risk Factors for Incident Age-Related Macular Degeneration." *Ophthalmology* 111(7): 1280–87.

Tortora, Gerard J., and Sandra Reynolds. Grabowski. 1993. *Principles of Anatomy and Physiology*. HarperCollinsCollege.

Tzekov, R.T., J. Checchi, C. Clemson, and S. Kaushal. 2010. 51 Investigative Ophthalmology & Visual Science *Investigative Ophthalmology & Visual Science.* [Association for Research in Vision and Ophthalmology, etc.].

Uddin, MD Imam et al. 2018. "Real-Time Imaging of VCAM-1 MRNA in TNF-α Activated Retinal Microvascular Endothelial Cells Using Antisense Hairpin-DNA Functionalized

Gold Nanoparticles." *Nanomedicine: Nanotechnology, Biology and Medicine* 14(1): 63–71.

Vavvas, Demetrios G. et al. 2016. "Regression of Some High-Risk Features of Age-Related Macular Degeneration (AMD) in Patients Receiving Intensive Statin Treatment." *EBioMedicine* 5: 198–203.

Vent-Schmidt, Ruanne Y.J. et al. 2017. "Opposing Effects of Valproic Acid Treatment Mediated by Histone Deacetylase Inhibitor Activity in Four Transgenic X. Laevis Models of Retinitis Pigmentosa." *The Journal of Neuroscience* 37(4): 1039.

Virgili, Gianni, and Alessandro Bini. 2007. "Laser Photocoagulation for Neovascular Age-Related Macular Degeneration." *Cochrane Database of Systematic Reviews* (3): CD004763.

Virtual medical center. 2018. "Human Eye Anatomy and How Vision Works Information." *myVMC*.

Wang, Lan et al. 2010. "Abundant Lipid and Protein Components of Drusen." *PloS one* 5(4): e10329.

Wassell, Julie, Sallyanne Davies, William Bardsley, and Mike Boulton. 1999. "The Photoreactivity of the Retinal Age Pigment Lipofuscin." *Journal of Biological Chemistry* 274(34): 23828–32.

Wee, Hyun, Hyun-Mee Oh, Jae-Hyeok Jo, and Chang-Duk Jun. 2009. "ICAM-1/LFA-1 Interaction Contributes to the Induction of Endothelial Cell-Cell Separation: Implication for Enhanced Leukocyte Diapedesis." *Experimental & Molecular Medicine* 41(5): 341. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2701983/ (July 17, 2019).

Whitcup, Scott M et al. 2013. "The Role of the Immune Response in Age-Related Macular Degeneration." *International journal of inflammation* 2013: 348092.

WHO. 2018. "Priority Eye Diseases." *WHO*.

Wolf, George. 2003. "Lipofuscin and Macular Degeneration." *Nutrition Reviews* 61(10): 342–46.

World Health Organization. 2010. “International Statistical Classification of Diseases, Injuries and Causes of Death. Tenth Revision.” *World Health Organization*.

———. 2018. “Blindness and Vision Impairment.” *World Health Organization*.

———. 2019. “Trachoma.” *World Health Organization*.

Wright, Carrie, Mohammed A. Tawfik, Michael Waisbourd, and Leslie J. Katz. 2016. “Primary Angle-Closure Glaucoma: An Update.” *Acta Ophthalmologica* 94(3): 217–25.

Xu, Xin-Rong et al. 2016. “Quercetin Phospholipid Complex Significantly Protects against Oxidative Injury in ARPE-19 Cells Associated with Activation of Nrf2 Pathway.” *European Journal of Pharmacology* 770: 1–8.

Yadav, Khushwant S., Rahul Rajpurohit, and Sushmita Sharma. 2019. “Glaucoma: Current Treatment and Impact of Advanced Drug Delivery Systems.” *Life Sciences* 221(February): 362–76.

Yee, K.L., V.M. Weaver, and D.A. Hammer. 2008. “Integrin-Mediated Signalling through the MAP-Kinase Pathway.” *IET Systems Biology* 2(1): 8–15.

Yip, Jennifer L. Y. et al. 2015. “Cross Sectional and Longitudinal Associations between Cardiovascular Risk Factors and Age Related Macular Degeneration in the EPIC-Norfolk Eye Study” ed. Alfred S Lewin. *PLOS ONE* 10(7): e0132565. http://www.ncbi.nlm.nih.gov/pubmed/26176222 (September 18, 2019).

Yu, Yamei et al. 2012. “Structural Specializations of α(4)β(7), an Integrin That Mediates Rolling Adhesion.” *The Journal of cell biology* 196(1): 131–46.

Yücel, Yeni H. et al. 2018. “Active Lymphatic Drainage From the Eye Measured by Noninvasive Photoacoustic Imaging of Near-Infrared Nanoparticles.” *Investigative Opthalmology & Visual Science* 59(7): 2699.

Zodpey, S P, S N Ughade, V A Khanolkar, and S N Shrikhande. 1999. “Dehydrational Crisis from Severe Diarrhoea and Risk of Age-Related Cataract.” *Journal of the Indian Medical Association* 97(1): 13–15, 24.