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Dynamic lipid turnover in photoreceptors and retinal pigment epithelium throughout life

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Abstract

The retinal pigment epithelium-photoreceptor interaction is one of the most studied cell-to-cell interfaces in nature. While photoreceptors use photosensitive pigments to convert light into electrical signals, the RPE supports photoreceptors in their function by phagocytizing shed photoreceptor tips, regulating the blood-retina barrier, and modulating inflammatory responses, as well as regenerating the 11-*cis*-retinal chromophore *via* the classical visual cycle. These processes involve multiple protein complexes, tightly regulated ligand-receptors interactions, and a plethora of lipids and protein-lipids interactions. The role of lipids in maintaining a healthy interplay between the RPE and photoreceptors has not been fully delineated. In recent years, novel technologies have resulted in major advancements in understanding several facets of this interplay, including the involvement of lipids in phagocytosis and phagolysosome function, nutrient recycling, and the metabolic dependence between the two cell types. In this review, we aim to integrate the complex role of lipids in photoreceptor and RPE function, emphasizing the dynamic exchange between the cells as well as discuss how these processes are affected in aging and retinal diseases.

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Introduction

Photoreceptor and retinal pigment epithelium (RPE) cells are crucially dependent on tightly controlled lipid homeostasis to maintain cellular function. Their interdependence relies on the constant incorporation and processing of each other's membranes. Each day, roughly a tenth of the rod outer segment (OS) is shed and phagocytized by the RPE, which is able to repurpose the membranes. This effort is expended to keep the membrane composition, as well as the protein inhabitants, physiologically optimized for the demanding process of vision. The rod OS has a unique organization of membranes that have been studied extensively for decades. Stacked disks pile on top of each other within the rod OS plasma membrane, creating a multi-layered photon-trap of rhodopsin-laden membranes. The effect is to create a highly sensitive system that can accommodate the activity of a para-crystalline membrane protein population (millimolar in concentration) that is both ordered and fluid. The oft-noted balance of order and fluidity maintained by photoreceptors is largely due to the composition of the membranes themselves. Each element plays a role in maintaining this delicate balance, and many will be discussed in this review, emphasizing the biogenesis, transportation, distribution, and metabolism of several vital components.

As noted by Boesze-Battaglia and Schimmel in their seminal review (1997) of membrane composition and distribution, "Membrane protein activity is influenced by the surrounding lipid matrix and specifically by the association between lipids and proteins at the lipid-protein interface." (Boesze-Battaglia and Schimmel, 1997). Since the publication of that review, more data has confirmed the ability of annular lipids to directly affect the function or stability of membrane proteins and complexes (Bechara et al., 2015; Schmidt et al., 2015). There has also been important work done identifying several "modes" of lipid binding by the Robinson group (Bechara and Robinson, 2015; Bolla et al., 2020). What this progress has yielded, though, is greater nuance of understanding regarding classical theories with respect to the purpose of the lipid components in membranes. It is well established that membrane proteins have varying degrees of contact with the surrounding membrane components. Lipids directly in contact with the transmembrane domains of proteins are said to be 'restricted' or non-annular if they do not also exchange with nearby lipids. Such interactions have been shown many times biochemically, and even structurally when lipids have been identified co-crystallizing in binding pockets of some transporters (Gonen et al., 2005; Gulati et al., 2017). Annular lipids form a shell around the protein, and, as discussed above, can have a substantial impact on membrane proteins without binding tightly to the protein itself.

Whether restricted, annular, or bulk, the lipid bilayers of most membranes consist of a few major classes, namely, phospholipids, sterols, and free fatty acids (FFAs). Phospholipids are usually discussed in the context of their charge/polarity; while phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) are all charged, phosphatidylethanolamine (PE) is not. This split makes PC/PS/PI suitable constituents for surfaces requiring good hydration and solubility, while PE-rich membranes can promote membrane fusion events and interfacial interactions between hydrophobic regions. These components are usually segregated to inner and outer leaflets of the plasma membrane, with PC on the outer layer and PE/PI on the inner. The segregation is maintained

via an ATP-transporter, and the increased presence of PS on the outer leaflet can signal apoptosis (Devaux, 1991; Mariño and Kroemer, 2013). Work done in the 1990s by Boesze-Battaglia and Albert (thanks to the development of a disk-plasma membrane separation protocol by Molday and Molday five years prior) gave the first indication of what the relative distribution of the four major phospholipids was in rod OS (Boesze-Battaglia and Albert, 1992; Molday and Molday, 1987). Interestingly, the rod OS disks showed a nearly 4-fold higher relative percent of PE than the photoreceptor plasma membrane (42% *vs.* 11%, roughly). The precise lipid sorting between the two membranes is particularly striking given that nascent disks themselves are made from the closure of plasma membrane invaginations at the base of the OS. (Spencer et al., 2020).

The sterol component of lipid membranes is mostly cholesterol, which helps create space in the membrane. Cholesterol often acts as a foil for the rest of the membrane. In warmer temperatures, the phospholipid components are much more dynamic than the bulky and locked cholesterol, and the fatty acid (FA) tails tend to allow for more movement when warmed. Cholesterol, then, acts as a buffer by retaining some of the rigidity and structure of the membrane. Conversely, when membranes are cooled, the phospholipids are less dynamic and may harm membrane protein function through stiffening of the membrane if not for cholesterol, which is able to maintain space between the contracting portions of the cooled membrane. Cholesterol's ability to mediate the extremes makes it a vital component of most membranes, including those of the photoreceptors and RPE (Albert and Boesze-Battaglia, 2005).

The effect of the FA chain length and saturation has been studied and reviewed several times, and it is a critical component of the unique environment used by photoreceptors. Docosahexaenoic acid (DHA, 22:6) is vitally important to many systems of the body, including the retina. DHA is incorporated into multiple lipid species and, given the high level of unsaturation, is able to create a very fluid environment in rod OS disks. Here again, Boesze-Battaglia and Albert were able to give the first indication that the disks were able to sort for a completely different profile of lipids, this time by FA chain length (Boesze-Battaglia and Albert, 1989). Of the components they could detect, DHA represented 35% of the isolated disk membranes, while the plasma membrane had about 5%. This was additional evidence that the hyper-specific and dynamic membrane environment of rhodopsin-laden membranes requires, or perhaps selects for, unique components able to facilitate phototransduction.

The structure and biophysical properties of membranes throughout the body are dictated by the composition of lipid building blocks - including both their chemical structures and stoichiometries. Even a single membrane can feature a dramatically different lipidome across its inner and outer leaflet (Lorent et al., 2020). Membrane properties depend on the lipid headgroup charge and size, as well as their acyl chains. These individual attributes affect aggregate membrane attributes such as viscosity, which is known to vary across cellular compartments (Saffman and Delbrück, 1975). Membranes rich in saturated lipids and cholesterol pack tightly and are rigid and ordered, whereas high unsaturation levels yield relatively fluid and more disordered membranes. Polyunsaturated FAs (PUFAs) reduce membrane viscosity but are also highly susceptible to peroxidation by reactive oxygen

species (ROS). Some PUFAs, most notably arachidonic acid (AA), can also be used for the synthesis of different signaling lipids, including eicosanoids, docosanoids, and elovanoids. These soluble compounds bind to cellular receptors, initiating important physiological processes ranging from inflammation to fertility (Bazan, 2018; Mouchlis and Dennis, 2019).

Lipids are also a source of energy and secondary signaling, both of which have been thoroughly reviewed elsewhere (Houten et al., 2016; Houtkooper and Vaz, 2008; Ploumi et al., 2017; Schönfeld and Wojtczak, 2016; van der Veen et al., 2017). To enable their transport to the inner mitochondrial membrane carnitine binds FAs, creating acyl-carnitine, where the FA is then subjected to a cycle of beta-oxidation to produce acetyl CoA and NADH/FADH$_2$. Those products are used in the generation of ATP *via* the electron transport chain. As mentioned, lipids also act as messengers in several essential signaling pathways. Lysophospholipid signaling has been shown to directly affect GPCR signaling in the Gi and Gq pathways (Jelsema, 1987). Phosphatidylinositol 4,5-diphosphate (PIP$_2$) can be cleaved by phospholipase C (PLC) to form two second messengers, diacylglycerol (DAG) and the soluble metabolite 1,4,5-inositol triphosphate (IP$_3$) (Berridge, 2009; Raucher et al., 2000). Signaling through IP$_3$ is essential for the release of intracellular calcium stores, among other duties (Berridge and Irvine, 1984). Of particular note to vision research is the light-dependent production of IP$_3$ in photoreceptors (Das et al., 1987; Fein et al., 1984; Ghalayini and Anderson, 1984; Hayashi and Amakawa, 1985; Millar et al., 1988). PLC activity has been shown to be the major cause of phototransduction in *Drosophila melanogaster*, but it is uncertain if IP3 or DAG is responsible for transmitting the signal with both having been hypothesized and contradicted in the literature (Ghalayini and Anderson, 1984; Wensel, 2020). The purpose of IP$_3$ production in the photoreceptor of mammals is not yet settled (Yoshioka et al., 1983).

The metabolism, transport, and function of all these membrane components will be discussed in more detail below (Figure 1). To put it briefly, all these processes accomplish a balance in support of phototransduction. The presence of each component relies upon diet, certainly, but also several classes of enzymes, primarily desaturases, elongases, dehydrogenases, and oxygenases. As will be discussed, the composition of photoreceptors and RPE membranes cannot be established without methods of transport through the body *via* proteins such as high-density and low-density lipoproteins (HDL and LDL, respectively). Transport then becomes a matter of joining the lipids to the target membranes, which is accomplished by several lipoprotein receptors. Other proteins like adiponectin receptor 1 (ADIPOR1) also play an important role in the photoreceptor and RPE lipid homeostasis. Being a regulator for DHA uptake and retention in photoreceptors and RPE, ADIPOR1 is indispensable for the healthy retina function. The rest of this review will address the uptake, synthesis, transport, and function of lipids in the retina and RPE, with a final discussion of the visual pathologies observed in cases of lipid dysregulation. While retinoids are certainly an important class of lipids in this context, we will not focus on them in this review and instead refer the reader to previous reviews (Kiser et al., 2014; Kiser and Palczewski, 2016; Palczewski and Kiser, 2020; Travis et al., 2007).

I - Role of lipids in the photoreceptors and RPE

I.1 Lipids as structural components

I.1.1. Rod outer segment membranes—The rod OS consists predominantly of glycerophospholipids (>90% by weight), cholesterol, and glycolipids. The phospholipids are dominated by PC (~30–40 mol%), PE (~30–40 mol%), PS (~10–12 mol%), and PI (~1–2 mol%). In addition, non-sialylated sphingolipids and gangliosides comprise about 1 mol% each, and cholesterol accounts for ~10 mol% of the total OS lipids (Boesze-Battaglia et al., 1989; Fliesler and Schroepfer, 1982). The retina as a whole contains 6% to 7% FAs that are N-linked to sphingosine, or roughly 11–13 mole% in comparison to phospholipids (Brush et al., 2010). Interestingly, OS membranes consist of an unusually high level of LC-PUFAs, especially DHA, which can represent up to 50% of the total acyl groups (Anderson and Maude, 1970; Aveldano and Bazan, 1983; Goldberg et al., 2016).

The rod OS plasma and disk membranes each have diverse functions that differ from one another, a fact reflected in their distinct lipid compositions. The disk membrane is the site of light absorption and phototransduction, whereas the plasma membrane primarily maintains appropriate ion permeability and hyperpolarization under dark and light conditions, respectively (Albert et al., 2016). As a cell limiting barrier, rod OS plasma membranes possess significantly higher rigidity/lower permeability than the disk membranes due to higher levels of saturated FA species, cholesterol, and PC. OS membranes also contain approximately twice as much PS as disks or typical mammalian plasma membranes (Boesze-Battaglia and Albert, 1992; Van Meer et al., 2008). The constitution of the rod OS plasma membrane gives it a high surface charge. It was postulated that increased PS exposure preceding rod shedding promotes rod OS tip phagocytosis by RPE (Ruggiero et al., 2012). PS also regulates Müller glia responses after retina injury by initiating phagocytosis, proliferation, and gliotic responses (Nomura-Komoike et al., 2020).

I.1.2. Disk OS membranes—Similar to other eukaryotic cell membranes, rod OS disks possess interleaflet phospholipid asymmetry, displaying elevated concentrations of PS on their outer/cytosolic leaflets (Hessel et al., 2000; Wu and Hubbell, 1993). One particular study reported that at least 73% of PS and PE are located in the cytosolic leaflet of disk membranes (Miljanich et al., 1981). Rhodopsin can preferentially bind to specific lipid species, thereby influencing membrane asymmetry (Palczewski, 2012). For instance, rhodopsin activation can change PS distribution between the intradiscal and cytosolic membrane leaflets (Hessel et al., 2001). Rhodopsin is localized in the center of rod OS disks and is surrounded by a protein-free lipid ring, which separates rhodopsin from disk rim composed of ATP-binding cassette protein, family A, 4 (ABCA4), rod outer membrane protein 1(ROM-1), and peripherin 2 (PRPH2) proteins (Buzhynskyy et al., 2011).

I.1.3. Compartmentalization of lipids and FAs in photoreceptor disks—A recent report from our groups has further distinguished the major membrane domains of the rod OS disks (Sander et al., 2021). Prior work by Falk and Fatt several decades ago on the ultra-structure of rod OS membranes showed that the outer rim region of rod OS disks resist disruption after OsO_4 fixation (Falk and Fatt, 1969). Their work indicated

that membranes in the rim region are distinct from the disk center, but concrete evidence in support of that idea was still lacking. We chose to study this difference more closely because the differences in membrane components between the rim and lamellar domains of rod OS disks could provide critical information regarding protein-lipid interactions supporting phototransduction. One question of particular interest was whether two forms of Stargardt disease (Table 1), namely STGD1 and STGD3, shared a common molecular etiology. STGD1 is caused by mutations in ABCA4, known as a lipid and retinoid flippase, and mutations in ABCA4 can lead to the accumulation of toxic levels of free retinal and bis-retinoids, later causing lipofuscin deposits to form and the RPE to deteriorate. However, STGD3 is caused by mutations in the elongation of very long FAs 4 (ELOVL4), an enzyme that elongates long-chain PUFAs (LC-PUFAs) into very long-chain PUFAs (VLC-PUFAs). The question naturally arises as to whether ABCA4 may have necessary interactions with VLC-PUFAs when functioning properly. If so, the two disease types could be tied together by the common element of ABCA4 dysregulation. VLC-PUFAs are relatively rare FAs, so their presence in rod OS disks has been studied for many decades. One such study found that VLC-PUFAs copurified with rhodopsin when both were extracted in hexanes, suggesting that the VLC-PUFAs may have a role in the center of rod OS disks as well (Aveldano, 1988). In turn, work by Hopiavuori et al. hypothesized that the saturated version of VLC-FAs (VLC-SFAs) may stabilize curved regions of membranes by reaching across the bilayer and forming enhanced van der Waals interactions that could then modulate the synaptic release of vesicles (Hopiavuori et al., 2019). Because the rod OS disk rim is a bulbous, curved structure, we initially hypothesized that a similar mechanism may be at work in stabilizing the disk rim. Until the advent of styrene maleic acid (SMA), however, there was little hope of resolving if VLC-PUFAs resided in a particular domain of rod OS disks.

SMA has rapidly become a popular agent for nanodisc creation from native membranes. When SMA is mixed with membranes, it forms SMA lipid particles (SMALPs) roughly 10–12 nm in diameter. Prior to our study, this method of proteoliposome nanodisc extraction had yet to be proven as a way to copurify native lipids with target membrane proteins of interest. To this end, there were many prior studies trying to address the lipid exchange dynamics of polymer-bound lipid nanodiscs (Cuevas Arenas et al., 2017; Danielczak and Keller, 2018; Schmidt and Sturgis, 2018). Those earlier studies of SMALPs and diisobutylene maleic acid lipid particles (DIBMALPs) showed that phospholipids can exchange more rapidly at ambient temperatures (*i.e.*, 20–30 °C) as compared to those prepared in large unilamellar vesicles (LUVs) or membrane scaffold protein (MSP) nanodiscs. The findings suggested that native membrane proteins, once extracted by SMA, might reside in a lipid environment that reflects the *average* lipid environment of the extracted tissue, not the domain from which it came. However, more recent data provided evidence that SMALPs of various bacterial proteins formed and isolated under lower temperature conditions (4 °C) have distinct lipid profiles (Teo et al., 2019), indicating the native local membrane environment composition is retained in samples prepared in this manner. With the addition of our manuscript, there have been no reports of lipid swapping in SMALP-protein nanodiscs. We further validated that the SMALP method could isolate distinct domains from within continuous membranes extracted by leveraging the known localizations of 3 proteins from

the rod OS disk. We not only checked for differences between rhodopsin (representing the center of the disks) and ABCA4 (which is found solely on the rim), but added the PRPH2/ROM1 complex, which helps maintain the disk rim curvature and is segregated to the rim region with ABCA4. We hypothesized that native distinctions between the center and rim samples should be more common than those between the rim samples. Our results confirmed this hypothesis, with over 20 statistically significant instances of the rim sample lipid species differing from the rhodopsin samples.

We isolated the central lamellar and rim regions of the disks by immunopurifying integral membrane proteins in SMALPs, and the copurifying lipids from each sample were extracted and analyzed for differences. We found systematic differences between the two rim samples (ABCA4 and PRPH2/ROM1) and the center samples (rhodopsin), the most striking being the proclivity of the rim to have short, saturated FAs (Figure 2). The center contained the vast majority of the LC- and VLC-PUFAs, including DHA. This finding helped clarify the outstanding question as to where VLC-PUFAs were having their effect in rod OS disks, near rhodopsin or perhaps primarily interacting with ABCA4 on the rim. We showed that VLC-PUFAs are nearly undetectable in ABCA4 and PRPH2/ROM1 native nanodiscs, lending credence that VLF-PUFAs exert the majority of their influence in the center of rod OS disks, likely interacting with rhodopsin.

The described lipid compartmentalization in the disk suggests the existence of a sorting mechanism that guarantees correct lipid distribution. This likely happens during the disk formation, as specific proteins assume their particular localization very early during the process (H. Y. Chen et al., 2021). One of the possibilities is that vesicles harboring disk proteins during disk formation/regeneration are already enriched in the population of lipids characteristic for each protein. This would suggest that lipid composition of the center and rim is established directly by protein composition. This hypothesis is partially supported by studies highlighting the role of PRPH2 in the initial disc formation and enclosure (Lewis et al., 2021; Salinas et al., 2017). Recent studies reinforced the role of proteins themselves being responsible for creating and maintaining the rim curvature (Pöge et al., 2021) further emphasizing the protein-dependent lipid composition of membrane microenvironments. Interestingly, it may suggest that disk shape and size might be regulated by the protein availability, i.e. high expression of rhodopsin is correlated with low expression of PRPH2 in order to allow growing of the flat center of the disc. How this process is regulated has yet to be explained. The most probable explanation is that proteins occupying the subcompartments of the disk are expressed in their correct proportions, possibly by a regulated feedback mechanism allowing proper daily photoreceptor regeneration. In addition, release of protein/lipid vesicles from the Golgi is likely dependent on FA availability, allowing another level of regulation of the disc formation.

I.1.4.   Rod OS cholesterol gradient—The lipid composition of the disk changes during disk transport from the rod OS basal to apical side, enabling functional regulation without affecting the protein composition. For instance, rhodopsin activation is affected by the lipid composition. In addition to the aforementioned lipid asymmetry across disk membrane leaflets, there are reports on changes in cholesterol and sphingomyelin concentrations from the basal to apical rod OS disks.

Cholesterol has a dual role in maintaining the cell membranes' function by changing the lipid bilayer's fundamental properties through interacting with phospholipids, as well as directly interacting with specific membrane proteins (Yeagle, 1991). Cholesterol increases the bilayer thickness and reduces the membrane permeability to small uncharged polar molecules and to ions (Jedlovszky and Mezei, 2003). It is suggested that the effective sealing of the plasma membrane with cholesterol against sodium ion leakage is an important factor in maintaining membrane hyperpolarization (Albert and Boesze-Battaglia, 2005). In the basal disks, there are cholesterol-rich, particle-free patches that appear to exclude rhodopsin. These patches had reduced prevalence in distal disks, reflecting a basal-to-distal cholesterol gradient (Andrews and Cohen, 1983, 1979). They contained concentrated cholesterol and sphingomyelin, which were shown to spontaneously segregate into ordered membrane phases called lipid rafts (Simons and Ikonen, 1997). The rod OS plasma membrane has similar cholesterol content to other cell membranes and the newly synthesized disks, which is ~30 mol%. However, a significant reduction in the cholesterol content is observed as the disks age, decreasing from ~30 mol% (basal) to ~5 mol% (distal) (Boesze-Battaglia et al., 1990, 1989).

Cholesterol has a higher affinity to saturated fatty acyl chains than unsaturated ones. The phospholipid species favored by cholesterol are primarily sphingomyelin, then PS, PC, and lastly PE (Yeagle and Young, 1986); and it is suggested that heterogeneous distribution of cholesterol in disk membranes, but also other membranes, is determined by phospholipid fatty acyl composition (Albert and Boesze-Battaglia, 2005). As the disks age during apical displacement, the phospholipid acyl chain unsaturation increases, creating unfavorable conditions for the cholesterol, which leads to decrease in its concentration in the disk membranes. The most notable alteration occurs within the PC fatty acyl chains, where the abundance of saturated palmitic (16:0) FA drops markedly, while the highly unsaturated DHA content doubles as disks age. On the other hand, PE, PS, and PI do not show significant changes in acyl chain composition, and the headgroups structure of the major phospholipid species stays largely unchanged during disk maturation (Albert et al., 2016). Cholesterol directly interacts with rhodopsin. The postulated function of the high cholesterol content in the newly synthesized disks is to stabilize rhodopsin by decreasing the partial free volume in the hydrocarbon core of the bilayer, which inhibits rhodopsin activation. As disks are apically displaced, there is a decrease in cholesterol but an increase in unsaturated FA concentrations favored by rhodopsin and resulting in its higher activity (Albert et al., 2016; Polozova and Litman, 2000).

I.1.5.   DHA in photoreceptors—DHA is the most abundant polyunsaturated FA in the mammalian body. In the human retina, DHA constitutes over 15% of total FAs and is the most abundant FA in rod OS (50–70% of FAs) (Bretillon et al., 2008; Fliesler and Anderson, 1983; Makrides et al., 1994). Seminal studies using dietary manipulation discovered the key role of DHA in visual function and established direct dependence of the amount of DHA precursors on DHA levels in the membranes and impact on electroretinogram responses (Benolken et al., 1973; Landis et al., 1973) setting the stage for studies trying to understand the mechanistic and biological role of DHA in the eye. The importance of DHA in vision has been further confirmed in many organisms, notably in non-human primates (Lin et al.,

1994; Reisbick et al., 1997) and in preterm and term infants (Birch et al., 1992; Uauy et al., 1992). It has been also found and confirmed by several laboratories that in situations of low DHA availability, this FA is recycled between the RPE and photoreceptors ((Stinson et al., 1991) and see chapter II), again putting DHA in the center of interest.

On the metabolic level, 24:6n-3, a molecule that is converted to DHA, is the shortest FA that undergoes beta oxidation in peroxisomes. At the same time, due to the enzyme specificity, DHA cannot be submitted to further beta-oxidation in peroxisomes and is quickly transported out and involved in further steps of lipids synthesis in the cell. Interestingly, DHA have never been found to be bound to carnitine for mitochondrial beta-oxidation which suggests another level of protection of this FA on the cellular level. Finally, the ratio of DHA to VLC-PUFA has to be tightly regulated, since both pathways use the same ELOVL2 product – 24:6n-3. It would be interesting to decipher this efficient mechanism of 24:6n-3 transport to peroxisomes in order to produce DHA and how modulate the pathway in order to produce more VLC-PUFAs in the cell.

On the biophysical, DHA is a particular molecule. With twelve, out of twenty-two, carbons involved in six double-bonds is the most unsaturated FA in human body. The double bond between two carbons, although by itself providing very rigid structure, allows the adjacent single bond bound carbon to change position with lower energy than if bound to the carbon involved with single bond bound carbon. Therefore, the flexible DHA chain easily changes the conformation, to efficiently support dynamic changes in structure of immersed receptors, channels and other membrane proteins. Of note, with highly abundant DHA levels, retina might be the only tissue in the body that contains double-PFA lipids in their membranes, quality that most probably make the rods disk membranes the most fluid membranes in the body. Our recent finding of the rhodopsin being associated with PUFAs in the disks is well aligned with series of studies showing the increased rhodopsin activity in artificial liposomes enriched with DHA containing phospholipids (Bush et al., 1991; Mitchell et al., 2001). It has been also shown that with concentration of PUFAs increasing towards apical part of rod OS (Albert et al., 1998), rhodopsin activity increases (Williams and Penn, 1985). Finally, several studies using animal models, have shown importance of DHA transporters (such as MFSD2A (Lobanova et al., 2019)) and enzymes involved in PUFA-containing-lipids biogenesis (for example ELOVL2, AGPAT3 (Chen et al., 2020; Shindou et al., 2017) position depends on age, disease state and diet, it is expected that rhodopsin activity in photoreceptor membranes changes as well. This intriguing possibility needs yet to be addressed experimentally.

I.1.6.   Lipid membranes microenvironments.—Small cholesterol- and sphingolipid-enriched membrane microdomains are organized into platforms and are resistant to extraction with nonionic detergents (detergent-resistant membrane fraction; DRM). They are involved in compartmentalization and regulation of vital cellular processes (Pike, 2006; Van Meer et al., 2008). Certain lipids interact more favorably with each other than with other lipids because of various chemical and geometric features. The most relevant interactions exist between saturated lipids, sphingolipids, and sterols, which preferentially interact with each other rather than highly unsaturated lipids (Almeida, 2009; Levental et al., 2020). These preferential interactions between lipids generate domains that can drive the sorting

of membrane proteins based on their transmembrane domains or lipid moiety modifications (Lorent and Levental, 2015). Lipid domains support membrane-based signaling and other processes by assembling all of the necessary protein and lipid components (Snead and Gladfelter, 2019).

Rhodopsin shows high affinity for specific lipid microenvironments upon light-dependent activation and binding to the G protein transducin. This quality of rhodopsin is dependent on its dimerization, as well as the presence of attached palmitoyl moieties, while monomeric or depalmitoylated rhodopsin favored different domains (Seno and Hayashi, 2017). This and other findings indicate that palmitoylation of many GPCRs plays a role in their compartmentalization (Kaneshige et al., 2020; Kobe et al., 2008; Zheng et al., 2012). Similarly, arrestin and a truncated form of transducin were mainly present in the detergent-soluble membrane fraction in the dark but, upon light exposure, were recruited to detergent-resistant lipid microenvironments (Perdomo and Bubis, 2020). The above reports show the importance of lipid membrane fluidity in regulating signalling pathways and underlies the potential role of liquid-liquid phase separation as a molecular mechanism(Su et al., 2021). It is yet to be discovered whether this biophysical process is involved in regulation of rhodopsin localization and activity.

I.2. Lipids as signaling molecules

Extracellular signals can drive hydrolysis of membrane lipids into lipid second messengers that intiate various downstream effects. Glycerolipid-derived signaling molecules include lysophospholipids, PI phosphates (PIPs), DAG, phosphatidic acid (PA), and FFAs (like AA). AA is the precursor of the signaling eicosanoids and endocannabinoids, and DHA is a substrate for the generation of docosanoids. PIP_2 can be further hydrolyzed to generate water-soluble IP_3 and DAG or phosphorylated to produce another essential second messenger, phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). The sphingolipid-derived signaling molecules include ceramide, sphingosine, and their phosphorylated forms - S1P and ceramide-1-phosphate (C1P).

I.2.1. Sphingolipids—Sphingolipids are a large family of lipids that are critical building blocks of cell membranes, playing important roles in regulating its barrier function, permeability, and fluidity. They also regulate various biological processes such as cell growth, proliferation, death, and survival (Hannun and Obeid, 2008; Simón et al., 2019; Wang and Bieberich, 2018). Sphingolipids constitute roughly 11–13 mol% of the total lipids in the bovine retina, making them the third most abundant type of lipid in the retina after phospholipids and sterols (Brush et al., 2010; Fliesler and Anderson, 1983). Sphingomyelin is the most abundant type of sphingolipid, amounting to about 2.5 mol%, whereas ceramide and glucosylceramide constitute less than 1 mol% of the total lipids.

Ceramide signaling in the retina: Ceramides are considered the base units for sphingolipids and the central hub in the sphingolipid biosynthesis pathways (Harrison et al., 2018; Kanehisa and Goto, 2000). They are comprised of a FA of variable chain length bound to an amino group of sphingosine or other sphingoid bases (Gault et al., 2010). C16-C24 ceramides, which are most common, have very low water solubility; they are

one of the least polar and most hydrophobic lipids in biological membranes (Castro et al., 2014). In addition to their structural role, ceramides are potent secondary signaling molecules that activate a diverse set of kinases and phosphatases. Increasing evidence suggests that ceramides can also control photoreceptor cell death decisions by activating the mitochondrial apoptotic pathway, subsequently activating the caspase cascade. This can increase calcium concentration in mitochondria and the cytosol that results in calpain-mediated apoptosis. Ceramides were also shown to trigger photoreceptor death in a caspase-independent manner through the generation of ROS, increase in mitochondrial permeability, activation of Poly(ADP-Ribose) Polymerase 1 (PARP-1) and calpain, accumulation of poly(ADP-ribose) polymers, and nuclear translocation of AIF, all of which are characteristic of the Parthanatos death pathway. Moreover, the inhibition of PARP-1 and calpain activity protected photoreceptors from ceramide-mediated death in a study done after PARP-1 enzyme activity was established as an important contributor in both rd1 and rd2 retinal degeneration mouse models (Prado Spalm et al., 2019; Sahaboglu et al., 2017, 2016). PARP-1 is also reciprocally regulated by calcium, which is one of the mediators of cell death (Bürkle and Virág, 2013; Virág et al., 1998).

Ceramide phosphorylation by ceramide kinase (CERK) leads to the generation of C1P, a sphingolipid with pleiotropic bioactivity (Bornancin, 2011). C1P is synthesized in the trans Golgi and then transported to the plasma membrane to be released for auto- or paracrine signaling (Simanshu et al., 2013). C1P stimulates proliferation, differentiation, and survival of photoreceptor progenitors *in vivo*, enhancing opsin and PRPH2 expression and promoting the development of OS (Miranda et al., 2011).

Increasing evidence suggests that ceramides are engaged in the onset of retina degeneration in mammals. While studying a rabbit model of retinal detachment, one study found that retinal apoptosis during induced retinal detachment is associated with *in vivo* production of ceramides (Ranty et al., 2009). Accumulation of ceramides due to deficient acid ceramidase *ASAH1* in Farber's disease leads to retinal dysplasia, inflammation, and rod photoreceptor dysfunction resulting in severe visual impairment (Yu et al., 2019). Many studies indicate that ceramide accumulation leads to retina degeneration, but overexpressing ceramidases or inhibiting ceramide generation pathways can rescue photoreceptor cells and prevent vision loss (Acharya et al., 2008, 2003; Fabiani et al., 2017; German et al., 2006; Piano et al., 2013; Sanvicens and Cotter, 2006; Stiles et al., 2016).

<u>Ceramide imbalance is detrimental for the RPE:</u> One of the critical functions of the RPE is trafficking and sorting cargo originating from the extracellular environment and plasma membrane using endosomes. Endosomes are derived from the RPE plasma membrane; therefore, the plasma membrane composition determines the number and size of nascent endosomes. Excessive accumulation of ceramides in the apical RPE membrane was recognized to play a role in macula diseases, such as age-related macular degeneration (AMD) and STGD (German et al., 2006; Tan et al., 2020; Victoria Simon et al., 2021; Zhu et al., 2010). Kaur and colleagues showed that increased ceramide levels promoted inward budding and fusion of early endosomes in the RPE (Kaur et al., 2018), but lowering ceramide levels with desipramine corrected these endosomal defects in a Stargardt's disease mouse model (Kaur et al., 2018).

In diabetic retinopathy, acid sphingomyelinase (ASMase, encoded by *SMPD1*) stimulation by high glucose and subsequent increase in ceramide levels have been shown to cause pathological alterations in RPE cells (Wang et al., 2016). Cholesterol-mediated activation of ASMase also disrupted autophagosome traffic and autophagic flux in the RPE due to ceramide-promoted tubulin acetylation and stiffening of microtubules (Toops et al., 2015). Furthermore, excessive neutral sphingomyelinase activity decreased RPE cells proliferation and increased apoptosis of ARPE-19 cells (Zhu et al., 2010). Another study shows that the addition of membrane-permeable ceramide C2 induced apoptosis-like cell death in the cultured human and rat RPE cells by increasing ROS production, mitochondrial membrane permeabilization, and caspase-3 activation (Kannan et al., 2004; Tomita et al., 2000).

Sphingosine and sphingosine-1-phosphate: Sphingosine is another secondary messenger, potently generated by apoptotic stimuli, that can induce photoreceptor cell death through mitochondrial membrane permeabilization and ROS formation (Rotstein et al., 2010). Reducing sphingosine generation or stimulating its phosphorylation to S1P prevents photoreceptor demise, emphasizing the role of sphingolipids in photoreceptor survival (Simón et al., 2019).

Sphingosine is phosphorylated to S1P through the action of two sphingosine kinases - SphK1 and SphK2. The local concentration of S1P in tissues depends on the balance between its synthesis and degradation. The main enzymes that degrade S1P are S1P lyases and S1P phosphatases (SPPs). SPPs have two isoforms, SPP1 and SPP2, which catalyze the dephosphorylation of S1P, generating sphingosine (Sph) that is then converted to ceramide by ceramide synthases (*CERS1–6*) (Levy and Futerman, 2010; Pyne et al., 2009) S1P lyase (*SGPL1*) irreversibly degrades S1P to hexadecenal and ethanolamine-1-phosphate, removing S1P from the process of sphingolipid metabolism (Bandhuvula and Saba, 2007).

S1P emerges as a mediator of photoreceptor development and survival, preventing photoreceptors from oxidative stress (Fabiani et al., 2017; Fang et al., 2018; Miranda et al., 2009). It was shown that cytokines, DHA, glial cell-derived neurotrophic factor, and growth factors such as transforming growth factor b and nerve growth factor stimulate the expression of SphK1, resulting in an increase of S1P that acts as a secondary messenger critical for photoreceptor neurogenesis and survival (Abrahan et al., 2010; Porter et al., 2018). In addition to its pro-survival effect, S1P also potently stimulates angiogenesis and inflammation. Overexpression of SphK2 increases S1P levels, resulting in accelerated retinal angiogenesis and increased neovascularization. Downregulation of S1P in SphK2 KO conditions reverses this effect (Eresch et al., 2018). S1P also promotes the recruitment of immune cells and retention of lymphocytes in inflamed tissues (Aoki et al., 2016). Thus, S1P action through S1P receptors is a driving force in the onset and progression of inflammation and angiogenesis in retinal inflammatory diseases (Simón et al., 2019). Following retinal injury, S1P promotes gliosis that alters the retinal structure, thereby enhancing visual dysfunction instead of preventing it (Lukowski et al., 2013; Swaney et al., 2008).

Ceramides and S1P appear to have opposite cellular roles; while ceramides induce cell cycle arrest or death, S1P promotes cell proliferation and survival (Cuvillier et al., 1996;

Gomez-Munoz et al., 1995). The metabolic interconnection of ceramides, C1P, Sph, and S1P, demonstrates how altering the balance of these mediators can affect cell fate. Enzymes interconverting sphingosine and ceramides are located close to each other in the plasma membrane (Gault et al., 2010). The proximity of these enzymes in the plasma membrane suggests that tight regulation of the formation and breakdown of these sphingolipids is critical. The balance between them often referred to as the "sphingolipid rheostat," can profoundly affect cell death or survival and differentiation (Hait et al., 2006; Lewis et al., 2018; Newton et al., 2015). Understanding how these pathways can be manipulated could lead to novel therapies for treating and preventing different retinal diseases, independent of the underlying etiology.

I.2.2. Signaling of glycerophospholipids—Glycerophospholipids are composed of a glycerol backbone, phosphate group, and two (or less frequently one) esterified FA group (Figure 3A). They account for about 90% (wt/wt) of the total lipids found in rod OS membranes (Fliesler and Anderson, 1983). Besides being a building block of cell membranes, they often act as cellular signal transducers to trigger intracellular signaling events (Van Meer et al., 2008).

Phosphatidylserine (PS) involvement in the phagocytosis signaling: When displayed on the surface of apoptotic cells, PS serves as a classic 'eat-me' signal recognized by surface receptors of many phagocytes (Segawa and Nagata, 2015). PS is concentrated in the cytoplasmic leaflet of OS membranes due to the action of PS flippases. One of the flippases keeping the PS distribution asymmetric in the OS membrane is ATPase phospholipid transporting 8A2 (*ATP8A2)*, a member of the P4-ATPase subfamily. ATP8A2 acts on PS and, to a lesser extent, PE without affecting other membrane lipids (Coleman et al., 2009). In the mammalian retina, renewal of the rod OS involves circadian shedding of distal rod OS tips, followed by their subsequent phagocytosis by the RPE. Rod OS tips have PS exposed to the extracellular space, primarily at the onset of light. (Ruggiero et al., 2012). Exposed PS is then bound by the glycoprotein milk fat globule factor-E8 (MFGE8), which is a ligand for αvβ5 integrin, located in the apical RPE (Akakura et al., 2004; Finnemann et al., 1997). OS phagocytosis by the RPE also requires binding of tetraspanin CD81 to integrin αvβ5 (Chang and Finnemann, 2007).

Out of phospholipids, PS have the highest ratio of di-PUFA species (Hopiavuori et al., 2017) which presumably, makes the PS molecule more susceptible to the oxidative stress, therefore prone to the conformational changes. These changes may impact the membrane organization and induce local disturbances. The exact mechanism regulating PS exposure to induce phagocytosis is not well understood. Studies deciphering the molecular mechanism of membrane repair show exposure of PS as one of the first signals of membrane break (Horn and Jaiswal, 2018). Several questions arise: 1. If the exposure of PS is induced similarly in photoreceptors, is it possible that photoreceptor membrane breaks preceed PS exposure also in phagocytosis? 2. Is the PS exposure dependent on oxidation of PS-bound PUFA species? 3. Does PS oxidation induce membrane breaks? These questions await experimentally driven answers.

Phosphatidylinositol phosphates (PIPs): Phosphatidylinositols are relatively abundant phospholipids in the retinal and RPE membranes (~4–7 mol% of total phospholipids), except for the rod OS where their content is estimated to be ~2 mol% (Anderson, 1970; Anderson et al., 1970). Phosphorylated forms of phosphatidylinositols called PIPs are found at much lower levels. PIPs are a potent class of signaling molecules playing important roles in mediating intracellular membrane trafficking of membrane proteins and lipids in response to environmental changes and cell demands (Wensel, 2020). Phosphatidylinositol-3-phosphate (PI(3)P) was found to regulate the trafficking of proteins to early and recycling endosomes (Carpentier et al., 2013) while also participating in the formation and maturation of autophagosomes (Dall'Armi et al., 2013). Phosphatidylinositol-4-phosphate (PI(4)P) was demonstrated as an essential factor in vesicle formation, lipid metabolism, and membrane trafficking within the trans-Golgi network (Lenoir and Overduin, 2013). PIP_2 is a key player in clathrin-dependent endocytosis (McLaughlin et al., 2002), as well as the regulation of ion channels, transporters, and enzymes in membranes (Falkenburger et al., 2010). PIP_2 is also a substrate and a regulator of PLC, which hydrolyzes PIP_2 to IP_3 and DAG – two critical second messengers described in this review (Fukami et al., 2010; Kadamur and Ross, 2013).

It was recently established that PIP2 and PI(4)P were the two major phosphorylated species of PI among retinal phosphoinositides, and 18:0/20:4 and 16:0/20:4 were the most common fatty-acyl chains (Finkelstein et al., 2020). Furthermore, Wensel's group showed that rods contain 10-fold higher levels of PI(4)P and PIP_2 than PI(3)P (Wensel, 2020). It was also demonstrated that PIP_2 was enriched in photoreceptor inner segment (IS) and synapses but was scarcely present in OS. PI(4)P was found in the OS, IS, and outer nuclear layer, as well as in the synaptic region to a smaller extent (Finkelstein et al., 2020). However, a study from Rajala lab showed that five PIPs species were found in the murine and bovine rod OS fractions, namely PI(3)P, PI(4)P, phosphatidylinositol 3,4-diphosphate, PIP_2, and PIP_3, but the PIP_2 was the most abundant and PIP_3 had the lowest levels (Rajala et al., 2020b). Unlike the retina, they noticed that RPE was the most enriched in PIP_3 compared to the other four species suggesting its high importance for RPE cells.

PIP_2 acts together with myosin, actin, rac1, and rab8 to regulate the fusion of rhodopsin transport carriers in retinal photoreceptors (Deretic et al., 2004). Impaired PIP metabolism causes dysfunction of the retina, as shown in animal models and human patients (Brooks et al., 2018; Hagstrom et al., 1998). Moreover, several reports suggested that the enzymes involved in PIPs metabolism in the retina are regulated by light (Giusto et al., 2000; Rajala, 2020; Schmidt, 1983a, 1983b). For instance, rod PI(3)P levels increased over 30-fold after the light activation of PI-3 kinase (He et al., 2016). Ablation of the PI-3 kinase Vps34 responsible for PI(3)P production resulted in an impaired fusion of endosomes and autophagosomes with lysosomes, accumulation of abnormal membrane structures, and ultimately photoreceptor degeneration (He et al., 2016), as well as loss of visual function and cone death (Rajala et al., 2020a, 2015). The PI-3 kinase-generated PIPs activate prosurvival Akt kinase, which regulates retinal mitochondrial integrity, triggering anti-apoptotic effects in photoreceptor cells (Li et al., 2007; Rajala et al., 2013), as well as protecting RPE from oxidative stress (Defoe and Grindstaff, 2004; Rajala, 2020). PIPs

are also indispensable in the RPE phagocytosis and autophagy processes; however, the molecular details are not yet fully understood (Intartaglia et al., 2021; Ravussin et al., 2021).

Inositol 1,4,5-trisphosphate (IP₃): The retina's PIP_2/IP_3/DAG metabolism, especially in the photoreceptors and RPE, is an under-studied component of this well-studied system, as discussed at length in recent studies review by Wensel (Wensel, 2020). The production of inositol polyphosphates during visual processes was first studied using biochemistry and electrophysiology in *Limulus polyphemus* (Brown et al., 1984). While it is now known that invertebrates such as *Limulus* and *Drosophila* use IP_3 signaling during phototransduction, comparatively recent work has led to speculation that IP_3 is involved in the termination of vertebrate phototransduction. Orisme et al. showed that visual arrestin (known at varying times as S-antigen or rod arrestin, and systematically as arrestin 1) translocates from the IS to OS without exposure to light in the presence of PLC and protein kinase C (PKC) agonists (Orisme et al., 2010). Furthermore, PLC and PKC antagonists reduced arrestin 1 translocation in the light, suggesting that those associated pathways were not merely sufficient but necessary for translocation.

Much earlier work on PIP_2, the phospholipid precursor of soluble IP_3, reported a light-dependent decrease of PIP_2 relative to PI that was attributable to PLC-mediated cleavage, suggesting the release of IP_3 upon exposure to light (Ghalayini and Anderson, 1984). Subsequent work found that there was indeed a decrease in PIP_2, but they did not detect increases in IP_3, which, as highlighted by Wensel, suggested that a phosphatase was acting on PIP_2, not PLC (Millar et al., 1988; Wensel, 2020). The aforementioned findings of Orisme et al. came as a surprise, then, as it suggested that PLC activity was responsible for prompting arrestin 1 translocation from the IS to OS. Because the major consequence of PLC activation is IP_3 production, there is renewed interest in IP_3 as a light-dependent secondary messenger in photoreceptors.

Recent work by one of our groups resolved the molecular details of IP binding to arrestin 1, including IP_3 (Sander et al., *accepted for publication*). Combined with the most complete basal structure to date, we were able to identify candidate residues involved in N-domain binding of the regulatory C-tail of arrestin 1 and which of those residues interact with IP_3. R171 and K298 bound both the C-tail and IP_3, and, when bound to IP_3, the C-tail was removed from the majority of the N-domain, leaving arrestin 1 in a primed conformation. Given the proposed tetrameric structure of arrestin 1, our basal structure suggests that the C-tails are close together in the NN contact sites within the tetramer (Figure 3C). Disturbance of those sites, possibly through IP_3 binding, could disrupt the tetramer sufficiently to allow arrestin to translocate from the inner to OS under a restriction-diffusion model as proposed previously. The difference here is that the restriction can potentially be the result of homotetramerization, wherein the protein population shifts in an average size and becomes sterically excluded from diffusion in the rod OS (Najafi et al., 2012; Malhotra et al., 2021).

These results have exciting implications for the PLC pathway in photoreceptor adaptation. First, the underlying mechanism that begins the light-dependent activation of PLC has not yet been explained. The experiments from Orisme, however, give strong evidence that arrestin 1 translocation is tied to PIP_2 cleavage by PLC and/or DAG signaling coupled to

PKC. If true, components of photoreceptor membrane lipids may play the role of secondary messengers for the termination of phototransduction through the translocation of arrestin 1 to the OS. Light-mediated reduction in PIP_2 levels has yet to be fully incorporated into the function of other light-dependent pathways in photoreceptors. Revisiting these older experiments with modern technology may help settle the debate over the levels of PIP_2 and IP_3 after exposure of photoreceptors to light. Furthermore, the possible connection between rhodopsin signaling and PIP_2 cleavage should be studied.

In contrast to photoreceptors, PIP_2-derived IP_3 has already been confirmed as an important signaling molecule for the essential functions of the RPE. Rodriguez de Turco et al. made an initial observation that light-stimulated RPE cells, at the time of phagocytosis, have increased IP_3 production (Rodriguez de Turco et al., 1992). Studies comparing rats without retinal degeneration to Royal College of Surgeons (RCS) rats, a model of retinitis pigmentosa (RP) that lacks phagocytosis, revealed that IP_3 signaling increases during RPE phagocytosis (Heth and Marescalchi, 1994). Subsequent work in RCS rats reported that exposure of cultured RCS RPE cells to carbachol (a muscarinic receptor agonist that stimulates IP_3 production) restored phagocytic behavior to RCS RPE cells (Heth et al., 1995). These findings were contradicted by Hall et al. one year later, where they reported that, while IP_3 increases are a likely result when exposed to carbachol, they could not confirm an increase in phagocytosis when it was incubated with RCS RPE cells (Hall et al., 1996).

The RPE also requires IP_3 for several other pathways, including growth factor and neuropeptide signaling (Kuriyama et al., 1991, 1992). $P2Y_1$-mediated inflammation and increased expression of nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) in cases of high NaCl levels surrounding the RPE was shown to be produced, in part, by IP_3 signaling (Prager et al., 2016). Ion channels also contribute to epithelial transport of Cl^- and secretion contain elements of IP_3 signaling as well (Strauss et al., 1996; Barro-Soria et al., 2012; Vainio et al., 2015; York et al., 2017; Hollborn et al., 2017), a phenomenon that has been reviewed previously (Constable, 2014).

Diacylglycerol (DAG): As mentioned above, PIP_2 cleavage by PLC generates IP_3 and DAG, which is a neutral glyceride containing two FA chains esterified to the glycerol molecule. DAG can also be generated through several other catabolic and anabolic reactions (Figure 3B) (Eichmann and Lass, 2015). DAG is found in cellular membranes as a building block for glycerophospholipids. It can also act as a second messenger at the plasma membrane while also regulating many cellular processes. These processes include insulin signaling, ion channel regulation, and neurotransmitter release (Eichmann and Lass, 2015; Ma et al., 2013; Schuhmacher et al., 2020).

DAG can bind and activate two out of three classes of PKC isozymes, conventional and novel PKC isoforms (Newton, 2018, 1993). DAG-sensitive PKC isozymes are activated at the membrane site, and their action needs to be tightly controlled to warrant healthy cell functions (Newton et al., 2016). DAG-activated PKC is present in rod OS (Kelleher and Johnson, 1985; Williams et al., 1997; Wolbring and Cook, 1991), where it phosphorylates rod OS proteins in a light-dependent manner. Proteins from the phototransduction

pathway that were shown to be phosphorylated by PKC isozymes are transducin, cGMP-phosphodiesterase (PDE6), rod cGMP-gated channel, arrestin and rhodopsin (Newton and Williams, 1993, 1991; Williams et al., 1997). Giusto group demonstrated that DAG and PA levels depend on the dark *vs.* light conditions in rod OS due to the light-driven regulation of the activity of enzymes producing these molecules (Pasquaré et al., 2008). Under illumination, there is an increase in DAG kinase activity (Huang et al., 2000) that generates higher PA levels, whereas in darkness DAG concentration increases due to elevated activity of phospholipase D (PLD)/ lipid phosphate phosphatases (LPP), that are otherwise inhibited by light (Salvador and Giusto, 2006). It is also suggested that DAG/PA ratio in rod OS is dependent on the LPPs activity, which is modulated differently based on the concentration of LPA, S1P, or C1P (Pasquaré et al., 2008).

The DAG kinase isoform epsilon (*DGKE*) is abundantly expressed in the human retina, especially in rods and cones (Figure 4), and a possible connection has been suggested with inherited RP; however, a pilot study did not reveal *DGKE* gene mutations (Tang et al., 1999). The increased *de novo* synthesis and abnormal DAG accumulation are driven by high glucose-induced PKC activation. This, in turn, causes microvascular pathologies in the retina of diabetic patients. Increased levels of DAG affect vascular blood flow and cause extracellular matrix buildup, basement membrane thickening, increased permeability, and neovascularization (Mérida et al., 2008; Way et al., 2001).

Phosphatidic acid (PA): Except for *de novo* synthesis, PA can be generated by acylation of lysophosphatidic acid (LPA), hydrolysis of PC by PLD, or produced from DAG through phosphorylation by DAG kinases (Figure 3B). Both PA and DAG are maintained in the cell at relatively low levels, and there is a dynamic interconversion between the two molecules (Moine and Vitale, 2019). It is proposed that the *de novo* pathway generates a pool of a 'structural' PA used as a precursor for other glycerophospholipids while the other metabolic pathways produce PA for signaling events (Kim and Wang, 2020). PA can increase its net negative charge from −1 to −2 depending on the pH, which allows for attracting and tethering proteins to membranes *via* electrostatic interaction. Moreover, PA can induce conformational changes within proteins upon binding, promote protein oligomerization, or hinder ligand binding, thus regulating enzyme catalytic activity (Kim and Wang, 2020; Kooijman et al., 2007; Zhukovsky et al., 2019). The average membrane displacement caused by the structure of PA enables it to induce negative (concave) curvature in the lipid bilayer, which aids membrane fusion and fission and the formation of secretory vesicles (Kooijman et al., 2003). PA is also one of the positive regulators of mTOR signaling (Ávila-Flores et al., 2005). Acting together with insulin, PA activates mTOR survival pathways and protects hyperglycemic neuronal cells from apoptosis in diabetic retinas (Fox et al., 2012).

Lysophosphatidic acid (LPA): Lysophosphatidic acid (LPA) is not a single entity but a family of naturally occurring glycerophospholipids composed of a glycerol backbone with an esterified single acyl chain and a phosphate group. LPA species can be derived from PA hydrolysis by phospholipase A1, but their primary source is the cleavage of LPC by autotaxin enzyme (encoded by *ENPP2* gene) (Yanagida and Valentine, 2020). Autotaxin is a secreted enzyme with lysophospholipase D activity that converts lysophospholipids to LPA

and which was found to be highly expressed in the retina (Uhlen et al., 2015). LPA exerts its functions by binding with its GPCR receptors (LPA1–6), and depending on the type of LPA receptor and the type of its coupled G-proteins, it can activate different signaling pathways (Chun et al., 2010). LPA regulates various biological functions such as cell proliferation, migration, inflammation, angiogenesis, metastasis, apoptosis, and others (Chun et al., 2010; Yanagida and Valentine, 2020).

LPA induced a rise in Ca^{2+} concentration in the neural retina of chick embryos during neurogenesis (Zhou et al., 1999), and promoted growth cone collapse throughout retina development in mice and chick (Birgbauer and Chun, 2010; Fincher et al., 2014). Moreover, LPA was suggested to alter different types of ion channel activity in human and bovine Müller cells (Kusaka et al., 1998). Another finding demonstrated that mutations in the *PNPLA6* gene encoding lysophospholipase, which deacetylates PC to LPC to glycerophosphocholine, led to retinal LPA accumulation and photoreceptor degeneration (Kmoch et al., 2015).

It was determined that treatment of the RPE with either PA or LPA stimulated cell proliferation, but the effect was much weaker when LPC was used (Thoreson et al., 1997). Using human pluripotent stem cell (hPSC)-derived retinal cells, it was shown that LPA regulates tight junctions in RPE in a receptor-dependent manner and increases the transepithelial electrical resistance of the RPE. Additionally, the high content of LPA decreased the efficiency of phagocytosis of photoreceptor OS by the RPE. LPA treatment of the stem cell-derived photoreceptors caused morphological changes and reorganization of the actin-myosin cytoskeleton (Lidgerwood et al., 2018).

Eicosanoids, docosanoids, and elovanoids.: Eicosanoids, docosanoids, and elovanoids are generated as breakdown products of membrane lipids. They act as secondary lipid messengers, modulating local inflammatory responses and homeostasis, and are often called bioactive lipids (BL).

Eicosanoids: Inflammatory events trigger the release of AA or other 20-carbon PUFAs (from either the n-3 or n-6 group) derived from membrane phospholipids by phospholipases A2, C, and D. These are further metabolized through cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P450 monooxygenase (CYP) pathways. From there, the resulting products can generate different types of eicosanoids (Bazan, 2018; Dennis and Norris, 2015; Wang et al., 2021).

Eicosanoids produced by COX include predominantly prostaglandins and thromboxanes. Prostaglandins regulate blood flow in the choroid and inner retina blood vessels. COX enzyme expression and activity is regulated by inflammatory signals, including ROS (Karaa et al., 2006; Li et al., 2011; Martín et al., 2012). In particular, COX2 expression is precisely regulated through the non-coding RNA mechanism and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) dependent initiation of transcription (Krawczyk and Emerson, 2014) and sustained inhibition of NFκB signaling pathway has been shown to protect neuroretina in Akita mice (Homme et al., 2021). It was suggested that prostaglandins could affect choroidal and retinal blood flow regulation in newborn animals, resulting in

surplus oxygen delivery and retinal microvascular damage in retinopathy of prematurity (Abran et al., 1995). Moreover, prostaglandin E_2 (PGE_2) effects on the blood vessels include increased oxidative stress, vasodilation, higher vascular permeability, and elevated production of proinflammatory cytokines (Huang et al., 2016).

The major LOX-derived eicosanoids are hydroxyeicosatetraenoic acids (HETEs), and leukotrienes (LTs). HETEs are involved in the degranulation of neutrophils, skin inflammation, and regulation of blood vessel dilation (Takayama et al., 1987; Tang et al., 1995; M. H. Wang et al., 2020). LTs promote leukocyte chemotaxis and degranulation while also enhancing oxidative stress, vascular permeability, and the production of proinflammatory cytokines (M. H. Wang et al., 2020). Interactions between retinal pericytes and polymorphonuclear leukocytes may lead to the production of sulfidopeptide LTs, which can alter microvascular permeability (Mcmurdo et al., 1998). LTA_4 has been considered a contributing factor in chronic inflammation due to diabetic retinopathy (Talahalli et al., 2010).

The CYP pathway comprises many enzymes that contain heme iron, and many of these enzymes are expressed in the liver, eye, and other tissues, where they inactivate and eliminate toxins and metabolites. Eicosanoids produced *via* the CYP pathway include epoxyeicosatrienoic acids (EETs) and 20-HETE that are recognized as anti-inflammatory, while downstream diHETEs are pro-inflammatory. Various EET species are produced in the retina (Hu et al., 2017). EETs have been implicated in angiogenesis by retinal endothelial cells, especially under hypoxic conditions (Michaelis et al., 2008). Various eicosanoids endogenously produced in damaged tissue initiated the inflammatory response by increasing vascular permeability and stimulating leukocyte chemotaxis in diabetic retinopathy. PGE_2, thromboxane B2 (TxB2), LTs, and 12-HETE were among the proinflammatory and angiogenic eicosanoids. In addition, EETs and LOX-derived lipoxins were also found in samples derived from several diabetic vitrei (Schwartzman et al., 2010). Precise regulation of oxygenases and CYP proteins, induced by external factors, is at the base of the composition of eicosanoids in the cell.

Docosanoids: Docosanoids are another group of bioactive lipid mediators derived from DHA or docosapentaenoic acid. Docosanoids have neuroprotective and pro-homeostatic properties. They aim to resolve inflammation by ceasing neutrophil recruitment, promoting tissue debris clearance by macrophages, counter regulating proinflammatory mediators, and stimulating tissue repair (Bazan, 2018; Serhan and Chiang, 2013; Serhan and Savill, 2005). Within this class of proresolving lipids, there are three families of distinct structures, namely protectins, resolvins, and maresins.

Under oxidative stress, DHA is released from membrane phospholipids by PLA2 and converted by 15-LOX-1 to dihydroxylated docosatriene, named neuroprotectin D1 (NPD1). NPD1 promotes photoreceptor and RPE survival by protecting them from oxidative stress, light damage, and inflammatory events (Bazan et al., 2011; Marcheselli et al., 2010; Mukherjee et al., 2004). Similar in structure, maresin 1 was demonstrated to have a beneficial effect on conjunctival goblet cells by maintaining optimal tear film mucin levels in

the healthy eye. It also attenuated mucin overproduction, as occurs in ocular allergy, through compensating calcium levels (Olsen et al., 2021).

Resolvins, protectins, and lipoxins, which are derived from various PUFAs, suppress the production of interleukin 6 (IL-6), tumor necrosis factor α (TNFα), and vascular endothelial growth factor (VEGF), and have anti-angiogenic effects. Thus, they were proposed as potential candidates for preventing and treating diabetic macular edema and retinopathy (Das, 2013). Resolvin D1 showed protective effects on primary retinal cells exposed to high glucose by reducing ROS levels, promoting mitochondrial DNA repair by 8-oxoguanine glycosylase (OGG1), and reducing apoptosis (Trotta et al., 2020). Resolvin D1 also showed a beneficial effect in rats with streptozotocin-induced diabetic retinopathy by inhibiting the activation of NLRP3 inflammasome and associated cytokine production (Yin et al., 2017).

Elovanoids: Elovanoids are another class of interesting lipid mediators. They are derived from VLC-PUFAs and show mainly protective functions. Elovanoids have been explicitly covered in the context of their general neuroprotective effects in the retina in several previous reviews (Bazan, 2021; Yeboah et al., 2021). Briefly, the VLC-PUFAs that result from elongation by the ELOVL enzymes can subsequently be incorporated into phospholipids. In a similar way to docosanoid creation beginning with the release from the *sn*-2 position of phospholipids by PLA2, VLC-PUFAs can be cleaved from phospholipids by PLA1 from the *sn*-1 position of a phospholipid, and elovanoids are then generated from the dihydroxylated derivatives of 32:6n-3 and 34:6n-3 (Jun et al., 2017). Elovanoids were found to protect RPE cells from uncompensated oxidative stress Bcl-2 and Bcl-xL upregulation and Bax, Bid, and Bim downregulation (Jun et al., 2017). A report in 2019 showed that elovanoids protect photoreceptors by counteracting oligomeric β-amyloid-induced gene expression in a model of simultaneous injection of elovanoids and oligomerized amyloid-β peptide, with subsequent topical administration of elovanoids, in 6-mo-old C57BL/6J WT mice (Do et al., 2019). Interestingly, although VLC-PUFAs are synthesized in situ by ELOVL4, they are also present in actively phagocytic RPE cells, where they can be further converted to elovanoids. Interestingly, because of their particular enzymatic requirements, elovanoids might be molecules present only in limited places in the organism (for example – central nervous system), making them potentially interesting molecule in searching specific pharmacological approaches. There is much work to be done to uncover how elovanoids exert their protective effects, however. Potential binding partners, receptors, and mechanisms involved in blunting the effects of oxidative stress will be important avenues of further research.

Many studies highlight the crucial role of FAs derivatives in cell and tissue biology. These molecules can act as ligands, signaling molecules, and structural components of membranes. The presence of each bioactive lipid is precisely regulated by a cohort of enzymes regulated by intrinsic and extrinsic signals, such as stress, nutrition changes, and others. In addition, the combination of these molecules can trigger different responses to the same stimulus. Complex interdependencies and "feed-forward" reactions, as well as autoregulatory loops, provide many opportunities for modulating the synthesis of eicosanoids, docosanoids and elovanoids. Further interdisciplinary studies involving chemistry, cell biology, and lipid experts are needed to understand the role of these interesting lipids in maintaining the

photoreceptor/RPE homeostasis. Such studies can then attempt to manipulate the network of enzymes and products to influence cell health.

I.3. Lipids as an energy source

The retina is one of the highest energy-consuming tissues in the body, requiring abundant energy substrates to support metabolic functions, including light sensing *via* phototransduction and maintenance of electrical gradients, production of the molecules and structures that allow vision, and managing the oxidative stress arising from these processes (Joyal et al., 2016). As a part of the central nervous system (CNS), it was assumed that the metabolism and energy requirements of the retina rely on glucose, similar to the brain (Mergenthaler et al., 2013). This was supported by studies where the lack of glucose and its transporter in the RPE was associated with photoreceptor degeneration (reviewed in (Hurley, 2021)). Besides glucose, Smith, and colleagues (Joyal et al., 2016) showed that lipids, especially FAs, can serve as another rich source of ATP through oxidative FA oxidation (FAO). The reader can find more details in a recently published, extensive review (Fu et al., 2021). In brief, during this process, FAs are oxidized in the mitochondria to acetyl-CoA and enter the Krebs cycle to produce energy. Lipid turnover plays an essential role in neuronal survival. Peroxisome proliferator-activated receptor-alpha (PPARα), a known lipid metabolism regulator, may be responsible for neovascularization in the retina (Pearsall et al., 2017). The *Ppara*$^{-/-}$ mouse model showed developing retinal degeneration and indicated that PPARα activity is required to internalize FAs into the mitochondria for oxidation and ATP production. Therefore, PPARα has been proposed to be a potential therapeutic target for treating neurovascular defects in retinal metabolic disorders (Fu et al., 2019; Pearsall et al., 2017). The mechanism underlying how cells balance between glucose and lipid energy sources is still not well understood. The involvement of pathways, such as mTOR, in the nutrient-sensing mechanism (Kim and Guan, 2019) is a great starting point for potential future studies.

Chapter II.   Lipid homeostasis in the photoreceptors and RPE

As mentioned earlier, lipids constitute a significant portion of photoreceptors and are especially enriched in the OS structures. Filled with the stacks of densely packed disk membranes, OS exists in an equilibrium between the proximal biosynthesis and elongation of lipid species balanced by distal OS shedding and phagocytosis by the adjacent RPE cells. The continuous renewal of the OS leads to a full renewal of the structure approximately every ten days in the vertebrate eye, drives increased demand for lipid anabolism in the photoreceptor IS, comparable to the levels observed in proliferating cells (Young, 1971, 1967; Young and Bok, 1969). To satisfy this demand, in addition to the systemic delivery of lipids and their precursors, lipid components of the shed OS undergo recovery and are delivered back to OS biogenesis sites, as will be discussed in the following section.

II.1   Lipid exchange with the blood

Transport of hydrophobic lipid compounds through the aqueous blood plasma is enabled by a variety of specialized protein carriers forming lipoproteins, a detailed description of which falls out of the scope of this review but has been extensively reviewed elsewhere

(Getz, 2018). In general, lipoproteins in the bloodstream form particles classified based on their size or density: from the smallest albumin complexes with the FFAs or their lysophospholipid derivatives to a wide range of differently sized spheres encapsulating the lipidic cargo in a hydrophilic shell. The latter include (in the order of size) HDL, LDL, intermediate-, and very-low-density lipoproteins (IDL and VLDL respectively) as well as the largest and lowest density chylomicrons (CM), which can exceed 1 μm in diameter.

Photoreceptor OS and IS form the avascular part of the retina is surrounded by two diffusion barriers. Junctional complexes between the Müller cell apical processes and the photoreceptor IS membranes form the outer limiting membrane, while on the other side the tightly joined RPE cell monolayer serves as the outer blood-retina barrier (BRB). The RPE, together with the opposed Bruch's membrane, separate the OS from a dense network of choroid vasculature, which mediates the majority of photoreceptor metabolite exchange with the systemic circulation. Large, retina-oriented fenestrations in the choriocapillaris facilitate the flow of plasma components into the extracapillary space, as well as further infiltration of the acellular Bruch's membrane layer. Studies of the permeability of the Bruch's membrane indicate that while the diffusion of macromolecules might be hindered to some degree by the inner collagenous portion of the membrane, even relatively large particles are capable of crossing it under native conditions (Cankova et al., 2011; Gordiyenko et al., 2004; Sørensen et al., 2019; Starita et al., 1997). Consequently, the RPE monolayer serves a prominent role in regulating the outer retina metabolite influx and efflux, including that of lipidic compounds.

II.1.1 Lipid influx to the RPE—The uptake of lipidic compounds to the RPE is facilitated by a range of specific receptors present in its cellular membranes (Figures 1 and 4). RPE cells express several lipoprotein cognate receptors, including the LDL receptor (LDLR) (Gordiyenko et al., 2004; Hayes et al., 1989; Tserentsoodol et al., 2006b) and VLDL receptor (VLDLR) (Hu et al., 2008), as well as broad-specificity scavenger receptor class B, member 1 (SCARB1, with alternative splicing variants I and II) (Duncan et al., 2009, 2002; Tserentsoodol et al., 2006a), and the cluster of differentiation 36 (CD36) (Kociok and Joussen, 2007; Ryeom et al., 1996). In addition, several other proteins present in RPE membranes were identified as important regulators of FA content and composition in the photoreceptor cells: major facilitator superfamily domain-containing protein 2a (MFSD2A) (Wong and Silver, 2020), ADIPOR1 (Rice et al., 2015), and membrane frizzled-related protein (MFRP) (Kautzmann et al., 2020). Since the tight junction barrier prevents membrane protein diffusion between the apical and basolateral aspects of the cell, both surfaces differ in their receptor composition and lipid uptake characteristics.

The choroid-oriented basolateral membrane is enriched in LDLR, and accordingly, it was observed that LDL constitutes a more efficient source for cholesterol uptake over the more abundant HDL pool (Tserentsoodol et al., 2006b). Loss of LDLR in mice results in lipid accumulation in the Bruch's membrane and gradual photoreceptor degeneration (Schmidt-Erfurth et al., 2008). This and several other lines of evidence suggest that LDL particles constitute a significant source of lipids taken up from the systemic circulation by RPE cells (Gordiyenko et al., 2004; Hayes et al., 1989; Rodríguez and Larrayoz, 2010; Tserentsoodol et al., 2006a). Loss of VLDLR, abundant in both photoreceptors and the RPE (Figure 4),

results in reduced FA levels in the retina, suggesting another important route for the lipid influx (Hu et al., 2008; Joyal et al., 2016). In addition, it was proposed that the influx of albumin-bound unesterified FAs can be to some extent driven by their spontaneous crossing of the cellular membrane followed by binding to the cellular FA transport proteins (FATP) and FA binding proteins (FABP), and subsequent quenching to a water-soluble acyl-CoA form (Hamilton and Brunaldi, 2007).

The systematic distribution of other lipid receptors is described in a single study from the Rodriguez group (Tserentsoodol et al., 2006a). Quantitative RT-PCR and immunohistochemistry analysis have shown that SCARB1 splice variant II and CD36 receptors are expressed on both the apical and basal side of RPE as well as on outer and inner segment of photoreceptors. In contrast, SCARB1 splice variant I is not expressed in the RPE and is only present in photoreceptor membranes (Figure 4). Other studies have shown that VLDLR is expressed highly in RPE cells (Hu et al., 2008); however, its exact distribution between apical and basal membranes was not investigated. Finally, the distribution of ApoE receptor 2 (APOER2), also known as LRP8, is confined to the limiting membrane area (Trotter et al., 2011), suggesting a role in communication with Müller Glia. These sparse but important data show the distribution differences between the membranes further underlying the high specification of RPE in lipid transport.

Interestingly, a dedicated uptake pathway involving the MFSD2A receptor seems to support the selective enrichment of DHA in the photoreceptor cells. MFSD2A, present in the RPE basolateral membrane, mediates the FA uptake primarily in the form of LPC, simultaneously contributing to the transcytosis suppression and the BRB permeability properties (Andreone et al., 2017; Nguyen et al., 2014; Wong et al., 2016). While DHA supplementation in the form of LPC effectively increases its retinal content (Sugasini et al., 2020), loss of Mfsd2a leads to the significant DHA depletion from the mouse retina. This depletion translates to a surprisingly modest retinal degeneration phenotype (Lobanova et al., 2019; Wong et al., 2016). Given the importance of DHA for photoreceptor health, it comes with no surprise that multiple delivery pathways coordinate its uptake to the neural retina. It is, however, intriguing that the retina depleted of nearly half of its original DHA content can retain relatively normal structural and functional characteristics, raising a question about the relation between the level of photoreceptor pathology and the retinal DHA content.

II.1.2   Lipid efflux from the RPE—The apical membrane of RPE mediates further lipid flux towards the neural retina. Thanks to its phagocytic capability, the same membrane mediates the daily clearance of photoreceptor OS tips concomitant with a significant lipid material ingestion by the RPE. Both processes, with opposite directionality, depend on a dedicated set of membrane receptors and transporters. While several proteins involved in the phagocytosis orchestration have been described in detail (see section II.3.4), the mechanisms by which the RPE apical membrane regulates lipid efflux towards the photoreceptors remain poorly understood.

The apical membrane contains ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1), which utilize ATP to flip various classes of lipids (cholesterol and phospholipids in particular) to the lipophilic acceptors located in the extracellular matrix (Figure 1)

(Ananth et al., 2014; Tserentsoodol et al., 2006a). RPE-specific depletion of both results in lipid accumulation in the form of lipid droplets in the RPE cells of young mice, coinciding with inflammatory response and retinal degeneration (Storti et al., 2019). Yet, the neural retinas of those animals do not present any major lipid deficiency, suggesting that certain redundancy and compensatory mechanisms exist at this level to ensure an adequate supply of lipids and essential FAs to the retina. Conversely, loss of either *AdipoR1* or *Mfrp*, two other transmembrane proteins abundant in the RPE apical membrane (Figure 1), leads to significant changes in the mouse retinal lipid profile (Kautzmann et al., 2020). These changes are reminiscent of the *Mfsd2a* KO phenotype (Lobanova et al., 2019), and characterized by significant depletion of multiple phospholipid species, most prominently containing DHA or VLC-PUFAs. Unlike *Mfsd2a* KO, however, *AdipoR1* or *Mfrp* deficient animals exhibit a gradual photoreceptor loss evident even at an early age (Rice et al., 2015; Won et al., 2008). While ADIPOR1 possesses an intrinsic ceramidase activity leading to the release of sphingosine and FFA (Holland et al., 2011), the role of MFRP in lipid metabolism or transport remains unresolved. Interestingly, ADIPOR1 deficiency has been recently proposed to affect the expression of *Elovl2* (Osada et al., 2021), a critical elongase contributing to the DHA synthesis (see Chapter II.2.1). Therefore, Osada and colleagues implicate that the primary cause of photoreceptor degeneration in *AdipoR1* KO mice might be related to DHA biosynthesis rather than its transport to the retina. Comparison of *Mfsd2a*, AdipoR1, and Mfrp KO phenotypes also suggests that a deeper look into the lipid compositional differences at single lipid species levels is needed to fully understand the role of DHA and VLC-PUFAs for retinal structural and functional homeostasis.

II.2 Lipid biosynthesis and metabolism *in situ*

In many ways, the RPE functions as a life support system for the outer retina, complementing the elevated metabolic requirements of both rods and cones. For example, a direct comparison between the retinal and RPE metabolite content showed elevated β-oxidation of FAs and sphingolipid biosynthesis intermediates in the RPE (Kanow et al., 2017; Sinha et al., 2020). Moreover, the expression of several essential enzymes involved in lipid biosynthesis and metabolism was confirmed in both RPE and photoreceptor cells (Figure 4), indicating the important role of lipid anabolism *in situ*, as will be further discussed in this chapter.

II.2.1 Free FAs—Photoreceptors exhibit a unique FA composition, being particularly enriched with DHA and other LC- and VLC-PUFAs. Early studies suggested that biosynthesis of DHA occurs predominantly in the liver, but it is also present in the retina and other parts of the CNS (Bazan et al., 2011; Scott and Bazan, 1989). In recent years more light has been shed on the presence, and consequently the role of PUFA biosynthesis enzymes in the retina (Figure 4). As mentioned earlier, all LC- and VLC-PUFAs can be biosynthesized in human body from their more abundant dietary precursors, like the α-Linolenic acid in case of the *n*-3 series, and part of the elongation reaction catalyzed by a family of ELOVL elongases constitutes the rate-limiting step in this process. ELOVL enzymes involved in PUFA elongation exhibit substrate selectivity: ELOVL5 elongates PUFAs 18–22 carbons in length, ELOVL2, 20–22 carbons, and ELOVL4 acts on VLC-PUFAs of 24 carbons or more (Deák et al., 2019). ELOVL5 exhibits high expression in

the liver and comparably low expression in the retina, where is it seemingly confined to the Müller cells. ELOVL2, as a critical enzyme for DHA synthesis, is highly expressed in the liver. In addition, it is expressed in the central nervous system, including retinal cones, and at low level in other cells (see chapter III). ELOVL4 is highly and specifically expressed in photoreceptors, mainly in the IS, and, in contrast to ELOVL2, is absent from the liver (Agbaga et al., 2008; Tikhonenko et al., 2010; Vasireddy et al., 2007). Thus, in great opposition to the DHA supply, biosynthesis of VLC-PUFAs by ELOVL4 occurs predominantly in the photoreceptor cells. ELOVL2 products of 24 carbons in length can serve a dual role as both DHA and VLC-PUFA precursors and, as such, are critical for the homeostasis of photoreceptors. In addition, it was shown that the RPE contains measurable amounts of the 22 carbon intermediate (22:5 n-3) that can be further converted into the DHA (Wang and Anderson, 1993) therefore confirming the presence of ELOVL2 in RPE. Accordingly, mutations in ELOVL4 and ELOVL2, the two most abundant elongases in the retina (Figure 4), affect the health of the photoreceptors. Loss of ELOVL4 significantly decreases the overall VLC-PUFA photoreceptor content, which likely contributes to the Stargardt-like macular dystrophy (STGD3) disease pathogenesis (see Table 1), as will be discussed later in this review (Bennett and Anderson, 2016). However, to this day, no genomic variants or mutations correlate with ocular phenotypes identified in *ELOVL2* (see more in chapter III).

To better understand the role of ELOVL2 in the eye, our group generated *Elovl2*[C234W] mutant mice lacking enzymatic ability to elongate DHA into its 24 carbon derivative (24:5 n-3), and showed that they exhibit accelerated vision decline as early as 6 months of age (Chen et al., 2020). In addition, we observed the development of sub-RPE deposits containing oxidized lipids and ApoE and altered composition of PUFAs in the retina, all of which are disease features reminiscent of the dry AMD. Intriguingly, our data also pointed to the potential role of ELOVL2 in the senescence induction. Furthermore, the analysis of *Elovl2*[C234W] transcriptomes from 4 months old animals (a stage before visual changes can be detected), showed downregulation of genes involved in oxidative phosphorylation (Figure 5A,B) and deregulation of genes engaged in oxidative-stress-induced senescence (Figure 5C). The analysis pointed out a potential connection between PUFAs and inflammation as retinas isolated from mutant mice had higher levels of inflammatory genes activated. This data agrees with studies performed on *Elovl2* KO animals (Talamonti et al., 2017), where authors noticed that macrophages isolated from the KO mice have a proinflammatory phenotype compared to healthy macrophages. Continuous inflammation can be the reason for several other phenotypes, including the presence of complement proteins in the sub-RPE deposits in mutant mice. Finally, we have recently obtained electron microscopy images of Bruch's membrane (BM) in 18 month-old wt and *Elovl2*[C234W] females. The data (Figure 5D) show several differences when compared to the WT tissues. First, the BM is distorted and thickened in the mutant animals. The collagen stripes are disturbed, and the elastin layer seems irregular. These phenotypes are accompanied by an accumulation of basal lamina deposits (BLamD) forming a layer on the BM and invading basal infoldings of RPE (Figure 5D, BLamD – red stars). Since the accumulation of BLamD can signify aging and stress (Ramrattan et al., 1994) we conclude that our *Elovl2*[C234W] animals express signs of

accelerated aging in the eye and as such the model may be considered to study an age component in age-related eye diseases including AMD.

II.2.2   Phospholipids—Phospholipids constitute a major fraction of the total retinal lipid content. Photoreceptors express components of both major pathways contributing to the diverse phospholipid composition: the *de novo* biosynthesis (Kennedy) pathway and the remodeling pathway (known as Land's cycle) (Figure 4). Biosynthesis first leads to the generation of a common precursor of all phospholipids, PA, through a multistep process involving glycerol-3-phosphate acyltransferase (GPAT) and 1-acylglycerol-3-phosphate acyltransferase (AGPAT, also known as lysophosphatidic acid acyltransferase: LPAAT) enzymes. AGPAT3, the most abundant AGPAT in both photoreceptors and RPE (Figure 4), is responsible for incorporating the majority of DHA into OS phospholipids. Mice devoid of *Agpat3* show a specific and almost complete lack of DHA and VLC-PUFA-containing phospholipids in the retina, leading to an abnormal morphology and organization of the OS disks (Shindou et al., 2017). Interestingly, the retinal degeneration and visual function decline associated with DHA and VLC-PUFA deficiency progress faster in those mice, compared to the previously mentioned *Mfsd2a* KO phenotype (Lobanova et al., 2019), suggesting a pivotal role of AGPAT3 in the retinal phospholipid supply. Such a conclusion, however, awaits further verification with the retina-specific genetic approaches as well as lipid supplementation studies. (Hishikawa et al., 2017; Nagata et al., 2021).

The FA composition of phospholipids can be further modified by the sequential action of PLA2 and lysophospholipid acyltransferase (LPLAT) families of enzymes, involved in the deacylation-reacylation cycles known as the Land's cycles (Figure 3B). LPCAT1, an LPC-specific LPLAT, was shown to facilitate the conversion of palmitoyl-LPC to dipalmitoyl-PC (DPPC) mainly distributed in the IS structures of the photoreceptor cells (Anderson et al., 2014). KO of *Lpcat1* leads to retinal degeneration (known as rd11 mouse model), confirming again the importance of *in situ* lipid biosynthesis in preserving photoreceptor integrity (Akagi et al., 2016; Friedman et al., 2010). Interestingly, an LRAT-related phospholipase A and acyltransferase 3 (PLAAT3, also known as HRASLS3 or AdPLA) (Golczak et al., 2015), occurs as the most abundant enzyme from the phospholipid metabolism pathway in the RPE layer (Figure 4). PLAAT3 shows both phospholipase A1 and A2 activity leading to the generation of LPC and LPE (Chatterjee et al., 2021), which suggests its important role in the lipolysis process, associated with both uptake and recycling of lipids, as will be described further in this review.

II.2.3   Sphingolipids—The first steps of sphingolipids *de novo* biosynthesis occur in the endoplasmic reticulum (ER) and involve condensation of serine and palmitoyl-CoA into a common precursor, dihydroceramide, through a sequential action of serine palmitoyltransferase (SPT; encoded by *SPTLC1–3*), 3-ketodihydrosphingosine reductase (KDSR), and ceramide synthases (CERS1–6) (Gault et al., 2010), all more abundant in RPE than rods or cones (Figure 4). Recently, TLCD3B, a novel ceramide synthase, has been discovered and associated with human recessive retinal dystrophy (Bertrand et al., 2021). SPT constitutes the rate-limiting enzyme in the process, while mutations in SPT lead to the accumulation of toxic atypical deoxysphingolipids (Table 1). Serine

deficiency, deoxysphingolipid accumulation, and SPT mutations were associated with macular telangiectasia, a rare macular disease that leads to loss of central vision (Chapter III), underscoring the importance of sphingolipid metabolism for photoreceptor health. Ceramides are further synthesized in the ER, but to begin sphingomyelin production, they must first be transported to the trans-Golgi region by the ceramide transferase (CERT1) or vesicular transport pathways (Hanada et al., 2009). In the trans-Golgi, ceramide can also be phosphorylated to C1P by CERK, an essential enzyme for photoreceptor health.

Apart from the *de novo* synthesis, ceramide generation can occur through the sphingomyelin hydrolysis or through the salvage pathway in which complex sphingolipids are converted back to sphingosine and ceramides. Catabolism of sphingomyelin and other complex sphingolipids involves the action of sphingomyelin phosphodiesterases (also known as sphingomyelinases; SMases). These enzymes differ in subcellular localization, optimal pH range, and cation dependence (Marchesini and Hannun, 2004). In contrast to the *de novo* pathway, the sphingomyelinase pathway can rapidly generate ceramides through a single type of enzyme upon activation by various stimuli, a fact which is essential for precise signal transduction (Chapter I). This prompt response is possible due to the abundance of sphingomyelin in the membrane (Canals et al., 2018). Mutations in *SMPD1*, the most abundant sphingomyelin phosphodiesterase (SMPD) in the photoreceptors and RPE (Figure 4), lead to human lysosomal storage disorder manifested by retinal stigmata, among many other symptoms, suggesting a direct link between photoreceptor health and the retinal sphingolipid hydrolysis capacity (Gault et al., 2010).

The salvage pathway of ceramide generation comes from the breakdown of complex sphingolipids, enzymatically degraded through non-SMase hydrolases such as cerebrosidases, galactosidases, and ceramidases in acidic compartments (late endosomes/ lysosomes). The resulting ceramide cannot be released from this compartment and must first be hydrolyzed by N-acylsphingosine amidohydrolase 1 (ASAH1), forming a free FA and sphingosine. The latter is then re-utilized in the ER to regenerate ceramides *via* CERS (Kitatani et al., 2008). The mouse model with deficient *Asah1* shows retinal dysplasia and optic nerve pathology accompanied by abnormal accumulation of ceramides and other sphingolipids in the retinal tissues, which is characteristic of Farber's disease (Table 1) (Yu et al., 2019). Exogenous ceramide can also be salvaged and converted to endogenous ceramide by the reverse activity of ceramidases (Novgorodov et al., 2011). C1P and S1P are generated through their phosphorylation by CERK or one of the two sphingosine kinases (SPHK), respectively (Hait et al., 2006; Wijesinghe et al., 2005). It was shown that adiponectin receptors (ADIPOR1 and ADIPOR2) possess high structural homology with intracellular alkaline ceramidase ACER3 (Vasiliauskaité-Brooks et al., 2018), and that adiponectin promotes ceramide degradation through its receptors (Holland et al., 2011). This observation was also confirmed by the Granier group, which demonstrated that both ADIPORs have ceramidase activity, which can be further stimulated through the binding of adiponectin (Vasiliauskaité-Brooks et al., 2017). Interestingly, ADIPOR1 mutations in humans are associated with syndromic and non-syndromic RP, and were also identified as a genetic risk factor for AMD in the Finish population (Kaarniranta et al., 2012; Xu et al., 2016; Zhang et al., 2016). Except for retina diseases mentioned above, impaired metabolism of sphingolipids, resulting in their dyshomeostasis, have been observed in AMD patients

with choroidal neovascularisation (CNV) and geographic atrophy, in patients with diabetic retinopathy, retinal ischemia and other pathologies (Fan et al., 2016; Shiwani et al., 2021; Wilmott et al., 2019).

II.3   Lipid cycling

II.3.1   Transport through the interphotoreceptor matrix (IPM)—Lipid transport
from the RPE to photoreceptor cells is by far the least understood part of the entire lipid cycle. Interphotoreceptor retinoid-binding protein (IRBP), a prevalent protein in the IPM initially recognized as a major retinoid carrier (Zeng et al., 2020), has been shown to efficiently bind various FFAs, including oleic acid and DHA (Chen et al., 1993; Ghosh et al., 2015; Semenova and Converse, 2003). In native conditions, IRBP associates with several FA chains and possesses two retinoid-binding sites, of which one has been shown to release 11-*cis*-retinal specifically upon DHA binding (Chen et al., 1996). IRBP undergoes rapid turnover in the IPM involving endocytosis into the RPE and photoreceptor IS, suggesting one intake route for the bound cargo (Cunningham and Gonzalez-Fernandez, 2003). The presence of albumin, the common FFA carrier of the blood, has also been postulated in the IPM (Adler and Edwards, 2000; Duncan et al., 2006). Unlike the RPE, both rods and cones lack MFSD2A while at the same time expressing ADIPOR1 at the highest levels in the retina (Figure 1). ADIPOR1 promotes DHA uptake and biosynthesis of VLC-PUFAs in the IS, mostly localizing to the OS structures of photoreceptor cells (Rice et al., 2015; Sluch et al., 2018). Proteins associated with lipoprotein particle formation and transport have also been identified in the subretinal space. Apolipoprotein A1 (APOA1), the major HDL constituent, is present in the apical portion of the RPE cells, rod IS, and the IPM (Tserentsoodol et al., 2006a). Its major transporter, ABCA1, is found in both the RPE apical membrane and photoreceptor IS membranes, suggesting an active HDL-mediated transport through the IPM (Ananth et al., 2014; Tserentsoodol et al., 2006a).

II.3.2   Lipid trafficking in photoreceptor cells—Upon delivery to photoreceptors,
FFAs preferentially accumulate in the IS, and their uptake is coupled with subsequent esterification into phospholipids (Rodriguez De Turco et al., 2009). Biosynthesis of phospholipids occurs at a high rate in the ER and Golgi system of the IS, and photoreceptor-expressed FABPs (FABP7 (Su et al., 2016), FABP12 (Liu et al., 2008) likely assist in channeling FFAs towards these sites. OS disk formation, which consumes a significant portion of the newly synthesized phospholipids, takes place in the base of the OS structure, separated from the IS by a narrow connecting cilium (CC). Vesicles transport lipids, as well as newly synthesized OS membrane proteins, across the IS to the periciliary ridge complex at the base of the CC structure (Besharse and Pfenninger, 1980; Papermaster et al., 1985). Both conventional and unconventional (*trans*-Golgi omitting) secretory pathways seem to be utilized in this process (Imanishi, 2019). Since OS proteins may directly interact with lipids, (a prominent example being rhodopsin's interaction with cholesterol and DHA (Albert et al., 1996; Grossfield et al., 2006; Soubias and Gawrisch, 2005)), it is plausible to speculate that just as vesicles provide means of transmembrane protein transport to OS, the proteins themselves may provide means of certain lipid trafficking. However, it was shown that some aspects of protein and lipid transport act independently of each other, as was the case for cholesterol precursors (Fliesler and Keller, 1997). The CC creates a barrier against

membrane protein and lipid diffusion between the IS and OS compartments (Nachury et al., 2010). Most rhodopsin molecules cross the CC *via* the ciliary plasma membrane, suggesting the same fate for at least a portion of the accompanying vesicular phospholipids (Burgoyne et al., 2015; Wolfrum and Schmitt, 2000). Nonetheless, vesicular structures have been observed within the CC lumen, implicating the existence of alternative protein and lipid transport modalities through the CC (Chuang et al., 2015; Gilliam et al., 2012). As revealed recently, nascent OS disks are formed through evaginations of the plasma membrane at the neck of OS structure, and this region is devoid of any vesicles (Spencer et al., 2020). Overall, it strongly suggests that most of the OS-destined lipid (and protein) transport occurs *via* the ciliary plasma membrane, continuous with the OS plasma membrane. Since the OS disk membrane differs in terms of both lipid and protein composition from the plasma membrane surrounding the structure (see Chapter I) it is evident that another sorting mechanism for both classes of molecules must be in place at the site of new disk formation (Nemet et al., 2014). Molecular details of this process, however, remain unknown.

II.3.3 Outer segment lipid dynamics—It takes approximately 10 days for the newly synthesized OS disk in the mammalian eye to reach the apical side of the OS structure, where it ultimately becomes detached and subjected to the RPE-mediated phagocytosis process (Young, 1967; Young and Bok, 1969). As a disk gradually moves along the OS longitudinal axis, its membrane proteins remain stably associated with that particular disk, without any measurable turnover (Hall et al., 1969). In stark contrast, using biochemical and autoradiography assays, disk lipids were shown to freely diffuse to other disks and the plasma membrane of the OS structure (Bibb and Young, 1974a, 1974b). Different rates of OS incorporation and turnover were observed for the individual phospholipid classes, as well as fatty acyl chains (Gordon and Bazan, 1990; Louie et al., 1988). Yet, the disk's membrane lipid composition undergoes significant changes as it moves from the basal to the apical side of the OS structure, most notably a decrease in cholesterol and increase in the unsaturated fatty acyl chain content (Chapter I). The mechanisms underlying this process are not known and may involve properties of certain proteins present in the OS to bind and translocate lipids between membranes, as well as lipid-lipid interactions. Significant differences in phospholipid headgroup composition between the OS disk and plasma membranes were suggested to favor gradual cholesterol transfer to the latter (Albert et al., 2016). In addition, cholesterol inherently segregates away from the unsaturated lipid acyl chains, DHA in particular, supporting the formation of their respective opposing gradients in the OS structure (Wassall and Stillwell, 2009). As discussed earlier in this review, the role of such gradients is not fully understood. However, since the lipid composition of a particular disk is directly correlated with its age (as opposed to the relatively stable protein composition), it could potentially serve a role in initiating the old disk clearance at the tip of the OS structure. Multiple synergizing factors make the OS membranes especially prone to lipid oxidation (Organisciak and Vaughan, 2010), as also discussed in the context of aging further in this review (Chapter III). Those factors include the exceptionally high PUFA content, light exposure, high metabolic rate of the photoreceptor cells, high overall oxygen tension in the outer retina, and possible lipofuscin accumulation in the RPE with age. In fact, similar reasons help to explain why OS structures are subject to such an intensive renewal process. While lipid oxidation products accumulate over time, it is yet to be determined what

their distribution is across the OS structure and whether the already described lipid gradients affect their generation process.

II.3.4 Phagocytosis—Every day the distal portion of the photoreceptor OS, equal to around 7–10% of the entire structure, is removed by the RPE cells through an evolutionarily conserved, multi-step process known as phagocytosis. The process has been the subject of several excellent recent reviews (Kwon and Freeman, 2020; Lakkaraju et al., 2020). PS externalized to the outer leaflet of the OS tip plasma membrane constitutes the earliest known hallmark of the phagocytosis initiation (Ruggiero et al., 2012). Asymmetric PS distribution to the inner leaflet in virtually all healthy cell membranes is tightly maintained by cellular flippases and only lost upon the apoptosis induction. Consequently, localized conditions resembling apoptosis initiation are suspected to occur diurnally at the OS tip, though the molecular details of this process remain unknown. It is further speculated that some form of diffusion barrier needs to be present to prevent the expansion of the externalized PS signal beyond the photoreceptor tip. Once externalized, PS is bound by a number of proteins secreted to the IPM, including growth arrest-specific protein 6 (GAS6), vitamin K-dependent protein S (PROS), and MFGE8. These in turn function as ligands for the two major receptors present in the RPE apical membrane known for their involvement in the phagocytosis initiation: Mer tyrosine kinase (MERTK) and αvβ5 integrin. While roles of MERTK-GAS6/PROS and αvβ5 integrin-MFG-E8 complexes with PS are well established in the context of RPE phagocytosis (Kwon and Freeman, 2020; Lakkaraju et al., 2020), additional pathways involving protein-lipid interaction likely play a supporting role in the orchestration of the process. Lipid scavenger receptor CD36, involved in the oxidized LDL cleanup from the subretinal space (Picard et al., 2010), was shown to specifically bind several oxidized PC and PS species present in the OS, and this binding was suggested to direct the engulfment of the photoreceptor tips by the RPE apical processes (Greenberg et al., 2006; Sun et al., 2006). Although intensely studied, the full mechanism of phagocytosis, the regulation of its onset and decline, as well as precise timing of ingestion *vs.* maturation of phagosomes have to be reevaluated using contemporary technologies.

II.3.5. Lipid recycling and repurposing in RPE—OS phagosomes formed at the apical membrane of the RPE undergo maturation as they gradually shift towards the basal side of the cell. The process involves fusion with endosomes and lysosomes aimed primarily at fast degradation of the large quantities of the OS material ingested every day. Each RPE cell contacts many photoreceptors, up to 30 in humans and 200 in mice (Gao and Hollyfield, 1992; Volland et al., 2015). Ultimate resolution of the phagolysosomes must occur at the end of each daily cycle to prevent the accumulation of cell debris while simultaneously allowing the recycling and repurposing of many nutrients. The RPE expresses several lysosomal lipid hydrolases at significantly higher levels than rods or cones, reflecting their important role in RPE biology. This includes pH-dependent phospholipases A1 (PLA1) and PLA2, which aid in the release of FFAs from phospho- and glycolipids (Swartz and Mitchell, 1973). The lysosomal acid lipase type A (LIPA) de-esterifies the cholesterol molecules. Breakdown of the ingested lipids constitutes an important step in phagosome maturation, and its inhibition results in both lipid and rhodopsin accumulation in the RPE. This observation, originating from the *Pnpla2* conditional knock-out in the RPE (Bullock et al., 2021), suggests that

lipase-mediated OS lipid breakdown is required for efficient hydrolysis of the accompanying OS proteins. The abundance of the released FAs comparable to the levels metabolized daily by hepatocytes creates a challenge, but also an opportunity for the postmitotic RPE layer. Similarly, like the liver, RPE expresses enzymes involved in energy production through the saturated and unsaturated FAO and ketogenesis pathways (Reyes-Reveles et al., 2017). At the same time, the presence of the high levels of major glucose transporter GLUT1 in both basal and apical membranes of the RPE combined with the known suppressive role of lactate in the RPE was suggested to support the notion of direct transfer of glucose from the choroid vasculature to photoreceptors instead of its catabolism by RPE cells (Kanow et al., 2017; Swarup et al., 2019). While the extent of glucose utilization by the RPE requires further investigation, the RPE is believed to use FAO to meet a significant portion of its energy requirements (Fu et al., 2021). However, the retina is known to preserve DHA content even upon prolonged n-3 FA deprivation, which early on suggested that DHA and likely other lipids are in part directly recycled back to the photoreceptor cells for non-energy purposes (Bazan et al., 1992; Chen and Anderson, 1993; Tinoco et al., 1977). Further studies utilizing biochemical and autoradiography assays showed that DHA ingested by the RPE upon phagocytosis is transiently converted into TAGs and quickly recycled back to the photoreceptor cells (Gordon et al., 1992; Rodriguez De Turco et al., 1999; Stinson et al., 1991). The process is selective towards the DHA over other FA species, however, it is unclear how this selectivity is obtained and which exactly proteins are involved in its orchestration.

Chapter III. Lipids in retinal aging and disease

As mentioned above (Chapter I), lipid composition affects membrane structure and properties, such as viscosity. Since viscosity is an essential factor in determining the rate of membrane-related reactions including cell signaling, cell adhesion, and enzyme binding/ activity, it plays a crucial role in aging processes. Both headgroup and tail chemistry can significantly modulate lipid diffusion (Seu et al., 2006). Therefore, it is not surprising that the the role of lipids, lipid composition and lipid metabolism in aging is under active investigation in several laboratories.

III.1. Lipids in aging

Aging is a complex, multifactorial process characterized by a progressive deterioration of tissue structure and function. Aging changes are driven by intrinsic (genetic) and extrinsic (environmental) factors. Because aging is the single greatest risk factor for the development of age-related eye diseases such as AMD and glaucoma, understanding the molecular mechanisms of aging presents an opportunity to find new therapeutic approaches in biomedical science. Changes in visual capability in later adulthood impacts patients' ability to perform common everyday visual tasks, thereby influencing a patient's quality of life and well-being. Several studies have focused on analyzing age-related changes in the lipid composition of human postmortem retinas and in mice.

Lipids play an important role in the mechanism of aging in the central nervous system (Layé et al., 2018; Singh et al., 2019; Wang and Michaelis, 2010). Multiple groups have measured

changes in various lipid levels to assess their association with healthy aging, longevity, and disease. Recent development of high-tech methods for detection and quantification of lipids has accelerated the development of the field. However, much is still to be done to parse lipids' causative *vs.* correlative roles in the aging process.

The change in membrane lipid composition with age has a number of characteristics. First, there is usually an increase of "rigid" lipids (cholesterol, sphingolipids, and degree of saturation in the FAs acyl chain). Second, several changes in lipid composition observed in one tissue can be translated to other tissues. For example, increases in cholesterol in plasma and decreases of PUFAs in plasma have been correlated to changes in cholesterol levels in the brain and eye. Lastly, it seems that changes in membrane fluidity materialize quite late compared to other cellular functions, which suggests that membrane function is tightly maintained and presents a metabolic challenge to the cell during the aging process (Skowronska-Krawczyk and Budin, 2020).

Photoreceptors—The photoreceptor OS is built from stacks of membranous discs with specific lipid composition (discussed in Chapter I), which fluctuates with age as reported by multiple laboratories. For example, changes in FA composition in the eye were quantified by several groups through the years (Bretillon et al., 2008; Liu et al., 2010; Prokopiou et al., 2019). Very little to no age-related change was observed in FAs such as 16:0, 18:0, 18:1, 18:2, 18:3, 20:4, 22:4, 22:5 and 22:6. The level of DHA is particularly well-maintained in the aging retina (Liu et al., 2010). Intriguingly, levels of VLC-PUFAs (>26C) were consistently lower in aged donors and severely reduced in AMD donor eyes. These changes were consistently accompanied by an altered ratio of n-3/n-6 PUFAs (Liu et al., 2010), with higher n-6 FAs correlating with decreased visual function.

Other changes have also been observed in aged retinas. For example, an age-dependent increase in membrane micro viscosity in rod OS has been reported, suggesting a decrease in membrane fluidity during aging (Ohia et al., 1994). This brings an important question, whether specific membrane composition changes affect the primary function of photoreceptors – the visual cycle. With our recent studies pointing to the specific lipidomic environments for different OS proteins (Sander et al., 2021), this question becomes even tractable experimentally.

Aging of the human retina is accompanied by elevated ROS levels and reduced antioxidant capacity (Ohia et al., 1994) adding to the already high oxygen tension in the outer retina, caused by low oxygen extraction for the venous blood leaving the eye. ROS are mainly produced in mitochondria during normal oxygen metabolism and signaling molecules through the redox pathway (Veal et al., 2007). However, excess ROS causes oxidative damage to lipids, proteins, and DNA, resulting in cell dysfunction and apoptosis. Highly abundant PUFAs in the retina are the primary targets of free radical-induced lipid peroxidation, since the susceptibility of unsaturated FAs to oxidation increases with the number of double bonds. Lipid peroxidation may affect the retina in several ways. Anderson's group observed the loss of PUFAs and the accumulation of lipid hydroperoxides in rod OS isolated from albino rats exposed to constant illumination (Wiegand et al., 1983). Therefore, they proposed that lipid peroxidation was an important factor in light-induced

retinal degeneration (Anderson et al., 1985, 1984; Wiegand et al., 1983). Palczewski group (Maeda et al., 2006) showed that retinol dehydrogenase 12 (RDH12) might play a role in the detoxification of lipid peroxidation products (Belyaeva et al., 2005; Lee et al., 2008). They concluded that RDH12 could protect photoreceptors from light-induced degeneration in mice because the $Rdh12^{-/-}$ mice showed increased sensitivity to light-induced photoreceptor apoptosis. Another group (Nag et al., 2019) detected changes in aged vessels, including degeneration of endothelial cells and pericytes, accumulations of lipofuscin granules, loss of filaments in pericytes, and thickening of the capillary endothelial and pericyte basal lamina. Immunohistochemistry for a biomarker of oxidative stress (4-hydroxy 2-nonenal [4-HNE]) was significantly localized in aged capillaries and arteries, suggesting that in the aged retina, elevated lipid peroxidation may damage vessels, followed by limitation of the energy supply to the neurons and eventually leading to neuronal loss in the inner retina. Oxidative damage of PUFAs generates different products, including malondialdehyde (MDA) and 4-HNE are observed in aged retinas but at increased levels in AMD (Castorina et al., 1992; Dhingra et al., 2018). Peroxidation of DHA-containing lipids generates carboxyethylpyrrole (CEP) easily detectable in AMD retina and in AMD patients plasma (Crabb et al., 2002; Gu et al., 2003). Noteworthy, immunization with a hapten generated by oxidative damage to the DHA present in the drusen is sufficient to produce AMD-like lesions in mouse retina, providing the most robust evidence for the role of these molecules in AMD genesis (Hollyfield et al., 2008). Other metabolites of LC-PUFA peroxidation present in the retina, such as isoprostanes and neuroprostanes, derived from AA and DHA, could contribute to retinal microvascular degeneration by directly inducing the death of retinovascular endothelial cells (Hardy et al., 2005). Finally, lipid peroxidation can also affect phagocytosis. Moderate oxidation induced-phototoxicity in ARPE-19 cells decreases their phagocytic activity (Pawlak et al., 2019). Similarly, in older retinas, increased lipid peroxidation is accompanied by a moderate decrease in the phagocytic activity of RPE cells (Inana et al., 2018).

Not surprisingly, the delivery of antioxidants is regarded as a promising approach to protecting the retina from light damage, aging, and AMD. Early studies of the Tso group have shown that several types of antioxidative molecules can accelerate the retina after photic injury recovery (Lam et al., 1990; Ll et al., 1993), while others have shown an increased lipid peroxidation upon lack of antioxidants, such as vitamin C or E and their protective role in response to retinal light damage (Li et al., 1985; Noell et al., 1987; Wiegand et al., 1986). These and other results precipitated the interest in using antioxidants to protect against age-related eye diseases such as AMD and were at the basis of formulation of the supplement used in Age-Related Eye Disease clinical Study (AREDS). AREDS was a randomized controlled trial set to evaluate the effects of high-dose zinc and antioxidants on the progression of AMD and vision loss. The study found that the proposed combination reduced the risk by 25% in those at high risk of developing advanced AMD and reduced the risk of moderate vision loss by 19%. No benefit was observed in patients with no AMD or early AMD after supplementation (Chew, 2013; Evans, 2008). Further studies have shown that supplementation with natural antioxidants, such as lutein and zeaxanthin (both used in AREDS supplement), can improve macular pigment and visual function in patients with

early AMD due to their ability to scavenge free radicals, absorb damaging blue light, and neutralize photosensitizers and ROS (Ma et al., 2012; Widomska, 2014).

Current studies are focused on finding new and more specific antioxidants. Recently, mitochondria-targeted antioxidant SkQ1 has proved effective in protecting rabbit retinas from light-induced oxidative stress (Baksheeva et al., 2019). Other efforts are focused on using deuterated DHA, where deuterium protects unsaturated bonds from oxidation, and only 20–25% deuteration prevents lipid peroxidation (Shchepinov, 2020). Several recent studies seem to confirm this hypothesis, for example, in RPE cell culture settings (Rosell et al., 2019) but it has not yet in animal models of retinal diseases. If successful, supplementation with deuterated DHA could provide an easy way to protect the retina from oxidative stress, and could be conveniently added to any supplement containing PUFAs and other antioxidants.

RPE aging—Age-related changes in the retina are concurrent with the aging of RPE. Aging RPE cells have been shown to change morphology accompanied by early documented cell loss in the fovea at the rate of approximately 0.3% per year(Panda-Jonas et al., 1996) and may increase the metabolic demand on the remaining cells. In addition, RPE endures significant aging-related changes such as the increase in senescence (as detected by senescence-associated b-galactosidase staining), telomere loss, DNA damage, and protein and lipids peroxidation (Cao et al., 2013; Matsunaga et al., 1999). These phagocytic cells have very long and thin microvilli on their apical surface intertwined with the tips of the rod and cone photoreceptor OS (Bosch et al., 1993). With aging, microvilli length is shorter (Bonilha et al., 2006), and the interaction with the photoreceptor OS is less interwoven (Katz and Robison, 1984; Lai and Rana, 1986). This observation is consistent with the hypothesis of change of lipids composition of RPE membrane in aging, but there is no data yet to suggest the potential age-related mechanism that could play a role in this process.

RPE cells are also under constant oxidative stress due to their unusually high levels of oxygen and exposure to light(Algvere et al., 2006; Bazan, 2008; Cai et al., 2000). In addition, the daily recycling of membranes that contain oxidized lipids and proteins as well as high exposure to toxic retinoids provides constant metabolic pressure and involves continuous activity of machinery controlling the stress levels (recently reviewed in (George et al., 2021)). The role of oxidative stress has been further shown in multiple studies with cultured primary or immortalized RPE cells and in many statistical studies correlating the cigarette smoking, i.e., oxidative environment, with RPE dysfunction (e.g. (Bertram et al., 2009; Espinosa-Heidmann et al., 2006) and more recent (Cano et al., 2021; L. Wang et al., 2020). Interestingly, smoke exposure in mice and cultured RPE cells has also been shown to induce lipid accumulation, as shown by the Roher group using several lipid staining procedures (Kunchithapautham et al., 2014). Most importantly, the use of antioxidants protect against the RPE cell death (e.g. (Cai et al., 2000; Kamoshita et al., 2016; Yu et al., 2012), however, whether these treatments are lowering the levels of oxidized lipids in the human retina still needs to be evaluated.

One of the most common age-related phenotypes of RPE is the accumulation of lipofuscin, also called "aging pigment", within lysosomes and melanolysosomes in the basal side of the

cell, accumulating in aged individuals (Feeney-Burns et al., 1984; Feeney, 1978). Specific distribution and number of specific granules have been recently observed by Bermond et al., suggesting different mechanisms of build-up between the fovea region and perifovea (Bermond et al., 2020). Although the exact composition of lipofuscin is not yet resolved, it is well documented that lipids, including peroxidized lipids, lipid-binding proteins, and toxic retinoids, are contained in these structures; however, the ratio of different components may differ depending on the region of the eye (Panda-Jonas et al., 1996). This accumulation of oxidative species in lipofuscine provides continuous low level of oxidative stress in the cell. Lysosomes, organelles extremely active in RPE, when filled with lipofuscin, are less effective in processing photoreceptor OS (Feeney-Burns et al., 1987; Shamsi and Boulton, 2001), delaying the hydrolysis of phospholipids (Finnemann et al., 2002), therefore disturbing the balanced lipid-based interaction between photoreceptors and RPE.

Lipofuscin is also abundant in extracellular deposits below RPE and in Bruch's membrane, which accumulation with age may be involved in drusen formation (Marshall et al., 1998). Additionally, age-dependent accumulation of lipids deposits has been observed underneath the RPE. These sub-RPE deposits also called basal lamina deposits (BLamD) (Curcio, 2018), increase with the progression of normal aging and are associated with an increased risk of developing AMD. Drusen are focal deposits located below RPE basal lamina. Accumulation of drusen deposits is aggravated in AMD patients where the phenotype is accompanied by thickening of the Bruch's membrane (Curcio et al., 2011; Li et al., 2005), activation of inflammatory events, and damage of the tissue. Dedicated studies were performed to describe and define the different types of deposits and correlate them with aging and progression of AMD (reviewed in (Curcio, 2018)). The presence and identity of lipids in drusen have been studied for many years. EM studies have detected different types of droplets and lipoproteins aggregates under the RPE. Although it is understood that part of the material in drusen consists of phagocytosed photoreceptor OS, the presence of lipid-like droplets in the exact location has been at the origin of a diet-based hypothesis of drusen (reviewed in (Bergen et al., 2019) and (Curcio, 2018)). While the accumulation of lipid droplet-like deposits in LDL-R KO mice seem to support the diet-origin of deposits in BM (Schmidt-Erfurth et al., 2008), detection of products of DHA oxidation, such as carboxyethylpyrroles (CEPs) and 4-Hydroxy-7-oxo-5-heptenoic acid (HOHA) lactone (Linetsky et al., 2020; Salomon et al., 2011) is congruent with the idea of the phagocytic debris origin of drusen (Bergen et al., 2019). Of note, the above-cited studies with antibody-based detection of CEP were only possible thanks to the development of an antibody specific for the molecule. The availability of such antibodies is scarce but highly desired in the field focused on understanding lipids' role on the cellular and sub-cellular levels. Finally, the composition of lipoproteins in the deposits also supports the phagocytic origin of lipids. Therefore, it is highly probable that both mechanisms add to the loss of functionality of BM and RPE in age. For example, due to the presence of RPE-derived deposits blocking the delivery of lipids from the circulation, local lipids and lipid-transporter proteins start to accumulate and form lipid-like droplets, adding to the local traffic. With time drusen undergo calcification and show accumulation of hydroxyapatite (Tan et al., 2018) which suggests the contribution of the vitamins and minerals exchange in the process of drusen aging.

One of the major consequences of accumulating deposits in the BM is a blockage of molecules exchange between circulation (choroidal vessels) and the retina. Multiple macromolecules are trafficking from circulation, and this process directly depends on the rate of diffusion of BM called hydraulic conductivity (HC). Studies measuring this process started already in the late 1970s, have established that HC of this compartment declines exponentially with age (Hussain et al., 2010; Starita et al., 1995; STARITA et al., 1996). Very early, it has been suggested that the age-related changes in BM could be responsible for this decrease (Starita et al., 1997). Aquaporin 1 expression has also been shown in the regions containing drusen, most probably to help the fluid transport. Unfortunately, studies involving measurements of HC ceased with time, leaving many questions unanswered. For example, it would be exciting to understand what components of deposits have the highest contribution to the age-related HC decrease. Additionally, population studies could address questions whether this biophysical property of BM is dependent on diet. Finally, it would be interesting to figure out whether any treatments could help increase HC in aging. As an example, Marshall's group showed that treatment with steroidal glycoside could slightly improve the diffusion rate in BM *in vitro* (Lee et al., 2015). Notably, lipid deposits in AMD precede disease symptoms; understanding these features is one of the current goals in eye research to better detect the disease and provide early treatment to patients.

Molecular mechanism—Very little is known about the molecular mechanism of age-related changes in lipid synthesis or metabolism regulation. Two recent studies shed light on potential mechanisms involved in lipid-dependent aging phenotype. It is well established that DNA methylation patterns progressively change during aging (Noroozi et al., 2021; Raj and Horvath, 2020). Within the top 10 markers of aging, four are localized in the regulatory element of *ELOVL2* gene (Hannum et al., 2013). Consequently, methylation of the *ELOVL2* regulatory region has been shown in many studies to correlate with the biological age of individuals (Hao et al., 2021; Sukawutthiya et al., 2021). The correlation has also been confirmed for rodents (Wang et al., 2017). DNA methylation impacts the properties of DNA and therefore affects the chromatin structure and function, implying the changes in gene expression (Campello et al., 2021; Telese et al., 2013). In WT mice, we demonstrated that the expression of *Elovl2* gradually declines in the aging retina, concomitant with reduced visual function (Chen et al., 2020). Taken together and with the data generated using the mouse lacking the ELOVL2 enzymatic activity (see chapter II) our work provides a potential direct genetic link between aging and age-related vision loss.

In another study, Swaroop and colleagues investigated epigenetic changes in aging photoreceptors (Corso-Díaz et al., 2020). The group also focused their efforts on the age-related pattern of DNA methylation, specifically in rods. After correlating differentially methylated regions with gene expression in aging rods, they noted a pattern of changes in genes associated with metabolism and mitochondrial oxidative respiration. Their results suggest that the changes in mitochondrial respiration are related to increased reliance on lipid beta-oxidation with age, therefore showing age-related changes in lipid homeostasis. Interestingly, mitochondrial membrane composition may affect mitochondrial function (Budin et al., 2018). Further studies should follow to understand the molecular basis of these changes, including the signals that promote this change in mitochondria metabolism.

Of note, the aforementioned Corso-Diaz et al. studies did not detect changes in Elovl2 expression most probably because of its low level of expression in rods (Figure 4) (Weir et al., 2021; Yan et al., 2020).

Overwhelming evidence of the role of lipids in retina aging and several variants or mutations in proteins involved in lipid metabolism (Table 1) brought the attention of researchers to the lipid transporters and lipid-binding proteins. Apolipoproteins, which are major components of lipids transport particles, are at the center of studies related to aging and age-related eye diseases. ApoE is a cholesterol particle transporting protein, expressed predominantly in RPE. Human ApoE protein exists in three variants, namely ApoE2, ApoE3, and ApoE4. Single amino-acid differences between these forms change their affinity to the LDL receptor as well as the ability to form HDL particles. In particular, ApoE2 protein has a low affinity to the LDLR receptors, therefore, it is not efficient in delivering cargo (mostly HDL) through this pathway. This isoform, however, still maintains the ability to bind to other lipid receptors. Population studies show that ApoE2 form correlates with longevity as higher frequencies of APOE2 in elderly individuals and centenarians were detected compared to younger populations, whereas the frequency of APOE4 is lower in the oldest individuals (Sebastiani et al., 2019). This particular correlation may be one of the key factors causing higher correlation of this variant with the risk of AMD (see III.2). Studies dividing the AMD patients into age groups to correlate ApoE2 variant frequency in each group are yet to be performed.

III.1.2. Bioactive lipids in aging

PUFAs and their metabolites, such as eicosanoids, docosanoids and elovanoids (as mentioned in chapter II) are the most common BLs. They are not only components of cellular membranes but also act as signaling molecules (Elmasry et al., 2019) . BLs are involved in several cellular functions and biological processes that are altered in aging. There is usually a decrease in the formation of PUFAs in age-related disorders (Liu et al., 2010), accompanied by an increase in the production of pro-inflammatory PGE_2, thromboxanes, and LTs, and a decrease in anti-inflammatory compounds including lipoxins, resolvins, protectins, and maresins (Beauchamp et al., 2001; Behl et al., 2016; Das, 2020; Schoenberger et al., 2012). The imbalance in the concentrations of pro- and anti-inflammatory BLs may result in enhanced local inflammation in aging and increase the risk of aging-associated diseases (Das, 1995; Gundala et al., 2017; Urli et al., 2020). For example, to understand the role of inflammation in aging, Steinle et al. compared the levels of inflammatory markers, including inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE2), and TNFα, in the retina and choroid in the young and old rat model. The results showed that PGE_2 (pro-inflammatory metabolites of AA) significantly increased in the aging retinas and choroids (Steinle et al., 2009). In other studies, qPCR analysis of lipoxin A4 (LXA4, anti-inflammatory metabolite of AA) biosynthetic enzyme and LXA4 receptor showed that LXA4 biosynthesis and signaling are significantly decreased in the end-stage of Retinal degeneration (RD) in RD1 mice model. Further study showed that LXA4 treated retina maintained the visual function of the mice through reducing photoreceptor apoptosis and inhibiting microglial overactivation (Lu et al., 2019). The above results indicate that metabolites of PUFAs are likely involved in the aging process in the retina and choroid.

It has been suggested that with advancing age, pro-inflammatory cytokines, such as TNFα and IL-6, block the activities of desaturases and affect the formation of PUFAs and their metabolites, leading to an imbalance of pro- and anti-inflammatory BLs (Mayer et al., 2002). Das summarized the potential relationship among AA and its metabolites with age (Das, 2020), indicating there is decreased concentration of AA and LXA4 accompanied by decreased activity of desaturases, and increase of the pro-inflammatory PGE_2, leukotriene B4 (LTB4), TNFα, and IL-6 in aging. In contrast, increased levels of DHA in the retina favor the production of docosanoids, neuroprotective molecules that leads to induction of reparative phenotype in macroglia, therefore helping the rescue of photoreceptors (Ebert et al., 2009). The failure to maintain the homeostasis of these molecules contributes to an increase of inflammatory events in aging and expansion of age-related diseases. This also suggests, that potential manipulations of the levels of PUFAs in the retina can still became a therapeuting options in some diseases.

III.1.4. Measuring lipids in the plasma – lipids supplementation

There is significant interest in studying the lipid composition of plasma isolated from patients as a marker of retinal health. There are several advantages to this approach: (i). Measuring lipid content can be done multiple times on the same model animal or patient; (ii) Access to the material is straightforward; (iii) The abundance of material helps in evaluating levels of several lipids; (iv) Longitudinal studies are more feasible; (v) The feasibility of measuring the impact of a given intervention on the lipid levels in the retina.

Multiple epidemiological studies have suggested that diets rich in n-3 LC-PUFAs are associated with lower rates of AMD. Low dietary intake of n-3 LC-PUFAs has conversely been associated with a higher risk of developing AMD (for example (Chong et al., 2008; Bénédicte M J Merle et al., 2015; van Leeuwen et al., 2018)). In addition, two extensive studies demonstrated that high plasma levels of n-3 LC-PUFAs were correlated with decreased risk of AMD (Merle et al., 2014, 2013). Interestingly, in the Age-Related Eye Disease Study (AREDS), a large prospective study investigating factors of progression to advanced AMD, subjects with the highest self-reported intake of foods rich in n-3 LC-PUFAS were 30% less likely to develop central GA and 50% less likely to develop AMD than subjects with the lowest self-reported intake (AREDS Research Group, 2009). Later, the impact of more defined n-3 LC-PUFA supplementation was investigated by two large prospective studies. The AREDS2 study and the nutritional AMD treatment study (NAT-2) examined the effect of n-3 PUFA supplementation to prevent progression to advanced AMD or wet AMD (Benedicte M J Merle et al., 2015; Souied et al., 2016). Surprisingly, neither study showed a significant difference between oral supplementation of PUFAs and the placebo in progression to wet AMD (Chew et al., 2012; Souied et al., 2013), suggesting that other FAs or other molecules present in foods rich in n-3 LC-PUFAs may have a preventive role in AMD progression. Similarly, Similarly in the openlabel study with (n-3) PUFA supplementation of 11 STDG3 patients, no significant beneficial effect has been observed; however, this study was limited by poor compliance with supplementation (Choi et al., 2018). Of note, recent studies showing the positive effect of oral supplementation of VLC-PUFA in animal models of STDG3 bring new hope in this line of pharmacological approaches(Gorusupudi et al., 2021). Future molecular studies focused on the specific roles

of enzymes involved in lipid metabolism should help understand these clinical findings' results.

Among all the lipids, cholesterol is gaining more attention since studies showed that failure to maintain cholesterol homeostasis is associated with many retina diseases, such as Bietti Crystalline Dystrophy, Bassen-Kornzweig syndrome (abetalipoproteinemia), Smith-Lemli-Opitz syndrome, and AMD (Table 1). Fluorescently-labeled lipoprotein particles injection indicated that the circulating cholesterol could cross the RPE and reach the retina with LDL as the major carrier (Tserentsoodol et al., 2006b). With aging, the clean-up of deposited lipids becomes less efficient, and the accumulation of cholesterol in Bruch's membrane is reported to be the primary reason for AMD (Curcio et al., 2001). A population-based study containing 963 elderly individuals showed that higher plasma high-density lipoprotein cholesterol was significantly associated with an increased risk of AMD (Cougnard-Grégoire et al., 2014). Accumulated oxidized cholesterol, such as 7-ketocholesterol, may also probably relate to the pathogenesis of AMD (Rodríguez and Larrayoz, 2010).

Lipids in body fluids are also assessed as a potential biomarker for aging and disease processes happening in the retina. Several correlative studies support this. For example, a plasma-based metabolomics study showed that AMD patients had altered plasma metabolomic profiles compared to the control group. Among them, the most significant metabolites map to the glycerophospholipid pathway, indicating the potential role of lipids as novel targets for early diagnosis of AMD (Laíns et al., 2018). A comparison of circulating n-3 FAs between AMD patients and controls showed lower serum eicosapentaenoic acid (EPA) and red blood cell membrane EPA/DHA levels in AMD patients, suggesting that circulating EPA and DHA may be related to the status of AMD. The differences are not sufficient as evidence that EPA and DHA represent markers for diagnosis of AMD) (Gorusupudi et al., 2016; Merle et al., 2014). However, strong evidences indicate that dietary intake of n-3 long-chain polyunsaturated fatty acid may decrease the risk of AMD and influence the development of advanced AMD (Cho et al., 2001; SanGiovanni et al., 2008).

The concentration of lipids composition in body fluids closely correlates with aging and disease processes happening in the retina. For example, a plasma-based metabolomics study showed that AMD patients had altered plasma metabolomic profiles compared to the control group. Among them, the most significant metabolites map to the glycerophospholipid pathway (Laíns et al., 2018). A comparison of circulating n-3 FAs between AMD patients and controls showed lower serum eicosapentaenoic acid (EPA) and red blood cell membrane EPA/DHA levels in AMD patients, suggesting that circulating EPA and DHA may be used as an indication for further diagnosis (Gorusupudi et al., 2016; Merle et al., 2014). Recent studies, aimed at describing the blood biomarkers correlating with AMD, pointed out the positive correlation of n-3 cholesteryl esters in the plasma compared to n-3 PUFAs levels in the retina. In contrast, n-6 cholesteryl esters levels seem to correlate with lower levels of n-3 in the retina, therefore a potential higher risk of disease (Acar et al., 2021). More extensive follow-up studies are needed to establish precise biomarkers that can be used to predict the disease.

Besides lipids, lipoproteins can also act as biomarkers for the aging retina and AMD. There is an increase in the serum concentration of total cholesterol and LDL accompanied by a decrease of HDL in AMD patients, suggesting they may be useful as markers of AMD (Reynolds et al., 2010). Fauser et al.(Fauser et al., 2011) reported elevated apolipoprotein B levels in AMD patients' serum. Curcio et al. (Curcio et al., 2010) addressed the role of apolipoprotein-B lipoproteins in the aging retina, especially its role in the formation of drusen associated with AMD. Some LC-PUFA peroxidation metabolites such as isoprostanes (IsoPs), which are derived from AA, are found to be applicable as diagnostic markers of oxidative stress in the mammalian eye due to their stability in healthy conditions and noticeably increased production upon exposure of the retina to oxidative stress (Dentchev et al., 2007; Njie-Mbye et al., 2013). This also suggests that since oxidative stress has been implicated as an integral part of aging processes, lipid peroxidation metabolites could also be used as potential indicators of aging in future studies.

III.2. Lipids in eye diseases

Several inherited diseases related to lipid metabolism and transport have been described. STGD is an autosomal recessive retinal disorder characterized by juvenile-onset macular degeneration, reduced light sensitivity or contrast sensitivity, and gradual loss of central vision (Allikmets et al., 1997b). Mutations in the *ABCA4* (STGD1), *ELOVL4* (STGD2, STGD3) (Small, 2001), *PROM1* (*STGD4*) (Yang et al., 2008), *BEST1* (Zolnikova et al., 2017), and *PRPH2* (STGD1) (Boon et al., 2007) genes have been detected in patients with autosomal recessive or dominant STGD. Among them, mutations in *ELOVL4* and *ABCA4* cause related lipid disorders in RPE and photoreceptors. Mutations in *ABCA4* result in the accumulation of A2E, a component of lipofuscin known to be toxic to RPE and photoreceptors. The autosomal dominant form of STGD3 is caused by mutations in *ELOVL4* (Molday and Zhang, 2010). Three independent mutations of *ELOVL4* are associated with juvenile macular degeneration causing STGD3 in humans (Bernstein et al., 2001; McMahon et al., 2007; Zhang et al., 2001). Due to the autosomal dominant nature of STGD3 mutations and the fact that VLC-PUFAs are associated with rhodopsin (Figure 2) (Sander et al., 2021), the lack of VLC-PUFAs in mutant animals may impair rhodopsin function, resulting in reduced visual function and photoreceptor degeneration. Because of the autosomal dominant nature of STGD3 mutations and the fact that VLC-PUFAs are associated with rhodopsin (Figure 2) (Sander et al., 2021), the lack of VLC-PUFAs in mutant animals may impair rhodopsin function, resulting in reduced visual function and photoreceptor degeneration. Interestingly, the dominant-negative mutant of ELOVL4 protein itself is not anchored to the ER but fills the OS of photoreceptors, impacting phagocytosis and recycling (Esteve-Rudd et al., 2018; Mandal et al., 2014). Further studies, however, are still needed to dissect the molecular steps affected and allow for looking for novel, effective strategies in treating patients. Mutation of *BEST1* was also identified in bestrophinopathy, which is one of the most common forms of macular degenerations. This disease was identified by abnormal accumulation of autofluorescent material within RPE cells was identified and leading to bilateral macular or multifocal lesions (Table 1) (Guziewicz et al., 2017).

Similarly, different species of lipids, including VLC-PUFAs, have been shown to play a role in AMD, which has been recently reviewed in depth (Skowronska-Krawczyk and Chao, 2019; van Leeuwen et al., 2018). Recently we have looked at the potential involvement of ELOVL2 in AMD disease. Our RNAscope analysis has shown that *ELOVL2* is abundantly expressed in cones, including the perifoveal subtype (Figure 7A). Therefore, we have looked at whether the DNA methylation of cg16867657, where methylation levels faithfully correlate with the person's age, changes in the samples isolated from AMD donors but could not detect any change (Figure 7B). In contrast, chromatin accessibility on *ELOVL2* promoter significantly decreases in macula and periphery (Figure 7C) of in retinas from AMD donors compared to age-matched healthy tissue. This change is specific to the promoter region of *ELOVL2* as the intron region accessibility does not change in both groups. This intriguing loss of accessibility in AMD patients may suggest the secondary, disease but not age-related impact on the chromatin in *ELOVL2* locus and further downregulation of the gene in the disease. For example, given the multifactorial character of AMD, it is not excluded that particular phenotype of the disease, such as oxidative stress, elicits an additional mechanism repressing *ELOVL2* promoter. Further studies need to establish what transcriptional and metabolic pathways are involved in age-related chromatin remodeling on *ELOVL2* locus.

To date, despite numerous genome-wide studies, no detected ELOVL2 mutations or variants are correlating with the risk of AMD. There are several possible explanations: 1. *ELOVL2* is an essential gene for population survival. It has been shown that complete deletion of the gene in a mouse model causes infertility in C57BL/6 mice that can be potentially overcome by keeping the genotype in the mixed background (Pauter et al., 2014). Therefore, variants that potentially can be correlated with the disease are rare and yet to be discovered. 2. *ELOVL2* is regulated on the transcriptional level, and the downregulation of its expression is not specific for AMD. Potential variants affecting the activity or expression of *ELOVL2* may correlate rather with the onset of the disease and not the disease itself. This type of analysis has yet to be performed. 3. Other ways to deregulate ELOVL2 transcription are employed and correlate with the disease. As shown in (Figure 7), the promoter accessibility significantly decreases in retinas isolated from AMD donors. What is the potential mechanism of this phenotype remains to be elucidated.

Accumulations of lipids, lipoproteins, and oxidized lipids within Bruch's membrane are considered as pathogenesis of AMD. Though the pathogenesis of AMD is complex and still unknown, many genetic variants in genes that regulate lipid metabolism and lipid transfer between lipoproteins have been proved to be associated with the risk of advanced AMD, such as *APOE2* (Baird et al., 2006), *CETP*, *LIPC*(Neale et al., 2010), *CFH* (Zareparsi et al., 2005), *ABCR* (Allikmets et al., 1997a), *IGF1*(Castellino et al., 2018), *ADIPOR1* (Castellino et al., 2018), and other. The molecular connection of AMD with lipid metabolism opened new opportunities to search for pharmaceutical and nutritional interventions for affected patients.

One of the most interesting cases is the association of ApoE variants and AMD (Baird et al., 2004). As mentioned above, human ApoE is encoded by three alleles – E2, E3, and E4. The association of APOE4 isoform with Alzheimer's disease has been very well established and

further correlated with the molecular mechanism of accumulation of beta-amyloid plaques (recently reviewed in (Yassine and Finch, 2020). Surprisingly, it is the APOE2 isoform that is associated with a higher risk of developing and progression of AMD (Baird et al., 2006; Levy et al., 2015b, 2015a). Several studies using the humanized mice expressing specifically E2, E3, or E4 isoforms have been performed to understand the mechanism of the disease in the presence of a high-fat diet (Ding et al., 2011; Malek et al., 2005). In particular, studies have shown that APOE isoforms are differentially associated with AMD-like phenotypes in mice than in humans. Opposite to what was observed in humans, mice carrying APOE4 allele had the highest levels of subRPE diffused and drusenoid deposits, and AMD-like RPE/retinal phenotypes. Several explanations are possible. In addition to the high-fat diet as an environmental factor, rarely considered in studies related to APOE alleles, specific differences in mice and humans physiology can help to explain the differences and help understand the disease. One of the main factors is the low levels of VLDL particles in the mice plasma, due to the extremely low levels of APOB100 expression, apolipoprotein indispensable for VLDL formation. High-fat diet and lack of ability to process the lipids *via* VLDL might have a specific impact on transmembrane lipid transport and lipid metabolism. Nevertheless, when knowing caveats, the humanized APOE mouse model can be used to test different therapies such as anti-amyloid therapy to rescue vision (Ding et al., 2011).

In parallel, studies focused on understanding the ApoE-dependent AMD phenotype in human iPS-RPE cell culture, and human tissues elegantly suggested that due to the conformational change upon oxidative stress, ApoE2 isoform poorly performs its primary function in the retina – cholesterol efflux from the RPE (La Cunza et al., 2021). This study connects mitochondrial stress to the APOE function, therefore bringing together two critical processes known to be at the basis of AMD development. This work also seems to support the RPE-origin of drusen (Bergen et al., 2019); however, as mentioned above, ApoE2 protein has very low or no affinity to the LDLR (Weisgraber et al., 1982), the most abundant receptor of the lipid particles on the basal side of the RPE (Tserentsoodol et al., 2006b, 2006a). It is, therefore, possible that uptake of HDL particles containing ApoE2 is slower, therefore ADDINg to the accumulation of the subRPE protein-lipid deposits.

One of the most known genetic variants associated with AMD is the tyrosine (Y) to histidine (H) substitution at amino acid 402 (Y402H) in CFH. It was believed that *CFH* variant induced AMD by activation of complement in the eye. Nevertheless, the limited success of the clinical trials of complement inhibitors in the treatment of AMD patients challenged this theory. To investigate the relationship between *Cfh* mutation and complement dysregulation and lipid homeostasis in BM, an aged, *Cfh* haploinsufficiency ($Cfh^{+/-}$), supplied with high fat, cholesterol-enriched diet mice model was developed (Toomey et al., 2015). Studies showed that this mice model presents many phenotypes resembling the human AMD phenotypes, including increased basal lamina deposits, accompanied by accumulation of lipoproteins and lipids in BM, RPE damage, and vision loss. This model revealed that rather than complement activation, *Cfh* variant caused the AMD-like symptoms through lack of activity and disturbing the complement pathway causing accumulation of lipoproteins and lipids and induction of sub-RPE deposit formation in aged mice (Landowski et al., 2019; Toomey et al., 2015). The use of high-fat, cholesterol-enriched diet (HFC) confirms the previous studies emphasizing the role of lipid balance in preserving vision. Fluorescence-

based measurement and Western blot showed that the CHF polymorphism affects the total level of plasma VLDLs and LDLs containing ApoB100 and ApoE, as well as ApoB48 and ApoA1 retention in aged murine BM in the eyecup (rather than complement activation) in response to the HFC diet. To date, this is the best animal model of AMD, and great hope in the field seeking for models to understand human disease.

RP is a group of inherited retinopathies characterized by tunnel vision (loss of peripheral vision) and night blindness due to the loss of rods from the retina. Over 70 genes have been associated with the development of RP so far (McColl and Converse, 1995). The panoply of genetic causes of RP has been reviewed elsewhere (Daiger et al., 2015). Interestingly, several mutations are located in genes encoding proteins involved in lipid metabolism and homeostasis. For example, variants in *MTTP* (encoding microsomal triglyceride transfer protein) were characterized in Bassen-Kornzweig syndrome, associated with low LDL-cholesterol, triglyceride and apolipoprotein, and a deficiency in fat-soluble vitamins and essential FAs. Batten disease caused by mutation of *CLN3* gene is characterized by accumulation of ceroid and lipofuscin in the neuronal system, including the retina. Variations in *PHYH* (encoding Phytanoyl-CoA Hydroxylase) and *PEX7* (encoding the Peroxisome targeting signal 2 (PTS2) receptor) were identified in Refsum's disease, causing deficiency in phytanic acid α-oxidation. Other RPs, including Zellweger syndrome and Usher syndrome, are also described as associated with lipid imbalance, such as abnormal saturated and unsaturated FA levels. Table 1 highlights the list of eye diseases associated with mutations in genes involved in lipid metabolism.

Macular telangiectasia is a rare degenerative retinal disease affecting the macula, causing loss of central vision. There are three types of Macular Telangiectasia: type 1, 2, and 3. Among them, macular telangiectasia type 2 (MacTel) is the most common of the three types, and it occurs in one out of 1,000 people over 40 years old. Phenotypic characterization of MacTel showed lack of foveolar reflex, reduced retinal transparency (graying), crystalline deposits and ectatic capillaries (Charbel Issa et al., 2013). Many familial cases of MacTel indicated genetic factors in the pathogenesis of this disease. Genetic and metabolism works showed a link between MacTel and serine metabolism (Scerri et al., 2017). However, the related genes were not described until very recently. Variant *SPTLC1* or *SPTLC2* (encoding a subunit of serine palmitoyltransferase) was identified through exome sequencing analysis of genomic DNA from MacTel patients and their family members (Gantner et al., 2019). The two variants led to elevated levels of circulating deoxysphingolipids, which negatively correlated with serine levels. A mouse model confirmed that the decreased level of serine is sufficient to cause increased retinal deoxysphingolipid levels and retina degeneration. Genome-wide association studies (GWAS) identified a new rare *PHGDH* (phosphoglycerate dehydrogenase) variant in MacTel patients (Fallon et al., 2018). *PHGDH* encodes a rate-limiting enzyme that performs the first step of the phosphorylated pathway of serine biosynthesis, converting 3-phosphoglycerate into phosphohydroxy pyruvate. The *PHGDH* variant also causes decreased serum levels of serine and accumulation of neurotoxic deoxysphingolipids (deoxySLs) in retinal pigmented epithelial cells, which is correlated with the extent of disease in MacTel (Eade et al., 2021; Gantner et al., 2019). These two findings help to understand the basis of the disease phenotype of MacTel and indicate the potential treatment targets.

Of the lipid storage disorders, Gaucher disease, Farber's disease and Sandhoff's disease are lysosomal storage disorders accompanied by the accumulation of harmful lipids and ocular phenotypes. For example, Gaucher disease is an inherited glycolipid storage disorder that affects many of the organs including retina. It is associated with mutation in *GBA* that encodes acid β-glucosidase (lysosome glucocerebrosidase) (GRABOWSKI, 1997). *L444P* is the most common mutation on *GBA* in Gaucher disease, it causes accumulation of glucosylsphingosine leading to tapeto-retinal degeneration, pigmented retinal lesions, intra-retinal white dots and inner retinal Gaucher cells (Table 1) (Winter et al., 2019). Optical coherence tomography (OCT) was utilized in a pilot study showed that retinal thinning can be detected in patients and carriers(McNeill et al., 2013). Farber's disease and Sandhoff's disease are rare lysosomal storage disorders. Farber's disease is caused by a mutation in *ASAH1* gene that encodes acid ceramidase enzyme (Koch et al., 1996). Deficiency of acid ceramidase causes ceramide accumulation in various tissues, including the retina (Sugita et al., 1972). Mouse model of *ASAH1* human mutation shows progressive retinal pathology, including progressive inflammation and retinal dysplasia accompanied by abnormal accumulation of ceramides and other sphingolipids followed by severe visual impairment (Yu et al., 2019). Sandhoff's disease, which is another disease related to pathologic sphingolipid build-up, is caused by variants in the hexosaminidase-B (HEXB) gene. The mutations led to the deficient hexosaminidase activity, resulting in the accumulation of GM2 ganglioside in the lysosomes of neuronal cells, progressive neural degradation, and vision deterioration (Mahuran, 1999; Sandhoff and Harzer, 2013). These two examples emphasize the importance of animal models when studying human diseases, especially for rare syndromes where gathering a substantial amount of data from patients is very limited.

Retinal diseases may also induce changes in membrane fluidity and further progression of retinal degeneration. For example, Smith-Lemli-Opitz syndrome (SLOS), caused by mutations in the *DHCR7* gene, is accompanied by abnormal accumulation of 7-dehydrocholesterol and decreased cholesterol levels, affecting most organ systems, including eye and brain, extremities, skin, lung, heart, and other (Porter, 2008). Fliesler and colleagues (Fliesler and Xu, 2018) generated a rat model by treating rats with AY9944, a selective inhibitor of the enzyme affected in SLOS. Steady-state anisotropy measurements of the model showed that the fluidity of rod OS membranes is significantly reduced, along with a marked decrease in membrane lipid unsaturation due to losses in DHA. Compromised rhodopsin regeneration and retinal degeneration have also been observed in this model (Table 1) (Boesze-Battaglia et al., 2008; Tulenko et al., 2006).

Also, other types of retinal diseases are connected to the lipids serving as co-factors in specific reactions. For example, mutation of the dehydrodolichyl diphosphate synthase (*Dhdds*) gene causes RP (RP59), which contains symptoms including night and peripheral vision loss, constriction of visual fields, and retinal degeneration (Zelinger et al., 2011). Animal experiments suggested that the knockdown of *Dhdds* in rod cells showed retinal dysfunction initiated by decreased retina dolichol levels, lipid molecule involved in N-glycosylation of proteins including rhodopsin. However, neither the human point mutation nor removal of *Dhdds* from rods affect the N-glycosylation of retinal proteins, which was considered as a key factor of the pathogenesis of RP59. (Ramachandra Rao et al., 2020a,

2020b). Further studies need to decipher whether the results are due to the differences between the mice and human or there are other factors contributing in the development of the disease in human.

Mutations of genes involved in lipid metabolism have been detected in other retinal phenotypes. For example, *ACBD5* (encoding Acyl-CoA binding domain-containing 5) mutation, a peroxisomal tail-anchored membrane protein, was observed in patients with a syndromic form of retinal dystrophy (Abu-Safieh et al., 2013). Genome editing experiment on patient-derived fibroblasts and HeLa cells indicated that ACBD5 deficiency causes defect in peroxisomal β-oxidation of very-long-chain FAs (VLCFAs) and accumulation of cellular phospholipids containing VLCFAs. It is proposed that ACBD5 plays a vital role in transporting VLCFA-CoAs from the cytosol into peroxisomes for subsequent β-oxidation (Yagita et al., 2017). Given the complexity of lipid metabolism and the vast number of enzymes involved therein, it is highly probable that a deficit in these pathways causes many eye pathologies.

Besides the imbalance of lipids, lipid peroxidation has been reported to contribute to degenerative ocular diseases like AMD, glaucoma, and diabetic retinopathy. For example, the age-dependent susceptibility of the macula to lipid peroxidation and its products is related to the attenuation of antioxidant defense systems with aging. Long-term oxidation of PUFAs results in impairment in the function and structure of cell membranes, leading finally to degeneration of photoreceptors (De La Paz and Anderson, 1992). With age, oxidized PUFAs are not efficiently digested in the lysosomes of aged RPE cells and become deposited in the form of lipofuscin (see above). Several clinical findings showed that excessive accumulation of lipofuscin granules initiates RPE cells damage (Kopitz et al., 2004; Malek et al., 2005; Sparrow et al., 2000). For instance, Batten's disease and Stargardt disease have been attributed to excessive accumulation of lipofuscin-like materials in the RPE, causing loss of visual function. Besides the imbalance of lipids, lipid peroxidation has also been reported to contribute to degenerative ocular diseases like AMD, glaucoma, and diabetic retinopathy. For example, the age-dependent susceptibility of the macula to lipid peroxidation and its products is related to the attenuation of antioxidant defense systems with aging. Long-term oxidation of PUFAs results in impairment in the function and structure of cell membranes, leading finally to degeneration of photoreceptors (De La Paz and Anderson, 1992). With age, oxidized PUFAs are not efficiently digested in the lysosomes of aged RPE cells and become deposited in the form of lipofuscin (see above). Several clinical findings showed that excessive accumulation of lipofuscin granules initiates RPE cells damage (Kopitz et al., 2004; Malek et al., 2005; Sparrow et al., 2000). For example, Batten's disease and Stargardt disease have been attributed to excessive accumulation of lipofuscin-like materials in the RPE, causing loss of visual function.

Lipid deposits are one of the most distinctive and earliest features of AMD. Studies indicate that unesterified and esterified cholesterol and triacylglycerols (Curcio et al., 2005), PC (Wang et al., 2010), and apolipoproteins (Anderson et al., 2001; Curcio et al., 2010) are abundant components in deposits and drusen of AMD eyes. Among them, esterified cholesterol and PC accounted for at least 40% of the drusen volume (Wang et al., 2010). As mentioned above, these lipids can be derived from the OS, the nutrient supply

system, or produced directly by RPE cells (Pikuleva and Curcio, 2014). Ultrastructural techniques indicated that esterified cholesterol is only located in Bruch's membrane, while unesterified cholesterol and phospholipids are located in nearby cellular membranes (Rudolf and Curcio, 2009). A high concentration of oxidized lipids in the drusen triggered several ideas regarding the involvement of inflammation in disease progression. Curcio et al. has proposed that the deposits may interact with ROS, form pro-inflammatory oxidized lipids, activate the complement system, and trigger angiogenesis (Curcio et al., 2011). Current studies focus on correlating subretinal lipid deposits with a patient's stage of AMD aim to discover potential pathways involved in deposit formation (L. Chen et al., 2021; Sura et al., 2020) to suggest the potential ways to interrupt this process in the aged retina.

Final comments

As is the case with all biomedical studies, a major challenge in the ophthalmology field is translating data from animal models to human subjects. Studies on mice and rats were instrumental in acquiring our current knowledge level, and studies on cone-rich retinas (*e.g.*, large animals, diurnal rodents) have allowed more accurate modeling of several human conditions. Research on primates, although extremely valuable, is limited due to the high cost. Studies on human organoids can answer questions relevant for human retina development but cannot yet be used in studying aging and age-related eye diseases. In short, there are still no attractive, comprehensive model with developed macula that would facilitate studies of macular function and degeneration. While there is an urgent need to develop appropriate animal models for these studies, current models, despite their shortcomings, can be extremely useful provided that steps are taken to limit the risk of overinterpreting data. With the development of the new technologies, new animal models and outstanding progress in systems biology approaches the field is poised to new discoveries in sufficient mechanistic detail to help initiate novel approaches to prevent and even reverse the age and genetic predisposition to lipid-related eye diseases.

Acknowledgments

Abbreviations

4-HNE	4-hydroxy 2-nonenal
AA	arachidonic acid
ABCA1	ATP-binding cassette transporters, family A, 1
ABCA4	ATP-binding cassette protein, family A, 4
ABCG1	ATP-binding cassette transporters, family G, 1
ACER3	Alkaline ceramidase 3

ADIPOR1	adiponectin receptor 1
AGPAT	1-acylglycerol-3-phosphate acyltransferase
AMD	age-related macular degeneration
APOA1	apolipoprotein A1
ApoE	apolipoprotein E
APOER2	ApoE receptor 2
AREDS	Age-Related Eye Disease Study
ASAH1	N-acylsphingosine amidohydrolase 1
ASMase	acid sphingomyelinase
ATP8A2	ATPase phospholipid transporting 8A2
BL	bioactive lipid
BLamD	basal lamina deposits
BM	Bruch's membrane
BRB	blood-retina barrier
C1P	ceramide-1-phosphate
CC	connecting cilium
CEP	carboxyethylpyrrole
CERK	ceramide kinase
CERS	ceramide synthases
CERT	ceramide transferase
CFH	complement factor H
CM	chylomicron
CNS	central nervous system
COX	cyclooxygenase
CPT1	choline phsphotransferase 1
CYP	cytochrome P450 monooxygenase
DAG	diacylglycerol
deoxySL	deoxysphingolipid
DGK	DAG kinase

DGKE	DAG kinase isoform epsilon
DHA	docosahexaenoic acid
DIBMALP	diisobutylene maleic acid lipid particles
DPPC	dipalmitoyl-phosphatidylcholine
DRM	detergent-resistant membrane fraction
EET	epoxyeicosatrienoic acid
ELOVL4	elongation of very long FAs 4
EPA	eicosapentaenoic acid
EPT1	ethanolamine phosphotransferase 1
ER	endoplasmic reticulum
FABP	fatty acid binding protein
FAO	fatty acid oxidation
FATP	fatty acid transfer protein
FFA	free fatty acid
GAS6	Growth arrest-specific protein 6
GLUT1	glucose transporter type 1
GPAT	glycerol-3-phosphate acyltransferase
HC	hydraulic conductivity
HDL	high-density lipoprotein
HETE	hydroxyeicosatetraenoic acid
hPSC	human pluripotent stem cell
IDL	intermediate-density lipoprotein
IL-6	interleukin 6
IP3	(1,4,5)-inositol triphosphate
IPM	interphotoreceptor matrix
IRBP	interphotoreceptor retinoid-binding protein
IS	inner segment
IsoP	isoprostane
KDSR	3-ketodihydrosphingosine reductase

KO	knock-out
LC-PUFA	long chain polyunsaturated fatty acid
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LIPA	lysosomal acid lipase type A
LOX	lipoxygenase
LPA	lysophosphatidic acid
LPA1–6	LPA-binding GPCR 1–6
LPAAT	lysophosphatidic acid acyltransferase
LPC	lysophosphatidylcholine
LPLAT	lysophospholipid acyltransferase
LPP	lipid phosphate phosphatases
LRAT	lecithin retinol acyltransferase
LT	leukotriene
LTA4	leukotriene A4
LUV	large unilamellar vesicles
LXA4	Lipoxin A4
MacTel	macular telangiectasia 2
MDA	malondialdehyde
MERTK	MER tyrosine kinase
MFGE8	milk fat globule factor-E8
MFRP	membrane frizzled-related protein
MFSD2A	major facilitator superfamily domain-containing protein 2a
MSP	membrane scaffold protein
mTOR	mechanistic target of rapamycin
n-3	omega-3
NAT-2	nutritional AMD treatment study
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor family pyrin domain-containing 3

NPD1	neuroprotectin D1
OGG1	8-oxoguanine glycosylase
OS	outer segment
PA	phosphatidic acid
PAP	phosphatidate phosphatase
PARP-1	Poly(ADP-Ribose) Polymerase 1
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PGE2	prostaglandin E2
PI(3)P	phosphatidylinositol-3-phosphate
PI(4)P	phosphatidylinositol-4-phosphate
PI	phosphatidylinositol
PIP	PI phosphate
PIP2	phosphatidylinositol 4,5-diphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKC	protein kinase C
PLAAT3	phospholipase A and acyltransferase 3
PLC	phospholipase C
PPARα	Peroxisome proliferator activated receptor-α
PRPH2	peripherin 2
PROS	vitamin K-dependent protein S
PS	phosphatidylserine
PTS2	peroxisome targeting signal 2
PUFA	polyunsaturated fatty acid
RCS	Royal College of Surgeons
RDH12	retinol dehydrogenase 12
ROM-1	rod outer membrane protein 1
ROS	reactive oxygen species
RP	retinitis pigmentosa

RPE	retinal pigment epithelium
S1P	sphingosine-1-phosphate
SCARB1	scavenger receptor class B, member 1
SLOS	Smith-Lemli-Opitz syndrome
SMA	styrene maleic acid
SMALP	styrene maleic acid lipid particle
SMase	sphingomyelinase
SMPD	sphingomyelin phosphodiesterase
Sph	sphingosine
SPHK	sphingosine kinase
SPP	S1P phosphatase
SPT	serine palmitoyltransferase
STGD	Stargardt-like macular dystrophy
TNFα	tumor necrosis factor α
TRP	transient receptor potential
TxB2	thromboxane B2
VEGF	vascular endothelial growth factor
VLC-PUFA	very long chain polyunsaturated fatty acid
VLC-SFA	very long chain saturated fatty acid
VLDL	very low density-lipoprotein
VLDLR	very low-density lipoprotein receptor
WT	wild-type

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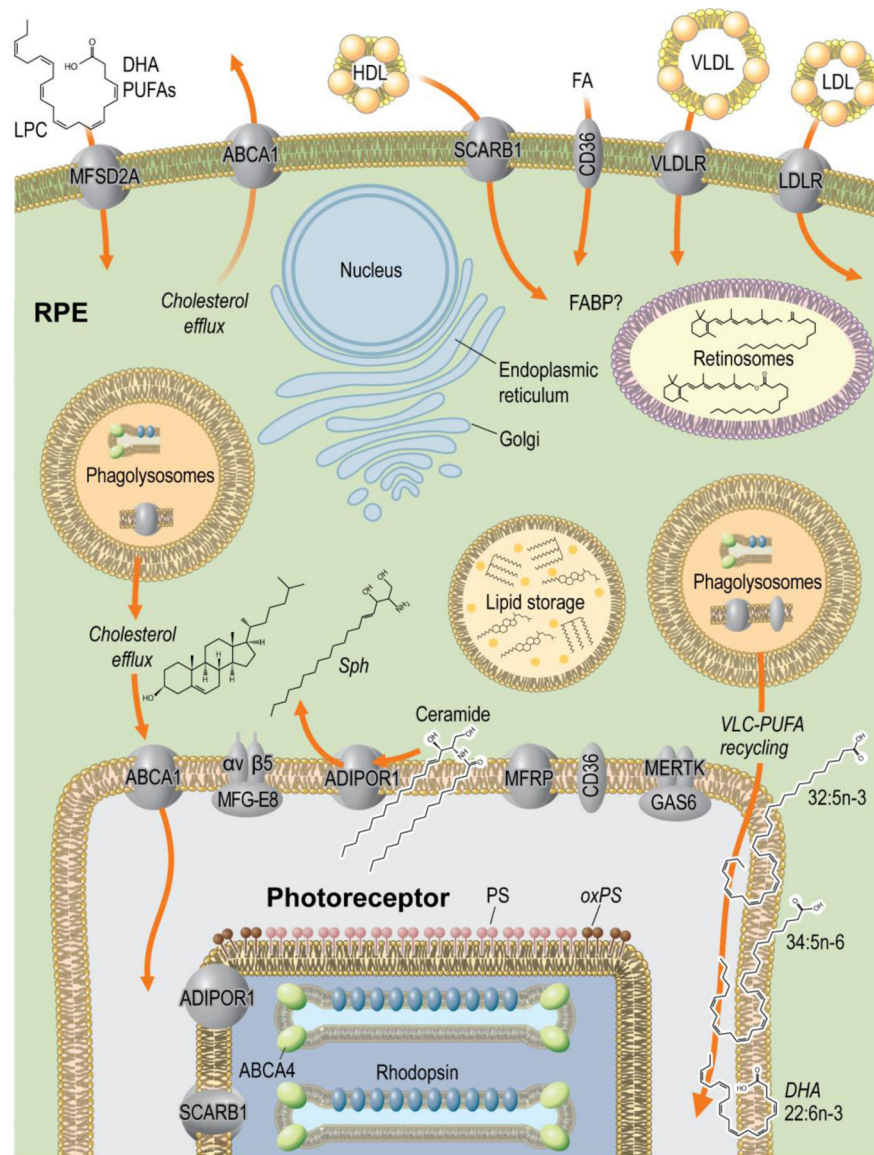
Figure 1. Lipid environment at the RPE/photoreceptor interface.

The dynamic interplay between photoreceptors and the RPE enables the efficient and necessary recycling and biogenesis of lipid components. Transporters on the basal side of RPE cells bring in several lipid components from circulation while also allowing for cholesterol efflux. The RPE also accepts lipids from photoreceptors from shedding disk tips, which become phagolysosomes in the RPE. The RPE uses both sources of lipid precursors to produced vital components of photoreceptor membranes, which are then transported back to photoreceptors for their incorporation and support of phototransduction.
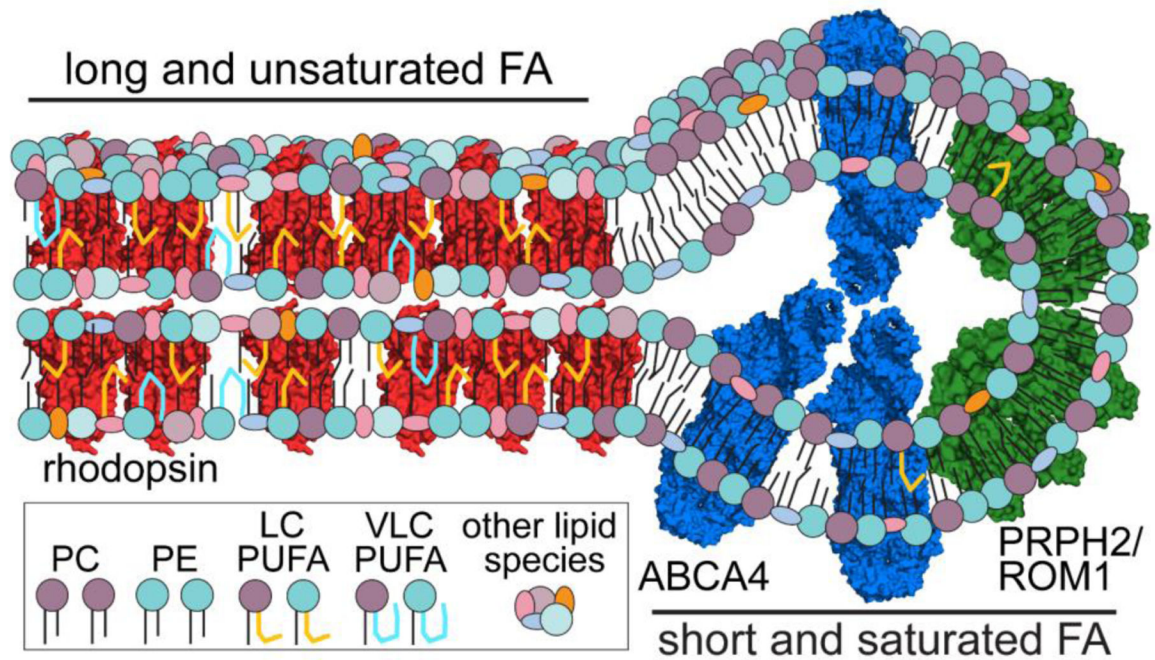
Figure 2. Rod OS disks have regionally distinct microenvironments.
The central region of rod OS disks, rich in rhodopsin (red, PDB: 1F88), has an abundance
of long and unsaturated FAs. Rim regions of rod OS disks containing ABCA4 (blue, PDB:
7LKP) and PRPH2/ROM1 (tetramer of human tetraspanin CD81 used as a model in green,
PDB: 5TCX) have relatively high amounts of short and saturated FAs. There are many other
distinctions in lipid species between the two regions, including relative amounts of PC and
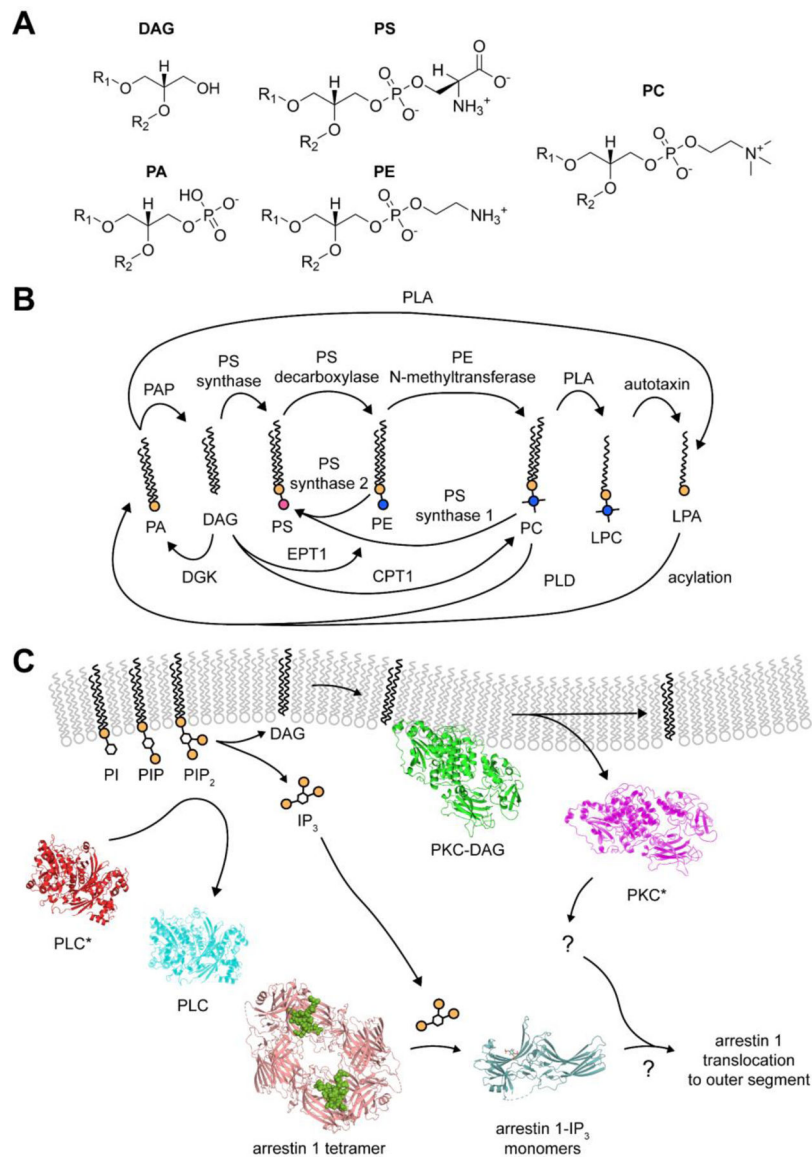PE. ©2021 Sander et al. Adapted from an article originally published in the Journal of Cell
Biology. https://doi.org/10.1083/jcb.202101063

Figure 3. Major glycerophospholipids – synthesis and potential signaling applications.
(**A**) Chemical structure of the five major glycerolipids. (**B**) Major glycerophospholipid pathways. Many enzymes are involved in the interconversion of each lipid species, including (**C**) Proposed involvement of IP$_3$ and DAG in arrestin 1 translocation. Structures from a recent study of arrestin 1 in complex with IPs showed their ability to displace the arrestin 1 C-terminus, which is essential for inner-segment localization. The combination of a new basal structure, IP-complex structures, and data from Orisme et al. suggest a possible molecular mechanism for arrestin 1 translocation in cases of PLC activity. PLC structures were made from PDB structure 2ZKM (Hicks et al., 2008). PKC structures made from model predicted using AlphaFold, Uniprot identifier P17252 (Jumper et al., 2021). Basal and IP$_3$-bound arrestin-1 structures taken from PDB structures 7JSM and 7JXA (Sander et al., *accepted for puvlication*).
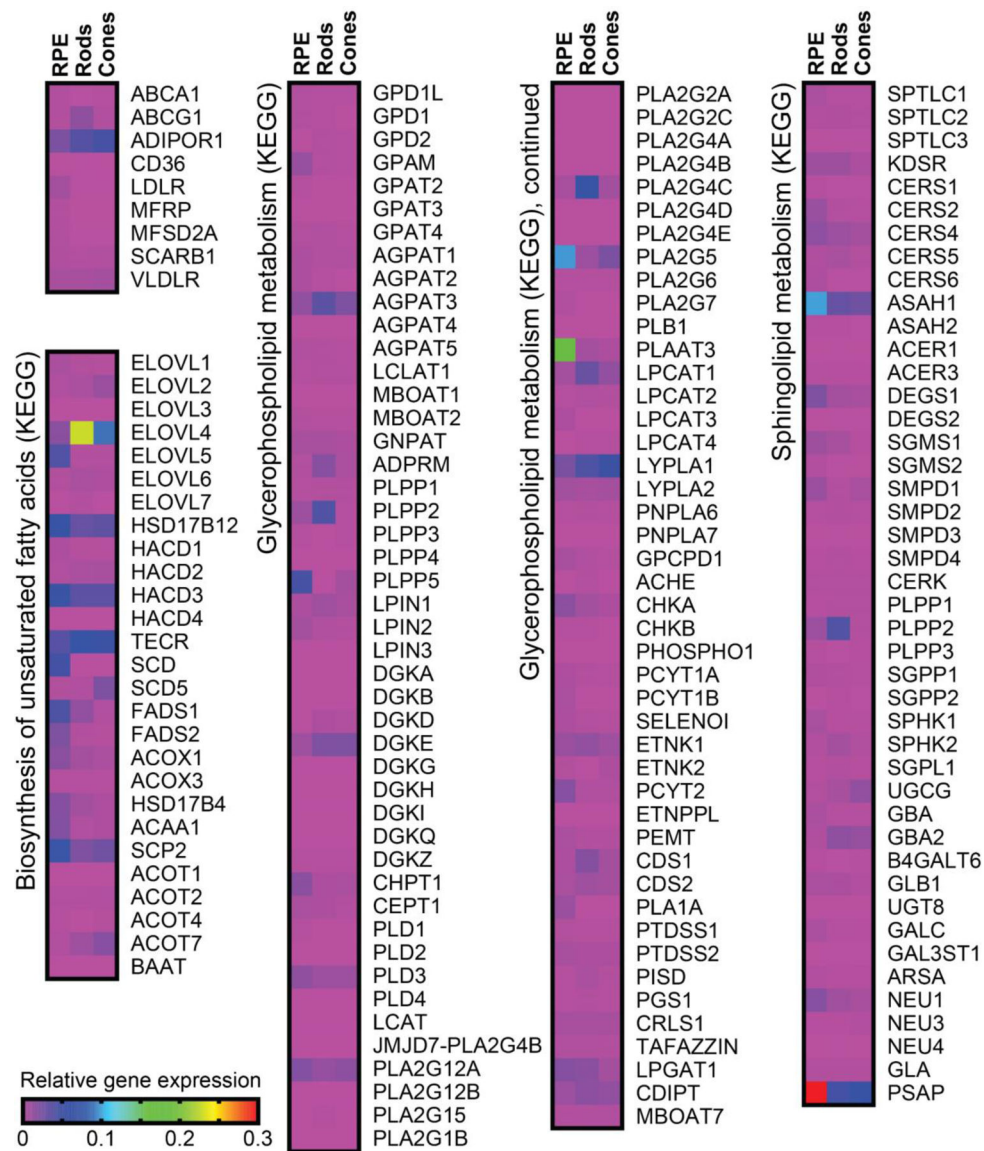
Figure 4. RPE, rods, and cones express select enzymes involved in lipid metabolism.
(**A**) Heatmaps representing gene expression profiles of genes and pathways involved in lipid uptake, biosynthesis, and metabolism. Data were sourced from the human eye single-cell RNA-seq gene expression database (Lu et al., 2020) and normalized to each cell GAPDH expression level.
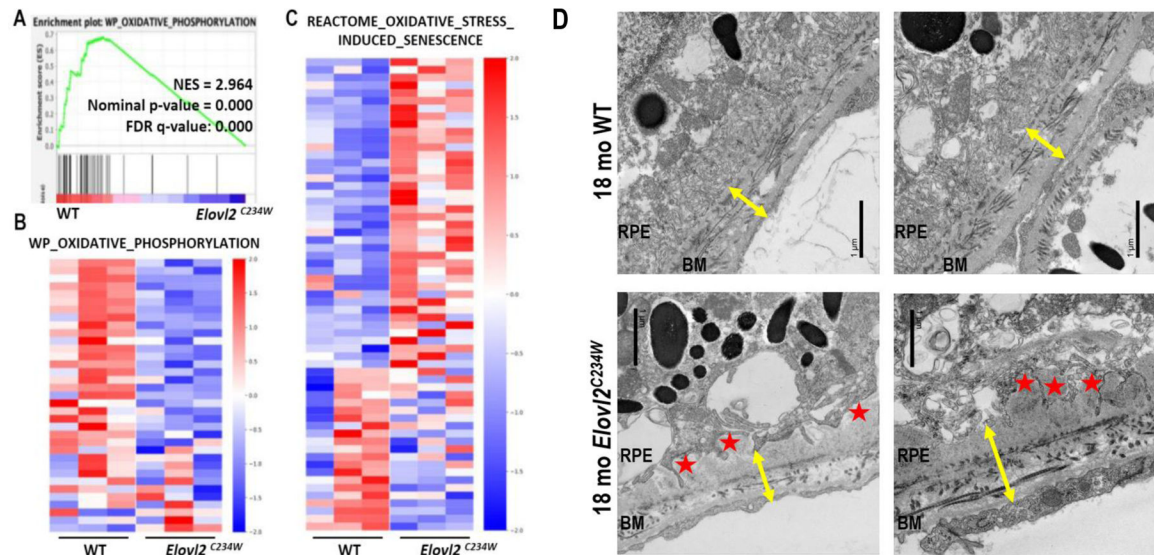
Figure 5. Metabolic and structural changes in ELOVL2 deficient animals.
Gene set enrichment analysis (GSEA) was performed on RNA-Seq data from the retina of *Elovl2^C234W^* and WT mice. **A.** "Oxidative Phosphorylation" pathway is significantly downregulated, as shown on the enrichment plot. **B.** Gene sets "Oxidative Phosphorylation" and **C.** "Oxidative Stress Induced Senescence'' with genes ranked by WT/*Elovl2^C234W^* in mean log2Fold Change of expression level. Color bars indicate row Z-score. In the analysis, we used 4-month-old mice (N=4 for each genotype) and Molecular Signatures Database (MSigDB). **D.** EM analysis of Bruch's Membrane (BM). Comparison of 18-month-old WT and *Elovl2^C234W^* RPE/BM surfaces show thickening and disorganization of BM (yellow arrows), including accumulation of basal lamina deposits (BLamD - red stars) and distorted RPE infoldings
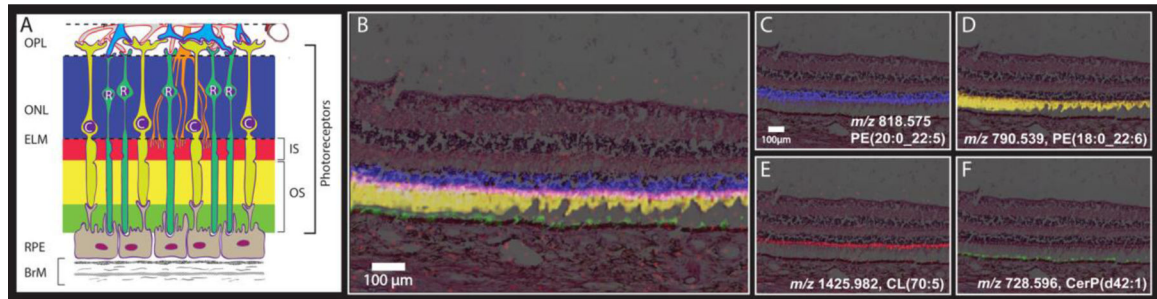
Figure 6. Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) signals consistent with localization to photoreceptor and RPE compartments.

(**A**) Schematic diagram of outer retina and Bruch's membrane. Blue, pink, yellow, and green bands indicate layers formed by highly compartmentalized and vertically aligned photoreceptors and RPE cells in panels **B** and **C**. Layers: OPL, outer plexiform layer; ONL, outer nuclear layer; ELM, external limiting membrane; RPE, retinal pigment epithelium; BrM, Bruch's membrane; R, Rod; C, cone photoreceptors. (**B–F**) Overlaid MALDI-MSI images and H&E stained cross-section of the peripheral retina display signals from multiple lipid classes that localize to specific subcellular compartments of the photoreceptor cells and RPE. (**B**) Overlay showing four separate signals defined in panels **C–F**, localized to (**C**) ONL, (**D**) photoreceptor inner and outer segments, (**E**) mitochondria-rich photoreceptor inner segments, and (**F**) RPE apical processes. ©2021 Anderson et al. Reprinted from an article originally published in J. Am. Soc. Mass Spectrom. https://doi.org/10.1021/jasms.0c00119.
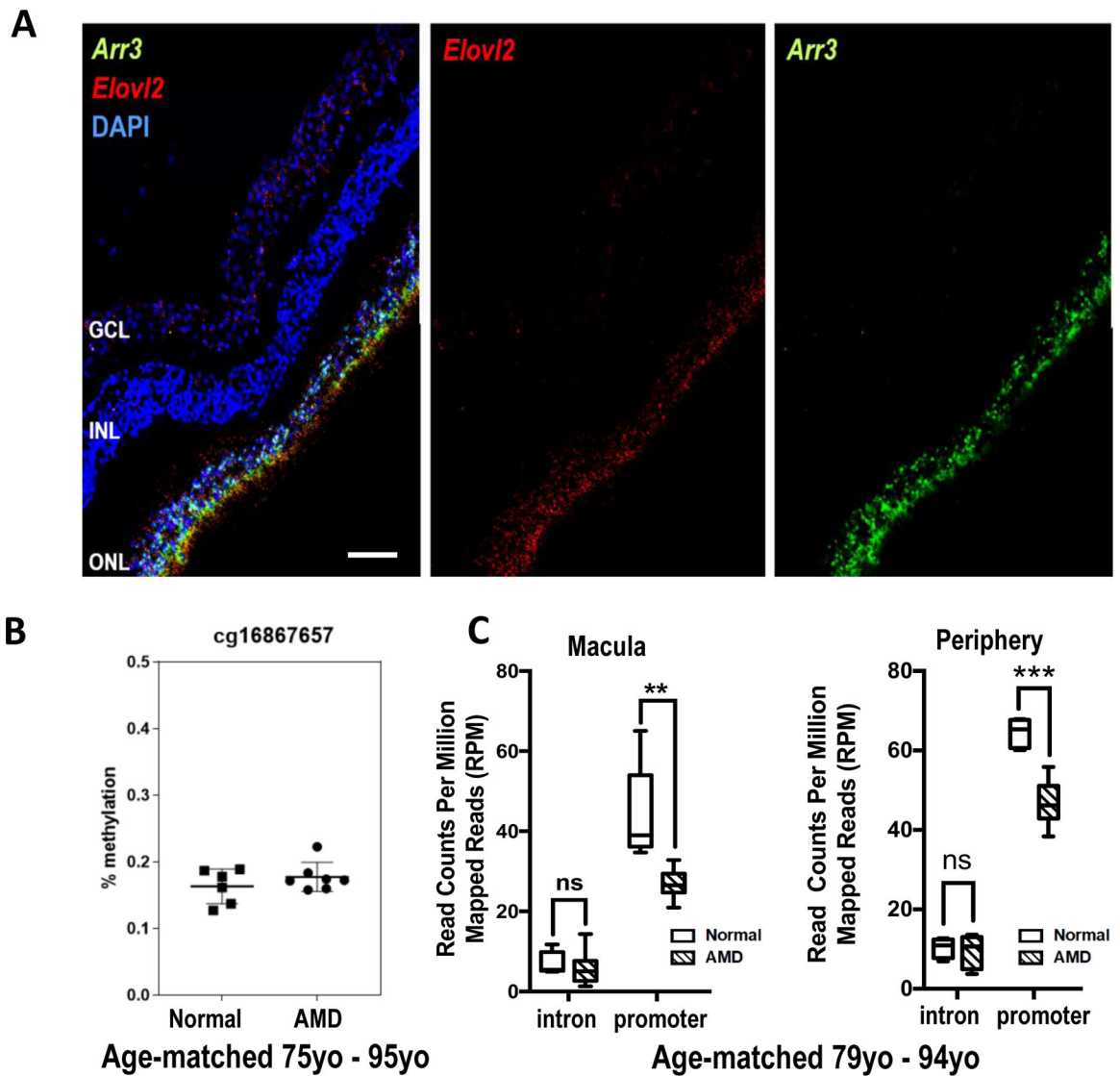
Figure 7. ELOVL2 and AMD.

(**A**) RNAscope detection of *Elovl2* (red) and *Arr3* (green) mRNA shows high expression of *Elovl2* in perifoveal cones and low expression in ganglion cells. (**B**) DNA methylation level of the cg16867657 in *ELOVL2* promoter is not increased in samples isolated from AMD donors (**GSE102952** (Oliver et al., 2015)); (**C**) Chromatin accessibility data measured by ATAC-seq (**GSE99287** (Wang et al., 2018)) shows significantly decreased signal of *ELOVL2* promoter in samples isolated from AMD donors when compared to age-matched healthy donors both in the macula and in the periphery. No change is observed in the intron region.

Table 1.

Eye disorders associated with lipid metabolism.

Disease	Genomic mutation	Lipids families affected	Phenotype	References
Age-related macular degeneration (AMD)	genetic associations: *APOE2, CETP, LIPC, CFH, ABCR, IGF1*	Lipid accumulation, lipid oxidation, drusen	Reduced light sensitivity or contrast sensitivity is the primary visual complaint, while in advanced disease, visual acuity loss is the main complaint.	(Ratnapriya and Chew, 2013) (Fletcher et al., 2014) (Rivera et al., 2000) (Jun et al., 2019) (Arroba et al., 2018) (Skowronska-Krawczyk and Chao, 2019)
Bestrophinopathy	*BEST1*	increase in unesterified cholesterol	Abnormal accumulation of autofluorescent material within RPE cells and bilateral macular or multifocal lesions.	(Guziewicz et al., 2017)
Bietti Crystalline dystrophy	*CYP4V2*	High levels of triglycerides and cholesterol storage, decreased metabolism of FA precursors into n-3 PUFAs	Multiple glistening intraretinal crystals scattered over the fundus, a characteristic degeneration of the retina, and sclerosis of the choroidal vessels, ultimately resulting in progressive night blindness and constriction of the visual field.	(Li et al., 2004) (Lee et al., 2001)
Farber's disease	*ASAH1*	Increased level of ceramide was found in retinas	Retinal impairment and vision loss	(Sugita et al., 1972) (Koch et al., 1996) (Yu et al., 2019)
Gaucher disease	*L444P*	Accumulation of glucosylsphingosine	Tapeto-retinal degeneration, pigmented retinal lesions, intra-retinal white dots and inner retinal Gaucher cells.	(Winter et al., 2019)
Macular telangiectasia 2	*SPTLC1 SPTLC2 PHGDH*	Elevated deoxysphingolipid levels in circulation and RPE	Gradual loss of central vision.	(Gantner et al., 2019) (Fallon et al., 2018) (Eade et al., 2021)
Niemann-Pick disease (NPD)	*ASM (SMPD1)-*Types A, B *NPC1, NPC2-*Type C	Accumulation of sphingomyelin (ceramide-phosphocholine), phospholipid, bis(monoacylglycero)phosphate (BMP), lysosphingomyelin (sphingosine phosphocholine) cholesterol, glucocerebroside, lactosylceramide, and gangliosides	Types A-cherry red macula Types B-retinal stigmata Type C- retinal axonal degeneration	(Schuchman and Desnick, 2017) (McGovern et al., 2004) (Vanier, 2015) (Havla et al., 2020)
Retinitis pigmentosa	changes in any one of more than 70 genes	Decreased retinal dolichol, hyperlipidemia or hypolipidemia, reductions in DHA, and differences in AA levels in the plasma	Night blindness and progressive vision loss. Progressive loss of rod photoreceptor cells, followed by loss	(McColl and Converse, 1995) (Ramachandra Rao et al., 2020b)

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				of cone photoreceptor cells.	
	Bassen-Kornzweig syndrome (abetalipoproteinemia: *MTTP*		Low cholesterol and triglyceride levels; absence of low-density lipoprotein, a deficiency in fat-soluble vitamins and essential FAs	Retinal degeneration, hearing impairment, renal cysts and hepatomegaly. Untreated individuals may develop atypical retina pigmentation that may present with progressive loss of night vision and/or color vision in adulthood. Neuromuscular findings in untreated individuals, including progressive loss of deep tendon reflexes, vibratory sense, and proprioception; muscle weakness; dysarthria; and ataxia typically manifest in the first or second decades of life.	(McColl and Converse, 1995) (Kayden, 1972)
	Batten's disease: *CLN3*		Neuronal ceroid lipofuscinoses	Affects the nervous system with increasing seizures, movement disorders, altered thought processes, and cognitive decline. Childhood neuronal ceroid lipofuscinoses include vision loss but adult-onset forms of the disease typically do not.	(Lerner et al., 1995)
	Refsum's disease: *PHYH* or *PEX7*		Deficiency in phytanic acid α-oxidation. As a result, toxic levels of phytanic acid build up in the brain, blood, and other tissues.	Night blindness, with eventual weakness in arms and legs or unsteadiness (cerebellar ataxia). Other common symptoms include a loss of sense of smell (anosmia), rough, scaly skin (ichthyosis) and after many years, deafness.	(van den Brink et al., 2003) (Jansen et al., 1997)
	RP59: *Dhdds*		Decreased retina dolichol levels	Night and peripheral vision loss, constriction of visual fields, and retinal degeneration	(Zelinger et al., 2011) (Ramachandra Rao et al., 2020b)
	RP26: *CERKL*		Decreased spgingolipids	photoreceptors neurodegeneration and progressive vision loss	(Tuson et al., 2004) (Mirra et al., 2021)
	Peroxisomal diseases (Zellweger syndrome) :	*PEX1* *PEX5* *PEX13* *PEX26*	The predominant VLC-PUFAs present in Zellweger brains are penta- and hexaenoic acids, whereas a normal brain contains C32 to C38 tetra- and pentaenoic acids. Zellweger brains also contained trace amounts of C40 *n*-6 VLC-PUFAs, which are absent in normal brains.	Psychomotor delay, dysmorphia, neonatal seizures, retinopathy, cataracts and hearing loss.	(FitzPatrick, 1996) (Ebberink et al., 2011) (Poulos et al., 1988)

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		PEX1	Elevated C26:0 lyso-PC, 18: 2 *n6* and 20: 3 *n6*, and very low levels of 22: 6 *n3*, 22: 5 *n3*, 22: 5 *n6* in retina. Elevated C26:0 and higher ratio of C24:0/C22:0 and C26:0/C22:0 in plasma	Progressive retinopathy leading to blindness.	(Argyriou et al., 2019) (Martinez, 1992)
	Usher syndrome: Usher 1B *MYO7A* Usher 1C *USH1C* Usher 1D *CDH23* Usher 1F *PCDH15* Usher 1G *USH1G*		Lower blood levels of long-chain polyunsaturated FAs (PUFAs).	Partial or total hearing loss and vision loss that worsens over time. Vision loss occurs as the light-sensing cells of the retina gradually deteriorate (RP).	(Geng et al., 2009) (Geng et al., 2009) (Maude et al., 1998) (Géléoc and El-Amraoui, 2020)
Sandhoff disease	*HEXB*		Deficient degradation of glycolipids, accumulation of GM2 ganglioside	The infantile forms are characterized by progressive muscular weakness, mental retardation, blindness, and death in early childhood	(Mahuran, 1999)
Smith-Lemli-Opitz syndrome (SLOS)	*DHCR7*		Depletion of cholesterol, abnormal accumulation of 7-dehydrocholesterol Deceased DHA in rod OS	Progressive retinal degeneration.	(Boesze-Battaglia et al., 2008) (Tulenko et al., 2006)
Stargardt-like macular dystrophy (STGD1)	*ABCA4*		Accumulation of bis(monoacylgylercoro)phosphate lipids, bisretinoid and lipofuscin	Juvenile macular degeneration - reduced light sensitivity or contrast sensitivity and gradual loss of central vision.	(Anderson et al., 2017) (Zhao et al., 2021) (Cideciyan et al., 2004)
Stargardt-like macular dystrophy (STGD3)	*ELOVL4*		Reduced levels of C28–C38 VLC-PUFAs	Juvenile macular degeneration - reduced light sensitivity or contrast sensitivity and gradual loss of central vision.	(Bernstein et al., 2001) (McMahon et al., 2007) (Zhang et al., 2001) (Agbaga et al., 2008)