The essential role of docosahexaenoic acid and its derivatives for retinal integrity

Daniëlle Swinkels[1] and Myriam Baes[1*]

[1] *Laboratory of Cell Metabolism, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, 3000 Leuven, Belgium.*

[*] Corresponding author: email; myriam.baes@kuleuven.be, address: Herestraat 49 O&N2 Box 823, Leuven, telephone: +32 16 33 08 53.

Abstract

The fatty acid composition of photoreceptor outer segment (POS) phospholipids diverges from other membranes, being highly enriched in polyunsaturated fatty acids (PUFAs). The most abundant PUFA is docosahexaenoic acid (DHA, C22:6n-3), an omega-3 PUFA that amounts to over 50% of the POS phospholipid fatty acid side chains. Interestingly, DHA is the precursor of other bioactive lipids such as elongated PUFAs and oxygenated derivatives. In this review, we present the current view on metabolism, trafficking and function of DHA and very long chain polyunsaturated fatty acids (VLC-PUFAs) in the retina. New insights on pathological features generated from PUFA deficient mouse models with enzyme or transporter defects and corresponding patients are discussed. Not only the neural retina, but also abnormalities in the retinal pigment epithelium are considered. Furthermore, the potential involvement of PUFAs in more common retinal degeneration diseases such as diabetic retinopathy, retinitis pigmentosa and age-related macular degeneration are evaluated. Supplementation treatment strategies and their outcome are summarized.

Keywords (max. 6)

- DHA
- VLC-PUFA
- Retina
- Mouse models
- Patients
- Docosanoids

Abbreviations

AdipoR1: adiponectin receptor 1, ALA: α-linolenic acid, AMD: age-related macular degeneration, CEP: carboxyethylpyrrole, CNV: choroidal neovascularization, D-DHA: deuterated docosahexaenoic acid, DHA: docosahexaenoic acid, DPA: docosapentaenoic acid, DR: diabetic retinopathy, ELOVL: elongase of very long chain fatty acids, EPA: eicosapentaenoic acid, ERG: electroretinogram, FA: fatty acid, FADS: fatty acid desaturase, IPM: inner photoreceptor matrix, IRBP: interphotoreceptor retinoid-binding protein, LA: linoleic acid, LC-PUFA: long chain polyunsaturated fatty acid, LPAAT: lysophosphatidic acid acyltransferase, LPC: lysophosphatidylcholine, M: month, Mfsd2a: Major facilitator super family domain containing protein 2a, MFP2: multifunctional protein 2, MFRP: membrane-type frizzle-related protein, n-3: omega-3, NPD1: neuroprotectin D1, P: postnatal day, PBD: peroxisome biogenesis disorder, PC: phosphatidylcholine, PEX: peroxin, PIS: photoreceptor inner segment, POS: photoreceptor outer segment, PUFA: polyunsaturated fatty acid, RD: retinal degeneration, RP: retinitis pigmentosa, RPE: retinal pigment epithelium, RPE65: retinal pigment epithelium 65 kDa protein, STGD3: Stargardt-like macular dystrophy 3, STZ: streptozotocin, TG: triglyceride, TMEM135: transmembrane protein 135, TPA: tetracosapentaenoic acid, VLCFA: very long chain fatty acid, VLC-PUFA: very long chain polyunsaturated fatty acid, W: week, Y: year.

Table of content

1. Introduction

The retina, together with the brain and testis, is highly enriched in omega-3 (n-3) long chain and very long chain polyunsaturated fatty acids (LC-PUFAs (C18-C22), VLC-PUFAs (≥C24)) (Yeboah, Lobanova, Brush, & Agbaga, 2021). The most abundant n-3 PUFA is docosahexaenoic acid (DHA, C22:6n-3). Over the years, the health benefits of DHA have been extensively studied, showing that it is essential throughout all stages of life, from fetal development to prevention of cardiovascular and cognitive diseases in elderly (J. Li, Pora, Dong, & Hasjim, 2021). It is generally accepted that this extraordinary lipid is pivotal for retinal functioning, but there are still many uncertainties and controversies concerning its acquirement in the retina and its specific roles.

The retina is the light sensitive layer in the eye and is roughly subdivided into the neural retina and the retinal pigment epithelium (RPE), which intensively interact to regulate vision (Hildebrand & Fielder, 2011; Masland, 2001). The neural retina consists of several specialized cell types including the photoreceptors, which are the light sensitive neurons of the retina as they convert photons into a nerve signal, through a process called phototransduction. The two types of photoreceptors, rods and cones, share the same principle of phototransduction, but they exhibit important differences in function and spatial distribution. In the human retina, rods outnumber the cones by approximately 20-fold and are predominantly localized to the periphery where they are responsible for night vision. Cones on the other hand, are localized to the central macula and regulate day vision (Hildebrand & Fielder, 2011; Masland, 2001). In the mouse retina, rods represent 97.2% and cones 2.8% of the photoreceptors and are intermingled (Jeon, Strettoi, & Masland, 1998). Each photoreceptor consists of an outer segment, inner segment, connecting cilium, nucleus, inner fiber, and synaptic terminal (Molday & Moritz, 2015). The photoreceptor outer segment (POS) is a modified sensory cilium, filled with densely packed lipid-rich disc membranes, carrying the visual pigments. The inner and outer segments are connected by a narrow connecting cilium (Baehr, et al., 2019). The organelles are located in the inner segments, where synthesis of components that populate the POS occurs. The inner fiber is the axon of the photoreceptor that transmits the light signal via the synaptic terminal to interneurons, which further relay with retinal ganglion cells to the brain (Hildebrand & Fielder, 2011; Masland, 2001).

The RPE is a monolayer of postmitotic, hexagonally shaped, dark pigmented cells that are connected through tight junctions (Strauss, 2005). Their main task is to maintain photoreceptor health by exerting several functions. Firstly, RPE cells act as important distributors of nutrients and waste products. In addition, they absorb scattered light via the melanosomes (Strauss, 2005). Furthermore, RPE cells assist in the visual cycle, as photoreceptors are unable to re-isomerize all-*trans*-retinol, which is formed during the phototransduction, back to 11-*cis*-retinal (a vitamin A derived chromophore) (Lakkaraju, et al., 2020; Strauss, 2005). Another important function of RPE cells is the phagocytosis of damaged POS (Kevany & Palczewski, 2010; Lakkaraju, et al., 2020). Photoreceptors are exposed to intense levels of light each day, leading to accumulation of photo-damaged proteins and lipids. To maintain excitability of photoreceptors, daily a part of these POS is shed by the photoreceptors, ingested and processed by the RPE. The lipid components are either degraded, converted to derivatives or recycled back to the photoreceptors. Of note, in the human retina, each RPE cell supports approximately 45 photoreceptors and it takes about 10 days to replace an entire outer segment (Strauss, 2005).

In this review, we first discuss the synthesis, distribution and roles of DHA and VLC-PUFAs in the retina. Next, we summarize the retinal pathologies in patients and mouse models in which retinal DHA levels are reduced due to either a defined genetic cause or a more general pathology (age-related macular degeneration (AMD), diabetic retinopathy (DR) or retinitis pigmentosa (RP)). We also pay attention to the impact on the RPE. Finally, the potential

contribution of deregulated lipids to retinopathy will be scrutinized and possible therapeutic options will be discussed.

2. PUFA synthesis and transport

2.1. Sources of polyunsaturated fatty acids

Because mammals are unable to synthesize PUFAs *de novo*, they need to acquire either the mature forms from the diet or their precursors (N. G. Bazan, 2007). Whereas eicosapentaenoic acid (EPA, C20:5n-3) and DHA are enriched in fatty fish, α-linolenic acid (ALA, C18:3n-3) and linoleic acid (LA, C18:2n-6) are present in green leaves, vegetable cooking oils, nuts and seeds (Calder, 2016). The latter are the precursors of the n-3 and n-6 series of PUFAs, respectively, and are converted to PUFAs with longer chains and more double bonds. This occurs primarily in the ER of the liver and is catalysed by fatty acid desaturases (FADS) and elongases of very long chain fatty acids (ELOVL) (Fig. 1). This pathway results in the formation of docosapentaenoic acid (DPA, C22:5n-3), which is expected to be converted to DHA via a Δ4-desaturase. However, the enzyme catalyzing this reaction is not found in mammals. Therefore, DPA is first elongated to tetracosapentaenoic acid (TPA, C24:5n-3), followed by Δ6-desaturation to introduce the last double bond, before shortening to DHA via one cycle of peroxisomal β-oxidation (Calder, 2016; Ferdinandusse, et al., 2001). This retroconversion is called the Sprecher pathway (Fig. 1, red arrows) (Sprecher, 2000; Sprecher, Luthria, Mohammed, & Baykousheva, 1995).

Further elongation to VLC-PUFAs is catalysed by ELOVL4 in certain tissues. Notably, the specific expression of ELOVL4 in tissues that are enriched in VLC-PUFAs (retina, testis, brain) or in saturated very long chain fatty acids (VLCFA) (skin, brain) points to the local synthesis of these fatty acids (Agbaga, Mandal, & Anderson, 2010; Yu, et al., 2012). Various PUFAs can be used as elongation substrates, but it was shown *in vitro* that ELOVL4 preferentially elongates EPA (Yu, et al., 2012). Nevertheless, EPA levels in the retina are low compared to DHA levels (10-fold less). Therefore, it was speculated that a retroconversion of DHA to EPA, via peroxisomal β-oxidation, could be an additional source of EPA for the synthesis of VLC-PUFAs in the retina (Fig. 1, dotted arrows) (Jun, et al., 2017; Yu, et al., 2012). However, it seems unlikely that the body would first spend energy synthesizing DHA from EPA in the liver, after which DHA is converted back to EPA in the photoreceptors and subsequently elongated. This issue was recently clarified by studying the retinal lipidome of a mouse model lacking the central enzyme of peroxisomal β-oxidation, multifunctional protein 2 (MFP2, encoded by the *HSD17b4* gene and also known as D-bifunctional protein), specifically in photoreceptors (*Crx-Mfp2*$^{-/-}$ mice) (Swinkels, et al., 2022). No shortage, but on the contrary an accumulation of VLC-PUFAs was found in the *Crx-Mfp2*$^{-/-}$ retina refuting the hypothesis that a retroconversion is required to synthesize these lipids in photoreceptors.

Important to note is that FAs with 24 carbons or more (including VLC-PUFAs) can only be broken down by peroxisomal and not by mitochondrial β-oxidation. On the other hand, DHA can be degraded in both organelles but the relative contribution may be cell type dependent (Van Veldhoven, 2010).

2.2. Retinal DHA: systemic supply versus local synthesis

Ever since Bazan and colleagues demonstrated with *in vivo* [1-^{14}C]18:3 labelling studies that DHA is synthesized in the liver followed by delivery to the retina, it is generally accepted that the retina acquires DHA via the systemic supply (Scott & Bazan, 1989). We recently confirmed these findings by studying a mouse model lacking functional peroxisomes in hepatocytes (*Alb-Pex5*$^{-/-}$ mice) (D. Swinkels and M. Baes, unpublished data). As expected, total DHA levels were more than 50% reduced in liver and plasma of *Alb-Pex5*$^{-/-}$ mice compared to wild-type

mice, which resulted in a significant reduction in DHA-containing phospholipid species in the neural retina (8 weeks (w)). This was accompanied by reduced photoreceptor length, survival and functioning (8w), confirming that the systemic supply of DHA is essential for retinal DHA levels and implying that DHA is crucial to maintain photoreceptor homeostasis.

However, besides the systemic supply, several studies involving labeled precursors and *ex vivo* experiments showed that DHA can be locally synthesized in photoreceptors and the RPE (H. E. Bazan, Careaga, Sprecher, & Bazan, 1982; Rotstein, Pennacchiotti, Sprecher, & Aveldaño, 1996; Simón, et al., 2016; Wang & Anderson, 1993). Given the Sprecher retroconversion pathway, this presumably requires peroxisomal β-oxidation. Indeed, peroxisomes and MFP2 are abundant throughout the entire retina, including the RPE and photoreceptors (Argyriou, et al., 2019; Daniele, et al., 2019; Das, et al., 2019; Das, Swinkels, Kocherlakota, et al., 2021; Smith, et al., 2016; Zaki, et al., 2016). However, loss of MFP2 in photoreceptors (*Crx-Mfp2*$^{-/-}$ mice) did not affect retinal DHA levels nor photoreceptor structure and function (see also section 4.1.2), demonstrating that local synthesis of DHA in photoreceptors is only of minor importance for the retinal DHA pool compared to the systemic supply (Swinkels, et al., 2022).

2.3.Transport of PUFAs: the big and small loop

After uptake from the diet or hepatic synthesis, DHA is transported in lipoproteins from the liver to the target organ, creating the big loop (Fig. 2) (N. G. Bazan, 2007). These lipoproteins contain apoliproteins on their surface, which can bind receptors on the target cells, such as the RPE (Mead, Irvine, & Ramji, 2002). Several transporters are involved in the uptake of DHA into the RPE, including the recently identified Major facilitator super family domain containing protein 2a (Mfsd2a) transporter (Lobanova, et al., 2019; Tachikawa, et al., 2018; Wong, et al., 2016). The adiponectin receptor 1 (AdipoR1) was also shown to import DHA by *in vivo* and *ex vivo* DHA-d5 functional studies (Rice, et al., 2015), but this was later contradicted by immunofluorescent studies that located AdipoR1 in the apical RPE membrane (Lewandowski, Foik, et al., 2022; Osada, et al., 2021; Sluch, et al., 2018). After transcellular transport of PUFAs in RPE cells, they are assumed to be excreted by AdipoR1 into the inner photoreceptor matrix (IPM) and subsequently taken up into the photoreceptor inner segments (PIS) via AdipoR1 (Rice, et al., 2015). While the Mfsd2a transporter shuttles DHA in its lysophosphatidylcholine (LPC) form (Nguyen, et al., 2014), it is not known which form is preferred by AdipoR1. Many other uncertainties remain in the transport of PUFAs from the blood supply to the photoreceptors (Fig. 2, orange question marks). In the PIS, LC-PUFAs can have several fates: conversion into protective mediators, catabolism by peroxisomal β-oxidation, elongation towards VLC-PUFAs, or esterification into the sn-2 position of phosphatidic acids by lysophosphatidic acid acyltransferase 3 (LPAAT3) (Shindou, et al., 2017). Subsequently, these phosphatidic acids are converted to phospholipids, such as phosphatidylcholine (PC), and distributed into the discs in the outer segments (N. G. Bazan, 2007). The composition of the phospholipids in the POS is quite unusual, as they often contain one VLC-PUFA moiety in the sn-1 position and one DHA moiety in the sn-2 position. Peculiar is PC(44:12), also called di-DHA, that contains two DHAs in both the sn-1 and sn-2 position (N. G. Bazan, 2007). To date, no VLC-PUFA transporters have been identified, again pointing to the *in situ* synthesis of these lipids in the photoreceptor inner segments.

The small loop consists of shedding of damaged POS by photoreceptors, followed by phagocytosis by the RPE and subsequent recycling of lipids back to the PIS/POS (N. G. Bazan, 2007). However, a portion of these lipids can be converted into protective mediators (docosanoids, elovanoids) or degraded for energy metabolism (Jun, et al., 2017; Mukherjee, Marcheselli, Serhan, & Bazan, 2004; Reyes-Reveles, et al., 2017). The RPE indeed relies on

fatty acids as an energy source. Furthermore, it was shown that eye cups incubated with POS released ketone bodies that can be used by photoreceptors (Reyes-Reveles, et al., 2017).

The turnover of DHA in the retina appears to be very slow. Recently, mice were supplemented with bis-allylic deuterated docosahexaenoic acid (D-DHA). It took 77 days before the retinal DHA content was replaced by D-DHA for 97% (RPE) and 92% (neural retina) and it took 74 days to reduce D-DHA again to 7% (RPE) and 10% (neural retina) when switched to a diet containing natural DHA (James, et al., 2022).

3. PUFAs exert diverse functions in the eye

3.1. DHA: an essential omega-3 fatty acid in the retina

Already half a century ago, the importance of DHA for the retina was demonstrated as insufficient intake of this PUFA caused impaired retinal functioning in rats (Benolken, Anderson, & Wheeler, 1973; Wheeler, Benolken, & Anderson, 1975), mice (Senapati, et al., 2018), guinea pigs (Weisinger, Vingrys, Abedin, & Sinclair, 1998; Weisinger, Vingrys, Bui, & Sinclair, 1999; Weisinger, Vingrys, & Sinclair, 1996a, 1996b), rhesus monkeys (Jeffrey, Mitchell, Gibson, & Neuringer, 2002; Neuringer, Connor, Lin, Barstad, & Luck, 1986; Neuringer, Connor, Van Petten, & Barstad, 1984) and humans (D. G. Birch, E. E. Birch, D. R. Hoffman, & R. D. Uauy, 1992; E. E. Birch, D. G. Birch, D. R. Hoffman, & R. Uauy, 1992; Uauy, Birch, Birch, Tyson, & Hoffman, 1990). This is not surprising given the abundance of DHA in the retina. With the recent advances in matrix assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) it was possible to visualize the localization of these lipids in the retina (D. M. Anderson, et al., 2014; D. M. G. Anderson, et al., 2020; Vidal, et al., 2020; Zemski Berry, Gordon, Murphy, & Bazan, 2014). DHA is especially concentrated in the POS, where it is present in the sn-2 position of glycerophospholipids, and amounts to over 50% of the phospholipid fatty acyl chains (Calder, 2016; Rice, et al., 2015). Here, DHA is involved in multiple processes, including photoreceptor biogenesis, function and survival (Fig. 3).

Photoreceptor development can be divided into five distinct steps and is mainly regulated by the interplay of several transcription factors (Swaroop, Kim, & Forrest, 2010). However, additional support by environmental signals, such as DHA, is necessary as well (Garelli, Rotstein, & Politi, 2006). The involvement of DHA in photoreceptor development was shown via *in vitro* experiments on rat retinal neuron cells. It was demonstrated that in the absence of DHA, photoreceptor cells degenerated, while addition of DHA selectively prevented photoreceptor cell death (Rotstein, Aveldaño, Barrantes, & Politi, 1996). The same research group showed in subsequent years that photoreceptor cells rely on DHA for differentiation, development and survival, via regulating photoreceptor specific gene expression, promoting axonal outgrowth, stimulating POS formation and preventing apoptosis (L. Politi, N. Rotstein, & N. Carri, 2001; L. E. Politi, N. P. Rotstein, & N. G. Carri, 2001; Rotstein, Aveldaño, Barrantes, Roccamo, & Politi, 1997; Rotstein, Politi, & Aveldaño, 1998; Rotstein, Politi, German, & Girotti, 2003).

Based on the physicochemical properties of DHA, i.e. the high abundance of unsaturated *cis*-oriented double bonds, it can be expected that they influence membrane characteristics (Hishikawa, Valentine, Iizuka-Hishikawa, Shindou, & Shimizu, 2017). Firstly, the structure of the polyunsaturated FA is more curved due to the double bonds, leading to a decrease in hydrophobic interactions and thus increase in membrane fluidity, which could influence cellular functions that depend on membrane dynamics (i.e. phototransduction). Furthermore, these double bonds allow rotation of vinyl-methylene bonds, which increases the flexibility of the membrane, consequently influencing membrane fusion and fission processes and conformational changes to membrane proteins. These double bonds also result in thinner membranes, affecting localization and trafficking of membrane proteins. Overall, these more

fluid, flexible and thinner membranes can also result in increased permeability to small polar molecules (Hishikawa, et al., 2017).

DHA is also considered to be essential for the phototransduction process. It is believed that DHA and rhodopsin interact with each other, as rhodopsin has specific binding sites for DHA. Furthermore, radio-labelling studies showed that newly synthetized DHA-containing phospholipids are co-transported with rhodopsin to the POS and they reach the tip of the POS at the same time (Gordon & Bazan, 1990; Grossfield, Feller, & Pitman, 2006; Rodriguez de Turco, Deretic, Bazan, & Papermaster, 1997; Soubias, Teague, & Gawrisch, 2006; Wiedmann, Pates, Beach, Salmon, & Brown, 1988). This interaction influences the functioning of rhodopsin, including its activation, regeneration and rhodopsin-transducin coupling (Mitchell, Niu, & Litman, 2001; Niu, Mitchell, & Litman, 2001; Sánchez-Martín, Ramon, Torrent-Burgués, & Garriga, 2013; Senapati, et al., 2018). Recently, using styrene maleic acid (SMA) extraction in combination with lipidomic analysis, the localization of the different lipids on the POS discs was identified. It revealed that the center regions of the POS discs, where rhodopsin is located, were highly abundant in LC- and VLC-PUFAs, while the rim regions, not containing the visual opsins, were enriched in shorter saturated FAs (Sander, et al., 2021). Interesting to note is that, compared to rod-dominant animals, the retinas and POS from cone-dominant animals contained almost 2-fold less PC(44:12) (di-DHA), suggesting that DHA could be more important for rhodopsin than cone opsin functioning (Agbaga, et al., 2018).

In addition, DHA improves the efficiency of the visual cycle, by regulating the affinity of the interphotoreceptor retinoid-binding protein (IRBP) for the visual cycle intermediate 11-*cis*-retinal. Near the RPE, where DHA levels are lower, IRBP preferentially binds 11-*cis*-retinal, while at the POS, where the DHA concentration is higher, 11-*cis*-retinal is released from IRBP (Y. Chen, Houghton, Brenna, & Noy, 1996).

Bazan et al. demonstrated that, under uncompensated oxidative stress, RPE cells can convert DHA to neuroprotection D1 (NPD1) via several rounds of lipoxidation and hydrolysis (N. G. Bazan, 2005). This derivative belongs to a group of lipid mediators, called docosanoids, which have anti-inflammatory and cytoprotective functions on the RPE and photoreceptors (N. G. Bazan, Molina, & Gordon, 2011; Mukherjee, et al., 2004). They can inactivate proapoptotic signaling by stimulating the antiapoptotic Bcl-2 and BclXL protein expression, attenuating the proapoptotic Bax and Bad and inhibiting caspase-3 activation (Asatryan & Bazan, 2017; N. G. Bazan, 2005; Mukherjee, et al., 2004). Moreover, two other groups of protective lipid mediators can be synthetized from DHA, namely resolvins and maresins (Calder, 2016). However, it remains heavily debated whether DHA only plays a protective role in the highly oxidative environment of the retina (German, Agnolazza, Politi, & Rotstein, 2015). Peroxidation products of DHA, such as 4-hydroxyhexenal (4-HHE) and 4-hydroxy-7-oxohept-5-enoic acid (HOHA), can lead to the formation of carboxyethylpyrrole (CEP), which are deleterious for photoreceptors (Gu, et al., 2003; Y. Liu, Zhang, Wu, & Ji, 2014; Tanito, et al., 2009; Tanito, Elliott, Kotake, & Anderson, 2005). It was suggested that the impact of DHA may depend on the circumstances such as the level of oxidative stress (German, et al., 2015).

3.2. VLC-PUFAs in the retina

VLC-PUFAs are believed to have similar functions in the retina as DHA (Fig. 3). They indeed share the same high degree of unsaturation, but in addition have longer carbon chains, which will affect their physicochemical properties. Although VLC-PUFAs are enriched in photoreceptors, it should be kept in mind that the levels of C24 PUFAs and longer are several orders of magnitude lower than the ubiquitous DHA (A. Liu, Terry, Lin, Nelson, & Bernstein, 2013). Firstly, VLC-PUFAs may affect the phototransduction process, as this is dependent on the lipid bilayer characteristics (Deák, Anderson, Fessler, & Sherry, 2019). Furthermore, VLC-PUFAs were shown to colocalize with rhodopsin and it was hypothesized that they interfere in

the recycling of 11-*cis*-retinal (Agbaga, et al., 2010; McMahon & Kedzierski, 2010; Sander, et al., 2021). Besides the enrichment in POS, VLC-PUFAs are also abundant in the photoreceptor ribbon synapses, where they were proposed to play a role in synaptic vesicle dynamics and functioning (Bennett, Hopiavuori, et al., 2014; McMahon & Kedzierski, 2010; Swinkels, et al., 2022). Lowered VLC-PUFA levels were shown to promote membrane fusion and vesicle content release (Deák, et al., 2019; Hopiavuori, Anderson, & Agbaga, 2019). It is unclear whether the VLC-PUFA are recycled from the RPE to the inner segments, similar to DHA. Lastly, it was recently shown that the VLC-PUFAs C32:6n-3 and C34:6n-3 can be released from the sn-1 position of PC and converted to protective mediators, called elovanoids, via two hydroxylation steps. This can occur both in photoreceptors and in RPE cells (Jun, et al., 2017). Like the docosanoids, the elovanoids are upregulated when the eye is exposed to stress, resulting in upregulation of the pro-survival proteins BCL-2 and BCL-XL and downregulation of the pro-apoptotic proteins BAX, BIM and BID. In addition, elovanoids were shown to stimulate protein levels of the pro-homeostatic sirtuin-1, pro-survival prohibitin and quality-control protein iduna (N. G. Bazan, 2021). Thus, the elovanoids play a pro-homeostatic role and protect both the photoreceptors and the RPE. It remains to be elucidated, how elovanoids complement the anti-inflammatory and neuroprotective properties of docosanoids.

4. Disorders with a genetic defect in the acquisition of retinal PUFAs

Although DHA and VLC-PUFAs seem to play an essential role in the retina, the mechanistic details of synthesis, trafficking and function remain unclear and there are still a lot of controversial findings. In recent years, novel players in retinal PUFA homeostasis have been identified, allowing to study the retinal consequences of loss-of-function approaches in mice. Here, the retinal phenotype of these mouse models with a primary defect in DHA or VLC-PUFA homeostasis will be discussed, together with the corresponding patients. Furthermore, attention will be given to the importance of PUFAs for RPE integrity. Data are summarized in Table I. It is important to note that in order to decipher the cause – consequence relation between DHA and retinal degeneration, it is essential to assess the lipidome before retinopathy starts. Indeed, a reduction in the number or length of photoreceptors, will evidently cause reduced levels of DHA in the neural retina.

4.1. Impaired generation of DHA-containing (phospho-)lipids

The generation of DHA involves various enzymes present in the ER and peroxisomes (Fig. 1). Although many mouse models were generated with deficiency in these enzymes (the peroxisomal ones were extensively reviewed by Das et al. (Das, Swinkels, & Baes, 2021)), only in a few both the retinal phenotype and DHA levels were reported.

4.1.1. Elongation of very long chain fatty acids 2

ELOVL2, acting on PUFAs with 20 and 22 carbons and necessary for the synthesis of DHA precursors, is highly expressed in the liver, but was also detected in photoreceptors and the RPE (D. Chen, et al., 2020). Several mouse models lacking functional ELOVL2 were generated. *Elovl2*$^{-/-}$ mice in the C57BL/6 background displayed defects in spermatogenesis and are infertile, which made them not suitable to use for breeding (Zadravec, et al., 2011). *Elovl2*$^{-/-}$ mice in the 129S2/Sv background (which were fertile) presented with a 90% reduction in both DHA-containing phospholipid and triglyceride (TG) species in the liver and total DHA levels in plasma, impairing the supply of DHA to the retina (Pauter, et al., 2014). Unfortunately, the retinal lipid levels and phenotype were not reported. Finally, *Elovl2*^{c234w} mice with a cysteine-to-tryptophan substitution destroying the elongase activity, displayed reduced retinal DHA

levels, which were accompanied by impaired scotopic electroretinogram (ERG) responses and RPE abnormalities including basal laminar and autofluorescent deposits, all at the age of 6 months (M) (D. Chen, et al., 2020).

To date, no individuals with mutations or genomic variants in *ELOVL2* were identified. Only recently a role for ELOVL2 in AMD was suggested, as *Elovl2* transcripts gradually decline in the retina in aging WT mice (D. Chen, et al., 2020) and chromatin accessibility on the *ELOVL2* promotor is significantly reduced in AMD retinas (Lewandowski, Sander, et al., 2022).

The lack of pathological information of the mouse models, hampers insights on the relation between DHA levels and retinal integrity, but the ERG data from the *Elovl2*^{c234w} mice do provide a first indication.

4.1.2. Peroxisomal defects

Given the essential role of peroxisomal β-oxidation in the Sprecher pathway to form DHA, we investigated the retinal phenotype of a mouse model lacking the pivotal enzyme MFP2 (*Mfp2*$^{-/-}$) (Das, Swinkels, Kocherlakota, et al., 2021). Extensive lipidome analysis on plasma and neural retina at 3w, revealed a significant reduction in DHA-containing phospholipid species, and a depletion of phospholipid species containing di-DHA, PC(44:12). Furthermore, the neural retina had a peculiar profile of the phospholipid species containing one DHA and one VLC-PUFA moiety. While PC species containing up to 56 carbons were severely abolished, those above 56 carbons accumulated. The lack of peroxisomal β-oxidation most likely enables uncontrolled elongation of PUFAs, despite the low DHA levels. These changes in lipid species were accompanied by an early onset retinal degeneration, consisting of impaired visual function at 3w, POS shortening already at 2w and progressive photoreceptor degeneration. Furthermore, some RPE abnormalities were observed, including RPE protrusions into the POS layer (9w) and microglia migration in the subretinal space (3w) (Das, Swinkels, Kocherlakota, et al., 2021).

As already mentioned, to investigate the cell-autonomous role of MFP2 in photoreceptors, mice with cell-type selective loss of MFP2 in photoreceptors and bipolar cells (*Crx-Mfp2*$^{-/-}$) were generated (section 2.2). Remarkably, the appearance, survival and functioning of photoreceptors were not affected till the age of 1 year (Y) (Swinkels, et al., 2022). However, from the age of 6M, loss of photoreceptor ribbon synapse integrity and impaired bipolar cell survival and functioning was seen. Full lipidome analysis on the neural retina of *Crx-Mfp2*$^{-/-}$ mice revealed normal levels of DHA-containing phospholipid species, while VLC-PUFAs accumulated. These data show that peroxisomes in photoreceptors are not important to maintain retinal DHA levels, but are essential to degrade VLC-PUFAs. Interestingly, the accumulation of VLC-PUFAs appears to affect the photoreceptor ribbon synapses and not the function of the POS. The major difference in phenotype between global *Mfp2*$^{-/-}$ and *Crx-Mfp2*$^{-/-}$ mice indicates that the early retinal abnormalities of global *Mfp2*$^{-/-}$ mice are caused by reduced retinal DHA levels, due to impaired systemic supply, and not by the VLC-PUFA accumulation.

Besides the *Mfp2* knockout mouse, several mouse models with a peroxisome biogenesis disorder (PBD) and thus broader peroxisome dysfunction were generated. Peroxisome biogenesis requires 13 peroxin proteins, encoded by *PEX* genes, that are involved in the formation of the peroxisomal membrane or in the import of matrix proteins (Wanders, Baes, Ribeiro, Ferdinandusse, & Waterham, 2022). The early postnatal death of peroxin knockout mice (Baes & Van Veldhoven, 2006) does not allow to assess the retina, except for *Pex1*^{G844D} mice, which mimic the most common and mild human PBD, *PEX1*^{G843D} (Argyriou, et al., 2019; Hiebler, et al., 2014). The *Pex1*^{G844D} mouse presented with severely impaired a- and b-wave responses from 2 weeks of age and reduced visual acuity at 11-13w. Interestingly, this was

accompanied by a severe reduction in the number of cones at 6w, while rods appeared to be unaffected. In addition, photoreceptor length was unaltered and at the ultrastructural level, photoreceptors showed normal POS, but disorganized PIS in 32-week-old mice (Argyriou, et al., 2019). The retinal levels of LPC(26:0) were elevated, but DHA and VLC-PUFAs were not reported, precluding insights in their contribution to the pathology.

Patients with a PBD or with MFP2 deficiency, present with a similar phenotype that can range from a pan-organ developmental pathology (Zellweger syndrome), resulting in early postnatal death, to milder pathologies with survival into adulthood (Wanders, et al., 2022). Patients are usually diagnosed by increased levels of saturated VLCFAs in plasma, but a reduction of plasma DHA levels was also reported, although less commonly (Ferdinandusse, et al., 2006). Severely affected patients present with multiple eye abnormalities, such as a flecked retina, nystagmus, cataract, atrophy of the optic nerve and ganglion cells (Bae, et al., 2020; Ferdinandusse, et al., 2006; Landau, et al., 2020). Milder mutations result in abnormal retinal pigmentation, retinopathy and reduced visual acuity and function (Berendse, et al., 2016; Ventura, et al., 2016). Only a few old histological reports on the retina of Zellweger syndrome patients are available. These revealed RPE atrophy, photoreceptor inner and outer segment shortening, reduced photoreceptor nuclei, atrophy of ganglion cells and the optic nerve and macrophage migration in the retina, optic nerve and vitreous (Cohen, et al., 1983; Glasgow, Brown, Hannah, & Foos, 1987). Lately, fundoscopy of a mild PBD patient showed a dull macula and abnormal retinal pigmentation. OCT revealed severe outer retina atrophy, hyperreflective spots, RPE atrophy and RPE bulging into the POS layer. Furthermore, dark- and light-adapted ERG responses were severely affected (Courtney & Pennesi, 2013). Importantly, in one PBD patient the retinal DHA levels were reported, revealing severely reduced levels (Martinez, 1992).

Taken together, despite the strong association between reduced DHA levels and neural retina degeneration in *Mfp2*$^{-/-}$ mice, there is currently no absolute evidence that altered PUFA levels contribute to the retinal pathology in peroxisomal disorders. Also, the outcome of DHA treatment studies to PBD patients was inconclusive (see section 6.1.1). Studying DHA-supplementation to newborn *Mfp2*$^{-/-}$ pups and its impact on the retinal phenotype would be very instructive to prove a causative link between DHA and retinal integrity in this model. Furthermore, better characterization of the retina of patients is desirable.

4.1.3. Transmembrane protein 135

The exact role of transmembrane protein 135 (TMEM135), which localizes to both mitochondria and peroxisomes, is unknown. Recently, it was postulated that it is involved in the export of DHA from peroxisomes (Landowski, et al., 2023) (Fig. 2). This was based on findings in *Tmem135* mutant mice (*Tmem135*$^{FUN025/FUN025}$, resulting in a non-functional protein) in which DHA levels were severely reduced in liver, plasma and retina, despite normal expression of all enzymes required for DHA synthesis. Given its location in the peroxisomal membrane and the fact that, to date, no such peroxisomal exporter is known, it was speculated that in the absence of TMEM135, DHA cannot leave the peroxisome and is degraded in the organelle by β-oxidation (Landowski, et al., 2023). However, direct evidence that TMEM135 is a DHA transporter in the peroxisomal membrane is required and would add an important insight into the path that n-3 PUFAs take in the retina.

Tmem135 mutant mice presented with retinal abnormalities, including reduced photoreceptor function (7M), survival (2M) and length (12M) (Landowski, et al., 2022; Lee, et al., 2016). The RPE phenotype consisted of RPE hypertrophy, autofluorescent deposits, reduced c-wave responses, accumulation of lipid droplets and microglia migration into the subretinal space (Landowski, et al., 2022; Landowski, et al., 2020). Patients with mutations in

TMEM135 have not been reported (Beasley, Rodman, Collins, Hinton, & Exil, 2021; Lee, et al., 2016).

Even though it remains to be proven that TMEM135 is a peroxisomal DHA exporter, the lowered retinal DHA levels in combination with the impaired retinal integrity in *Tmem135* mutant mice are undisputable. Nevertheless, it needs to be elucidated whether the impaired photoreceptor integrity is caused by lowered systemic DHA levels or by the RPE degeneration. Also, the impact on VLC-PUFA levels remains unsolved.

4.1.4. Lysophosphatidic acid acyltransferase 3

LPAAT3, also called acylglycerol-3-phosphate-O-acyltransferase (AGPAT3) is responsible for the esterification of PUFAs, including DHA, into the sn-2 position in lysophospholipids (Harayama, et al., 2014; Koeberle, Shindou, Harayama, & Shimizu, 2010). Interestingly, *in situ* hybridization staining for *Lpaat3* revealed that it is localized to the inner segments of the photoreceptors. Therefore, it was hypothesized that it is involved in the incorporation of DHA into LPC species in the PIS, after which they are integrated into the POS discs (Shindou, et al., 2017). Indeed *Lpaat3*$^{-/-}$ mice displayed a striking retinal phospholipid profile. Already from postnatal day (P) 11, phospholipid species containing two DHA moieties (PA(44:12), PC(44:12), PE(44:12), PS(44:12)) were almost ablated (Shindou, et al., 2017). Also, phospholipid species containing most likely one DHA and one VLC-PUFA moiety were severely reduced. Despite the early deficiency of DHA-containing phospholipid species, POS shortening was only seen starting from 3w of age. Furthermore, 6-week-old mice exhibited loss of photoreceptor nuclei and at 8w both light- and dark-adapted a- and b-wave responses were severely impaired. Electron microscopy analysis revealed severely disorganized POS, while the PIS and connecting cilium were spared. Unfortunately, the RPE phenotype of the *Lpaat3*$^{-/-}$ mouse was not described. LPAAT3/AGPAT3 has not been associated with any human disease (Takeuchi & Reue, 2009).

The *Lpaat3*$^{-/-}$ mice thus confirm that DHA-containing phospholipid species are crucial for photoreceptor integrity and functioning, as there are no confounding factors influencing the phenotype other than reduced levels of these phospholipids.

4.2. Deficient trafficking of DHA in the retina

4.2.1. Adiponectin receptor 1

AdipoR1 was first identified as a receptor for adiponectin, an adipokine that influences the insulin response. However, by phenotypic screening, retinal degeneration was detected in *AdipoR1*$^{-/-}$ mice, which was not recapitulated in *adiponectin*$^{-/-}$ mice, suggesting a different mechanism underlying the retinopathy (Rice, et al., 2015).

The retinal phenotype of *AdipoR1*$^{-/-}$ mice of three different sources was investigated in depth, and their retinal phenotype concurred with each other (Lewandowski, Foik, et al., 2022; Osada, et al., 2021; Rice, et al., 2015; Sluch, et al., 2018). Histological assessment revealed a progressive retinal degeneration, with loss of photoreceptor nuclei starting from P28. Although not mentioned by the authors, shortened POS were already visible at P14. Moreover, *AdipoR1*$^{-/-}$ mice exhibited severely reduced ERG responses at 3-4w (Osada, et al., 2021; Rice, et al., 2015). This was accompanied by a reduction of photoreceptor specific proteins, downregulation of visual system pathways and upregulation of inflammatory and immune system markers at 3w (Sluch, et al., 2018). Interestingly, loss of AdipoR1 also downregulates retinal *Elovl2* levels (Osada, et al., 2021). With regard to PUFA levels, *AdipoR1*$^{-/-}$ mice displayed a severe reduction of total and unesterified retinal DHA levels (P20), and near ablation of phospholipid species containing di-DHA (PC(44:12)) or a combination of DHA and VLC-PUFA (Osada, et al., 2021; Rice, et al., 2015). Importantly, these lipid changes occurred before the retinal degeneration. Therefore, it was hypothesized that the AdipoR1 receptor could

be involved in retinal DHA uptake and retention. This was investigated via three approaches. (i) Systemic delivery of labelled DHA (DHA-d5) to P14-old mice, before the onset of retinal degeneration, resulted in less incorporation in the *AdipoR1*$^{-/-}$ retina compared to WT mice. (ii) *AdipoR1*$^{-/-}$ eyecups took up lesser DHA-d5 compared to WT eyecups. (iii) Overexpression of AdipoR1 in ARPE-19 cells enhanced DHA uptake (Rice, et al., 2015). Remarkably, despite the convincing evidence that AdipoR1 is a basally-located RPE transporter, immunohistochemical stainings by three groups located AdipoR1 to the apical side (Lewandowski, Foik, et al., 2022; Osada, et al., 2021; Sluch, et al., 2018). Therefore, it remains to be clarified with double stainings, whether AdipoR1 is expressed on the apical or basal side of the RPE and in which form DHA is taken up in the RPE.

Next, the cell-autonomous role of AdipoR1 in the RPE and photoreceptors was investigated via subretinal injection of AAV-VMD2-Cre or AAV-IRBP-Cre vectors respectively, into 9M old floxed *AdipoR1* mice, and retinal phenotyping 5M post-injection (Sluch, et al., 2018). Remarkably, only rhodopsin and retinal pigment epithelium 65 kDa protein (RPE65) levels were significantly reduced in the photoreceptor specific knockout mouse (*IRBP-AdipoR1*$^{-/-}$), while other phototransduction proteins were unaffected. In contrast, loss of *AdipoR1* specifically in the RPE (*VMD2-AdipoR1*$^{-/-}$) resulted in significant reduction of all investigated phototransduction proteins, with the exception of RPE65 (Sluch, et al., 2018). These data point to an essential role of AdipoR1 in the RPE. However, caution is warranted with the interpretation of these results, as subretinal injections are subject to variation in injection efficiency. Therefore, these data need to be confirmed by crossing the floxed mice with appropriate Cre lines.

During the initial characterization of the *AdipoR1*$^{-/-}$ mouse, several RPE abnormalities were described, including autofluorescent deposits, infiltration of F4/80 labeled macrophages and undigested POS debris (20w) (Rice, et al., 2015). However, Lewandowski et al. revealed that the RPE phenotype started much earlier. Already at P20, *AdipoR1*$^{-/-}$ RPE flatmounts showed loss of hexagonal shape, enlarged RPE cells and microglia activation. By 3M, *AdipoR1*$^{-/-}$ RPE cells started to detach from the monolayer (own interpretation) (Lewandowski, Foik, et al., 2022). In addition, transcriptome analysis on RPE cells of 30-day-old *AdipoR1*$^{-/-}$ mice revealed significant upregulation of pathways involved in phagocytosis and microglia activation (Lewandowski, Foik, et al., 2022). At this point it is unclear whether the RPE pathologies are a consequence of the photoreceptor dysfunction, or whether they are caused in a cell-autonomous way. Unfortunately, the histology of the *VMD2-AdipoR1*$^{-/-}$ mouse was not reported, which would have been insightful to explore the role of AdipoR1 in the RPE (Sluch, et al., 2018).

Recently, it was proposed that the retinal phenotype of the *AdipoR1*$^{-/-}$ mice was not only caused by depleted DHA levels, but that accumulating ceramides played a role as well (Lewandowski, Foik, et al., 2022; Lewandowski, Sander, et al., 2022). It was previously shown that AdipoR1 contains an intrinsic ceramidase activity (Holland, et al., 2011; Vasiliauskaité-Brooks, et al., 2017). Indeed, treatment with desipramine/L-cycloserine (DC), known inhibitors of ceramide-generating pathways, increased photoreceptor survival by 37% compared to non-treated *AdipoR1*$^{-/-}$ mice. However, more investigation is warranted into the relative contribution of ceramide accumulation versus DHA reduction to the retinal phenotype.

Interestingly, even before the findings in the *AdipoR1* knockout mouse models, it was reported that several RP patients have a mutation in the *AdipoR1* gene (Xu, et al., 2016; J. Zhang, et al., 2016). These include an autosomal dominant mutation affecting structure and protein folding, resulting in mislocalization of the protein. The first noticeable symptom was night blindness, with a variable age of onset between childhood and twenties. Other ocular symptoms include reduced visual acuity, impaired ERG responses, pigmentary retinopathy, RPE atrophy and thinning of the outer nuclear layer (ONL) (J. Zhang, et al., 2016).

Furthermore, a patient with a homozygous frameshift mutation already showed signs of pigmentary retinopathy and impaired myopic refractive error by 7 years of age. By 27 years, the patient did not have functional visual field or peripheral vision and further investigation revealed cataracts, vascular attenuation, waxy optic nerve pallor and extensive pigmentary retinopathy in both eyes (Xu, et al., 2016). In addition, a study in the Finnish population associated AMD with a single nucleotide polymorphism (SNP) in an intron in *ADIPOR1* (Kaarniranta, et al., 2012). The authors suggested that this SNP could be associated with the adiponectin activity of AdipoR1, as reduced adiponectin levels are a known risk factor for AMD. This is, however, in disagreement with the absence of retinal pathology in *adiponectin*$^{-/-}$ mice, indicating that more research is required (Rice, et al., 2015). Finally, AdipoR1 expression is upregulated in the retinas of DR patients (Lin, et al., 2013).

Combining the retinal phenotype of the AdipoR1 deficient mouse model and patients, it is clear that this DHA-transporter is essential to maintain retinal DHA homeostasis. As DHA levels were already reduced before retinal degeneration occurred, it seems that this is the underlying cause for the observed retinal phenotype. However, some questions remain with regard to the localization of AdipoR1 in the RPE (apical vs basal), in which form it transports DHA and to what extent the ceramidase activity plays a role.

4.2.2. Major facilitator superfamily domain-containing protein 2a

Importantly, loss of the AdipoR1 transporter does not completely ablate retinal DHA levels, suggesting that this is not the sole DHA transporter in the basal RPE. Indeed, Mfsd2a, which was initially reported as the primary LPC(DHA) transporter across the blood-brain-barrier (Nguyen, et al., 2014), is also located in the RPE, where it is involved in the transport of LPC(DHA) over the blood-retinal-barrier (Wong, et al., 2016). In agreement, uptake of intravenously injected radiolabeled LPC-[^{14}C]DHA was reduced by 50% in *Mfsd2a*$^{-/-}$ eyes (Wong, et al., 2016). Furthermore, lipidome analysis on whole eyes of *Mfsd2a*$^{-/-}$ mice showed a striking reduction of DHA-containing phospholipids (8w). Interestingly, shortening of the POS was already visible at P13 and discs were disorganized and misfolded, suggesting a defect in photoreceptor development and maturation. At later ages, *Mfsd2a*$^{-/-}$ mice showed loss of photoreceptor nuclei, reduced rhodopsin levels and defective rhodopsin trafficking. Strikingly, despite photoreceptor shortening and death, and reduced DHA levels, only a small non-significant, reduction in ERG responses was measured (4M), which is in clear contrast with previously discussed models (Wong, et al., 2016).

A few years later, Arshavsky's lab published the characterization of an independently generated *Mfsd2a*$^{-/-}$ mouse, displaying a less severe retinal degeneration (Lobanova, et al., 2019). A small loss (12%) of photoreceptor nuclei was visible at 1M, which progressively worsened with age. Unexpectedly, POS length and structure appeared normal and single-cell rod responses were not altered. It has to be noted that, unlike full-field ERG, single-cell recordings do not take into account the fraction of rods that died (Lobanova, et al., 2019). Despite the mild retinal pathology, levels of LPC(DHA), PC(44:12) and PC most likely containing one DHA and one VLC-PUFA moiety (PC(>46:12)) were more severely reduced than in the previously described *Mfsd2a*$^{-/-}$ mouse.

Interestingly, microglia migration into the subretinal space was already observed at an early age in both *Mfsd2a*$^{-/-}$ mouse models (P14 – 1M) (Lobanova, et al., 2019; Wong, et al., 2016). At later ages undigested POS accumulated in the RPE (4M) and microvilli were lost (5M) (Wong, et al., 2016).

The expression of the Mfds2a transporter was recently also investigated in the human retina, demonstrating that it is present in all neural retina cell types (Ruiz-Pastor, et al., 2022). Until now, four families with homozygous mutations in the *Mfsd2a* gene have been reported (Alakbarzade, et al., 2015; Guemez-Gamboa, et al., 2015; Harel, et al., 2018). The identified

mutations caused inactivation of transport activity, while maintaining stable expression and localization of the transporter to brain plasma membranes. Interestingly, elevated plasma LPC(DHA) levels were reported in all patients, as these lipids cannot be taken up by the Mfsd2a transporter (Alakbarzade, et al., 2015; Guemez-Gamboa, et al., 2015; Harel, et al., 2018). Remarkably, funduscopic examination was normal, but esotropic strabismus was reported in two patients (Harel, et al., 2018).

It is unclear why the two Mfsd2a deficient mouse models differ in their phenotype. It was proposed that the first model (Wong, et al., 2016) expressed the spontaneously occurring retinal degeneration 8 (rd) mutation. However, this seems unlikely as wild-type mice appeared normal and the phenotype is milder than would be expected of an rd8 mutation. The second *Mfsd2a*$^{-/-}$ mouse model (Lobanova, et al., 2019) does seem to be in coherence with the mild retinal phenotype of *Mfsd2a* patients. It remains unresolved why ERG responses are unaltered in both models, despite extensive reduction of retinal DHA levels. Another unexpected finding is the severely reduced retinal DHA levels in both the *Mfsd2a*$^{-/-}$ and *AdipoR1*$^{-/-}$ mouse models, as both are claimed to be DHA transporters. It appears that lack of one of the transporters does not cause a compensatory increase in activity of the other. Therefore, it would be informative to gain insight into the relative contribution of each transporter for DHA uptake and to investigate the levels of both transporters in the two *Mfsd2a* deficient mouse models.

4.2.3. Membrane-type frizzle-related protein

Rd6 mice were first described as a new mouse model to study RP and were later found to have a 4 bp deletion in the membrane-type frizzle-related protein (*Mfrp*) gene (Hawes, et al., 2000; Kameya, et al., 2002). This protein localizes to the apical side of the RPE, however its function in the retina is still unresolved (Won, et al., 2008). More recently, it was discovered that AdipoR1 levels were obliterated on both RNA and protein level in *Mfrp*$^{rd6/rd6}$ retinas and thus it was hypothesized that the phenotype of mutant *Mfrp* mice was due to impaired uptake and retention of retinal DHA (Sluch, et al., 2018).

The retinal pathology of *Mfrp*$^{rd6/rd6}$ and *AdipoR1*$^{-/-}$ mice are indeed very similar. Histological assessment of *Mfrp*$^{rd6/rd6}$ mice revealed developmental problems as early as P14 in both POS length and organization. Furthermore, there was an extensive loss of photoreceptor nuclei from P28, which coincided with severely impaired a- and b-wave responses at P25. On the other hand, cone function and morphology seemed to be less affected (Won, et al., 2008). The importance of MFRP for maintenance of genes involved in the visual cycle (*Rpe65*, *Lrat*, *Rgr*), phototransduction (*Rgs, Guca1b, Pde6a*) and structural components of rods (*RpGrip1, Fscn2*) was shown via microarray analysis on P14-old retinas (Soundararajan, et al., 2014). Interestingly, lipidome analysis on *Mfrp*$^{rd6/rd6}$ retinas revealed that phospholipid species containing either two DHA moieties (PC(44:12)) or one DHA and one VLC-PUFA moiety were almost eliminated (Kautzmann, et al., 2020). Unfortunately, the age at which this lipidome analysis was performed, was not mentioned. Therefore, it is impossible to conclude whether the reduction in these lipid species are the cause or consequence of the retinal degeneration.

Also, the RPE, where MFRP is located, was thoroughly investigated. Already at P14, lesser and disorganized RPE microvilli were seen in *Mfrp*$^{rd6/rd6}$ mice, which worsened with age. This impaired the efficiency of POS uptake by the RPE. However, caution is warranted for the interpretation of these results, as POS phagocytosis was investigated in 2-month-old *Mfrp*$^{rd6/rd6}$ mice, at which age the POS were already severely shortened (Won, et al., 2008). Interestingly, autofluorescent deposits, microglia activation, RPE protrusions and ezrin mislocalization to the basolateral side were also present (own interpretation of published data) (Soundararajan, et al., 2014; Won, et al., 2008). Furthermore, visual cycle genes, such as *rpe65* and *lrat*, were already significantly downregulated at P14 (Soundararajan, et al., 2014; Won, et al., 2008).

A considerable number of patients lacking MFRP that were diagnosed with eye problems were reported. However, phenotype, expressivity and age of onset of the syndrome varies among and within families. Furthermore, both homozygous base-pair insertions or deletions and heterozygous point mutations or base-pair deletions have been described, all resulting in a premature truncation of the protein. In the most severe cases, age of onset was childhood and patients presented with reduced visual acuity, impaired ERG responses (extinguished rod responses and moderate to severely impaired cone responses), retinitis pigmentosa, RPE atrophy, posterior microphthalmos, foveoschisis and optic nerve drusen (Ayala-Ramirez, et al., 2006; Crespí, et al., 2008; Godinho, et al., 2020; Mukhopadhyay, et al., 2010; Neri, et al., 2012; O'Connell, et al., 2022; Ren, et al., 2022; Ritter, et al., 2013; Wasmann, et al., 2014). In milder cases, retinitis pigmentosa, foveoschisis and/or optic nerve head drusen did not occur (Almoallem, et al., 2020; Matsushita, Kondo, & Tawara, 2012; Mukhopadhyay, et al., 2010; Zenteno, Buentello-Volante, Quiroz-González, & Quiroz-Reyes, 2009). Furthermore, in rare cases glaucoma developed during adolescence (Crespí, et al., 2008; Godinho, et al., 2020; Ren, et al., 2022). Later, an association between primary angle closure glaucoma (PACG) and MFRP was found (Zukerman, et al., 2020).

Combining the severe and early-onset retinal phenotype of the MFRP deficient mouse model and patients, it is clear that this protein is essential to maintain retinal homeostasis. It remains, however, to be elucidated whether reduced retinal DHA levels due to loss of AdipoR1 are the root cause of the pathology.

4.3. Impact of impaired VLC-PUFA homeostasis for retinal health

The relatively high abundance of VLC-PUFAs in the retina is suggestive that they play an important role as well. In the previously discussed models, it can be assumed that besides the loss of DHA, also the VLC-PUFA derivatives were reduced, but these were often not analysed. In this section, the specific role of VLC-PUFAs is discussed by assessing mouse models and patients lacking ELOVL4, which is not involved in the synthesis of DHA, but is necessary for the formation of VLC-PUFAs.

4.3.1. Elongation of very long chain fatty acids 4

As ELOVL4 plays an essential role for the synthesis of VLCFA-containing ceramides, which are required for the skin's barrier function, homozygous *Elovl4*$^{mut/mut}$ and *Elovl4*$^{-/-}$ mice are not viable. Therefore, *Elovl4*$^{+/mut}$, *Elovl4*$^{+/-}$ and cell-type selective knockout mice were studied and their retinal phenotype was recently extensively reviewed (Hopiavuori, et al., 2019). There is large phenotypic variability between the mouse models, even those with a similar genotype. Furthermore, in some models it is unsure whether the lack of VLC-PUFAs or a gain-of-function of the mutant ELOVL4 is the cause for the retinal degeneration (Hopiavuori, et al., 2019; Yeboah, et al., 2021). As the main goal here is to understand the role of VLC-PUFAs for retinal homeostasis, only the photoreceptor specific knockout mice will be discussed.

The first group to generate photoreceptor specific knockout mice of *Elovl4* was Harkewicz et al. (Harkewicz, et al., 2012). *Opsin-Elovl4*$^{-/-}$ mice (rod-specific) presented with a severe reduction in VLC-PUFA-containing phospholipid species, which was accompanied by a small loss of photoreceptors (age >10M) and significant reduction in the scotopic b-wave response (5M). On the other hand, the reduction of VLC-PUFAs was lesser in *HRGP-Elovl4*$^{-/-}$ mice (cone-specific) and no morphological abnormalities were reported. Later on, it was established that this *Opsin* promotor results in incomplete deletion of the ELOVL4 protein (Barabas, et al., 2013). Therefore, the *Opsin-iCre75-Elovl4*$^{-/-}$ mice were generated, resulting in 89% reduction of *Elovl4* mRNA levels and a striking 98% loss of VLC-PUFAs (Barabas, et al., 2013). Remarkably, this did not result in any functional or morphological abnormalities

until 10M of age. Important to note is that this *Opsin-iCre75* promotor starts to excise floxed genomic DNA only from P7, while *Elovl4* expression starts at embryonic day (E) 7. Therefore, it is possible that the *Opsin-iCre75-Elovl4*$^{-/-}$ mice had sufficient VLC-PUFA levels during the formation of the retina. Lastly, *Chx10-Elovl4*$^{-/-}$ mice were generated, resulting in loss of *Elovl4* in both rods and cones starting from E16.5 (Bennett, Brush, et al., 2014; Bennett, Hopiavuori, et al., 2014). Even though VLC-PUFAs were barely detectable at 8w, a significant loss of rod photoreceptors and function was only seen at 12M. Again, cone morphology and function were not affected (Bennett, Brush, et al., 2014). Interestingly, the same group hypothesized that besides a role of VLC-PUFAs for rod structure and function, they were also essential for synaptic functioning (see section 3.2), as they demonstrated that VLC-PUFAs were significantly enriched in the photoreceptor synaptic ribbon. Indeed, loss of *Elovl4* in both rods and cones resulted in retraction of pre-synaptic terminals into the ONL and a reduction in synaptic vesicle number and diameter in rod pre-synaptic terminals (Bennett, Hopiavuori, et al., 2014).

At a later timepoint (15M), absence of ELOVL4 causes RPE abnormalities, including accumulation of lipofuscin and lipid droplets (Harkewicz, et al., 2012). However, it remains to be determined whether this is due to a primary deficiency of VLC-PUFA levels in the RPE or a secondary effect of phagocytising POS with an altered lipid profile.

Patients with base-pair deletions or mutations in the *ELOVL4* gene suffer from autosomal dominant Stargardt-like macular dystrophy 3 (STGD3) (Yeboah, et al., 2021). Clinical presentation is early-onset vision loss, macular degeneration, flecked retina and high lipofuscin levels in the RPE (Bernstein, et al., 2001; Donato, et al., 2018; Edwards, Donoso, & Ritter, 2001; Vasireddy, Wong, & Ayyagari, 2010; K. Zhang, et al., 2001).

The latest conditional knockout mouse, *Chx10-Elovl4*$^{-/-}$ mice (Bennett, Brush, et al., 2014; Bennett, Hopiavuori, et al., 2014), seems to be the most reliable to draw conclusions on the role of VLC-PUFAs. Surprisingly, loss of VLC-PUFAs has only a late impact on rod functioning and survival in mice, but it affects the synaptic transmission of rods to the bipolar cells at an earlier age. Possibly, VLC-PUFAs do play a more important role in human photoreceptors (Yeboah, et al., 2021).

Table 1: Retinal phenotype of mouse models with altered (VLC-)PUFA levels

	Mouse model		Neural retina phenotype			RPE phenotype				
		Retinal DHA and VLC-PUFA levels	POS shortening	PR death	Impaired PR function	Lipid droplets	Hyper-trophy	RPE protrusions	Microglia activation	Autofluorescent deposits
Impaired (phospho-) lipid synthesis	*Elovl2*^{c234w}	DHA↓ (*age n.k.*)	*n.k.*	*n.k.*	X *(6M)*	*n.k.*	*n.k.*	*n.k.*	*n.k.*	X *(6M)*
	Mfp2$^{-/-}$	LPC(DHA)↓ PL(44:12)↓ PL(<C56)↓ PL(>C56)↑ *(3w)*	X *(2w)*	X *(8w)*	X *(3w)*	*n.k.*	*n.k.*	X *(9w)*	X *(3w)*	*n.k.*
	Tmem135 *FUN025/ FUN025*	PL(DHA)↓ *(2,5M)*	X *(12M)*	X *(2M)*	X *(7M)*	X *(3M)*	X *(7M)*	X *(7M)*	X *(7M)*	X *(7M)*
	Lpaat3$^{-/-}$	PL(44:12)↓ PL(<C58)↓ *(P11)*	X *(3w)*	X *(6w)*	X *(8w)*	*n.k.*	*n.k.*	*n.k.*	*n.k.*	*n.k.*
Defective lipid trafficking	*AdipoR1*$^{-/-}$	PL(44:12)↓ PL(<C58)↓ *(3-4w)*	X *(P14)*	X *(4w)*	X *(3-4w)*	X *(4w)*	X *(P20)*	X *(3M)*	X *(P20)*	X *(20w)*
	Mfsd2a$^{-/-}$ (2016)	PL(DHA)↓ *(8w)*	X *(P13)*	X *(4M)*	- *(4M)*	*n.k.*	*n.k.*	*n.k.*	X *(P14)*	*n.k.*
	Mfsd2a$^{-/-}$ (2019)	LPC(DHA)↓ PL(44:12)↓ PL(<C58)↓ *(6w)*	- *(1M)*	X *(1M)*	- *(P70)*	*n.k.*	*n.k.*	*n.k.*	X *(1M)*	*n.k.*
	Mfrp$^{rd6/rd6}$	PL(44:12)↓ PL(<C56)↓ *(age n.k.)*	X *(2w)*	X *(P28)*	X *(P25)*	*n.k.*	*n.k.*	X *(5M)*	X *(2M)*	X *(4M)*
Impaired VLC-PUFA synthesis	*Chx10-Elovl4*$^{-/-}$	VLC-PUFA↓	-	X *(12M)*	X *(12M)*	*n.k.*	*n.k.*	*n.k.*	*n.k.*	*n.k.*

AdipoR1: adiponectin receptor 1, DHA: docosahexaenoic acid, ELOVL: very long chain fatty acid elongase, LPAAT3: lysophosphatidic acid acyltransferase 3, LPC: lysophosphatidylcholine, MFP2: multifunctional protein 2, MFRP: membrane frizzled-related protein, Mfsd2a: major facilitator super family domain-containing protein 2a, PL—phospholipid (including PC, PE and PS); POS—photoreceptor outer segment; PR—photoreceptor; RPE—retinal pigment epithelium; Tmem135: transmembrane protein 135, VLC-PUFA—very long chain polyunsaturated fatty acid; n.k.—not known.

5. Other retinal degeneration models with loss of PUFAs in the retina

Besides the mouse models with a defect in the acquisition of DHA in the retina, there are numerous other models with retinal pathologies in which retinal DHA levels were found to be reduced. Here, it should be taken into account that lowered DHA levels in the retina might not be the basis of the retinopathy. Intriguingly, reduced plasma DHA levels were found in patients with the following eye disorders.

5.1. Age-related macular degeneration

AMD is the most prevalent form of blindness worldwide, affecting mostly the elderly population, causing degeneration of the central part of the retina, i.e. the macula. It is difficult to study AMD due to the late onset, complex genetics, the influence of environmental factors, and especially the location of the degeneration, as rodents lack a macula. Still, mostly rodents are used to study AMD (Ramkumar, Zhang, & Chan, 2010).

The AMD murine models can be classified into three groups: genetically engineered, naturally occurring strains, and immunologically manipulated (immunized with CEP) (Ramkumar, et al., 2010). Overall, mice presented with RPE abnormalities, including lipofuscin accumulation, basal laminar deposits, RPE atrophy, choroidal neovascularization (CNV) and consequential impairment of photoreceptor length, survival and function and reduced retinal DHA levels at later ages (Ramkumar, et al., 2010).

In patients, different stages of AMD can be defined. In the early stages of AMD, the pathology is confined to the RPE and Bruch's membrane. Typical observations are lipofuscin accumulation, decrease in the number of RPE cells in the macula, thickening of Bruch's membrane and drusen. In advanced AMD, which can be divided into the dry (non-neovascular) and wet (neovascularization) form, the neural retina is affected as a consequence of the RPE abnormalities. In dry AMD, the RPE atrophy is accompanied by severe degeneration of both rod and cone photoreceptors in the parafovea, which can progress to total photoreceptor loss. Wet AMD is characterized by CNV, retinal inflammation, hemorrhages (detachment of either the RPE or neural retina), serous exudates, and edema in the neuroretina (Ramkumar, et al., 2010). Interestingly, DHA and VLC-PUFA levels were reduced in plasma, RPE and neural retina of AMD patients, independent of the disease stage (Gorusupudi, Liu, Hageman, & Bernstein, 2016; A. Liu, Chang, Lin, Shen, & Bernstein, 2010). This is different from the normal aging retina in which DHA levels were similar in teenagers and people in their seventies (A. Liu, et al., 2010). Therefore, decreased plasma DHA levels were proposed to be a biomarker for AMD (Orban, et al., 2015).

The role of DHA in AMD is however rather complicated. On the one hand, lower plasma DHA levels are associated with higher risk for AMD, suggesting that higher DHA intake could alleviate the retinal symptoms. On the other hand, the levels of CEP, a byproduct formed due to oxidation of DHA, were advocated to be a biomarker of AMD, implying that increasing DHA supply could aggravate the disease (Gu, et al., 2003).

5.2. Diabetic retinopathy

DR is a common complication in diabetic patients and is influenced by both genetic and environmental factors. The main causative factor is chronic hyperglycemia, affecting the retinal vascular network (Aizu, Oyanagi, Hu, & Nakagawa, 2002).

Many animal models are available to study the retinal consequences of diabetes, including genetic mutations or induction. The most commonly studied model is the single intraperitoneal injection of streptozotocin (STZ) in rodents, as it results in the fastest rate of disease development. The neural retinal phenotype is characterized by early loss of photoreceptor survival, length and function and increased inflammation (1M post STZ-

injection). Furthermore, RPE vacuolization, a wavy apical side and reduced RPE65 levels were observed (Aizu, et al., 2002; Énzsöly, et al., 2014).

Interesting to note is that insulin deficiency affected the expression levels of elongases and desaturases that are involved in the synthesis of DHA already 3-6 weeks after STZ injection (Tikhonenko, et al., 2010). In the liver, diabetes induced a decrease in *Elovl2*, *Elovl6* and *Δ9 desaturase* transcripts, while *Elovl2* and *4* were reduced in the retina. This resulted in a significant loss of both plasma and retinal, but not liver, total DHA levels (3w post injection). Furthermore, levels of DHA-containing phospholipid species (PC(18:0/22:6), PC(22:6/22:6), PC(32:6/22:6)) were reduced in the retina (Tikhonenko, et al., 2010).

The hallmark of diabetic retinopathy is retinal neovascularization. However, various visual deficits can occur before these vascular changes, including loss of color and contrast sensitivity and reduced ERG responses, which can be used as predictors of progression (Aizu, et al., 2002; Yee, Weymouth, Fletcher, & Vingrys, 2010). Interestingly, both LC- and VLC-PUFA levels were significantly lower in plasma and retinal punches of DR patients (Fort, et al., 2021; Gorusupudi, Chang, Nelson, Hageman, & Bernstein, 2019).

5.3. Retinitis pigmentosa

RP is a group of inherited retinal degeneration disorders, characterized by primary rod cell death, followed by cone degeneration and progressive night-blindness. So far, more than 65 genes have been associated with RP and mutations can be either autosomal dominant, autosomal recessive or X-linked (R. E. Anderson, et al., 2002; Hoffman, DeMar, Heird, Birch, & Anderson, 2001; Nakazawa, Hara, & Ishiguro, 2019). Due to the genetic heterogeneity of RP, the cause of photoreceptor death, age of onset and progression differs significantly. Remarkably, one study reported that X-linked RP patients have significantly lower plasma DHA levels, which correlated with retinal function (Hoffman & Birch, 1995). Unfortunately, the affected genes were not specified, making it difficult to interpret the cause and consequences of the reduced plasma DHA levels.

In general, RP animal models also present with significant loss of rod and consequent cone photoreceptors, impaired ERG responses and shortened POS. Furthermore, retinal and plasma DHA levels are significantly reduced (R. E. Anderson, Maude, Alvarez, Acland, & Aguirre, 1991; R. E. Anderson, Maude, & Bok, 2001; R. E. Anderson, et al., 2002).

6. Therapeutic options to rescue retinal PUFA shortage

If a deficiency of PUFAs is the root cause or contributes to retinal degeneration, it seems obvious to increase their levels by nutritional supplementation in order to alleviate the pathology. Furthermore, PUFA derivatives with protective functions have been administered to counter the degeneration process. Depending on the underlying mechanism of the retinal degeneration, alternative therapeutic approaches might be necessary.

6.1. Therapies for diseases with a genetic defect in retinal PUFA acquisition

In patients with a mutation in the genes involved in the synthesis of DHA (*ELOVL2*, *TMEM135* and *HSD17B4*) or VLC-PUFAs (STGD3), supplementation of the respective lipid would be the most plausible option. For *LPAAT3* patients, supplementation of phospholipid-containing DHA could be explored, although it remains to be determined if these lipids are able to pass the blood-retinal-barrier. However, for patients with defective DHA transport (*AdipoR1*, *Mfds2a* and *MFRP* gene defects), this approach will most likely not be effective.

We here summarize the PUFA supplementation studies that were done in patients and in mouse models as well as gene therapy approaches that are being developed.

6.1.1. Dietary supplementation of DHA to peroxisomal disorders

DHA was supplemented to patients with peroxisome biogenesis defects and one patient with MFP2 deficiency (Noguer & Martinez, 2010; Paker, et al., 2010). In an open trial, DHA ethyl ester supplementation led to disappearance of nystagmus and stabilization or even improvement of retinal appearance, visual acuity and visual function (Noguer & Martinez, 2010). In contrast, a randomized, double blind and placebo-controlled clinical trial supplementing DHA in the TG-form, showed that in both the treated and the non-treated patients, the ERG stabilized or even improved after one year in surviving patients (Paker, et al., 2010). The small number of patients with a variable disease severity and a poorly documented natural history (Bose, et al., 2022) hinder the drawing of conclusions on the effectiveness of DHA supplementation to patients with peroxisomal disorders.

6.1.2. Supplementation studies to STGD3

Although STGD3 is caused by defects in ELOVL4 that converts DHA into VLC(-PU)FA, supplementation of DHA to STGD3 mice and patients has been attempted several times. These studies were based on the finding that there was an inverse association between plasma DHA and severity of STGD3 (Hubbard, Askew, Singh, Leppert, & Bernstein, 2006) and the protective role of DHA in the retina. However, DHA supplementation to both mice (Dornstauder, et al., 2012; F. Li, et al., 2009) and patients (Choi, Gorusupudi, & Bernstein, 2018) did not prevent retinal degeneration. This could be due to several reasons: (i) poor compliance of the patients, (ii) VLC-PUFA levels are still ablated, (iii) the possibly toxic mutant ELOVL4 was still present, or (iv) supplementation started too late.

Recently, an oral supplementation study with the VLC-PUFA, C32:6n3, to *rod-cone-Elovl4*$^{-/-}$ (*E4cKO*) mice was attempted showing promising results (Gorusupudi, et al., 2021). Firstly, C32:6n3 levels increased to WT levels in both retina and RPE of supplemented *E4cKO* mice. Furthermore, visual acuity and scotopic/photopic a- and b-wave responses improved compared to non-supplemented *E4cKO* mice, and even normalized to WT levels for the highest intensity in the scotopic b-wave response. These data reveal that supplementation of VLC-PUFAs to STGD3 patients would be a possible approach.

6.1.3. DHA transporters

When a DHA transporter in the basolateral RPE is defective, it could be explored whether overstimulation of the unaffected transporter, by supplementation of DHA in the appropriate form, can enhance the retinal DHA levels. For *AdipoR1* patients, supplementation with LPC-DHA would be an option as this is transported by Mfds2a. Unfortunately, information is lacking in which form AdipoR1 takes up DHA, therefore it remains to be determined how DHA should be supplemented to *Mfds2a* patients.

With regard to MFRP deficiency, for which the precise role in retinal DHA homeostasis was not elucidated yet, a more permanent gene therapy approach was evaluated in *Mfrp*$^{rd6/rd6}$ mice resulting in variable results (Ali, 2012; Dinculescu, et al., 2012; Dinculescu, Min, Deng, Li, & Hauswirth, 2014; Y. Li, et al., 2014; Velez, et al., 2017). Subretinal delivery of the *Mfrp* gene using an AAV8 or AAV2/8 vector at either P5 or P14, completely prevented retinal degeneration, loss of visual function and hyperopia (Dinculescu, et al., 2012; Y. Li, et al., 2014; Velez, et al., 2017). When an AAV2 vector was used, only retinal degeneration was prevented, while visual function declined (Dinculescu, et al., 2014). These data provide a proof-of-concept that gene addition to *MFRP* patients before the onset of degeneration is a possible therapeutic approach.

6.2. Supplementation of DHA to retinal degeneration models

6.2.1. The effect of DHA supplementation to prevent retinal degeneration is disputed

As comprehensively reviewed (German, et al., 2015; Lewandowski, Sander, et al., 2022), many studies have tried to alleviate or prevent the progression of AMD, RP and DR patients, by supplementing DHA. This was inspired by reduced levels of DHA in plasma of these patients in addition to its presumed anti-oxidant and anti-inflammatory properties. However, clinical trials resulted in divergent results. Firstly, several studies showed that higher intake of DHA-rich diets and higher plasma DHA levels are associated with lower risk of AMD (Chong, Kreis, Wong, Simpson, & Guymer, 2008; Merle, et al., 2014; Merle, et al., 2013; Merle, Silver, Rosner, & Seddon, 2015; van Leeuwen, et al., 2018). Furthermore, the large prospective study Age-Related Eye Disease Study (AREDS), reported that patients with higher self-reported intake of n-3 LC-PUFAs rich food were less likely to develop AMD (SanGiovanni, et al., 2008). However, the AREDS2 and nutritional AMD treatment (NAT-2) studies could not show a difference between the n-3 PUFA supplementation and placebo group on progression of wet AMD (Chew, et al., 2012; Merle, Richard, et al., 2015; Souied, et al., 2013). Furthermore, DHA supplementation studies to RP patients did not show beneficial effects in four different clinical trials (Berson, et al., 2004a; Birch, 2005; Sacchetti, Mantelli, Merlo, & Lambiase, 2015; Schwartz, Wang, Chavis, Kuriyan, & Abariga, 2020). Only one study of DHA in combination with vitamin A supplementation resulted in a slower decline in field sensitivity and ERG amplitude in the first two years, but not at later time points (Berson, et al., 2004b). Also, DHA supplementation studies to prevent DR resulted in conflicting results. While a recent study showed an inverse relationship between plasma DHA levels and severity of DR (MESA and GOLDR cohorts) (Weir, et al., 2023), a randomized double-blind placebo-controlled study did not show significant differences between the DHA (TG) and placebo treated groups on the disease progression of DR (Piñas García, Hernández Martínez, Aznárez López, Castillón Torre, & Tena Sempere, 2022).

It remains unexplained why some clinical trials showed positive effects, while others did not. Timing of supplementation with regard to the disease-stage, adequate setup of the clinical trial (primary and secondary outcomes) and compliance of the patients are possible explanations. However, other reasons could be that (i) reduced DHA levels are not the root cause for the retinal degeneration therefore only providing a temporary solution, (ii) DHA supplementation aggravated the disease progression (CEP formation in AMD patients) or (iii) the bioavailability of the supplied DHA was different. Not only is there great variation in the DHA concentration of the different supplements, the bioavailability is also crucial for the efficacy of DHA supplements (J. Li, et al., 2021). Mostly, the fatty acids in the DHA supplements (fish oils) are esterified into triglycerides, however some formulations consisted of the ethyl esters. Previous studies showed that the TG form is not only more stable, it is also more rapidly absorbed by the body compared to the ethyl esters (Ahonen, Damerau, Suomela, Kortesniemi, & Linderborg, 2022). Furthermore, the bioavailability of DHA is dependent on the excipient, where emulsification of DHA in oils or the food matrix can significantly increase the bioavailability (J. Li, et al., 2021).

6.2.2. A new therapeutic avenue: deuterated DHA

Recently, a different approach for DHA supplementation was explored, taking into account the many oxidation sites within DHA. Deuteration of DHA (D-DHA) at the bis-allylic position reduces its susceptibility to oxidative damage, therefore providing an interesting tool to prevent the formation of DHA-derived toxic products (James, et al., 2022; Y. Liu, et al., 2022). Indeed, orally supplemented D-DHA protected against iron-induced oxidative damage (Y. Liu, et al., 2022). It would be interesting to evaluate the effect of D-DHA supplementation to AMD

patients, as it will prevent the formation of the toxic oxidation products of DHA (CEP), which are elevated in AMD patients (Y. Liu, et al., 2022).

6.2.3. Supplementation of NPD1 as anti-oxidant/anti-inflammation agent

Another possible therapeutic avenue that is being explored is the delivery of the DHA-derived protective mediator, neuroprotectin D1 to the eye. Indeed, this has been attempted by delivering NPD1 to the eye via intraperitoneal injection (Connor, et al., 2007; Sheets, et al., 2010), topical administration (Cortina, He, Russ, Bazan, & Bazan, 2013; Rajasagi, Reddy, Mulik, Gjorstrup, & Rouse, 2013; Sheets, et al., 2013) or via tail vein injection (Qin, Patil, Gronert, & Sharma, 2008). Interestingly, promising results were obtained in the attenuation of laser-induced CNV (Sheets, et al., 2013; Sheets, et al., 2010), retinal angiogenesis (Connor, et al., 2007) and corneal damage after either experimental surgery (Cortina, et al., 2013) or Herpes Simplex Virus infection (Rajasagi, et al., 2013). Furthermore, retinal ganglion cell survival upon axotomy was significantly improved in the NPD1-treated condition, compared to the non-treated condition (Qin, et al., 2008). These studies are encouraging as they provide evidence that (i) it is possible to synthesize NPD1 in sufficient levels to be effective, and (ii) neuroprotectin D1 is able to reach the retina.

7. Discussion

This overview reveals that there are still a number of unsolved mysteries concerning the role of PUFAs in the retina in health and disease, which need to be elucidated in order to successfully develop therapeutic interventions.

The recently generated mouse models with a primary defect in DHA metabolism or transportation in the retina reinforce the notion that DHA is essential for the formation of photoreceptor outer segments, for photoreceptor function and integrity. However, some discrepancies remain, as both *Mfsd2a*$^{-/-}$ mouse models displayed normal ERG responses, despite severe reduction in retinal DHA levels and loss of photoreceptors. An important obstacle in the comparison of the different mouse models is the erratic description of the phenotypes. A detailed morphological and functional characterization of the retina, in combination with comprehensive PUFA lipidomics at different time points, certainly including the onset of retinal degeneration, is required.

With regard to degenerative retinal diseases, such as AMD, DR and RP, it is less clear whether reduced DHA levels cause, contribute to or are a consequence of the retinal degeneration. Despite several indications that reduced supply of DHA could play a role, DHA supplementation studies to these patients resulted in contradictory results. Some uncertainties should first be solved using animal models, such as the dosage and the form in which DHA should be supplied (ethyl ester, TG or D-DHA). An important consideration with regard to using mouse models to predict effectiveness of DHA treatment, is that mice lack a macula and it was recently shown that the lipid content of rods and cones is different, where cones contain lesser DHA (Agbaga, et al., 2018). Therefore, caution is warranted for the translatability of the mouse models to the patients.

Overall, it is clear from this overview that DHA plays an essential role in both retinal development and maintenance. However, it remains to be determined to what extent pathologies depend on lowered DHA levels *per se* or on altered levels of other lipid species provoked by the DHA shortage. i) Firstly, as a consequence of reduced retinal DHA levels, the retina upregulates the n-6 PUFA arachidonic acid (AA, C20:4n-6), to preserve retinal PUFA desaturation (Lewandowski, Sander, et al., 2022). Although AA is an essential component of cell membranes (Carlson, Werkman, Peeples, Cooke, & Tolley, 1993), this n-6 PUFA has also been linked to inflammation as it can give rise to the pro-inflammatory mediators, eicosanoids

(extensively reviewed by (Lewandowski, Sander, et al., 2022). Hence, the compensatory upregulation of AA, in response to reduced retinal DHA levels, might accelerate the retinal degeneration. ii) As DHA is the precursor for n-3 VLC-PUFAs, it can be assumed that their levels will be lowered as a consequence of reduced retinal DHA levels. Although VLC-PUFAs were not important for retinal development, both a lack and excess of VLC-PUFAs correlated with impaired photoreceptor integrity at later stages (Bennett, Brush, et al., 2014; Bennett, Hopiavuori, et al., 2014; Swinkels, et al., 2022). iii) In addition, lowered retinal DHA levels probably hinders generation of the protective lipid mediators NPD1, which can be derived from DHA in conditions of uncompensated oxidative stress (N. G. Bazan, 2005), and elovanoids, which originate from the n-3 VLC-PUFAs, C32:6n-3 and C34:6n-3, that are elongation products of DHA (Jun, et al., 2017). It is currently unsolved whether these protective lipid derivates of DHA, contribute to the beneficial effects of DHA. Although reduced levels of NPD1 (Miyagishima, et al., 2021) and elovanoids (Do, et al., 2019; Jun, et al., 2017) were reported in some instances, it appears that a stress response needs to be inflicted to reliably measure these mediators. Interestingly, treatment with NPD1 was performed in several occasions, revealing promising results with regard to the ability to reach the retina and prevention of degeneration (Connor, et al., 2007; Cortina, et al., 2013; Qin, et al., 2008; Rajasagi, et al., 2013; Sheets, et al., 2013; Sheets, et al., 2010). Furthermore, in models of oligomeric Aβ-induced damage to photoreceptors and the RPE, administration of elovanoids counteracted the effects and protected these cells (Do, et al., 2019). Although further research is needed on the specific beneficial effects of DHA and the derived docosanoid and elovanoid mediators, there is potential for these lipids as therapeutic strategies.

Another therapeutic avenue that could be explored is ocular gene therapy, which was successful in preclinical studies on *Mfrp*$^{rd6/rd6}$ mice. Nevertheless, important to consider is that in the models with reduced synthesis of DHA in the liver, local delivery of the affected gene will not be sufficient. In these cases, a combination therapy of DHA supplementation with gene therapy will be necessary.

The role of VLC-PUFAs in photoreceptors is also still obscure. Their absence caused a milder retinal phenotype than expected only impacting on photoreceptor survival and functioning at a later age (1Y) (Bennett, Brush, et al., 2014; Bennett, Hopiavuori, et al., 2014). However, the surprising finding that the photoreceptor synaptic ribbon was already affected at an earlier time point, alerted that PUFAs are not only important in the POS (Bennett, Hopiavuori, et al., 2014). The critical role of VLC-PUFAs in the ribbon synapse, was underscored by a mouse model in which VLC-PUFAs accumulated (*Crx-Mfp2*$^{-/-}$ mice), also displaying synaptic abnormalities (Swinkels, et al., 2022).

Finally, several gaps remain in the knowledge of DHA transport from the circulation via the RPE to the photoreceptors and the recycling of DHA in ingested POS from the RPE to the photoreceptors. Labelling studies to track the fate of PUFAs are badly needed to gain better understanding of their trajectories. Furthermore, the DHA-deficient mouse models presented with several RPE abnormalities and it remains to be determined whether these are due to a primary deficiency of DHA levels in the RPE or a secondary effect of neural retina degeneration. Quite interesting was the finding that in the *Mfrp*$^{rd6/rd6}$ mouse model, microvilli were affected already at P7, before other retinal abnormalities were observed. As loss of RPE integrity and functioning can give rise to consequential retinal degeneration, it seems worthwhile to further explore the potential role of DHA in the RPE as well.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. *Synthesis of n-3 (V)LC-PUFAs starting from ALA (18:3n-3). First, in the ER of the liver, ALA is desaturated to stearidonic acid (18:4n-3) via Δ6-desaturase, followed by elongation to eicasotetraenoic acid (20:4n-3), Δ5 desaturation to eicosapentaenoic acid (EPA, 20:5n-3) and elongation to docosapentaenoic acid (DPA, 22:5n-3). This is retroconverted in the Sprecher pathway (red arrows) by consecutive elongation to tetracosapentaenoic acid (TPA, 24:5n-3), desaturation to tetracosahexaenoic acid (THA, C24:6n-3), and transport to the peroxisome where one β-oxidation step will result in the formation of docosahexaenoic acid (DHA, C22:6n-3). The hypothesized retroconversion of DHA to EPA in photoreceptors is indicated with dotted arrows. Subsequent synthesis of n-3 VLC-PUFAs by ELOVL4 occurs in the ER of photoreceptors and they are degraded via peroxisomal β-oxidation. ACOX: acyl-CoA oxidase, des: desaturase, ELOVL: elongase of very long chain fatty acids, MFP2: multifunctional protein 2, RPE: retinal pigment epithelium. Figure created with BioRender.com.*

Figure 2. *PUFA trafficking in the body. Big loop: mature PUFAs are either supplied by diet (fatty fish) or synthesized in the liver starting from ALA. Next, lipids are transported via lipoproteins and taken up by the RPE. After translocation to the apical side, PUFAs are excreted into the inner photoreceptor matrix (IPM) and transported into photoreceptor inner segments. Here, PUFAs (i.e. DHA) are either elongated towards VLC-PUFAs, converted into protective mediators, metabolized or integrated into phospholipids, which are the backbone of the outer segments. Small loop: After POS phagocytosis, DHA is either recycled back to the inner segment, converted to protective mediators or metabolized. Question marks indicate processes that need further investigation. AdipoR1: adiponectin receptor 1, ALA: α-linolenic acid, DHA: docosahexaenoic acid, ELOVL: very long chain fatty acid elongase, Elv: elovanoids, LPL: lysophospholipids, LPAAT3: lysophosphatidic acid acyltransferase 3, MFP2: multifunctional protein 2, MFRP: membrane frizzled-related protein, Mfsd2a: major facilitator super family domain containing protein 2a, NPD1: neuroprotectin D1, PL: phospholipid, RPE: retinal pigment epithelium, THA: tetracosahexaenoic acid, Tmem135: Transmembrane protein 135. Figure created with BioRender.com.*

Figure 3. *Roles of DHA and VLC-PUFAs in the retina. DHA: docosahexaenoic acid, IRBP: interphotoreceptor retinoid-binding protein; NPD1: neuroprotectin D1, VLC-PUFA: very long chain polyunsaturated fatty acid. Figure created with BioRender.com.*

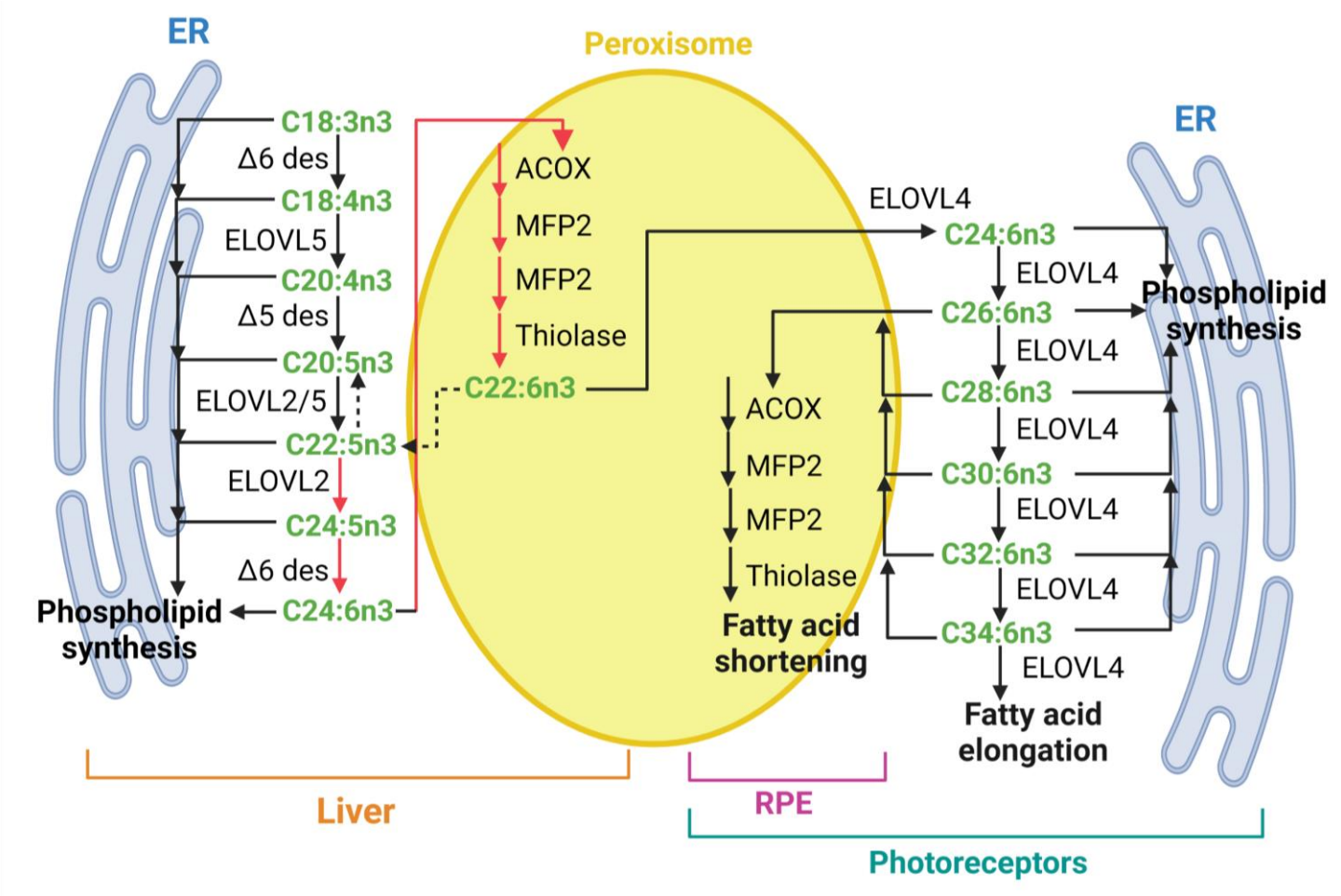


Figure 1. Synthesis of n-3 (V)LC-PUFAs starting from ALA (18:3n-3). First, in the ER of the liver, ALA is desaturated to stearidonic acid (18:4n-3) via Δ6-desaturase, followed by elongation to eicasotetraenoic acid (20:4n-3), Δ5 desaturation to eicosapentaenoic acid (EPA, 20:5n-3), elongation to docosapentaenoic acid (DPA, 22:5n-3) and further elongation to tetracosapentaenoic acid (TPA, 24:5n-3). After desaturation to THA this is transported to the peroxisome where one β-oxidation step will result in the formation of docosahexaenoic acid (DHA, C22:6n-3), also known as the Sprecher pathway (red arrows). Retroconversion of DHA to EPA is indicated with dotted arrows. Subsequent synthesis of n-3 VLC-PUFAs by ELOVL4 will occur cell type-specific in the ER and their catabolism can only occur via peroxisomal β-oxidation. Figure generated via biorender.

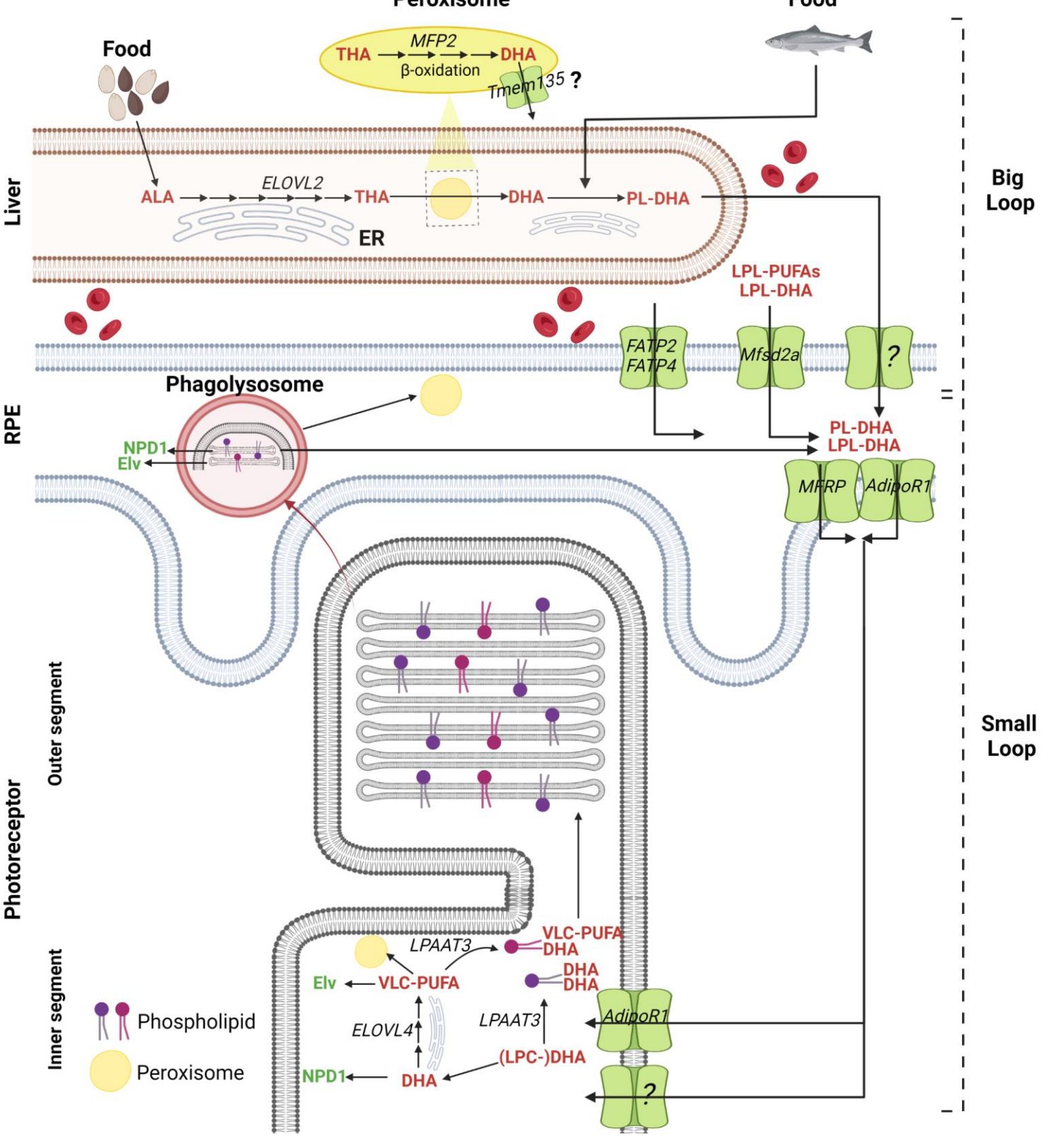
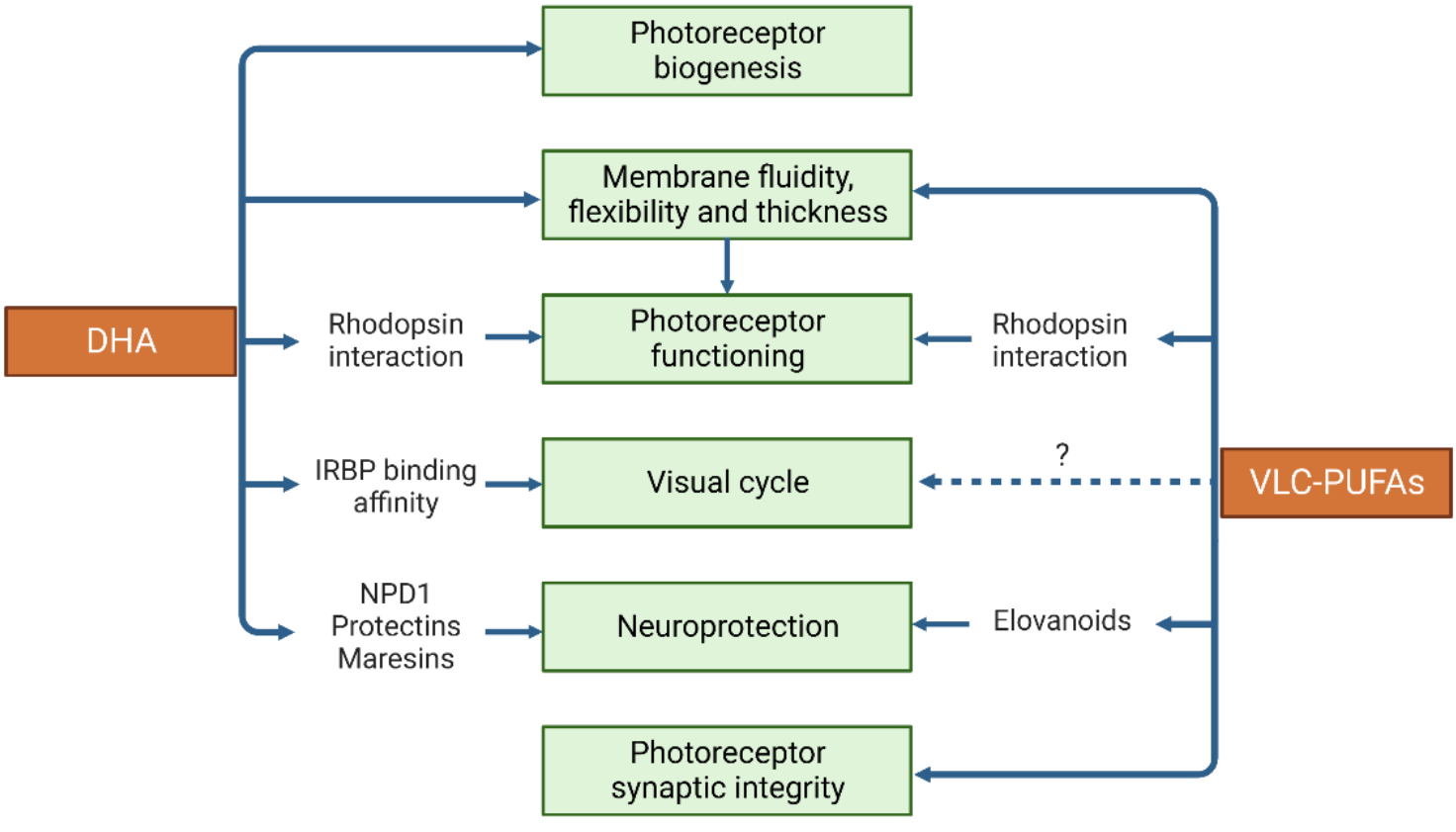


Figure 2. PUFA trafficking in the body. Big loop: mature PUFAs are either supplied by diet (fatty fish) or synthesized in the liver starting from ALA. Next, lipids are transported to the RPE, via lipoproteins, and subsequently taken up. RPE cells excrete PUFAs into the inner photoreceptor matrix (IPM), after which they reach the photoreceptor inner segments. In the inner segments, PUFAs (i.e. DHA) are either elongated towards VLC-PUFAs, converted into protective mediators, metabolized or integrated into phospholipids, which are the backbone of the outer segments. Small loop: After POS phagocytosis, DHA is either recycled back to the inner segment, converted to protective mediators or metabolized. AdipoR1: adiponectin receptor 1, ALA: α-linolenic acid, DHA: docosahexaenoic acid, ELOVL: very long chain fatty acid elongase, Elv: elovanoids, FATP: fatty acid transport protein, IPM: inner photoreceptor matrix, LPL: lysophospholipids, LPAAT3: lysophosphatidic acid acyltransferase 3, Mfsd2a: major facilitator super family domain containing protein 2a, NPD1: neuroprotectin D1, PL: phospholipid, RPE: retinal pigment epithelium, THA: tetracosahexaenoic acid, Tmem135: Transmembrane protein 135. Figure generated with Biorender



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