HHS Public Access

Author manuscript

*Pharmacol Ther*. Author manuscript; available in PMC 2024 September 01.

Published in final edited form as:

*Pharmacol Ther*. 2023 September ; 249: 108482. doi:10.1016/j.pharmthera.2023.108482.

# Retina and RPE Lipid Profile Changes Associated with ABCA4 Associated Stargardt's Maculopathy

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## Abstract

Stargardt maculopathy, caused predominantly by mutations in the ABCA4 gene, is characterized by an accumulation of non-degradable visual pigment derivative, lipofuscin, in the retinal pigment epithelium (RPE) - resulting in RPE atrophy. RPE is a monolayer tissue located adjacent to retinal photoreceptors and regulates their health and functioning; RPE atrophy triggers photoreceptor cell death and vision loss in Stargardt patients. Previously, ABCA4 mutations in photoreceptors were thought to be the major contributor to lipid homeostasis defects in the eye. Recently, we demonstrated that ABCA4 loss of function in the RPE leads to cell-autonomous lipid homeostasis defects. Our work underscores that an incomplete understanding of lipid metabolism and lipid-mediated signaling in the retina and RPE are potential causes for lacking treatments for this disease. Here we report altered lipidomic in mouse and human Stargardt models. This work provides the basis for therapeutics that aim to restore lipid homeostasis in the retina and the RPE.

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Conceptualization, K.B., M.F; Methodology, K.B., M.F., B.B., and N.B.; Investigation M.F., F.B., L.N., M.B., D.B., B.J., R.P., K.D., W.G., M.K., T.C., and B.B.; Formal Analysis, M.F., F.B., L.N., and B.J.; Project Administration, M. F.; Writing – Original Draft, M.F., Writing - Review & Editing, M.F., and K.B.; Funding Acquisition, K.B.; Resources, K.B., B.B., N.B.; Supervision, K.B.



[7]·Declaration Of Interests

The authors declare no competing interests.

**Keywords**

ABCA4 loss of function; lipidomic; lipid homeostasis; ABC transporters; macular degeneration

## 1. Introduction

Understanding cellular physiology under healthy conditions and how a dysfunction in that physiology relates to a diseased state requires a thorough understanding of the cellular and tissue molecular composition – the transcriptome, the proteome, and the lipidome of a cell. Lipids are an essential component of biological activities and physiology of most cells across the body and, in particular, play a crucial role in retinal function (David MG Anderson, et al., 2020). Changes in the lipidomic profile and lipid homeostasis of the retina have been reported in various monogenic and polygenic retinal degenerative diseases (Allikmets, Singh, et al., 1997; Zhang, et al., 2001). Despite their significance in normal cellular physiology and as causative and diagnostic molecules for several retinal diseases, little is known about the lipid profiles in the normal and diseased retina.

Two genes encoding lipid-processing proteins have been linked to hereditary retinal degenerative disorders (Allikmets & Consortium, 2000; Allikmets, Shroyer, et al., 1997; Zhang, et al., 2001). Mutations in the gene encoding the elongase enzyme ELOVL4, involved in the elongation of very long-chain fatty acids, are associated with autosomal dominant Stargardt-like macular dystrophy disease (STDG3, OMIM 600110) (Zhang, et al., 2001). Patients with STGD3, a juvenile form of macular degeneration, develop a loss of central vision at a young age (Bennett, et al., 2014). The enzyme ELOVL4 catalyzes the first condensation phase in the elongation of polyunsaturated fatty acids (PUFA) with more than 26 carbons (C26) to very long chain PUFA (VLC-PUFA; C28 and larger) (Marchette, et al., 2014). Retinal phosphatidylcholine VLC-PUFA analysis of the conditional KO (Cre+/*Elovl4*$^{f/f}$) retinas showed reduced VLC-PUFA and a loss of photoreceptors, mainly affecting rod photoreceptors (Bennett, et al., 2014).

STGD4 (OMIM No. 603786) is another autosomal dominant Stargardt-like macular dystrophy (Kniazeva, et al., 1999). STGD4 was identified in 1999 and is linked to mutations in the PROM1 gene, a common mutation being p.R373C (Kniazeva, et al., 1999). PROM1 is generally found in the retina's photoreceptor outer segments (Yang, et al., 2008). STGD4 patients with PROM1 p.R373C mutations may have ocular phenotypes similar to STGD1 and STGD3 and also cone-rod dystrophy (Kniazeva, et al., 1999; Michaelides, et al., 2010; Palejwala, et al., 2016; Yang, et al., 2008).

ABCA4, an ATP-binding cassette, sub-family A, member 4, gene mutations result in autosomal recessive Stargardt macular degeneration (STDG1, OMIM 248200) and related retinal degenerative disorders (Allikmets, Shroyer, et al., 1997; Allikmets, Singh, et al., 1997; Cremers, et al., 1998; de Jong & Allikmets, 2000). Most STDG1 cases show a progressive bilateral appearance of yellow-orange flecks (lipofuscin) in and around the macula (central retina) and eventual atrophy of the retinal pigment epithelium (RPE) and photoreceptors (PR) cell death in that region (K. L. Anderson, et al., 1995; Lambertus, et al.,

2015) .In fact, lipofuscin accumulation in the RPE is a hallmark phenotype in the eyes of STGD1 patients and the *Abca4*$^{-/-}$ mice model of Stargardt disease (Kong, et al., 2008).

ABCA4 was previously demonstrated to be preferentially expressed in the PR outer segment (POS) disc (Molday, Zhong, & Quazi, 2009). However, recent studies suggest that ABCA4 is also expressed on the plasma membrane of mouse and human RPE cells (Farnoodian, et al., 2022; Lenis, et al., 2018). In PRs, ABCA4 transposes a retinal phospholipid compound known as N-retinylidene-phosphatidylethanolamine (N-ret-PE) across POS disc membranes after photoexcitation, enabling the removal of this potentially toxic compound from photoreceptor cells (Molday, 2007; Sun, Molday, & Nathans, 1999; Weng, et al., 1999). A similar function is proposed in RPE cells where ABCA4 may perform ATP-dependent translocation of N-ret-PE from the luminal to the cytoplasmic side of lysosomes or phagosomes (Lenis, et al., 2018). In Stargardt patients with ABCA4 dysfunction, N-ret-PE accumulates inside the lumen of the POS disc and leads to the formation of the A2E precursor (A2PE). When A2PE containing POS are phagocytosed by RPE cells; inside RPE lysosomes, A2PE is converted to N-retinylidene-N-retinyl-ethanolamine (A2E), a compound that cannot be degraded by any enzymes in the body. This A2E is thought to react with other lipids and convert into lipofuscin deposits (Boyer, et al., 2012; Molday, et al., 2009; Sparrow, et al., 2003). In support of this idea, lipofuscin deposits were discovered in the RPE cells of *Abca4*$^{-/-}$ and *Abca4*$^{+/-}$ mice subjected to continuous or cyclic illumination (Kim, et al., 2007; Mata, Weng, & Travis, 2000; Sparrow & Boulton, 2005). Similar A2E rich molecules have been discovered in lipofuscin deposits from people with Stargardt disease (Delori, et al., 1995; Eldred & Lasky, 1993). Historically, all alterations in key lipids have been attributed to phagocytosis of Stargardt POS by RPE and A2E accumulation (Molday, et al., 2009; Sparrow & Boulton, 2005). That's because until recently, it was thought that ABCA4 predominantly functions in POS of PR, and atrophy of the RPE is an indirect phenomenon due to the accumulation of toxic A2E and lipofuscin (Molday, 2007; Molday, et al., 2009). However, recent evidence suggests that ABCA4 is critical for lipid homeostasis in RPE cells independent of its known function in PRs. For instance, Stargardt patient stem cell-derived and ABCA4$^{-/-}$ stem cell-derived RPE show cell-autonomous lipid handling defects without the presence of Stargardt POS (Farnoodian, et al., 2022). This observation further underscores the critical role of ABCA4 in lipid homeostasis in the retina and the RPE.

Over 1000 mutations are known to affect ABCA4 function and are associated with a broad spectrum of Stargardt's disease phenotypes (Quazi, Lenevich, & Molday, 2012; Quazi & Molday, 2013). Thus, it is enticing to speculate that mutations in different ABCA4 domains may affect RPE and PR differently – suggesting that ABCA4 may have slightly different functions in the two cell types. A genotype-phenotype study with iPSCs-derived RPE and PRs with such different mutations may help further clarify this hypothesis. Furthermore, cells with different mutations provide an important tool for understanding the differences in severity of disease phenotype in individuals harboring these mutations. Here we focus on lipidomic alteration due to ABCA4 loss of function and review Stargardt POS dependent and independent lipid profile alterations in *Abca4* $^{-/-}$ mice and *in vitro* human Stargardt disease models and discuss the relative contributions of those changes to disease pathogenesis. Highlighting specific lipid metabolism defects in the RPE and the retina

suggest a potential therapeutic mechanism for Stargardt diseases involving lipid-lowering drugs. This review adds to our understanding of cellular lipid metabolism events in response to ABCA4 loss of function, which may contribute to Stargardt disease pathogenesis.

## 2. Stargardt POS Dependent Lipidomic Changes in Retina and RPE

### 2.1. *Abca4*$^{-/-}$ mouse model

Mice lacking a functional *Abca4* gene show specific phenotypes of human ABCA4 retinopathy, particularly lipofuscin deposits in the RPE (Charbel Issa, et al., 2013). Characterization of the *Abca4*$^{-/-}$ mouse retina also showed delayed dark adaptation, elevated levels of all-trans-retinal, and increased phosphatidylethanolamine (Weng, et al., 1999). The *Abca4*$^{-/-}$ mice show a normal rate of all-trans-retinal conversion to 11-cis retinal during the visual cycle for functional rhodopsin regeneration. However, *Abca4*$^{-/-}$ animals exhibit significant light-dependent alterations in lipids (Mata, et al., 2000). Compared to age-matched wild-type (WT) mice or *Abca4*$^{-/-}$ animals kept in the dark, the outer segments of *Abca4*$^{-/-}$ mice subjected to cyclic or continuous lighting had enhanced levels of pathological visual cycle intermediates all-trans-retinal, N-ret-PE, and PE and decreased levels of functional visual cycle intermediates all-trans-retinol and all-trans-retinal esters (Mata, et al., 2001). Albino *Abca4*$^{-/-}$ animals exhibit the buildup of bisretinoids such as A2E in the RPE, the deposition of luminous lipofuscin granules in RPE cells, and the gradual degeneration of photoreceptors (Radu, et al., 2011). Below we discuss the pathological consequence of lipofuscin accumulation in the RPE and its contribution to Stargardt disease pathogenesis.

### 2.2. Lipofuscin and A2E lipid derivatives

Lipofuscin (also known as "aging pigment") refers to intracellular, luminous pigment granules that develop mostly in postmitotic cells such as neurons, heart muscle, and the RPE. This substance differs from ceroids, the lipo-pigments found in mitotic cells in different tissues that accumulate in neurodegenerative lysosomal storage diseases (Seehafer & Pearce, 2006). Lipofuscin is a heterogeneous mixture of oxidized proteins, lipids, carbohydrates, and metals (mostly iron), and its composition varies greatly depending on the tissue of origin (Brunk & Terman, 2002; Terman & Brunk, 2004). Lipofuscin is known to fluoresce at approximately 600 nm wavelength, but the spectral range depends on the tissue from which it was isolated. The significance of lipofuscin in general cellular function is unknown. Ocular lipofuscin is highly photoreactive, producing reactive oxygen species upon exposure to visible light, which has been hypothesized to disrupt lysosomal activity and other cellular functions and is a potential mechanism of age-related macular degeneration and Stargardt disease pathology (Yin, 1996).

The RPE lipofuscin is highly fluorescent. (Mata, et al., 2000; Sparrow, Parish, Hashimoto, & Nakanishi, 1999; Yin, 1996). *Ex vivo*, RPE lipofuscin has an excitation spectrum with peaks at 450-490 nm and maximum fluorescence emission at 600 nm (Sparrow, Wu, et al., 2010). As stated above, POS phagocytosed by RPE cells is believed to be the primary source of this lipofuscin. Proteomic analysis of the RPE revealed that lipofuscin granules contained little (~2%) protein (K.-P. Ng, et al., 2008). Up to this point, approximately 25

bis-retinoid components of lipofuscin, including several photo-oxidized compounds, have been reported for lipofuscin isolated from the RPE (Sparrow & Yamamoto, 2012). A detailed explanation of the above summary of the established mechanism for bisretinoid production in the RPE is that the clearance of retinaldehyde produced from bleached visual pigments in rod POS is slowed due to the loss of N-ret-PE flippase activity in Stargardt patients with ABCA4 mutations (Lenis, et al., 2018). This promotes secondary condensation of N-ret-PE with another retinaldehyde molecule to produce a phospholipid-conjugated bisretinoid, dihydro-N-retinylidene-N-retinyl-phosphatidylethanolamine (A2PE-H2) or its oxidized version (A2PE). These bisretinoids are thought to be transformed into the main lipofuscin fluorophore A2E in the acidic environment of RPE phagolysosomes with continued diurnal phagocytosis of POS (Lenis, et al., 2018; Mata, et al., 2000; Young & Bok, 1969, 1970). Consistent with this idea, the formation of RPE bisretinoids is increased several-fold in the *Abca4*$^{-/-}$ mouse (Mata, et al., 2000; Sparrow & Boulton, 2005; Weng, et al., 1999; Y. Wu, Fishkin, Pande, Pande, & Sparrow, 2009). This increased bisretinoid formation is associated with photoreceptor cell degeneration that is readily detectable at 8-9 months old animals (L. Wu, Nagasaki, & Sparrow, 2010). Furthermore, Matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI IMS), a powerful tool for detecting and identifying biomolecules in tissues, revealed a high relative abundance of A2E metabolites, including A2GPE in *Abca4* $^{-/-}$ mice (D. M. Anderson, et al., 2014; Kubo, Kajimura, & Suematsu, 2012). A2E is both cationic and amphiphilic and is known to accumulate in lysosomes (Sparrow, et al., 2003; Sparrow, Kim, & Wu, 2010). A2E accumulation in lysosomes is proposed as a potential cause of elevated Bis(monoacylglycero)phosphate (BMP) lipids level in the RPE of *Abca4*$^{-/-}$ mice (D. M. G. Anderson, et al., 2017). *In vitro* studies have demonstrated that A2E inhibits the lysosomal degradation of POS, leading to alterations in cholesterol and ceramide levels and delayed lipid degradation (Finnemann, Leung, & Rodriguez-Boulan, 2002; Lakkaraju, Finnemann, & Rodriguez-Boulan, 2007; Toops, Tan, Jiang, Radu, & Lakkaraju, 2015). Furthermore, accumulation of A2E in the RPE of *Abca4*$^{-/-}$ mice and Stargardt-iRPE cultures was shown to activate the alternate complement pathway, increase inflammatory markers, and cause oxidative stress (E. S. Y. Ng, et al., 2022; Radu, et al., 2011).

### 2.3. Bis(monoacylglycero)phosphate lipids

Bis(monoacylglycero)phosphate lipids (BMP) are a structurally distinct family of lipids that are highly enriched in the intraluminal vesicles of late endosomes/lysosomes (Kobayashi, et al., 2002). BMP is negatively charged at acidic lysosomal pH and can operate as a docking platform that recruits positively charged lipid hydrolases to intraluminal vesicles, hence improving lipid cargo breakdown, which is critical for the degradation of lipids and membranes in lysosomes (Grabner, et al., 2020). Furthermore, through its interactions with cholesterol transport and sphingolipid activator proteins, BMP is a key cofactor in lysosomal cholesterol and sphingolipid metabolism (Enkavi, Mikkolainen, Güngör, Ikonen, & Vattulainen, 2017; Locatelli-Hoops, et al., 2006). Multiple studies have found that BMP lipids regulate cellular cholesterol content in the endosome-lysosome compartment (Chevallier, et al., 2008; Gallala & Sandhoff, 2011). BMP lipids are enriched in a variety of endosomal/lysosomal storage diseases (Chevallier, et al., 2008; Walkley & Vanier, 2009). BMP lipids have a unique structural variation in the stereospecific numbering (sn) position

of fatty acid chains (Akgoc, et al., 2015). Fatty acids are esterified to three stereospecific sites on the glycerol backbone in the original TAG molecule (Karupaiah & Sundram, 2007). Accumulation of BMP lipids with varying fatty acid chains, such as BMP (20:4 22:6) at m/z 841.50, BMP (20:4 20:4), and BMP (18:2 22:6) at m/z 817.50, was found in the RPE of *Abca4*$^{-/-}$ mice using MALDI IMS. Targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) in both negative ion mode (to identify the fatty acid chains) and positive ion mode (to validate the sn configuration of the fatty acid chains) is used to distinguish lipid species, especially differentiating BMP lipids from others (Duffin, Obukowicz, Raz, & Shieh, 2000). MALDI IMS combined with LC-MS/MS analysis revealed the presence of BMP lipids in the aging human retina (D. M. G. Anderson, et al., 2017; Hankin, Murphy, Barkley, & Gijón, 2015). Notably, the majority of the fatty acid constituents of BMP lipids are polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) (22:6), a fatty acid that is known to be highly abundant in photoreceptors and to be essential for photoreceptor cell recycling and survival (D. M. G. Anderson, et al., 2017; N. G. Bazan, 2006). DHA in the endosomes, in the form of BMP lipids, is known to prevent the production of neuroprotectin D1 (NPD1) - which protects RPE cells from oxidative stress-induced apoptosis (N. G. Bazan, 2006). Despite some understanding of BMP lipid structure, pathways leading to the formation of BMP lipids are not well studied. It is thought that the accumulation of phospholipids in drug-induced phospholipidosis results from interactions between cationic amphiphilic drugs and endogenous lipids to produce indigestible metabolites or the direct inhibition of enzymes involved in lipid recycling and degradation (Hullin-Matsuda, Luquain-Costaz, Bouvier, & Delton-Vandenbroucke, 2009). The formation of BMPs is most likely due to endogenous lipid interactions that produce indigestible metabolites or due to direct inhibition of the enzymes involved in lipid recycling and degradation. In *Abca4*$^{-/-}$ mice, the accumulation of A2E most likely triggers the formation of BMP (D. M. G. Anderson, et al., 2017). Since A2E is both cationic and amphiphilic, and it accumulates in lysosomes (Sparrow, Kim, et al., 2010). Previous studies have demonstrated that A2E accumulation can inhibit lipid degradation and the phagocytosis of photoreceptor outer segments and result in free and esterified cholesterol (Finnemann, et al., 2002; Kaur, et al., 2018; Lakkaraju, et al., 2007; Poliakov, et al., 2014). These findings suggest that BMP accumulation in the RPE due to the disrupted lipid metabolism associated with lysosomal dysfunction can contribute to the Stargardt pathogenesis; BMP accumulation in the Stargardt patient retina further highlights the role of BMP and similar lipids in Stargardt disease pathogenesis (D. M. G. Anderson, et al., 2017; Hankin, et al., 2015).

### 2.4. Cholesterol and Ceramide

The majority of sterol part of lipid membranes is cholesterol, which acts as a buffer to create space in the membrane (Lewandowski, et al., 2021). Cholesterol regulates membrane biophysical properties and plays a crucial role in lipid and protein trafficking. Furthermore, cholesterol is the predominant sterol in the retina, especially in POS. It can be produced within the retina. However, cholesterol synthesis is a slow process (A. J. Fliesler & Anderson, 1983; Keller, Fliesler, & Nellis, 1988). In the vertebrate retina, cholesterol stabilizes rhodopsin in POS disks and slows visual cycle kinetics (Albert & Boesze-Battaglia, 2005). Daily phagocytosis and processing of POS impose a substantial metabolic burden on RPE cells. RPE cells express several receptors and cholesterol

transporters to regulate cholesterol homeostasis and metabolism (Tserentsoodol, et al., 2006). In the RPE, cholesterol can be metabolized to ceramide by acid sphingomyelinase (ASMase) (Toops, et al., 2015). High levels of A2E and other bisretinoids are shown to result in cholesterol metabolism defects leading to excess cholesterol in the RPE (Toops, et al., 2015). The A2E-induced lysosomal cholesterol storage activates ASMase by sequestering BMP, an ASMase cofactor, leading to increased ceramide levels in the RPE (Toops, et al., 2015). Ceramide is a metabolite of sphingolipids, a family of membrane lipids that play essential structural roles in the fluidity regulation and subdomain structure of the lipid bilayer, particularly lipid rafts (Martin, Elliott, Brush, & Anderson, 2005; Tsui-Pierchala, Encinas, Milbrandt, & Johnson, 2002). Ceramide also plays key functional roles in receptor function, cell–cell interactions, and pathogen internalization (Hannun & Obeid, 2008; Huwilera, 2000). *In vitro* studies have established ceramide as a crucial second mediator in activating photoreceptor apoptosis (German, Miranda, Abrahan, & Rotstein, 2006). In addition, ceramide involvement in the activation of RPE cell death was shown previously (Barak, Goldkorn, & Morse, 2005; Barak, Morse, & Goldkorn, 2001; Kannan, Jin, Gamulescu, & Hinton, 2004; Zhu, Sreekumar, Hinton, & Kannan, 2010). Furthermore, increased ceramide at the apical surface of the RPE promotes endosome enlargement, which results in the internalization of complement protein C3 into the RPE and the formation of intracellular C3a fragments. In turn, elevated C3a activates the mammalian target of rapamycin (mTOR), a regulator of metabolic processes, including autophagy, leading to decreased autophagosome biogenesis and chronic metabolic reprogramming in the RPE (Kaur, et al., 2018). These findings suggest that ceramide accumulation disrupts autophagosome biogenesis, and reduces autophagosome traffic, and autophagic flux, all of which contribute to Stargardt disease pathogenesis (Kaur, et al., 2018; Lakkaraju, et al., 2007; Toops, et al., 2015).

### 2.5. New discoveries: Major Phospholipids/Fatty acids

Proper composition of membrane phospholipids, cholesterol, and fatty acids is critical in the function of membrane receptors, ion channels, and membrane-bound enzymes involved in 'RPE's ability to phagocytose POS and transport visual pigment to PRs (Kwon & Freeman, 2020; Lewandowski, et al., 2021). The lipid content of RPE cells differs from that of the retina, with a lower phospholipid content (60% <RPE vs. >85% retina) and a higher level of cholesteryl esters (19% RPE vs. 1.7% retina) (Bretillon, et al., 2008). Most membrane lipid bilayers comprise three major classes: phospholipids, sterols, and free fatty acids (FFAs) (Lewandowski, et al., 2021). Phospholipids are typically discussed in terms of charge/polarity. For example, while phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) are charged, phosphatidylethanolamine (PE) is not. These lipids play a fundamental role in retinal function and disease. The predominant long-chain PUFAs (LC-PUFAs) in the retina and POS membranes of all vertebrate species studied thus far are Docosahexaenoic Acid (DHA; 22:6n3), a highly unsaturated fatty acid, and arachidonic acid (AA; 20:4n6) (A. J. Fliesler & Anderson, 1983; SanGiovanni & Chew, 2005). For example, PUFAs with DHA (22:6, 22 carbons with 6 double bonds) are representative of the retina, accounting for approximately 50% of the fatty acids in photoreceptors (Gu, et al., 2003). The accumulation of such large amounts of DHA in retinal membranes makes them fluid-like, favoring efficient conformational changes during phototransduction. RPE

cell-autonomous functions are meditated in specific lipid signaling crucial for photoreceptor integrity (N. G. Bazan, 2007). RPE synthesizes a DHA-derived lipid mediator that prompts balancing cell-protective, anti-inflammatory, and pro-survival repair signaling called NPD1 (N. G. Bazan, 2005, 2006; Nicolas G Bazan, Gordon, & Rodriguez de Turco, 1992).

Small changes in the dietary level of phospholipids/fatty acids (AA and/or DHA) and VLC-PUFA-containing AA and/or DHA are important determinants of visual cell membrane fatty acid composition during the development of the retina (Suh, Wierzbicki, Lien, & Clandinin, 2000; Uauy, Hoffman, Peirano, Birch, & Birch, 2001).

To determine the impact of ABCA4 loss of function (LOF) on major phospholipids/fatty acids lipids, we performed LC-MS/MS analysis on RPE/choroid and retina samples from 11-month-old WT (C57BL/6J) and *Abca4*$^{-/-}$ mice. For the phospholipid analysis semi–targeted analysis was performed. For PC and SM, parent ion scans were performed for the fragment m/z 184; for PE, PS parent ion scans were performed for the neutral loss of fragments m/z 141 and m/z 185, respectively. The sum of the peak area for one class of phospholipids was used to determine the percentage for each individual species. This percentage was then normalized to the WT. The Multiple reaction monitoring (MRM) method was used to analyze fatty acids. Retention time and mass transition were determined using fatty acid standards (Cayman, Ann Arbor, MI, USA), and the data were used to identify/quantify fatty acid species in samples.

Our analysis revealed an altered lipid profile in the RPE's major phospholipids/fatty acids in the absence of *Abca4* (Figure 1). *Abca4*$^{-/-}$ mice exhibited a 30% decrease in PC38:6 (DHA/16:0) and PC40:6 (DHA/18:0), whereas PC38:4 and PC36:4 (16:0/20:4) were marginally (10-20%) higher relative to WT mice (Figure 1A). The changed PC phospholipid profile was associated with a decrease in VLC-PUFA-containing PC (PC50:12) and DHA-containing species in *Abca4*$^{-/-}$ RPE and an increase in AA-containing species (Table 1). Relevant changes identified in the most common PC species (with a concentration range of 4-13 nmol/eye) in *Abca4*$^{-/-}$ RPE are shown in Figure 1A. PE is the second main type of glycerophospholipid class in the retina (the first being PC) (S. J. Fliesler, 2010). PE phospholipids may also participate in the movement of the retinoid in the membrane (Ahn, Wong, & Molday, 2000). PE (40:6) (DHA/18:0) was 30% lower in *Abca4*$^{-/-}$ RPE as compared to the WT RPE, while PE (36:1) and PE (38:7) were 15-30% higher (Figure 1B). Figure 1B shows variations in the most frequent PE species (with a concentration range of 2-19 nmol/eye) in *Abca4*$^{-/-}$ RPE. Similar to PC species, a large reduction of DHA-containing PE species was observed in the *Abca4*$^{-/-}$ RPE, while AA-containing species were noted to increase (Table 1, Figure 1B). Table 1 shows changes in the less common PC species with a concentration (<4 nmol/eye) and PE species with a concentration (<2 nmol/eye) in the *Abca4*$^{-/-}$ RPE.

PS phospholipid involvement in phagocytosis signaling has been shown previously (Lewandowski, et al., 2021). The observed differences in specific molecular species of PS included a 30% reduction of DHA-containing species in the *Abca4*$^{-/-}$ RPE and a 30-50% increase in AA-containing species (Figure 1C). The commonly found PS species (with a concentration range of 1.5-10 nmol/eye) are shown in Figure 1C. PS (46:12) was 30%

lower in the *Abca4*$^{-/-}$ than in WT animals, while PS (38:4) (18:0/20:4) and PS (38:5) were 30-50% higher (Figure 1C). The levels of PS are higher in RPE compared to the retina. In addition, sphingolipids, crucial lipids for the retina, are higher in the RPE, indicating sphingolipid metabolism is a major component of the RPE (Sinha, Naash, & Al-Ubaidi, 2020). Analysis of sphingomyelin (SM) species in RPE and retina revealed no difference between *Abca4*$^{-/-}$ and WT (data not shown).

Phospholipid PC and PE did not show clear differences between *Abca4*$^{-/-}$ and WT retina, including DHA-containing or VLC-PUFA-containing species (data not shown). The most frequent PS phospholipid species in the retina (with a concentration range of 1.5-4.5 nmol/eye) are shown in Figure 1D. PS (38:4) and PS (42:9) were 10-30% lower in the *Abca4*$^{-/-}$ than in WT animals, while PS (46:11) (18:0/20:4) were 20 % higher (Figure 1D).

Elovanoids (ELVs) are formed as a result of the breakdown of membrane lipids. They are derived from VLC-PUFAs and have primarily protective properties (Lewandowski, et al., 2021). In addition, ELVs have been discussed explicitly regarding their general neuroprotective effects on the retina (Nicolas G Bazan, et al., 1992). The majority of DHA in RPE cells is a component of photoreceptor disc membrane phospholipids that are recycled as part of outer segment renewal following phagocytosis (Nicolas G Bazan, 2006). It has been previously shown that RPE cells utilize free DHA for NPD1 production when exposed to oxidative stress. These signals activate phospholipase A2 or A1 to cleave docosahexaenoic chains or VLC-PUFAs from POS disk membrane phospholipids, leading to NPD1 or ELVs production, respectively (N. G. Bazan, 2018; Mukherjee, Marcheselli, de Rivero Vaccari, et al., 2007). These findings imply that DHA-NPD1- ELVs signaling enhances photoreceptor and RPE cellular integrity and function. The active secretion of NPD1 and ELVs from RPE cells shows that these lipid mediators have autocrine and paracrine bioactivity. The disruption of this homeostatic DHA-NPD1-ELVs regulation could play a role in the onset and progression of retinal degenerative disorders (N. G. Bazan, 2007; Miyagishima, et al., 2021; Mukherjee, Marcheselli, Barreiro, et al., 2007; Mukherjee, Marcheselli, de Rivero Vaccari, et al., 2007). Free VLC-PUFAs (FA32:6 n3 and FA34:6 n3) were more abundant in the WT RPE compared with the *Abca4*$^{-/-}$ mice, so were the monohydroxy-derivatives, 27-monohydroxy 32:6 and 29-monohydroxy 34:6, which are the stable metabolites in the pathway of ELVs Synthesis (data not shown).

The polyunsaturated long-chain fatty acid concentration varies with cell type, age, and ocular diseases (Agbaga, et al., 2018; Liu, Chang, Lin, Shen, & Bernstein, 2010). For example, DHA, AA, and other VLC-PUFAs were significantly lower in AMD retina and RPE/choroid compared to age-matched normal donors (Liu, et al., 2010). Our findings indicate that the loss of ABCA4 function leads to an altered lipid profile in DHA-containing or VLC-PUFA-containing species, primarily in RPE, with minimal impact on the retina. Therefore, a potential disruption of homeostatic DHA-NPD1- ELVs regulation due to the loss of ABCA4 function in the RPE needs further investigation. In addition, the effects of changes in lipid species and specific metabolic enzymes involved in these processes remain unknown and merit further investigation.

## 3. Stargardt POS Independent Lipidomic Change

### 3.1. *In vitro* Stargardt Disease Model

*In vitro* model for Stargardt disease using ABCA4 mutant induced pluripotent stem cell (iPSC)-derived RPE (Stargardt-iRPE) demonstrated disease phenotype of intra/subcellular lipid deposition and progressive RPE atrophy (Farnoodian, et al., 2022). Previously intra/subcellular lipid and cholesterol deposits in Stargardt patients and *Abca4*$^{-/-}$ mice were thought to be caused by A2E-induced lipid metabolism defects. A2E increases cholesterol's chemical activity and displacement from membranes, providing a biophysical mechanism for cholesterol sequestration leading to aberrant cholesterol metabolism in RPE cells (Lakkaraju, et al., 2007). We recently showed Stargardt-iRPE could reproduce the same lipid handling defects cell-autonomously without exposure to Stargardt POS. We replicated cholesterol and ceramide accumulation without exogenously adding A2E or Stargardt POS to our Stargardt-iRPE cultures, indicating a Stargardt POS-independent pathway for lipid homeostasis in RPE cells. The LC-MS/MS analysis confirmed increased cholesterol and its metabolites and ceramide species in the Stargardt-iRPE compared to the WT-iRPE treated with WT POS for seven days (Figures 2A and B). Our findings suggest that ABCA4 LOF in iRPE cells induces phagolysosomal dysfunction, resulting in ineffective POS digestion (Farnoodian, et al., 2022).

Bisretinoids and their oxidation products elicit a robust alternate complement response in RPE cells in culture (Zhou, Kim, Westlund, & Sparrow, 2009). In addition, A2E accumulation in the RPE in *Abca4*$^{-/-}$ mice is shown to activate complement, induce inflammatory responses, and result in oxidative stress (Radu, et al., 2011). Recent research published by Radu's group revealed complement dysregulation in ABCA4-deficient patient-iRPE cells. They discovered a strong correlation between aging RPE and MAC deposition resulting in RPE cell loss, similar to the RPE phenotype observed in *Abca4*$^{-/-}$ mice (E. S. Y. Ng, et al., 2022).

Stargardt *in vitro* disease model using human iRPE cells reproduced similar phenotypes and lipid alteration observed in *Abca4*$^{-/-}$ mice (Figure 2 C and D), suggesting the use of these models to better understand mechanisms involved in lipid metabolism and lipid handling defects that contribute to the pathogenesis of Stargardt disease.

## 4. Potential Therapeutic Mechanisms Targeting Lipid Homeostasis Defects

It is commonly acknowledged that lipid metabolism plays a critical role in therapeutic processes (Islam & Manna, 2019). Consistently, several studies have tried ameliorating Stargardt disease phenotype by targeting the vitamin A–dependent nature of A2E biosynthesis (Radu, et al., 2005). In the case of lacking ABCA4 function, retinaldehyde-PE conjugates may react to create vitamin A dimers (A2E and ATR-dimer, among others), which are then deposited in the RPE after photoreceptor outer segment phagocytosis, resulting in lipofuscin accumulation and retinal degeneration (Lamb & Simon, 2004; Sparrow, et al., 2003). Radu et al. showed that N-(4-hydroxyphenyl) retinamide (HPR),

known to diminish serum retinol and retinol-binding protein reversibly, effectively inhibited the development of A2E and other lipofuscin fluorophores with no adverse effects on visual function or retinal morphology (Radu, et al., 2005). In addition, *ABCA4* mutant albino mice treated with vitamin A deuterated at the C20 position (C20-D3-vitamin A), which inhibits vitamin A's innate reactivity to dimerize, exhibited a decrease in A2E and fundus autofluorescence, resulting in decreased lipofuscin accumulation and improved eye function (Ma, Kaufman, Zhang, & Washington, 2011). Modulating cholesterol efflux in RPE cells to reduce cholesterol and cholesteryl esters accumulation has also been considered a potential therapeutic target. Activating the liver X receptor (LXR)/peroxisome proliferator-activated receptor (PPAR) pathway restores cholesterol homeostasis in A2E-laden RPE cells (Lakkaraju, et al., 2007). Furthermore, LXR agonist is shown to rescue the cell autonomous lipid-handling defect seen in Stargardt-iRPE (Farnoodian, et al., 2022). ASMase inhibition could be a viable therapeutic target for lowering cholesterol and ceramide accumulations induced by lipofuscins bisretinoid. Desipramine, a functional inhibitor of ASMase, lowers ASMase activity and ceramide levels, restoring autophagic flux in RPE with the lipofuscin bisretinoid A2E(Kaur, et al., 2018; Toops, et al., 2015). These approaches further underscore our observation of broad lipid homeostasis defects as the key disease inducing feature in Stargardt patients.

## 5. Conclusions

There is currently inadequate information on the complexity of changes in lipid composition, homeostasis, metabolism, and lipid-mediated signaling in the RPE, leading to ABCA4 retinopathy. The development of novel therapies targeting Stargardt disease is enabled by identifying and investigating the pathobiological processes implicated in Stargardt. Here we reviewed two pathways leading to lipidomic changes in the RPE and retina and their contributions to Stargardt disease pathogenesis (Figure 3). Furthermore, we discussed how lipofuscin and its metabolites, including A2E, may cause cascading changes in the RPE lipid profile, including elevated BMP, cholesterol, and ceramide, contributing to abnormal RPE phenotype. These lipid pathway changes are potential therapeutic targets to rescue disease phenotype in RPE cells (Figure 3). Published work in Stargardt disease pathogenesis and our recent findings underscore the need for further investigation of lipid metabolism and lipid-mediated signaling in the retina and RPE and their contributions to disease pathogenesis. These findings are expected to lead to new, effective treatments for a number of retinal degenerative diseases, including Stargardt maculopathy and AMD.

## Abbreviations:

| | |
|---|---|
| **RPE** | retinal pigment epithelium |
| **POS** | photoreceptor outer segment |
| **BMP** | Bis(monoacylglycero)phosphate |
| **ASMase** | acid sphingomyelinase |
| **DHA** | docosahexaenoic acid |

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|---|---|
| **FA** | fatty acid |
| **LC-PUFA** | long-chain polyunsaturated fatty acid |
| **LPC** | lysophosphatidylcholine |
| **NPD1** | neuroprotectin D1 |
| **PC** | phosphatidylcholine |
| **PS** | phosphatidylserine |
| **PI** | phosphatidylinositol |
| **PE** | phosphatidylethanolamine |
| **AA** | Arachidonic Acid |
| **LOF** | loss of function |
| **PUFA** | polyunsaturated fatty acid |
| **VLCFA** | very long chain fatty acid |
| **VLC-PUFA** | very long-chain polyunsaturated fatty acid |
| **ELVs** | Elovanoids |

## 8. References


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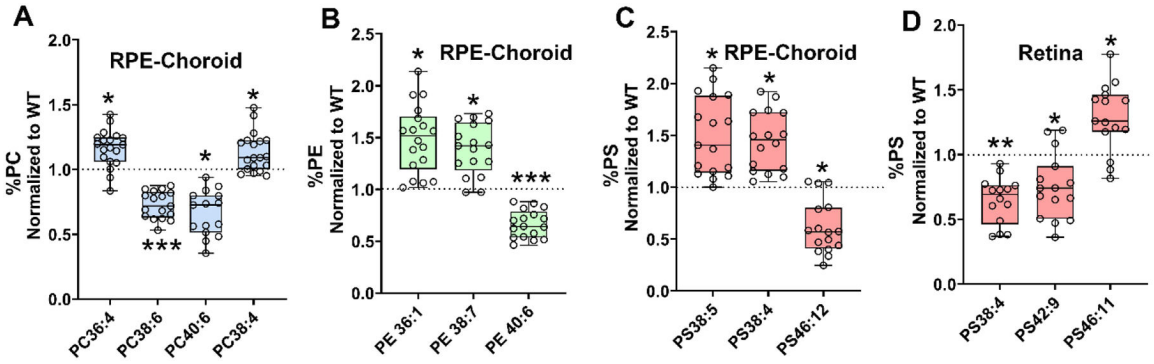


**Figure 1: ABCA4 loss of function altered major phospholipids/fatty acids lipids in the RPE.**

LC-MS/MS analysis on the RPE/choroid and retina tissues from 11M WT and *Abca4*$^{-/-}$ mice (n=16 eye/animal). LC-MS/MS analysis was performed in a Xevo TQ - S equipped with Acquity I class UPLC with a flow-through needle (Waters, Milford, MA, USA). **A:** A box plot showing relevant changes in the most common PC species (with a concentration > 4-13 nmol/eye) in the RPE. Note decreased levels of PC38:6 (DHA/16:0) and PC40:6 (DHA/18:0) and increased PC36:4 and PC38:4 in *Abca4*$^{-/-}$ mice (*: $p<0.05$, ***: $p<0.001$). **B:** Box plot showing variations in the most frequent PE species (concentrations > 2-19 nmol/eye) in *Abca4*$^{-/-}$ RPE. Note decreased PE (40:6) (DHA/18:0) and increased PE (36:1) and PE (38:7) in *Abca4*$^{-/-}$ mice (*: $p<0.05$, ***: $p<0.001$). **C:** The most common phosphatidylserine(PS) species (concentrations > 1.5-10 nmol/eye) are shown using a box plot. Note decreased PS (46:12) and increased PS (38:5), PS (38:4) (18:0/20:4) in the *Abca4*$^{-/-}$(*: $p<0.05$). D: Box plots showing relevant changes in the most common PS species phospholipids (concentrations > 1.5-4.5 nmol/eye) in the retina (*: $p<0.05$, **: $p<0.01$). n represents independent samples from each group. The statistical analysis was performed using GraphPad Prism software (La Jolla, CA).

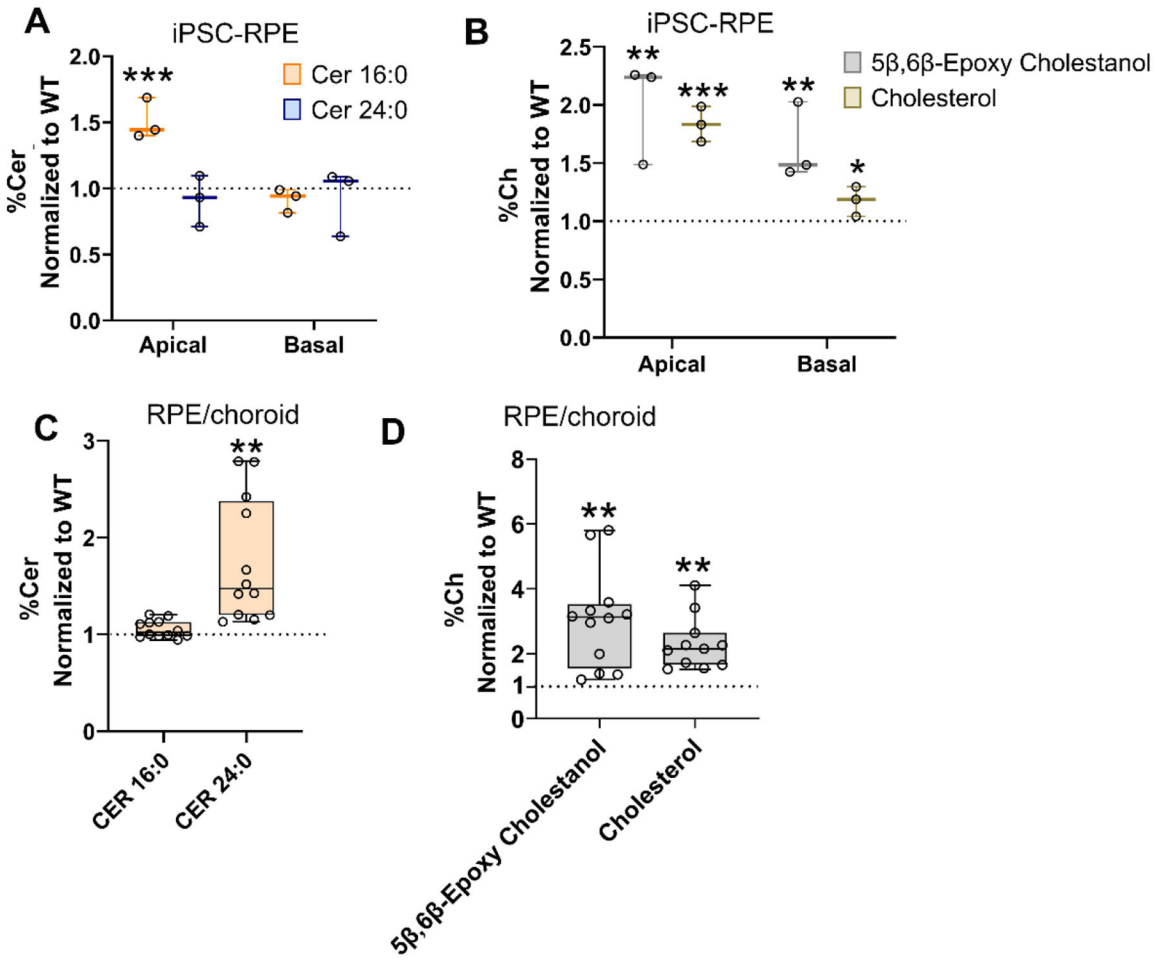


**Figure 2: ABCA4 loss of function increased cholesterol, its metabolite, and ceramide species in the Stargardt-iRPE cultures and *Abca4*$^{-/-}$-RPE/choroid.**

LC-MS/MS analysis on t Stargardt-iRPE treated with POS for seven days (n=3) and the RPE/choroid tissues from 11M WT and *Abca4*$^{-/-}$ mice (n=12 eye/animal). **A:** A box plot showing relevant changes in the ceramide species in the Stargardt-iRPE treated with POS. Note Increased levels of Cer 16:0 in the collected apical medium from Stargardt-iRPE ( ***: p<0.001). **B:** A box plot showing relevant changes in the cholesterol and its metabolite in the Stargardt-iRPE treated with POS. Note Increased levels of cholesterol and 5, 6-Epoxy Cholestanol in the collected medium from the apical and basal side in Stargardt-iRPE (*: p<0.05, **: p<0.01, ***: p<0.001). **C:** Box plot showing variations in the ceramide species in *Abca4*$^{-/-}$ RPE. Note Increased levels of Cer 24:0 in the *Abca4*$^{-/-}$ RPE ( **: p<0.01). D: Box plots showing relevant changes in the cholesterol and its metabolite in the *Abca4*$^{-/-}$ RPE. Note Increased levels of cholesterol and 5, 6-Epoxy Cholestanol in the *Abca4*$^{-/-}$ RPE ( **: p<0.01). n represents independent samples from each group.

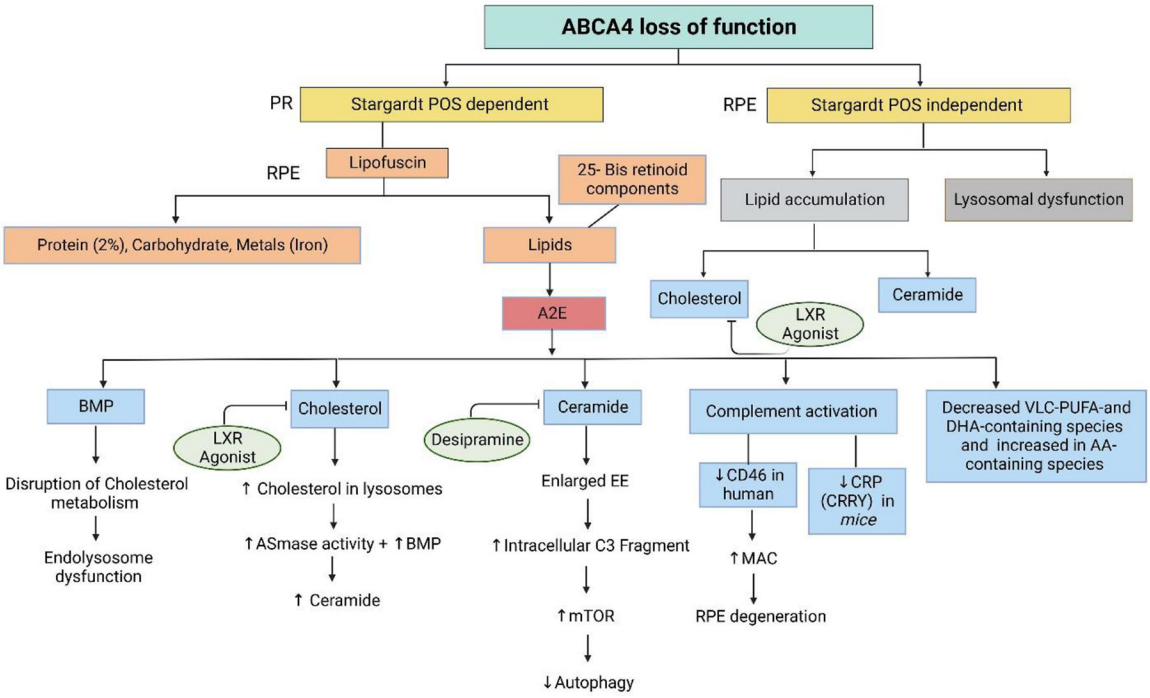


**Figure 3. Schematic design of summarizing lipidomic change in Stargardt disease.**

In Stargardt POS-dependent pathway, ABCA4 loss of function results in the ongoing intracellular accumulation of lipofuscin material, including A2E, which has been associated with lysosomal dysfunction, activating the complement system and autophagy down-regulation through elevated BMP, cholesterol, and ceramide level in the RPE cells. As shown in the figure, these defects can be restored by targeting cholesterol transport and the ceramide pathway. ABCA4 deficiency can also cause lipid handling defects cell autonomously in the RPE cells. In addition, the accumulated cholesterol and ceramide are associated with lysosome dysfunction in iRPE cells. Diagram created with BioRender.com.

**Table 1.**

LC-MS/MS analysis on the RPE/ Choroid from 12M WT and *Abca4*$^{-/-}$ mice showing alerted phospholipids/ fatty acids lipids including PC, and PE species phospholipids [ (n=16 eye/animal), (*: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001)]. n represents independent samples from each group.

| **Phosphatidylcholine (PC)** | | **Phosphatidylethanolamine (PE)** | |
|---|---|---|---|
| **Increased** | **Decreased** | **Increased** | **Decreased** |
| PC54:10 (*) | PC50:12 (*) | PE 38:8 **(**)** | PE 44:10 **(***)** |
| PC36:00 (**) | PC44:11 **(**)** | PE 38:1 **(*)** | |
| | PC46:8 **(***)** | PE 38:2 **(**)** | |
| | PC38:7 **(***)** | PE 40:9 **(*)** | |