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Plasmalogen deficiency is associated with major polyunsaturated fatty acid (PUFA) alterations in the mouse retina☆

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Abstract – Background: Plasmalogens are ether-phospholipids that play essential roles in cell physiology. They serve as reservoirs for polyunsaturated fatty acids (PUFAs), which regulate retinal vascular development. Plasmalogen deficiency is associated with severe ocular defects in mice, including abnormal retinal vascularization. However, the impact of plasmalogen deficiency on retinal fatty acid composition remains to be determined. **Objectives:** This study evaluated the effects of plasmalogen deficiency on mouse retinal fatty acid composition and whether dietary supplementation of lactating mothers with a plasmalogen precursor, alkylglycerol, could prevent these alterations in offspring. Because the retina is an extension of the central nervous system, the brain cortex was also analyzed to compare tissue-specific responses to plasmalogen deficiency. **Methods:** Lactating mice were fed a diet enriched with 2% alkylglycerols. Retinal and brain fatty acid compositions were determined in pups at postnatal day 21 using gas chromatography with flame ionization detection (GC-FID). **Results:** Plasmalogen deficiency was associated with significant alterations in retinal fatty acid composition. Total PUFAs content was significantly reduced, while the n−6:n−3 ratio was strongly increased. These changes were accompanied by modest but significant increases in saturated and monounsaturated fatty acids. Alterations of PUFAs composition were also observed in the cortex. Maternal alkylglycerol supplementation showed only a slight trend toward improvement and was insufficient to restore retinal plasmalogen levels, PUFA content, or n−6:n−3 ratio. In the brain, alkylglycerol supplementation had no effect, suggesting potential tissue-specific differences in precursors uptake/metabolism.**Conclusions:** Plasmalogen deficiency significantly disrupts the fatty acid composition of the retina, primarily through PUFA depletion and an increased n−6:n−3 ratio. These alterations may contribute to the pathological retinal phenotype observed in plasmalogen-deficient mice. Maternal alkylglycerol supplementation during early postnatal life is insufficient to correct these defects. Further studies are needed to clarify the role of altered lipid composition in retinal vascular development.

Keywords: plasmalogens / retina / development / nutrition / alkylglycerol / PUFAs

Résumé – La déficience en plasmalogènes est associée à une altération significative du profil rétinien en acides gras polyinsaturés (AGPI) chez la souris. Contexte : Les plasmalogènes sont des éther-phospholipides essentiels à la physiologie cellulaire. Ils constituent un réservoir d'acides gras polyinsaturés (AGPIs), qui sont notamment impliqués dans le développement vasculaire rétinien. Chez la souris, la déficience en plasmalogènes est associée à de nombreuses anomalies oculaires, notamment du développement vasculaire rétinien. L'impact de la déficience en plasmalogènes sur la composition rétinienne en acides gras (AG) reste toutefois à déterminer. **Objectifs :** Cette étude visait à évaluer les effets de la déficience en plasmalogènes sur le profil en AG de la rétine de souriceaux, ainsi que la capacité d'une supplémentation maternelle en alkylglycérols, précurseurs des plasmalogènes, à prévenir ces altérations.

☆ Contribution to the Topical Issue: "Lipids and health / Lipides et santé".
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Le cortex cérébral a également été analysé afin d'identifier d'éventuelles spécificités tissulaires.**Méthodes :** Les souris allaitantes ont reçu un régime enrichi à 2% d'alkylglycérols. Les compositions en AG de la rétine et du cortex cérébral des souriceaux âgés de 21 jours ont été déterminées par chromatographie en phase gazeuse couplée à un détecteur à ionisation de flamme (GC-FID). **Résultats :** La déficience en plasmalogènes était associée à une diminution significative des AGPIs et une augmentation du ratio n−6: n−3 dans la rétine, accompagnées d'une hausse modérée des AG saturés et monoinsaturés. Des altérations du profil en AGPIs ont également été observées dans le cortex cérébral. Malgré une légère tendance, la supplémentation maternelle en alkylglycérols n'a restauré ni les niveaux de plasmalogènes ni le profil en AGPIs. Elle n'a par ailleurs eu aucun effet dans le cerveau, suggérant de potentielles différences tissulaires dans l'absorption et/ou le métabolisme de ces précurseurs. **Conclusion :** La déficience en plasmalogènes altère significativement la composition en AGPIs de la rétine, ce qui pourrait contribuer aux anomalies du développement vasculaire rétinien de ce modèle. La supplémentation maternelle précoce en alkylglycérols n'a pas permis de corriger ces altérations.

Mots-clés : plasmalogènes / rétine / développement / nutrition / alkylglycérol / AGPIs

Highlights

- Plasmalogen deficiency significantly modifies retinal and cortical lipid composition in mice, with reduced PUFA levels and increased n−6/n−3 ratio. Maternal alkylglycerol supplementation during lactation only marginally improves these abnormalities.

1 Introduction

Plasmalogens are a subgroup of membrane phospholipids characterized by a vinyl-ether bond at the *sn*−1 position of the glycerol backbone. They are particularly abundant in nervous tissues where they can represent up to 30% of ethanolamine phospholipids (Nagan and Zoeller, 2001), suggesting a specific role in these tissues' physiology. As an extension of the central nervous system (CNS), the retina was also shown to exhibit high concentrations of plasmalogens, especially in Müller cells (Acar *et al.*, 2007), the main glial cells of the retina (Bringmann *et al.*, 2006). Plasmalogens are involved in several major functions of cell physiology such as membrane structure and dynamics, antioxidative status maintenance and cell signaling. Several studies suggested that plasmalogens are involved in the development and physiology of the eye and the retina and: indeed, plasmalogen deficiency is associated with several major eye defects in the mouse such as microphthalmia, optic nerve hypoplasia, cataract, persistence of hyaloid vessels during adult life, and abnormal vascular development of retinal vessels (Brites *et al.*, 2011; Rodemer *et al.*, 2003; Saab, Buteau *et al.*, 2014). These abnormalities in retinal vascularization take place in a two-phase manner during the post-natal development of the retina: first a delayed outgrowth of vessels at post-natal day 7 (PN7), followed by a second phase of proliferative angiogenesis at post-natal day 14 (PN14) (Saab, Buteau *et al.*, 2014). However, the mechanisms responsible for these abnormalities remain to be determined. One such mechanism could involve polyunsaturated fatty acids (PUFAs). Plasmalogens are indeed considered as a PUFAs reservoir (Braverman and Moser, 2012; Nagan and Zoeller, 2001), and several studies have interestingly shown the role of plasmalogens in the regulation of tissular PUFA status, including in nervous tissues (Gaposchkin and Zoeller, 1999a; Martinez, 1992b; Martinez and Mougan, 1999a). Furthermore, other studies have highlighted the importance of PUFAs on the vascular development of the retina. For instance, DHA (docosahexaenoic acid), the main n−3 PUFA in the retina, was shown to inhibit pathological neovascularization in a mouse model of oxygen-induced retinopathy (OIR), whereas ARA (arachidonic acid), the main n−6 PUFA in the retina, exerted the opposite effect (Connor *et al.*, 2007). In humans, we have shown that children developing retinopathy of prematurity (ROP) display several alterations of blood PUFAs levels, with a potential accumulation of n−6 over n-3 PUFAs with advancing gestational age (Pallot *et al.*, 2019) that may be partly due to plasmalogens (Karadayi *et al.*, 2022). Previous studies have shown that a nutritional supplementation with 2% of alkylglycerols (a plasmalogens precursor) was able to restore plasmalogen levels and to prevent at least partly several abnormalities in the plasmalogen deficient mouse (Rasmiena *et al.*, 2015; Todt *et al.*, 2020), including those related to lens development (Brites *et al.*, 2011). However, the exact mechanisms underlying these abnormalities are still undocumented. Therefore, our study aimed to expand our understanding of the mechanisms by which plasmalogens regulate the fatty acid profile of the retina during its development. Using nutritional and chromatographic approaches, we determined the consequences of plasmalogen deficiency on the fatty acid profile of the mouse retina *in vivo* and assessed if a nutritional supplementation of the pups with alkylglycerol through lactation is able to restore plasmalogen levels and the fatty acid composition of the retina. Finally, considering that the retina is a direct extension of the CNS and that the brain and retina share common physiological features, including the enrichment in PUFAs, we also investigated lipid changes in the brain cortex.

2 Materials and methods

2.1 Animals

Experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology statements and with French legislation (authorization number 21CAE086 for N.A. and animal quarters agreement number

A21231010 EA), after approval by the local ethics committees (#105 Comité d'Ethique de l'Expérimentation Animale Grand Campus Dijon). DAPAT$^{-/-}$ pups were studied indistinctively of their sex distribution.

While the gene encoding for the first enzyme responsible for plasmalogen synthesis is usually known as *Gnpat* (or eventually *Dhapat*), the previous studies conducted on this mouse model were referring to these mice as DAPAT mice. Accordingly, plasmalogen-deficient mice will be referred to as DAPAT$^{-/-}$ mice in this article. DAPAT heterozygous (DAPAT$^{+/-}$) mutants were kindly provided by Prof. W.W. Just (Heidelberg, Germany). They were crossed to generate DAPAT$^{+/+}$, DAPAT$^{+/-}$ and DAPAT knock-out (DAPAT$^{-/-}$) mice. Animals were genotyped as previously described (Saab, Buteau, *et al.*, 2014). Briefly, DNA was extracted from tail samples using the Archive Pure DNA Cell/Tissue kit (5 Prime GmbH, Catalog no 2300820; Gaithersburg, MD). Then, 2 ng of genomic DNA, 100 pmol of each primer in reaction buffer and 2.5U of Taq polymerase (BiotaqTM DNA Polymerase, BIO-21040, Bioline, Paris, France) were used in a total volume of 25µl to amplify DNA. Polymerase-chain reaction (PCR) was performed using the primers neomycin-forward (CGCATCGCCTTCTATCGCCTTCTTG, Eurofins MWG Operon, Ebersberg, Germany), exon7-forward (CGATACC-TACTTTGTCCCAATTAGC, Eurofins) and exon7-reverse (GCTGGTCTCAAACAGCTACGTAGCTGA, Eurofins). PCR was performed on a C1000TM Thermo Cycler (Biorad Laboratories, Hercules, CA, USA), with an initial 2 min denaturation step (95 °C) followed by 35 cycles of 94 °C for 30 s and 57 °C for 1 min, followed by a final extension step at 72 °C for 1 min. This resulted in a 650-bp product for the wild-type gene and an 860-bp product for the neomycin-recombinant DAPAT gene. PCR products were determined by electrophoresis in 2% agarose gels that were further visualized on a Geldoc 2000 (Biorad) for analysis.

2.2 Diets

Starting from delivery, mothers were fed with diets containing either 2% alkylglycerol (AKG) or 0% AKG (Tab. 1). The lipid fraction of the diets was prepared by using sunflower oil and canola oil in order to supply linoleic acid (LA, C18:2n−6) and α-linolenic acid (ALA, C18:3n−3), respectively. High oleic sunflower oil and palm oil were added to balance the levels of oleic acid and palmitic acid, respectively. Fish oil was used to balance docosahexaenoic acid (DHA, C22:6n−3) and eicosapentaenoic acid (EPA, C20:5n−3) levels. Finally, shark liver oil (SLO) (Polaris, France) was used to provide AKG. The detailed composition of dietary lipids is presented in Table 2.

2.3 Lipid quantification

Animals were euthanized by decapitation at PN21. Cortex isolated from the brain and retinas carefully excised from the ocular globes were instantly frozen in liquid nitrogen and then stored at −80 °C until further analyses. Total lipid from retinas and cortex were extracted according to the method described by Folch and collaborators using a mixture of chloroform/methanol (2:1, v:v) (Folch *et al.*, 1957). Lipid extracts were stored at −20 °C under inert gas. Total lipids were transmethylated using boron trifluoride (BF3) in methanol according to Morrison and Smith (Morrison and Smith, 1964). Fatty acid methyl esters (FAMEs; formed by the trans-methylation of fatty acids at *sn*−1 and *sn*−2 positions of diacylglycerophospholipids and the *sn*−2 of plasmalogens) and dimethylacetals (DMAs; formed by the transmethylation of the aldehyde aliphatic groups on *sn*−1 position of plasmalogens) were subsequently extracted with hexane and analyzed by gas chromatography on a Trace 1310 gas chromatograph (Thermo Scientific, Les Ulis, France) using a CPSIL-88 column (100 m × 0.25 mm i.d. film thickness 0.20 µm; Varian, Le Plessis-Robinson, France) equipped with a flame ionization detector. Hydrogen was used as the carrier gas (inlet pressure 200 kPa). The oven temperature was held at 60 °C for 5 min, increased to 165 °C at a rate of 15 °C/min, held for 1 min, then increased again to 225 °C at 2 °C/min, and finally held at 225 °C for 17 min. FAMEs and DMAs were identified by comparison with commercial and synthetic standards. Data were processed using the Chromeleon 7 software (Thermo Scientific) and reported as a percentage of total FAMEs and DMAs. Plasmalogen levels were calculated as 2 x (% of total DMAs) as previously described by Acar and collaborators (Acar *et al.*, 2007).

Table 1. Composition of diets.

Ingredients	Amount (g/kg diet)
Casein	180
Cornstarch	460
Sucrose	230
Cellulose	20
Fat[a]	50
Mineral mix[b]	50
Vitamin mix[c]	10

[a] Represented by oil mixes prepared with variable amounts of sunflower oil, canola oil, high oleic sunflower oil, palm oil, and fish oil.

[b] Composition (g/kg diet): sucrose, 5.535; CaCO3, 12; K2HPO4, 10.75; CaHPO4, 10.75; MgSO4, 7H2O, 5; NaCl, 3; MgO, 2; FeSO4, 7H2O, 0.4; ZnSO4, 7H2O, 0.35; MnSO4, H2O, 0.1; CuSO4, 5H2O, 0.05; Na2SiO7, 3H2O, 0.025; AlK(SO4)2, 12H2O, 0.01; K2CrO4, 0.0075; NaF, 0.005; NiSO4, 6H2O, 0.005; H2BO3, 0.005; CoSO4, 7H2O, 0.00025; KIO3, 0.002; (NH4)6Mo7O24, 4H2O, 0.001; LiCl, 0.00075; Na2SeO3, 0.00075; NH4VO3, 0.0005.

[c] Composition (g/kg diet): sucrose, 5.4945; retinyl acetate, 0.01; cholecalciferol, 0.0025; DL-a-tocopheryl acetate, 0.2; phylloquinone, 0.001; thiamin HCl, 0.01; riboflavin, 0.01; nicotinic acid, 0.05; calcium pantothenate, 0.025; pyridoxine HCl, 0.01; biotin, 0.01; folic acid, 0.002; cyanobalamin, 0.025; choline HCl, 2; DL-methionin, 2; p-aminobenzoic acid, 0.05; inositol, 0.1.

2.4 Statistical analyses

Data were processed using GraphPad Prism v6.05 (GraphPad software. USA). Statistical analyses were performed using a one-way ANOVA with post-hoc Tukey's multiple comparisons test. Data are shown as mean ± SEM. An adjusted p value lower than 0.05 was considered as statistically

Table 2. Fatty acid composition of dietary lipids.

Fatty acids	Control diet	AKG-supplemented diet
Total alkylglycerols	0%	2%
Total saturated fatty acids	26.55%	28.70%
Total mono-unsaturated fatty acids	55.18%	52.87%
C18:2n−6 (LA)	16.16%	16.14%
C20:4n−6 (ARA)	0.001%	0.02%
Total n−6 fatty acids	16.16%	16.18%
C18:3n−3 (ALA)	1.37%	1.32%
C20:5n−3 (EPA)	0.47%	0.43%
C22:6n−3 (DHA)	0.27%	0.29%
Total n−3 fatty acids	2.11%	2.16%
Total n−6 + n−3 fatty acids	18.27%	18.34%
LA/ALA ratio	11.81	12.20
n−6/n−3 ratio	7.66	7.48

significant and noted by one star (*). Two (**) and three stars (***) were used for p values lower than 0.01 and 0.001, respectively.

3 Results

3.1 Plasmalogen deficiency is associated with major alterations of the fatty acid profile of the retina that are partially prevented by AKG supplementation

The complete results of fatty acid composition of mouse pup retinas at PN21 are presented in Table 3. First, we confirmed the lack of plasmalogens in the plasmalogen-deficient DAPAT$^{-/-}$ mice (Fig. 1A). Secondly, our data revealed that supplementing mothers' diet with the AKG-enriched shark liver oil (SLO) is associated with a small but not significant increase in the plasmalogen content of DAPAT$^{-/-}$ retinas, which remained significantly lower than in WT mice (1.03 ± 2.17% *vs.* 6.3 ± 0.79% in KO + AKG and WT animals, respectively) (Figs. 1A and 1B), suggesting that 21 days of AKG supplementation through lactation was not sufficient to fully restore plasmalogen content of the retina in DAPAT$^{-/-}$ pup. Our data also showed that AKG supplementation tended to raise the levels of 16:0, 18:0 and 18:1n−9 plasmalogens without reaching significancy, while 18:1n−7 plasmalogens levels showed no difference between WT and supplemented (KO + AKG) mice, suggesting a significant effect of the SLO nutritional supplementation in restoring these specific plasmalogen species in the retina (Fig. 1B). Interestingly, plasmalogen deficiency was also associated with several alterations in the fatty acid profile of the pups' retinas, such as a significant decrease of polyunsaturated fatty acids (PUFAs) (−42,4%; $p < 0.01$) that was balanced by a significant increase of saturated fatty acids (SFAs) (+20%; $p < 0.001$) and monounsaturated fatty acids (MUFAs) (+22,3%; $p < 0.5$) (Fig. 1C; Tab. 4). AKG supplementation was associated with a significant decrease of SFA levels in KO mice (−8.8%; $p < 0.05$) but remained significantly higher than in WT mice (56.4% *vs.* 51.5%; $p < 0.05$). The decrease in SFA was not sufficient to restore MUFA or PUFA levels, which remained significantly different than in WT mice (Fig. 1C; Tab. 4). The increase in SFA was mainly (but not only) driven by palmitic acid (C16:0; +25%; $p < 0.01$) and stearic acid (C18:0; +13%; $p < 0.05$) (Fig. 2A), while the increase in MUFAs was essentially driven by oleic acid (C18:1 n−9; +24%; $p < 0.001$) (Fig. 2B). Among PUFAs, n−6 and n−3 FA displayed opposite regulations. The concentrations of arachidonic acid (ARA, C20:4n−6), the main n−6 PUFA of the retina, were significantly decreased (−43.7%, $p < 0.05$) while those of linoleic acid (LA, C18:2n−6) were significantly increased (+74%; $p < 0.01$) (Fig. 2C), leading to a slight but statistically not significant reduction (−14%; $p = 0.2$) in total n−6 PUFA concentrations (Fig. 2E). Conversely, the concentration of docosahexaenoic acid (DHA, C22:6n−3), the main n−3 PUFA of the retina, was dramatically reduced in plasmalogen-deficient mice (-76%; $p < 0.001$) (Fig. 2D), leading to both a significant decrease of the total n−3 content (−67%, $p < 0.001$) (Fig. 2F) and a strong increase of the n−6:n−3 PUFAs ratio in DAPAT$^{-/-}$ retinas (+148%; $p < 0.001$) (Fig. 2G). Interestingly, AKG supplementation could restore the retinal levels of ARA (Fig. 2C) but not those of DHA (Fig. 2D), which remained about two-fold lower when compared to DAPAT$^{+/+}$ animals, and which were associated with a 25% decrease of the n−6:n−3 PUFAs ratio when compared to DAPAT$^{-/-}$ mice (Fig. 2G). As a consequence, AKG-supplemented DAPAT$^{-/-}$ mice exhibited a n−6:n−3 PUFAs ratio that was still significantly higher than that of WT mice (1.7 ± 0.56% *vs.* 0.9 ± 0.26% in KO+AKG and WT mice, respectively; $p < 0.01$) (Fig. 2G).

3.2 Plasmalogen deficiency is associated with major alterations of the cortex fatty acid profile that are not prevented by AKG supplementation

The complete results of fatty acid composition of mice pups' cortices at PN21 are presented in Table 4. In contrast to the retina, AKG supplementation didn't induce any change in the concentration of brain cortex plasmalogens, neither in total plasmalogens (Fig. 3A) nor in any plasmalogen subspecies (Fig. 3B). Unlike to the retina, the loss of plasmalogens in the cerebral cortex was not associated with any significant changes in the levels of total SFAs, MUFAs or PUFAs (Fig. 3C). However, our data suggest some modifications in the composition of each molecular species. For instance, in the SFA class, there is a small but significant increase in stearic acid (C18:0) (+11.5%, $p < 0.001$) in plasmalogen deficient mice, while palmitic acid levels remained statistically unchanged despite a small trend toward a decrease. On the other end, minor species such as arachidic levels (C20:0) were significantly reduced in plasmalogen deficient mice (−48%, $p < 0.01$) (Fig. 4A). Concerning MUFAs, while their overall levels remained unchanged in DAPAT −/−, our data reveal small but significant increases in oleic acid levels (+5%, $p < 0.001$) and vaccenic acid (C18:1n−7) (+2.5%, $p < 0.05$), that are compensated by significant decreases in other minor species like C20:1n−9 ($p < 0.01$) or C22:1n−9 ($p < 0.01$). (Fig. 4B). Within cortex PUFAs, plasmalogen deficiency was associated with a significant increase of ARA concentrations (+36%, $p < 0.001$) (Fig. 4C), as well as other minor species (such as C20:3n−6) which led to a significant increase in total

Table 3. Fatty acid composition of retinas.

	DAPAT$^{+/+}$		DAPAT$^{-/-}$		DAPAT$^{-/-}$ + AKG	
	mean	*SD*	mean	*SD*	mean	*SD*
	(n = 8)		*(n = 4)*		*(n = 10)*	
C14:0	0.27	*0.07*	0.49	*0.17*	0.58**	*0.26*
C15:0	0.18	*0.08*	0.27	*0.09*	0.28	*0.10*
DMA16:0	1.10	*0.09*	0.0***	*0.00*	0.16***	*0.33*
C16:0	24.63	*2.08*	30.85**	*2.30*	27.67	*3.42*
C16:1n−9	0.37	*0.09*	0.53	*0.06*	0.66***	*0.14*
C16:1n−7	0.54	*0.17*	0.89*	*0.14*	1.05***	*0.22*
C17:0	0.19	*0.04*	0.23	*0.09*	0.22	*0.05*
DMA18:0	1.42	*0.22*	0.0***	*0.00*	0.14***	*0.3*
DMA18:1n−9	0.25	*0.03*	0.0***	*0.00*	0.04***	*0.09*
DMA18:1n−7	0.37	*0.13*	0.0	*0.00*	0.17	*0.37*
C18:0	25.59	*1.77*	29.02*	*1.04*	26.94	*1.86*
C18:1*trans*	0.20	*0.05*	0.37**	*0.09*	0.32*	*0.09*
C18:1n−9	12.78	*1.50*	15.84***	*0.96*	15.81***	*1.56*
C18:1n−7	3.30	*0.25*	3.51	*0.10*	3.95**	*0.43*
C18:2n−6	3.14	*1.00*	5.46**	*1.08*	4.15	*1.27*
C20:0	0.47	*0.18*	0.77	*0.14*	0.50	*0.26*
C20:1n−9	0.66	*0.26*	0.60	*0.04*	0.60	*0.13*
C18:3n−3	0.46	*0.22*	0.96*	*0.24*	0.74	*0.29*
C20:2n−6	0.46	*0.11*	0.36	*0.06*	0.35*	*0.07*
C20:3n−9	0.17	*0.04*	0.19	*0.04*	0.29**	*0.09*
C22:0	0.16	*0.04*	0.21	*0.02*	0.19	*0.03*
C20:3n−6	0.49	*0.11*	0.29*	*0.05*	0.37	*0.10*
C22:1n−9	0.52	*0.29*	0.77	*0.19*	0.75	*0.33*
C20:4n−6	7.05	*1.27*	3.97*	*1.26*	6.28	*2.49*
C20:5n−3	0.32	*0.06*	0.28	*0.03*	0.33	*0.07*
C22:4n−6	0.88	*0.15*	0.30***	*0.09*	0.48***	*0.15*
C22:5n−6	0.29	*0.14*	0.20	*0.05*	0.17*	*0.04*
C22:5n−3	0.32	*0.09*	0.25	*0.13*	0.26	*0.09*
C22:6n−3	13.27	*4.64*	3.23***	*1.07*	6.40**	*3.37*
Total SFA	51.5	*3.87*	61.8***#	*2.87*	56.4*#	*3.11*
Total MUFA	18.4	*2.33*	22.5*	*0.99*	23.1***	*2.36*
Total PUFA	26.9	*5.50*	15.5**	*2.45*	19.8*	*5.09*
Total DMA	3.1	*0.39*	0.0***	*0.00*	0.5***	*1.08*
Total n−6 PUFA	12.3	*1.79*	10.6	*1.46*	11.8	*1.87*
Total n−3 PUFA	14.4	*4.51*	4.7***	*1.02*	7.7**	*3.22*
n−6 PUFA /n−3 PUFA	0.9	*0.26*	2.3***	*0.26*	1.7**	*0.56*

Results are expressed as% of total FAMEs and DMAs.
AKG: alkylglycerol; SD: standard deviation; DMA: dimethylacetal; SFA: saturated fatty acids: MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids
*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significantly different when compared to DAPAT$^{+/+}$, one-way ANOVA with post-hoc Tukey's multiple comparisons test
# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$: significantly different between DAPAT$^{-/-}$ and DAPAT$^{-/-}$ + AKG, one-way ANOVA with post-hoc Tukey's multiple comparisons test

n−6 PUFAs (+22%, p <0.001) (Fig. 4E). By contrast (and similarly to the retina), plasmalogen deficiency was associated with a significant reduction of DHA levels (−32%, p <0.001) (Fig. 4D), which was the main contributor to the reduction of total n−3 PUFAs levels (−32%, p <0.001) (Fig. 4F). As a consequence, the n−6:n−3 PUFAs ratio was significantly increased by 81% in the brain cortex of plasmalogen deficient mice (p <0.001) (Fig. 4G). Unlike what was observed in the retina, supplementing mothers' diet with the AKG-enriched shark liver oil did not induce any change in cortices lipid composition, suggesting physiological differences between the brain cortex and the retina.

4 Discussion

Previous studies, including those from our team, described several eye defects in the mouse model of plasmalogen deficiency (DAPAT$^{-/-}$ mice), including microphthalmia, optic nerve hypoplasia, cataract, persistence of hyaloid vessels in adult life, and abnormalities in the vascular architecture of the

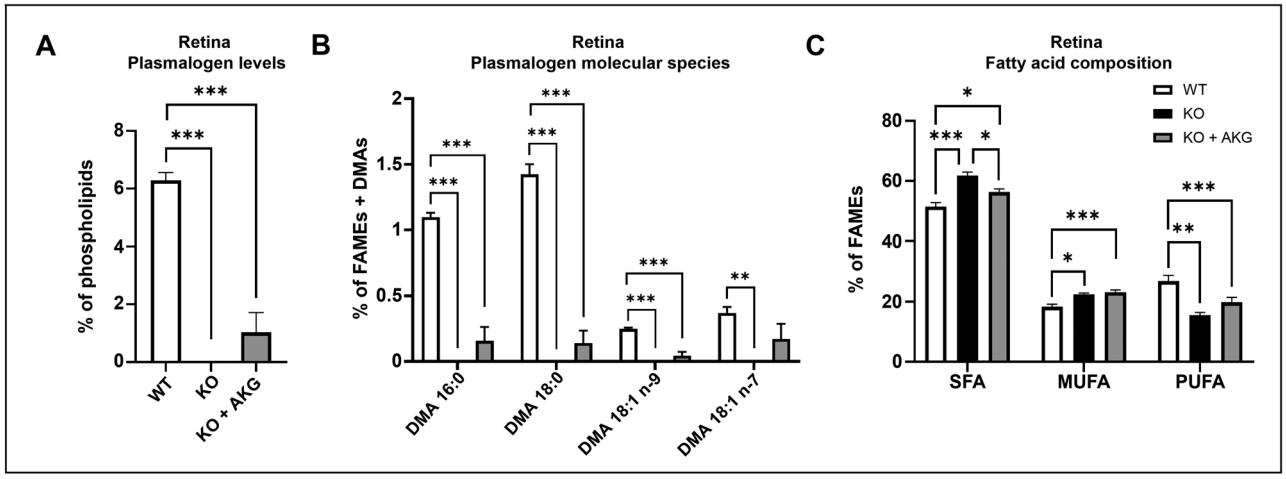

Fig 1. AKG supplementation partially restores the plasmalogen and fatty acid profile of the pups plasmalogen-deficient retina. A) Plasmalogen deficient (DAPAT$^{-/-}$) mice retina were completely devoid of plasmalogens, while they represent 6% of total phospholipids in WT mice retina. 3 weeks of alkylglycerol-enriched shark liver oil (plasmalogen precursor) nutritional supplementation through lactation was able to significantly increase plasmalogen levels in the retina of plasmalogen deficient pups (DAPAT$^{-/-}$ + AKG), but remained significantly lower than in WT mice. B) Plasmalogen molecular species. The main plasmalogen species are saturated. Interestingly, SLO maternal supplementation increased the concentration of 18:1n−7 but not those of 16:0, 18:0 and 18:1n−7 plasmalogens. C) Fatty acid profile of the retina. Plasmalogen deficiency was associated with a significant increase in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFAs), while polyunsaturated fatty acids (PUFAs) were significantly decreased. SLO maternal supplementation partially restored SFA and PUFA concentrations. * p <0.05; ** p <0.01; ***p <0.001. n = 4–8. One-way ANOVA with post-hoc Tukey's multiple comparisons test. FAMEs: Fatty acid methyl esters; DMAs: Dimethylacetals.

retina (Brites *et al.*, 2011; Rodemer *et al.*, 2003; Saab, Buteau, *et al.*, 2014). Alteration of retinal vasculature is a hallmark of several retinopathies that can lead to visual impairments or even vision loss. Interestingly, the retinal vascular abnormalities of the DAPAT$^{-/-}$ mice develop in two phases, similar to what is observed in retinopathy of prematurity (ROP) (Saab, Buteau, *et al.*, 2014; Saab, Mazzocco *et al.*, 2014). Previous studies showed that supplementing mouse diets with the plasmalogen precursor alkylglycerol (AKG) can at least partially restore tissue levels of plasmalogens and prevent some of the ocular alterations associated with plasmalogen deficiency (Brites *et al.*, 2011; Das *et al.*, 1992). Digestion of AKG follows the same path as regular dietary lipids, as they are absorbed by intestinal cells and then found in the lymph. It seems that up to 30–50% of AKG are functionally absorbed and detected in the lymph lipids (Paul *et al.*, 2021). Several other studies showed that orally administered AKG significantly raise plasma AKG levels after a single intake and ultimately tissue plasmalogen levels with various duration, up to several months (Paul *et al.*, 2021; Tham *et al.*, 2018; Todt *et al.*, 2020; Yu *et al.*, 2019). Furthermore, Oh and Jadhav showed a dose-dependent relationship of AKG levels in the milk of AKG-supplemented lactating mothers, with AKG levels that keep increasing with supplementation duration (Oh and Jadhav, 1994). Yu et al also found that plasma AKG levels mirrored those in breast milk in neonate mice (Yu *et al.*, 2019). Therefore, we decided to investigate whether post-partum maternal supplementation with an AKG-enriched diet for 3 weeks would be effective in preventing the retinal abnormalities that take place during the vascular development and maturation of the retina in DAPAT$^{-/-}$ pups. Shark liver oil (SLO) was chosen as a potent source of AKG since it can contain more than 30% of AKG, depending on the manufacturer. Using shark liver oil containing 60% of AKG, we were able to design a balanced diet that contains a total of 2% of AKG. This concentration was chosen as it is one of the most used concentrations of dietary AKG in previously published studies (Brites *et al.*, 2011; Todt *et al.*, 2020). Furthermore, trying to reach higher concentrations of AKG may be problematic. Indeed, AKG-enriched SLO are difficult to obtain, especially above 20% AKG. Secondly, adding more SLO in our mix would significantly have impaired the nutritional balance of our diet, especially regarding lipid profiles.

Our results suggest that the AKG-enriched diet was able to induce a small increase of plasmalogen concentration in the pups retina, confirming that they can (at least partially) get AKG through the milk of supplemented lactating mothers as previously described (Das *et al.*, 1992). However, the increase in retinal plasmalogen concentrations was small and inconsistent. Brites and collaborators showed that 8 weeks of post-natal supplementation with 2% of AKG are necessary to reach plasmalogen levels that match those of controls (Brites *et al.*, 2011). On the other hand, our results also showed high variability in retinal plasmalogen concentrations, suggesting that some pups did not display any increase in their plasmalogen levels at all. This could be the consequence of a competition between pups to get maternal milk, as plasmalogen-deficient pups are very weak and fragile during the first 3 weeks of life. Indeed, plasmalogen-deficient pups display many pathological features besides the ocular abnormalities described earlier: they are about 40% underweight compared to WT and are characterized by a shortening of proximal limbs. All these defects are associated with a

Table 4. Fatty acid composition of brain cortex.

	DAPAT$^{+/+}$		DAPAT$^{-/-}$		DAPAT$^{-/-}$ + AKG	
	mean *(n = 8)*	*SD*	mean *(n = 8)*	*SD*	mean *(n = 2)*	*SD*
C14:0	0.17	*0.07*	0.21	*0.10*	0.33	*0.00*
C15:0	0.10	*0.03*	0.07	*0.02*	0.09	*0.00*
DMA16:0	2.73	*0.12*	0.0***	*0.00*	0.0***	*0.00*
C16:0	20.82	*0.99*	20.62	*0.67*	21.37	*0.40*
C16:1n−9	0.26	*0.09*	0.35	*0.11*	0.52*	*0.04*
C16:1n−7	0.49	*0.06*	0.86***	*0.08*	0.85***	*0.01*
C17:0	0.16	*0.01*	0.16	*0.01*	0.14*#	*0.01*
DMA18:0	3.24	*0.33*	0.0***	*0.00*	0.0***	*0.00*
DMA18:1n−9	1.10	*0.16*	0.0***	*0.00*	0.0***	*0.00*
DMA18:1n−7	0.92	*0.23*	0.0***	*0.00*	0.0***	*0.00*
C18:0	19.89	*0.49*	24.08***	*0.90*	23.34***	*0.01*
C18:1*trans*	0.10	*0.04*	0.06	*0.02*	0.11	*0.02*
C18:1n−9	13.53	*0.76*	15.51***	*0.59*	14.84	*0.19*
C18:1n−7	3.64	*0.24*	4.05*	*0.30*	3.56	*0.03*
C18:2n−6	0.75	*0.23*	0.99	*0.26*	0.81	*0.01*
C20:0	0.35	*0.11*	0.20**	*0.02*	0.16*	*0.01*
C20:1n−9	1.01	*0.20*	0.73**	*0.10*	0.55**	*0.04*
C18:3n−3	0.33	*0.06*	0.30	*0.04*	0.20*#	*0.03*
C20:2n−6	0.26	*0.06*	0.17*	*0.06*	0.18	*0.01*
C20:3n−9	0.14	*0.02*	0.16	*0.02*	0.33***###	*0.01*
C22:0	0.25	*0.02*	0.22	*0.03*	0.17*#	*0.02*
C20:3n−6	0.53	*0.07*	0.58	*0.10*	0.66	*0.01*
C22:1n−9	0.15	*0.02*	0.11**	*0.02*	0.08***	*0.01*
C20:4n−6	11.10	*0.81*	16.49***	*0.97*	17.77***	*0.12*
C24:0	0.34	*0.04*	0.38	*0.05*	0.39	*0.02*
C24 :1n−9	0.42	*0.13*	0.43	*0.11*	0.22	*0.04*
C22:4n−6	2.59	*0.15*	2.36**	*0.07*	2.12***#	*0.04*
C22:5n−6	0.41	*0.08*	0.35	*0.02*	0.49#	*0.01*
C22:5n−3	0.15	*0.01*	0.16	*0.01*	0.14	*0.00*
C22:6n−3	14.09	*0.50*	10.38***	*0.40*	10.65***	*0.25*
Total SFA	42.1	*0.73*	46.0***	*0.43*	46.0***	*0.33*
Total MUFA	19.9	*1.25*	22.4**	*0.99*	20.9	*0.27*
Total PUFA	30.0	*1.11*	31.6*	*1.00*	33.1**	*0.08*
Total DMA	8.0	*0.61*	0.0***	*0.00*	0.0***	*0.00*
Total n−6 PUFA	15.6	*1.28*	20.9***	*1.35*	22.0*	*0.16*
Total n−3 PUFA	14.2	*0.49*	10.5***	*0.40*	10.8*	*0.25*
n−6 PUFA /n−3 PUFA	1.1	*0.11*	2.0***	*0.20*	2.0*	*0.06*

Results are expressed as% of total FAMEs and DMAs.
AKG: alkylglycerol; SD: standard deviation; DMA: dimethylacetal; SFA: saturated fatty acids: MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids
*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significantly different when compared to DAPAT$^{+/+}$, one-way ANOVA with post-hoc Tukey's multiple comparisons test
# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$: significantly different between DAPAT$^{-/-}$ and DAPAT$^{-/-}$ + AKG, one-way ANOVA with post-hoc Tukey's multiple comparisons test

significant mortality rate of about 40% for KO animals within the first 4–6 weeks of age (Rodemer *et al.*, 2003). In complete transparency, earlier points PN7 and PN14 were initially considered for the study. However, considering that PN21 pups did not display a sufficient increase in plasmalogen levels, and given the high mortality rate of DAPAT$^{-/-}$ pups, we decided to stop the study with the PN21 time point, as earlier time points wouldn't show a better increase in plasmalogen levels. However, we do acknowledge that PN7 and PN14 time points would have been an interesting source of information to understand the time-course of the retinal fatty acid profile dysregulations, especially regarding PUFAs.

Moreover, comparing the fatty acid compositions of retinas and brain cortices shows that dietary intervention had a very limited effect on the brain cortex (Figs. 3, and 4) when compared to the retina (Figs. 1, and 2). This could confirm earlier observations including from our group that the retina and the brain display different sensitivity to dietary lipids

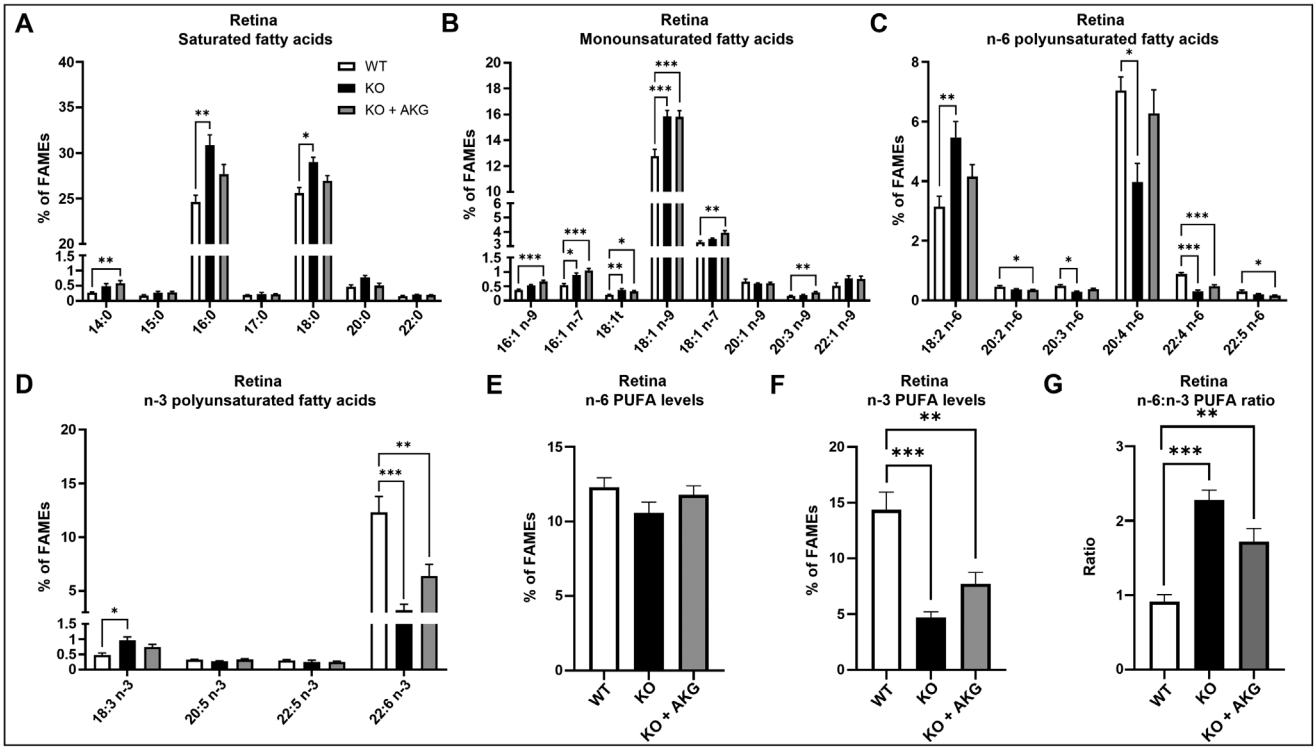

Fig 2. Plasmalogen deficiency is associated with major alterations of fatty acid species in the pups retina that are partially prevented by maternal AKG supplementation. A) Saturated fatty acids profile. Plasmalogen deficiency was associated with an increase in palmitic acid (16:0) and stearic acid (18:0) concentrations. AKG maternal supplementation partially restores their levels. B) Monounsaturated fatty acids profile. Plasmalogen deficiency was associated with an increase in several monounsaturated chains, especially in oleic acid (18:1n−9), that are not restored by AKG maternal supplementation. C) n−6 polyunsaturated fatty acids (PUFA) profile. Plasmalogen deficiency was associated with several dysregulations, especially with an increase in C18:2n−6 (LA) and a decreased C20:4n−6 (ARA) levels, that are restored through maternal supplementation with AKG. D) n−3 PUFA profile. Plasmalogen deficiency was associated with several dysregulations, mostly characterized by a decrease in C22:6n−3 (DHA) levels, that was not restored through maternal supplementation with AKG. E) Plasmalogen deficiency was associated with a slight but not significant decrease in total n−6 PUFA. F) Plasmalogen deficiency was associated with a significant and dramatic decrease in total n−3 PUFA, that was not restored by AKG maternal supplementation. G) Plasmalogen deficiency was associated with a significant increase of the n−6:n−3 PUFA ratio in the retina. AKG nutritional supplementation partially restored the n−6:n−3 PUFAs ratio but remained significantly higher than in WT mice retina. FAMEs: Fatty acid methyl esters. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n = 4–8. One-way ANOVA with post-hoc Tukey's multiple comparisons test.

(Abedin *et al.*, 1999; Acar *et al.*, 2006; Alberghina *et al.*, 1994; Fliesler and Bretillon, 2010; Gorusupudi *et al.*, 2021; Lewandowski *et al.*, 2024; Pikuleva and Curcio, 2014; Su *et al.*, 1996). Besides, Brites et al also showed that while oral supplementation of lactating mothers with AKG was able to significantly increase plasmalogen levels in pups' eyes in another model of plasmalogen-deficient mice (Pex7 KO mice), plasmalogen levels remained unchanged in the cerebrum (Brites *et al.*, 2011). Similarly, Das and Hajra showed that oral supplementation with I-0-heptadecyl-glycerol (20 mg/g of food) was significantly incorporated into tissue plasmalogens (>50% in kidney, heart, lunger, liver or intestine), its brain incorporation was relatively low (8%) (Das and Hajra, 1988). However, we cannot rule out that the lack of effect of AKG supplementation in the brain cortex displayed in our data is due to the small number of cortices samples in the group of AKG-supplemented pups (n = 2). We also acknowledge that even though several studies showed that AKG supplementation was effectively absorbed by lactating mother and transferred to the pups through their milk, it would have been useful to collect and analyze the mother's milk fatty acid composition to eventually help us put our data in perspective. Further studies will be needed to conclude about potential absorption of AKG by the brain cortex.

The present study shows that plasmalogen deficiency is associated with a significant increase in the retinal n−6:n−3 PUFAs ratio, mainly due to a dramatic decrease in DHA levels. This suggests that in the plasmalogen-deficient retina, ARA levels may be maintained through a potential compensation within other phospholipids species while DHA originating from plasmalogens cannot be balanced, thereby strongly highlighting plasmalogens as an essential player in the n−3 PUFA content of the retina. While we acknowledge that this is a hypothesis that cannot be proven without a direct causal mechanism, we have some confidence in this hypothesis. Indeed, several studies have shown that phospholipids from PE class, and not PC, are compensating for the lack of plasmalogens, including in the DAPAT mouse model (Dorninger *et al.*, 2015; Todt *et al.*, 2020). More interestingly, plasmalogen deficiency seems to specifically favor the

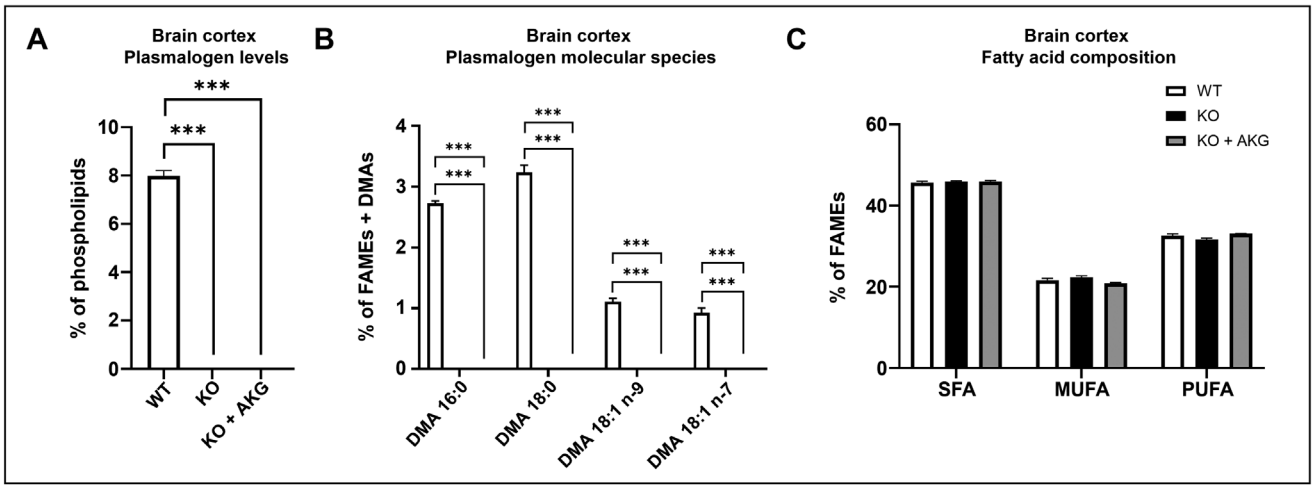

Fig 3. Plasmalogen deficiency is associated with major alterations of the brain cortex plasmalogen and fatty acid profiles that are not restored by AKG supplementation. A) Plasmalogen deficient (DAPAT$^{-/-}$) mice cortices were completely devoid of plasmalogens, while they represented 8% of total phospholipids in WT mice. 3 weeks of AKG-enriched nutritional supplementation through lactation was not able to increase plasmalogen levels in the brain cortex of plasmalogen deficient pups (KO + AKG). B) Plasmalogen profile of the brain cortex. The repartition of plasmalogen species was similar to that of the retina, but unlike to the retina no plasmalogens are detected in the cortices of supplemented pups. C) Fatty acid profile of the brain cortex. Plasmalogen deficiency was not associated with any significant change in saturated fatty acids (SFA), monounsaturated fatty acids (MUFAs), or polyunsaturated fatty acids (PUFAs) levels. AKG maternal supplementation does not affect any of the fatty acids concentrations in the pups cortices. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. $n = 2$–8. One-way ANOVA with post-hoc Tukey's multiple comparisons test. FAMEs: Fatty acid methyl esters; DMAs: Dimethylacetals.

incorporation of ARA into PE, thereby reducing DHA levels (Dorninger *et al.*, 2015). This is in line with results from previous studies which showed that patients affected by plasmalogen deficiency due to peroxisomal disorders like Zellweger's syndrome also display a dramatic decrease of DHA levels but not those of ARA, including in the retina (Gaposchkin and Zoeller, 1999b; Martinez, 1992a; Martinez and Mougan, 1999b). These similarities between mouse and human suggest evolutionary conserved mechanisms of PUFA level regulation and reinforce the value of mice models to study the pathophysiology of plasmalogen deficiency-related disorders. Interestingly, supplementing maternal diet with AKG was able to partially restore the n−6:n−3 PUFAs ratio (1.7 in DAPAT$^{-/-}$ + AKG *vs.* 2.3 in DAPAT$^{-/-}$) (Fig. 2G), suggesting that an incomplete restoration of retinal plasmalogen levels is sufficient to improve PUFAs composition of the retina.

However, our study also suggests that brain and retinal PUFAs may be (at least partly) regulated through different mechanisms. Indeed, while PUFA levels were significantly downregulated in the plasmalogen-deficient retina (Fig. 1C), no such alteration was found in plasmalogen-deficient cortices (Fig. 3C). This surprising result seems to be linked with n−6 PUFA levels: while plasmalogen deficiency was associated with a significant reduction in n−3 PUFA and DHA levels in both the retina (Figs. 2D–2F) and the brain cortex (Figs. 4D–4F), plasmalogen deficiency was associated with unchanged n−6 PUFA levels in the retina (Fig. 2E) but was associated with a significant increase in brain cortex n−6 PUFA levels (+23%, $p < 0.001$) (Fig. 4E). When looking into detailed compositions of n−6 PUFA, we could see that this discrepancy was mainly due to both ARA (C20:4n−6) and LA (C18:2n−6). On the one hand, in the retina, plasmalogen deficiency was associated with a significant decrease in ARA levels (−44%, $p < 0.05$) that was compensated by a significant increase in LA levels (+73%, $p < 0.01$) (Fig. 2C). On the other hand, in the brain cortex, plasmalogen deficiency was associated with a significant increase in ARA levels (+37%, $p < 0.001$), while LA levels remained unchanged (Fig. 4C). Furthermore, our data also show that LA levels are significantly higher in the retina than in the brain cortex (3.14% *vs.* 0.75%, respectively) (Tabs. 3, and 4). The reasons behind these differences are unknown to us and would need additional investigations to help us fully understand the mechanisms regulating PUFA levels in these tissues.

Our analysis showed that SLO also contained unusual FA, some of them at high concentrations like cetoleic acid (CA), erucic acid (EA) and nervonic acid (NA). CA represented 8.92% of the SLO fatty acids, and 0.67% of our supplemented diet total fatty acids (*vs.* 0% for the control diet). While still poorly understood, CA has been associated with several biological effects. It may positively affect neurological health, bioavailability of DHA and EPA, and improve cholesterol status (Mjaatveit *et al.*, 2024; Tang *et al.*, 2026). There are currently no studies on direct uptake of CA through the blood-retinal barrier (BRB) or the blood-brain barrier (BBB). However, it was shown that dietary supplementation of rats was able to increase tissular CA levels, except for the brain (Rimmen *et al.*, 2025). Erucic acid (EA) represented 3.94% of the SLO fatty acids, and 0.34% of our supplemented diet total fatty acids (*vs.* 0% for the control diet). As CA, the biological roles of EA are poorly understood. It was labeled as a cardiotoxic fatty acid decades ago, but recent studies suggest that these conclusions may not be true (Galanty *et al.*, 2023). Rather, EA may have some beneficial properties, such as anti-inflammatory effects or in the prevention of neurodegenerative

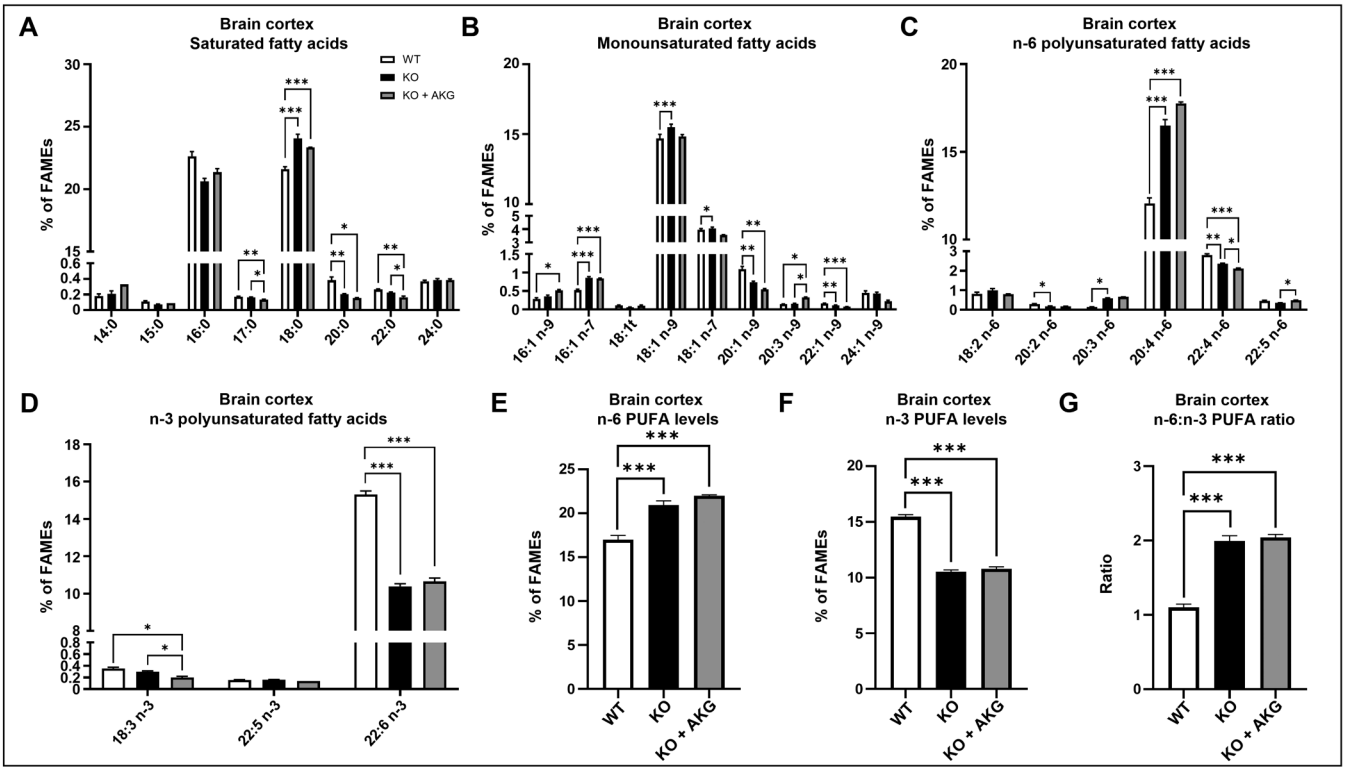

Fig 4. Plasmalogen deficiency is associated with several alterations of cortex fatty acid species that are not prevented by AKG supplementation. A) Saturated fatty acids profile. Plasmalogen deficiency was mainly associated with an increase in stearic acid (C18:0). AKG maternal supplementation did not affect any of the saturated chains concentrations. B) Monounsaturated fatty acids profile. Plasmalogen deficiency was associated with small but significant increases in several monounsaturated chains, especially in 18:1n−9 and 18:1n−7, that were partially restored by AKG maternal supplementation as they were no longer different when compared to WT. C) n−6 polyunsaturated fatty acids (PUFA) profile. Plasmalogen deficiency was especially associated with a significant increase in C20:4n−6 (ARA) levels, that were not restored through maternal supplementation with AKG. D) n−3 PUFA profile. Plasmalogen deficiency was associated with a strong and significant decrease in C22:6n−3 (DHA) levels, that was not restored through maternal supplementation with AKG. E) Plasmalogen deficiency was associated with a significant increase in total cortex n−6 PUFA, that was not modified by AKG maternal supplementation. F) Plasmalogen deficiency was associated with a significant decrease in total cortex n−3 PUFA, that was not modified by AKG maternal supplementation. G) Plasmalogen deficiency was associated with a strong and significant increase of the n−6:n−3 PUFA ratio in the brain cortex. AKG nutritional supplementation of lactating mothers had no effect on the n−6:n−3 PUFAs ratio of the pups' cortices. * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. n = 2–8. One-way ANOVA with post-hoc Tukey's multiple comparisons test. FAMEs: Fatty acid methyl esters.

disorders, potentially through PPAR-mediated pathways (Altinoz and Ozpinar, 2019; Galanty *et al.*, 2023; Goyal *et al.*, 2024). However, its' incorporation in the CNS is still up to debate: one study showed that EA can cross the BBB and reach the brain, where it was mainly oxidized (Golovko and Murphy, 2006), while another study couldn't find any uptake in the brain or in the retina (Wang *et al.*, 1992). Nervonic acid (NA) represented 31.52% of the SLO fatty acids, and 2.09% of our supplemented diet total fatty acids (*vs.* 0.05% for the control diet). Unlike CA or EA, NA has been more extensively studied. It is one of the main components of sphingomyelins, and is associated with CNS development and regeneration, mainly through (re)myelination (Namieciska *et al.*, 2024). Results of dietary supplementation with NA are still unclear, especially about BBB crossing, and no data can be found about a potential uptake in the retina. For these reasons, we think that the effects of CA, EA or NA may be negligeable in the outcomes of our study, especially regarding PUFAs.

Overall, while the AKG-enriched diet limited some of the alterations associated with plasmalogen deficiency, our results suggest that supplementing lactating mothers with a diet containing 2% of the plasmalogen precursor AKG for 21 days post-partum is not sufficient to completely restore plasmalogen levels of the retina in DAPAT$^{-/-}$ mice at PN21. Increasing the concentration of AKG might help to better prevent these abnormalities, but technical issues may arise. For instance, SLO enriched in AKG at a level of more than 20% is quite rare on the market and it may be difficult to obtain sufficient amounts of 60% AKG-enriched SLO. Finally, it would be of particular interest to assess if a DHA supplementation alone could restore the vascular phenotype of the DAPAT mouse retina, which would confirm if the plasmalogen deficiency-associated alterations are directly caused by a significant decrease in DHA levels and bioavailability. Further studies are required to decipher the precise mechanisms by which DHA originating from plasmalogens is involved in the development and the physiology of the mouse retina.

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Conflicts of interest

The authors declare they have no conflict of interest related to the data presented in this manuscript.

Author contribution statement

NA: funding acquisition, supervision. RK, NA: conceptualization, project administration. RK, BL, SG, NA: methodology, investigation, formal analysis, data curation. RK, AMB, NA: writing – original draft preparation. All authors: writing – review & editing.

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