



Decreased very long chain polyunsaturated fatty acids in sperm correlates with sperm quantity and quality

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Received: 5 March 2019 / Accepted: 26 April 2019 / Published online: 9 May 2019
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Abstract

Purpose To determine if levels of very long chain polyunsaturated fatty acids (VLC-PUFA; ≥ 28 carbons; 4–6 double bonds) in human sperm correlate with sperm quantity and quality as determined by a complete semen analysis.

Methods Ejaculates from 70 men underwent a complete semen analysis, which included volume, count, motility, progression, agglutination, viscosity, morphology, and pH. For lipid analysis, sperm were pelleted to remove the semen. Lipids were extracted from the cell pellet and methyl esters of total lipids analyzed by gas chromatography. The sphingolipids were enriched and sphingomyelin (SM) species measured using tandem mass spectrometry. Pair-wise Pearson correlation and linear regression analysis compared percent VLC-PUFA-SM and percent docosahexaenoic acid (DHA) to results from the semen analysis.

Results VLC-PUFA-SM species having 28–34 carbon fatty acids were detected in sperm samples, with 28 and 30 carbon VLC-PUFA as most the abundant. The sum of all VLC-PUFA-SM species comprised 0 to 6.1% of the overall SM pool (mean 2.1%). Pair-wise Pearson analyses showed that lower levels of VLC-PUFA-SM positively correlated with lower total motile count (0.68) and lower total count (0.67). Total VLC-PUFA-SM and mole % DHA (22:6n3) were not strongly correlated (-0.24). Linear regression analysis confirmed these findings.

Conclusion This study revealed a positive correlation between the levels of VLC-PUFA with sperm count and total motile count and suggests that both sperm quality and quantity may depend on the presence of VLC-PUFA. The lack of correlation between VLC-PUFA and DHA suggests that low VLC-PUFA levels do not result from inadequate PUFA precursors.

Keywords Sperm lipids · Sperm · Sphingomyelin · Fatty acids · Very long chain polyunsaturated fatty acids · Motility · Sperm count · Fertility

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10815-019-01464-3>) contains supplementary material, which is available to authorized users.

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Introduction

Semen analysis is the initial step for infertility evaluation of the male partner. However, the cause of abnormal semen analysis is often times unexplained even after urologic evaluation for anatomic and hormonal causes. The lipid composition of the sperm membrane plays a major role in sperm structure and function and could relate to the semen analysis abnormalities and ultimately fecundability. The lipids of sperm are relatively unique in that they contain many different sphingolipids including sphingomyelin (SM) that have long-chain (LC) polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA, 22:6n3), eicosapentaenoic acid (EPA, 20:5n3), and arachidonic acid (AA, 20:4n6), as well as very long chain PUFA (VLC-PUFA; ≥ 28 carbons) [1, 2]. There is precedent for linking polyunsaturated fatty acid (PUFA) deficiencies to

infertility as levels of DHA, docosapentaenoic acid (DPA), and AA were reduced when an important enzyme in PUFA biosynthesis, delta-6-desaturase ($\Delta 6$ D), was knocked out in mice. These mice were infertile [3] and PUFA supplementation restored their fertility [4]. One type of fatty acid that is particularly unique to sperm is the VLC-PUFA (≥ 28 carbons) in the sperm membrane, which are synthesized via the enzyme elongation of very long chain fatty acids-4 (ELOVL4) from dietary essential fatty acids and incorporated into sphingolipids, primarily sphingomyelin [5]. The biosynthetic

pathways for synthesis on n3 and n6 VLC-PUFA are presented in Fig. 1.

VLC-PUFA are found in the testis, sperm, and the retina [1, 6–9]. While studying several different transgenic and knock-in/knockout rodent models of ELOVL4 (juvenile-onset Stargardt-like macular dystrophy-3), we discovered that some of the affected males bred poorly or not at all, depending on the severity of the expression of the mutation. Consequently, we examined the VLC-PUFA in their testes and discovered that the poor-breeding ELOVL4-affected animals had

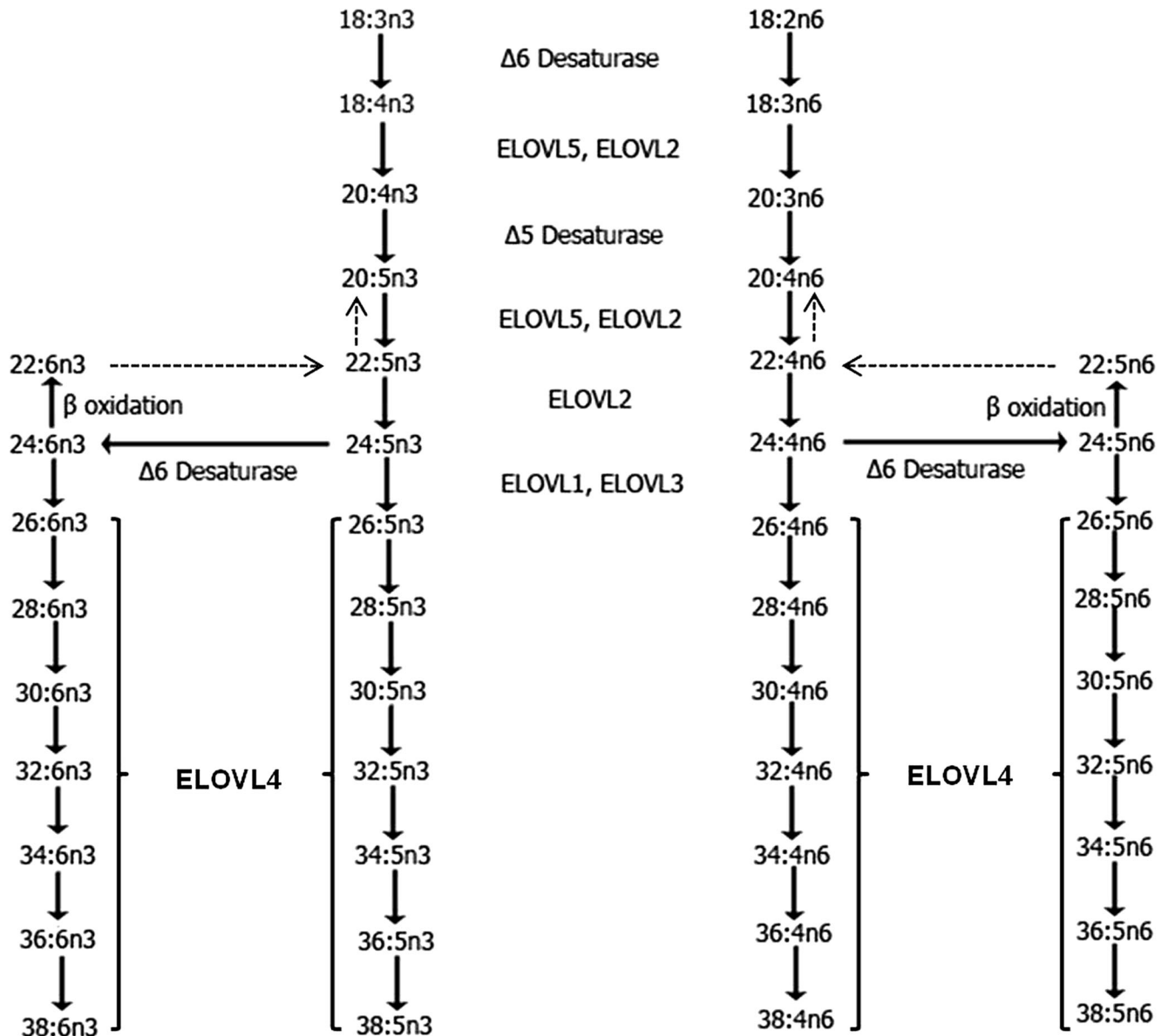


Fig. 1 Schematic of the biosynthesis of n3 and n6 VLC-PUFA from shorter chain polyunsaturated fatty acids. Pathways of in vivo very long chain polyunsaturated fatty acid (VLC-PUFA) biosynthesis from essential fatty acids (EFA) 18:3n3 and 18:2n6 mediated by ELOVL4 and other ELOVL enzymes. Desaturation and elongation of the EFAs are performed by fatty acid desaturase-1 (FADS1 or $\Delta 5$ desaturase), fatty acid

desaturase-2 (FADS2 or $\Delta 6$ desaturase), and ELOVL1–5. Apart from ELOVL4, which mediates C28–C36 VLC-PUFA biosynthesis, other ELOVL enzymes are nonspecific or multifunctional and act at several steps. Reproduced with permission from Man Yu et al., (2012). *J. Lipid Res.* 53:(3) 494–504

significantly reduced levels (unpublished data). Knowing that lipids play integral roles in energy production and membrane structure, we hypothesized that a subset of human males who suffer from poor fertility would have comparatively low levels of VLC-PUFA in their sperm. The objective of our study was to determine if levels of VLC-PUFA in the sperm correlate with sperm quantity and quality as determined by a complete semen analysis.

Materials and methods

Institutional review board approval was received from the University of Oklahoma Health Sciences Center and the study has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. This prospective cohort study enrolled males age 18–55 presenting for semen analysis at an academic fertility center. Subjects were excluded if they were unable to provide a sample, had a current history of sexually transmitted disease, used testosterone or anabolic steroids in the last 2 years, had a history of vasectomy or known testicular obstruction, or had a history of retrograde ejaculation.

In all cases, semen was collected by masturbation and processed after liquefaction had occurred. Sperm count and motility was conducted according to the World Health Organization (WHO) 5th edition for semen evaluation [10]. After thorough mixing, semen volume was determined using a serological pipette and pH was determined using a commercial strip of pH paper (pHydriion, Micro Essential Laboratory, Brooklyn, New York) by comparison to a color standard. Each sample was given a viscosity score of 0, 1, or 2 (normal, slightly thick, and thick, respectively) using a wide-bore pipette and assessing flow of the semen column. Seven microliters of semen was placed in a pre-warmed Microcell counting chamber (VitroLife, Göteborg, Sweden) and allowed to equilibrate for at least 1 min on a 37 °C stage warmer. Sperm concentration (million/ml) and motility (percent) were then determined. The rate of forward progression (RFP) was scored as 0 to 4; (0, no motility; 1, sluggish or poor motility; 2, moderate motility; 3, good motility and 4, vigorous or rapid motility), according to the American Association of Bioanalysts (AAB)/College of Reproductive Biology (CRB) andrology proficiency testing standards. Each sample was given an agglutination score of 0, 1, or 2 for none, some/mixed, and complete, respectively. The total motile sperm count (TMSC) was calculated using the formula “volume × count × motility.” For strict morphology (Kruger) [11], approximately 10 µl of semen was placed on a clean glass slide, spread evenly, and allowed to dry completely and then stained with Stat III andrology stain (Mid-Atlantic Diagnostics, Mount Laurel, New Jersey). A minimum of 200 sperms were then counted at 100× under oil immersion and the

percent normal forms was recorded. Following clinical semen evaluation, the remaining sample was stored at –80 °C.

Lipid extraction A 100 µl aliquot of sample was spun at 4 °C for 12 min at 9000×g to pellet the sperm cells, and the supernatant was discarded. Total lipids were extracted from the cell pellets using the method of Folch et al. [12] and stored under nitrogen at –20 °C.

Sphingomyelin analysis Total lipid extracts were subjected to mild base hydrolysis to hydrolyze phospholipids. The resulting mixture was then extracted with chloroform:methanol to obtain the intact sphingolipids [13]. The resulting lipid extract was dried under nitrogen and resuspended in 200 µl of 2-propanol/methanol/chloroform (4:2:1 v/v/v) containing 20 mM ammonium formate and 0.5 µM SM (d18:1/12:0) as internal standard. Samples were introduced into a TSQ Ultra triple quadrupole mass spectrometer (Thermo Scientific, Waltham, Massachusetts) using a nanomate chip-based nano-ESI source (Advion Biosciences, Ithaca, New York) operating in infusion mode and SM species were measured using precursor ion scanning of m/z 184 [14]. Abundances of SM molecular species were calculated using the lipid mass spectrum analysis (LIMS) software (University of Helsinki) and are represented as a relative percent of the sum based upon their response values.

Fatty acid analysis Fatty acid profiles were determined for total lipid extracts. The internal standards 15:0 and 17:0 were added and the lipid extracts were subjected to acid hydrolysis/methanolysis to generate fatty acid methyl esters (FAME) [15]. FAME were quantified using an Agilent Technologies (Santa Clara, California) 7890B gas chromatograph equipped with a flame ionization detector [16].

Statistical analyses Descriptive statistics such as mean, median, and variance were determined for each continuous variable. Frequency counts were determined for categorical variables. Pair-wise Pearson correlation was calculated for each measured clinical and lipid parameter. Values greater than |0.5| are considered significantly correlated. The relationship between each pair of variables was examined using linear and logistic regression models with and without consideration for race and age.

Results

Seventy males were consented with an average age of 33 years old (range 23–53). The racial makeup of all participants was as follows: American Indian 2.7%, Asian 2.7%, African American 2.7%, Native Hawaiian/Pacific Islander 0.9%, White 81.3%, and unreported 9.8%. The mean semen analysis parameters are listed in Table 1. The standard deviations were

Table 1 Semen analysis ($n = 70$)

Sperm parameters	Mean \pm Stdev ^d	Range
Volume (ml)	3.0 \pm 1.5	0.1–6.5
pH	7.5 \pm 0.4	6.6–8.0
Count (million/ml)	37.2 \pm 33.7	0.1–176
% Motility	46.9 \pm 15.6	0–73
Rate of forward progression ^a	2.0 \pm 0.3	0–3
Agglutination ^b	0.5 \pm 0.5	0–2
Viscosity ^c	0.4 \pm 0.6	0–2
Total motile sperm count (million)	52.8 \pm 49.5	0–216.2
Morphology (% normal)	4.6 \pm 2.9	0–11

^a The rate of forward progression (RFP) was scored as 0 to 4)

^b Each sample was given an agglutination score of 0, 1, or 2 for none, some/mixed, and complete, respectively

^c Viscosity score of 0, 1, or 2 was assigned for normal, slightly thick, and thick, respectively

^d StDev = standard deviation

large in most categories, especially in count and TMSC, reflecting the heterogeneity of the population being studied.

Analysis of the SM molecular species of sperm cells from 70 human participants was performed. The relative percent of each SM species for each sample was calculated (Fig. 2; Online Resource 1) to determine the amount of VLC-PUFA-SM present. VLC-PUFA-SM species having 28–34 carbon

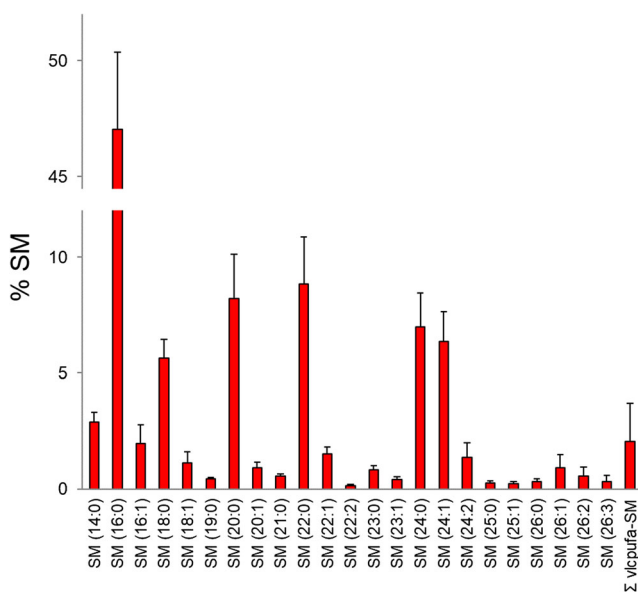


Fig. 2 Molecular species composition of human sperm sphingomyelin ($n = 70$). Values are relative percent of each sphingomyelin (SM) species \pm standard deviation. The value in parenthesis represents the amide-bound fatty acid in the SM molecule. The number to the left of the colon represents the number of carbons in the fatty acid and the number to the right of the colon represents the number of double bonds in the fatty acid. Σ VLC-PUFA-SM = sum of VLC-PUFA-containing sphingomyelin (SM) species

fatty acids were detected, with 28 and 30 carbon VLC-PUFA being the most abundant. For comparison purposes, the percentages of all individual VLC-PUFA-SM were summed (Σ VLC-PUFA-SM). The sum of all VLC-PUFA-SM species made up anywhere from 0 to 6.1% of the overall SM pool, with the average being 2.1%. The most abundant SM species was SM (16:0), averaging 47.1%. Saturated fatty acid-containing SM collectively made up 82.1% of the overall SM pool, on average. The most abundant monounsaturated fatty acid-containing SM was SM (24:1), averaging 6.4% of the overall SM pool. Interestingly, DHA (22:6n3) was not a component of SM.

The fatty acid profile of the sperm cell pellet total lipid extracts was likewise measured (Fig. 3; Online Resource 2). The most abundant fatty acids were the saturates 16:0 (46.6%) and 18:0 (23.4%). DHA (22:6n3) was the third most abundant fatty acid with an average value of 9.1 relative mole percent.

Pair-wise Pearson analysis (Online Resource 3) was used to compare the Σ VLC-PUFA-SM to results from the semen analysis (volume, count, motility, RFP, agglutination, viscosity, TMSC, morphology, and pH) performed on the same sample to look for correlations. The pair-wise Pearson analyses showed that lower levels of VLC-PUFA-SM positively correlated with lower TMSC (0.68; $p < 0.0001$) and lower total count (0.67; $p < 0.0001$), with no differences attributed to race or age. Linear regression analysis confirmed these findings (Fig. 4), showing a positive correlation between VLC-PUFA in SM and sperm count ($p < 0.0001$) and sperm motility ($p < 0.0001$).

Discussion

We found that lower levels of VLC-PUFA strongly correlated with decreased sperm count and decreased total motile sperm count. The chance to conceive increases with

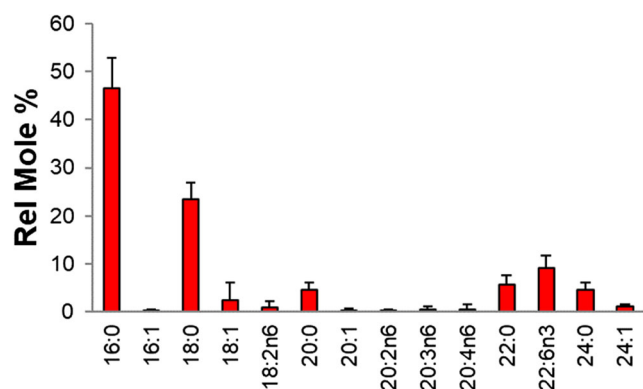


Fig. 3 Fatty acid profile of sperm pellet total lipid extract ($n = 70$). Values are relative mole percent of each species \pm standard deviation. The number to the left of the colon represents the number of carbons in the fatty acid and the number to the right of the colon represents the number of double bonds in the fatty acid

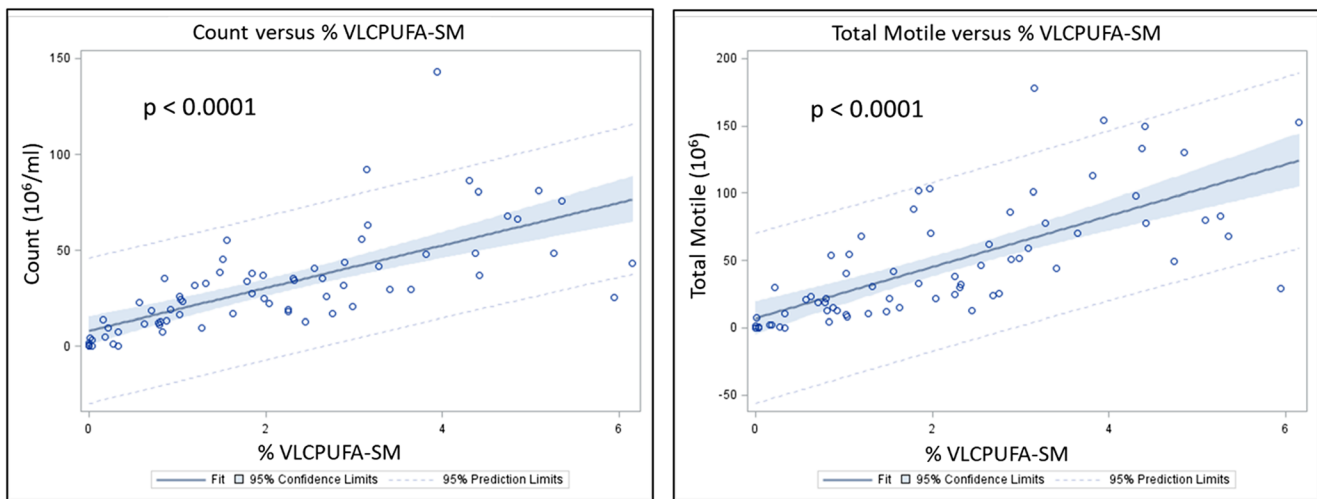


Fig. 4 Linear regression analysis of % VLC-PUFA-SM (sum of VLC-PUFA-containing sphingomyelin) versus sperm count (million/ml) and total motile sperm count (volume \times count \times motility)

higher sperm concentrations [17, 18]. Total motile sperm count also positively correlates with the chance of pregnancy in infertility treatments such as intrauterine insemination (IUI), a treatment frequently recommended for idiopathic male infertility [19, 20]. The semen analysis is a superficial external view of the sperm quantity and quality. There are not reliable and reproducible tests for sperm function such as capacitation, the acrosome reaction, binding and penetration of the zona pellucida, and ultimately fertilization [21]. VLC-PUFA-containing sphingomyelin are concentrated in the head of the spermatozoa and undergo changes with capacitation, thereby playing a role in fertilization [22]. The VLC-PUFA content of the sperm plasma cell membrane is crucial for stability of the membrane, and varying levels of VLC-PUFA have resulted in changes in the cell membrane fluidity, flexibility, and/or rigidity [23, 24]. Certainly, the components of the sperm plasma membrane play an important role in sperm development as well as sperm function.

The lipids of sperm are relatively unique in that they contain many different sphingolipids that have long-chain polyunsaturated fatty acids (LC-PUFA) such DHA (22:6n3), EPA (20:5n3), and AA (20:4n6), as well as very long chain PUFA containing 28 or more carbons [1, 2]. Some studies in humans found an association between sperm quality and the sperm levels of n3 PUFA [25, 26]. In one study published in *Clinical Nutrition* [27] proven fertile men ($n = 78$) were found to have higher blood and spermatozoa levels of n3 PUFA. Infertile men ($n = 82$) had significantly higher AA (n6)/DHA(n3) and AA (n6)/EPA(n3) ratios that were both negatively correlated with total sperm count, sperm motility, and sperm morphology. Another study reported a positive correlation ($p < 0.001$) between sperm motility and DHA levels [27]. Esmaili et al. [5] reviewed the literature on dietary fatty acid effects on sperm quality and concluded that there were

differences in n3 and n6 PUFA levels in sperm of fertile and infertile men and that supplementation with DHA could improve the quality of human sperm, although this was not shown in all publications. Interestingly, there have been no reported clinical trials to determine the efficacy of DHA supplementation on male fertility using pregnancy and live births as an outcome measure. Neither has there been any detailed analysis of VLC-PUFA in human sperm from fertile and infertile males.

VLC-PUFA are essential fatty acids (EFA) and must be synthesized from shorter chain EFA that are derived from the diet (Fig. 1). Delta-6 desaturase is an important enzyme in PUFA biosynthesis and plays a key role in converting 18:3n3 to 18:4n3, and 24:5n3 to 24:6n3; the latter is then retroconverted in peroxisomes to DHA (22:6n3). We collaborated with a group at the University of Illinois Urbana and found that male mice lacking delta-6 desaturase activity were infertile and showed an arrest in spermatogenesis [4]. We showed in this same study that dietary supplementation with DHA, a precursor of VLC-PUFA, completely reversed the infertility and sperm defects, which clearly established an essential role for DHA in mouse fertility. However, at that time, we did not appreciate the potential role of VLC-PUFA, products of elongation of DHA, in fertility. Zadavec et al. [28] showed that deletion of ELOVL2, the enzyme that converts C-22 PUFA to C-24/26 PUFA (precursors of VLC-PUFA), also resulted in mouse male infertility. In these mice, providing DHA *did not* restore fertility, indicating that DHA deficiency in and of itself is not the problem. Likely, it is the lack its elongation products (VLC-PUFA). Rabionet et al. [29] were the first to show the importance of VLC-PUFA in sperm maturation in mice; they characterized a number of sphingolipids, mostly fucosylated, several of which were composed primarily of VLC-PUFA. Depletion of *ceramide synthase-3* led to a significant decrease in VLC-PUFA-containing sphingolipids and a loss of

spermatogenesis. However, they did not confirm infertility with breeding studies.

To our knowledge, this is the first time that a correlation has been made between the health of human sperm and VLC-PUFA. Future studies should evaluate if fertilization rates in *in vitro* fertilization correlate with levels of sperm VLC-PUFA. Additionally, we need to evaluate pregnancy and live birth as a consequence of VLC-PUFA and see if this improves with supplementation. Future animal studies will focus on ELOVL4 knockout mice and determine if fertility can be restored through VLC-PUFA supplementation in their diet. If successful, we would anticipate that some forms of human male infertility could be reversed with VLC-PUFA treatment.

Acknowledgments We thank Sonny Icks of the Dean McGee Eye Institute for his invaluable assistance with all matters related to IRB approvals and updates. We also thank Michelle Scruggs, RN, and Kisha Turner, RN, for their assistance in recruitment, consenting, and data management. And finally, we thank Dr. Sixia Chen for statistical assistance.

Funding This work was supported by the Presbyterian Health Foundation Team Science Grant (L.B.C, R.E.A and M-P.A), the National Institutes of Health Grants EY04149 (R.E.A.) and P30EY021725 (R.E.A); an unrestricted grant from Research to Prevent Blindness to the University of Oklahoma Health Sciences Center's Department of Ophthalmology; OU College of Medicine Alumni Association, BrightFocus Foundation, and Oklahoma Center for Advancement of Science and Technology to M-P.A; and the Oklahoma Shared Clinical and Translational Resource Institute (NIGMS U54 GM104938).

Compliance with ethical standards

Conflict of interest LB Craig, MT Sullivan, and MT Zavy declare that they have no conflicts of interest. RS Brush, MP Agbaga and RE Anderson have a US Patent for biological synthesis of VLC-PUFA (United States Patent 8,021,874 B2).

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

Research involving human participants Research involved human participants. Institutional Review Board approval was obtained prior to initiating the trial.

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