Brief Report

Lipid Signature of Motile Human Sperm: Characterization of Sphingomyelin, Ceramide and Phospholipids with a Focus on Very Long Polyunsaturated Fatty Acid

Gerardo M. Oresti[1,2]*, Jessica M. Luquez[1,2], and Silvia A. Belmonte[3,4]*

[1] Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Bahía Blanca, Argentina; gmoresti@criba.edu.ar; jluquez@criba.edu.ar
[2] Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur (UNS), Bahía Blanca, Argentina; gmoresti@criba.edu.ar; jluquez@criba.edu.ar
[3] Instituto de Histología y Embriología de Mendoza (IHEM) "Dr. Mario H. Burgos", CONICET, Universidad Nacional de Cuyo, Centro Universitario, M5502JMA, Mendoza, Argentina; silvia.bemonte@fcm.uncu.edu.ar
[3] Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina; silvia.bemonte@fcm.uncu.edu.ar

* Correspondence: GMO, gmoresti@criba.edu.ar; SAB, silvia.bemonte@fcm.uncu.edu.ar

Abstract:

Sperm membrane lipids play a crucial role in male fertility, influencing sperm motility, viability, and functional competence. This study provides a comprehensive characterization of the phospholipid and sphingolipid composition in highly motile human spermatozoa obtained through the swim-up method, a widely used technique in assisted reproductive technology (ART). Using two-dimensional thin-layer chromatography and phosphorus analysis, we identified choline glycerophospholipids (CGP, 45%), ethanolamine glycerophospholipids (EGP, 26%), and sphingomyelin (SM, 17%) as predominant phospholipids, with minor components including cardiolipin, lysophospholipids, phosphatidylinositol, phosphatidylserine, phosphatidic acid, and neutral lipids. Gas chromatography analysis of glycerophospholipids (GPL) revealed a high long chain (C20-C22) polyunsaturated fatty acid (PUFA) content (46.3%), particularly docosahexaenoic acid (DHA, 22:6n-3), which was more abundant in CGP (46%) than EGP (26%). Sphingolipid analysis indicated that ceramide (Cer) and SM shared similar fatty acid profiles due to their metabolic relationship, with very long-chain (VLC) PUFA (≥C26) being more prevalent in SM (10%) than in Cer (6%). Additionally, argentation chromatography allowed the identification of highly unsaturated VLCPUFA species in Cer, including 28:3n-6, 28:4n-6, and 30:4n-6, which had not been previously quantified in motile human spermatozoa. Given the essential function of sphingolipid metabolism in spermatogenesis, capacitation, and acrosomal exocytosis, our findings suggest that the balance of VLCPUFA-containing SM and Cer emerges as a key factor in sperm performance and fertilization potential. This study provides novel insights into the lipid signature of human sperm and highlights the relevance of membrane lipid remodeling for male fertility and ART outcomes.

Keywords: sphingolipids; ceramide; sphingomyelin; phospholipids; VLPUFA; human sperm

1. Introduction

The importance of the lipid composition of the sperm plasma membrane lies in its unique characteristics, which are closely related to the ability of these fully differentiated cells to successfully fertilize the oocyte [1]. In both murine and large mammal spermatozoa, the high content of C20-C22 polyunsaturated fatty acids in their glycerophospholipids (GPL) [2-4] and the presence of sphingolipids (SLs) with very-long-chain polyunsaturated fatty acids ≥C26 (VLCPUFA) stand out [5, 6]. It is worth noting that these VLCPUFA are exclusively bound to sperm sphingomyelins (SM) and not to other phosphoglycerolipids [5]. Notably, SM with VLCPUFA has been found to be exclusively located in the heads of bull and rat spermatozoa [6, 7]. The VLCPUFAs are produced through the elongation and desaturation of n-3 and n-6 dietary C18-C22 PUFA by a series of elongase and desaturase enzymes that involve the sequential action of ELOVL2, ELOVL5, and especially ELOVL4. The latter is expressed in a limited number of tissues, including the skin, brain, retina, Meibomian glands, and testes [8, 9].

In the rat testicular environment, Santiago Valtierra et al. (2018) demonstrated that the expression and activity of ELOVL4 correlate with the abundance of VLCPUFA. These unusual molecular species are incorporated into SLs compounds through the enzymatic action of ceramide synthase 3 (CerS3) in mice testis [10]. When CerS3 is conditionally deleted, it results in an almost-complete absence of SL products harboring VLCPUFA, increased apoptosis during meiosis, and spermatogenic arrest that culminates in infertility [11].

The requirement of VLCPUFA in human fertility is underscored by observations correlating diminished levels of these fatty acids in sperm with reduced quantity and quality of spermatozoa, as reported by Craig et al. in 2019 [12]. Although the majority of these investigations have focused on the total semen samples from various mammalian species, including human, our study aims to elucidate the fatty acid composition of main GPL, SM, and Cer, within the membranes of human sperm isolated by swim up commonly used for in vitro fertilization treatment. This issue has not yet been explored. Our findings have prompted us to investigate the relationship between lipids and the human sperm functions we previously studied. Maintaining lipid homeostasis, which is crucial for cell health, significantly impacts sperm viability, development, and performance during fertilization.

2. Results

2.1. Phospholipid composition and fatty acid of total glycerophospholipids (GPL) in non-capacitated motile human sperm

To assess the qualitative and quantitative phospholipid profile of motile non-capacitated human sperm we first resolved the lipids in a two-dimensional TLC (Figure 1A). After the exposure of the plates to iodine vapors, we transferred the silica gel containing each lipid class to different tubes. Then, we quantified the phospholipids by phosphorus analysis. The results showed in Figure 1A and B revealed the lipid composition of sperm

membranes, which are choline glycerophospholipids (CGP), diphosphatidylglycerol, commonly known as "cardiolipin" (DPG), ethanolamine glycerophospholipids (EGP), free fatty acids (FFA), lysophosphatidylcholine (LPC), lysophosphoethanolamine (LPE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), phosphatidic acid (PA), seminolipid (SL), neutral lipids (NL, e.g. cholesterol and their esters) and some unknown lipids indicated with a question mark.

As expected, we found that major PL were CGP, EGP, and SM that accounted on average 45%, 26% and 17%, respectively, and minor percentages (3% or less) of lysoPLs (LPC and LPE), PI, PS, DPG followed of negligible amounts of PA (Figure 1C). The CGP/EGP and CGP/SM were 1,73 and 2,64, respectively.

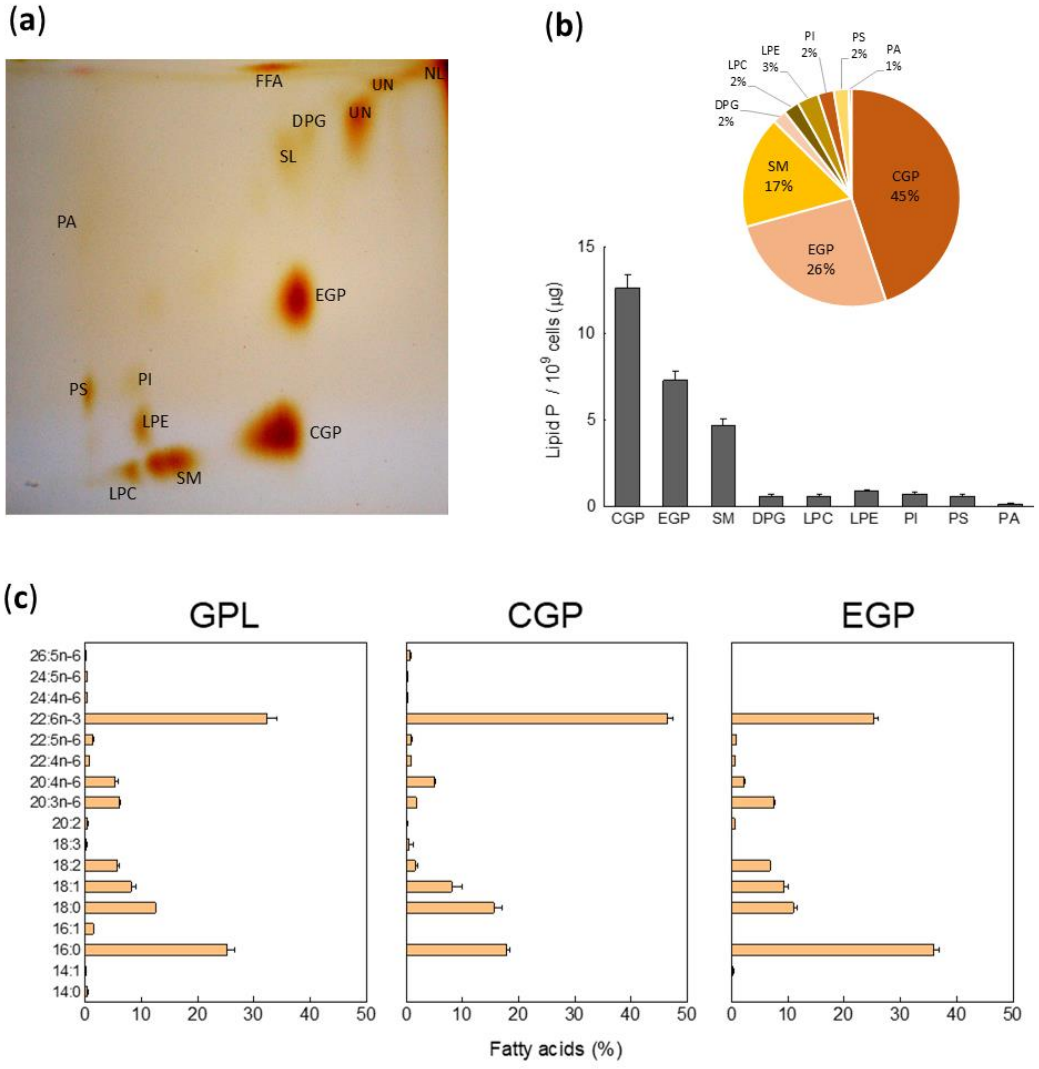

Figure 1. Qualitative phospholipid and fatty acid profile of human spermatozoa obtained by swim-up. (**a**) shows a representative TLC to resolve phospholipids. To perform this two-dimensional TLC two solvents were used, chloroform/methanol/ammonia (65:25:5) and chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5 by vol). The lipids were located by exposure of the plates to iodine vapors. (**b**) Phospholipid composition (upper panel) and amounts per cell estimated from lipid phosphorus (bottom panel) of each phospholipid constituens of human spermatozoa. (**c**) Fatty acid composition of total glycerophospholipids (GPL), choline and ethanolamine glycerophospholipids (CGP and EGP, respectively). Other abreviattions: DPG, diphosphatidylglycerol (cardiolipin); FFA, free fatty acids; LPC, lysophosphatidylcholine; LPE, lysophosphoethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PA, phosphatidic acid ; SL, Seminolipid; UN, Unknown lipids.

Then, aliquots of isolated total PL from human sperm membranes were analyzed by GC to determine the fatty acid composition of the GPL. As shown in Figure 1C, saturated FA represented 37.9 ± 1.5 %, being the 16:0 and 18:0 the most prevalent in human sperm samples. Further, we found MFA (9.5 % ± 1.1, mainly 18:1) and DFA (5.9 % ± 0.4, mainly 18:2). Strikingly, PUFA constituted 46.3 % of the total sperm GPL fatty acids and as observed in the Figure 1C, 22:6n-3 is the most abundant PUFA (32%), followed by minor amounts of 20:3n-6 and 20:4n-6. In addition, we found that only 0.5% of the total FA are C24-26 VLCPUFA in human sperm GPL. This fatty acid composition pattern, with some differences, was similar in major GPL, CGP and EGP. Notably, the percentage of 22:6n-3 in CGP reached 46%, while in EGP was 26% (Figure 1C). The latter was characterized by high amounts of molecular species of 16:0, about 36% of total fatty acids (Figure 1C).

2.2. Fatty acid composition in sphingomyelin (SM) and ceramide (Cer) from human motile spermatozoa

Our investigation was particularly directed towards the fatty acid composition of the SM and specially Cer molecular species isolated from the membranes of human motile spermatozoa obtained via swim up. In Figure 2, representative chromatograms of Cer and SM are shown, demonstrating that both lipids had the same fatty acids, which is expected due to their metabolic relationship. The total content of SM per unit of lipid phosphorus is 6.5 times greater than that of Cer (Figure 2B), and the latter exhibited the same molecular species of VLCPUFAs with ≥C26 carbon atoms as SM. Furthermore, when comparing the proportions of fatty acid groups, no differences were observed between the two lipids, with saturated (S), monoenoic (M), and dienoic (D) fatty acids, ranging from 56-58%, 25-30%, and 6-8% respectively. However, VLCPUFAs (V) were proportionally more abundant in SM than in Cer, at 10% compared to 6%, respectively ($p<0.05$).

To concentrate individual molecular species of ≥C26 VLCPUFAs from the minor Cer, we resolved the fatty acid methyl esters (FAME) using argentation TLC. This method allowed us to separate total FAME into discrete bands containing saturated, monoenoic, and dienoic fatty acids, as well as those containing trienoic, tetraenoic, and pentaenoic VLCPUFA series. Each molecular species was then identified by GC (Figure 2C). Notably, 28:3n-6, 28:4n-6, and 30:4n-6 were the most abundant VLCPUFAs found in human sperm Cer (Figure 2C and Figure 3). Additionally, the longest VLCPUFA detected (Figure 2C, lower panel) and quantified (Figure 3) was 32:4n-6.

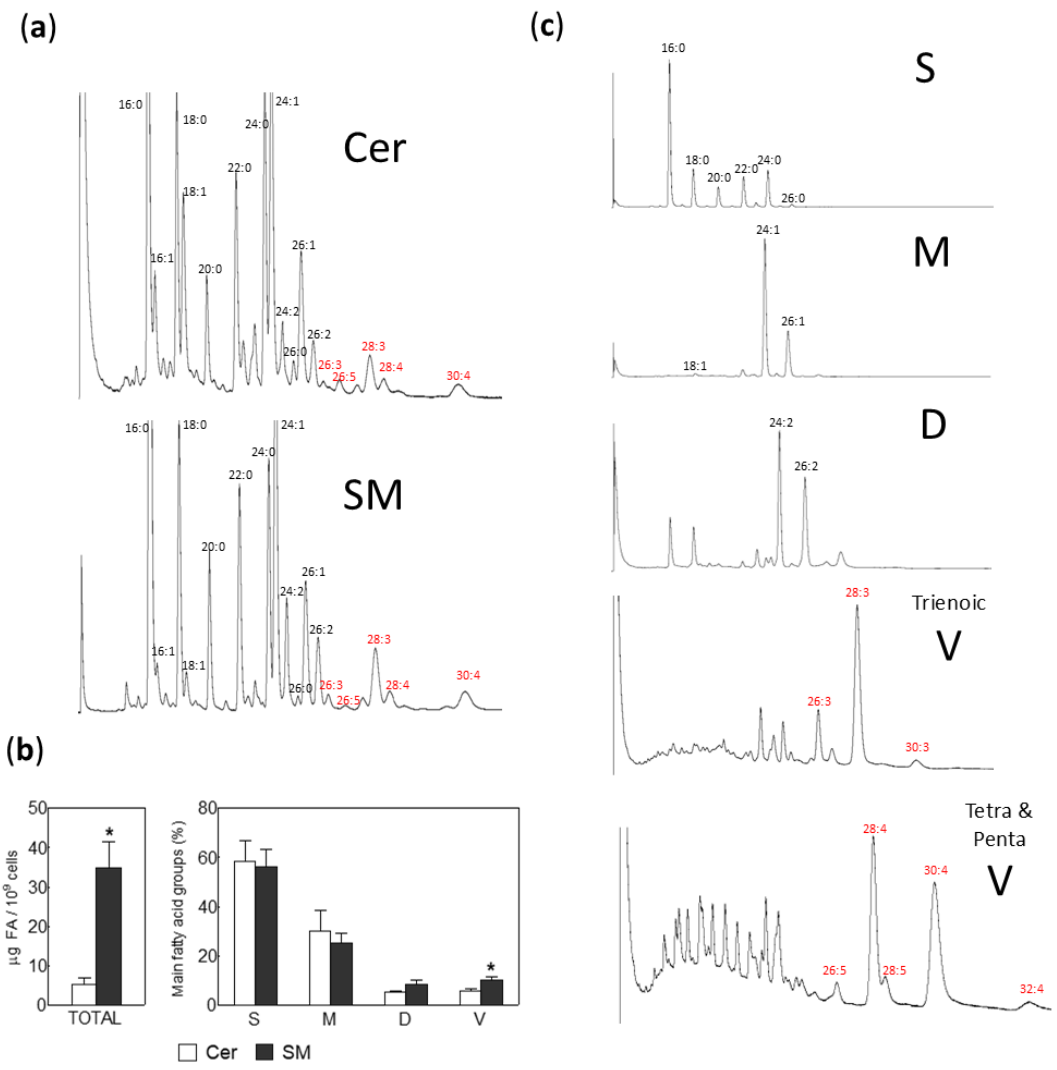

Figure 2. Ceramide (Cer) and sphingomyelin (SM) fatty acid profile of human spermatozoa obtained by swim-up. (A) shows a representative chromatograms showing the qualitative presence of ≥C26 VLCPUFA. (B) Content of Cer and SM. To compare them, the lipids were quantified by their fatty acids and expressed as µg per cells. Left panel: amounts of total fatty acids; rigth panel, percentages of main fatty acids, grouped into saturated (S), monoenoic (M), dienoic (D), and total VLCPUFA (V) (*p<0.01). (C) Enrrichment of different groups of fatty acids from sperm Cer, each chromatograms represent different fraction of fatty acids separated using AgNO3-TLC, VLCPUFA fractions were composed by tri-, tetra- and pentaenoic fatty acids.

In the quantitative comparison of fatty acid profiles of Cer and SM in human male gametes, we observed a predominance of saturated and monounsaturated fatty acids, ranging from 16 to 26 carbon atoms (Figure 2C and Figure 3). Notably, palmitic acid (16:0) was the most abundant, accounting for 18.0% of Cer and 32.5% of SM fatty acids. As shown in Figure 3, the proportion of 16:0 in SM is nearly double that of Cer. In contrast, Cer exhibited approximately double the proportions of the longer fatty acids 22:0 and 24:0 compared to SM, with 9.5% vs. 4.7% and 13.1% vs. 5.4%, respectively.

Within the individual molecular species of ≥C26 VLCPUFAs, no qualitative differences were found, but quantitative differences were observed. In both lipids, the predominant molecular species of ceramides (Cer) and sphingomyelins (SM) were 28:3n-6, 28:4n-

6, and 30:4n-6, although the proportions were slightly different: 4.4/1.6/2.3 in SM, while they were 2.5/1.0/1.4 in Cer (Figure 3).

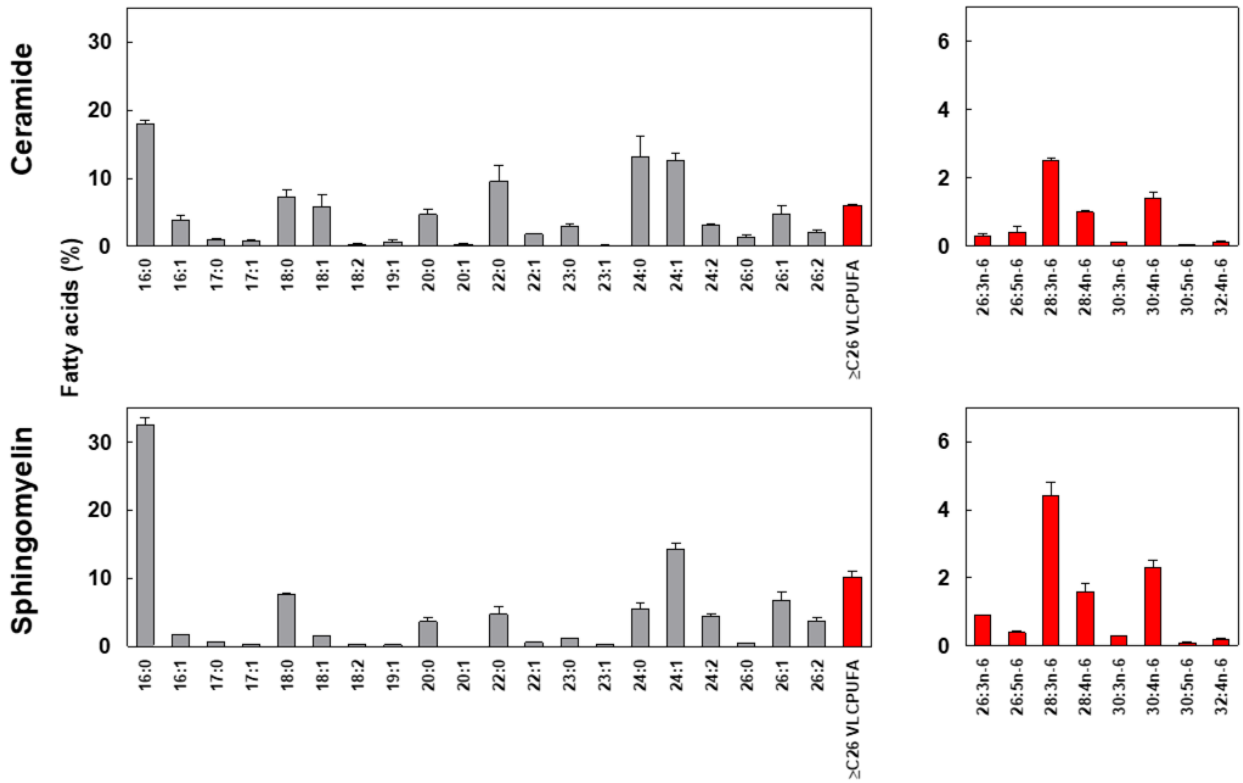

Figure 3. Cer and SM main individual fatty acids. Left panels: percentages of individual saturated, monoenoic and dienoic fatty acids in comparison with total ≥26VLCPUFA. Right panels: percentages of individual ≥C26 VLCPUFA are showed. Notably, in both Cer and SM from human sperm 28:3n-6, 28:4n-6, and 30:4n-6 are the main VLCPUFA molecular species.

3. Discussion

This study aimed to elucidate the comprehensive lipid composition of human sperm membranes of motile spermatozoa before the capacitation. Maintaining lipid homeostasis is crucial for sperm health, influencing the survival and functionality of male gametes during fertilization [13]. Thus, we characterized membrane lipids in human spermatozoa with high motility obtained by swim-up that are commonly used for in vitro fertilization assays, with a special focus on molecular species of GPL and the major sphingolipids SM and Cer.

Here, we showed that alongside the abundant GPL rich in 22:6n-3 and the "common" SM and Cer with saturated and monounsaturated fatty acids, molecular species of SM and Cer incorporating VLCPUFA are quantitatively critical constituents of human sperm membranes. In addition, this is the first report showing the quantification of endogenous molecular species of Cer pool in motile human sperm cells obtained by the same method used in assisted reproductive technology procedures. Interestingly, we found that SM from motile spermatozoa had higher percentage of VLCPUFA (about 10% of total fatty acid) in comparison with previous reports on total sperm cells, where VLCPUFA containing SM comprised 0 to 6.1% of the overall SM pool (mean 2.1%) [12, 14]. This is likely attributable to our analysis being conducted on a selected sperm population with the highest motility. Our findings stress the importance of the VLCPUFA concentration in SM for sperm functionality.

This prospect prompts intriguing inquiries into the characteristics and significance of VLCPUFA within SL in human sperm membranes. Recognizing the presence of significant amounts of SM and Cer containing "unusual" fatty acids in spermatozoa could facilitate exploration of their role in male reproductive physiology, potentially serving as key players in fertilization-related processes. Indeed, Craig and colleagues [12] investigated this issue in the human sperm total cell pool, noting that lower levels of VLCPUFA containing SM were strongly associated with reduced sperm count and total motility.

Sphingoglycolipids and the sphingophospholipid, SM, constitute the main SLs within eukaryotic cells, playing crucial roles in shaping membrane structure and functionality [15]. Over the past decade, there has been a lot of interest in these lipids due to certain metabolites they generate, such as sphingoid bases, phosphorylated sphingoid bases, and Cer, which are now essential messengers in cell signaling [15-20]. SM and Cer alterations in their molar ratio impact membrane physical properties, influencing microdomain formation, vesicular trafficking, membrane fusion, as well as, different processes involving membrane dynamics [21, 22]. Specific sphingomyelinases catalyze the hydrolysis of human sperm SM [20] generating most of the ceramide present in the male gamete. In rodents and boar sperm, the enzyme activity rises just before fertilization hydrolyzing SM, to produce ceramides during the acrosome reaction, with a consistent SM drop [23-26]. Consequently, the steady-state levels of Cer in cells can be modulated by a set of enzymes, which remove or modify the SM or Cer. Cer is a minor lipid class in most animal tissues and cells but is essential for human sperm function and fertilizing capability [18, 20, 27, 28].

We hypothesize that the fatty acid composition of these SLs plays a crucial role in their biological function in membrane fusion. In secretory cells, SM is primarily regarded as a structural lipid, yet it can also generate metabolites with highly bioactive properties like Cer, ceramide 1-phosphate (C1P), or sphingosine 1-phosphate (S1P) [29, 30]. Furthermore, it remains unclear whether the incorporation of a VLCPUFA into the molecular structure of the distinctive sperm ceramides affects their bioactivity, and if so, in what manner.

Some authors have demonstrated the accumulation of VLCPUFA-rich SM, Cer and, even glycosphingolipids (e.g. fucosylated GSL) in normal spermatogenic cells across different species [6, 10, 11, 31-33]. This finding underscores the incorporation of these sphingolipids into the spermatozoa where they play crucial physiological roles. The importance of different sphingolipids in human male reproductive physiology has been demonstrated. Our laboratory has elucidated the signaling cascades mediated by different sphingolipids during sperm acrosomal exocytosis [17, 18, 20]. Some enzymes involved in SL metabolism, such as neutral-sphingomyelinase, ceramidases, ceramide synthase [20], sphingosine kinase 1[17], and, ceramide kinase [18], are present in fully differentiated and terminal cells like the human sperm. We determined how some of these enzymes and SL regulate exocytosis. Cer induces the acrosome reaction and enhances the gamete response to progesterone. Furthermore, we outlined the signaling sequence linking ceramide to the internal and external mobilization of calcium during acrosome secretion [20], demonstrating that some enzymes involved in sphingolipid metabolism are active. For example, the ceramide kinase is able to synthesize C1P from sperm ceramide under a calcium increase during spermatozoa physiological function [18]. In addition, the ceramide effect is mainly due to C1P synthesis. On the other hand, progesterone needs ceramide kinase activity to rise intracellular calcium and induce the acrosome release. S1P, a bioactive sphingolipid,

initiates the acrosome reaction by binding to Gi-coupled receptors, thereby activating extracellular calcium influx and calcium efflux from intracellular reserves. [17].

Numerous studies in sperm and testis of different animal species stress the importance of VLCPUFA in the reproductive physiology. The C20-C24 PUFA and ≥C26 VLCPUFA in spermatogenic cells originate from fatty acids derived from the diet, including those from the n-6 series derived from linoleic acid, and the n-3 series derived from linolenic acid (Figure 4). The PUFA elongases Elovl5 and Elovl2 are recognized for synthesizing C18-C22 and C20-C24 PUFAs of the n-3 and n-6 series, respectively, operating both sequentially and in conjunction with position-specific fatty acid desaturases. These enzymes are essential for the major PUFAs (C20 and C22) found in the glycerophospholipids of mammalian cells [34, 35]. Previously, it has been demonstrated that Lysophosphatidic acid acyltransferase 3 (LPAAT3) is essential for incorporating 22:6n-3 into the membrane GPLs of differentiating germ cells. In LPAAT3-KO mice, there is a drastic and specific decrease in DHA-containing phospholipids, leading to male infertility due to a failure in spermiogenesis [36]. In addition, PUFA-rich GPLs, especially those containing DHA, facilitate membrane reshaping and flexibility [37], a crucial characteristic for spermatogenic cells to progress through spermiogenesis and generate spermatozoa. The mature gamete retains these GPL molecular species until their final destination in the oviduct. This suggests that maintaining this membrane deformability is important even up to the moment of fertilization.

It has been shown that this enzyme group can elongate C20-C24 PUFAs up to C32 n-V, a process mediated by the elongase Elovl4 [38], in rat seminiferous tubules [39] and, more recently, in isolated germ cells [38]. The critical role of Elovl2 in PUFA and VLCPUFA synthesis in germ cell lipids is highlighted by the fact that Elovl2 –/– mice are sterile, with their testes containing only spermatogonia and primary spermatocytes [40].

The production of sphingolipids with VLCPUFA in spermatogenic cells relies on the Cers3 gene [10]. When Cers3 is specifically deleted in the germ cells of mice, leading to the lack of VLCPUFA-containing Cer, SM, GlcCer, and complex GSL, it causes a halt in spermatogenesis due enhanced apoptosis during meiosis and formation of multinuclear giant cells [11]. Consequently, in rodents, both simple and complex sphingolipids containing VLCPUFA are crucial for normal spermatogenesis and male fertility. Interestingly, CerS3-mRNA and SLs with VLCPUFA were absent in infertile human patients with Sertoli cell-only syndrome, and normal adult human testes contain the same molecular species of VLCPUFA-containing Cer and SM that we observed in motile spermatozoa [11]. This demonstrates that these lipids, as well as VLCPUFA themselves, play a role early in testis physiology and are conserved during epididymal transit to function in fully differentiated, fertilization-ready spermatozoa.

Conversely, changes in the SM/Cer ratio (including those molecular species with VLCPUFA) induce changes in rat sperm membrane stability [41]. Our results show that human sperm Cer share the same fatty acids as sphingomyelins (SM), suggesting that Cer may originate from the action of sphingomyelinase on pre-existing SM. In systems containing SM where the Cer ratio is progressively increased by sphingomyelinase activity, the formation of Cer leads to changes in lipid bilayer properties. Cer molecules spontaneously associate to form Cer-enriched microdomains that fuse into large Cer-rich membrane platforms as Cer concentrations increase [42]. These Cer structures segregate from the bulk liquid-crystalline fluid phase and exhibit gel-like properties. These properties depend not only on the amount but also on the acyl chain length and unsaturation of the

generated Cer [43]. In agreement varied levels of Cer with VLCPUFA induced changes in cell membrane biophysical properties [23, 41], which could promote, along with other enzymatic and signaling processes, the development of motility and/or acrosomal reaction (Figure 4).

In summary, the constituents of sperm membranes play a crucial role in development and sperm function. Recent lipidomics studies have highlighted the importance of membrane lipids—such as sulfogalactosylglycerolipid (SGG, seminolipid), cholesterol sulfate, and GPL with PUFAs—as key predictors of semen quality [44]. Notably, SLs with VLCPUFA have emerged as particularly significant.

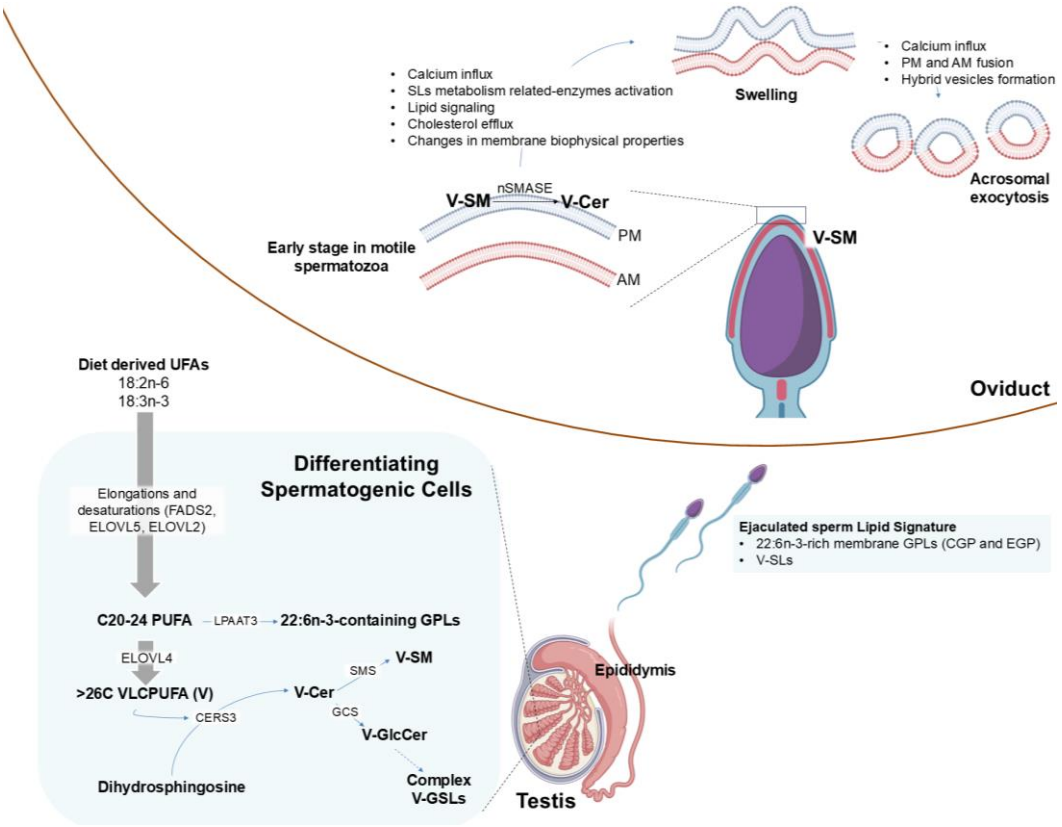

Figure 4. The scheme summarizes the biosynthetic pathways of long (C20-C22) and very long-chain (≥C26) polyunsaturated fatty acids (PUFA and VLCPUFA, respectively) and their incorporation into differentiating spermatogenic cell GPLs and SLs in the testis. After leaving the testis and passing through epididymal transit, ejaculated spermatozoa experience lipid remodeling but conserve 22:6n-3-rich GPLs (CGP and EGP) and VLCPUFA-containing SM. Upon ejaculation, sperm cells carry these molecular species of lipids into the female reproductive tract, where additional lipid remodeling occurs in the oviduct, including the V-SM → V-Cer reaction. These final modifications facilitate membrane fluidity and the ability of spermatozoa to undergo capacitation and the acrosome reaction, both of which are essential for fertilization. Abbreviations: AM, acrosomal membrane; Cer, ceramide; CERS3, ceramide synthase 3; CGP and EGP, choline and ethanolamine glycerophospholipids, respectively; ELOVL, elongation of very-long-chain fatty acid protein; FADS2, Δ6 desaturase; GPL, glycerophospholipids; GlcCer, glucosylceramide; GCS, GlcCer synthase; GSLs, glycosphingolipids; LPAAT3, lysophosphatidic acid acyltransferase 3; nSMASE, neutral sphingomyelinase; PM, plasma membrane; SL, sphingolipids; SMS, SM synthase; SM, sphingomyelin; UFAs, unsaturated fatty acids; V, VLCPUFA. The scheme was created with BioRender.com.

4. Materials and Methods

4.1. Ethics statement and human sperm preparation.

Twelve healthy male donors provided ejaculates by masturbation, after at least 48 h of sexual abstinence. We used only semen samples that accomplished the World Health Organization (WHO, 2021) specifications, for the experiments shown here. Data collection adheres to the guidelines established in Argentina (ANMAT 5330/97) and the International Declaration of Helsinki. All donors signed an informed consent according to supply semen samples. The protocol for semen manipulation was accepted by the Ethics Committee of the School of Medicine, National University of Cuyo. After semen liquefaction (30–60 min at 37° C) and highly motile sperm were recovered after a swim-up separation for 1 h in HTF (5.94 g/liter NaCl, 0.35 g/liter KCl, 0.05 g/liter $MgSO_4.7H2O$, 0.05 g/liter KH_2PO_4, 0.3 g/liter $CaCl_2.2H_2O$, 2.1 g/liter $NaHCO_3$, 0.51 g/liter glucose, 0.036 g/liter sodium pyruvate, 2.39 g/liter sodium lactate, 0.06 g/liter penicillin, 0.05 g/liter streptomycin, 0.01 g/liter phenol red supplemented with 5 mg/ml of BSA) at 37 °C in an atmosphere of 5% CO2, 95% air. Cell concentration was then adjusted with HTF to 10×10^6 sperm/ml. We developed the protocol as described in our publications[17, 18, 20, 45-47].

4.2. Lipid separation and analysis

The bulk of sperm suspensions obtained were then subjected to a gentle centrifugation (5 min at 400 x g). After collecting cells, lipid extracts were prepared and partitioned according to Bligh and Dyer [48]. Aliquots from these extracts were taken to determine the total phospholipid (PL) content in the samples by measuring the amount of lipid phosphorus[49].

For preparative isolation of lipid classes, most of the lipid extracts were spotted on TLC plates (500 μm, silica gel G) under N_2, along with commercial standards (Sigma Chemical Co, MO, USA). The polar lipids remained at the origin of the plates, and the neutral lipids were resolved in two steps. Chloroform: methanol: aqueous ammonia (90:10:2 by vol) was run up to the middle of the plates to separate the ceramides. Then, these solvents were evaporated and the plates were developed again by running n-hexane:diethyl ether (80:20, by vol.) up to the top of the plates to resolve minor neutral lipids (e.g. cholesterol esters and/or triacylglycerols).

The total phospholipid fraction was subjected to further separations and analyses. Aliquots were set aside to study the total GPL fatty acid composition. Most of the rest was used for preparative isolation of SM and major GPL using chloroform: methanol:acetic acid:0.15 mol/L NaCl (50:25:8:2.5, by vol). The phospholipids were resolved into classes by two-dimensional TLC [50]. The spots containing each phospholipid class were scraped from the plates followed by elution and quantification by phosphorus analysis in eluate aliquots. After drying, other aliquots of choline glycerophospholipids (CGP) and ethanolamine glycerophospholipids (EGP) were subjected to study fatty acid composition.

A mild alkali treatment was performed on the SM and Cer samples in order to remove any potential lipid contaminant containing ester-bound fatty acids. Both lipids were taken to dryness and treated (under N_2) with 0.5 N NaOH in anhydrous methanol at 50ºC for 10 min. After this alkaline treatment, SM and Cer were recovered again by TLC. This procedure, involving alkaline methanolysis, was also used to obtain, as methyl esters, the fatty acids ester-bound to total GPL (thus excluding the fatty acids amide-bound to SM from this group). All solvents used in this study were HPLC-grade (JT Baker, NJ, USA; UVE, Dorwill, Argentina), and most procedures were carried out under N_2.

With the exception of the data shown in Fig. 1, in which lipid classes were located with iodine vapors, lipids were located under UV light after spraying the plates with dichlorofluorescein, scraped from the plates and collected into tubes for elution. This was achieved by three successive extractions of the silica support by vigorously mixing it with chloroform:methanol:water (5:5:1, by vol.), centrifuging, collecting the solvents, and partitioning the resulting solvent mixtures with four volumes of water to recover the lipids in the organic phases.

After elution, fatty acid analysis of Cer and SM was performed after converting the lipids to fatty acid methyl esters (FAME), followed by gas-chromatography (GC), using the conditions and instrumentation described in previous work [51]. Briefly, after adding appropriate internal standards, transesterification was performed with 0.5N H_2SO_4 in N_2-saturated anhydrous methanol by keeping the samples overnight at 45°C under N_2 in screw-capped tubes. The resulting FAME were routinely purified by TLC on pre-cleaned silica Gel G plates using hexane:ether (95:5, by vol) and then injected in the GC instrument.

To resolve according unsaturation, aliquots of FAME from Cer were subjected to argentation thin-layer chromatography (TLC) (Silica Gel G:$AgNO_3$, 80:20 by weight, and chloroform:methanol, 90:10 by vol), which separated the esters in different bands containing saturates and different unsaturated fractions. After elution, these fractions were analyzed by GC/FID to identify the VLCPUFA linked to human sperm Cer.

4.3. Statistical analysis

Data are presented as mean values ± SD from at least three samples pool, each obtained from independent cell preparations. Statistical analysis was performed using the Graph Pad Prism software, version 5.0 © (San Diego, CA). The Student t-test was used to compare differences between two data. P values <0.05 were considered significant.

Author Contributions: Conceptualization, S.A.B.; methodology, G.M.O. and J.M.L; validation, S.A.B., G.M.O., and J.M.L; formal analysis, G.M.O.; investigation, S.A.B.; G.M.O., and J.M.L.; writing—original draft preparation, S.A.B.; writing—review and editing, G.M.O. and S.A.B.; visualization, J.M.L.; supervision, S.A.B. and G.M.O.; project administration, S.A.B. and G.M.O; funding acquisition, S.A.B. and G.M.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PICT-2015-1222 to S.A.B and PICT-2017-2535 to G.M.O.), CONICET, Argentina (PIP11220210100232 to S.A.B and PIP11220210100420 to G.M.O), SIIP-Universidad Nacional de Cuyo, Argentina (06/J003-T1 to S.A.B.) and SGCyT-Universidad Nacional del Sur, Argentina (PGI-24/B341 to G.M.O.).

Institutional Review Board Statement: The studies involving human participants were reviewed and approved by the Ethics Committee of the Medical School, Universidad Nacional de Cuyo. The Committee approved the signed informed consent and the protocol for semen handling (CUDAP: EXP-CUY:0025793/2016). Data collection adheres to the guidelines established in Argentina (ANMAT 5330/97) and followed the principles outlined in the Declaration of Helsinki.

Informed Consent Statement: All donors signed an informed consent agreeing to supply their own anonymous information and semen samples. The participants provided their written informed consent to participate in this study.

Data Availability Statement: All relevant data are contained within the manuscript.

Acknowledgments: The authors thank Dr. L. Suhaiman for excellent technical assistance in samples preparation.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results

Abbreviations

The following abbreviations are used in this manuscript:

ART	Assisted reproductive technology
Cer	Ceramide
CERS3	Ceramide synthase 3
CGP	Choline glycerophospholipids
DHA	Docosahexaenoic acid
EGP	Ethanolamine glycerophospholipids
ELOVL	Elongases
FADS2	Δ6 desaturase
GCS	Glucosylceramide synthase
GluCer	Glucosylceramide
GPL	Glycerophospholipids
GSLs	Glycosphingolipids
LPAAT3	Lysophosphatidic acid acyltransferase
LPC	Lysophosphatidylcholine
LPE	Lysophosphoethanolamine
NL	Neutral lipids
nSMASE	Neutral sphingomyelinase
PA	Phosphatidic acid
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
SL	Seminolipid
SLs	Sphingolipids
SM	Sphingomyelin
SMS	Sphingomyelin synthase
UFAs	Unsaturated fatty acids
VLCPUFA	Very long-chain polyunsaturated fatty acid

References

1. Flesch, F.M. and B.M. Gadella, *Dynamics of the mammalian sperm plasma membrane in the process of fertilization.* Biochim. Biophys. Acta, 2000. **1469**(3): p. 197-235.
2. Aveldano, M.I., N.P. Rotstein, and N.T. Vermouth, *Occurrence of long and very long polyenoic fatty acids of the n-9 series in rat spermatozoa.* Lipids, 1992. **27**(9): p. 676-80.
3. Carro, M.L.M., et al., *Desmosterol Incorporation Into Ram Sperm Membrane Before Cryopreservation Improves in vitro and in vivo Fertility.* Front Cell Dev Biol, 2021. **9**: p. 660165.
4. Carro, M., et al., *PUFA-rich phospholipid classes and subclasses of ram spermatozoa are unevenly affected by cryopreservation with a soybean lecithin-based extender.* Theriogenology, 2022. **186**: p. 122-134.
5. Poulos, A., et al., *Occurrence of unusual molecular species of sphingomyelin containing 28-34-carbon polyenoic fatty acids in ram spermatozoa.* Biochem J, 1987. **248**(3): p. 961-4.
6. Furland, N.E., et al., *Very long-chain polyunsaturated fatty acids are the major acyl groups of sphingomyelins and ceramides in the head of mammalian spermatozoa.* J. Biol. Chem, 2007. **282**(25): p. 18151-18161.
7. Oresti, G.M., et al., *Uneven distribution of ceramides, sphingomyelins and glycerophospholipids between heads and tails of rat spermatozoa.* Lipids, 2011. **46**(12): p. 1081-90.
8. Sandhoff, R., *Very long chain sphingolipids: tissue expression, function and synthesis.* FEBS Lett, 2010. **584**(9): p. 1907-13.

9. Yeboah, G.K., et al., *Very long chain fatty acid-containing lipids: a decade of novel insights from the study of ELOVL4.* J Lipid Res, 2021. **62**: p. 100030.
10. Rabionet, M., et al., *Male germ cells require polyenoic sphingolipids with complex glycosylation for completion of meiosis: a link to ceramide synthase-3.* J Biol Chem, 2008. **283**(19): p. 13357-69.
11. Rabionet, M., et al., *Male meiotic cytokinesis requires ceramide synthase 3-dependent sphingolipids with unique membrane anchors.* Hum Mol Genet, 2015. **24**(17): p. 4792-808.
12. Craig, L.B., et al., *Decreased very long chain polyunsaturated fatty acids in sperm correlates with sperm quantity and quality.* J Assist Reprod Genet, 2019. **36**(7): p. 1379-1385.
13. Cheng, X., et al., *Lipidomics profiles of human spermatozoa: insights into capacitation and acrosome reaction using UPLC-MS-based approach.* Front Endocrinol (Lausanne), 2023. **14**: p. 1273878.
14. Gavrizi, S.Z., et al., *Sperm very long-chain polyunsaturated fatty acids: relation to semen parameters and live birth outcome in a multicenter trial.* Fertil Steril, 2023. **119**(5): p. 753-760.
15. Hannun, Y.A. and L.M. Obeid, *Principles of bioactive lipid signalling: lessons from sphingolipids.* Nat. Rev. Mol. Cell Biol, 2008. **9**(2): p. 139-150.
16. Futerman, A.H. and Y.A. Hannun, *The complex life of simple sphingolipids.* EMBO Rep, 2004. **5**(8): p. 777-82.
17. Suhaiman, L., et al., *Sphingosine 1-phosphate and sphingosine kinase are involved in a novel signaling pathway leading to acrosomal exocytosis.* J. Biol. Chem, 2010. **285**(21): p. 16302-16314.
18. Vaquer, C.C., et al., *The pair ceramide 1-phosphate/ceramide kinase regulates intracellular calcium and progesterone-induced human sperm acrosomal exocytosis.* Front Cell Dev Biol, 2023. **11**: p. 1148831.
19. Belmonte, S.A. and L. Suhaiman, *Optimized protocols to analyze sphingosine-1-phosphate signal transduction pathways during acrosomal exocytosis in human sperm.* Methods Mol. Biol, 2012. **874**: p. 99-128.
20. Vaquer, C.C., et al., *Ceramide induces a multicomponent intracellular calcium increase triggering the acrosome secretion in human sperm.* Biochim Biophys Acta Mol Cell Res, 2020. **1867**(7): p. 118704.
21. Kolesnick, R.N., F.M. Goni, and A. Alonso, *Compartmentalization of ceramide signaling: physical foundations and biological effects.* J Cell Physiol, 2000. **184**(3): p. 285-300.
22. van Blitterswijk, W.J., et al., *Ceramide: second messenger or modulator of membrane structure and dynamics?* Biochem J, 2003. **369**(Pt 2): p. 199-211.
23. Ahumada-Gutierrez, H., et al., *Mechanical properties of bilayers containing sperm sphingomyelins and ceramides with very long-chain polyunsaturated fatty acids.* Chem Phys Lipids, 2019. **218**: p. 178-186.
24. Penalva, D.A., et al., *Membrane Restructuring Events during the Enzymatic Generation of Ceramides with Very Long-Chain Polyunsaturated Fatty Acids.* Langmuir, 2018. **34**(14): p. 4398-4407.
25. Zanetti, S.R., et al., *Differential involvement of rat sperm choline glycerophospholipids and sphingomyelin in capacitation and the acrosomal reaction.* Biochimie, 2010. **92**(12): p. 1886-1894.
26. Zanetti, S.R., et al., *Ceramides with 2-hydroxylated, very long-chain polyenoic fatty acids in rodents: From testis to fertilization-competent spermatozoa.* Biochimie, 2010. **92**(12): p. 1778-1786.
27. Furse, S., et al., *Relative Abundance of Lipid Metabolites in Spermatozoa across Three Compartments.* Int J Mol Sci, 2022. **23**(19).
28. Suhaiman, L. and S.A. Belmonte, *Lipid remodeling in acrosome exocytosis: unraveling key players in the human sperm.* Front Cell Dev Biol, 2024. **12**: p. 1457638.
29. Gafurova, C.R., et al., *beta2-Adrenergic Regulation of the Neuromuscular Transmission and Its Lipid-Dependent Switch.* Mol Neurobiol, 2024.
30. Tsentsevitsky, A.N., et al., *Sphingomyelinase modulates synaptic vesicle mobilization at the mice neuromuscular junctions.* Life Sci, 2023. **318**: p. 121507.
31. Furland, N.E., et al., *Mild testicular hyperthermia transiently increases lipid droplet accumulation and modifies sphingolipid and glycerophospholipid acyl chains in the rat testis.* Lipids, 2011. **46**(5): p. 443-54.

32. Oresti, G.M., et al., *Differentiation-related changes in lipid classes with long-chain and very long-chain polyenoic fatty acids in rat spermatogenic cells.* J Lipid Res, 2010. **51**(10): p. 2909-21.
33. Furland, N.E., et al., *Ceramides and sphingomyelins with high proportions of very long-chain polyunsaturated fatty acids in mammalian germ cells.* J. Biol. Chem, 2007. **282**(25): p. 18141-18150.
34. Guillou, H., et al., *The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice.* Prog Lipid Res, 2010. **49**(2): p. 186-99.
35. Gregory, M.K., et al., *Elongase reactions as control points in long-chain polyunsaturated fatty acid synthesis.* PLoS One, 2011. **6**(12): p. e29662.
36. Iizuka-Hishikawa, Y., et al., *Lysophosphatidic acid acyltransferase 3 tunes the membrane status of germ cells by incorporating docosahexaenoic acid during spermatogenesis.* J Biol Chem, 2017. **292**(29): p. 12065-12076.
37. Pinot, M., et al., *Lipid cell biology. Polyunsaturated phospholipids facilitate membrane deformation and fission by endocytic proteins.* Science, 2014. **345**(6197): p. 693-7.
38. Santiago Valtierra, F.X., et al., *Elovl4 and Fa2h expression during rat spermatogenesis: a link to the very-long-chain PUFAs typical of germ cell sphingolipids.* J Lipid Res, 2018. **59**(7): p. 1175-1189.
39. Aveldano, M.I., et al., *Long and very long chain polyunsaturated fatty acids of the n-6 series in rat seminiferous tubules. Active desaturation of 24:4n-6 to 24:5n-6 and concomitant formation of odd and even chain tetraenoic and pentaenoic fatty acids up to C32.* J Biol Chem, 1993. **268**(16): p. 11663-9.
40. Zadravec, D., et al., *ELOVL2 controls the level of n-6 28:5 and 30:5 fatty acids in testis, a prerequisite for male fertility and sperm maturation in mice.* J Lipid Res, 2011. **52**(2): p. 245-55.
41. Oresti, G.M., et al., *Lipid Biochemical and Biophysical Changes in Rat Spermatozoa During Isolation and Functional Activation In Vitro.* Biol Reprod, 2015. **93**(6): p. 140.
42. Bollinger, C.R., V. Teichgraber, and E. Gulbins, *Ceramide-enriched membrane domains.* Biochim Biophys Acta, 2005. **1746**(3): p. 284-94.
43. Pinto, S.N., et al., *Changes in membrane biophysical properties induced by sphingomyelinase depend on the sphingolipid N-acyl chain.* J Lipid Res, 2014. **55**(1): p. 53-61.
44. Di Nisio, A., et al., *Lipidomic Profile of Human Sperm Membrane Identifies a Clustering of Lipids Associated with Semen Quality and Function.* Int J Mol Sci, 2023. **25**(1).
45. Suhaiman, L., et al., *Different Approaches to Record Human Sperm Exocytosis.* Methods Mol Biol, 2021. **2233**: p. 139-168.
46. Lopez, C.I., et al., *Diacylglycerol stimulates acrosomal exocytosis by feeding into a PKC- and PLD1-dependent positive loop that continuously supplies phosphatidylinositol 4,5-bisphosphate.* Biochim. Biophys. Acta, 2012. **1821**(9): p. 1186-1199.
47. Pelletan, L.E., et al., *ADP Ribosylation Factor 6 (ARF6) Promotes Acrosomal Exocytosis by Modulating Lipid Turnover and Rab3A Activation.* J. Biol. Chem, 2015.
48. Bligh, E.G. and W.J. Dyer, *A rapid method of total lipid extraction and purification.* Can J Biochem Physiol, 1959. **37**(8): p. 911-7.
49. Holub, B.J. and C.M. Skeaff, *Nutritional regulation of cellular phosphatidylinositol.* Methods Enzymol, 1987. **141**: p. 234-44.
50. Rouser, G., S. Fkeischer, and A. Yamamoto, *Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots.* Lipids, 1970. **5**(5): p. 494-6.
51. Santiago Valtierra, F.X., et al., *Sphingomyelins and ceramides with VLCPUFAs are excluded from low-density raft-like domains in differentiating spermatogenic cells.* J Lipid Res, 2017. **58**(3): p. 529-542.