Levels of L-carnitine in human seminal plasma are associated with sperm fatty acid composition

Mario Iliceto1,2, Mette Haug Stensen1, Jorunn M Andersen2, Trine B Haugen2, Oliwia Witczak2

The fatty acid composition of spermatozoa has been shown to be important for their function, and L-carnitine is crucial for fatty acid metabolism. Its levels in the seminal plasma positively correlate with semen quality, whereas high body mass index (BMI) is associated with both reduced semen quality and altered sperm fatty acid composition. Here, we examined the associations between free seminal L-carnitine levels and sperm fatty acid composition as well as BMI. Semen samples were collected and analyzed from 128 men with unknown fertility status and with BMI ranging from 19 kg m⁻² to 63 kg m⁻². Sperm fatty acid composition was assessed by gas chromatography, while free seminal L-carnitine analysis was performed using high-performance liquid chromatography. Multiple linear regression analysis showed a positive correlation of free seminal L-carnitine levels with the amount of sperm palmitic acid ($β = 0.21; P = 0.014$), docosahexaenoic acid (DHA; $β = 0.23; P = 0.007$), and total n-3 polyunsaturated fatty acids ($β = 0.23; P = 0.008$) and a negative correlation of free seminal L-carnitine levels with lignoceric acid ($β = −0.29; P = 0.001$) and total n-6 polyunsaturated fatty acids ($β = −0.24; P = 0.012$) when adjusted for covariates. There was no relationship between free seminal L-carnitine levels and BMI. Since free seminal L-carnitine levels are associated with semen quality, the absence of a correlation with BMI suggests that reduced semen quality in obese men is independent of seminal L-carnitine.

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INTRODUCTION

Among the many metabolic changes related to obesity, changes in semen quality including total sperm count,1–3 concentration,1–4 motility,2,3 morphology, and vitality4,4 have been observed. The composition of sperm fatty acids is also influenced by obesity.3 In obese men, levels of sperm polyunsaturated fatty acids (PUFAs) including docosahexaenoic acid (DHA; C22:6 n-3) are reduced. Interestingly, the sperm fatty acid constituents are important for their function.1,8 The levels of sperm PUFAs with 22 carbon atoms or more correlate positively with semen quality.9 The sperm fatty acid composition has been shown to be modified during spermatogenesis and epididymal maturation; however, its regulation in spermatozoa is not well understood.

L-carnitine, a ubiquitous quaternary ammonium cation derived from lysine and methionine, plays a vital role in fatty acid metabolism by transporting long-chain fatty acids to the mitochondria for beta-oxidation.10 In mammalian cells, carnitine is present as both free L-carnitine and acylcarnitine esters produced by carnitine acyltransferases using various fatty acids as substrates. L-carnitine also prevents oxidative stress by regulating nitric oxide levels, cellular respiration,11 and the activity of enzymes involved in the defense against oxidative damage.12 Abnormalities in carnitine regulation are implicated in complications including diabetes mellitus and malnutrition.13,14 Most of L-carnitine in humans is derived from dietary intake,15 and carnitine supplementation has been shown to have beneficial effects on obesity and weight loss.16 However, the association between body mass index (BMI) and serum L-carnitine levels in humans shows conflicting results.17–19

In the male reproductive tract of mammals, free L-carnitine is transported by the organic cation/carnitine transporter 2 (OCTN2) and the carnitine transporter 2 (CT2) from the systemic circulation into the epididymal lumen,20,21 where it accumulates22–24 and could play a role in sperm maturation and metabolism.25 High levels of L-carnitine originating from the epididymis are found in both spermatozoa and seminal plasma.26,27 The uptake of free L-carnitine into epididymal spermatozoa is suggested to be both passive26 and active, mediated by OCTN1, OCTN2, and OCTN3.29,30 In animal studies, fatty acids from the breakdown of phospholipids30 can be oxidized in epididymal spermatozoa in a carnitine-dependent manner.31 Jeulin et al.32 reported parallel increases in the percentage of spermatozoa with progressive motility and free L-carnitine contents of distal corpus spermatozoa of the boar. Furthermore, in humans, levels of free seminal L-carnitine have been found to positively correlate with progressive sperm motility, sperm count,23,33 and sperm morphology.34 In addition, a study suggests that L-carnitine-mediated oxidation of fatty acids in the human spermatozoa is involved in motility.14

The aim of this study was to examine the relation between the amount of free L-carnitine distribution in the seminal plasma and levels of sperm fatty acids. Furthermore, we hypothesized that one of the mechanisms behind reduced semen quality observed in men with high BMI could result from altered seminal L-carnitine levels.
PARTICIPANTS AND METHODS

Study population
Men over the age of 18 years were recruited between August 2008 and December 2013 in South-Eastern Norway as previously described. Upon entry, weight (in kg) of the participants was recorded using a digital scale (Soehnle Professional, Backnang, Germany). Height (in cm) was measured by a wall-mounted stadiometer. In addition, information about medical treatment, dietary supplements, as well as history of cryptorchidism or previous cancers, which were exclusion criteria, was recorded. No prior knowledge of semen quality was required, and no further criteria were required for inclusion. Samples with spillage were excluded. For the present study, semen samples for L-carnitine analysis from 128 men were included, of which 90 participants were recruited from the general population by advertisement, 24 men were recruited from couples with female factor infertility from a fertility clinic (Fertility Department Soer, Telemark Hospital Trust, Skien, Norway) and 14 men from obesity clinics (The Morbid Obesity Centre, Vestfold Hospital Trust, Tonsberg, Norway, and Department of Morbid Obesity and Bariatric Surgery, Oslo University Hospital, Oslo, Norway). Men from the fertility clinic were included to achieve a wider age distribution in the group of men with BMI ≤ 27 kg m⁻², while men from the obesity clinic were included to obtain a broader BMI distribution. The study was approved by the Regional Committee for Medical and Health Research Ethics (REK), South East, Norway (REK number: 2008/3957 and 2010/2721), and all participants provided written informed consent.

Blood sampling and biochemical analysis
Fasting blood samples were collected before 10 a.m. Venous samples were centrifuged 30 min after collection at 1800g for 10 min, and serum and ethylenediaminetetraacetic acid (EDTA)-plasma were aliquoted and stored until further analyses were performed. Levels of low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol, and triglycerides (TG) were measured in the serum, while glycated hemoglobin (HbA1c) was measured in the blood at Fürst Medical Laboratory, Oslo, Norway. Upon arrival, fasting glucose concentration was analyzed in 105 capillary samples using HemoCue 201+ Glucose Analyzer (HemoCue, Ängelholm, Sweden) at OsloMet (Oslo Metropolitan University, Oslo, Norway) and in 77 venous samples at Fürst Medical Laboratory. In fifty samples, glucose levels were analyzed using both methods, and since strong correlation between the two methods was found (r = 0.939), results from both were included in the analysis.

Semen analysis
Semen analysis was performed at OsloMet in the period of 2008–2013 according to the World Health Organization (WHO) guidelines as previously described. Semen samples were obtained by masturbation and collected on-site or at home in a preweighed sterile container. Participants were asked to abstain from ejaculating for 2–7 days before sample collection and to report the length of abstinence time. When collected at home, participants were asked to avoid cooling of the sample during transportation. For participants (n = 24) with long travel distance to the laboratory, the sample was delivered after more than 1 h. Out of 128 semen samples, 83 were collected on-site, 39 were collected at home, and the place of collection was not registered for 6 samples. Time to semen analysis was recorded. Out of 128 semen samples, 104 were analyzed within 1 h, 16 between 1 h and 2 h, and 8 before 4 h. Motility was not assessed in samples that were analyzed after more than 2 h. Semen samples were analyzed after completion of the liquefaction: on-site-collected semen samples were incubated for 30 min at 37°C, while samples collected at home were incubated for 10 min at 37°C. The ejaculated volume was estimated by sample weight. Sperm concentration was determined using a Neubauer improved hemocytometer (Hecht Assistant, Sondheim vor der Rhön, Germany) and morphology was determined according to the Tygerberg strict categorization after Papanicolaou staining. Laboratory personnel performing semen analysis at OsloMet participates in the external quality program for semen assessment organized by the European Society of Human Reproduction and Embryology. Sperm pellets for fatty acid analysis and seminal plasma for free L-carnitine analysis were prepared from the remaining ejaculate by centrifugation at 500g for 15 min. The samples were stored at −80°C.

Sperm chromatin structure assay
Sperm chromatin structure assay (SCSA) was performed in 2013 at Reproductive Medicine Center, Skåne University Hospital, Malmö, Sweden. The method is described previously. Briefly, DNA fragmentation index (DFI) in the spermatozoa was analyzed using FACSScan flow cytometer (Becton Dickinson, San Jose, CA, USA), equipped with an air-cooled argon ion laser. A total of 10,000 events were accumulated for each measurement at a flow rate of 200–300 cells s⁻¹. Flow cytometric data were analyzed by the SCSA Soft software (SCA Diagnostics, Brookings, SD, USA). All samples were run in the same batch by one technician with an intralaboratory coefficient of variation (CV) of 4.5%.

Fatty acid analysis
Fatty acid levels in the spermatozoa samples were analyzed in 2015, by gas chromatography at a commercial laboratory in Oslo (Vitas AS, Oslo, Norway) as previously described. Briefly, frozen sperm pellets were thawed, methylated with 3 mol l⁻¹ methanolic HCl, and neutralized with 3 mol l⁻¹ KOH in water. Fatty acid methyl esters were extracted with hexane and analyzed on an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA). A SP-2380 column (Supelco, Bellefonte, PA, USA) was used for separation. Data were obtained for the most relevant and/or abundant individual fatty acids of spermatozoa and are presented in Supplementary Table 1. Categories of fatty acids were also calculated including saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and PUFAs. PUFAs were additionally divided into n-3 and n-6 categories (Supplementary Table 1). Results for fatty acids are expressed as weight percentage (wt%) and accounted for 87.3 wt% of the total amount of fatty acids analyzed by gas chromatography.

Free L-carnitine analysis
Free L-carnitine in the seminal plasma samples were analyzed in 2018 for the purpose of this study using liquid chromatography/tandem mass spectrometry. Seminal plasma stored at −80°C was used and analysis of the samples was performed at a commercial laboratory in Oslo. A total of 10 µl seminal plasma was thawed at room temperature and diluted with labeled internal standard L-carnitine d9 (Sigma Aldrich, Taufkirchen, Germany) before undergoing butylation by methyl chlorofomate and butanol. Samples were liquid-liquid extracted before analysis on an Agilent 1260 HPLC coupled to an Agilent 6460 QQQ mass spectrometer operated in Multiple Reaction Monitoring Mode (Agilent Technologies). Separation was performed on a Phenomenex Kinetex C18 100 mm × 4.6 mm × 2.7 µm analytical column (Phenomenex Inc., Torrance, CA, USA), and quantification was performed using a 7-point calibration curve covering a range of 5–200 µg ml⁻¹. Results for free seminal L-carnitine are expressed in µg ml⁻¹. Total free L-carnitine in
the seminal plasma (in μg per ejaculate) was calculated by multiplying free seminal L-carnitine concentration with semen volume.

**Statistical analyses**

All participant variables were summarized as median (range). BMI was calculated as weight in kg divided by height in meters squared. For the background characteristics, we categorized men into tertiles on the basis of the distribution of BMI (18.8–25.8 kg m⁻²; 25.9–31.2 kg m⁻²; and 31.3–62.7 kg m⁻²) in order to maintain an equal number of participants in each group. Statistical differences in these characteristics by BMI tertiles were assessed by Kruskal–Wallis test. Total free L-carnitine in the ejaculate was entered in the analysis of background characteristics to account for interindividual differences in sex gland fluid contribution. To visualize differences in the sperm DHA levels and BMI across the total free L-carnitine distribution, total free L-carnitine levels were categorized into tertiles (<110.8 μg per ejaculate, 110.8–218.6 μg per ejaculate, and >218.6 μg per ejaculate), and the data were presented as Box–Whiskers plot. The Mann–Whitney U test was used to compare differences between the tertiles.

Variable distributions were evaluated by histograms. Skewed variables were log-, square-root-, squared-, or inverse-transformed. Multiple linear regression analysis was used to estimate associations between levels of free seminal L-carnitine and levels of sperm fatty acids and between levels of free seminal L-carnitine and semen characteristics. All variables were continuous, except for time to semen analysis that was dichotomized.

To estimate associations between levels of free seminal L-carnitine and levels of sperm fatty acids, covariates were included in the multivariate models if they were statistically significant at \( P < 0.1 \). Covariates included in the model were age (in year), sexual abstinence time (in day), and BMI (in kg m⁻²), as these values varied among the participants. Associations between levels of free seminal L-carnitine and semen characteristics were also adjusted for time to semen analysis (<1 h or >1 h), since not all samples were delivered and analyzed within 1 h after ejaculation. Motile sperm concentration (MSC; 10⁶ ml⁻¹) was obtained by multiplying the sperm concentration and the percentage of motile spermatozoa divided by 100%. The MSC variable was entered in the regression analysis.

The Mann–Whitney U test was used for (i) comparison of free seminal L-carnitine levels in the samples delivered and analyzed within 1 h after ejaculation or later, (ii) comparison of free seminal L-carnitine levels and semen parameter values between participants recruited from the general population and from the fertility clinic, and (iii) comparison of age, free seminal L-carnitine levels, and semen parameter values between participants from the fertility clinic and a subgroup of men from the general population with comparable BMI ≤27 kg m⁻².

The level of statistical significance was set at 0.01 or lower. Statistical analyses were performed by using IBM SPSS Statistics 20 (IBM, Chicago, IL, USA).

**RESULTS**

**Study population**

The characteristics of the study population are described in Table 1. Only nine participants (7.0%) reported use of dietary supplements; however, none of the supplements included L-carnitine. When stratified by BMI tertiles, the factors closely related to obesity varied across the groups as expected, apart from total cholesterol. Neither free nor total free seminal L-carnitine levels showed difference across BMI tertiles. Among the men in the first (18.8–25.8 kg m⁻²) and second (25.9–31.2 kg m⁻²) BMI tertile, respectively, 18 (42.8%) and 6 (13.9%) were recruited from the fertility clinic from couples with female factor infertility. Fourteen (32.6%) men in the third BMI tertile (31.3–62.7 kg m⁻²) were from the obesity clinic. The remaining men were from the general population.

Subanalysis was performed to investigate if free seminal L-carnitine levels were different in samples that were delivered and analyzed within 1 h after ejaculation or later (Supplementary Table 2). No such difference was observed.

Men from the fertility clinic were included in the study population to achieve a wider age distribution in the group of men with BMI ≤27 kg m⁻². Subanalysis was done to examine whether there were differences in free seminal L-carnitine levels and semen parameter values between participants recruited from the general population (n = 104) and those from the fertility clinic (n = 24; Supplementary Table 3). Levels of free seminal L-carnitine did not differ between the two groups. Significant differences in semen parameter values were only seen in the length of sexual abstinence time (\( P = 0.004 \)) and in proportion of spermatozoa with normal forms (\( P = 0.001 \)).

In addition, subanalysis was also performed to compare age, seminal L-carnitine levels, and semen parameter values between men from the fertility clinic and men from the general population with comparable BMI ≤27 kg m⁻² (n = 33; Supplementary Table 4). As expected, there was a significant difference in age \( (P < 0.001) \), as men from the fertility clinic were older. There was no difference in seminal L-carnitine levels nor in semen parameter values between these two groups, except for seminal volume \( (P < 0.001) \) and sexual abstinence time \( (P = 0.008) \).

**Table 1: Background characteristics of men in the study population by BMI tertiles**

| Variable                  | BMI tertiles (kg m⁻²), median (range) | \( P \)    |
|---------------------------|--------------------------------------|-----------|
|                           | Lowest tertile (n=42) | Middle tertile (n=43) | Highest tertile (n=43) |
| BMI (kg m⁻²)              | 23.3 (18.8 – 25.8)    | 28.2 (25.9 – 31.2)    | 34.7 (31.3 – 62.7)     | <0.001* |
| Age (year)                | 35 (24 – 51)          | 37 (22 – 59)          | 39 (22 – 61)           | 00.07*  |
| TG (mmol l⁻¹)             | 0.8 (0.4 – 5.2)       | 1.1 (0.6 – 10.7)      | 1.6 (0.8 – 3.3)        | <0.001* |
| Total cholesterol (mmol l⁻¹) | 4.8 (3.5 – 7.2)   | 5.4 (3.0 – 8.0)       | 5.0 (3.6 – 7.2)        | 0.021   |
| LDL cholesterol (mmol l⁻¹) | 2.8 (1.6 – 4.5)     | 3.5 (1.7 – 5.6)       | 3.3 (1.9 – 4.9)        | 0.005*  |
| HDL cholesterol (mmol l⁻¹) | 1.3 (0.8 – 2.1)      | 1.2 (0.7 – 3.5)       | 1.1 (0.7 – 1.8)        | <0.001* |
| Fasting blood glucose (mmol l⁻¹) | 5.0 (4.3 – 7.1)   | 5.6 (4.7 – 7.0)       | 5.8 (4.4 – 14.7)       | 0.001*  |
| HbA1c (%)                 | 5.5 (4.6 – 5.8)       | 5.4 (4.7 – 6.5)       | 5.7 (4.8 – 9.5)        | 0.001*  |
| Free seminal L-carnitine (μg ml⁻¹) | 39.9 (8.6 – 168.3) | 52.9 (8.9 – 159.8)    | 44.4 (10.8 – 326.3)    | 0.302   |
| Total free seminal L-carnitine (μg) | 184.3 (33.7 – 551.8) | 138.8 (22.3 – 695.5)  | 181.5 (35.4 – 569.4)   | 0.898   |

*Missing/not reported values (n=1); **missing/not reported values (n=2). Variables are presented as median (range). BMI: body mass index; TG: triglyceride; LDL: low-density lipoprotein; HDL: high-density lipoprotein; HbA1c: glycated hemoglobin.
Sperm motility was not assessed on the semen samples of the participants recruited from the fertility clinic.

**Correlations between levels of free L-carnitine in seminal plasma and levels of sperm fatty acids**

Sperm fatty acid composition is shown in Table 2. The two main fatty acids were palmitic acid (median: 24.6 wt%) and DHA (median: 23.4 wt%). The amount of free L-carnitine in the seminal plasma correlated positively with levels of sperm palmitic acid ($P = 0.014$), DHA ($P = 0.007$), and total n-3 PUFAs ($P = 0.008$) and negatively with lignoceric acid ($P = 0.001$) and total n-6 PUFAs ($P = 0.012$) when adjusted for covariates. After adjustment for possible covariates, the relation between the amount of eicosenoic acid and total PUFAs in spermatozoa and free seminal L-carnitine levels did not maintain statistical significance. Unadjusted levels of sperm DHA distributed by tertiles of total free L-carnitine in seminal plasma are shown in Figure 1a. Compared with the first tertile, sperm DHA levels were higher in the second and third tertile of total free L-carnitine distribution in the seminal plasma ($P < 0.001$).

**Correlations between levels of free L-carnitine in seminal plasma, body mass, and semen parameter values**

Levels of free L-carnitine in the seminal plasma were not correlated with BMI (Table 3). Unadjusted distribution of BMI in tertiles of free total L-carnitine in the seminal plasma is shown in Figure 1b. The amount of free L-carnitine in the seminal plasma correlated positively with age ($P = 0.013$) and sexual abstinence time ($P = 0.007$) in the unadjusted model (Table 3). Free seminal L-carnitine correlated positively with sperm concentration ($P < 0.001$), total sperm count ($P < 0.001$), progressive motility ($P = 0.002$), and MSC ($P < 0.001$), and negatively with semen volume ($P = 0.003$), when adjusted for covariates.

**DISCUSSION**

As far as we know, the relationship between levels of free seminal L-carnitine and sperm fatty acid content in humans has not been previously investigated. The composition of sperm fatty acid is important for semen quality. We and others have shown that sperm DHA is positively associated with sperm concentration, total count, progressive motility, vitality, and normal morphology. In dog sperm, higher concentration of DHA is observed in cauda epididymis where mature spermatozoa are stored, than that in other epididymal segments. L-carnitine is also found at high levels in the epididymal fluid, and dietary supplementation with L-carnitine results in improved semen quality in infertile men, as well as in increased levels of total n-3 PUFAs and improved semen quality in the rooster. Here, we found that levels of free seminal L-carnitine correlated positively with the amount of total PUFAs, total n-3 PUFAs, palmitic acid, and DHA levels, and negatively with levels of total n-6 PUFAs and lignoceric acid in spermatozoa. Several of these sperm fatty acids have been previously shown to correlate with semen quality. Our results underline the importance of sperm fatty acid composition in humans and suggest a potential specific role of seminal L-carnitine in sperm maturation in the epididymis.

**Table 2: Associations between levels of free L-carnitine in seminal plasma (μg ml⁻¹) and levels of sperm fatty acids (n=128; wt%, of the total fatty acids analyzed by gas chromatography)**

| Variable                  | Median (range) | Unadjusted | Adjusted |
|---------------------------|----------------|------------|----------|
| Free seminal L-carnitine  | 44.4 (8.6 – 326.3) | 0.13 (0.134) | 0.01 (0.075) |
| Myristic acid C14:0        | 1.2 (0.5 – 2.9) | 0.13 (0.134) | 0.01 (0.075) |
| Palmitic acid C16:0        | 24.6 (12.6 – 30.0) | 0.25 (0.004) | 0.21 (0.014) |
| Stearic acid C18:0         | 9.5 (6.7 – 13.9) | 0.11 (0.226) | 0.03 (0.073) |
| Arachidic acid C20:0       | 0.64 (0.01 – 3.97) | 0.08 (0.357) | 0.05 (0.585) |
| Behenic acid C22:0         | 1.7 (0.1 – 3.7) | 0.17 (0.056) | 0.15 (0.091) |
| Lignoceric acid C24:0      | 1.2 (0.1 – 8.5) | <0.01 (0.001) | <0.01 (0.001) |
| SFAs total                 | 38.8 (29.7 – 50.2) | 0.56 (0.112) | 0.08 (0.414) |
| Palmitoleic acid C16:1 n-7  | 0.90 (0.04 – 3.27) | 0.38 (0.398) | 0.16 (0.162) |
| Oleic acid C18:1 n-9       | 10.01 (0.01 – 29.54) | 0.08 (0.089) | 0.13 (0.147) |
| Eicosenoic acid C20:1 n-9  | 0.4 (0.2 – 1.0) | 0.02 (0.002) | 0.04 (0.043) |
| Nervonic acid C24:1 n-9    | 1.7 (0.1 – 5.1) | 0.18 (0.048) | 0.20 (0.044) |
| MUFAs total                | 13.0 (3.4 – 34.1) | 0.19 (0.030) | 0.16 (0.061) |
| LA C18:2 n-6               | 4.0 (2.4 – 6.7) | 0.07 (0.420) | 0.09 (0.309) |
| ALA C18:3 n-3              | 0.07 (0.02 – 0.57) | 0.04 (0.656) | 0.07 (0.651) |
| GLA C18:3 n-6              | 0.03 (0.01 – 1.28) | 0.14 (0.105) | 0.15 (0.100) |
| EDA C20:2 n-6              | 0.6 (0.2 – 1.3) | 0.13 (0.146) | 0.10 (0.259) |
| DGLA C20:3 n-6             | 2.4 (0.9 – 8.2) | 0.04 (0.628) | 0.06 (0.563) |
| AA C20:4 n-6               | 2.6 (1.0 – 8.4) | 0.17 (0.058) | -0.20 (0.036) |
| EPA C20:5 n-3              | 0.07 (0.02 – 0.66) | 0.14 (0.24) | 0.21 (0.024) |
| DPA C22:5 n-6              | 1.2 (0.5 – 2.7) | 0.10 (0.265) | 0.01 (0.090) |
| DHA C22:6 n-3              | 23.4 (3.6 – 31.3) | 0.28 (0.002) | 0.23 (0.007) |
| PUFAs total                | 35.11 (11.7 – 43.1) | 0.25 (0.005) | 0.19 (0.022) |
| PUFAs n-3 total            | 25.1 (4.9 – 33.1) | 0.28 (0.002) | 0.23 (0.008) |
| PUFAs n-6 total            | 10.0 (5.6 – 16.2) | 0.08 (0.028) | -0.24 (0.012) |

*P<0.01 were considered statistically significant. Multiple linear regression analysis was used to determine associations between sperm fatty acids as dependent variables and free L-carnitine in seminal plasma as independent variable adjusted for age, sexual abstinence, and BMI. Free seminal L-carnitine and BMI were log-transformed. Log-transformed variable; *inverse-transformed variable. Variables are presented as median (range). LA: linoleic acid; ALA: alpha-LA; GLA: gamma-LA; DGLA: dihomo-gamma-LA; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; B: regression coefficient; CI: confidence interval; Wt%: weight percentage.
Table 3: Associations between levels of free seminal L-carnitine (μg ml⁻¹; n=128) and semen parameter values

| Variable                  | n  | Median (range) | Unadjusted | Adjusted | B (95% CI) | β    | P  |
|---------------------------|----|----------------|------------|----------|------------|------|----|
| Age (year)                | 128| 36 (22–61)     | 6.68 (1.41–11.95) | 0.22 | 0.013*     |      |    |
| BMI (kg m⁻²)              | 128| 28.4 (18.8–62.7)| 0.002 (−0.06–0.06) | 0.01 | 0.958      |      |    |
| Sexual abstinence (day)   | 125| 3 (0–7)        | 1.20 (0.33–2.08) | 0.24 | 0.007*     |      |    |
| Semen volume (ml)         | 128| 3.8 (1–10)     | −0.35 (−0.58–−0.11) | −0.25 | 0.004*    | −0.37 | −0.13 | 0.27 | 0.003*     |      |    |
| Sperm concentration x10⁶ ml⁻¹ | 128| 56 (1–350)    | 7.39 (5.62–9.17) | 0.59 | <0.001* | 6.71  | 4.85–8.57 | 0.54 | <0.001*    |      |    |
| Total sperm count x10⁶     | 128| 196 (6–1290)  | 11.56 (7.82–15.30) | 0.48 | <0.001* | 10.49 | 6.70–14.28 | 0.43 | <0.001*    |      |    |
| Progressive motility (%)  | 93 | 43 (1–76)     | 18.98 (5.63–32.30) | 0.28 | 0.006* | 20.46 | 7.69–33.24 | 0.31 | 0.002*     |      |    |
| Non-progressive motility (%) | 93 | 27 (2–54)   | −12.33 (−19.45–−5.21) | −0.34 | 0.001* | −11.53 | −19.30–−3.76 | −0.32 | 0.004*     |      |    |
| MSC x10⁶ ml⁻¹             | 93 | 49 (1–294)    | 5.66 (3.58–7.74) | 0.49 | <0.001* | 5.02  | 2.93–7.12   | 0.44 | <0.001*    |      |    |
| Vitality (%)              | 122| 87 (40–97)    | −0.42 (−0.81–−0.04) | −0.19 | 0.032 | −0.19  | −0.56–0.18  | −0.09 | 0.318      |      |    |
| Normal forms (%)          | 121| 3 (0–11)      | −0.16 (−0.59–0.26) | −0.07 | 0.450 | −0.09  | −0.53–0.35  | −0.04 | 0.697      |      |    |
| Head defects (%)          | 121| 97 (85–100)   | 0.80 (−1.19–2.79) | 0.07 | 0.428 | 0.36   | −1.67–2.40  | 0.03 | 0.724      |      |    |
| Neck-mid-piece defects (%) | 121| 25 (7–72)     | −0.52 (−1.18–−0.15) | −0.14 | 0.124 | −0.39  | −1.11–0.33  | −0.11 | 0.283      |      |    |
| Principal piece defects (%) | 121 | 13 (3–52) | −0.06 (−0.23–0.10) | −0.07 | 0.456 | −0.18  | −0.33–0.02  | −0.19 | 0.026      |      |    |
| DFI (%)                   | 99 | 15 (3–85)     | 0.14 (−0.02–0.31) | 0.17 | 0.090 | 0.03   | 0.13–0.18   | 0.03 | 0.747      |      |    |

*P<0.01 were considered statistically significant. MSC was calculated by multiplying the sperm concentration and the percentage of motile spermatozoa divided by 100. Multiple linear regression analysis was used to determine associations between semen parameter values as dependent variables and free L-carnitine in seminal plasma as independent variable adjusted for age, sexual abstinence, time to semen analysis and BMI. All variables were continuous except for time to semen analysis that was dichotomized (≤1 h or >1 h). Free seminal L-carnitine was log-transformed. Log-transformed variables; square-root transformed variables. Variables are presented as median (range). BMI: body mass index; MSC: motile sperm concentration; DFI: DNA fragmentation index; B: regression coefficient; CI: confidence interval; β: standardized beta.

L-carnitine is known to have a central role in cellular aerobic metabolism by transporting long-chain fatty acids across the mitochondrial membranes for beta-oxidation. Analysis of the sperm tail proteome showed that 24% of the proteins involved in metabolism and energy production consist of enzymes involved in lipid metabolism, including mitochondrial beta-oxidation and carnitine shuttle system. In spermatozoa of several species, oxidation of fatty acids can provide the energy required for motility. Accordingly, incubation of spermatozoa with etomoxir, an inhibitor of fatty acid metabolism and energy production consist of enzymes involved in lipid metabolism, including mitochondrial beta-oxidation and carnitine shuttle system. Furthermore, Banhani et al. reported a significant increase in sperm motility in vitro in human semen samples supplemented with L-carnitine that could result from the involvement of L-carnitine in the beta-oxidation of fatty acids.

We found that levels of free seminal L-carnitine correlated with levels of sperm fatty acids important for sperm function. The mechanism underlying this association remains unknown. However, we cannot rule out a causal relationship between sperm fatty acid composition and free seminal L-carnitine levels. During transit of spermatozoa through the epididymis, sperm maturation involves remodeling of sperm membrane phospholipids with increase in DHA levels and in the ratio of PUFA to SFAs in humans. A knockout study in mice points toward an involvement of group III secreted phospholipase A2 (Pla2g3) expressed in epididymal epithelium in the rearrangement of fatty acids in spermatozoa during maturation. In Pla2g3−/− mice, the sperm phospholipid composition showed higher SFA and lower n-3 PUFA levels compared to wild type mice, and sperm from Pla2g3−/− had impaired oocyte fertilization ability. Because of the changing fatty acid composition during maturation, fatty acids released from the spermatozoa membrane could function as signaling molecules in the epididymal epithelium to increase carnitine transport mediated by OCTN2 and OCTN3. Accordingly, palmitic acid upregulated the expression of OCTN2 and OCTN3 genes in the TM4 murine Sertoli cell line. In addition, both human and animal OCTN2 genes are regulated by the fatty acid-activated nuclear receptor, peroxisome proliferator-activated receptor alpha (PPARα), in hepatoma and kidney cell lines. Our results showed a positive association between fatty acids important for sperm function and free seminal L-carnitine levels. This could be explained by a release of fatty acids from spermatozoa that can regulate L-carnitine transport from the bloodstream into the epididymal lumen.

In our study and as shown by others, levels of free L-carnitine in the seminal plasma were correlated positively with sperm concentration, total count, and progressive motility and negatively with semen volume. In addition, we observed a positive relation between MSC and seminal L-carnitine levels. No correlation between free seminal L-carnitine and DFI was observed in our study, although supplementation with a combined formulation containing L-carnitine has previously been shown to reduce sperm DNA fragmentation. In infertile men, administration of L-carnitine increased both sperm motility and concentration. The total oxyradical scavenging capacity of semen increased after L-carnitine supplementation and rats that received L-carnitine intraperitoneally during testicular...
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Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.

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 gamma-irradiation showed a reduction in germ cell apoptosis. Thus, L-carnitine could still have a positive effect on sperm concentration and total sperm count by its antioxidant and anti-apoptotic effects preserving membrane and DNA integrity of spermatozoa.

We have previously suggested that high BMI may be involved in the mechanism underlying reduced semen quality observed in obese men. Since L-carnitine is crucial for normal lipid metabolism, we investigated if there was a relation between free seminal L-carnitine levels and BMI but found no association. We have not found other studies that have investigated seminal L-carnitine in overweight or obese persons. Studies describing blood serum L-carnitine levels in relation to BMI show conflicting results and are difficult to be compared with ours, as we did not measure L-carnitine in blood samples.

Strengths of this study are the broad BMI range of the study population and the fact that all semen samples were analyzed in the same laboratory according to the WHO standards. Moreover, none of the participants reported having consumed dietary supplements containing L-carnitine. Limitations of this study are that only levels of free, not total or acetylated, L-carnitine were measured in seminal plasma and that no measurement of L-carnitine was performed in spermatozoa. Detailed analysis of the levels of these molecules could give more insights regarding the relationship between seminal L-carnitine and sperm fatty acids. In addition, seminal fatty acid content was not measured. The seminal fatty acid composition might have given an indication of the fatty acids of the epididymal epithelium. Another limitation of this study is that not all the semen samples were analyzed within 1 h after ejaculation.

It is worth noting that 18.8% of our participants were recruited at a fertility clinic. By using female infertility and not semen quality as inclusion criteria, we hypothesized that these men would reflect the general population and align with the rest of our study population. This was supported by our subanalysis. Significant differences between the two subgroups were seen in the length of sexual abstinence time and in normal sperm morphology. It is important to note that participants did not report their diet in this study and an influence of diet on the fatty acids’ composition of spermatozoa and free seminal L-carnitine cannot be excluded.

In this study, we found that levels of free seminal L-carnitine positively correlated with the amount of palmitic acid, DHA, and total n-3 PUFA of spermatozoa. Free seminal L-carnitine levels were not associated with BMI, indicating that reduced semen quality in obesity is independent of L-carnitine levels. Our findings are in line with the notion that the epididymis plays an active part in sperm maturation and that beta-oxidation might provide for energy production in mature spermatozoa. Further studies with causal research design are needed to investigate if free seminal L-carnitine may affect the fatty acid composition of the spermatozoa and if seminal L-carnitine is involved in energy production via fatty acids oxidation and oxidative phosphorylation in spermatozoa.

AUTHOR CONTRIBUTIONS
Conception of the study was initiated by MI. MI, OW, MHS, and TBH contributed to the study design. OW, MI, and JMA contributed substantially to the data collection. MI performed the statistical analysis. MI and OW drafted the manuscript. All authors contributed to interpretation of the data and critical revision of content. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declared no competing interests.
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Supplementary Table 1: Overview of sperm fatty acids included in the dataset

| Saturated fatty acids | Monounsaturated fatty acids | Polyunsaturated fatty acids |
|-----------------------|-----------------------------|-----------------------------|
| Trivial name          | Symbol                      | Trivial name                | Symbol                      | Trivial name | Symbol |
| Myristic acid         | C14:0                       | Palmitoleic acid            | C16:1 n-7                   | LA           | C18:2 n-6                  |
| Palmitic acid         | C16:0                       | Oleic acid                  | C18:1 n-9                   | ALA          | C18:3 n-3                  |
| Stearic acid          | C18:0                       | Eicosenoic acid             | C20:1 n-9                   | GLA          | C18:3 n-6                  |
| Arachidic acid        | C20:0                       | Nervonic acid               | C24:1 n-9                   | EDA          | C20:2 n-6                  |
| Behenic acid          | C22:0                       |                                |                             | DGLA         | C20:3 n-6                  |
| Lignoceric acid       | C24:0                       |                                |                             | AA           | C20:4 n-6                  |

LA: linolenic acid; ALA: alpha-LA; GLA: gamma-LA; DGLA: dihomo-gamma-LA; EDA: Eicosadienoic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid

Supplementary Table 2: Comparison of free seminal L-carnitine levels between samples delivered within one hour after ejaculation or later

| Variable                          | ≤1 h after ejaculation | >1 h after ejaculation | P       |
|-----------------------------------|------------------------|------------------------|---------|
| Free seminal L-carnitine (μg mL⁻¹) | 45.7 (8.6–326.3)       | 36.4 (11.7–134.3)      | 0.204   |

P<0.01 were considered statistically significant; Mann–Whitney U test was performed to compare the groups; Variables are presented as median with minimum and maximum values in brackets.

Supplementary Table 3: Comparison of free seminal L-carnitine levels and semen parameter values between participants from the general population and from a fertility clinic

| Variable                          | General population | Fertility clinic** | P       |
|-----------------------------------|--------------------|-------------------|---------|
| Free seminal L-carnitine (μg mL⁻¹) | 44.2 (8.6–326.3)   | 41.4 (11.7–146.8) | 0.475   |
| BMI (kg m⁻²)                      | 29.7 (18.8–62.7)   | 24 (20.7–27.1)    | <0.001* |
| Age (years)                       | 35 (22–61)         | 38 (35–51)        | 0.142   |
| Sexual abstinence (days)*         | 3 (0–7)            | 3 (1–7)           | 0.004*  |
| Semen volume (mL)                 | 4 (1–10)           | 3 (2–6)           | 0.132   |
| Sperm concentration (10⁶ mL⁻¹)    | 62 (3–350)         | 42 (1–222)        | 0.388   |
| Total sperm count (10⁹)           | 213 (6–1290)       | 146 (7–867)       | 0.241   |
| Vitality (%)                      | 87 (40–97)         | 88 (63–96)        | 0.726   |
| Normal forms (%)                  | 3 (0–10)           | 6 (2–11)          | 0.001*  |

*P<0.01 were considered statistically significant; Mann–Whitney U test was performed to examine the differences in the variables between groups; **men from couples where female factor infertility was an inclusion criterion; not semen quality; Variables are presented as median with minimum and maximum values in brackets. BMI: body mass index

Supplementary Table 4: Comparison of free seminal L-carnitine levels and semen parameter values between participants from the fertility clinic and from a subgroup of men from the general population with comparable body mass index (≤27 kg m⁻²)

| Variable                          | General population | Fertility clinic** | P       |
|-----------------------------------|--------------------|-------------------|---------|
| Free seminal L-carnitine (μg mL⁻¹) | 42.3 (8.6–168.3)   | 41.4 (11.7–146.8) | 0.961   |
| BMI (kg m⁻²)                      | 23.7 (18.8–27.4)   | 24 (20.7–27.1)    | 0.599   |
| Age (years)                       | 29 (22–51)         | 38 (35–51)        | <0.001* |
| Sexual abstinence (days)*         | 4 (0–7)            | 3 (1–7)           | 0.008*  |
| Semen volume (mL)                 | 5 (2–8)            | 3 (2–6)           | 0.001*  |
| Sperm concentration (10⁶ mL⁻¹)    | 70 (5–187)         | 42 (1–222)        | 0.210   |
| Total sperm count (10⁹)           | 345 (14–808)       | 146 (7–867)       | 0.021   |
| Vitality (%)                      | 90 (75–97)         | 88 (63–96)        | 0.119   |
| Normal forms (%)                  | 5 (0–10)           | 6 (2–11)          | 0.238   |

*P<0.01 were considered statistically significant; Mann–Whitney U test was performed to examine the differences in the variables between groups; **men from couples where female factor infertility was an inclusion criterion; not semen quality; Variables are presented as median with minimum and maximum values in brackets. BMI: body mass index