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Lipid concentrations and semen quality: the LIFE study

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SUMMARY

The decline in sperm count rates over the last 50 years appears to parallel the rising prevalence of obesity. As lipid levels are strongly associated with obesity, high lipids levels or hyperlipidaemia may thus play an important role in the decline in fertility in addition to other environmental or lifestyle factors. The objective of this population based cohort study was to evaluate the association between men's serum lipid concentrations and semen quality parameters among 501 male partners of couples desiring pregnancy and discontinuing contraception. Each participant provided prospectively up to two semen samples (94% of men provided one or more semen samples, and 77% of men provided a second sample approximately 1 month later). Linear mixed effects models were used to estimate the associations between baseline lipid concentrations and semen quality parameters, adjusted for age, body mass index and race. We found that higher levels of serum total cholesterol, free cholesterol and phospholipids were associated with a significantly lower percentage of spermatozoa with intact acrosome and smaller sperm head area and perimeter. Our results suggest that lipid concentrations may affect semen parameters, specifically sperm head morphology, highlighting the importance of cholesterol and lipid homeostasis for male fecundity.

INTRODUCTION

There is great controversy over the decline in male fertility and semen quality around the globe (Joffe, 2000; Jensen *et al.*, 2002; Sallmen *et al.*, 2005; Joffe *et al.*, 2006). Some data have shown that sperm count rates have declined over the last 50 years with current reference ranges of approximately 15 million/mL (Fisch *et al.*, 1996; Swan *et al.*, 1997, 2000; Bonde *et al.*, 1998; Swan & Elkin, 1999; Cooper *et al.*, 2010; Rolland *et al.*, 2013). However, other data are equivocal with regard to global semen quality parameters (Fisch *et al.*, 1996). This potential decline in semen parameters appears to parallel the rising prevalence of obesity. The prevalence of obesity has also increased over the same time period. In fact, the prevalence of obesity (defined as a body mass index ≥30) has increased from 12.8% in 1976–1980 to 35.5% by 2009–2010 (Flegal *et al.*, 1998; Ogden *et al.*, 2012). A global hyperlipidaemia epidemic paralleled obesity rates during this time period, although of late hyperlipidaemia rates are declining in some developed countries (Carroll, 2012). As lipids levels are strongly associated with obesity, high lipids levels or hyperlipidaemia may thus play an important role in the decline in fertility in addition to other environmental or lifestyle factors (Charlton, 2009; Crawford *et al.*, 2010).

A potential link between lipids and human fecundity is plausible given that cholesterol is the main substrate for steroid synthesis, and also has been shown to play a crucial role in steroidogenesis and associated downstream effects including spermatogenesis (Gwynne & Strauss, 1982). Moreover, there is ample evidence from animal studies linking cholesterolaemia, steroidogenesis and male fertility. Hypercholesterolaemia in rabbits lowers Leydig and sertoli cell secretory function and lowers serum testosterone response to hCG (Yamamoto *et al.*, 1999). Cholesterol fed rats and rabbits have reductions in spermatid cell population, seminiferous tubules and Leydig's cell nuclear dimensions (Gupta & Dixit, 1988). Even mild hyperlipidaemia induced by cholesterol feeding significantly reduced sperm motility and density in the cauda epididymides and testis

(Purohit & Daradka, 1999; Bataineh & Nusier, 2005). However, there are a lack of population level studies evaluating lipid concentrations and semen quality among men of couples seeking pregnancy without fertility treatment.

The purpose of this study is to evaluate the link between lipid concentrations and semen quality parameters independent of obesity in a population based prospective cohort study. We designed the recently completed Longitudinal Investigation of Fertility and the Environment (LIFE) Study to address environmental influences on human fecundity. These hypotheses are of great interest as there is a need to identify modifiable risk factors to improve male fecundity, particularly at the population level.

METHODS

Design and study population

The LIFE Study was a prospective cohort study designed to investigate environmental influences on human fecundity and fertility, and its design and methods have been described previously in detail (Buck Louis *et al.*, 2011). In brief, 501 male partners of couples discontinuing contraception for the purposes of becoming pregnant were recruited from 16 counties in Michigan and Texas from 2005–2009 using sampling frameworks tailored for each State allowing for the identification of couples planning pregnancy in the near future. Eligible men were aged 18–51 years in a committed relationship; were able to communicate in English or Spanish; and were not surgically or medically sterile. Full human subjects' approval was granted prior to obtaining informed consent from all participants.

Data collection

Upon enrolment, in-person interviews were conducted with each male partner to ascertain health, demographic and reproductive histories, as well as physical activity, and medication and supplement use (including lipid lowering drugs use). All data and biospecimens were collected in the home, and baseline interviews were followed by a standardized anthropometric assessment for determination of body mass index (BMI) as conducted by research nurses (Lohman *et al.*, 1988). The research nurse obtained non-fasting blood (~2 mL) for quantification of serum lipids. Samples were transported on ice to the site laboratories for processing where they were centrifuged for 15 min after approximately 2 h and aliquoted according to the protocol. Samples were frozen at −20° or colder until shipment on ice to the CDC laboratory for analysis of serum lipids.

Laboratory analysis of serum lipids

All assays were completed using a Hitachi Model 912 clinical analyser at the Centres for Disease Control and Prevention Environmental Health Laboratory. Total cholesterol was analysed with the Roche Cholesterol/HP method (Roche Diagnostics, Indianapolis, IN, USA), an enzymatic colorimetric determination using cholesterol esterase and cholesterol oxidase. Free cholesterol used the Wako Free Cholesterol C method (Wako Chemicals USA, Inc., Richmond, VA, USA), an enzymatic colorimetric assay which uses cholesterol oxidase and peroxidase but omits cholesterol esterase. Triglycerides were analysed with the Roche Triglycerides/GPO method without blanking, and phospholipids were measured by using the Wako Phospholipids B enzymatic colorimetric method. The Wako phospholipids method uses phospholipase D and choline oxidase for the analysis, so it measures specifically the major choline-containing phospholipids including lecithin, lysolecithin and sphingomyelin (Takayama *et al.*, 1977). All analyses were performed according to the directions of each kit. All unknown samples were analysed concurrently with quality control (QC) materials with known concentrations. QC materials were evaluated using published QC rules (Caudill *et al.*, 2008). All reported results were from runs found to be in control by standard statistical methods. Overall, the coefficients of variation range between 3–9%.

Semen collection and analysis

A semen sample was obtained at baseline followed by a second sample approximately 1 month apart (to identify azoospermia) irrespective of couples' pregnancy status. Men collected semen samples through masturbation without the use of any lubricant following a recommended 2 days of abstinence using home collection kits (actual abstinence time: median 3 days, mean 4.12 days) (Royster *et al.*, 2000; Turner & Schrader, 2006). At collection, a glass capillary tube was placed into the semen, and each subject recorded the duration of abstinence, time of semen collection and any information regarding sample collection loss or spillage. Semen samples were shipped via Federal Express overnight to the study's andrology laboratory for semen analysis. Semen delivered to a central andrology laboratory by overnight mail in insulated mailing kits have been successful in maintaining specimens for other studies (Royster *et al.*, 2000). Semen analysis after home collection has been reported to be reliable for all semen parameters with the exception of motility parameters (Stovall *et al.*, 1994; Morris *et al.*, 2003). A percentage of spermatozoa are alive after 24 h and a next day motility assessment still can be made and may provide important information on sperm function and survivability (Stovall *et al.*, 1994).

We quantified 35 semen parameters including five reflecting general characteristics (volume, straw distance, sperm concentration, total sperm count, hypo-osmotic swollen), eight motility measures, 12 morphometry measures, eight morphology measures and two sperm chromatin stability assay measures, using established laboratory protocols inclusive of ongoing quality assurance and control procedures (American Society of Andrology, 1996).

The initial evaluation of the sample was conducted when the sample arrived at the laboratory consisting of recording the temperature, turbidity, colour, liquefaction and volume of the semen. A temperature logging monitor (Maxim Integrated, San Jose, CA, USA) placed on the collection jar determined the temperatures to which the semen had been exposed since collection. Motility assessments, viability estimates, sperm concentrations, the preparation of slides and preservation of seminal plasma were conducted at this time. The glass capillary tube was evaluated on with a microscope to determine the distance the most progress spermatozoa had travelled. Semen volume was measured to the nearest 0.1 mL. An aliquot of semen was heated to 37 °C, placed in a 20 micron deep chamber, and sperm motility was assessed using the HTM-IVOS (Hamilton Thorne Biosciences, Beverly, MA) computer assisted semen analysis system (CASA). Sperm concentration was measured using the IVOS system and the IDENT™ stain (Zinaman *et al.*, 1996). Sperm viability was conducted by hypo-osmotic swelling (HOS assay) (Jeyendran *et al.*, 1984). The HOS assay determines the

structural and functional integrity of the cell membrane. Four microscope slides were prepared for sperm morphology and morphometry assessments. An aliquot of the whole semen was diluted in TNE buffer with glycerol and frozen for SCSA® analysis.

Sperm morphology was determined on a fixed, stained semen smear. Sperm morphology was classified by the two widely accepted classification systems; WHO 3rd Edition (traditional morphology) and WHO 5th Edition (strict morphology) (World Health Organization, 1992, 2010). The main difference between these classification systems is how they classify a 'borderline normal' spermatozoa. They are reported as normal with the traditional scheme and abnormal with the strict scheme (Rothmann *et al.*, 2013). Morphometric analyses were conducted HTM-IVOS CASA (Hamilton Thorne Biosciences, Beverly, MA, USA) and provided objective assessments of individual sperm head size and shape.

Progressive sperm motility was assessed by placing a flat capillary tube filled with hyaluronic acid placed into the fresh ejaculate and the progression of the vanguard spermatozoa was measured when the specimen arrived at the laboratory the next day (Turner & Schrader, 2006). SCSA® was assayed according to the methods of Evenson, as modified by Breitenstein. 100 μL of whole semen were diluted into 500 μL TNE buffer and keep frozen at −70 °C until analysis (Evenson *et al.*, 1991; Breitenstein *et al.*, 1994). The SCSA® procedure was conducted on a Coulter Epics Elite Flow Cytometer using the SCSA® program (SCSA diagnostics, Brookings, SD, USA).

The second sample did not have the full range of measurements taken compared with the first sample, due to budgetary constraints. In the second sample, the analysis was limited to exclusively measurement of volume, concentration and motility.

Statistical analysis

Five men were found to be azoospermic on both samples and were excluded from this analysis and were referred to clinical care. Descriptive analysis included the inspection of missing data and influential observations. The study cohort was assessed by select characteristics for male partners and quartiles of total cholesterol. Differences in characteristics between quartiles of total cholesterol were assessed using ANOVA and Fisher's exact test, where appropriate. Linear mixed effects models were used to estimate the associations between lipid concentrations and semen quality parameters. Mixed modelling techniques were used to incorporate the inter-sample correlations for semen quality endpoints measured in both samples (volume, concentration, motility and sperm head morphology). Models were adjusted for age (years), BMI (kg/m^2) and race (non-Hispanic White, non-Hispanic Black, Hispanic, other) (Ramlau-Hansen *et al.*, 2007; Keltz *et al.*, 2010; Colaci *et al.*, 2012).

Models were also adjusted for fish consumption (as a proxy for dietary intake), and use of lipid lowering drugs (any report on the daily journal of taking a lipid lowering drug during the study follow-up), serum cotinine and alcohol consumption although adjustment for these factors did not appreciably change the results and were not included in the final models for parsimony.

A sensitivity analysis was conducted to evaluate the possible impact of unmeasured confounding by diet (e.g., dietary fat intake) or environmental factors in estimating the association between serum lipids and semen quality. Dietary fat intake could be considered as a potential unmeasured confounder, as intake may be correlated with lipid concentrations and has been associated with reduced semen quality (Attaman *et al.*, 2012; Jensen *et al.*, 2013). We simulated such a variable for a range of correlations between the unmeasured factor and semen quality (from half to double the observed effect), and between the unmeasured factor and lipids ($\rho = 0$–0.9), to represent mild to severe potential confounding factor. We compared the results of our final adjusted models to sensitivity models adjusting for this simulated dietary factor.

Semen quality parameters were also considered with Box-Cox transformation to achieve normality assumption in the linear mixed models. Following Handelsman, (Evenson *et al.*, 2002) we found the optimal transformation parameter for each semen quality outcome, transformed the semen outcome, and reran the analyses to determine whether the obtained results were different from the primary analyses using untransformed semen outcomes. Specifically, for pre-specified lambda values ranging from 0 to 1, by 0.1, we applied the Box-Cox transformation formula. In this step, $\lambda = 0$ is for the logarithm transformation and $\lambda = 1$ is for no transformation. For each transformed semen quality outcome, we estimated the Shapiro-Wilks W statistic and the transformation corresponding to the largest W value is the optimal transformation.

RESULTS

The LIFE Study cohort comprised 501 male partners of couples attempting to become pregnant, among whom 347 (69%) achieved pregnancy. The mean average of male partners was (31.8 ± 4.9) years; the majority of men were college educated (68%) and self-identified as non-Hispanic white race (72%). Age was significantly associated with total cholesterol levels (Table 1). Lipids were quantified for 491 (98.0%) males, with no differences in various characteristics by availability of blood (data not shown). The main reason for the absence of serum was insufficient volume following the analysis of environmental chemicals. A higher percentage of Hispanic than non-Hispanic men was in the upper quartile of total cholesterol as compared with the other quartiles. BMI was not associated with total cholesterol levels among study participants.

Select lipid levels were associated with some but not all semen parameters. Specifically, a negative association was observed between total cholesterol and semen volume ($\beta = -0.004$, $p < 0.05$) and percent hypo-osmotic swollen ($\beta = -0.026$, $p < 0.05$) decrease after adjustment for age, BMI, study site and race, while free cholesterol and was negatively associated with percent sperm head with acrosome ($\beta = -0.043$, $p < 0.05$), sperm head area ($\beta = -0.008$, $p < 0.05$) and sperm head perimeter ($\beta = -0.005$, $p < 0.05$) (Table 2). Phospholipids also were negatively associated with sperm head area ($\beta = -0.002$, $p < 0.05$) and percent with acrosome ($\beta = -0.014$, $p < 0.05$).

Our sensitivity analysis supported the robustness of our results in the presence of a possible mild to moderate unmeasured confounder (Fig. 1). Still, we cannot entirely dismiss the possibility that an unmeasured dietary fat intake may account for the results. However, the unmeasured factor would need to exert twice the effect as the observed association with lipids, while being highly correlated with lipids ($\rho = 0.6$ or larger) to completely explain away the observed association between cholesterol levels and semen quality. A degree of confounding this

Table 1 Sociodemographic description of male partners at baseline by quartile of total serum cholesterol concentrations, LIFE Study, 2005–2009

Characteristics: N (%)	Males Total cholesterol (mg/dL)					
	Overall	Q1: <167 118 (24)	Q2: 167–190 127 (26)	Q3: 191–216 123 (25)	Q4: >216 123 (25)	*p*-Value
Age, years; mean (SD)	31.8 (4.9)	31.3 (4.4)	30.4 (4.5)	32.6 (5.4)	33.0 (4.9)	<0.001
BMI, kg/m^2; mean (SD)	29.8 (5.6)	29.1 (6.2)	30.0 (5.9)	29.5 (5.4)	30.4 (4.8)	0.34
Self-identified Race/Ethnicity						
Non-Hispanic White	384 (78)	100 (85)	99 (78)	101 (82)	84 (68)	
Non-Hispanic Black	23 (5)	6 (5)	6 (5)	3 (2)	8 (7)	0.01
Hispanic	45 (9)	2 (2)	10 (8)	14 (11)	19 (15)	
Other	39 (8)	10 (8)	12 (9)	5 (4)	12 (10)	
Education						
<High School/Equivalent	47 (10)	7 (6)	15 (12)	11 (9)	14 (11)	
Some College or Tech	141 (29)	32 (27)	29 (23)	44 (36)	36 (29)	0.22
College Graduate or Higher	303 (62)	79 (67)	83 (65)	68 (55)	73 (59)	
Baseline serum cotinine (ng/mL)						
No exposure (0, 9.99)	384 (78)	90 (77)	108 (85)	98 (80)	88 (72)	
Passive smoking (10, 99.99)	22 (4)	6 (5)	3 (2)	6 (5)	7 (6)	0.18
Active smoking (100, 299.99)	44 (9)	11 (9)	9 (7)	6 (5)	18 (15)	
Heavy smoking (300, 595.31)	40 (8)	10 (9)	7 (6)	13 (11)	10 (8)	
Baseline alcohol (per month)						
<Once per month	29 (7)	9 (10)	7 (6)	5 (5)	8 (8)	
Once per month	39 (9)	11 (12)	9 (8)	12 (11)	7 (7)	
2–3 days per month	80 (19)	15 (16)	22 (20)	22 (20)	21 (20)	
Once a week	104 (25)	23 (24)	28 (25)	26 (24)	27 (25)	0.98
2–3 times per week	127 (30)	29 (31)	32 (29)	33 (30)	33 (31)	
4–6 times per week	28 (7)	4 (4)	7 (6)	9 (8)	8 (8)	
Every day	12 (3)	3 (3)	5 (5)	2 (2)	2 (2)	
Baseline report of participation in a vigorous exercise program during the last 12 months	207 (42)	51 (43)	59 (46)	53 (43)	44 (36)	0.37
Any use of lipid lowering drugs during the study reported on the daily journals	18 (4)	8 (7)	5 (4)	3 (2)	2 (2)	0.16

extreme seems implausible. Sensitivity analysis was used to evaluate the effects under the Box-Cox family of transformations and results were similar (Table S1). Specifically, all except one of the primary significant findings were obtained in the sensitivity analysis. The sensitivity analysis also revealed five more significant findings, mostly in cholesterol with sperm head. We remain conservative with our reporting given that Box-Cox transformations are known to increase the type I error.

DISCUSSION

Overall, we observed that total and free cholesterol and phospholipid concentrations were negatively associated with several sperm head morphology parameters independently of BMI. Total cholesterol concentrations also were associated with reductions in semen volume and live/total count. These results highlight the role of serum lipids in male fecundity, and should be of concern given the rising prevalence of obesity and dyslipidaemia.

There are many stages in the development of spermatozoa where cholesterol and lipid homeostasis could potentially influence multiple semen quality parameters and ultimately the fertilization competency of spermatozoa (Keber *et al.*, 2013; Maqdasy *et al.*, 2013). The mammalian sperm plasma membrane primarily comprises phospholipids and cholesterol, which undergo several modifications upon removal from the testis. Cholesterol efflux from the sperm plasma membrane, referred to as capacitation, is responsible for changes in membrane fluidity necessary for the acrosome reaction and fertilization competence (Cross, 1998). In rodent models, dietary cholesterol and hypercholesterolaemia impair testicular function, (Gupta & Dixit, 1988) and in rabbit models sperm head membrane lipids are altered by hypercholesterolaemic diets (az-Fontdevila & Bustos-Obregon, 1993). Cholesterol and polyunsaturated acid enriched diets may also slow the kinetics of the acrosome reaction in rabbit spermatozoa and cause detrimental effects on spermatogenesis and overall sperm fertilizing capacity.

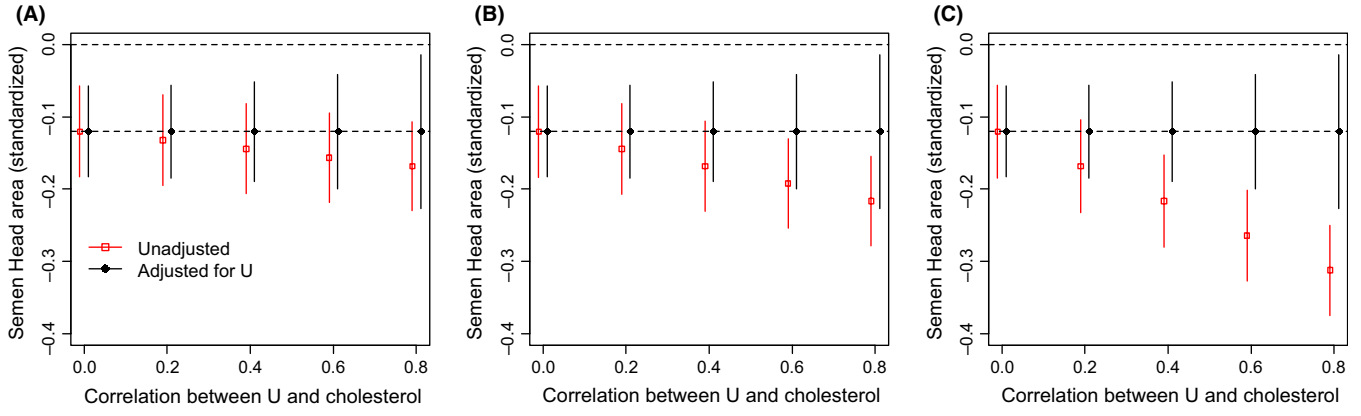
Our results indicate that higher levels of serum total cholesterol, free cholesterol and phospholipids are associated with a significantly lower percentage of sperm with intact acrosome, smaller sperm head area and perimeter. These results are consistent with spermatozoa that are further along the acrosome reaction and capacitation path. These results seemingly conflict with findings from animal studies that report higher levels of cholesterol being associated with a slower acrosome reaction (Cross, 1998). In very small cross-sectional human studies, total cholesterol has been shown to be higher and phospholipid classes lower, in the seminal plasma of azoospermic men compared with normo- and oligospermic men (Sebastian *et al.*, 1987). Two small studies among infertile men showed declines in semen quality associated with lipid abnormalities (Padron *et al.*, 1989; Ergün *et al.*, 2007). Hyperlipoproteinamic patients were also observed to have low semen quality (Padron *et al.*, 1989). Our data support these findings and represent the first and largest population based evidence for serum lipid concentrations and semen quality.

Table 2 Associations between male serum lipid concentrations and semen quality outcomes

	Semen quality outcome (unit)	Cholesterol β (SE)	Free cholesterol β (SE)	Phospholipids β (SE)	Triglycerides β (SE)	Total lipid β (SE)
Overall	Volume (mL)	**−0.004 (0.002)**	−0.010 (0.006)	−0.003 (0.002)	−0.001 (0.000)	**−0.001 (0.000)**
	Sperm concentration ($^a10^6$/mL)	−0.021 (0.068)	−0.041 (0.203)	−0.004 (0.065)	0.014 (0.017)	0.006 (0.012)
	Total sperm count ($^a10^6$/mL)	−0.292 (0.225)	−0.983 (0.673)	−0.164 (0.215)	−0.016 (0.057)	−0.028 (0.041)
	Distance spermatozoa travelled in straw (mm)	−0.002 (0.009)	0.013 (0.027)	0.002 (0.008)	0.002 (0.002)	0.001 (0.002)
	Hypo-osmotic swollen (%)	**−0.026 (0.012)**	−0.030 (0.035)	−0.011 (0.011)	0.003 (0.003)	−0.000 (0.002)
Motility	Amplitude of lateral head displacement (μm)	−0.001 (0.001)	−0.002 (0.003)	−0.001 (0.001)	0.000 (0.000)	0.000 (0.000)
	Avg. path velocity (μm/sec)	0.002 (0.010)	0.024 (0.029)	−0.005 (0.009)	0.001 (0.002)	0.001 (0.002)
	Beat cross frequency (Hz)	−0.006 (0.005)	−0.013 (0.015)	−0.006 (0.005)	0.000 (0.001)	−0.000 (0.001)
	Curvilinear velocity (μm/sec)	0.005 (0.017)	0.058 (0.051)	−0.005 (0.016)	0.005 (0.004)	0.003 (0.003)
	Linearity (%)	0.000 (0.008)	0.001 (0.025)	0.001 (0.008)	0.000 (0.002)	0.000 (0.001)
	Percent motility (%)	0.007 (0.015)	0.040 (0.046)	−0.006 (0.015)	0.004 (0.004)	0.002 (0.003)
	Straightness (%)	−0.000 (0.008)	0.006 (0.025)	−0.000 (0.008)	0.002 (0.002)	0.001 (0.002)
	Straight-line velocity (μm/sec)	0.003 (0.009)	0.024 (0.027)	−0.003 (0.009)	0.002 (0.002)	0.001 (0.002)
Morphometry	Sperm head with acrosome (%)	−0.017 (0.006)	**−0.043 (0.018)**	**−0.014 (0.006)**	−0.001 (0.001)	−0.002 (0.001)
	Elongation factor-width/length (%)	−0.002 (0.007)	0.007 (0.020)	−0.003 (0.006)	0.001 (0.002)	0.001 (0.001)
	Sperm head area (μm^2)	−0.003 (0.001)	**−0.008 (0.003)**	**−0.002 (0.001)**	−0.000 (0.000)	−0.000 (0.000)
	Sperm head length (μm)	−0.000 (0.000)	−0.002 (0.001)	−0.000 (0.000)	−0.000 (0.000)	−0.000 (0.000)
	Sperm head perimeter (μm)	−0.002 (0.001)	**−0.005 (0.002)**	−0.001 (0.001)	−0.000 (0.000)	−0.000 (0.000)
	Sperm head width (μm)	−0.000 (0.000)	−0.001 (0.001)	−0.000 (0.000)	0.000 (0.000)	−0.000 (0.000)
	Round (%)	0.000 (0.002)	−0.006 (0.007)	−0.002 (0.002)	−0.001 (0.001)	−0.001 (0.000)
	Pyriform (%)	0.001 (0.008)	−0.006 (0.024)	0.009 (0.008)	0.000 (0.002)	0.001 (0.001)
	Megalo head (%)	−0.002 (0.002)	−0.011 (0.007)	−0.003 (0.002)	−0.001 (0.001)	−0.001 (0.000)
	Micro head (%)	0.003 (0.002)	0.007 (0.005)	0.002 (0.002)	−0.000 (0.000)	0.000 (0.000)
	Coiled tail (%)	0.015 (0.015)	0.049 (0.043)	0.011 (0.014)	0.001 (0.004)	0.001 (0.003)
	Other tail abnormalities (%)	−0.007 (0.005)	−0.028 (0.016)	−0.004 (0.005)	−0.002 (0.001)	−0.002 (0.001)
Morphology	Amorphous (%)	0.016 (0.014)	0.019 (0.042)	0.013 (0.014)	−0.004 (0.004)	−0.001 (0.003)
	Bicephalic (%)	0.002 (0.003)	−0.003 (0.008)	−0.001 (0.003)	**−0.001 (0.001)**	−0.001 (0.000)
	Cytoplasmic droplet (%)	−0.009 (0.007)	−0.032 (0.020)	−0.007 (0.007)	−0.002 (0.002)	−0.001 (0.001)
	Immature sperm (#immature)	0.011 (0.025)	0.015 (0.073)	0.016 (0.023)	−0.001 (0.006)	0.001 (0.005)
	Neck & midpiece abnormal (%)	−0.001 (0.013)	−0.004 (0.039)	0.001 (0.013)	−0.003 (0.003)	−0.002 (0.002)
	Strict criteria (%)	−0.005 (0.013)	0.012 (0.039)	−0.003 (0.012)	0.004 (0.003)	0.002 (0.002)
	Taper (%)	−0.001 (0.004)	−0.004 (0.010)	0.000 (0.003)	−0.001 (0.001)	−0.000 (0.001)
	WHO normal (%)	−0.007 (0.016)	0.007 (0.048)	−0.005 (0.015)	0.005 (0.004)	0.002 (0.003)
Sperm chromatin stability	DNA fragmentation (%)	0.001 (0.013)	−0.039 (0.039)	−0.005 (0.012)	−0.004 (0.003)	−0.002 (0.002)
	High DNA stainability (%)	0.004 (0.007)	0.011 (0.020)	0.006 (0.006)	−0.001 (0.002)	−0.000 (0.001)

SE, standard error. Semen outcomes are not transformed. Mixed effects model (for volume, concentration, 24-hour motility and sperm head morphology) and linear regression model (for the others) were used, and adjusted for age (years), BMI (kg/m^2), study site (Texas/Michigan) and race (non-Hispanic White, non-Hispanic Black, Hispanic, other). Bolded: $p < 0.05$. ^{a}Next day semen analysis.

Figure 1 Sensitivity analyses of lipid concentrations (X) and semen quality (standardized sperm head area) unadjusted and adjusted for a hypothesized unmeasured confounding factor U (e.g., standardized dietary factor that could influence both cholesterol levels and semen quality). Simulations are data-driven based on results from models of male standardized total cholesterol (i.e., mean 0, standard derivation 1) levels on semen quality. b/a is the ratio of the strength of the association between the unmeasured factor and semen quality compared with the association between total cholesterol levels and semen quality (A: b/a = 0.5, B: b/a = 1, C: b/a = 2).



The relation between the lipid-associated changes in sperm morphology observed in our study and male fecundity are unknown, given the absence of research involving men not seeking clinical investigation. In a previous clinical study of couples undergoing IVF, smaller median sperm head area and greater uniformity of sperm head size was observed in men

without a history of an unassisted pregnancy (Aziz *et al.*, 1998). A similar statistically significant difference in sperm head area was observed in a previous examination of the predictive value of live sperm head morphometry for the achievement of pregnancy in vivo (Irvine *et al.*, 1994). We observed similar results in previous work using the LIFE study in that individual semen quality parameters, especially sperm head parameters, were positively and negatively significantly associated with time to pregnancy, although not after adjusting for couples' ages and BMI (Buck Louis *et al.*, 2014). Improvements in semen quality through lifestyle modifications to improve lipid concentrations may therefore improve overall couple fecundity.

There is less information regarding the direct associations of serum triglycerides with sperm parameters independent of hypercholesterolaemia. At least one study however has shown that increased triglycerides may have deleterious effects on spermatiogenesis (Ergün *et al.*, 2007). Increased serum VLDL, total triglycerides and testosterone levels were significantly correlated with decreased sperm motility in a group of infertile men. Similarly, negative correlations between total triglycerides and VLDL levels with multiple spermatozoa, lydig cell and seminiferous tubule parameters were documented in the goat (Monfared, 2013). The mechanism of these associations is not clear however one line of evidence indicates that hormone sensitive lipase (HSL) may play a key role. HSL is a multifunctional enzyme that liberates fatty acids from triglycerides and cholesteryl esters for energy production. *HSL* gene knockout mice appear normal but are sterile due to oligospermia (Osuga *et al.*, 2000). Any spermatozoa that were found in the epididymis of these mice were nonmotile. Furthermore, the epididymal epithelia cells were heavily vacuolated, presumable with lipid. Interestingly, steroidogenic tissues other than testes were not affected indicating that the disruption of the *HSL* gene caused direct effects on spermatogenesis rather than effects secondary to hormone insufficiency (Saltiel, 2000). As HSL catalyses the hydrolysis of cholesteryl esters and triglycerides it is therefore reasonable that the resulting free cholesterol and/or fatty acids are important for spermatogenesis. As previously discussed, the profound remodelling of sperm cholesterol during maturation may be related to this process. Fatty acids are not only energy rich molecules, they are also powerful signal molecules. Free fatty acids are agonists for peroxisome proliferation activated receptors (PPARs) via heterodimerization with the retinoid x receptors (RXRs) (Kliewer *et al.*, 1997). Interestingly, disruption of RXRs in mice leads to abnormal spermatogenesis (Kastner *et al.*, 1996).

If corroborated, our findings have both clinical and public health relevancy across men's lifespan. For example, monitoring serum lipids with appropriate lifestyle and/or pharmacological intervention may improve health and wellness across the lifespan. This paradigm would include the promotion and maintenance of reproductive health irrespective of pregnancy intentions, given growing recognition of the interrelatedness between male fecundity and later onset adult diseases (Skakkebaek *et al.*, 2001; Buck Louis *et al.*, 2013). Moreover if corroborated, our findings underscore the role of the clinicians in preconception care. With growing recognition of the importance of preconception guidance and care for women (http://www.cdc.gov/reproductivehealth/infertility/publichealth.htm), our findings underscore the importance of including male partners in such care or even targeting such guidance for men.

This study has several strengths, including a large number of male participants recruited irrespective of serum lipids or pregnancy outcome and for whom serum lipids were individually quantified. In addition, anthropometric assessments were conducted with all men allowing for valid and reliable BMI measurement. Our thorough sensitivity analysis demonstrated that our results were robust to unmeasured confounding due to diet or other environmental or lifestyle factors, lessening concerns regarding residual confounding. Although we exemplified dietary fat as a potential unmeasured factor, these results are generalizable to any other factor of the same magnitude. When interpreting the results, it is important to keep in mind that our findings are limited by reliance on a single non-fasting serum sample, no measurement of high/low density lipoprotein or standardized dietary assessment, and the cross-sectional nature of the lipids and semen quality parameter. However, the fasting status is not likely to be related to semen quality and, therefore, any introduced bias introduced would likely be non-differential and would only affect the precision of the estimates. Another important study limitation is the use of next day semen quality analysis, which is not ideal for time-sensitive endpoints such as motility and viability (Buck Louis *et al.*, 2014). We were able to globally assess the presence of motile spermatozoa at collection through the glass straw methods described above. However, we recognize that the lack of findings between cholesterol concentrations and motility may be a result of using the next day analysis. Although the variability in measurement is increased (reducing efficiency), there is no evidence to support that the use of the next day analysis introduces bias as the laboratory staff were blinded to the fecundity status of the male and their lipid levels. Moreover, there is no empirical evidence to support a systematic difference in the integrity of semen samples collected in the home by a couples' time-to-pregnancy. We recognize that the next day analysis is not suitable for clinical purposes, but is utilized here for large population-based studies. In addition, no differences were observed between various semen endpoints (excluding motility) between samples collected at home the night before compared with samples analysed within 1.5 h (Luben *et al.*, 2007; Olshan *et al.*, 2007). Study participation was not dependent upon serum lipids minimizing the selective recruitment of men with higher/lower lipid concentrations including for a range of lipid subcomponents. Our sensitivity analyses are not consistent with the presence of unmeasured or residual confounding from diet or other unmeasured factors, unless the effect was incredibly strong. We recognize the need for future work that times serum lipid quantification relative to sensitive window for spermatogenesis as we assumed that mean lipid concentrations were approximately constant over a short period of time (such as spermatogenesis). It is also important to recognize the exploratory nature of this study and the potential for spurious findings given the large number of comparisons being made. However, these findings are biologically plausible and should be replicated in other study populations.

In conclusion, our findings demonstrate that serum lipids may affect semen quality parameters, specifically sperm head morphology, highlighting the importance of cholesterol and lipid homeostasis for male fecundity. The exact mechanisms remain elusive, but effects on sperm head characteristics suggest a possible male mediated effect on couple fecundity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Association between male serum lipid concentrations and semen quality outcome with Box-Cox transformation.