INTRODUCTION

Two meta-analyses1,2 and several single-center studies3–7 have documented a downward trend in semen quality in Western countries. Some have also reported a concomitant downward trend in circulating testosterone levels among men.8–11 These downward trends have coincided with numerous changes in environmental and lifestyle factors that may directly contribute to diminished testicular function. Most notably, increased exposure to endocrine-disrupting chemicals,12–15 increased worldwide prevalence of obesity,16,17 and worsening diet quality in Western countries18–21 have been previously related to semen quality, suggesting that these may explain some of the downward trends in semen quality and testosterone levels.

Most of the published work about fat intake and markers of male testicular function have been focused on semen quality parameters.22–26 and only a few on reproductive hormones. In addition, studies examining the relation between fat consumption and male sex hormones have focused on middle-aged and older men,27–30 and to our knowledge, there are no data on the association between dietary fats and reproductive hormones in young men. Moreover, the potential effect of dietary fat intake on testicular volume has not been explored so far. To address these gaps, we examined the relations between dietary fats with serum reproductive hormones and testicular volumes among young healthy men in Spain.

MATERIALS AND METHODS

Study population

The Murcia Young Men’s Study (MYMS) was a cross-sectional study conducted between October 2010 and November 2011 in the Murcia Region of Spain. Healthy young university students born in Spain, aged 18 to 23 years, were eligible to join the study. Recruitment flyers were posted at university campuses to invite students to participate in this study. A total of 240 students contacted the study staff, 17 of whom...
were ineligible. Of the remaining 223 men, 215 completed a study visit and agreed to participate in the study. We further excluded 6 men who reported implausible total caloric intake leaving 209 men (97.2%) for the current analysis. With this sample size, we had 80% statistical power to detect differences of 35% standard deviations of the outcome measures between men in extreme quartiles of dietary factors. Men received a €50 gift card for their participation. During the study visit, men completed questionnaires on lifestyle, diet, and smoking, underwent a physical examination, and provided blood and semen samples. Written informed consent was obtained from all cases. The Research Ethics Committee of the University of Murcia approved this study.

**Physical examination and testicular volume assessment**

Body weight and height were measured using a digital scale (Tanita SC 330-S, London, UK). Body mass index (BMI) was calculated as weight in kilograms divided by square height in meters. The presence of varicocele or other scrotal abnormalities was also evaluated, and classified as grade I (only detected during Valsalva maneuver), grade II (palpable), or grade III (visible). Testicular volume was measured using a Prader orchidometer (Andrology Australia, Clayton, Victoria, Australia). Mean testicular volume was calculated as the mean of right and left testicular volumes. All physical examinations were performed by the same investigator (Jaime Mendiola) to minimize variability in study procedures.

**Semen quality assessment**

Men were asked to abstain from ejaculation for at least 48 h before sample collection but were not excluded if they failed to follow this instruction (n = 30). Abstinence time was recorded as the time between current and previous ejaculation as reported by the study subject. Men collected semen samples by masturbation at the clinic; no lubricants were used. Ejaculate volume was estimated by specimen weight, assuming a semen density of 1.0 g ml⁻¹. Sperm concentration was evaluated by hemocytometer (Improved Neubauer; Hauser Scientific, Inc., Horsham, PA, USA). Spermatozoa were classified as either motile or immotile according to the World Health Organization (WHO) criteria, and the percentage of motile spermatozoa (progressive + nonprogressive) was calculated. Smears for morphology were prepared, air-dried, fixed, Papanicolaou stained, and assessed using strict criteria. Total sperm count (volume × sperm concentration) was also calculated. The same specialist biologist carried out all the semen analyses. An external quality control on semen samples throughout the study period was carried out in collaboration with the University of Copenhagen’s Department of Growth and Reproduction. To assess interlaboratory variation in sperm concentration analysis, five sets of duplicate semen samples (600 ml each) were sent by mail during the study period from the University of Copenhagen’s Department of Growth and Reproduction to the Murcia Andrology Laboratory. The specimens were coded, undiluted fresh sperm samples from regular semen donors that were preserved by adding 10 ml of a 3 mol l⁻¹ sodium azide solution per 1 ml of the ejaculate after liquefaction. No systematic differences in the results were identified. The mean interexaminer coefficient of variation was 4.0%, ranging between 1.7% and 7.1%.

**Dietary assessment**

We used a validated 101-food item semi-quantitative food frequency questionnaire (FFQ) to assess the usual intake of foods and nutrients (available at: http://bibliodieta.umh.es/files/2011/07/CFA101.pdf). Men were asked to report how often, on average, they had consumed each food item over the past year. Serving sizes were specified for each food item in the FFQ. The questionnaire offered nine options for frequency of consumption for each food, ranging from never or less than once a month to 6 or more times per day. Nutrient values for each food in the questionnaire were obtained from food composition tables of the US Department of Agriculture and supplemented with Spanish sources. 35,36 *Trans* fatty acid intake was derived using published sources for Spanish foods. 37,38 We calculated nutrient intakes (saturated fatty acids [SFA], monounsaturated fatty acids [MFA], polyunsaturated fatty acids [PUFA], omega-3 [n3], and omega-6 [n6]) by multiplying the frequency of use for each food by the nutrient composition of the portion size specified on the FFQ and by addition across all foods to obtain a total nutrient intake for each individual. Intake of energy-bearing nutrients was adjusted for total energy intake using the multivariate nutrient density method while non-energy-bearing nutrients were adjusted using the nutrient residual method. 39

The reproducibility and validity of this FFQ is comparable to other widely used FFQs. 40–43 The average of correlation coefficients between nutrient intakes estimated using four one-week prospectively collected diet records and those estimated with the FFQ were 0.44 for validity and 0.40 for reproducibility. 44 This FFQ also showed satisfactory biochemical validity when compared to plasma levels of carotenoids and vitamin C. 45

**Reproductive hormones measurement**

Blood samples were drawn from participants’ cubital veins in the afternoon and centrifuged; the serum was separated, stored, and frozen at –80°C. Serum samples were then shipped to Copenhagen, Denmark, on dry ice and stored at –20°C until hormone analysis was performed at Rigshospitalet. The methods have been described previously. 45 Briefly, hormone assessments were performed simultaneously to reduce interlaboratory variations. Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone-binding globulin (SHBG) were determined using time-resolved immunofluorometric assays (DELFIA; PerkinElmer, Skovlund, Denmark). Intra- and inter-assay variations were <5% in each of the three assays. Serum total testosterone (TT) levels were determined using a time-resolved fluoroimmunoassay (DELFIA; PerkinElmer) with intra- and inter-assay variation of <8%. Estradiol (E2) was measured by radioimmunoassay (Pantex, Santa Monica, CA, USA) with an intraassay variation of <8% and an interassay variation of <13%. Inhibin B levels were determined by a specific two-sided enzyme immunoassay (Oxford Bio-Innovation Ltd., Bicester, UK) with intra- and inter-assay variation of 13% and 18%, respectively. When inhibin B was above approximately 100 pg ml⁻¹, the intraassay variation was <7% and the interassay variation was <6%. The majority of the men had levels above 100 pg ml⁻¹. Calculated free testosterone (cT) was determined using the equation of Vermeulen and colleagues assuming a fixed albumin of 43.8 g l⁻¹. 46

**Statistical analyses**

Because the reproductive hormones, fat intakes, and the men’s characteristics had a skewed distribution, the median and 5th and 95th percentiles were used to describe those variables. FSH and E2 concentrations were transformed using the natural log (ln) before analysis because they showed nonnormal distributions. Left and right testicular volumes were averaged, and the mean volume was used for analysis. Men were divided into quartiles of intake for total and major subtypes of dietary fat. Men with the lowest intake of each fat category
were considered as the reference group. To test for associations across quartiles of intake, Kruskal-Wallis tests were used for continuous variables, and χ²-tests for categorical variables. Linear regression was used to examine the association of each fatty acid category with testicular volume and reproductive hormone levels. Tests for linear trend were performed using the median values of fat categories in each quartile as a continuous variable and reproductive hormones as the response variable. We used analysis of covariance (ANCOVA) to calculate adjusted testicular volume and reproductive hormone levels for each quartile by relevant covariates. Regression coefficients for outcomes that were log-transformed for analysis (FSH and E2 serum levels) were exponentiated ("back-transformed") to allow the presentation of adjusted means in the scale variables that were originally measured. In addition, the presence of nonlinear associations was tested modeling fat intake as a continuous variable and modeling them as a linear and quadratic term. We considered that an association was present when we found a linear trend across quartiles <0.05.

Confounding was assessed using a hybrid method that combines previous knowledge using directed acyclic graphs (DAGs) and a statistical method on change in point estimate. The variables considered as potential confounders included factors previously related to semen quality or reproductive hormones in this or other studies, and factors associated with fat intake and reproductive outcomes in this study, regardless of whether they had been previously described as predictors of male reproductive health. Using this method, final models included terms for BMI (kg m⁻²), smoking (current smoker versus not current smoker), time to blood sampling (min), mean daily alcohol intake (g day⁻¹), and mean daily caffeine intake (mg day⁻¹). Mean daily intakes of micronutrients previously related to semen quality in this population (vitamin C, β-cryptoxanthin, lycopene, and β-carotene) were forced into the model. In order to adjust for total energy intake, terms for total calorie intake, intake of protein, and the remaining types of fat were included in the model, regardless of statistical significance, to allow the interpretation of the regression parameter of interest as the isocaloric substitution of a specific type of fat for the same amount of energy from carbohydrates. Statistical analyses were performed with the IBM Statistical Package for the Social Sciences 19.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

Almost all the participants were Caucasian (98%) with a median (interquartile range [IQR]) age of 20.4 (19.6, 21.4) years. Sixty-eight (33%) of the men were smokers and the median (IQR) BMI was 23.7 (21.8, 25.5). Four (1.9%) men had a history of cryptorchidism and 32 (15.3%) men had a left varicocele diagnosed during the examination. Men in the highest quartile of total fat intake had lower intake of carbohydrates, vitamin C, cryptoxanthin, and alcohol, compared with men in the lowest quartile (Table 1).

Table 2 shows the multivariate-adjusted associations between fatty acid intake and reproductive hormones. In these models, MFA intake was inversely related with blood levels of cFT, TT, and inhibin B (Ptrans < 0.05). There was a positive relation between intakes of PUFAs, particularly of omega-6, and LH concentrations. In addition, intake of trans fatty acids was associated with lower cFT and TT concentrations (Ptrans = 0.01 and 0.02, respectively). Relative to men in the lowest quartile of trans fatty acid intake, the adjusted difference (95% Confidence Interval [CI]) of TT blood levels for men in the 2nd, 3rd, and 4th quartiles were −0.3 (−3.0 to 2.5), −0.4 (−3.2 to 2.4), and −3.4 (−6.4 to −0.4) nmol L⁻¹, respectively.

We then evaluated the relation between fatty acid intake and testicular volume. Intake of omega-3 PUFAs was positively related to testicular volume while intakes of omega-6 PUFAs and trans fatty acids were inversely related to testicular volume (Figure 1). The adjusted median (95% CI) total testicular volumes (ml) for men in increasing quartiles of fatty acid intake were 20.7 (19.7, 21.7), 21.0 (20.1, 22.0), 20.9 (20.0, 21.9), and 22.9 (21.9, 23.9) for omega-3 fatty acids; 22.2 (21.1, 23.3), 21.9 (21.0, 22.8), 21.5 (20.6, 22.4), and 20.0 (18.9, 21.0) for omega-6 fatty acids; and 21.3 (20.3, 22.3), 22.0 (21.1, 22.9), 21.9 (20.9, 22.8), and 20.4 (19.5, 21.4) for trans fatty acids. Intake of other fatty acids was not related to testicular volume (data not shown). Further adjustment by physical activity did not modify the associations (data not shown).

DISCUSSION

We examined the cross-sectional associations of fat intake with reproductive hormone levels and testicular volume in a population of young healthy Spanish men. We found inverse associations of monounsaturated fat intake with cFT and TT and inhibin B concentrations. Intake of omega-6 PUFAs was positively associated with LH concentrations and inversely related to testicular volume. Similarly, intake of trans fatty acids was inversely related to cFT and TT levels and testicular volume. In addition, we found a positive association between intake of omega-3 PUFAs and testicular volume. The findings for trans fatty acids are of particular interest given our previous report of an association between trans fat intake and lower sperm count in these men and previous experimental work in rodents showing a deleterious effect of trans fats on testicular function supporting a causal interpretation of this association.

We found inverse associations of MFA intake with three reproductive hormones (inhibin B, cFT, and TT concentrations). In this population, monounsaturated fats are primarily consumed from animal products (27%: from red and processed meats and dairy products) and from olive oil (20%). Jensen and colleagues found a borderline positive association of monounsaturated fatty acid intake and morphologically normal sperm among Danish men from general population. Jensen’s finding could be interpreted as consistent with our results, suggesting that monounsaturated fat intake may impair male reproductive health. However, we have previously reported no association between monounsaturated fat intake and semen quality in this population and others have found no relation between monounsaturated fats and reproductive hormones in older men.
Nevertheless, intake of red and processed meats and of dairy foods has also been previously related to poor semen quality, \(^{33,34}\) suggesting that the observed relation in this study could reflect the relation with these foods rather than with monounsaturated fats \(\text{per se}\). Given the paucity of data on the relation between fat intake with reproductive hormone concentrations and testicular volume in men, additional work is necessary to evaluate these relations further.

We observed a positive association between intake of omega-6 PUFAs and LH levels coupled with an inverse relation between intake of these fats and testicular volume. Elevated LH levels are characteristic of primary Leydig cell failure. While none of the men in the study had abnormally high LH levels and all had a testicular volume within the normal range, the co-occurrence of higher LH levels and lower testicular volume among men with the highest intake of omega-6 PUFAs may suggest that these fats could negatively influence testicular function acting directly on the testis. As we did not observe a relation to testosterone, it might indicate a situation with a compensated state (i.e., unchanged testosterone on a background of increased stimulation with LH). We did not observe a relation between intake of omega-6 PUFAs with FSH (expected in the case of spermatogenic dysfunction) raising the possibility that the observed relation to testis volume could be due to chance. The literature on the relation between these fats and markers of testicular function is sparse. PUFA intake was unrelated to sex hormones among 696 British men aged 70 years and older\(^{44}\) and also in middle-aged US men.\(^{22}\) We also found that intake of omega-3 PUFAs was not associated with reproductive hormones, but it was positively related to testicular volume. Although there is little consistency in the relation between omega-3 PUFA intake and reproductive hormones among men,\(^{22,39}\) it seems that omega-3 consumption may have a positive effect on semen quality parameters, in agreement with our finding regarding testicular volume. Afiche and colleagues have recently found that fish intake, an important source of omega-3 fatty acids, was positively associated with sperm counts and morphologically normal sperm among men attending a fertility center, and that relation appeared to be mediated through intake of long-chain omega-3 fats.\(^{21}\) In agreement with these results, omega-3 fatty acid supplementation increased the total percentage of sperm with normal morphology among oligoasthenoteratospermic Iranian men,\(^{23}\) and Jensen et al.\(^{31}\) found a positive and borderline association between omega-3 fat intake and semen volume among the general population \((P_{\text{trend}} = 0.05)\). Moreover, experimental models also showed a positive effect of omega-3 PUFAs on male reproductive health.\(^{55,56}\)

We are not aware of previous reports between the relation of \(\text{trans}\) fat intake and testosterone levels and testicular volume. Nevertheless, our findings are in agreement with our previous report on the relation between intake of these fats and total sperm count.
in this study population.28 Specifically, we had previously reported that men in the highest quartile of intake had 37% lower total sperm count;29 these same men were also found to have 15% lower T levels and 4% lower testicular volume than men in the lowest quartile of intake. These findings are also in agreement with a study among fertility patients where sperm intake levels, a biomarker of rodent models of intake, were inversely related to sperm concentration.29 Moreover, rodent models of fat supplementation provide strong evidence supporting a causal interpretation of these relations. Supplementation of the diet of male mice with fats in the testis,31,40 decreased serum testosterone concentrations and sperm counts, and at the highest levels of supplementation, arrest of spermatogenesis and testicular degeneration.28,31,41 Findings from these rodent models closely parallel our findings in humans. However, given the scarcity of data in humans, it is important that these relations are further evaluated in different populations.

We cannot discount the limitations of our study. Reverse causation is possible due to the cross-sectional study. However, we selected young men with untested fertility and we did not communicate the results of the semen analyses or reproductive hormones to them, reducing the possibility of this bias. Second, as is the case in all observational epidemiologic studies, we cannot exclude the possibility of unmeasured confounding. Nevertheless, we adjusted for a large number of known and suspected confounders. Adjustment for these factors had a minimal impact on the results, suggesting that any unmeasured confounding is unlikely to have a major impact on the results. Based on the number of tested associations, we cannot completely rule out the possibility that unmeasured confounding is possible due to the cross-sectional study. However, we selected young men with untested fertility and we did not communicate the results of the semen analyses or reproductive hormones to them, reducing the possibility of this bias. Second, as is the case in all observational epidemiologic studies, we cannot exclude the possibility of unmeasured confounding. Nevertheless, we adjusted for a large number of known and suspected confounders. Adjustment for these factors had a minimal impact on the results, suggesting that any unmeasured confounding is unlikely to have a major impact on the results. Based on the number of tested associations, we cannot completely rule out the possibility that observed associations may result from chance. Third, misclassification of dietary intakes is possible as well. However, we used a previously
validated FFQ. Third, associations with testicular volume are more difficult to evaluate since little is known about how much this outcome could be influenced by environmental factors, such as diet, among adult men. Whether and to what extent testicular volume might change in response to modifiable environmental factors remains an open question. Interpretation of these findings since we used an orchidometer to assess testicular volume which limited the precision of the measurements. Fifth, only one serum sample was obtained for each man opening the possibility of error due to within-person variability in reproductive hormones. Nevertheless, previous work has shown that a single sample can be used to classify men’s reproductive hormone function. Sixth, the intra- and inter-assay variation for assessing inhibin B levels could be considered as high (13%-18%); however, the majority of the men had inhibin B levels above 100 pg ml$^{-1}$, which is translated to a lower intra- and inter-assay variation (<7%).

In summary, we found that omega-3 fatty acids may be positively associated with testicular function as indicated by testicular volume whereas intake of omega-6 fatty acids and trans fatty acids appear to be negatively related to it. Some of these findings, most notably those associated with testicular function as indicated by testicular volume translated to a lower intra- and inter-assay variation (<7%).

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**COMPETING INTERESTS**

The authors declared no competing interests.

**REFERENCES**


