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ORIGINAL ARTICLE

Male Health

Histologically proven hepatic steatosis associates with lower testosterone levels in men with obesity

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Men with obesity often present with low testosterone (T) and sex hormone-binding globulin (SHBG) levels. Several mechanisms for this have been proposed, but as SHBG is secreted by hepatocytes and sex steroids undergo hepatic metabolization, this study investigates whether severity and histological components of nonalcoholic fatty liver disease (NAFLD) are associated with sex steroid levels in obese men. This cross-sectional study included 80 obese men (age: 46 ± 11 years; body mass index: 42.2 ± 5.5 kg m^{-2}). Serum levels of total T and estradiol (E_2) were measured using liquid chromatography coupled with tandem mass spectroscopy (LC/MS-MS) and SHBG and gonadotropins by immunoassay. Liver biopsies were evaluated using Steatosis, Activity, and Fibrosis scoring. Participants with steatohepatitis had similar median (1st quartile–3rd quartile) total T levels (7.6 [5.0–11.0] nmol l^{-1} *vs* 8.2 [7.2–10.9] nmol l^{-1}; $P = 0.147$), lower calculated free T (cFT) levels (148.9 [122.9–188.8] pmol l^{-1} *vs* 199.5 [157.3–237.6] pmol l^{-1}; $P = 0.006$), and higher free E_2/T ratios (10.0 [6.4–13.9] x10^{-3} *vs* 7.1 [5.7–10.7] x10^{-3}; $P = 0.026$) compared to men with only nonalcoholic fatty liver. Among the histological components of NAFLD, only steatosis was independently associated with total T ($r_s = -0.331$, $P = 0.003$) and cFT levels ($r_s = -0.255$, $P = 0.025$). Obese men with steatohepatitis have even lower cFT levels compared to those without, an association mainly driven by grade of steatosis. Whether this reflects a subgroup of men with a more severe obesity-related phenotype or results from direct relations between hepatic steatosis and sex steroid metabolism needs further investigation.

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INTRODUCTION

Men with obesity regularly present with lower serum testosterone (T) and sometimes higher estradiol (E_2) levels compared to a healthy control population.[1–6] Although these lower total T levels are largely explained by lower sex hormone-binding globulin (SHBG) levels, calculated free T (cFT) levels also decrease with increasing obesity.[2,5–7] Remarkably, these changes are not accompanied by a rise in gonadotropin levels, suggesting central downregulation of the hypothalamic–pituitary–gonadal axis. Moreover, this disturbed sex steroid profile is (partially) restored when these men lose weight.[8–11] Adiposity-related mechanisms, such as an increase in aromatase activity and inhibitory effects of leptin on T production, have been suggested,[12–15] but the exact pathophysiologic mechanisms and clinical relevance of these hormonal changes remain unclear. However, as the prevalence of obesity keeps rising and sex steroids are involved in the regulation of muscle mass,[16] bone,[17] and adipose tissue,[18] there is a need to understand the causes and consequences of this disturbed sex steroid profile in men with obesity.

Obesity also often leads to nonalcoholic fatty liver disease (NAFLD), which covers a broad histological spectrum from simple steatosis to nonalcoholic steatohepatitis (NASH), and can progress into fibrosis, cirrhosis, and even hepatocellular carcinoma.[19] As sex steroids undergo hepatic metabolization and their serum levels depend in part on hepatic SHBG secretion, NAFLD could be related to the observed changes in sex steroid levels in men with obesity. Indeed, several studies reported differences in sex steroid levels between healthy controls and patients with NAFLD.[3,20–29] However, some of the results were contradictory and most of these studies did not use state-of-the-art mass spectrometry-based sex steroid assays, were not based on biopsy-proven NAFLD, or did not account for disease severity. Therefore, this study aims at investigating the relation between biopsy-proven NAFLD, focusing on overall severity and its histological components, and sex steroid serum levels in men with obesity.

PATIENTS AND METHODS

Study design and subjects

One cross-sectional study (registration No. B67020084018) recruited 71 men with obesity scheduled for gastric bypass surgery (GBS) at the Ghent University Hospital (Ghent, Belgium). These men met the national refund criteria for GBS because they had either a body mass index (BMI) >40 kg m^{-2} or a BMI >35 kg m^{-2} with at least one of the

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following comorbidities: type 2 diabetes (T2D), obstructive sleep apnea, and therapy-resistant arterial hypertension. Exclusion criteria were as follows: having malignancies, drinking more than 3 units alcohol per day, having other known liver pathologies than NAFLD, and having a recent diagnosis of hypo- or hyperthyroidism or a recent change in their medication. In total, seven patients were excluded from the analysis due to use of choriogonadotropin ($n = 1$), refusing liver biopsy ($n = 1$), no scored liver biopsy ($n = 4$), and no blood samples ($n = 1$) available.

In addition, 16 men with obesity were recruited in another study (registration No. B670201526667) which had the same inclusion and exclusion criteria. Three of them followed a conservative weight loss program and the other 13 subjects underwent GBS. In both studies, none of the subjects used Vitamin E supplements, thiazolidinediones, or SLGT2 inhibitors and two used glucagon-like peptide 1 (GLP1) analogs. During the previsit period, body weight was stable, and no subjects used a very low caloric diet.

Thus, a total of 80 men with obesity who underwent a liver biopsy were available for analysis. Finally, an age-matched control group consisted of 80 nonobese men participating in ongoing studies recruiting healthy men, *i.e.*, 69 men from the Siblings Osteoporosis Study (SIBLOS)[30] and 11 men from the European Network for the Investigation of Gender Incongruence (ENIGI) study (registration No. B67020097465).[31] All participants gave written informed consent to participate in these studies, which were approved by the Ethical Review Board of the Ghent University Hospital (Ghent, Belgium) and conducted according to the principles of the Declaration of Helsinki.

Anthropometrics and general characteristics

For all subjects, standing height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. Body weight was measured to the nearest 0.1 kg on a calibrated scale in light indoor clothing without shoes. Subsequently, BMI was calculated. Medication use was retrieved from the patients' records and double checked by questioning the patient. T2D was defined according to the American Diabetes Association criteria.[32]

Hormonal and biochemical measurements

Blood samples from patients undergoing GBS were collected prior to surgery, after overnight fasting. Blood samples from the three men with obesity adhering a weight loss program and from control subjects were retrieved during a clinical visit before 10 a.m. after overnight fasting. All blood samples were centrifuged, and serum was fractionated and stored at −80°C until further analysis.

Serum levels of fasting glucose were analyzed by the hexokinase method (COBAS, Roche Diagnostics, Mannheim, Germany). Insulin levels were determined with electrochemiluminescence using the immunoanalyzer COBAS e411 (Roche Diagnostics). Homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated with following formula: HOMA-IR = (fasting glucose [mmol l^{-1}] × fasting insulin [μU ml^{-1}])/22.5.[33] C-reactive protein (CRP) and triglycerides (TG) were routinely determined using standard laboratory assays (COBAS 8000 modular analyser series, Roche Diagnostics). Nonesterified fatty acids (NEFA) were determined with the standard enzymatic colorimetric method (P-modular, Roche Diagnostics).

Commercial immunoassays were used to determine follicle-stimulating hormone (FSH), luteinizing hormone (LH), and SHBG levels (Elecsys LH, FSH, and SHBG immunoassay, Roche Diagnostics). Total T and E_2 serum concentrations were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS; AB Sciex, Toronto, Canada). Total T concentrations were analyzed on an AB Sciex 5500 triple-quadrupole mass spectrometer as previously described.[34] The interassay coefficient of variation (CV) for T is 6.5% at 3 ng dl^{-1} (104 pmol l^{-1}) ($n = 30$) and the limit of quantification (LOQ) is 1 ng dl^{-1} (35 pmol l^{-1}). Total E_2 concentrations were analyzed using 2D-LC-MS/MS on an AB Sciex 5500 triple-quadrupole mass spectrometer (AB Sciex).[35] The assay characteristics were as follows: an LOQ of 1.03 pmol l^{-1} (CV <20%, $n \geq 6$) and an intra- and inter-assay CV of 9.8% at 4.15 pmol l^{-1} and 4.0% at 77.8 pmol l^{-1}, respectively. cFT serum levels were calculated by the formula of Vermeulen *et al.*[36] with albumin set as a constant at 4.3 g dl^{-1}. Calculated free E_2 (cFE2) concentrations were calculated from total serum E_2, SHBG, and albumin as described by Szulc *et al.*[37] The ratios of cFE_2 to cFT (cFE_2/cFT) and cFT to LH (cFT/LH) were calculated.

Hepatic histopathological analysis

Liver biopsies were performed in 80 men with obesity. Liver biopsies from the three patients following the conservative approach were taken, guided by ultrasonography using a biopsy gun with an 18-Gauge needle (Bard Magnum, Tempe, AZ, USA) after local anesthesia with 2% xylocaine. In the other obese participants, a liver biopsy was performed at the end of the GBS procedure, which was at least 5 mm × 5 mm and taken from the lateral edge of the left liver lobe. All biopsies were immediately fixed in formalin (buffered 4% paraformaldehyde solution, Klinipath, Leuven, Belgium) at room temperature for microscopic analysis. The formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin (H and E) and Sirius Red and scored by experienced pathologists (Prof. Marleen Praet and AH). Steatosis was assessed by the percentage of hepatocytes containing large- and medium-sized intracytoplasmic lipid droplets (but not foamy microvesicles), on a scale of 0–3 (0: steatosis <5%; 1: 5%≤ steatosis ≤33%; 2: 33%< steatosis ≤66%; and 3: steatosis >66%). Lobular inflammation was scored at ×20 magnification ranging from 0–3 (0: none; 1: <2 foci per ×20 field; 2: 2–4 foci per ×20 field; and 3: >4 foci per ×20 field). Hepatocellular ballooning was scored from 0–2 (0: none; 1: few; and 2: many) and fibrosis from 0–4 (0: none; 1: perisinusoidal or [peri]portal; 2: perisinusoidal and [peri]portal; and 3: bridging fibrosis; and 4: cirrhosis).[38] Based on this, the Steatosis, Activity, and Fibrosis (SAF) score could be used to categorize patients into three groups: those without NAFLD; those with nonalcoholic fatty liver (NAFL), which comprises patients with simple steatosis and steatosis with associated liver lesions such as minor inflammation or ballooning;[39] and those with NASH. All patients who received a diagnosis of NASH had >5% steatosis in hepatocytes and a grade of activity A ≥2.[40,41]

Statistical analyses

Data were evaluated for normality of distribution and if necessary, logarithmically transformed. Data are presented as mean ± standard deviation (s.d.) for normally distributed data and median (1st quartile–3rd quartile) for non-Gaussian distributed data. Differences in general characteristics and sex steroid levels between cases and controls were evaluated using a Mann–Whitney U test. Within the obese participants, Kruskal–Wallis tests with Mann–Whitney U *post hoc* tests for continuous variables and the Fisher's exact test for categorical variables were used for comparison between groups according to histological grading. Spearman's correlations were performed to investigate possible associations between liver parameters and sex steroid levels. Furthermore, a one-way analysis of covariance (ANCOVA) was conducted to determine a statistically significant

difference between the different steatosis levels and different categories according to the SAF score on total T and cFT levels and cFE_2/cFT ratios controlling for age, BMI, T2D status, or HOMA-IR. For ANCOVA, the dependent variable was logarithmically transformed, and for each ANCOVA, output residual plots were made and outliers were removed from analysis. Patients who used insulin ($n = 6$), GLP1 analogs ($n = 1$), or both ($n = 1$) were excluded from the analyses with HOMA-IR, TG, and NEFA. Histological subgroups with less than four patients were excluded from all analyses. Test results were considered statistically significant at $P < 0.05$. IBM SPSS statistics (version 25, Chicago, IL, USA) was used for all statistical analyses.

RESULTS

General characteristics and sex steroid levels of obese men and age-matched controls are shown in **Supplementary Table 1**. As expected, the obese men present with a less favorable sex steroid profile with lower LH, FSH, SHBG, total T, and cFT levels and higher cFE_2 levels and E_2/T and cFE_2/cFT ratios in comparison with the control group.

Among the 80 obese subjects, 3 men had no NAFLD, 30 men had NAFL, and 47 men had NASH according to SAF classification. Their general characteristics and sex steroid levels are shown in **Table 1**. Obese men with NASH were nonsignificantly older and had nearly 10% lower BMI ($P = 0.006$) compared to obese men with NAFL. The prevalence of T2D was higher in participants with NASH as compared to those with NAFL (48.9% *vs* 6.7%, $P < 0.001$). Further, obese men with NASH presented with lower cFT levels ($P = 0.006$), a higher cFE_2/cFT ratio ($P = 0.026$), and a lower cFT/LH ratio ($P = 0.043$) compared to those with only NAFL. These differences persisted after adjusting for possible confounders such as age, BMI, HOMA-IR, and/or T2D status (**Table 2**). In contrast, no differences in SHBG, (cF)E_2, or gonadotropin levels between both groups were observed.

Subsequently, sex steroid levels were analyzed as a function of grade of steatosis, ballooning, lobular inflammation, and fibrosis. Distribution of participants according to histopathological grading is given in **Table 3**. Obese men with steatosis score 2 and 3 had lower total T and cFT levels as compared to men with steatosis score 1 (**Figure 1a** and **1b**). Moreover, Spearman's correlations indicated significant correlations between steatosis score and total T ($r_s = -0.331$, $P = 0.003$) and cFT ($r_s = -0.255$, $P = 0.025$) levels (**Table 4**), and correcting for possible confounders such as age, BMI, HOMA-IR, and/or T2D status, did not affect these findings (**Table 5**). Evaluating participants according to lobular inflammation, ballooning, or fibrosis scores revealed nonsignificant trends in cFT levels ($r_s = -0.214$, $P = 0.059$; $r_s = -0.217$, $P = 0.053$; and $r_s = -0.191$, $P = 0.100$; respectively). Furthermore, no associations were found between any histological component and SHBG, (cF)E_2, and gonadotropins (all $P > 0.05$).

DISCUSSION

This is the first biopsy-based study investigating a possible relation between NAFLD severity and sex steroid levels within a male population with obesity. Specifically, in men with obesity, subjects with NASH have lower cFT levels and a higher cFE_2/cFT ratio as compared

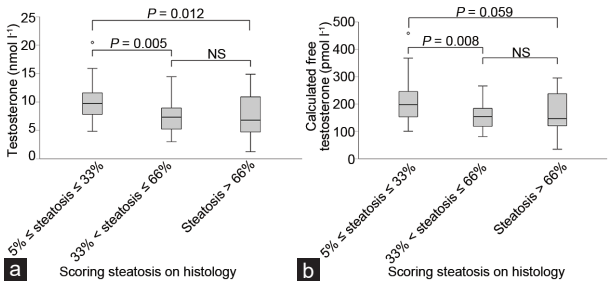

Figure 1: (**a**) The total T concentration for the different steatosis groups, a Kruskal–Wallis test was performed to indicate possible differences in total T levels between groups ($P = 0.007$). (**b**) The cFT concentration for the different steatosis groups, a Kruskal–Wallis test was performed to indicate possible differences in cFT levels between groups ($P = 0.022$). For both panels, steatosis group with less than 5% steatosis was removed from analysis and figures as only three patients were included. Statistically significant differences are indicated in the figures. NS: not significant; T: testosterone, cFT: calculated free testosterone.

Table 1: Descriptives of the obese cohort categorized according to the steatosis, activity, and fibrosis score

Characteristic	*NAFL (n=30)*	*NASH (n=47)*	*P*
Age (year), mean±s.d.	43±12	48±9	0.074
BMI (kg m^{-2}), mean±s.d.	44.5±6.1	40.7±4.8	0.006*
T2D, yes/no (*n*)	2/28	23/24	<0.001**
CRP (nmol l^{-1}), median (Q1–Q3)	41.4 (28.6–65.7)	27.6 (13.3–53.3)	0.097
TG (nmol l^{-1})[a], median (Q1–Q3)	2.05 (1.63–2.21)	2.02 (1.65–2.95)	0.208
NEFA (nmol l^{-1})[a], median (Q1–Q3)	0.564 (0.488–0.652)	0.647 (0.519–0.780)	0.251
HOMA-IR[a], median (Q1–Q3)	3.22 (1.97–4.12)	3.64 (2.07–7.43)	0.217
LH (U l^{-1}), median (Q1–Q3)	3.75 (3.01–4.14)	3.92 (2.52–5.10)	0.856
FSH (U l^{-1}), median (Q1–Q3)	3.48 (2.86–5.09)	3.54 (2.79–5.43)	0.901
SHBG (nmol l^{-1}), median (Q1–Q3)	23.6 (19.6–31.4)	25.2 (17.6–36.8)	0.500
E_2 (pmol l^{-1}), median (Q1–Q3)	73.8 (56.4–100.1)	84.7 (54.6–99.5)	0.832
cFE_2 (pmol l^{-1}), median (Q1–Q3)	1.48 (1.16–1.92)	1.5 (1.1–1.9)	0.920
T (nmol l^{-1}), median (Q1–Q3)	8.2 (7.2–10.9)	7.6 (5.0–11.0)	0.147
cFT (pmol l^{-1}), median (Q1–Q3)	199.5 (157.3–237.6)	148.9 (122.9–188.8)	0.006*
E2/T ($\times 10^{-3}$), median (Q1–Q3)	8.4 (6.6–12.0)	10.8 (7.5–15.8)	0.090
cFE_2/cFT ($\times 10^{-3}$), median (Q1–Q3)	7.1 (5.7–10.7)	10.0 (6.4–13.9)	0.026*
cFT/LH, median (Q1–Q3)	51.60 (40.89–77.06)	38.60 (29.75–60.27)	0.043*

Data from three patients without NAFLD are not shown separately. *P* values are shown for differences between the NAFL and NASH group and significant *P* were indicated with *$P<0.05$ and **$P<0.005$. [a]Eight patients were excluded from analysis due to use of insulin ($n=6$), GLP1 analogs ($n=1$), or both ($n=1$). GLP1: glucagon-like peptide 1; NAFLD: nonalcoholic fatty liver disease; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; BMI: body mass index; T2D: type 2 diabetes; CRP: C-reactive protein; TG: triglycerides; NEFA: nonesterified fatty acids; LH: luteinizing hormone; FSH: follicle-stimulating hormone; SHBG: sex hormone-binding globulin; E_2: estradiol; cFE_2: calculated free estradiol; T: testosterone; cFT: calculated free testosterone; HOMA-IR: homeostasis model assessment-estimated insulin resistance; Q1–Q3: 1st quartile–3rd quartile; s.d.: standard deviation

Table 2: Analysis of covariance evaluating whether calculated free testosterone levels and calculated free estradiol/calculated free testosterone ratios (dependent variables) are significantly associated with steatosis, activity, and fibrosis classification while controlling for different independent variables

Independent variables	*cFT*[a]			*cFE_2/cFT*[a]		
	F (df1, df2)	*P*	*n*	*F (df1, df2)*	*P*	*n*
SAF classification						
No independent variables	F(1,75)=6.875	0.011*	77	F(1,67)=5.678	0.020*	69
+ age	F(1,74)=4.473	0.038*	77	F(1,66)=4.862	0.031*	69
+ BMI	F(1,72)=10.713	0.002**	75	F(1,65)=19.136	<0.001**	68
+ T2D	F(1,73)=4.451	0.038*	76	F(1,65)=5.974	0.017*	68
+ age + BMI	F(1,71)=7.598	0.007*	75	F(1,64)=17.154	<0.001**	68
+ age + BMI + HOMA-IR	F(1,61)=4.967	0.029*	66	F(1,55)=16.809	<0.001**	60
+ age + BMI + T2D	F(1,70)=8.102	0.006*	75	F(1,62)=16.406	<0.001**	67

[a]Dependent variables. The dependent variables are log transformed. Significant *P* were indicated with *$P<0.05$ and **$P<0.005$. SAF: steatosis, activity, and fibrosis; BMI: body mass index; T2D: type 2 diabetes; HOMA-IR: homeostasis model assessment-estimated insulin resistance; cFT: calculated free testosterone; df: degree of freedom; cFE_2: calculated free estradiol

Table 3: Distribution of the subjects according to the histological components of nonalcoholic fatty liver disease

Histological components NAFLD	*n*
Steatosis	
Steatosis <5%	3
5%≤ steatosis ≤33%	31
33%< steatosis ≤66%	24
Steatosis >66%	22
Ballooning	
No balloon cells	29
Few balloon cells	32
Many balloon cells	19
Lobular inflammation	
No inflammatory foci (per ×20 field)	25
<2 inflammatory foci	44
2–4 inflammatory foci	10
>4 inflammatory foci	1
Fibrosis[a]	
No fibrosis	13
Perisinusoidal or (peri)portal fibrosis	40
Perisinusoidal and (peri)portal fibrosis	22
Bridging fibrosis	3
Cirrhosis	1

[a]One patient did not have a fibrosis score. NAFLD: nonalcoholic fatty liver disease

to those with only NAFL, independently from BMI and other possible confounders. In addition, steatosis seems to be the NAFLD component mainly associating with these lower T levels, while our results cannot reject or confirm the association of lobular inflammation, ballooning, and fibrosis with low T levels. Further, no histological components are related with E_2, gonadotropin, or SHBG levels.

Previous studies indeed showed lower T levels in patients with NAFLD as compared to healthy controls, which is in line with our results.[20–23,25,26,28] However, only two ultrasound-based studies investigated the relation between NAFLD severity and sex steroid levels and the results were equivocal. In agreement with our findings, Tian *et al.*[22] reported lower cFT levels in Chinese men with moderate-to-severe NAFLD compared to men with only mild NAFLD. In contrast, Shin *et al.*[29] did not find differences in measured free and total T levels between Korean men with mild or with moderate-to-severe NAFLD. Further, neither NAFLD severity nor its individual components were associated with (cF)E_2 or SHBG levels in our cohort, contradicting the results of Shin *et al.*[29] who found a negative and Tian *et al.*[22] who found a positive association between SHBG and NAFLD severity. Comparison with these studies, however, is difficult as they used ultrasound to asses NAFLD, did not use state-of-the-art mass spectroscopy to determine sex steroid levels, assessed different confounders, and were conducted in an Asian population or in men without a severe grade of obesity.

Our finding of even lower cFT levels in men with NASH within this group of men with obesity could merely indicate a subgroup of men with a more severe obesity-related phenotype, both in terms of hepatic manifestations and hypothalamic–pituitary–gonadal axis dysregulation. Supporting this hypothesis, participants with NASH presented with a 10% lower mean BMI. This finding is in line with a meta-analysis of Lu *et al.*[42] who concluded that there is no relation between grade of obesity and NASH in patients with NAFLD. However, there are also some physiologic mechanisms which could contribute to direct relations between NAFLD and sex steroid metabolism. First, T is a potent stimulator of hepatic lipase activity.[43] Hence, lower T exposure could lead to a decrease in lipase activity resulting in increasing hepatic fat accumulation. Second, the liver is the primary source for circulating SHBG, which plays an important role in the serum concentration of sex steroids.[42,43] However, SHBG levels were unrelated to NAFLD severity or grade of steatosis and can thus not explain the lower cFT levels in participants with NASH. Third, sex steroids undergo hepatic metabolization with, among others, hepatic P-450 enzymes being involved in the hydroxylation of T.[44] However, despite the lower cFT levels, participants with NASH presented with comparable gonadotropin levels and a lower cFT/LH ratio, suggesting hypothalamic–pituitary downregulation instead of increased metabolization as an underlying mechanism. Participants with NASH did present with a higher cFE_2/cFT ratio, which might suggest a relatively higher aromatization in these men. Aromatization mostly takes place in adipose tissue and is considered to contribute at least partially to the disturbed sex steroid profile in men with obesity.[15] However, BMI was lower in obese men with NASH and the association of T levels with steatosis was independent of BMI, which raises the question whether NASH as such might contribute to overall T aromatization and/or vice versa.

Taken together, our data confirm the importance of assessing cFT besides total T levels when evaluating gonadal status in obese men with NASH. Further, we confirm that there is no linear relation between grade of excess adiposity, NAFLD severity, and obesity-related low T. In contrast, participants with NASH had an even lower BMI than those with NAFL. The strength of this study lies in the use of LC-MS/MS for measuring sex steroid concentrations and biopsy-based NAFLD diagnosis, allowing evaluation of individual components of

Table 4: Correlations between individual nonalcoholic fatty liver disease components and sex steroids

Sex steroid	*Steatosis (n=77)*	*Ballooning (n=80)*	*Inflammation (n=79)*	*Fibrosis (n=75)*
LH (U l^{-1})	0.001 (0.938)	–0.037 (0.756)	0.062 (0.608)	0.075 (0.548)
FSH (U l^{-1})	0.005 (0.968)	–0.005 (0.964)	0.041 (0.735)	0.006 (0.960)
SHBG (nmol l^{-1})	–0.206 (0.073)	0.037 (0.745)	0.063 (0.579)	0.068 (0.564)
E_2 (pmol l^{-1})	–0.132 (0.278)	–0.060 (0.620)	0.039 (0.747)	0.043 (0.735)
cFE_2 (pmol l^{-1})	–0.098 (0.424)	–0.058 (0.632)	0.000 (0.999)	0.005 (0.967)
T (nmol l^{-1})	–0.331 (0.003**)	–0.119 (0.292)	–0.096 (0.400)	–0.117 (0.316)
cFT (pmol l^{-1})	–0.255 (0.025*)	–0.217 (0.053)	–0.214 (0.059)	–0.191 (0.100)
E_2/T	0.189 (0.120)	0.108 (0.371)	0.118 (0.329)	0.148 (0.235)
cFE_2/cFT	0.145 (0.235)	0.126 (0.295)	0.186 (0.122)	0.200 (0.107)
cFT/LH	–0.192 (0.114)	–0.197 (0.100)	–0.207 (0.085)	–0.177 (0.156)

Data are represented as r_s (*P*). For analysis of steatosis, subjects with a score 0; of lobular inflammation, subjects with a score 3; and of fibrosis, subjects with score 3 and 4; were excluded because of the low number of subjects per group (n <4). One patient did not have a fibrosis score, so could not be included in the analysis. Significant *P* were indicated with *P<0.05 and **P<0.005. NAFLD: nonalcoholic fatty liver disease; LH: luteinizing hormone; FSH: follicle-stimulating hormone; SHBG: sex hormone-binding globulin; E_2: estradiol; cFE_2: calculated free estradiol; T: testosterone; cFT: calculated free testosterone

Table 5: Analysis of covariance evaluating whether total testosterone and calculated free testosterone levels (dependent variables) are significantly associated with steatosis grade while controlling for different independent variables

Independent variables	*T*			*cFT*		
	F(df1, df2)	*P*	*n*	*F(df1, df2)*	*P*	*n*
Steatosis score						
No independent variables	F(2,73)=5.561	0.006*	76	F(2,73)=4.880	0.010*	76
+ age	F(2,72)=5.258	0.007*	76	F(2,72)=3.614	0.032*	76
+ BMI	F(2,71)=5.241	0.008*	75	F(2,71)=4.380	0.016*	75
+ T2D	F(2,72)=4.832	0.011*	76	F(2,72)=3.982	0.023*	76
+ age + BMI	F(2,70)=4.800	0.011*	75	F(2,70)=3.047	0.054	75
+ age + BMI + HOMA-IR	F(2,59)=5.519	0.006*	65	F(2,59)=2.890	0.063	65
+ age + BMI + T2D	F(2,69)=4.417	0.016*	75	F(2,69)=2.939	0.060	75

[a]Dependent variables. The dependent variables, T and cFT, are log transformed. Significant *P* values were indicated with *P<0.05. BMI: body mass index; T2D: type 2 diabetes; HOMA-IR: homeostasis model assessment-estimated insulin resistance; T: testosterone; cFT: calculated free testosterone; df: degree of freedom; s.d.: standard deviation

NAFLD. Until now, it is the largest study using liver biopsies in obese men to address this topic. Moreover, we strengthened our findings as we controlled for several possible confounders. It is limited by its cross-sectional design and unavailability of body adiposity distribution estimates. Moreover, no group of obese men without NAFLD was included; therefore, no conclusions concerning the influence of NAFLD alone on sex steroid levels could be made.

CONCLUSION

Among obese men with NAFLD, those with NASH have even lower cFT levels compared to those with only NAFL, an association mainly driven by grade of steatosis. Whether this finding reflects a subgroup of men with a more severe obesity-related phenotype or results from direct relations between hepatic steatosis and sex steroid metabolism needs further investigation.

AUTHOR CONTRIBUTIONS

FVdV helped with the concept and design of the study, study implementation, data collection and analysis, and drafted and revised the manuscript. MB helped with the concept and design of the study, study implementation, data collection, and revision of the manuscript. AH, GT, TF, and JMK helped with the concept and design of the study, data analysis, and revision of the manuscript. AG helped with the concept and design of the study, data collection, and revision of the manuscript. BL and YVN helped with the concept and design of the study, study implementation, data collection and analysis, and revision of the manuscript. All authors read and approved the final manuscript and agreed with the order of presentation of the authors.

COMPETING INTERESTS

All authors declared no competing interests.

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REFERENCES

1 Derby CA, Zilber S, Brambilla D, Morales KH, McKinlay JB. Body mass index, waist circumference and waist to hip ratio and change in sex steroid hormones: the Massachusetts Male Ageing Study. *Clin Endocrinol* 2006; 65: 125–31.
2 Mohr BA, Bhasin S, Link CL, O'Donnell AB, McKinlay JB. The effect of changes in adiposity on testosterone levels in older men: longitudinal results from the Massachusetts Male Aging Study. *Eur J Endocrinol* 2006; 155: 443–52.
3 Kley HK, Edelmann P, Krüskemper HL. Relationship of plasma sex hormones to different parameters of obesity in male subjects. *Metabolism* 1980; 29: 1041–5.
4 Rohrmann S, Shiels MS, Lopez DS, Rifai N, Nelson WG, *et al.* Body fatness and sex steroid hormone concentrations in US men: results from NHANES III. *Cancer Causes Control* 2011; 22: 1141–51.
5 Wu FC, Tajar A, Pye SR, Silman AJ, Finn JD, *et al.* Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: the European Male Aging Study. *J Clin Endocrinol Metab* 2008; 93: 2737–45.
6 Giagulli VA, Kaufman JM, Vermeulen A. Pathogenesis of decreased androgen levels in obese men. *J Clin Endocrinol Metab* 1994; 79: 997–8.

7 Nielsen TL, Hagen C, Wraae K, Brixen K, Petersen PH, *et al.* Visceral and subcutaneous adipose tissue assessed by magnetic resonance imaging in relation to circulating androgens, sex hormone-binding globulin, and luteinizing hormone in young men. *J Clin Endocrinol Metab* 2007; 92: 2696–705.
8 Pellitero S, Olaizola I, Alastrue A, Martínez E, Granada ML, *et al.* Hypogonadotropic hypogonadism in morbidly obese males is reversed after bariatric surgery. *Obes Surg* 2012; 22: 1835–42.
9 Rao SR, Kini S, Tamler R. Sex hormones and bariatric surgery in men. *Gend Med* 2011; 8: 300–11.
10 Grossmann M. Low testosterone in men with type 2 diabetes: significance and treatment. *J Clin Endocrinol Metab* 2011; 96: 2341–53.
11 Zhu C, Zhang Y, Zhang L, Gao J, Mei F, *et al.* Changes in sex hormones after laparoscopic sleeve gastrectomy in Chinese obese men: a 12-month follow-up. *Obes Surg* 2019; 29: 869–77.
12 Michalakis K, Mintziori G, Kaprara A, Tarlatzis BC, Goulis DG. The complex interaction between obesity, metabolic syndrome and reproductive axis: a narrative review. *Metabolism* 2013; 62: 457–78.
13 Teerds KJ, de Rooij DG, Keijer J. Functional relationship between obesity and male reproduction: from humans to animal models. *Hum Reprod Update* 2011; 17: 667–83.
14 Cobo G, Cordeiro AC, Amparo FC, Amodeo C, Lindholm B, *et al.* Visceral adipose tissue and leptin hyperproduction are associated with hypogonadism in men with chronic kidney disease. *J Ren Nutr* 2017; 27: 243–8.
15 Bekaert M, Van Nieuwenhove Y, Calders P, Cuvelier CA, Batens AH, *et al.* Determinants of testosterone levels in human male obesity. *Endocrine* 2015; 50: 202–11.
16 Rossetti ML, Steiner JL, Gordon BS. Androgen-mediated regulation of skeletal muscle protein balance. *Mol Cell Endocrinol* 2017; 447: 35–44.
17 Golds G, Houdek D, Arnason T. Male hypogonadism and osteoporosis: the effects, clinical consequences, and treatment of testosterone deficiency in bone health. *Int J Endocrinol* 2017; 2017: 4602129.
18 Newell-Fugate AE. The role of sex steroids in white adipose tissue adipocyte function. *Reproduction* 2017; 153: 133–49.
19 Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, *et al.* The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. *Gastroenterology* 2012; 142: 1592–609.
20 Kley HK, Nieschlag E, Wiegelmann W, Solbach HG, Krüskemper HL. Steroid hormones and their binding in plasma of male patients with fatty liver, chronic hepatitis and liver cirrhosis. *Acta Endocrinol* 1975; 79: 275–85.
21 Völzke H, Aumann N, Krebs A, Nauck M, Steveling A, *et al.* Hepatic steatosis is associated with low serum testosterone and high serum DHEAS levels in men. *Int J Androl* 2010; 33: 45–53.
22 Tian GX, Sun Y, Pang CJ, Tan AH, Gao Y, *et al.* Oestradiol is a protective factor for non-alcoholic fatty liver disease in healthy men. *Obes Rev* 2012; 13: 381–7.
23 Li Y, Liu L, Wang B, Chen D, Wang J. Nonalcoholic fatty liver disease and alteration in semen quality and reproductive hormones. *Eur J Gastroenterol Hepatol* 2015; 27: 1069–73.
24 Wang N, Zhai H, Zhu C, Li Q, Han B, *et al.* Combined association of vitamin D and sex hormone binding globulin with nonalcoholic fatty liver disease in men and postmenopausal women. *Medicine* 2016; 95: e2621.
25 Lazo M, Zeb I, Nasir K, Tracy RP, Budoff MJ, *et al.* The association of endogenous sex hormones with liver fat – Multi-Ethnic Study of Atherosclerosis (MESA). *Clin Gastroenterol Hepatol* 2015; 13: 1686–93.
26 Hua X, Sun Y, Zhong Y, Feng W, Huang H, *et al.* Low serum hormone-binding globulin is associated with nonalcoholic fatty liver disease in type 2 diabetic patients. *Clin Endocrinol* 2014; 80: 877–83.
27 Kim S, Kwon H, Park JH, Cho B, Kim D, *et al.* A low level of serum total testosterone is independently associated with nonalcoholic fatty liver disease. *BMC Gastroenterol* 2012; 12: 69–77.
28 Seo NK, Koo HS, Haam J, Kim HY, Kim MJ, *et al.* Prediction of prevalent but not incident non-alcoholic fatty liver disease by levels of serum testosterone. *J Gastroenterol Hepatol* 2015; 30: 1211–6.
29 Shin JY, Kim SK, Lee MY, Kim HS, Ye BI, *et al.* Serum sex hormone-binding globulin levels are independently associated with nonalcoholic fatty liver disease in people with type 2 diabetes. *Diabetes Res Clin Pract* 2011; 94: 156–62.
30 Lapauw BM, Taes Y, Bogaert V, Vanbillemont G, Goemare S, *et al.* Serum estradiol is associated with volumetric BMD and modulates the impact of physical activity on bone size at the age of peak bone mass: a study in healthy male siblings. *J Bone Miner Res* 2009; 24: 1075–85.
31 Dekker MJ, Wierckx K, Van Caenegem E, Klaver M, Kreukels BP, *et al.* A European network for the investigation of gender incongruence: endocrine part. *J Sex Med* 2016; 13: 994–9.
32 American Diabetes Association. Standards of medical care in diabetes – 2016. *Diabetes Care* 2016; 39 Suppl 1: S1–112.
33 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, *et al.* Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; 28: 412–9.
34 Fiers T, Delanghe J, T'Sjoen G, Van Caenegem E, Wierckx K, *et al.* A critical evaluation of salivary testosterone as a method for the assessment of serum testosterone. *Steroids* 2014; 86: 5–9.
35 Fiers T, Casetta B, Bernaert B, Vandersypt E, Debock M, *et al.* Development of a highly sensitive method for the quantification of estrone and estradiol in serum by liquid chromatography tandem mass spectrometry without derivatization. *J Chromatogr B Analyt Technol Biomed Life Sci* 2012; 893–894: 57–62.
36 Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab* 1999; 84: 3666–72.
37 Szulc P, Claustrat B, Munoz F, Marchand F, Delmas PD. Assessment of the role of 17β-oestradiol in bone metabolism in men: does the assay technique matter? The MINOS study. *Clin Endocrinol* 2004; 61: 447–57.
38 Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; 41: 1313–21.
39 Bedossa P. Pathology of non-alcoholic fatty liver disease. *Liver Int* 2017; 37: 85–9.
40 Bedossa P, Poitou C, Veyrie N, Bouillot JL, Basdevant A, *et al.* Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology* 2012; 56: 1751–9.
41 Bedossa P; the FLIP Pathology Consortium. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. *Hepatology* 2014; 60: 565–75.
42 Lu FB, Hu ED, Xu LM, Chen L, Wu JL, *et al.* The relationship between obesity and the severity of non-alcoholic fatty liver disease: systematic review and meta-analysis. *Expert Rev Gastroenterol Hepatol* 2018; 12: 491–502.
43 Herbst KL, Amory JK, Brunzell JD, Chansky HA, Bremner WJ. Testosterone administration to men increases hepatic lipase activity and decreases HDL and LDL size in 3 wk. *Am J Physiol Endocrinol Metab* 2003; 284: E1112–8.
44 Waxman DJ, Attisano C, Guengerich FP, Lapenson DP. Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6β-hydroxylase cytochrome P-450 enzyme. *Arch Biochem Biophys* 1988; 263: 424–36.

Supplementary Table 1: General descriptives and sex steroid levels of the control and obese cohort

Characteristics	*Control (n=80)*	*Obesity (n=80)*	*P*
Age (year)	46±11	46±11	0.913
BMI (kg m^{-2})	23.8±2.1	42.2±5.5	<0.001**
LH (U l^{-1})	4.57 (3.34–5.85)	3.88 (2.69–5.03)	0.001**
FSH (U l^{-1})	4.71 (3.22–6.74)	3.48 (2.79–5.46)	0.026*
SHBG (nmol l^{-1})	45.8 (32.8–58.1)	24.6 (17.9–33.0)	<0.001**
E_2 (pmol l^{-1})	78.9 (60.3–93.3)	80.7 (56.4–100.1)	0.526
cFE_2 (pmol l^{-1})	1.35 (1.00–1.66)	1.48 (1.06–1.97)	0.019*
T (nmol l^{-1})	20.1 (16.3–23.8)	7.9 (6.0–10.9)	<0.001**
cFT (pmol l^{-1})	349.1 (261.0–415.9)	168.0 (135.7–235.9)	<0.001**
E_2/T ($\times 10^{-3}$)	3.7 (3.0–4.6)	10.1 (6.7–14.8)	<0.001**
cFE_2/cFT ($\times 10^{-3}$)	3.8 (2.9–4.6)	8.7 (6.0–13.1)	<0.001**
cFT/LH	76.74 (58.06–110.77)	46.35 (32.37–64.12)	<0.001**

Data represented as mean±SD or median (1st quartile–3rd quartile). Significant *P* values were indicated with *$P<0.05$ and **$P<0.005$. LH: luteinizing hormone; FSH: follicle-stimulating hormone; SHBG: sex hormone-binding globulin; E_2: estradiol; cFE_2: calculated free estradiol; T: testosterone; cFT: calculated free testosterone; s.d.: standard deviation