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Research paper

Sex steroids and sex hormone-binding globulin in postmenopausal women with nonalcoholic fatty liver disease

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ABSTRACT

OBJECTIVE: The evaluation of serum sex steroids and sex hormone-binding globulin (SHBG) levels in postmenopausal women with nonalcoholic fatty liver disease (NAFLD) and their association to the disease severity. DESIGN: Twenty-two postmenopausal women with biopsy-proven NAFLD and 18 matched controls were recruited. Blood samples for serum SHBG, total testosterone, estradiol levels and standard biochemical tests were obtained after overnight fasting. Free androgen index (FAI), calculated free (cFT) and bioavailable testosterone were estimated by standard formulas. RESULTS: The NAFLD group had lower serum SHBG levels and higher values of cFT, bioavailable testosterone and FAI, despite exhibiting similar to controls levels of serum total testosterone and estradiol. Serum SHBG levels (adjusted odds ratio [aOR]=0.912; 95% CI 0.854-0.973), bioavailable testosterone (aOR=1.254; 95% CI 1.010-1.556) and FAI (aOR=2.567; 95% CI 1.153-5.716), but not cFT, were associated with NAFLD independently of age, body mass index (BMI) and waist circumference. Serum estradiol levels were associated with the presence of nonalcoholic steatohepatitis (NASH) independently of age, BMI and waist circumference (aOR=0.727; 95% CI 0.537-0.985). CONCLUSIONS: Low SHBG levels and high metabolically active testosterone fractions were independently associated with NAFLD. Among NAFLD patients, serum estradiol levels were independently associated with NASH. However, these results need further validation from large-scale studies.

Key words: Estradiol, Free androgen index, Insulin resistance, Metabolic syndrome, Nonalcoholic fatty liver disease, Nonalcoholic steatohepatitis, Sex hormone-binding globulin, Testosterone

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is considered to be the hepatic manifestation of insulin resistance (IR) syndrome, given that IR plays a pivotal role in its pathogenesis.[1] NAFLD ranges from simple nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), comprising steatosis, inflammation and fibrosis; advanced NASH may ultimately result in liver cirrhosis, subacute liver failure and hepatocellular carcinoma.[2]

In the setting of IR, NAFLD shares common pathogenetic mechanisms with other IR-related morbidities, including type 2 diabetes mellitus (T2DM), obesity, dyslipidemia, hypertension and polycystic ovary syndrome (PCOS), all of which increase the risk for cardiovascular disease and mortality, the endpoints of IR syndrome.[3] PCOS is considered to be the ovarian manifestation of IR syndrome and may co-exist with NAFLD.[4] Although androgen and sex hormone-binding globulin (SHBG), which transports androgens and estrogens in the blood and regulates their access to target tissues, have a distinct role in the pathogenesis and diagnosis of PCOS, there are only limited data regarding androgen and SHBG levels in the NAFLD populations; most of the relevant studies refer to premenopausal women with PCOS, while the definite diagnosis of NAFLD in these studies was not based on liver biopsy.[5-12]

The primary endpoints of this study were the evaluation of serum sex steroids and SHBG levels in postmenopausal women with biopsy-proven NAFLD and their association with the disease severity. Secondary endpoints were the association of serum sex steroids and SHBG levels with clinical or circulating parameters related to NAFLD.

PATIENTS AND METHODS

This was a single center, cross-sectional study. Postmenopausal women with NAFLD and controls were recruited on an outpatient basis at the Second Medical Clinic, Ippokration Hospital, Aristotle University of Thessaloniki, between June 2008 and November 2010. Determination of eligibility was based on medical history, physical examination, liver function tests (serum aspartate transaminase [AST], alanine transaminase [ALT], gamma-glutamyl transferase [GGT], total alkaline phosphatase [ALP], total and indirect bilirubin) and liver ultrasound imaging performed during the screening visit. All participants provided informed consent. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local ethics committee. Inclusion criteria for the NAFLD patients were: 1) female gender; 2) age >45 years; 3) last menstruation >24 months; and 4) bright liver on ultrasound imaging and increased liver function tests for at least 6 months before liver biopsy.

Age- gender- and weight-matched individuals were recruited for the control group. The control group consisted of apparently healthy individuals subjected to regular check-up for professional needs. Inclusion criteria for controls were: 1) female gender; 2) age >45 years; 3) last menstruation >24 months; 4) normal liver ultrasound imaging and normal liver function tests. Controls did not undergo a liver biopsy because of obvious ethical considerations.

Exclusion criteria for both groups were: 1) ethanol consumption >20 g/day; 2) liver cirrhosis; 3) other liver disease (viral hepatitis, autoimmune hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis and overlap syndromes, drug-induced liver disease, hemochromatosis, Wilson's disease, α1-antitrypsin deficiency); 4) type I diabetes mellitus; 5) pancreatitis; 6) uncontrolled hypothyroidism or hyperthyroidism; 7) adrenal insufficiency; 8) renal failure; 9) thrombotic disorders; 10) cancer; 11) premature ovarian failure; 12) addiction to any drug; 13) use of the following medications within a 12-month period before screening: estrogens, progestins, glucocorticosteroids, thiazolidinediones, insulin, sibutramine, orlistat, rimonabant, vitamin E, vitamin C, ursodeoxycholic acid, ferrum, interferon, tamoxifene, amiodarone, biologic agents, folate or vitamin B supplements, antibiotic, any medication against tuberculosis, epilepsy or viruses, or any medication affecting hemostasis, such as antiplatelet agents, aspirin or oral anticoagulants; and 14) use of intravenous glucose administration or parenteral nutrition within a 1-month period before screening. The patients were initially selected on the basis of their medical history, family history and previously performed laboratory exams, if any. In those initially selected, a set of serum tests

was performed at screening visit, as follows: HBsAg, anti-HCV, anti-mitochondrial antibody, anti-nuclear antibody, anti-cardiolipin antibody, anti-neutrophil cytoplasmic antibody, anti-smooth muscle antibody, alpha-1 antitrypsin, thyroid stimulating hormone, iron, ferritin, prothrombin time, partial thromboplastin time, platelets count. Furthermore, in specific NAFLD patients, Perl stain, orcein Shikata stain or periodic acid stain were selectively performed on histologic samples, if hemochromatosis, Wilson's disease or α1-antitrypsin deficiency were suspected.

Morning (8-9 am) fasting blood samples were collected 1-2 h prior to liver biopsy, which was performed under computed tomography-guidance by an experienced radiologist (EZ) and interpreted by two experienced pathologists (KP, EK). Serum AST, ALT, GGT, ALP, triglycerides, high-density lipoprotein cholesterol (HDL-C), albumin and glucose were measured within 1 h after blood drawing with standard methods using an automated analyzer (Olympus AU2700; Olympus, Hamburg, Germany). Sera were also immediately frozen at -30°C for the measurement of insulin, estradiol, total testosterone, SHBG, cortisol, dehydroepiandrosterone sulfate (DHEAS), ferritin, tumor necrosis factor (TNF)-α, total and high molecular weight (HMW) adiponectin levels. Serum insulin, estradiol, total testosterone, SHBG, cortisol and DHEAS levels were measured with immunochemiluminescence on a Immulite 2500 immunoassay system (Siemens Healthcare Diagnostics, Deerfield, IL; insulin: intra-assay coefficient of variation [CV] 3.3-5.5%, total CV 4.1-7.3%; estradiol: intra-assay CV 4.3-9.9%, total CV 6.7-16.0%; total testosterone: intra-assay CV 5.1-11.7, total CV 7.2-13.0; SHBG: intra-assay CV 2.4-4.4, total CV 3.7-7.0; cortisol: intra-assay CV 5.2-7.4, total CV 7.2-9.4; DHEAS: intra-assay CV 4.9-9.8, total CV 7.9-13.0). Serum ferritin levels were measured with immuno-chemiluminescence on an ADVIA Centaur CP analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA; intra-assay CV 2.1-3.0%, inter-assay CV 2.7-5.4%). Serum TNF-α, total and HMW adiponectin levels were measured with enzyme-linked immunosorbent assay (ELISA) on a ELx800 Absorbance Microplate Reader automated analyzer (BioTek, Winooski, VT, USA), by using the following commercial kits, respectively: TNF-α human ELISA kit (R&D Systems, Minneapolis, MN, USA; intra-assay CV 4.2-5.2%, inter-assay CV 4.6-7.4%), adiponectin human ELISA kit (Phoenix Europe GmbH, Karlsruhe, Germany; intra-assay CV 5.0%, inter-assay CV 6.0%); adiponectin multimeric ELISA kit (ALPCO Immunoassays, Salem, NH, USA; intra-assay CV 3.3-5.0%, inter-assay CV 5.7%).

Body mass index (BMI) was calculated by the formula: body weight (kg) / height^2 (m^2). IR was quantified by homeostatic model of assessment - insulin resistance (HOMA-IR) using the formula HOMA-IR = glucose (mmol/L) * insulin (μU/mL) / 22.5.[13] FAI was calculated by the formula: total testosterone (nmol/L) / SHBG (nmol/L) * 100. Calculated free testosterone and bioavailable testosterone were calculated based on the Vermeulen et al. formula (http://www.issam.ch/freetesto.htm).[14] Given that testosterone circulates in plasma unbound, bound (with high binding affinity) to SHBG and bound (with weak binding affinity) to non-specific proteins, such as albumin, free testosterone represents the unbound fraction and bioavailable testosterone the unbound plus weakly bound to albumin fraction. It is considered that cFT, bioavailable testosterone and FAI more accurately reflect the level of metabolically active fraction of testosterone than the measurement of serum total testosterone levels.

NAFLD patients were classified into those with NAFL or NASH according to the criteria of the NAFLD Activity Score (NAS).[15] Steatosis grade, fibrosis stage, lobular and portal inflammation and ballooning were categorized based on the classification of the NASH Clinical Research Network.[15] Regarding fibrosis stage, cirrhosis (grade 4) was not included since it was an exclusion criterion.

Statistical Analysis

Continuous data are presented as median (25-75 percentile). Categorical data are presented as frequencies. The Kolmogorov-Smirnov test was used to check the normality of distributions of continuous variables. The independent sample t-test or Mann-Whitney test was used for between group comparisons in cases of two groups of continuous variables. Spearman's coefficient (r_s) was used for binary correlations. Binary logistic regression analysis was used to identify whether serum sex hormones or SHBG levels were independently associated with NAFLD, NASH or

specific histological lesions (the "enter" method). For the need of these analyses, if any of the included variables did not follow normal distribution, they were logarithmically transformed. Statistical analysis was performed with SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). Significance was set at $P<0.05$. *Post-hoc* power analysis was performed by G*Power software (v.3.1.5.1. for Macintosh; University of Heinrich-Heine, Düsseldorf, Germany).

RESULTS

Twenty-two postmenopausal women with NAFLD (10 with NAFL and 12 with borderline or definite NASH) and 18 age-matched controls were included in this series. Comparative data of the study groups are presented in Table 1. There were no statistically significant differences between groups in age, age at menopause, weight and BMI, although BMI tended to be higher in the NAFLD group. Waist circumference

Table 1. Comparative data of study groups

	Control group (n=18)	NAFLD group (n=22)	*p*-value*	Reference values
Age (years)	56.5 (53.8-61.0)	55.0 (53.8-63.0)	0.978	-
Menopause (years)	49.5 (47.0-52.0)	50.0 (46.0-52.3)	0.870	-
Weight (kg)	75 (70.8-81.0)	78 (71.0-75.0)	0.391	
BMI (kg/m^2)	29.4 (28.7-31.4)	32.0 (29.7-37.8)	0.057	20-25
Waist circumference (cm)	99.0 (94.5-101.5)	105.0 (98.3-113.0)	0.017	≤80
AST (U/L)	19.0 (17.0-21.3)	31.0 (23.0-40.5)	<0.001	10-31
ALT (U/L)	16.5 (13.0-20.3)	38.5 (22.8-57.3)	<0.001	10-34
GGT (U/L)	13.0 (10.8-18.3)	34.5 (25.0-53.3)	<0.001	0-38
ALP (U/L)	63.0 (51.0-75.0)	80.5 (67.3-93.5)	0.004	30-120
Triglycerides (mg/dL)	96.5 (79-139.3)	165.5 (127.8-275.3)	0.002	<150
HDL-C (mg/dL)	56.0 (49.3-70.0)	49.5 (44.8-55.3)	0.029	>50
Albumin (g/dL)	4.4 (4.2-4.5)	4.4 (4.3-4.7)	0.061	3.5-5.2
Ferritin (ng/mL)	41.0 (19.0-73.8)	102.0 (47.3-144.0)	0.003	10-291
Glucose (mg/dL)	88.5 (80.8-94.0)	105.0 (90.0-121.3)	0.003	60-100
Insulin (μU/mL)	4.3 (2.0-6.2)	12.9 (6.5-23.2)	0.001	6-27
HOMA-IR	0.87 (0.53-1.51)	3.21 (2.04-6.63)	<0.001	na
Costisol (μg/dL)	13.3 (10.7-16.7)	12.4 (8.6-15.8)	0.550	5-20
DHEAS (μg/dL)	85.5 (45.3-117.0)	66.9 (31.1-98.0)	0.221	35-430
SHBG (nmol/L)	57.7 (44.1-71.1)	29.0 (21.9-43.2)	<0.001	na
Estradiol (pg/mL)	25.1 (21.5-33.8)	23.3 (20.0-29.4)	0.251	12-56
Total testosterone (ng/dL)	29.3 (20.0-38.5)	21.1 (20.0-35.5)	0.308	49-113
cFT (ng/dL)	0.36 (0.26-0.47)	0.48 (0.34-0.67)	0.027	na
Bioavailable testosterone (ng/dL)	8.4 (6.3-10.9)	11.8 (8.1-16.4)	0.010	na
FAI	1.7 (1.3-2.3)	3.4 (1.9-4.4)	0.001	na
Total adiponectin (μg/mL)	9.8 (4.7-11.8)	4.4 (3.5-7.1)	0.006	na
HMW adiponectin (μg/mL)	4.1 (2.8-5.2)	2.3 (1.7-3.6)	0.026	na
TNF-α (pg/mL)	9.4 (7.8-13.2)	14.7 (11.8-19.4)	0.007	na

Data are presented as median (25-75 percentile)

*: Between groups comparison (independent sample t-test or Mann-Whitney test).

ALT: alanine transaminase; ALP: alkaline phosphatase; AST: aspartate transaminase; BMI: body mass index; cFT: calculated free testosterone; GGT: gamma-glutamyl transferase; FAI: free androgen index; HDL-C: high density lipoprotein cholesterol; HMW: high molecular weight; HOMA-IR: homeostatic model of assessment insulin resistance; na: not available; NAFLD: nonalcoholic fatty liver disease; SHBG: sex hormone-binding globulin; TNF: tumor necrosis factor

was higher in the NAFLD than in the control group. The NAFLD group had statistically lower serum SHBG levels and higher cFT, bioavailable testosterone and FAI (Figure 1; Table 1), despite exhibiting similar serum total testosterone and estradiol levels. As expected, AST, ALT, GGT, ALP, triglycerides, glucose, insulin, HOMA-IR, ferritin and TNF-α were higher, whereas HDL-C, total and HMW adiponectin were lower in the NAFLD compared to the control group. There were no statistically significant differences in serum cortisol, DHEAS and albumin levels between groups, although albumin tended to be higher in the NAFLD group (Table 1). Serum SHBG levels, bioavailable testosterone and FAI (model 1, 2 and 3, respectively; Table 2) were separately associated with NAFLD independently of age, BMI and waist circumference in binary logistic regression analysis. On the other hand, serum estradiol, total testosterone levels and cFT were not independently associated with NAFLD.

Between NAFL and NASH subgroups, there were no statistically significant differences in serum SHBG (35.7 [26.2-43.6] vs. 26.1 [18.7-41.4] nmol/L; p=0.286), estradiol 26.6 [21.7-31.5] vs. 21.7 [20.0-25.9] pg/mL; p=0.096; Figure 2), total testosterone (26.4 [20.2-39.9] vs. 20.1 [19.3-31.1] ng/dL; p=0.324), cFT (0.51 [0.33-0.78] vs. 0.46 [0.35-0.59] ng/dL; p=0.522), bioavailable testosterone (13.2 [8.0-18.6] vs. 11.4 [8.4-14.8] ng/dL; p=0.499) and FAI (3.4 [1.9-4.6] vs. 3.4 [2.0-3.9]; p=0.831), respectively. Notably, serum estradiol levels were associated with NASH independently of age, BMI and waist circumference in binary logistic regression analysis (Table 3). In contrast, serum SHBG and total testosterone levels or cFT, bioavailable testosterone and FAI were not independently associated with NASH.

Comparison of the NAFLD subgroups according to steatosis grade, fibrosis stage, ballooning, lobular and portal inflammation showed that there were no statistically significant differences in serum SHBG, estradiol and total testosterone levels or cFT, bioavailable testosterone and FAI (Table 4); however, SHBG tended to be higher in the absence of lobular inflammation. None of these parameters was associated with any of the histological lesions independently of age, BMI and waist circumference.

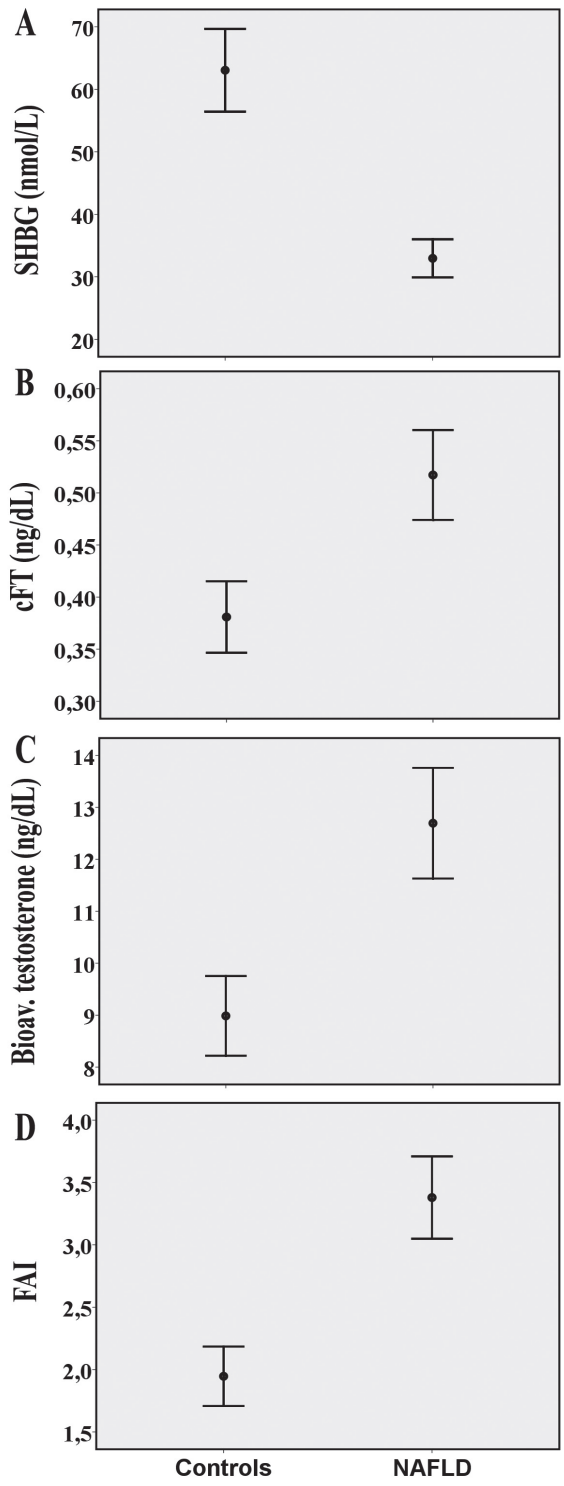

Figure 1. Error bars (mean ± standard error of the mean) showing SHBG (A), cFT (B), bioav. testosterone (C), and FAI in control and NAFLD groups. Abbreviations: bioav. testosterone, bioavailable testosterone; cFT, calculated free testosterone; NAFLD, nonalcoholic fatty liver disease; SHBG, sex hormone-binding grobulin.

Table 2. Logistic regression analysis to assess the association between NAFLD and serum SHBG levels (model 1) or bioavailable testosterone (model 2) or FAI (model 3) after adjustment for age, BMI and waist circumference

Variables	Beta	*p*-value	Adjusted odds ratio	95% CI for adjusted odds ratio
Model 1: SHBG				
Age (years)	0.071	0.402	1.073	0.910-1.267
BMI (kg/m^2)	0.105	0.564	1.111	0.777-1.589
Waist circumference (cm)	0.024	0.818	1.025	0.832-1.261
SHBG (nmol/L)	-0.093	0.005	0.912	0.854-0.973
Model 2: bioavailable testosterone				
Age (years)	0.080	0.247	1.083	0.946-1.239
BMI (kg/m^2)	-0.130	0.431	0.878	0.636-1.213
Waist circumference (cm)	0.134	0.132	1.144	0.960-1.362
Bioavailable testosterone (ng/dL)	0.226	0.040	1.254	1.010-1.556
Model 3: FAI				
Age (years)	0.081	0.249	1.084	0.945-1.245
BMI (kg/m^2)	-0.073	0.664	0.929	0.667-1.294
Waist circumference (cm)	0.105	0.267	1.111	0.923-1.338
FAI	0.943	0.021	2.567	1.153-5.716

*: Control group was rated as 0 and NAFLD group as 1 within dependent variable
BMI: body mass index; CI: confidence interval; FAI: free androgen index; NAFLD: nonalcoholic fatty liver disease; SHBG: sex hormone-binding globulin

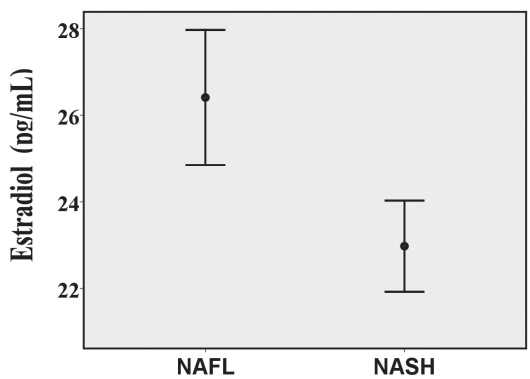

Figure 2. Error bars (mean ± standard error of the mean) showing serum estradiol in NAFL and NASH groups. Abbreviations: NAFL, nonalcoholic fatty liver (simple steatosis); NASH, nonalcoholic steatohepatitis.

Correlations between serum SHBG and sex steroids levels are presented in Table 5. The correlations between SHBG and adiponectin, HOMA-IR, GGT and triglycerides are selectively depicted in Figure 3.

DISCUSSION

In this pilot study, patients with NAFLD had lower serum SHBG levels and higher cFT, bioavailable testosterone and FAI, despite exhibiting similar to controls serum total testosterone and estradiol levels. Serum SHBG levels, which play an expanded role in the pathophysiology of IR and T2DM, cFT, bioavailable testosterone and FAI were all associated with the presence of NAFLD independently of age, BMI and waist circumference. However, only serum estradiol levels were independently associated with the presence of NASH. Data on SHBG and sex steroids in NAFLD populations are limited in the literature. To our knowledge, this is the first study evaluating serum sex steroids and SHBG levels in patients with biopsy-proven NAFLD. In all previous clinical studies that assessed sex steroids or SHBG (most of which in premenopausal PCOS women), NAFLD diagnosis was not based on liver biopsy, which is the gold standard, but on ultrasonographic and/or biochemical criteria.[5-12] Bioavailable testosterone has not been evaluated in NAFLD patients as yet. Generally, similar data were shown in the previous studies, whereas any differences may be attributed mostly to gender and menopause status differences among studies. However, large-scale studies in biopsy-proven NAFLD populations are needed to confirm these findings.

Table 3. Logistic regression analysis to assess the association between NASH and serum estradiol levels after adjustment for age, BMI and waist circumference

Variables	Beta	*p*-value	Adjusted odds ratio	95% CI for adjusted odds ratio
Age (years)	-0.118	0.164	0.888	0.752-1.050
BMI (kg/m^2)	-0.094	0.638	0.910	0.616-1.346
Waist circumference (cm)	0.091	0.347	1.095	0.906-1.324
Estradiol (pg/mL)	-0.318	0.040	0.727	0.537-0.985

*: NAFL group was rated as 0 and NASH group as 1 within dependent variable
BMI: body mass index; CI: confidence interval; NAFL: simple nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; SHBG: sex hormone-binding globulin.

Table 4. Comparative data of SHBG, total testosterone, estradiol, cFT, bioavailable testosterone and FAI within specific histological lesions of NAFLD subgroups according to NASH Clinical Research Network classification[15]

Histological lesion	Patients (N)	SHBG (nmol/L)	Estradiol (pg/mL)	Total testosterone (ng/dL)	cFT (ng/dL)	Bioavailable testosterone (ng/dL)	FAI
Steatosis grade (p-value)*		0.155	0.438	0.858	0.477	0.569	0.434
≤33%	13	36.0 (25.4-43.7)	23.8 (20.3-30.6)	19.5 (19.1-36.3)	0.46 (0.33-0.71)	11.9 (7.8-16.9)	3.1 (1.8-4.5)
>33%	9	23.2 (17.6-39.9)	22.1 (20.1-27.5)	22.2 (19.8-37.2)	0.49 (0.40-0.68)	11.8 (11.3-16.8)	3.5 (2.8-4.3)
Fibrosis stage (p-value)*		0.591	0.075	0.530	0.474	0.420	0.531
Absent	5	32.4 (25.6-42.4)	24.2 (23.1-32.0)	23.9 (20.0-46.8)	0.60 (0.37-0.81)	14.9 (9.1-19.1)	3.9 (2.8-4.6)
Present	17	29.0 (20.4-43.2)	22.1 (19.7-28.9)	20.3 (19.7-34.3)	0.47 (0.34-0.62)	11.4 (8.0-15.2)	3.3 (1.9-4.2)
Lobular inflammation (p-value)*		0.082	0.352	0.422	0.942	0.971	0.562
Absent	14	36.7 (26.2-43.6)	23.8 (20.1-30.1)	23.1 (20.8-38.5)	0.51 (0.33-0.69)	13.2 (7.8-16.4)	3.3 (1.9-4.6)
Present	8	22.3 (18.7-34.5)	22.0 (19.5-27.4)	20.8 (19.9-31.1)	0.46 (0.38-0.70)	11.4 (9.9-16.6)	3.5 (2.6-3.9)
Portal inflammation (p-value)*		0.833	0.151	0.247	0.291	0.259	0.324
None to minimal	11	32.1 (24.0-43.2)	24.2 (22.3-29.6)	23.9 (19.9-40.5)	0.57 (0.33-0.76)	14.1 (8.1-18.0)	3.9 (1.9-4.6)
Greater than minimal	11	29.0 (19.4-43.3)	20.1 (19.6-28.5)	20.2 (19.6-33.8)	0.45 (0.34-0.51)	11.4 (7.8-13.2)	3.2 (1.9-3.8)
Ballooning (p-value)*		0.421	0.367	0.649	0.366	0.365	0.421
Absent	4	35.7 (28.4-44.4)	27.0 (20.6-32.1)	28.9 (20.1-51.2)	0.73 (0.34-0.84)	17.5 (8.2-19.6)	4.4 (1.9-4.6)
Present	18	28.4 (20.9-43.1)	23.3 (20.0-28.7)	21.1 (19.7-34.0)	0.48 (0.34-0.61)	11.6 (7.9-15.1)	3.3 (1.9-4.1)

Data are presented as median (25-75 percentile)
*: Between groups comparison (independent sample t-test or Mann-Whitney test)
cFT: calculated free testosterone; FAI: free androgen index; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; SHBG: sex hormone-binding globulin

Table 5. Correlations between serum SHBG levels, cFT, bioavailable testosterone or FAI and selective study parameters.

Parameter		SHBG (nmol/L)	cFT (ng/dL)	Bioavailable testosterone (ng/dL)	FAI
Age (years)	r_s	0.098	-0.359	-0.335	-0.299
	p-value	0.555	0.025	0.037	0.064
Waist circumference (cm)	r_s	-0.294	0.351	0.346	0.361
	p-value	0.069	0.028	0.031	0.024
AST (U/L)	r_s	-0.455	0.259	0.272	0.359
	p-value	0.004	0.111	0.093	0.025
ALT (U/L)	r_s	-0.509	0.254	0.268	0.365
	p-value	0.001	0.119	0.099	0.022
GGT (U/L)	r_s	-0.640	0.311	0.352	0.460
	p-value	<0.001	0.154	0.028	0.003
Triglycerides (mg/dL)	r_s	-0.639	0.218	0.266	0.401
	p-value	<0.001	0.182	0.101	0.011
HDL-C (mg/dL)	r_s	0.440	-0.256	-0.308	-0.358
	p-value	0.006	0.116	0.057	0.025
Ferritin (ng/mL)	r_s	-0.453	0.340	0.387	0.448
	p-value	0.004	0.034	0.015	0.004
Glucose (mg/dL)	r_s	-0.354	0.083	0.132	0.195
	p-value	0.027	0.616	0.425	0.233
Insulin (μU/mL)	r_s	-0.437	0.340	0.389	0.425
	p-value	0.006	0.037	0.016	0.008
HOMA-IR	r_s	-0.441	0.296	0.349	0.394
	p-value	0.006	0.071	0.032	0.014
DHEAS (μg/dL)	r_s	-0.110	0.490	0.496	0.398
	p-value	0.506	0.002	0.001	0.012
Total adiponectin (μg/mL)	r_s	0.507	-0.273	-0.314	-0.379
	p-value	0.001	0.092	0.052	0.017
HMW adiponectin (μg/mL)	r_s	0.444	-0.288	-0.319	-0.365
	p-value	0.005	0.075	0.048	0.022

Data are presented as Spearman's coefficient of correlation (r_s) and p-value
ALT: alanine transaminase; AST: aspartate transaminase; BMI: body mass index; cFT: calculated free testosterone; GGT: gamma-glutamyl transferase; FAI: free androgen index; HDL-C: high density lipoprotein cholesterol; HMW: high molecular weight; HOMA-IR: homeostatic model of assessment insulin resistance; SHBG: sex hormone-binding globulin.

More specifically, in a cross-sectional study with premenopausal PCOS women, serum SHBG levels were lower and FAI higher in PCOS women with hepatic steatosis versus either PCOS women without steatosis or controls, and were both independently associated with hepatic steatosis; however, total testosterone was similar between PCOS women with or without hepatic steatosis.[5] In another cross-sectional study, serum SHBG levels were lower in PCOS women with than those without hepatic steatosis, but serum total testosterone levels and FAI were similar between groups.[8] In a third study, serum SHBG levels were lower and FAI higher in PCOS women with higher ALT levels than PCOS women with lower ALT levels, whereas both serum total testosterone and estradiol levels were similar between groups.[7] In a fourth study, FAI was higher in PCOS women with higher cytokeratin-18 (M30) than PCOS women with lower cytokeratin-18 levels (cytokeratin-18 is an apoptotic marker related to NASH).[11] In a fifth

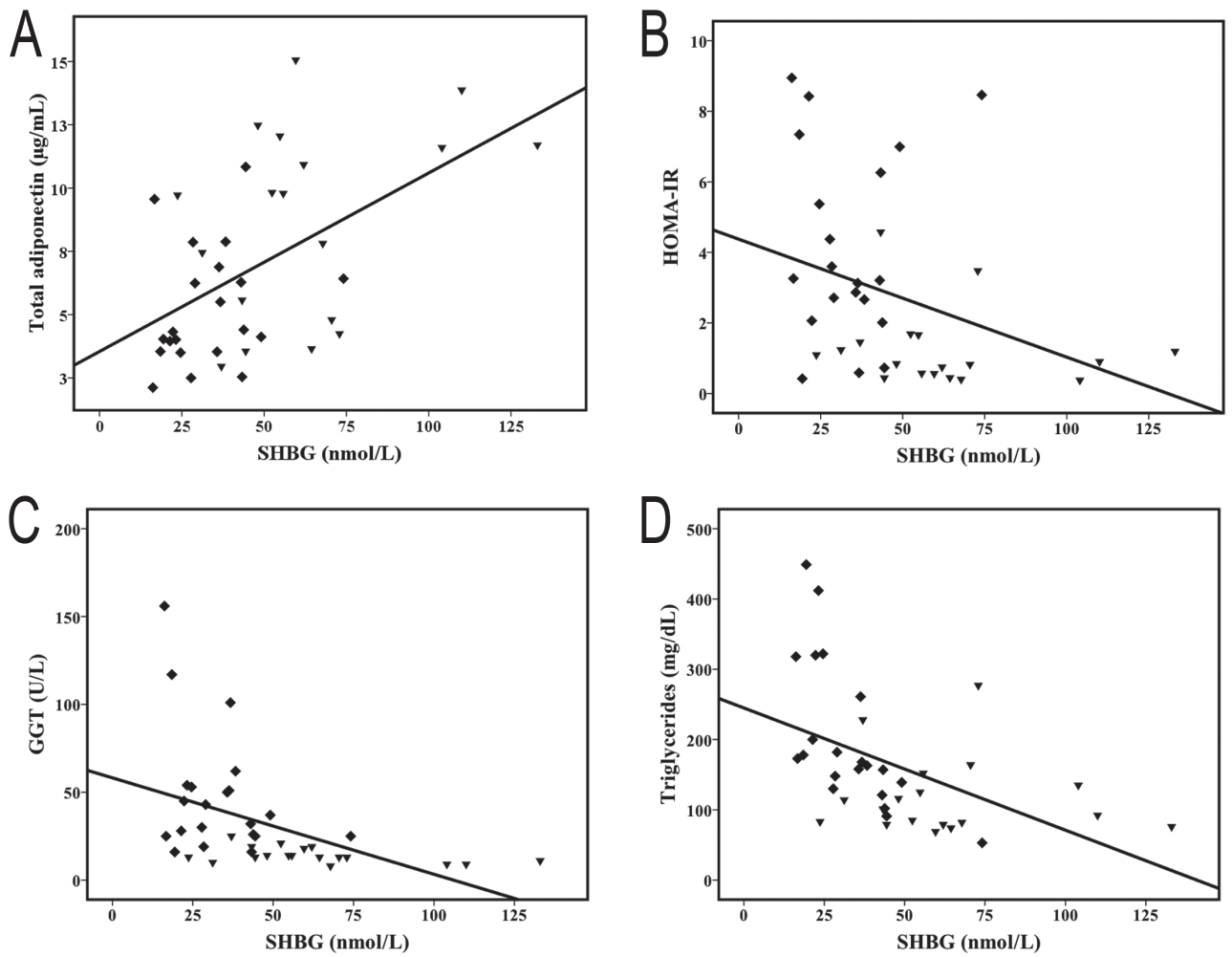

Figure 3. Correlations between SHBG and total adiponectin (A), HOMA-IR (B), GGT (C) or triglycerides (D). Triangles represent control and rhombuses NAFLD individuals. Abbreviations: GGT, gamma-glutamyl transferase; HOMA-IR, homeostatic model of assessment insulin resistance; NAFLD, nonalcoholic fatty liver disease; SHBG, sex hormone-binding grobulin.

study, postmenopausal women with or without hepatic steatosis had similar estradiol levels; however, serum estradiol levels were lower in premenopausal PCOS women with hepatic steatosis compared to those without it.[10]

In a cross-sectional study with T2DM patients, serum SHBG levels were lower in patients with ultrasonographic high grade of hepatic steatosis compared to those with normal liver and were independently associated with it. Serum free testosterone levels were higher in patients with high grade of hepatic steatosis, but were not independently associated with it, whereas serum total testosterone and estradiol levels were similar between groups.[6]

In a cross-sectional study with apparently healthy men, serum SHBG levels were higher, whereas cFT and serum estradiol levels lower in parallel to the severity of (ultrasonographically-proven) hepatic steatosis; however, serum total testosterone levels were similar between groups.[9] On the other hand, serum total testosterone levels were lower in apparently healthy men with hepatic steatosis rather than those without in another cross-sectional study. Men in the low serum testosterone quintile were at a higher risk for NAFLD than men in the highest serum testosterone quintile.[12]

In a meta-analysis of 52 observational studies evaluating serum total and free testosterone levels

in IR syndrome, the presence of a sex-dependent association between serum testosterone levels and IR syndrome was supported: both serum total and free testosterone levels were lower in men with IR syndrome, whereas they were higher in women with IR syndrome. In contrast, serum SHBG levels were lower in both men and women with IR syndrome.[16] Low serum SHBG levels have been proposed as a biomarker for IR syndrome, T2DM, PCOS and cardiovascular disease risk.[16] Furthermore, there is evidence suggesting that hepatic lipogenesis reduces serum SHBG levels.[17] Therefore, SHBG, which is mostly produced in the liver, might be affected by both IR and hepatic lipogenesis. This speculation could be further supported by the significant correlations between serum SHBG levels and glucose, insulin, HOMA-IR, liver function tests, HDL-C and triglycerides shown in this study. Regarding the correlations between serum SHBG and total or HMW adiponectin or ferritin levels, a cause-effect relationship cannot be implied by this study; however, these correlations could represent an epiphenomenon, with IR being the common denominator. Similar correlations have previously been shown in other than NAFLD populations.[18,19] Given that SHBG regulates sex steroids access to tissues and that it is highly correlated with cFT, bioavailable testosterone and FAI (because SHBG is one of the input parameters in the formulas), the correlations of these three calculated parameters with other parameters were expected to have inverse relationship with SHBG, as was shown in this study (Table 5).

Regarding the role of estradiol in NAFLD, this study showed for the first time that serum levels were associated with the presence of NASH in postmenopausal women independently of age, BMI and waist circumference. The role of estrogen in the pathogenesis of NAFLD has recently been gaining increasing interest. In a randomized placebo-controlled trial with T2DM women, hormone replacement therapy (HRT) improved liver function tests in women assigned to HRT, but not to placebo.[20] Similarly, the phytoestrogen genistein had an inhibitory effect on hepatic steatosis in a mouse model with NAFLD.[21] Estrogen deficiency worsens NASH in mice fed with high-fat and high-cholesterol diet.[22] Estradiol has also been shown to attenuate saturated fatty acid diet-induced liver injury in ovariectomized mice by up-regulating hepatic senescence marker protein-30.[23] Moreover, a selective estrogen receptor α agonist was reported to ameliorate hepatic steatosis in male aromatase knockout mice.[24] On the other hand, the human liver expresses estrogen and androgen receptors and experimentally both androgens and estrogens have been implicated in stimulating hepatocyte proliferation and might act as liver cancer inducers or promoters.[25,26] In this regard, estrogen might play a critical role in female hepatocarcinogenesis.[27]

Similarly to serum total testosterone levels, no difference was found in the serum levels of the adrenal androgen DHEAS between the NAFLD and control group in this study. Other studies have reported similar serum DHEAS levels between biopsy-proven NAFLD and control groups;[28-30] however, lower DHEAS was reported in some of the NASH patients with advanced NAFLD or fibrosis.[29,30]

Similarly to previous studies, serum cortisol levels were not different between NAFLD and control groups.[10,31] However, some,[31] but not all,[32] authors proposed that hepatic 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which converts cortisone to the active cortisol, is increased in NASH patients, thereby playing a local role in NASH pathogenesis.

Waist circumference was higher in patients than in controls in this study. Abdominal adiposity is a factor known to be associated with IR, IR syndrome and NAFLD.[1] Furthermore, waist circumference is between included parameters (together with BMI, and serum triglycerides and GGT levels) in the Fatty Liver Index (FLI), which provides a non-invasive prediction of hepatic steatosis[33] and is positively associated with fibrosis stage in NAFLD patients.[34]

This study has certain limitations: 1) the sample size was small; we did not perform an *a priori* power analysis, but serum SHBG difference between the NAFLD and control group, provided a *post-hoc* power of 98.6%, for type α error 0.05 (two-sided; difference between two independent means); 2) findings regarding the histological lesions of fibrosis and ballooning should be cautiously interpreted because of skewed distribution; 3) the controls were not subjected to liver biopsy due to obvious ethical considerations; and 4) serum free testosterone levels were not directly

measured; however, methods commonly used for the measurement of serum free testosterone levels do not correlate with the results of equilibrium dialysis, the gold standard for the measurement of free testosterone levels, which are only performed in very limited specialized centers. On the other hand, cFT is well correlated with the results of equilibrium dialysis.[35]

In conclusion, this study showed, for the first time in a biopsy-proven NAFLD population, that NAFLD patients had significantly lower SHBG levels and higher metabolically active testosterone fractions, despite having similar serum total testosterone and estradiol levels, compared to age-, gender- and weight-matched controls. Furthermore, serum SHBG levels, bioavailable testosterone and FAI, but not cFT, were separately associated with NAFLD independently of age, BMI and waist circumference. Importantly, serum estradiol levels were associated with the presence of NASH independently of age, BMI and waist circumference, whereas no other parameter was. Although a cross-sectional study cannot imply a cause-effect association, it could be speculated that low serum SHBG levels and high metabolically active testosterone fractions may represent some more factors contributing to the pathogenesis of NAFL, though not to the progression from NAFL to NASH. On the other hand, low estradiol levels may be a factor contributing to NASH progression. However, these results need further validation from large-scale studies.

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