RESEARCH

The hepatic lipidome and HNF4α and SHBG expression in human liver

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Abstract

Low plasma levels of sex hormone-binding globulin (SHBG) are a marker for obesity, insulin resistance, non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes. The transcription factor HNF4α is a major determinant of hepatic SHBG expression and thereby serum SHBG levels, and mediates in part the association of low SHBG with hyperinsulinemia and hepatic steatosis. We analyzed the lipidome in human liver specimens from a cohort of patients who underwent hepatic resection as a treatment for cancer, providing insight into hepatic lipids in those without extreme obesity or the clinical diagnosis of NAFLD or non-alcoholic steatohepatitis. Both steatosis and high HOMA-IR were associated with higher levels of saturated and unsaturated FA, other than arachidonic, with the most dramatic rise in 18:1 oleate, consistent with increased stearoyl-CoA desaturase activity. Individuals with low HOMA-IR had low levels of total hepatic fatty acids, while both low and high fatty acid levels characterized the high HOMA-IR group. Both insulin resistance and high levels of hepatic fat were associated with low expression levels of HNF4α and thereby SHBG, but the expression of these genes was also low in the absence of these determinants, implying additional regulatory mechanisms that remain to be determined. The relationship of all FA studied to HNFα and SHBG mRNAs was inverse, and similar to that for total triglyceride concentrations, irrespective of chain length and saturation vs unsaturation.

Key Words
- SHBG
- HNF4α
- fatty acids
- insulin resistance
- metabolic syndrome

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Introduction

Sex hormone-binding globulin (SHBG) is a 90–100 kDa homodimeric glycoprotein that transports testosterone and other steroids in the blood plasma, reduces their metabolic clearance, and regulates their access to target tissues (1). SHBG levels are lower with obesity (2) and in patients with type 2 (T2DM) but not type 1 diabetes (3, 4), and in those with the metabolic syndrome (MetS) (5). Moreover, a low level of SHBG is associated with an increased risk for developing MetS (6), gestational diabetes (7) and T2DM (8, 9, 10), and SHBG is often studied as an early biomarker for these disorders (11). In addition, obese, insulin-resistant patients with low SHBG levels often have fatty liver disease (12, 13).

However, the mechanism(s) linking low SHBG to these metabolic disorders remains incompletely understood. Early studies established a relationship between hyperinsulinemia and low SHBG (14). Polymorphisms in the SHBG gene (15) and experiments using SHBG-transgenic mice (16) have also implicated SHBG in the pathogenesis of T2DM and NAFLD.

Hepatocyte nuclear factor-4 (HNF4α) is a transcription factor that activates the promoters of multiple genes expressed in liver that function in lipid metabolism (17), and overexpression of HNF4α increased SHBG transcription in Hep-G2 hepatocarcinoma cells (18), suggesting that hepatic expression of HNF4α may underlie

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the metabolic associations found with circulating SHBG. Based on those findings, we studied human liver samples, and demonstrated a strong positive correlation between mRNA levels for HNF4α and SHBG, and found an inverse relationship with the amount of liver triglyceride (19). It is now known that HNF4α expression is reduced in rodents fed a high fat diet (20), and in liver samples from human patients with NASH (21).

Study of the crystallized ligand-binding domain of rat HNF4α revealed binding of long-chain FA, which were presumed to represent endogenous ligands (22, 23). Moreover, Hertz *et al.* found that radiolabeled FA CoA thioesters bound to HNF4α protein, and that the transcriptional activity of HNF4α in Cos-7 cells by long-chain fatty acid-CoAs varied by chain length, with activation by C:16 palmitate but suppression by C:18 stearoyl-CoA, while FA shorter than C16 were inactive (24). In other experiments using HepG2 cells, however, palmitoyl-CoA was reported to suppress HNF4α transcription (25). In the current research, we analyzed the lipidome in human liver specimens from the foregoing patients in order to better understand the relationship between insulin, insulin resistance by HOMA, and hepatic long chain fatty acids and the expression of HNF4α and SHBG in human liver. We hypothesized that both insulin resistance and total hepatic fat would be associated with lower levels of SHBG mRNA, and based on the results of Hertz *et al*. (24) there would be a positive association between SHBG mRNA and hepatic palmitate but an inverse relationship with stearate implying a direct role for FA in the control of SHBG expression.

Materials and methods

Subjects

Adult men ($n=25$) and women ($n=23$) undergoing hepatic resection as treatment for cancer were recruited for this study that was approved by the Institutional Board of the University of Louisville. Their diagnoses included colorectal carcinoma ($n=27$), hepatocellular carcinoma ($n=7$), renal cell cancer ($n=3$) and other ($n=11$). Subjects were ECOG performance status 0: fully active, and able to carry on all pre-disease performance without restriction. Subjects with other liver diseases, such as hepatitis C, were excluded, as were two women treated with oral estradiol and one with tamoxifen due to marked increases in SHBG. 19 patients had diabetes and 17 were being treated for dyslipidemia. The time from diagnosis to surgery was 1–5 weeks. During this time, there was a median change in weight of −5 lbs (range −20 to +10 lbs). Patients were not instructed to take nutritional supplements before surgery, and no patients received chemotherapy or X-irradiation. Following informed consent, the patient's medical history was reviewed and anthropometric data were collected, and a fasting blood sample was obtained in which glucose was measured in a biomedical panel and an aliquot was frozen at −70°C for the measurement of SHBG and insulin. After surgical resection of the liver specimen, tumor was separated for analysis by the pathologist, and normal liver as distant as possible from the tumor was frozen immediately in an effort to prevent changes due to warming, and stored at −70°C for subsequent study, or was stored in RNAlater (Life Technologies).

RNA isolation and real-time reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from liver tissue using RNAeasy columns (Qiagen), and analyzed for HNG4α and SHBG mRNAs by qPCR as described previously (19).

Immunoassays

SHBG levels were measured with an ELISA kit from ALPCO Diagnostics (Salem, NH, USA). Insulin was measured using an ELISA kit from Mercodia (Winston-Salem, NC, USA).

Lipid profiles

Tissue was homogenized in diluent (100 mg/0.4 mL) containing 10 µL/mL protease inhibitors (RIPA Lysis buffer, Santa Cruz Biotechnology), and samples were diluted 1:5 for assay. Free fatty acids in the tissue homogenates were analyzed at the Mouse Metabolic Phenotyping Center at the University of Cincinnati (NIH U24 DK059630). Samples were saponified and methylated for gas chromatography analysis. The extracted solution was injected into the GC and retention times were compared to those of known standards.

Data analysis

Data are presented as the mean ± S.D. or as the median and interquartile range when continuous variables were not normally distributed or had unequal variance, SigmaStat (San Jose, CA, USA). HOMA-IR was calculated as fasting insulin (mU/L) × glucose (mg/dL)/405. Student's *t*-test was used to assess differences between two groups with

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equal variance, and the Mann–Whitney rank-sum test to compare groups when a skewed distribution was found. ANOVA followed by Dunn's test was used to determine differences among multiple groups. Bivariate general linear regression models were performed to determine the association of liver fat chain length/saturation with HNF4α and SHBG mRNA expression levels. All statistical tests were two-sided and performed at the 0.05 level of significance .

Results

Normal liver samples surrounding tumor were used to quantitate a spectrum of long-chain fatty acids, and to determine their relationship to expression levels of HNF4α and SHBG. Liver samples from the individuals in this cohort contained a wide range of FA concentrations. The mean (±S.D.) FA level was 58.1 ± 55.1 mg/g liver, with a range of 17.5–251 mg/g. Total fatty acid concentrations were slightly higher in men (median 42.9 mg/g, interquartile range 29.9–80.5) than in women (median 31.3 mg/g, interquartile range (25.3–40.6). Three women and four men had levels >10% FA/g.

Twenty-eight FA was quantified in each sample. Of these, seven FA, three saturated and four non-saturated, accounted for 91.6% of total FA, and were studied further. The most abundant FA was oleic (18:1) and palmitic (16:0) followed by linoleic (18:2), stearic (18:0), arachidonic (20:4), palmitoleic (16:1) and myristic (14:0) acids (Table 1).

Subjects were divided into two groups based on the traditional biochemical diagnosis of steatosis of >5% fat content by weight ($n=17$) or controls ($n=29$). By this criterion 37% of this population had steatosis which is comparable to prior estimates by magnetic spectroscopy for United States urban adults (26). As summarized in Table 1 those subjects who met the criteria for steatosis had a higher BMI ($P < 0.001$) and HOMA-IR ($P = 0.017$). The concentrations of myristic, palmitic, stearic, palmitoleic, oleic and linoleic acids were all significantly higher ($P < 0.001$) in those with steatosis while the level of arachidonic acid was similar ($P = 0.15$) in the two groups.

Figure 1 compares the levels of each of the seven FA in the two patient groups as a percent of total liver triglyceride levels. From this perspective, four FA were increased (14:0, 16:0, 16:1 and 18:1) and three were lower (18:0, 18:2 and 20:4) in the steatosis group (all $P < 0.001$). The most prominent increase was in oleic 18:1, which accounted for 31% of total FA in the steatosis subjects. The ratios of oleic/stearic, and palmitoleic/palmitic (Table 1) were calculated as indices of stearoyl-CoA desaturase activity (27). Both ratios were higher ($P < 0.001$) in the steatosis subjects, although the fold increase was 3.80 for the 18:0 FA compared to 2.2-fold for the 16:0 FA pair, consistent with the greater activity of stearoyl-CoA desaturase for stearic than palmitic as substrate,

Table 1 Clinical characteristics and hepatic fatty acid composition in individuals with steatosis compared to the low-fat group.

	Total cohort ($n=48$)	**<5% fat content** ($n=32$)	**>5% fat content** ($n=16$)	***P* value**
Age (years)	61.8 ± 11.2	62.6 ± 11.8	58.9 ± 9.7	0.29
Sex	21F/25M	17F/15M	6F/10M	0.475
BMI (kg/m^2)	28.9 ± 6.4	26.6 ± 5.4	33.2 ± 6.0	<0.001
HOMA-IR	2.49 ± 2.17	1.99 ± 0.36	3.62 ± 0.57	0.017
Total fat (mg/g)	58.1 ± 55.1	29.9 (25.7–38.1)	98.1 (54.0–196)	<0.001
Myristic 14:0 mg/g	0.78 ± 1.3^{d}	0.105 (0.065–0.23)	1.91 (0.61–3.43)	<0.001
Palmitic 16:0 mg/g	15.5 ± 17.0^{a}	6.80 (5.38–9.34)	31.7 (13.8–51.1)	<0.001
Palmitoleic 16:1 mg/g	1.75 ± 2.60^{cd}	0.28 (0.18–0.58)	3.52 (1.01–6.90)	<0.001
Stearic 18:0 mg/g	4.96 ± 1.74^{b}	4.08 (3.51–4.83)	6.05 (5.26–8.50)	<0.001
Oleic 18:1 mg/g	16.2 ± 21.6^{ab}	4.88 (3.57–7.19)	31.8 (15.6–67.9)	<0.001
Linoleic 18:2 mg/g	10.7 ± 9.0^{a}	6.42 (5.60–7.40)	14.3 (10.3–30.5)	<0.001
Arachidonic 20:4 mg/g	3.31 ± 0.91^{c}	3.41 (2.67–3.87)	2.86 (2.38–3.70)	0.151
16:1/16:0	0.072 ± 0.049	0.04 (0.031–0.065)	0.095 (0.074–0.145)	<0.001
18:1/18.0	2.63 ± 2.30	1.23 (0.90–1.75)	5.44 (3.29–7.57)	<0.001
SHBG (nmol/L)	80.4 ± 51.3	73.4 (48.1–96.2)	48.1 (35.9–90.9)	0.07
SHBG mRNA (×10^6) copies/μg RNA	1.04 ± 0.63	1.05 ± 0.66	0.79 ± 0.50	0.048
HNF4α mRNA (×10^7) copies/μg RNA	1.19 ± 0.86	1.10 (0.68–1.75)	0. 97 (0.35-1.41)	0.19

Results represent the mean ± S.D., or the median and interquartile range. FA that share a common superscript are p=NS. *P* values compare subgroup with <5% and >5% fat content.

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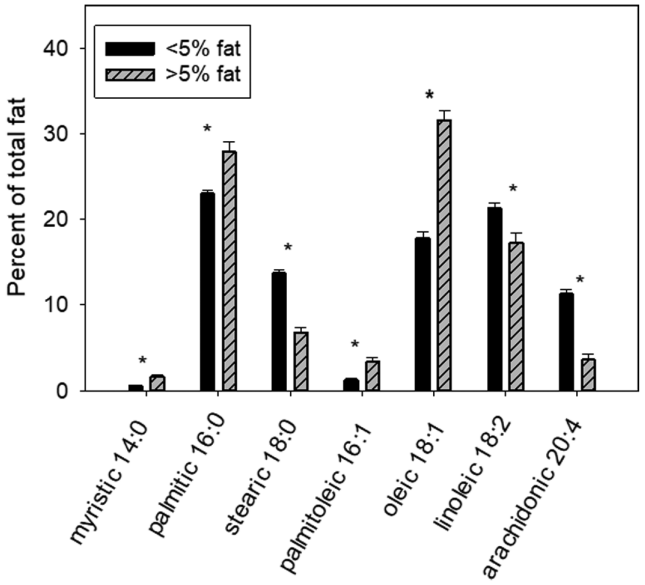

Figure 1
Liver composition of fatty acids represented as percent of total fatty acids measured in individuals with less than or greater than 5% liver fat. Percent myristic (14:0), palmitic (16:0), palmitoleic (16:1) and oleic (18:1) were higher, while stearic (16:0), linoleic (18:2) and arachidonic (20:4) were lower in subjects with NAFLD. *$P < 0.01$.

based on the partially purified enzyme from rat liver microsomes (28).

The relationship between hepatic fat and HOMA-IR is shown in Fig. 2. It is clear that a low HOMA-IR was a determinant of low hepatic triglycerides. On the other hand, the hepatic triglyceride concentration was highly variable in insulin-resistant subjects among whom 6/23 were being treated with a lipid-lowering drug. Nevertheless, total hepatic fat was significantly ($P = 0.014$) greater with insulin resistance when subjects were divided into two groups based on HOMA-IR (Table 2). As with the analysis by steatosis, all saturated and unsaturated FA analyzed, other than arachidonic (20;4) were higher in the high HOMA-IR group. The ratios of oleic/stearic and palmitoleic/palmitic were also higher in the high HOMA group, with a higher ratio for the 18 than for the 16 carbon FA pairs. Individuals with high HOMA-IR also had lower levels of SHBG and HNF4α mRNAs in liver.

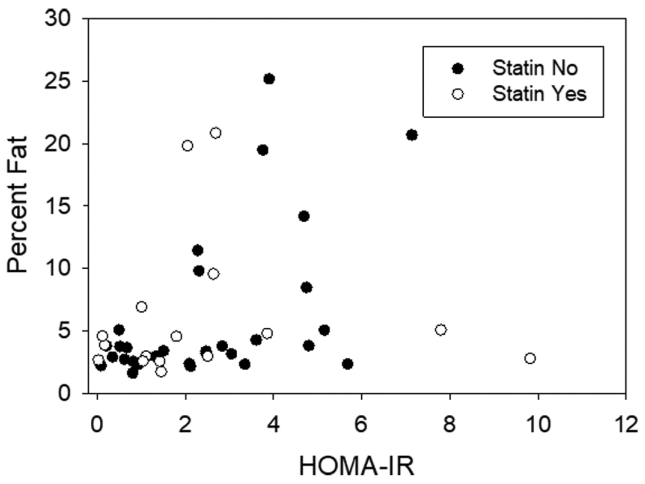

Figure 2
Relationship between percent hepatic fat and HOMA-IR. Subjects being treated with statins are indicated by open circles. Insulin resistance by HOMA-IR was found in the absence or presence of steatosis, whereas total lipid levels were low with insulin sensitivity.

Figure 3 illustrates the relationship between SHBG and HNF4α mRNAs and hepatic triglyceride concentrations for the low HOMA-IR and high HOMA-IR subgroups (A), and with HOMA-IR for those with TGA <5% vs >5% (B). Subjects with >50 mg/g hepatic triglyceride had lower levels of SHBG and HNF4α mRNAs; however, low mRNA levels were also found in liver without steatosis. Furthermore, most individuals with HOMA-IR >2.5 had low SHBG and HNF4α mRNA concentrations, but low mRNA levels were also found in individuals with HOMA-IR <2.5 as well as TGA < 5%.

Each of the lipid signatures was next compared with SHBG and HNF4α mRNA levels. Figure 4 illustrates the relationships between myristic 14:0, palmitic 16:0, stearic 18:0 and oleic 18:1 with SHBG mRNA. In each case, high FA levels were associated with suppressed SHBG as well as HNF4α mRNAs, whereas low HNF4α and SHBG mRNAs were also found in individuals without high levels of these fatty acids. As summarized in Table 3, in unadjusted models, levels of FA of 14–18 carbons were inversely associated with SHBG and HNF4α expression. In contrast to our initial hypothesis, in each case, irrespective of chain length and saturation vs unsaturation, the relationship to HNFα and SHBG mRNAs was inverse and similar to that for total triglyceride concentrations.

Discussion

This research examined the relationships between hepatic triglycerides, insulin resistance and the expression levels of SHBG and HNF4α in surgical human liver samples in part to determine if a unique relationship exists based on fatty acid chain length or saturation. To the best of our knowledge, we also provide the largest quantitative assessment of the human hepatic lipidome for individuals who do not have a clinical diagnosis of NAFLD or NASH, or did not undergo liver biopsy because of extreme obesity

Table 2 Hepatic fatty acid composition, and HNF4α and SHBG mRNA levels based on HOMA-IR.

	HOMA-IR		*P* value
	0.3–2.09 ($n = 23$)	2.12–9.82 ($n = 23$)	
	0.82 (0.35–1.42)	3.61 (2.51–4.80)	0.001
Total fat (mg/g)	29.8 (25.8–38.8)	48.1 (21.8–114)	0.009
Myristic 14:0 mg/g	0.105 (0.065–0.395)	0.598 (0.150–2.08)	0.006
Palmitic 16:0 mg/g	6.79 (5.23–9.43)	11.7 (6.95–39.0)	0.012
Palmitoleic 16:1 mg/g	0.284 (0.161–0.700)	1.17 (0.747–3.62)	0.002
Stearic 18:0 mg/g	4.12 (3.51–5.00)	5.24 (3.72–6.70)	0.033
Oleic 18:1 mg/g	4.88 (3.50–9.55)	12.6 (6.28–34.0)	0.005
Linoleic 18:2 mg/g	6.42 (5.50–8.03)	9.60 (6.36–22.6)	0.016
Arachidonic 20:4 mg/g	3.57 (2.90–3.91)	2.72 (2.45–3.33)	0.047
Oleate 18:1/stearic 18:0	1.25 (0.90–2.04)	2.65 (1.54–6.00)	0.005
Palmitoleic 16:1/palmitic 16:0	0.044 (0.031–0.067)	0.086 (0.051–0.118)	0.002
HNF4α mRNA × 10^7	1.64 ± 0.98	0.80 ± 0.57	0.001
SHBG mRNA × 10^6	1.29 ± 0.59	0.78 ± 0.61	0.006

Results are mean ± s.d., or median and interquartile range. Total subjects are 46 due to missing fasting insulin levels.

in conjunction with gastric bypass surgery. We found that both insulin resistance based on high HOMA-IR, and hepatic steatosis, are associated with low HNF4α and SHBG mRNA levels, but low levels were also found in the absence of these regulators, implying that other mechanisms also lead to suppressed expression of these genes. In addition, neither chain length nor saturation influenced substantially the relationship between long-chain FAs with SHBG or HNF4α mRNAs. These results extend the findings of Luo *et al.* (29) and Sáez-Lopez *et al.* (30), who reported that SHBG mRNA and protein levels correlate negatively with hepatic triglyceride content in patients with benign hepatic tumors or obese patients with NAFLD undergoing bariatric surgery, respectively, and suggest that down-stream signaling by increased hepatic fatty acids, rather than the fatty acid *per se*, is responsible for the low levels of SHBG in subjects with metabolic syndrome.

This study provides data from a unique patient cohort in which results for subjects fulfilling the traditional criteria for NAFLD of >5% hepatic fat were compared to a comparable cohort with <5% hepatic fat. The BMI of those with steatosis was significantly higher. Steatosis was not found in subjects with normal HOMA-IR, whereas excess hepatic triglyceride accumulation was found in some but not all those with insulin resistance. Both treatment with statins (31), and the impact of dietary fructose and fat (32), which were not assessed, may have contributed to these results.

As in previous studies (33, 34, 35), the predominant FA in liver was palmitic (16:0) followed by oleic (18:1) and

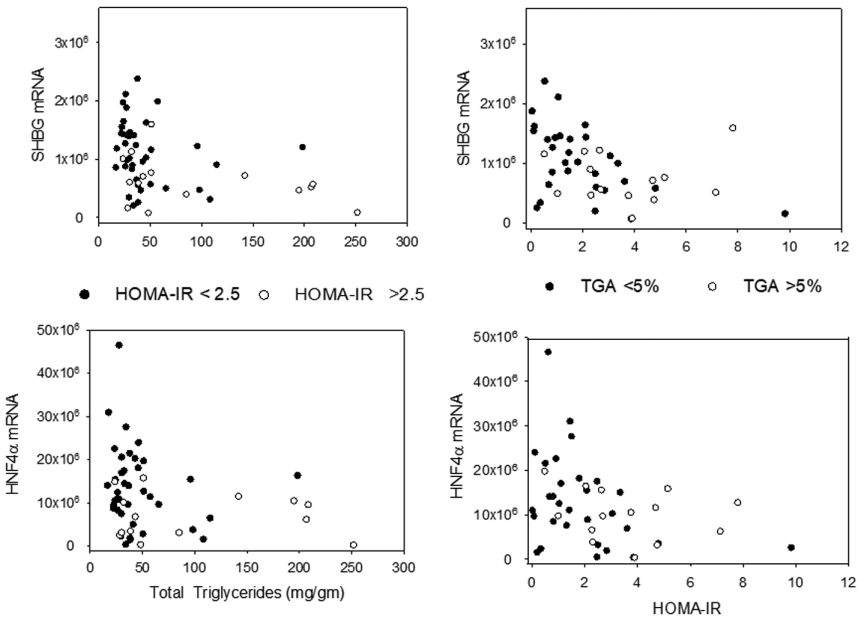

Figure 3
Relationship between total triglycerides and SHBG (A) and HNF4α (B) mRNA levels, and between HOMA-IR and SHBG (C) and HNF4α mRNA (D) levels. In A and B those with HOMA-IR of < or >2.5 are noted while in C and D those with hepatic triglyceride of < or >5% are shown. There is a curvilinear relationship between these variables, with high HOMA-IR or hepatic triglycerides associated with lower HNF4α and SHBG mRNAs. It is noteworthy that certain individuals have low SHBG or HNF4α mRNA levels with <5% hepatic triglycerides as well as HOMA-IR <2.5.

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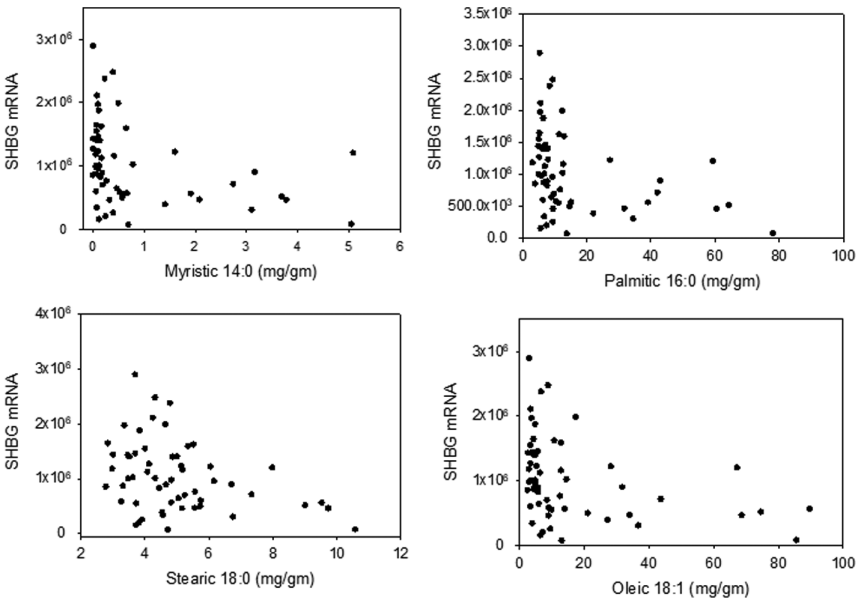

Figure 4
Relationship between hepatic FA and SHBG mRNA concentrations. Spearman correlation coefficients relating hepatic FA levels to SHBG mRNA were: myristic 14:0 ($r = -0.344$; $P = 0.009$), palmitic 16:0 ($r = -0.356$; $P = 0.007$), stearic 18:0 ($r = -0.345$; $P = 0.009$) and oleic 18:1 ($r = -0.354$; $P = 0.007$), as well as (not shown) linoleic 18:2 ($r = -0.28$, $P = 0.036$); palmitoleic 16:1 ($r = -0.354$; $P = 0.007$) and arachidonic ($r = -0.113$, $P = 0.409$). Neither type of FA, chain length nor saturation was found to have a unique relationship to the expression level of SHBG.

stearic (18:0). The concentrations of myristic, palmitic, stearic, palmitoleic, oleic and linoleic acids were all significantly higher in those with steatosis while the level of arachidonic acid in the two groups was similar perhaps because arachidonic acid is not metabolically determined but rather is derived from the dietary essential linoleic acid through Δ6-desaturation (36) and is esterified in membrane phospholipids (37). With steatosis, there was a striking increase in oleic 18:1, the product of stearoyl-CoA reductase, which accounted for 31% of total FFA among the steatosis group.

Because of lipotoxicity (38), several previous studies have carefully examined the spectrum of FA in liver in NAFLD and NASH patients, and controls, but with variable results. Araya *et al.* (39) studied liver from 19 patients undergoing bariatric surgery (10 with NAFLD and 9 with NASH), and 11 patients undergoing anti-reflux surgery as controls. While total triglycerides and levels of 16:0 and 18:1 were high in NAFLD and NASH subjects, 18:0 and 18:2 were similar while 14:0 and 20:4 were lower in patients than controls. Some subjects had consumed a 25 kcal/kg diet before the surgery. Puri *et al.* (33) performed a core needle liver biopsy in individuals diagnosed with NAFLD ($n=9$) or NASH ($n=9$). The control group ($n=9$) was undergoing abdominal surgery with no symptoms or signs of liver disease, but were noted to be obese with the metabolic syndrome. They found increased total lipids, diacyglycerol and triacylglycerol in the NAFLD and NASH groups, but no differences in the levels of individual saturated or non-saturated FFA among their study groups. Allard *et al.* (34) studied 73 patients undergoing liver biopsy for elevated liver enzymes and suspected NAFLD. Of these, 17 with normal liver biopsies and <5% steatosis were designated controls, but they had similar BMI, waist circumference and percent diabetes as those diagnosed with NAFLD by biopsy. They reported FA composition as percent of total FA, and found higher values for 16:1 and 18:1 only in those with NASH, but no difference in 16:0 and 18:0, and like our findings, lower percentage of 20:4 than in those without steatosis. Lukkonenn *et al.* (40)

Table 3 Relation of liver fat chain length/saturation with SHBG and HNF4α expression levels.

	SHBG		HNF4α	
	Standardized beta coefficients	*P* value	Standardized beta coefficients	*P* value
Unadjusted				
Oleic 18:1	−0.36061	0.0101	−0.23114	0.1063
Palmitic 16:0	−0.35985	0.0103	−0.24728	0.0834
Stearic 18:0	−0.35060	0.0126	−0.22461	0.1168
Palmitoleic 16:1	−0.35004	0.0127	−0.26400	0.0639
Myristic 14:0	−0.34976	0.0128	−0.25287	0.0764
Linoleic 18:2	−0.29208	0.0396	−0.17212	0.2320
Arachidonic 20:4	0.13327	0.3562	0.22717	0.1126

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studied 125 adults undergoing laparoscopic bariatric surgery and divided them into low vs high HOMA-IR subgroups. Hepatic concentrations of palmitic, stearic and oleic FA were all statistically higher in the high HOMA group. Kumashiro *et al.* (35) found higher levels of all hepatic FA measured in subjects with high HOMA-IR, but the numbers of subjects was small (4/group), and the between-group difference were not statistically significant. Not only sample size, but also differences in diet and control groups, as well as methodological details are among the factors contributing to these variable results.

Stearoyl-CoA desaturase (SCD) is the rate-limiting enzyme in the bioconversion of saturated to monounsaturated FA (41). We found a marked rise in oleic 18:1 in our steatosis patients among whom it represented 31.6% of total hepatic fat. In agreement with the results from Kotronen *et al.* (42), the ratio of product to precursor 18:1–18:0, as well as 16:1–16:0, as measures of SCD activity, were higher in our subjects with hepatic steatosis, as well as those with high HOMA-IR, with higher values for the 18:1/18:0 pair, the preferential substrate. Stefan *et al.* (43) did not find up-regulation of SCD-1 expression or activity in subjects with fatty liver or insulin resistance while Peter *et al.* (44) found that SCD1 mRNA levels in human liver samples obtained at surgery for a variety of clinical conditions were positively associated with the ratio of 16:1–16:0 in the VLDL triglyceride fraction separated by TLC but not with the ratio 18:1–18:0. Our data support the notion that insulin resistance and hepatic steatosis in humans are associated with increased SCD activity.

HNF4α is an orphan nuclear receptor that is a master regulator of liver development and function, including genes involved in triacylglycerol, cholesterol and lipoprotein metabolism (17, 45), and is thought to play a central role in fatty liver disease (46). Our results reveal suppressed HNF4α expression not only with steatosis but also with insulin resistance. While the direction of causality between variables cannot be determined from a cross-sectional study, our results support the notion of a feed-forward mechanism in which hepatic HNF4α expression is decreased by the steatosis that results from genetically determined insulin resistance in adipose tissue leading to increased plasma and hepatic FA (47, 48), and reduced HNF4α expression, together with hepatokines, increase liver fat which worsens insulin resistance (49). This sequence is further amplified inasmuch as HNF4α regulates its own level of expression (50).

Many studies have shown that NALFD in men and women is associated with low serum SHBG levels (51). Fatty acids and their lipotoxic metabolites increase the production of cytokines, including TNF, IL-6 and IL-1b, which initiate the production of pro-inflammatory signals, including nuclear factor-κB (NFκb) and c-jun n-terminal kinase (JNK) (52). Selva and colleagues, using HepG2 hepatocarcinoma cells, showed that TNFα suppresses SHBG expression by decreasing HNF4α through a mechanism involving NF-κB (53), and that IL1β reduces SHBG mRNA through HNF-4α via the MAPK kinase-1/2 and JNK signaling pathways (54), and we reported a strong positive correlation between the expression levels of HNF4α and SHBG using the human liver samples in this study (19). We then considered the possibility that fatty acids might also regulate HNF4α and thereby SHBG directly since fatty Acyl CoAs have been reported to stimulate or inhibit HNF4α transactivation depending on chain length and saturation (24). Our findings show, however, that a high fat content in human liver samples is associated with low levels of SHBG and HNF4α mRNAs irrespective of chain length and saturation.

The association of high HOMA-IR with reduced HNF4α and SHBG expression in human liver support and extend the association of high insulin or c-peptide levels with low serum SHBG levels in patients (55), and with experimental evidence linking insulin resistance with hyperinsulinemia to low levels of SHBG by suppressing HNF4α. Specifically, Xie *et al.* (56) showed that HNFα mRNA in liver is decreased in diabetic hyperinsulinemic db/db mice but not in mice rendered diabetic by streptozotocin-induced hypoinsulinemia, and that insulin inhibits HNF4α expression by stimulating transcription of SREBP. Thus both high insulin and hepatic fat down-regulate HNF4α and thereby SHBG expression through distinct mechanisms.

Several limitations of the current study should be acknowledged. First, liver samples were obtained from patients with cancer, and although their clinical performance was ECOG grade 0 – fully active – and they had not received chemo- or radiation-therapy, some impact of their cancer diagnosis or self-management is possible. As a cross-sectional correlative study, the direction of causality cannot be proven. Gas chromatography was used to separate and quantify FA, and quantification by mass spectrometry may be more sensitive and accurate (57). While the tissue samples studied were at least 1 cm^3 in volume, the lipid content of the liver is not homogeneous, and the distribution of FA differs within lipid droplets and outside of steatotic vesicles (58). DAG within lipid droplets was the strongest predictor of insulin resistance in the study by Kumashiro *et al.* (35).

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In summary, we describe the distribution of fatty acids in surgical liver specimens from subjects with a wide range of values and insulin sensitivity by HOMA-IR. Because of ethical considerations, most previous studies of human liver have been performed in obese subjects undergoing bariatric surgery or in those with the diagnosis of NAFLD or NASH. Our purpose was to further determine how hepatic FA and insulin resistance might regulate HNF4α expression in humans, and thereby SHBG, a plasma biomarker for metabolic syndrome, T2DM, and NALFD. We provide evidence that FA and insulin resistance are both important determinants of HNF4α and thereby SHBG expression, but show that other yet to be discovered factors appear to also cause low SHBG levels. Neither FA side chain length nor saturation altered these relationships.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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