Bioscience Reports (2018) **38** BSR20171274
https://doi.org/10.1042/BSR20171274

PORTLAND PRESS

Research Article

Serum, liver and bile sitosterol and sitostanol in obese patients with and without NAFLD

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Background and aims: Non-alcoholic fatty liver disease (NAFLD) associates with low levels of serum plant sterols in cross-sectional studies. In addition, it has been suggested that the hepatic sterol transport mechanisms are altered in NAFLD. Therefore, we investigated the association between serum, liver and bile plant sterols and sitostanol with NAFLD.
Methods: Out of the 138 individuals (age: 46.3 ± 8.9, body mass index: 43.3 ± 6.9 kg/m^2, 28% men and 72% women), 44 could be histologically categorized to have normal liver, and 94 to have NAFLD. Within the NAFLD group, 28 had simple steatosis and 27 had non-alcoholic steatohepatitis. Plant sterols and sitostanol were measured from serum (n=138), liver (n=38), and bile (n=41). The *mRNA* expression of genes regulating liver sterol metabolism and inflammation was measured (n=102).
Results: Liver and bile sitostanol ratios to cholesterol were higher in those with NAFLD compared to those with histologically normal liver (all $P<0.022$). Furthermore, liver sitostanol to cholesterol ratio correlated positively with histological steatosis and lobular inflammation ($r_s > 0.407$, $P<0.01$ for both). In contrast, liver sitosterol to cholesterol ratio correlated negatively with steatosis ($r_s = -0.392$, $P=0.015$) and lobular inflammation ($r_s = -0.395$, $P=0.014$). Transcriptomics analysis revealed suggestive correlations between serum plant sterol levels and mRNA expression.
Conclusion: Our study showed that liver and bile sitostanol ratios to cholesterol associated positively and liver sitosterol ratio to cholesterol associated negatively with liver steatosis and inflammation in obese individuals with NAFLD..

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver injury in Western countries [1]. NAFLD can present as simple steatosis, but it can also proceed into nonalcoholic steatohepatitis (NASH), and ultimately to liver fibrosis and cirrhosis [2]. Currently, the mechanisms regulating the progression from steatosis to NASH are poorly defined.

NAFLD associates with low levels of serum plant sterols in cross-sectional studies [3,4] and plant sterols are suggested to prevent the progression of NAFLD [5]. Plant sterols and plant stanols are normal components of plants. They cannot be synthesized in humans and are therefore completely derived from food. The most frequent plant sterols present in humans are campesterol, sitosterol and avenasterol, and the most frequent plant stanol is sitostanol [6]. Thus, the serum levels of plant sterols, especially as ratios to serum cholesterol concentration, are used as biomarkers of cholesterol absorption efficiency [7-9]. Accordingly, their low serum levels reflect decreased intestinal absorption of sterols, e.g. in insulin resistant states [10] including NAFLD and NASH [3,4].

Received: 20 August 2017
Revised: 08 March 2018
Accepted: 12 March 2018

Accepted Manuscript Online: 14 March 2018
Version of Record published: 20 April 2018

Bioscience Reports (2018) **38** BSR20171274
https://doi.org/10.1042/BSR20171274

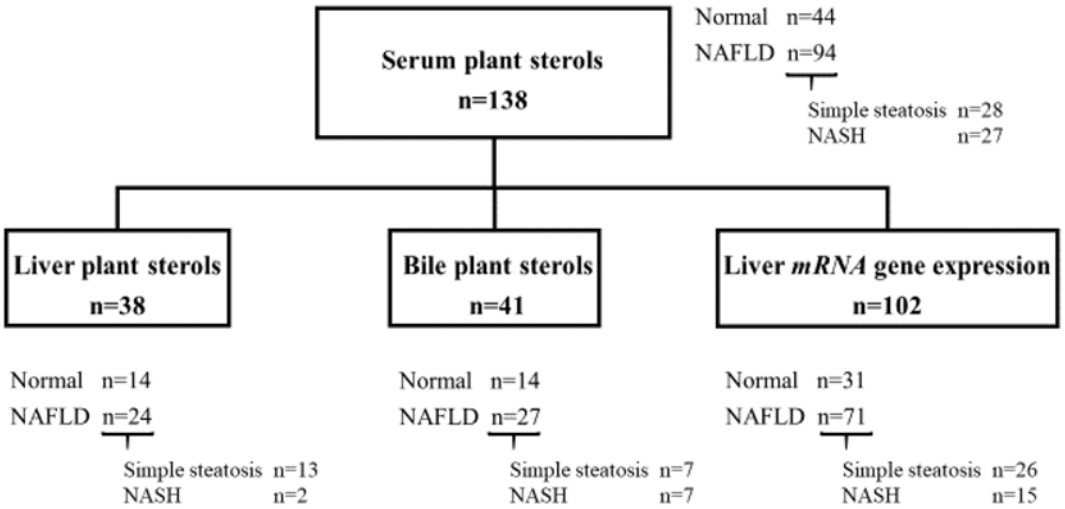

Figure 1. A chart demonstrating the study subjects in groups that had serum, liver and bile measurements of plant sterols and liver mRNA expression available

Of the original cohort of 150 subjects that had serum plant sterol measurements available, a distinct liver phenotype could be recognized in 138 subjects [normal liver (normal, *n*=44) and nonalcoholic fatty liver disease (NAFLD, *n*=94)]. Of those in NAFLD group, 28 had simple steatosis and 27 had nonalcoholic steatohepatitis (NASH). Of the 138 subjects who had serum plant sterol measurements, liver (*n*=38) and bile (*n*=41) sterol measurements, and liver mRNA expression (*n*=102) were performed. Liver and bile sterol measurements were from different subjects.

Absorption of sterols from the small intestine and biliary excretion from the liver and bile are regulated by transporter genes Niemann–Pick C1-Like 1 (*NPC1L1*), ATP-Binding Cassette, Subfamily G, Member 5 (*ABCG5*), and ATP-Binding Cassette, Subfamily G, Member 8 (*ABCG8*) [11,12]. For example, *ABCG5/8* deficiency reduces cholesterol excretion from the liver into the bile [13-15] and increases cholesterol absorption in mice [15] and in humans [14]. On the other hand, normally functioning NPC1L1 transporter located at the hepatic canalicular membranes actively transports sterols into hepatocytes [16]. Interestingly, liver protein expression of ABCG8 and ABCG5 has been suggested to be higher and expression of NPC1L1 to be lower in those with steatosis and NASH compared to those with normal liver [17,18]. On the other hand, both the mRNA and protein expression of ABCG8 has been reported to be lower in those with NAFLD or NASH than in those with normal liver [19]. Taken together, these results suggest a link between altered sterol/stanol export mechanisms and NAFLD.

To clarify the mechanisms for altered plant sterol and plant stanol metabolism in NAFLD and NASH, we investigated serum, liver and biliary plant sterol (campesterol, sitosterol, and avenasterol) and sitostanol levels in 138 obese individuals participating in the Kuopio Obesity Surgery Study (KOBS).

Materials and methods

Subjects

All patients undergoing obesity surgery in Kuopio University Hospital are recruited into our ongoing study investigating the metabolic consequences of obesity surgery (Kuopio Obesity Study, KOBS) [20,21].

The study group included 138 individuals from the KOBS [mean age: 46.3 $\pm$ 8.9, body mass index (BMI): 43.3 $\pm$ 6.9 kg/m^2, 38 (28%) men and 100 (72%) women], of whom the measurements of serum plant sterols were available and the histological liver phenotype was either normal or NAFLD. Subjects using cholesterol lowering medications were excluded. Forty-four of the 138 participants had histologically normal liver and 94 had NAFLD. From those who had NAFLD, 28 had simple steatosis and 27 had NASH, and the remaining 39 participants with NAFLD had an intermediate phenotype between simple steatosis and NASH and were thus excluded from the study groups with specified phenotypes (Figure 1). Plant sterols and sitostanol were measured from serum (*n*=138), liver (*n*=38), and bile (*n*=41). The mRNA expression of genes *NPC1L1*, *ABCG5* and *ABCG8,* and several other genes regulating inflammation and lipid metabolism in the liver, was measured from liver samples of 102 individuals (Figure 1).

The study protocol confirms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a prior approval by the institution's human research committee, and has been approved by the Ethics

Committee of the Northern Savo Hospital District (54/2005, 104/2008, and 27/2010). Written informed consent was obtained from each patient included in the study.

Laboratory measurements

Cholesterol and triglycerides from serum were assayed by an automated enzymatic method (Roche Diagnostics, Mannheim, Germany), as described before [10,21]. Plant sterols (campesterol, sitosterol, and avenasterol) and sitostanol were measured in serum (n=138), liver (n=38), and bile (n=41) by gas–liquid chromatography (GLC) with a 50 m long capillary column (Ultra 2; Agilent Technologies, Wilmington, DE) as described earlier [21] with 5α-cholestane as the internal standard. To standardize the varying cholesterol levels, the plant sterols and sitostanol concentrations in serum, liver, and bile are presented as ratios to cholesterol by dividing the plant sterol and sitostanol concentrations with the respective cholesterol concentration of the same GLC run. Dietary phytosterol intake (DPI) was considered by calculating the ratio serum campesterol/cholestanol [22]. The serum plant sterol and sitostanol values are expressed as 10^2 mmol/mol cholesterol (the multiplication with 10^2 was used to reduce the decimals), those of liver as μg/100 mg of liver cholesterol, and those of bile as μg/100 mg of cholesterol respectively.

Liver biopsies, bile samples and histological study groups

Liver biopsies were obtained using Trucut needle (Radiplast AB, Uppsala, Sweden) or with the ultrasonic scissors during elective laparoscopic Roux-en-Y gastric bypass (RYGB) operation. Overall the histological assessment of liver biopsy samples was performed by one pathologist according the standard criteria [23,24]. According to histology, patients were divided into two main study groups: normal liver (no steatosis, inflammation, ballooning, or fibrosis) and NAFLD (>5% of the hepatocytes have lipid droplets). From those who had NAFLD, a subdivision was possible for simple steatosis (>5% steatosis without inflammation, ballooning, or fibrosis) and NASH, as previously described [25]. Thirty-nine subjects could not be categorized to specified phenotypes with simple steatosis and NASH (Figure 1). However, all study subjects were included in correlation analyses (Table 2). Bile sample was taken transhepatically from the gall bladder during the operation with a fine needle aspiration.

Liver gene expression

All samples for gene expression analysis were immediately frozen in liquid nitrogen. Total RNA from the liver tissue was extracted using Tri-Reagent (Applied Biosystems [ABI] Foster City, CA) and reverse-transcribed using the High Capacity cDNA Reverse Transcriptional KIT (ABI) according the manufacturer's protocol. Quantitative real-time polymerase chain reaction (PCR) was carried out with the Applied Biosystems 7500 Real Time PCR System using KAPA SYBR FAST qPCR Universal Master Mix (KAPA Biosystems, Woburn, MA). Primers are listed in Supplementary Table S1. Relative expression was normalized to *RPLP0*. A gene panel of TruSeq Targeted RNA Expression (TREx) platform with MiSeq system (Illumina, San Diego, CA, U.S.A.) was also used for measuring gene expression levels in the liver at baseline of the KOBS study, as previously described [25].

For the TREx analysis, total RNA from the liver (150 ng) was reverse-transcribed using the ProtoScript II Reverse Transcriptase (New England BioLabs). The oligo pool targeted regions of interest were hybridized to cDNA. Next, hybridized cDNA was extended by DNA polymerase followed by ligation using DNA ligase. The extension–ligation products were amplified with PCR and AMPure XP beads (Beckman Coulter) were used to clean up the PCR products. Equal volumes of the products were pooled together and quantitated with DNA 1000 chip (Agilent Technologies). Finally, the pooled sample was diluted, denatured, and sequenced with MiSeq.

Statistical analysis

All analyses were conducted via IBM SPSS Statistics for Windows, Version 21, (Armonk, NY: IBM Corp). Data are presented as mean ± standard deviation (SD). Differences between the study groups were examined by the $\chi 2$ (in categorical variables) and by nonparametric Kruskal–Wallis test (continuous variables). The Spearman rank correlation was used for correlation analysis. For the TREx analysis, the expression levels for each gene per sample in the gene panel were normalized based on the total number of aligned reads of the corresponding sample.

Results

Clinical characteristics

Table 1 demonstrates characteristics of the 138 participants (38 men and 100 women) in the study groups with normal liver and NAFLD. Age and BMI did not differ between the groups. Serum alanine aminotransferase (ALT) (P=0.007), fasting plasma glucose, and insulin levels were higher in those with NAFLD compared to those with normal liver

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Table 1 Clinical characteristics (mean ± SD) of study subjects divided to those with normal liver and nonalcoholic fatty liver disease (NAFLD)

	Normal liver 44	NAFLD 94	*P* over the groups
Gender (male/female)	12/32	26/68	0.962
Age (years)	44.2 ± 8.4	47.2 ± 9.0	0.069
Body mass index (kg/m^2)	43.5 ± 5.7	43.3 ± 7.4	0.911
ALT (U/L)	39.7 ± 28.3	54.3 ± 34.7	**0.007**
Fasting glucose (mmol/l)	5.7 ± 0.8	6.8± 2.3	**0.001**
Fasting insulin (mU/l)	14.2 ± 7.3	22.0 ± 11.9	**0.0004**
Total cholesterol (mmol/l)	4.4 ± 0.7	4.5 ± 1.0	0.831
HDL cholesterol (mmol/l)	1.1 ± 0.3	1.1 ± 0.3	0.987
LDL cholesterol (mmol/l)	2.7 ± 0.6	2.7± 0.9	0.805
Total triglycerides (mmol/l)	1.5 ± 0.6	1.6 ± 0.7	0.204
DPI*(dietary phytosterol intake)	0.96 ± 0.4	0.98 ± 0.4	0.971

$P<0.05$ compared with normal liver; *DPI (dietary phytosterol intake, serum campesterol to cholestanol ratio).

Table 2 Spearman correlations of serum and liver plants sterols and sitostanol (ratio to total cholesterol) with liver histology

	Steatosis grade	Fibrosis stage	Lobular inflammation	Ballooning
Serum (*n*=138)				
Campesterol	−0.025	−0.002	−0.025	0.092
Sitosterol	−0.027	0.029	−0.028	0.159
Avenasterol	0.092	0.099	0.086	0.128
Sitostanol	0.100	0.026	0.098	0.024
Liver (*n*=38)				
Campesterol	0.013	0.137	−0.052	0.119
Sitosterol	**−0.392***	−0.097	**−0.395***	0.054
Avenasterol	0.041	0.086	−0.025	−0.107
Sitostanol	**0.650†**	0.215	**0.407***	0.059

Significant correlations are bolded, *$P<0.05$, †$P<0.01$.

($P<0.001$). DPI was not different between the study groups (Table 1). The characteristics of study subjects in subgroups that had plant sterol and plant stanol measurements available from liver (n=38) and bile (n=41) are shown in Supplementary Table S2.

Serum plant sterols and sitostanol do not associate with liver histology

Serum plant sterols and sitostanol ratios to cholesterol did not differ between the study groups (Supplementary Figure S1). Accordingly, serum levels of plant sterols and sitostanol did not correlate with histological parameters (Table 2).

Liver sitosterol and sitostanol ratios to cholesterol associate with liver steatosis and inflammation

Liver sitosterol ratio to cholesterol was lower and that of liver sitostanol was higher in those subjects with NAFLD compared to individuals with normal liver (P=0.049 and P=0.004) (Figure 2). Accordingly, liver sitosterol ratio to cholesterol correlated inversely with steatosis and lobular inflammation ($r_s < -0.392$, $P<0.015$ for both), whereas liver sitostanol ratio to cholesterol correlated positively with liver steatosis and inflammation ($r_s > 0.407$, $P < 0.011$ for both) (Table 2). Liver avenasterol and campesterol ratios to cholesterol did not associate with NAFLD (data not shown), nor did they correlate with steatosis or inflammation (Table 2). Liver and serum campesterol, sitosterol, and avenasterol ratios to cholesterol correlated with each other (n=38, r_s = 0.544–0.488, $P<0.02$ for all), but liver and serum sitostanol ratios to cholesterol did not correlate with each other (Supplementary Table S3).

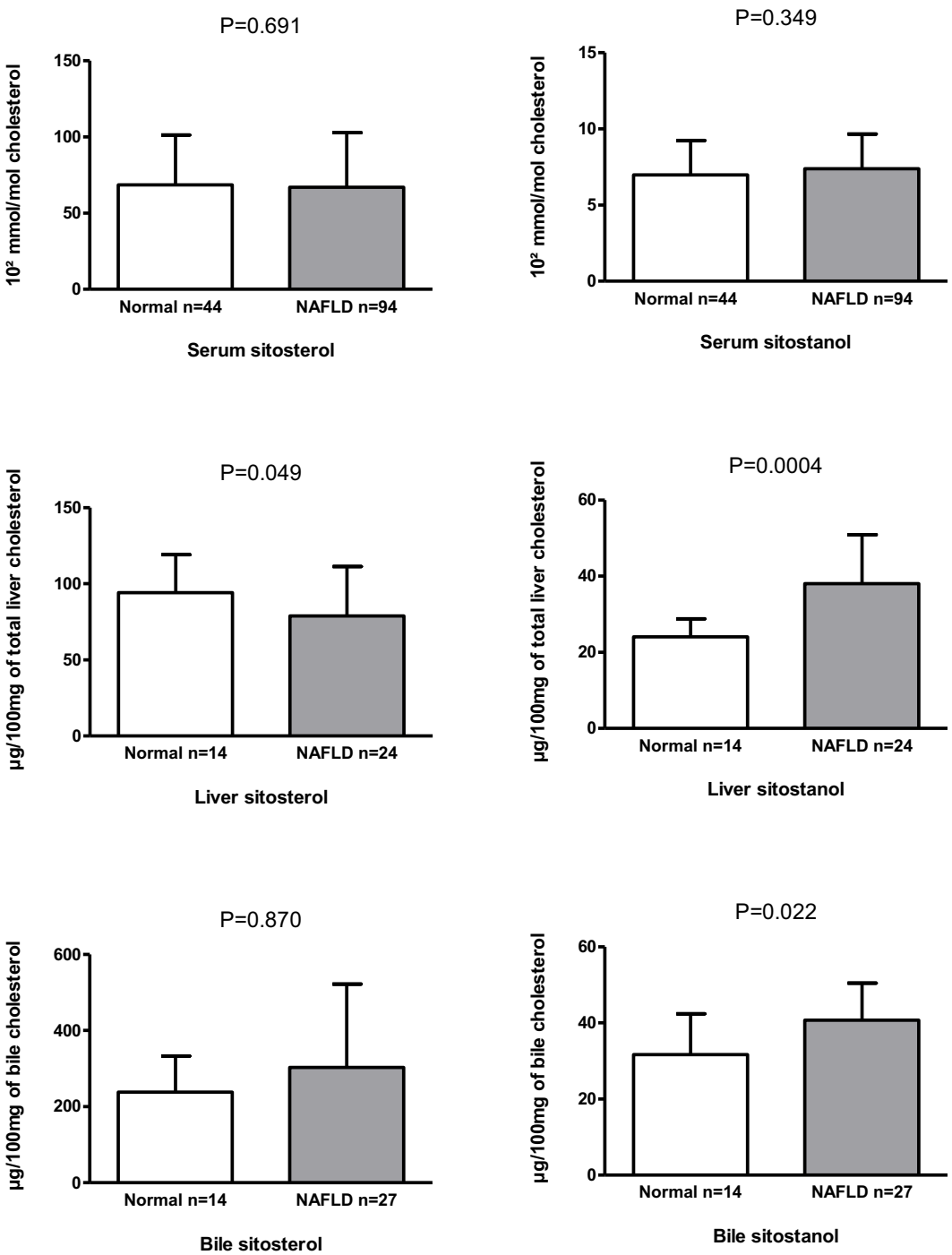

Figure 2. Serum, liver and bile sitosterol and sitostanol ratios to cholesterol (mean ± SD) in individuals with normal liver and nonalcoholic fatty liver disease (NAFLD).

Biliary sitostanol ratio to cholesterol is increased in individuals with steatosis

Finally, we measured plant sterols and sitostanol from the bile (n=41). Sitostanol ratio to cholesterol was higher in those with NAFLD than those with normal liver (P=0.022, Figure 2) while biliary sitosterol ratio to cholesterol did not differ between the study groups (Figure 2). In addition, there was a strong positive correlation between serum

Bioscience Reports (2018) **38** BSR20171274
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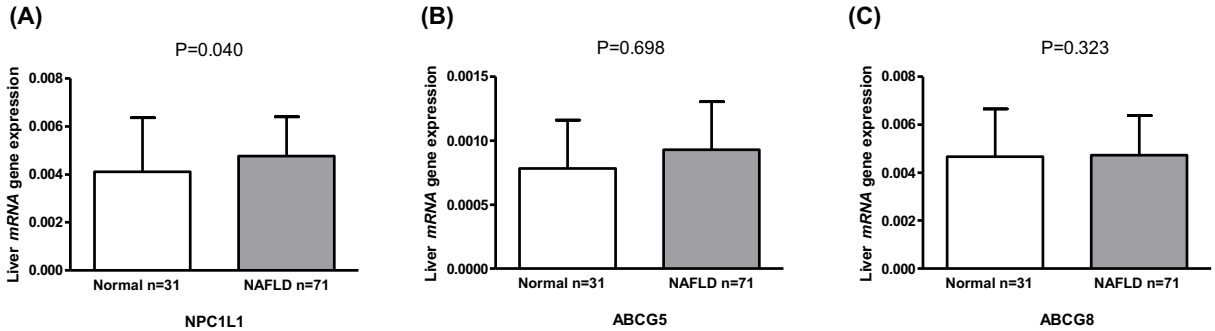

Figure 3. Liver mRNA expression analyzed with qPCR

(mean ± SD) of *NPC1L1* (Niemann–Pick C1-Like 1) (**A**), *ABCG5* (ATP-Binding Cassette, Subfamily G, Member 5) (**B**), and *ABCG8* (ATP-Binding Cassette, Subfamily G, Member 8) (**C**) in individuals with normal liver and nonalcoholic fatty liver disease (NAFLD).

and biliary sitosterol ($r_s = 0.795$, P=1.45 × 10^{-9}), but not between serum and bile sitostanol ratios to cholesterol (Supplementary Table S3). Campesterol and avenasterol were unmeasurable in the biliary samples.

Liver mRNA expression with plant sterols and liver histology

Next, we investigated if the differences in sitostanol and sitosterol levels could be related to the liver mRNA expression of transporters *NPC1L1*, *ABCG5*, and *ABCG8* (n=102). First, we observed that the hepatic mRNA expressions of *NPC1L1* was higher in those with NAFLD compared to those with normal liver (P=0.040) (Figure 3A). *ABCG5* and *ABCG8* were not different between the study groups (Figure 3B,C). Next, we correlated the liver *mRNA* expression of these genes with sitosterol and sitostanol ratios to cholesterol in serum (n=102), liver (n=38), and bile (n=41) (Supplementary Tables S4 and S5). The mRNA expression of *NPC1L1* correlated negatively with serum sitosterol (r_s = −0.210, P=0.032) and positively with serum sitostanol ($r_s = 0.248$, P=0.011), but not with liver or bile sitosterol or sitostanol ratios to cholesterol. Finally, we did a correlation analysis between the expression of several other known genes regulating inflammation, cholesterol and lipid metabolism, and the ratios to cholesterol of serum, liver and bile sitosterol and sitostanol (Supplementary Table S5). This analysis revealed several suggestive differences in correlations between mRNA expression and the sitostanol and sitosterol levels. However, due to the multiple testing of correlations none of the correlations were strongly significant and thus require further replication.

Discussion

Our main finding was that liver sitosterol and sitostanol ratios to cholesterol associated differentially with normal liver and NAFLD in obese individuals (Figure 2). In contrast, we did not observe an association between liver histology and the levels of plant sterols and sitostanol in serum (Table 2). This suggests that serum sitosterol and campesterol ratios to cholesterol, are not primarily affected in NAFLD. More likely, a differential regulation of sitosterol and sitostanol contents in the liver may exist between those with normal liver and NAFLD.

There are several potential explanations why liver sitosterol and sitostanol were differentially associated with NAFLD in our study. Even though serum and liver plant sterols correlated with each other, serum and liver sitostanol did not correlate suggesting different regulation of sitostanol (Supplementary Table S3). In addition, there was a strong positive correlation between serum and biliary sitosterol, but not between serum and biliary sitostanol suggesting that serum sitostanol levels do not reflect hepatic and biliary levels of sitostanol (Supplementary Table S3). First, this might be due to different chemical structures of sitosterol and sitostanol, which affect the solubility regulating their absorption and secretion [26-29]. Second, the positive correlation of liver sitostanol and negative correlation of liver sitosterol with liver inflammation (Table 2) suggest that their abilities to take part in inflammatory processes may differ. It is not yet clear how plant sterols and plant stanols can regulate inflammation in humans [30-32]. Plant sterols and stanols have been suggested to reduce inflammation in asthma both *in vitro* [33,34] and in animal models [33,35]. In addition, sitosterol and sitostanol markedly decreased the mRNA levels of *MCP-1* and *IL-1*β in cultured myofibroblasts from stenotic hearth valves [32]. Plant sterols and plant stanols have been reported to attenuate inflammatory responses via T-lymphocytes in cell models [34,36] and in humans [37], and via cytokines in animal and *in vitro* studies [33,36].

The finding of the association between NASH-related histological parameters and sitostanol was supported by remarkable positive correlations of liver sitostanol with steatosis and lobular inflammation. At the same time serum sitostanol levels did not correlate with histology (Table 2). Besides liver sitostanol biliary sitostanol levels are also positively associated with NAFLD (Figure 2). This is in line with experimental models in rats demonstrating that perfused sitostanol was taken into the isolated liver and secreted to bile [33,36]. Thus, our observation using bile samples in the analysis strengthens the conclusion that liver sitostanol metabolism is altered in NAFLD. Taken together, these results suggest that transport of sitosterol and sitostanol from gut to serum and further from the liver to bile may be differentially regulated in NAFLD compared with normal liver.

Our results of the liver mRNA expression of known genes involved in cholesterol, lipid, and inflammation metabolism suggest differences in sterol export mechanisms in NAFLD. Previously, the expression findings related to sterol exporters have been controversial in humans with NAFLD. ABCG5/8 protein expression was reported to be higher in those with steatosis compared to those with normal liver [17], a finding not confirmed in our study. In another study, the mRNA expression of *ABCG8* was found to be lower in humans with NASH compared to those with NAFLD while no difference in the expression of *ABCG5* was observed [19]. On the other hand, *NPC1L1* expression has been reported to be lower in those with NAFLD compared to those with normal liver [17]. This was opposite to our findings demonstrating that the liver mRNA expression of *NPC1L1* was higher in those with NAFLD compared to those with normal liver, whereas *ABCG5* and *ABCG8* were not changed (Figure 3A–C). Accordingly, serum sitosterol correlated negatively and sitostanol positively with the liver gene expression of *NPC1L1* (Supplementary Table S4), suggesting a link between our results and *NPC1L1* expression in the liver. However, our key finding that liver/bile sitostanol ratio to cholesterol was higher in those with NAFLD could not be linked to mRNA expression of export genes, supporting the possibility of a more complex dysregulation in NAFLD.

Our large-scale analysis of mRNA expression using Truseq methodology suggested other potential divergent metabolism between human serum, liver and bile metabolism of plant sterols and plant stanols. We saw differential correlations of sitosterol and sitostanol with the liver mRNA expression of known genes involved in inflammation, cholesterol, and lipid metabolism (Supplementary Table S5).

We recognize the following limitations in our study. Our study subjects were morbidly obese and thus our results cannot be generalized to normal weight subjects. However, it would be ethically challenging to obtain liver biopsies and bile samples from lean and healthy individuals. Unfortunately, we only had two individuals with NASH, as compared to 13 with simple steatosis, with liver samples available for liver analysis of plant sterols and sitostanol. Thus, we could not investigate the independent associations of liver sitostanol with steatosis and NASH.

In conclusion, our study is the first to demonstrate that both liver and bile sitostanol ratio to cholesterol associate with NAFLD, even though serum sitostanol ratio to cholesterol does not in obese individuals. The mechanisms related to altered sitostanol metabolism in NAFLD should be clarified in experimental studies.

Clinical perspectives

- Association between plant sterols, sitostanol, and NAFLD is not clear. Thus, we studied serum, liver, and bile plant sterols in obese individuals with and without NAFLD.
- The main findings were that liver and bile, but not serum, sitostanol was higher in those with NAFLD compared to those with normal liver. Accordingly, liver sitostanol correlated positively with steatosis and lobular inflammation.
- The mechanisms related to altered sitostanol metabolism in NAFLD should be clarified in experimental studies.

Acknowledgments

We thank Päivi Turunen, Tiina Sistonen, and Matti Laitinen for their work in patient recruitment and laboratory analyzes, and Leena Kaipiainen for the sterol analyzes.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This study was supported by the Finnish Diabetes Research Foundation (to J.P.). J.P. had an Academy of Finland Clinical Researcher fellowship related to this work [138006 2011-2013] and support from the Kuopio University Hospital Project grant (EVO/VTR to J.P.). M.V. received also support from VTR, Finnish Cultural Foundation and Finnish Diabetes Research Foundation and M.T. received a grant from Finnish Cultural Foundation and TUJ-grant from Kuopio University Hospital.

Author Contribution

M-M.T. researched the data and wrote the manuscript in guidance with V.M. and H.G. D.K. and M.V. performed gene expression analyzes. V.K. was responsible for the histological analysis of the liver samples. S.V. and P.K. took the liver biopsies and bile samples. J.P. was responsible for the clinical and molecular studies, researched data, and had full access to all the data to take responsibility for the integrity and for the accuracy of the analyses.

Abbreviations

NAFLD, Non-Alcoholic Fatty Liver Disease; NASH, Non-Alcoholic Steatohepatitis; KOBS, Kuopio Obesity Surgery Study; NPC1L1, Niemann-Pick C1-Like 1; ABCG5, ATP-Binding Cassette, Subfamily G, Member 5; ABCG8, ATP- Binding Cassette, Subfamily G, Member 8; RYGB, Roux-en-Y Gastric Bypass; ALT, Alanine aminotransferase; DPI, Dietary phytosterol intake.

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Serum, liver and bile sitosterol and sitostanol in obese patients with and without NAFLD

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Table of contents

Supplementary Table 1. Sequences of qPCR primers.

Gene	Forward primer (5’ – 3’)	Reverse primer (5’ – 3’)
NPC1L1	TACTGTGCCAATGCCCCGCT	GGGAAGACAGGGGCCCCGTA
ABCG5	TCTCATCTTTGACCCCCGGA	GATGTGATGTCCCACCAGGG
ABCG8	GGAACCTGGAGGGAACAATAAC	GGCATCTTGCTGGTACATCTT
RPLP0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC

Supplementary Table 2. Clinical characteristics (mean±SD) of study subjects in groups that had plant sterol and plant stanol measurement available from serum, liver and bile and had either histologically normal liver or NAFLD. *P<0.05 compared to those with serum measurements. DPI (dietary phytosterol intake, serum campesterol to cholestanol ratio).

	Serum	**Liver**	**Bile**	**P serum vs. liver**	**P serum vs. bile**
	138	**38**	**39**		
Gender (male/female)	38/100	14/24	9/30	0.133	0.463
Age (years)	46.3±8.9	45.8±9.1	48.2±9.0	0.746	0.180
Body mass index (kg/m²)	43.3±6.9	43.8±5.2	41.6±4.9	0.662	**0.021**
ALT (U/L)	49.6±33.4	49.9±42.1	48.4±30.0	0.629	0.903
Fasting glucose (mmol/L)	6.5±1.9	6.3±1.1	6.4±1.7	0.604	0.932
Fasting insulin (mU/L)	18.6±10.1	18.5±10.5	18.8±9.5	0.694	0.673
Total cholesterol (mmol/L)	4.5±0.9	4.3±1.0	4.5±0.7	**0.031**	0.301
HDL cholesterol (mmol/L)	1.1±0.3	1.1±0.3	1.2±0.3	0.683	**0.004**
LDL cholesterol (mmol/L)	2.7±0.8	2.5±0.8	2.6±0.7	0.085	0.849
Triglycerides (mmol/L)	1.3±0.5	1.3±0.5	1.7±0.9	0.166	0.898
DPI (dietary phytosterol intake)	1.0±0.4	0.9±0.4	1.0±0.5	0.577	0.526

Supplementary Table 3. Spearman correlations of serum, liver and bile plant sterol and sitostanol. **P<0.01 and *P<0.05. Bile avenasterol and campesterol were undetected.

	Serum			
	Campesterol	Sitosterol	Avenasterol	Sitostanol
Liver (n=38)	**0.544****	**0.488****	**0.517***	0.214
Bile (n=41)		**0.795****		0.236

Supplementary Table 4. Spearman correlations of liver mRNA gene expression (qPCR assay) with plant sterols and sitostanol ratios to cholesterol in serum, liver and bile. *P<0.05.

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	NPC1L1	***ABCG5***	***ABCG8***
SERUM (n=102)			
Campesterol	-0.112	-0.042	-0.090
Sitosterol	**-0.210***	-0.069	-0.172
Avenasterol	-0.046	0.117	-0.110
Sitostanol	**0.248***	**0.243***	-0.016
LIVER (n=38)			
Campesterol	-0.059	0.027	-0.194
Sitosterol	-0.168	-0.073	-0.310
Avenasterol	0.133	-0.025	**-0.383***
Sitostanol	0.215	0.190	0.012
BILE (n=41)			
Sitosterol	-0.295	-0.138	-0.209
Sitostanol	0.227	0.247	0.376

Supplementary Table 5. Correlations of the inflammation, lipid and cholesterol metabolism associated genes (liver *mRNA* expression, qPCR assay) with sitosterol and sitostanol ratios to cholesterol in serum (n=102), liver (n=38) and bile (n=41). *P<0.05, **P<0.009 in bold. Spearman`s correlation analysis. N/A: data not available.

Inflammation-related genes		Serum n=102		Liver n=38		Bile=41	
		Sitosterol	Sitostanol	Sitosterol	Sitostanol	Sitosterol	Sitostanol
Gene name	Gene symbol	10**2 mmol/mol of chol		ug/100 mg chol		ug/100mg chol	
ADAM Metallopeptidase Domain 8	*ADAM8*	-0,036	-0,069	-0,281	0,309	0,088	-0,211
Apoptotic Peptidase Activating Factor 1	*APAF1*	**,199***	0,054	0,174	**-,394***	0,227	-0,008
BCL2-Associated Agonist of Cell Death	*BAD*	-0,187	**-,205***	-0,088	0,065	**-,368***	0,050
BCL2-Associated Athanogene 6	*BAG6*	0,008	**-,386****	-0,262	0,064	-0,146	-0,200
B-Cell CLL/Lymphoma 3	*BCL2*	0,034	0,070	-0,185	0,243	0,067	0,178
Complement Component 3	*C3*	-0,105	**-,348****	-0,299	-0,013	-0,069	-0,107
Caspase 1, Apoptosis-Related Cysteine Peptidase	*CASP1*	-0,008	0,036	-0,255	0,203	-0,020	-0,077
Caspase 3, Apoptosis-Related Cysteine Peptidase	*CASP3*	-0,116	-0,001	0,043	0,043	-0,096	0,020
Caspase 8, Apoptosis-Related Cysteine Peptidase	*CASP8*	0,080	-0,165	-0,114	-0,091	0,013	-0,139
Caspase 9, Apoptosis-Related Cysteine Peptidase	*CASP9*	-0,013	**-,306****	-0,323	0,044	0,277	-0,102
Chemokine (C-C Motif) Ligand 2	*CCL2*	0,130	0,142	0,202	-0,133	0,079	-0,173
CD68 Molecule	*CD68*	-0,040	-0,039	-0,275	0,120	-0,147	0,273
C-Reactive Protein, Pentraxin-Related	*CRP*	-0,092	-0,020	-0,188	0,206	-0,022	-0,014
Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like 1	*EMR1=ADGRE1*	0,041	-0,024	0,085	0,105	-0,054	0,060
Fas (TNFRSF6)-Associated Via Death Domain	*FADD*	0,153	-0,017	0,042	-0,001	0,130	-0,170
Fas Cell Surface Death Receptor	*FAS*	**,238***	-0,030	0,064	-0,011	-0,036	-0,249
Guanylate Binding Protein 5	*GBP5*	-0,151	-0,009	-0,163	-0,117	-0,276	0,049
Heme Oxygenase 1	*HMOX1*	0,025	0,008	-0,024	0,282	-0,139	**,359***
Interleukin 10	*IL10*	-0,032	0,167	-0,119	0,266	0,310	0,294
Interleukin 18	*IL18*	0,041	-0,146	-0,144	0,116	-0,065	0,264
Interleukin 1, Beta	*IL1B*	-0,106	-0,068	-0,217	**,389***	-0,027	-0,162
Interleukin 1 Receptor, Type I	*IL1R1*	0,034	-0,125	-0,147	-0,172	0,151	0,016
Interleukin 1, Receptor, Type II	*IL1RN*	-0,175	0,066	-0,332	0,153	0,028	0,029
Interleukin 2	*IL2*	-0,040	0,067	-0,043	-0,216	0,025	0,041
Interleukin 32	*IL32*	-0,116	0,077	-0,281	**,528****	-0,062	0,003
Interleukin 6	*IL6*	-0,015	0,007	N/A	N/A	0,047	0,219
Integrin, Alpha M (Complement Component 3 Receptor 3 Subunit)	*ITGAM*	-0,018	-0,125	**-,437****	**,507****	0,120	-0,040
MHC Class I Pylypeptide-Related Sequence A	*MICA*	0,069	**-,240***	-0,158	-0,013	-0,120	**-,321***
Matrix Metallopeptidase 9	*MMP9*	-0,020	-0,010	-0,088	0,163	0,116	0,191
Nuclear Factor of Kappa Light Polypeptidase Gene Enhancer in B-Cells 1	*NFKB1*	-0,019	0,104	-0,016	0,239	-0,168	-0,235
nlr Family, Pyrin Domain Containing 3	*NLRP3*	-0,144	0,077	0,111	-0,129	-0,180	-0,055
Platelet Derived Growth Factor Beta Polypeptidase	*PDGFB*	-0,052	**-,194***	-0,289	0,099	**-,321***	0,180
PYD and Card Domain Containing	*PYCARD*	-0,023	0,023	0,104	-0,031	-0,116	0,236
Receptor (TNFRSF)-Interacting Serine-Threnonine Kinase 1	*RIPK1*	**,193***	0,190	0,228	-0,232	0,096	-0,059
Superoxide Dismutase 2, Mitochondrial	*SOD2*	-0,023	**,210***	**,361***	-0,119	-0,148	0,056
Signal Transducer and Activator of Transcription 5B	*STAT5B*	-0,007	0,042	-0,109	0,208	0,131	0,113
Toll-Like Receptor	*TLR4*	0,007	-0,143	**-,405***	**,391***	-0,035	-0,023
Tumor Necrosis Factor Alpha	*TNFα*	-0,060	0,038	-0,158	0,191	-0,172	0,051
Tumor Necrosis Factor Receptor Superfamily, Member 1A	*TNFRSF1A*	0,069	**-,213***	-0,123	-0,333	-0,037	-0,162
Tumor Necrosis Factor Receptor Superfamily, Member 1B	*TNFRSF1B*	-0,098	0,063	-0,310	0,112	-0,101	0,185
Tumor Necrosis Factor Receptor Superfamily, Member 10	*TNFSF10*	-0,008	**-,372****	-0,159	-0,290	0,112	-0,076
Tumor Protein 53	*TP53*	-0,032	-0,032	-0,215	0,160	0,164	-0,050
TNFRSF1A-Associated Via Death Domain	*TRADD*	-0,093	**-,222***	0,025	0,055	-0,086	-0,002
TNF Receptor-Associated Factor 2	*TRAF2*	-0,120	-0,088	-0,278	0,012	-0,305	0,027
Thioredoxin Interacting Protein	*TXNIP*	0,017	**,285****	0,193	0,126	0,070	0,245

Lipid metabolism - related genes		Serum n=102		Liver n=38		Bile=41	
		Sitosterol	Sitostanol	Sitosterol	Sitostanol	Sitosterol	Sitostanol
Gene name	Gene symbol	10**2 mmol/mol of chol		ug/100 mg chol		ug/100mg chol	
Peroxisome Proliferator-Activated Receptor Alpha	*PPARA*	-0,045	0,192	0,006	-0,040	-0,062	**-,365***
Peroxisome Proliferator-Activated Receptor Gamma (total)	*PPARG_tot*	-0,086	**-,273****	**-,370***	-0,036	-0,270	-0,057
Peroxisome Proliferator-Activated Receptor Gamma (variant 1A)	*PPARGC1A*	0,082	**-,302****	-0,286	-0,155	-0,033	0,086
Peroxisome Proliferator-Activated Receptor Gamma (variant 1B)	*PPARGC1B*	**-,204***	**-,432****	**-,428***	0,109	-0,181	-0,082
Sterol Regulatory Element Binding Transcription Factor 1	*SREBF1_tot*	-0,072	-0,070	-0,253	-0,121	-0,131	0,118
ATP Citrate Lyase	*ACLY*	-0,006	-0,033	-0,202	0,242	-0,054	0,265
Acetyl-CoA Carboxylase Alpha	*ACACA=ACC*	-0,093	**-,271****	-0,300	-0,025	0,045	-0,108
Fatty Acid Synthetase	*FASN*	-0,052	0,004	-0,072	0,247	-0,101	0,269
Fatty Acid Elongase 1	*ELOVL1*	-0,069	0,001	**-,414***	**,496****	-0,125	0,122
Fatty Acid Elongase 2	*ELOVL2*	-0,047	**,331****	0,118	0,321	0,049	0,300
Fatty Acid Elongase 3	*ELOVL3*	0,062	0,098	-0,004	0,128	0,175	0,070
Fatty Acid Elongase 4	*ELOVL4*	-0,125	0,083	-0,118	0,094	-0,066	0,094
Fatty Acid Elongase 5	*ELOVL5*	-0,060	**,306****	0,314	0,074	-0,246	**,352***
Fatty Acid Elongase 6	*ELOVL6*	0,110	0,134	0,185	-0,218	0,303	0,014
Fatty Acid Elongase 7	*ELOVL7*	0,070	-0,043	-0,244	0,152	-0,048	0,015
Stearoyl-CoA Desaturase (Delta-9 Desaturase)	*SCD*	0,040	-0,174	-0,202	0,095	0,007	0,054
Fatty Acid Desaturase 1	*FADS1*	-0,061	0,022	0,109	0,332	-0,291	-0,128
Fatty Acid Desaturase 2	*FADS2*	-0,092	-0,064	-0,018	0,306	-0,207	-0,083
Fatty Acid Desaturase 3	*FADS3*	-0,015	**-,233***	0,011	0,095	-0,234	-0,152
Carnitine Palmitoyltransferase 1A (Liver)	*CPT1A*	-0,046	0,170	-0,161	**,375***	0,146	-0,066
Carnitine Palmitoyltransferase 1B (Muscle)	*CPT1B*	0,170	0,164	-0,201	0,228	0,128	0,156
Glyserol-3 Phosphate Acyltransferase, Mitochondrial	*GPAM=GPAT1*	-0,047	-0,063	-0,303	0,183	-0,031	0,164
Glyserol-3 Phosphate Acyltransferase 2, Mitochondrial	*GPAT2*	0,021	-0,147	-0,272	0,276	-0,082	-0,100
1-Acylglycerol-3-Phosphane O-Acyltransferase 1	*AGPAT1*	**-,270****	0,065	-0,329	0,218	-0,010	0,084
1-Acylglycerol-3-Phosphane O-Acyltransferase 2	*AGPAT2*	-0,065	**-,199***	-0,090	0,059	0,001	0,181
1-Acylglycerol-3-Phosphane O-Acyltransferase 3	*AGPAT3*	0,102	-0,006	0,054	-0,043	0,253	-0,205
1-Acylglycerol-3-Phosphane O-Acyltransferase 4	*AGPAT4*	0,025	-0,007	-0,025	0,141	-0,165	0,058
1-Acylglycerol-3-Phosphane O-Acyltransferase 5	*AGPAT5*	-0,014	-0,077	-0,004	-0,031	-0,210	-0,202
1-Acylglycerol-3-Phosphane O-Acyltransferase 9	*AGPAT9=GPAT3*	0,008	-0,095	-0,034	-0,275	0,153	-0,252
1-Acylglycerol-3-Phosphane O-Acyltransferase 6	*AGPAT6=GPAT4*	-0,014	**-,294****	0,007	-0,321	-0,209	-0,222
Lipin 1	*LPIN1*	-0,082	**-,406****	-0,334	-0,058	0,023	**-,340***
Lipin 2	*LPIN2*	0,155	**-,238***	0,133	**-,444****	0,078	-0,251
Lipin 3	*LPIN3*	-0,001	-0,186	**-,373***	-0,187	-0,138	0,045
Diacylglycerol O-Acyltransferase 1	*DGAT1*	**-,204***	0,047	-0,278	0,174	-0,127	-0,110
Diacylglycerol O-Acyltransferase 2	*DGAT2*	-0,090	0,014	-0,167	0,222	-0,123	0,172
Lipoprotein Lipase	*LPL*	0,000	0,082	-0,019	**,393***	0,179	-0,091
Patatin-Like Phospholipase Domain Containing 2	*PNPLA2=ATGL*	-0,088	-0,010	-0,270	0,048	0,038	-0,111
Lipase, Hormone Sensitive	*LIPE=HSL*	0,056	**,197***	-0,071	-0,013	0,062	-0,169
Perlipin 1	*PLIN1*	-0,005	-0,006	-0,230	0,322	0,028	0,210
Patatin-Like Phospholipase Domain Containing 3	*PNPLA3*	0,026	-0,190	-0,239	0,081	-0,114	-0,020
Phospolipid Phosphatase 2A	*PPAP2A*	0,128	**,318****	0,233	0,118	-0,008	0,104
Phospolipid Phosphatase 2B	*PPAP2B*	-0,135	0,078	-0,167	0,128	0,001	0,290
Phospolipid Phosphatase 2C	*PPAP2C*	0,120	0,139	0,024	0,267	0,135	0,052
Uncoupling Protein 1 (Mitochondrial, Proton Carrier)	*UCP1*	-0,028	0,033	N/A	N/A	-0,137	-0,094
Uncoupling Protein 2 (Mitochondrial, Proton Carrier)	*UCP2*	**-,229***	-0,049	-0,179	**,433***	-0,313	**,343***

Cholesterol metabolism - related genes		Serum n=102		Liver n=38		Bile=41	
		Sitosterol	Sitostanol	Sitosterol	Sitostanol	Sitosterol	Sitostanol
Gene name	Gene symbol	10**2 mmol/mol of chol		ug/100 mg chol		ug/100mg chol	
Nuclear Receptor Subfamily 1, Group H, Member 2	*NR1H2=LXRB*	-0,077	**-,201***	-0,125	0,122	-0,203	0,219
Sterol Regulatory Element Binding Transcription Factor 2	*SREBF2*	-0,105	-0,052	-0,029	**,354***	-0,135	0,205
Acyl-CoA Synthetase Short-Chain Family Member 2	*ACSS2*	-0,013	-0,022	-0,060	0,186	-0,181	-0,122
Acetyl-CoA Acetyltransferase 1	*ACAT1*	-0,048	0,143	-0,050	0,263	0,090	0,228
3-Hydroxy-3-Methylglutaryl-CoA Synthetase 1 (Soluble)	*HMGCS1*	0,046	0,055	0,230	0,179	0,191	0,155
3-Hydroxy-3-Methylglutaryl-CoA Synthetase 3 (Mitochondrial)	*HMGCS2*	-0,084	**,285****	-0,169	-0,016	-0,078	0,180
3-Hydroxy-3-Methylglutaryl-CoA Reductase	*HMGCR*	-0,135	**-,213***	-0,117	0,091	0,037	0,219
Farnesyl-Diphosphate Farnelystransferase 1	*FDFT1*	-0,004	-0,112	-0,018	0,219	-0,018	-0,145
Squalene Epoxidase	*SQLE*	-0,074	-0,100	-0,042	0,308	0,002	0,063
Lanosterol Synthetase (2,3-Oxidosqualene-Lanosterol Cyclase)	*LSS*	-0,019	-0,112	-0,107	0,191	-0,200	**-,324***
Transmembrane 7 Superfamily Member 2	*TM7SF2*	0,003	-0,011	0,068	0,159	-0,067	-0,199
Emopamil Binding Protein (Sterol Isomerase)	*EBP*	0,125	**-,233***	-0,042	-0,134	0,104	**-,331***
Methylsterol Mono-oxygenase 1	*MSMO1=SC4MO*	-0,101	-0,030	0,157	-0,127	-0,046	-0,035
Sterol C5-Desaturase	*SC5DL*	-0,023	**,232***	0,151	0,050	-0,062	0,094
24-Dehydrocholesterol Reductase	*DHCR24*	**-,237***	-0,127	-0,150	-0,110	0,068	0,057
7-Dehydrocholesterol Reductase	*DHCR7*	-0,124	**,224***	-0,083	**,569****	0,122	0,143
ATP-Binding Cassette, Sub-Family A (ABC1), Member 1	*ABCA1*	-0,076	**-,287****	**-,366***	-0,026	-0,031	-0,007
ATP-Binding Cassette, Sub-Family G (WHITE), Member 1	*ABCG1*	0,006	-0,073	-0,207	0,060	0,028	-0,034
ATP-Binding Cassette, Sub-Family G (WHITE), Member 5	*ABCG5*	-0,069	**,243***	-0,073	0,190	-0,138	0,190
ATP-Binding Cassette, Sub-Family G (WHITE), Member 8	*ABCG8*	-0,172	-0,016	-0,310	0,012	-0,209	0,146
Niemann-Pick C1-Like 1	*NPC1L1*	**-,210***	**,248***	-0,168	0,215	-0,295	0,227
Low Density Lipoprotein Receptor	*LDLR*	-0,009	-0,030	0,045	0,005	0,057	-0,244
Proprotein Convertase Subtilisin/Kexin Type 9	*PCSK9*	**-,200***	0,005	-0,186	0,047	-0,038	-0,026
3-Oxoacid CoA Transferase 1	*OXCT1*	-0,011	0,127	-0,249	0,201	0,062	0,301
3-Hydroxybutyrate Dehydrogenase, Type 1	*BDH1*	-0,102	**,229***	-0,119	0,120	0,013	0,026
Scavenger Receptor Class B, Member 1	*SCARB1=SRB1*	-0,128	0,024	-0,139	0,116	-0,158	0,149
Fibroblast Growth Factor 21	*FGF21*	-0,047	-0,135	-0,222	0,111	0,205	**,347***
Cytocrome P450, Family 51, Subfamily A, Polypeptide 1	*CYP51A1*	0,066	**,196***	0,188	0,157	0,032	**-,321***

Supplementary Figure. 1. Serum plant sterols and sitostanol ratios to cholesterol (mean ± SD) in individuals with normal liver (n=44) and NAFLD (n=94). Serum campesterol, sitosterol, avenasterol or sitostanol were not different between normal liver and NAFLD.