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**Fatty liver is an integral feature of familial combined hyperlipidemia:
relation with fat distribution and plasma lipids**

Short running title: fatty liver in familial combined hyperlipidemia

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Abstract

Overproduction of VLDL-particles is an important cause of familial combined hyperlipidemia (FCHL). It was recently shown that the VLDL-production is driven by the amount of hepatic fat.

The present study was conducted to determine the prevalence of fatty liver in relation to the different fat compartments and lipid parameters in FCHL.

Sixty-eight FCHL patients, 110 normolipidemic relatives and 66 spouses underwent ultrasound of the abdominal region to estimate the amount of subcutaneous, visceral and hepatic fat. Skinfold callipers were used to measure subcutaneous fat of the biceps, triceps, subscapular and supra-iliacal regions.

Fatty liver was observed in 18% of the spouses, 25% of the normolipidemic relatives and 49% of the FCHL patients. After adjustment for age, gender and body mass index, the prevalence of fatty liver was significantly higher in FCHL patients versus spouses ($p=0.03$, OR:3.1), and also in the normolipidemic relatives versus spouses ($p=0.02$, OR:4.0), whereas no differences were observed between FCHL patients and normolipidemic relatives ($p=0.58$, OR:0.8). In the normolipidemic relatives and FCHL patients combined, both visceral fat mass and subcutaneous abdominal fat were independent predictors of fatty liver ($p<0.001$ for both fat compartments; FCHL status corrected). Of interest, fatty liver stages were correlated with both VLDL-apo B and VLDL-triglycerides in a representative subset of patients and relatives ($n=69$; $r^2=0.12$, $p=0.006$ and $r^2=0.18$, $p=0.001$, respectively).

These data show that fatty liver is a central aspect of FCHL, i.e. patients and normolipidemic relatives. Both visceral and subcutaneous adiposity contribute to its three to four fold higher risk in FCHL.

Introduction

More than 30 years ago, familial combined hyperlipidemia (FCHL) was delineated as a genetic hyperlipidemia, characterized by the presence of different hyperlipidemic phenotypes, i.e. isolated hypercholesterolemia, isolated hypertriglyceridemia or the combination of both, within one family [1]. It was estimated that FCHL is highly prevalent (1:100) in the general population and even more abundant (1:10) among survivors of a premature myocardial infarction [1].

More recently it was shown that the presence of small-dense LDL-particles and elevated apolipoprotein B levels are consistent features of FCHL [2, 3]. Furthermore, most FCHL subjects exhibit many features of the metabolic syndrome, such as insulin resistance [4] and abdominal obesity [5, 6]. Several studies have demonstrated that, although the expression of FCHL very much depends on the amount of (abdominal) fat [7], FCHL patients are still more hyperlipidemic when corrected or matched for the degree of obesity [7, 8]. These observations underline the complex pathogenesis of FCHL: both environmental and genetic factors are responsible for the FCHL phenotype.

The characteristic hyperlipidemia of FCHL is the consequence of both a hepatic overproduction of very low-density lipoproteins (VLDL) and an impaired clearance of remnant particles [9-11]. Adiels et al. very recently reported that the overproduction of VLDL-particles is driven by the amount of fat that is accumulated in the liver in normal subjects and patients with type 2 diabetes mellitus [12]. Of interest, we recently demonstrated that the prevalence of an increased amount of hepatic fat, i.e. fatty liver, was drastically increased in a small group of outpatient FCHL patients in comparison with community controls [13]. Given these recent findings, it is anticipated that the fatty liver trait will play a very important role in the expression of the FCHL phenotype.

The aim of the present study was to extend and refine current knowledge of fatty liver in FCHL. For this we estimated the prevalence of fatty liver not only in a larger sample of FCHL patients, but also in their normolipidemic relatives, derived from our 21 well-defined FCHL families. These prevalence numbers were subsequently compared with their spouses, who are exposed to a similar environment. Furthermore, analogous to the

reported contribution of abdominal obesity to the expression of FCHL [7], we investigated whether abdominal obesity, more specifically visceral and abdominal subcutaneous obesity, contribute to the expression of fatty liver in FCHL. Finally, we correlated plasma insulin, apolipoprotein B and triglycerides levels, three hallmarks of FCHL [2, 3, 14], with the different stages of fatty liver.

Research methods and procedures:

Study population

FCHL family members (i.e. FCHL patients and their normolipidemic relatives) and spouses of ages ranging between 20 and 70 years were invited to participate in this study. Diagnosis of FCHL was established as described previously [15]. The FCHL affected state was defined as plasma levels of total cholesterol > 6.5 mmol/L and/or triglycerides > 2.3 mmol/L (classical criteria [1]). More recently it was proposed to redefine FCHL according to plasma triglycerides levels > 1.5 mmol/L and apolipoprotein (apo) B > 1.2 g/L [14]. Some of our analyses were repeated with these newly proposed criteria to exclude the possibility that our observations depend on one particular definition of the affected and non-affected state.

Subjects visited the clinical research centre after an overnight fast and abstinence from alcohol for the last three days. Lipid lowering medication was stopped two weeks prior to the visit. Furthermore, subjects had a stable weight, did not take any medication associated with the development of fatty liver [16], and consumed no more than 20 g of alcohol daily. One subject found to be seropositive for hepatitis C and was therefore excluded from the present study.

The study protocol was approved by the Human Investigations Review Committee at Maastricht University/Academic Hospital Maastricht. All subjects gave written informed consent.

Anthropometric measurements

Subjects were weighed in their underwear, height was determined by a stadiometer and body mass index (BMI) was calculated by weight (kg) divided by height (m) squared.

Hip circumference was determined in standing position at the level of the trochanter major. Skinfold thickness was measured with plastic calliper at left biceps, triceps, subscapular and suprailiacal site. Waist circumference and sagittal diameter were measured in supine position at the level midway between the iliacal crest and lowest rib at the end of a normal expiration. Sagittal diameter was measured with a sliding beam calliper. Waist to hip ratio (WHR) was calculated as the ratio between waist and hip circumference.

Ultrasound

All ultrasound measurements were performed with an ATL 9 HDI ultrasound (US) system (Bothel, USA). Both visceral adipose tissue (VAT-US) and subcutaneous adipose tissue (SAT-US) were determined at the same level as waist circumference and sagittal diameter. SAT-US was measured at the midline as the distance between skin and linea alba, using a L5-10 transducer. VAT-US was determined as the distance between the anterior of the vertebrate body and the peritoneum, exactly as described by Stolk et al [17]. All measurements were done by the same researcher with minimal pressure exerted on the probe and at the end of a normal expiration. The intra-observer variability, as determined in 30 subjects, was 1.7%.

Fatty liver was detected by ultrasound as well, using a C7-4 and C4-2 transducer. Standardized images of the liver and right kidney were recorded on videotape and examined by an independent radiologist unaware of the subject's clinical characteristics. The severity of fatty liver was graded into mild, moderate and severe fatty liver by using the criteria of posterior beam attenuation, increased echogenicity ('bright liver') and decreased visualization of intrahepatic vessels, as described previously [18, 19]. The intra-observer agreement expressed as kappa, determined in 30 random scans, was substantial for both the presence of fatty liver (present/absent) ($\kappa = 0.74$) and the different fatty liver stages (normal, mild, moderate, severe) ($\kappa = 0.68$). This is in agreement with earlier studies [18].

Laboratory measurements

Plasma total cholesterol, triglyceride, apo B and insulin concentrations were measured as previously described [20]. VLDL isolation was done as described by Redgrave et al. [21]. Apolipoprotein B concentration in this fraction was quantified by gel electrophoresis according to the method of Karpe et al [22].

Statistical analyses

Differences in continuous and binary variables between spouses, normolipidemic relatives and FCHL patients, included as dummy variables, were tested with multivariate linear and logistic regression, respectively.

Univariate correlations of anthropometric variables, plasma insulin, triglycerides and apo B levels with the different stages of fatty liver were calculated with ordinal regression in all FCHL family members. Several multivariate ordinal regression models were constructed to study the independent effects of different anthropometric variables on the prediction of fatty liver. The FCHL patients and normolipidemic relatives were included in these models as dummy variable. The overall goodness of fit of the model was expressed as Nagelkerke's pseudo r-squared that serves the same function as r-squared in linear regression models.

SPSS 13.0 for Windows was used for all statistical analyses (SPSS Inc., Chicago, Illinois).

Results

General and anthropometric characteristics of FCHL patients, normolipidemic relatives and spouses

In total, 178 FCHL family members (68 FCHL patients and 110 normolipidemic relatives), and 66 spouses from 21 families participated in this study. Table 1 shows their general and anthropometric characteristics. Since the normolipidemic (NL) relatives were significantly younger than the spouses and FCHL patients, all analyses were adjusted for age and gender. By definition, FCHL patients had higher total cholesterol and serum triglycerides values compared with NL relatives and spouses. Furthermore, FCHL

patients were more obese. This was explained, at least in part, by an increased visceral fat depot reflected by increased waist circumference, WHR, sagittal diameter, and more directly assessed by increased VAT-US. Furthermore, the subcutaneous fat compartment also seemed to be increased, as expressed by increased SAT-US, biceps and subscapular skinfolds. The latter two were only significantly different from the spouses. However, other markers of subcutaneous adipose tissue, i.e. hip circumference, triceps skinfold and suprailiacal skinfold, were not statistically different between the three groups. Of interest, both WHR and VAT-US were significantly higher in NL relatives in comparison with spouses (Table 1).

Prevalence of fatty liver in FCHL patients, NL relatives and their spouses

The prevalence of fatty liver measured with ultrasound was 49% in FCHL patients, which was significantly higher in comparison with the 18% in spouses ($p = 0.003$, Odds ratio [OR]: 2.9; 95% CI: 1.5 – 5.5), but not with the 25% as observed in the NL relatives ($p = 0.18$, OR: 1.6; 95% CI: 0.8 – 3.3), after correction for age and gender (Table 1). Of interest, when these analyses were repeated with further adjustment for obesity, measured as BMI, the prevalence of fatty liver remained elevated in FCHL patients in comparison with spouses (Odds ratio [OR]: 3.1; 95% CI: 1.1 – 8.7; $p = 0.03$). Moreover, a significant difference in prevalence was also observed for NL relatives versus spouses (OR: 4.0; 95% CI: 1.3 – 12.3; $p = 0.02$), whereas the difference between FCHL patients and NL relatives was completely abolished after correction for BMI (OR: 0.8; 95% CI: 0.3 – 1.9; $p = 0.6$).

Of note, very similar results were obtained when FCHL patients were defined according to recently proposed criteria of plasma triglycerides > 1.5 mmol/L and apo B levels > 1.2 g/L [14] (OR: 4.2, $p = 0.02$ for FCHL patients vs spouses; OR: 4.7, $p = 0.006$ for relatives vs spouses). In addition, similar trends were observed when only one FCHL patient or one NL relative from each family was taken for analysis (data not shown).

Independent contribution of different fat compartments to fatty liver in FCHL patients and NL relatives

In all FCHL family members, i.e. FCHL patients and NL relatives combined, all anthropometric variables were significantly correlated with the different stages of fatty liver in univariate analysis (Table 2). Subsequently, age and gender corrected multivariate models were constructed to study the independent contribution of the different fat compartments to the fatty liver stages. As shown in Table 3, the prediction models that included either overall obesity, represented by BMI (model 1), or abdominal obesity (model 2), resulted in very similar correlation coefficients of 0.46 and 0.44, respectively. A model with only visceral obesity, i.e. VAT-US measured at the same level as waist circumference, yielded a substantially lower coefficient (model 3). Of interest, when SAT-US was added to VAT-US (model 4a), the correlation coefficient very much approached the r^2 of the model that included waist circumference (model 2). Replacing SAT-US by subscapular skinfold resulted in a similar model (model 4b), whereas biceps skinfold only marginally added to the contribution of VAT-US (model 4c versus model 3). Combining VAT-US, subscapular and biceps skinfold into one model (model 5) showed that both visceral and subcutaneous obesity of the trunk, but not of the upper limb, independently contributed to the prediction of the different stages of fatty liver. Of note, correction for the hyperlipidemic state, i.e. being FCHL patient or normolipidemic relative (classical [1] or newly proposed [14] criteria), did not influence these results.

Relation of plasma triglycerides, apolipoprotein B and insulin levels with fatty liver stages in FCHL patients and normolipidemic relatives

In univariate, ordinal regression analysis, insulin levels were positively correlated with the different stages of fatty liver ($r^2 = 0.32$, $p < 0.001$, Figure 2), independent of the affected state.

Of the two newly proposed parameters to diagnose FCHL [14], only plasma triglycerides were significantly related with the fatty liver stages ($r^2 = 0.22$, $p < 0.001$), whereas plasma apo B levels were not ($r^2 = 0.002$, $p = 0.6$).

Since plasma triglycerides are mainly secreted by the liver as VLDL-particles, we subsequently decided to measure VLDL-triglycerides and VLDL-apo B concentrations in a representative subset of relatives and patients ($n = 69$) and to relate these parameters with fatty liver. VLDL-triglycerides were similarly correlated with fatty liver stages as total plasma triglycerides ($r^2 = 0.18$, $p = 0.001$, Figure 3, panel A). Of interest, VLDL-apo B did show a correlation with fatty liver in contrast to total plasma apo B ($r^2 = 0.12$, $p = 0.006$, Figure 3, panel B).

Discussion

Our laboratory and others have previously demonstrated that familial combined hyperlipidemia develops against a background of abdominal obesity [7, 8]. The present study was conducted to relate these findings to fatty liver, a condition that is known to drive the production of VLDL-particles [12], which is an important feature of FCHL [9, 10].

The present study showed that both NL relatives and FCHL patients exhibited a three to four times increased risk (as expressed by the odds ratio) of having fatty liver in comparison with spouses. The prevalence of fatty liver was considerably higher in FCHL patients in comparison with spouses (49% versus 18%). This number seems to be somewhat lower than the previously reported prevalence of 76% in an outpatient FCHL population [13]. This discrepancy is likely to be explained by the higher co-morbidity that is often seen in outpatient populations in comparison with a non-outpatient population, as was predominantly the case in the present study. Furthermore, the previous study was conducted in a relatively small sample, thus yielding prevalence numbers with large confidence intervals. The prevalence estimate from the present larger population is therefore expected to be more reliable.

In all FCHL family members, obesity was an important contributor to the grade of fatty liver: 46% of the variation in fatty liver grade was accounted for by BMI. Of interest, a very similar correlation was observed for the abdominal compartment, quantified as waist

circumference, indicating that it is specifically this region that predicts fatty liver in FCHL. A more detailed study in this region revealed that both the visceral as well as the subcutaneous fat depot, both measured with ultrasound, were independent predictors of fatty liver grade. Furthermore, the combination of these two compartments very much approached the model that included only waist circumference. The association of visceral fat with fatty liver has been demonstrated in several other conditions associated with the metabolic syndrome [23-25], whereas the correlation of the subcutaneous adipose tissue with fatty liver is more controversial [23, 24]. Nielsen et al. have elegantly shown that both visceral adipose tissue and subcutaneous adipose tissue contribute to the flux of free fatty acids to the liver [26, 27], which is thought to be the most important process that drives hepatic fat accumulation [28]. The present study also suggests that the independent contribution of the subcutaneous fat depot in FCHL is confined to the truncal region. Of interest, Abate et al. have demonstrated that subcutaneous truncal but not peripheral fat is associated with the hepatic glucose production [29], a process that is related with hepatic fat accumulation [23].

The importance of obesity in the relation with fatty liver was also illustrated by the observation that BMI completely abolished the seemingly marked difference in prevalence of fatty liver between FCHL patients and NL relatives (49% versus 25%). This indicates that FCHL patients and their NL relatives are very similar regarding the prevalence of fatty liver, with the only exception that the patients are more obese and older. In contrast, the almost threefold higher prevalence of fatty liver in FCHL patients in comparison with spouses remained significant after correction for age, gender and BMI. Additionally, the apparently small prevalence difference between NL relatives and spouses became significant after correction for age, gender and BMI. These data imply an increased susceptibility of FCHL family members in general for the development of fatty liver, independent of the affected state (defined by both the classical [1] and the more recently proposed [14] criteria). Genetic factors could account for this lower threshold, since FCHL family members and spouses share the same environment.

The central role of fatty liver in FCHL was further emphasized by its association with both insulin levels and plasma triglycerides. Apo B levels, another hallmark of FCHL, were not related with the different fatty liver stages. These results are in concordance with Toledo et al. who reported very similar outcomes in a diabetes population [30]. Of interest, in that report the relation of fatty liver with plasma triglycerides was mainly explained by VLDL-particle size [30], whereas our study suggested that VLDL-particle number, as reflected by VLDL-apo B levels, was associated with the different stages of fatty liver. It should be noted that an increase in VLDL-particle number could be due to an increased VLDL-production, but also to an impaired clearance.

The apparent discrepancy between our findings in FCHL and previous observations in type 2 diabetes mellitus [30] may be explained by the study populations that were under investigation. Previous studies have suggested that VLDL-particles are larger in patients type 2 diabetes mellitus, but of normal size in FCHL patients [31, 32].

In the present study, we used liver ultrasound to detect hepatic fat accumulation. The gold standard, liver biopsy, was not ethically accepted in this relatively healthy population. Liver ultrasound was therefore used as an alternative, validated method to detect hepatic fat accumulation [18, 19]. Since ultrasound is less sensitive to detect mild stages of fatty liver [18], it is anticipated that the presently observed prevalence of fatty liver in FCHL is somewhat underestimated. Of note, the prevalence of fatty liver in the presently studied spouses was similar to the prevalence of fatty liver detected by histology in a general population [33, 34].

In summary, we showed that not only FCHL patients, but also their normolipidemic relatives have a three times higher risk of developing fatty liver compared to spouses. Within the FCHL population, BMI is an important contributor to fatty liver grade, and both the subcutaneous truncal and the visceral fat depots independently contributed to this association. The relation of fatty liver with plasma insulin, VLDL-triglycerides and VLDL-apo B levels further underlines the central role of fatty liver in the pathogenesis and expression of FCHL.

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Table 1: General descriptives and anthropometry of the study population

	Spouses	NL relatives	FCHL patients
Male / Female	34 / 32	50 / 60	31 / 37
Age, years	48.0 (43.0-56.3)	37.5 (28.8-52.0)*	54.0 (46.5-60.0)
Total Cholesterol, mmol/L	5.3 ± 1.0	5.0 ± 0.8	7.2 ± 2.0 ^{†‡}
Triglycerides, mmol/L	1.1 (0.8-1.7)	1.1 (0.9-1.5)	2.6 (1.6-3.5) ^{†‡}
Apolipoprotein B, g/L	1.0 ± 0.2	1.0 ± 0.3	1.3 ± 0.3 ^{†‡}
Insulin, U/L	4.6 (2.0-8.7)	5.4 (2.0-8.9)	9.5 (6.9-13.7) ^{†‡}
Lipid medication, %	6	10	50 ^{†‡}
BMI, kg/m ²	25.4 ± 4.2	25.2 ± 4.3	28.2 ± 3.9 ^{†‡}
Waist circumference (cm)	89.2 ± 12.4	88.2 ± 12.1	97.5 ± 9.9 ^{†‡}
Hip circumference (cm)	100.6 ± 8.2	100.4 ± 8.0	104.1 ± 8.0
WHR	0.88 ± 0.08	0.88 ± 0.08 [†]	0.94 ± 0.06 ^{†‡}
Sagittal diameter (cm)	19.6 (17.9-23.1)	19.5 (17.8-21.8)	22.8 (21.0-25.1) ^{†‡}
SAT-US, cm	2.5 ± 1.1	2.5 ± 1.2	2.9 ± 1.1 ^{†‡}
VAT-US, cm	6.7 (5.6-9.0)	6.9 (5.5-8.6) [†]	8.4 (7.3-10.5) ^{†‡}
SKF biceps, mm	8 (6-11)	9 (6-14)	11 (7-17) [†]
SKF triceps, mm	15 (10-20)	16 (11-23)	16 (10-24)
SKF subscapular, mm	18 (13-22)	20 (14-25)	24 (18-30) [†]
SKF supra-iliacal, mm	22 (16-32)	25 (16-32)	26 (22-31)
Fatty liver % (mild, moderate, severe)	18 (5, 7, 6)	25 (15, 7, 3)	49 (24, 12, 13) [†]

Data are presented as mean ± SD or as median (interquartile range); abbreviations: BMI: body mass index; WHR: waist to hip ratio; SAT-US: subcutaneous adipose tissue

measured with ultrasound; VAT-US: visceral adipose tissue measured with ultrasound;
SKF: skinfold

* $p < 0.05$ in comparison with spouses and FCHL patients

† $p < 0.05$ in comparison with spouses, age and gender adjusted

‡ $p < 0.05$ in comparison with NL relatives, age and gender adjusted

All analyses are Hochberg corrected

Table 2: Univariate correlations of anthropometric parameters with fatty liver stages in all FCHL family members

	r^2	p-value
BMI	0.44	< 0.001
Waist circumference	0.43	< 0.001
Hip circumference	0.24	< 0.001
Waist to hip ratio	0.27	< 0.001
Sagittal diameter	0.41	< 0.001
VAT-US	0.32	< 0.001
SAT-US	0.07	0.001
Biceps SKF	0.08	< 0.001
Triceps SKF	0.05	0.008
Subscapular SKF	0.28	< 0.001
Supra-iliacal SKF	0.07	< 0.001

Analysed with ordinal regression; abbreviations: BMI: body mass index; SAT-US: subcutaneous adipose tissue measured with ultrasound; VAT-US: visceral adipose tissue measured with ultrasound; SKF: skinfold

Table 3 : Prediction models of fatty liver stages with different fat compartments in all FCHL family members

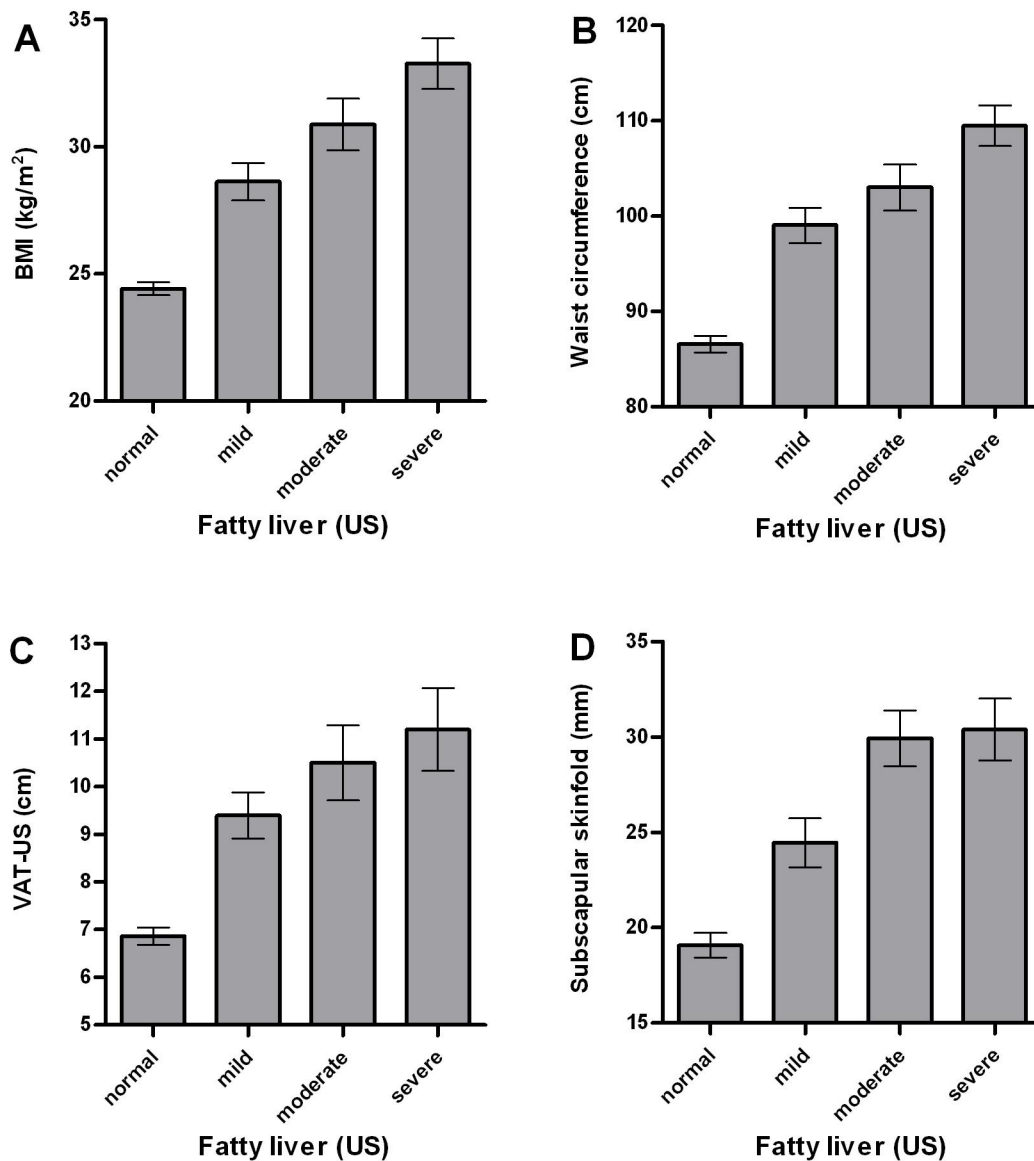
Model		Model r^2	Variables entered	P-value
1. Overall obesity		0.46	BMI	< 0.001
2. Abdominal obesity		0.44	Waist circumference	< 0.001
3. Visceral obesity		0.35	VAT-US	< 0.001
4. Visceral + subcutaneous obesity	a	0.43	VAT-US SAT-US	< 0.001 < 0.001
	b	0.40	VAT-US Subscap SKF	< 0.001 < 0.001
	c	0.36	VAT-US Biceps SKF	< 0.001 0.01
5. Visceral + subcutaneous (limb + trunk) obesity		0.41	VAT-US Subscapular SKF Biceps SKF	< 0.001 0.002 0.20

Analysed with ordinal regression with inclusion of age and gender as covariates;
 abbreviations: BMI: body mass index; SAT-US: subcutaneous adipose tissue measured

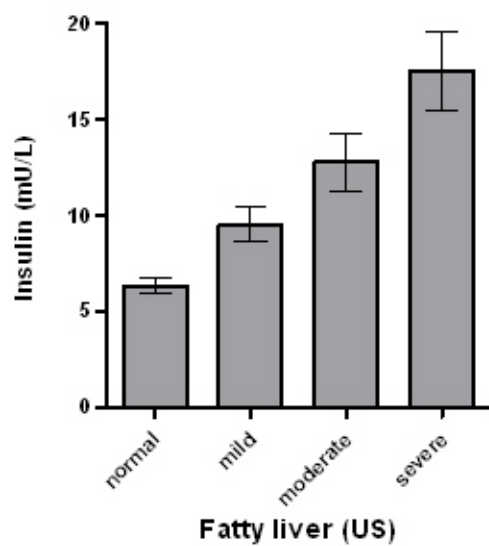
with ultrasound; VAT-US: visceral adipose tissue measured with ultrasound; SKF: skinfold

Including the FCHL status, i.e. being a FCHL patient or normolipidemic relative, as a covariate did not influence the results.

Figure 1

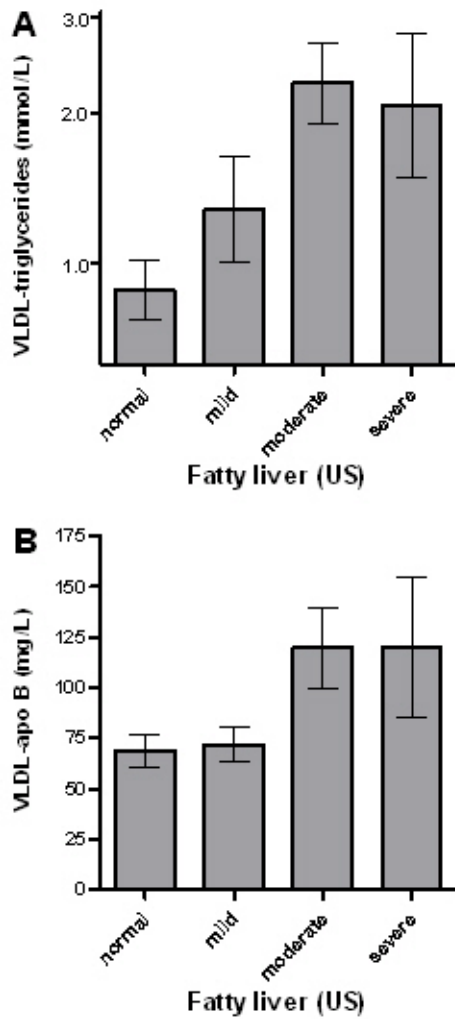


Relationship of fatty liver stages with body mass index (BMI) (A), waist circumference (B), visceral adipose tissue measured with ultrasound (VAT-US) (C) and subscapular skinfold (SKF) (D) in all FCHL family members, i.e. patients and relatives combined (number of FCHL family members for each fatty liver stage: 120 normal, 53 mild, 16 moderate, 11 severe fatty liver). Error bars represent SEM.

Figure 2

Relation of fatty liver stages with plasma insulin levels in all FCHL family members, i.e. patients and relatives combined. Error bars represent SEM.

Figure 3



Relation of fatty liver stages with VLDL-triglycerides (panel A) and VLDL-apo B (panel B) in a subset of FCHL family members (n = 69). Error bars represent SEM.