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Original article

Clinical and metabolic characterization of obese subjects without non-alcoholic fatty liver: A targeted metabolomics approach

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ABSTRACT

Introduction. – As a small proportion of obese individuals do not develop metabolic complications and non-alcoholic fatty liver disease (NAFLD), this study aimed to provide a comprehensive clinical, metabolic and genetic description of obese subjects with healthy livers.

Methods. – A total of 183 subjects were stratified, according to BMI, presence of metabolic syndrome, biochemical liver tests and hepatic steatosis on ultrasound, into: (i) lean controls (n = 69); (ii) obese healthy (n = 50); and (iii)obese NAFLD (n = 62) groups.

Detailed clinical, genetic and metabolic evaluations were then performed.

Results. – Obese healthy subjects did not differ in glucose parameters from lean controls, and had a lower rate of minor *TM6SF2* gene variants compared with obese NAFLD (2/49 vs. 11/60, respectively; P = 0.035) and lean controls (13/64; P = 0.035), but significantly higher leptin concentrations than lean controls ($P < 0.001$); they also higher adiponectin concentrations ($P < 0.001$), and lower TNF-α and IL-6 concentrations (P = 0.01 and $P < 0.001$, respectively), than obese NAFLD subjects. Also, metabolomic studies identified ether- and ester-containing phospholipids [PC ae C44:6, PC ae C42:5, PC aa C40:4; $P < 0.001$, corrected by the false discovery rate (FDR) method] and found that the amino-acids lysine, glycine and isoleucine (FDR < 0.001) differed between the two obese groups, but not between lean controls and obese healthy subjects.

Conclusion. – Obese people with healthy livers are characterized by intact glucose homoeostasis, lower pro-inflammatory cytokine levels, and higher adiponectin and leptin concentrations compared with obese people with NAFLD. In addition, the major allele of *TM6SF2*, a set of phosphatidylcholines and several amino acids are associated with healthy livers in obesity.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) affects approximately one-third of the population in Western societies [1]. At the population level, NAFLD is closely linked to overweight and visceral obesity [2]. While approximately 10–30% of the obese population have no evidence of metabolic complications and so are referred to as the 'metabolically healthy obese', about half of this population will progress to the metabolically unhealthy phenotype over 5–10 years, suggesting that metabolic health in obesity may, in many cases, represent only a transient stage towards the

Abbreviations: NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; FDR, false discovery rate; OGTT, oral glucose tolerance test; NFS, NAFLD fibrosis score; FIB4, fibrosis 4 score; MetS, metabolic syndrome; SNP, single nucleotide polymorphism; T2DM, type 2 diabetes mellitus; IFG, impaired fasting glucose; US, ultrasound examination; IPAQ, international physical activities questionnaire; PC, phosphatidylcholine; SM, sphingomyelin; lysoPC, lysophosphatidylcholine.

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development of metabolic complications [3]. Liver fat, along with insulin resistance (IR) and visceral fat accumulation, is a key determinant of metabolic health in the context of obesity [4].

The pathogenesis of NAFLD involves both genetic and environmental factors [2]. NAFLD is characterized by profound changes in lipid and glucose homoeostasis and, in particular, systemic and hepatic IR [5]. Several genome-wide association studies (GWAS) and candidate gene analyses have identified single nucleotide polymorphisms (SNPs) associated with NAFLD, among which the patatin-like phospholipase domain-containing 3 protein gene (*PNPLA3*) I148M polymorphism and transmembrane 6 superfamily member 2 (*TM6SF2*) gene have been consistently linked to NAFLD and disease progression [6]. Thus, the purpose of the present study is to comprehensively describe those obese subjects who have no clinical or biochemical evidence of fatty liver.

2. Methods

2.1. Definition of study groups

Of the 3800 people from a health-screening programme, the Salzburg Colon Cancer Prevention Initiative (SAKKOPI), 312 (8.0%) of them [95 men (30.5%), 216 women (69.5%)] met the criteria for lean controls, and the random selection of the present study subjects was made from these 312 (see below). A total of 693 (18%) subjects were obese, of which 554 [297 men (53%), 257 women (46%)] had evidence of fatty liver on ultrasound examination. Of these, 110 men and 136 women consumed no alcohol and, from these, 62 obese NAFLD subjects (22 female, 40 male) were randomly selected for our study. In total, 50 obese individuals [7.2%; 14 men (28%), 36 women (72%)] had neither elevated liver tests nor steatosis on ultrasound, nor fulfilled any criteria for the metabolic syndrome (MetS), and were therefore selected as obese healthy subjects in our study; these were all people who fulfilled criteria for their respective groups from the whole of the background population (1.3%).

Ultimately, data from 181 Caucasians were included in our analysis, and each group was defined as follows:

- lean control subjects (n = 69) had body mass index (BMI) Scores $\leq$ 25 kg/m^2, normal liver tests, normal ultrasound and no components of the MetS, according to the US Third Report of the Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults (ATP III) criteria [7];
- obese healthy (n = 50) subjects had BMIs $\geq$ 30.0 kg/m^2, normal liver tests, normal ultrasound and no MetS ($\leq$ 2 components noted; obesity and one additional criterion of MetS were allowed) according to ATP III criteria; and;
- obese NAFLD (n = 62) subjects had BMIs $\geq$ 30.0 kg/m^{2}and unequivocal ultrasound evidence of fatty liver with or without elevated transaminases.

Normal liver tests were defined as alanine transaminase (ALT) and aspartate transaminase (AST) levels $\leq$ 40 U/L for women and $\leq$ 50 U/L for men, with or without increases in gamma-glutamyl transpeptidase (GGT) on at least two occasions at least 4 weeks apart. Although NAFLD is a diagnosis of exclusion that, by definition, requires liver biopsy, this was not ethically acceptable in a population-based study. Thus, our limited definition reflects common clinical practice, but is also supported by the European Association for the Study of the Liver (EASL) – European Association for the Study of Diabetes (EASD) – European Association for the Study of Obesity (EASO) clinical practice guidelines for NAFLD [8].

Study exclusion criteria were laboratory or clinical evidence of autoimmune, viral (viral hepatitis, human immunodeficiency virus) or hereditary causes (Wilson's disease, hereditary haemochromatosis, alpha-1 antitrypsin deficiency) of liver disease, malignancy and clinically relevant alcohol consumption ($>$ 20 g/day for men, $>$ 10 g/day for women). None of the eligible subjects had been previously diagnosed with type 2 diabetes mellitus (T2DM), and none were allowed to be taking potentially steatogenic medications. Subjects were also selected because they were not taking regular medications in order to be representative of the gender and age distributions of all subjects fulfilling their respective group criteria. Antihypertensive and cholesterol-lowering drugs, however, were being used by a small proportion of obese subjects (13/112, 11.6%). From the remaining subjects, lean controls and obese NAFLD subjects were randomly selected.

This study was approved by the local ethics committee (Ethikkommission des Landes Salzburg), and written informed consent was obtained from all participants.

2.2. Clinical and laboratory assessment and ultrasound examination

Details of the participants' clinical, laboratory and ultrasound examinations have been previously reported elsewhere [9], but are also described here in the Appendix (see supplementary materials associated with this article online).

2.3. Genotyping

Genomic DNA was extracted from peripheral blood samples according to standard procedures as reported previously [10]. The *PNPLA3* rs738409, *TM6SF2* rs58542926, *PPP1R3B* rs4240624, *GCKR* rs6834314 and *LYPLAL1* rs12137855 SNPs were assessed.

2.4. Metabolomics

These analyses were performed using the targeted metabolomics approach of combined direct flow injection and liquid chromatography with mass spectrometry (LC-MS/MS; AbsoluteIDQ® p180 Kit; BIOCRATES Life Sciences AG, Innsbruck, Austria), according to the manufacturer's instructions. Methodological details have been reported previously [9] and are here also provided in the supplementary appendix online.

2.5. Assessment of lifestyle and dietary habits

A questionnaire on consumption of meat, vegetables, fruit, fast food and alcohol was used for assessment of dietary habits. The amount of one serving as well as its fibre content were calculated according to recommendations of the American Heart Association. Physical activity was assessed by the short-form International Physical Activity Questionnaire (IPAQ-SF) [11], which classifies populations into three physical-activity categories: high; intermediate; and low. The high category included those performing vigorous activity on $\geq$ 3 days/week and achieving a minimum of 1500 metabolic equivalents of task (METs)-min/week, or any combination of walking and moderate or vigorous activity on at least 7 days/week to achieve a minimum of 3000 METs-min/week. The intermediate IPAQ category grouped participants doing $\geq$ 20 min/day of vigorous activity for $\geq$ 3 days/week, or $\geq$ 30 min/day of walking or moderate activity for $\geq$ 5 days/week or a minimum of 600 METs-min/week of any combination of walking, moderate or vigorous activity for $\geq$ 5 days/week. The low IPAQ category included those not meeting any of the criteria for either of the previous two groups. In addition, smoking status was divided into current smokers, never smokers and former smokers.

2.6. Statistical analysis

For all analyses, the obese healthy group was compared with lean controls and the obese NAFLD group. Statistical calculations were performed using the R statistics environment (R Foundation for Statistical Computing, Vienna, Austria) and SPSS Statistics version 22.0 software (IBM Corp., Armonk, NY, USA). A two-sided P-value < 0.05 was considered statistically significant. Continuous data were expressed as means ± standard deviation (SD) or medians (interquartile range, IQR), and analyzed using analysis of variance (ANOVA) or Kruskal–Wallis tests according to the distribution. Categorical variables were reported as frequencies and compared using Pearson's Chi^2 test. Group differences in genetic variables were assessed by Chi^2 analysis.

For exploration of metabolomics data, the publicly accessible online MetaboAnalyst 3.0 platform was used [12,13]. For analytes measured as below the limit of detection and for missing values, half the minimum positive measured value of the respective metabolite was used instead. Metabolites with > 30% missing values were excluded from the analysis. To identify significantly different metabolites, P-values were adjusted for multiple testing, using the Benjamini–Hochberg procedure for conceptualizing the false discovery rate (FDR). Controlling multiple testing for the FDR is a way to identify a large number of significant features while allowing a relatively low proportion of false positives [14]. Manifestations of metabolite subgroups were visualized by heat maps created with the R 'gplots' package. To identify potential group predictors, variables that were significant on univariate analysis were included in a multiple logistic regression analysis. All possible models were computed and compared using the generalized linear models ('glm') package for R statistics [15]. As a selection criterion to determine the model, the small-sample-size corrected version of the Akaike information criterion (AICc) was used to measure goodness of fit while at the same time avoiding overfitting [16]. The results of different models are presented as receiver operating characteristic (ROC) curves and their respective areas under the curve (AUCs) and 95% confidence intervals (CIs), all calculated and created by the R statistics 'pROC' package [17]. To analyze differences in ROC curves, DeLong's test for ROC curves was applied.

3. Results

3.1. Clinical characteristics

All three groups had similar age distributions. The obese healthy had a higher proportion of women than did the obese NAFLD group, but were similar to lean controls in their waist–hip ratio (WHR), oral glucose tolerance test (OGTT) results, cholesterol concentrations, ALT and GGT activities. High-density lipoprotein cholesterol (HDL-C) concentrations and homoeostasis model assessment for insulin resistance (HOMA-IR) in the obese healthy subjects were in between those for lean controls and obese NAFLD subjects. Cytokine and adipokine analyses revealed similar serum concentrations of interleukin (IL)-6, tumour necrosis factor (TNF)-α and adiponectin in obese healthy subjects and lean controls. Details of the clinical and biochemical characteristics are summarized in Table 1.

3.2. Genotyping NAFLD risk loci

To characterize the genetic underpinnings of obese subjects with normal livers, SNPs previously linked to NAFLD in GWAS were

Table 1
Demographic, clinical, anthropometric and laboratory characteristics of the study population.

	Lean controls (L) (n = 69)	Obese healthy (OH) (n = 50)	Obese NAFLD (ON) (n = 62)	P	Post-hoc P		
					L vs. OH	L vs. ON	OH vs. ON
Age (years)	58 ± 9.6	60 ± 11.0	62 ± 8.1	0.107			
Gender (female n/male n)	44/25	36/14	22/40	< 0.001	0.345	0.002	< 0.001
Body mass index (kg/m^2)	22.7 (2.1)	31.6 (2.7)	32.7 (4.4)	< 0.001	< 0.001	< 0.001	0.071
Waist (cm)	86 ± 7.9	106 ± 9.5	114 ± 8.0	< 0.001	< 0.001	< 0.001	< 0.001
Hip (cm)	96 (7.7)	114 (8.0)	114 (9.7)	< 0.001	< 0.001	< 0.001	0.647
Waist-to-hip ratio	0.90 ± 0.1	0.93 ± 0.1	0.99 ± 0.1	< 0.001	0.138	< 0.001	0.001
ALT (μkat/L)	0.28 (0.13)	0.28 (0.19)	0.47 (0.30)	< 0.001	0.511	< 0.001	< 0.001
AST (μkat/L)	0.33 (0.10)	0.32 (0.11)	0.39 (0.17)	< 0.001	0.788	< 0.001	< 0.001
GGT (μkat/L)	0.28 (0.28)	0.30 (0.20)	0.55 (0.56)	< 0.001	0.648	< 0.001	< 0.001
Fibrosis-4 Index	1.1 (0.6)	1.1 (0.5)	1.1 (0.5)	0.943			
NAFLD fibrosis Score	-2.26 ± 0.98	-1.03 ± 1.2	-0.66 ± 1.3	< 0.001	< 0.001	< 0.001	0.287
Total cholesterol (mmol/L)	5.8 ± 1.0	5.8 ± 0.9	5.6 ± 1.1	0.648			
HDL-C (mmol/L)	1.74 (0.60)	1.49 (0.35)	1.18 (0.33)	< 0.001	0.008	< 0.001	< 0.001
LDL-C (mmol/L)	3.48 ± 0.95	3.74 ± 0.84	3.72 ± 0.90	0.234			
TG (mmol/L)	0.98 (0.46)	1.2 (0.5)	1.9 (0.9)	< 0.001	0.010	< 0.001	< 0.001
FG (mmol/L)	5.1 (0.6)	5.4 (0.8)	6.0 (1.4)	< 0.001	0.036	< 0.001	< 0.001
1-h OGTT (mmol/L)	7.6 (2.5)	8.3 (2.1)	11 (4.4)	< 0.001	0.058	< 0.001	0.004
2-h OGTT (mmol/L)	5.9 (1.4)	6.2 (1.6)	6.8 (2.7)	0.008	0.301	0.005	0.045
HOMA-IR	1.2 ± 1.4	2.1 ± 1.6	3.7 ± 8.1	< 0.001	< 0.001	< 0.001	< 0.001
HOMA ≥ 2.5 [n (%)]	5 (7.2)	13 (26)	50 (82)	< 0.001	< 0.001	< 0.001	< 0.001
MetS components [n (n)]	0 (0)	2 (1)	3 (1)	< 0.001	< 0.001	< 0.001	< 0.001
Interleukin-6 (IU/mL)	0.36 (0.22)	0.34 (0.28)	0.62 (0.39)	< 0.001	0.250	< 0.001	< 0.001
TNF-α (IU/mL)	0.64 (0.19)	0.64 (0.23)	0.75 (0.25)	0.003	0.383	0.012	0.002
Leptin (nmol/L)	0.31 (0.41)	1.7 (1.7)	1.1 (1.1)	< 0.001	< 0.001	< 0.001	0.077
Adiponectin (μg/mL)	14 (5.9)	12 (4.7)	9 (4.3)	< 0.001	0.065	< 0.001	< 0.001
Low physical activity (%)	7/69 (10)	16/49 (33)	16/62 (26)	0.007	0.003	0.034	0.302
Fast-food ≥ 1/week (%)	6/68 (8.8)	6/44 (14)	13/50 (26)	0.034	0.534	0.021	0.198

Data are means ± standard deviation or medians (interquartile range) according to distribution unless otherwise indicated; P-values assessed by ANOVA or Kruskal–Wallis test, or post-hoc analysis or chi-squared test as statistically appropriate.
NAFLD: non-alcoholic fatty liver disease; ALT/AST: alanine/aspartate transaminase; GGT: gamma-glutamyl transferase; HDL-C/LDL-C: high-density lipoprotein/low-density lipoprotein cholesterol; TG: triglyceride; FG: fasting glucose; OGTT: oral glucose tolerance test; HOMA-IR: homoeostasis model assessment for insulin resistance; MetS: metabolic syndrome; TNF: tumour necrosis factor.

analyzed. For all genes, when the proportion of subjects homozygous for the major allele was compared with the proportion of those with at least one minor allele, the rate of subjects with at least one *TM6SF2* minor allele was lower in the obese healthy than in the lean controls and obese NAFLD subjects. No statistically significant differences were observed for any of the other risk alleles, nor did *PNPLA3* variants differ between groups. All subjects had at least one *PPP1R3B* risk allele, so this was not considered further. Details of these genetic analyses are presented in Table 2.

3.3. Serum metabolome analysis

The metabolomic profile included acylcarnitines, amino acids, phosphatidylcholines (PCs), biogenic amines, sphingomyelin (SM) and lysophosphatidylcholines (lysoPCs). The complete results for all group comparisons of metabolite concentrations with FDR-corrected $P < 0.05$ values are presented in Table S1 (see supplementary materials associated with this article online). Comparison of the obese healthy with the obese NAFLD subjects revealed that 57 components differed between them. Differences in metabolite profiles, as visualized on heat maps, are expressed as ratios of metabolite concentrations in the obese healthy to the corresponding means in obese NAFLD subjects (Fig. S1; see supplementary materials associated with this article online). The most significant differences were found for lysine, SM C22:3 and aa C30:2 (all $P < 0.001$); their absolute serum concentrations and *P*-values are presented in Table S1. ROC analysis showed the AUCs for asymmetrical dimethylarginine (ADMA; AUC: 0.89), lysine (AUC: 0.84), SM C22:3 (AUC: 0.83) and PC aa C30:2 (AUC: 0.810).

Comparison of the lean controls with the obese healthy identified 62 components that were different. Ratios of metabolite concentrations in obese healthy to mean lean controls are shown in Fig. S2 (see supplementary materials associated with this article online). Lysine, ADMA and C4:1 (all $P < 0.001$) differed most significantly between these two groups (Table S1). As single components, ADMA (AUC: 0.88), lysine (AUC: 0.83) and C4:1 (AUC: 0.79) had the highest AUCs to discriminate between lean controls and obese healthy subjects.

However, as our specific aim was to identify plasma components linked to the healthy liver phenotype in obesity, our search was narrowed down to components that were different between the two obese groups yet, at the same time, similar between the obese healthy and lean controls. Ultimately, 19 components fulfilled these criteria to potentially serve as indicators for liver health in the context of obesity. These included low concentrations of branched-chain amino acids (BCAAs) and higher concentrations of ether- and ester-containing phospholipids (PC ae) and sphingolipids (Table 3).

3.4. Multivariate analysis

To identify potential group predictors, variables that were significant on univariate analysis were included in a multiple logistic regression analysis, which found that lysine, isoleucine, SM C22:3, adiponectin and IL-6 concentrations effectively differentiated obese healthy from obese NAFLD subjects (Table 4AA). Multiple ROC analyses for these components calculated an AUC of 96.7% for these components in combination to discriminate between obese healthy and obese NAFLD subjects (Fig. 1), while DeLong's test for ROC curves showed a significant difference ($P = 0.002$) between the two curves. Moreover, multiple logistic regression analysis revealed that phosphate, IL-6, leptin, kynurenine, SM C22:3 and *TM6SF2* discriminated between lean controls and the obese healthy (Table 4B); indeed, the combination of leptin, *TM6SF2*, IL-6, kynurenine and SM C22:3 differentiated lean controls from obese healthy subjects with an AUC of 97.6% (Fig. 2), and DeLong's test for ROC curves showed a significant difference ($P = 0.002$) between the two curves. These findings did not change after adjusting the analysis by gender (not shown here).

3.5. Lifestyle and dietary habits

Detailed assessments of dietary composition and smoking status revealed no differences between obese NAFLD and obese healthy subjects, whereas a greater frequency of fast-food consumption was found in obese NAFLD subjects compared with lean controls ($P = 0.021$). Low physical activity was also less frequent in lean controls compared with healthy obese and NAFLD subjects ($P = 0.003$ and $P = 0.034$, respectively), but no differences were found for physical activity between obese healthy and obese NAFLD subjects. The significant results of our lifestyle and dietary evaluations are presented in Table 1.

4. Discussion

In the present study, our aim was to characterize the clinical features, genetic backgrounds, metabolic profiles and lifestyle habits of obese Caucasian subjects with no clinical or biochemical evidence of NAFLD compared with lean controls and obese subjects with unequivocal NAFLD, as detailed investigation of the factors linked to preservation of metabolic fitness in the context of obesity could throw more light on the pathophysiology of obesity-related complications. The key findings of the study were that obese people without fatty liver are clinically characterized by a low degree of abnormal glucose homoeostasis and less pronounced visceral adiposity and, in particular, less adipose tissue dysfunction. In terms of genetics, the obese healthy have a higher carrier rate of the

Table 2
Prevalence of minor alleles of non-alcoholic fatty liver disease (NAFLD) and analysis of association between single nucleotide polymorphism (SNP) genotypes in the three study cohorts.

Genes (alleles)	Lean controls (L) (n = 64)	Obese healthy (OH) (n = 49)	Obese NAFLD (ON) (n = 60)	*P*	Post-hoc *P*		
					L vs. OH	L vs. ON	OH vs. ON
PNPLA3 (G, GG/CC)	21/64 (32.8%)	19/49 (38.8%)	30/60 (50.0%)	0.144	0.511	0.156	0.482
TM6SF2 (CT, TT/CC)	13/64 (20.3%)	2/49 (4.1%)	11/60 (18.3%)	0.039	0.035	0.780	0.045
NCAN (CT, TT/CC)	11/64 (17.2%)	2/49 (4.1%)	11/60 (18.3%)	0.063	0.067	0.867	0.067
GCKR (CT, TT/CC)	40/64 (62.5%)	34/49 (69.4%)	41/60 (68.3%)	0.693	1.000	1.000	1.000
LYPLAL1 (T, TT/CC)	24/64 (37.5%)	20/49 (40.8%)	22/60 (36.7%)	0.898	1.000	1.000	1.000
PPP1R3B (AG/AA)	11/64 (17.2%)	7/49 (14.3%)	15/60 (25.0%)	0.326	0.676	0.571	0.497

P-values assessed by chi-quadrat test or post-hoc analysis as statistically appropriate.
PNPLA3: patatin-like phospholipase domain-containing 3; *TM6SF2*: transmembrane 6 superfamily member 2; *NCAN*: neurocan; *GCKR*: glucokinase regulatory protein; *LYPLAL1*: lysophospholipase-like 1.

Table 3
Components of the metabolomics analysis characteristic of obese healthy (OH) subjects.

	Lean controls (L) (μmol/L)	Obese healthy (OH) (μmol/L)	Obese NAFLD (μmol/L)	*P*	L vs. OH	L vs. ON	OH vs. ON
Acylcarnitines							
C5	0.11 (0.04)	0.00 (0.25)	0.15 (0.04)	< 0.001	0.902	< 0.001	< 0.001
Amino acids and biogenic amines							
Glycine	276 (80)	284 (66)	227 (56)	< 0.001	0.919	< 0.001	< 0.001
Alanine	348 (76)	366 (121)	437 (113)	< 0.001	0.095	< 0.001	< 0.001
Isoleucine	64 (22)	64 (18)	82 (18)	< 0.001	0.943	< 0.001	< 0.001
Valine	194 (45)	195 (27)	221 (48)	< 0.001	0.448	< 0.001	< 0.001
Leucine	143 (49)	136 (38)	170 (39)	< 0.001	0.964	< 0.001	< 0.001
Lysophosphatidylcholines							
LysoPC C16:0	135 (53)	135 (49)	118 (36)	0.007	0.880	0.006	0.011
Phosphatidylcholines							
PC ae C32:1	2.8 (0.96)	2.8 (1.2)	2.3 (0.64)	0.002	0.651	0.002	0.002
PC ae C32:2	0.70 (0.20)	0.68 (0.30)	0.58 (0.17)	0.007	0.646	0.003	0.017
PC ae C34:1	9.3 (3.0)	8.8 (3.4)	7.7 (2.3)	< 0.001	0.357	< 0.001	0.007
PC ae C40:6	4.9 (1.9)	4.5 (1.7)	3.7 (1.1)	< 0.001	0.128	< 0.001	< 0.001
PC ae C42:3	0.92 (0.37)	0.82 (0.29)	0.68 (0.50)	0.001	0.244	< 0.001	0.021
PC ae C42:5	2.5 (0.84)	2.4 (0.85)	2.1 (0.74)	< 0.001	0.505	< 0.001	< 0.001
PC ae C44:4	0.46 (0.16)	0.45 (0.19)	0.39 (0.15)	0.003	0.820	0.002	0.005
PC ae C44:6	1.3 (0.53)	1.3 (0.66)	1.0 (0.36)	< 0.001	0.875	< 0.001	< 0.001
Sphingolipids							
SM OH C14:1	8.9 (2.8)	8.9 (4.2)	6.8 (2.6)	< 0.001	0.491	< 0.001	< 0.001
SM OH C16:1	4.1 (1.6)	4.4 (1.7)	3.3 (1.4)	< 0.001	0.829	< 0.001	< 0.001
SM OH C22:2	14 (7.1)	16 (6.2)	11 (4.4)	< 0.001	0.129	< 0.001	< 0.001
SM C16:0	122 (35)	128 (31)	102 (33)	< 0.001	0.454	< 0.001	< 0.001

Data are expressed as medians (interquartile range); *P*-values assessed by chi-quadrat test or by post-hoc analysis as statistically appropriate.
NAFLD: non-alcoholic fatty liver disease; ae: acyl–alkyl (residue sum); SM: sphingomyelin.

Table 4
Multivariable logistic regression analysis of (A) the most significantly different factors between obese healthy (OH) and obese non-alcoholic fatty liver disease (ON) subjects and (B) between lean controls (L) and OH subjects.

A	OR (95% CI)	*P*
OH vs. ON		
Interleukin-6 (IU/mL)	0.67 (0.43–1.05)	0.081
Adiponectin (μg/mL)	1.37 (1.02–1.85)	0.039
Isoleucine (μmol/L)	0.94 (0.89–0.99)	0.033
Lysine (μmol/L)	1.04 (1.01–1.08)	0.003
SM C22:3 (μmol/L)	2.02 (0.86–4.73)	0.106
B		
L vs. OH		
Phosphate (μmol/L)	0.00 (0.00–0.11)	0.006
Interleukin-6 (IU/mL)	0.78 (0.64–0.95)	0.016
Leptin (IU/mL)	1.25 (1.10–1.41)	0.000
Kynurenine (μmol/L)	4.22 (1.51–11.77)	0.006
SM C22:3 (μmol/L)	3.34 (1.39–8.04)	0.007
TM6SF2 (CT/TT)	6.88 (0.51–92.45)	0.146

OR: odds ratio; SM: sphingomyelin.

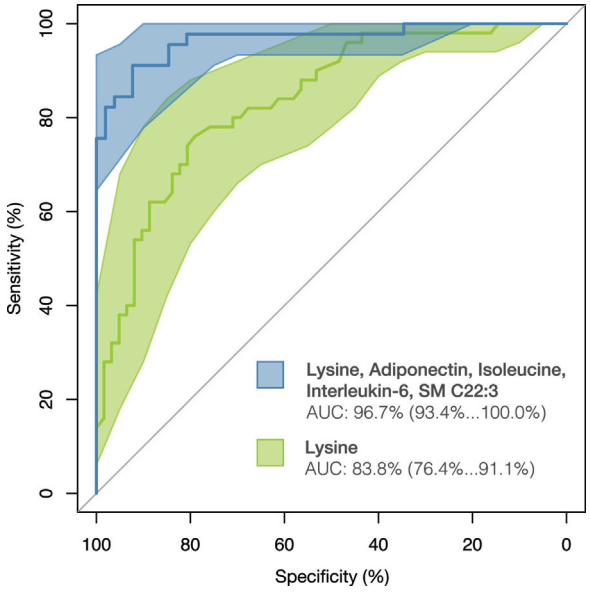

Fig. 1. Metabolomics analysis of obese healthy and obese non-alcoholic fatty liver disease (NAFLD) subjects: receiver operating characteristic (ROC) curve analysis of components significant on multiple logistic regression analysis shows the group prediction (blue) and top component of a single ROC analysis (green), based on area under the curve (AUC) values as percentages and 95% CIs. DeLong's test for ROC curves found a significant difference ($P = 0.002$) between the two curves.

TM6SF2 major allele. In addition, several identified metabolites could serve as indicators of pathways linked to health and to the development of complications in obesity, as no differences in either physical activity or dietary habits were found between obese healthy and obese NAFLD subjects. Moreover, lifestyle and dietary habits were generally found to differ between lean and obese subjects.

One limitation of our study was that the absence or presence of NAFLD was not determined by liver histology, but on clinical grounds. However, more elaborate and sensitive non-invasive tests such as liver stiffness measurement, including continuation attenuation parameter determination and magnetic resonance imaging (MRI), were either not available at the time our study began or were not part of the study protocol. However, that notwithstanding, our study was still in line with international guidelines, which state that normal transaminase and ultrasound findings in the absence of MetS components are sufficient to rule out clinically relevant NAFLD [8]. Although a considerable proportion of NAFLD subjects typically present with normal ALT levels, even in cases of underlying steatohepatitis, our very low ALT levels, which were identical in both our lean controls and obese healthy subjects, support the idea that such obese subjects can reliably be considered to have healthy livers as adjudged both clinically and biochemically.

In addition, when the NAFLD fibrosis Score (NFS) and Fibrosis (FIB)-4 Index were calculated for our study population, the FIB-4 Score was shown to be similar across all three groups with a mean value < 1.4, thereby further indicating the absence of progressive stages of disease. The BMI-based NFS was similar between the obese healthy and obese NAFLD groups because of the impact of

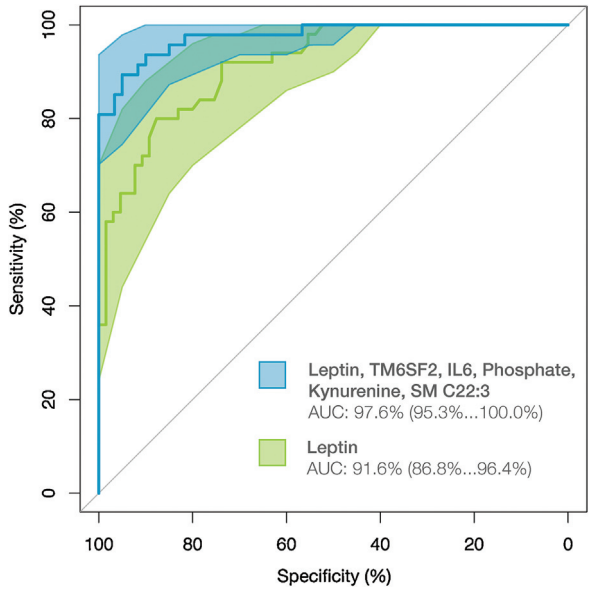

Fig. 2. Metabolomics analysis of lean controls and obese healthy subjects: ROC analysis of components significant on multiple logistic regression analysis shows the group prediction (blue) and top component of a single ROC analysis (green), based on area under the curve (AUC) values as percentages and 95% CIs. DeLong's test for ROC curves found a significant difference (P = 0.013) between the two curves.

BMI on the calculation. NAFLD has been established as the hepatic manifestation of MetS [18], and our results confirm the tight link between NAFLD and the MetS, as the lack of biochemical and clinical signs of NAFLD was linked to a wider absence of clinical, lipid and glucose abnormalities of MetS, thereby suggesting maintenance of systemic metabolic health. Although it is not clear to what extent the liver is causally involved in maintaining metabolic health, our results suggest that the absence of fatty liver in obese subjects might at least serve as a strong marker of overall metabolic fitness. In this context, our genetic findings appear noteworthy, as the near absence of the *TM6SF2* polymorphism for NAFLD risk in obese healthy subjects may indicate that intact *TM6SF2* function may be sufficient to handle the increased metabolic stress in conditions like obesity. The gene is also involved in lipid export and, as such, it is biologically plausible that its proper function may become increasingly relevant during times of energy excess and high systemic lipid turnover [19]. However, as *TM6SF2* has so far only been implicated in the NAFLD phenotype and not in other components of MetS, this genetic finding may be pointing towards causal involvement of the liver in preserving metabolic health in the context of excess adipose tissue. Moreover, twin studies have suggested that liver fat is crucial for the metabolic phenotype of obesity, which might also suggest that *TM6SF2* has a causal role [4].

Adipose tissue (AT) function has been well studied in the metabolically healthy obese phenotype. These subjects present with less visceral and ectopic fat compared with the unhealthy obese despite having similar total fat mass, and also less systemic inflammation [20]. Although AT dysfunction was not directly evaluated in our study – no AT biopsies were performed – changes in concentrations of cytokines like adiponectin, TNF-α, IL-6 and WHR as clinical measures of AT distribution are nonetheless useful indicators of AT function and dysfunction. Most prominently, high adiponectin levels are associated with the metabolically healthy obese phenotype [21], and our present data for serum concentrations of inflammatory cytokines and adipokines are in line with those studies, as our obese healthy subjects had levels similar to those in lean controls. Interestingly, leptin was elevated in the obese healthy compared with lean controls, indicating that leptin concentrations are primarily determined by total fat mass and may not reflect AT function in our study population. However, adiponectin was an indicator of metabolic health in our obese subjects, which is in line with its known effects towards maintaining physiological function in expanding subcutaneous AT as well as insulin sensitivity, and ameliorating inflammation [22].

Regarding glucose homoeostasis, the obese healthy showed a low degree of abnormalities with OGTT after 2 h similar to those of lean controls. On the other hand, fasting glucose and HOMA-IR results in the obese healthy were in between those of the lean controls and obese NAFLD subjects. Similar results were shown in the Cremona study by Calori et al. [23], a population-based study of around 2000 individuals. This might be an indicator of the relatively high risk of obese healthy subjects to deteriorate metabolically over time [3]. To attain further insight into metabolic health associated with obesity, serum metabolome analysis was performed, and revealed that the obese healthy phenotype was associated with lower concentrations of BCAAs and higher concentrations of several PCs and sphingolipids. BCAAs, including leucine, isoleucine and valine, are essential amino acids. Yet, lower concentrations of BCAAs were not linked to lower dietary protein intake in that group. Previous studies, however, have reported an association between BCAAs and IR, obesity, T2DM and NAFLD [24,25], and AT and skeletal muscle are capable of catabolizing circulating BCAAs [26], while recent studies have also shown that obesity and IR may induce down-regulation of BCAA catabolizing enzymes in AT, thereby increasing BCAA concentrations [27]. The subsequent accumulation of BCAAs increases BCAA catabolism in the liver and skeletal muscle [24]. Thus, high levels of BCAAs may hypothetically be associated with higher concentrations of acylcarnitines C5 and C3 in obese subjects as intermediates in the catabolism of BCAAs in muscle, which was also previously confirmed in our group [28]. BCAA metabolism in liver leads to accumulation of catabolic intermediates and incomplete oxidation of fatty acids and glucose [24]. Higher concentrations of fatty acids and their toxic intermediates lead to mitochondrial dysfunction in pancreatic β cells, an important factor in the metabolic unhealthy phenotype [29]. Our present findings may further support the hypothesis that impaired BCAA metabolism may be causally involved in the development of adverse health outcomes in obesity and may therefore also merit further study in the context of NAFLD.

Low concentrations of some PC ae (acyl–alkyl PCs) and sphingolipids were characteristic of obese NAFLD subjects whereas, in the obese healthy and lean controls, such concentrations were similar. Indeed, similar findings were also reported by Böhm et al. [30] for cultured human adipocytes from healthy and unhealthy obese subjects. Also, a direct correlation of certain PC ae and sphingolipid concentrations with cardiorespiratory fitness was reported by Floegel et al. [31] in a population-based study of 2500 participants, whereas obesity was inversely linked to PC ae concentrations. Sphingolipids and PCs are phospholipids that are biochemically related, and both are key components of cell membranes. Synthesis of sphingolipids requires transfer of a phosphocholine head group of PCs to ceramide [32]. Phospholipids are required for intact formation of lipoproteins, and are also substrates for triglycerides and fatty acids in the liver [33]. A mouse model of MetS suggested that lower PC concentrations may be due to increases in turnover and size of adipocytes necessitating high PC amounts for membrane production [34]. In obesity, a higher turnover of adipocytes has been documented, as has also an association between adipocyte hypertrophy and metabolic complications like NAFLD [35]. In the context of these reports, our findings provide evidence that higher concentrations of PCs may indicate intact AT function and metabolic health.

Our investigation into dietary habits and smoking status revealed no differences between obese NAFLD and obese healthy

subjects, although differences were observed between lean and obese subjects, as reported previously [36].

However, multiple logistic regression analyses found that lysine, isoleucine, SM C22:3, adiponectin and IL-6 concentrations effectively discriminated the obese healthy from obese NAFLD subjects, whereas ROC analysis indicated that these parameters may be useful for developing a Score to rule out NAFLD in obese subjects. Unfortunately, our small-sample size was not appropriate for the development of a validated Score.

Another goal of our study was to assess the metabolic and genetic characteristics that reflected the background population, which led to a larger proportion of women with obesity but healthy livers. Such a gender imbalance was previously reported with men being more likely to develop visceral adiposity and a fatty liver [37]. Nevertheless, the results of our group comparisons did not change significantly when the number of men in the obese NAFLD group or number of women in the obese healthy group was artificially reduced.

At this time, our intention is to follow-up all our groups to conclusively determine the biological relevance of the obese healthy phenotype to the development of adverse health sequelae and causes of death. This is because the natural course of so-called metabolically healthy obesity has been incompletely defined, as it may represent only a transitory state on the way to metabolically unhealthy obesity [38].

5. Conclusion

Our present study has shown that obese subjects with clinically defined healthy livers are largely devoid of the systemic metabolic complications of obesity. Also, as lower concentrations of BCAAs are closely linked to the healthy phenotype, this suggests that their metabolism may be causally involved in the pathways maintaining metabolic health independently of dietary habits and physical activity. In addition, presence of the *TM6SF2* major allele may serve as a prerequisite of the ability to preserve a healthy liver when obesity has already developed. However, the long-term natural course of our well-defined subjects still needs to be elucidated.

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Author contributions

AF, SKE – data analysis, drafting and writing of manuscript; TKF, BP, MS, SZ, LS, MS, GS, WP, DW, USH, DN – patient recruitment, data acquisition and revision of manuscript for important intellectual content; CD, EA – study concept and design, analysis and interpretation of data, outlining and revising the manuscript.

Disclosure of interest

The authors declare that they have no competing interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.diabet.2018.09.003.

References

[1] Younossi ZM, Blissett D, Blissett R, Henry L, Stepanova M, Younossi Y, et al. The economic and clinical burden of non-alcoholic fatty liver disease in the United States and Europe. Hepatology 2016;64:1577–86.
[2] Pallayova M, Taheri S. Non-alcoholic fatty liver disease in obese adults: clinical aspects and current management strategies. Clin Obes 2014;4:243–53.
[3] Soriguer F, Gutierrez-Repiso C, Rubio-Martin E, Garcia-Fuentes E, Almaraz MC, Colomo N, et al. Metabolically healthy but obese, a matter of time? Findings from the prospective Pizarra study. J Clin Endocrinol Metab 2013;98:2318–25.
[4] Naukkarinen J, Heinonen S, Hakkarainen A, Lundbom J, Vuolteenaho K, Saarinen L, et al. Characterising metabolically healthy obesity in weight-discordant monozygotic twins. Diabetologia 2014;57:167–76.
[5] Maximos M, Bril F, Portillo Sanchez P, Lomonaco R, Orsak B, Biernacki D, et al. The role of liver fat and insulin resistance as determinants of plasma aminotransferase elevation in non-alcoholic fatty liver disease. Hepatology 2015;61:153–60.
[6] Sookoian S, Pirola CJ. Genetic predisposition in non-alcoholic fatty liver disease. Clin Mole Hepatol 2017;23:1–12.
[7] Expert Panel on Detection E. Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult treatment panel III). Jama 2001;285:2486.
[8] European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO clinical practice guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388–1402.
[9] Feldman A, Eder SK, Felder TK, Kedenko L, Paulweber B, Stadlmayr A, et al. Clinical and metabolic characterization of lean Caucasian subjects with non-alcoholic fatty liver. Am J Gastroenterol 2017;112:102–10.
[10] Kedenko L, Lamina C, Kedenko I, Kollerits B, Kiesslich T, Iglseder B, et al. Genetic polymorphisms at SIRT1 and FOXO1 are associated with carotid atherosclerosis in the SAPHIR cohort. BMC Med Genet 2014;15:112.
[11] Craig CL, Marshall AL, Sjostrom M, Bauman AE, Booth ML, Ainsworth BE, et al. International physical activity questionnaire: 12-country reliability and validity. Med Sci Sports Exerc 2003;35:1381–95.
[12] Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res 2009;37(Web Server issue):W652–60.
[13] Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0 – making metabolomics more meaningful. Nucleic Acids Res 2015;43(W1):W251–7.
[14] Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. Stat Med 1990;9:811–8.
[15] Calcagno V, de Mazancourt C. glmulti: an R package for easy automated model selection with (generalized) linear models. Journal of statistical software 2010;34:1–29.
[16] McQuarrie AD, Tsai C-L. Regression and time series model selection. World Scientific, Singapore; 1998.
[17] Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011;12:77.
[18] Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, et al. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes 2001;50:1844–50.
[19] Rinella ME, Sanyal AJ. NAFLD in 2014: genetics, diagnostics and therapeutic advances in NAFLD. Nat Rev Gastroenterol Hepatol 2015;12:65–6.
[20] Koster A, Stenholm S, Alley DE, Kim LJ, Simonsick EM, Kanaya AM, et al. Body fat distribution and inflammation among obese older adults with and without metabolic syndrome. Obesity (Silver Spring) 2010;18:2354–61.
[21] Aguilar-Salinas CA, Garcia EG, Robles L, Riano D, Ruiz-Gomez DG, Garcia-Ulloa AC, et al. High adiponectin concentrations are associated with the metabolically healthy obese phenotype. J Clin Endocrinol Metab 2008;93:4075–9.
[22] Stern Jennifer H, Rutkowski Joseph M, Scherer Philipp E. Adiponectin, leptin, and fatty acids in the maintenance of metabolic homeostasis through adipose tissue crosstalk. Cell Metab 2016;23:770–84.
[23] Calori G, Lattuada G, Piemonti L, Garancini MP, Ragogna F, Villa M, et al. Prevalence, metabolic features, and prognosis of metabolically healthy obese Italian individuals: the Cremona study. Diabetes Care 2011;34:210–5.
[24] Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. Cell Metab 2012;15:606–14.
[25] Goffredo M, Santoro N, Trico D, Giannini C, D'Adamo E, Zhao H, et al. A branched-chain amino acid-related metabolic signature characterizes obese adolescents with non-alcoholic fatty liver disease. Nutrients 2017;9:642 [piiE642].
[26] Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB. Adipose tissue branched-chain amino acid (BCAA) metabolism modulates circulating BCAA levels. J Biol Chem 2010;285:11348–56.
[27] Pietilainen KH, Naukkarinen J, Rissanen A, Saharinen J, Ellonen P, Keranen H, et al. Global transcript profiles of fat in monozygotic twins discordant for BMI: pathways behind acquired obesity. PLOS Med 2008;5:e51.
[28] Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 2009;9:311–26.

[29] Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol 2014;10:723–36.
[30] Bohm A, Halama A, Meile T, Zdichavsky M, Lehmann R, Weigert C, et al. Metabolic signatures of cultured human adipocytes from metabolically healthy versus unhealthy obese individuals. PLOS One 2014;9:e93148.
[31] Floegel A, Wientzek A, Bachlechner U, Jacobs S, Drogan D, Prehn C, et al. Linking diet, physical activity, cardiorespiratory fitness and obesity to serum metabolite networks: findings from a population-based study. Int J Obes (Lond) 2014;38:1388–96.
[32] Gault CR, Obeid LM, Hannun YA. An overview of sphingolipid metabolism: from synthesis to breakdown. Adv Exp Med Biol 2010;688:1–23.
[33] van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. Biochim Biophys Acta 2017;1859:1558–72.
[34] Schafer N, Yu Z, Wagener A, Millrose MK, Reissmann M, Bortfeldt R, et al. Changes in metabolite profiles caused by genetically determined obesity in mice. Metabolomics 2014;10:461–72.
[35] Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, et al. Dynamics of fat cell turnover in humans. Nature 2008;453:783–7.
[36] Hankinson AL, Daviglus ML, Van Horn L, Chan Q, Brown I, Holmes E, et al. Diet composition and activity level of at risk and metabolically healthy obese American adults. Obesity (Silver Spring) 2013;21:637–43.
[37] Samaras K, Campbell LV. The non-genetic determinants of central adiposity. Int J Obes Relat Metab Disord 1997;21:839–45.
[38] Munoz-Garach A, Cornejo-Pareja I, Tinahones FJ. Does metabolically healthy obesity exist? Nutrients 2016;8:E320.