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Circulating Triacylglycerol Signatures in Nonalcoholic Fatty Liver Disease Associated With the I148M Variant in PNPLA3 and With Obesity

We examined whether relative concentrations of circulating triacylglycerols (TAGs) between carriers compared with noncarriers of PNPLA3^{I148M} gene variant display deficiency of TAGs, which accumulate in the liver because of defective lipase activity. We also analyzed the effects of obesity-associated nonalcoholic fatty liver disease (NAFLD) independent of genotype, and of NAFLD due to either PNPLA3^{I148M} gene variant or obesity on circulating TAGs. A total of 372 subjects were divided into groups based on PNPLA3 genotype or obesity. Absolute and relative deficiency of distinct circulating TAGs was observed in the PNPLA3$^{148MM/148MI}$ compared with the PNPLA3^{148II} group. Obese and 'nonobese' groups had similar PNPLA3 genotypes, but the obese subjects were insulin-resistant. Liver fat was similarly increased in obese and PNPLA3$^{148MM/148MI}$ groups. Relative concentrations of TAGs in the obese subjects versus nonobese displayed multiple changes. These closely resembled those between obese subjects with NAFLD but without PNPLA3^{I148M} versus those with the I148M variant and NAFLD. The etiology of NAFLD influences circulating TAG profiles. 'PNPLA3 NAFLD' is associated with a relative deficiency of TAGs, supporting the idea that the I148M variant impedes intrahepatocellular lipolysis rather than stimulates TAG synthesis. 'Obese NAFLD' is associated with multiple changes in TAGs, which can be attributed to obesity/insulin resistance rather than increased liver fat content per se.

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Nonalcoholic fatty liver disease (NAFLD) is defined as steatosis, which is not caused by excess alcohol consumption or other known causes (1). It refers to a spectrum of hepatic pathology, which may progress from simple steatosis to nonalcoholic steatohepatitis, fibrosis, and even cirrhosis (2). NAFLD has become a leading cause of chronic liver disease worldwide (3). Recent studies have, however, shown that NAFLD is heterogeneous and at least two distinct forms exist.

The increase in the prevalence of NAFLD has paralleled that of obesity (1). Although not all obese subjects have

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See accompanying commentary, p. 42.

NAFLD, and NAFLD may also be observed in 'nonobese' subjects, 'obese NAFLD' is characterized by hepatic insulin resistance (4), which impairs the ability of insulin to inhibit hepatic glucose and VLDL production. This leads to hyperglycemia, hyperinsulinemia, hypertriglyceridemia, and a low HDL cholesterol concentration. Thus, 'obese NAFLD' closely resembles the metabolic/insulin resistance syndrome (4).

In 2008, Romeo et al. (5) described a single nucleotide polymorphism (I148M) in the PNPLA3 (adiponutrin) gene, which was strongly associated with NAFLD. In subsequent studies, the variant allele has been identified to robustly associate with hepatic triglyceride content in at least 10 different ethnic groups (5,6). In a meta-analysis of 16 studies by Sookoian and Pirola (7), carriers of the minor G allele ($PNPLA3^{148MM}$) had a 73% higher liver fat content than weight-matched subjects homozygous for the C allele ($PNPLA3^{148II}$). In the study by Romeo et al. (5) and the studies included in the meta-analysis (7), $PNPLA3^{148MM/148MI}$ was not accompanied by insulin resistance, hyperglycemia, hypertriglyceridemia, or a low HDL cholesterol concentration (4,7). Consistent with lack of insulin resistance and in contrast to obese NAFLD, $PNPLA3^{148MM/148MI}$ has not been shown to be associated with an increase in the prevalence of type 2 diabetes (5).

In humans, PNPLA3 is expressed predominantly in the liver (8). In vitro studies using recombinant purified human PNPLA3 have shown that the wild-type enzyme hydrolyzes triglycerides and that the I148M substitution abolishes this activity (9). These data suggested that the I148M substitution is a loss-of-function mutation impairing triglyceride hydrolysis. Consistent with these data, overexpression of the human $PNPLA3^{I148M}$ variant in the murine liver recapitulated the fatty liver phenotype (10), whereas overexpression of PNPLA3 or its variant in adipose tissue did not affect fat cell morphology; body weight; or circulating concentrations of lipids, glucose, or fatty acids (10). On the other hand, recent data have suggested that PNPLA3 may also have lysophosphatidic acid acyltransferase (LPAAT) activity, and that the I148M substitution increases LPAAT activity (i.e., it is a gain-of-function mutation), resulting in hepatic steatosis by increasing triacylglycerol (TAG) synthesis (10).

PNPLA3 has a strong preference for TAGs in which the acyl group is oleic acid (9). Consistent with this substrate preference, the livers of transgenic mice overexpressing the $PNPLA3^{I148M}$ variant are enriched by 18:1 and 16:1 containing TAGs compared with wild-type mice (9). If this also occurred in humans, one might hypothesize that deficiency of monounsaturated fatty acid containing TAGs characterizes the relative concentration of serum TAGs in subjects carrying the $PNPLA3^{I148M}$ allele compared with those without the variant allele. On the other hand, if the I148M substitution increased intrahepatocellular TAG synthesis by increasing LPAAT activity, one would not expect a reduced amount of TAGs in the circulation (10). Whether and how NAFLD is attributable to the I148M substitution or obesity independent of the genotype changes the distribution of TAGs in humans has not been studied.

In the current study, we examined whether the circulating lipid signature differs between carriers and noncarriers of the I148M variant in PNPLA3. We also analyzed how 'common NAFLD' (i.e., NAFLD due to obesity) influences the lipid signature compared with less obese subjects with a similar PNPLA3 genotype. Finally, we searched for subjects with NAFLD due to the I148M variant and for obese subjects with NAFLD but without the I148M variant to examine how obesity/insulin resistance changes serum TAGs in the face of a similar increase in liver fat content.

RESEARCH DESIGN AND METHODS

Subjects and Study Design

The metabolic studies were performed at the University of Helsinki and the University of Turku. The subjects (n = 372) were recruited by newspaper advertisements or by contacting occupational health services or from among subjects referred to the Department of Gastroenterology due to chronically elevated serum transaminase concentrations using the following inclusion criteria: *1*) age 18 to 75 years; *2*) no known acute or chronic disease except for obesity or type 2 diabetes based on medical history, physical examination, and standard laboratory test results (blood counts, serum creatinine, thyroid-stimulating hormone, electrolyte concentrations), and electrocardiogram findings; and *3*) alcohol consumption of $<$20 g/day. Hepatitis B and C serology, transferrin saturation, anti–smooth muscle antibodies, antinuclear antibodies, and anti–mitochondrial antibodies were measured in patients referred to the gastroenterologist as a result of chronically elevated liver function test results. Exclusion criteria included use of glitazones and pregnancy. Elevated levels of liver enzymes (serum alanine aminotransferase [ALT] or aspartate aminotransferase [AST]) were not exclusion criteria. The study protocol was approved by the ethics committees of the University Hospital of Helsinki and the University Hospital of Turku. Each participant provided written informed consent.

In eligible subjects, a blood sample was taken after an overnight fast for lipidomic analyses and for measurement of fasting plasma glucose, fasting serum (fS) insulin, fS-LDL cholesterol, total serum cholesterol, fS-HDL cholesterol, fS-TAG, fS-AST, fS-ALT, and fS–γ-glutamyl transferase concentrations, as previously described (11). On this visit, body weight was recorded to the nearest 0.1 kg using a calibrated weighting scale with subjects standing barefoot and wearing light indoor clothing. Waist circumference was measured midway between spina iliaca superior and the lower rib margin. Body height was recorded to the nearest 0.5 cm using a ruler attached to the scale. Blood pressure was measured in the sitting position after 10–15 min of rest

using a random-zero sphygmomanometer (ERKA, Bad Tölz, Germany).

Definition of Subgroups Based on PNPLA3 Genotype and Obesity

To study the effect of the PNPLA3 genotype on the circulating lipid signature, the study subjects were divided into groups of subjects who either carried the PNPLA3 I148M variant ($PNPLA3^{148MM/148MI}$) or were homozygous for the wild-type allele ($PNPLA3^{148II}$), or into two groups based on their median BMI (33.5 kg/m^2), although the nonobese is somewhat of a misnomer as this group included some obese subjects. The latter groups are referred to as obese (BMI greater than the median) and 'nonobese' (BMI less than the median), although the nonobese group included some obese subjects (BMI $\geq$ 30 kg/m^2). To determine whether obesity and the $PNPLA3^{I148M}$ gene variant are associated with differences in serum TAG profile in the face of a similar increase in liver fat content, we searched for subjects with NAFLD due to the I148M variant ('PNPLA3 NAFLD') and for obese subjects with NAFLD lacking the I148M variant ('obese NAFLD').

Liver Fat Content

In 75% of the subjects, liver fat content was measured using ^{1}H-magnetic resonance spectroscopy as previously described (11). This measurement has been validated against histologically determined lipid content (12) and against estimates of fatty infiltration by computed tomography (13). The reproducibility of repeated measurements of liver fat in nondiabetic subjects as determined on two separate occasions in our laboratory is 11% (14). In 25% of subjects, liver fat was measured using a liver biopsy. The fat content of the liver biopsy specimens (the percentage of hepatocytes with macrovesicular steatosis) was determined using hematoxylin-eosin staining. The percent of macrovesicular steatosis was converted to the liver fat percentage, measured by ^{1}H-magnetic resonance spectroscopy and liver histology, as previously described (12). NAFLD was defined as liver fat $\geq$55.6 mg triglyceride per gram of liver tissue or $\geq$5.56% of liver tissue weight (15).

Lipidomic Analysis by Ultra-Performance Liquid Chromatography-Mass Spectrometry

An established platform for ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-MS), based on ACQUITY UPLC (Waters), was applied to analyze serum or plasma samples (16). The samples analyzed by the UPLC-MS included citrate plasma (78% of the samples) as well as EDTA plasma and serum (16 and 7%, respectively). Studies comparing citrate and EDTA plasma and serum samples from the same subjects showed comparable lipidomics data (Supplementary Data). The data were processed by using MZmine 2 software (17), and the lipid identification was based on an internal spectral library or on de novo identification using tandem MS (16). Details of sample analysis are given in the Supplementary Data.

Definition of the Metabolic Syndrome

The metabolic syndrome was defined according to criteria of the International Diabetes Federation (18).

Statistical Analyses

Statistical Analysis of Clinical Data

The clinical data (i.e., the variables shown in Table 1) consisted of some discrete binary variables and some continuous variables. The association of each binary variable with PNPLA3 genotype or obesity was tested using Pearson χ^2 tests. Shapiro-Wilks tests indicated that all continuous variables significantly deviated from normal distribution. Therefore, to compare values between PNPLA3 variant and wild-type allele groups and obese and nonobese groups, Wilcoxon rank sum tests were applied. Medians and 95% CIs and means and SEMs were computed for all four subgroups based on PNPLA3 genotype and obesity.

Cluster Analysis of Lipidomics Data

In order to find groups of lipids with similar profiles in all study samples, we applied Bayesian model-based clustering using an R (http://www.r-project.org/) package, mclust version 4.0 (19). For this analysis, the lipidomics data were $\log_2$-transformed, and each lipid variable was scaled to zero mean and unit variance. An average profile for each resulting cluster was calculated by taking the mean value of all variables in it, sample by sample. Mean values of the cluster profiles were compared between PNPLA3 variant and wild-type allele groups and obese and nonobese groups using Student *t* tests with the t.test function of the R package, stats. The comparisons of cluster profiles were visualized as bar plots using barplot2 function of the R package, gplots, after antilogging the cluster profiles.

Analysis of TAG Abundances as a Function of Fatty Acid Chain Lengths and Saturation

Mean values and SEMs of the TAG abundances were calculated in all four subgroups based on PNPLA3 genotype and obesity. Student *t* tests were used to compare the mean values of TAG abundances between the pair of PNPLA3 genotype groups after the data were $\log_2$-transformed. Multiple hypotheses testing has been addressed by using the false discovery rate method of Benjamini and Hochberg (20) to calculate *q* values. The obese groups were also compared similarly. The comparisons of the abundances of TAG molecules were visualized with respect to their fatty acid chain lengths and the number of double bonds using heatmaps created using an R package, ihm (http://code.google.com/p/ihm). The data values visualized in the heatmap are $\log_2$ of the ratio of the mean values of the case group divided by the

Table 1—Clinical characteristics between PNPLA3^{148II} and PNPLA3$^{148MM/148MI}$, and the obese and the nonobese groups

Variables	PNPLA3^{148II} (*N* = 196)	PNPLA3$^{148MM/148MI}$ (*N* = 176)	Nonobese (*N* = 165)	Obese (*N* = 165)	PNPLA3 NAFLD (*N* = 47)	Obese NAFLD (*N* = 51)
Age (years)	47 (44–49)	48 (45–50)	48 (45–51)	47 (45–50)	51 (44–53)	47 (41–53)
Male sex (%)	37	36	36	39	53	31*
PNPLA3 genotype (%)†	0/0/100	14/86/0	6/41/53	8/41/51	17/83/0	0/0/100
Type 2 diabetes (%)	25	34	26	36	36	33
Metabolic syndrome (%)	73	76	66	84‡	83	82
BMI (kg/m^2)	35.7 ± 6	36.2 ± 0.7	28.9 ± 0.3	42.1 ± 0.5‡	29.6 ± 0.4	41.0 ± 0.9‡
Liver fat (%)	8.6 ± 0.6	10.5 ± 0.7*	7.5 ± 0.5	11.6 ± 0.7‡	15.8 ± 1.2	15.9 ± 1.2
fS-Insulin (mU/L)	12.5 ± 0.5	12.9 ± 0.7	9.9 ± 0.5	15.6 ± 0.7‡	12.3 ± 0.9	15.7 ± 1.0*
fS-ALT (IU/L)	31 (29–33)	33.5 (29–36)	29 (26–33)	33 (30–37)	46 (34–64)	33 (29–44)*
fS-AST (IU/L)	28 (26–30)	30 (28–33)	27.5 (26–30)	30 (28–32)	34 (30–45)	31 (27–35)*
fS-Triglycerides (mmol/L)	1.51 (1.40–1.70)	1.36 (1.25–1.50)§	1.4 (1.3–1.6)	1.5 (1.4–1.6)	1.60 (1.43–1.75)	1.60 (1.45–1.97)
fS-HDL cholesterol (mmol/L)	1.21 (1.16–1.27)	1.21 (1.17–1.28)	1.31 (1.24–1.41)	1.16 (1.11–1.21)‡	1.16 (1.05–1.25)	1.17 (1.05–1.26)
fS-LDL cholesterol (mmol/L)	2.89 (2.70–3.06)	2.7 (2.54–2.88)	2.96 (2.85–3.10)	2.60 (2.32–2.80)‖	3.03 (2.71–3.30)	2.98 (2.57–3.28)

Data are median (95% CI) or mean ± SEM unless otherwise stated. *$P < 0.05$. †PNPLA3 genotype at rs738409 for pairs GG/CG/CC, where C is the common allele and G is the variant allele (I148M). ‡$P < 0.0005$. §$P = 0.10$. ‖$P < 0.005$.

control group. The cells in the heatmap are also marked to represent the significance of the difference in the mean. All data analyses of the in vivo data were performed using an R package, metadar (http://code.google.com/p/metadar), as the interface.

RESULTS

Characteristics of the Study Groups

PNPLA3 Subgroups

The PNPLA3$^{148MM/148MI}$ and the PNPLA3^{148II} groups were comparable with respect to age, sex, and BMI (Table 1 and Fig. 1). The PNPLA3$^{148MM/148MI}$ group had a significantly higher liver fat content (10.5 ± 0.7%) than the PNPLA3^{148II} group (8.6 ± 0.6%, $P < 0.05$). Serum insulin (Table 1 and Fig. 1), HDL, and LDL cholesterol concentrations (Table 1) were comparable between the groups. Serum total TAG concentrations were slightly but not significantly ($P = 0.10$) lower in the PNPLA3$^{148MM/148MI}$ than the PNPLA3^{148II} group (Table 1).

Obese Versus Nonobese Subgroups

The obese and the 'nonobese' groups were comparable with respect to age, sex, and PNPLA3 genotype (Table 1). The mean BMI in the obese group was 42.1 ± 0.5 kg/m^2, and in the 'nonobese' group, 28.9 ± 0.3 kg/m^2 (Table 1 and Fig. 1). Liver fat content was significantly ($P < 0.0005$) higher in the obese group (11.6 ± 0.7%) than in the 'nonobese' group (7.5 ± 0.5%) (Table 1 and Fig. 1). The obese group also had significantly higher serum insulin (15.6 ± 0.7 vs. 9.9 ± 0.5 mU/L, $P < 0.0005$) (Table 1 and Fig. 1) and TAG concentrations, and lower HDL and LDL cholesterol concentrations than the 'nonobese' group (Table 1). The obese group is therefore denoted as being obese/insulin-resistant in the discussion.

'PNPLA3 NAFLD' Versus 'Obese NAFLD'

We identified 47 subjects with 'PNPLA3 NAFLD' and 51 with 'obese NAFLD.' The 'PNPLA3 NAFLD' and the 'obese NAFLD' groups were comparable with respect to liver fat (Table 1). The mean BMI in the 'PNPLA3 NAFLD' group was 29.6 ± 0.4 kg/m^2, and in the 'obese NAFLD' group it was 41.0 ± 0.9 kg/m^2 ($P < 0.0001$). The 'obese NAFLD' group had significantly ($P = 0.005$) higher mean serum insulin concentrations (15.7 ± 1.0) than the 'PNPLA3 NAFLD' group (12.3 ± 0.9 mU/L, $P < 0.001$). Serum total TAG, HDL, and LDL cholesterol concentrations were similar between the NAFLD subgroups (Table 1).

Cluster Analysis

Using the UPLC-MS based analytical platform, a total of 413 molecular lipids were measured. Of these, 161 were identified. The global lipidome was first surveyed by clustering the data into a subset of clusters using Bayesian model-based clustering (19). The lipidomic platform data were decomposed into 11 lipid clusters (LCs). The clusters largely followed different lipid functional or structural groups (data not shown). In the PNPLA3$^{148MM/148MI}$ group, compared with the PNPLA3^{148II} group, only LC1 differed significantly (Fig. 2). This LC included only TAGs (Supplementary Table 1), which were therefore the focus for further analysis. Three

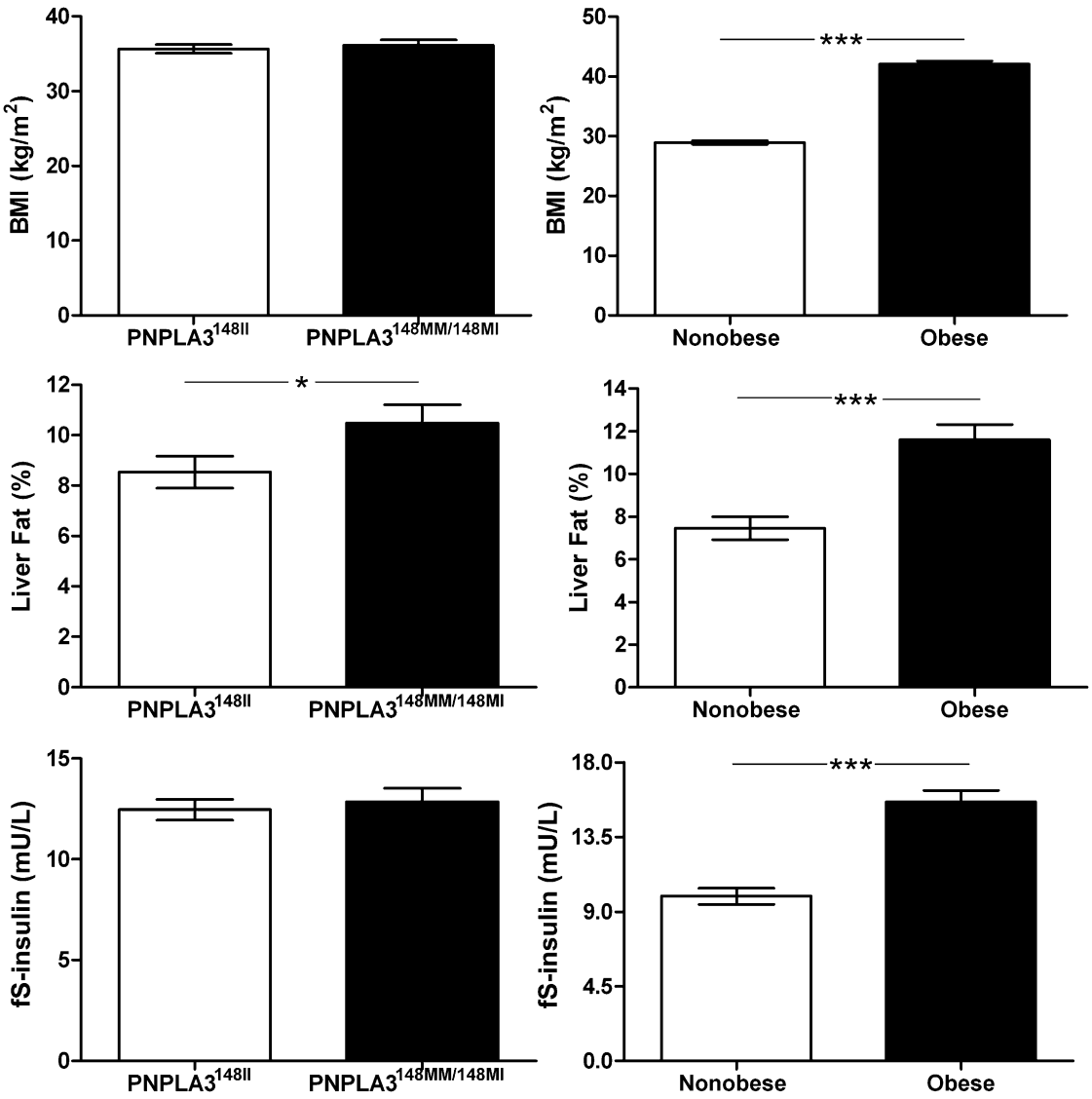

Figure 1—BMI (*top panel*), liver fat content (*middle panel*), and fS-insulin concentrations (*bottom panel*) between PNPLA3^{148II} and PNPLA3$^{148MM/148MI}$ groups, and between obese and 'nonobese' groups. Data are shown as mean ± SEM. *P < 0.05, ***P < 0.0005 for the comparisons shown in the figure.

LCs differed between the obese and nonobese groups, but will not be discussed further.

Absolute Concentrations of TAGs

Comparison of the absolute concentrations of circulating TAGs between the PNPLA3$^{148MM/148MI}$ and PNPLA3^{148II}, obese and 'nonobese,' and 'PNPLA3 NAFLD' and 'obese NAFLD' groups are shown in Supplementary Tables 1–3 and as heatmaps (Figs. 3,4,5, *left panels*).

The absolute concentrations of several TAG species were significantly lower in the PNPLA3$^{148MM/148MI}$ compared with the PNPLA3^{148II} group. These TAGs included both major (most abundant) and minor TAG species, of which most contained an 18:1 fatty-acyl group (Supplementary Table 1).

In the obese group, compared with the 'nonobese' group, the absolute concentrations of multiple TAGs were significantly increased (Supplementary Table 2). The most abundant TAG, in terms of absolute TAG concentrations, was TAG (16:0/18:1/18:1), which was increased in the obese subjects compared with the 'nonobese' subjects (168 ± 7 vs. 148 ± 5 μmol/L, P = 0.0042). The same most abundant TAG (16:0/18:1/18:1) was lower in the PNPLA3$^{148MM/148MI}$ than the PNPLA3^{148II} group (148 ± 4 vs. 169 ± 6 μmol/L, P = 0.026). The absolute concentrations of several other TAGs with 51–58 carbon bonds and 2–9 double bonds were also significantly higher in the obese group than in the 'nonobese' group, as shown in Supplementary Table 2. The absolute concentrations of short-chain TAGs (42–44 carbon bonds) were lower in the obese group than in the 'nonobese' group (Fig. 4, *left panel*).

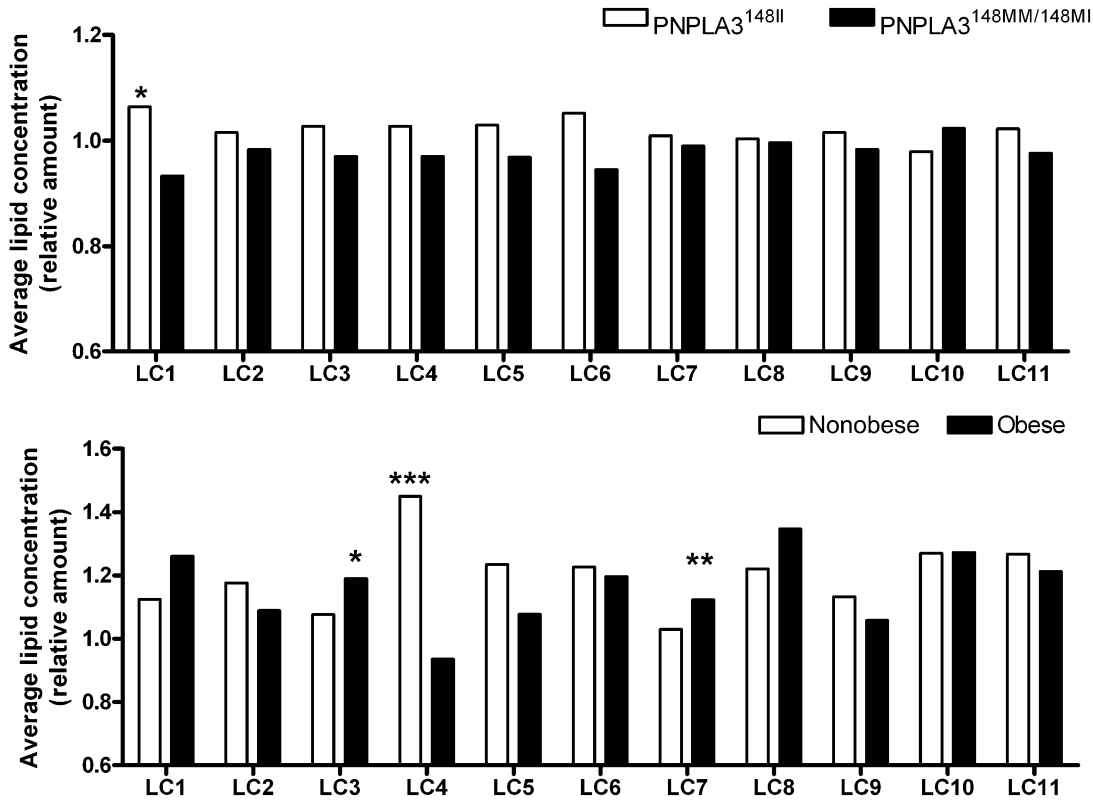

Figure 2—Mean lipid concentrations within each cluster between PNPLA3^{148II} and PNPLA3$^{148MM/148MI}$ groups (*top panel*) and between obese and 'nonobese' groups (*bottom panel*). Statistical comparison performed by two-sided *t* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

The differences in absolute concentrations of TAGs between the NAFLD subgroups closely resembled those observed between the obese and nonobese groups (Fig. 5 and Supplementary Table 3).

Relative Concentrations of Triglycerides

The relative distribution of triglycerides (concentration of an individual TAG/all TAGs measured by UPLC-MS) between the PNPLA3$^{148MM/148MI}$ and PNPLA3^{148II} groups is shown in Fig. 3. The PNPLA3$^{148MM/148MI}$ group had significantly decreased concentrations (Fig. 3, blue) of three long-chain TAGs: 52:1 (TAG [16:0/18:0/18:1]), 53:2 (TAG [17:0/18:1/18:1]), and 54:2 (TAG [18:0/18:1/18:1] or TAG [16:0/18:1/20:1]) (Fig. 3, *right panel*). This distribution pattern was clearly different from that observed between the obese and nonobese subgroups (Fig. 4, *right panel*). The relative TAG profile in the obese group was characterized by relative enrichment of long-chain polyunsaturated TAGs, whereas the relative concentrations of short-chain TAGs with a low number of double bonds were downregulated (Fig. 5). Analysis of TAGs using their relative concentrations rather than absolute concentrations abolished the differences observed in absolute concentrations of TAGs with 51–54 carbons and 2–3 double bonds between the obese and nonobese groups (Fig. 4).

Comparison of the relative distributions of serum TAGs between the two NAFLD subgroups (Fig. 5) closely resembled that observed between the obese and 'nonobese' subgroups (Fig. 4, *right panel*) with the exception of the two most abundant TAGs (52:2 and 52:3) (Supplementary Table 3 and Fig. 5).

DISCUSSION

The current study shows that the circulating TAG profiles depend on whether NAFLD is associated with the PNPLA3 I148M variant independent of obesity, or by obesity independent of the PNPLA3 genotype. Comparison of 'PNPLA3 NAFLD' and 'obese NAFLD' groups showed that human NAFLD includes subtypes that have distinct effects on both the absolute and relative distribution of circulating TAGs (Fig. 5), and that the differences can be attributed to obesity/insulin resistance rather than total liver TAG concentrations. The relative distribution of TAGs was analyzed to enable comparison between subtypes of NAFLD independent of the differences in absolute TAG concentrations.

We divided ~400 subjects based on their PNPLA3 genotype at rs738409 into two groups of roughly equal size, one carrying one or two of the variant alleles in PNPLA3 (PNPLA3$^{148MM/148MI}$) whereas the other was homozygous for the C allele (PNPLA3^{148II}). As expected,

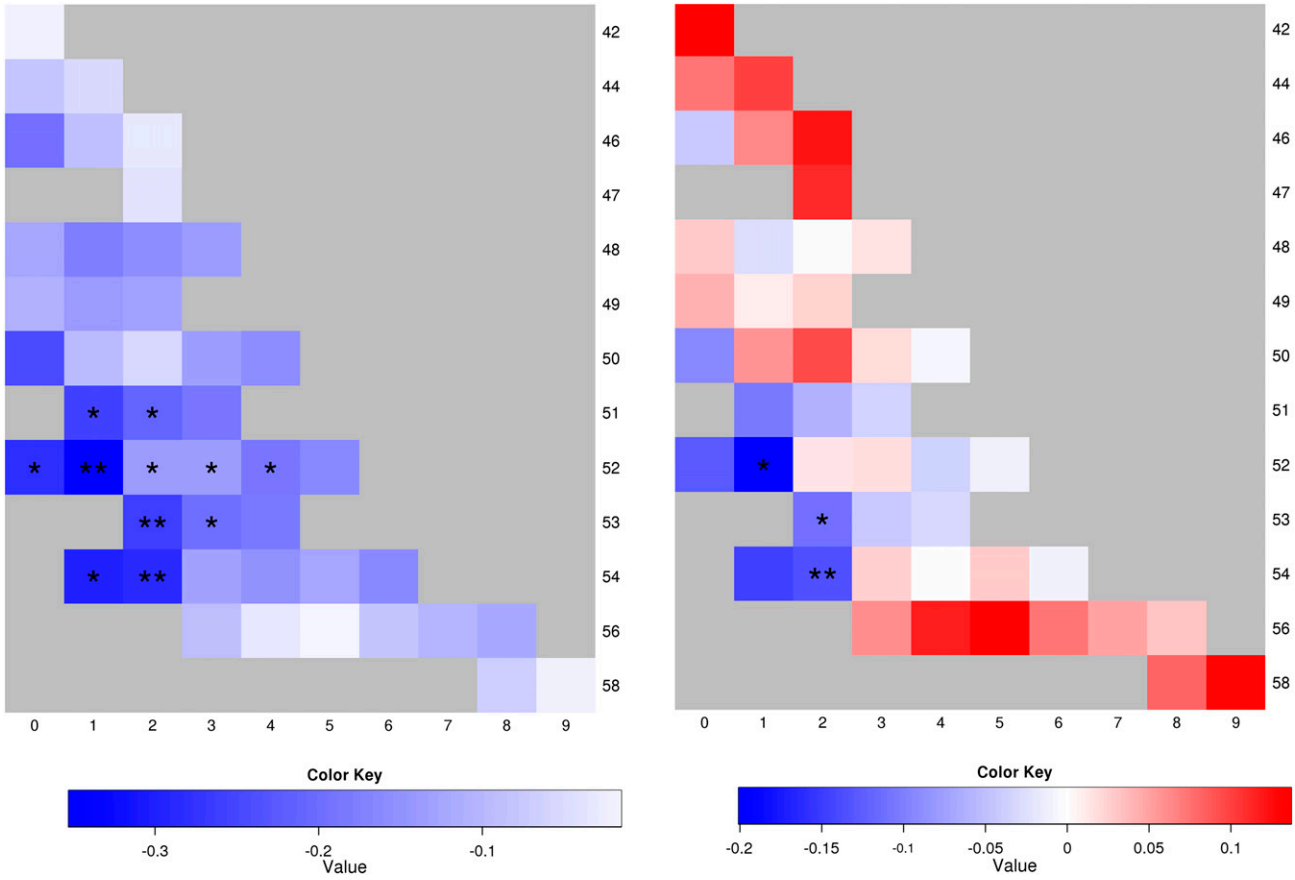

Figure 3—Absolute (*left panel*) and relative (*right panel*) concentrations of TAGs between the PNPLA3$^{148MM/148MI}$ and PNPLA3^{148II} groups. The color code denotes the log of the ratio between means of the groups for an individual TAG. The *y*-axes denote the number of carbons, and the *x*-axes the number of double bonds. Blue denotes a decrease in the variant allele carriers (PNPLA3$^{148MM/148MI}$) compared with nonvariant allele carriers (PNPLA3^{148II}). The absolute and relative concentrations of individual TAGs and the significances between the groups are shown in Supplementary Table 1.

the PNPLA3$^{148MM/148MI}$ group had significantly higher liver fat content than the PNPLA3^{148II} group, although not all subjects in this group had NAFLD. The PNPLA3$^{148MM/148MI}$ group did not display hypertriglyceridemia or a low HDL cholesterol concentration. This is in line with most previous studies showing that carriers of the I148M variant lack the lipid changes usually accompanying increased liver fat content (5,21–31). Serum total triglycerides tended to be even slightly lower in the PNPLA3$^{148MM/148MI}$ than in the PNPLA3^{148II} group (Table 1), which is in keeping with recent studies including those performed in Danes (32), Japanese (33), and morbidly obese Swedes (34).

The increase in liver fat in the PNPLA3$^{148MM/148MI}$ group was not accompanied by an increase in fasting insulin concentrations (Fig. 2). Fasting insulin is a good surrogate for hepatic insulin sensitivity, although ideally hepatic insulin sensitivity should have been measured directly (35). Lack of hyperinsulinemia in subjects with 'PNPLA3 NAFLD' is consistent with data from the seven studies that reported data on fasting insulin in a meta-analysis (7) and from subsequent studies (32,36). These include our previous study, in which hepatic insulin sensitivity was directly measured in 109 subjects using the euglycemic-hyperinsulinemic clamp technique combined with infusion of [3-^{3}H] glucose (31) and that of Kantartzis et al. (21), who also performed direct measurements of insulin sensitivity. At variance with these, Palmer et al. (34) reported insulin concentrations to be increased in morbidly obese Swedish subjects carrying the I148M variant compared with noncarriers. The PNPLA^{I148M} variant was also associated with hyperinsulinemia by Wang et al. (37), but in that study, the variant allele carriers were more obese than the noncarriers. Thus, the majority of the data suggests that steatosis is dissociated from insulin resistance in 'PNPLA3 NAFLD' as it is in, for example, steatosis associated with familial hypobetalipoproteinemia (38).

In the PNPLA3$^{148MM/148MI}$ group, only one of the LCs differed from that in the PNPLA3^{148II} group. This LC exclusively contained TAGs (Fig. 2) and was therefore the focus of further analysis. Comparison of the relative distribution of individual circulating TAG species between the groups showed that TAGs preferred as

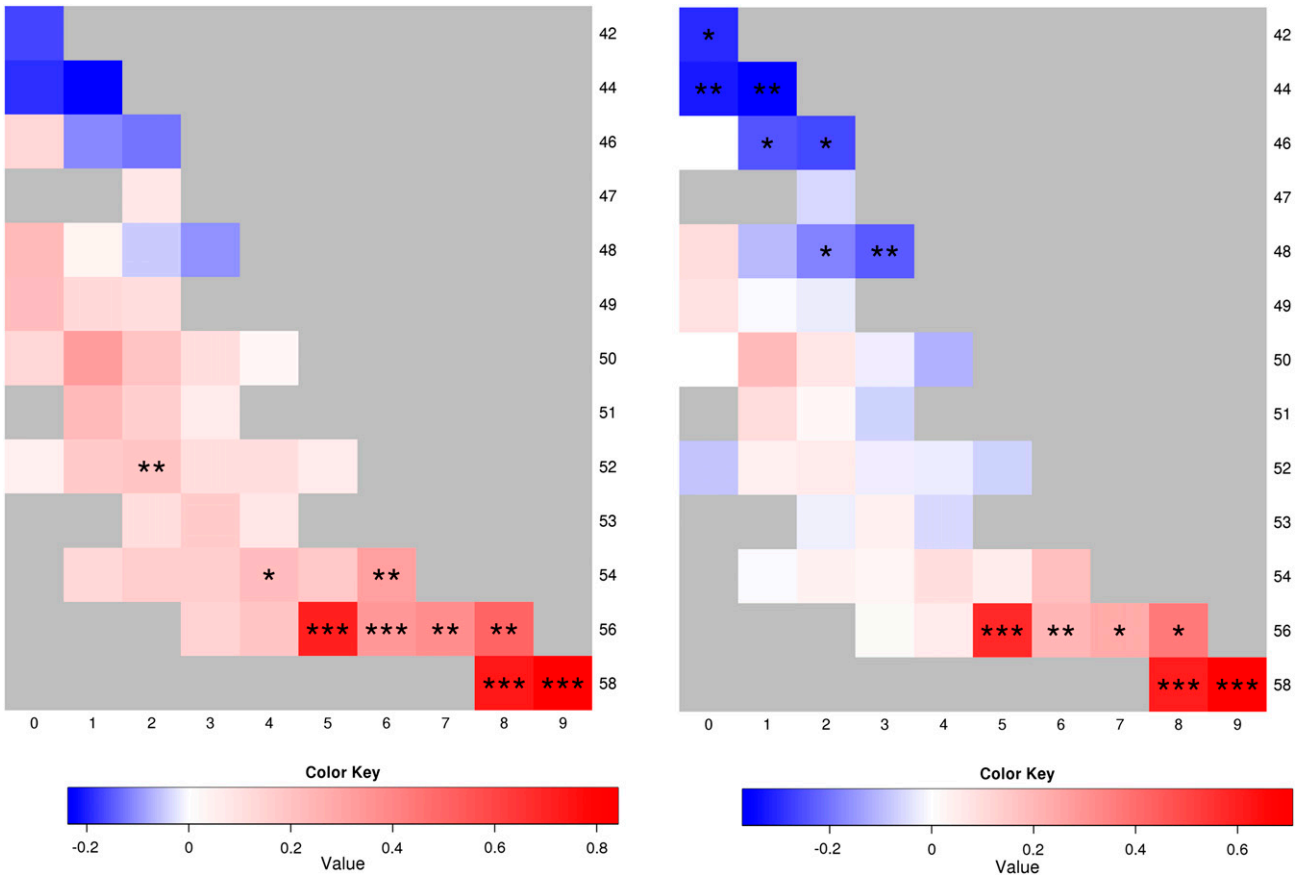

Figure 4—Absolute (*left panel*) and relative (*right panel*) concentrations of TAGs between obese and nonobese groups. The color code denotes the log of the ratio between means of the groups for an individual TAG. The *y*-axes denote the number of carbons, and the *x*-axes the number of double bonds. Blue denotes a decrease and red an increase in the obese group compared with the nonobese group. The absolute and relative concentrations of individual TAGs and the significances between the groups are shown in Supplementary Table 2.

substrates by PNPLA3 in the liver of mice overexpressing the human PNPLA3^{I148M}, such as 18:1 fatty acid–containing TAGs (9), were significantly depleted in the variant allele carriers (Fig. 3). The differences were observed in relatively minor TAG species (Supplementary Table 1). There was, however, also a trend (Fig. 5, blue) for the more abundant TAG species containing 18:1 fatty acids such as 54:2 (TAG [18:0/18:1/18:1] or TAG [16:0/18:1/20:1]) and 52:1 (TAG [16:0/18:0/18:1]) to be relatively depleted (Supplementary Table 3) in the PNPLA3 NAFLD group. Thus, the close coupling between liver fat content and hepatic VLDL production found in obese NAFLD subjects (39) may not characterize carriers of the PNPLA3^{I148M} variant. Data showing that rates of $VLDL_1$ and apolipoprotein B100 production rather than clearance in human carriers of the PNPLA3^{I148M} are lower than in noncarriers at any given liver fat content support this interpretation (39). We have also directly measured insulin sensitivity of lipolysis using [$^2H_5$] glycerol and shown that insulin sensitivity of lipolysis is similar in homozygous carriers of PNPLA3^{I148M} and in noncarriers (40). This result is in keeping with mouse data showing that overexpression of human PNPLA3^{I148M} in adipose tissue in contrast to the liver does not affect liver fat content (41). The relative depletion of a subset of circulating TAGs thus supports the idea that in vivo in humans, the I148M variant, impedes hydrolysis of intrahepatocellular TAGs (41) rather than stimulates TAG synthesis (10).

The exact mechanisms by which the I148M variant disrupts the coupling between TAGs stored in lipid droplets and VLDL synthesis are incompletely understood. However, it is known that most TAGs in lipid droplets will undergo hydrolysis before being reassembled into TAGs in the endoplasmic reticulum (42). The PNPLA3 variant could impede lipolysis of TAGs at the surface of lipid droplets possibly by modifying the activity of adipose triglyceride lipase (43), thereby decreasing VLDL synthesis. Consistent with this hypothesis, cells overexpressing the I148M mutant have a higher neutral lipid content and lower rates of apolipoprotein B secretion than cells overexpressing wild-type PNPLA3 (39). The reason for the lack of insulin resistance in 'PNPLA3 NAFLD' subjects is unknown. Hypothetically, defective lipolysis of TAGs could increase the size of metabolically inert TAGs sequestered in the lipid droplets

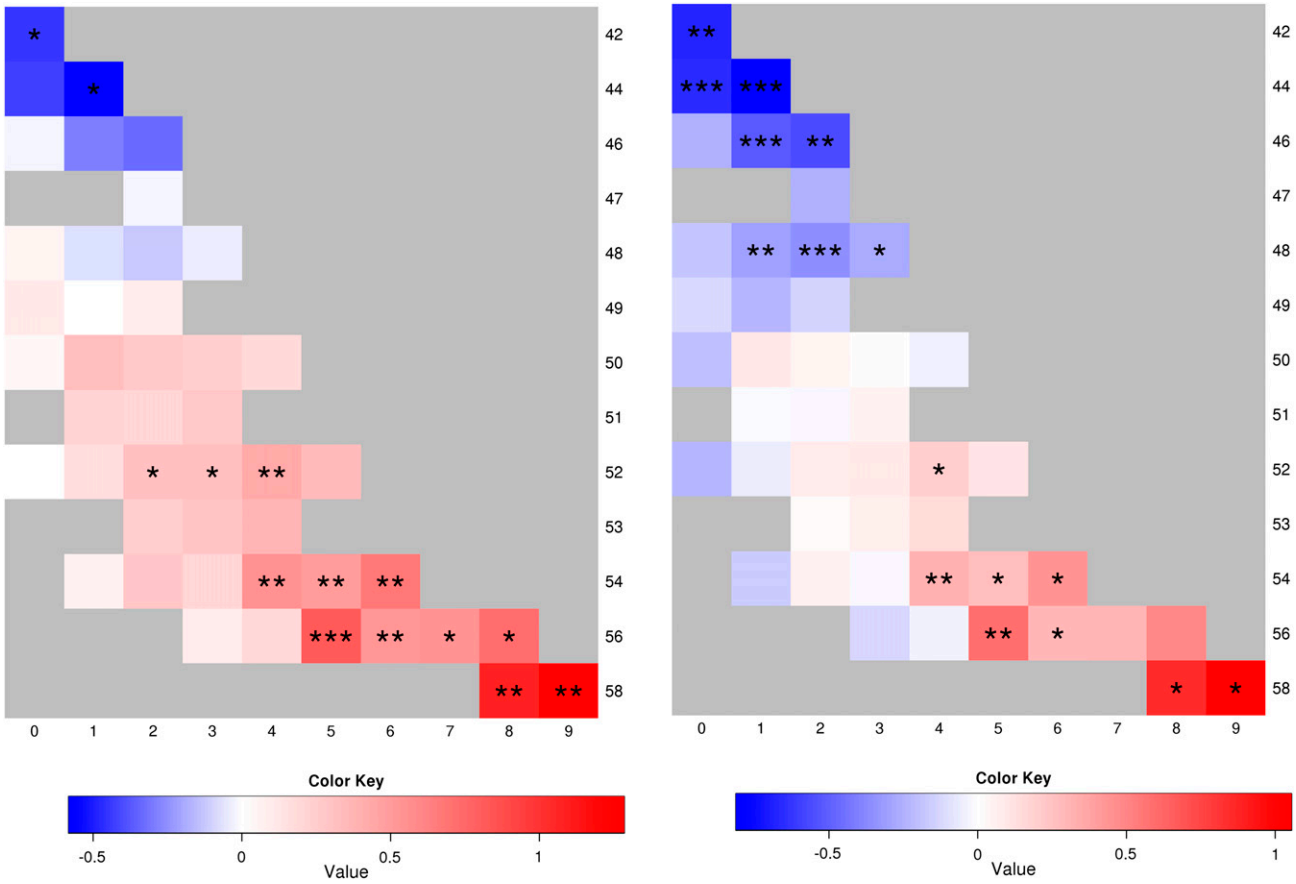

Figure 5—Absolute (*left panel*) and relative (*right panel*) concentrations of TAGs between PNPLA3 NAFLD and obese NAFLD. The color code denotes the log of the ratio between means of the groups for an individual TAG. The *y*-axes denote the number of carbons, and the *x*-axes the number of double bonds. Blue denotes a decrease and red an increase in the PNPLA3 NAFLD group compared with the obese NAFLD group. The absolute and relative concentrations of individual TAGs and the significances between the groups are shown in Supplementary Table 3.

and reduce the intrahepatocellular concentrations of harmful lipid intermediates such as diacylglycerols and ceramides (42). Consistent with this, the size of lipid droplets is increased in cells overexpressing the PNPLA3 variant (43).

When essentially the same ~400 subjects were divided into obese and nonobese groups based on their median BMI, liver fat content was found to be similarly increased in the obese group as in the PNPLA3$^{148MM/148MI}$ group (Table 1). The obese group compared with the 'nonobese' group displayed, however, extensive alterations in circulating LCs (Fig. 2) and in the relative distribution of TAGs (Fig. 4). These changes could have been caused by obesity/insulin resistance or the higher liver fat content. When 'obese NAFLD' and 'PNPLA3 NAFLD' groups were compared, very different TAG profiles were still observed. The difference in the relative TAG profile between the NAFLD subgroups (Fig. 5) closely resembled that between obese and 'nonobese' groups (Fig. 4), implying that the changes in the TAG profiles were not due to an increase in liver fat per se. The deficiencies of the TAGs observed when comparing PNPLA3 subgroups were not observed when comparing 'obese NAFLD' and 'PNPLA3 NAFLD' groups. This suggests that obesity/insulin resistance has a much more profound influence on the TAG profile than the relative minor changes observed with 'PNPLA3 NAFLD.' The differences in the TAG profiles characterizing obesity/insulin resistance could be due to either clearance of VLDL or its production (44). The latter possibility is more likely as an inability of insulin to normally suppress VLDL production in the liver appears to be the main mechanism underlying hypertriglyceridemia in obese NAFLD subjects (45). The composition of VLDL in NAFLD subjects also closely mimics the composition of intrahepatocellular TAGs (46). Thus, in 'obese NAFLD' subjects, the liver oversecretes TAGs in direct proportion to increased TAG synthesis (46).

We have previously measured the lipid and fatty acid composition in lipoprotein fractions in subjects who exhibited a broad range of insulin sensitivity (47) and determined the rate of production of individual TAGs across the splanchnic bed in NAFLD subjects (47). In serum, the relative concentrations of 16:0, 16:1, and 18:0

esterified fatty acids were positively correlated with insulin resistance, and those of essential fatty acids were inversely correlated with insulin resistance (48). Similar data were observed when liver fat was plotted against the splanchnic production rate of TAGs (47). TAGs containing saturated or monounsaturated fatty acids also predominate in liver biopsy samples of subjects with increased compared with normal liver fat content (49). Consistent with these data, the absolute serum concentrations of the abundant TAGs containing monounsaturated and saturated fatty acids were significantly increased in the obese group compared with the 'nonobese' group (Supplementary Table 2). However, the relative concentrations of these abundant TAGs did not differ between the obese and 'nonobese' subgroups. The differences were, rather, observed in the minor, polyunsaturated TAG species, which were relatively enriched, and the short-chain species with few double bonds, which were de-enriched in the obese group (Figs. 4 and 5). The mechanism underlying these changes cannot be determined from the current study.

We conclude that NAFLD, which is still clinically regarded as a uniform entity, is heterogeneous. 'PNPLA3 NAFLD' is characterized by absolute and relative deficiencies of distinct circulating TAGs, which support in vitro data suggesting that the I148M variant impairs lipolysis rather than stimulates synthesis of intrahepatocellular TAGs. 'Obese NAFLD,' compared with 'PNPLA3 NAFLD,' is associated with multiple changes in absolute and relative TAG concentrations (Fig. 5). These changes are not due to an increased liver fat content per se, but to obesity/insulin resistance.

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