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ARTICLE

Adipose tissue is inflamed in NAFLD due to obesity but not in NAFLD due to genetic variation in *PNPLA3*

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Abstract
Aims/hypothesis The rs738409 C>G single-nucleotide polymorphism in *PNPLA3* leads to a missense mutation (I148M) which increases liver fat but does not cause insulin resistance. We hypothesised that patients with non-alcoholic fatty liver disease (NAFLD) due to the *PNPLA3* variant ('PNPLA3 NAFLD'=PNPLA3-148MM) do not have adipose tissue (AT) inflammation in contrast with those with NAFLD due to obesity ('obese NAFLD').
Methods Biopsy specimens of AT were taken, and *PNPLA3* genotype and liver fat (^{1}H-magnetic resonance spectroscopy) were determined in 82 volunteers, who were divided into groups based on either median BMI (obese 36.2±0.7 kg/m^2; non-obese 26.0±0.4 kg/m^2) or *PNPLA3* genotype. All groups were similar with respect to age and sex. The *PNPLA3* subgroups were equally obese (PNPLA3-148MM, 31.1±1.3 kg/m^2; PNPLA3-148II, 31.2±0.8 kg/m^2), while the obese and non-obese subgroups had similar *PNPLA3* genotype distribution. Gene expression of proinflammatory (*MCP-1*, *CD68*) and anti-inflammatory (*Twist1*, *ADIPOQ*) markers was measured using quantitative real-time RT-PCR.
Results Liver fat was similarly increased in obese NAFLD (9.5±1.3% vs 5.1±0.9%, obese vs non-obese, $p=0.007$) and PNPLA3 NAFLD (11.4±1.7% vs 5.3±0.8%, PNPLA3-148MM vs PNPLA3-148II, $p<0.001$). Fasting serum insulin was higher in the obese than the non-obese group (76±6 vs 47±6 pmol/l, $p<0.001$), but similar in PNPLA3-148MM and PNPLA3-148II (60±8 vs 62±5 pmol/l, NS). In obese vs non-obese, *MCP-1* and *CD68* mRNAs were upregulated, whereas those of *Twist1* and *ADIPOQ* were significantly downregulated. AT gene expression of *MCP-1*, *CD68*, *Twist1* and *ADIPOQ* was similar in PNPLA3-148MM and PNPLA3-148II groups.
Conclusions/interpretation PNPLA3 NAFLD is characterised by an increase in liver fat but no insulin resistance or AT inflammation, while obese NAFLD has all three of these features.

Keywords Adipose tissue · Inflammation · NAFLD · *PNPLA3* genotype

Abbreviations

^{1}H-MRS	Proton magnetic resonance spectroscopy
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AT	Adipose tissue
CD68	Cluster of differentiation 68
MCP-1	Monocyte chemoattractant protein-1
NAFLD	Non-alcoholic fatty liver disease

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PNPLA3 Patatin-like phospholipase domain-containing 3
Twist1 Twist-related protein 1

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a rapidly increasing liver disorder covering a range of conditions from clinically benign steatosis to non-alcoholic steatohepatitis and cirrhosis [1]. The increase in prevalence of NAFLD has paralleled that of obesity [2]. This common form of NAFLD (‘obese NAFLD’) is closely associated with all features of the metabolic syndrome [3]. This is because the liver, once fatty, is insulin resistant and overproduces glucose and VLDL-cholesterol, which in turn induces hyperinsulinaemia and a low HDL-cholesterol concentration [4]. As NAFLD and the metabolic syndrome can be observed even in non-obese people, the condition could also be called ‘metabolic NAFLD’.

In 2008, Romeo et al [5] described a single-nucleotide polymorphism (rs738409; C>G/I148M) in the *PNPLA3* (also known as adiponutrin) gene. The I148M variant is either a lack-of-function mutation, which impairs triacylglycerol hydrolysis, or a gain-of-function mutation, which enhances lipogenesis, or both [6, 7]. A meta-analysis of 16 studies has shown that people homozygous for the variant allele (PNPLA3-148MM) have, on average, 73% higher liver fat content than weight-matched people homozygous for the wild-type allele (PNPLA3-148II) [8]. People with PNPLA3-148MM also have more steatosis, fibrosis and inflammation in their liver biopsy specimens than those with PNPLA3-148II [8]. However, unlike obese NAFLD, ‘PNPLA3 NAFLD’ is not characterised by features of the metabolic syndrome, i.e. hyperinsulinaemia, hypertriacylglycerolaemia and a low HDL-cholesterol concentration [1, 8].

In obese insulin-resistant people, adipose tissue (AT) is inflamed compared with that of non-obese people [9, 10]. AT inflammation is characterised by macrophage infiltration and increased levels of proinflammatory chemokines, such as monocyte chemoattractant protein-1 (MCP-1), and macrophage markers, such as cluster of differentiation 68 (CD68) [9–12]. Levels of anti-inflammatory markers such as Twist-related protein 1 (Twist1) and adiponectin are significantly lower in obese insulin-resistant than in non-obese insulin-sensitive people [13–15]. These changes have been suggested to be causally linked to hepatic steatosis and insulin resistance [16]. If AT inflammation is, indeed, important for development of insulin resistance in obesity-related NAFLD, then one would predict that there would be no AT inflammation in people with PNPLA3 NAFLD.

In the present study, we examined whether AT is inflamed in volunteers with PNPLA3 NAFLD, i.e. in volunteers homozygous for the variant allele I148M at rs738409 of the *PNPLA3* gene (PNPLA3-148MM) compared with those without, i.e. volunteers homozygous for the wild-type allele (PNPLA3-148II). For this purpose, subcutaneous AT biopsy samples were taken from 82 volunteers whose liver fat (proton magnetic resonance spectroscopy, ^{1}H-MRS) and *PNPLA3* genotype at rs738409 were determined. Gene expression of the proinflammatory chemokine, MCP-1, the macrophage marker, CD68, the anti-inflammatory transcription factor, Twist1, and the anti-inflammatory and insulin-sensitising adipokine, adiponectin, was determined by quantitative real-time RT-PCR. Fasting serum adiponectin and NEFAs were also measured, as these molecules may mediate the crosstalk between AT and the liver [1, 17].

Methods

Participants and study design

The volunteers for this cross-sectional study were recruited from those who had been previously genotyped at rs738409 in the PNPLA3 gene in our laboratory [18]. Exclusion criteria included: (1) heterozygous for the variant allele (PNPLA3-148MI); (2) pre-existing liver disease other than NAFLD (i.e. autoimmune, viral or drug-induced liver disease); (3) significant disease other than obesity based on medical history and physical examination and laboratory tests as detailed below; (4) excessive use of alcohol (over 20 g/day); (5) pregnancy or lactation. We invited all volunteers who were homozygous for the variant allele (PNPLA3-148MM). The remaining volunteers were selected at random. The purpose, nature and potential risks of the study were explained to the volunteers before their written informed consent was obtained. The study protocol was approved by the ethics committee of the Helsinki University Central Hospital.

All volunteers who were eligible based on a telephone interview were invited for a metabolic study. The measurements were carried out in the clinical research centre where patients arrived in the morning after an overnight fast. At this visit, medical history was taken and a physical examination performed. Weight and height were recorded, and blood samples were taken for measurement of: complete blood count; creatinine, HDL- and LDL-cholesterol, triacylglycerol, NEFA, glucose, HbA_{1c}, adiponectin and insulin concentrations; alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ-glutamyltransferase (γGT) activity; transferrin saturation; antibodies against hepatitis A, B (core) and C, and anti-smooth muscle, anti-nuclear and anti-mitochondrial antibodies. A pregnancy test was performed in women of child-bearing age. A needle aspiration AT biopsy sample was taken from abdominal subcutaneous AT as detailed below. Measurement of liver fat content by ^{1}H-MRS was conducted within a few days of the metabolic study visit.

Measurement of liver fat content

Liver fat content was measured by ^{1}H-MRS using a 1.5 T clinical scanner (Siemens, Erlangen, Germany). The intensity differences arising from the acquisition parameters and localisation techniques were normalised as previously described, and liver fat content was expressed as a mass fraction [18]. We and others have previously validated the ^{1}H-MRS measurement against the histologically determined hepatic fat content [19, 20]. NAFLD was defined as liver fat ≥5.56% by ^{1}H-MRS as in the Dallas Heart Study [21].

Measurement of body composition

Body weight was recorded to the nearest 0.1 kg using a calibrated digital scale (Soehnle, Monilaite-Dayton, Finland) with participants barefoot and wearing light indoor clothing. Height was recorded to the nearest 0.5 cm using a non-stretchable tape. BMI was defined as (weight [kg]/height [m^2]). Body circumference was measured with a non-stretchable band for the waist midway between the lower rib margin and the iliac crest and, for the hip circumference, over the greater trochanters, and recorded to the nearest 0.5 cm.

Subcutaneous AT biopsies

Needle aspiration biopsy specimens of subcutaneous abdominal AT were taken under local anaesthesia with 1% lidocaine at the metabolic study visit as previously described [22]. The AT samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Analysis of AT gene expression

Total RNA was isolated from subcutaneous AT by using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA). The RNA (500 ng) was reverse-transcribed by using the SuperScript VILO cDNA synthesis kit (Invitrogen/Life Technologies, Carlsbad, CA, USA). Each sample was amplified in duplicate for quantification of mRNA concentration of the proinflammatory (*MCP-1*, *CD68*) and anti-inflammatory (*Twist1*, *ADIPOQ*) genes, and the housekeeping gene, *36B4*, on a 7000 Sequence Detection System (Applied Biosystems/Life Technologies) using the manufacturer's SYBR-Green kit. Relative quantification for the gene of interest was carried out using normalisation to the *36B4* mRNA concentration using a $2^{-\Delta\Delta C_t}$ method [23]. Sequences of the PCR primers used are listed in Electronic supplementary material (ESM) Table 1.

Analytical procedures

Fasting plasma glucose was measured using a hexokinase method on an autoanalyser (Roche Diagnostics Hitachi 917; Hitachi, Tokyo, Japan). Fasting serum insulin concentration was determined by time-resolved fluoroimmunoassay using the Insulin Kit (AUTOdelfia; Wallac, Turku, Finland). HOMA insulin resistance was calculated using models described by Matthews et al [24]. HbA_{1c} (%) was measured by HPLC using a fully automated Glycosylated Hemoglobin Analyzer System (BioRad, Richmond, CA, USA), and HbA_{1c} (mmol/mol) was calculated from the formula: (HbA_{1c} [%]−2.15)×10.929. LDL- and HDL-cholesterol and triacylglycerol concentrations were measured with the respective enzymatic kits from Roche Diagnostics using an autoanalyser (Roche Diagnostics Hitachi 917). Serum creatinine, ALT, AST, ALP and γGT were determined as recommended by the European Committee for Clinical Laboratory Standards. NEFA were measured by an enzymatic colorimetric assay (NEFA-HR[2]; Wako Chemicals, Neuss, Germany) using a Konelab 60i Analyzer (Thermo Electron Corporation, Vantaa, Finland). Serum adiponectin was measured using the Human Adiponectin ELISA kit from B-Bridge International (Cupertino, CA, USA).

Statistical analysis

Distribution of continuous variables was tested for normality using a Kolmogorov–Smirnov test. Normally distributed data are shown as mean±SEM, and non-normally distributed data are shown as median followed by the 25th and 75th centile. The study groups were compared using Fisher's exact test for categorical variables, and unpaired *t* test or Mann–Whitney test, as appropriate, for continuous variables. Two-tailed p values <0.05 were considered significant. GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses.

Results

Characteristics of the study groups

Obese NAFLD The obese and non-obese groups were comparable with respect to age, sex and *PNPLA3* genotype (Table 1). BMI averaged 36.2±0.7 kg/m^2 in the obese and 26.0±0.4 kg/m^2 in the non-obese group (Fig. 1a). Liver fat content was significantly higher in the obese than the non-obese group (9.5±1.3% vs 5.1±0.9%, respectively, p=0.007) (Fig. 1b). The obese group also had significantly higher serum insulin (76±6 vs 47±6 pmol/l, p<0.001) (Fig. 1c) and triacylglycerol (Table 1) concentrations than the non-obese group.

PNPLA3 NAFLD The PNPLA3-148MM and the PNPLA3-148II groups were comparable with respect to age, sex and BMI (Table 1, Fig. 1d). The PNPLA3-148MM (PNPLA3

Table 1 Characteristics of the study groups

Characteristic	Obese NAFLD		PNPLA3 NAFLD	
	Non-obese (n=41)	Obese (n=41)	PNPLA3-148II (n=55)	PNPLA3-148MM (n=27)
Men/women	13/28	6/35	12/43	7/20
PNPLA3-148II/ PNPLA3-148MM	26/15	29/12	55/0	0/27***
Age (years)	47 (32–58)	50 (39–61)	48 (37–60)	50 (34–63)
Weight (kg)	75.3±1.6	101.7±2.4***	88.1±2.6	89.5±3.4
Waist circumference (cm)	91.3±1.5	113.3±1.6***	102.3±2.0	102.3±3.1
WHR	0.89 (0.84–0.96)	0.94 (0.90–1.00)**	0.92 (0.86–0.97)	0.92 (0.88–0.97)
Fasting plasma glucose (mmol/l)	5.4 (5.1–5.8)	5.7 (5.4–6.2)	5.6 (5.2–5.9)	5.5 (4.9–5.9)
HbA_{1c} (%)	5.6 (5.2–5.8)	5.7 (5.5–5.9)	5.7 (5.5–5.9)	5.7 (5.2–5.9)
HbA_{1c} (mmol/mol)	38 (33–40)	39 (37–41)*	39 (37–41)	39 (33–40)
HOMA-IR index	1.3 (0.2–2.0)	2.6 (0.3–3.7)***	1.8 (0.2–3.0)	2.0 (0.4–2.7)
Fasting plasma triacylglycerol (mmol/l)	0.9 (0.6–1.5)	1.3 (1.2–1.9)***	1.3 (0.8–1.8)	1.2 (0.8–1.7)
Fasting plasma HDL-cholesterol (mmol/l)	1.6 (1.2–2.1)	1.4 (1.2–1.7)	1.6 (1.2–1.8)	1.4 (1.2–1.7)
Fasting plasma LDL-cholesterol (mmol/l)	2.7±0.1	3.2±0.2*	2.9±0.1	3.0±0.2
Plasma AST (µkat/l)	0.45 (0.38–0.68)	0.43 (0.38–0.63)	0.42 (0.35–0.50)	0.52 (0.43–0.89)***
Plasma ALT (µkat/l)	0.35 (0.28–0.69)	0.47 (0.37–0.74)	0.38 (0.30–0.58)	0.63 (0.37–1.50)**
Plasma AST/plasma ALT	1.1±0.06	0.9±0.04	1.1±0.05	0.9±0.06
Plasma γGT (µkat/l)	0.37 (0.25–0.67)	0.45 (0.29–0.71)	0.43 (0.27–0.63)	0.37 (0.28–0.78)
Relative mRNA quantity				
MCP-1	1.0 (0.5–1.4)	1.2 (0.9–1.6)*	1.0 (0.6–1.4)	0.9 (0.6–1.3)
CD68	1.0 (0.4–1.8)	1.3 (0.8–2.2)	1.0 (0.4–1.8)	1.0 (0.7–1.6)
Twist1	1.0 (0.6–2.0)	0.8 (0.5–1.0)*	1.0 (0.6–1.4)	1.2 (0.8–1.8)
ADIPOQ	1.0 (0.9–1.4)	0.9 (0.7–1.2)*	1.0 (0.8–1.4)	1.1 (0.9–1.5)

Data are shown as mean±SEM or median (25th–75th centile)

*p<0.05, **p<0.005, ***p<0.0005

NAFLD) group had significantly higher liver fat content (11.4±1.7%) than the PNPLA3-148II group (5.3±0.8%, p<0.001) (Fig. 1e). Serum insulin (Fig. 1f) and triacylglycerol (Table 1) concentrations were comparable between the groups.

Expression of proinflammatory and anti-inflammatory genes in subcutaneous AT

Obese NAFLD In the obese group, the mRNA concentration of *MCP-1* was significantly higher than in the non-obese group (Table 1, Fig. 2a). Gene expression of the macrophage marker *CD68* was slightly but not significantly higher in the obese than the non-obese group (Table 1, Fig. 2b). Expression of the anti-inflammatory genes, *Twist1* and *ADIPOQ*, was significantly lower in the obese than the non-obese group (Table 1, Fig. 2c, d).

PNPLA3 NAFLD There were no statistically significant differences in expression of the proinflammatory or anti-inflammatory genes between the PNPLA3-148MM and PNPLA3-148II groups (Table 1, Fig. 2).

Serum NEFA and adiponectin

Fasting serum NEFA were comparable between the obese (515 [403–594] µmol/l) and non-obese (450 [361–657] µmol/l) and between the PNPLA3-148MM (474 [367–582] µmol/l) and PNPLA3-148II (462 [384–635] µmol/l) groups. Fasting serum NEFA correlated with liver fat in all participants (r=0.28, p=0.02), and in the obese NAFLD (r=0.41, p=0.01) and PNPLA3-148II (r=0.36, p<0.02) groups, but not in the non-obese or PNPLA3-148MM (PNPLA3 NAFLD) groups.

Fasting serum adiponectin was slightly but not significantly lower in the obese (9.2±0.7 µg/ml) than the non-obese (10.2±1.0 µg/ml) group. There was no difference in fasting serum adiponectin between the PNPLA3-148II (9.5±0.6 µg/ml) and PNPLA3-148MM (10.1±1.4 µg/ml) groups. Serum adiponectin correlated inversely with liver fat (r=−0.41, p<0.001).

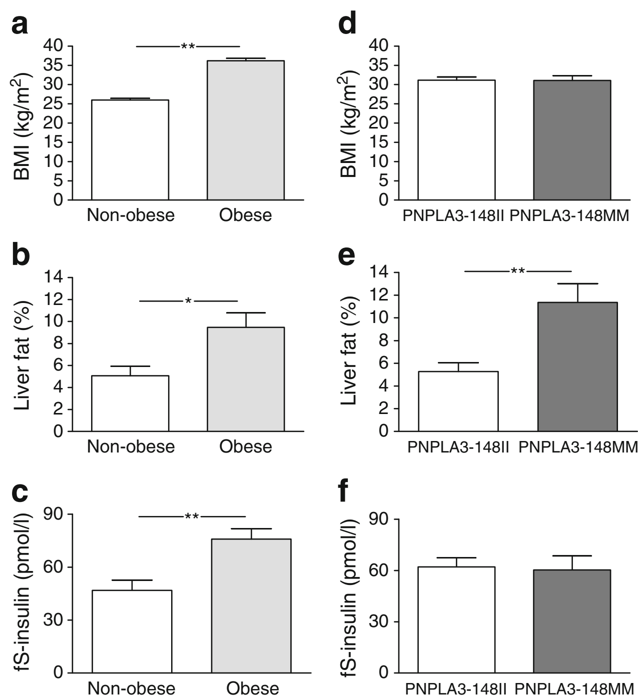

Fig. 1 BMI, liver fat content and fasting serum (fS) insulin concentration in obese NAFLD (non-obese, obese; **a**–**c**) and PNPLA3 NAFLD (PNPLA3-148II, PNPLA3-148MM; **d**–**f**) subgroups. Data are shown as mean±SEM. *$p<0.005$, **$p<0.0005$

Discussion

In this study, we examined whether AT is inflamed in volunteers with PNPLA3 NAFLD compared with those without. For this purpose, we studied 82 volunteers who were divided into groups either by categorising them as obese or non-obese based on median BMI (obese NAFLD subgroups) or the same individuals categorised as PNPLA3-148MM (PNPLA3 NAFLD) or PNPLA3-148II based on their *PNPLA3* genotype at rs738409. We found that obese NAFLD was associated with features of the metabolic syndrome, such as hypertriacylglycerolaemia and hyperinsulinaemia, and AT inflammation as judged by increased expression of the proinflammatory genes, *MCP-1* and *CD68*, as well as decreased expression of the anti-inflammatory genes, *Twist1* and *ADIPOQ*. In contrast, PNPLA3 NAFLD was not characterised by increased fasting serum insulin and triacylglycerol concentrations or AT inflammation.

Several studies have linked the development of NAFLD and insulin resistance to the genes chosen for analysis in this study. Transgenic mice overproducing MCP-1 in AT are known to be insulin resistant, and display increased macrophage infiltration into AT as well as hepatic steatosis [25]. In mice, deficiency of the proinflammatory cytokine, MCP-1, protects against macrophage infiltration into AT and insulin resistance [26]. Also, deficiency of the MCP-1 receptor reduces hepatic steatosis, macrophage accumulation and AT inflammation, increases expression of the *Adipoq* gene in AT and circulating adiponectin concentrations, and improves insulin sensitivity in obese mice [26]. In humans, mRNA concentration of *MCP-1* in AT correlates positively with circulating MCP-1 concentration in the plasma and with BMI [27]. We have previously found insulin to increase MCP-1 gene and protein expression more in insulin-resistant than in insulin-sensitive people, and to decrease serum concentrations of MCP-1 in insulin-sensitive but not insulin-resistant people [28]. Furthermore, mRNA and protein expression of MCP-1 in AT was found to be higher in obese than in non-obese individuals, and closely

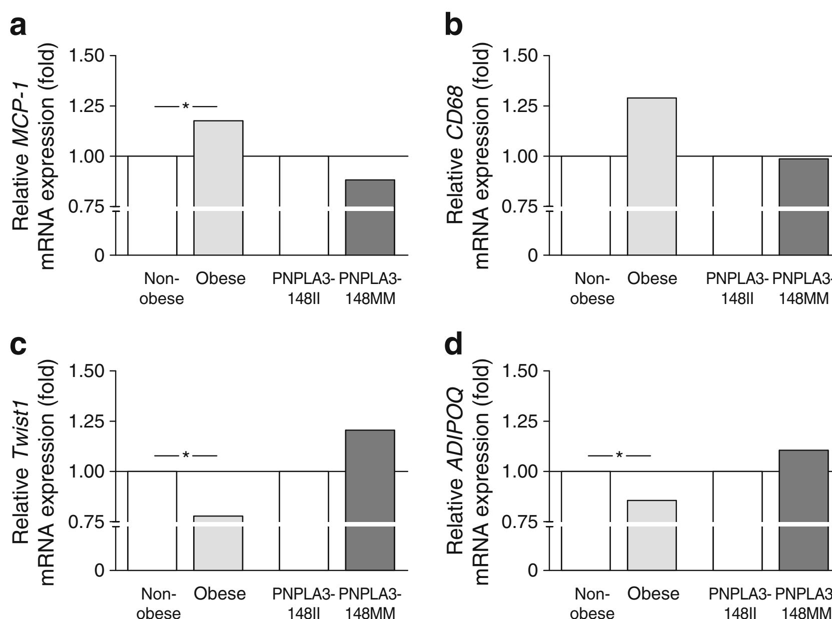

Fig. 2 mRNA expression of proinflammatory (*MCP-1*, *CD68*; **a**, **b**) and anti-inflammatory (*Twist1*, *ADIPOQ*; **c**, **d**) genes relative to mRNA expression of the housekeeping gene (*36B4*) in subcutaneous AT of obese NAFLD (non-obese, obese) and PNPLA3 NAFLD (PNPLA3-148II, PNPLA3-148MM) subgroups. *$p<0.05$

related to the number of macrophages present in AT [29]. In keeping with these data, we found that the mRNA concentration of *MCP-1* in AT was significantly higher in the obese than the non-obese group, whereas expression was similar in the PNPLA3-148MM and PNPLA3-148II groups.

Gene expression of the macrophage marker, *CD68*, has been reported to be significantly higher in AT of obese than non-obese people, and mRNA expression of *CD68* to correlate strongly with the number of macrophages in AT in both mice [10] and humans [12]. We have also shown mRNA expression of *CD68* to correlate closely with liver fat content in humans [12]. In the present study, gene expression of *CD68* was slightly, but not significantly, higher in the obese than the non-obese group, and it was similar in the PNPLA3-148MM and PNPLA3-148II groups.

Studies in knockout mice have shown that the transcription factor, Twist1, decreases proinflammatory cytokine expression by creating a negative feedback loop which inhibits the nuclear factor-kappaB-dependent cytokine pathway [30]. In humans, *Twist1* is expressed mainly in adipocytes of AT, but not in the liver, pancreas or skeletal muscle [31]. mRNA and protein expression of Twist1 in human AT are lower in obese than non-obese individuals [15]. Moreover, it has been reported that low mRNA and protein expression of Twist1 is associated with increased expression of proinflammatory cytokines and decreased insulin sensitivity in humans [15]. In accordance with the latter study in human AT, we found gene expression of *Twist1* to be significantly lower in the obese than the non-obese group. mRNA expression of *Twist1* was similar in the PNPLA3-148MM and PNPLA3-148II groups.

Adiponectin knockout mice display moderate insulin resistance in the liver and impaired glucose tolerance [32], and have activated macrophages in AT with increased secretion of proinflammatory cytokines such as MCP-1 compared with macrophages of wild-type mice [17, 33]. Adiponectin knockout mice also appear to have more hepatic steatosis and activated Kupffer cells than wild-type mice [34, 35]. The liver of transgenic mice overexpressing *Adipoq* is characterised by increased fatty acid oxidation leading to decreased hepatic triacylglycerol content and increased hepatic and systemic insulin sensitivity [17, 32]. Overexpression of *Adipoq* in obese mice decreases infiltration of macrophages into AT and release of proinflammatory cytokines from adipocytes and stromal vascular cells [36]. In humans, gene expression of *ADIPOQ* in AT and circulating concentrations of adiponectin are decreased in obese and insulin-resistant compared with non-obese and insulin-sensitive individuals [32, 37]. In keeping with such data, in the present study, the obese group had significantly lower gene expression of *ADIPOQ* in AT than the non-obese group. In contrast, there was no statistically significant difference in expression of *ADIPOQ* between the PNPLA3-148II and PNPLA3-148MM group.

An increase in circulating NEFA may contribute to obese NAFLD [38]. In keeping with this, we found a significant correlation between fasting NEFA and liver fat in the obese NAFLD and the PNPLA3-148II but not the PNPLA3-148MM, i.e. the PNPLA3 NAFLD, group. The latter is in keeping with our previous studies, in which we showed, by direct measurement of glycerol turnover using stable isotopes [39] and insulin action on serum NEFA [40], that lipolysis is not increased in individuals with increased liver fat due to the PNPLA3-I148M variant. These human data are in line with recent data from Li et al [41], who showed that overexpression of this variant in mouse AT does not change liver fat content, whereas overexpression in the liver does.

In conclusion, we show that NAFLD due to obesity (obese NAFLD) but not that due to the PNPLA3-I148M variant (PNPLA3 NAFLD) is accompanied by insulin resistance and AT inflammation. Together with the mechanistic studies linking increased *MCP-1* expression and macrophage infiltration, as well as low levels of Twist1 and adiponectin, to insulin resistance, the present data suggest that lack of AT inflammation may contribute to the lack of features of the metabolic syndrome in individuals with NAFLD due to the PNPLA3-I148M variant. The data also show that liver fat per se may not be deleterious, as increased liver fat in PNPLA3 NAFLD is not accompanied by insulin resistance.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement KS, VMO and HY-J designed the study. All authors contributed to acquisition or interpretation of the data. SL, KS and HY-J wrote the article. All authors contributed to critical revision of the manuscript and approved its final version. HY-J supervised the whole study.

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