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Insulin resistance is an integral feature of MASLD even in the presence of PNPLA3 variants

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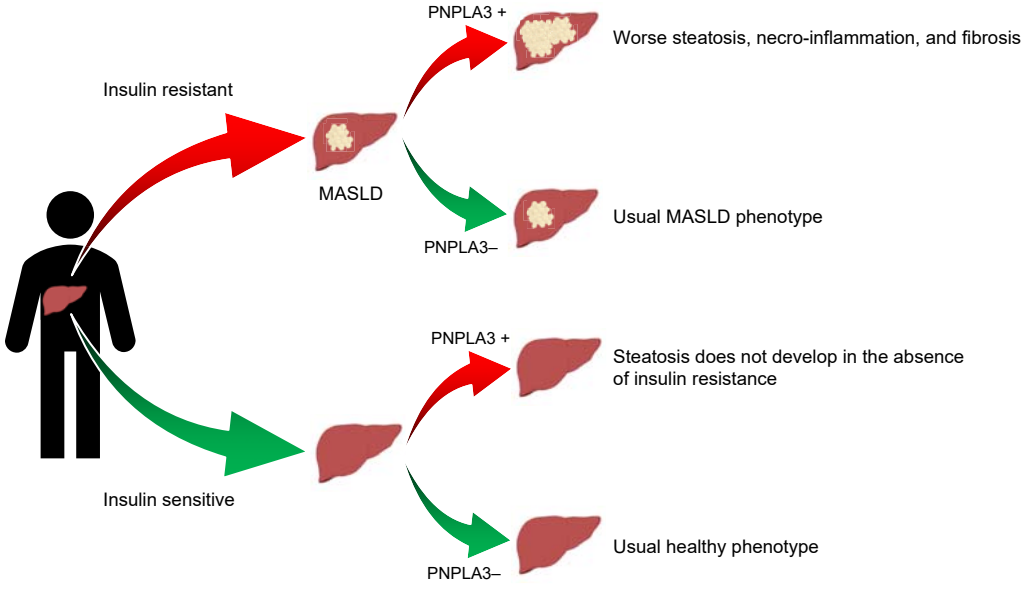
Graphical abstract



Highlights:

- Patients with PNPLA3 variants had more steatosis and worse liver histology with similar insulin resistance (IR).
- The presence of MASLD in patients with PNPLA3 variants was associated with insulin resistance.
- Changes in whole-body and adipose tissue IR were similar between MASLD and non-MASLD irrespective of PNPLA3 alleles.
- Liver fat accumulation was associated with whole-body and adipose tissue IR irrespective of PNPLA3 alleles.

Impact and implications:

It has been proposed that the *PNPLA3* G allele is associated with the presence of metabolic dysfunction-associated steatotic liver disease (MASLD) in the absence of insulin resistance. However, our results suggest that regardless of *PNPLA3* alleles, the presence of insulin resistance is necessary for the development of MASLD. This calls for reframing patients with "PNPLA3 MASLD" not as insulin sensitive, but on the contrary, as an insulin-resistant population with increased hepatic susceptibility to metabolic insults, such as obesity or diabetes.

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Insulin resistance is an integral feature of MASLD even in the presence of PNPLA3 variants

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Background & Aims: It has been postulated that carriers of PNPLA3 I148M (CG [Ile/Met] or GG [Met/Met]) develop metabolic dysfunction-associated steatotic liver disease (MASLD) in the absence of insulin resistance or metabolic syndrome. However, the relationship between insulin resistance and MASLD according to the *PNPLA3* allele has not been carefully assessed.

Methods: A total of 204 participants were recruited and underwent *PNPLA3* genotyping, an oral glucose tolerance test, liver proton magnetic resonance spectroscopy and percutaneous liver biopsy if diagnosed with MASLD. A subgroup of patients (n = 55) had an euglycemic hyperinsulinemic clamp with glucose tracer infusion.

Results: As expected, patients with the CG/GG genotype had worse intrahepatic triglyceride content and worse liver histology. However, regardless of *PNPLA3* genotype, patients with a diagnosis of MASLD had severe whole-body insulin resistance (Matsuda index, an estimation of insulin resistance in glucose metabolic pathways) and fasting and postprandial adipose tissue insulin resistance (Adipo-IR index and free fatty acid suppression during the oral glucose tolerance test, respectively, as measures of insulin resistance in lipolytic metabolic pathways) compared to patients without MASLD. Moreover, for the same amount of liver fat accumulation, insulin resistance was similar in patients with genotypes CC *vs.* CG/GG. In multiple regression analyses, A1c and Adipo-IR were associated with the presence of MASLD and advanced liver fibrosis, independently of *PNPLA3* genotype.

Conclusions: *PNPLA3* variant carriers with MASLD are equally insulin resistant as non-carriers with MASLD at the level of the liver, muscle, and adipose tissue. This calls for reframing "PNPLA3 MASLD" as an insulin-resistant condition associated with increased hepatic susceptibility to metabolic insults, such as obesity or diabetes, wherein early identification and aggressive intervention are warranted to reverse metabolic dysfunction and prevent disease progression.

Introduction

Non-alcoholic fatty liver disease (NAFLD), recently renamed metabolic dysfunction-associated steatotic liver disease (MASLD), is characterized by insulin resistance and strongly influenced by acquired factors such as sedentary lifestyle, obesity and type 2 diabetes mellitus (T2D).[1–3] However, genetic and epigenetic factors have been shown to play a role in modulating the severity of liver disease.[4] Single nucleotide polymorphisms (SNPs) in several genes have been associated with the development and severity of MASLD and metabolic dysfunction-associated steatohepatitis (MASH) (formerly non-alcoholic steatohepatitis [NASH]), such as patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), *TM6SF2, GCKR, MBOAT7, HSD17B13* among others.[5,6] Of these, PNPLA3 is probably the best characterized SNP and the one most strongly associated with the development of steatohepatitis, advanced fibrosis, cirrhosis, decompensation and liver-related death in patients with MASLD.[7–12]

The rs738409 [G], encoding I148M variant of PNPLA3 (CG [Ile/Met] or GG [Met/Met]), is found in ∼40% of individuals and with greater predominance among Hispanics.[13,14] Carriers with obesity or diabetes appear to be at a greater risk of poor liver-related outcomes.[15,16] Moreover, it has been postulated that I148M carriers develop MASLD in the absence of insulin resistance or metabolic syndrome,[17–19] although cross-sectional studies have reported minimal or no differences in insulin sensitivity between carriers and non-carriers using homeostatic model assessment for insulin resistance (HOMA-IR).[12,20] However, the relationship between insulin resistance in patients with and without the PNPLA3 I148M variant has not been carefully assessed in larger studies. HOMA-IR largely examines fasting hepatic insulin sensitivity, not skeletal muscle, or adipose tissue, and cannot distinguish elevated insulin levels from insulin resistance as opposed to the reduced insulin clearance typical of individuals with MASLD/MASH.[21,22] Some early studies examined insulin sensitivity by the gold-standard euglycemic insulin clamp technique in patients with or without the PNPLA3 variants (although relatively limited as few were homozygous for the I148M variant) in youth[23] and adults.[17,24] Because patients with

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the I148M variant had more liver fat accumulation for any degree of insulin sensitivity, this has led many to conclude that PNPLA3 is associated with MASLD independent of insulin resistance. In a study by Kantartzis *et al.*,[17] all patients with MASLD, regardless of their *PNPLA3* genotype, had insulin resistance when compared to patients without MASLD. Furthermore, studies from Taiwan[25] and South Korea[26] using HOMA-IR reported a close relationship between PNPLA3 I148M polymorphism and having more severe insulin resistance.

Therefore, the aim of this study was to carefully assess the relationship between insulin resistance and MASLD according to *PNPLA3* genotype. Specifically, to focus on determining whether patients with MASLD and the PNPLA3 variant are insulin sensitive relative to patients with MASLD without this genetic trait. This distinction has important clinical implications. If those with "PNPLA3 MASLD" are insulin sensitive (or more than non-carriers), it would strongly support the notion that *PNPLA3* genotype can alter the natural history of the disease relatively independent of metabolic dysfunction. If both groups are equally insulin resistant, this would instead imply that "PNPLA3 MASLD" should still be seen as an important and distinctive trait for individuals with MASLD, but only in the context of insulin resistance and metabolic dysfunction, not independent of them. This subtle paradigm shift would call to change current clinical practice by calling for the early determination of PNPLA3 status so that more aggressive lifestyle and pharmacological intervention are recommended for people with the *PNPLA3* variant genotype, or at minimum in subgroups at higher risk, such as those with T2D and/or "at risk MASH" (NAFLD activity score ≥4 and F≥2). It would also help clinicians to fully grasp the concept that insulin resistance and metabolic dysfunction are the fundamental characteristics of all patients with MASH, regardless of *PNPLA3* genotype.

Materials and methods

Participants

Participants were recruited using newspaper ads, flyers, or from hepatology and endocrinology clinics at the University of Texas Health Science Center at San Antonio in San Antonio, TX or the University of Florida in Gainesville, FL. Participants underwent research procedures as part of their screening for potential participation in epidemiological and clinical trial studies.[27–29]

Inclusion criteria were BMI >25 kg/m^2 after exclusion of secondary liver diseases (autoimmune hepatitis, hemochromatosis, viral hepatitis, Wilson's disease), significant alcohol consumption (≥30 g/day for males and ≥20 g/day for females), type 1 or other forms of diabetes other than T2D, or use of medications known to affect liver fat content (*i.e.*, amiodarone, glucocorticoids, methotrexate, tamoxifen, olanzapine, protease inhibitors, vitamin E, pioglitazone, SGLT-2 inhibitors, GLP-1 agonists, weight loss medications). Patients with or without T2D were included in the study. All allowed glucose-lowering medications (*i.e.*, metformin, sulfonylureas, and insulin) were required to be stable for at least 3 months prior to participation in the study, as were physical activity and diet. The study was approved by the institutional review boards at the University of Florida and University of Texas Health Science Center at San Antonio, and written informed consent was obtained from each patient prior to their participation.

Study design

As part of this cross-sectional study, participants underwent routine bloodwork, determination of *PNPLA3* alleles, and an oral glucose tolerance test (OGTT) – to stratify individuals on the presence/absence of diabetes and to measure fasting and postprandial insulin sensitivity. Screening for MASLD was performed with proton magnetic resonance spectroscopy (^{1}H-MRS), and patients with a diagnosis of MASLD were offered a percutaneous liver biopsy to determine the presence of MASH and staging of liver fibrosis. A subgroup of patients had an euglycemic hyperinsulinemic clamp with measurement of glucose turnover using a radioactive isotope.

Procedures

OGTT

A 2-hour OGTT was performed with 75 g of glucose. Blood draws were obtained at minutes -15, 0, and every 30 min for 2 h. Plasma glucose was measured bedside by the glucose oxidase method (Analox Instruments, Hammersmith, UK). Plasma insulin, and free fatty acids (FFAs) were also measured in these samples by radioimmunoassay and standard colorimetric methods, respectively. Whole-body insulin sensitivity was estimated during the OGTT by Matsuda index,[30] which is a reflection of insulin sensitivity of glucose metabolic pathways in skeletal muscle and liver. Fasting adipose tissue insulin sensitivity was calculated as fasting plasma insulin multiplied by the fasting FFA concentration (Adipo-IR). Postprandial adipose tissue insulin sensitivity was estimated as the suppression of FFAs after 60-120 min of the OGTT. Unlike the Matsuda index, these indices of insulin sensitivity assess the response to insulin in lipolytic pathways.

^{1}H-MRS and percutaneous liver biopsy

^{1}H-MRS was performed as previously reported.[31] Briefly, three liver areas of 3x3x3 cm each were selected avoiding vessels and bile ducts. Intrahepatic triglyceride content was estimated as area under the curve of fat peak/(area under the curve of fat + water peaks) * 100, using NUTS (Acorn NMR Inc., CA, USA). Percutaneous liver biopsies were performed under ultrasound guidance in patients with MASLD. Biopsies were evaluated by a pathologist who was unaware of the individual's identity or clinical information. Histologic characteristics were determined using standard criteria.[32] Definite MASH was defined by the concomitant presence of zone 3 accentuation of macrovesicular steatosis (of any grade), hepatocellular ballooning (any degree) and lobular inflammatory infiltrates (any amount).

Euglycemic hyperinsulinemic clamp with 3-[^{3}H]-glucose tracer

A primed (25 μCi x [fasting glucose/100])–continuous (0.25 μCi/min) infusion of 3-[^{3}H]-glucose (DuPont-NEN, Boston, MA) was maintained throughout the study. After a 3-hour equilibration period, insulin was administered as a primed-continuous infusion at 10 mIU/(m^2 · minute) for 120 min to assess suppression of endogenous glucose production

(EGP). Afterwards, insulin infusion was increased to a rate of 80 mIU/(m^2 · minute) for 120 min to assess skeletal muscle insulin stimulated glucose disposal (R*d*). A dextrose 20% infusion was titrated every 5-10 min based on the negative feedback principle to maintain a plasma glucose concentration of 90-100 mg/dl. EGP and *R*d were calculated using non-steady state equations, as previously reported.[21,33]

DNA isolation and PNPLA3 genotyping

The genomic DNA from blood white blood cells was isolated using a commercially available kit (QiaGen, MD, USA). The genotyping for *PNPLA3* (rs738409) gene SNPs was performed using TaqMan allelic discrimination genotyping method on the Quant- Studio 12K Flex System according to the manufacturer's recommendations (Life Technologies, Thermo Fisher Scientific, Waltham, MA) at the University of Florida Center for Pharmacogenomics and Precision Medicine.

Statistical analysis

Data was summarized as mean ± SD (or mean ± SE in figures) or as percentages. Comparisons between three or more groups were performed with ANOVA (with Bonferroni's adjustment for pairwise comparisons) and Chi-square (or Fisher's exact test when appropriate). Comparisons between two groups were done with t-test and Chi-square (or Fisher's exact test when appropriate). Multivariate logistic regression analysis with forward selection was used to assess the effect of metabolic and demographic co-variates (*e.g.*, age, sex, race, BMI, fasting plasma glucose, A1c, fasting and postprandial measurements of insulin sensitivity) on the presence of MASLD, MASH, or advanced fibrosis. A two-tailed *p* value <0.05 was considered to indicate statistical significance. Analyses were performed with Stata 15.1 (StataCorp LP, College Station, TX) and graphs with Prism 8.1.2 (GraphPad Software, Inc., La Jolla, CA).

Results

Patient characteristics

A total of 204 individuals were included in this study. Baseline clinical characteristics based on their rs738409 *PNPLA3* genotype (CC, *vs.* CG, *vs.* GG) are summarized in Table 1. The frequency of the minor G allele was higher in Hispanics compared to other racial/ethnic groups (0.57 *vs*. 0.26, 0.30, and 0.34, for Hispanic, non-Hispanic Black, other, and non-Hispanic White, respectively). The distribution of the rs738409 *PNPLA3* genotype was in Hardy-Weinberg equilibrium in all races/ethnic groups (Table S1).

As can be observed in Table 1, patients had similar age, sex, BMI, presence of diabetes (and A1c), fasting plasma glucose

Table 1. Patients' characteristics based on *PNPLA3* alleles.

	***PNPLA3* rs738409**			
	CC (n = 79)	**CG (n = 80)**	**GG (n = 45)**	***p* value**
Age, years	57 ± 9	56 ± 9	56 ± 9	0.56
Sex, male/female %	76%/24%	72%/28%	71%/29%	0.81
Ethnicity				<0.001
Caucasian, %	58%	50%	31%	
Hispanic, %	20%	41%	60%	
African-American, %	18%	8%	7%	
Other, %	4%	1%	2%	
Body mass index, kg/m^2	34.2 ± 5.6	33.8 ± 4.8	32.7 ± 5.3	0.32
Presence of obesity/overweight, %	70/30%	80/20%	64/36%	0.132
Presence of impaired glucose tolerance, %	11%	18%	31%	0.159
Presence of diabetes, %	72%	68%	56%	0.166
Knowledge of prior diabetes diagnosis, %	100%	87%	88%	0.008
A1c, %				
In patients with diabetes	7.2 ± 1.1	6.9 ± 1.0	6.8 ± 0.8	0.157
In patients without diabetes	5.6 ± 0.4	5.6 ± 0.4	5.8 ± 0.5	0.192
Use of diabetes medications				
Metformin, %	57%	44%	49%	0.32
Sulfonylureas, %	35%	32%	20%	0.26
Insulin, %	11%	17%	13%	0.59
Fasting plasma glucose, mg/ml	135 ± 41	130 ± 34	122 ± 31	0.170
Fasting plasma insulin, μU/ml	14 ± 12	15 ± 13	13 ± 8	0.75
2-hour glucose, mg/dl	238 ± 88	236 ± 83	212 ± 71	0.24
Total cholesterol, mg/dl	169 ± 38	180 ± 44	165 ± 42	0.086
LDL-C, mg/dl	94 ± 31	104 ± 38	99 ± 34	0.187
HDL-C, mg/dl	43 ± 14	43 ± 14	40 ± 9	0.50
Triglyceride, mg/dl	130 (93-193)	136 (94-194)	126 (85-160)	0.33
Statin use, %	60%	60%	66%	0.78
Aspartate aminotransferase, U/L	31 ± 23	39 ± 24	40 ± 19	0.030
Alanine aminotransferase, U/L	38 ± 32	52 ± 38	52 ± 31	0.016
NAFLD activity score	3.5 ± 1.5	4.2 ± 1.5	4.4 ± 1.3	0.010
Steatosis grade	1.5 ± 0.7	1.9 ± 0.9	2.0 ± 0.8	0.022
Inflammation grade	1.5 ± 0.6	1.7 ± 0.5	1.7 ± 0.5	0.34
Ballooning grade	0.4 ± 0.5	0.6 ± 0.5	0.7 ± 0.5	0.024
Fibrosis stage	0.8 ± 0.9	1.1 ± 1.1	1.1 ± 1.0	0.170
Percentage of patients with clinically significant fibrosis (F2–F4), %	15%	23%	24%	0.44

and insulin, and lipid profile, across all *PNPLA3* genotypes. Patients with CG or GG had higher plasma levels of liver aminotransferases, as well as worse liver histology (significant for steatosis, ballooning and overall NAFLD activity score; Table S2). Fig. 1 shows that patients with GG and CG had higher intrahepatic triglyceride (IHTG) content by ^{1}H-MRS (16 ± 12% *vs.* 14 ± 10% *vs.* 9 ± 9% for GG, CG, and CC, respectively, p = 0.002) and a higher prevalence of definite MASH (70% *vs.* 60% *vs.* 42%, respectively, p = 0.024) compared to patients with CC. This remained true even when limiting the analyses to patients with obesity (BMI ≥30 kg/m^2): IHTG content by ^{1}H-MRS was 17 ± 13% *vs.* 16 ± 11% *vs.* 11 ± 8% for GG, CG, and CC, respectively, p = 0.033, and prevalence of definite MASH was 68% *vs.* 64% *vs.* 41%, respectively, p = 0.040. Similarly, among patients without obesity, IHTG content by ^{1}H-MRS was also higher in patients with GG and CG (14 ± 10% *vs.* 8 ± 7% *vs.* 5 ± 8% for GG, CG, and CC, respectively, p = 0.018). Not enough patients without obesity underwent a liver biopsy to provide analysis on prevalence of MASH among this group. In order to minimize the possibility of race playing a role in observed differences, we also repeated the analyses only in Caucasian patients (*i.e.*, IHTG content by ^{1}H-MRS was 16 ± 14% *vs.* 16 ± 12% *vs.* 9 ± 8% for GG, CG, and CC, respectively, p = 0.023, and prevalence of definite MASH was 50% *vs.* 52% *vs.* 38%, respectively, p = 0.54).

Insulin sensitivity across *PNPLA3* genotypes in patients with or without MASLD

In Fig. 2, whole-body insulin sensitivity measured with the Matsuda index during an OGTT (a composite estimate of hepatic and muscle insulin sensitivity) is reported based on *PNPLA3* genotype and presence or absence of MASLD. As can be observed, patients with MASLD were equally insulin resistant, independently of *PNPLA3* genotype (p = 0.90). Similarly, across all *PNPLA3* genotypes, the difference (delta) in insulin sensitivity in patients with *vs.* without MASLD was similar across groups, with a ∼50% reduction in the Matsuda index in all genotypes (p ≤0.002 for all). Similar results were obtained if only patients with T2D were included; p ≤0.011 for all). When limiting the analysis to patients with obesity, we observed similar results in CC patients (53% reduction in Matsuda index with MASLD, p = 0.008). Groups were too small for formal comparisons in the CG and GG groups, but reductions were 31% and 59%, respectively. Results were unchanged when excluding different races from the analyses (p <0.001 for all).

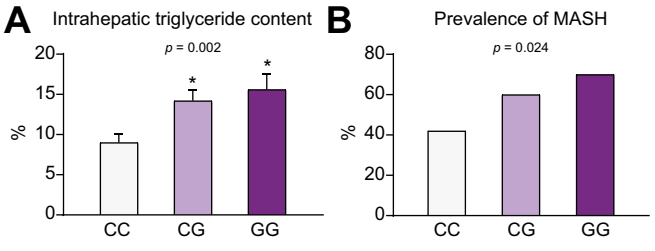

Fig. 1. Intrahepatic triglyceride content and prevalence of MASH based on *PNPLA3* alleles. *p <0.02 *vs.* CC group after Bonferroni adjustment for multiple comparisons. MASH, metabolic dysfunction-associated steatohepatitis.

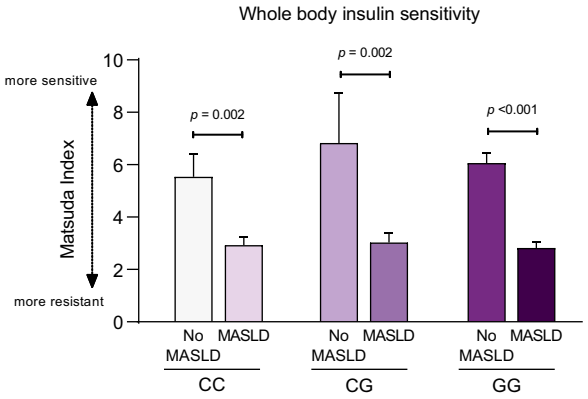

Fig. 2. Whole-body insulin sensitivity measured by Matsuda index during an oral glucose tolerance test based on *PNPLA3* allele and presence of MASLD. MASLD, metabolic dysfunction-associated steatotic liver disease.

As can be seen in Fig. 3A, when fasting adipose tissue insulin sensitivity was measured as Adipo-IR index, patients with MASLD were equally insulin resistant, independently of *PNPLA3* genotype (p = 0.53). While the difference in Adipo-IR index between patients with or without MASLD was similar for all genotypes (CC: 4.88 [2.26 to 7.51]; CG: 4.69 [-0.05 to 9.44]; GG: 4.02 [0.51 to 7.54]), it was only statistically significant for CC (p <0.001) and GG (p = 0.026) groups, with a p value of

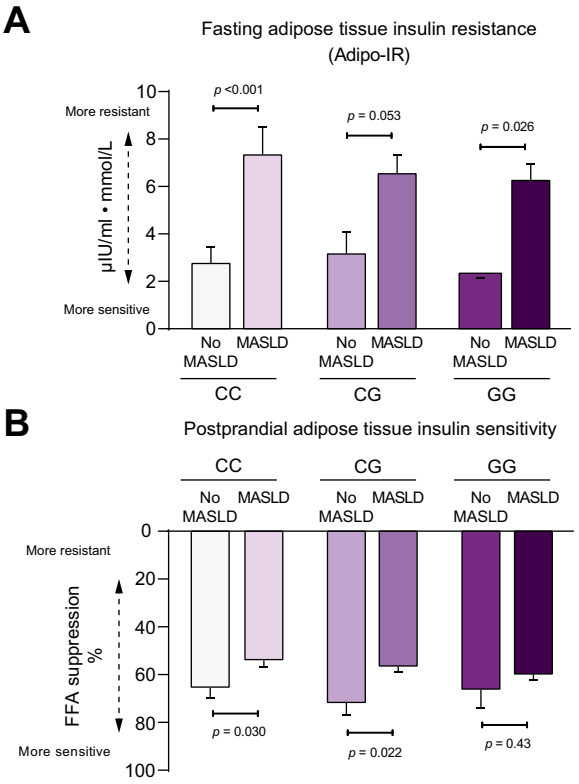

Fig. 3. Adipose tissue insulin sensitivity based on *PNPLA3* alleles and presence of MASLD. Measured in the fasting (A; measured by the Adipo-IR index) and postprandial (B; measured by suppression of plasma FFA during an OGTT) states based on *PNPLA3* alleles and presence of MASLD. Adipo-IR, adipose tissue insulin resistance; FFA, free fatty acid; OGTT, oral glucose tolerance test; MASLD, metabolic dysfunction-associated steatotic liver disease.

0.053 in the CG group. In Fig. 3B, data on adipose tissue insulin sensitivity measured in the postprandial period was presented. Patients with MASLD had similar suppression of FFA during the OGTT regardless of their *PNPLA3* genotype (p = 0.62). In patients with *PNPLA3* genotypes CC and CG, there was a significant difference in postprandial adipose tissue insulin sensitivity in patients with *vs.* without MASLD. However, no significant difference was detected in patients with the GG genotype (results were similar when only patients with diabetes or obesity were included, although some p values in CG and GG groups were unable to be obtained due to small sample size; data not shown). Results were unchanged when excluding different races from the analyses, except for loss of significance in Adipo-IR and suppression of FFA during the OGTT in the CG group (p <0.05 for all others).

Determinants of MASLD based on *PNPLA3* genotype

In order to assess which factors are associated with the presence of MASLD according to *PNPLA3* genotype, we performed a multivariate logistic regression analysis. As can be observed in Table 2, several metabolic factors, as well as sex were associated with the presence of MASLD in the univariate analyses. However, only adipose tissue insulin sensitivity (*i.e.*, Adipo-IR, odds ratio [OR] 1.49; 95% CI 1.20-1.86), diabetes control (*i.e.*, A1c OR 2.00; 95% CI 1.24-3.26), African-American race (OR 0.30; 95% CI 0.10-0.92), and *PNPLA3* G allele (OR 2.49; 95% CI 1.36-4.56) remained significant in the multivariate analysis. In agreement with these findings, Adipo-IR was significantly correlated with IHTG content by ^{1}H-MRS in patients with CC or CG/GG genotypes (r= 0.38, p = 0.003; and r= 0.25, p = 0.025, respectively). When patients were divided into four groups based on the amount of IHTG content (Fig. 4), presence of MASLD was associated with significant insulin resistance in adipose tissue (Fig. 4A) as well as in liver and skeletal muscle (Matsuda index) (Fig. 4B), irrespective of *PNPLA3* genotype.

Insulin sensitivity across *PNPLA3* genotypes in a subgroup of patients during an insulin clamp

We also measured liver and skeletal muscle insulin sensitivity independently in 55 participants with MASLD using the gold-standard euglycemic hyperinsulinemic clamp during standardized low-dose (for hepatic insulin sensitivity) and high-dose (for skeletal muscle insulin sensitivity) insulin infusions (see Methods) (Fig. S1). Six controls without MASLD, obesity, or T2D served to establish 'normal' values (dotted lines in Fig. S1). No significant difference was observed in hepatic insulin sensitivity (assessed as suppression of EGP during the low-dose euglycemic hyperinsulinemic clamp) with different *PNPLA3* genotypes (CC: -45 ± 21%; CG: -37 ± 19%; and GG: -45 ± 20%, p = 0.35). Of note, they were all lower than in non-obese patients without MASLD and

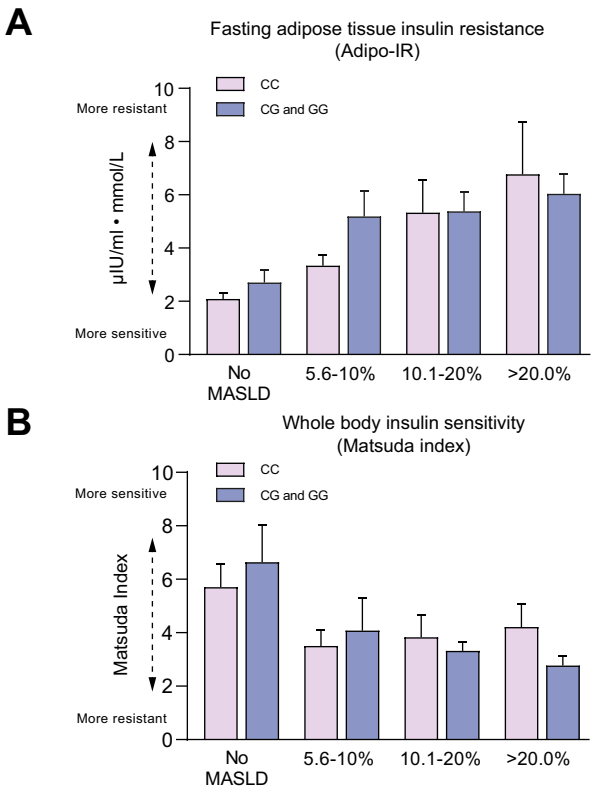

Fig. 4. Adipose tissue insulin resistance and whole-body insulin sensitivity depending on the degree of liver fat accumulation by ^{1}H-MRS. (A) Adipose tissue insulin resistance and (B) whole-body insulin sensitivity. ^{1}H-MRS, proton magnetic resonance spectroscopy.

Table 2. Multiple logistic regression analysis to identify factors associated with the presence of MASLD depending on *PNPLA3* genotype.

	Univariate analysis		Multivariate analysis	
	OR	p value	OR	p value
Age	0.98 (0.94-1.01)	0.22		
Sex, male	1.64 (0.83-3.24)	0.153		
Hispanic	1.94 (0.97-3.87)	0.060		
African-American	0.22 (0.09-0.53)	0.001	0.30 (0.10-0.92)	0.034
BMI	1.13 (1.06-1.21)	<0.001		
FPG	1.01 (0.99-1.02)	0.091		
A1c	1.73 (1.20-2.49)	0.003	2.00 (1.24-3.26)	0.005
Hypertension	0.70 (0.22-2.20)	0.54		
Dyslipidemia	3.45 (1.60-7.47)	0.002		
Adipo-IR	1.58 (1.28-1.94)	<0.001	1.49 (1.20-1.86)	<0.001
Suppression of FFA - OGTT	0.97 (0.95-0.99)	0.004		
Matsuda index	0.75 (0.65-0.87)	<0.001		
PNPLA3, number of G alleles	2.18 (1.37-3.47)	0.001	2.49 (1.36-4.56)	0.003

Adipo-IR, adipose tissue insulin resistance; FFA, free fatty acid; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; MASLD, metabolic dysfunction-associated steatotic liver disease; OR, odds ratio.

without diabetes (-63 ± 11%; *p* values: 0.072, 0.004, and 0.049, respectively).

Insulin-stimulated muscle uptake during the high-dose euglycemic hyperinsulinemic clamp revealed that all groups independent of *PNPLA3* genotype were extremely insulin resistant when compared to non-obese controls without MASLD or diabetes (R*d* controls: 12.70 ± 3.71 mg/kg/min, with *p* values of <0.001 compared to CC, CG and GG), although slightly less in patients with GG genotype (CC: 2.96 ± 0.92 *vs.* CG: 3.25 ± 1.42 *vs.* GG: 4.72 ± 1.84 mg/kg/min, $p = 0.002$; pairwise comparisons after Bonferroni's adjustment GG *vs.* CC: $p = 0.012$ and GG *vs.* CG: $p = 0.007$; Fig. S1).

Relationship between insulin sensitivity and liver histology according to *PNPLA3* genotype

We then explored if adipose tissue insulin resistance was associated with worse liver histology independently of *PNPLA3* genotype. Regardless of *PNPLA3* genotype, adipose tissue insulin resistance index quartiles were not associated with NAFLD activity score (Fig. 5A). However, among patients with *PNPLA3* CC genotype, patients in the highest quartile of adipose tissue insulin resistance (Q4) showed a significantly higher mean fibrosis stage compared to other quartiles (Fig. 5B). Patients with genotypes CG and GG showed similar mean fibrosis stage in all adipose tissue insulin resistance quartiles, suggesting that adipose tissue insulin resistance may have a larger impact on histology among patients without *PNPLA3* G alleles. The mean NAFLD activity score (Fig. 5C) did not show differences based on quartiles of Matsuda index in either PNPLA3 carriers or non-carriers. However, fibrosis stage was significantly higher with worsening (decreasing) Matsuda index quartiles, regardless of the *PNPLA3* genotype (Fig. 5D). As sensitivity analyses, we have presented the data including only patients with obesity (Fig. S2) and only among patients with T2D (Fig. S3).

To further characterize the impact of all these variables on the presence of MASH or advanced liver fibrosis, we performed multivariate logistic regression analyses (Table 3). Only Hispanic ethnicity was independently associated with definite MASH, with a strong trend for presence of *PNPLA3* allele variants (OR 1.59, $p = 0.055$). Advanced fibrosis was independently associated with adipose tissue insulin resistance, A1c and presence of G alleles in *PNPLA3*.

Discussion

A growing body of evidence indicates that people with the *PNPLA3* genotype rs738409 [G], encoding I148M, carry a greater risk of developing more severe steatohepatitis and cirrhosis, although the mechanisms remain incompletely understood.[7–12] This higher risk has led to the categorization of patients into two groups: one where the disease is perceived as being predominantly driven by insulin resistance and its

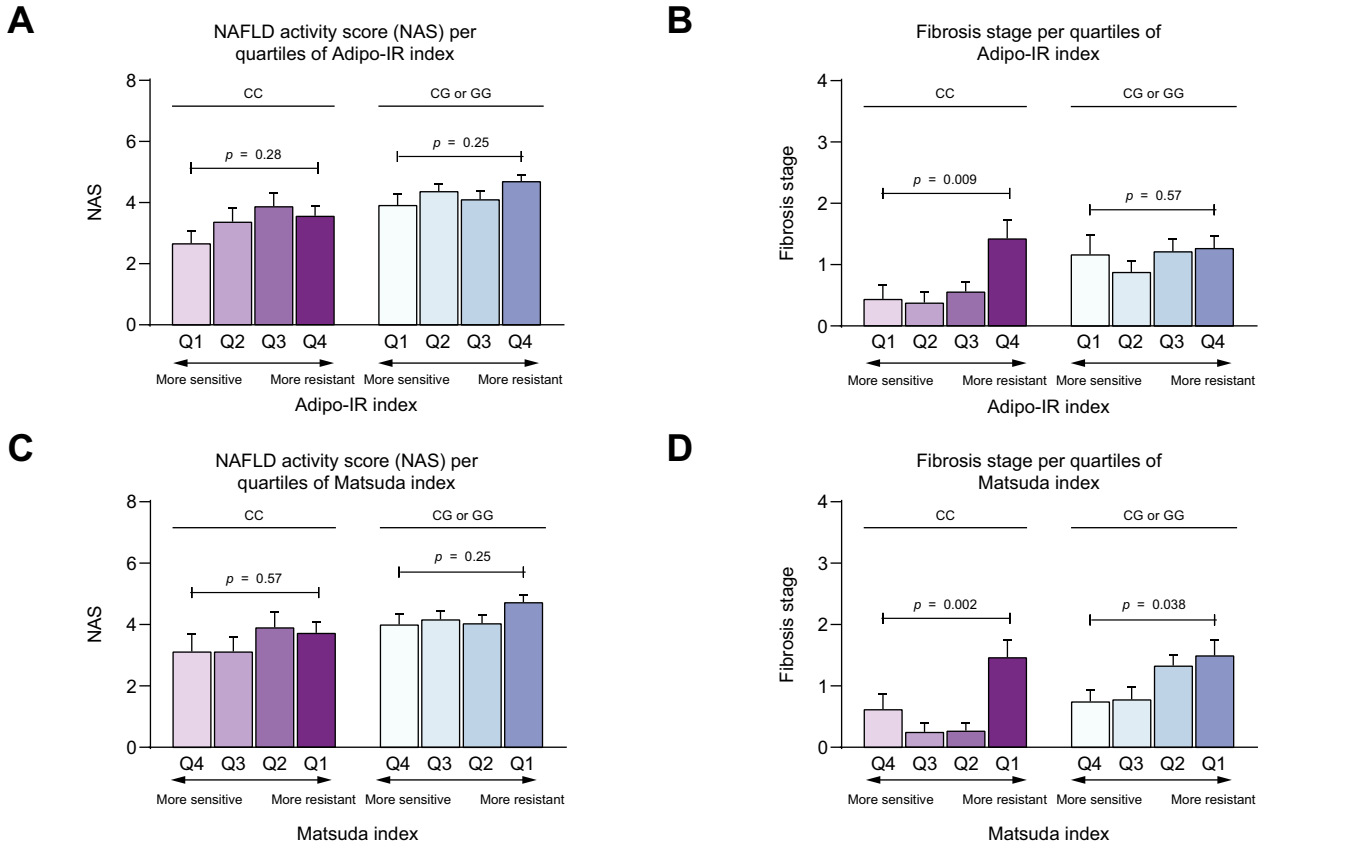

Fig. 5. Mean NAS and fibrosis stage based on fast adipose tissue insulin resistance, and hepatic and muscle insulin sensitivity. Mean NAS and fibrosis stage based on (A,B) quartiles of fasting adipose tissue insulin resistance or (C,D) quartiles of hepatic and muscle insulin sensitivity in patients with or without a *PNPLA3* variant. NAS, NAFLD activity score.

Table 3. Multiple logistic regression analysis to identify factors associated with the presence of definite MASH and those with MASH and advanced fibrosis (F3 or F4).

	Univariate analysis		Multivariate analysis	
	OR	*p* value	OR	*p* value
Definite MASH				
Age	0.94 (0.91-0.98)	0.007		
Sex, male	0.52 (0.22-1.23)	0.136		
Hispanic	3.36 (1.63-6.93)	0.001	2.85 (1.35-6.01)	0.006
African-American	0.35 (0.10-1.23)	0.102		
BMI	1.01 (0.94-1.09)	0.69		
FPG	0.99 (0.98-1.00)	0.069		
2-hour OGTT glucose	1.00 (0.99-1.00)	0.074		
A1c	0.86 (0.63-1.16)	0.31		
Adipo-IR	1.03 (0.98-1.09)	0.24		
Suppression of FFA - OGTT	1.00 (0.99-1.02)	0.56		
Matsuda index	0.88 (0.72-1.07)	0.21		
PNPLA3, number of G alleles	1.84 (1.17-2.90)	0.008	1.59 (0.99-2.55)	0.055
Advanced fibrosis (F3–F4)				
Age	1.02 (0.96-1.08)	0.56		
Sex, male	2.16 (0.47-9.98)	0.32		
Hispanic	2.13 (0.79-5.77)	0.138		
BMI	0.99 (0.89-1.10)	0.86		
FPG	1.00 (0.99-1.01)	0.92		
2-hour OGTT glucose	1.00 (1.00-1.01)	0.29		
A1c	1.82 (1.18-2.79)	0.006	2.19 (1.25-3.84)	0.006
Adipo-IR	1.11 (1.04-1.18)	0.001	1.11 (1.03-1.19)	0.005
Suppression of FFA - OGTT	0.98 (0.95-0.99)	0.042		
Matsuda index	0.57 (0.36-0.91)	0.018		
PNPLA3, number of G alleles	1.45 (0.75-2.78)	0.27	2.27 (1.04-4.93)	0.038

Adipo-IR, adipose tissue insulin resistance; FFA, free fatty acid; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; MASH, metabolic dysfunction-associated steatohepatitis; OR, odds ratio.

associated "metabolic dysfunction" ("Metabolic MASLD"), and another, in patients with *PNPLA3* genotype rs738409 [G], where MASH is predominantly attributed to their genetic risk and not to insulin resistance ("PNPLA3 MASLD"). We aimed to reexamine this premise, and carefully determine whether those with "PNPLA3 MASLD" had normal insulin sensitivity, or at least were more insulin sensitive than those matched individuals not carrying the PNPLA3 variant, and examine how their genetic background influenced liver disease. We reasoned that if the "PNPLA3 MASLD" group was insulin sensitive, it would strongly support the notion of a *PNPLA3* genotype playing a major role in the natural history of the disease, independent of metabolic dysfunction. Instead, if both groups were equally severely insulin resistant, this would call for a shift where "PNPLA3 MASLD" is no longer seen as a distinctive, non-insulin-resistant form of the disease, but rather as a liver susceptibility trait, within the context of insulin resistance/metabolic dysfunction, which would remain as the cardinal characteristic of all patients with MASLD/MASH. Our results are important in clarifying this conundrum. Individuals with "PNPLA3 MASLD" did have a higher IHTG content, and worse steatohepatitis and fibrosis across the spectrum of insulin resistance, but all individuals with MASLD had severe insulin resistance at the level of the liver, whether they had or not the *PNPLA3* variant genotype. Moreover, we extended this observation to establish that severe insulin resistance was not confined to the liver, but clearly present in muscle (measured either by Matsuda index or the gold-standard euglycemic insulin clamp) and adipose tissue, and that insulin resistance was fully established even with modest degrees of hepatic steatosis. Taken together, our observations carry major clinical implications as they provide strong evidence for the role of insulin resistance and metabolic dysfunction as a key target of treatment, independent of the genetic background.

Our study carefully characterizes a rather large group of people and provides a novel analysis of the role of insulin resistance in the setting of the *PNPLA3* allele, the best studied genetic factor in MASH. Our population was representative of the overall population of people with MASLD/MASH, having the known distribution of the *PNPLA3* allele and genotype frequency by race and ethnic group (*e.g.*, more prevalent in Hispanics; Tables 1 and 3, and Table S1). Moreover, other than the expected higher plasma aminotransferases associated with more severe steatohepatitis, carriers compared to non-carriers were not different in other clinical or laboratory parameters, such as diabetes or hyperglycemia, hypertension, or atherogenic dyslipidemia or statin use. Within this context, we confirmed prior observations of greater IHTG content and prevalence of MASH based in carriers of the *PNPLA3* allele (Fig. 1). In the current study, we decided to perform a more detailed analysis of insulin resistance than in prior epidemiological studies that resorted only to fasting parameters like HOMA-IR, which primarily is an index of hepatic insulin resistance derived from the fasting plasma glucose (dependent on the rate of hepatic glucose production) and the fasting plasma insulin,[3,30] which is also affected by impaired insulin clearance in MASLD and in MASH.[21,22] The Matsuda index derived from the OGTT instead gives a broader picture of both hepatic and skeletal muscle insulin sensitivity.[30] As expected, individuals with MASLD had a >50% reduction in insulin sensitivity. However, there was no evidence that people with "PNPLA3 MASLD" were less insulin resistant than non-carriers of the *PNPLA3* mutation (Fig. 2). Moreover, we confirmed the severe impairment in hepatic and skeletal muscle (*R*d) insulin

sensitivity by means of an euglycemic hyperinsulinemic clamp with a stable isotope glucose infusion in a representative subset of individuals (Fig. S1). Taken together, these results offer robust evidence that individuals with the *PNPLA3* genotype are as insulin resistant as non-carriers who are labelled as "Metabolic MASLD", perhaps a misnomer as insulin resistance and metabolic dysfunction are the same and central to the pathophysiology of the disease in all patients. Prior studies have provided similar evidence of insulin resistance in individuals with the *PNPLA3* genotype variant, but in most studies based only on HOMA-IR.[12] For example, in the study by Dongiovanni *et al.*,[34] among patients with higher genetic risk score for MASLD, a close relationship between liver fat and HOMA-IR was noted in two out of the three cohorts included, but no relationship was noted in the Dallas Heart Study cohort. In line with this, population-level genome-wide association studies confirmed that genes associated with MASLD increase the risk of type 2 diabetes.[35,36]

Some investigators performed more in-depth metabolic studies but with few patients homozygous for the *PNPLA3* allele.[24] However, a perception for *PNPLA3* carriers as being insulin sensitive, or at least more insulin sensitive than non-carriers, may have arisen from misinterpretation of available evidence.[17,12,20] Conversely, a higher IHTG content may have been considered less metabolically harmful if not associated with more insulin resistance, despite the worse liver histology. However, this interpretation ignores the fact that hepatic insulin resistance does not follow a linear relationship with IHTG content. Rather hepatic insulin resistance develops rapidly and is associated with even mild increases in hepatic steatosis (*e.g.*, ~5%) and that metabolic harm is rather fully manifested in both tissues once IHTG is above this threshold.[37,38] Therefore, the impact of a "PNPLA3 MASLD" genotype is not really at the level of worsening IHTG accumulation or insulin resistance (already severe, independent of *PNPLA3* carrier status) but rather at the molecular level where disturbed lipid droplet trafficking affects triglyceride and overall lipid composition (*i.e.*, predominance of unsaturated fatty acids),[39] metabolic pathways (*i.e.*, *de novo* lipogenesis)[18] or impacts tricarboxylic acid cycle or overall mitochondrial function.[18,40] In support of a pathogenic role of PNPLA3 in the progression of liver disease, in patients with MASLD the association between MASLD genetic risk score and hepatocellular carcinoma was independent of HOMA-IR.[41] This is an important concept as drugs in development targeting lipid droplet metabolism[42] or even available diabetes medications, such as GLP-1 receptor agonists[43] or pioglitazone that improve DNL[44] and mitochondrial function,[45] may prove to be more effective in this population.

Beyond the severe hepatic and skeletal muscle insulin resistance in both *PNPLA3* allele variant carriers or not, we examined if in addition to the reported intrinsic defects in hepatocyte lipid droplet trafficking,[42] the higher IHTG content could be, at least in part, due to alterations in lipolysis and increased substrate (*e.g.*, FFA) supply to the liver by dysfunctional, insulin-resistant adipose tissue. Recent work by our group has reported a strong link between insulin-resistant adipose tissue and development of liver fibrosis in people with T2D.[46] We measured adipose tissue insulin sensitivity in the fasting (Fig. 3A) and postprandial (Fig. 3B) state and again stratified participants based on the presence or not of *PNPLA3* variant alleles. As anticipated from prior work,[21,37,47] people with MASLD had marked adipose tissue insulin resistance both in the fasting and postprandial state. However, its severity was similar regardless of the genetic makeup (Fig. 3), expanding on an early elegant study by Kotronen *et al.*[24] that suggested no differences but that had only a small number (n = 18) of individuals homozygous (GG) for the *PNPLA3* allele variant. These results are also consistent with a recent study where insulin sensitivity and adipose tissue lipolysis were measured in 41 obese individuals, divided into groups of equal sizes with either low (1-4 risk alleles) or high (5-8 risk alleles) genetic risk (number of risk alleles in *PNPLA3, TM6SF2, MBOAT7, HSD17B13* and *MARC1*).[19] While IHTG was higher in those with more risk alleles, groups were similar with respect to HOMA-IR and insulin sensitivity of adipose tissue (*i.e.*, rates of lipolysis), as determined by the suppression of plasma FFAs and whole-body glycerol turnover during euglycemic hyperinsulinemia. Unfortunately, a limitation of the study was that among the 284 patients divided into groups based on genetic risk score, only 16 individuals were homozygous (GG) for the *PNPLA3* allele variant. When a separate non-bariatric cohort of participants (n = 252) was also divided into groups based on the genetic risk score, again all had similar HOMA-IR.[19] Overall, in the entire cohort of 846 individuals, when divided by *PNPLA3* and the number of risk alleles, those with more alleles had higher IHTG, but also a trend towards more insulin resistance (by HOMA-IR), mitochondrial dysfunction and impaired DNL.[19] Another study found no differences in expression of proinflammatory or anti-inflammatory genes in adipose tissue between individuals who were carriers or not of the *PNPLA3* variant.[48] We further examined if we could have overlooked differences based on the severity of hepatic steatosis, but no significant differences were apparent across the spectrum of liver triglyceride accumulation. Moreover, adipose tissue insulin resistance (Fig. 4A) or whole-body (liver and muscle) insulin sensitivity (Fig. 4B) were abnormal whether liver fat content was 5.6-10%, 10.1-20% or >20%, independent of the presence or not of the *PNPLA3* allele. Individuals with a *PNPLA3* variant genotype appeared to have slightly worse adipose tissue insulin resistance in the very early stages of steatosis (*i.e.*, IHTG 5.6-10%; Fig. 4A), but this requires further examination as one cannot rule out a random finding from this small subgroup. However, early adipose tissue insulin resistance with its associated lipotoxicity, leading to broader metabolic dysfunction, would be an attractive hypothesis to account for the association of the *PNPLA3* variant allele with worse steatohepatitis and fibrosis in many prior studies.[7–11]

To fully assess the clinical implications of insulin resistance in people with the *PNPLA3* G allele, we examined liver histology across the spectrum of adipose tissue, liver and muscle insulin sensitivity (Fig. 5). Of interest, whether the *PNPLA3* G allele was present or not, the severity of steatohepatitis (*i.e.*, NAFLD activity score) did not differ in either group with worsening adipose tissue or liver/muscle insulin resistance. This appears to expand on prior studies that did not examine the impact of modest insulin resistance on disease activity or fibrosis. However, compared to those without the *PNPLA3* G allele, individuals with the *PNPLA3* variant had a trend towards worse steatohepatitis for any degree of metabolic dysfunction, and even with mild degrees of adipose tissue (Fig. 5A) or liver and muscle (Fig. 5C) insulin resistance. In patients without any G allele, severity of fibrosis stage appeared to increase among

patients in the highest quartile of insulin resistance (both in adipose tissue and whole-body). In patients with *PNPLA3* variants, fibrosis seemed to appear even at earlier degrees of insulin resistance and lipotoxicity (Fig. 5B,D), and increased with worsening Matsuda index. These results are in line with prior studies, which have also shown that indices of insulin resistance derived from the OGTT (*e.g.*, oral glucose insulin sensitivity index or OGIS) are closely related to liver fibrosis in patients with MASLD, but not in other chronic liver conditions.[49,50] However, these studies did not provide information regarding PNPLA3 status of the patients.

Worse steatohepatitis and fibrosis in carriers of the *PNPLA3* G allele has been extensively reported before.[7–11] However, prior reports have often overlooked or minimized that individuals with the *PNPLA3* G allele are not insulin sensitive but rather have severe metabolic dysfunction. Taken together, metabolic factors appear to be as important drivers of fibrosis in "PNPLA3 MASLD" as in "Metabolic MASLD", although the natural history is clearly worsened by the *PNPLA3* genotype. This view aligns well with some studies where non-obese individuals with less severe metabolic dysfunction are not as affected by being *PNPLA3* variant allele carriers compared to those with obesity.[15] The clinical implication of our findings is that individuals with "PNPLA3 MASLD" should not be seen as without metabolic disease, but rather as a subtype where one should more aggressively treat obesity, diabetes, and metabolic dysfunction to prevent accelerated disease. There appears to be a significant interaction between total energy intake and *PNPLA3* genotype in the development of high-risk MASH,[51] obesity[15] and T2D.[16] Moreover, in light of this increased liver susceptibility to insulin resistance and metabolic dysfunction, one may wonder if it is not time to systematically screen patients with MASLD for the *PNPLA3* G allele, or at least do so in all people with T2D and/or "at risk MASH" (*e.g.*, MASH with F≥2), who are at a much greater risk of developing cirrhosis. Early identification may be even more important if lifestyle modification has a greater benefit in *PNPLA3* variant allele carriers, although at present the available data from small, pilot studies is insufficient to support this notion.[52]

In summary, our study offers an in-depth, novel examination of the role of insulin resistance and its associated metabolic dysfunction in individuals with MASLD and "PNPLA3 MASLD". Our findings indicate that people who are *PNPLA3* allele variant carriers are equally severely insulin resistant as non-carriers at the level of the liver, skeletal muscle and adipose tissue. This calls for reframing "PNPLA3 MASLD", not as an insulin sensitive population, but as an insulin resistant one that has more liver susceptibility to metabolic insults, such as obesity and T2D. The clinical implication is that early identification/genotyping and more aggressive use of lifestyle modification and pharmacological treatments (*e.g.*, GLP-1 receptor agonists, pioglitazone, novel MASH therapeutic agents) should be mandatory in this population to reverse metabolic dysfunction and prevent their relentless progression to cirrhosis.

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Abbreviations

[1]H-MRS, proton magnetic resonance spectroscopy; Adipo-IR, adipose tissue insulin resistance; DNL, *de novo* lipogenesis; EGP, endogenous glucose production; FFA, free fatty acid; HOMA-IR, homeostatic model assessment for insulin resistance; IHTG, intrahepatic triglyceride; MASH, mnetabolic dysfunction-associated steatotic liver disease; MASLD, metabolic dysfunction-associated steatotic liver disease; OGTT, oral glucose tolerance test; OR, odds ratio; PNPLA3, patatin-like phospholipase domain-containing protein 3; SNP, single nucleotide polymorphisms; T2D, type 2 diabetes.

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Conflict of interest

No conflict of interests to disclose related to the current manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

FB: Patient recruitment and follow-up; data acquisition, analysis, and interpretation; statistical analysis; writing and editing of the manuscript. He is one of the guarantors of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; SK, RF: Laboratory measurements, assay performance, data interpretation, critical revision of the manuscript; RL, EGL: Patient recruitment, data acquisition, critical revision of the manuscript; KC: Study design and funding; patient recruitment and follow-up; data acquisition, analysis and interpretation; writing, critical revision and editing of the manuscript. KC is also the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data availability statement

Data available upon request to corresponding author; prior approval by IRB may be required.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2024.101092.

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Author names in bold designate shared co-first authorship

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