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Effects of *PNPLA3* I148M on hepatic lipid and very-low-density lipoprotein metabolism in humans

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Abstract. Borén J, Adiels M, Björnson E, Matikainen N, Söderlund S, Rämö J, et al. Effects of *PNPLA3* I148M on hepatic lipid and very-low-density lipoprotein metabolism in humans *J Intern Med.* 2022;**291:**218–223.

Background. The phospholipase domain-containing 3 gene (*PNPLA3*)-148M variant is associated with liver steatosis but its influence on the metabolism of triglyceride-rich lipoproteins remains unclear. Here, we investigated the kinetics of large, triglyceride-rich very-low-density lipoprotein (VLDL), ($VLDL_1$), and smaller $VLDL_2$ in homozygotes for the *PNPLA3*-148M variant.

Methods and results. The kinetics of apolipoprotein (apo) B100 (apoB100) and triglyceride in VLDL subfractions were analysed in nine subjects homozygous for *PNPLA3*-148M and nine subjects homozygous for *PNPLA3*-148I (controls). Liver fat was >3-fold higher in the 148M subjects. Production rates for apoB100 and triglyceride in $VLDL_1$ did not differ significantly between the two groups. Likewise, production rates for $VLDL_2$-apoB100 and -triglyceride, and fractional clearance rates for both apoB100 and triglyceride in $VLDL_1$ and $VLDL_2$, were not significantly different.

Conclusions. Despite the higher liver fat content in *PNPLA3* 148M homozygotes, there was no increase in VLDL production. Equally, VLDL production was maintained at normal levels despite the putative impairment in cytosolic lipid hydrolysis in these subjects.

Keywords: triglycerides, lipid metabolism, lipoproteins, fatty liver

Abbreviations: apo, apolipoprotein; ATGL, adipose triglyceride lipase; DNL, de novo lipogenesis; NAFLD, non-alcoholic fatty liver disease; PNPLA3, patatin-like phospholipase domain-containing 3; TM6SF2, transmembrane 6 superfamily member 2; VLDL, very-low-density lipoprotein.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has a multifactorial aetiology, but epidemiological and human genomic studies reveal that there is a strong genetic contribution to the risk of developing the condition [1]. The patatin-like phospholipase domain-containing 3 gene (*PNPLA3*) I148M (rs738409) polymorphism is the most common variant associated with NAFLD identified to date, with a prevalence of around 23% for the rarer allele (148M) in Caucasian populations. Consequently, it seems to be the most important genetic determinant of hepatic fat content [1]. However, whilst the inheritance of the *PNPLA3 148M* variant disposes to the development of NAFLD and non-alcoholic steatohepatitis (NASH), it is not associated with metabolic co-morbidities including abnormalities in plasma lipid levels [2, 3]. The mechanism by which *PNPLA3* I148M induces hepatic steatosis is

still debated, but results from genetically modified mice indicate that the variant reduces the lipolysis rate of stored hepatic triglycerides by preventing the interaction between adipose triglyceride lipase (ATGL), the major lipase in the liver and its cofactor [4, 5].

There is emerging consensus that NAFLD is positively associated with an increased risk of atherosclerotic cardiovascular disease [6], but the nature of the link between hepatic fat accumulation and atherogenesis is unclear. In a large cohort of overweight/obese subjects, we demonstrated that hepatic steatosis leads to overproduction of large triglyceride-rich very-low-density lipoproteins (VLDLs; $VLDL_1$) by the liver and consequent development of an atherogenic dyslipidaemia [7, 8]. However, we also recently reported that the transmembrane 6 superfamily member 2 gene variant transmembrane 6 superfamily member 2 (*TM6SF2)* E167K, another variant linked to NAFLD, is associated with lower plasma triglycerides due to impaired secretion of $VLDL_1$ [9]. Thus, it is important to evaluate separately the impact on plasma lipoprotein metabolism of individual genetic variants predisposing to NAFLD [10].

In the present report, we investigated the impact of *PNPLA3* I148M on VLDL production. We show that individuals who are homozygous for *PNPLA3* 148M have substantially increased liver fat but VLDL production rates that are comparable to those of body mass index (BMI)-matched controls with the wild-type *PNPLA3* 148II genotype. This finding offers an explanation as to why the *PNPLA3-I148M* variant is not accompanied by atherogenic changes in the plasma lipoprotein profile [11].

Materials and methods

Subjects

This study included 18 subjects: nine (all men) homozygous for *PNPLA3* 148M and nine (seven men and two postmenopausal women) homozygous for *PNPLA3* 148I. Of these, 17 were homozygous for the wild-type *TM6SF2* 167E genotype, and one was heterozygous for *TM6SF2* E167K. Thirteen of the 18 subjects were identified from the THL Biobank in Finland having participated in previous studies exploring genetic determinants of lipid metabolism. The remaining five subjects were from previous kinetic study cohorts [12]. Inclusion criteria were age 18−70 years, non-smoking status and BMI <35 kg/m^2. Exclusion criteria included any condition affecting lipid levels and premenopausal status in women. The protocol was approved by the Ethics Committee of Helsinki University Central Hospital, Helsinki, Finland. ClinicalTrials.gov Identifier: NCT04209816. All study participants signed a written informed consent form before any study procedures were initiated.

Study design

Kinetic investigations were performed as described previously [9, 13]. On the evening before the kinetic study, ^2H_2O(2 g/kg) was given to measure the contribution of hepatic de novo lipogenesis (DNL) to $VLDL_1$-triglyceride [14, 15]. Subjects returned at 7.30 AM to the research unit of the Helsinki University Hospital after a 12 h overnight fast. A bolus injection was then administered containing 500 mg of [$^2H_5$]-glycerol and 7 mg/kg body weight of [$^2H_3$]-leucine. Blood samples were drawn before tracer injection and at frequent intervals thereafter [9, 13].

Lipoprotein isolation, biochemical analyses and lipidomics

Lipoprotein isolation and biochemical analyses were performed as described [13]. Lipid extraction and lipidomics were performed as described [9].

Imaging

Liver fat content was measured using proton magnetic spectroscopy (1.5 T whole-body instrument) [7].

Modelling

The multi-compartmental model used to simultaneously analyse VLDL-apolipoprotein (apo) B100 (apoB100) and -triglyceride kinetics was constructed using SAAM–II (The Epsilon Group) as described [9].

Statistics

Statistical calculations were performed using R version 3.6.3. *P*-values were calculated using the Kruskal–Wallis test. *P*-values < 0.05 were considered statistically significant.

Results

Subject characteristics

The two groups of subjects (nine *PNPLA3* 148M homozygotes and nine *PNPLA3* 148I homozygotes

Table 1. *Characteristics of subjects in the phospholipase domain-containing 3 gene (PNPLA3) 148II (control) and 148MM groups*

	PNPLA3 (mean ± SD)		*PNPLA3* (median [min-max])			
	148II	148MM	148II	148MM	Fold	*p*-Value
Age (years)	53,9 ± 9	56,3 ± 8,3	50 [43-69]	56 [43-68]	1,04	0,54
BMI (kg/m^2)	27,0 ± 2,5	28,7 ± 3,3	27,5 [22,8-29,7]	29,2 [23,7-34,4]	1,06	0,30
Body weight (kg)	82,9 ± 10,3	96 ± 14,1	83,6 [58,3-92,3]	96,3 [78,6-119,5]	1,16	0,11
Waist (cm)	97,1 ± 7,5	105 ± 9,3	97 [82-110]	105 [94-121]	1,08	0,17
Liver fat (%)	2,8 ± 3,7	8,6 ± 6,2	1 [0,6-10,6]	6,8 [1-21]	3,07	0,01
Glucose (mmol/L)	5,5 ± 0,5	5,6 ± 0,4	5,5 [4,9-6,4]	5,5 [5-6,1]	1,02	0,53
Insulin (mU/L)	6,8 ± 4,8	10,4 ± 3,7	6,6 [1,3-17,5]	9,2 [6,4-16,9]	1,53	0,11
HOMA-IR	1,7 ± 1,2	2,6 ± 1	1,9 [0,3-4,4]	2,2 [1,4-4,5]	1,53	0,09
ALT (U/L)	25 ± 14	54 ± 50	19 [11-50]	40 [18-183]	2,16	0,06
ALP (U/L)	66 ± 11	66 ± 10	67,5 [44-80]	66 [45-83]	1,00	0,85
AST (U/L)	28 ± 4	45 ± 29	28 [22-34]	34 [24-118]	1,61	0,06
Total cholesterol (mmol/L)	5,2 ± 0,5	4,6 ± 1	5,4 [4,4-5,7]	4,8 [2,6-5,9]	0,88	0,14
LDL cholesterol (mmol/L)	3,4 ± 0,5	3,1 ± 0,9	3,5 [2,5-4]	3,2 [1,2-4,4]	0,91	0,44
HDL cholesterol (mmol/L)	1,3 ± 0,4	1,2 ± 0,3	1,2 [0,7-1,9]	1,1 [0,9-1,7]	0,92	0,45
Fasting TG (mmol/L)	1,4 ± 0,3	1,1 ± 0,5	1,2 [1-1,9]	0,8 [0,6-2,1]	0,79	0,16
$VLDL_1$-TG (mmol/L)	0,6 ± 0,3	0,5 ± 0,4	0,6 [0,2-0,9]	0,3 [0,1-1]	0,83	0,41
$VLDL_2$-TG (mmol/L)	0,2 ± 0,1	0,2 ± 0,1	0,2 [0,1-0,4]	0,1 [0,1-0,3]	1,00	0,30
Plasma apoB (mg/dl)	107 ± 20	91 ± 28	106 [81-142]	86 [37-134,4]	0,85	0,18
$VLDL_1$-apoB (mg/dl)	1,9 ± 1	1,5 ± 1,2	1,5 [0,6-3,7]	0,6 [0,4-3,1]	0,79	0,30
$VLDL_2$-apoB (mg/dl)	3,1 ± 1,9	2,1 ± 1	2,5 [1,1-7,1]	2,2 [0,7-3,6]	0,68	0,30
Plasma apoC-III (mg/dl)	12,2 ± 2,5	11,5 ± 5,6	12,3 [8,9-16,1]	9,2 [4,7-19,4]	0,94	0,66
DNL in $VLDL_1$ (%)	7,4 ± 5,2	2,6 ± 1,9	7,5 [0-16,9]	2,2 [0,3-6]	0,35	0,03
β-OH butyrate (mg/dl)	0,7 ± 0,3	1,0 ± 0,9	0,7 [0,2-1,2]	0,5 [0,1-2,9]	1,43	0,73

Note: Data are reported as mean ± SD; fold indicates the ratio of means in the *PNPLA3* 148MM group versus the *PNPLA3* 148II group; *p*-values are calculated by the Kruskal–Wallis test.
Abbreviations: HOMA-IR, homeostatic model assessment for insulin resistance; apo, apolipoprotein; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; β-OH butyrate, β-hydroxy butyrate; DNL, de novo lipogenesis; TG, triglycerides; VLDL, very-low-density lipoprotein.

as controls) were matched for overall BMI (Table 1). As expected, liver fat content was >3-fold higher in the *PNPLA3* 148M homozygotes than in the controls (P = 0.01; Table 1). There were no significant differences in lipid profile, total apoB and apoC-III concentrations and mean concentrations of apoB and triglyceride in $VLDL_1$ and $VLDL_2$ between the two groups (Table 1). Lipidomic analysis of $VLDL_1$ and $VLDL_2$ showed that the major lipid classes in these lipoproteins did not differ between the two groups (Table 2).

VLDL kinetics

The production rates of $VLDL_1$- and $VLDL_2$-apoB100 (which reflect the number of particles secreted from the liver as each particle contains only one apoB100) did not differ between *PNPLA3* 148M homozygotes and controls (Table 3). Similarly, the production rates of $VLDL_1$- and $VLDL_2$-triglyceride were the same in the two groups.

Fractional catabolic rates of $VLDL_1$ and $VLDL_2$-apoB100 (a measure of lipoprotein particle clearance rate) were not significantly different between the groups (Table 3). Likewise, the fractional catabolic rates of $VLDL_1$- and $VLDL_2$-triglyceride (a measure of intravascular lipolysis rate) were similar (Table 3). *PNPLA3* 148M homozygotes exhibited a lower contribution of hepatic DNL to $VLDL_1$-triglyceride whilst plasma β-hydroxybutyrate levels (a marker of hepatic β-oxidation) did not differ between the two groups (Table 3). However, if the 148M variant results in impaired recruitment of triglycerides from the lipid droplets into $VLDL_1$ as suggested [4], the DNL

Table 2. *$VLDL_1$) and $VLDL_2$ composition by lipid class (molecules per mole apoB)*

	PNPLA3			
	148II	148MM	Fold	*p*-Value
$VLDL_1$				
CE	15,483 ± 7261	14,019 ± 9157	0,91	0,72
LPC	640 ± 394	684 ± 584	1,07	0,93
PC	11,440 ± 5726	11,479 ± 6831	1,00	0,43
PE	635 ± 206	682 ± 457	1,07	0,54
SM	2109 ± 1075	2205 ± 1268	1,05	0,54
TG	41,934 ± 17026	47,510 ± 28,539	1,13	0,25
$VLDL_2$				
CE	12,923 ± 4654	9436 ± 7883	0,73	0,60
LPC	299 ± 177	236 ± 213	0,79	0,79
PC	4538 ± 1502	3566 ± 2974	0,79	0,79
PE	117 ± 52	91 ± 86	0,78	0,54
SM	1887 ± 896	1163 ± 900	0,62	0,43
TG	10,563 ± 3719	8038 ± 6644	0,76	0,54

Note: The number of lipid molecules per lipoprotein particle (1 apoB peptide is present on each particle of $VLDL_1$ or $VLDL_2$). Data are reported as mean ± SD; fold indicates the ratio of means in the *PNPLA3* 148MM group versus the *PNPLA3* 148II group; *p*-values are calculated using the Kruskal–Wallis test.
Abbreviations: CE, cholesteryl esters; LPC, lysophosphatidylcholines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; SM, sphingomyelins.

Table 3. *Production rates and fraction clearance rates of $VLDL_1$ and $VLDL_2$ apoB and triglycerides*

PNPLA3	148II	148MM	Fold	*p*-Value
ApoB				
Total VLDL production (mg/day)	1037 ± 230	976 ± 378	0,94	0,60
$VLDL_1$ production (mg/day)	787 ± 223	684 ± 329	0,87	0,73
$VLDL_1$ FCR (pools/day)	14,2 ± 8,5	16,7 ± 11,7	1,18	0,60
$VLDL_2$ production (mg/day)	250 ± 64	292 ± 111	1,17	0,19
$VLDL_2$ FCR (pools/day)	6,4 ± 2,2	8,9 ± 3,3	1,39	0,11
Triglycerides				
Total VLDL production (mg/day)	33,350 ± 7240	33,120 ± 10,940	0,99	0,74
$VLDL_1$ production (mg/day)	29,290 ± 7410	29,430 ± 10,370	1,00	0,96
$VLDL_1$ FCR (pools/day)	20,6 ± 15,8	27,7 ± 22,1	1,34	0,74
$VLDL_2$ production (mg/day)	4063 ± 1142	3687 ± 1682	0,91	0,54
$VLDL_2$ FCR (pools/day)	15,1 ± 5,2	16,7 ± 4,7	1,11	0,54

Note: Data are reported as mean ± SD; fold indicates the ratio of means in the *PNPLA3* 148MM group versus the *PNPLA3* 148II group; *p*-values are calculated by the Kruskal–Wallis test.
Abbreviation: FCR, fractional clearance rate.

measurements might be technically invalid as we measure the enrichment of the stable isotope in $VLDL_1$-triglycerides.

Discussion

This study investigated the influence of the *PNPLA3* I148M polymorphism on VLDL metabolism. We observed the expected (>3-fold) increase in liver fat in homozygotes for the 148M variant, compared to matched controls (148I homozygotes), but no difference in hepatic secretion rates of either large triglyceride-rich $VLDL_1$ or smaller $VLDL_2$ particles between the groups. Clearance rates for $VLDL_1$ and $VLDL_2$ particles from the circulation were, likewise, similar in the two groups.

We have previously shown that $VLDL_1$ secretion increases in parallel with liver fat content both in a large cohort of overweight/obese subjects [8] and in heterozygous *PNPLA3* 148M carriers [16]. Here, we showed that $VLDL_1$ assembly and secretion in *PNPLA3* 148M homozygotes was maintained at near-normal rates despite (i) an increase in liver fat that would normally lead to a rise in $VLDL_1$ production and (ii) the proposed inefficient release of triglyceride from cytoplasmic lipid stores conferred by this genetic variant [4] that could be expected to lead to decreased $VLDL_1$ secretion. The phenotype in *PNPLA3* 148M homozygotes also contrasted with the increase in liver fat but reduction in $VLDL_1$ secretion observed previously in *TM6SF2* E167K homozygotes and potentially explained by impairment of the bulk lipidation process that converts $VLDL_2$-sized precursor particles to $VLDL_1$ [9]. Additionally, we did observe decreased DNL in the 148M homozygotes, which may have been a component of the metabolic phenotype, but we cannot rule out technical reasons confounding this interpretation.

Triglycerides required for $VLDL_1$ assembly have a number of potential sources: mobilisation from cytoplasmic droplets, synthesis by DNL or uptake of fatty acids or chylomicron remnants from the bloodstream [17, 18]. Our current results, combined with earlier findings [16], suggest that VLDL assembly *per se* is not impaired in *PNPLA3* 148M homozygotes but that the mobilisation of triglycerides from lipid droplets for VLDL assembly is reduced. Thus, for a given liver fat content, less VLDL can be produced in *PNPLA3* 148M versus 148I homozygotes. The near-normal rates of $VLDL_1$ assembly and secretion observed in *PNPLA3* 148M homozygotes could indicate that: (i) *PNPLA3* 148M does not completely inhibit ATGL-mediated lipolysis, and its effects can be overcome by the expansion of the substrate pool, (ii) other lipolytic enzymes such as hormone-sensitive lipase can compensate, albeit at reduced efficiency [19] or (iii) other sources of fatty acids for triglyceride synthesis (e.g., body fat stores) are involved.

A strength of this study was that we were able to recruit *PNPLA3* 148M homozygotes and exclude the effect of the *TM6SF2* 167E allele. The main limitations are the number of subjects and the fact that the majority of participants were men. Since we excluded premenopausal women, the findings cannot be generalized to women. Our results of investigations by others show that genetic variants predisposing to NAFLD are not consistently associated with hepatic $VLDL_1$ overproduction and increased risk of atherosclerotic cardiovascular disease. It is therefore inappropriate to use genetic approaches [11] to test the hypothesis that high liver fat is a causal risk factor for ischemic heart disease if the population under study includes *PNPLA3* I148M or *TM6SF2* E167K carriers.

Conflict of interest

The authors declare that there is no conflict of interest.

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