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Healthy *PNPLA3* Risk Allele Carriers Present with Unexpected Body Fat Composition. A Study of One Thousand Subjects

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ABSTRACT

Introduction: The common *PNPLA3* (adiponutrin) variant p.I148M represents a major genetic driver of progression in non-alcoholic fatty liver disease (NAFLD). NAFLD is commonly associated with traits of the metabolic syndrome, therefore it is mostly suspected in obese individuals. Here, we investigate the association between the *PNPLA3* variant and anthropometric traits in a cohort of healthy individuals.
Patients and methods: We recruited 1,000 (500 females; age 18 - 66 years) healthy blood donors. The *PNPLA3* variant was genotyped using TaqMan assays. All individuals were phenotyped with respect to anthropometric characteristics. We also determined the percentage of total fat (F%) and active tissue (TA%) of body weight.
Results: Healthy carriers of the *PNPLA3* [IM] and [MM] genotypes, although not differing in height from individuals with the genotype [II], displayed significantly lower body weight and lower BMI (both $P = 0.005$), higher TA% ($P = 0.03$) but lower F% ($P = 0.03$) and smaller waist, chest and shin circumferences (all $P < 0.05$). Separate analysis for males and females demonstrated an association between the [IM] and [MM] genotypes and higher TA% but lower F% ($P = 0.04$) in females. In males, BMI and total weight were significantly ($P = 0.04$) lower among carriers of the [M] allele.
Discussion: Healthy individuals carrying the prosteatotic *PNPLA3* allele p.I48M may be leaner as compared to the carriers of the common allele. Hence in clinical practice they might be overlooked since they do not necessarily present with the anthropometric characteristics commonly associated with severe hepatic steatosis.

Key words: autotaxin – body mass index – obesity – single nucleotide polymorphism.

Abbreviations: ATX - autotaxin; BMI - body mass index; F% - total fat of body weight in %; Fkg - total fat of body weight in kilograms; GWAS - genome-wide association study; LPA - lysophosphatidic acid; NAFLD, non-alcoholic fatty liver disease; NASH - non-alcoholic steatohepatitis; PA - phosphatidic acid; PNPLA3- patatin-like phospholipase domain containing 3 (adiponutrin); TA% - active tissue of body weight in %; TAkg - active tissue of body weight in kilograms; WHR - waist-to-hip ratio.

INTRODUCTION

The common *PNPLA3* (adiponutrin) variant p.I148M has been identified in recent years as the major genetic determinant of non-alcoholic fatty liver disease (NAFLD) [1]. Indeed, a genome-wide association study (GWAS) published in 2008 [2] demonstrated that the amino acid substitution [I] > [M] at position 148 of the *PNPLA3* protein is associated with higher liver fat contents [2]. This association, replicated subsequently in pediatric [3, 4] and additional adult NAFLD cohorts [1], was also extended to patients with alcoholic [5, 6] and viral liver diseases [7, 8]. Carriers of the *PNPLA3* p.I48M allele are characterized by increased hepatic fat content and are prone to progressive liver fibrosis [7, 9], cirrhosis [6, 7, 9], and hepatocellular carcinoma [10], which all render this variant the first common genetic risk factor for severe forms of chronic liver diseases [11]. Although several functional studies have been performed, the functional mechanisms underlying this association have not been fully elucidated. Increased intracellular synthesis of triglycerides in the liver might be one of the mechanisms triggering the *PNPLA3*-related phenotypes. As demonstrated by Kumari et al [12], *PNPLA3* metabolizes lysophosphatidic acid (LPA) into

phosphatidic acid (PA), which can be used in the synthesis of triglycerides in the liver. The p.148M *PNPLA3* variant, in turn, might be a 'gain-of-function' mutation [12], resulting in enhanced synthesis of hepatic lipids. Others reported that the *PNPLA3* variant leads to decreased hepatic secretion of very low-density lipoprotein (VLDL) and decreased lipolysis which might contribute to increased hepatic lipid accumulation [13, 14]. Interestingly, a significant association between lipid and glucose metabolism and the *PNPLA3* p.I148M variant was demonstrated in selected cohorts only [15-17].

Increased hepatic fat accumulation in patients who do not consume excessive quantities of alcohol is often detected during routine abdominal ultrasound in asymptomatic individuals without any apparent liver disease [18]. In general, fatty liver is regarded to be a benign condition. However, in patients with non-alcoholic steatohepatitis (NASH) it may progress to liver cirrhosis and deterioration of liver function. Indeed, NASH is claimed to be one of the leading causes for cryptogenic cirrhosis [19]. Fatty liver disease is commonly associated with traits that fall into the spectrum of the so-called metabolic syndrome, in particular central obesity and diabetes [20]. Hence, fatty liver is mostly suspected in obese individuals, whereas its presence in lean patients might easily be overlooked.

Previous reports focused on investigating the *PNPLA3* polymorphism in individuals with liver diseases. In the current study we investigate the effects of the *PNPLA3* variant on several traits of body composition, in particular the amount of fat tissue and body mass index (BMI), in a large cohort (n = 1,000) of healthy individuals.

PATIENTS AND METHODS

Patients

A cohort of 1,000 Caucasian (median age 24 years; mean 27 years; range 18 - 66 years) blood donors from the Regional Blood Donor Center in Szczecin (Poland) was investigated. In total, 500 females (median age 22.5 years; mean 27.1±10.2 years; range 18 - 66 years) and 500 males (median age 26 years; mean 27.8±8.3 years; range 18 - 62 years) were included. All subjects had a medical checkup, and a good state of health was a prerequisite to qualify for blood donation. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) and was approved by the Ethics Committee of Pomeranian Medical University.

Anthropological examination

Anthropometric measurements, taken with a medical scale, an anthropometer and an anthropological centimeter (body mass, height, hip, chest, shin, and forearm measurements), served as a basis for calculating BMI, the total fat of body weight in percentage (F%) and kilograms (Fkg), and the active tissue of body weight in percentage (TA%) and kilograms (TAkg). The following formula was applied: BMI = body mass (kg) / (height)^2 (m). The BMI values were stratified according to the WHO (1995) classification. Adipose tissue distribution was assessed using the waist-to-hip ratio (WHR). In accordance with the anatomical classification of obesity, two types of obesity were defined: android and gynoid.

Active tissue (TA) was determined using Piechaczek's formula [21], i.e. for men: TAkg = -103,85484 + 0,446921x_1 + 0,13343x_2 + 0,458056x_3 + 0,838393x_4, and for women: TAkg = -61,719697 + 0,339491x_1 + 0,540846x_4 + 0,26024x_3 + 0,407343x_5, where x_1 – body height (cm), x_2 – hip size (cm), x_3 – chest size (cm), x_4 – shin size, x_5 – forearm size.

Adipose tissue in kilograms (Fkg) was calculated using the formula: body mass (kg) - TAkg. Active tissue of body weight in percentage (TA%) was calculated as follows: TA (kg) / body mass (kg) x 100%. Total fat of body weight in percentage (F%) was calculated as 100% - TA%.

Genotyping

In all individuals we genotyped the *PNPLA3* variant p.I148M (rs738409) as described previously [9]. In brief, genomic DNA was isolated from EDTA anticoagulated blood using the DNeasy Blood & Tissue Kit (Qiagen). PCR reactions contained 20 ng DNA, 900 nM of each primer, 1 × TaqMan Universal Master Mix, and 200 nM of VIC-labelled and FAM-labelled probes in 25 μL-reactions. Amplification conditions were as follows: 95°C for 10 min, 40 cycles of 92°C for 15 s, and 60°C for 1 min. Primer and probe sequences are listed in our previous publication [9]. The fluorescence data were analyzed with allelic discrimination 7500 Software v.2.0.2.

Statistical analysis

All statistical analyses were performed using StatView software (Carry, NC, US). Hardy-Weinberg equilibrium (HWE) was checked by exact tests (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). The genotype and allele frequencies, as well as anthropometric variables were compared between female and male individuals using Fisher's PLSD test. Phenotypic quantitative data were expressed as means ± standard deviation. P value < 0.05 was considered to be statistically significant

RESULTS

The distribution of genotype and allele frequencies of the *PNPLA3* variant p.I148M is presented in Table I. We achieved a 100% genotyping success. In the whole cohort, a total of 61.0% subjects had the genotype [II], while others were heterozygous [IM] (33.3%) or homozygous [MM] (5.7%). The analysis of allelic and genotype frequencies of the *PNPLA3* polymorphism demonstrated that [I] represented the major allele. The genotype and allele frequencies in the studied cohort were in line with the genotype and allele frequencies deposited with the *Entrez* database and with frequencies presented in previous reports. As presented in Table I, the minor allele [M] was significantly (P < 0.05) more prevalent among females as compared to males. We did not identify any departure from the HWE either in the whole cohort or in the separate analyses performed in males and females (all P > 0.05) proving a robust genotyping.

Overall, 37.6% of studied individuals were overweight, i.e. presented with BMI ≥ 25 kg/m^2. As demonstrated in Fig. 1, we detected an increased prevalence of overweight individuals (i.e. BMI 25 ≥ kg/m^2) among carriers of the genotype [II] as compared to individuals with the genotypes [IM] or [MM] (females: 60.3% vs. 39.7%; males: 65.3% vs. 34.7%). Overall,

Table I. Distribution of the *PNPLA3* alleles and genotypes

Allele Genotype	Whole cohort (n = 1000)	Females (n = 500)	Males (n = 500)
[I]	1553 (77.7)	753 (75.3)*	800 (80.0)
[M]	447 (22.3)	247 (24.7)*	200 (20.0)
[II]	610 (61.0)	289 (57.8)*	321 (64.2)
[IM]	333 (33.3)	175 (35.0)	158 (31.6)
[MM]	57 (5.7)	36 (7.2)	21 (4.2)

Abbreviations: I, isoleucine; M, methionine (prosteatotic allele); *PNPLA3* - adiponutrin. * P < 0.05 between females and males.

obesity was more prevalent among males as compared to females (49.6 % and 25.6%, respectively). The anthropometric analyses showed that 44.4% of females and only 10.4% of males were at a higher risk of android obesity, while the others were at a risk of or suffered from gynoid obesity. As demonstrated in Table II, individuals with genotypes [IM] and [MM], although they did not differ in height from the carriers of the [II] genotype, had lower weight and BMI, higher TA%, lower F% and smaller chest, waist, hip shin and forearm circumferences. These differences were significant for weight and BMI (both P = 0.005), chest circumference (P = 0.01), waist circumference (P = 0.03), shin circumference (P = 0.002), TA% (P = 0.03), and F% (P = 0.03). Thus, carriers of the *PNPLA3* allele [M], which is associated with liver steatosis, were overall leaner and demonstrated lower whole body fat content as compared to carriers of the major allele. The strength of this correlation differed, when women and men were examined separately. Results of this analysis are presented in Table III. Fisher's correlation coefficient values demonstrated significant differences between carriers of the genotype [II] and individuals with genotypes [IM] + [MM] for TA% and F% (both P = 0.04) in females. In males, only BMI and total weight were significantly lower among carriers of the [M] allele. Moreover, the shin circumference differed significantly (P = 0.02).

Table II. Association of the *PNPLA3* polymorphism with anthropometric variables

	PNPLA3		
	[II]	[IM] + [MM]	P
Height (m)	1.7±0.9	1.7±0.1	0.2
Weight (kg)	75.4±16.1	72.5±14.9	0.005
BMI (kg/m^2)	24.9±4.2	24.2±3.8	0.005
WHR	0.84±0.4	0.83±0.1	0.3
Active tissue (kg)	50.3±7.9	49.0±7.3	0.001
Fat (kg)	25.1±9.3	23.5±8.7	0.01
Active tissue (%)	67.6±5.5	68.4±6.3	0.03
Fat (%)	32.4±5.5	31.6±6.3	0.03
Chest circumference (cm)	86.5±11.8	84.6±10.9	0.01
Waist circumference (cm)	85.4±13.4	83.6±12.2	0.03
Hip circumference (cm)	117.8±15.6	101.5±8.12	0.4
Shin circumference (cm)	37.1±3.7	36.4±3.6	0.002
Forearm circumference (cm)	25.9±3.6	25.5±4.7	0.1
Age (years)	28±10	27±9	0.3
Female / male (n)	289/321	211/179	0.04

Data are shown as means ± standard deviation. [M] represents the risk allele for hepatic steatosis. Abbreviations: BMI, body mass index; I, isoleucine; M, methionine; PNPLA3, adiponutrin; WHR, waist-hip ratio.

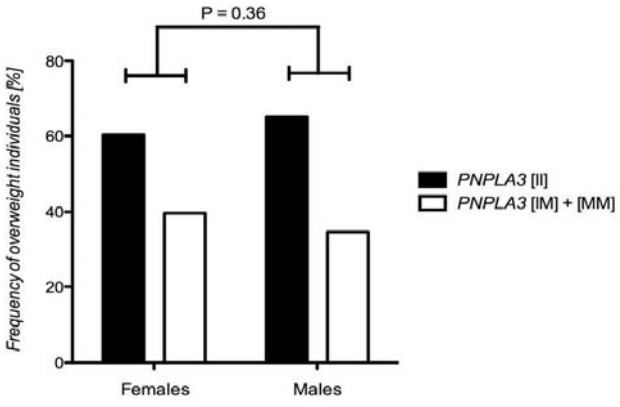
Table III. Association of the *PNPLA3* polymorphism with anthropometric variables among females (A) and males (B)

(A)

	PNPLA3		
	[II]	[IM] + [MM]	P
Height (m)	1.7±0.1	1.7±0.1	0.4
Weight (kg)	65.7±11.2	64.4±11.1	0.2
BMI (kg/m^2)	23.8±3.8	23.2±3.9	0.1
WHR	0.8±0.6	0.8±0.1	0.4
Active tissue (kg)	44.6±5.0	44.3±5.2	0.6
Fat (kg)	21.1±7.1	20.0±7.6	0.1
Active tissue (%)	68.6±5.6	69.7±6.5	0.04
Fat (%)	31.4±5.5	30.3±6.5	0.04

(B)

	PNPLA3		
	[II]	[IM] + [MM]	P
Height (m)	1.8±0.1	1.8±0.1	0.3
Weight (kg)	79.4±16.3	76.8±14.8	0.04
BMI (kg/m^2)	25.3±4.3	24.9±3.6	0.04
WHR	0.87±0.1	0.86±0.1	0.3
Active tissue (kg)	52.7±7.7	51.4±7.2	0.04
Fat (kg)	26.7±9.6	25.3±9.0	0.07
Active tissue (%)	66.7±5.3	66.8±5.7	0.8
Fat (%)	33.3±5.3	33.2±5.7	0.8



Fi. 1. Frequencies of overweight (BMI ≥ 25 kg/m^2, n = 377) individuals among carriers of different *PNPLA3* genotypes: higher prevalence of overweight individuals among carriers of the genotype [II] as compared to individuals with the prosteatotic genotypes [IM] and [MM] of the adiponutrin polymorphism.

DISCUSSION

Since the *PNPLA3* p.148M variant represents a major risk factor for liver steatosis, one would expect that carriers of the risk variant present with increased whole body fat content as well. Here, we demonstrate that healthy individuals carrying

the prosteatotic *PNPLA3* allele p.148M might be leaner and have lower amounts of whole body fat as compared to the carriers of the common allele. Therefore, they may lack the anthropometric characteristics that are commonly associated with hepatic steatosis.

Several reports have previously investigated the effects of the *PNPLA3* variant on metabolic phenotypes. For example, Palmer et al [17] demonstrated that severely obese individuals carrying the [M] allele are prone to diabetes mellitus and insulin resistance but present with lower serum triglyceride levels. In our previous study, we also detected an association between the *PNPLA3* polymorphism and serum fasting glucose concentrations [16]. However, other studies in extensive cohorts did not underline any link between the *PNPLA3* variant and serum glucose levels, BMI or insulin resistance in the general population or in patients with NAFLD [2, 22-24]. On the other hand, a large meta-analysis demonstrated the link between the *PNPLA3* p.I148M mutation and serum cholesterol but not lipid levels [15]. Although we do not posses data on metabolic profiles of individuals included in the current study, our results emphasize the notion that healthy individuals carrying the prosteatotic *PNPLA3* variant might be overall leaner as compared to carriers of the common variant. To date, the function of adiponutrin and the effects of the p.I148M mutation remain controversial [12-14]. One explanation of the association between the *PNPLA3* mutation and whole body fat composition might be based on studies indicating that LPA, a potential substrate of *PNPLA3* [12], could be involved in the regulation of metabolic pathways. Indeed, previous studies in mouse models and cell lines show that LPA signaling modulates adipogenesis and the expansion of adipose tissue [25]. However, further studies in carriers of specific *PNPLA3* genotypes are required to delineate the pathobiological mechanisms.

In our current study we investigated a cohort of blood donors, who *per se* are in good health and present without any viral or non-viral liver diseases or elevated serum liver enzymes. Since obese carriers of the adiponutrin polymorphism would most likely develop hepatic phenotypes, including elevated serum liver enzyme activities, that excluded them from the pool of blood donors, our cohort might be biased towards lean individuals in comparison to the general population. This could also explain the increased number of female carriers of the risk allele, since women, due to overall healthier life style [26], are more likely to be accepted as blood donors as compared to males. In contrast, our findings indicate that the combination of the *PNPLA3* risk variant and protective environmental factors can prevent or delay NAFLD manifestation. Given the current epidemics of obesity, the prevalence of fatty liver is predicted to rise dramatically. Since liver disease often progresses insidiously in patients with NASH an early detection of individuals who are at-risk of severe lipid accumulation in the liver is crucial to prevent the progression of liver disease. As pointed out previously [11], patients with the adiponutrin variant are predisposed to rapid progression of chronic liver diseases, but may present without any additional traits that indicate the presence of this critical genetic risk factor.

CONCLUSION

Our study indicates that a subgroup of the carriers of the *PNPLA3* mutation might actually be leaner than individuals carrying the common allele. Hence, they might easily be overlooked in clinical practice, since they do not necessarily present with the anthropometric characteristics that are commonly associated with severe hepatic steatosis.

Conflicts of interest: We declare that we have no conflict of interest.

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REFERENCES

1. Sookoian S, Pirola CJ. Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (*PNPLA3*) on the susceptibility and histological severity of nonalcoholic fatty liver disease. Hepatology 2011;53:1883-1894.
2. Romeo S, Kozlitina J, Xing C, et al. Genetic variation in *PNPLA3* confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008;40:1461-1465.
3. Santoro N, Kursawe R, D'Adamo E, et al. A common variant in the patatin-like phospholipase 3 gene (*PNPLA3*) is associated with fatty liver disease in obese children and adolescents. Hepatology 2010;52:1281-1290.
4. Valenti L, Alisi A, Galmozzi E, et al. I148M patatin-like phospholipase domain-containing 3 gene variant and severity of pediatric nonalcoholic fatty liver disease. Hepatology 2010;52:1274-1280.
5. Stickel F, Buch S, Lau K, et al. Genetic variation in the *PNPLA3* gene is associated with alcoholic liver injury in caucasians. Hepatology 2011;53:86-95.
6. Tian C, Stokowski RP, Kershenobich D, Ballinger DG, Hinds DA. Variant in *PNPLA3* is associated with alcoholic liver disease. Nat Genet 2010;42:21-23.
7. Valenti L, Rumi M, Galmozzi E, et al. Patatin-like phospholipase domain-containing 3 I148M polymorphism, steatosis, and liver damage in chronic hepatitis C. Hepatology 2011;53:791-799.
8. Viganò M, Valenti L, Lampertico P, et al. Patatin-like phospholipase domain-containing 3 I148M affects liver steatosis in patients with chronic hepatitis B. Hepatology. 2013;58:1245-1252.
9. Krawczyk M, Grünhage F, Zimmer V, Lammert F. Variant adiponutrin (*PNPLA3*) represents a common fibrosis risk gene: non-invasive elastography-based study in chronic liver disease. J Hepatol 2011;55:299-306.
10. Trépo E, Nahon P, Bontempi G, et al. Association between the *PNPLA3* (rs738409 C>G) variant and hepatocellular carcinoma: evidence from a meta-analysis of individual participant data. Hepatology 2013 Oct 1.
11. Krawczyk M, Portincasa P, Lammert F. *PNPLA3*-associated fatty steatohepatitis: toward a gene-based classification of fatty liver disease. Semin Liver Dis 2013;33: 369-379.
12. Kumari M, Schoiswohl G, Chitraju C, et al. Adiponutrin functions as a nutritionally regulated lysophosphatidic acid acyltransferase. Cell Metab 2012;15: 691-702.
13. Pirazzi C, Adiels M, Burza MA, et al. Patatin-like phospholipase domain-containing 3 (*PNPLA3*) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro. J Hepatol 2012;57: 1276-1282.

14. Li JZ, Huang Y, Karaman R, et al. Chronic overexpression of *PNPLA3*I148M in mouse liver causes hepatic steatosis. J Clin Invest 2012;122:4130-4144.
15. Kollerits B, Coassin S, Beckmann ND, et al. Genetic evidence for a role of adiponutrin in the metabolism of apolipoprotein B-containing lipoproteins. Hum Mol Genet 2009;18:4669-4676.
16. Krawczyk M, Grünhage F, Mahler M, Tirziu S, Acalovschi M, Lammert F. The common adiponutrin variant p.I148M does not confer gallstone risk but affects fasting glucose and triglyceride levels. J Physiol Pharmacol 2011;62: 369-375.
17. Palmer CN, Maglio C, Pirazzi C, et al. Paradoxical lower serum triglyceride levels and higher type 2 diabetes mellitus susceptibility in obese individuals with the *PNPLA3* 148M variant. PloS One 2012;7: e39362.
18. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology 2012; 142: 1592-1609.
19. Caldwell SH, Oelsner DH, Iezzoni JC, Hespenheide EE, Battle EH, Driscoll CJ. Cryptogenic cirrhosis: clinical characterization and risk factors for underlying disease. Hepatology 1999;29: 664-669.
20. de Alwis NM, Day CP. Non-alcoholic fatty liver disease: the mist gradually clears. J Hepatol 2008;48 Suppl 1: S104-S112.
21. Piechaczek H. Marking total body mass by the densitometric and anthropometric methods. Materiały i Prace Antropologiczne 1975;89: 3-48.
22. Kantartzis K, Peter A, Machicao F, et al. Dissociation between fatty liver and insulin resistance in humans carrying a variant of the patatin-like phospholipase 3 gene. Diabetes 2009;58: 2616-2623.
23. Speliotes EK, Butler JL, Palmer CD, et al. *PNPLA3* variants specifically confer increased risk for histologic nonalcoholic fatty liver disease but not metabolic disease. Hepatology 2010;52: 904-912.
24. Speliotes EK, Yerges-Armstrong LM, Wu J, et al. Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. PLoS Genet 2011;7: e1001324.
25. Rancoule C, Dusaulcy R, Tréguer K, et al. Involvement of autotaxin/lysophosphatidic acid signaling in obesity and impaired glucose homeostasis. Biochimie 2014;96:140-143.
26. Gulland A. Europe's life expectancy rises by five years, but health inequalities remain. BMJ 2013;346: f1687.