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Conflict of interest:

None declared

Abstract

Introduction:

The prevalence of non-alcoholic fatty liver disease (NAFLD) and the closely associated metabolic syndrome is high and is related to risk factors such as obesity and type 2 diabetes. A genetic basis for NAFLD has been suggested, but only few causal genes have been identified. The most significant association reported to date is the robust association of the *PNPLA3* I148M variant with susceptibility to NAFLD. We therefore hypothesized that the *PNPLA2* gene might also be involved in NAFLD pathogenesis, because of its close sequence similarity with *PNPLA3* and its possible involvement in ectopic fat accumulation.

Methods: In this study, we investigated the association of *PNPLA2* polymorphisms with the development of non-alcoholic fatty liver disease in a prospectively recruited Belgian obese population comprising 633 individuals with varying degrees of fatty liver disease. We selected 3 *PNPLA2* SNPs for genotyping, including 2 tagSNPs that cover most information on common genetic variation in the selected region.

Results: After performing linear regression analysis, we found that 2 of the analyzed *PNPLA2* SNPs were associated with anthropometric and metabolic parameters. In our subcohort of patients that underwent liver biopsy (n=372/633 or 58.7%), we assessed the influence of the *PNPLA2* variants on the severity of histologically determined liver damage, but we did not find convincing evidence for association.

Conclusion: Although we found evidence for moderate association between *PNPLA2* tagSNPs and anthropometric and metabolic parameters in our cohort, no evidence for association between polymorphisms in the *PNPLA2* gene and the presence and severity of NAFLD was identified.

Keywords: *PNPLA2*; NAFLD; genetics; association study

Introduction

The global increase in the prevalence of obesity has led to a rise in the associated incidences of the metabolic syndrome and non-alcoholic fatty liver disease (NAFLD). The metabolic syndrome is an important risk factor for type II diabetes and cardiovascular disease [1, 2] and recent findings suggest an important role for hepatic steatosis and abdominal adipose tissue in the development of the syndrome. Furthermore, the metabolic syndrome appears to be strongly associated with the development of NAFLD, which is considered to be the hepatic manifestation or hepatic component of the metabolic syndrome [3, 4]. NAFLD has become the most common chronic liver disease in Western countries, with an estimated prevalence of 20-30% in adults in developed countries. The prevalence of hepatic steatosis increases with BMI, reaching a prevalence of 65-85% in obese and morbidly obese individuals [5, 6]. NAFLD comprises a disease spectrum of hepatic disorders ranging from simple steatosis (triglyceride accumulation) to steatohepatitis, liver cirrhosis, and hepatocellular carcinoma [7]. Several studies show that NAFLD relates to obesity and its metabolic consequences such as insulin resistance and dyslipidemia [8], but the underlying mechanisms explaining this relationship are still poorly understood.

The heritability of NAFLD is estimated between 20 and 39 percent [9, 10], indicating that genetic factors play an important role in the etiology of the disease. In order to identify DNA sequence variations that contribute to inter-individual differences in NAFLD, Romeo et al. carried out a genome wide association study. In this study, a nonsynonymous variant (rs738409 – I148M) in the *PNPLA3* (patatin-like phospholipase domain-containing 3 or adiponutrin) gene was found to be associated with increased hepatic fat levels and hepatic inflammation and thus susceptibility to NAFLD [11]. This original association was found using a liver phenotype based on magnetic resonance spectroscopy, but has been robustly replicated in several independent studies, including our own study and was also confirmed in subjects with histologically characterized NAFLD [12-15].

PNPLA3 is a member of the patatin-like phospholipase domain containing protein (PNPLA) family, most closely resembling PNPLA2 (also known as adipose triglyceride lipase (ATGL) or desnutrin) [16, 17], which is a major triglyceride lipase in adipose tissue [18]. Because of its close sequence similarity with PNPLA3 and its possible involvement in ectopic fat accumulation [19], the lipase PNPLA2 has caught our attention as it is likely to be involved in NAFLD pathogenesis. Results from animal experiments have shown that liver-specific deletion of *PNPLA2* renders mice to be more prone to hepatic steatosis [20]. Furthermore, polymorphisms in the *PNPLA2* gene have been found to be associated with plasma free fatty acids, triglycerides and type 2 diabetes [21], indicating involvement of PNPLA2 in pathways related to the development of metabolic syndrome.

To the best of our knowledge, no prior association study exploring the role of *PNPLA2* in the pathogenesis of NAFLD has already been performed. In this study, we therefore aim to evaluate the contribution of *PNPLA2* gene polymorphisms to the development of NAFLD using an extensive and well-phenotyped cohort of individuals with varying degrees of fatty liver disease.

Materials and methods

Study population

A total of 633 obese individuals (182 men and 451 women), presenting with a problem of overweight or obesity were consecutively recruited from the outpatient obesity clinic at the Antwerp University Hospital. All patients underwent a standard metabolic work-up combined with a liver-specific program. Patients known to have diabetes were excluded from the study. Patients were also excluded from further analysis in case of significant alcohol consumption (>20 g/day) [22] or if another liver disease was diagnosed. All study subjects are of Belgian Caucasian origin. Population characteristics are summarized in table 1. The study was approved by the local ethics committee and all participants gave their written informed consent once the aim and design of the study had been explained.

Metabolic work-up

A detailed questionnaire was completed by all patients and a clinical examination including anthropometry measures was performed. All measurements were performed under fasting conditions. Height was measured to the nearest 0.5 cm and body weight was measured with a digital scale to the nearest 0.2 kg. BMI was calculated as weight in kilograms over height in meters squared. Waist circumference was measured at the mid-level between the lower rib margin and the iliac crest. Hip circumference was measured at the level of the trochanter major. Waist to hip ratio (WHR) was calculated by dividing waist circumference by hip circumference. A blood analysis included lipid profile (total and high density lipoprotein cholesterol (HDL-C), and triglycerides) and liver tests [AST, ALT, lactate dehydrogenase (LDH), gamma glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), total bilirubin and fractions]. A 3-h oral glucose tolerance test (75g of glucose) including insulin and c-peptide analysis was also performed. The cross sectional area of total abdominal adipose tissue (TAT), visceral abdominal adipose tissue (VAT) and subcutaneous abdominal adipose tissue (SAT) was measured by computed tomography (CT) at level L4-L5 according to previously described methods [23].

Hepatological work-up

The liver specific program included additional blood analysis, to exclude the classical etiologies of liver disease (e.g. viral hepatitis and autoimmune disease), a Doppler ultrasound of the abdomen with parameters of liver and spleen volume and liver vascularization, a liver–spleen scintigraphy [24] and an aminopyrine breath test as a measure for liver metabolic reserve [25].

Suspicion of non-alcoholic fatty liver disease was defined by one or more of the following elements: abnormal liver tests (AST and/or ALT and/or GGT and/or ALP), ultrasound abnormality of the liver (enlarged or steatotic liver) [26, 27], signs of parenchymal liver disease on liver–spleen scintigraphy [24], abnormal aminopyrine breath test [25].

Liver biopsy

When one or more of the above criteria were met, a liver biopsy was proposed [28]. In patients undergoing bariatric surgery the liver biopsy was performed peri-operatively. The remaining patients were proposed for transjugular or percutaneous liver biopsy.

The liver biopsy specimen was stored in formalin aldehyde. Haematoxylin–eosin stain, Sirius red stain, periodic acid Schiff stain after diastase, reticulin stain and Perl's iron stain were routinely performed on all biopsies and subsequently analyzed by an experienced pathologist blinded for any clinical data. The diagnosis of NASH was made according to Chalasani et al. requiring the association of some degree of steatosis, some degree of ballooning and some degree of lobular inflammation [29]. The different features were scored according to the NASH Clinical Research Network Scoring System [30]. The NASH Activity Score was calculated as the sum of the scores for steatosis, ballooning and lobular inflammation [30].

Genotyping

Blood samples from all patients were obtained for extraction of genomic DNA by standard procedures [31].

For our association study we used the presence of linkage disequilibrium (LD) between SNPs to reduce the number of SNPs needed to cover all variation in the gene. We selected a region of 14.74kb surrounding *PNPLA2* including 6kb upstream (up to the *RPLP2* gene) and 2.4kb downstream (up to the *EFCAB4A* gene) of the gene (HapMap Data Rel 28 phaseII+III, August10, on NCBI B36 assembly, dbSNP b126; Chr11:802,876..817,618) and SNP genotyping data from the CEPH population was downloaded into Haploview [32] in order to select the appropriate tagSNPs. Only SNPs with a minor allele frequency ≥ 0.05 were included in the Tagger analysis, using aggressive tagging of 2- and 3-marker haplotypes and with r^2 and LOD thresholds at 0.8 and 3.0, respectively [33, 34]. Furthermore, we included one extra coding polymorphism (rs1138693, L481P) that was not included in HapMap at the time. Genotypes of SNPs were determined using TaqMan SNP Genotyping assays (ABI, Foster City, USA). Assays were performed according to instructions by the kit manufacturers

and analysis of TaqMan assays was done on a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). Blank samples and samples with known genotype were included as negative and positive controls, respectively. The genotyping success rate was >99% for all SNPs.

Statistical analyses

For each SNP, Hardy-Weinberg equilibrium (HWE) was calculated using the De Finetti program [35]. All SNPs were in Hardy-Weinberg equilibrium. Linear regression, adjusted for age and BMI, was used to quantify the effect of a SNP on anthropometric and metabolic parameters. All analyses were performed under an additive mode of inheritance. Significance level was set at $p = 0.05$. Genotype frequencies of the selected SNPs were compared using Pearson's chi square analysis to detect an effect on liver histology. Again, significance level was set at $p = 0.05$. All statistical analyses were performed using SPSS version 20 (SPSS, Chicago, IL, USA). The program Quanto [36] was used for power calculations. Using this study population, we are able to obtain more than 80 % power to detect small allelic effects of 1.18%. Correction for multiple testing was performed using the Bonferroni correction for number of SNPs genotyped.

Results

Single nucleotide polymorphism (SNP) selection was based on the data provided by the HapMap project (Data Rel 28). After downloading the data into Haploview and running the program, the output showed that genotyping of 2 tagSNPs (rs1138714 and rs28633403) was sufficient to capture all informative SNPs (MAF ≥ 5%) in the selected region with $r^2 > 0.8$. We also included one extra coding polymorphism (rs1138693, L481P). Genotypes for the 3 selected SNPs were obtained for 633 individuals. Minor allele frequencies were 49.7%, 49.2% and 31.2% for rs1138714, rs28633403 and rs1138693 respectively.

Linear regression analyses for metabolic parameters and parameters of liver disease showed that several SNPs were associated (table 2). Rs1138714 was shown to be associated with fat mass

percentage (p = 0.018), subcutaneous adipose tissue (p = 0.029) and fasting triglycerides (p = 0.038). For rs28633403, we found association with fat mass percentage (p = 0.014), subcutaneous adipose tissue (p = 0.008), glucose metabolism (p = 0.019) and alkaline phosphatase (p = 0.031) The third SNP, rs1138693, was only found to be associated with AST levels (p = 0.042). Only the associations with fat mass percentage and subcutaneous adipose tissue for SNP rs28633403 survive Bonferroni correction for multiple testing.

We also assessed the influence of the *PNPLA2* variants on the severity of histologically determined liver damage in overweight and obese patients. In our subcohort with liver biopsy (n=372/633 or 58.7%), we only found association between rs1138714 and lobular inflammation (p = 0.038), while no other parameters of liver disease showed association with the selected variants (table 3).

Discussion

While the role of PNPLA2 in adipose tissue as the rate limiting enzyme for triglyceride hydrolysis is well known, its function in liver lipid metabolism is less understood. Being a closely related homolog to the *PNPLA3* gene [16, 17] and causing ectopic lipid storage upon mutation [19], we hypothesized that the *PNPLA2* gene could be an important candidate gene to be involved in the pathogenesis of NAFLD.

With the current study, we were able to identify a limited effect of *PNPLA2* SNPs on triglyceride levels, adipose tissue characteristics and liver enzymes, indicating that PNPLA2 might indeed play a role in pathways involved in components of the metabolic syndrome.

To the best of our knowledge, only one study investigating the role of common genetic variation in the *PNPLA2* gene has been reported. In this study by Schoenborn *et al*, 12 polymorphisms from the *PNPLA2* gene region were analyzed for association with plasma free fatty acids (FFAs) as primary analysis, as well as triglycerides and glucose as a secondary analysis. Polymorphisms in the *PNPLA2* gene were found to be associated with plasma free fatty acids, triglycerides and type 2 diabetes in a cohort of 2434 participants from 3 recruited groups of subjects (recruited for severe obesity,

coronary artery disease or as a general population sample) [21]. While in our study, we were unable to investigate the association of the selected *PNPLA2* tagSNPs and free fatty acid levels, we did analyze the effect on fasting triglycerides and glucose metabolism. In the study by Schoenborn *et al,* they reported a moderate effect of *PNPLA2* variations on plasma triglycerides and a highly significant association with an increase in fasting glucose concentrations [21]. However, in the present study, we were only able to identify a modest association of rs1138714 with fasting triglycerides ($p = 0.038$). Furthermore, no association with fasting glucose was found for any of the investigated SNPs, while SNP rs28633403 was found to be associated with blood glucose levels at 2h in an oral glucose tolerance test (OGTT).

As PNPLA2 shares the greatest homology with adiponutrin (PNPLA3), this raised the possibility that these two molecules share function and/or regulation [37]. *PNPLA3* has initially been found to be associated with fatty liver and hepatic inflammation in the first genome-wide association study (GWAS) on NAFLD [11]. Furthermore, these results have been further robustly replicated in independent candidate gene studies by our and other groups [12-15]. Additionally, it was repeatedly demonstrated that *PNPLA3* was also associated with histological disease severity [13-15, 38, 39]. Based on these results and the sequence similarity between the two homologous proteins, we hypothesized that PNPLA2 might also play a role in the development of non-alcoholic fatty liver disease. However, when analyzing *PNPLA2* polymorphisms for association with liver steatosis and liver tests measuring ALT and AST, no convincing evidence for association was found (tables 2 and 3). Furthermore, *PNPLA3* gene variants have been reported not to be associated with anthropometric and metabolic parameters [15, 38]. In contrast, we did observe significant associations of *PNPLA2* tagSNPs with anthropometric parameters (fat mass and subcutaneous adipose tissue) as well as moderate associations with parameters involved in lipid and glucose metabolism. The absence of association of *PNPLA2* polymorphisms with NAFLD despite the clear influence on metabolic and antropometric features might be explained by the absence of association with visceral adipose

tissue, which is known to be associated with NAFLD. Overall, our results disprove the hypothesis that PNPLA3 and PNPLA2 might have similar functions or working mechanisms.

Human studies on NAFLD and NASH may suffer from various methodological issues. While liver biopsy is still the gold standard for the accurate diagnosis of NAFLD and NASH, it remains an invasive procedure, limiting the number of included patients [40]. Therefore, most of the studies on NAFLD and NASH are retrospective. In addition, patients in most of these studies are highly selected, as they are referred to specialised hepatology clinics because of elevated transaminases or, in case of bariatric surgery series, only represent one extreme of the spectrum. This results in patient series with relative high prevalence of more advanced disease. Moreover, in some studies a matched control group is used, the selection of which is also subject to bias and in which histological data are usually lacking. The approach used in this study differs from most other studies as all patients presenting to the obesity clinic for a problem of overweight where prospectively assessed. There was hence no *a priori* suspicion of liver disease on entrance in the programme. All patients underwent a series of comprehensive tests aiming at detecting any sign of liver affection. If there was any indication of NAFLD, a liver biopsy was proposed. We assume that this prospective approach avoids bias and allows us to confidently apply the obtained results to the overall population of overweight patients. Moreover, this approach resulted in a patient series with a representation of all degrees of NAFLD severity. The patients who ultimately appeared to have a normal liver histology were used as an internal control group, avoiding the need to compose an external group of matched controls. We believe that these methodological considerations strengthen the validity of the obtained results.

In conclusion, we can report that we found evidence for association between *PNPLA2* tagSNPs and anthropometric and metabolic parameters in this large and prospectively recruited Belgian obese population. However, no evidence for association was found between polymorphisms in the *PNPLA2* gene and the presence and severity of NAFLD.

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Table1

Parameter	
N	633
Female/male	451/182
Age (years)	42.9 ± 12.6
BMI (kg/m²)	38.6 ± 6.3
Fat mass (%)	49.1 ± 7.5
TAT (cm²)	793.9 ± 187.4
VAT (cm²)	197.8 ± 96.6
SAT (cm²)	600.2 ± 160.6
Fasting triglycerides (mg/dL)	148.6 ± 82.0
Cholesterol (mg/dl)	203.6 ± 40.3
Hdl (mg/dl)	50.9 ± 14.6
Ldl (mg/dl)	123.1 ± 36.5
Fasting glucose (mg/dL)	86.3 ± 19.8
Fasting insulin (mIU/L)	16.7 ± 11.2
HOMA-IR	3.57 ± 3.34
AST (U/L)	29.2 ± 14.7
ALT (U/L)	41.7 ± 23.6
GGT (U/L)	41.2 ± 37.2
Liver biopsy performed	372 (58.7%)

Characteristics of the study cohort. Mean value ± standard deviation is given.
TAT, total adipose tissue; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue.

Table 2

	PNPLA2_rs1138714	**PNPLA2_rs28633403**	**PNPLA2_rs1138693**
	B – 95%CI – p-value	B – 95%CI – p-value	B – 95%CI – p-value
Anthropometry and visceral fat			
Weight	0.419	0.200	0.051
Height	0.803	0.181	0.325
Fat mass (%)	0.840; 0.15-1.53; **0.018**	0.893; 018-1.60; **0.014**	0.111
VAT (cm^2)	0.594	0.719	0.843
SAT (cm^2)	11.637; 1.22-22.05; **0.029**	14.458; 3.80-25.12; **0.008**	0.391
Lipid metabolism			
Fasting triglycerides (mg/dL)	-9.538; -18.56 to -0.51; **0.038**	0.117	0.279
cholesterol	0.813	0.430	0.272
Hdl	0.314	0.399	0.629
ldl	0.697	0.607	0.280
Glucose metabolism			
Fasting glucose (mg/dL)	0.899	0.195	0.422
Glucose 120’	0.233	-5.778; -10.59 to -0.97 **0.019**	0.493
Fasting insulin (mIU/L)	0.666	0.964	0.417
HOMA-IR	0.440	0.738	0.352
Parameters of liver disease			
AST (U/L)	0.304	0.340	-1.820; -3.57 to -0.07; **0.042**
ALT (U/L)	0.414	0.686	0.399
GGT (U/L)	0.870	0.913	0.657
Alkaline phosphatase (ALP)	0.125	2.932; 0.26-5.60; **0.031**	0.135
NAFLD score (Kotronen) [41]	0.170	0.716	0.277
Liver fat % (Kotronen) [41]	0.238	0.711	0.342

Results for linear regression for all tagSNPs. The effect of each SNP is indicated by regression coefficient B, 95% confidence interval (95% CI) and p-value. All given p-values are BMI and age-adjusted. Significant nominal p-values ($p < 0.05$) are indicated in bold. P-values that withstand Bonferroni correction for multiple testing ($p < 0.05/3 = 0.017$) are underlined. VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue.

Table 3

	PNPLA2_rs1138714	PNPLA2_rs28633403	PNPLA2_rs1138693
Histological parameters of liver disease			
Steatosis grade	0.556	0.414	0.193
Lobular inflammation	**0.038**	0.155	0.399
Ballooning	0.939	0.641	0.923
NAS (median-range)	0.277	0.886	0.836
NASH diagnosis	0.830	0.735	0.850
Fibrosis stage	0.612	0.333	0.284

Results for linear regression for all tagSNPs in the subgroup of patients who underwent liver biopsy (n=372/633 or 58.7%). Nominal P values for Pearson chi square analysis are given for each tagSNP. Significant nominal p-values ($p < 0.05$) are indicated in bold.