

## PNPLA3, the triacylglycerol synthesis/hydrolysis/storage dilemma, and nonalcoholic fatty liver disease

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### Abstract

Genome-wide and candidate gene association studies have identified several variants that predispose individuals to developing nonalcoholic fatty liver disease (NAFLD). However, the gene that has been consistently involved in the genetic susceptibility of NAFLD in humans is patatin-like phospholipase domain containing 3 (*PNPLA3*, also known as adiponutrin). A nonsynonymous single nucleotide polymorphism in *PNPLA3* (rs738409 C/G, a coding variant that encodes an amino acid substitution I148M) is significantly associated with fatty liver and histological disease severity, not only in

adults but also in children. Nevertheless, how *PNPLA3* influences the biology of fatty liver disease is still an open question. A recent article describes new aspects about *PNPLA3* gene/protein function and suggests that the I148M variant promotes hepatic lipid synthesis due to a gain of function. We revise here the published data about the role of the I148M variant in lipogenesis/lipolysis, and suggest putative areas of future research. For instance we explored in silico whether the rs738409 C or G alleles have the ability to modify miRNA binding sites and miRNA gene regulation, and we found that prediction of *PNPLA3* target miRNAs shows two miRNAs potentially interacting in the 3' UTR region (hsa-miR-769-3p and hsa-miR-516a-3p). In addition, interesting unanswered questions remain to be explored. For example, *PNPLA3* lies between two CCCTC-binding factor-bound sites that could be tested for insulator activity, and an intronic histone 3 lysine 4 trimethylation peak predicts an enhancer element, corroborated by the DNase I hypersensitivity site peak. Finally, an interaction between *PNPLA3* and glycerol-3-phosphate acyltransferase 2 is suggested by data miming.

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**Key words:** Adiponutrin; Nonalcoholic fatty liver disease; miRNA; Glycerol-3-phosphate acyltransferase 2; Systems biology; Rs738409; Epigenetics

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## INVITED COMMENTARY ON HOT ARTICLES

We have read with great interest the recent article by Kumari *et al.*<sup>[1]</sup> describing new aspects about patatin-like phospholipase domain containing 3 (*PNPLA3*, also known as adiponutrin) gene function. Notably, the authors reported that human and murine *PNPLA3* exhibit increased lysophosphatidic acid acyltransferase (LPAAT) activity leading to increased cellular lipid accumulation. Kumari *et al.*<sup>[1]</sup> concluded that the I148M substitution promotes hepatic lipid synthesis due to a gain of function, and that this property provides a plausible biochemical mechanism for the development of liver steatosis in subjects carrying the 148M variant (rs738409, allele G) as discussed below.

These results give new answers to the previous conflicting data around the question of whether the rs738409 is associated with a gain or loss of gene/protein function (Figure 1). For instance, several functional studies were elegantly performed to decipher whether the I148M substitution interferes with hepatic triglyceride (TG) hydrolysis and thus promoting hepatic steatosis<sup>[2]</sup>. Based on an *in vitro* structural model of the patatin-like domain of PNPLA3 protein, He *et al.*<sup>[2]</sup> showed that the 148M variant interferes with TG hydrolysis, affecting the association of PNPLA3 with the lipid droplets. Thus, the resulting mutant enzyme is inactive against its substrates. In addition, Huang *et al.*<sup>[3]</sup> recently demonstrated that PNPLA3 plays a role in the hydrolysis of glycerolipids, and the I148M substitution causes a loss of function.

Several animal studies were conducted to evaluate the effect of the PNPLA3 loss of function by using knockout models, for instance *PNPLA3*<sup>-/-</sup> mice by gene targeting<sup>[4]</sup>. Surprisingly, loss of PNPLA3 does not cause fatty liver, liver enzyme elevation, or insulin resistance in mice under a standard or a high-sucrose/high-fat diet<sup>[4]</sup>; a finding replicated by global targeted deletion of the *PNPLA3* gene<sup>[5]</sup>.

Interestingly, a human study demonstrated that the rs738409 PNPLA3 G allele is associated with morphological changes in adipocyte cell size<sup>[6]</sup>, and this concept strongly supports a role of the variants in the liver fat remodeling; unfortunately, these findings were not replicated.

Finally, Kumari *et al.*<sup>[1]</sup> confirmed that PNPLA3 functions as a TG hydrolase in mice and humans but found that the specific TG hydrolase activity is much lower than that of adipose triglyceride lipase (ATGL).

### Can a loss or gain of function in a single protein explain such a strong effect in the biology of nonalcoholic fatty liver disease?

The question as to whether the role of the rs738409 in the pathobiology of nonalcoholic fatty liver disease (NAFLD) is associated with a gain or loss of function is hard to answer because the biological function attributed to the PNPLA3 in lipid-related pathways is shared with other related proteins. For example, almost all members of the PNPLA family have established TG hydrolases and phospholipases activities, and PNPLA3 shares, as expected, protein domains with the PNPLA family (PNPLA1, PNPLA2, and PNPLA4-8), and also with

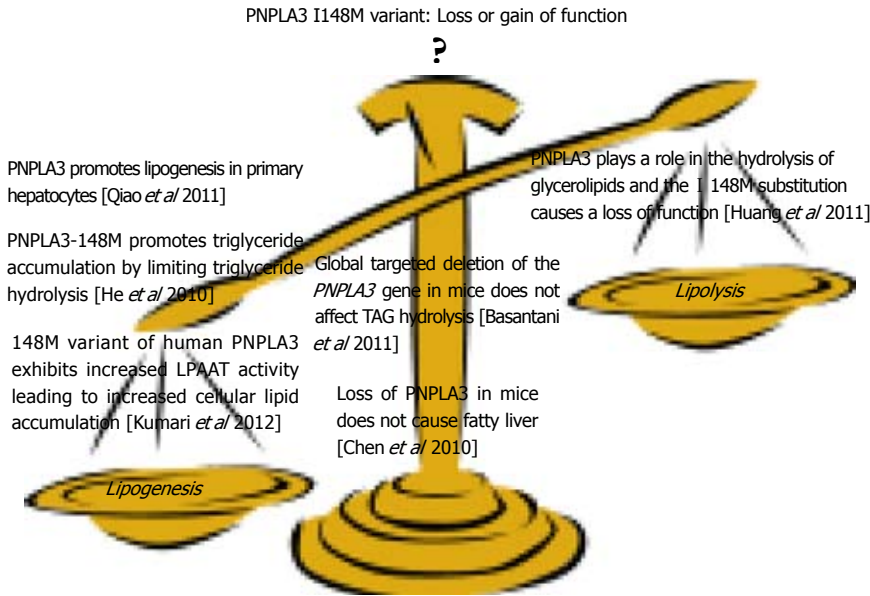
PLA2G6 (phospholipase A2, group VI cytosolic, calcium-independent, also known as PNPLA9). *In silico* prediction of PNPLA3 protein function by imputation of functional association data<sup>[7]</sup> shows that only PNPLA2, PNPLA3, PNPLA8, and PLA2G6 have carboxylesterase and lipase activities ( $P < 4.8$  and  $9.4 \times 10^{-5}$ ) and PNPLA3, PNPLA8, and PLA2G6 have phospholipase A2 activity ( $P < 1.6 \times 10^{-4}$ ). As we mentioned recently, these similarities with enzymes with the potential of releasing arachidonic acid as the precursor of potent inflammatory substances such as prostaglandins may have therapeutic implications<sup>[8]</sup>.

In addition, neutral lipid, triglyceride, or glycerolipid catabolic processes are shared by only PNPLA2 and PNPLA3 ( $P < 0.05$ ). This finding is also consistent with the notion that PNPLA3 plays a role in the hydrolysis of glycerolipids as shown by Huang *et al.*<sup>[3]</sup>.

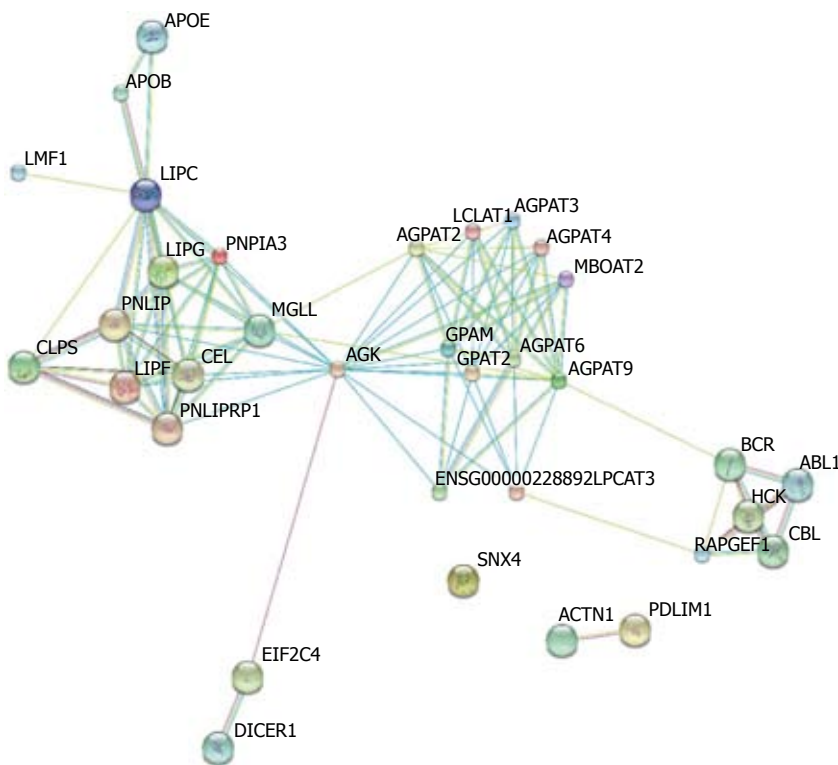
Furthermore, *in silico* prediction of the protein network related to PNPLA3 is beyond the PNPLA family (Figure 2), suggesting that some other interesting and still unexplored issue about the putative role of the rs738409 in liver steatosis are waiting for answers. For instance, the putative interaction between PNPLA3 and genes in the related networks, particularly glycerol-3-phosphate acyltransferase 2 (GPAT2, mitochondrial), whose protein esterifies acyl-group from acyl-ACP to the sn-1 position of glycerol-3-phosphate, an essential step in glycerolipid biosynthesis<sup>[9]</sup>. We wonder to what extent the PNPLA3 and GPAT2 interaction modulates hepatic fat content. We used a data integration and data mining platform to explore the putative interaction between PNPLA3 and GPAT2, and as shown in Figure 3, PNPLA3 and GPAT2 are involved in shared metabolic pathways, including triglyceride biosynthetic process, glycerolipid metabolism pathways, and acyltransferase activity, which may explain an interaction in the pathogenesis of NAFLD. This prediction is biologically plausible as GPAT2 catalyzes the initial and rate-limiting step in glycerolipid synthesis, and overexpression and knock-out studies suggest that GPAT isoforms can play important roles in the development of hepatic steatosis, insulin resistance, and obesity<sup>[10]</sup>.

### Impact of rs738409 on genetic risk of NAFLD

The first description about the association between a nonsynonymous single nucleotide polymorphism (SNP) of PNPLA3, the rs738409 C/G, (a coding variant that encodes an amino acid substitution I 148M as described) and fatty liver was reported by Romeo *et al.*<sup>[11]</sup> by performing a genome-wide association study (GWAS) of nonsynonymous sequence variations ( $n = 9229$ ) in a large multiethnic population-based epidemiological study of fatty liver. This initial finding was further replicated in several populations confirming that the G allele in the forward strand is significantly associated with increased risk of hepatic triglyceride accumulation and fatty liver disease, as reviewed recently in a meta-analysis<sup>[12]</sup>. Likewise, the rs738409 variant was associated with fatty liver in pediatric patients with NAFLD<sup>[13-16]</sup>, and patients with NAFLD related comorbidity, such as type 2 diabetes<sup>[17-19]</sup> or morbid



**Figure 1 Patatin-like phospholipase domain containing 3 rs738409 and its role in nonalcoholic fatty liver disease.** Summary of the evidence from human and rodent studies. PNPLA3: Patatin-like phospholipase domain containing 3.



**Figure 2 Protein network around patatin-like phospholipase domain containing 3 by the Search Tool for the Retrieval of Interacting Genes/Proteins resource (<http://string-db.org/>).** The interactions include direct (physical) and indirect (functional) associations; they are derived from four sources: Genomic Context, High-throughput Experiments, Conserved Coexpression and Previous Knowledge. PNPLA3: Patatin-like phospholipase domain containing 3; LIPF: Lipase, gastric; SNX4: Sorting nexin 4; AGPAT9: 1-acylglycerol-3-phosphate O-acyltransferase 9; MGLL: Monoglyceride lipase; GPAM: Glycerol-3-phosphate acyltransferase, mitochondrial; AGPAT3: 1-acylglycerol-3-phosphate O-acyltransferase 3; LIPC: Lipase, hepatic; MBOAT2: Membrane-bound O-acyltransferase domain containing 2; LCLAT1: Lysocardiolipin acyltransferase 1; AGPAT4: 1-acylglycerol-3-phosphate O-acyltransferase 4; LPCAT3: Lysophosphatidylcholine acyltransferase 3; AGK: Acylglycerol kinase; PNLIPRP1: Pancreatic lipase-related protein 1; GPAT2: Glycerol-3-phosphate acyltransferase 2, mitochondrial; PNLIP: Pancreatic lipase; PDLIM1: PDZ and LIM domain 1; AGPAT2: 1-acylglycerol-3-phosphate O-acyltransferase 2; CEL: Carboxyl ester lipase; EIF2C4: Eukaryotic translation initiation factor 2C, 4; HCK: Hemopoietic cell kinase; AGPAT6: 1-acylglycerol-3-phosphate O-acyltransferase 6; ENSG00000228892: 1-acyl-sn-glycerol-3-phosphate acyltransferase.

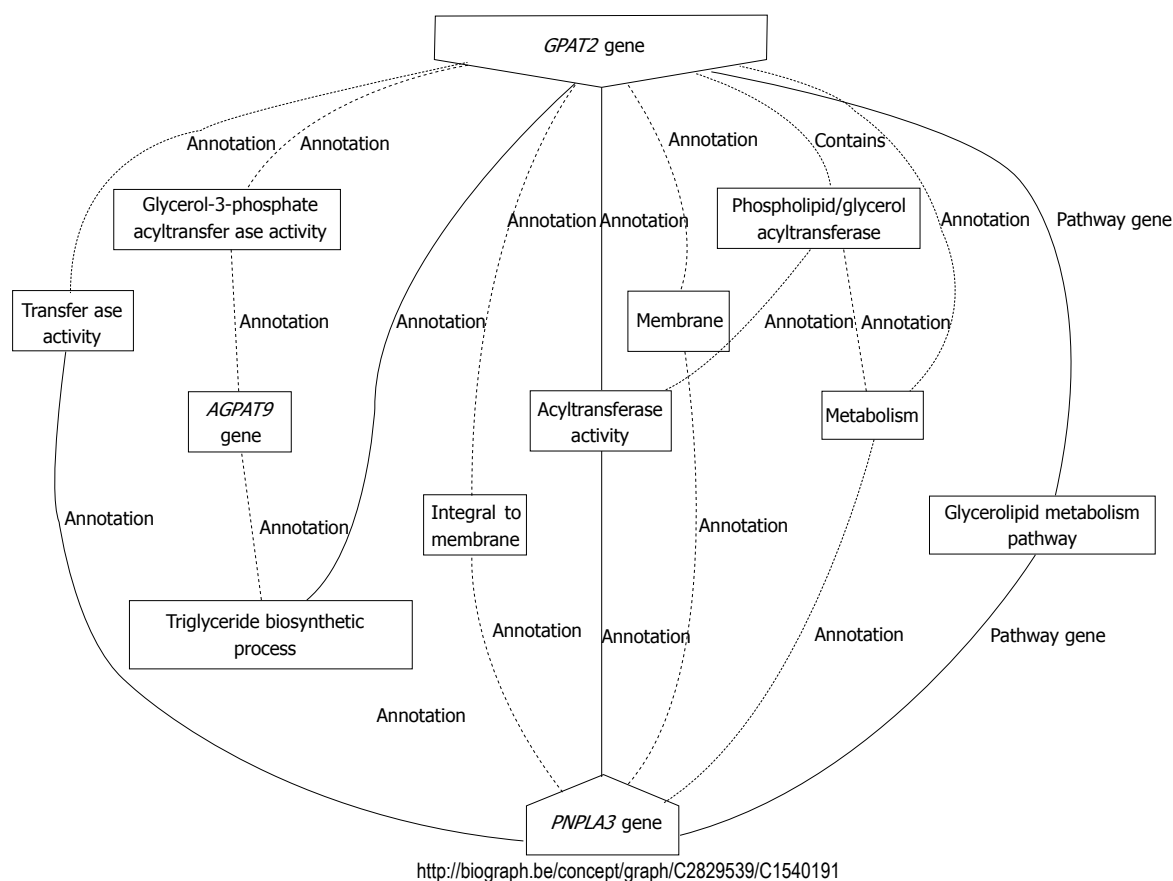
obesity<sup>[15]</sup>, although it remained to be explored whether these associations are truly independent of NAFLD<sup>[12]</sup>.

Interestingly, we demonstrated for the first time<sup>[20]</sup> that the rs738409 was also significantly associated with histological disease severity; a finding that was replicated by others<sup>[21-23]</sup>. In fact, the G allele significantly increases the risk of progressive liver disease (odds ratio 1.88 per G allele; 95% CI: 1.03-3.43;  $P < 0.04$ )<sup>[20,12]</sup>.

Overall, the most remarkable conclusion about the impact of the genetic variation in PNPLA3 on NAFLD-related outcomes is the strong effect that the rs738409 has on the susceptibility of fatty liver, because the pro-

portion of the total variation attributed to the SNP genotypes is about 5.3%<sup>[20]</sup>. This effect is perhaps one of the strongest ever reported for a common variant modifying the genetic susceptibility for a complex disease.

Furthermore, the rs738409 variant not only modifies the biology of NAFLD but also has a considerable impact on the genetic susceptibility to alcoholic liver disease<sup>[24-26]</sup>, and hepatitis-C-virus-induced fatty liver<sup>[23]</sup>, and is a strong predictor of hepatocellular carcinoma occurrence among patients with cirrhosis<sup>[27,28]</sup>, indicating that these diverse liver diseases may share common physiological pathways.



**Figure 3 Interaction between patatin-like phospholipase domain containing 3 and glycerol-3-phosphate acyltransferase 2.** Prediction was performed by the tool BioGraph (<http://biograph.be/about/welcome>), a data mining framework that allows for the automated formulation of comprehensible functional hypotheses relating a context to targets. *PNPLA3*: Patatin-like phospholipase domain containing 3; *GPAT2*: Glycerol-3-phosphate acyltransferase 2; *AGPAT9*: Acylglycerol-3-phosphate acyltransferase isoform.

### ***PNPLA3*: Short summary about gene structure and variation**

*PNPLA3* gene is located in chromosome 22 (22q13.31) and has nine exons; its transcript length is 2805 bp and it is translated to a protein of 481 amino acids. There are 34 SNPs with reported frequency and heterozygosity information in the HapMap ([www.hapmap.org](http://www.hapmap.org)). Nevertheless, among all known *PNPLA3* variants in coding or noncoding regions, there are three SNPs that have shown association with NAFLD-related phenotypes, including the rs738409. One is the rs6006460 (S453I) that after gene resequencing is associated with lower hepatic fat content in African Americans<sup>[11]</sup>. The other SNP is the nonsynonymous rs2294918 (Lys434Glu) that is significantly associated with serum alanine aminotransferase levels<sup>[20]</sup>. Haplotype analysis of *PNPLA3* shows that the rs738409 is in moderate linkage disequilibrium (LD) ( $r^2$ : 0.65) with the other variants, including rs2294918 (Figure 4). Thus, this scenario precludes any imputation across the *PNPLA3* locus centered on rs738409, and suggests that the I148M variant might be the casual variant in the susceptibility of fatty liver. However, Figure 5 shows annotation of nearby SNPs in LD (proxies) with rs738409 based on HapMap data and a nearby locus, such as SAMM50 that is a component of the sorting and assembly machinery (SAM) complex of the outer mitochondrial membrane. The SAM complex has a role in integrating  $\beta$ -barrel proteins into

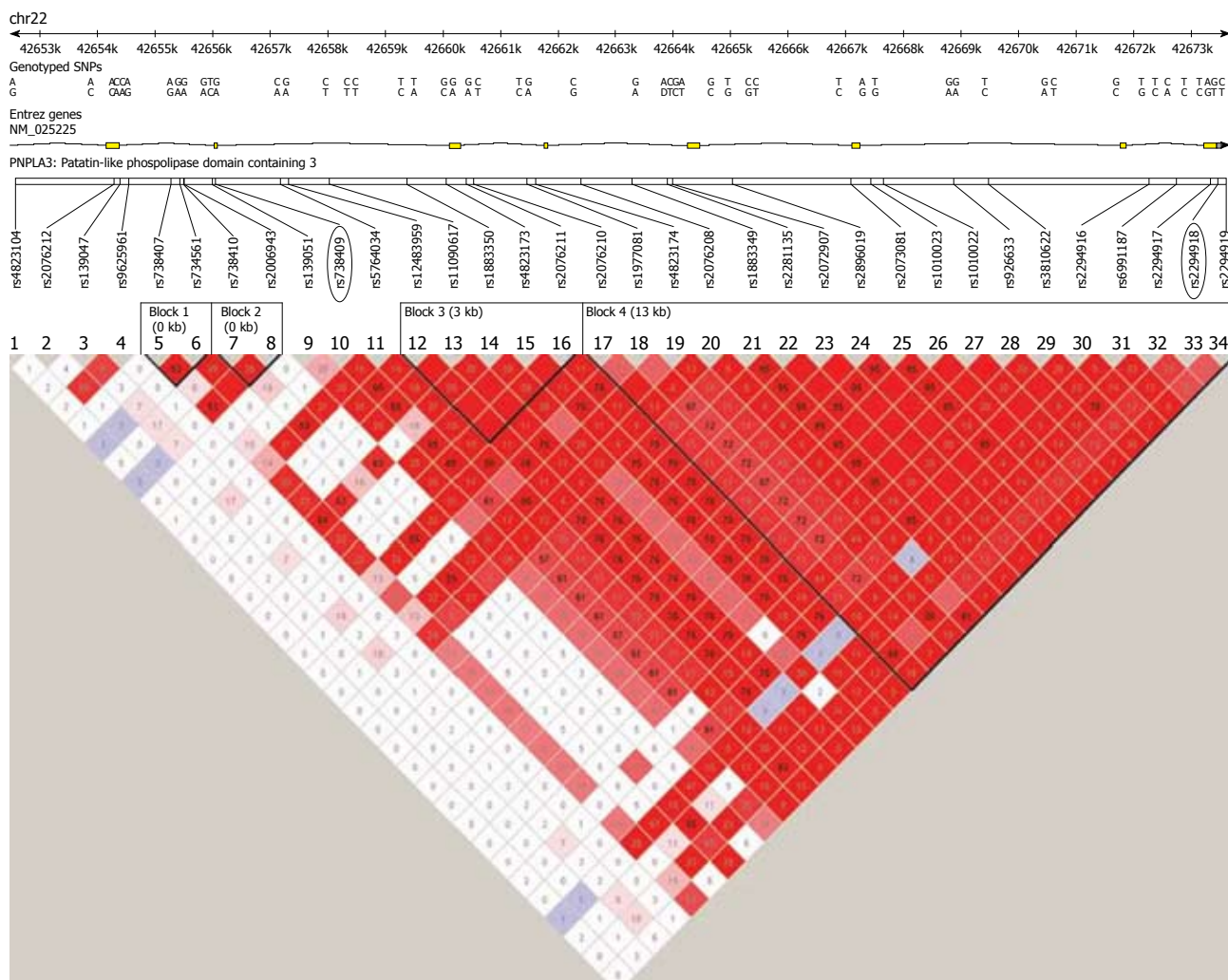
the outer mitochondrial membrane, and makes SAMM50 a good candidate because mitochondrial dysfunction may play a major role in NAFLD pathogenesis, as suggested by experimental models<sup>[30]</sup> and human studies<sup>[31]</sup>; nevertheless, this issue deserves further investigation.

Another interesting feature to highlight is the population diversity of rs738409. Genome diversity data extracted from <http://www.ncbi.nlm.nih.gov/SNP/> shows that the risk allele G is highly prevalent around the world, with an average prevalence close to 0.70 from Caucasian to Yoruban populations. Negative selection of the ancestral allele that seems to be the C allele (<http://www.ensembl.org>), which is shared by chimpanzees, orangutans, macaques, and other species, suggests that environmental pressures have exerted a strong influence. However, this picture probably reflects a sort of confusion about the reference strand and probably these annotation data refer to the minus strand when the gene is located in the plus strand, and the real frequency of the risk allele G is < 50% in all populations. In fact, published data<sup>[12]</sup> from rs738409 association studies show that the most prevalent allele is certainly the reference allele C.

### ***PNPLA3*: Mechanisms of control of gene and protein expression and unexplored areas of research**

*PNPLA3* is a multifunctional enzyme that has both triac-





**Figure 4** Linkage disequilibrium plot across the patatin-like phospholipase domain containing 3 gene. The horizontal white line depicts the DNA segment of chromosome 22q13.31. The rs738409 location is highlighted in a black ellipse. An LD plot is depicted in the bottom part of the figure: each diamond represents the magnitude of LD for a single pair of markers, with colors indicating strong LD (red  $r^2 = 1.0$ ) and no LD (white,  $r^2 = 0$ ) as the extremes (different red tones indicate intermediate LD). Numbers inside the diamonds stand for  $r^2$  values. Haplotype analysis of PNPLA3 shows that the rs738409 is in moderate LD (no more than  $r^2: 0.65$ ) with other variants. Plot was obtained by the software Haploview 2.0 freely available at [www.broad.mit.edu/mpg/haploview/](http://www.broad.mit.edu/mpg/haploview/). LD: Linkage disequilibrium; PNPLA3: Patatin-like phospholipase domain containing 3.

**Table 1** Patatin-like phospholipase domain containing 3 protein features and functions

*PNPLA3* belongs to the IPLA2/lipase family. The protein encoded by *PNPLA3* is a triacylglycerol lipase that mediates triacylglycerol hydrolysis in adipocytes. Multifunctional enzyme that has both triacylglycerol lipase and acylglycerol O-acyltransferase activity (<http://genatlas.medecine.univ-paris5.fr/>) Lipid hydrolase with an unusual folding topology that differs from classical lipases<sup>[38]</sup>

The enzyme is highly regulated by changes in energy balance: nutritional control of *PNPLA3* being affected by a feed-forward loop<sup>[36]</sup>

*PNPLA3* mRNA increases during differentiation of rat adipocytes in an insulin-dependent manner<sup>[39]</sup>

*PNPLA3* mRNA expression is upregulated by tri-iodothyronine in adipocytes *in vitro*, in humans and rats, and *in vivo* in rat WAT<sup>[39]</sup>

*PNPLA3* mRNA expression is upregulated by tri-iodothyronine in adipocytes *in vitro*, in humans and rats, and *in vivo* in rat white adipose tissue<sup>[40]</sup>

Promoter region of the human adiponutrin/*PNPLA3* gene is regulated by glucose and insulin<sup>[33]</sup>

Fasting significantly downregulates *PNPLA3* mRNA expression in liver and adipose tissue<sup>[35]</sup>

Feeding significantly upregulates *PNPLA3* mRNA in liver and fat<sup>[34]</sup>

Liver *PNPLA3* mRNA is expressed in human liver in higher levels compared with adipose tissue<sup>[36]</sup>

*PNPLA3* mRNA is highly expressed in liver of b/ob mice<sup>[40]</sup> and visceral and subcutaneous adipose issue in obese humans<sup>[41]</sup>

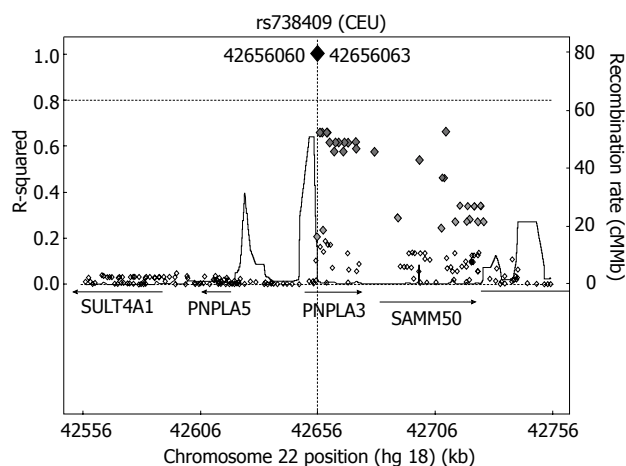
*PNPLA3* mRNA is expressed in hepatocytes but not in Kupffer cells<sup>[35,36]</sup>

*PNPLA3* is expressed in hepatocytes but not in liver endothelial and Kupffer cells; microarray-based gene profiling showed that the expression level of *PNPLA3* in hepatocytes is correlated with that of genes associated with the lipogenic pathway such as ME1, SPOT14, and SCD1<sup>[35]</sup>

*PNPLA3* is regulated in human hepatocytes by glucose *via* ChREBP<sup>[42]</sup>

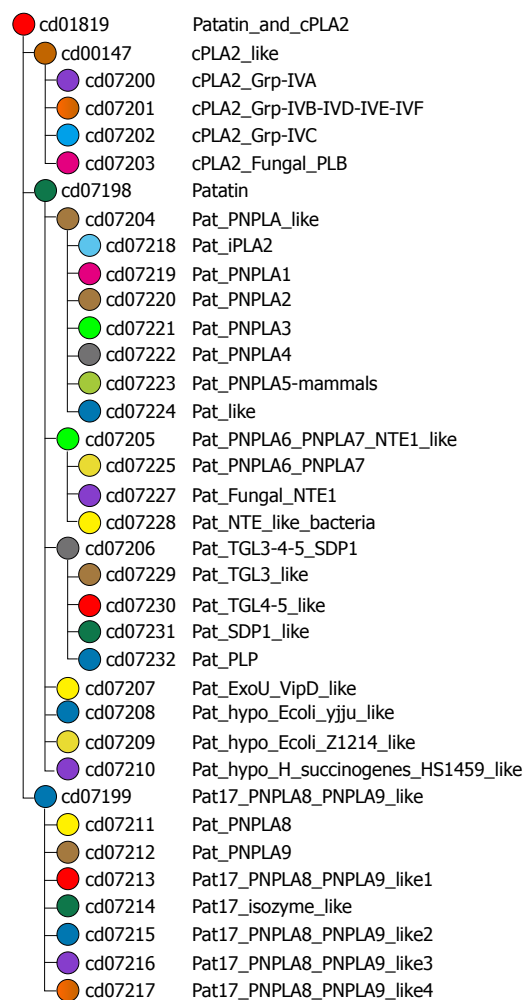
SREBP1c is able to induce *PNPLA3* expression in human immortalized hepatocytes and in HepG2 hepatoma cells<sup>[37]</sup>

PNPLA3: Patatin-like phospholipase domain containing 3; SREBP1c: Sterol regulatory element binding protein 1; ChREBP: Carbohydrate-responsive element-binding protein; ME1: Malic enzyme; SCD1: Stearoyl-CoA desaturase 1.



**Figure 5 Regional Linkage disequilibrium plot for the rs738409 at chromosome 22 (22q13.31).** The single nucleotide polymorphisms are plotted with their proxies (shown as diamonds) (based on HapMap data for CEU) as a function of genomic location, annotated by the recombination rate across the locus and nearby genes. The regional association plot was performed by the SNAP server, available at <http://www.broad.mit.edu/mpg/snap/>. SULT4A1: Sulfotransferase-4A1; PNPLA5: Patatin-like phospholipase domain containing 5; PNPLA3: Patatin-like phospholipase domain containing 3; SAMM50: Sorting and assembly machinery component 50 homolog.

ylglycerol lipase and acylglycerol O-acyltransferase activity<sup>[32]</sup>; subfamily hierarchy of PNPLA3 protein is shown in Figure 6. A summary of PNPLA3 protein characteristics and mechanisms of gene regulation is shown in Table 1. A very interesting feature of PNPLA3 protein is that is induced by several nutritional factors, such as oleic acid, C18:2 fatty acid, palmitic acid, glucose, insulin and lactone. Moreover, it was shown that the promoter activity of PNPLA3 is upregulated by glucose concentrations in a dose-dependent manner<sup>[33]</sup>. Adiponutrin was initially regarded as a dietary and obesity-linked protein<sup>[34]</sup>. Furthermore, Hoekstra *et al.*<sup>[35]</sup> suggested that PNPLA3 plays an important role in hepatic lipid metabolism under conditions of lipid excess. In the light of these findings, subsequent studies were conducted to evaluate how *PNPLA3* is transcriptionally regulated, and most of the experiments were focused to answer the question whether the lipogenic transcription factor sterol regulatory element binding protein 1 (SREBP-1) was orchestrating these events. Interestingly, Huang *et al.*<sup>[36]</sup> demonstrated in mice that *PNPLA3* mRNA levels are regulated by SREBP-1c as they found a SREBP1c binding site mapped to intron 1 of *PNPLA3*. In particular, Huang *et al.*<sup>[36]</sup> observed robust transcriptional upregulation of *PNPLA3* expression with carbohydrate feeding mediated by activation of liver X receptor/retinoic acid receptor (RXR) and increased levels of SREBP1c. In agreement with this finding, we observed in a high-fat-induced rat NAFLD model that liver transcript levels of RXR- $\alpha$  were significantly upregulated in fatty liver in comparison with normal liver (RXR- $\alpha$  mRNA/TATA box binding protein mRNA ratio:  $11.71 \pm 1.11$  vs  $6.48 \pm 1.15$ , respectively,  $P = 0.008$ ), data adjusted by HOMA-IR (homeostatic model assessment-insulin resistance) using analysis of covariance, (unpublished data).



**Figure 6 Domain family hierarchy of patatin-like phospholipase domain containing 3 protein.** Data extracted from NCBI-curated domains at <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>. This picture provides data about how patterns of residue conservation and divergence in a family relate to functional properties. In this particular case, picture shows the patatin-like phospholipase family. Patatin is a storage protein, but it also has the enzymatic activity of a lipid acyl hydrolase, catalyzing the cleavage of fatty acids from membrane lipids. Members of this family have also been found in vertebrates. This family also includes the catalytic domain of cytosolic phospholipase A2 (PLA2; EC 3.1.1.4) hydrolyzes the sn-2-acyl ester bond of phospholipids to release arachidonic acid. At the active site, cPLA2 contains a serine nucleophile through which the catalytic mechanism is initiated. PNPLA3: Patatin-like phospholipase domain containing 3; TGL: Triglyceride lipase; PLP: Pyridoxal phosphate.

In addition, *in vitro* data show that mouse but not human *PNPLA3* gene expression is under the transcriptional control of carbohydrate-responsive element-binding protein (ChREBP)<sup>[37]</sup>.

### Unexplored mechanism of gene expression regulation

As shown in Figure 7, the promoter of *PNPLA3* gene has a typical chromatin structure [a peak of histone 3 lysine 4 trimethylation (H3K4me3) between the bimodal peaks of H3K4me1] and is DNase hypersensitive. The gene lies between two CCCTC-binding factor (CTCF)-bound sites that could be tested for insulator activity. An intronic H3K4me1 peak predicts an enhancer element, corroborated by the DNase I hypersensitivity site peak. The presence of a CpG island is typical of an active

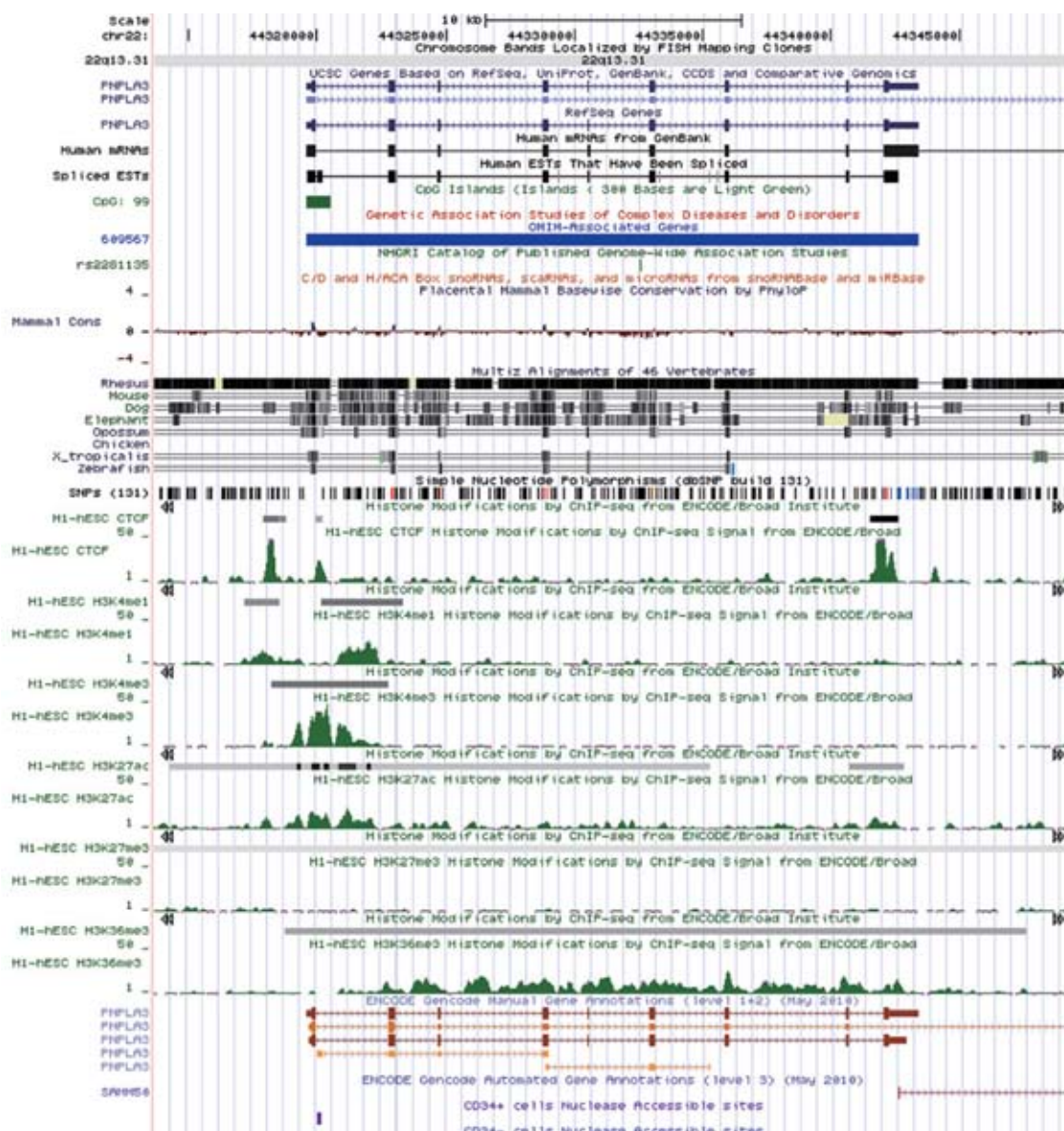


Figure 7 Prediction of patatin-like phospholipase domain containing 3 gene structure extracted from The University of California-Santa Cruz (UCSC) Genome Browser. <http://www.genome.ucsc.edu/>.

Table 2 *In silico* prediction of rs738409 C or G alleles as putative and differential target sites of microRNA

rs738409-C miRNA	rs738409-G miRNA
hsa-miR-1233	hsa-miR-1181, hsa-miR-1295, hsa-miR-1298, hsa-miR-133a, hsa-miR-133b
hsa-miR-1249	hsa-miR-135a, hsa-miR-136, hsa-miR-139-5p, hsa-miR-190b, hsa-miR-222
hsa-miR-129-5p	hsa-miR-297, hsa-miR-324-5p, hsa-miR-331-5p, hsa-miR-34c-5p, hsa-miR-362-5p
hsa-miR-155	hsa-miR-370, hsa-miR-370, hsa-miR-376b, hsa-miR-384, hsa-miR-412, hsa-miR-432
hsa-miR-365	hsa-miR-449a, hsa-miR-449b, hsa-miR-452, hsa-miR-455-5p, hsa-miR-509-3-5p
hsa-miR-433	hsa-miR-511, hsa-miR-513a-5p, hsa-miR-513b, hsa-miR-515-3p, hsa-miR-516a-3p
hsa-miR-498	hsa-miR-516b, hsa-miR-518d-5p, hsa-miR-519b-5p, hsa-miR-519c-5p, hsa-miR-519e
hsa-miR-517a	hsa-miR-526a, hsa-miR-550, hsa-miR-552, hsa-miR-554, hsa-miR-557, hsa-miR-579
hsa-miR-517c	hsa-miR-582-5p, hsa-miR-584, hsa-miR-593, hsa-miR-600, hsa-miR-601
hsa-miR-578	hsa-miR-609, hsa-miR-613, hsa-miR-623, hsa-miR-623, hsa-miR-623, hsa-miR-629
hsa-miR-586	hsa-miR-632, hsa-miR-636, hsa-miR-642, hsa-miR-642, hsa-miR-654-5p, hsa-miR-659
hsa-miR-874	hsa-miR-661, hsa-miR-663b, hsa-miR-664, hsa-miR-668, hsa-miR-760, hsa-miR-875-5p

Prediction was assessed by PITA miRNA prediction tool. Each allele was represented by at least 60 bp around the polymorphic base using a seed of 7 bp.

promoter. The coding sequence is well conserved across species and there is no known alternative splicing of the



**Table 3** Results of search for miRNA target sites on the 3' untranslated region of the patatin-like phospholipase domain containing 3 gene by DIANA-microT v3.0 prediction tool

miRNA name	Binding category	UTR start	UTR stop	UTR binding NTs
hsa-miR-769-3p	8mer (pos 1)	89	117	GCC GAC U G GGAUCCAG
hsa-miR-769-3p	7mer (pos 2)	389	417	GAGA GAUCCA
hsa-miR-769-3p	7mer (pos 1)	699	727	G CAG GGC CGG AUCCAG
hsa-miR-769-3p	7mer (pos 1)	834	862	G CA CCUG AUCCAG
hsa-miR-516a-3p	8mer (pos 1)	128	156	UGAA AGGAAGCA
hsa-miR-516a-3p	7mer (pos 2)	216	244	GCU UUGGG AGGAAGC
hsa-miR-516a-3p	8mer (pos 2)	591	619	UG AAGGAAGC

mRNA. PNPLA3 is an OMIM-related gene (number 609567). These data were extracted from The University of California-Santa Cruz (UCSC) Genome Browser.

### Concluding remarks and future research directions: Possible role for rs738409 alleles in modifying miRNA target sites

SNPs associated with polygenetic disorders, such as NAFLD, can destroy or create miRNA binding sites. We hypothesize that disruption of miRNA target binding by rs738409 alleles may play a role in the effect of the gene variant on fatty liver disease susceptibility. To test this, we analyzed *in silico* whether the rs738409 C or G alleles have the ability to modify miRNA binding sites and miRNA gene regulation by using the PITA microRNA prediction tool ([http://genie.weizmann.ac.il/pubs/mir07/mir07\\_prediction.html](http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html)). We observed that rs738409 alleles show potentially different miRNA binding sites (Table 2), suggesting a putative different role in regulation of gene regulation; a hypothesis that has to be proven experimentally. Finally, although these data do not explain the association of the rs738409 variant with NAFLD, it is worth noting that prediction of PNPLA3 target miRNA by DIANA-microT v3.0 (<http://diana.cs-lab.ece.ntua.gr/microT/>) shows two miRNAs potentially interacting in the 3'UTR region (hsa-miR-769-3p and hsa-miR-516a-3p) (Table 3).

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