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Article type : Editorial

To be, or not to be: the Quest for *PNPLA3* p.I148M function

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Funding:

Italian Ministry of Health (Ministero della Salute) Ricerca Finalizzata RF-2016-02364358 (LV)

Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Ricerca corrente (LV)

Fondazione IRCCS Ca' Granda "Liver BIBLE" (PR-0391) (LV)
Innovative Medicines Initiative 2 joint undertaking of European Union's Horizon 2020 research and innovation programme and EFPIA European Union (EU) Programme Horizon 2020 (under grant agreement No. 777377) (LV)
The European Union ERC2020, under grant agreement "101016726" (LV)
Gilead_IN-IT-989-5790 (LV)

Word count: 1018; ***References***: 10; ***Figure***: 1

Understanding the mechanisms underpinning the association between rs738409 C>G encoding for the common p.I148M variant of patatin-like phospholipase domain-containing 3 (*PNPLA3*) with fat accumulation in hepatocytes may hold the key to unravel new treatments for liver disease. The I148M variant is the main inherited determinant of both nonalcoholic and alcoholic fatty liver disease, now the leading cause of liver related mortality. Furthermore, the p.I148M genotype accounts for a large fraction of the interethnic and interindividual susceptibility to hepatic fat accumulation, and for as much as 16 and 27% of cirrhosis and hepatocellular carcinoma variability in the European population[1,2]. Indeed, after the variant identification as the main inherited determinant of liver fat[3], it was shown to translate into increased risk of fibrosis, hepatocellular carcinoma, and liver related mortality in clinical and then in population-based cohorts[1,4]. Therefore, correction of the molecular pathway underlying the phenotypic expression this causal factor may represent a next step to reduce the burden of liver disease, after the successes in the prevention and treatment of viral hepatitis.

It was initially hypothesized that the p.I148M variant contributed directly to intracellular fat retention because it leads to a loss of triglyceride hydrolase activity in lipid droplets[1,5], *Pnpla3* deletion in rodents failed to recapitulate the liver phenotype, suggesting the alternative hypothesis that the variant may acquire a new function hindering lipid remodeling and metabolism[1]. Along this line, a series of elegant studies from Helen Hobbs' group showed that, at least in female mice engineered to carry *PNPLA3* p.I148M in hepatocytes, steatosis development was dependent on the accumulation of the mutant protein which becomes resistant to ubiquitylation on lipid droplets. Accumulation of the mutant PNPLA3 sequestered ABHD5/CGI-58, an essential cofactor for the major triglyceride lipase PNPLA2, leading to lipid droplet enlargement[6,7]. These findings were buttressed by circumstantial evidence from human genetics that other genetic variants that mitigate the detrimental impact of p.I148M were linked to reduced PNPLA3 expression[1], and also by the absence of other loss-of-function *PNPLA3* variants in patients with severe liver disease[1]. In addition, *PNPLA3* silencing by antisense oligonucleotides (ASO) reduced hepatic fat and fibrogenesis in mice fed steatogenic diets, more so in mice expressing the p.I148M variant[8]. These discoveries support the conclusion that the I148M variant is a gain-of-function allele and suggest that strategies including ASO-mediated silencing of the

PNPLA3 p.I148M variant might be a potential therapeutic approach for carriers at high risk of liver disease.

However, a new study by Tilson and coworkers published in *Hepatology* challenges this conceptual framework[9]. To overcome limitations related to disease modeling in rodents, these authors developed an *in vitro* model using human induced pluripotent stem cells (hiPSC), with either *PNPLA3* knock-out (KO) or p.I148M knock-in (KI) in wild-type (WT) cells using CRISPR/Cas9 editing. This was an important step and complementary to the previous efforts, because humans and mice show profound differences in the regulation of lipid metabolism, of PNPLA3 expression, and in the sequence of the PNPLA3 protein itself[1,9]. Next, they differentiated hiPSC towards a hepatocellular phenotype, and exposed them to excess fatty acids (either oleic acid or palmitic acid) to model nonalcoholic fatty liver disease, comparing the impact of PNPLA3-KO and PNPLA3-KI to PNPLA3-WT on lipid accumulation and lipotoxicity. Importantly, they showed that PNPLA3 was expressed at physiological levels, although it was apparently not regulated by metabolic cues as previously shown in rodents. A key finding was that, especially following exposure to palmitic acid, PNPLA3-KO cells exhibited increased intracellular fat accumulation as compared to PNPLA3-WT, whereas PNPLA3-KI exhibited an intermediate phenotype. In addition, they found that PNPLA3-KO cells were more susceptible to other triggers of fatty liver disease including excess ethanol and methotrexate. A potential confounder was that PNPLA3-KO hepatocytes were less susceptible to lipotoxicity induced by palmitic acid and other saturated fatty acids, leaving open the possibility that increased intracellular fat in this background was dependent on a higher tolerance to this stressor. However, another important novel observation was that even in basal conditions and irrespective of intracellular fat content PNPLA3-edited cells tended to accumulate triglycerides enriched in polyunsaturated fatty acids (PUFA). These results are consistent with findings in patients homozygous for the p.I148M variant and with the preferential enzymatic activity of PNPLA3 towards unsaturated triglycerides[1,5,10]. Of note, Luukkonen *et al*. had previously reported that, in human hepatoma cells, *PNPLA3* p.I148M behaves as a loss-of-function regarding its ability to remodel triglycerides in a polyunsaturated direction, by impairing the hydrolysis or transacylation of PUFA from diacylglycerol to phosphatidylcholine[10]. It could therefore be speculated that altered phospholipid monolayer composition affects the properties of the lipid droplet surface. Despite some discrepancies, the overall results of this new study[9]

argue against the *PNPLA3* p.I148M being a gain-of-function and the authors conclude that the variant exerts its predisposition to fatty liver disease though the loss of enzymatic activity[9].

It should be noted that despite the novel approach, the findings of Tilson *et al*.[9] have limitations. Notably, these include the use of single cell lineage models, induction of hepatic fat accumulation by isolated stressors, lack of detailed characterization of the enzymatic and molecular mechanism leading to the observed phenotypes, including rescuing the PNPLA3-edited phenotype by overexpression of the wild-type version, and finally of characterization of the model for genetic modifiers of *PNPLA3* p.I148M. Notwithstanding these concerns, they provide compelling evidence that a gain-of-function mechanism is unlikely to fully account for the detrimental impact of *PNPLA3* p.I148M in human hepatocytes.

Eventually, with evidence accumulating from new technological approaches, many biological processes prove more complex than initially imagined. It would therefore be perhaps not surprising if a combination of gain and loss of protein functions, possibly loss of PUFA and retinol remodeling coupled with enhanced protein stability leading to negative transactivation of other lipases, together explain the impact of *PNPLA3* p.I148M on liver disease[1]. A graphical overview of these alternative hypothesis is presented in Figure 1. Future goals to understand the mechanisms underlying this epidemiological association is not simply an academic exercise, but an essential step towards the identification of the most effective and safe targets and approaches to modulate therapeutically a pathway that plays a major role in liver disease development at global level.

REFERENCES

1. Trepo E, Valenti L. Update on NAFLD genetics: From new variants to the clinic. *J Hepatol*. 2020;72(6):1196-1209.

2. Jamialahmadi O, Bianco C, Pelusi S, Romeo S, Valenti L. Reply to: "Polygenic risk score: A promising predictor for hepatocellular carcinoma in the population with non-alcoholic fatty liver disease". *J Hepatol*. 2021;74(6):1494-1496.

3. 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.

4. Valenti L, Al-Serri A, Daly AK, et al. Homozygosity for the PNPLA3 / adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. *Hepatology*. 2010;51:1209-1217.

5. Pingitore P, Pirazzi C, Mancina RM, et al. Recombinant PNPLA3 protein shows triglyceride hydrolase activity and its I148M mutation results in loss of function. *Biochim Biophys Acta*. 2014.

6. BasuRay S, Smagris E, Cohen JC, Hobbs HH. The PNPLA3 variant associated with fatty liver disease (I148M) accumulates on lipid droplets by evading ubiquitylation. *Hepatology*. 2017;66(4):1111-1124.

7. BasuRay S, Wang Y, Smagris E, Cohen JC, Hobbs HH. Accumulation of PNPLA3 on lipid droplets is the basis of associated hepatic steatosis. *Proc Natl Acad Sci U S A*. 2019;116(19):9521-9526.

8. Linden D, Ahnmark A, Pingitore P, et al. Pnpla3 silencing with antisense oligonucleotides ameliorates nonalcoholic steatohepatitis and fibrosis in Pnpla3 I148M knock-in mice. *Mol Metab*. 2019;22:49-61.

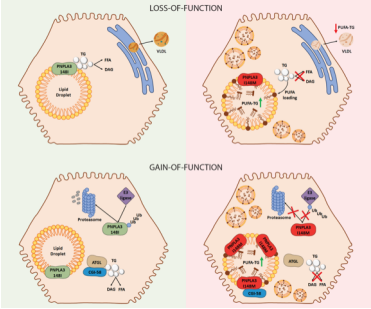
9. Tilson SG, Morell CM, Lenaerts AS, et al. Modelling PNPLA3-Associated Non-Alcoholic Fatty Liver Disease Using Human Induced Pluripotent Stem Cells. *Hepatology*. 2021:in press.

10. Luukkonen PK, Nick A, Holtta-Vuori M, et al. Human PNPLA3-I148M variant increases hepatic retention of polyunsaturated fatty acids. *JCI Insight*. 2019;4(16).

FIGURE LEGEND

Figure 1. Hypothesis of mechanism linking the *PNPLA3* p.I148M genetic variant with fatty liver disease in hepatocytes. A) Gain-of-function model; B) Loss-of-function model. See the text for a detailed explanation.

ATGL: adipose triglyceride lipase (also known as PNPLA2); CGI-58: comparative gene identification-58 (also known as ABHD5); DAG: diacylglycerol; FFA: free fatty acids; PNPLA3: patatin-like phospholipase domain-containing 3 protein; PUFA: polyunsaturated fatty acids; TG: triglycerides; Ub: ubiquituin; VLDL: very low-density lipoprotein.



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