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PNPLA3 as a therapeutic target for fatty liver disease: the evidence to date

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Abstract

Introduction: An interaction between metabolic triggers and inherited predisposition underpins the development and progression of nonalcoholic Fatty Liver disease (NAFLD) and fatty liver disease in general. Among the specific NAFLD risk variants, *PNPLA3* rs738409 C>G, encoding for the p.I148M protein variant, accounts for the largest fraction of liver disease heritability and is being intensively scrutinized. It promotes intrahepatic lipid accumulation and is associated with lipotoxicity and the more severe phenotypes, including fibrosis and carcinogenesis. Therefore, PNPLA3 appears as an appealing therapeutic target to counter NAFLD progression.

Areas covered: The scope of this review is to briefly describe the PNPLA3 gene and protein function before discussing therapeutic approaches for fatty liver aiming at this target. Literature review was carried out searching through PubMed and clinicaltrials.gov website and focusing on the most recent works and reviews.

Expert opinion: The main therapeutic strategies under development for NAFLD have shown variable efficacy and side-effects likely due to disease heterogeneity and lack of engagement of the main pathogenic drivers of liver disease. To overcome these limitations, new strategies are

becoming available for targeting *PNPLA3* p.I148M, responsible for a large fraction of disease susceptibility.

Keywords: PNPLA3, HSD17B13, Precision medicine, Nonalcoholic fatty liver disease, Liver organoids

Article Highlights

- *PNPLA3* exerts a key role in hepatic lipid droplet remodeling
- *PNPLA3* rs738409 C>G variant (p.I148M) accounts for the largest fraction of both non-alcoholic and alcoholic fatty liver disease heritability
- Experimental studies suggested that the accumulation of *PNPLA3* I148M induced hepatic fat accumulation; the pathological phenotype can be rescued by silencing PNPLA3
- Due to its importance in diseases onset and progression, PNPLA3 is an attractive target for fatty liver disease with a wide array of direct and indirect approaches
- Several molecules modulating mutated PNPLA3 expression are already being investigated in clinical trials, yet further research is required to develop novel precision medicine therapeutic approaches

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide (affecting ≈25% of population), and is defined by an excess of the amount of fat stored in the liver (≥ 5% of weight) unrelated to alcohol intake or secondary causes [1]. NAFLD encompasses a wide spectrum of liver pathologies ranging from uncomplicated steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis. Due to the increasing global prevalence of the metabolic triggers related to obesity and type 2 diabetes, NAFLD is expected to become the leading cause of liver transplantation and hepatocellular carcinoma (HCC), in the near future [2, 3, 4, 5]. Progression to severe disease is usually accompanied by several pathophysiological events including metabolic dysregulation, development of lipototoxicity, infiltration of the liver by inflammatory cells, and activation of fibrogenesis (so called nonalcoholic steatohepatits - NASH) [6]. Moreover, the diagnosis of NAFLD is associated with increased risk of cardiac morbidities and extra-hepatic malignancies (e.g. type 2 diabetes mellitus and chronic kidney disease) and thus poses a serious threat to the global health.

Despite the high prevalence of NAFLD, we still have a limited knowledge of the biological mechanisms involved in disease progression [7]. Indeed, NAFLD represents a multifactorial disease triggered by environmental stressors related to unhealthy lifestyle and diet. These triggers lead to increased adiposity, development of insulin resistance and diabetes, and synergize with genetic predisposition and epigenetic modifiers to induce liver disease [8]. In the last years it has been shown that changes in the gut bacterial microbiome may also contribute to NAFLD pathogenesis [9, 10, 11]. The heritability of NAFLD and hepatic fat accumulation is large generally estimated between 20 to 70%, depending on the ethnicity, the study design and methodology used to investigate it [12]. Among the specific genetic risk variants, the patatin-like phospholipase domain–containing 3 (*PNPLA3*) rs738409 C>G single nucleotide polymorphism (SNP), encoding for p.I148M protein variation, has been widely demonstrated to represent the main genetic risk variant for hepatic fat accumulation, NAFLD and other forms of fatty liver disease, including alcoholic liver disease. At the same time, this variant represents a main determinant of NAFLD progression to cirrhosis and HCC [13, 14, 15, 16].

Besides genetic variants, other factors are strong modifiers in NAFLD heterogeneity including other inherited factors such as gender [17], aging [5]. Indeed, female sex has been demonstrated to protect against NAFLD, yet after menopause the risk of developing fibrosis is higher than in males, likely due to sex-specific distribution of fat and changes in estrogen levels

after menopause. The main environmental triggers of NAFLD are increased adiposity, insulin resistance and type 2 diabetes. Obese individual are four times more at risk to develop NAFLD, and high BMI predisposes to NASH, fibrosis and HCC. The endocrine activity of enlarged visceral adipose tissue favors insulin resistance and the release of inflammatory mediators (e.g. IL-6 and TNF-α) that promote steatosis and hyperglycemia [18] [1]. Alterations in the gut permeability and bacterial microbiome [10, 11, 19], and various degrees of alcohol consumption has also been involved in the pathogenesis of fatty liver [20]. All these factors could result in a different NAFLD sub-phenotypes with a different prognosis and response to therapeutic treatments [21]. To improve the identification of individuals at high risk of liver disease due to metabolic comorbidities, in the last years several researchers have proposed to replace the nomenclature of NAFLD with metabolic associated fatty liver disease (MAFLD) [3, 22, 23, 24, 25].

In this narrative review, we have summarized the recent findings that point to the impact of the inherited *PNPLA3* variants predisposing to fatty liver disease on the biological functions of the PNPLA3 protein, and the latest data on therapeutic strategies that aim at exploiting these discoveries to prevent and treat liver disease. In order to identify the main findings, a literature search in PubMed was carried out, selecting studies published since 2008, when the *PNPLA3* variant was first identified as a risk factor for liver disease. Clinical trials evaluating drugs targeting PNPLA3 were retrieved from the www.clinicaltrial.gov website.

2. Impact of PNPLA3 gene variation on liver disease

Among *PNPLA3* variants, rs738409 C>G, resulting in an aminoacidic substitution of methionine for isoleucine at position 148 (p.I148M) of the protein, was first identified as a genetic risk factor for hepatic fat accumulation and NAFLD at exome-wide level in the general population [13], and next with NAFLD-related phenotypes, including NASH [14, 26]. It soon became evident that the impact of the p.I148M variant on liver fat translated into a more severe liver disease, first on the severity of fibrosis, which is the main determinant of the risk of suffering from complication of liver disease [27], including the risk of HCC development [28]. The detrimental impact of the p.I148M variant on liver disease was evident not only in NAFLD but in several liver diseases, especially those associated with hepatic fat accumulation (fatty liver) [15, 29]. The impact of variant carriage is clinically significant, as it was recently shown that in a US population study characterized by a high prevalence of dysmetabolism, it increased not only liver-related mortality, but translated into increased overall mortality as well [30]. We recently showed that the p.I148M

variant accounts for ~16% of the interindividual susceptibility to cirrhosis and ~25% of that to HCC in a Northern European population [31]. This figure is an underestimation of the p.I148M variant's impact on global health, as *PNPLA3* variation accounts for almost 70% of interethnic variation in the susceptibility to fatty liver disease [13, 32].

Indeed, other *PNPLA3* variants affecting liver disease natural history were identified, including rs6006460 G>T p.S453I which has a protective effect or rs2294918 G>A p.E434K that reduces PNPLA3 expression [33]. On the other hand, no rare variants predicted to damage PNPLA3 were enriched in patients with severe phenotypes related to NAFLD, such as children with severe NASH or patients progressing to severe fibrosis or HCC [33, 34].

Overall, these human genetics data indicate that different *PNPLA3* variants may result in opposite phenotypes, and in particular the main p.I148M responsible for a large fraction of liver disease susceptibility may behave as a gain-of-function. We therefore initially raised the hypothesis that *PNPLA3* p.I148M may represent a therapeutic target [35].

Among the other genetic risk variants for fatty liver disease, variation in *HSD17B13* has recently been reported to influence the risk of both alcoholic and nonalcoholic liver diseases by interacting with *PNPLA3* [36, 37]. Indeed, Abul-Husn and colleagues showed that *HSD17B13* rs72613567:TA leading to a loss-of-function, provides a strong protection against steatohepatitis in patients with *PNPLA3* p.I148M variants, and was linked with reduced PNPLA3 expression [36]. Pirola and colleagues, in line with showed by Abul-Husn et al, reported that *HSD17B13* rs72613567:TA plays also a protective role against ballooning degeneration, lobular inflammation and liver fibrosis [38], suggesting HSD17B13 as new promising target for NAFLD treatment.

3. PNPLA3 biology and involvement in liver disease pathophysiology

PNPLA3 was first described as a lipase that hydrolyzes glycerolipids, preferentially acting on monounsaturated fatty acids (FAs), and the p.I148M variant hampers its hydrolytic activity resulting in a loss of function variant [39]. Moreover, in 2014 Pingitore confirmed that PNPLA3 also has a weak acyltranferase activity, and confirmed that p.I148M determines a loss of function of both activities [40]. In another proposed model PNPLA3 transfers polyunsaturated fatty acids (PUFAs) from diacylglycerol (DAG) to phosphatidylcholines or, alternatively, it acts as a lipase hydrolyzing PUFAs from DAG to provide substrates to synthesize PUFA-containing phosphatidylcholines. This hypothesis is supported by the fact that homozygosity for the *PNPLA3* p.I148M variant results in intrahepatic accumulation of PUFAs, behaving as loss of function

relatively to this specific phenotype since it phenocopies PNPLA3-KO [41], [42]. However, in experimental models in mice the p.I148M variant acquired also new biological functions (neomorph variant), as it becomes resistant to ubiquitination-dependent degradation [43] leading to accumulation of the protein around lipid droplets, entrapping lipids within cells. The mechanism is mediated by sequestering ABHD5/CGI-58, an essential cofactor of ATGL/PNPLA2, the main TAG lipase in hepatocytes [43, 44]. Along this line, the overall evidence indicates that the p.I148M variant behaves as both a loss-of-function (since enzymatic activity is compromised) and gain-of-new function (neomorph) with negative transactivation activity (since the mutation hampers protein degradation causing lipid accumulation) [45]. A model of the possibile mechanisms linking the PNPLA3 p.I148M variant with liver damage in hepatocytes is presented in Figure 1.

3.1 Gene expression and cellular localization

The human PNPLA3 is mainly expressed in retina and in the liver, in hepatic stellate cells (HSCs) and hepatocytes, but also in the adipose tissue (adipocytes), and in the kidney (perivascular cells) [46]. In hepatocytes, insulin receptor signaling leads to heterodimerization of LXR with RXR, activating the transcription factor sterol regulatory element binding protein 1c (SREBP1c) [47, 48], which in turn increases the expression of PNPLA3. Moreover, the presence of glucose response elements at the *PNPLA3* promoter suggests that its expression can be also regulated by the transcription factor carbohydrate responsive element-binding protein (ChREBP) [49]. This fine tuning of *PNPLA3* transcription suggests that may be physiologically involved in accommodating increased amount of lipids in a safe form in post-prandial conditions [50]. Whether carriage of the p.I148M may confer some specific advantage in individuals without increased adiposity and dysmetabolism, thereby accounting for the apparent increase in the prevalence with population migrations outside Africa during human evolution [32] remains to be determined.

Importantly, Helen Hobbs's lab reported in a series of elegant studies in experimental models in mice and *in vitro* that *PNPLA3* p.I148M mutant protein prevents its ubiquitylation at several lysine residues avoiding proteasomal degradation, leading to the accumulation of the protein on the surface of lipid droplets which does not allow other proteins to metabolize TAG in hepatocytes [43, 44]. Yang et al showed that PNPLA3 strongly interacts with ABHD5/CGI-58, with efficiency higher than ATGL/PNPLA2, the main lipid droplet TAG lipase, leading to lipid droplet enlargement [51]. This hypothesis is in line with the aforementioned human genetic data suggesting that the

p.I148M variant behaves as a gain-of-function with negative transactivation activity on other lipases in hepatocytes, triggering TAG accumulation in lipid droplets.

4. Targeting PNPLA3 to treat liver disease

Since the common *PNPLA3* p.I148M variant is a key driver of the risk of progressive fatty liver disease, and due to the increasing global spread of dysmetabolism as well as of at-risk alcohol intake, this represents an appealing target to prevent and treat this condition, reducing the burden of liver disease worldwide. This may represent the first precision medicine application targeted at a common genetic variant for a non-rare condition. Here we present the latest approaches aimed at targeting PNPLA3 at the RNA, protein, and metabolic pathways (such as HSD17B13) levels, as summarized in Figure 2. Finally, we have also put the spotlight on new preclinical models to study precision medicine approaches targeting PNPLA3.

4.1 RNA interference

Targeting *PNPLA3* (p.I148M) at RNA levels by small hairpin RNAs (shRNAs) or antisense oligonucleotides (ASOs) could provide a significant advantage to achieve long-lasting suppression of the expression of the risk variant in carriers, possibly also trying to avoid affecting the wild type allele in heterozygous individuals. ASOs are a novel therapeutic approach to target the cognate mRNA sequences, modulating gene expression or translation of protein in question [52, 53]. The efficacy of ASOs can be further improved by targeted tissue delivery via conjugation to a ligand specific to selected cell types, but are especially effective for liver expressed genes. For instance, triantennary N-acetyl galactosamine $GalNAc_3$ conjugation to ASOs improved hepatocyte uptake and therapeutic efficacy by targeting the asialoglycoprotein receptor 1 (ASGR1) on hepatocytes [54, 55]. This strategy has already proven successful in experimental models. In 2013, Kumashiro et al. already showed a ~50% reduction of hepatic DAG content in high-fat fed rats after *Pnpla3* knockdown with ASO [56]. With a similar approach but different chemistry, BasuRay and colleagues exploited adenoviral vectors bearing PNPLA3-targetting shRNAs to revert steatosis in mice fed a high sucrose diet with promising results. Despite the study was only conducted in $PNPLA3^{148M/M}$ mice, hepatic TGs levels were reduced after shRNA administration [43]. More recently, Banini *et al.* replicated similar results in a NASH murine model overexpressing p.I148M PNPLA3 by rescuing the NASH phenotype via siRNA targeting of *Pnpla3* [57].

More recently, Linden et al proposed a new ASO strategy to target *Pnpla3* using a homozygous p.I148M knock-in model in mice, which were fed steatogenic diets: a high-sucrose diet to mimic simple steatosis, and a Westernized diet to induce steatohepatitis and liver fibrosis [58]. The authors showed that *Pnpla3* silencing caused a significantly reduction of liver steatosis in the first model, and of inflammation and fibrosis in male mice fed with a NASH-inducing diet. The beneficial impact of *Pnpla3* silencing was remarkably larger in mice knock-in for the p.I148M variant, but was also observed in wild type mice. Based on these promising results, an ASO compound called ION839 (also known as AZD2693), is currently under investigation in a phase 1 trials (NCT04142424, NCT04483947) in overweight NASH participants homozygous for *PNPLA3* p.I148M variant.

Overall, ASOs can be easily administered subcutaneously and directly target mRNA molecules producing a quicker and more lasting response than the direct inhibition of protein. This approach may reduce the frequency of administration (weekly to once every several months) when compared to small molecule inhibitors, which usually require at least daily administrations. Moreover, their flexibility in design due to Watson-Crick base recognition, accompanied by optimized synthesis procedures and several chemical-modification increasing their stability, safety and tissue-specific targeting, have led in the last years to a number of FDA and EMA approval for clinical applications ranging from treatment of hypelipidemia, rheumatoid arthritis, psoriasis, cancer and Crohn's disease [59].

Despite ASOs represent a highly promising class of drug for precision medicine, mild-to-moderate toxicity may still be observed when they are used chronically and at high doses, including splenomegaly, lymphoid hyperplasia and diffused multi-organ mononuclear cell infiltrates [60, 61]. In addition, GalNac-ASO are engineered to specifically target hepatocytes, PNPLA3 is also highly expressed in HSC, where the mutation may facilitate liver disease by determining a loss-of-function on the ability to release retinol, which was mimicked by *PNPLA3* silencing. Therefore, widespread PNPLA3 silencing might potentially hamper the beneficial impact of hepatocellular PNPLA3 silencing by facilitating HSC activation. Considering the risk benefit ratio seen in preclinical models and initial clinical studies and the high risk of liver related events, *PNPLA3* silencing with ASOs remain an interesting strategy for precision medicine, worth of further studies.

4.2 Small molecule inhibitors

Although to date no therapeutic approach directly targeting the PNPLA3 protein has yet been developed, because the localization of PNPLA3 on lipid droplets makes it difficult to reach the protein via antibodies or small molecule approaches, PNPLA3 degradation could be a viable therapeutic intervention.

In 2019, BasuRay et al proposed a new strategy to accelerate degradation of PNPLA3 via heterobifunctional proteolysis-targeting chimera (PROTAC3) system [43]. This mechanism improves the affinity for E3 ligase increasing the ubiquitination of PNPLA3 and its recruitment towards the proteasome. Despite this is not a viable approach for therapy, other mechanisms to force PNPLA3 degradation (such as ubiquitination, proteasomal degradation or autophagy) are worthy of further investigations. Another challenge to overcome would be to selectively degrade only the mutated protein, while maintaining the functionality of the wild-type counterpart in the case of heterozygous carriers, if the mutation has a partial loss-of-function impact on liver disease (e.g. on transacylation of phospholipids or in the release of retinol from HSCs).

As previously discussed, *PNPLA3* expression is regulated not only by liver fat content, but also by glucose and insulin [62] by means of transcription factors that directly bind the *PNPLA3* promoter. Therefore, reduction of PNPLA3 gene expression may also be achieved by targeting upstream actors that drive *PNPLA3* transcription. Among these, SREBP-1c (sterol regulatory element binding protein-1c) is a transcription factor induced by insulin as well as by LXR agonists to drive hepatic lipogenesis. So, silencing of hepatic SREBP-1c have potential beneficial effects in patients with NAFLD.

In 2020, Schwartz et al purposefully screened a library of 18 clinical-stage small molecules to identify modulators of *PNPLA3* expression. Among them, momelotinib, previously identified to treat myeloproliferative neoplasm by inhibiting JAK1 and JAK2 [63], emerged as a strong inhibitor of PNPLA3 expression in a dose dependent manner in human hepatocytes [64]. Using a series of chromatin-based assays, authors showed that momelotinib downregulates PNPLA3 mRNA through the inhibition of BMP receptor (ACVR1)-SMAD signaling pathway. Nevertheless, momelotinib and JAK2 inhibition in general have side effects, with cough, diarrhea, and nausea being the most common [64]. On the other hand, the reduction of *PNPLA3* mRNA was also observed in mice treated with high-sucrose diet [64], representing a new interesting and rapid therapeutic approach for NASH. Considering recent findings [57] linking the *PNPLA3* p.I148M with STAT3 pathway activation, momelotinib may also reduce inflammation and HSCs activation by blocking JAK1-2-mediated STAT3-signaling. Further studies are required to corroborate this approach.

4.3 Modulation of PNPLA3-dependent pathways

Recently, the rs62305723 and rs72613567 *HSD17B13* variants have been shown to result in loss-of-function of enzymatic activity [36, 37, 65] and protection against liver disease. HSDB17B13 has retinol dehydrogenase (RDH) activity and is expressed on lipid droplets in hepatocytes. In keeping , inhibition of HSD17B13 may represent an interesting therapeutic target. Indeed, very recent data have been reported on a new first-in-class small molecule, able to target HSD17B13 in *in vitro* and *in vivo* models of fatty liver disease, which led to improvement of lipid profile and decreasing liver damage [66]. Initial results, still reported in preliminary form, suggest that its short term administration is safe, able to achieve profound suppression of hepatic HSD17B13 expression, and may lead to amelioration of fatty liver disease [67]. In addition, a phase I clinical trial study, based on double-stranded RNAi called ARO-HSD, have already been registered (NCT04202354) to evaluate safety, tolerability and pharmacokinetics/pharmacodynamics effects in healthy individuals and patients with NASH or suspected NASH. Despite HSD17B13 enzymatic activity is not yet fully characterized, it has other lipid substrates, so that long-term effects of inhibition are difficult to predict, overall, these initial results sparkle some enthusiasm on the possibility to progress to further stages of clinical development of approaches targeting this liver disease pathway.

4.4 New preclinical models for testing precision medicine approaches to PNPLA3

Despite mouse models have been extensively employed to model liver diseases, they do not fully recapitulate all the features of human NASH. Hence, testing new therapeutic approaches in human cells/tissues may increase the rate of final success, especially when a specific human variant is targeted.

To overcome these limits, human liver chimeric mice were developed to study human drug metabolism, excretion, and toxicity, overcoming the differences between human and animals [68, 69, 70, 71, 72, 73]. Recently, Bissig-Choisat and colleagues established and characterized a humanized TIRF (transgene-free, $Il2rg^{-/-}$, $Rag2^{-/-}$, $Fah^{-/-}$) mouse model, able to replicate the pathophysiology and histology of human NAFLD when fed with a high fat/sucrose diet for 12 weeks [74]. These tools have a promising potential to study the response of human hepatocytes to

diet-induced NAFLD and to accelerate the development of new therapies improving the translation of preclinical drug test.

In the past few years, another promising *in vitro* tool, called organoids, has emerged to study liver diseases. Usually, experimental models were limited to the use of 2D cultures of primary or immortalized cell lines. However, whilst primary cells have a limited lifespan and will stop dividing (or senesce) after a certain number of cell divisions, on the other hand there are no continuously replicating cell lines that provide normal levels of metabolic activity over a wide range of functions. Moreover, 2D culture systems do not recapitulate the complexity and heterogeneity of the tissue *in vivo*. Organoids allow to overcome all these limits in an *ex vivo* context. They are unique because are a self-organizing 3D system, supported by an extracellular matrix, derived from tissue-resident stem/progenitor cells, embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) able to mimic and in some cases completely recapitulate the *in vivo* tissue counterpart both at the physiological and architectural levels [75, 76, 77, 78, 79, 80]. Accordingly, they have become groundbreaking tools for basic and translational studies to recapitulate more closely the human pathophysiology, as well as to develop personalized medicine approaches that would not be possible with animal models. In the liver field, Ouchi et al generated an iPSC-derived hepatocyte organoid model able to recapitulate the development of NAFLD and some aspect of NASH, including lipid accumulation and fibrosis [81]. More recently, Qin's lab proposed a human NAFLD-on-a-chip model combining iPSC-derived organoids with organ-on-chips, providing a platform for improving organoids' applications in disease modeling and drug testing [80]. Finally, Ramli et al generated a stem cell-derived organoid, showing a surprisingly contiguous canaliculi network similar to the in vivo counterpart, providing a promising model of intrahepatic cholestatic diseases [82]. As they can be generated starting from human tissues, liver organoids represent a complementary approach to animal models for studying different aspects of liver diseases and drug-related response, allowing to stratify the analyses for carriage of specific genetic risk variants for fatty liver disease and in particular for the *PNPLA3* p.I148M.

5. Conclusion

NAFLD remains a considerable challenge to global public health, because it encompasses a wide range of chronic liver disorders including uncomplicated fatty liver (steatosis), NASH, fibrosis and may evolve to cirrhosis and HCC [2, 3, 4, 5]. Genome wide studies led to the identification of the main common genetic risk variants for NAFLD [13, 14, 26]. Among them, the *PNPLA3* p.I148M

variant accounts for the largest fraction of variation in liver disease interindividual susceptibility [13, 27, 83]. The molecular mechanisms underlying its pathogenicity seem to be related to an altered enzymatic activity, resulting in the accumulation of the variant protein and altering lipid droplets dynamics and fat accumulation in hepatocytes and altered retinol metabolism and to an inflammatory/fibrogenic phenotype in HSCs.

Concerning possible therapeutic approaches, inhibition of *PNPLA3* p.I148M and HSD17B13 expression by ASO or direct inhibition of HSD17B13 led to promising results in proof-of-principle studies, and the first clinical trials have already been registered. Table 1 provides an overview over therapeutic studies on the compounds to target PNPLA3 discussed in this review.

6. Expert opinion

The liver is a central hub for several physiological processes, including the regulation of systemic glucose and lipid metabolism, the major source of energy for human body. During the last decades, the global diffusion of a lifestyle characterized by increased caloric intake, Western diet and physical inactivity led to the increasing prevalence of obesity, type 2 diabetes and NAFLD [6]. NAFLD can progress to more worrying conditions such as NASH, cirrhosis and HCC. Moreover, prevalence is steeply rising, jointly with the ongoing obesity and metabolic dysfunction epidemic: indeed, NAFLD poses a serious threat to worldwide health.

The mainstay of NAFLD treatment is weight loss through diet and lifestyle modification, while no pharmacological treatment has yet been approved for this condition. Promising approaches are directed toward the targeting of excess adiposity and consequently insulin resistance such as via GLP-1 agonist (Exenatide, Semaglutide and Liraglutide) [84, 85, 86, 87]. Drugs with a direct impact on cholesterol and bile acids metabolism such as statins and FXR agonists can reduce liver damage improving fibrosis [88, 89, 90, 91]. However, some of these compounds have potentially limiting detrimental side effects, including an unfavorable impact on cardiovascular risk profile, affecting their long-term safety. New drug classes are coming, including pan-PPAR agonists and TRB-β receptor agonists, which may improve liver damage by improving lipid catabolism via oxidation. Finally, as lipotoxicity plays a key role in NAFLD, antioxidants such as vitamin E (tocopherol) have shown some beneficial effect its antioxidant properties [84]. Despite the recent progresses, exploration of novel therapeutic routes is therefore of paramount importance.

The advent of next generation studies, WES and GWAS highlighted an initial panel of variants associated with severe NAFLD, confirming that genetic factors influence the natural course of this condition. Over the years, the causal link between *PNPLA3* p.I148M and progressive NAFLD has become more and more established. Of note, carriage of the *PNPLA3* p.I148M variant hampers the response to at least some of the therapeutic options under clinical evaluation (e.g. statins and N3-PUFA) [92]. This was shown in a clinical trial (NCT00885313) conducted on 60 children carrying rs738409 variant, clearly respond to a lesser extent than wild-type patients to docosahexaenoic acid [93].

On the other hand, PNPLA3 itself represents an appealing therapeutic target for NAFLD and fatty liver disease in general, due to its seminal role in disease onset and progression. Based on these premises, adopting a precision medicine approach focused on PNPLA3 related pathways can be a precious contribution to expand the array of therapeutic options. Recent results obtained in preclinical models by targeting PNPLA3 have shown encouraging results, arousing interest among researchers.

Therefore, modulation of the PNPLA3 pathway in carriers of the p.I148M variant may allow to prevent and cure various forms of liver disease in the subset at highest risk of progression to severe outcomes (Figure 2). Although this approach is currently being pursued for some rare genetic disorders, this would be first precision medicine application targeting a common genetic risk variant responsible for a common chronic degenerative condition, representing a paradigm shift in routine clinical management. Among the novel approaches, ASO and siRNA have gained enormous interest to regulate the mRNA translation [94, 95]. Moreover, these compounds could be easily modified, both in chemical structure and in ligand conjugation, to increase its stability and improving the uptake to disease-associated cells [96]. Indeed, pharmaceutical companies started to develop drug using GalNac-conjugated oligonucleotide products to specifically target the liver, some of which are currently under investigation in a phase 1 trial to treat patients affected NASH.

Another promising target highly linked to PNPLA3 is HSD17B13, that may also be involved in STAT3 signaling, driving inflammation and fibrosis. Interestingly, the HSD17B13 rs72613567 loss of function variant reduces p.I148M PNPLA3 levels: thus, ASO targeting HSD17B13 can be employed as a precision therapy in p.I148M variant carriers. The implementation of this precision medicine approach will require preclinical testing in *in vivo/in vitro* models that recapitulate human genetic variability to understand the molecular mechanisms causing NAFLD and discovery

of new potential therapeutic compounds, remain a major challenge. Organoids isolated from clinical samples are becoming a robust tool to study human diseases due to their attractive features and may therefore help in targeted drug screening hopefully reducing the rate of failure in clinical testing.

Despite *PNPLA3* p.I148M variant role as a major NAFLD severity modifier is well established, additional studies are needed to characterize the molecular mechanism by which *PNPLA3* p.I148M leads to liver disease. In particular, it would be key to determine whether the detrimental impact of the *PNPLA3* is accounted for by the loss-of-function of the enzymatic activity, or to a gain-of-new function leading to inhibition of lypolysis, whether the main effect is exerted in hepatocytes or encompasses also modulation of HSC function (Figure 1). Unfortunately, up to now most studies to understand the molecular mechanisms of PNPLA3 were conducted in mouse models, which show profound differences in the regulation of lipid metabolism, PNPLA3 expression, and PNPLA3 sequence itself as compared to humans. However, these last are limited to mimic the extremely complex systems of the human body. The implementation of more complex *in vitro* systems like organoids may help to define PNPLA3 molecular function.

In the next five years, we expect that the mechanism underlying the pathological phenotype of the p.I148M variant will be clarified, and the first early phase clinical trials evaluating compounds specifically designed to directly or indirectly target PNPLA3 p.I148M will be completed granting us insight on whether PNPLA3 is a viable therapeutic target for NAFLD and fatty liver diseases.

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Reviewer Disclosures

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Figure Legend

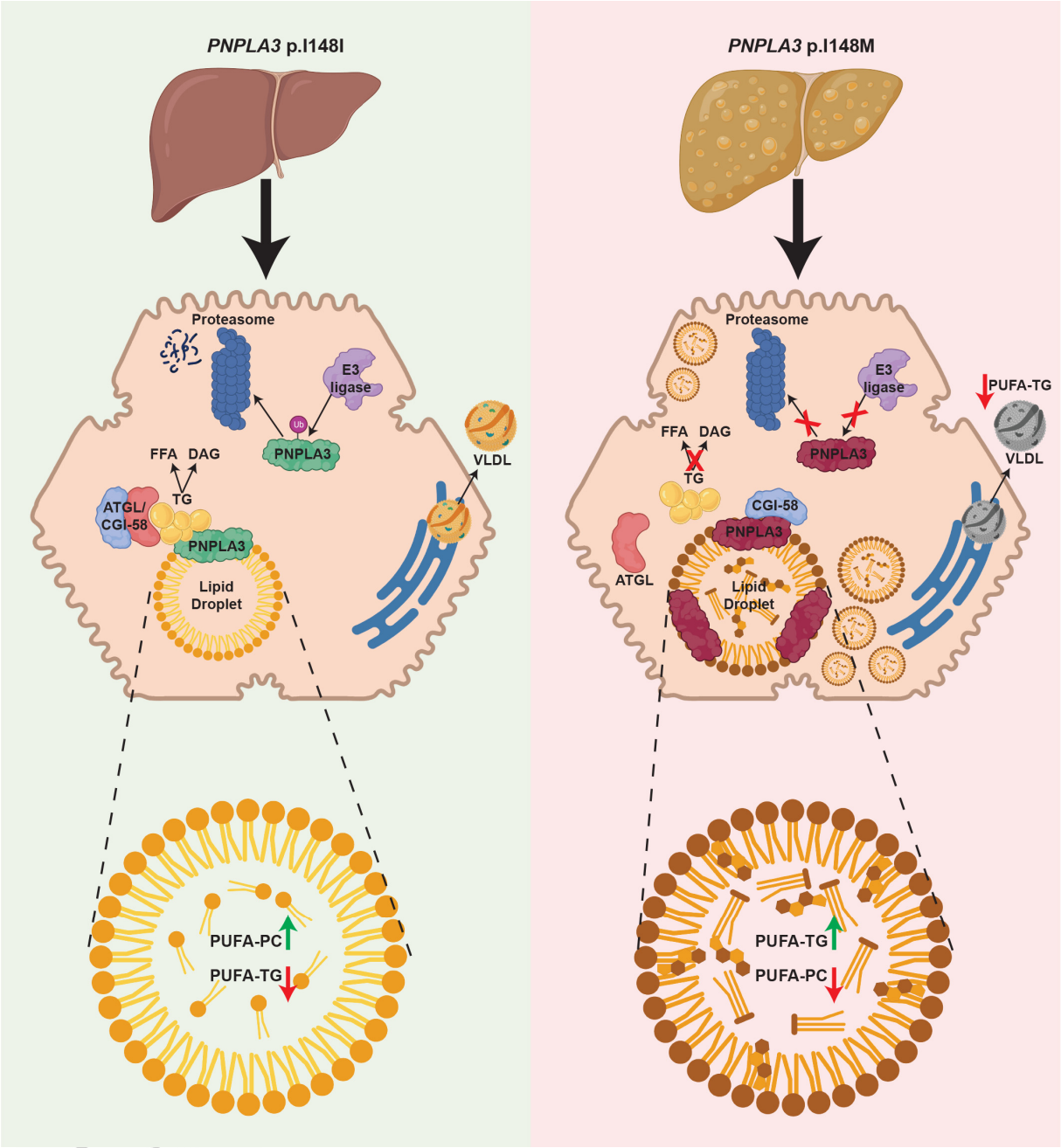

Figure 1. Schematic representation of mechanism linking the *PNPLA3 rs738409* p.I148M genetic variant with fatty liver disease in hepatocytes.

In physiological conditions, PNPLA3 is located on lipid droplets and it has hydrolase activity towards triglycerides (TAG), and possibly trans-acylation activity from TAG to phospholipids, with preferential activity towards unsaturated fatty acids. PNPLA3 p.I148I can be ubiquitinated by E3 ligases, leading to its recruitment toward the proteasome and subsequently to its degradation. The PNPLA3 p.I148M protein variant results in opposite phenotypes: a loss-of-function of triglyceride hydrolase and trans-acylase activity in lipid droplets leading to accumulation of PUFAs

in TAG, and reduction of their secretion within VLDLs from hepatocytes; a gain-of function (neomorph), as it becomes resistant to ubiquitination-dependent degradation leading to accumulation of the protein around lipid droplets. Here PNPLA3 competes with ATGL/PNPLA2, the main TAG lipase in hepatocytes, sequestering its cofactor ABHD5/CGI-58, thereby leading to lipid droplet enlargement due to reduced lipolysis. The figure was created with BioRender.com.

Abbreviations: ATGL, dipose triglyceride lipase (also known as PNPLA2); CGI-58: comparative gene identification-58 (also known as ABHD5); DAG, diacylglycerol; FFA, free fatty acids; PC, phospatidylcholine; PNPLA3, Patatin Like Phospholipase Domain Containing 3; PUFA, polyunsaturated fatty acids; TG, triglycerides; Ub, ubiquituin; VLDL, very low-density lipoprotein.

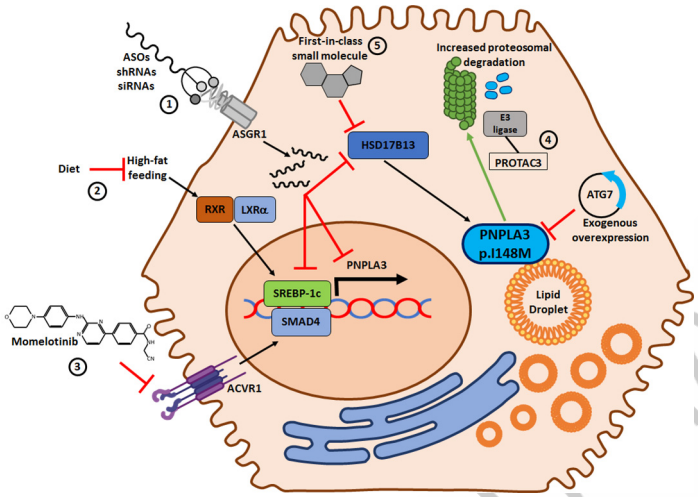

Figure 2. Schematic representation of potential PNPLA3 therapeutic approaches reviewed in this article.

1) Small hairpin RNAs (shRNAs) or antisense oligonucleotides (ASOs) conjugate with triantennary N-acetyl galactosamine ($GalNAc_3$), target ASGR1 receptor on hepatocytes, silencing PNPLA3 expression. 2) Under high-fat diet, insulin receptor signaling leads to heterodimerization of LXR with RXR, activating SREBP-1c, which in turn increases the expression of PNPLA3. Silencing of SREBP-1c have potential beneficial effects in patients with NAFLD by reducing the expression of PNPLA3. 3) Momelotinib emerged as a strong inhibitor of PNPLA3 expression in human hepatocytes, by inhibition of BMP receptor (ACVR1)-SMAD signaling pathway. 4) PROTAC3 system improves the affinity for E3 ligase increasing the ubiquitination of PNPLA3 and its recruitment towards the proteasome. 5) Inhibition of the activity of HSD17B13 by the new first-in-class small molecules or by ASOs, results in the improvement of liver damage.

Abbreviations: ACVR1, Activin A Receptor Type 1; ASOs, antisense oligonucleotides; ATG7, Autophagy Related 7; HSD17B13, Hydroxysteroid 17-Beta Dehydrogenase 13; LXR, liver X receptor;

PNPLA3, Patatin Like Phospholipase Domain Containing 3; PROTAC3, PROteolysis TArgeting Chimera 3; RXR, retinoid X receptor; shRNAs, short hairpin RNAs; siRNAs, small interfiring RNAs; SREBP-1c, sterol regulatory element-binding protein 1.

Target	Compound	Action	Stage of development	Clinical Trials ID	Reference
PNPLA3 (I148M)	PNPLA3 knockdown with ASO molecule	Reduction (~50%) of DAG	Preclinical: High-fat fed (HFF) rats		Kumashiro, 2013
PNPLA3 (I148M)	ASO compound called ION839 (also known as AZD2693)	Reduction of liver steatosis, inflammation and fibrosis in preclinical study	Preclinical: Phase 1 trial in overweight NASH participants with fibrosis stage 0 to 3 and homozygous for PNPLA3 I148M variant	NCT04142424 NCT04483947	Linden, 2019
PNPLA3	shRNAs	TGs levels were reduced	Preclinical: PNPLA3 148M/M mice		BasuRay, 2019
PNPLA3	Proteolysis-targeting chimera (PROTAC3) system	PNPLA3 degradation	Preclinical: PNPLA3 I148M mice Mainly for preclinical validation of the therapeutic approach		BasuRay, 2019
PNPLA3 promoter	Transcription factors (SREBP-1)	Reduction of PNPLA3 gene expression	Preclinical: Obese and diabetic mice		Quiao, 2011
Signaling pathways acting upstream of PNPLA3 expression	Momelotinib	Reduction of PNPLA3 mRNA	Preclinical: Mice treated with high-sucrose diet		Schwartz, 2020
HSD17B13	ARO-HSD	Reduction of HSD17B13	Preclinical: Phase 1 trial clinical in healthy adult volunteers and in patients with NASH or suspected NASH	NCT04202354	Gane, 2021
HSD17B13	Compound A	Inhibition of HSD17B13 and significantly of α-SMA mRNA level	Preclinical: Mice and treated with choline-deficient, L-amino acid-defined high-fat diet (CDAHFD)		Choi, 2021

Table 1