Sex-specific effects of PNPLA3 I148M

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Abstract.

Metabolic dysfunction-associated steatotic liver disease (MASLD, previously termed NAFLD, non-alcoholic fatty liver disease), is a complex multifactorial disease showing generally higher prevalence and severity in men than in women. With respect to women, men are also more prone to develop metabolic dysfunction–associated steatohepatitis (MASH), fibrosis, and liver-related complications. Several genetic, hormonal, environmental, and lifestyle factors may contribute to sex differences in MASLD development, progression and outcomes. However, after menopause, the sex-specific prevalence of MASLD shows an opposite trend between men and women, pointing to the relevance of estrogen signalling in the sexual dimorphism of MASLD.

The patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene, that encodes a triacylglycerol lipase that plays a crucial role in lipid metabolism, has emerged as a key player in the pathogenesis of MASLD, with the I148M variant being strongly associated with increased liver fat content and disease severity. Recent advances indicate that carrying the *PNPLA3* I148M variant can be a risk factor for MASLD especially for women. To elucidate the molecular mechanisms underlying the sex-specific role of *PNPLA3* I148M in the development of MASLD, several *in vitro*, *ex vivo* and *in vivo* models have been developed.

In this review, we provide an overview of the current state of knowledge regarding the sex-specific effects of *PNPLA3* I148M in the pathophysiology of MASLD.

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), defined as an excess of the amount of fat stored in the liver (≥ 5% of weight), represents a spectrum of liver disease ranging from hepatic fat accumulation without inflammation to steatohepatitis (metabolic dysfunction-associated steatohepatitis, MASH), fibrosis, cirrhosis and potentially hepatocellular carcinoma (HCC) in the absence of excessive alcohol consumption [1]. MASLD is characterized by substantial inter-patient variability in terms of severity and rate of progression [1]. However, the early identification of individuals at high risk of progression remains an important unmet need in the clinical context. Genetics plays an important role influencing both, the onset of steatosis and the worsening of liver disease [2]. Moreover, the liver is characterized by sexual dimorphism, and the pathogenic mechanisms involved in the onset and progression of MASLD differ, at least in part, according to sex and sex-related factors [3]. This observation has been further corroborated by a recent large-scale analysis of hepatic transcriptomic profiles that emphasized the sexually

dimorphic nature of MASLD and its association with the progression of liver disease suggesting the importance to consider sex as one of the main tools for patients' risk stratification [4].

Overall, the prevalence of MASH and hepatic fibrosis is higher in men compared to women. Similarly, the incidence of advanced hepatic fibrosis and cirrhosis complications such as the MASH-related hepatocellular carcinoma (HCC) is 2-4 fold higher in men than women [5]. However, the molecular mechanisms underlying sex differences are not yet fully elucidated. What is known is that sex hormones, mainly estrogens, exert a protective effect on the liver as demonstrated by several studies showing that the prevalence of MASLD and MASH in post-menopausal women with a mean age of 51 years is comparable to that observed in men [6,7]. Overall, menopause in women is associated with advanced fibrosis ($F \geq 2$) [8], while conflicting data are reported on the impact of estrogens in the progression of liver disease in fertile and pre-menopausal women [9,10]. Further studies are necessary to understand the effective contribute of such hormones species to the progression of MASLD.

The patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene, that encodes a triacylglycerol lipase that plays a crucial role in lipid metabolism, has emerged as a key player in the pathogenesis of MASLD, with the I148M variant being strongly associated with increased liver fat content and disease severity [11]. Specifically, patients carrying the GG risk homozygosis for the rs738409 single nucleotide polymorphism (SNP) have a higher risk of developing hepatic fibrosis and MASLD/MASH-related HCC compared to those with the wild-type allele [12]. Recently, the interaction between the I148M variant in the *PNPLA3* gene and the estrogen receptor alpha (ERα) has been described and seems to explain at least in part the sexual dimorphism in the context of MASLD [13]. Unfortunately, clinical longitudinal studies are scanty and few data on the impact of genetics on the development of clinical events over time are available at present.

In this review, we provide an overview of the current state of knowledge regarding the sex-specific effects of *PNPLA3* I148M variant in the pathophysiology of MASLD from clinical to *in vitro* and *in vivo* studies (Figure 1).

Understanding the sex-specific role of *PNPLA3* in MASLD: an update from clinical studies

From a clinical point of view, the strong association between the *PNPLA3* I148M polymorphism and sexual dimorphism dates back over ten years, when a meta-analysis showed a negative association between the genetic variant on hepatic steatosis and male sex, suggesting its possible effect on MASLD susceptibility [14]. Specifically, the meta-analysis analysed 16 studies selected for the common aim to assess the strength of the effect of the rs738409 SNP on MASLD across different populations, as well as on intermediate phenotypes such as insulin resistance and

overweight/obesity [14]. Just a year later, Li and colleagues observed a strong association between the rs738409 variant and elevated ALT levels in males from the Cameron County Hispanic Cohort (a Mexican American cohort including 1532 individuals with a high prevalence of obesity, diabetes and MASLD), confirming previous results [15]. In addition, the study reported a significant association between the rs738409 genetic variant and ALT levels only in women aged ≤ 50 compared to those older than 50 years. Considering the differences in estrogen levels between the two groups, the authors hypothesized the existence of an interaction between the I148M variant and estrogen levels that, in turn, led to the onset and worsening of liver disease [15]. Sexual dimorphism of the rs738409 SNP was further observed in a study on 121 patients with primary sclerosing cholangitis (PSC). Specifically, in male with severe liver disease, the carriage of the G risk allele was a risk factor for reduced survival [16]. Recently, a longitudinal study performed in a large European cohort of biopsied-proven MASLD patients, identified a group of individuals consisting of non-obese females with 50 years or older carrying the I148M GG risk genotype who had a higher risk of developing liver-related events during follow-up compared to the counterpart [17].

All the evidences suggest that a careful follow-up should be important in specific subgroup of individuals and that *PNPLA3* genotyping and sex should be considered in the clinical setting for patients' risk stratification. However, additional studies are required to validate previous results in external and independent cohorts.

Understanding the sex-specific role of Pnpla3 in MASLD: insights from *in vitro* studies

Although sex hormones, such as estrogens and androgens, are known to influence adiposity and insulin resistance [18], results of *in vitro* studies are generally not contextualized and interpreted in a unisex way. Several *in vitro* studies reported that *PNPLA3* I148M, with a critical role in homeostasis of lipid metabolism, plays a key role in the development of MASLD. However, mechanisms explaining sex biological specificities in liver disease susceptibility are largely unknown.

Some evidence shows that the *PNPLA3* gene is under nutritional control, being in strong relationship with the steroid regulator element binding protein (SREBP)-1c through liver X receptor, two important factors involved in the regulation of fatty acid synthesis [19]. Contextually, sex hormones, like estrogens, modulate lipogenic genes such as SREBP family, changing lipid metabolism and then contributing to the onset of liver steatosis [20].

Recently, Cherubini and colleagues reported existence of an interaction between female hormones and *PNPLA3* I148M variant in determining MASLD progression [13]. The authors identified the presence of an estrogen receptor response element (ERE), that is highly conserved in mammals, in *PNPLA3* promoter. In this study, immortalized cell line and tissue-derived liver organoids of both sexes were treated with ERα agonists, observing *PNPLA3* transcription induction. Next, they exposed cells to excess fatty acids (either oleic acid or palmitic acid) to model liver disease, showing an accumulation of PNPLA3 I148M protein on lipid droplets, intracellular lipid accumulation and collagen deposition by stellate cells in response to ERα agonists. To corroborate these findings, the authors removed the *PNPLA3*-ERE by CRISR/Cas9 showing a reduction of ERα agonists effects on lipid accumulation and collagen deposition. All in all, this evidence demonstrates for the first time an axis between female hormones, genetic variant and altered lipid metabolism. However, further *in vitro* studies that consider sex-specific approaches are needed to better understand sex differences in MASLD susceptibility and progression.

Understanding the sexual dimorphism in MASLD: insights from mouse models

Several diet-induced and genetic mouse models for MASLD have been developed to investigate the molecular drivers accounting for MASLD susceptibility, development, and progression [21]. Recently, a wide-ranging retrospective review tried to rank diet-induced mouse models for MASLD according to metabolic phenotype, liver histopathology, transcriptome benchmarked based on their proximity to human MASLD [22].

Besides mouse models can recapitulate most of the features of human MASLD, including MASH and fibrosis, the majority of the studies has been limited to the male sex [22], with female mice being excluded from analysis for the potential confounding effects ascribable to fluctuating levels of estrogens typical of estrous cycle [23].

Although very few, the comparative studies between the two sexes have highlighted the sexually dimorphic nature of MASLD, with males generally developing more severe pathogenic features of MASLD, including steatosis, insulin resistance, and inflammation compared to females [7,10,18]. When exposed to an excess of dietary lipids (high-fat diet, HFD), female mice, contrary to males, counteract liver lipid deposition thank to the regulatory activity of estrogens, acting mainly through ERα at the hepatic level [10]. The lack of a proper estrogen signalling, indeed, impairs liver metabolic homeostasis, leading to hepatic lipid deposition and inflammation in ovariectomized (OVX) as well as in liver-specific ERα knockout (LERKO) females [10,24].

Such a female-specific and ERα-dependent ability to counteract MASLD may be likely the consequence of the strict interplay between metabolism and reproduction gained during evolution

by the female liver [23] and aimed to modulate the hepatic metabolism according to nutrient availability and to the energy requirements characterizing each reproductive stage [25]. The peculiar role exerted by ERα in the female liver contributes to sex differences in the regulation of hepatic metabolism under several nutritional and fasting conditions [10,26].

Given the nutritional regulation of PNPLA3 and given the interaction between ERα and PNPLA3 I148M variant in women with MASLD [13,19], studies with mice of both sexes carrying this genetic variant may represent a useful research tool for investigating the sex-specific role of PNPLA3 in MASLD pathogenesis.

Several animal models, including PNPLA3 I148M knock-in (KI) mice, which carry the human PNPLA3 I148M variant, have been developed to elucidate the role of PNPLA3 I148M variant in hepatic triglyceride (TG) metabolism and its association with MASLD.

PNPLA3 I148M KI mice had normal levels of hepatic fat on a chow diet, but under dietary challenges (i.e. high-sucrose or high-fat diet) develop hepatic steatosis, inflammation, and fibrosis, recapitulating key features of human MASLD/MASH [27]. The increased in liver fat in PNPLA3 I148M KI mice is associated with the accumulation of catalytically inactive PNPLA3 on the surfaces of lipid droplets (LDs) [27]. Studies using these mice have revealed that the I148M variant disrupts ubiquitylation and proteasomal degradation of PNPLA3, leading to accumulation of PNPLA3 I148M and impaired mobilization of TG from LDs resulting in aberrant lipid accumulation in the liver [28].

The I148M variant acts as a loss-of-function mutation, disrupting the mobilization of poly-unsaturated fatty acids (PUFAs) from intracellular TG pools, thus impairing the hepatic secretion of very-large low-density lipoproteins [29]. These last findings derive from a study where several mouse models (PNPLA3 I148M KI mice; mice overexpressing PNPLA3 I148M; liver KO *Pnpla3* mice; ASO-treated mice) have been used, without performing sex-based comparative analysis.

Although the majority of the studies on PNPLA3 I148M KI mice has been limited to males, research indicates that there are sex-specific differences in the impact of PNPLA3 I148M variant on liver metabolism and disease progression, pointing to the relevance of this variant in accounting for sexual dimorphism in MASLD development and progression [18]. As occurs for women, female mice carrying the PNPLA3 I148M variant tend to have higher liver fat content compared to PNPLA3 I148M KI males after 4 weeks on a high sucrose diet [27]. In another report, PNPLA3 I148M KI mice of both sexes were studied to investigate their susceptibility to MASLD; however, the analysis of differences between the two sexes is not suitable since PNPLA3 I148M KI females were fed with a high-sucrose diet (70%) for 15 weeks, while PNPLA3 I148M KI males were fed with a NASH-inducing diet for 26 weeks [30].

Several other metabolic parameters such as glucose metabolism, insulin sensitivity, lipid profiles, and gene expression profiles related to lipid metabolism, inflammation, and fibrosis may vary between male and female PNPLA3 I148M KI mice, potentially influencing the development and progression of MASLD in a sex-specific manner.

Further research is needed to uncover the intricate molecular mechanisms by which the PNPLA3 I148M variant contributes to MASLD pathogenesis. Studies with mice of both sexes carrying the PNPLA3 I148M variant may represent a valuable tool to elucidate the sex-specific role of PNPLA3 I148M in MASLD and to disentangle the contribution of genetic and hormonal drivers accounting for the sexually dimorphic nature of MASLD. Additionally, exploring sex-specific potential therapeutic strategies targeting PNPLA3 and its downstream effectors in the context of MASLD holds promise for the development of therapeutic strategies useful to hamper MASLD in both sexes, even also according to hormonal status.

Conclusion

MASLD is a progressive wide spectrum pathology that involves several factors; among them, gene variants and sex hormones play a critical role. Here, we tried to summarize the sex differences reported in the role of *PNPLA3* I148M variant on disease progression, reviewing recent literature from the perspective of clinical, *in vitro* and *in vivo* studies. Particularly, results obtained so far pave the way for future precision medicine approaches targeted to women or in individual with higher estrogen levels carrying *PNPLA3* I148M variant. However, to elucidate the underlying mechanisms and extend them to other genetic variants, further studies will need to be conducted in which males and females are included as separate groups and compared with each other.

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Key points

- Metabolic dysfunction-associated steatotic liver disease (MASLD) prevalence and incidence are higher in men then in pre-menopausal women, while tend to become more common in women after menopause.
- Women carrying *PNPLA3* I148M variant affected by MASLD show a larger impact in all liver damage outcomes than men.
- Female mice show higher hepatic *Pnpla3* expression during the follicular phase of the cycle characterised by high estradiol levels than during the luteal phase and then in males.
- Hepatic *in vitro* model carrying *PNPLA3* I148M variant show lipid droplet accumulation and collagen deposition when exposed to estrogen receptor a agonists.

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Figure legend

Figure 1. Recent advances indicate that carrying the PNPLA3 I148M variant can be a risk factor for MASLD and accounts for sex differences in MASLD susceptibility, progression and outcomes. To elucidate the molecular mechanisms underlying the sex-specific role of PNPLA3 I148M in the pathophysiology of MASLD, further clinical and pre-clinical studies with *in vitro* and *in vivo* models are needed.