Sex and Menopause Modify the Effect of Single Nucleotide Polymorphism Genotypes on Fibrosis in NAFLD

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The development of fibrosis in nonalcoholic fatty liver disease (NAFLD) is influenced by genetics, sex, and menopausal status, but whether genetic susceptibility to fibrosis is influenced by sex and reproductive status is unclear. Our aim was to identify metabolism-related single nucleotide polymorphisms (SNPs), whose effect on NAFLD fibrosis is significantly modified by sex and menopausal status. We performed a cross-sectional, proof-of-concept study of 616 patients in the Duke NAFLD Clinical Database and Biorepository. The primary outcome was nonalcoholic steatohepatitis–Clinical Research Network (NASH–CRN) fibrosis stage. Menopause status was self-reported; age 51 years was used as a surrogate for menopause in patients with missing menopause data. The Metabochip was used to obtain 98,359 SNP genotypes in known metabolic pathway genes for each patient. We used additive genetic models to characterize sex and menopause-specific effects of SNP genotypes on NAFLD fibrosis stage. In the main effects analysis, none of the SNPs were associated with fibrosis at $P < 0.05$ after correcting for multiple comparisons. Twenty-five SNPs significantly interacted with sex/menopause to affect fibrosis stage (interaction $P < 0.0001$). After removal of loci in linkage disequilibrium, 10 independent loci were identified. Six were in the following genes: *KCNIP4* (potassium voltage-gated channel interacting protein 4), *PSORS1C1* (psoriasis susceptibility 1 candidate 1), *KLHL8* (Kelch-like family member 8), *GLRA1* (glycine receptor alpha 1), *NOTCH2* (notch receptor 2), and *PRKCH* (protein kinase C eta), and four SNPs were intergenic. In stratified models, four SNPs were significant in premenopausal and postmenopausal women, three only in postmenopausal women, two in men and postmenopausal women, and one only in premenopausal women. *Conclusion:* We identified 10 loci with a significant sex/menopause interaction with respect to fibrosis. None of these SNPs were significant in all sex/menopause groups, suggesting modulation of genetic susceptibility to fibrosis by sex and menopause status. Future studies of genetic predictors of NAFLD progression should account for sex and menopause. (*Hepatology Communications* 2021;5:598-607).

Nonalcoholic fatty liver disease (NAFLD) is a significant global health concern. One-quarter of adults worldwide are estimated to have NAFLD.[1] About 25% of patients with NAFLD have nonalcoholic steatohepatitis (NASH), characterized by progressive hepatocyte damage and hepatic inflammation. Patients with NASH develop hepatic fibrosis, and eventually cirrhosis and hepatocellular carcinoma, but at variable rates. NAFLD-related cirrhosis is a rapidly growing cause of

Abbreviations: BMI, body mass index; GLRA1, glycine receptor alpha 1; GWAS, genome-wide association study; KCNIP4, potassium voltage-gated channel interacting protein 4; KLHL8, Kelch-like family member 8; LD, linkage disequilibrium; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NASH–CRN, nonalcoholic steatohepatitis-Clinical Research Network; NOTCH2, notch receptor 2; PC, principal component; PNPLA3, *patatin-like phospholipase domain-containing protein 3;* PRKCH, *protein kinase C eta;* PSORS1C1, *psoriasis susceptibility 1 candidate 1; SNP, single nucleotide polymorphism;* TM6SF2, *transmembrane 6 superfamily member 2; VLDL, very low density lipoprotein.*

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orthotopic liver transplantation (OLT) in the United States and is expected to become the most common OLT indication.[2] It is also associated with increased risk for cardiovascular disease, higher all-cause mortality, and a shortened life span, and represents a significant health care burden.[3]

NAFLD is a multifactorial disease, and its progression is likely affected by both genetic and environmental (or acquired) factors. Many single nucleotide polymorphisms (SNPs) have been associated with NAFLD, NASH, hepatic fibrosis, and/or hepatocellular carcinoma. However, identified SNPs and their effect sizes vary from study to study, likely due to differences in study populations. rs738409 in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene and rs58542926 in the transmembrane 6 superfamily member 2 (*TM6SF2*) gene have been associated with presence of NAFLD, NASH histology, and hepatic fibrosis, findings that have been replicated in independent and diverse cohorts.[4-10] Of note, both of these SNPs are implicated in lipid metabolism; *TM6SF2* plays a role in hepatic very low density lipoprotein (VLDL) export as well as intestinal lipid clearance,[11-13] whereas *PNPLA3* possesses lipase and retinyl-esterase activities and regulates triglyceride accumulation in the hepatocyte as well as hepatic stellate cell activation and matrix remodeling.[14] One large genome-wide association study (GWAS) found an association between NASH activity score and rs2645424 on farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*), an enzyme involved in cholesterol biosynthesis.[15] Multiple SNPs in the choline metabolism pathway have also been associated with NAFLD severity.[16] SNP genotyping may improve existing clinical prediction models for NAFLD progression and guide a personalized therapeutic approach, providing valuable information for a disease in which risk stratification and treatment continue to be challenging.

Sex and sex hormones modulate key mechanisms involved in NAFLD pathobiology.[17] Insulin resistance, hepatic lipid influx, and hepatic adaptation to lipids (e.g., regional fat distribution, visceral adiposity, low-grade systemic inflammation, sarcopenia, hepatic beta-oxidation and triglyceride synthesis in the liver) are all affected by sex and sex hormones.[18,19] We hypothesize that SNPs associated with metabolic traits affect NAFLD progression in sex and menopausal status-specific manners. We expect the effects of metabolism-related SNPs on NAFLD will differ significantly based on the patient's sex and menopausal status (i.e., potential effect modifiers) (Fig. 1). Supporting our hypothesis, rs738409 on PNPLA3 has been found to have sex-specific effects on disease progression in primary sclerosing cholangitis,[20] and a recent study of about 18,000 Taiwanese subjects demonstrated that autosomal genetic effects on various metabolic traits are sexually dimorphic and are influenced by a proxy of menopause.[21] Thus, the specific aim of this proof-of-concept study was to identify metabolism-related SNPs whose effect on hepatic fibrosis in NAFLD is significantly modified by sex and menopausal status. As our focus was on SNPs linked to metabolism, the present study was not a GWAS, but rather a targeted analysis of metabolism-related SNPs.

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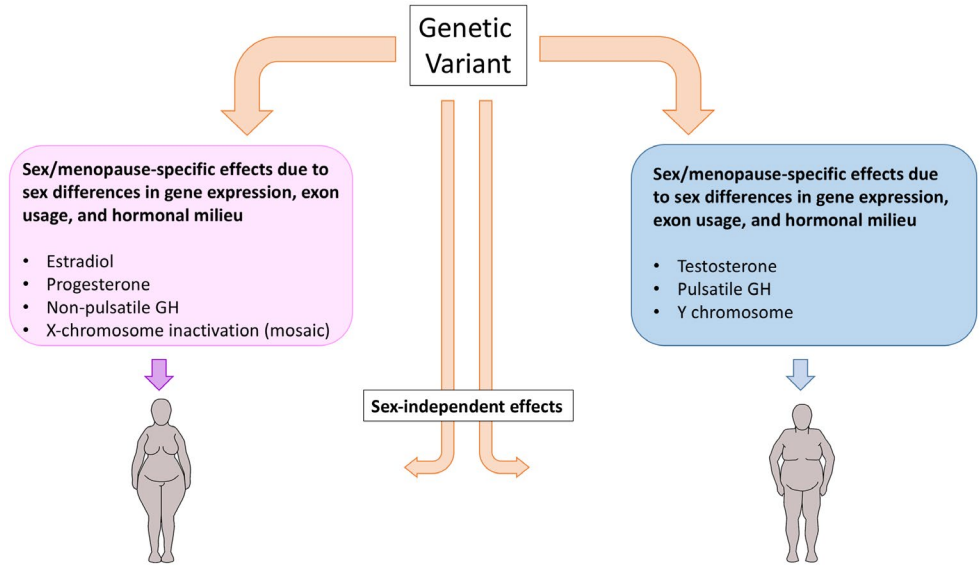

FIG. 1. Sex and reproductive status modify the effects of genetic variants on disease severity. The liver is a sexually dimorphic organ. Due to sex differences in gene expression, exon use, and hormonal milieu, men and women exhibit diverse gene expression despite the same genetic code, which may exert different effects on the disease severity, by sex and reproductive status. Abbreviation: GH, growth hormone.

Methods

STUDY POPULATION

We used DNA samples and data from the Duke University Health System NAFLD Clinical Database and Biorepository, which is an ongoing clinical database study established in 2005.[22] Written, informed consent was obtained from all participants before study enrollment. Blood samples, liver tissue, and clinical information were collected at the time of liver biopsy or bariatric surgery in a standardized manner. Patients with a history of alcohol abuse or significant alcohol use (more than 14 servings per week for men and more than 7 servings for women), or serologic or histologic evidence of other chronic liver diseases (e.g., viral hepatitis, primary biliary cirrhosis, autoimmune hepatitis, hemochromatosis) were excluded. Patients separately consented for genomic analysis at the time of study entry. This study was approved by the Duke University Institutional Review Board and is conducted in accordance with the Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects.

CLINICAL DATA AND OUTCOMES

The primary outcome was fibrosis stage (NASH–CRN scoring system).[23] Demographics and clinical data including age, sex, self-reported race/ethnicity, body mass index (BMI), diagnosis of diabetes, and histologic features were retrieved from the database. The population was classified into men, premenopausal women, and postmenopausal women based on self-reported surgical history, menstruation, and reproduction data. Natural menopause was defined as the lack of menstruation for 12 continuous months. Reported bilateral oophorectomy was considered surgical menopause. Self-reported regular or irregular menstruation was considered premenopausal. When the menopause category was uncertain (n = 180 of 406 women [44%]), an age of 51 years, the average age at menopause in the United States, was used to define a menopausal state.[24]

SNP ASSAYS AND GENOTYPES

DNA was extracted from whole-blood samples and was stored at −80°C as a part of the database study. Metabolism-related SNPs were analyzed using the Metabochip (consortium version; Illumina,

San Diego, CA).[25] Briefly, the Metabochip was designed to enable cost-effective replication and fine-mapping of 217,965 loci previously associated with cardiometabolic phenotypes including type 2 diabetes, coronary artery disease, and myocardial infarction, as well as related traits like BMI, glucose and insulin levels, lipid levels, and blood pressure. We chose to focus our analysis on these loci, as well as SNPs in the major histocompatibility complex, with minor allele frequency >5% and Hardy-Weinberg equilibrium P value > 10^{-6} due to the limited size of our sample. As such, 98,359 SNPs remained in our targeted analysis. Only SNPs on autosomal chromosomes were included in this analysis. GenomeStudio software was used to determine SNP genotypes. Samples were required to have a call rate >99% and no gender discrepancy to pass initial quality control. Identity by state analysis was performed using PLINK[26]; duplicate samples and first-degree relatives were subsequently removed.

STATISTICAL ANALYSIS

Clinical characteristics were compared among the sex/menopause categories using analysis of variance or chi-square tests in SAS v9.4 (SAS Institute, Cary, NC). To classify the population based on SNP profiles, independent of self-reported race/ethnicity, we performed principal component analysis using EIGENSOFT and evaluated resulting eigenvectors in relation to the self-reported race/ethnicity.[27] Five principal components (PCs) were deemed necessary to account for population structure in the genetic data and were subsequently included as covariates in all statistical models.

We used additive genetic models to characterize sex and menopause-specific effects of SNP genotypes on NAFLD fibrosis stage. The proportional odds assumption was tested before the analysis. We performed ordinal logistic regression (outcome: fibrosis stage 0, 1, 2, 3, 4) when the proportional odds assumption was met, using an R plug-in (R package ordinal) to PLINK.[28] When the proportional odds assumption was not met, binary logistic regression (outcome: fibrosis stage 0-1 or 2-4) was performed using PLINK. SNP as a primary predictor, age, five PCs, sex/menopausal categories as covariates, and the interaction term (SNP*sex/menopausal categories) were included in the models. We elected not to include BMI or the diagnosis of diabetes mellitus as covariates in the models, because we intended to identify SNPs associated with such metabolic traits that are impactful on hepatic fibrosis in NAFLD in a sex/menopausal status-specific manner. To reduce redundancy among SNPs, we performed linkage disequilibrium (LD) clumping in PLINK using previously published thresholds (p1 = 1, p2 = 1, r^2 = 0.25, 500-kb window).[29] Statistical significance was defined by P value < 0.0001 for the interaction between SNP genotype and sex/menopause, without adjusting for multiple comparisons. When significant interactions between SNPs and sex/menopausal categories were identified, separate models including age and five PCs as covariates were developed for men, premenopausal women, and postmenopausal women to compute sex/menopausal status-specific estimates. The main effect of SNPs on fibrosis was assessed in models including age, five PCs, and the sex/menopausal categories as covariates.

As a secondary analysis, we computed the effects of SNPs that are reportedly associated with fibrosis in the literature in premenopausal women, postmenopausal women, and men separately. For this secondary analysis, the SNPs were identified using a MeSH search with the terms "polymorphism, single nucleotide" and "no-alcoholic fatty liver disease." Peer-reviewed articles associating SNPs with NAFLD severity (histology, fibrosis, or other clinical parameters) were included. We used ordinal or binary logistic regression models as appropriate, including age and PCs as covariates. We did not adjust for multiple comparisons in this secondary analysis due to the descriptive nature of the analysis.

Results

BASELINE CHARACTERISTICS

A total of 624 subjects in the biorepository and clinical database with biopsy-proven NAFLD had SNP data evaluated. Of these, 8 were excluded due to poor quality of SNP data. Therefore, 616 subjects were included in the final analysis: 216 premenopausal women, 190 postmenopausal women, and 210 men. Clinical characteristics of the study population are given in Table 1. As expected, the mean age was

higher in postmenopausal women (41 ± 8 years for premenopausal women, 58 ± 6 years for postmenopausal women, and 48 ± 11 years for men). Self-reported Caucasian race was more prevalent among men (88%), compared with premenopausal women (78%) and postmenopausal women (84%). Fibrosis stage was higher for postmenopausal women compared to premenopausal women, with 23% of postmenopausal women having advanced fibrosis (stage 3-4), compared to only 9% of premenopausal women. Of women in whom the age surrogate of 51 years was applied for unknown menopause status (n = 180), 64 women (36%) were age 45 and younger, 55 women were between age 46 and 49, 24 women (13%) were between the ages of 50 and 52, and 37 women (21%) were age 55 and older.

IDENTIFICATION OF METABOLISM-RELATED SNPs THAT EXERT SEX/MENOPAUSE-SPECIFIC EFFECTS ON NAFLD FIBROSIS

Of the 98,359 SNPs evaluated, 97,488 SNPs met the proportional odds assumption, while 871 SNPs did not. We identified 25 SNPs that significantly interact with the sex/menopause group to affect fibrosis stage (interaction $P < 0.0001$). After performing LD clumping to remove genomic correlation, 10 independent results remained, the most significant of which was rs12501548 in the potassium voltage-gated channel interacting protein 4 (*KCNIP4*) gene ($P = 3.97 \times 10^{-6}$, q = 0.1634) (Table 2). Other significant SNPs reside in the following genes: psoriasis susceptibility 1 candidate 1 (*PSORS1C1*), Kelch-like family member 8 (*KLHL8*), glycine receptor alpha 1 (*GLRA1*), notch receptor 2 (*NOTCH2*), and protein kinase C eta (*PRKCH*). Four of the 10 significant SNPs were intergenic. All 10 models satisfied the proportional odds assumption. When we stratified these models by sex/menopause group, we observed that four SNPs were significant in premenopausal and postmenopausal women, three SNPs were significant only in postmenopausal women, two were significant in men and postmenopausal women, and one was significant only in premenopausal women.

SNPs were also assessed for main effect on fibrosis in the entire population, adjusting for age, five PCs, and the sex/menopausal categories. In the main effect analysis, none of the SNPs were associated with fibrosis at $P < 0.05$ after correcting for multiple comparisons.

TABLE 1. CLINICAL CHARACTERISTICS OF THE STUDY POPULATION BY SEX AND MENOPAUSAL STATUS

	Men (n = 210)	Premenopausal (n = 216)	Postmenopausal (n = 190)	*P* Value
Age, years	48 ± 11	41 ± 8	58 ± 6	<0.001*
White race, %	88	78	84	0.02†
BMI, kg/m^2	38 ± 9	45 ±10	40 ± 10	<0.001*
Diabetes mellitus, %	40	38	51	0.02†
Hypertension, %	72	60	80	<0.0001†
Fibrosis stage				<0.0001†
Stage 0	21	30	26	
Stage 1	32	44	28	
Stage 2	29	17	23	
Stage 3	17	9	17	
Stage 4	1	0	6	
Lipid panel				
Total cholesterol	182 ± 44	190 ± 49	194 ± 48	0.05*
Triglycerides	173 ± 97	167 ± 114	162 ± 109	0.28*
HDL	37 ± 13	41 ± 15	45 ± 17	<0.0001*

Note: Data are presented as mean ± SD for continuous variables or a percentage for categorical variables.
*Kruskal-Wallis test.
†Cochran-Mantel-Haenszel test.

TABLE 2. METABOLISM-RELATED SNPs WHOSE EFFECTS ON NAFLD FIBROSIS ARE SIGNIFICANTLY MODULATED BY SEX AND/OR MENOPAUSE

			Interaction		Men (n = 210)		Premenopausal Women (n = 216)		Postmenopausal women (n = 190)	
Chr	SNP Probe	Gene	*P* Value	FDR q-Value	Beta	*P* Value	Beta	*P* Value	Beta	*P* value
4	rs12501548	*KCNIP4*	3.97×10^{-6}	0.23	–0.35	0.06	**–0.75 ↓**	**4.5×10^{-5}**	**0.57 ↑**	**0.003**
6	rs3778638	*PSORS1C1*	1.15×10^{-5}	0.24	0.33	0.19	0.48	0.06	**–1.08 ↓**	**1.64×10^{-4}**
4	chr4:88356546	*KLHL8*	2.4×10^{-5}	0.24	**–0.45 ↓**	**0.01**	0.38	0.05	**0.62 ↑**	**0.001**
20	rs742748	Intergenic	2.66×10^{-5}	0.24	**0.38 ↑**	**0.04**	–0.25	0.19	**–0.78 ↓**	**7.83×10^{-5}**
5	rs2071220	*GLRA1*	3.48×10^{-5}	0.24	0.26	0.13	**–0.39 ↓**	**0.04**	**–0.67 ↓**	**5.61×10^{-4}**
6	rs10499276	Intergenic	4.58×10^{-5}	0.24	–0.47	0.06	**0.62 ↑**	**0.02**	**–1.07 ↓**	**8.39×10^{-4}**
3	rs349715	Intergenic	4.74×10^{-5}	0.24	–0.13	0.52	**–0.83 ↓**	**4.26×10^{-4}**	**0.47 ↑**	**0.03**
1	rs4659248	*NOTCH2*	5.17×10^{-5}	0.24	–0.18	0.41	–0.24	0.27	**0.80 ↑**	**0.001**
12	rs2358942	Intergenic	5.32×10^{-5}	0.35	–0.51	0.12	**1.04 ↑**	**0.002**	–0.74	0.051
14	rs1033910	*PRKCH*	8.72×10^{-5}	0.23	0.37	0.051	0.04	0.85	**–0.59 ↓**	**0.005**

Note: Estimates are computed after LD clumping, to reduce redundancy among SNPs (see "Methods"). Bold-faced values indicate statistical significance. Negative beta indicates a protective effect (green), whereas positive beta indicates a detrimental effect (orange) with respect to fibrosis.
Abbreviation: Chr, chromosome.

EVALUATION OF SNPs FROM THE LITERATURE

Of the 102 SNPs identified as being associated with NAFLD presence, histologic severity, or fibrosis by our search of the literature, 16 were included on the Metabochip and were tested for association with fibrosis stage in each sex/menopause group separately.[30-42] Four SNPs were nominally significant ($P < 0.05$) in at least one sex/menopause group: two SNPs in premenopausal women only, one SNP in women only (premenopausal and postmenopausal), and one in men and postmenopausal women only. These results are found in Table 3. Notably, rs2076211 in *PNPLA3*, previously strongly identified with NAFLD prevalence,[38] was significant in men and postmenopausal women (but not premenopausal women), whereas rs58542926 in *TM6SF2*, associated with NASH susceptibility,[40] was significant in premenopausal and postmenopausal women but not men. Several important variants, including rs738409 on *PNPLA3*, are not included in the Metabochip; therefore, these were not evaluated.

Discussion

Our analysis demonstrated that among 98,359 metabolism-related SNPs, 25 SNPs (10 independent loci after LD clumping) were significantly associated with fibrosis stage in a sex/menopausal status-specific manner. Some of the SNPs showed opposite effects on hepatic fibrosis, depending on the patient's sex and menopausal status. Of note, none of these SNPs displayed a significant main effect ($P < 0.05$) on hepatic fibrosis. Our analysis also demonstrated that four of the 16 SNPs associated with hepatic fibrosis in the literature that were included in the Metabochip showed sex and menopausal status–dependent effects. Despite the preliminary nature of this proof-of-concept analysis, these findings strongly support our hypothesis that metabolism-related genetic variants affect NAFLD progression in sex and menopausal status–specific manners. Our results also highlight the importance of consideration of sex/menopause in investigating risk factors for progression in NAFLD and in future GWAS studies, given the robust evidence to suggest multifaceted sexual dimorphism in NAFLD.

The genes harboring SNPs associated with fibrosis in sex/menopausal status–specific manners include

TABLE 3. SEX/MENOPAUSAL STATUS-SPECIFIC EFFECTS ON SNPs ASSOCIATED WITH NAFLD FIBROSIS IN THE LITERATURE

			All (n = 616)		Men (n = 212)		Premenopausal Women (n = 216)		Postmenopausal Women (n = 190)	
Chr	SNP	Gene	Beta	*P* Value	Beta	*P* Value	Beta	*P* Value	Beta	*P* Value
1	rs2228145	IL6R	0.21	0.06	0.16	0.34	0.11	0.60	0.28	0.17
1	rs1342387	ADIPOR1	0.002	0.99	–0.05	0.78	0.17	0.37	–0.02	0.91
2	rs780094	GCKR	–0.026	0.80	–0.14	0.44	0.09	0.62	–0.06	0.75
3	rs1801282	PPARG	–0.15	0.41	0.13	0.66	–0.31	0.39	–0.38	0.25
3	rs2101247	SCAP	–0.009	0.93	–0.10	0.56	0.29	0.12	–0.17	0.35
3	rs3774261	ADIPOQ	–0.16	0.12	–0.28	0.11	0.15	0.40	–0.34	0.10
4	rs2231142	ABCG2	–0.14	0.42	–0.08	0.78	0.21	0.49	–0.46	0.13
6	rs1800562	HFE	0.33	0.13	–0.38	0.33	**0.99**	**0.01**	0.29	0.41
6	rs762623	CDKN1A	0.04	0.81	–0.34	0.19	0.36	0.24	0.11	0.71
8	rs4240624	LOC157273	**0.39**	**0.01**	0.51	0.05	0.42	0.11	0.43	0.13
10	rs717620	ABCC2	0.02	0.88	0.39	0.08	–0.45	0.10	–0.02	0.93
10	rs2862954	ERLIN1	–0.16	0.15	0.05	0.79	–0.20	0.29	–0.37	0.06
10	rs7903146	TCF7L2	0.05	0.66	–0.12	0.53	0.05	0.82	0.20	0.35
19	rs2228603	NCAN	**0.57**	**0.001**	0.19	0.51	**1.12**	**0.001**	0.52	0.09
19	rs58542926	TM6SF2	**0.63**	**0.0003**	0.32	0.27	**0.84**	**0.009**	**0.72**	**0.02**
22	rs2076211	PNPLA3	**0.47**	**6.70×10^{-5}**	**0.49**	**0.01**	0.36	0.10	**0.63**	**0.003**

Note: Estimates are computed in multiple ordinal logistic or logistic regression models, adjusting for age and five PCs. Bold-faced text indicates statistical significance. Positive beta indicates a detrimental effect, while negative beta indicates a protective effect.
Abbreviation: Chr, chromosome.

PSORS1C1, *GLRA1*, *NOTCH2*, *PRKCH*, *KLHL8*, and *KCNIP4*. Differences observed in the effects of the SNPs among the sex/menopause categories vary significantly among the loci. Some variants exhibited sex-specific effects: opposite direction in men versus women, or women-specific effects showing a consistent or an opposite trend in premenopausal versus postmenopausal women. A few SNPs were only significantly associated with hepatic fibrosis in postmenopausal women.

The potential mechanism of the association between many of the genes represented by these SNPs and fibrosis in patients with NAFLD has yet to be elucidated. However, a few aspects are noteworthy for discussion. *NOTCH2* has been implicated in NAFLD pathogenesis and progression. NASH severity and treatment response are associated with Notch signaling in the liver.[43] Activation of Notch in mouse models of NAFLD induces fibrosis by increasing SRY (sex determining region Y)-box 9–dependent osteopontin expression and secretion from hepatocytes.[20] Further, Notch signaling is known to have metabolic effects, increasing intrahepatic triglyceride synthesis.[44] Thus, there is evidence that Notch signaling has pleiotropic effects on NAFLD progression, ranging from dysregulation of metabolism to fibrogenesis. Intriguingly, our analysis showed that a SNP in *NOTCH2* was significantly associated with hepatic fibrosis only in postmenopausal women. The precise mechanisms underlying this association remain unclear. No studies investigating the interplay between hepatic *NOTCH2* and estradiol were found in the literature; however, there is one study showing the downregulation of *NOTCH2* by estradiol in human umbilical vein endothelial cells.[45] Further studies are needed to investigate Notch signaling in NAFLD, accounting for possible sexual dimorphism and modification by physiological estrogen levels.

PSORS1C1 is implicated in susceptibility to psoriasis, systemic sclerosis, and other autoimmune diseases, and has been linked to hepatocellular carcinoma through *NM23*, a metastasis suppressor gene.[46] No previous studies identified this gene or related SNPs as a risk factor of NAFLD, but a sex-specific association with psoriasis has been suggested in the literature.[47] In our analysis, SNPs in *KLHL8* showed beneficial

effects among men but detrimental effects among postmenopausal women, with a similar trend among premenopausal women. *KLHL8* is involved in protein ubiquitination,[23] and its expression level in peripheral blood mononuclear cells has been negatively associated with serum triglycerides and BMI, although there have been no studies to date that account for sex/menopausal status.[48] Of note, the SNPs that emerged from our analysis include both exonic and intronic variants. Intronic SNPs may have important implications in NAFLD, as they can in other complex diseases. There are many potential mechanisms for the impact of intronic SNPs on a complex phenotype. First, the SNP could be in linkage disequilibrium with a coding variant; therefore, the association we observe could in fact reflect an association impacting the protein. Likewise, the intronic variant could lie in a splice site and therefore alter expression of the gene. Finally, the intronic SNP could have regulatory function and act as an enhancer or repressor. Additional functional work will be needed to uncover the true mechanisms.

Despite a current lack of known causal connections, our findings have an implication for future research in the field. A body of evidence suggests that NAFLD pathogenesis and outcomes are influenced by sex and menopause.[18,19] Furthermore, a study using RNA sequencing data from humans, chimpanzees, and rhesus macaques demonstrated that expression of many metabolism-related genes, especially those involved in lipid metabolism, RNA processing, and key pathways in NAFLD (e.g., JNK, Wnt signaling) was conserved and sexually dimorphic. Genes related to immune responses were enriched among genes with conserved sexually dimorphic exon use.[49] Thus, genomic variants in these specific functional categories may exert different effects on men and women's health and diseases.

There are several limitations to our study. This was a single-center study, and our cohort was predominantly Caucasian. Our study population was relatively small for analyzing effect modifications by sex and menopause in a SNP array and too small for evaluating high-level interactions (e.g., sex/menopause *SNP* other metabolic traits, sex/menopause *SNP* race/ethnicity). The SNPs analyzed in the study are limited to metabolism-related genes and did not include genes on sex chromosomes. We were also not able to analyze several well-established variants (such as rs738409 in *PNPLA3*), as these were not included on the Metabochip. Finally, menopause status in this study was categorized using self-reported reproductive status or age surrogate when the information was not available (44% of female subjects). This may have led to misclassification. However, of the women with unknown menopause status, only 13% were between the ages of 50 and 52 years, near the age cutoff.

In summary, our findings suggest that sex and menopause modify the effects of metabolic traits on NAFLD severity. No variants showed significant associations with hepatic fibrosis in the entire population. Thus, it is critical to formulate an analytic approach considering possible sex differences and modulation by reproductive status, when investigating contribution of metabolic traits to NAFLD. Incorporating sex and menopause status may elucidate sex-specific mechanisms that allow further risk stratification of patients with NAFLD and promote individualized disease management.

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