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Genetic variants and metabolic diseases

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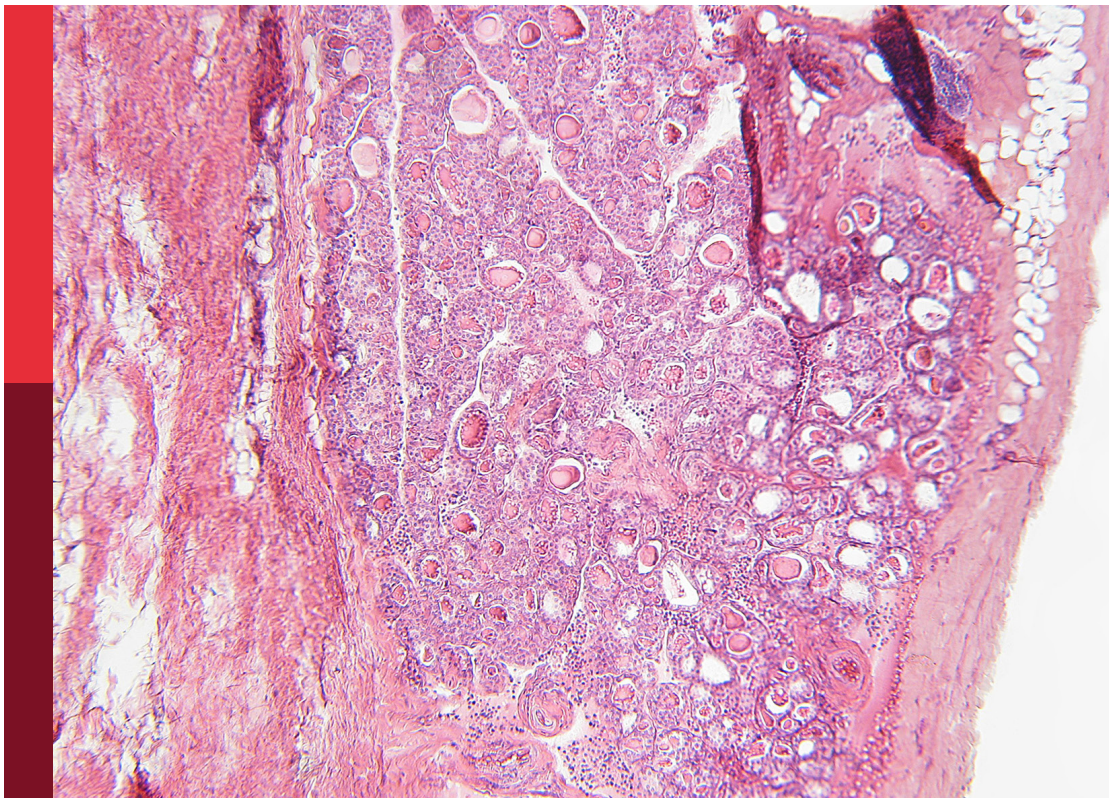

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Genetic variants and metabolic diseases

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SYSTEMATIC REVIEW
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Correlation of Adiponectin Gene Polymorphisms *rs266729* and *rs3774261* With Risk of Nonalcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis

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Background: Increasing evidence has suggested an association of adiponectin gene polymorphisms rs1501299, rs2241766, rs266729 and rs3774261 with risk of nonalcoholic fatty liver disease (NAFLD). This correlation has been extensively meta-analyzed for the first two polymorphisms, but not the second two.

Methods: The PubMed, EMBASE, Google Scholar, and China National Knowledge Infrastructure databases were searched for relevant literature. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.

Results: A total of 10 case-control studies on rs266729 (2,619 cases and 1,962 controls) and 3 case-control studies on rs3774261 (562 cases and 793 controls) were included. Meta-analysis showed that rs266729 was associated with significantly higher NAFLD risk based on the following five models: allelic, OR 1.72, 95% CI 1.34-2.21, $P < 0.001$; recessive, OR 2.35, 95% CI 1.86-2.95, $P < 0.001$; dominant, OR 1.84, 95% CI 1.34-2.53, $P < 0.001$; homozygous, OR 2.69, 95% CI 1.84-3.92, $P < 0.001$; and heterozygous, OR 1.72, 95% CI 1.28-2.32, $P < 0.001$. This association between rs266729 and NAFLD risk remained significant for all five models among studies with Asian, Chinese and Caucasian samples. The rs2241766 polymorphism was associated with significantly higher NAFLD risk according to the recessive model (OR 1.87, 95% CI 1.15-3.04, $P = 0.01$).

Conclusion: Polymorphisms rs266729 and rs3774261 in the adiponectin gene may be risk factors for NAFLD. These findings may pave the way for novel therapeutic strategies, but they should be verified in large, well-designed studies.

Keywords: adiponectin, polymorphism, nonalcoholic fatty liver disease, system review, meta-analysis

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), also known as metabolism-associated fatty liver disease (1), is rapidly becoming the most common liver disease worldwide. The primary characteristic of NAFLD is hepatocellular macrovesicular steatosis. NAFLD can progress to hepatic injury, which can range from simple steatosis or nonalcoholic steatohepatitis (NASH), to fibrosis, cirrhosis, and even hepatocellular carcinoma or end-stage liver disease (2–6). NAFLD and its progression have been linked to diet (7), insulin resistance (8, 9), lipotoxicity (10), inflammation (11, 12), genetic predisposition and increases in compounds produced by gut microbes (13, 14). Genetic factors, for example, can alter hepatic lipid metabolism. In this way, NAFLD is a complex metabolic state to which lifestyle and genetic factors contribute (15, 16).

Adiponectin is a protein specific to adipose tissue that regulates insulin sensitivity, glucose homeostasis, and lipid metabolism (17). Decreased levels of adiponectin in plasma are associated with NAFLD as well as obesity, type 2 diabetes, and coronary artery disease (18, 19). Adiponectin is encoded by the 16-kb *AMP1* gene on human chromosome 3q27, and it consists of three exons and two introns. Genetic and epigenetic changes in the adiponectin gene may reduce adiponectin levels in plasma and dysregulate hepatic lipid metabolism, which may help explain differences in NAFLD risk among individuals (20, 21). Thus, single-nucleotide polymorphisms (SNPs) in the adiponectin gene may alter levels of the protein in circulation, in turn affecting lipid metabolism and NAFLD risk.

The two adiponectin SNPs most thoroughly investigated for their association with NAFLD risk are rs2241766, which leads to genomic mutation T45G, and rs1501299, which leads to mutation G276T (22–31). Indeed, these two associations have been extensively reviewed and meta-analyzed (32–35). In contrast, much less is known about potential associations of the polymorphisms rs266729 (-11377C>G) and rs3774261 with NAFLD risk (36–47).

Thus, we meta-analyzed here the relevant literature on potential associations of rs266729 and rs3774261 with NAFLD risk.

MATERIAL AND METHODS

Search Strategy

The PubMed, EMBASE, Google Scholar, Web of Science and China National Knowledge Infrastructure (CNKI) databases were searched up to October 20, 2021 without language restrictions using the following search terms: (a) *adiponectin, ADIPOQ, APMI, -11377, -11377C>G, rs266729* and *rs3774261*; (b) those seven terms in combination with *polymorphisms, SNP, variant, variants, variation, genotype, genetic* or *mutation;* and (c) all of the above terms in combination with nonalcoholic fatty liver disease or NAFLD. Only studies involving humans were considered. Reference lists in original and review articles were searched manually to identify additional studies. In the case of multiple studies involving overlapping samples, only the largest study was retained.

Inclusion and Exclusion Criteria of the Studies

Studies were included if they met the following criteria: (a) studies had a case-control design to assess the association of adiponectin rs266729 or rs3774261 with NAFLD risk; (b) all patients were diagnosed with NAFLD based on the following diagnostic criteria: abnormal levels of aspartate aminotransferase and alanine aminotransferase persisting for at least 6 months, or evidence of fatty liver based on ultrasonography and/or evidence of diffuse fatty liver based on other imaging examinations, or liver histology; (c) the full text was available and it reported genotype frequencies in cases and controls, or sufficient data to estimate odds ratios (ORs) and 95% confidence intervals (CIs).

Studies were excluded if they: (a) were not a case-control study; (b) did not report precise genotypes; (c) were duplicate publications of data from the same study; (d) were meta-analyses, letters, reviews, or editorial articles; (e) investigated other polymorphisms of adiponectin.

Data Extraction

Two authors (YTZ and LYL) independently selected eligible studies and extracted the following data: first author's name, year of publication, ethnicity, country, sample size, type of controls, genotyping method, genotype distribution, *P* value for Hardy-Weinberg equilibrium among controls, and matched parameters.

Assessment of Methodological Quality

The quality of included studies was assessed independently by two investigators (YTZ and LYL) using the Newcastle–Ottawa Scale (48). Scores of 0-4 were considered to indicate poor methodological quality; scores of 5-9, high quality (49). Any disagreements about scoring were resolved through comprehensive reassessment by the other authors. Only high-quality studies were included in the meta-analysis.

Statistical Analysis

The strength of association of rs266729 and rs3774261 with NAFLD risk was calculated in terms of unadjusted ORs with 95% CIs based on genotype frequencies in cases and controls. The significance of pooled ORs was determined using the Z test, with $P < 0.05$ defined as significant. Meta-analysis was conducted using a fixed-effect model when $P > 0.10$ for the Q test, indicating lack of heterogeneity among studies; otherwise, meta-analysis was conducted using a random-effect model. All statistical tests for meta-analyses were performed using Review Manager 5.3 (Cochrane Collaboration). Publication bias was assessed using Begg's funnel plot and Egger's weighted regression in Stata 12.0 (Stata Corp, College Station, TX, USA), with $P < 0.05$ considered statistically significant.

RESULTS

Characteristics of Primary Studies

The search strategy retrieved 313 potentially relevant studies, 277 of which were excluded on the basis of titles and abstracts (**Figure 1**). Another 17 studies were excluded because they investigated other polymorphisms of the adiponectin gene, one study was excluded because it enrolled only cases (50), three studies were excluded because they were review articles (51–53), and one study was excluded because it did not report precise genotypes (30). Two publications were based on the same participants, so they were considered as one study (38, 54). Ultimately, 12 case-control studies (36–47) were included in the meta-analysis (**Table 1**).

Ten studies (36–45) focused on rs266729 and three (45–47) on rs3774261. The distribution of genotypes in controls was consistent with Hardy-Weinberg equilibrium in all but three studies (36, 42, 47). The mean Newcastle-Ottawa score for the 12 studies was 6.83 (range, 6-7). Thus the overall quality of the included studies was adequate.

Quantitative Data Synthesis

rs266729 and NAFLD Risk

Meta-analysis of data from 2,619 cases and 1,962 controls indicated that rs266729 was associated with significantly increased NAFLD risk according to the following five models: allelic, OR 1.72, 95% CI 1.34-2.21, $P < 0.001$; recessive, OR 2.35, 95% CI 1.86-2.95, $P < 0.001$; dominant, OR 1.84, 95% CI 1.34-2.53, $P < 0.001$; homozygous, OR 2.69, 95% CI 1.84-3.92, $P < 0.001$; and heterozygous, OR 1.72, 95% CI 1.28-2.32, $P < 0.001$ (**Table 2** and **Figures 2A–E**).

This association remained significant when we meta-analyzed only the eight studies involving 2,433 Asian cases and 1,776 Asian controls (36–43). Again, significance was obtained with all five models: allelic, OR 1.76, 95% CI 1.31-2.37, $P < 0.001$;

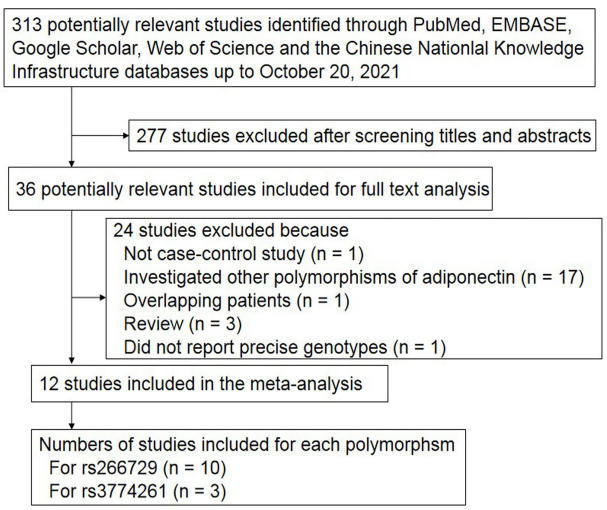

FIGURE 1 | Flowchart of study selection.

TABLE 1 | Characteristics of the included studies and genotype distributions.

First author	Year	Ethnicity	Country	Sample size		Genotyping method	*P* for HWE	Source of controls	No. of cases			No. of controls			NOS score	Matched parameters
				Cases	Controls											
rs266729									**CC**	**CG**	**GG**	**CC**	**CG**	**GG**		
Gupta (36)	2012	Asian	India	137	250	PCR	0.003	HB	77	53	7	156	92	2	7	Age, Sex, BMI
Hashemi (37)	2013	Asian	India	83	93	Tetra ARMS-PCR	0.107	PB	27	53	3	49	41	3	6	Undetermined
Ye (38)	2014	Asian	China	130	130	PCR-RFLP	0.458	HB	77	47	6	83	40	7	7	Age, Sex
Hsieh (39)	2015	Asian	Taiwan, China	350	209	TaqMan	0.545	T2DM without NAFLD	175	126	49	113	79	17	7	Age, Sex
Cheng (40)	2015	Asian	China	338	280	PCR	0.715	HB	158	149	31	164	102	14	7	Age, Sex, Height
Zhang (41)	2016	Asian	China	302	310	PCR-RFLP	0.619	HB	152	126	24	197	102	11	7	Age, Sex, Drinking, Smoking
Zhang (42)	2016	Asian	China	600	200	PCR-RFLP	<0.001	HB	180	202	218	143	28	29	7	Age, Sex, Ethnicity, Birthplace
Du (43)	2016	Asian	China	493	304	PCR	0.068	HB	278	175	40	219	73	12	7	Age, Sex
Mahmoud (44)	2019	Caucasian	Egypt	100	100	PCR	0.539	HB	38	44	18	47	45	8	7	Age, Sex
Hasan (45)	2021	Caucasian	Egypt	86	86	PCR-RFLP	0.236	HB	28	53	5	44	38	4	6	Age
rs3774261									**AA**	**AG**	**GG**	**AA**	**AG**	**GG**		
Zhang (46)	2012	Asian	China	119	350	PCR-RFLP	0.808	PB	41	50	28	107	171	72	7	Sex
Li (47)	2015	Asian	China	357	357	PCR-RFLP	0.044	HB	48	179	130	131	155	71	7	Age, Sex
Hasan (45)	2021	Caucasian	Egypt	86	86	PCR-RFLP	0.954	HB	11	55	20	39	38	9	6	Age

T2DM, diabetes mellitus type 2; NAFLD, nonalcoholic fatty liver disease risk; ARMS, amplification refractory mutation system; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; HB, hospital-based; PB, population-based; BMI, body mass index; HWE, Hardy-Weinberg equilibrium; NOS, Newcastle–Ottawa Scale.

TABLE 2 | Meta-analysis of associations of rs266729 or rs3774261 with risk of nonalcoholic fatty liver disease.

Genetic model	OR [95% CI]	Z (*P* value)	Heterogeneity of study design			Meta-analysis model
			χ^2	df (*P* value)	I^2 (%)	
Adiponectin rs266729 polymorphism						
Adiponectin rs266729 polymorphism in total population from 10 case control studies (36–45) (2,619 cases and 1,962 controls)						
Allelic model (G-allele vs. C-allele)	1.72 [1.34, 2.21]	4.26 (<0.001)	53.95	9 (<0.001)	83	Random
Recessive model (GG vs. CG + CC)	2.35 [1.86, 2.95]	7.25 (<0.001)	10.29	9 (0.33)	13	Fixed
Dominant model (CG + GG vs. CC)	1.84 [1.34, 2.53]	3.74 (<0.001)	55.59	9 (<0.001)	84	Random
Homozygous model (GG vs. CC)	2.69 [1.84, 3.92]	5.11 (<0.001)	18.42	9 (0.03)	51	Random
Heterozygous model (CG vs. CC)	1.72 [1.28, 2.32]	3.55 (<0.001)	42.95	9 (<0.001)	79	Random
Adiponectin rs266729 polymorphism in Asian population from 8 case-control studies (36–43) (2,433 cases and 1,776 controls)						
Allelic model (G-allele vs. C-allele)	1.76 [1.31, 2.37]	3.75 (<0.001)	53.01	7 (<0.001)	87	Random
Recessive model (GG vs. CG + CC)	2.38 [1.86, 3.03]	6.98 (<0.001)	9.48	7 (0.22)	26	Fixed
Dominant model (CG + GG vs. CC)	1.85 [1.28, 2.69]	3.26 (0.001)	54.56	7 (<0.001)	87	Random
Homozygous model (GG vs. CC)	2.70 [1.73, 4.23]	4.35 (0.001)	18.00	7 (0.01)	61	Random
Heterozygous model (CG vs. CC)	1.75 [1.23, 2.47]	3.15 (0.002)	41.11	7 (<0.001)	83	Random
Adiponectin rs266729 polymorphism in Chinese population from 6 case-control studies (38–43) (2,213 cases and 1,433 controls)						
Allelic model (G-allele vs. C-allele)	1.74 [1.20, 2.52]	2.94 (0.003)	52.49	5 (<0.001)	90	Random
Recessive model (GG vs. CG + CC)	2.35 [1.83, 3.01]	6.72 (<0.001)	7.01	5 (0.22)	29	Fixed
Dominant model (CG + GG vs. CC)	1.91 [1.21, 3.00]	2.78 (0.005)	50.92	5 (<0.001)	90	Random
Homozygous model (GG vs. CC)	2.58 [1.57, 4.24]	3.75 (<0.001)	16.55	5 (0.005)	70	Random
Heterozygous model (CG vs. CC)	1.79 [1.18, 2.73]	2.71 (0.007)	37.20	5 (<0.001)	87	Random
Adiponectin rs266729 polymorphism in Caucasian population from 2 case-control studies (44, 45) (186 cases and 186 controls)						
Allelic model (G-allele vs. C-allele)	1.55 [1.14, 2.10]	2.79 (0.005)	0.02	1 (0.90)	0	Fixed
Recessive model (GG vs. CG + CC)	2.07 [0.99, 4.30]	1.94 (0.05)	0.70	1 (0.40)	0	Fixed
Dominant model (CG + GG vs. CC)	1.74 [1.15, 2.63]	2.61 (0.009)	0.90	1 (0.34)	0	Fixed
Homozygous model (GG vs. CC)	2.51 [1.16, 5.44]	2.33 (0.02)	0.16	1 (0.68)	0	Fixed
Heterozygous model (CG vs. CC)	1.60 [1.04, 2.46]	2.14 (0.03)	1.80	1 (0.18)	45	Fixed
Adiponectin rs3774261 polymorphism						
Adiponectin rs3774261 polymorphism in total population from 3 case-control studies (45–47) (562 cases and 793 controls)						
Allelic model (G-allele vs. A-allele)	1.76 [0.98, 3.18]	1.86 (0.06)	22.72	2 (<0.001)	91	Random
Recessive model (GG vs. AG + AA)	1.87 [1.15, 3.04]	2.51 (0.01)	5.20	2 (0.07)	62	Random
Dominant model (AG + GG vs. AA)	2.55 [0.81, 7.98]	1.60 (0.11)	31.99	2 (<0.001)	94	Random
Homozygous model (GG vs. AA)	3.29 [0.97, 11.15]	1.91 (0.06)	22.63	2 (<0.001)	91	Random
Heterozygous model (AG vs. AA)	2.25 [0.75, 6.76]	1.45 (0.15)	26.19	2 (<0.001)	92	Random
Adiponectin rs3774261 polymorphism in Chinese population from 2 case-control studies (46, 47) (476 cases and 707 controls)						
Allelic model (G-allele vs. A-allele)	1.49 [0.66, 3.35]	0.97 (0.33)	19.79	1 (<0.001)	95	Random
Recessive model (GG vs. AG + AA)	1.70 [0.89, 3.25]	1.60 (0.11)	4.69	1 (0.03)	79	Random
Dominant model (AG + GG vs. AA)	1.78 [0.41, 7.68]	0.77 (0.44)	25.73	1 (<0.001)	96	Random
Homozygous model (GG vs. AA)	2.28 [0.48, 10.85]	1.03 (0.30)	19.02	1 (<0.001)	95	Random
Heterozygous model (AG vs. AA)	1.56 [0.39, 6.27]	0.63 (0.53)	20.11	1 (<0.001)	95	Random

OR, odds ratio; 95% CI, 95% confidence interval.

recessive, OR 2.38, 95% CI 1.86-3.03, $P < 0.001$; dominant, OR 1.85, 95% CI 1.28-2.69, $P = 0.001$; homozygous, OR 2.70, 95% CI 1.73-4.23, $P = 0.001$; and heterozygous, OR 1.75, 95% CI 1.23-2.47, $P = 0.002$ (**Table 2**).

Next, this association remained significant when we meta-analyzed only the eight studies involving 2,213 Chinese cases and 1,433 Chinese controls (38–43). Again, significance was obtained with all five models: allelic, OR 1.74, 95% CI 1.20-2.52, $P = 0.003$; recessive, OR 2.35, 95% CI 1.83-3.01, $P < 0.001$; dominant, OR 1.91, 95% CI 1.21-3.00, $P = 0.005$; homozygous, OR 2.58, 95% CI 1.57-4.24, $P < 0.001$; and heterozygous, OR 1.79, 95% CI 1.18-2.73, $P = 0.007$ (**Table 2**).

Lastly, this association remained significant when we meta-analyzed only the eight studies involving 186 Caucasian cases and 186 Caucasian controls (44, 45). Again, significance was obtained with all five models: allelic, OR 1.55, 95% CI 1.14-2.10, $P = 0.005$; recessive, OR 2.07, 95% CI 0.99-4.30, $P = 0.05$; dominant, OR 1.74, 95% CI 1.15-2.63, $P = 0.009$; homozygous, OR 2.51, 95% CI 1.16-5.44, $P = 0.02$; and heterozygous, OR 1.60, 95% CI 1.04-2.46, $P = 0.03$ (**Table 2**).

rs3774261 and NAFLD risk

Meta-analysis of three studies (45–47) involving 562 cases and 793 controls showed that rs3774261 was associated with significantly increased NAFLD risk according to the recessive model (OR 1.87, 95% CI 1.15-3.04, $P = 0.01$; **Table 2** and **Figure 2F**). But this association could not be found in the Chinese population (**Table 2**).

Sensitivity Analysis

To assess the reliability of the outcomes in the meta-analysis, we repeated the meta-analysis after excluding, one by one, three studies in which the *P* value associated with Hardy-Weinberg equilibrium was less than 0.05 (36, 42, 47).

After excluding the study by Gupta et al. (36), the results did not differ substantially either in total or in Asian population for rs266729 polymorphism (**Supplementary Table S1**).

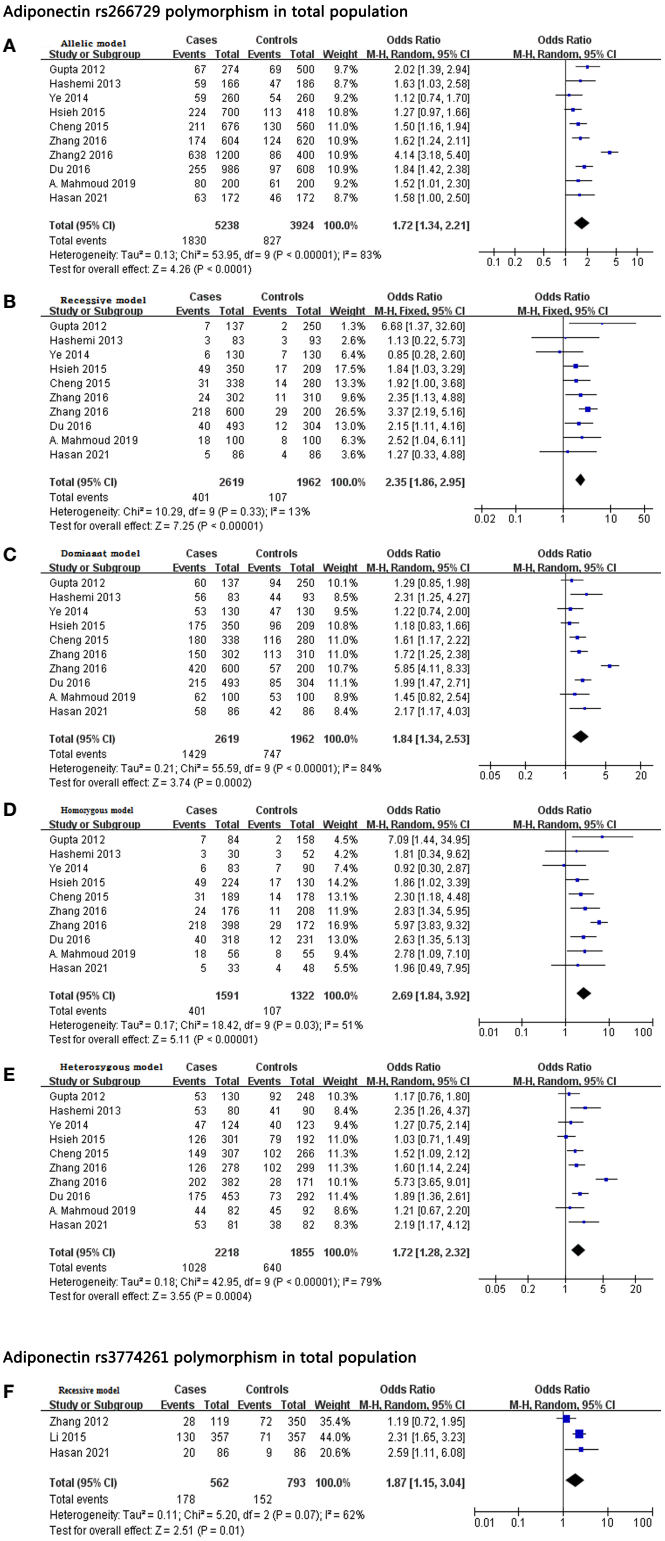

FIGURE 2 | **(A–E)** Forest plot showing the relationship between rs266729 polymorphism and NAFLD risk in the total population according to different genetic models: **(A)** allelic (G-allele vs. C-allele), **(B)** recessive (GG vs. CG + CC), **(C)** dominant (CG + GG vs. CC), **(D)** homozygous (GG vs. CC), or **(E)** heterozygous (CG vs. CC). **(F)** Forest plot showing the relationship between rs3774261 polymorphism and NAFLD risk in the total population according to the recessive model (GG vs. AG + AA). CI, confidence interval; *df*, degree of freedom; M-H, Mantel-Haenszel; NAFLD, nonalcoholic fatty liver disease.

After excluding the study by Zhang et al. (42), the results did not differ substantially in total, Asian or Chinese population for rs266729 polymorphism (**Supplementary Table S2**).

After excluding the study by Li et al. (47), the results were altered in recessive model in total population for rs3774261 polymorphism (**Supplementary Table S3**). Therefore, the results for rs3774261 polymorphism should be interpreted with caution.

Publication Bias

Begg's funnel plot and Egger's test were performed to detect potential publication bias in our meta-analysis. Funnel plots showed no obvious asymmetry in the dominant model of the rs266729 polymorphism (**Figure 3A**), and the result for Egger's test was not significant (**Figure 3B**). Similar results were obtained with the dominant model of the rs3774261 polymorphism (**Figures 3C, D**).

DISCUSSION

The physiological roles of adiponectin remain unclear, but it has been associated with obesity, insulin resistance, type 2 diabetes, atherosclerosis, hypertension, coronary artery disease, various inflammatory diseases, metabolic syndrome and NAFLD (18, 19, 55, 56). In fact, high levels of adiponectin may protect against NAFLD (56), perhaps by activating AMPK and peroxisome proliferator-activated receptor γ to improve insulin sensitivity, reduce fatty acid synthesis and enhance fatty acid oxidation (57). Here we provide additional evidence that adiponectin levels may influence onset of NAFLD by demonstrating associations between two SNPs in the adiponectin gene and risk of the disorder.

We found that rs266729 was significantly related to elevated NAFLD risk across all ethnic groups examined, as well as specifically in Asian, Chinese and Caucasian populations. Consistent with our findings, a previous meta-analysis (35) of three case-control studies (30, 36, 37) suggested a similar association among Asians. We included two of those case-control studies in the present meta-analysis but not one (30) because it did not report precise genotypes. Another Chinese study (43) reported an association between rs266729 and elevated NAFLD risk, as well as elevated risk of coronary artery disease among NAFLD patients. Our results extend the findings of a previous study linking rs266729 to elevated NAFLD risk in a southeastern Iranian population (37). However, our results contrast with a study (38) that failed to link rs266729 to NAFLD risk among Han Chinese. The relatively large sample in our meta-analysis may make our findings more reliable.

We also found that rs3774261 was significantly related to elevated NAFLD risk across all ethnic groups examined. The fact that our meta-analysis contained only three case-control studies involving 562 cases and 793 controls emphasizes the need for further research. Indeed, further research is needed into the potential association of the adiponectin SNPs rs17300539 (G–11391A) (24, 58) and rs822393 (42) and risk of NAFLD. We were unable to include those SNPs in our meta-analysis because of the limited data available.

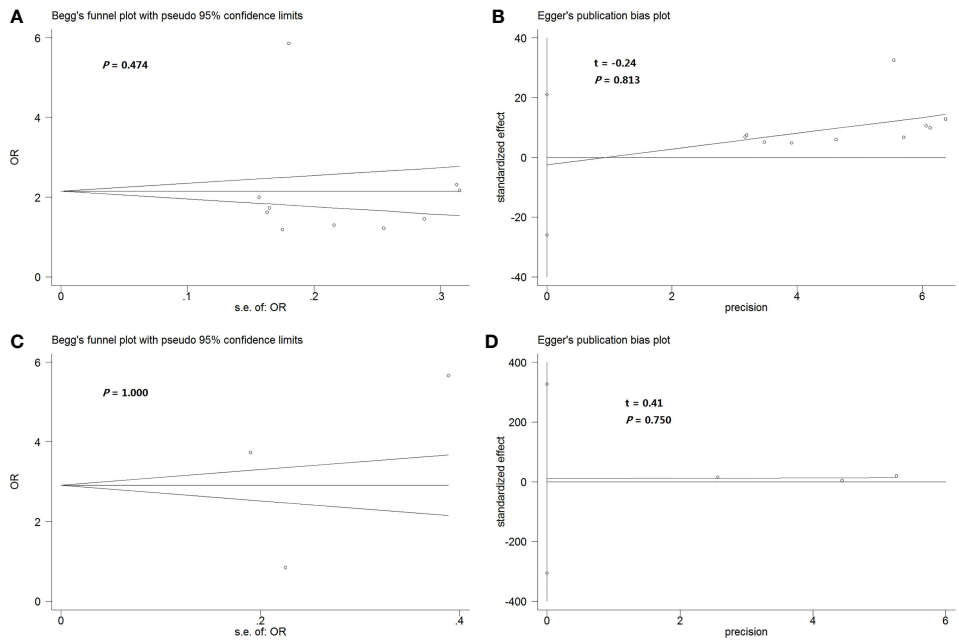

FIGURE 3 | **(A, B)** Begg's funnel plot **(A)** and Egger's test **(B)** to assess publication bias in the meta-analysis of the association between rs266729 polymorphism and NAFLD risk in the total population according to the dominant genetic model. **(C, D)** Begg's funnel plot **(C)** and Egger's test **(D)** to assess publication bias in the meta-analysis of the association between rs3774261polymorphism and NAFLD risk in the total population according to the dominant genetic model. NAFLD, nonalcoholic fatty liver disease; OR, odds ratio.

Our results should be interpreted with caution in light of several limitations. First, the controls in one study (39) had diabetes mellitus type 2, so they may not be comparable to healthy controls in other studies. Second, the *P* value associated with Hardy-Weinberg equilibrium was < 0.001 in three studies (36, 42, 47), suggesting a lack of generalizability to the broader population. Nevertheless, excluding each of those studies one at a time did not substantially alter the meta-analysis. Third, the robustness of our meta-analysis may be reduced by the fact that studies used genotyping methods differing in sensitivity and specificity, and by confounding due to sex, age, insulin resistance, family history of type 2 diabetes, obesity, coronary artery disease, hypertension and metabolic syndrome. We were unable to account for those factors in our meta-analysis because the original studies either did not report their frequencies or they aggregated the factors in different ways.

CONCLUSION

The available evidence suggests that SNPs rs266729 and rs3774261 in the adiponectin gene are risk factors for NAFLD. If our results can be verified in large, well-designed studies, they may help pave the way for novel therapeutic strategies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Designed the study: L-YL and Y-TZ. Searched databases and collected full-text papers: T-MX and C-XW. Extracted and analyzed the data: L-YL and Y-TZ. Statistical analyses: J-YC. Wrote the manuscript: Y-TZ. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.798417/full#supplementary-material

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Circulating microRNAs Are Associated With Metabolic Markers in Adolescents With Hepatosteatosis

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Background: Altered hepatic microRNA (miRNA) expression may play a role in the development of insulin resistance (IR) and non-alcoholic fatty liver disease (NAFLD). Circulating miRNAs could mirror the liver metabolism.

Objective: This study aimed to assess the relationship between serum miRNA profile in children with obesity, IR, and NAFLD.

Methods: Adolescents with obesity (n = 31) were stratified based on insulin resistance and NAFLD status. One-hundred seventy-nine miRNAs were determined in the serum by quantitative RT-PCR. Differentially expressed miRNAs were compared between groups, and log-transformed levels correlated with metabolic markers and intrahepatic triglyceride.

Results: Serum miR-21-5p, -22-3p, -150-5p, and -155-5p levels were higher in children with IR and NAFLD, and their expression levels correlated with hepatic fat and serum triglyceride. In patients with NAFLD, miR-155-5p correlated with ALT ($r = 0.68$, $p<0.01$) and AST ($r = 0.64$, $p<0.01$) and miR-21-5p and -22-3p levels correlated with plasma adiponectin ($r = -0.71$ and $r = -0.75$, respectively, $p<0.05$) and fibroblast growth factor-21 ($r = -0.73$ and $r = -0.89$, respectively, $p<0.01$). miR-27-3a level was higher in children without IR and NAFLD.

Conclusions: Several miRNAs are differentially expressed in children with IR and NAFLD. Determining their mechanistic roles may provide newer diagnostic tools and therapeutic targets for pediatric NAFLD.

Keywords: childhood obesity, insulin resistance, liver disease, MRI, microRNA

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferases; BMI, body mass index; FFA, free fatty acids; FGF21, fibroblast growth factor 21; GGT, Gamma-Glutamyl Transferase; IHTG, intrahepatic triglycerides; IR, insulin resistance; MRI, magnetic resonance imaging; NAFLD, nonalcoholic fatty liver disease.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a broad spectrum of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) with or without fibrosis (1). NAFLD is the most common cause of chronic liver disease in children and is believed to follow a more aggressive course compared to adult disease due to early-onset and distinct histological features. Yet, the prevalence of pediatric NAFLD has been difficult to assess clinically and there is no approved pharmacotherapy for its treatment.

The etiopathogenesis of NAFLD in children is complex. It is regarded as the hepatic manifestation of the metabolic syndrome given its strong association with insulin resistance (IR), type 2 diabetes (T2D), and dyslipidemia, but is also an independent risk factor for cardiovascular morbidity and mortality. Early diagnosis through screening followed by treating associated comorbidities, i.e., obesity and IR, is the standard of care. However, the commonly available screening tests have major limitations, for example serum ALT has low specificity and liver ultrasonography has low sensitivity, whereas reference standards such as liver biopsy and MRI are expensive and not readily accessible in most centers (2). Additional research is needed to identify molecular mechanisms and/or novel biomarkers to improve diagnostic accuracy and provide potential targets for pharmacotherapy. Recent research has highlighted the role of epigenetic factors on NAFLD development. MicroRNAs (miRNAs), a type of short non-coding RNAs in the length of 19-28 nucleotides, are implicated in the epigenetic regulation of gene expressions involved in the pathogenesis of NAFLD (3). Dysregulation of hepatic miRNAs is associated with NAFLD (4–6). For instance, decreased hepatic miR-122 promotes hepatic *de novo* lipogenesis, which is implicated in the development of steatosis and progression to NASH (7); circulating miR-122 and miR-192 may distinguish NAFLD patients from healthy controls; and miRNA-34a may differentiate NASH from steatosis (8). Interestingly, one clinical cohort demonstrated that the classification performance of validated miRNAs (or their ratios) for NASH is better than that reached by ALT or aspartate aminotransferase (AST) (9). In addition, cross-validated models combining both clinical and miRNA variables showed an enhanced prediction of NAFLD. Taken together these findings show that circulating miRNAs correlate to the molecular events contributing to the pathogenesis of NAFLD and monitoring miRNAs may improve the accuracy of diagnostic screening tools.

Pediatric studies investigating the relationship between miRNA expressions and clinical and metabolic markers in children at-risk for NAFLD are limited. Most studies utilized healthy children without obesity as controls (6, 10–12) and one study (5) compared miRNA profile among pre-pubertal children. Considering the puberty-associated IR and strong association between onset of puberty and NAFLD, a more appropriate control group, one with similar risk factors is needed to decipher the effect of obesity or associated metabolic complications such as IR on miRNA profile in patients with NAFLD. In the present study, we aim to determine the associations between circulating miRNAs and metabolic and hepatic features of NAFLD and serum levels of insulin, adiponectin, and fibroblast growth factor (FGF)-21 in children with obesity and varying degrees of IR and intrahepatic triglyceride (IHTG).

MATERIALS AND METHODS

Study Design and Subject Recruitment

This miRNA expression study was a secondary analysis of data collected to examine the role of FGF21 in NAFLD and prediction of changes in intrahepatic triglyceride (IHTG) percent in children with obesity presenting an outpatient weight management clinic during a 6-month observational study (13). The Institutional Review Board of the University of Arkansas for Medical Sciences approved the study. Parental consent and participant assent from all participants < 18 years old were obtained as previously described. Briefly, sixty-one pubertal children (aged 10-17 years, Tanner stage II and up) with a body mass index (BMI) ≥ 95th percentile for age and sex, with no underlying medical problems including diabetes and liver diseases, were randomly recruited. Fasting serum samples were collected, and liver magnetic resonance imaging (MRI) was performed in all participants at baseline and 6 months later. Forty-nine children completed the study at 6 months. Serum miRNA expressions were determined in thirty-one children only at 6-month due to sample availability. As such, all-comparative analyses were performed on the data obtained at 6 months.

Anthropometric Measurements, Body Composition and Clinical Analyte Detection

Anthropometric measurements, including weight, height, BMI, age, and sex-adjusted BMI percentile, were collected. The clinical analytes, including serum concentrations of glucose, insulin, triglycerides (TG), ALT, AST, gamma-glutamyl transferase (GGT), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined using a clinical analyzer (Siemens Atellica, Malvern, PA, USA) at the Arkansas Children's Hospital Chemistry Laboratory. Fasting free fatty acids (FFA) were measured *via* a chemistry analyzer (Randox Daytona, Holliston, MA, USA) in the Metabolism and Bioenergetics Core at the Arkansas Children's Research Institute (ACRI). Fasting serum leptin (Human Leptin Quantikine ELISA), adiponectin (Human Total Adiponectin/Acrp30 Quantikine ELISA), and FGF21 (Human FGF21 Quantikine ELISA) were measured according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) in the Metabolism and Bioenergetics Core at the ACRI. Insulin resistance was estimated using the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), calculated using the formula insulin (μIU/mL) × glucose (mg/dL)/405. A HOMA score of 4 was used to classify subjects with IR (HOMA-IR > 4), given that the risk of developing Type 2 Diabetes is relatively low below this HOMA-IR level (14).

Quantification of Intrahepatic Triglyceride

We estimated IHTG percent using magnetic resonance imaging (MRI) as previously described (13). In brief, a multi-echo multi-slice gradient-echo pulse sequence with TR 150 ms, flip angle 25 degrees, and echo times of 2.3 ms, 4.6 ms, and 9.2 ms with breath-hold were used to acquire in/out of phase images of the whole liver using a 1.5T MRI scanner (Philips Healthcare, Best, The Netherlands). The confounding effects of intrinsic T2/T1 relaxation in the liver fat quantification were controlled by the triple-echo method. Raw MRI images were downloaded to a workstation with MATLAB software (The MathWorks Inc., Natick, MA, USA) and customized scripts for data analysis. Two raters first sketched a region-of-interests (ROI) for each subject which included the whole liver as much as possible but avoided intrahepatic vessels and perihepatic fat as well at all edges. The average signal intensity in the selected ROI for each echo time was computed, and the liver fat concentration for the subject was calculated from these signal intensities as described (13). Participants were diagnosed with NAFLD *via* MRI if liver fat content was ≥ 5%. Raters were blinded to the anthropometric and biochemical data of the subjects to avoid interpretation bias in the MRI data analysis.

Serum RNA Extraction and miRNA Profiling

Total RNA, including microRNA, in 200 uL serum were extracted using miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA, USA) per the manufacturer's instruction. One microliter of RNA (total 14 uL) was reverse transcribed using the miRCURY LNA RT Kit (Qiagen). miRNA was amplified with 179 different primers on the Human Serum/Plasma miRCURY LNA miRNA Focus PCR panels (96-well format panel I& II, YAHS-106Y; Qiagen) using locked nucleic acids (LNA) technology and miRCURY LNA SYBR Green PCR kit (Qiagen). Spike-in controls: *C.elegans* miRNA (cel-miR-39-3p), UniSp6, UniSp2, UniSp4, and Unisp5 were added to each plate for monitoring RNA isolation, cDNA synthesis, and PCR amplification. Sample quality and hemolysis were assessed using miScript PCR Controls (Qiagen). The miRNA profiles were performed using a quantitative RT-PCR (qRT-PCR) on a Fast 7500 Real-time PCR System, Applied Biosystems (Life Technologies, Foster City, CA, USA). Amplicons were analyzed for distinct melting curves, and the T_m was checked to the within known specifications for the assays. qRT-PCR data were analyzed using the ΔCt method and normalized to a normalization factor calculated based on GeNorm methodology from the entire panel (15). All the miRNAs were assessed for the least variance across all samples in groups. According to the GeNorm analysis, selecting miR-486-5p, -193-5p, -101-3p, and let-7a-5p as normalizers showed the least variance and was confirmed by comparison of the five suggested spike-in controls. UniSp3 was used to do inter-plate calibration and correct for variances across plates. miRNA with Ct values > 35 in at least 65% of samples was excluded.

Statistical Analysis

Data are presented as mean ± standard deviation except where otherwise indicated. Categorical proportions (e.g., sex and ethnicity) were determined by Fisher's exact test. For multiple-group comparison, one-way ANOVA was conducted, followed by Tukey or Dunn all pairwise comparisons *post hoc* analysis to compare groups to each other. For two-group comparison, Student's t-test was used for analytes that were normally distributed and a Mann-Whitney test for analytes not normally distributed (as defined by $p < 0.05$, determined by D'Agostino-Pearson normality test). Correlations between miRNA expression levels (-log2) and clinical and biological parameters (independent variables) were determined using Pearson's correlation coefficients for normally distributed data or Spearman correlation coefficients for not normally distributed data. All statistical analyses were performed using GraphPad Prism7 (GraphPad Software, Inc., La Jolla, CA, USA). Significance was considered as $p < 0.05$.

RESULTS

Characteristics of Participants

We have previously shown that IR in adolescents with obesity is associated with a specific miRNA signature (16). Therefore, given the wide-range of insulin concentrations and HOMA-IR levels in the non-NAFLD group, we stratified the non-NAFLD group into i) Non-NAFLD without IR (n = 7), and ii) Non-NAFLD with IR (n = 8) to explore the effects of IR and NAFLD on miRNA expression pattern in the circulation. The clinical and metabolic characteristics of participants based on IR and NAFLD status are summarized in **Table 1**. As per design, subjects in the NAFLD group (n = 16) had a higher mean hepatic fat percentage than the groups without NAFLD (median IHTG 10.43%, range 5.04 – 23.09%). In addition, all subjects in the NAFLD group had IR (i.e., HOMA-IR > 4). The groups were similar regarding sex distribution, mean age, BMI, and BMI percentile ($p > 0.05$ for all). Serum insulin and HOMA-IR levels were higher in the NAFLD group ($p < 0.05$ for all). Other metabolic markers including serum glucose, cholesterol (Total, HDL, LDL), FFA, liver enzymes (ALT, AST, GGT), FGF21, adiponectin, and leptin were not different between the groups.

miRNA Expression Pattern

In miRNA profiling, we identified five miRNAs that were differentially expressed between the three groups (Non-NAFLD without IR, Non-NAFLD with IR, and NAFLD), including miR-21-5p, -22-3p, -150-5p, -155-5p, and -27a-3p (**Figure 1**).

Expression levels of miR-21 and -22 were significantly higher in subjects with NAFLD compared to non-NAFLD without IR group (10.2-fold [$p = 0.011$] and 3.6-fold [$p = 0.038$] increase, respectively) **(Figures 1A, B),** but they were not significantly higher compared to non-NAFLD with IR group (4.4-fold increase for miR-21, $p = 0.142$, and 4-fold increase for miR-22, $p = 0.841$). miR-150 and miR-155 were both higher in the NAFLD (3.5-fold [$p = 0.030$] and 5.6-fold [$p = 0.034$] increase, respectively) and non-NAFLD with IR (4.0-fold [$p = 0.012$] and 3.5- fold [$p = 0.025$] increase, respectively) groups compared to non-NAFLD without IR group (**Figures 1C, D**). In contrast, miR-27a-3p expression was significantly higher in the non-

TABLE 1 | Characteristics of participants according to insulin resistance and NAFLD status.

	Non-NAFLD		NAFLD
	without IR (n=7)	with IR (n=8)	(n=16)
Age (years)	14 ± 1.4	14.1 ± 2.4	14.1 ± 2.1
Sex (Male : Female)	4:3	4:4	8:8
BMI (kg/m^2)	35.7 ± 5.5	36.2 ± 4.7	37.1 ± 4.5
BMI percentile	99 ± 0.64	98.9 ± 0.62	99.5 ± 1.04
IHTG (%)	2.9 ± 1.3	3.4 ± 1.1	13.1 ± 7[a, b]
Glucose (mg/dL)	88 ± 11	94 ± 9	93 ± 10
Insulin (μIU/mL)	15.2 ± 3.1	27.6 ± 7.5[a]	52.2 ± 33.3[a, b]
HOMA-IR	3.3 ± 0.7	6.3 ± 1.7[a]	12.4 ± 8.5[a, b]
Leptin (pg/mL)	42 ± 29	56 ± 20	51 ± 24
Adiponectin (ng/mL)	6.5 ± 3.8	7.4 ± 2.9	8.2 ± 5.2
FGF21 (pg/mL)	181 ± 164	202 ± 136	242 ± 174
Triglyceride (mg/dL)	67 ± 27	126 ± 74	169 ± 61[a]
Total Chol (mg/dL)	133 ± 10	165 ± 40	152 ± 24
HDL-Chol (mg/dL)	44 ± 6	40 ± 7	37 ± 7
LDL-Chol (mg/dL)	75 ± 7	101 ± 42	80 ± 21
ALT (IU/L)	34 ± 10	34 ± 22	47 ± 29
AST (IU/L)	26 ± 4	27 ± 8	33 ± 13
GGT (IU/L)	27 ± 10	20 ± 8	29 ± 13
FFA (mmol/L)	4.6 ± 1.9	4.3 ± 1.6	4.2 ± 1.7

Data are expressed as mean ± standard deviation (SD). Significant differences between groups were determined by one-way ANOVA followed by post-hoc all-pairwise comparison. Labeled (a or b) means difference for comparison of two groups following the ANOVA analysis. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FFA, free fatty acids; FGF, fibroblast growth factor; GGT, gamma-glutamyl transferase; IHTG, intrahepatic triglyceride.

[a]$p < 0.05$ compared with non-NAFLD without IR group; [b]$p < 0.05$ compared with non-NAFLD with IR group.

NAFLD without IR group compared to non-NAFLD with IR (4.5-fold decrease, [p=0.002]) and NAFLD (3.7-fold decrease [p = 0.018]) groups (**Figure 1E**).

Associations Between Differentially Expressed miRNAs and Metabolic Markers of IR and IHTG

The correlation analyses across three groups showed that expression profiles of five miRNAs (-21-5p, -22-3p, -150-5p, -155-5p, -27a-3p) were correlated with serum markers of IR and lipid metabolism, and hepatic enzyme levels in the circulation (**Table 2**). In the non-NAFLD without IR group, miR-150-5p was positively correlated with TC, miR-155-5p was negatively correlated with HDL-C, and miR-27a-3p was negatively correlated with TG. In the non-NAFLD with IR group, miR-150-5p was positively correlated with TC and LDL-C, miR-155-5p was positively correlated with insulin but negatively with adiponectin, miR-22-3p level was negatively correlated with adiponectin, and miR-27a-3p was negatively correlated with insulin and TG. In the NAFLD group, miR-150-5p was positively correlated with TG, miR-155-5p was positively correlated with insulin, TG, and TC, miR-21-5p and miR-22-3p were both positively correlated with TG and LDL-C and negatively correlated with adiponectin, and miR-27a-3p was negatively correlated with insulin and TG. In the NAFLD group only, while IHTG was positively correlated with miR-21-5p, -22-3p, -150-5p, and -155-5p, it was negatively correlated with miR-27a-3p expression. There was a negative correlation between FGF21 and miR-22-3p in the non-NAFLD with IR and NAFLD groups, and FGF21 and miR-21-5p only in the NAFLD group. Finally, ALT was negatively correlated with miR-27a-3p in the Non-NAFLD without IR group, and ALT and AST were both positively correlated with miR-155-5p in the NAFLD group (**Table 2**).

DISCUSSION

Hepatic miRNAs are involved in lipid and glucose metabolic pathways; however, little is known about associations of miRNA expression in pediatric NAFLD. In this secondary analysis, we investigated the relationships between serum miRNA profile, metabolic biomarkers (insulin, lipid profile, adipokines, and liver enzymes), and IHTG percent in a well-described cohort of pubertal children with obesity and varying degrees of IR. We provided new evidence that serum concentrations of miR-21-5p, -22-3p, -150-5p, and -155-5p were higher in patients with NAFLD, and the metabolic profile of adolescents with IR were comparable among those with and without NAFLD. We also demonstrated that miR-27-3a expression was higher in adolescents without IR compared to those with IR regardless of NAFLD status.

A growing body of evidence suggests that the expression pattern of several miRNAs, including miR-21 and -22, are associated with the severity of NAFLD. miR-21 and -22 are two of the most abundantly expressed liver miRNAs. miR-21 is hypothesized to regulate the genes in hepatic cholesterol and triglyceride metabolisms (17). Furthermore, ablation of hepatic miR-21 was shown to decrease hepatic inflammation and improve fibrosis (18). In our study, we showed that serum miR-21 and -22 were both positively correlated with IHTG, TG, and LDL-C only in the NAFLD group. No correlation was observed between these miRNAs and liver enzymes in any of the groups. Based on these results it is tempting to speculate that in children with obesity and IR, increased expression of miR-21 and -22 in the circulation could

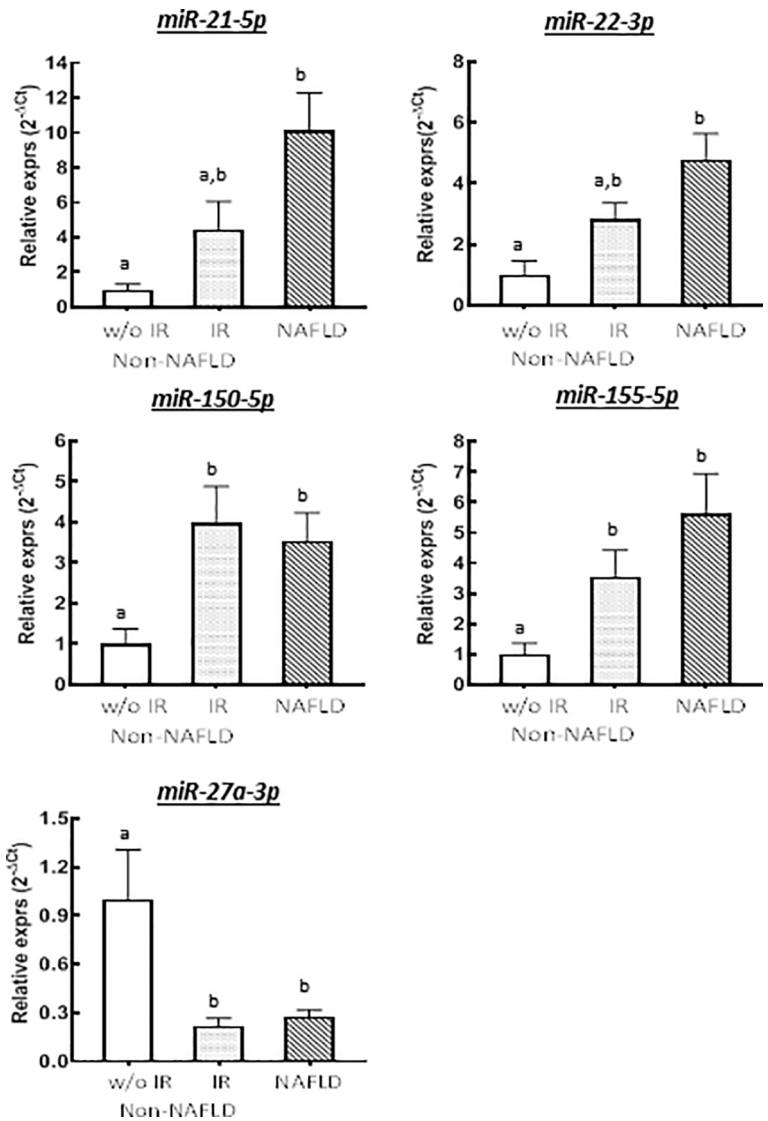

FIGURE 1 | Relative expressions of differentially expressed miRNAs across groups stratified by IR and NAFLD status (n = 7 for Non-NAFLD without IR, n = 8 for Non-NAFLD with IR, and n = 15 for NAFLD groups). Group means were compared by one-way ANOVA. Labeled (a or b) means difference for comparison of two groups following the ANOVA analysis. Bars represent standard error of the means.

be a harbinger of impending NAFLD. However, a limited number of studies in adults have reported contradictory results about miR-21 expression pattern. Sun et al. demonstrated decreased concentration of miR-21 in the circulation in patients with NAFLD (19), while Becker and colleagues did not find any difference between the control and NAFLD groups, but higher levels in patients with NASH (7). Moreover, while the role of miR-22 in the progression of steatosis to advanced stages such as fibrosis and cirrhosis is recognized (20), its role in the development of steatosis is less understood. Further validation studies are needed to determine if miR-21 and -22 are predictive markers for NAFLD progression in pediatric populations.

In the current study, we also showed that miR-22 expression negatively correlated with FGF21 and adiponectin levels in adolescents with IR regardless of NAFLD status. There were no differences in FGF21 levels among the groups with or without IR or NAFLD. We speculate that increased miR-22 blunts hepatic FGF21 synthesis and secretion in children with NAFLD, and therefore promotes worsening of steatosis. In a recent study, Hu et al. (21) utilizing a human liver cell line and fatty liver specimens demonstrated that increased hepatic miR-22 expression inhibited FGF21 expression and promoted lipogenesis through directly suppressing fibroblast growth factor receptor 1 (FGFR1) while reducing PPARα and PPARγ coactivator 1α (PGC1α), two transcriptional factors regulating FGF21 expression. Moreover, they also showed inhibition of miR-22 eliminates alcohol-induced steatosis in murine models, possibly due to restoring FGF21 expression. Considering the

TABLE 2 | Correlation analysis between serum miRNAs and metabolic markers among the groups.

Variables	Non-NAFLD without IR (n=7)					Non-NAFLD with IR (n=8)					NAFLD (n=16)				
	miR-21-5p r	miR-22-3p r	miR-150-5p r	miR-155-5p r	miR-27a-3p r	miR-21-5p r	miR-22-3p r	miR-150-5p r	miR-155-5p r	miR-27a-3p r	miR-21-5p r	miR-22-3p r	miR-150-5p r	miR-155-5p r	miR-27a-3p r
Insulin (μIU/mL)	0.26	0.36	0.53	-0.46	0.36	-0.19	-0.10	-0.39	***0.82****	***-0.78****	-0.34	-0.14	0.23	***0.73****	***-0.74****
FGF21 (pg/mL)	-0.43	-0.23	-0.22	0.31	-0.13	-0.54	***-0.74****	-0.54	0.60	0.48	***-0.73****	***-0.89****	0.18	0.43	0.52
Adiponectin (ng/mL)	0.39	0.19	-0.38	0.71	-0.09	-0.53	***-0.79****	0.51	***-0.86****	0.32	***-0.71****	***-0.75****	-0.15	***-0.82****	0.22
IHTG (%)	0.21	0.34	0.64	0.34	-0.32	0.42	0.34	-0.35	-0.01	-0.56	***0.75****	***0.78****	***0.62****	***0.64****	***-0.68****
Triglyceride (mg/dL)	0.41	0.46	0.12	0.21	***-0.86****	0.54	0.34	-0.36	0.28	***-0.78****	***0.74****	***0.85****	***0.64****	***0.69****	***-0.72****
Total Chol (mg/dL)	0.52	0.22	***0.82****	-0.66	0.21	0.38	0.18	***0.80****	-0.51	-0.26	-0.51	-0.13	-0.05	***0.54****	-0.14
HDL-Chol (mg/dL)	0.28	0.48	*0.63*	***-0.87****	0.40	0.15	0.40	0.36	-0.31	0.54	-0.46	-0.26	0.50	-0.44	0.36
LDL-Chol (mg/dL)	0.14	0.34	0.58	-0.45	0.64	0.32	0.20	***0.81****	-0.52	-0.36	***0.68****	***0.72****	-0.29	0.38	0.46
ALT (IU/L)	0.48	0.48	0.37	-0.08	***-0.64****	0.42	0.46	0.04	-0.41	-0.46	0.35	0.35	-0.35	***0.68****	-0.35
AST (IU/L)	-0.23	-0.53	0.07	-0.46	-0.53	0.32	0.47	-0.08	-0.39	-0.03	0.46	0.26	-0.26	***0.64****	0.25

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; FGF, fibroblast growth factor; IHTG, intrahepatic triglyceride; IR, insulin resistance; NAFLD, Non-Alcoholic Fatty Liver Disease. miRNA levels were transformed into a log scale (-log2). r represents correlation coefficient, shown in bold italic when the p-value is less than 0.05. *represents p < 0.05; **represents p < 0.01.*

beneficial properties of FGF21 in hepatic lipid metabolism (22), these findings provide insight on the mechanistic role of miR-22 in NAFLD development.

Altered hepatic and serum levels of miR-150 have been reported in animal models and patients with insulin resistance and liver diseases. However, whether miR-150 exerts a protective role in NAFLD or promotes development and progression of the steatosis into more advanced stages is yet to be determined. Zhuge et al. (23) demonstrated elevated levels of miR-150 in the serum of adult patients with NAFLD compared to healthy controls, and in the liver of mice fed with high-fat diet compared to chow-fed mice. They also reported that miR-150 knock-out mice were protected from developing hepatic steatosis and insulin resistance even when fed high fat diet. In line with these findings, Huang et al. showed that increased miR-150 expression promotes steatosis in human fetal hepatocyte line (24). On the contrary, Ying et al., also utilizing miR-150 deficient mice, showed that miR-150 plays an important regulatory role in adipose tissue inflammation and that deficiency of miR-150 in mice is associated with severe systemic inflammation and insulin resistance (25). Our findings did not support any relationship between miR-150 and markers of insulin resistance such as insulin, adiponectin, or FGF21, but showed that miR-150 might be an important regulator in the cholesterol and triglyceride metabolisms.

The mechanistic role of miR-155 in hepatic lipid metabolism is less understood and debated. Earlier studies suggested a protective role for miR-155, through its downstream effector liver X receptor, for the development of steatosis (26). In line with these reports, Johnson et al. (27) suggested that miR-155 expression in white adipose tissue is pivotal to prevent progression of obesity-associated inflammatory response. However, Ying et al. (28) reported that macrophage-derived exosomal miR-155 obtained from the adipose tissue of obese mice could induce IR in lean mice *via* its target PPARγ. More recently, Bala et al. (29) showed that miR-155 knock-out mice displayed less steatosis and fibrosis compared to wild type mice. Studies in humans are scarce and contradictory. While Wang et al. (30) demonstrated decreased liver and serum miR-155 levels in adult patients with NAFLD, Zhou et al. (31) showed increased serum levels in children with NAFLD. In the current study, we found positive correlations between serum miR-155 level and insulin and adiponectin concentrations in children with IR regardless of NAFLD status, and IHTG and liver enzymes in children with NAFLD only. Taken together, these findings may suggest that elevated miR-155 may play a role in the development and progression of NAFLD through induction or exacerbation of IR.

miR-27 is another understudied and potentially overlooked miRNA in the pathophysiology of pediatric NAFLD. In an *in vitro* study, Ji and colleagues (32) showed that overexpression of miR-27a and -27b were influential in fat accumulation in hepatic stellate cells. This was supported by the findings of Singaravelu et al. (33) who demonstrated that overexpression of miR-27b results in larger and more abundant lipid droplets in hepatocytes infected with hepatitis C virus, likely due to inhibition of PPARα. Increased expression levels of miR-27b were also shown in humans with NAFLD compared to healthy controls (4). On the contrary, Zhang et al. (34) showed that miR-27a, through the inhibition of two important regulatory genes in the fatty acid synthesis and thereby decreasing the rate of *de novo* lipogenesis, decreases lipid accumulation in the hepatocytes. In line with this report, we found increased serum miR-27 levels in adolescents without IR regardless of the NAFLD status. We observed negative correlations between miR-27 and TG for all groups, miR-27 and insulin for groups with IR, and finally miR-27 and IHTG for groups with NAFLD. In contrast to adult studies, our comparison group was composed of children with similar degrees of obesity and body fat percent. Since IR is considered the first-hit in the development of NAFLD, we speculate miR-27 plays a protective role and prevents excessive hepatic lipid deposition *via* regulation of hepatic insulin signaling pathways.

In conclusion, our results add to the growing body of literature showing miRNAs are important regulators of systemic glucose and

hepatic lipid metabolism in children with obesity and varying degrees of IR. These findings may have clinical implications. Particularly miR-21-5p, -22-3p, -150-5p, and -155-5p may be used to identify patients with IR at-risk for developing NAFLD, while miRNA-27-3a could be used to identify those who have a low risk of developing IR and hence NAFLD. We acknowledge that the small cohort may not equally distribute potential confounders such as race/ethnicity or pubertal developmental stages among the groups being compared. In addition, given the cross-sectional design, a cause-and-effect relationship cannot be established in this association study. However, our findings do address the current need for more accurate diagnostic tools for determining patients with IR at-risk for NAFLD. Further characterization of the mechanical roles of these miRNAs in the pathogenesis of IR and NAFLD may help develop targeted pharmacotherapies.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The data that support the findings of this study (all of the individual participant data collected during the study - after deidentification, statistical analysis plan, and analytic code) are available from the corresponding author immediately following publication with the researchers who provide a methodologically sound proposal. Requests to access these datasets should be directed to ET, etas@uams.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of Arkansas for Medical Sciences. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HL and ET designed the research. ET conducted the study. HL, KEM, XO, KM, RB, EB, and ET processed and interpreted the clinical and imaging data. HL and ET performed the statistical analyses. HL and ET wrote the manuscript. ET had primary responsibility for final content and edits. All authors read and approve the final manuscript.

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Characterization of Genetic Variants of Uncertain Significance for the *ALPL* Gene in Patients With Adult Hypophosphatasia

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Hypophosphatasia (HPP) a rare disease caused by mutations in the *ALPL* gene encoding for the tissue-nonspecific alkaline phosphatase protein (TNSALP), has been identified as a potentially under-diagnosed condition worldwide which may have higher prevalence than currently established. This is largely due to the overlapping of its symptomatology with that of other more frequent pathologies. Although HPP is usually associated with deficient bone mineralization, the high genetic variability of *ALPL* results in high clinical heterogeneity, which makes it difficult to establish a specific HPP symptomatology. In the present study, three variants of *ALPL* gene with uncertain significance and no previously described (p.Del Glu23_Lys24, p.Pro292Leu and p.His379Asn) were identified in heterozygosis in patients diagnosed with HPP. These variants were characterized at phenotypic, functional and structural levels. All genetic variants showed significantly lower *in vitro* ALP activity than the wild-type (WT) genotype (p-value <0.001). Structurally, p.His379Asn variant resulted in the loss of two Zn^{2+} binding sites in the protein dimer which may greatly affect ALP activity. In summary, we identified three novel *ALPL* gene mutations associated with adult HPP. The correct identification and characterization of new variants and the subsequent study of their phenotype will allow the establishment of genotype-phenotype relationships that facilitate the management of the disease as well as making it possible to individualize treatment for each specific patient. This would allow the therapeutic approach to HPP to be personalized according to the unique genetic characteristics and clinical manifestations of each patient.

Keywords: alkaline phosphatase, bone, hypophosphatasia, mineralization, genetic variant, enzymatic activity, pyridoxal 5´ phosphate

1 INTRODUCTION

Hypophosphatasia (HPP) is a genetic disease characterized mainly by a lack of bone and tooth mineralization (1). In 2011 the prevalence of HPP in Europe was estimated at 1/300,000 for severe forms and 1/6,370 for moderate forms (2). In 2019, Garcia-Fontana et al. showed that, the estimated prevalence of mild HPP could be double that of previously reported estimation for Spanish population (1/3,100 vs. 1/6,370), and there could be more than 15,000 potential patients affected with mild forms of HPP underdiagnosed due to a lack of recognition in clinical practice (3). The clinical manifestations and prevalence of HPP has changed significantly between 1997 and 2019 from a rare and mostly recessive bone disease to a multisystemic, mostly dominant and frequent disease in its moderate and mild form (4).

In humans, the *ALPI, ALPP, ALPPL2* and *ALPL* genes encode intestinal (IALP), placental (PALP), tissue-specific germ cell (GCALP) and non-tissue-specific (TNSALP) ALP isoenzymes, respectively (4). The role of ALP is to hydrolyze phosphomonoesters with the release of inorganic phosphate (5). HPP is caused by the presence of loss-of-function mutations in the *ALPL* gene, resulting in decreased enzyme activity and consequent accumulation of the natural substrates of the enzyme (6). The extracellular substrates of TNSALP are pyridoxal 5′ phosphate (PLP) and inorganic pyrophosphate (PPi) (7, 8). Recent studies also suggest that adenosine triphosphate (ATP), di-phosphoryl lipopolysaccharide (LPS), and phosphorylated osteopontin (p-OPN) are also natural substrates of TNSALP (7, 9, 10).

The *ALPL* gene is ubiquitously expressed in the body participating TNSALP in numerous pleiotropic processes (11, 12). However, it is particularly expressed in bone, liver and kidney. The most frequent clinical manifestations of HPP are at the bone level. A hallmark of HPP is increased rates of fragility fractures and recurrent bone marrow injuries due to defective bone mineralization (13). In addition, generalized TNSALP deficiency is often associated with a rare form of rickets and osteomalacia (13–16). This does not apply to all patients due to the wide variety of genetic mutations and to the different forms of inheritance, which classifies HPP as a multisystemic disease due to its extraordinary heterogeneity. Phenotypes range from total absence of bone mineralization and fetal death mainly due to thoracic deformities and lung hypoplasia (17) to a wide variety of manifestations in different organs and systems including dental (13, 18–20), skeletal (13, 18–20), muscular (21), respiratory (22), neurological (23), renal (24) and rheumatological (25) and/or neurological symptoms (26) (**Figure 1**). However, some HPP patients seems to be asymptomatic, presenting non clinical manifestations often in adult HPP form.

According to the latest update of the Leiden Open Variation Database (LOVD) web site, about 500 loss-of-function variants of the *ALPL* gene have been described to date (https://databases.lovd.nl/shared/genes/ALPL) (6). The large variety of nonsense variants and the dominant negative effect of some variants contribute greatly to the clinical heterogeneity (6). This diverse clinical feature has led to the classification of HPP in different clinical forms from more severe to milder forms into perinatal lethal HPP, infantile HPP, childhood-onset HPP, adult HPP, odontohypophosphatasia and benign perinatal HPP (14, 17). In general, earlier onset subtypes are more severe and are inherited recessively, while moderate to mild late onset subtypes can be inherited in an autosomal recessive or dominant pattern (26).

The American College of Medical Genetics and Genomics (ACMG) together with the Association of Molecular Pathology and the College of American Pathologists have proposed new terminology in the interpretation of nucleotide changes occurring in genes for various pathologies (27). Pathogenic variant: variant that is certain to disrupt gene function or to be disease causing; probably pathogenic variant: the nucleotide change has a greater than 90% chance of being pathogenic; variant of uncertain significance (VUS): it is not possible to define whether it is pathogenic or not; variant probably non-pathogenic: the nucleotide change has a greater than 90% probability of not being pathogenic; non-pathogenic variant: a variant that has been shown to have no functional effect. Currently, there are computer tools as VarSome that classify variants in the criteria previously described (28).

Identifying and characterizing each genetic variant non previously described in order to determine whether particular variants give rise to particular phenotypes it would be interesting to know with greater certainty the possible effects it could have on the carrier patient. In this context, the aim of this study is to characterize at functional and structural level, three new VUS of the *ALPL* gene in HPP patients. In this way, genotype-phenotype relationships could be established for each patient. This would allow HPP therapy to be more targeted and personalized according to the genetic and clinical characteristics of each patient.

2 MATERIAL AND METHODS

2.1 Study Population

The subjects included were evaluated at the Clinical Analysis Unit of the University Hospital San Cecilio of Granada following the algorithm detailed by García-Fontana et al. (3). The clinical records of selected subjects were reviewed to exclude those subjects with low ALP levels caused by other causes different to HPP as certain therapies, malnutrition, zinc or magnesium deficiency, celiac disease among other, that could generate transiently or precipitously low ALP levels (29, 30). Subjects selected signed an informed consent and an individualized and personal interview about potentially related HPP symptoms was conducted. Two venous blood samples were collected from each subject at the Clinical Analysis Unit for PLP determination and for the genetic study. The present study was approved by the ethics committee of the University Hospital San Cecilio of Granada in accordance with the principles of the World Medical Association Declaration of Helsinki (Project ID: 0777-M1-20. Research Ethics Committee of Granada Center (CEI-Granada) on 8 May 2019).

2.2 Biochemical Analyses

2.2.1 Alkaline Phosphatase (ALP)

ALP activity was measured in blood samples by absorption spectrophotometry on AU5800 analyzers (Beckman Coulter,

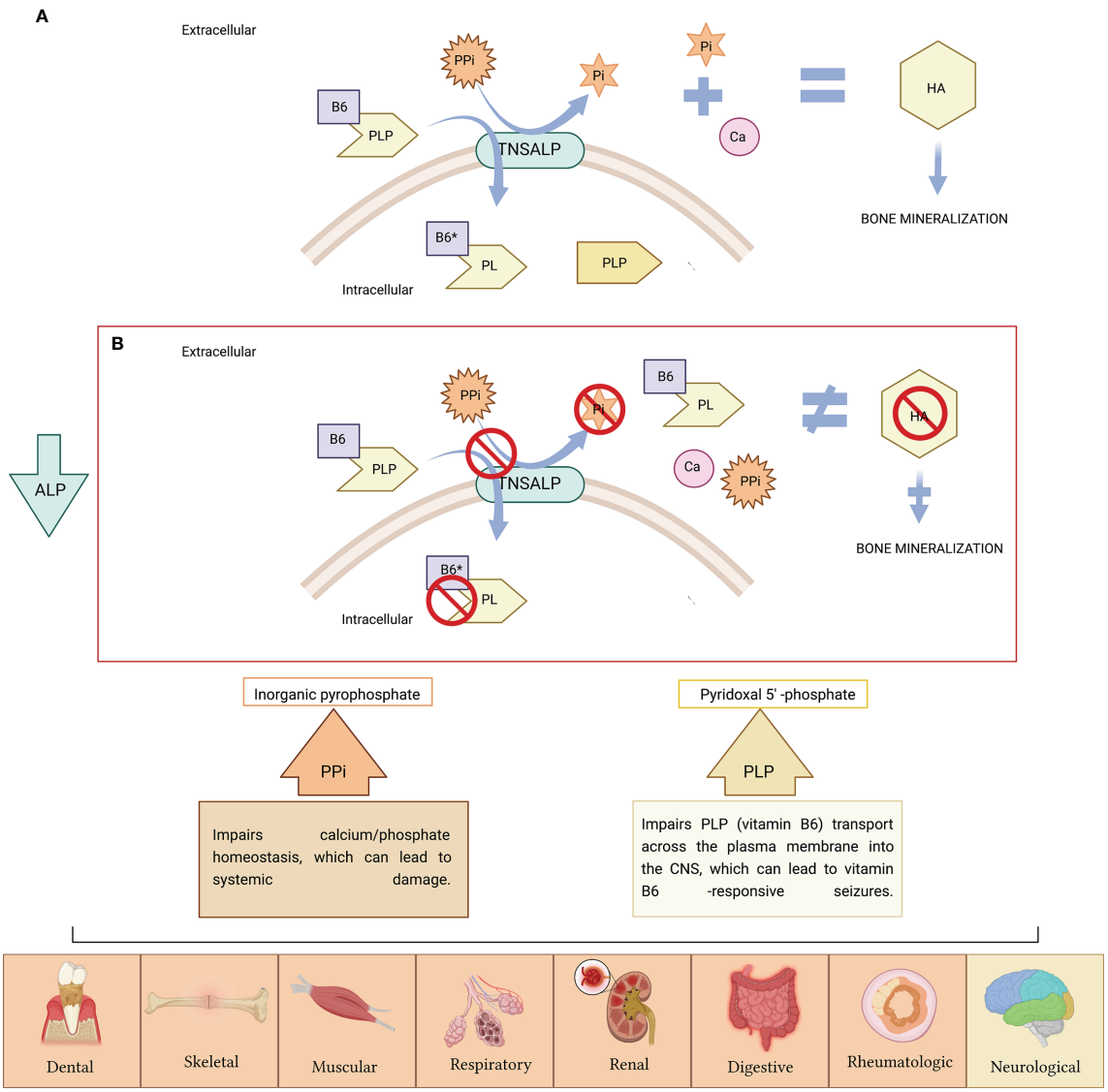

FIGURE 1 | **(A)** TNSALP is attached to the cell membrane, where it degrades inorganic pyrophosphate (PPi) to inorganic phosphate (Pi), needed for hydroxyapatite (HA) crystal formation and further bone mineralization. TNSALP hydrolyses PLP (active form of B6) to pyridoxal (PL) in order to cross the cell membranes, after which is, then intracellularly rephosphorylated into PLP. **(B)** PPi is not degraded to Pi. Excess of PPi inhibits HA crystal formation with further inhibition of bone mineralization. PLP is not hydrolyzed to pyridoxal (PL), and accumulates extracellularly. The low activity of ALP causes the accumulation of PPi and PLP, causing symptoms in various organs of the human body (the symptoms related to PPi accumulation appears in orange boxes while symptoms related to PLP accumulation as vitamin B6 deficiency are indicated in yellow box). Figure created with BioRender.com.

California, USA) according to the method recommended for the "International Federation for Clinical Chemistry" (IFCC). ALP activity was determined by measuring the conversion rate of p-nitrophenyl phosphate (pNPP) to p-nitrophenol (pNP) in the presence of magnesium and zinc ions and of 2-amino-2-methyl-1-propanol (AMP) as a phosphate acceptor at pH 10.4. The rate of change in absorbance due to pNP formation was measured bichromatically at 410/480 nm Routine ALP determinations were performed at the Clinical Analysis Laboratory of the University Hospital Clínico San Cecilio of Granada. ALP activity reference values were 38-106,5 IU/L.

2.2.2 Pyridoxal 5´ Phosphate

Plasma PLP concentrations, the active form of vitamin B6 and one of the main substrates of TNSALP, were determined by high-pressure liquid chromatography (HPLC) in the Clinical Analysis Unit of the University Hospital Niño Jesús (Madrid). Chromatographic determination was performed in an isocratic HPLC system with a fluorescence detector. The excitation and

emission wavelengths were 320 nm and 415 nm, respectively. PLP reference values were 5 to 50 µg/L.

2.3 Sequencing of *ALPL* Gene

DNA extraction from peripheral blood lymphocytes was performed followed by amplification of the *ALPL* gene by polymerase chain reaction (PCR) according to the methodology described by Riancho et al. (31). Subsequent Sanger sequencing was performed using the PCR product, in order to know the sequence of the coding regions and exon-intron junctions of the *ALPL* gene, using the truncated sequence NM_000478.5 as a reference. The sequencing reaction product was read on an automated sequencer by capillary electrophoresis. Next, a copy number variant study was performed by multiple ligation probe amplification (MLPA) (MRCHolland) and subsequent analysis of results using the SeqPilot program (JSI Medical Systems). The results of the sequencing of the *ALPL* gene in peripheral blood DNA were provided by the Biomedical Diagnostic Center of the Clinic Hospital of Barcelona.

2.4 Cell Culture

In this study human embryonic kidney cell line (HEK293T) were cultured in 75cm^2 filter cap cell culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) High Glucose with pH = 7.2 (Biowest), and 10% fetal bovine serum (FBS) (Capricorn scientific), 5% Ham's F-12 (Biowest) and 1% of 100X antibiotic-antimicotic (Biowest). The cell culture flasks were maintained in an incubator at 37°C with 5% CO_2 until the cells reached confluence of 70–80%. To suspend cells, 3mLof 10X Trypsin-EDTA solution (Sigma Aldrich) was used and the cells were successfully transferred to the 24-well cell culture plates. All cell culture plates were incubated at 37°C with 5% CO_2.

2.5 Plasmids Design and Amplification

The vectors used throughout this study were constructed by modifying the plasmid pcDNA3.1. The *ALPL* gene with the different mutations under study (pcDNA3.1:*ALPL* c.Del69_74, pcDNA3.1:*ALPL* c.1135C>A and pcDNA3.1:*ALPL* c.875C>T) was inserted into this plasmid. The plasmid pcDNA3.1 with the *ALPL* wild-type (WT) gene insert (pcDNA3.1:*ALPL*) was used as a control to functionally characterize the identified variants. The plasmid without any insert (empty pcDNA3.1) was used as an internal control to check the basal expression of the *ALPL* gene at the cellular level. The *ALPL* gene sequences and their corresponding gene variants were each inserted independently into the multicloning site, with flanking sequences for the *HindIII* and *BamHI* enzymes. All vectors were supplied by GenScript.

To amplify the plasmid concentration, 1 µg of each plasmid vector was transferred separately to 50 µL of competent *E. coli* JMC109 strain by using heat shock method. Transformed *E. coli* cells were grown overnight on LB agar medium containing ampicillin at concentration of 100 mg/mL at 37°C. Then, all plasmid vectors were extracted by using kit NucleoBond® Xtra Midi EF (Macherey-Nagel™) according to the manufacturers` instructions. The purity and concentration of the extracted plasmids was determined by spectrophotometric reading, using the NanoDrop Spectrophotometer (bioNovan). Samples with absorbance ratios 280/260 nm around 1.8 were selected. To ensure the complete integrity of the different plasmids, a PCR was performed confirming that the *ALPL* genes with the mutations and *ALPL* WT were correctly inserted into the plasmid. For this purpose, the primers *ALPL*-F: 5′-TGGCAC CTGCCTTACTAACT-3′ and *ALPL*-R: 5′-CACGTTGGTGT TGAGCTTCT-3′ for plasmids containing some *ALPL* variant and the primers pCDNA3.1-F: 5'-CGTCACGCTGTAGGTAT CTCAGTTC-3' and pCDNA3. 1-R: 5'-GCCTACATACCTC GCTCTGCTAATC-3' for empty pCDNA3.1 plasmid, were used. The PCR reaction was performed using the Horse-power Taq DNA Polymerase kit (Canvax) and following the protocol established by the manufacturer. Visualization of the process was performed by 2% agarose gel electrophoresis.

2.6 Transfection Studies

ALPL WT, *ALPL* mutants and pcDNA plasmids were transiently transfected into HEK293T cells for 48 hours. Transfections were performed by the lipofection method with LipoD293 DNA *In Vitro* Transfection Reagent (SignaGen Laboratories) following the manufacturer's instructions. Specifically, 150,000 cells/well were seeded in 24-well plates and allowed to grow for 24 hours at 37°C and 5% CO_2. For transfection, 1.5 µL of LipoD293, 500 ng of plasmid of interest and non-supplemented Advance DMEM were added to complete a final volume of 50 µL per well to be transfected.

2.7 Gene Expression Determination

RT-qPCR experiments were performed to control transfection and *ALPL* exogenous expression. The total RNA was isolated from each transfected culture using a *RNeasy*® Mini Kit (QIAGEN). RNA was treated with DNAse (Qiagen), then, cDNA was synthesized from 600 ng of total RNA using the iScript cDNA synthesis kit (BioRad) following the manufacturers' instructions. Quantitative PCR was performed using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) in a CFX96 Real Time thermocycler (BioRad). The set of primers designed to amplify a 121 bp fragment of the *ALPL* gene from both WT and mutant plasmids are the following: ALPL-F: 5′-TGGCACCTGCCTTACTAACT-3′ and ALPL-R: 5′-CACGTTGGTGTTGAGCTTCT-3′. Gene expression data were normalized to the expression of the reference gene Ribosomal Protein L13 (RPL13) and reported as normalized *ALPL* expression. The following set of primers was used to amplify the reference gene: RPL13-F: 5′-CGTAAGATCC GCAGACGTAAGGC-3′ and RPL13-R: 5′-GGACTTGTTC CGCCTCCTCGGAT-3′.A standard line was run at known cDNA concentrations (ng/µL) to determine the total amount of cDNA in our samples.

2.8 ALP Activity in Transfected Cells

The ALP activity was determined in triplicate using Alkaline Phosphatase Detection Kit (Abnova) following the methodology recommended by the manufacturer. The enzyme activity of ALP was measured with a spectrophotometer (Dynex Technologies) at 450 nm to detect the chromogenic product as a result of the ALP activity.

2.9 Cell Viability

The cell viability was analyzed in triplicate using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) following the steps indicated by the manufacturer. Viable cells are FITC Annexin V and propidium iodide (PI) negative and death cells are both FITC Annexin V and PI positive. Subsequently, a reading was performed on the BD FACSAriaTM II Cell Sorter flow cytometer.

2.10 Three-Dimensional (3D) Structural Modeling

To check the effect of the new mutations identified in this study, the 3D modeling of WT and TNSALP mutants was completed using SWISS MODEL (https://swissmodel.expasy.org/). The structure modeling is based on the sequence homology between TNSALP and the PALP (PDB ID: 1EW2)determined at a resolution of 1.8 Å (7, 32).The amino acid sequence of the human TNSALP molecule is 57% identical and 74% homologous with the human PLAP molecule (33). The new pdb files for WT, p.Del Glu23_Lys24, p.Pro292Leu, and p.His379Asn TNSALP are available upon request. Ribbon representations and hydrophobic surface representations were obtained using UCSF Chimera software. Mutation-related residues in the present study were positioned using the open source https://swissmodel.expasy.org/repository/uniprot//P05186.35.

2.11 Statistical Analyses

The ALP activity measure was calculated based on absorbance at 450 nm in three independent experiments in transfected cells, and Shapiro-Wilk test was used to study the distribution of this variable. ANOVA was performed to compare the differences between groups. Statistical significance was set at $p < 0.05$ (two-tailed). Statistical analysis was performed using SPSS version 22.0.

3 RESULTS

3.1 Biochemical and Genetic Results

3.1.1 Biochemical Analysis of ALP and PLP Levels in Serum Samples

ALP and PLP levels were analyzed from serum samples of subjects with suspected diagnosis of HPP due to low levels of ALP following the protocol described by García-Fontana et al. (3). Blood samples were sequenced in order to identify genetic variants of *ALPL* gene. The results of ALP and PLP values of the patients in whose a genetic variant of the *ALPL* gene was identified are summarized in **Table 1**.

The p.Del Glu23_Lys 24 variant resulted in ALP activity values close to the lower limit of the normal range, while PLP levels were within the normal range (34μg/L). On the other hand, p.Pro292Leu and p.His379Asn variants showed abnormal levels of both ALP and PLP activity in blood. Particularly significant is the p.His379Asn variant, which has a very low ALP activity (10 IU) as well as drastically elevated levels of serum PLP (462 μg/L) (**Table 1**).

TABLE 1 | Biochemical determinations of ALP and PLP associated with mutations of uncertain significance.

Samples	c.Del69_74/ p.Del Glu23_Lys24	c.875C>T/ p.Pro292Leu	c.1135C>A/ p.His379Asn	Reference range
ALP activity (IU/L)	23	16	10	38-106,5
PLP (μg/L)	34	119	462	5-50

The reference values were extrapolated from the values defined by the Clinical Analysis Unit of the University Hospital Niño Jesús (Madrid).

3.1.2 Identification of Variants of Uncertain Significance

The mutations analyzed in this study were obtained from patients evaluated at the Clinical Analysis Unit of the University Hospital Clínico San Cecilio of Granada (Spain). Three mutations in the *ALPL* gene were identified by the sequencing service of the Center for Biomedical Diagnosis of the Hospital Clínic of Barcelona.

The 27-year-old patient presented a c.69_74del VUS in exon 3 and in heterozygosis, involving a deletion of glutamine 23 and lysine 24 in the TNSALP protein (p.Del Glu23_Lys24).

The 62-year-old patient presented the c.875C>T VUS in exon 9 and in heterozygosis, coding for the amino acid change from proline to leucine at position 292 of the TNSALP protein (p.Pro292Leu).

The 45-year-old patient presented a c.1135C>A VUS in exon 10 in heterozygosis, coding for the amino acid change from a histidine to an asparagine at position 379 of the TNSALP protein (p.His379Asn).

These three mutations were categorized as VUS by VarSome tool (https://varsome.com).

3.2 Clinical Manifestations

The symptomatology of these patients was highly heterogenous. Nevertheless, in all three cases, articular pain was observed, especially in the knees (**Table 2**).

The most severe symptomatology is attributed to the p.His379Asn variant, while the clinical profiles derived to the p.Del Glu23_Lys24 and p.Pro292Leu variants were associated with a milder symptomatology. Interestingly, none of the 3 affected patients showed a clinical history of fractures, chondrocalcinosis or dentition abnormalities, symptoms characteristic of the moderate phenotypes associated with adult HPP, but presented with other complaints not associated, *a priori*, with HPP (**Table 2**).

According to the age of diagnosis of the different clinical manifestations in affected patients, the p.His379Asn variant has the earliest symptomatologic onset. Thus, the episodes of muscle weakness and pain in the upper and lower extremities began in childhood. From the age of 30 the patient presented episodes of extreme weakness and fatigue with inability to move the extremities and palpebral ptosis in addition to dysphonia related to exertion. At the age of 42 the patient suffered episodes of paresthesia in face and upper limbs as well as visual disturbances, kidney cramps, achalasia, profuse salivation and thyroid alterations (hypothyroidism). The patient was treated with meniston, corticoids, azathioprine, immunoglobulins and

TABLE 2 | Clinical manifestations of patients affected with variants of uncertain significance of *ALPL* gene.

Samples	c.Del69_74/p.Del Glu23_Lys24	c.875C>T/p.Pro292Leu	c.1135C>A/p.His379Asn
Pathognomonic symptoms	Knee and lower back pain	Gonalgia Meniscopathy	Episodes of paresthesia in face and upper limbs Elbow joint pain and fatigue Hip and lower limb pain Muscle weakness
Non-pathognomonic symptoms	Thyroid bulge	Benign prostatic hyperplasia	Drowsiness and tiredness Unfocused vision Achalasia and profuse salivation Kidney cramps Hypothyroidism

plasmapheresis without improvement. Additionally, the affected patient received treatment with carnicor, ubiquinol, bisoprolol, lexatin, melatonin, acfol, auxin A, collagen+Mg and probiotics with slight improvement. The current treatment includes eutirox only and has recently started with asfotase alfa therapy, which has resulted in a significant improvement, evidencing that most of the clinical manifestation of this patient may be related to HPP.

Regarding the p.Del Glu23_Lys24, the affected patient presented with knee and lumbar pain at the age of 28 years, without treatment. In addition, at the age of 23 she was diagnosed with bulge without thyroid hormone involvement and therefore did not require treatment.

The patient affected with p.Pro292Leu variant presented episodes of gonalgia, meniscopathy and benign prostatic hyperplasia at the age of 61 years, receiving treatment with topical anti-inflammatory drugs.

3.3 ALP Activity of Variants of TNSALP in An *In Vitro* Model

The ALP activity was measured in HEK293T cells transfected with plasmids containing the *ALPL* WT or the *ALPL* mutants. Empty pcDNA3.1 plasmid was used to measure basal ALP activity of the cells.

Cells containing *ALPL* WT had a statistically significant higher expression compared to the rest of the transfected cells. The p.Pro292Leu and p.His379Asn variants had no significant differences in the enzyme activity compared to TNSALP basal expression control.

The p.Del Glu23_Lys24 variant showed significantly higher activity than the other variants and pcDNA3.1 control. The results are shown in **Figure 2**.

3.4 Absolute mRNA Expression of VUS of *ALPL* in an *In Vitro* Model

The determination of the absolute expression of VUS-associated mRNA was performed by qPCR of the different groups of HEK293T transfected with the corresponding plasmids.

The transfection and expression studies performed to verify the validity of the experiment demonstrated that the *ALPL* transcriptional expression was similar in cells transfected with plasmids containing the *ALPL* WT or mutants (**Figure 3**). Statistically significant *ALPL* gene expression was found in *ALPL* WT cells or *ALPL* mutant cells compared to cells transfected with empty vector (**Figure 3**). The latter represents the endogenous expression of the *ALPL* gene in HEK293T cells (p-value<0.05).

3.5 Cell Viability Assay

Cell viability was assessed by flow cytometry. As depicted in **Figure 4**, there were no statistically significant differences in cell viability between the groups. All groups had a survival rate of

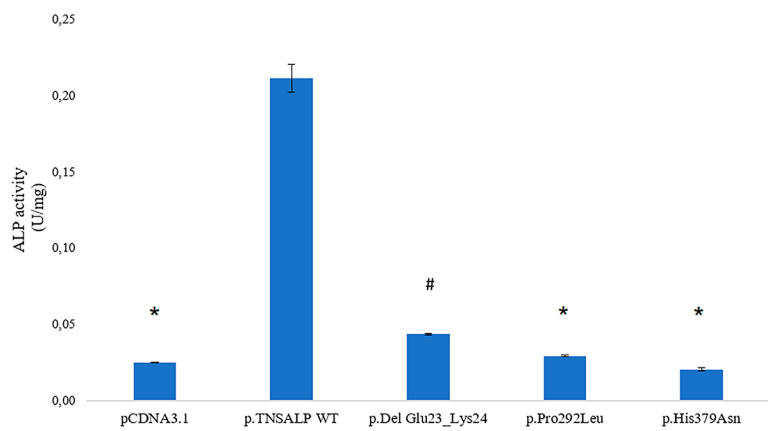

FIGURE 2 | ALP activity in HEK293T cells transfected with pcDNA 3.1 or pcDNA 3.1+ insert. Quantitative results of the ALP assay expressed in U/mg are shown. Results are expressed as mean ± SEM of TNSALP activity. ANOVA was used for comparisons between groups. $^{*}p < 0.001$ vs WT; $^{\#}p < 0.001$ vs all groups.

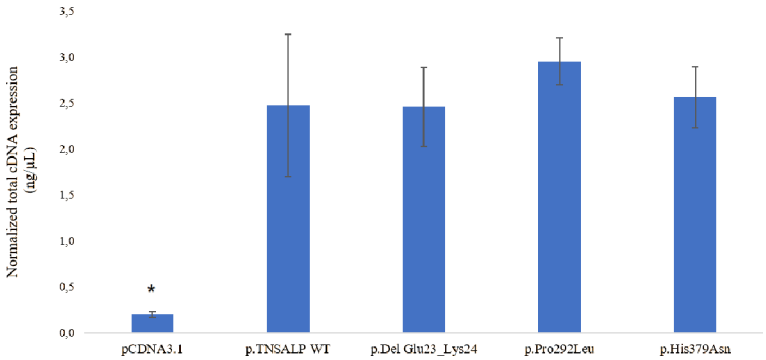

FIGURE 3 | Total mRNA expression. The results are expressed as the percentage of expression of the Cts ± SEM normalized with the expression values of the constitutive gene RPL13. ANOVA corrected by Tukey's test was used for comparisons between groups. to empty pcDNA3.1 are indicated with an *$p < 0.05$ vs all groups.

around 85-90% (**Figure 4A**) and an apoptosis rate around of 5-10% (see **Figure 4B**).

3.6 Three-Dimensional Modelling of the Structure of the Variants of TNSALP

In order to predict the effect of the three newly identified mutations on the TNSALP structure, a 3D model based on the sequence homology between TNSALP and the PALP was obtained using the web-server SWISS MODE. **Figure 5A** shows the structure of the TNSALP WT protein. The yellow arrows and green dots indicate the locations of the mutations in the different variants described in this study

The p.Del Glu23_Lys24 variant showed significant changes at the end of the α-helix anchored to the N-terminal end, where the deletion occurs, leading to a shortening of the protein structure (**Figure 5B**). Secondly, the p.Pro292Leu variant showed an affectation of two of the β-strands that constitute the β-sheet, which acts as a structural domain and an integral part of the calcium-binding domain. This variant resulted in the elongation of these chains and reduction of the extension of the loops between them (**Figure 5C**). Finally, the p.His379Asn variant affected one of the two Zn^{2+} binding sites of each of the protein monomers. Although it seems to have no important structural impact, this change implies a reduction of the enzyme's Zn^{2+} atom binding capacity by half to carry out its catalytic function (**Figure 5D**).

The study of the protein polarity related to these genetic variants revealed significant changes in the hydrophobicity and folding in the area surrounding the mutation in the structure of the protein encoded by p.Del Glu23_Lys24 variant (**Figure 6**).This causes the exposure of two highly hydrophobic residues to the outside (see **Figure 6B**). The p.Pro292Leu variant caused polarity changes in the immediate vicinity of the mutation and at the homodimer interface leading to an increased gapping in the region as is represented in **Figure 6C**. Finally, the polarity changes related to the p.His379Asn variant were minimal, causing a little compaction of some exposed hydrophobic residues in the protein (**Figure 6D**). Notably, the three genetic variants resulted in a change in the lower region of the homodimer interface, affecting one of the characteristic clusters of hydrophilic residues present in TNSALP WT.

4 DISCUSSION

In the present study we identified three uncertain variants into adult patients classified as VUS, as there was not yet sufficient evidence to categorize them according to its pathogenicity.

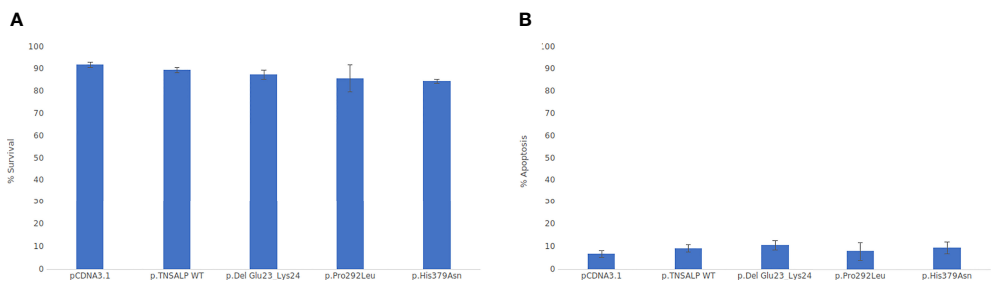

FIGURE 4 | Viability cell assay graphs. ANOVA corrected by Tukey's test was used for comparisons between groups Statistically significant differences was set at a *p*-value <0.05. **(A)** Percentage of cell viability. **(B)** Percentage of apoptotic cells.

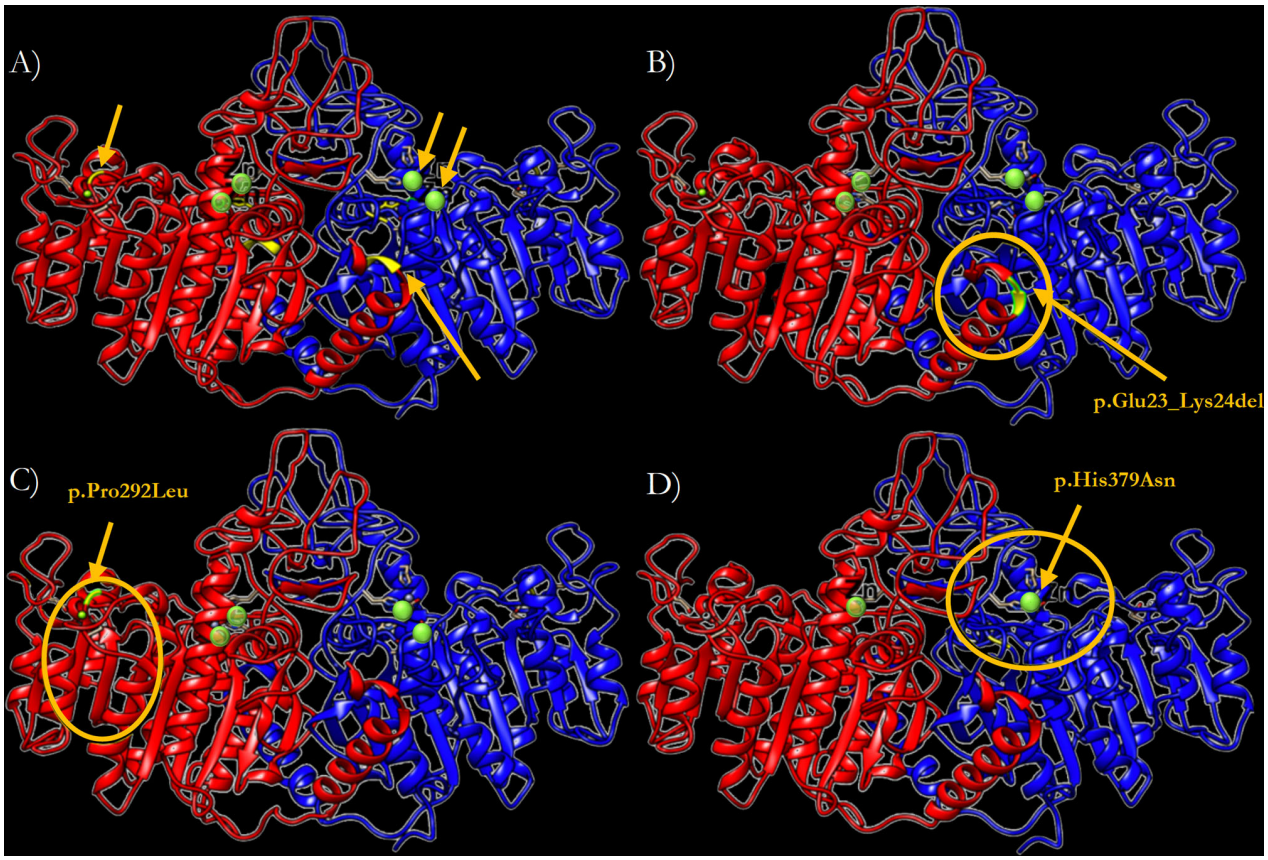

FIGURE 5 | Three-dimensional representation of the TNSALP protein in the form of ribbons. **(A)** TNSALP WT. Zn^{2+} atoms bound to the corresponding Zn^{2+} binding sites are highlighted in green. The arrows indicate the location of the different mutations identified in this study. **(B)** TNSALP with the genetic variant p.Del Glu23_Lys24. The deletion is marked in yellow and the major structural changes are highlighted with a yellow circle. **(C)** TNSALP with the genetic variant p.Pro292Leu. The amino acid substitution is marked in yellow and the major structural changes are highlighted with a yellow circle. **(D)** TNSALP with the genetic variant p.His379Asn. The loss of 2 of the 4 Zn^{2+} binding sites in the dimeric protein is highlighted with a yellow circle in one of the protein monomers.

However, the low serum levels observed in patients carrying these variants raise the suspicion that they could probably be pathogenic. Functional studies supported the results of serum determinations, showing significantly decreased enzyme activity in all three variants compared to TNSALP WT, showing the p.Del Glu23_Lys24 variant the higher enzymatic activity and the p.His379Asn variant the lower activity values. Regarding PLP levels, as expected, we found an inverse correlation with ALP activity, with gradually increasing serum PLP values associated with gradually decreasing ALP enzyme activity. Considering only the biochemical data, it seems difficult to establish a clear relationship between the severity of clinical manifestations and ALP enzyme activity, since of the two variants showing a major reduction in enzyme activity (p.Pro292Leu and p.His379Asn) only the latter is associated with a severe HPP phenotype. However, it appears that serum levels of PLP may better reflect the prognosis of the disease, and drastically elevated PLP levels may act as a marker of HPP severity.

Structural characterization allowed us to observe that p.Del Glu23_Lys24, the least severe variant at biochemical level, was strongly affected both structurally and in terms of polarity, particularly in the region of the N-terminal domain. The N-terminal domain region is composed of 22 residues with an important role in protein dimerization and is the anchoring site of glycosylphosphatidylinositol (GPI) to the TNSALP molecule (34). This deletion results in a shortening of the protein structure with a loss of the hydrophobic structure that protects the 22 residues of the N-terminal domain leading to the exposition of a hydrophilic residue to the surface which may affect the stability of the protein. The N-terminal domain of the monomeric subunits of TNSALP surrounds the active site of the enzyme (11). In fact, the correct folding of the N-terminal domain and the interaction with its microenvironment is crucial for both the structural integrity of the protein and for intramolecular transitions, so mutations in this area could lead to influence the allosteric performance of TNSALP as well as enzymatic catalysis of the active center (11, 35). Therefore, the p.Del Glu23_Lys24 mutation could compromise structural stability and alter interactions with the microenvironment leading to disturbed catalysis as our results showed. At clinical level, this

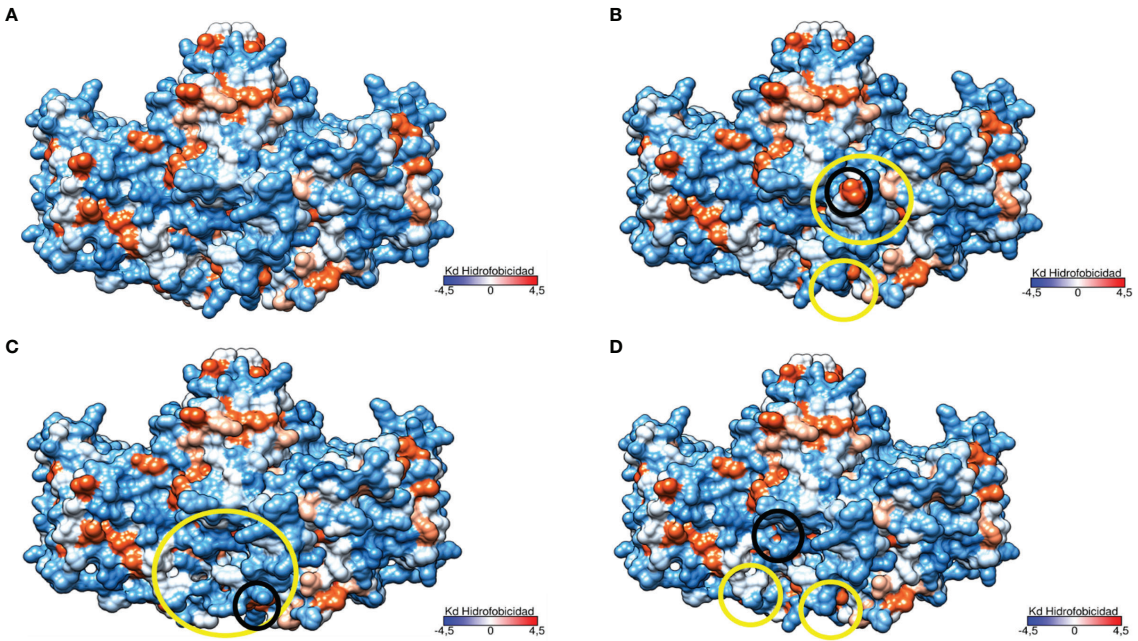

FIGURE 6 | Three-dimensional representation of the TNSALP protein according to its hydrophobicity. Hydrophobicity is represented on the Kyte and Doolittle hydrophobicity scale (Kd scale); from the most hydrophilic amino acids in light blue to the most hydrophobic in red. Changes to more hydrophilic and hydrophobic amino acids are marked in yellow and black circles respectively. **(A)** TNSALP WT. **(B)** TNSALP with the genetic variant p.Del Glu23_Lys24. **(C)** TNSALP with the genetic variant p.Pro292Leu. **(D)** TNSALP with the genetic variant p.His379Asn.

variant seems to have a mild symptomatology although the early onset of the articular manifestations could imply a worsening of the symptomatology over time, so this patient should be followed up to study the phenotype evolution.

Although this variant was initially classified as VUS, based on our functional and structural characterization results as well as on the symptomatology of the carrier patient, we consider that the p.Del Glu23_Lys24 variant could be considered as likely pathogenic.

The structural change of TNSALP associated with p.Pro292Leu variant affects the structural domain involved in calcium binding. Although calcium binding is crucial for proper folding and assembly of the TNSALP molecule (15), and it appears to be fundamental to TNSALP activity in bone mineralization (36), the structural and functional significance of the calcium binding site in TNSALP is not yet entirely clear to date (37). Two variants at the same amino acid position have been described in compound heterozygosis in patients affected with HPP: [c.(98C>T); (874C>A) and c.(815G>T); (874C>T)] related to severe forms of HPP (infant HPP and lethal perinatal HPP, respectively) (38, 39).

On the other hand, the structural change in TNSALP derived from this variant mainly affect the β-strand generating the elongation of this chain which causes instabilities in the hydrophobic region. Other mutations previously described affecting parts of this β-strand (p.Val217Ala or p.Val217Lys) has been associated with perinatal lethal form of HPP (40), so in general, mutations in this strand of the protein seem to be associated to severe phenotypes.

The variant identified in our study (p.Pro292Leu), appears to be associated with a mild phenotype of HPP in contrast to the severe phenotypes associated with other mutations in the same region. This variant seems to be the least pathogenic of the three identified (at clinical level) since, in addition to presenting few symptoms related to HPP, the age of symptomatologic onset was quite late.

These results suggest that the severity of symptomatology appears to be closely related to the amino acid encoded at that position. In the variant identified in our study, the mild phenotype observed in the affected patient could be explained by the similarity in stereochemistry and charge of proline with leucine, leading to a less severe amino acid substitution. However, it must be considered that the severity of the other variants in the same position may be due to compound heterozygosity and different degrees of penetrance.

The p.His379Asn variant seems to be the one associated with a more severe form of HPP, and although at the structural level there are no significant changes in terms of structure or polarity, the amino acid change in the protein sequence seems to have an important functional repercussion. This variant results in an amino acid change that causes the loss of one of the Zn^{2+} binding sites of the protein. This cation located in the active site of TNSALP acts as a cofactor of the metalloenzyme. Therefore, the main cause of such a drastic decrease in enzyme activity seems to

be associated with the 50% reduction in Zn^{2+} binding capacity. Accordingly, one of the characteristic symptoms of acrodermatitis enteropathica, a disease caused by a Zn^{2+} deficiency due to a mutation in SLC39A4 which encodes the zinc transporter Zip4, is hypophosphataemia (41). Based on this, we consider that this amino acid change close to the active center is particularly significant and helps to understand the severity of this variant. However, the heterozygous inheritance of this variant requires additional studies to rule out the presence of other mutations in non-coding regions of the *ALPL* gene that may explain the severity of the disease in the affected patient.

Despite the p.His379Asn variant has been associated to adult HPP the beginning of clinical manifestations in the affected patient was during childhood increasing the severity with age, ranging from fatigue and muscle pain in the lower limbs to hypotonia and paresthesias. This fact suggests that p.His379Asn variant could be associated with a form of infantile HPP, but due to the lack of knowledge of the HPP, its low prevalence and the absence of bone symptoms, there was a long delay in the diagnosis leading an accumulated damage until it was diagnosed as adult HPP (42).

Although some mutations have been identified in the other 5 residues that make up both Zn^{2+} binding sites (p.His341, p.His454, p.Asp337, p.Asp60, p.Asp378) that have been classified as potentially pathogenic, none of them had been characterized at the molecular level to date. However, the p.Asp378 variant has been associated with mild to severe phenotype of infantile HPP. Similarly, our findings supported by biochemical and clinical data as well as functional and structural characterization, reveal that p.His379Asn variant is clearly associated with a moderate to severe phenotype of HPP.

According to our results, of the three variants c.69_74del; p.(Del Glu23_Lys24), c.875C>T; p.(Pro292Leu) and c.1135C>A; p.(His379Asn) initially classified according to ACMG recommendations as VUS, the first has been reclassified as likely pathogenic and the two last as pathogenic in Varsome web search (https://varsome.com/) (28).

Interestingly, none of the patients had any fracture event in their clinical history. As mentioned above, this may be because TNSALP is not only involved in the mineralization process but also has pleiotropic functions in the body (11, 12).

Although this study is more oriented to find a genotype-phenotype relationship between HPP and mutations in the ALPL gene, it is important to point out that there are other less common situations that are also associated with low levels of ALP which must be considered to make a correct diagnosis. In this line, iron and ferritin has been shown as potent inhibitors of osteogenesis, significantly inhibiting ALP activity considering the ferroxidase activity of ferritin as the central element of this inhibition (43). In accordance with this, there are some studies that shows the involvement of other factors in TNSALP regulation such as the transcription factor RUNX2 (44). Similarly, it has been described other regulatory factors as PHOSPHO1, a factor responsible for generating Pi for HA crystallization with non-redundant role for TSNALP or ENPP1 that acts as a phosphatase in the absence of TNSALP (45). In this context, the existence of other modifier genes not yet known or identified cannot be excluded regarding the development of the heterogeneous clinical manifestations in the HPP patients.

In addition, the epigenetic modifications could contribute to the severity grade of the clinical behavior of the disease. In this line, the study of Delgado-Calle et al. shows an important role of DNA methylation in the regulation of ALPL expression through the osteoblast-osteocyte transition (46). Additionally, different lifestyles or behaviors should be considered as modifiers factors of HPP phenotype since seem to be a direct effect in ALP levels. Thus, the physical activity has been directly related to increased levels of ALP (47, 48). Regarding the study of phenotype associated to HPP, maybe it worthy to explore the role of these regulatory factors as well as the contribution of external factors as these lifestyles or behaviors.

This study highlights the importance of making a correct diagnosis of HPP in addition to establishing a geno-phenotypic relationship whenever possible. This will allow on the one hand to avoid erroneous or late diagnoses such as the case of the patient with p.His379Asn variant or erroneous treatments such as treatment with bisphosphonates, which worsen the symptoms derived from the hypomineralization of HPP by decreasing the activity of TNSALP (49); on the other hand, it will improve the knowledge of this metabolic disorder to make it accessible to the scientific community, allowing a better management of the disease through the establishment of a personalized medicine based on to the unique genetic characteristics and clinical manifestations of each patient.

5 CONCLUSIONS

Due to the high heterogeneity in the symptoms of HPP, it is necessary to characterize the new genetic variants that are identified in order to establish a genotype-phenotype relationship, which allows the most appropriate therapeutic measures to be carried out in each case. Functional and structural characterization studies showed that the p.Del Glu23_Lys24 and p.Pro292Leu variants are associated with mild adult HPP despite the marked reduction in enzymatic ALP activity. The variant p.Pro292Leu appears to be associated with the mildest symptomatology, with clinical manifestations debuting at an advanced age. Variant p.Del Glu23_Lys24, despite of presenting a mild symptomatology, its phenotype has developed at an early age (28 years), so it is convenient to follow up this patient in order to study the evolution of the symptomatology. The p.His379Asn variant is associated with a phenotype from moderate to severe of HPP probably childhood onset without bone impairment. The drastic decrease of the ALP activity could be explained mainly by the loss of Zn-binding capacity of TNSALP. The treatment of the patient affected with this variant with asfotase alpha therapy has shown an important improvement of the clinical manifestations suggesting that the main symptoms of this patient are related to HPP disease.

Supporting our results, the VarSome web has reclassified the study variants as likely pathogenic (p.Del Glu23_Lys24) and pathogenic (p.Pro292Leu; p.His379Asn).

The characterization and subsequent classification of new *ALPL* gene mutations found in HPP patients may facilitate disease management by healthcare professionals. This allows the disease to be addressed avoiding misdiagnosis or/and mistreatment and improving the quality of life of patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because they have been generated by a hospital service under a privacy clause. Requests for access to the datasets should be addressed to the corresponding author Cristina Garcia-Fontana.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the University Hospital San Cecilio of Granada in accordance with the principles of the World Medical Association Declaration of Helsinki (Project ID: 0777-M1-20. Research Ethics Committee of Granada Center (CEI-Granada) on 8 May 2019). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study design: MM-T, BG-F,VC-B, RS-dT, CG-F, JMV-S, FA-V, SG-S, II-B, and LM-H. Study conduct: JV-S, CG-F, FA-V, BG-F, SG-S, GM-N, PR, MC-S, II-B, RS-dT, and LM-H. Data collection: JMV-S, CG-F, BG-F, FA-V, and SG-S. Data analysis: JMV-S, CG-F, BG-F, FA-V, SG-S, II-B, RS-dT, and VC-B. Data interpretation: FA-V, RS-dT, LM-H, SG-S, JMV-S, CG-F, BG-F, and MM-T. Drafting manuscript: RS-dT, BG-F, CG-F, FA-V, MM-T, and II-B. Revising manuscript: MM-T, BG-F, CG-F, II-B, and RS-dT. All authors contributed to the article and approved the submitted version.

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The Value of Rare Genetic Variation in the Prediction of Common Obesity in European Ancestry Populations

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Polygenic risk scores (PRSs) aggregate the effects of genetic variants across the genome and are used to predict risk of complex diseases, such as obesity. Current PRSs only include common variants (minor allele frequency (MAF) ≥1%), whereas the contribution of rare variants in PRSs to predict disease remains unknown. Here, we examine whether augmenting the standard common variant PRS (PRS_{common}) with a rare variant PRS (PRS_{rare}) improves prediction of obesity. We used genome-wide genotyped and imputed data on 451,145 European-ancestry participants of the UK Biobank, as well as whole exome sequencing (WES) data on 184,385 participants. We performed single variant analyses (for both common and rare variants) and gene-based analyses (for rare variants) for association with BMI (kg/m^2), obesity (BMI ≥ 30 kg/m^2), and extreme obesity (BMI ≥ 40 kg/m^2). We built $PRSs_{common}$ and $PRSs_{rare}$ using a range of methods (Clumping+Thresholding [C+T], PRS-CS, lassosum, gene-burden test). We selected the best-performing PRSs and assessed their performance in 36,757 European-ancestry unrelated participants with whole genome sequencing (WGS) data from the Trans-Omics for Precision Medicine (TOPMed) program. The best-performing PRS_{common} explained 10.1% of variation in BMI, and 18.3% and 22.5% of the susceptibility to obesity and extreme obesity, respectively, whereas the best-performing PRS_{rare} explained 1.49%, and 2.97% and 3.68%, respectively. The PRS_{rare} was associated with an increased risk of obesity and extreme obesity ($OR_{obesity}$ = 1.37 per SD_{PRS}, $P_{obesity}$ = 1.7x10^{-85}; $OR_{extremeobesity}$ = 1.55 per SD_{PRS}, $P_{extremeobesity}$ = 3.8x10^{-40}), which was attenuated, after adjusting for PRS_{common} ($OR_{obesity}$ = 1.08 per SD_{PRS}, $P_{obesity}$ = 9.8x10^{-6}; $OR_{extremeobesity}$= 1.09 per SD_{PRS}, $P_{extremeobesity}$ = 0.02). When PRS_{rare} and PRS_{common} are combined, the increase in explained variance attributed to PRS_{rare} was small (incremental Nagelkerke R^2 = 0.24% for obesity and 0.51% for extreme obesity). Consistently, combining PRS_{rare} to PRS_{common} provided little improvement to the prediction of obesity (PRS_{rare} AUC = 0.591; PRS_{common} AUC = 0.708; $PRS_{combined}$ AUC = 0.710). In summary, while rare variants show convincing association with BMI, obesity and extreme obesity, the PRS_{rare} provides limited improvement over PRS_{common} in the prediction of obesity risk, based on these large populations.

Keywords: polygenic risk score, rare variants, obesity risk, burden score, PRS-CS, lassosum, C+T, BMI - body mass index

INTRODUCTION

With an estimated prevalence of 12% among adults worldwide and up to 42% in the US (1, 2), obesity is a growing epidemic, causing major public health concerns (1, 3). Risk prediction and early prevention of weight gain is key to reducing the personal and global burden of obesity and its comorbidities (4). Developing obesity across the lifespan is the result of an interaction between environmental and innate biological factors, encoded by our genomes. Twin and family studies have reported heritability estimates of obesity that range between 40 - 70% (5).

In the past 15 years, genome-wide association studies (GWAS) have identified thousands of variants associated with obesity-related traits (6). Polygenic risk scores (PRSs), which are based on GWAS summary statistics, represent an individual's overall genetic predisposition to obesity. In recent years, PRSs have been studied for their use in the prediction of future obesity and the identification of individuals at risk of obesity early on in life (7). The promise is that accurate estimation of people's genetic

predisposition would allow more targeted lifestyle intervention for those at risk. However, current PRSs, which are based on traditional GWAS, have been shown to be suboptimal, with unsolved challenges remaining (8). For example, existing methods to develop PRSs only include common variants (MAF ≥ 1%), they explain little of the variation (< 10%) in BMI and, thus, have limited ability to predict obesity (7, 9). There is a pressing need to incorporate rare variants (MAF < 1%), which have been shown to capture a proportion of the 'missing heritability' (10), and are currently not considered in the PRS construction.

Including rare variants in the PRS may improve the accuracy with which we estimate individuals' genetic predisposition. Because of the large sample size of studies, such as the UK Biobank, association summary statistics for rare variants (0.1% ≤ MAF < 1%) can be assessed by single variant testing (11). However, for ultra-rare variants (MAF < 0.1%), which occur by definition very infrequently in the population, even current large-scale studies are not large enough to study their individual effects (12). The accuracy of the PRS depends largely on the power of the discovery GWAS summary statistics (13). Therefore, aggregating ultra-rare variants in genes, based on their predicted functional consequences, offers a potentially powerful complementary approach to the single variant testing (14) and subsequently, building rare variant PRSs.

The aim of our study is to leverage sequencing data from the UK Biobank and the Trans-Omics for Precision Medicine (TOPMed) program to build obesity PRSs that use rare variants ($PRSs_{rare}$) and test their associations with obesity and extreme obesity. In addition, we will test the predictive power of $PRSs_{rare}$ for obesity outcomes alone or in combination $PRSs_{common}$.

MATERIALS AND METHODS

Study Design

We built and tested PRSs from common variants (MAF ≥ 1%), rare variants (MAF < 1%) and ultra-rare variants (MAF < 0.1%) for three traits; BMI, obesity and extreme obesity. We used data from the UK Biobank to conduct single variant GWAS analyses and gene burden analyses (ultra-rare variants). Then, the GWAS summary statistics, calculated using the UK Biobank data, were used to build PRSs for which we tested the predictive performance in the TOPMed program (**Figure 1**).

Study Populations

UK Biobank

All GWAS analyses were performed using data of the UK Biobank, a prospective cohort study with extensive genetic and phenotypic data collected in approximately 500,000 individuals, aged between 40–69 years (11). Briefly, participants were

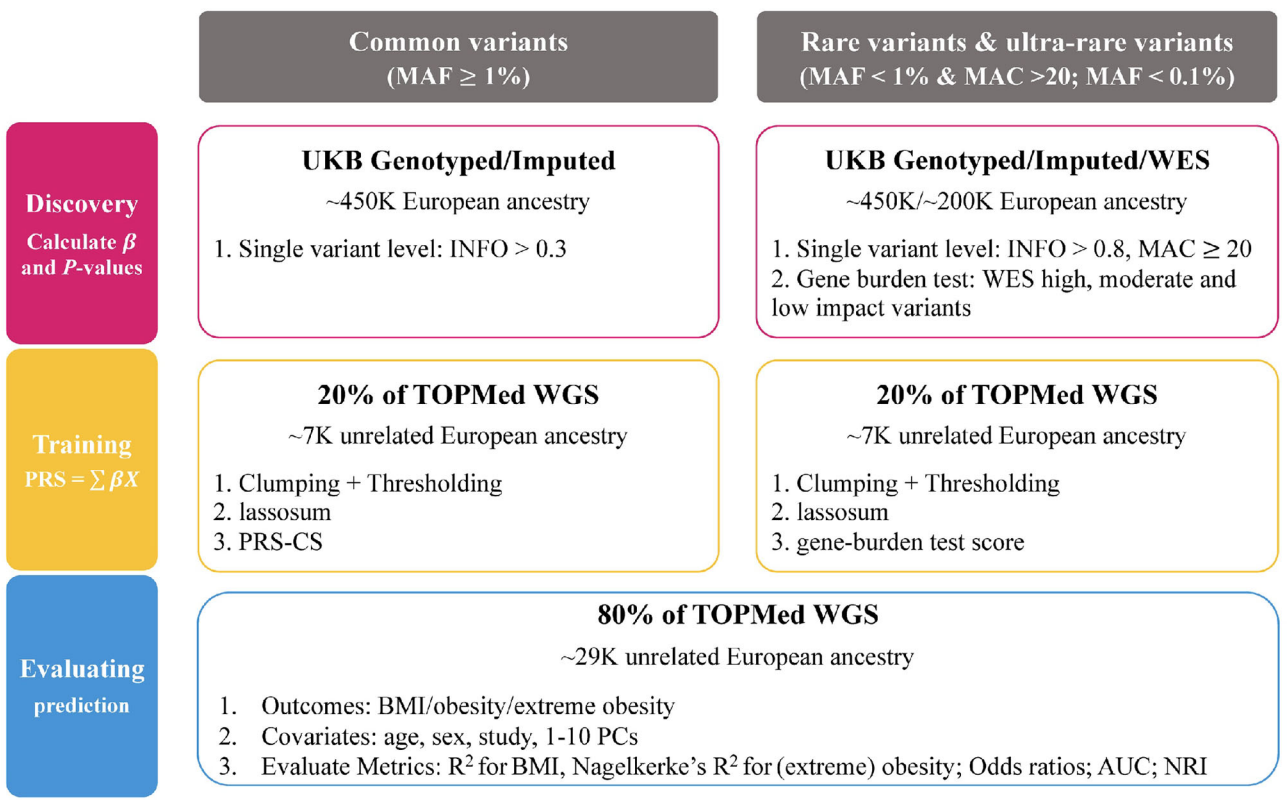

FIGURE 1 | Overview of the study framework.

enrolled from 2006 to 2010 at one of 22 assessment centers across the UK to provide baseline information, physical measures, and biological samples according to standardized procedures (11, 15). All participants provided written informed consent. We restricted analyses to individuals of European ancestry (described in detail below), excluded individuals who underwent weight loss surgery before recruitment and women who were pregnant at the time of recruitment. Data for 451,145 individuals was available for analyses.

TOPMed

For constructing and testing the PRS, we used data from 22 parent studies of the TOPMed program (**Supplementary Table 1**). We restricted analyses to 43,251 individuals of European ancestry that have cleaned phenotype data (described in detail below) and Whole Genome Sequencing (WGS) data. We removed one individual from each related pair (N_{excl} = 6,494; genetic relatedness ≥.0625). In addition, we removed Data for a total of 36,757 individuals were available for analyses (**Supplementary Table 1**).

Phenotype Definitions

UK Biobank

Height and weight, used to calculate BMI as weight (kg) divided by height squared (m^2), were collected at the baseline visit. BMI was used to categorize individuals with underweight (BMI < 18.5 kg/m^2), normal weight (18.5 kg/m^2 ≤ BMI < 25 kg/m^2), overweight (25 kg/m^2 ≤ BMI < 30 kg/m^2), obesity (BMI ≥ 30 kg/m^2) or extreme obesity (BMI ≥ 40 kg/m^2). More details can be found elsewhere (11, 15).

TOPMed

Data on height and weight, used to calculate BMI, were harmonized across studies by the TOPMed Anthropometry Working Group. BMI was calculated based on weight and height measurements, collected from the participating studies. We excluded individuals with known pregnancy at measurement, with implausibly high BMI values (> 100 kg/m^2), and those < 18 years old. In the presence of duplicated samples, the sample with the highest sequencing depth was retained.

Genotyping, Imputation and Sequencing Data

UK Biobank

All UK Biobank participants were genotyped using the UK Biobank Axiom Array. More than 800,000 variants were directly genotyped and > 90 million variants were imputed, using the Haplotype Reference Consortium or UK10K + 1000G reference panels (11). Variants with imputation INFO score of ≥ 0.3 for common (MAF ≥ 1%), and imputation INFO score of ≥ 0.8 for rare variants (MAF < 1%) were included in analyses.

We identified individuals of European ancestry based on their genetic information, using k-means clustering. First, we calculated principal components and their loadings for 488,377 genotyped UK Biobank participants based on the intersection of ~121,000 variants after quality control and 1000G Phase 3v5 reference panel. Reference ancestries are 504 European (EUR), 347 American Admixed (AMR), 661 African (AFR), 504 East Asian (EAS) and 489 South Asian (SAS) samples (overall 2504). We projected the 1000G reference panel dataset based on the calculated PCA loadings from UK Biobank. We then used k-means clustering with a pre-specified amount of 4 clusters to the UK Biobank PCA and the projected 1000G reference panel dataset. Individuals that clustered within the EUR individual cluster from the 1000G reference panel were assigned as individuals of European ancestry (N = 453,812). Because PRSs based on current methods generalize poorly across other ancestries, and because of the smaller sample sizes of non-European ancestry population, we performed analyses only in European ancestry populations.

In addition to the genotyped and imputed data, we used data of the first release of exome sequencing (N=184,385). The approach used to perform exome sequencing and quality control is described in detail elsewhere (16, 17). We annotated variants using Variant Effect Predictor (VEP) v104.3 with genome build GRCh38 (18).

TOPMed

WGS, targeting a mean depth of >30X coverage, was performed at seven different Sequencing Centers. For this study, we used WGS data from Freeze 8 release (19). Information about genome sequencing, variant calling, and quality control procedures can be accessed through the TOPMed website (20). The genetic relationship was estimated using the PC-Relate algorithm (21). We removed one from each pair of the individuals with genetic relationship closer than 3rd degree (≥.0625) of relatedness (21).

Population groups in TOPMed were based on a combination of participants' self-reported race/ethnicity and genetic ancestry represented by PCs. When participants' self-reported race/ethnicity values were "Other", "Multiple" or missing, the HARE method was used to classify individuals into "Asian", "Black", "White", or "Hispanic/Latino" subgroups using the first nine PC-AiR PCs (22). For this project, we limited our analyses to those either self-identified as "White" or they had overall genetic ancestry that closely resembled groups of European ancestry (HARE strata classified as 'White").

Genome-Wide Association Testing: Single Variant and Gene Burden Tests in UK Biobank

BMI residuals were generated in men and women separately, adjusting for age, age^2, and the first 10 genetic principal components (PCs). Residuals underwent inverse normal transformation, to achieve a normal distribution with a mean of 0 and a standard deviation of 1.

Single Variant Association Testing

Association analyses of the inverse normal BMI residuals, obesity, and extreme obesity were carried out using a (generalized) linear mixed-model approach in BOLT-LMM (23) and REGENIE (24). Models were adjusted for age, age^2, sex and first 10 PCs for obesity and extreme obesity. For all single variant association testing, variants with a minor allele count of

≤20 were excluded. We performed single variant association testing using [1] genotyped and imputed variants, and [2] WES data, separately.

Gene Burden Testing

We aggregated ultra-rare variants (MAF < 0.1%) from the WES data for gene burden testing. For each gene, we considered five categories of masks (i.e. variant sets considered in burden test): [M1] a strict burden of rare loss-of-function (LoF) variants (i.e. splice_acceptor, splice_donor, stop_gained, frameshift, stop_lost, and start_lost), [M2] a permissive burden of rare LoF variants and inframe indels, [M3] a more permissive burden of all high and moderate impact rare variants (including LoF, inframe indels, and missense variants) [M4] moderate impact variants (inframe indels and missense variants), and [M5] high, moderate and low impact variants (LoF, inframe indels, missense and synonymous variants, **Figure 2**). We aggregated MAF ≤ 0.1% variants for each of these masks, that is up to 5 burden tests per gene.

Polygenic Risk Score Derivation in TOPMed

Based on the single variant association testing and gene burden testing results in UK Biobank, we generated $PRSs_{common}$ and $PRSs_{rare}$ using three different approaches (PRS_{common}: Clumping + Thresholding [C+T], PRS-CS (18), lassosum (25); PRS_{rare}: C+T, lassosum, gene-burden test) in 36,757 unrelated individuals of European ancestry of TOPMed. Summary statistics from GWAS of the UK Biobank were filtered for variants present in TOPMed (**Figure 2**).

C+T denotes the Linkage Disequilibrium (LD) clumping and *P* value thresholding method, which was conducted using the PRSice-2 software (26). For clumping, we used the entire sample of 36,757 unrelated individuals of European ancestry as the reference panel for LD and set clumping parameters to R^2 = 0.2, 0.5 and 0.8, with each region being 250kb in size. We varied the *P* value thresholds from $5x10^{-5}$ to 0.8, with a step-wise increase of $1x10^{-4}$. The C +T method was used to build both PRS_{common} and PRS_{rare}.

PRS-CS is a Bayesian method that infers the posterior mean effect size of each variant using GWAS summary statistics and external LD (27), but is distinct from previous methods by placing a continuous shrinkage (CS) prior on the variant effect sizes (27). A 1000G LD reference panel for European ancestry populations was provided by the developers. We followed the PRS-CS author recommended protocol by removing ambiguous A/T or G/C variants and restricting to common variants (MAF ≥ 1%) included in HapMap3. Therefore, this method was used only to build PRS_{common}. We considered the shrinkage prior (phi = $1x10^{-3}$, $1x10^{-4}$) and the PRS-CS auto option, which allows the software to learn the continuous shrinkage prior from the data.

lassosum is an approach that uses penalized regression on summary statistics and accounts for LD using an external reference panel or target sample to produce more accurate weights for building PRSs (25). To accurately assess the LD –

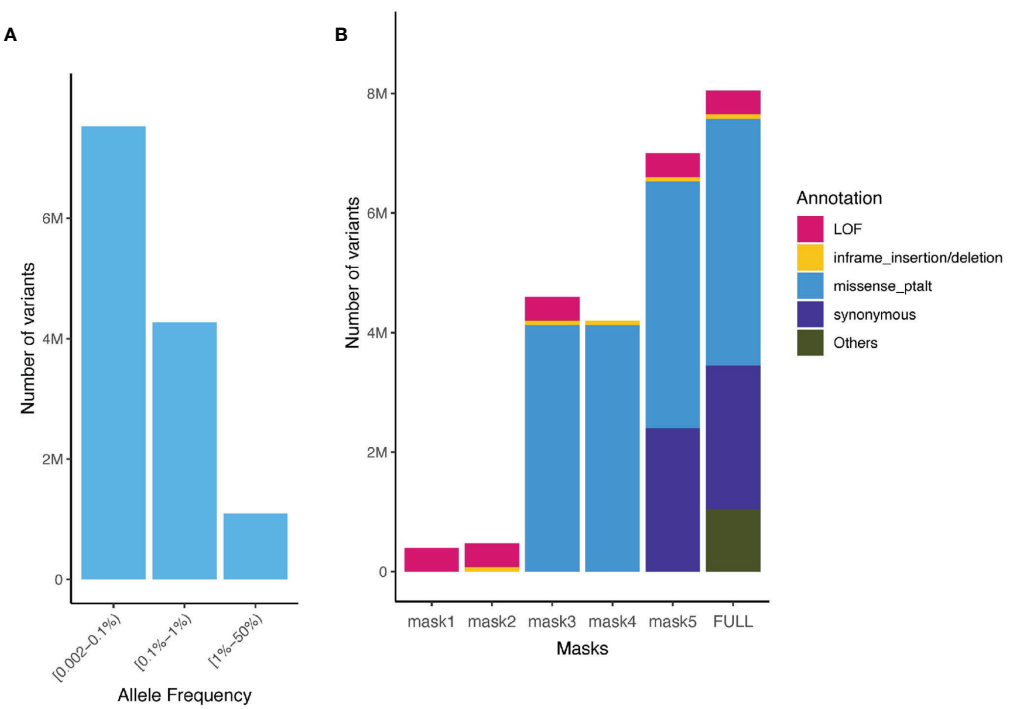

FIGURE 2 | Allele frequency spectrum of imputed variants and number of aggregated sequenced variants captured in the UK Biobank and the TOPMed. **(A)** Minor allele frequency spectrum of imputed variants present in the UK Biobank (rare variants imputation INFO ≥ 0.8, common Hapmap3 variants imputation INFO ≥ 0.3) and TOPMed; **(B)** Number of variants for different functional class of variants and masks (aggregation model) in the UK Biobank WES ultra-rare variants (MAF < 0.1%).

particularly important for rare variants – we used the entire sample of 36,757 unrelated individuals of European ancestry TOPMed as the reference panel. lassosum's model parameters (s, the shrinkage parameter: 0.2, 0.5, 0.9 and 1; and λ, the penalty parameter: varied from 0.001 to 0.1) were tuned. We applied the lassosum method to common and rare variants separately to build PRS_{common} and PRS_{rare}.

Lastly, we built ultra-rare variant burden scores using the gene burden test results from the UK Biobank. For each of the five masks, we tested the following *P* value threshold of gene burden tests; P = 0.05, 0.001, 0.0001, 10^{-5}, and $2.8x10^{-6}$ (i.e. exome-wide significance level). For assigning weights to variants within each gene, we tested two methods: 1) a simple method, which assigned the same weights to all variants in the same mask (i.e. using the aggregate effect size estimated from LoF (mask1) gene A in UK Biobank to the LoF (mask1) variants in gene A in the TOPMed samples); 2) a nested method, which assigned a weight to each variant equal to the aggregate effect size of variants with annotation at least as severe as the variant (**Supplementary Figure 1** provides an example to illustrate the nested method).

For each individual in the testing sets (TOPMed), PRSs were calculated as the sum of the dosages multiplied by the given weight at each variant. Taken together, we generated six sets of PRSs ($PRS_{common\text{-}C+T}$, $PRS_{common\text{-}lassosum}$, $PRS_{common\text{-}PRS\text{-}CS}$, $PRS_{rare\text{-}C+T}$, $PRS_{rare\text{-}lassosum}$, and $PRS_{rare\text{-}burden}$) for each trait (BMI, obesity and extreme obesity) using the different methods under a range of tuning parameters.

Statistical Analyses

BMI in TOPMed was inverse rank normalized, in men and women separately. We split unrelated individuals in TOPMed by randomly selecting 20% for PRS training (N=7,433, tuning parameter and selecting the best performing PRS) and 80% for evaluation (N=29,324, validating R^2 and predicting performance). For each PRS method applied, we calculated adjusted R^2 values for BMI and Nagelkerke R^2 values for (extreme) obesity. Models were adjusted for age, sex, the first ten PCs and study. 95% confidence intervals were calculated using bootstrapping. We selected the best-performing PRS for each method and PRS combination (i.e. the largest variance explained (adjusted R^2 values or Nagelkerke R^2), resulting in six best-performing PRSs in total (one for each from $PRS_{common\text{-}C+T}$, $PRS_{common\text{-}lassosum}$, $PRS_{common\text{-}PRS\text{-}CS}$, $PRS_{rare\text{-}C+T}$, $PRS_{rare\text{-}lassosum}$, and $PRS_{rare\text{-}burden}$).

In the 80% withheld TOPMed individuals, we tested the association between each PRS and obesity/extreme obesity status using logistic regression. The best-performing PRS_{common} and PRS_{rare} across multiple methods were then combined to study the joint effects of PRS_{common} and PRS_{rare} to predict obesity. To evaluate the prediction performance of PRS_{rare}, we calculated the area under the receiver operator curve (AUC) in a Cox regression model with the obesity/extreme obesity status as the outcome. We also assessed the net reclassification index (NRI) and the Integrated Discrimination Increment (IDI), which evaluated the model improvement in discrimination and reclassification.

RESULTS

Best-Performing Polygenic Risk Scores Based on Common Variants (PRSscommon)

Using BMI-GWAS summary statistics derived in the UK Biobank (**Supplementary Figure 2**), the PRS_{common} built with the lassosum method (**Supplementary Table 2** and **Figure 3**) explained the most variation in BMI (R^2 = 10.1%, 95% CI = 9.4-10.7%).

Similarly, the best-performing $PRSs_{common}$ based on summary statistics of obesity and extreme obesity GWASs, was built using lassosum (Nagelkerke R^2 = 16.7% for obesity and 20.7% for extreme obesity, **Supplementary Table 2** and **Figure 3**). Of interest is that that the PRS_{common} based on BMI-GWAS summary statistics explained more of the variation in (extreme) obesity (Nagelkerke R^2 = 18.3% for obesity and 22.5% for extreme obesity) than the PRS_{common} based on (extreme) obesity GWAS summary statistics (**Figure 3**). This likely reflects the relatively higher power of the BMI GWAS.

Best-Performing Polygenic Risk Scores Based on Rare Variants (PRSsrare) at Single Variant Level

The best-performing PRS_{rare} for BMI was built using the lassosum method, based on BMI-GWAS summary statistics, explaining 1.49% of variation in BMI (95% CI = 1.23-1.77%, **Supplementary Table 2** and **Figure 3**). Consistent with our observations for the $PRSs_{common}$, a PRS_{rare} based on BMI-GWAS summary statistics explained more of the variance for (extreme) obesity liability (Nagelkerke R^2 = 2.97% for obesity and 3.68% for extreme obesity) than a PRS_{rare} based on (extreme) obesity GWAS (Nagelkerke R^2 = 2.28% for obesity and 2.55% for extreme obesity) (**Figure 3**).

Best-Performing Polygenic Risk Score Based on Ultra-Rare Variants (PRSrare-Burden) Using Gene Burden Score

Aggregating variants using mask1 (LoF variants) with an association significance of $P < 2.8x10^{-6}$ resulted in the best-performing $PRS_{rare\text{-}burden}$, explaining a mere 0.03% (95%CI = 0.002-0.08%) of variation in BMI (**Methods, Supplementary Figure 3** and **Supplementary Figure 4**). However, this $PRS_{rare\text{-}burden}$ aggregated LoF variants in only two genes (*MC4R* and *UBN2*) and identified 2,957 individuals (8% of the TOPMed population) with non-zero values of the score (**Supplementary Figure 4**).

We repeated the gene burden score approach using summary statistics of obesity and extreme obesity (**Supplementary Figure 5**), yielding slightly improved results than for a $PRS_{rare\text{-}burden}$ based on BMI summary statistics. Mask3, which aggregates variants in genes that reached exome-wide significance—only *MC4R* meets this P-value threshold ($P < 2.8x10^{-6}$)—provided the best-performing $PRS_{rare\text{-}burden}$ score, explaining 0.08% of variation in obesity and 0.39% of variation in extreme obesity liability.

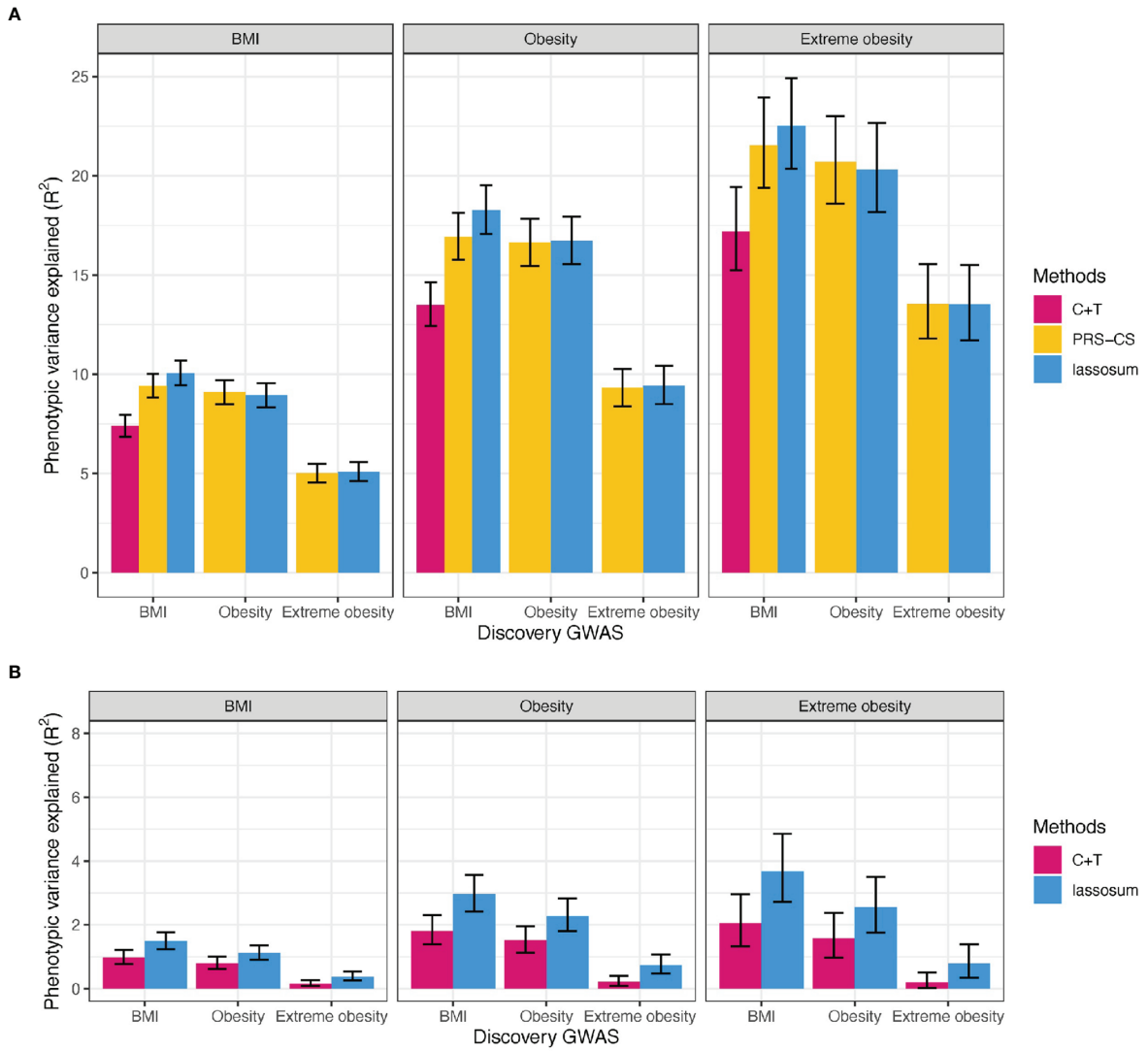

FIGURE 3 | Variance explained by PRS for BMI, obesity, and extreme obesity in BMI, obesity and extreme obesity. **(A)** PRScommon **(B)** PRSrare, We reported adjusted R^2 for BMI, Nagelkerke's R^2 for (extreme) obesity on top of covariates including age, sex, study and PCs. C+T: Clumping and Thresholding method. Error bars indicates 95% CI.

Association of PRSscommon and PRSsrare With Risk of Obesity

We next tested the association of the best-performing PRSs (i.e. $PRS_{common\text{-}lassosum}$ and $PRS_{rare\text{-}lassosum}$ based on BMI-GWAS summary statistics and $PRS_{rare\text{-}burden}$ based on obesity-GWAS summary statistics) with obesity outcome.

Each SD increase in the BMI-GWAS based $PRS_{rare\text{-}lassosum}$ was associated with a 1.37 ($P = 1.7x10^{-85}$) increase in the odds of obesity (**Supplementary Table 3**). Adding $PRS_{common\text{-}lassosum}$ to the model substantially attenuated the association between $PRS_{rare\text{-}lassosum}$ and risk of obesity (OR = 1.08 per SD, $P = 9.8x10^{-6}$). This attenuation is likely due to the correlation between $PRS_{rare\text{-}lassosum}$ and $PRS_{common\text{-}lassosum}$ (r = 0.31). Each 0.1 increase in obesity-GWAS based $PRS_{rare\text{-}burden}$ (range: 0 - 0.41) was associated with a 1.83 higher odds of obesity ($P = 0.02$). Adding the $PRS_{common\text{-}lassosum}$, (r = 0.008) and/or $PRS_{rare\text{-}lassosum}$ (r=0.01) had little impact on the association (**Supplementary Table 3**). We observed a similar pattern for the PRSs' associations with extreme obesity (**Supplementary Table 3**). Consistently, adding both $PRS_{rare\text{-}lassosum}$ and $PRS_{rare\text{-}burden}$ in addition to model with PRS_{common} was extremely small (incremental Nagelkerke R^2 0.24% for obesity and 0.51% for extreme obesity, **Supplementary Table 3**).

Using the $PRS_{common\text{-}lassosum}$ and $PRS_{rare\text{-}lassosum}$ to identify individuals at high risk of obesity (top PRS decile), we observe that, relative to the reference group (deciles 1-9), individuals in the top decile for both PRSs had the highest risk of obesity and extreme obesity (OR [95%CI] = 5.3 [4.2-6.7], 13.5 [9.6-18.9], respectively), as compared to individuals that were defined as high risk by only one of the two PRSs (**Figure 4**).

Using PRScommon and PRSrare to Predict Common Obesity

Adding both $PRS_{rare\text{-}lassosum}$ and $PRS_{rare\text{-}burden}$ to $PRS_{common\text{-}lassosum}$ in the prediction model did not improve the prediction of obesity (PRS_{common} only AUC [95%CI] 0.708 [0.701 – 0.716] *vs* all three PRSs 0.710 [0.702 – 0.717], **Figure 5**). Adding both $PRS_{rare\text{-}lassosum}$ and $PRS_{rare\text{-}burden}$ to a model with $PRS_{common\text{-}lassosum}$ only slightly improved the discrimination of the model (IDI= 0.0014 [0.0008 - 0.0019], **Supplementary Table 4**). Knowledge of individuals' $PRS_{rare\text{-}lassosum}$ and $PRS_{rare\text{-}burden}$, in addition to the $PRS_{common\text{-}lassosum}$, would only reassign 0.9% of individuals to their appropriate risk category (NRI=0.9%; 95% CI= 0.49-1.32%; $P = 2x10^{-5}$). Using extreme obesity as the outcome yielded similarly small improvements in predictive accuracy (**Supplementary Table 4**, **Supplementary Figure 6**).

DISCUSSION

In this study, we examined the contribution of rare variants to the polygenic prediction of obesity by leveraging data from 451,145 European-ancestry individuals in UK Biobank and 36,757 in TOPMed. We observed that $PRSs_{rare}$ were associated with an increased risk of obesity and extreme obesity, partially independent of PRS_{common}. Nevertheless, their explained variance (up to 1.49%) as well as predictive accuracy were small (AUC 0.591 for obesity and 0.630 for extreme obesity), and particularly limited when considered in combination with PRS_{common}.

As PRSs are becoming a standard tools in translational research and clinical practice, there has been an increasing interest to study the role of rare variants, in addition to common ones, for a range of common diseases, such as breast cancer, prostate cancer, coronary artery disease (CAD) and obesity (28–31). Most previous studies that have reported on the contribution of rare variants studied the role of pathogenic variants in one or few high-penetrance genes and did not investigate their predictive accuracy at a population level (28, 29, 31). Consistent with our findings, though, these studies demonstrated that rare variants act—at least in part—independently from common variant PRSs and add to people's polygenic susceptibility to disease (28, 29, 31). Thus, knowing an individuals' PRS_{rare}, in addition to PRS_{common}, may contribute to identifying individuals at high risk of obesity. However, given the

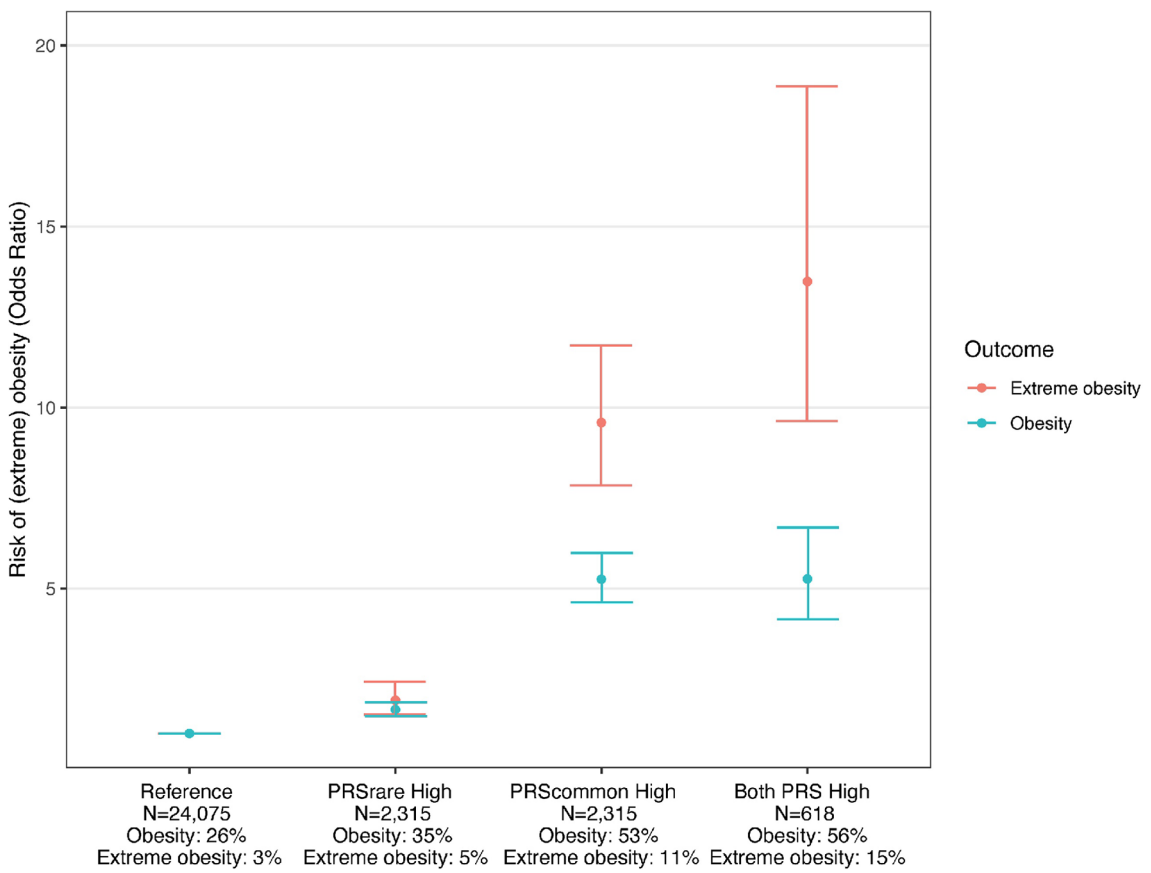

FIGURE 4 | Risk of obesity among individuals with high PRSrare and PRScommon. Reference: deciles 1-9 of PRS_{common} and PRS_{rare}, PRS_{rare} High: top decile of PRS_{rare}, PRS_{common} High: top decile of PRS_{common}, Both PRS High: top decile of PRS_{common} and PRS_{rare}.

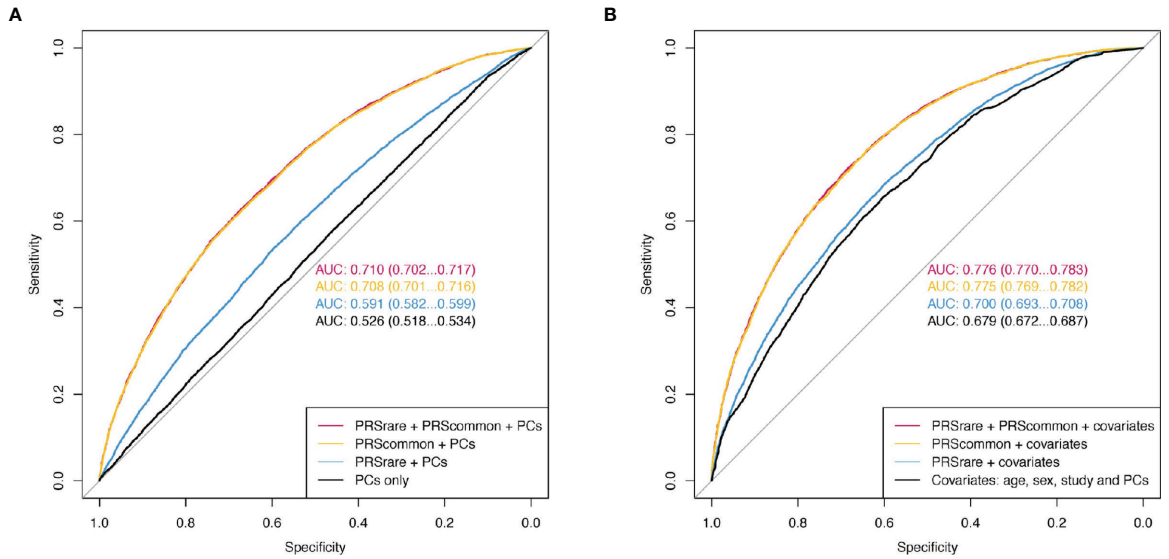

FIGURE 5 | The receiver operating characteristic curve (ROC) of obesity. **(A)** Model only included PCs as baseline covariates. **(B)** Additionally included age, sex, and study. PRSrare includes $PRS_{rare\text{-}lassosum}$ and $PRS_{rare\text{-}burden}$.

limited explained variance observed in our analyses, we expect that few individuals will indeed score high on both scores. Nevertheless, for these few individuals, knowing their high risk may be valuable.

Recently, a new framework was developed to aggregate rare variant burden into a rare variant PRS (30). As an example, a rare variant genetic risk score for CAD was built, using UK Biobank data. Similar to our findings for obesity and extreme obesity, a significant association of this PRS_{rare} with risk of CAD was observed, although the explained variation was only 0.1% of the population variance (30). We report a similar explained variance of 0.2% for obesity and 0.5% for extreme obesity. The reasons why the PRS_{rare}'s explained variance is small, in particular in addition to the PRS_{common}, are threefold. First, the PRS_{rare} was not completely independent from PRS_{common}, even after including only non-overlapping variants. It is likely that the true causal (rare) variants were tagged by common variants in LD. Second, any new (rare) variant added to the PRS increases the PRS' uncertainty due to statistical noise associated with estimating a new weight (32). The PRS_{rare} might have suffered more from this, as accurately estimating weights for rare variants requires larger sample size in general. Third, rare variants, although more likely to have larger effects (12), are too rare to explain much of the obesity epidemic in the general population.

Consistent with the low variance explained, the predictive power by the PRS_{rare} over that of the PRS_{common} was limited. The improvement in AUC for obesity (from 0.708 to 0.710) was negligible, although the AUC for the PRS_{rare} alone was up to 0.59. This supports our observation that the predictive power of the PRS_{rare} in part overlapped with that of the PRS_{common}. So far, no other studies have reported on the contribution of $PRS_{rare,}$ in the presence of PRS_{common}.

In addition to using BMI summary statistics to build PRSs and test their predictive performance for obesity and extreme obesity, we built $PRSs_{common}$ and $PRSs_{rare}$ based on obesity and extreme obesity GWAS summary statistics. The PRS_{common} and PRS_{rare} based on BMI-GWAS summary statistics outperformed those based on obesity or extreme obesity GWAS summary statistics, which is in line with previous findings that PRS_{common} based on the full distribution explains a larger proportion of the variance than when based on the tails of the distribution (33). For the ultra-rare variants, the $PRS_{rare\text{-}burden}$ based on obesity summary statistics performed better than the those based BMI-based summary statistics, which maybe be due to the role of ultra-rare variants in (extreme) obesity, but less in BMI. Our discovery GWASs were conducted in a relatively healthy and less deprived UK Biobank population (34), which may have limited our ability to capture the genetic contribution of rare variants for obesity and extreme obesity.

We acknowledged that our samples for analyses were restricted to one ancestry only. We focused our analyses on European-ancestry populations for which the most data are available. Because allele frequencies, LD patterns, and effect sizes, differ between ancestries, the accuracy of European-derived PRSs decays rapidly when applied to other ancestries (35). PRSs derived from other ancestries are currently underpowered because of relatively small sample sizes. As more data becomes available for other ancestries, both GWAS as well as sequencing data, the here described analyses should be performed to examine whether observation are generalizable across ancestries. Furthermore, we focused solely on obesity, a common multifactorial trait that is moderately heritable. While many complex traits have similar feature, we cannot guarantee that our observations can be extrapolated to other outcomes as

the genetic architecture, explained variance from common variants, and contribution from rare pathogenic variants may differ (36).

Taken together, we demonstrate that while rare variants, aggregated in $PRSs_{rare}$, have been shown to independently associate with obesity risk, they provide a minimal improvement in prediction accuracy over PRS_{common} in predicting obesity risk in the general population. Our findings cast an important light on the potential value of rare variants in the prediction of complex diseases, such as obesity.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. UK Biobank data can be found here: UK Biobank (https://www.ukbiobank.ac.uk/). All TOPMed data for each participating study can be accessed through dbGaP with the corresponding accession number listed in Acknowledgments.

ETHICS STATEMENT

All phenotypic and genetic data were collected with approval from the Institutional Review Board with patient consent at each institution. This study was approved by the Institutional Review Board (IRB) of the Icahn School of Medicine at Mount Sinai in New York, New York.

AUTHOR CONTRIBUTIONS

Study concept and design: ZW and RL. Acquisition of cohort level data: EB, RL, ZW, NC, MF, SR, BP, JAB, JCB, ES, M-LM, ER, WK, RV, C-TL, RM, LY, RRK, DA, RK, KN, AJ, SH, MA, JR, XG, LL, SSR, PE, SL, JB, MS, DD, MG, CA, DC, CK, RJ, and AR. Statistical analysis: ZW and SC. Interpretation of data: ZW, PFO, and RL. Manuscript writing group: ZW, PFO, SC, and RL. Supervision: PFO and RL. All authors contributed to the article and approved the submitted version.

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performed at the Broad Institute of MIT and Harvard (3R01HL092577-06S1). WGS for "NHLBI TOPMed: The Vanderbilt Atrial Fibrillation Registry" (phs001032.v3.p2) was performed at the Broad Institute of MIT and Harvard (3R01HL092577-06S1). WGS for "NHLBI TOPMed: Walk-PHaSST Sickle Cell Disease (SCD)" (phs001514.v2.p1) was performed at the Baylor Human Genome Sequencing Center (HHSN268201500015C). WGS for "NHLBI TOPMed: The Women's Genome Health Study" (phs001040.v3.p1) was performed at the Broad Institute of MIT and Harvard (3R01HL092577-06S1). WGS for "NHLBI TOPMed: Women's Health Initiative (WHI)" (phs001237.v1.p1) was performed at the Broad Institute of MIT and Harvard (HHSN268201500014C). Core support including centralized genomic read mapping and genotype calling, along with variant quality metrics and filtering were provided by the TOPMed Informatics Research Center (3R01HL-117626-02S1; contract HHSN268201800002I). Core support including phenotype harmonization, data management, sample-identity QC, and general program coordination were provided by the TOPMed Administrative Coordinating Center (R01HL-120393; U01HL-120393; contract HHSN268201800001I). We gratefully acknowledge the studies and participants who provided biological samples and data for TOPMed.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.863893/full#supplementary-material

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Identification and Characterization of Two Novel Compounds: Heterozygous Variants of *Lipoprotein Lipase* in Two Pedigrees With Type I Hyperlipoproteinemia

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Background: Type I hyperlipoproteinemia, characterized by severe hypertriglyceridemia, is caused mainly by loss-of-function mutation of the *lipoprotein lipase* (*LPL*) gene. To date, more than 200 mutations in the *LPL* gene have been reported, while only a limited number of mutations have been evaluated for pathogenesis.

Objective: This study aims to explore the molecular mechanisms underlying lipoprotein lipase deficiency in two pedigrees with type 1 hyperlipoproteinemia.

Methods: We conducted a systematic clinical and genetic analysis of two pedigrees with type 1 hyperlipoproteinemia. Postheparin plasma of all the members was used for the LPL activity analysis. *In vitro* studies were performed in HEK-293T cells that were transiently transfected with wild-type or variant *LPL* plasmids. Furthermore, the production and activity of LPL were analyzed in cell lysates or culture medium.

Results: Proband 1 developed acute pancreatitis in youth, and her serum triglycerides (TGs) continued to be at an ultrahigh level, despite the application of various lipid-lowering drugs. Proband 2 was diagnosed with type 1 hyperlipoproteinemia at 9 months of age, and his serum TG levels were mildly elevated with treatment. Two novel compound heterozygous variants of *LPL* (c.3G>C, p. M1? and c.835_836delCT, p. L279Vfs*3, c.188C>T, p. Ser63Phe and c.662T>C, p. Ile221Thr) were identified in the two probands.

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The postheparin LPL activity of probands 1 and 2 showed decreases of 72.22 ± 9.46% ($p<0.01$) and 54.60 ± 9.03% ($p<0.01$), respectively, compared with the control. *In vitro* studies showed a substantial reduction in the expression or enzyme activity of LPL in the *LPL* variants.

Conclusions: Two novel compound heterozygous variants of *LPL* induced defects in the expression and function of LPL and caused type I hyperlipoproteinemia. The functional characterization of these variants was in keeping with the postulated *LPL* mutant activity.

Keywords: lipoprotein lipase (LPL), type 1, hypertriglyceridemia, variants, pedigree

1 INTRODUCTION

Type I hyperlipoproteinemia, also known as familial lipoprotein lipase (LPL) deficiency, is characterized by very severe hypertriglyceridemia with episodes of abdominal pain, recurrent acute pancreatitis, eruptive cutaneous xanthomata, and hepatosplenomegaly (1). The prevalence of type I hyperlipoproteinemia in the general population is estimated to be 1–2 per million (2, 3). Inherited in an autosomal recessive manner, type I hyperlipoproteinemia is caused mainly by the loss-of-function mutation of the *LPL* gene (4).

The *LPL* gene encodes a secreted glycoprotein, LPL, containing 448 amino acids (5). Synthesized by cells such as fat cells, macrophages, and muscle cells, LPL is an important rate-limiting enzyme for triglyceride degradation, which can hydrolyze triglycerides into fatty acids and glycerol to regulate lipid metabolism (6, 7). Defective *LPL* can cause the accumulation of triglyceride-rich chylomicrons and very low-density lipoproteins and further lead to severe hypertriglyceridemia. To date, more than 200 mutations in the *LPL* gene have been reported to result in type I hyperlipoproteinemia in the Human Gene Mutation Database (HGMD), while only a limited number of mutations have been evaluated for pathogenesis (8, 9).

In this study, two patients with severe hypertriglyceridemia were confirmed to have type I hyperlipoproteinemia by whole-exome sequencing (WES), and two novel compound heterozygous variants of the *LPL* gene were identified. We showed through bioinformatics analysis and *in vitro* experiments that these variants can affect the enzymatic activity, production, and/or secretion of LPL and cause type I hyperlipoproteinemia in both cases. Our research provides evidence for elucidating the molecular mechanisms of mutant *LPL* and helps improve the genetic diagnosis rate and precise treatment of this disease.

2 MATERIALS AND METHODS

2.1 Ethics

This study was approved by the Ethics Committee of Shandong Provincial Hospital affiliated with Shandong University. The study protocol was in line with the Declaration of Helsinki (as revised in Brazil 2013). The consent obtained from all the participants was both informed and written.

2.2 Subjects and Follow-Up Studies

Two patients diagnosed with severe hypertriglyceridemia from our hospital were involved in the study. All patient data were collected at the first visit. All family members received specific physical and laboratory examinations in Shandong Provincial Hospital. Peripheral blood specimens were collected from each member for genetic analysis.

We followed these two patients from their first presence and closely tracked their clinical and biochemical information.

2.3 Whole-Exome Sequencing and Sanger Sequencing

Using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), genomic DNA was isolated from peripheral blood leukocytes. Next, DNA from peripheral blood was used for WES. We performed genomic DNA fragmentation, paired-end adaptor ligation, amplification, and purification and then captured human exons using a SeqCap EZ Med Exome Enrichment Kit (Roche NimbleGen, Madison, WI, USA). By postcapture amplification and purification, a DNA library was generated and then sequenced with the Illumina HiSeq sequencing platform. To obtain the coverage and mean read depth of target regions, sequence data alignment to the human genome reference (hg19) and variant calling were performed using NextGene V2.3.4 software. The average coverage of the exome was >100×, which allowed a deep examination of the target region to accurately match >99% of the target exons. Mutations with low coverage in the target area were screened and filtered to ensure the accuracy of data analysis.

In addition, the frequency of normal populations [data from Genome Aggregation Database (GnomAD), Exome Aggregation Consortium (ExAC), Trans-Omics for Precision Medicine (TOPMED), Human Gene Mutation Database (HGMD), Clinvar and Online Mendelian Inheritance in Man (OMIM) databases] was obtained by NextGene V2.3.4 and our in-house scripts. A variant was identified as a mutation when it was not found in 500 Chinese controls, in dbSNP (http://www.ncbi.nlm.nih.gov/snp/) and the exome variant server (http://evs.gs.washington.edu/EVS/), or when the allele frequency was <0.001 in the database. According to Standards and Guidelines for the Interpretation of Sequence Variants published by the American College of Medical Genetics (ACMG) in 2015, pathogenic variants were determined with the Human Genome Variation Society (HGVS) nomenclature.

Candidate variants were detected by WES. Pathogenic or suspected pathogenic variants were verified by Sanger sequencing. Universally tagged sequencing primers were designed by using Primer3 version 1.1.4 (http://www.sourceforge.net) and GeneDistiller 2014 (http://www.genedistiller.org/). Polymerase chain reaction (PCR) was performed in a 50-μl system including 5 μl 10 × PCR buffer, 4 μl dNTPs, 4 μl genomic DNA, 1 μl forward and reverse primers, and 0.3 μl Taq Hot Start (Takara Bio, Otsu, Japan). The PCR conditions were as previously described (10). Amplicons were sequenced using an ABI 3730 system (Applied Biosystems, Foster City, CA, USA) and were analyzed by the autoassembler software Chromas 2.6 and visual inspection.

2.4 The Activity of LPL in Plasma

Preheparin blood samples were taken after fasting overnight. Then, heparin was injected intravenously (60 IU/kg), and the postheparin blood was collected from the contralateral arm 10 min later. The subjects stopped taking insulin and other medications in the morning to eliminate the interference caused by medications. The blood sample was centrifuged at 3,000 rpm for 10 min at 4°C to obtain plasma. A Lipoprotein Lipase Activity Assay Kit (Fluorometric, Biovision, Milpitas, CA, USA) was used to measure LPL activity. Mouse (C57BL/6) postheparin plasma was used as a positive control.

2.5 *In Vitro* Functional Analysis of *LPL* Variants

2.5.1 Construction of Overexpression Plasmids Containing the Target *LPL* Mutation

By GeneArt Gene Synthesis (Thermo Fisher Scientific, Rockford, IL, USA), wild-type *LPL* cDNA (pcDNA3.1- *LPL*-WT) was synthesized and cloned into the pcDNA3.1 vector with a V5 epitope tag. *LPL* variants (pcDNA3.1-*LPL*-MU: p. M1?, p. L279Vfs*3, p. Ser63Phe, or p. Ile221Thr) were obtained by site-directed mutagenesis. To obtain the mutant *LPL* cDNA, we used the following primers: forward (wild type) GACCCAATAAGCTTCGTCAGAATTTTG TAATACGACTCACTATAGG, reverse (wild type) ATTGGGTC AAGCTTATGTTTTGTAAAAGTTACTTCCTCCACT; forward (p. M1)? CAGAGGGACGCGCCCCGAGATCGAGAGCAAAG CCCTGCTCGTGC, primer reverse (p. M1)? AGCACGAG CAGGGCTTTGCTCTCGATCTCGGGGCGCGTCCCTCTG; primer forward (p. L279Vfs*3) GACCCAATAAGCTTC GTCAGAATTTTGTAATACGACTCACTATAGG, primer reverse (p. L279Vfs*3) ATTGGGTCAAGCTTGGAACTGCAC CTGTAGGCCTTACTTGGA; primer forward (p. Ser63Phe) CACCTCATTCCCGGAGTAGCAGAGTTCGTGGC TACCTGTCATTTCA, primer reverse (p. Ser63Phe) TGAAATGACAGGTAGCCACGAACTCTGCTACTCCGGG AATGAGGTG; primer forward (p. Ile221Thr) GAGGGTCCCCTGGTCGAAGCACTGGAATCCAGAAA CCAGTTGG, primer reverse (p. Ile221Thr) CCCAACTGGTTTCT GGATTCCAGTGCTTCGACCAGGGGACCCTCT. All the primers used were purchased from GenScript (Cayman Islands, UK). Polymerase chain reaction (PCR) was carried out in a 50-μl reaction system including 5 μl 10 × PCR buffer, 4 μl genomic DNA, 4 μl dNTPs, 1 μl forward and reverse primers, and 0.3 μl Taq Hot Start (Takara Bio, Otsu, Japan). The PCR conditions consisted of an initial denaturation step (95°C for 5 min), followed by 40 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and elongation (68°C, 30 s). The presence and fidelity of pcDNA3.1-*LPL*-MU were confirmed by the ABI 3730 sequencing system (Applied Biosystems, Foster City, CA, USA).

2.5.2 Transient Transfection

In high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 5% penicillin–streptomycin, and 2 mM L-glutamine, human embryonic kidney 293T/17 (HEK293T/17) cells obtained from the National Collection of Authenticated Cell Cultures were cultured. Next, HEK293T/17 cells were transiently transfected with plasmids containing pcDNA3.1- *LPL*-WT and pcDNA3.1-*LPL*-MU by using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific). As previously described (10), the transfection concentration of a single plasmid was 2.5 μg/ml, and the concentration of each plasmid was 1.5 μg/ml at the cotransfection of the two plasmids.

After 48 h, the cells were lysed with mammalian protein extraction reagent (Thermo Fisher Scientific) containing protease inhibitor cocktail (Sigma-Aldrich). HEK293T/17-cell lysates were used to analyze the protein expression of LPL by Western blotting. We used Amicon® Ultra15 ultrafiltration centrifuge tubes (Millipore-Sigma, Burlington, MA, USA) to concentrate the medium 80 times. The concentrated medium was used for LPL activity analysis.

2.5.3 The Activity of LPL in Cell Culture Medium

The Lipoprotein Lipase Activity Assay Kit (Fluorometric, Biovision) was used to determine LPL activity in the culture medium of HEK293T/17 cells transfected with *LPL* wild-type plasmids and plasmids containing *LPL* variants.

2.5.4 Immunoblotting

We boiled the cell lysates mixed with Laemmli buffer containing 2-mercaptoethanol for 5 min at 99°CC. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 100 V, 90 min) and then transferred onto a nitrocellulose membrane (400 mA, 1 h). Membranes were incubated overnight with primary antibodies and washed three times for 10 min with 0.2% Tris-buffered saline Tween (TBST). Then, the membranes were incubated with secondary antibodies for 1 h and washed three times for 10 min with 0.2% TBST. Next, a 5-min incubation between chemiluminescent horseradish peroxidase (HRP) substrate (Millipore Corporation, Billerica, MA, USA) and membranes was conducted, and bands were visualized by a ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). The following antibodies were used: rabbit monoclonal anti-LPL antibody (Abcam Ab91606) and mouse monoclonal anti-GAPDH (glyceraldehyde-3-phosphatee dehydrogenase) antibody (Abcam Ab8245).

2.6 Statistical Analysis

Statistical analysis was performed using the SPSS 24.0 software package (SPSS Inc., Chicago, IL, USA). The Kolmogorov–

Smirnov test was used to determine the distribution of continuous variables. Continuous variables with a normal distribution are given as the mean ± standard deviation (SD) and were compared by the Mann–Whitney U test. The results were considered statistically significant when the p-value was <0.05.

3 RESULTS

3.1 Clinical Manifestations

Proband 1, female, was hospitalized with acute necrotizing pancreatitis and underwent abdominal surgery at the age of 24. At 25 years old, she was found to have high triglyceride (TG) levels (approximately 17 mmol/L). However, TG levels increased further after she was administered some lipid-lowering drugs (including fenofibrate, rosuvastatin, Chinese medicine, etc.). In recent years, TG levels fluctuated at 13.63–33.94 mmol/L, and TC levels fluctuated at 4.92–10.51 mmol/L after drug withdrawal (**Figures 1A, B**). At the age of 49, the proband presented symptoms of hyperglycemia with fasting plasma glucose at 15.22 mmol/L and developed vomiting and diarrhea after oral administration of Chinese medicine decoction. Abdominal computerized tomography (CT) scan (**Figure 1C**) showed that the pancreas was irregular in shape; no obvious abnormality was observed in other parts. After giving patient 1 the low-fat diet, insulin, and liver protection drugs, the last follow-up in June 2020 showed that the TG, TC, and fasting plasma glucose were 15.37, 9.43, and 6.01 mmol/L, respectively (**Table 1**). The family history of the patient showed that the patient's parents and her sister were deceased and could not be tested. However, it was recalled that her sister had also suffered from recurrent pancreatitis (**Figure 2A**).

Proband 2 came to our hospital for treatment because his blood sample showed severe hyperlipemia during the physical examination at 9 months old. On the second day of admission, his TG level was 20.35 mmol/L, and his TC level was 8.89 mmol/L. A low-fat diet and 4 U of continuous intravenous infusion of insulin were ordered to lower blood lipids. After 3 days, the TG level was 22.77 mmol/L, and the TC was 7.60 mmol/L. After 7 days, the TG level was 12.32 mmol/L, and the TC level was 4.55 mmol/L. After 10 days, the proband was discharged, his blood lipids improved significantly, with TG at 10.25 mmol/L and TC at 4.93 mmol/L. After being discharged from the hospital, he continued to consume a low-fat diet without any special

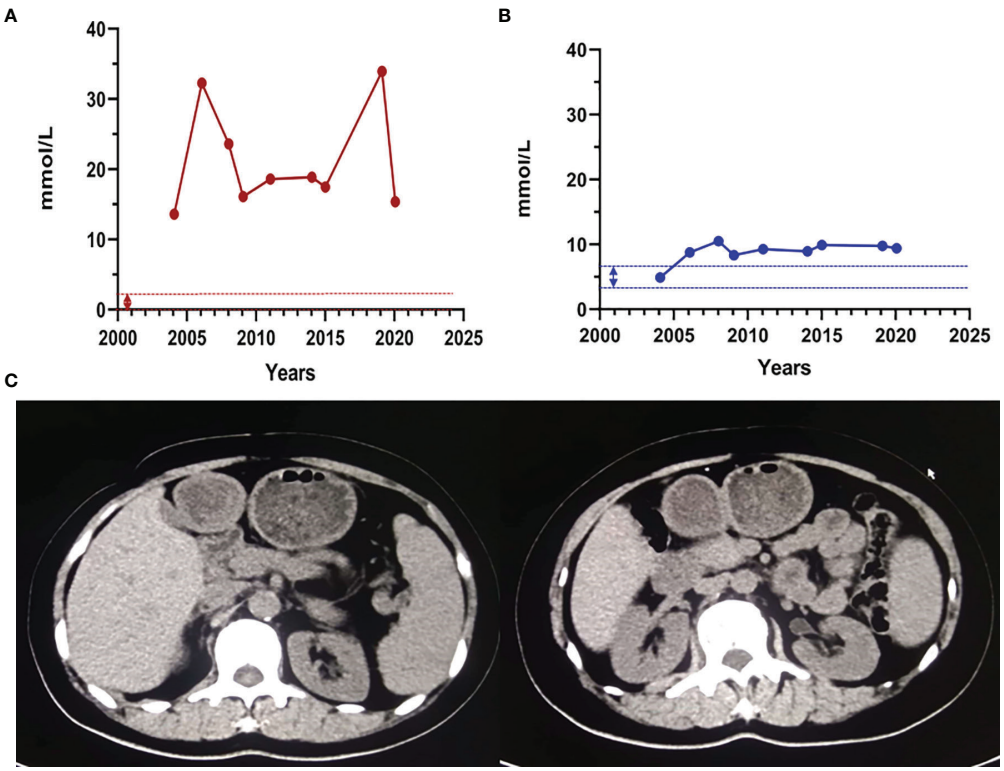

FIGURE 1 | The clinical characteristics of proband 1. **(A)** The proband 1 triglyceride and total cholesterol levels. The dashed line indicates the normal reference range. Abbreviations: TG, triglycerides; TC, total cholesterol. Normal range: TG: 0.4–1.8 mmol/L; TC: 3.6–6.2 mmol/L. **(B)** Abdomen and lower limbs of patient 1. **(C)** Abdominal CT scan of patient 1. The pancreas was irregular in shape; the liver was normal in shape and size; no abnormal density shadow was observed in the parenchyma; no expansion of the bile ducts in and outside the liver was observed; the shape and size of the gallbladder were fine; no abnormal density shadows were found in the gallbladder; and the spleen was normal in shape and size.

TABLE 1 | Clinical features and lipoprotein profile of the individuals screened for *LPL* variants.

Variable	Patient 1	Patient 2
Age (years)	49	5
Gender (male/female)	Female	Male
BMI (kg/m^2)	18.8	17.6
Alcohol intake (yes/no)	No	No
Diabetes mellitus (yes/no)	Yes	No
History of pancreatitis (yes/no)	Yes	No
Number of pancreatitis	2	0
Pregnancy	Yes	No
TG (mmol/L)	15.37	3.35
TC (mmol/L)	9.43	2.72
HDL-c (mmol/L)	0.57	0.75
LDL-c (mmol/L)	3.30	1.42
FBG (mmol/L)	6.01	5.60
Treatment	Low-fat diet, statin, fibrate, Chinese medicine, insulin, and liver protection drugs	Low-fat diet, insulin

BMI, body mass index; TG, triglycerides; TC, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; FBG, fasting blood glucose. Normal range: TG: 0.4–1.8 mmol/L; TC: 3.6–6.2 mmol/L; HDL-c: 0.8–1.5 mmol/L; LDL-c: 0.5-3.36; FBG, 3.9–6.3 mmol/L.

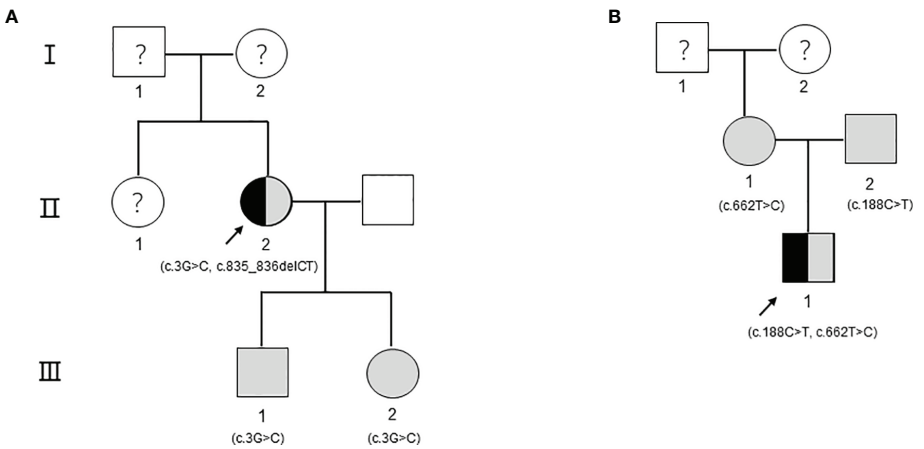

FIGURE 2 | The pedigree including the patients. The arrow refers to the proband. Black indicates that the person has type I hyperlipoproteinemia. The shading indicates that the person carries gene mutations but has a healthy phenotype. Circles indicate female individuals reared, and squares indicate male individuals reared. The question mark means that it cannot be determined whether the individual carries a genetic mutation. **(A)** The pedigree of patient 1. **(B)** The pedigree of patient 2.

discomfort. The last follow-up conducted in June 2020 showed that the serum TG was 3.35 mmol/L, the TC was 2.72 mmol/L, and the fasting plasma glucose was 5.60 mmol/L (**Table 1**). The family history of the patient shows that the parents of patient 2 have thus far been healthy (**Figure 2B**).

3.2 Variant Detection

To further identify disease-causing genes to facilitate diagnosis, we subsequently applied the WES technique for genetic analysis of the two pedigrees. According to HGMD, we found a novel compound variant of *LPL* (c.3G>C and c.835_836delCT, **Figure 3A**) in proband 1, which may cause truncation mutations (p. M1)?, and frameshift mutations (p. L279Vfs*3) leading to premature amino acid stop codes for protein synthesis, respectively. The p. M1? variant has been reported in a related clinical case, and its region is the translation initiation codon of this protein (11). The p. L279Vfs*3 variant is absent in the GnomAD, TOPMED, and ExAC databases. Notably, the son and daughter of proband 1 carry the p. M1? variant derived from the maternal line, as verified by Sanger sequencing, but their lipid levels and other phenotypes were normal.

We also identified proband 2 as carrying a novel compound variant of *LPL* (c.188C>T and c.662T>C, **Figure 3B**) that resulted in a change in the 63rd amino acid of the encoded protein from serine to phenylalanine (p. Ser63Phe) and a change in the 221st amino acid of the encoded protein from isoleucine to threonine (p. Ile221Thr), respectively. The p. Ser63Phe variant is absent in the GnomAD, TOPMED, and ExAC databases, while the p. Ile221Thr variant has been reported in three unrelated probands causing lipoprotein lipase deficiency (12). In addition, the parents of proband 2 each carried a variant. The Ser63Phe

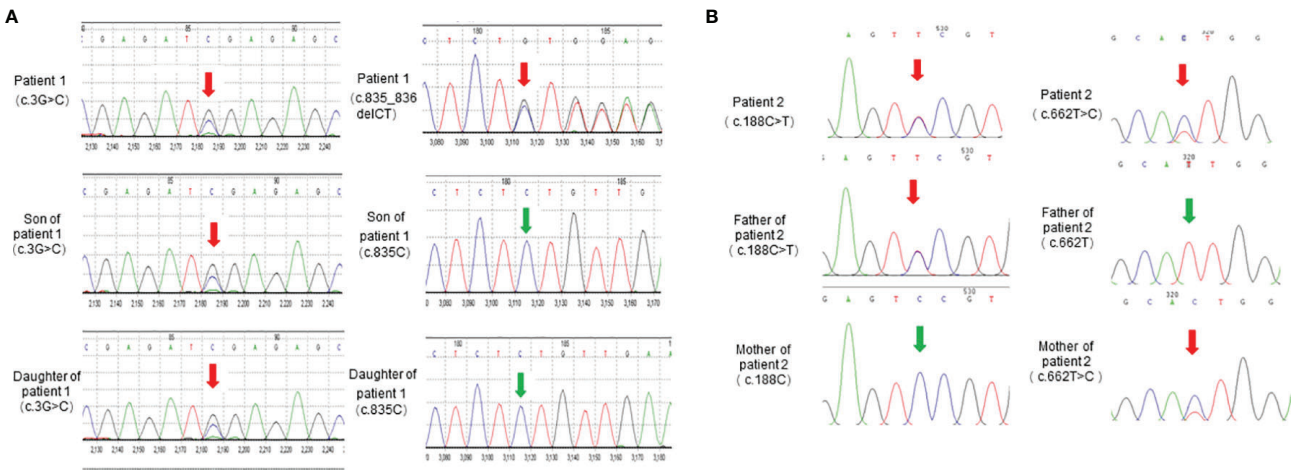

FIGURE 3 | *LPL* gene sequencing diagram. **(A)** *LPL* gene (reference sequence NM_000237) sequencing diagram of patient 1 and her children. **(B)** *LPL* gene (reference sequence NM_000237) sequencing diagram of patient 2 and his parents.

variant comes from the father, and the p. Ile221Thr variant comes from the mother, but they have thus far been healthy.

3.3 The Activity of LPL in Plasma

Accordingly, the postheparin LPL activity of patient 1 (0.46 ± 0.13 mU/ml) and patient 2 (0.75 ± 0.11 mU/ml) actually showed a decrease of 72.22 ± 9.46% ($p<0.01$) and 54.60 ± 9.03% ($p<0.01$), respectively, compared with the control (1.66 ± 0.09 mU/ml). The differences between the son and daughter of proband 1, the parents of proband 2, and the control seem to be negligible

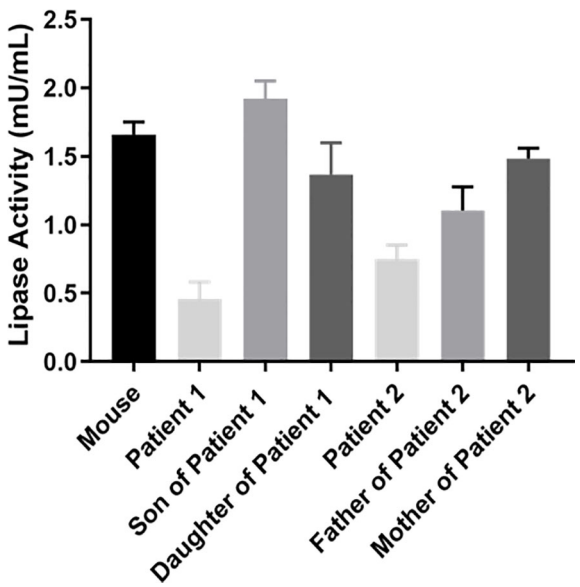

FIGURE 4 | Analysis of LPL activity in plasma. Peripheral blood was collected at 10 min after heparin injection (60 IU/kg) to assay lipase activity by the enzyme-fluorescent method.

(**Figure 4**). Therefore, two novel compound heterozygous variants of *LPL* may cause changes in LPL enzyme activity in the patient's plasma, leading to defects in triglyceride metabolism and resulting in extremely elevated serum triglycerides.

3.4 LPL Protein Expression in Cells

To understand the effect of the identified *LPL* variants at the protein level, we transiently transfected the plasmids containing pcDNA3.1-*LPL*-WT and pcDNA3.1-*LPL*-MU into HEK293T/17 cells. HEK293T/17 cells transfected with the p. M1? mutation, p. L279Vfs*3 mutation, or p. M1? and p. L279Vfs*3 mutation showed virtually no protein in the cell lysate ($p<0.01$, **Figure 5**). Compared with cells transfected with the plasmids containing the pcDNA3.1-*LPL*-WT, there was no significant difference in the amount of protein reduction produced by the cells transfected with the p. S63F mutation or the two variants (p. S63F and p. I221T), but protein synthesis was reduced by 48.33 ± 1.78% in cells transfected with the p. I221T variant ($p<0.01$, **Figure 5**).

3.5 The Activity of LPL in Cell Culture Medium

Next, we tested LPL activity in media of HEK 293T/17 cells. Wild-type LPL medium was used as a positive control. Except for the p. L279Vfs*3 mutation, LPL activity in the medium transfected with other plasmids containing pcDNA3.1-*LPL*-MU was significantly reduced compared with the LPL activity of the wild type. Specifically, cells transfected with the p. M1? or the two variants (p. M1? and p. L279Vfs*3) showed reductions in protein activity of approximately 55.93 ± 3.28% ($p<0.01$) or 59.84 ± 4.47% ($p<0.01$), while LPL activity was reduced by 62.76 ± 9.90% ($p<0.01$) or 63.25 ± 10.55% ($p<0.01$) in the media of HEK 293T/17 cells transfected with p. Ser63Phe or the two variants (p. Ser63Phe and Ser63Phe+Ile221Thr). The media of cells transfected with p. Ile221Thr demonstrated a maximum

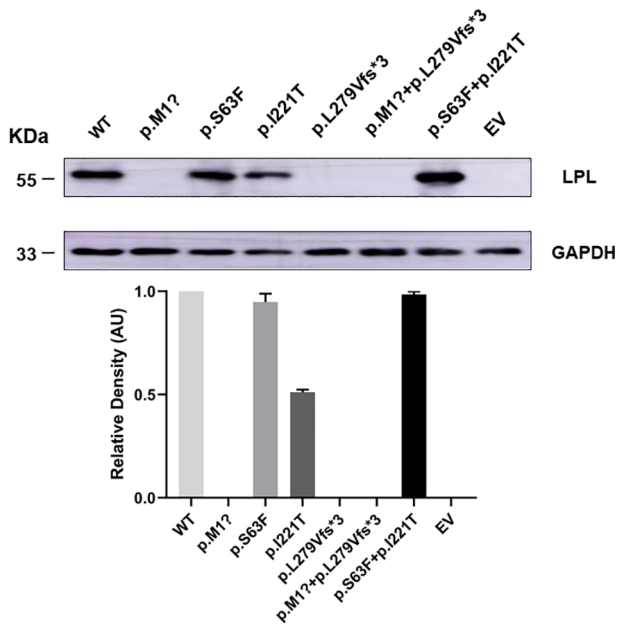

FIGURE 5 | HEK 293T/17 cells transiently transfected showed a reduction in the production of *LPL* variants. HEK293T/17 cells transfected with the p. M1? mutation, p. L279Vfs*3 mutation, or p. M1? and p. L279Vfs*3 mutation showed virtually no protein in the cell lysate ($p<0.01$). Compared with cells transfected with the plasmids containing the pcDNA3.1-*LPL*-WT, there was no significant difference in the amount of protein reduction produced by the cells transfected with the p. S63F mutation or the two variants (p. S63F and p. I221T), but protein synthesis was reduced by 48.33 ± 1.78% in cells transfected with the p. I221T variant ($p<0.01$). Abbreviations: EV, empty vector; WT, wild type; LPL, lipoprotein lipase.

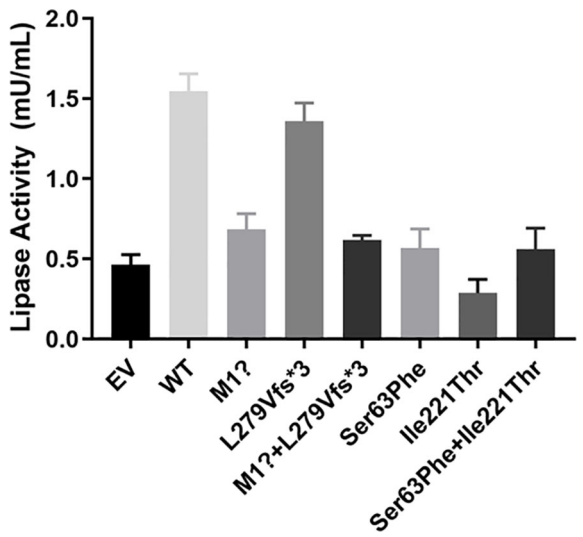

FIGURE 6 | Functional analysis of *LPL* mutants in the medium. Except for the p. L279Vfs*3 mutation, LPL activity in the medium transfected with other plasmids containing pcDNA3.1-*LPL*-MU was significantly reduced compared with the wild type. Specifically, cells transfected with the p. M1? or the two variants (p. M1? and p. L279Vfs*3) showed reductions in protein activity of approximately 55.93 ± 3.28% ($p<0.01$) or 59.84 ± 4.47% ($p<0.01$), while LPL activity was reduced by 62.76 ± 9.90% ($p<0.01$) or 63.25 ± 10.55% ($p<0.01$) in the media of HEK 293T/17 cells transfected with p. Ser63Phe or the two variants (p. Ser63Phe and Ser63Phe+Ile221Thr). The media of cells transfected with p. Ile221Thr demonstrated a maximum reduction in LPL activity of approximately 81.56 ± 4.35% ($p<0.01$).

reduction in LPL activity of approximately 81.56 ± 4.35% ($p<0.01$, **Figure 6**).

4 DISCUSSION

In the present study, we systematically identified and characterized two novel compound heterozygous variants of the *LPL* gene in patients with type I hyperlipoproteinemia. This harmful blood lipid metabolism disorder prompted us to solve this problem by determining the activity of LPL in plasma after heparin and analyzing the activity and quality defects of LPL *in vitro*. The functional characterization of these variants was in keeping with the postulated *LPL* mutant activity.

LPL is the rate-limiting enzyme in normal triglyceride metabolism, which plays a central role in the hydrolysis of triglycerides present in very-low-density lipoproteins and chylomicrons (13). Therefore, defective LPL can cause severe hypertriglyceridemia, such as type I hyperlipoproteinemia, a rare autosomal recessive genetic disease (14). In patients with hypertriglyceridemia, low plasma LPL activity and quality have been found (4, 15), and this phenomenon is usually caused by pathogenic *LPL* variants (16–18). The LPL protein consists of a large N-terminal domain (amino acid residues 1–315) and a small C-terminal domain (amino acid residues 316–448). The N-terminal domain contains the catalytic center, active site region, substrate-binding site, and heparin-binding site (19, 20). In addition to participating in the binding of heparin to the substrate, the C-terminal domain has been considered to be critical to the formation and stability of the LPL head and tail non-covalent homodimers (21, 22). Although LPL has always been considered to be active only as a homodimer, there are reports of biochemical data showing that LPL in complex with GPIHBP1 can be active as a monomer 1:1 complex (23).

Interestingly, we detected a novel compound variant of *LPL* (p. M1? and p. L279Vfs*3) in a Chinese patient with type I hyperlipoproteinemia. This patient exhibited severe hypertriglyceridemia and recurrent acute pancreatitis. In particular, the p. M1? variant, an initiation codon mutation, has previously been identified in an 18-year-old patient from China who also carried the previously reported heterozygous substitution of glutamic acid at residue 242 with lysine (p. Glu242Lys) (11). *In vitro* experiments found that the p. M1? had approximately 3% protein mass and 2% activity, whereas p. Glu242Lys had normal mass but undetectable activity; that is, the p. M1? variant rather than the heterozygous p. Glu242Lys variant is mainly responsible for the phenotypic expression of type I hyperlipoproteinemia in this patient. Here, our *in vitro* expression of both mutations separately or in combination displayed virtually no protein in the cell lysate. Except for the p. L279Vfs*3 mutation, the LPL activity in the

medium transfected with p. M1? or both mutations in combination was significantly reduced compared with the LPL activity of the wild type. Therefore, we speculate that the p. M1? mutation will have a significant impact on the quality and activity of LPL, but p. L279Vfs*3 has little effect on the activity. What needs illustration is that the son and daughter also carry the p. M1? variant derived from proband 1, but their lipid levels and other phenotypes were normal. Compared with the control, the postheparin LPL activity in the plasma of the son and daughter was not different. Hence, what caused proband 1 to have severe hyperlipidemia? This requires more in-depth research to explore.

Proband 2 also carries a novel compound variant of *LPL* (p. Ser63Phe and p. Ile221Thr). The p. Ile221Thr variant has been reported in three unrelated probands causing lipoprotein lipase deficiency (12). The synthesis and secretion of a catalytically defective protein induced by the p. Ile221Thr mutation were confirmed through *in vitro* experiments in COS-1 cells. In accordance with the previous data, two new compound *LPL* variants of patient 2 in our study caused changes in the LPL enzyme activity in the plasma of the patient, whereas the differences in enzyme activity between the parents of proband 2 and the control seemed to be negligible. Curiously, the parents each carried a variant of proband 2. Sanger sequencing confirmed that the Ser63Phe variant comes from the father and the p. Ile221Thr variant comes from the mother, but the parents have thus far been healthy. Our *in vitro* experiments to understand the effect of the two *LPL* variants at the protein level indicated that the p. I221T variant can reduce approximately 48.33 ± 1.78% mass and approximately 81.56 ± 4.35% activity, and the LPL activity was reduced by 62.76 ± 9.90% or 63.25 ± 10.55% in the media of HEK 293T/17 cells transfected with p. Ser63Phe or the two variants (p. Ser63Phe and Ser63Phe+Ile221Thr).

Functional studies have potential benefits for the diagnosis and treatment of severe hypertriglyceridemia and can promote the mechanistic study of the occurrence of type I hyperlipoproteinemia. Ultimately, the findings may lead to the development of more effective drugs for the precise intervention for such patient rather than limited symptomatic treatment. In this study, differences in the activity of LPL would affect the corresponding clinical research, and interested researchers may summarize more cases and find some rules, such as mutations in one exon region having a greater impact on LPL activity, while mutations in another exon region have little impact on LPL activity.

This study has some limitations. For instance, in our study, we could not analyze whether the parents of patient 1 had hypertriglyceridemia because they had passed away. Second, we have not been able to conduct a more in-depth study on why the son and daughter of proband 1 and the parents of proband 2 carry a certain variant, but the phenotype is normal. Our patients did not have two harmful lifestyle factors, namely, severe obesity and severe tobacco abuse. Obesity is not only related to primary hypertriglyceridemia but also a risk factor for secondary hypertriglyceridemia (24). Tobacco can affect fat metabolism in the liver and increase TG levels due to defects in the lipolysis system (25). In short, it can basically rule out the possibility that our two probands had secondary hypertriglyceridemia.

5 CONCLUSIONS

To conclude, we have described two novel compound heterozygous variants of *LPL* in patients with type I hyperlipoproteinemia, showing that all these variants are pathogenic by disrupting LPL mass and the enzymatic activity of LPL. Although *LPL* variants are rare, WES is important to discover the monogenic and oligogenic genetic patterns in severe hyperlipoproteinemia, a challenge now predigested by the access to next-generation sequencing.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GenBank, 2561205.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SW and YC designed the experiments, researched the data, and wrote the manuscript. YS contributed to the statistical analysis. LG and WZ contributed to the experiments and technical supporting. LF, XJ, XH, and QS to the data Interpretation and Technical Assistance. GL, JZ and CX conceived the study, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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SYSTEMATIC REVIEW
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Association of Serum Bilirubin With Metabolic Syndrome and Non-Alcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis

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Objective: Metabolic syndrome (MetS) and non-alcoholic fatty liver disease (NAFLD) are the leading chronic diseases worldwide. There are still many controversies about the association between serum bilirubin and MetS or NAFLD. This study aims to evaluate the association of serum total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL) with MetS and NAFLD.

Methods: Multiple databases were searched for relevant studies until November 2021. Randomized controlled trials, cross-sectional and cohort studies evaluating the association between serum bilirubin levels and MetS or NAFLD were included.

Results: Twenty-four cross-sectional and cohort studies with 101, 517 participants were finally analyzed. Fifteen studies and 6 studies evaluated the association between bilirubin and MetS or NAFLD in health screening population, respectively, while 3 studies evaluated the association between bilirubin and non-alcoholic steatohepatitis (NASH) in NAFLD patients. Random effect model analysis showed the inverse association between TBIL and MetS in male (95%CI=0.71-0.96) and gender-neutral (95%CI=0.61-0.91) group. However, no significant association was found in females. Notably, the inverse association between DBIL and MetS was noticed in male (95%CI=0.36-0.75), female (95%CI=0.16-0.58) and gender-neutral population (95%CI=0.67-0.92). IBIL level was inversely associated with MetS in females (95%CI=0.52-0.96), whereas no statistical correlation presented in males. TBIL was not statistically correlated with NAFLD in gender-neutral or male subgroup. Similarly, there were no association between DBIL or IBIL and NAFLD in gender-neutral subgroup. However, the negative correlation between DBIL and NAFLD existed in males (95%CI=0.76-0.96). In NAFLD patients, IBIL analysis showed an inverse association with NASH (95%CI=0.01-0.12).

Conclusion: Serum TBIL and DBIL levels, especially DBIL levels, assume an inverse correlation with MetS in healthy population. Serum IBIL is inversely associated with the onset and degree of NASH in NAFLD patients. Exogenous bilirubin supplement may be a potential strategy to assist in lowering the risk of developing MetS and NAFLD.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42021293349

Keywords: serum bilirubin, metabolic syndrome, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, meta-analysis

INTRODUCTION

With the development of urbanization and the improvement of living standards, the incidence of diseases related to metabolic disorders is steadily increasing, which makes them serious diseases threatening human health (1–3). Metabolic syndrome (MetS) is defined as a group of complex metabolic disorders characterized by insulin resistance, hypertension, atherogenic dyslipidemia and abdominal obesity, etc. Several societies harmonize that the MetS can be defined when any three or more of the following factors are met: (a) elevated waist circumference based on population-and country-specific definitions; (b) elevated triglycerides or being previously diagnosed as hypertriglyceridemia and taking antihypertriglyceridemia medication; (c) reduced high-density lipoprotein cholesterol (HDL-C) or being previously diagnosed as reduced HDL-C and taking medication for reduced HDL-C; (d) elevated blood pressure (BP) or being previously diagnosed as hypertension and taking antihypertensive medication; (e) raised fasting plasma glucose level or being previously diagnosed as type 2 diabetes and taking antiglycemic medication (4, 5). Non-alcoholic fatty liver disease (NAFLD), a kind of metabolic stress liver injury closely related to insulin resistance and genetic susceptibility, has been regarded as the leading chronic liver disease and primary cause of abnormal liver biochemical indexes found in physical examination (6). The prevalence of NAFLD is increasing worldwide, with an average prevalence of about 24% (7). The close correlation between NAFLD and MetS, and the reciprocal causality between them has been reported. Therefore, some of risk factors and serum diagnostic markers for both NAFLD and MetS may be consistent (8, 9). Current literature has shown there are no drugs available for the treatment of MetS and NAFLD. Owing to significant increased incidence of MetS and NAFLD, it's essential to seek for new therapeutic agents or targets for those.

Serum bilirubin, mainly originating from the catabolism of hemoglobin in senescent erythrocyte, is commonly used as a biochemical index for the diagnosis of hepatobiliary and metabolic diseases. The conjugation between free bilirubin and UDP-glucuronosyltransferase (UGT) 1A1, which catalyzes the transfer of glucuronic acid, leads to the generation of conjugated bilirubin. For a long time, bilirubin has been deemed as a metabolic waste of iron porphyrin compounds, which means no beneficial effects can be provided by bilirubin. However, latest studies have shown that mildly elevated bilirubin, such as that found in Gilbert's syndrome(GS), may serve as an important endogenous tissue protector. Meanwhile, it can act as a physiological modulator of oxidative stress and chronic inflammation in MetS (10, 11). In a meta-analysis including 9 observational studies, serum bilirubin levels are demonstrated to be inversely associated with adverse metabolic outcomes. Unfortunately, subgroup analysis was not performed considering that fewer studies were included. In addition, the lack of information about direct bilirubin (DBIL) and indirect bilirubin (IBIL) limits the value of that meta-analysis to evaluate which kind of bilirubin is associated with MetS (12). Both MetS and NAFLD seem to be associated with serum bilirubin, including total bilirubin (TBIL), DBIL, and IBIL. And the increase in bilirubin levels has been demonstrated to be negatively correlated with the prevalence of NAFLD (8, 13–16). Nevertheless, the alternative study based on Mendelian randomization analysis did not find a causal relationship between bilirubin levels and the risk of NAFLD (17, 18). Similarly, Bellarosa et al. reported that bilirubin does not provide protection against MetS and NAFLD in children population with severe obesity (8). What is noteworthy is that the association between bilirubin and NAFLD or MetS in normal-weight adults remains controversial. Importantly, there are no meta-analysis evaluating the association between serum bilirubin and NAFLD currently. In this context, we conducted this meta-analysis to clarify the relationship between serum bilirubin levels and the MetS or NAFLD.

MATERIALS AND METHODS

This meta-analysis was designed and implemented according to the Preferred Reporting Items for Systematic Reviews and Meta –Analyses(MOOSE) guidelines [**Supplementary Material 1**], and the search strategy [**Supplementary Material 2**], eligibility criteria and outcomes had been registered in the PROSPERO database (CRD42021293349).

Search Strategy

Pubmed, Embase and Cochrane Library databases were searched for analyzing the association between serum bilirubin and MetS or NAFLD until November 2021. Subject terms included 'Non-alcoholic Fatty Liver Disease', 'Metabolic Syndrome', and 'Bilirubin', and the random combination of these words were utilized for retrieval. The detailed literature search strategy was

shown in supplementary material. Besides, relevant references were also manually searched. The preliminary screening of collected studies was conducted by scanning titles and abstracts. Then, full text was read through to identify the studies that met the inclusion criteria.

Inclusion Criteria

Studies meeting the following criteria were included (1): randomized controlled trials (RCTs), cross-sectional studies, case-control studies or cohort studies which evaluated the association between TBIL, DBIL or IBIL and MetS or NAFLD (2); similar or identical research protocols were adopted (3); diagnostic criteria of cases were definite (4); comprehensive statistical indicators were provided, such as odds ratio (OR), relative risk (RR), or hazard ratio (HR) with their 95% credible interval (CI).

Data Extraction and Quality Assessment

Two investigators independently carried out data extraction and quality assessment. Disagreements were reconciled by a third investigator when different opinions exist. Following data were extracted from initial studies: study characteristics (first author, study type, year of publication, country, and follow-up time), patient characteristics (sample size, cases, gender, age, and adjusted covariates), and outcome indicators (OR, RR, HR with their 95% CI). Since the enrolled studies were cross-sectional or cohort studies, Newcastle-Ottawa Quality Assessment Scale (NOS) was used to evaluate the literature quality by two independent reviewers (19). Studies with an NOS score ≥7, 5-6, and <5 were considered as high, fair, and low quality. A score ≥5 indicated adequate quality for inclusion in the present review (12).

Statistical Analysis

Meta-analysis was implemented using Review Manager 5.3 software (The Cochrane Collaboration, Software Update, Oxford, UK). OR, RR, and HR were defined as the effect indicators, and the point estimates with 95%CI were calculated for these effect indicators. Heterogeneity was assessed using both the chi-square test and I^2 index, the value of $P<0.1$ and $I^2>50\%$ was considered significant. Subsequently, random-effects model was created to incorporate effect indicators when significant heterogeneity existed, otherwise the fixed-effects model would be aqdopted. Subgroup and sensitivity analysis were performed to find out the source of heterogeneity and verify the accuracy of analysis results, respectively. Sensitivity analysis was carried out by excluding studies one by one to identify the studies with significant heterogeneity. Funnel plots was used to assess the possibility of publication bias.

RESULTS

Study Selection and Study Characteristics

A total of 2307 studies were identified based on retrieval strategy, and 43 repetitive articles were excluded by NoteExpress software. After scanning the titles and abstracts, 61 studies were left. Then, we read through the remaining studies in full-text. After that, 101, 517 healthy persons from 24 observational studies including cross-sectional and cohort studies were included in this meta-analysis (13–18, 20–37). The flow diagram of literature screening was shown in **Figure 1**. All included studies were dual-arm studies, of which 15 studies including 11, 696 cases and 6 studies including 9,813 cases evaluated the association between serum bilirubin and MetS (20–34) or NAFLD (13, 14, 17, 18, 35, 36) in health screening population, respectively, while the remaining 3 studies (15, 16, 37) including 997 cases evaluated the association between serum bilirubin and NASH in NAFLD patients. Of note, 15 studies on MetS were conducted in Asian countries included China, Korea and Japan (20–34). Four studies on NAFLD derived from Western countries (15, 16, 18, 35), while the remaining 5 studies derived from Eastern ones (13, 14, 17, 36, 37). The NOS scores of all included literatures were no less than 7 points, indicating high literature quality. The characteristics of included studies were shown in **Table 1**.

The Association Between Serum Bilirubin and MetS

A total of 15 studies evaluated the association between TBIL, DBIL or IBIL with MetS (20–34). Among them, two studies included both cross-sectional and cohort population (21, 29). To eliminate the influence of gender on analysis results, population were divided into male group (contain only males), female group (contain only females) and gender-neutral group (contain both males and females) for meta-analysis, respectively. When enough studies were included, they were redivided into cross-sectional group and cohort group for subgroup analysis.

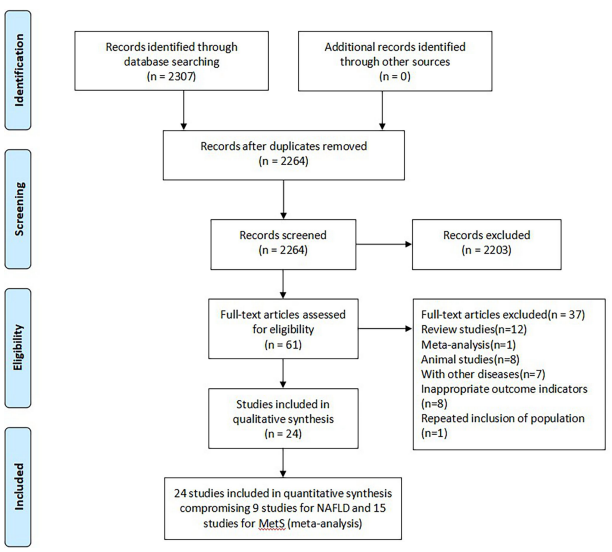

FIGURE 1 | Flow diagram of literature screening and selection. MetS, metabolic syndrome; NAFLD, non-alcoholic fatty liver disease.

TABLE 1 | Characteristics of included studies.

Author, year of type/publication (years)	Country	Study follow-up	Year(s) of study	Age (years)	Sample size	Gender (M/F)	Cases	Adjusted covariates	Quality (NOS)
Metabolic syndrome (MetS)									
Hao 2020 (20)	China	Cohort/5.72 ±1.49	2009-2012	40.3±11.9	565	221/344	204	Age, gender	8
Kawamoto	Japan	Cross-sectional	2014-2017	70±9	893	893/0	451	Age, smoking, alcohol, exercise, presence of CVD,LDL-C, SUA, eGFR, GGT, ALT	
2019 (21) Kawamoto 2019 (21)	Japan	Cohort/4	2014-2017	66±9	288	288/0	46		9
Li 2017 (22)	China	Cohort/5	2011-2016	45.6±12.7	1339	1339/0	117	Age, alcohol, smoking, exercise,TG, LDL-C	8
Zhong 2017(23)	China	Cross-sectional	2012	73.1±6.6	1728	744/984	484	Age, gender, exercise, smoking, alcohol, TC,ALT	7
Lee 2016 (24)	Korea	Retrospec tive/>4	2006-2012	50.9 (18-89)	11613	6890/ 4723	2439	Age, smoking, medication history, ALT, SUA, eGFR, FPG, diabetes, SBP, WC, BMI	8
Chen 2016(25)	China	Cohort/5	2006-2011	45.6±10.0	5258	3262/ 1996	831	Age, ALT, AST, BUN, WBC, GGT, SUA, gender,	7

The Association Between TBIL and MetS

Among male group, 10 studies evaluated the association between TBIL and MetS, including 7 cross-sectional studies (21, 23, 28–30, 32, 33) and 5 cohort studies (21, 22, 24, 27, 29). There was obvious heterogeneity between included studies, so random effect model was used for analysis. According to our results, TBIL level was inversely associated with MetS in cross-sectional subgroup (OR=0.81, 95% CI=0.70-0.94, P=0.005), whereas no statistical correlation was found in cohort subgroup (OR=0.91, 95%CI=0.54-1.53, P=0.72). Moreover, the pooled results from cross-sectional and cohort studies showed a negative correlation between TBIL and MetS (OR=0.83, 95% CI=0.71-0.96, P=0.01) (**Figure 2A**). Seven cross-sectional studies (23, 28–33) and 2 cohort studies (24, 29) assessed the relationship between TBIL and MetS in female group. Random effect model was adopted for meta-analysis, and the results of cross-sectional subgroup, cohort subgroup and comprehensive analysis displayed negative correlation (OR=0.69, 95%CI=0.57-0.84, P=0.0002), no correlation (OR=1.28, 95%CI=0.40-4.06, P=0.68) and no correlation (OR=0.78, 95%CI=0.60-1.02, P=0.06), respectively (**Figure 2B**) between TBIL and MetS. Random effect model analysis for gender-neutral population showed inverse association between TBIL and MetS (OR=0.75, 95%CI=0.61-0.91, P=0.004) (20, 23, 28, 30, 34) (**Figure 2C**).

The Association Between DBIL and MetS

Two cross-sectional studies (32, 33) and 3 cohort studies (22, 25, 26) evaluated the association between DBIL and MetS in male group. Random effect model was adopted and meta-analysis showed that DBIL were negative correlated with MetS in both cross-sectional (OR=0.50, 95%CI=0.36-0.69, P<0.0001) and cohort subgroup (OR=0.50, 95%CI=0.27-0.93, P=0.03) (**Figure 3A**). Additionally, the inverse association between DBIL and MetS was found in male (OR=0.52, 95%CI=0.36-0.75, P=0.0004) (22, 25, 26, 32, 33), female (OR=0.31, 95%CI=0.16-0.58, P=0.0003) (25, 32, 33)(**Figure 3B**) and gender-neutral population (OR=0.78, 95%CI=0.67-0.92, P=0.002) (20, 25) (**Figure 3C**), regardless of the study type.

The Association Between IBIL and Met

Three studies (22, 32, 33) including male population only and 2 studies (32, 33) including female population only analyzed the association between IBIL and MetS. As a result, IBIL level was inversely associated with MetS in female group (OR=0.71, 95%CI=0.52-0.96, P=0.03) (**Figure 4B**), whereas no statistical correlation was noticed in male group (OR=0.92, 95%CI=0.60-1.42, P=0.71) (**Figure 4A**).

The Association Between Serum Bilirubin and NAFLD

Nine studies evaluated the association between TBIL, DBIL or IBIL and NAFLD (13–18, 35–37). In view of the inconsistence in recruited populations and enough sample size, study population was subdivided into gender-neutral and male divisions for TBIL and DBIL sub-groups. And the random effect model was utilized for separate meta-analysis. There was no obvious heterogeneity in IBIL subgroup, so fixed effect model was adopted.

The Association Between TBIL and NAFLD

Four studies (13, 14, 17, 18)and 2 studies (18, 36), respectively, evaluated the association between TBIL and NAFLD in gender-neutral and male subgroup. Random effect model analysis showed no statistical correlation between TBIL and NAFLD, regardless of the subgroups, OR=0.89, 95%CI=0.78-1.02, P=0.09 for gender-neutral subgroup; OR=0.89, 95%CI=0.75-1.06, P=0.20 for male subgroup (**Figure 5A**).

The Association Between DBIL and NAFLD

In gender-neutral subgroup, 2 studies analyzed the connection between DBIL and NAFLD (14, 17), showing no statistical association (OR=0.89, 95%CI=0.67-1.18, P=0.41). Nevertheless,

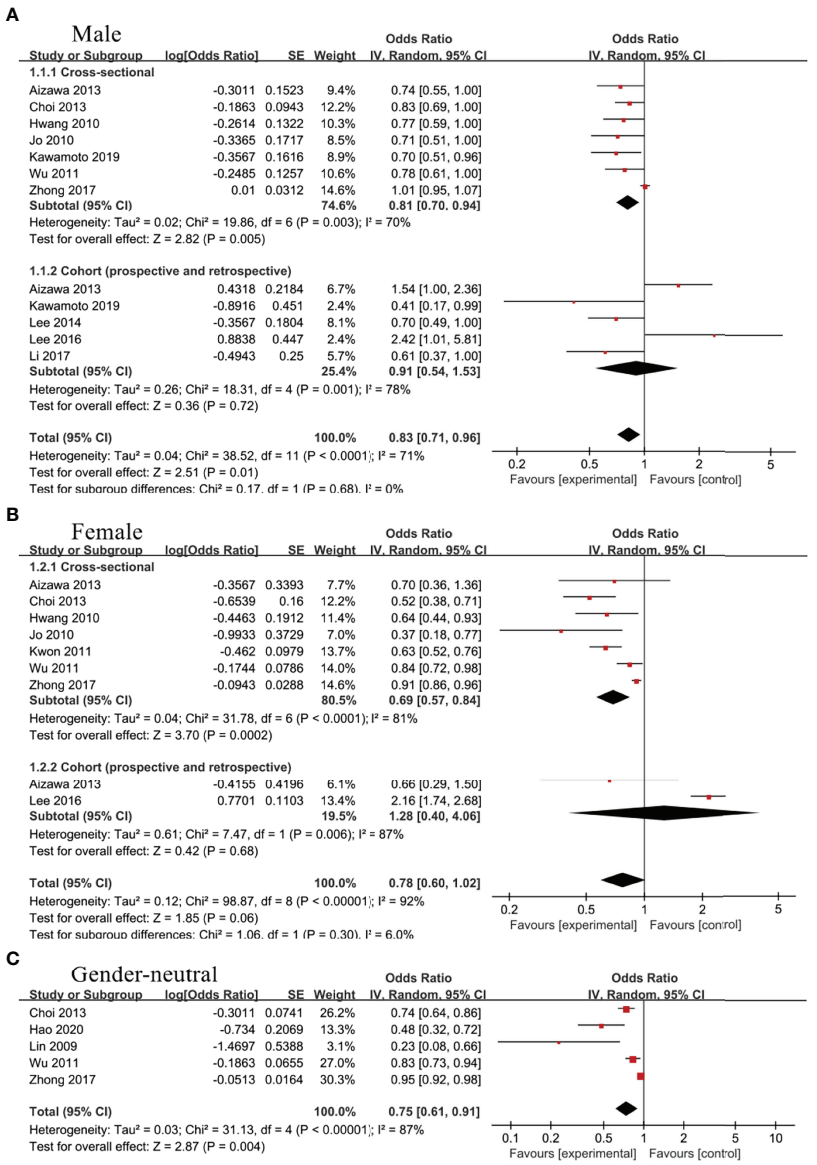

FIGURE 2 | Association of metabolic syndrome (MetS) with total bilirubin (TBIL) among **(A)** male group, **(B)** female group, and **(C)** gender-neutral group.

negative correlation between DBIL and NAFLD (OR=0.85, 95% CI=0.76-0.96, P=0.01) was noticed in male subgroup (14, 36)(**Figure 5B**).

The Association Between IBIL and NAFLD

Three studies evaluated the association between IBIL and NAFLD in health screening population (14, 17, 36). Meta-analysis revealed no statistical association between IBIL and the incidence rate of NAFLD (OR=1.02, 95%CI=0.95-1.09, P=0.58) (**Figure 5C**). Subsequently, further analysis was conducted to explore the correlation between IBIL and non-alcoholic steatohepatitis (NASH) in NAFLD patients. Random effect model analysis including 3 studies (15, 16, 37) manifested inverse association between IBIL and NASH (OR=0.03, 95% CI=0.02-0.05, P<0.00001) (**Figure 6**).

Sensitivity Analysis

To estimate the influence of single study on overall results of meta-analysis, sensitivity analysis was carried out by excluding studies one by one. In association analysis between TBIL and NAFLD, we temporarily excluded Luo's study (P=0.30, I^2=17%) and re-analyzed remaining studies. Fixed effect model analysis based on remaining studies showed negative correlation between TBIL and NAFLD in gender-neutral subgroup (OR=0.86, 95%CI=0.80-0.92, P<0.0001), which was different than previous analysis. Additionally, sensitibity analysis based on other outcome indicators all showed no significant

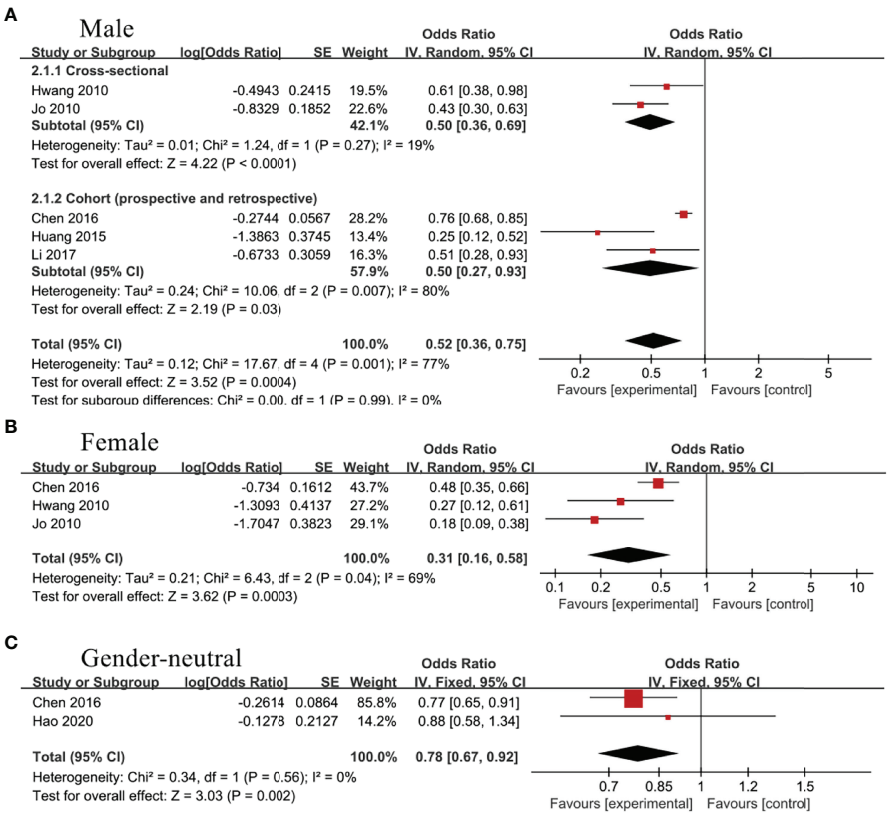

FIGURE 3 | Association of metabolic syndrome (MetS) with direct bilirubin (DBIL) among **(A)** male group, **(B)** female group, and **(C)** gender-neutral group.

changes after deleting each trial, which confirmed the rationality and reliability of our meta-analysis.

Publication Bias

Publication bias analysis based on the association between TBIL and MetS in male group is more convincing and accurate. Funnel plot was drawn for MetS with TBIL in male group as an outcome indicator, and it was found that the left and right distributions of each study site were asymmetrical, suggesting the possible existence of publication bias (**Figure 7**). The other results of publication bias analysis are shown in **Supplementary Material 3**.

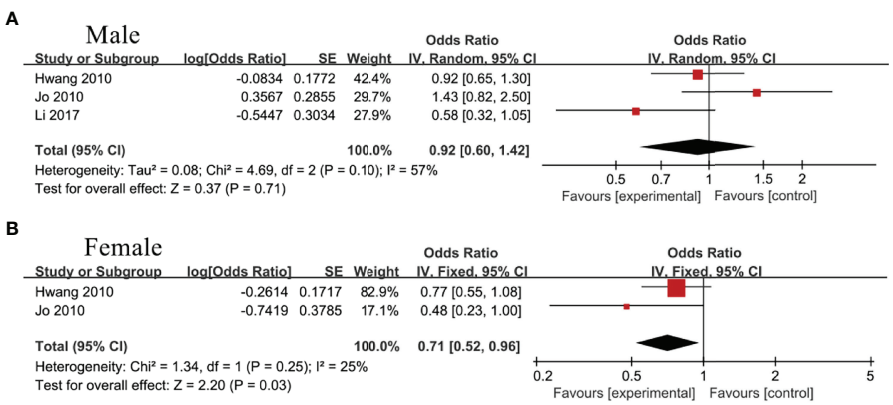

FIGURE 4 | Association of metabolic syndrome (MetS) with indirect bilirubin (IBIL) among **(A)** male group and **(B)** female group.

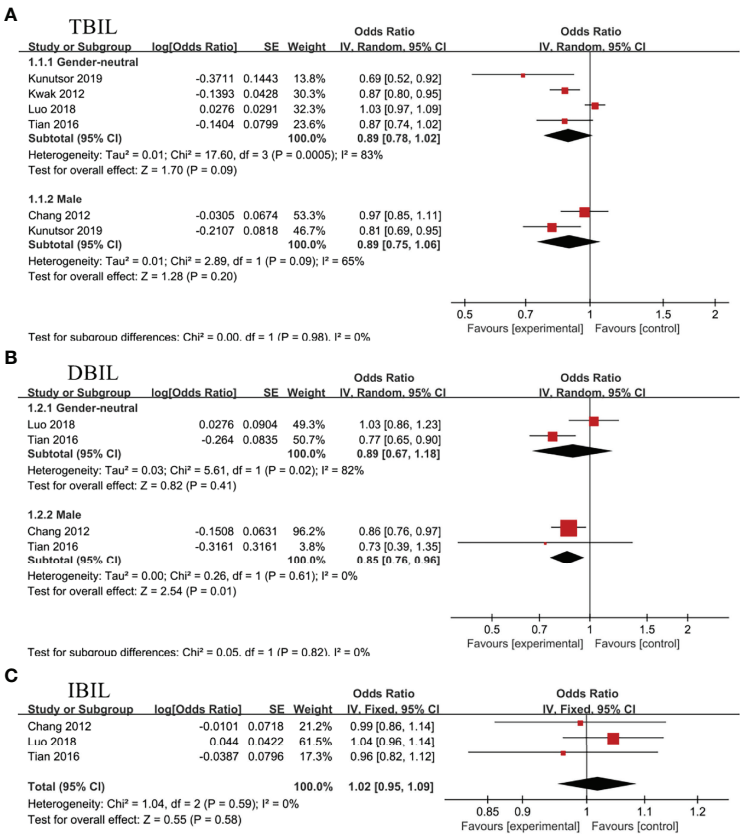

FIGURE 5 | Association of non-alcoholic fatty liver disease (NAFLD) with **(A)** total bilirubin (TBIL), **(B)** direct bilirubin (DBIL), and **(C)** indirect bilirubin (IBIL).

DISCUSSION

MetS is associated with an increased risk of cardiovascular disease and all-cause mortality (1). NAFLD, characterized by fat degeneration and accumulation in hepatocyte, is regarded as the "liver manifestation of metabolic syndrome". Owing to significant increased incidence of MetS and NAFLD (1, 38), it's essential to seek for new therapeutic agents or targets for MetS and NAFLD. At present, hyperbilirubinemia is considered to play a beneficial protective role in numerous oxidative stress and inflammation-related diseases, such as coronary heart disease, diabetes, and stroke (11, 39, 40). What relations have been existed between bilirubin and MetS or NAFLD, and whether hyperbilirubinemia could lower the risk of MetS or NAFLD, however, needs further discussion.

Except for MetS, this study is the first to systematically review and summarize published studies in order to assess the correlation between bilirubin subtypes and NAFLD through

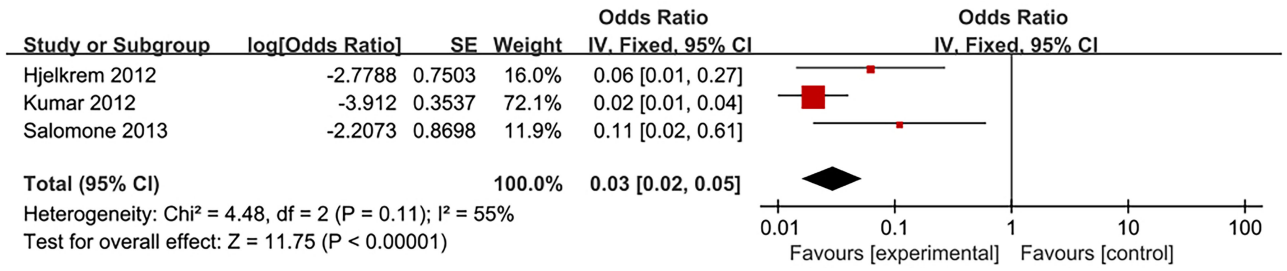

FIGURE 6 | Association of non-alcoholic steatohepatitis (NASH) with indirect bilirubin (IBIL).

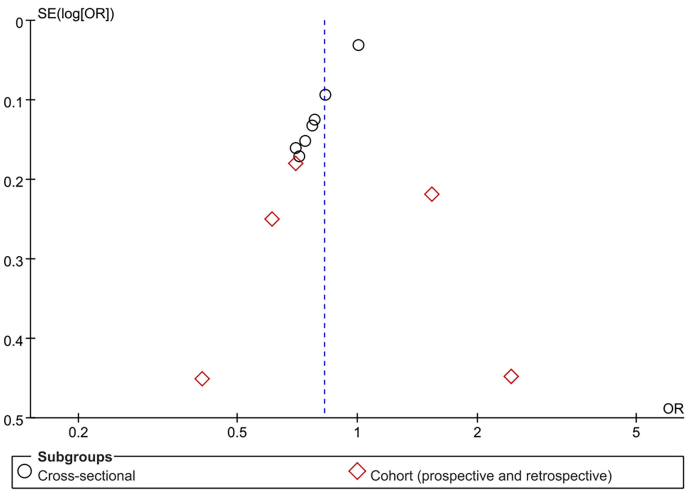

FIGURE 7 | Funnel plot for association of metabolic syndrome (MetS) with total bilirubin (TBIL) among male group.

meta-analysis. Serum TBIL is inversely associated with MetS in male and gender-neutral group, but not in female. However, the inconsistency existed for the analysis results derived from the cross-sectional and cohort study when we evaluated the association between TBIL and MetS in male or female group There were 7 cross-sectional studies in both male and female group, while 5 cohort studies were included in male and 2 cohort studies in female, respectively. Thus, we speculated that the reason for the inconsistency may be ascribed to the differences in the number of cross-sectional and cohort studies. Fewer cohort studies might bring about the inconsistency. DBIL exhibits an inverse association with MetS, regardless of gender. IBIL displays a negative correlation with MetS in females but not in males. No stastistical correlation is found between TBIL and NAFLD. DBIL is negatively correlated with NAFLD in male subgroup. IBIL manifests an inverse association with NASH in NAFLD patients.

Bilirubin and its precursor biliverdin can increase the antioxidant activity of vascular endothelial cells (41). Bilirubin can also increase insulin sensitivity by regulating levels of cholesterol metabolism, adipokines and peroxisome proliferator-activated receptor γ (PPARγ) (39). What's more, it can selectively bind to proliferator-activated receptor α (PPARα), causing the decrease in lipid accumulation by increasing the number and function of mitochondria (40, 42, 43). In this analyses, an inverse association was found between TBIL levels and MetS among male and gender-neutral group. In females, TBIL tends to be negatively associated with MetS, although no statistical correlation is found between them (P=0.06). In addition, an inverse association between TBIL and MetS exists in cross-sectional subgroup, but not in cohort subgroup. Insufficient number of included studies in cohort subgroup could affect the accuracy of overall results. Therefore, in line with previous reports, we confirm a protective role of TBIL in MetS (12).

An obvious negative correlation between DBIL and MetS was revealed in all subgroups, regardless of gender and study type. Moreover, correlation between DBIL and MetS is closer than that between IBIL and MetS. As we know, DBIL tends to build looser bound with albumin than IBIL. Hence, it is easier for DBIL to separate from albumin than IBIL. As a result, DBIL might directly act on target organs and molecules (44). Collectively, DBIL may possess better prognostic value than IBIL according to the analysis conducted by we and other investigators (32, 33).

In general, average bilirubin levels in males are slightly higher than those in females. GS, an inherited metabolic liver disease characterized by IBIL elevation, is also most frequently occurs in young males. This discrepancy may be explained by a gap in life style and the effect of sex hormines on the glucuronic acid (45, 46). Hwang et al. demonstrated that all 3 types bilirubin levels are inversely relevant with MetS in females, while DBIL exhibits significant inverse association with MetS in males, after adjusting for the confounding variables including lifestyle (32). Another investigation indicated that all bilirubin subtypes are negatively correlated with MetS in Korean men and women. Nevertheless, this significant inverse correlation between TBIL or IBIL and MetS vanishes according to the adjusted multivariate analysis model (adjusting for age, smoking status, alcohol consumption and so on) (33). In this analysis, we adjusted for the potential confounding factors such as age, gender, life styles, etc. Our data showed that all the studies type of DBIL were inversely related with the MetS in male whereas the IBIL was only inversely related with the MetS in female. These results likely indicate that DBIL is more related to the MetS than the other subtypes of bilirubin in male, and the protective effect of IBIL against MetS is more pronounced in female group. This is partially consistent with Hwang's findings (32). Apart from uridine diphosphate-glucuronosyltransferase (UGT1A1), the protective effect of bilirubin may be also related to other metabolic enzymes that can regulate the bilirubin metabolism, such as heme oxygenase

(HO). Bilirubin is produced under the action of HO, the rate-limiting enzyme of heme catabolism. Downregulation of HO activity inhibits bilirubin production (47). Notably, the gender difference exists in HO activities, which is related to oxidative stress (48) or high iron storage (49). This finding can be used, at least in part, to explain the reasons for the different effects of bilirubin on men and women. However, the exact mechanism is still needed to be explored. Besides, limited number of included studies may reduce the analysis accuracy.

NAFLD does not only refer to the fat accumulation in the liver caused by excessive free fatty acids, but also involves numerous metabolic problems such as oxidative stress, insulin resistance, and mitochondrial dysfunction. Furthermore, a wide spectrum of histological lesions ranging from pure hepatic steatosis to NASH are included in NAFLD. Various laboratory and clinical studies have demonstrated that bilirubin most likely reduces hepatic lipid accumulation by increasing PPARα activation and inhibiting PPARγ transcriptional activity in humanized mice with the Gilbert's polymorphism (HuUGT*28) or humans with GS (39, 40, 50, 51). According to our analysis, the negative correlation between DBIL and NAFLD only exists in male subgroup, while no significant associations between all bilirubin subtypes with NAFLD is found in gender-neutral group. Furthermore, DBIL rather than other bilirubin subtypes manifests significiant inverse association with MetS or NAFLD in male population. Although no significant relation between IBIL and NAFLD is noticed in health screening population, an inverse association is reported between IBIL and NASH occurring in NAFLD patientsis. These findings are not compatible with the results provided by Luo et al. We think this inconsistence can be ascribed to the following factors. In Luo's study, NAFLD was diagnosed based on liver ultrasonography rather than liver biopsy which is regarded as,the gold standard for NAFLD diagnosis (52, 53). As a result, mild fatty liver might be missed considering the insensitiveness originating from ultrasonography. In addition, Luo's study mainly enrolled mid-aged adults with lower incidence of NAFLD, therefore bringing about the risk to draw an biased conclusion (17). In this context, three separate investigation diagnosed NAFLD based on liver biopsy results, and the authors found that IBIL is inversely associated with the severity of liver damage in NASH patients (15, 16, 37). Oxidative stress has been documented to promote the progression from hepatic steatosis to NASH (38, 53). In view of the potent oxidation resistance, it is conceivable that IBIL may provide a protective effect through antioxidant activity in lipotoxic diseases such as NAFLD. Even through, the association between serum IBIL and NAFLD and the underlying mechanism behind this association still need to be explored. Nowadays, studies on association between TBIL or DBIL and NASH in NAFLD patients is insufficient.

Women at reproductive period have a different metabolic status from those at post-menopause. It may reflect the effects of decreased estrogen levels, which have a certain impact on lipid metabolism and insulin resistance. In addition, estrogen deficiency hastens the development of hepatic steatosis and the progression of hepatic fibrosis (53, 54). Serum bilirubin levels in post-menopause may be higher than pre-menopause, which is also related to estrogens deficiency (45, 46). Elevated bilirubin levels have been reported to be closely related to decreased prevalence rate of MetS or NAFLD (12, 13). And our results partially support this finding. Nevertheless, elevated bilirubin levels may not counteract the effect of estrogen deficiency in postmenopausal women. Thus, the prevalence rates of MetS and NAFLD are significantly higher in postmenopausal women than pre-menopausal women (1, 2, 7). For premenopausal women, not only bilirubin but also estrogen can protect from developing MetS and NAFLD. Because of the lack of detailed information for determining whether a female participant is in post-menopause or not, it is unfeasible to conduct subgroup analysis based on this factor. Further research is needed to be carried out to clarify this issue.

Why the association is inconsistent between serum bilirubin and MetS or NAFLD? The possible reasons are as follows: Primarily, except for the liver, other factors such as other tissues, gene, et. are also involved important role in the pathogenesis and development of MetS or NAFLD (55). For instance, steatosis in PNPLA3-associated NAFLD is not accompanied by features of MetS, while PNPLA3-uncorrelated NAFLD closely resembles MetS with regards to its causes and consequences (56). There is a basic research showed that bilirubin deficiency renders mice susceptible to hepatic steatosis in the absence of insulin resistance. It adopted a kind of contrarian strategy to prove that the pathogenesis of MetS and NAFLD is not exactly identically (57). In addition, the variety of definitions of MetS and the variety of diagnostic methods for NAFLD in different studies is also make it challenging that assess the consistent-association of serum bilirubin with MetS and NAFLD.

The inverse correlation between serum bilirubin and MetS or NAFLD suggests that bilirubin might be utilized as a potential and promising strategy to assist in lowering the risk of developing MetS and NAFLD. Exogenous bilirubin supplement is the most direct way to prevent the occurrence of MetS and NAFLD. So far, several studies have attempted to carry out targeted therapy for cancer, inflammation and vascular diseases utilizing bilirubin nanoparticles (BRNP) or bilirubin coated stents (58). And the preliminary efficacy is promising. Moreover, basic research shows that BRNP reduces diet-induced hepatic steatosis (59). On the other hand, increasing endogenous bilirubin production is also a feasible treatment strategy. In this regard, inducing "iatrogenic Gilbert syndrome" by uricosuric drug-probenecid has been demonstrated to be capable of reducing the liver gluconaldehyde acidification activity followed by the increase in serum bilirubin (60). Curcumin supplemented by diet has been documented to increase bilirubin levels through targeting HO-1. These strategies have been proven to be effective and safe *in vitro* and *in vivo*. Therefore, increasing bilirubin levels may be an advantageous treatment strategy for MetS and NAFLD (47).

Although the stratification has been executed as far as possible, the shortcomings exist in this meta-analysis. Firstly, the absence of

RCTs or prospective cohort studies brings down the credibility of analysis results. Secondly, the classification criteria for serum bilirubin are not identical among different studies, which may partially account for the existence of heterogeneity. Thirdly, insufficient studies on the association between TBIL or DBIL and NASH in NAFLD patients that impairs the credibility and clinical value of this analysis. Fourthly, the studies on MetS were all conducted in Asian countries included China, Korea and Japan. There are no obvious difference in geographical location, dietary patterns, figures, and life styles among these coutryies. Thus, the dietary patterns may have little impact on the results related to MetS in this meta analysis. In terms of NAFLD, four studies derived from Western countries and five studies derived from Eastern countries. Nevertheless, it is unfeasible to conduct subgroup analysis in view of lacking detailed information on diet. Fifthly, in terms of age, most studies included in our meta analysis refer to populations at all ages. However, most original studies had adjusted age as a covariate. For the remaining studies, the lack of detailed information on age makes it infeasible to carry out subgroup analysis on age. Meanwhile, lacking of detailed information on menopause, it is infeasible to conduct subgroup analysis on pre or post-menopause. Finally, follow-up time for each included study is inconsistent or unclear, therefore underscoring the incidence of MetS or NAFLD. Consequently, more high-quality, large-scale, prospective and long-term follow-up studies are urgently needed.

CONCLUSION

In brief, our meta-analysis indicates that serum TBIL and DBIL levels, especially serum DBIL levels, supporting an inverse connection with MetS, Moreover, serum IBIL could decrease the onset of NASH in NAFLD patients. Therefore, appropriately elevated serum bilirubin levels seem to reduce the risk of MetS and NAFLD. Regulation of bilirubin metabolic pathways may be a potential strategy and exogenous bilirubin supplement may be a medicine to assist in lowering the risk of developing MetS and NAFLD. Bilirubin is still far from being used in the clinic at present. Large-scale prospective and high-quality animal or clinical studies are required to establish to investigate the association and potential prevention of bilirubin on MetS or NAFLD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTION

CL and ZY wrote and amended the original draft. LB and WH participated in extracting and analyzing the data. ST and WZ searched literature and produced the tables, figures. XC, ZH and ZD gave critical revisions, and final approval of the article. SZ contributed to design the study, interpretation of data, and the final approval of the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.869579/full#supplementary-material

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Integrated Bioinformatic Analysis of the Shared Molecular Mechanisms Between Osteoporosis and Atherosclerosis

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Background: Osteoporosis and atherosclerosis are common in the elderly population, conferring a heavy worldwide burden. Evidence links osteoporosis and atherosclerosis but the exact underlying common mechanism of its occurrence is unclear. The purpose of this study is to further explore the molecular mechanism between osteoporosis and atherosclerosis through integrated bioinformatic analysis.

Methods: The microarray data of osteoporosis and atherosclerosis in the Gene Expression Omnibus (GEO) database were downloaded. The Weighted Gene Co-Expression Network Analysis (WGCNA) and differentially expressed genes (DEGs) analysis were used to identify the co-expression genes related to osteoporosis and atherosclerosis. In addition, the common gene targets of osteoporosis and atherosclerosis were analyzed and screened through three public databases (CTD, DISEASES, and GeneCards). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed by Metascape. Then, the common microRNAs (miRNAs) in osteoporosis and atherosclerosis were screened out from the Human microRNA Disease Database (HMDD) and the target genes of whom were predicted through the miRTarbase. Finally, the common miRNAs–genes network was constructed by Cytoscape software.

Results: The results of common genes analysis showed that immune and inflammatory response may be a common feature in the pathophysiology of osteoporosis and atherosclerosis. Six hub genes (namely, COL1A1, IBSP, CTSD, RAC2, MAF, and THBS1) were obtained *via* taking interaction of different analysis results. The miRNAs–genes network showed that has-let-7g might play an important role in the common mechanisms between osteoporosis and atherosclerosis.

Conclusion: This study provides new sights into shared molecular mechanisms between osteoporosis and atherosclerosis. These common pathways and hub genes may offer promising clues for further experimental studies.

Keywords: osteoporosis, atherosclerosis, bioinformatics, molecular mechanism, inflammation, immune

INTRODUCTION

More and more pieces of evidence show that vascular system diseases are related to bone metabolism. Some scholars presented a concept of a bone-vascular axis to explore the correlation between them (1, 2). They supported that there exited cellular, endocrine, and metabolic signals that flow bidirectionally between the vasculature and bone, and vascular and skeletal disease may occur concurrently when dysmetabolic states perturbed the bone–vascular axis (2). Osteoporosis and atherosclerosis are the most common skeletal and vascular disease while also being the chronic systemic diseases and major public health problems worldwide (3). Epidemiology studies have linked osteoporosis and atherosclerosis, suggesting that postmenopausal women with osteoporosis are often accompanied by atherosclerosis (4). Decreased bone mineral density and osteoporotic fractures were significantly associated with the development of echogenic plaques in carotid artery (5). Regardless of no widely consensus on the exact cellular and molecular basis underlying the high comorbidity between osteoporosis and atherosclerosis, it has been testified that they shared risk factors, common pathogenesis and genetic factors, and a causal association (6).

Several risk factors involving ageing, postmenopausal status, smoking habit, physical inactivity, and alcohol intake were considered as the common factors shared by osteoporosis and atherosclerosis (7). Osteoblast activity and survival decline with aging, whereas osteoclast activity increases, contributing to the age-associated decline of bone mass (8). Simultaneously, atherosclerosis, a high prevalence and incidence disease in the elderly causing most heart attacks and strokes, is considered a hallmark of aging process (9). However, there is growing evidence that a potential link exists between osteoporosis and atherosclerosis beyond aging (10). Several pathophysiological mechanisms including inflammatory cytokines, lipid oxidation products, and vitamin D and K deficiency were identified for the interplay between the skeletal and vascular systems (7, 11). Inflammatory cytokines [including tumor necrosis factor–alpha (TNF-α) and interleukin (IL)-1, IL-6, and IL-17] and chemokines have been shown to be associated with atherosclerosis, increased cardiovascular morbidity and mortality, and increased bone loss (12, 13). Dyslipidemia, including elevated level of total serum cholesterol, triglycerides, and low-density lipoprotein (LDL) cholesterol, was considered to promote atherosclerosis progression and also influenced bone metabolism (14). In addition, there also exists similarity of the treatment for osteoporosis and atherosclerosis. Statins, cholesterol-lowering drugs in preventing and treating cardiovascular disease, have potential positive effects on bone mineral density and decreasing osteoporotic fracture (15). Bisphosphonate therapy for osteoporosis reduces progression of vascular calcification (16). These findings strongly suggested the interaction of these two pathological conditions, simultaneously exerting an influence on the development of osteoporosis and atherosclerosis. However, they were mainly from clinical perspectives, and few studies have investigated genomic relationship between osteoporosis and atherosclerosis.

Contemporary, the quick development of bioinformatics approaches allows us to get a better grasp of disease pathobiology more deeply from the genetic level (17). Recently, a system-level analysis of whole blood genome–wide expression data identified several enriched biological pathways and three genes (NOSIP, GXYLT2, and TRIM63) that were significantly associated with early traits of both osteoporosis and atherosclerosis, supporting the idea that they were comorbid based on transcriptomic evidence (18). However, the representativeness of their samples was limited, and studies integrating gene data from public databases with both osteoporosis and atherosclerosis are lacking. In view of this, the purpose of this study is to integrate and analyze gene data related to the pathogenesis of osteoporosis complicated with atherosclerosis from the public databases, which provide new insights into the biological mechanisms of these two diseases, and it will help to develop dual-purpose prevention methods. The research flowchart of this research was shown in **Figure 1**.

METHODS

Data Source

Microarray datasets were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), which contain a great deal of high-throughput sequencing and expression microarray data. The keywords "osteoporosis" and "atherosclerosis" were used to search related gene expression datasets and non-human tested specimens were excluded. Finally, the datasets numbered GSE22829, GSE56815, GSE35958, GSE100927, GSE43292, and GSE35956 were downloaded from GEO database. In addition, the co-expressed genes of osteoporosis and atherosclerosis were screened through three disease database, including Comparative Toxicogenomics Database (CTD) (http://ctdbase.org/) (19), GeneCards (https://www.genecards.org/) (20), and DISEASES (https://diseases.jensenlab.org/) database (21).

Weighted Gene Co-Expression Network Analysis

A system biological approach called Weighted Gene Co-Expression Network Analysis (WGCNA) analysis co-expressed gene modules that have high biological significance and explores the relation between gene networks and diseases (22). Therefore, the WGCNA was used to analyze GSE56815 dataset and GSE22829 dataset to obtain the osteoporosis and atherosclerosis associated modules. Using R language, the Hclust function was used prior to the analysis of eliminating outlier samples from the hierarchical clustering analysis. Based on the criterion of $R^2 > 0.85$, an appropriate soft-thresholding power β (ranged from 1 to 20) is calculated to achieve the scale-free topology. Then, through hierarchical clustering, co-expression modules were identified, and the hierarchical clustering tree was obtained. The minimum number of module genes was set as 25, and the modules were merged for the second time according to the

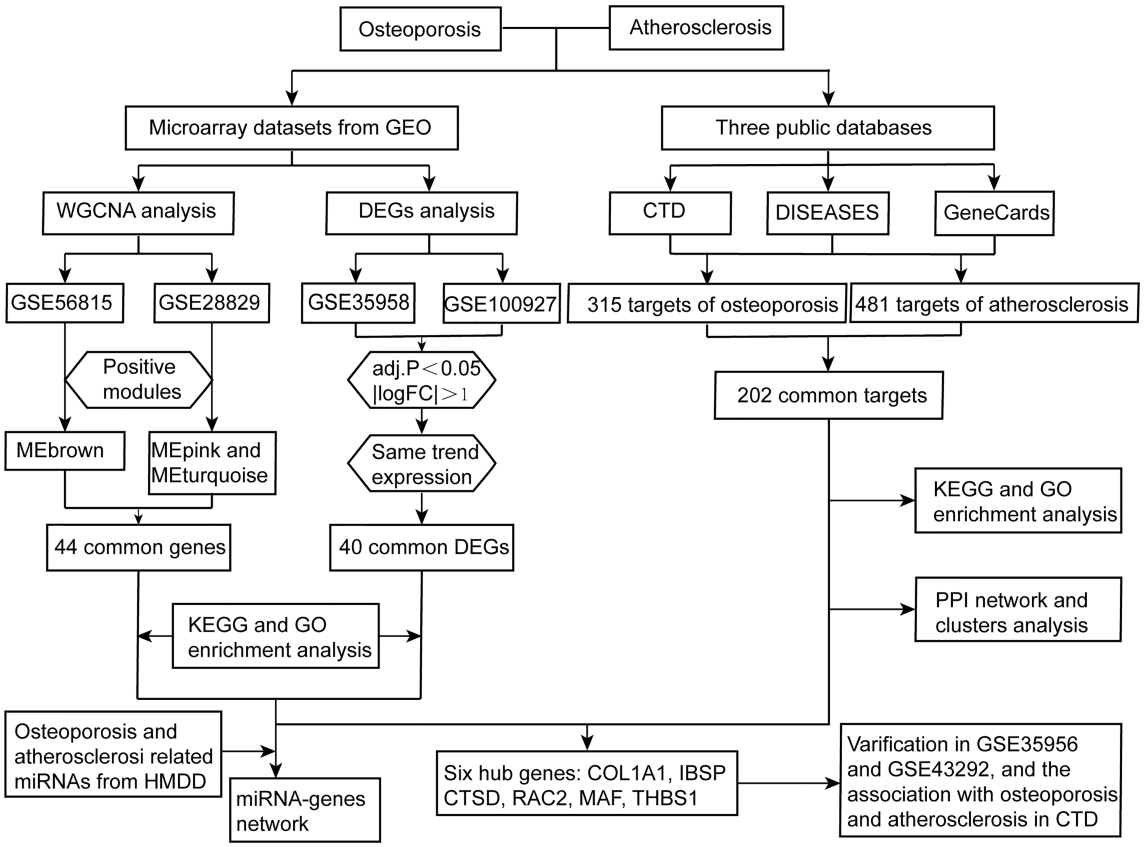

FIGURE 1 | Research design flow chart.

modules with a correlation greater than 50%. Finally, the module eigengene and the correlation between the module eigengene and clinical features were calculated to obtain the expression profiles of each module. As a result, we focused on modules with high correlation coefficients with clinical features and later selected genes from these modules for further analysis.

Analysis of Gene Modules Through WGCNA Analysis

Using the Pearson correlation coefficient and the *P*-value of eigengenes and disease traits of each module, we identified key modules in osteoporosis and atherosclerosis. Then, the genes in key modules positively associated with osteoporosis and atherosclerosis were used to obtain shared genes *via* ImageGP (23).

Identification of Common Genes Through DEGs Analysis

GEO2R (www.ncbi.nlm.nih.gov/geo/ge2r) is an online analysis tool developed based on two R packages (GEO query and Limma) (24). The differentially expressed genes (DEGs) in GSE35958 and GSE100927 datasets were determined by comparing gene expression profiles between the diseased and control groups using GEO2R. Log|FC| > 1 and adj. *P*-value < 0.05 were considered to indicate statistical significance. The common DEGs were obtained by Venn diagram.

Enrichment Analysis, PPI Network Construction, and Module Analysis

In order to analyze the biological functions and pathways involved in common genes, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using Metascape, which is a web-based portal designed to provide a comprehensive gene list annotation and analysis resource for experimental biologists (25). Min overlap = 3 and Min Enrichment = 1.5 were the screening conditions. The *P*-value < 0.01 was considered significant.

The protein–protein interaction (PPI) network was analyzed using the Search Tool for the Retrieval of Interacting Genes (STRING; http://string-db.org). An interaction with a combined score > 0.4 was selected and used to construct a PPI network with Cytoscape software (version 3.7.0). The cluster analysis used Cytoscape's plug-in molecular complex detection technology (MCODE) with default parameters: K-core = 2, degree cutoff = 2, max depth = 100, and node score cutoff = 0.2.

Shared Gene Targets Obtained From Public Database

The common osteoporosis-related genes and the atherosclerosis-related genes that were shared between the three public databases were obtained using the Venn diagram. Then, taking the intersection of them, we obtained the common gene targets between osteoporosis and atherosclerosis.

Hub Gene Selection and Validation

Common gene targets between osteoporosis and atherosclerosis were obtained through disease database screening. At the same time, shared genes were also gained from WGCNA and DEG analysis. Then, the intersection of common targets from disease databases and genes from WGCNA and DEG analysis was selected as hub genes. Furthermore, the expression of hub genes was verified in GSE43292 and GSE35956. The comparison between the two sets of data was performed with the t-test. *P*-value < 0.05 was considered significant.

Hub Gene Interaction With Diseases

In order to explore the relationship between hub genes and diseases, CTD was used to isolate the inference score and reference count of hub genes associated with osteoporosis and atherosclerosis. Interaction between hub genes and atherosclerosis, cardiovascular diseases, vascular diseases, osteoporosis, bone diseases, and bone resorption was analyzed in CTD. The inference score and reference count were visualized by the Histogram.

Identified the Common miRNAs

Small non-coding RNAs called microRNAs can modulate gene expression by promoting or inhibiting mRNA degradation and translation (26). We therefore investigate whether some miRNAs share a common regulatory mechanism and development process in osteoporosis and atherosclerosis. Osteoporosis-associated and atherosclerosis-associated miRNAs were obtained from the Human microRNA Disease Database (HMDD), which presents more detailed and comprehensive annotations to the human miRNA-disease association data, including miRNA-disease association data from the evidence of genetics, epigenetics, circulating miRNAs, and miRNA-target interactions (27). Based on a published literature, we further identified the expression levels of these miRNAs in osteoporosis and atherosclerosis, and only miRNAs with the same disorder types were further analyzed. In particular, GO analysis of these common miRNAs was performed using the online software mirPath (v.3) from DIANA tools. The GO terms with *P*-values < 0.01 were considered significant.

The Common miRNAs–Genes Network Construction

Target gene information of common miRNAs was collected from miRTarbase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php), which is an experimentally validated miRNA-target interactions database (28). The intersection of target genes of common miRNAs and shared genes in osteoporosis and atherosclerosis was used to construct the miRNAs–genes regulated network. Cytoscape software was used to visualize the network.

RESULTS

GEO Information

Six GEO datasets (namely, GSE56815, GSE28829, GSE100927, GSE35958, GSE43292, and GSE35956) were selected in all. Detailed information of these six datasets is shown in **Table 1**, such as GSE number, detection platforms, and samples. GSE56815 and GSE28829 were paired used for the WGCNA analysis, GSE35958 and GSE100927 were paired used for the DEG analysis, and GSE35956 and GSE43292 were used to verify the hub gene expression levels.

The Co-Expression Modules in Two Diseases

As shown in **Figures 2A–C**, gene modules associated with the bone mineral density phenotype were obtained by WGCNA analysis of GSE56815 dataset, including circulating monocytes from females with 40 high bone mineral density and 40 low bone mineral density. Clustering analysis of GSE56815 showed that the GSM1369791 sample was poorly clustered (**Supplementary Figure 1A, B**). Therefore, this sample was excluded as an outlier in the WGCNA analysis. The analysis of soft threshold selection revealed that gene associations were maximally consistent with the scale-free distribution and when $\beta = 7$ (scale free $R^2 = 0{:}85$). Then, a total of eight modules were identified in the weighted gene co-expression network by merging modules with feature factors greater than 0.5 and setting the minimum number of genes in a module to 25. We found that, as shown in **Figure 2C**, MEbrown module ($r = 0.462$, $p = 0.0031$) was positively correlated with osteoporosis in non-gray modules. Genes in the MEbrown module were further used to the analysis, including 257 genes.

As shown in **Figures 2D–F**, a total of 12 gene modules were obtained through the WGCNA analysis of GSE28829 dataset, including 16 advanced atherosclerotic segments and 13 early atherosclerotic segments from human. Clustering analysis showed that no sample was excluded as an outlier in the WGCNA analysis (**Supplementary Figures 1C, D**). Similarly, a heatmap was mapped about module–trait relationships according to the Spearman's correlation coefficient to evaluate the association between each module and the disease. Among

TABLE 1 | Detailed information of GEO datasets.

ID	GSE number	Platform	Samples	Disease
1	GSE56815	GPL96	40 patients and 40 controls	osteoporosis
2	GSE35958	GPL570	4 patients and 5 controls	osteoporosis
3	GSE35956	GPL570	5 patients and 5 controls	osteoporosis
4	GSE28829	GPL199	16 patients and 13 controls	atherosclerosis
5	GSE100927	GPL17077	69 patients and 35 controls	atherosclerosis
6	GSE43292	GPL6244	32 patients and 32 controls	atherosclerosis

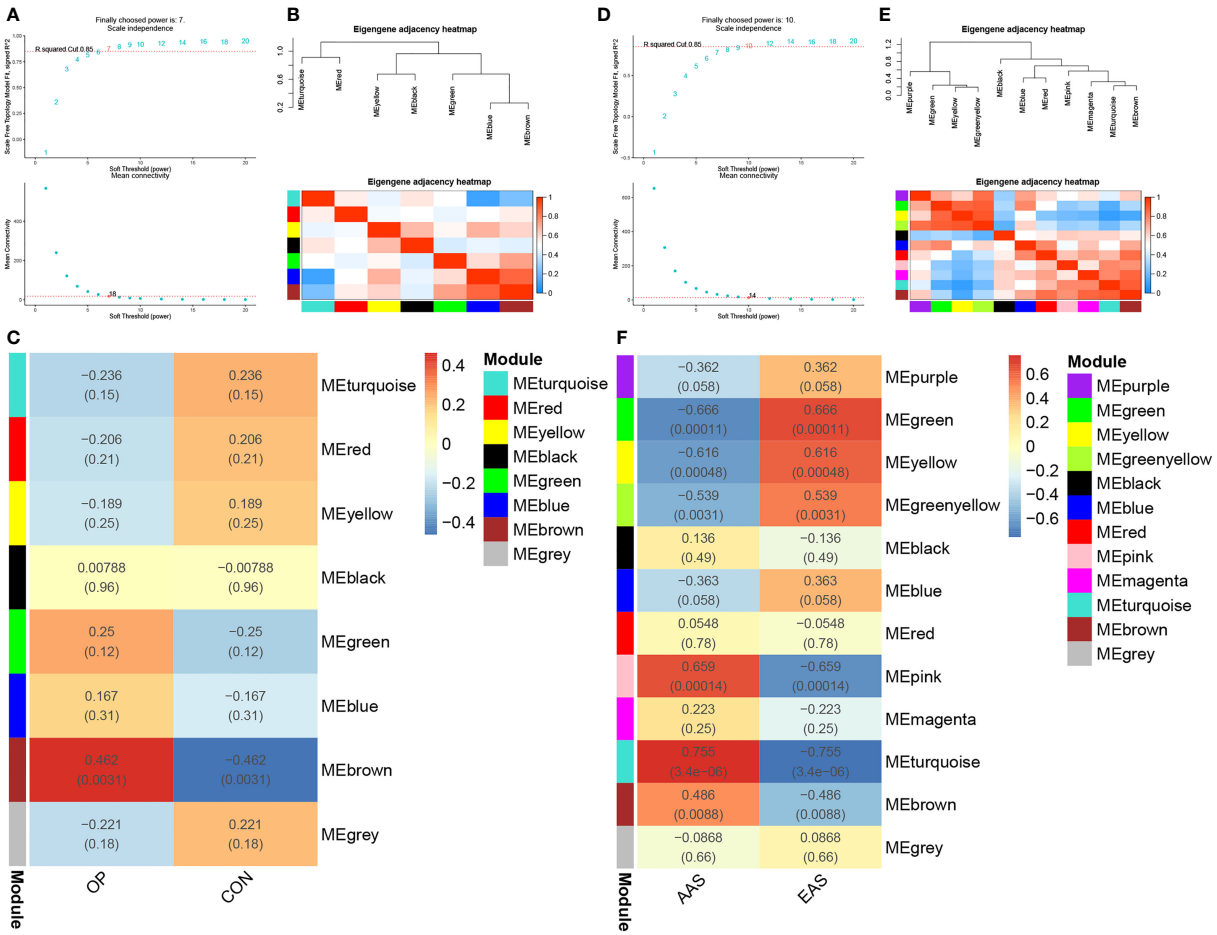

FIGURE 2 | Weighted genes correlation network analysis (WGCNA) of GSE56815 and GSE28829 datasets. **(A)** Soft threshold analysis in osteoporosis. **(B)** Module correlations in osteoporosis. **(C)** Heatmap of the module–trait relationship in osteoporosis. Each cell contains the corresponding correlation and *P*-value. **(D)** Soft threshold analysis in atherosclerosis. **(E)** Module correlations in atherosclerosis. **(F)** Heatmap of the module–trait relationship in atherosclerosis. Each cell contains the corresponding correlation and *P*-value. OP, osteoporosis; CON, control; AAS, advanced atherosclerosis; EAS, early atherosclerosis.

these 12 modules, the correlations of two modules "MEturquoise" and "MEpink" was high and they were positively correlated with atherosclerosis (MEturquoise module: r = 0.755, p = 3.4e-06; MEpink modules: r = 0.659, p = 0.00014), including 446 and 69 genes, respectively (**Figure 2F**).

Enrichment Analysis of Common Genes from WGCNA

The common genes were screened between atherosclerosis positively related modules (MEturquoise and MEpink modules) and osteoporosis positively related modules (MEbrown module). Then, 44 common genes were identified in the three positivity related modules of osteoporosis and atherosclerosis (**Figure 3A**). PPI network was further constructed by Cytoscape (**Figure 3B**). To explore the potential functions of these genes, GO and KEGG enrichment analyses were performed using Metascape. Results showed that these common genes were mainly enriched in leishmaniasis, immune receptor activity, inflammatory response, tertiary granule, and regulation of leukocyte activation, which indicated that most of them were involved in immune- and inflammatory-related functions (**Figure 3C**).

The Unique Gene Signatures in Two Diseases

In order to investigate the possible pathogenesis of osteoporosis and atherosclerosis, enrichment analysis of genes in their positive modules was performed. Function enrichment analysis showed that the genes in osteoporosis MEbrown module were mainly associated with immune and inflammatory response. Interestingly, we also found that they were also mainly enriched in lipid and atherosclerosis pathway, which is closely associated with the development and progression of atherosclerosis. Therefore, it may hint that a close link exists between osteoporosis and atherosclerosis at the molecular level (**Figure 4A**).

The MEturquoise and MEpink modules were closely associated with atherosclerosis, and the genes shared with

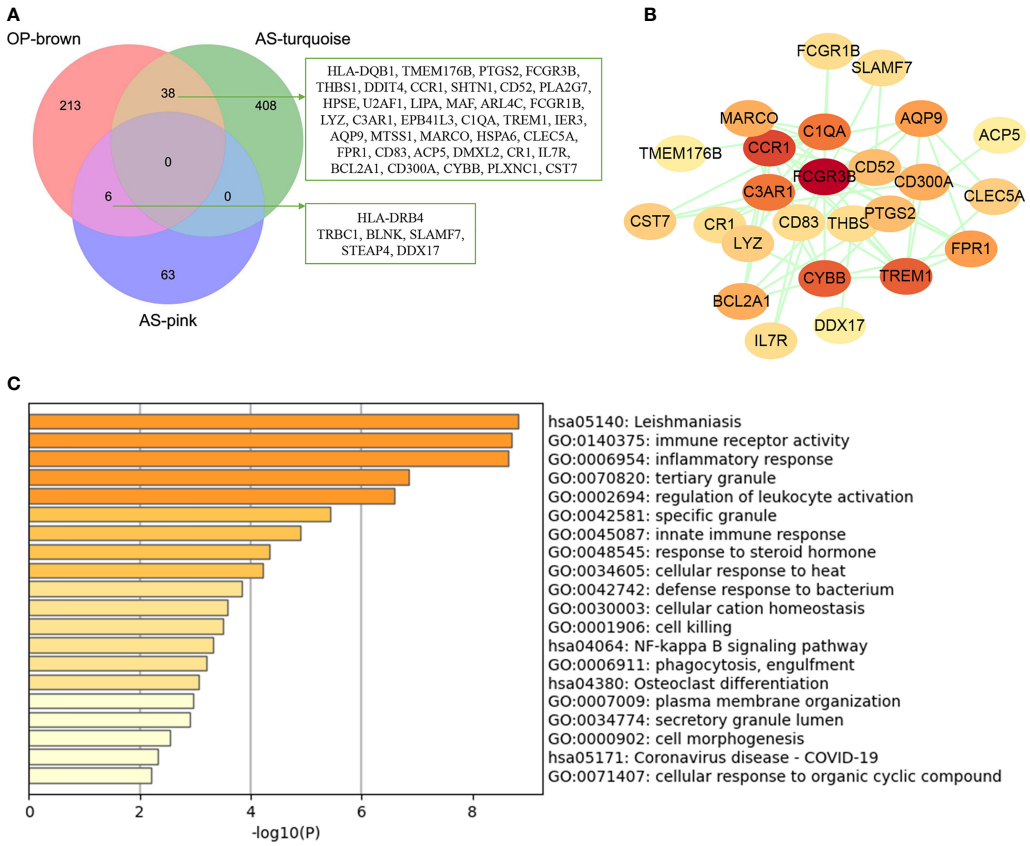

FIGURE 3 | Analysis of shared genes through WGCNA. **(A)** The shared genes between the MEpink and MEturquoise modules of atherosclerosis and MEbrown module of osteoporosis by overlapping them. **(B)** The PPI network of the shared genes. **(C)** GO and KEGG enrichment analysis of the shared genes. OP, osteoporosis; AS, atherosclerosis.

osteoporosis were mainly located in the MEturquoise module. As shown in the **Figures 4B, C**, the turquoise and pink modules were also mainly related to inflammatory response, immune effector process, and leukocyte activation. The results described above indicated that immune and inflammatory response may play important roles in both osteoporosis and atherosclerosis and be major contributors to atherosclerosis complicted with osteoporosis.

Identification and Analysis of Common DEGs

GSE35958 and GSE100927 datasets were used to DEG analysis. The GSE35958 dataset contains four osteoporosis mesenchymal stem cell samples and five non-osteoporosis mesenchymal stem cell samples from human bone marrow. GSE100927 consists of 69 atherosclerotic plaque samples and 35 control artery samples from human peripheral arteries. After analysis of GEO2R, a total of 575 and 2,619 DEGs were identified in GSE100927 and GSE35958, respectively. The overall distribution of DEGs (fold change > 1 and adj. *P*-value < 0.05) was reflected by the volcanic map (**Supplementary Figure 2**). After taking the intersection of the Venn diagram, 40 common upregulated DEGs in both datasets were obtained (**Figure 5A**). PPI network showed that TNF, ITGB2, CTSD, and LAPTM5 had high degree (**Figure 5B**). Function enrichment analysis indicated that these common DEGs were enriched in regulation of cell adhesion, lysosomal lumen, specific granule, and inflammatory response (**Figure 5C**). Interestingly, we found that immune and inflammatory response–related functions were significantly enriched again, which were consistent with the results of WGCNA analysis.

Analysis of Common Gene Targets From Three Public Databases

In order to integrate the reported biological data, we combined osteoporosis- and atherosclerosis-related genes available in CTD, DISEASES, and GeneCards databases by Venn diagram software, respectively. Then, 315 genes related to osteoporosis and 481 genes to atherosclerosis were selected, and 202 common gene targets between osteoporosis and atherosclerosis were mapped, which hints that osteoporosis and atherosclerosis share a large common set of genes (**Figures 6A–C**). The list of 202 common gene targets of osteoporosis and atherosclerosis was showed in **Supplementary Table 1**.

KEGG enrichment analysis of these 202 common genes revealed that most of the shared genes related pathways are linked to lipid and atherosclerosis, PI3K-Akt signaling pathway, and some immune and inflammation-related pathways,

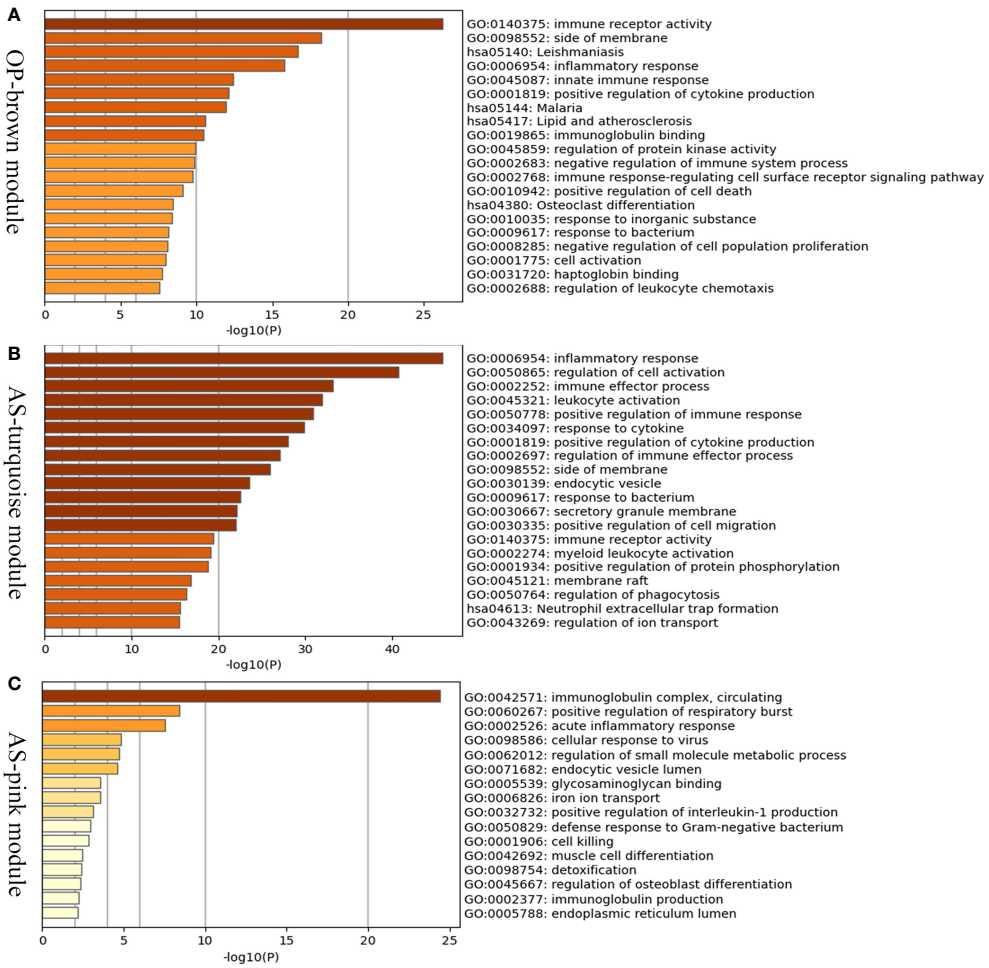

FIGURE 4 | Enrichment analysis of positive related modules in osteoporosis and atherosclerosis. **(A)** GO and KEGG enrichment analysis of MEbrown module in osteoporosis. **(B)** GO and KEGG enrichment analysis of MEturquoise module in atherosclerosis. **(C)** GO and KEGG enrichment analysis of MEpink module in atherosclerosis. OP, osteoporosis; AS, atherosclerosis.

including cytokine–cytokine receptor interaction, IL-17 signaling pathway, and T-cell differentiation, which were consistent with our enrichment analysis results above. **Figure 6D** contains the top 15 results and **Supplementary Table 2** contains the top 20 signalling pathways. **Figure 6E** contains the top five results of three GO enrichment analysis, including biological process (BP), cellular component (CC), and molecular function (MF). **Supplementary Table 3** contains the top 20 results of BP analysis results.

The PPI network of the common targets was constructed with combined scores greater than 0.4 using Cytoscape, which contained 200 nodes and 1,697 edges (**Figure 7A**). Three closely connected gene modules were obtained through MCODE plug-in of Cytoscape (**Figures 7B–D**). Enrichment analysis results of these three clusters showed that the immune and inflammatory response–related functions were enriched again.

Identification and Analysis of Hub Genes

After taking the intersection of three sets, six hub genes (COL1A1, IBSP, CTSD, RAC2, MAF, and THBS1) were identified (**Figure 8**). In order to verify the reliability of these hub gene expression levels, we then selected GSE35956 dataset to analyze the expression levels of these hub genes in osteoporosis, and GSE43292 dataset to analyze the expression levels of hub genes in atherosclerosis. Interestingly, all hub genes were significantly upregulated in both the osteoporosis and atherosclerosis group compared with the control group (**Figures 9A, B**).

In addition, we find that these six hub genes were associated with not only osteoporosis but also atherosclerosis with different inference scores and reference counts in CTD (**Table 2**). The information of interaction between hub genes and diseases was shown in **Figures 9C, D**.

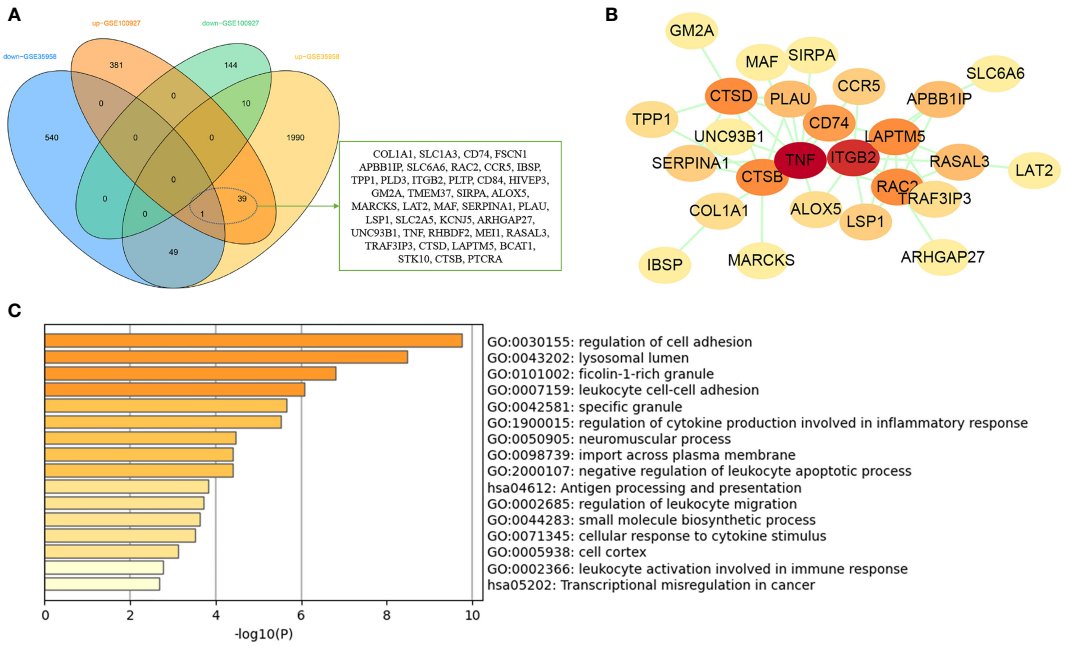

FIGURE 5 | Analysis of common DEGs between osteoporosis and atherosclerosis. **(A)** The Venn diagram of the common DEGs in GSE35958 and GSE100927. **(B)** The PPI network of the common DEGs. **(C)** GO and KEGG enrichment analysis of the common DEGs.

Identification and Analysis of Common miRNAs in Two Diseases

A total of 119 miRNAs associated with atherosclerosis and 32 miRNAs associated with osteoporosis were screened out from the HMDD database (**Supplementary Data 1**). After taking the intersection of them, 17 common miRNAs between osteoporosis and atherosclerosis were obtained. According to the published literature provided by the HMDD database, we obtained the disorder types of these common miRNAs; there were six miRNAs (hsa-miR-133b, hsa-miR-205-5p, hsa-miR-21-3p, hsa-miR-320a, hsa-miR-23b-3p, and hsa-miR-181a-5p) upregulated and two miRNAs (hsa-miR-150-5p and hsa-let-7g-5p) downregulated in both osteoporosis and atherosclerosis. Then, the eight miRNAs were further studied. The enrichment analysis showed that the functions of these miRNAs were involved in multiple BPs including immune response related functions (**Supplementary Figure 3**).

The common miRNAs-shared genes network

A total of 5,015 target genes of eight common miRNAs were predicted using miRTarbase, and the miRNAs–genes network was constructed by taking the intersection of them and shared genes (obtained from WGCNA and DEGs). Finally, the miRNAs–genes network contained seven miRNAs and 23 shared genes, including three hub genes (CTSD, COL1A1, and THBS1) (**Figure 10**). It is evident from the network that hsa-let-7g-5p regulated the most downstream target genes. Moreover, target genes predicted by has-let-7g were closely related to the inflammatory and immune response using the online software mirPath (v.3) from DIANA tools. Therefore, we speculate that has-let-7g might function importantly in the common mechanism of osteoporosis and atherosclerosis.

DISCUSSION

Osteoporosis and atherosclerosis are both widely prevalent disorders and often seen concurrently, exerting a severe impact on public health (29). The common pathophysiological mechanisms between osteoporosis and atherosclerosis have been attracting intense research interest around the world. Previous studies identified some molecular lipid species associated with early markers of both osteoporosis and atherosclerosis based on the Young Finns Study cohort, supporting the osteoporosis and atherosclerosis comorbidity hypothesis (14, 30). Latest research found that platelet-derived growth factor–BB (PDGF-BB), secreted from preosteoclasts, worked as an important mediator of vascular stiffening in response to aging and metabolic stress (31). Extracellular vesicles derived from aged bone matrix during bone resorption promote bone marrow mesenchymal stem cells adipogenesis rather than osteogenesis and augment calcification of vascular smooth muscle cells (32). These results have deepened our knowledge of the concept of a bone-vascular axis and may help to reveal the molecular mechanism between osteoporosis and atherosclerosis. However, it seems that few studies have explore the common pathogenesis of osteoporosis and atherosclerosis on a genetic level. To our knowledge, this is the first time to investigate osteoporosis and atherosclerosis

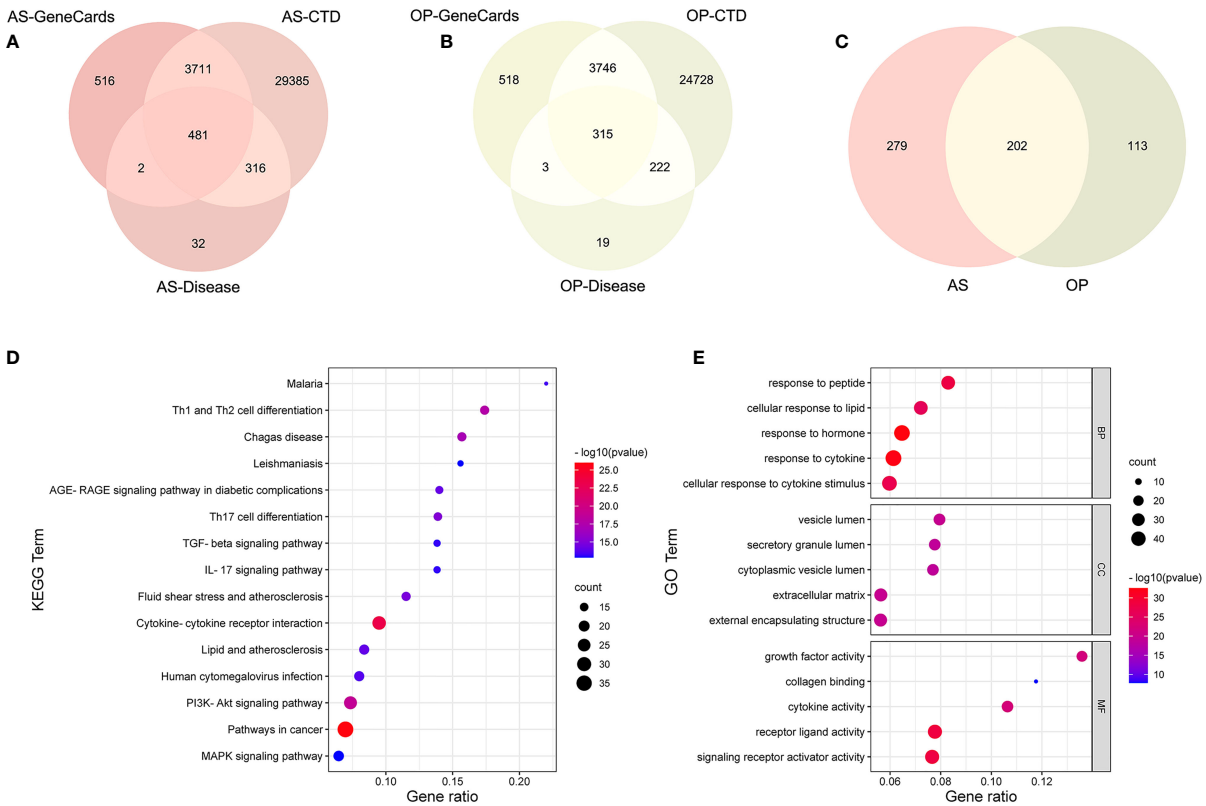

FIGURE 6 | Analysis of common targets between osteoporosis and atherosclerosis from three public databases. **(A–C)** Venn diagram of common targets between atherosclerosis and osteoporosis. **(D)** KEGG enrichment analysis of the common targets. **(E)** GO enrichment analysis of the common targets.

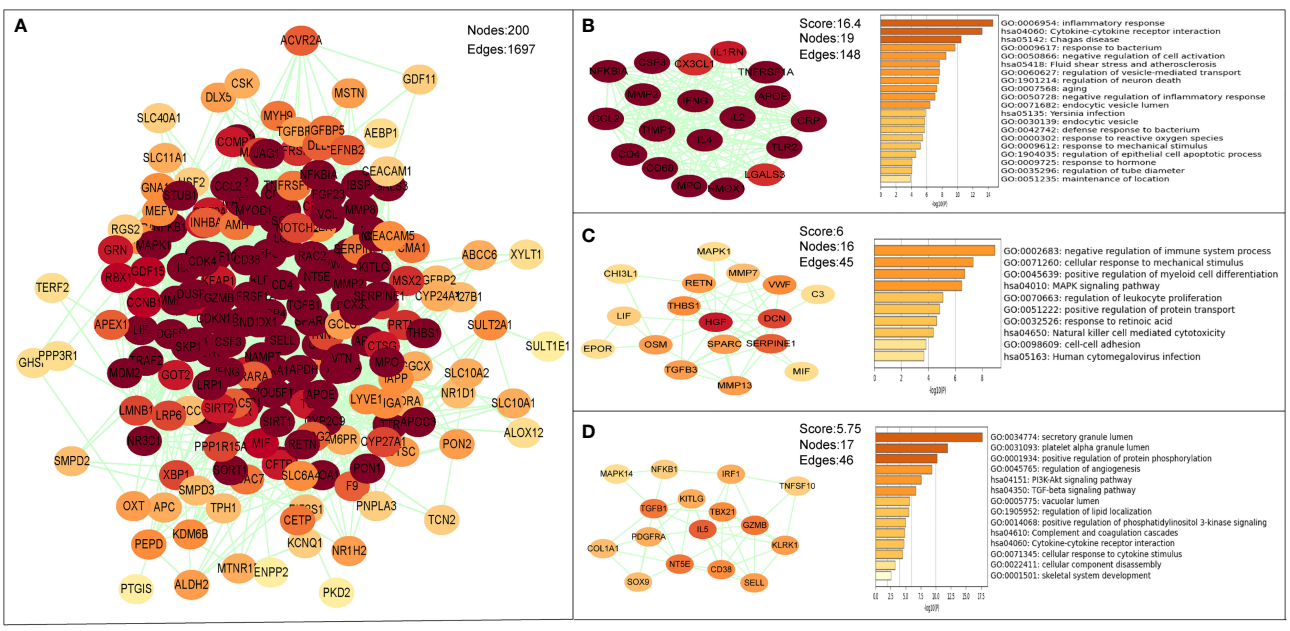

FIGURE 7 | The PPI network and clusters analysis of common targets. **(A)** PPI network of 202 common targets. **(B–D)** Three significant gene clustering modules and enrichment analysis of the modular genes.

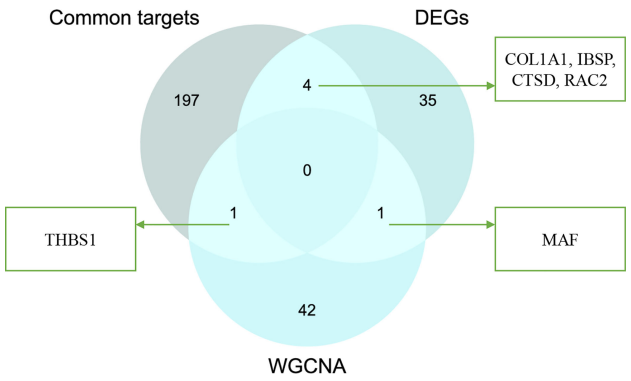

FIGURE 8 | Venn diagram of interaction between common targets and genes from WGCNA and DEG analysis.

comorbidity hypothesis by integrating data from a variety of public databases to identify the common mechanisms of osteoporosis and atherosclerosis.

Global gene expression studies can help us better understand the specific pathobiology between osteoporosis and atherosclerosis. The results of WGCNA and DEG analysis showed that immune and inflammatory response–related functions may play an important role in both osteoporosis and atherosclerosis. At the same time, we found that T-cell differentiation and inflammatory signaling pathways were also enriched in the enrichment analysis results of common genes from public disease databases. Therefore, an inflammatory environment caused by the immune and inflammatory response may be a common feature in the pathophysiology of osteoporosis and atherosclerosis.

Under physiological conditions, the bone homeostasis is regulated by osteoblast-mediated bone formation and osteoclast-mediated bone resorption through receptor activator of nuclear factor–κB (RANK), ligand for a RANK receptor (RANKL), and osteoprotegerin (OPG) interactions (33). Any imbalance of this control leads to an increase in the activity of osteoclast, resulting in osteoporosis. Although initially, it was thought that hormonal imbalance was the leading cause of osteoporosis, the role of the immune system in osteoporosis slowly came into view with advanced studies (34, 35). In pathological states such as rheumatoid arthritis, multiple sclerosis, osteoarthritis, and bone tumors, a host of inflammatory cytokines and activated immune cells disrupts this balance in favor of osteoclast-mediated bone resorption (36). Accumulated pieces of evidence suggested that immune and inflammatory response induced by both innate and adaptive immune cells can affect bone metabolism through several pathways.

Immune cell activation often goes along with inflammatory mediators' production, such as reactive oxygen species, and pro-inflammatory cytokines and chemokines, which directly or indirectly influence bone metabolism and promote the development of osteoporosis (37). In innate immune system, pro-inflammatory cytokines such as TNF-a and IL-6 can stimulate the polarization of macrophages into M1 macrophages (both an inflammatory phenotype and a precursor of osteoclast), associating with bone catabolic activity (38). The monocytes show high levels of C-C chemokine receptor 2 (CCR2) in inflammatory microenvironment and can serve as osteoclast precursor, participating in bone remodeling by producing cytokines (39). In addition, dendritic cells and neutrophils can activate T cells, and the activated T cells produce cytokines and soluble factors that participate in bone resorption process (40). In adaptive immune system, T cells are major players of it and the results of our enrichment analysis were also involved in T_H1, T_H2, and T_H17 cell differentiation and inflammatory factor–related pathways. Previous studies have found that T_H1 and T_H2 cells inhibit osteoclastogenesis *via* secreting IFN-γ and IL-4 cytokines and thus act as an osteoprotective role (41). Lower serum levels of IFN-γ and IL-4 cytokines in postmenopausal osteoporotic patients further suggest its osteoprotective role (42). However, a study suggested that IFN-γ can also promote osteoblasts generation by inducing expression of RANKL on activated T cells and thus plays a dual role in bone remodeling (43). T_H17 cells are osteoclastogenic subsets of T cells and can produce proinflammatory cytokine, including RANKL, TNF-α, IL-17, and IL-6, all of which augment osteoclastogenesis (44). Moreover, enhancement in the number of T_H17 cells and enhanced expression of proinflammatory cytokines (IL-6, TNF-α, RANKL, and IL-17) were observed in osteoporotic mice (45). Recent research suggested that "pyroptosis" of osteoblast (a programmed cell death mechanism) correlates with inflammation and contributes to excessive differentiation of osteoclasts *via* producing NLRP3 and also IL-1β and IL-18 (46). In summary, the concept of osteoimmunology was consistent with the enrichment results above in our study. Zhang et al. (47) performed a GO analysis of genes corresponding to differentially expressed proteins in osteoporosis and also found that immune inflammation and related functions were significantly enriched, consistent with our analysis.

Atherosclerosis is a chronic disease with an autoimmune component due to its accompaniment with a chronic, low-grade inflammatory response that attracts cells of the innate and adaptive immune systems into the atherosclerotic plaque (48). The important role of immunity and inflammation in atherosclerosis has been demonstrated by overwhelming experimental and clinical evidence. In our study, the analysis of positively correlated modules in atherosclerosis also supported this view. A genome-wide association study for coronary artery disease identified some locus linked to inflammation (49). The most robust genetic association has been identified for single-nucleotide polymorphisms in the 9p21 locus, which has been implicated in regulation of IFN-γ signaling (50). Oxidized LDL (oxLDL), the well-established precursor particles of atherosclerosis, can trigger inflammation of the arterial wall by binding to Toll-like receptors (TLRs) (51), and it can also reduce osteoblast viability (52).

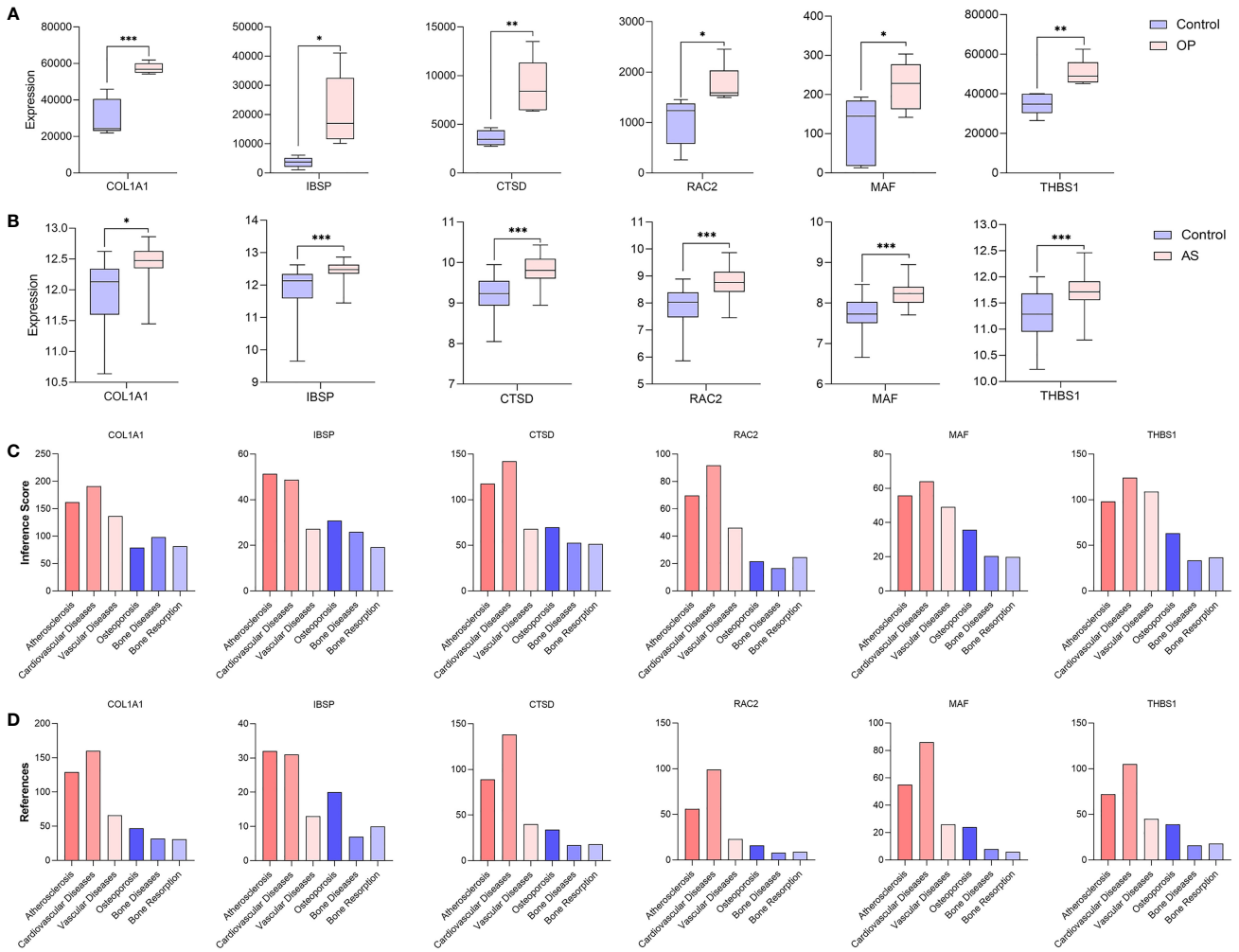

FIGURE 9 | Verification of hub genes. **(A)** The expression level of hub genes in GSE35956. **(B)** The expression level of hub genes in GSE43292. The comparison between the two sets of data uses the mean t-test; *P*-value < 0.05 was considered statistically significant. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Inference score **(C)** and reference count **(D)** between hub genes and atherosclerosis, cardiovascular diseases, vascular diseases, osteoporosis, bone diseases, and bone resorption in CTD.

In the vessel wall, oxLDL and inflammatory statement elicit an influx of monocytes that differentiate into macrophages and then into foam cells, accumulate intracellular cholesterol, and produce inflammatory mediators (53). $CD4^+$ T cells are also recruited to the forming lesion, and single-cell data from human atherosclerotic plaques showed that the majority of $CD4^+$ T cells in the plaque are T_H1 and T_H2 cells (54). In our study, we similarly found a significant enrichment for T_H1 and T_H2 cell differentiation pathway. Proinflammatory mediators such as IFN-γ, TNF-α, IL-2, and IL-3, produced by T_H1 and T_H2 cells, can activate macrophages and other plaque cells and thereby accelerate the inflammatory response (54). Knocking IFN-γ and its receptor protects mice from atherosclerosis (55). In addition, T_H17 cells also can be induced by oxLDL and constitute a minor population in plaques, promoting the vascular inflammation (56). Ongoing inflammatory and hemodynamic assaults on the atherosclerotic lesion might eventually cause local dysfunction or breakdown of endothelial integrity. One study identified the DEGs in atherosclerosis, compared with healthy controls. The bioinformatics analysis showed that immune response, inflammatory response, and vascular smooth muscle

TABLE 2 | The hub genes associated with atherosclerosis and osteoporosis in CTD.

Gene/ Disease	Atherosclerosis		Osteoporosis	
	Inference Score	References	Inference Score	References
COL1A1	162	129	79.32	47
RAC2	69.82	56	21.68	16
THBS1	98.13	72	63.13	39
MAF	55.78	55	35.76	24
CTSD	117.68	89	69.85	34
IBSP	51.38	32	30.75	20

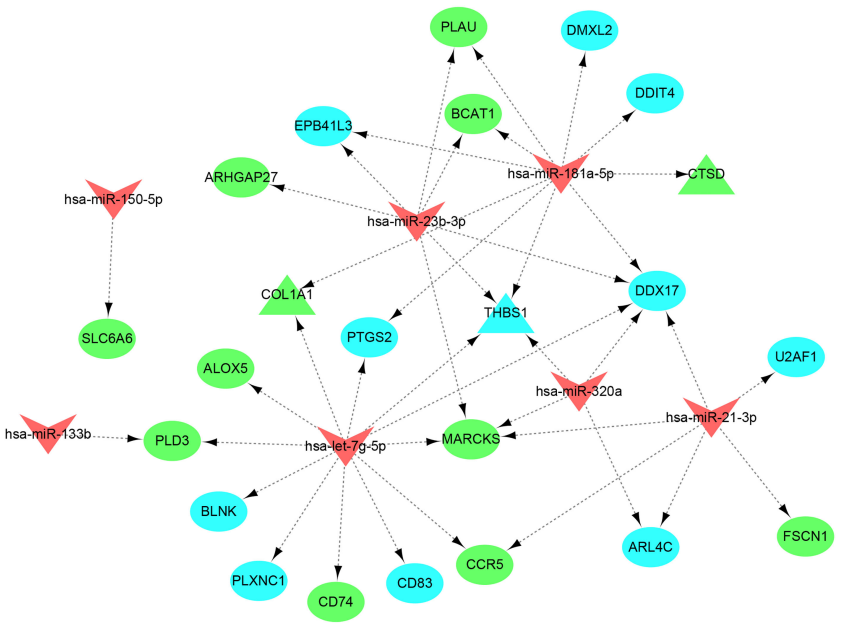

FIGURE 10 | miRNAs–shared genes regulatory network. The V-shape represents miRNA, ellipse shape represents gene, and triangle shape represents hub gene. Blue represents gene from WGCNA and green represent gene from DEGs.

contraction were the unique gene signatures in atherosclerosis, which were consistent with our analysis (57).

As mentioned above, we found that immune and inflammatory response were enrich in both atherosclerosis and osteoporosis, and most of common genes obtained from WGCNA, DEGs, and three public databases were associated with immune response. The immune-associated genes in both atherosclerosis and osteoporosis from WGCNA mainly included TREM1, CYBB, CCR1, CD83, CD52, IL7R, and THBS1. The immune-associated genes from DEGs mainly included TNF, ITGB2, CD74, CCR5, and MAF. The immune-associated genes from three public databases mainly included IFNG, IL2, IL4, CCL2, TGFB1, and TLR2. Therefore, the occurrence and development of atherosclerosis and osteoporosis are complicated and these genes may provide clues for the common underlying mechanisms between them. Suppression of inflammation may have beneficial effects on bone and on vasculature.

Then, six hub genes (COL1A1, IBSP, CTSD, RAC2, MAF, and THBS1) were obtained through the integration of multisource databases and they were all significantly upregulated in both the osteoporosis and atherosclerosis group compared with the control group. According to CTD, COL1A1 was closely associated with osteoporosis and atherosclerosis and it had the highest inference score and reference count. COL1A1 is mainly involved in bone matrix formation, coding for collagen type 1 which is the most abundant extracellular protein in bone and serves as an indicator of bone formation (58). Mutations in COL1A1 gene have been demonstrated to be responsible for the autosomal dominant form of osteogenesis imperfecta, with severe osteoporosis (59). Studies have confirmed that COL1A1 is overexpressed in artery at animals with atherogenic diet and the increase was contributed to the inflammatory process and the activate the cytokine TGF-β, which supports the fibrotic process through expressing COL1A1 (60). In addition, previous studies have shown that COL1A1 may influence the prognosis in tumors by affecting infiltrating immune cells (61). Therefore, COL1A1 may be involved in the pathogenesis of osteoporosis and atherosclerosis by influencing bone formation, vascular fibrosis, immune response, but these related molecular mechanisms warrant additional investigation. Thrombospondin-1 (THBS1), an immune-associated gene, can affect endothelial cell proliferation, migration, and apoptosis by antagonizing the activity of VEGF, participating in the regulation of vascular formation (62). The latest research suggests that $CD68^{+}$ macrophages activate TGF-β1 by expression and secretion of THBS1 and consequently induce bone information (63). Importantly, THBS1 was closely related to inflammatory response and is found to be elevated in inflammatory processes (64). However, few studies directly analyze the role of THBS1 in osteoporosis and atherosclerosis, which emphasizes its importance in future research. Other hub genes were also involved in the pathogenesis of osteoporosis and atherosclerosis by regulating different BPs and believe that the specific mechanism is worthy of further exploration.

Finally, by constructing miRNAs–genes network, we found that let-7g had the most target genes and it also involved in immune and inflammatory response. It was reported that the let-7g was significantly downregulated in patients with recent osteoporotic fractures and it could enhance osteoblast formation *in vitro* and *in vivo* by targeting HMGA2 (65). In addition, it also can regulate osteoblast formation by targeting

COL1A2 (66). More importantly, high-fat diet can suppress let-7g expression (67) and activate NF-κB signaling pathway, which serves as a pivotal mediator of inflammatory responses (68). let-7g can reduce macrophage transformation and alleviates foam cell apoptosis by suppressing NF-κB pathways to prevent atherosclerosis (69). Because the expression of let-7g is regulated by a number of cellular elements which are associated with inflammatory states and chemokine release (70), it is possible that inflammatory conditions contribute to the relative paucity of let-7g resulting in reducing osteogenic formation and increasing endothelial and foam cell apoptosis, in favor of both osteoporosis and atherosclerosis. In view of the critical role of let-7g in inflammatory response, let-7g might be an important potential target for the treatment of osteoporosis and atherosclerosis.

In conclusion, our work revealed that the immune and inflammatory response might be a common susceptible factor for both osteoporosis and atherosclerosis and identified novel gene candidates who could be used as biomarkers or as potential therapeutic targets. It may provide some clues for detailed molecular mechanisms underlying bone–vascular axis. However, the results of our study need to be further verified in cell or animal experiments, which will be critical direction for future research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LM, CM, and YL contributed to the conception of the study; LM, CM, ZW, and JL performed the analysis; CZ, WN, ZC, and WH contributed significantly to analysis and manuscript preparation; LM and CM performed the data analyses and wrote the manuscript; LM, WH, ZC, CZ and YL helped perform the analysis and constructive discussions. YL, CZ, and ZC gained the founding; All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.950030/full#supplementary-material

Supplementary Figure 1 | Sample clustering analysis **(A)** and clustering diagram **(B)** of GSE56815; Sample clustering analysis **(C)** and clustering diagram **(D)** of GSE28829. OP: osteoporosis; CON: control; AAS: advanced atherosclerosis; EAS: early atherosclerosis.

Supplementary Figure 2 | Volcanic map of DEGs (|logFC| > 1 and adj. p-value < 0.05) in GSE35958 **(A)** and in GSE100927 **(B)**.

Supplementary Figure 3 | The functional enrichment analysis of eight common miRNAs through HMDD database.

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The role of obesity, type 2 diabetes, and metabolic factors in gout: A Mendelian randomization study

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Background: Several epidemiological studies have reported a possible correlation between risk of gout and metabolic disorders including type 2 diabetes, insulin resistance, obesity, dyslipidemia, and hypertension. However, it is unclear if this association is causal.

Methods: We used Mendelian randomization (MR) to evaluate the causal relation between metabolic conditions and gout or serum urate concentration by inverse-variance-weighted (conventional) and weighted median methods. Furthermore, MR-Egger regression and MR-pleiotropy residual sum and outlier (PRESSO) method were used to explore pleiotropy. Genetic instruments for metabolic disorders and outcome (gout and serum urate) were obtained from several genome-wide association studies on individuals of mainly European ancestry.

Results: Conventional MR analysis showed a robust causal association of increasing obesity measured by body mass index (BMI), high-density lipoprotein cholesterol (HDL), and systolic blood pressure (SBP) with risk of gout. A causal relationship between fasting insulin, BMI, HDL, triglycerides (TG), SBP, alanine aminotransferase (ALT), and serum urate was also observed. These results were consistent in weighted median method and MR-PRESSO after removing outliers identified. Our analysis also indicated that HDL and serum urate as well as gout have a bidirectional causal effect on each other.

Conclusions: Our study suggested causal effects between glycemic traits, obesity, dyslipidemia, blood pressure, liver function, and serum urate as well as gout, which implies that metabolic factors contribute to the development of gout *via* serum urate, as well as potential benefit of sound management of increased serum urate in patients with obesity, dyslipidemia, hypertension, and liver dysfunction.

KEYWORDS

gout, urate, Mendelian randomization, causal relationship, metabolic factors

Introduction

Gout is a disorder of purine metabolism which results from monosodium urate crystals in and around the joints caused by long-standing hyperuricemia (1). In developed countries, the prevalence of gout in men and women is 3%–6% and 1%–2%, respectively, which increases with age but stabilizes after the age of 70 (2). The concentration of uric acid is affected not only by environmental factors but also by inheritance. Several genome-wide association studies (GWAS) determined the relationship between SLC2A9, ABCG2, and SLC17A3 gene polymorphisms and uric acid concentration as well as gout (3, 4). Although the major cause of gout is well known, the understanding of its pathogenesis is still incomplete (5).

Several epidemiological studies have repeatedly indicated that gout is associated with obesity (6, 7). In addition, it has been reported that several metabolic diseases and factors including type 2 diabetes mellitus (T2DM), insulin resistance, dyslipidemia, and hypertension were associated with increased risk of gout or serum urate (8–10). Moreover, general obesity in women and hypertriglyceridemia in men may potentiate a hyperuricemia effect for gout development (11). Evidence indicated that hyperuricemia is associated with increased prevalence, incidence, and disease severity of non-alcoholic fatty liver disease (NAFLD), while NAFLD can predict hyperuricemia as well (12). Although these metabolic diseases and factors have been associated with gout or elevated serum urate in epidemiological studies, whether these associations are causal remains unclear because the associations may be confounded by an unhealthy diet or other behavioral or environmental risk factors.

To clarify the causal relationships between the observed associations, a Mendelian randomization is performed (13). There are three key assumptions for Mendelian randomization (MR) analysis: first, the genetic variants used as instrumental variables [single-nucleotide polymorphisms (SNPs)] should be robustly associated with the risk factor of interest (relevance assumption); second, the used genetic variants should not be associated with potential confounders (independent assumption); and third, the genetic variants should affect the risk of the outcome only through the risk factor, not *via* alternative pathways (exclusion restriction assumption) (14). In that way, MR evaluates the causal effect of an exposure on the outcome of interest by using genetic variants.

In the current study, we performed a two-sample Mendelian randomization analysis to investigate the causal relationship between metabolic exposures (including T2DM, obesity, blood lipid, blood pressure, and liver function) and serum urate or gout in individuals of mainly European ancestry. For this, summary level data from the GWAS on obesity, T2DM, metabolic factors, gout, and serum urate were included (13, 15–19).

Materials and methods

Study design and data sources

We investigate whether predisposition to metabolic traits [T2DM, fasting glucose, fasting insulin, body mass index (BMI), waist-to-hip ratio adjusted for body mass index (WHRadjBMI), blood lipid, blood pressure, and liver function] is likely to have an impact on gout and serum urate level, using MR.

A meta-analysis combining three GWAS data sets of European ancestry (62,892 T2DM and 596,424 controls) was used to identify genetic loci for T2DM (19). Results from the MAGIC (the Meta-Analyses of Glucose and Insulin-related traits Consortium) consortium were used to identify genetic proxies for glycemic traits (fasting glucose, fasting insulin) including 133,010 and 108,557 non-diabetic individuals of European ancestry, respectively (17). Summary statistics on BMI and WHRadjBMI were extracted from the Genetic Investigation of ANthropometric Traits consortium (GIANT) including 322,154 and 210,088 European ancestry individuals, respectively (15, 18).

Data (joint analysis of Metabochip and GWAS data) from the GWAS of Global Lipids Genetic Consortium (GLGC) were used to identify genetic loci for blood lipids [high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), total cholesterol (TC), triglycerides (TG)] including 188,577 individuals mostly of European ancestry (20). Summary statistics for the association between SNP and blood pressure traits (systolic, diastolic, pulse pressure) were extracted from the largest genetic association study over one million people of European ancestry (13). Genetic instruments for liver function were identified from a recent genetic analysis in European-ancestry individuals (16).

The genetic data of outcomes (gout and serum urate) were derived from the Global Urate Genetics Consortium (GUGC). These data included 2,115 cases and 67,259 normal individuals from 14 European studies (21).

Selection of instrumental variables

SNPs for each exposure trait were selected as instrumental variables (IV) according to the fundamental principle of MR. Each IV was independently [linkage disequilibrium (LD) $r^2 < 0.01$] associated with the exposure traits at a genome-wide significance threshold ($P < 5 \times 10^{-8}$) in a previously published GWAS. Suitable proxy SNPs were chosen with the linkage disequilibrium ($r^2 > 0.8$) to ensure that proxy SNP and target SNP have a strong correlation (22). Instrument strength in MR was evaluated with the F statistic derived from a measure of the exposure variance explained by each SNP. SNPs with instrument strengths (F) larger than 10 were selected (23).

According to the principle mentioned above, we finally selected multiple independent SNPs strongly associated with each exposure trait and details of the included traits are displayed in Table 1.

Statistical analysis

In the main Mendelian randomization analyses, the inverse-variance-weighted (IVW) method was used to assess the causal associations (24). The Cochran Q test was used to assess heterogeneity between instrumental variables in the MR. We used random-effect models if the p value of the Cochran Q test was less than 0.05; otherwise, fixed-effect models were used (25). MR-Egger and the weighted median method were conducted to supplement the result of IVW. The MR-Egger method provided an estimate of horizontal pleiotropy from the intercept of a linear regression of SNP–outcome and SNP–exposure association estimate (26). In addition to the MR-Egger intercept, the MR-pleiotropy residual sum and outlier (PRESSO) method was also used to evaluate pleiotropy (27). Furthermore, MR-PRESSO is able to identify outlier variants based on their observed distance from the regression line and estimate results after correction of outliers (27). To control for false positive findings due to multiple testing, a conservative Bonferroni correction adjusted for the number of primary exposures analyzed in the study was applied, and *P*-values less than 0.003 were considered statistically significant. *P* values between 0.003 and 0.05 were deemed suggestive evidence of possible associations. Statistical analysis was conducted by R version 4.0.5. The R package "TwoSampleMR" version 0.5.6 and "MRPRESSO" were applied.

Results

Genetic association of type 2 diabetes and glycemic traits with gout and urate

The individual instrument-exposure (T2DM, fasting glucose, and fasting insulin) is shown in Supplementary Table 1. IVW MR using associated instrumental SNPs indicated that T2DM (β = 0.027, 95% CI: -0.007-0.061, *P* = 0.116) was not causally associated with serum urate level and gout, which was also supported by weighted median and MR-Egger analysis (Figures 1, 2 and Supplementary Figure 1). However, post-removal of outliers identified by MR-PRESSO suggested a significance of the association between T2DM and increased serum urate (β = 0.652, 95% CI: 0.029–0.080, *P* < 0.001) (Table 2).

In the IVW analysis, fasting insulin-associated SNPs in Europeans showed no causal effect on increased serum urate and risk of gout (*P* = 0.287 and *P* = 0.635, respectively). After removing rs780094 in the GCKR gene according to leave-one-out analysis, recalculating the main IVW estimate suggested a causal effect of fasting insulin on increased serum urate (β = 0.802, 95% CI: 0.514–1.090, *P* < 0.001) and risk of gout (OR = 3.293, 95% CI: 1.030–10.532, *P* = 0.045) (Figures 1, 2 and

TABLE 1 Related information of included traits in the Mendelian randomization analyses.

Trait	Variable type	Consortium	Ancestry
Type 2 diabetes	Exposure	eQTLGen	European
Fasting glucose	Exposure	MAGIC	European
Fasting insulin	Exposure	MAGIC	European
Body mass index	Exposure	GIANT	European
Waist-to-hip ratio	Exposure	GIANT	European
High-density cholesterol	Exposure	GLGC	Trans-ancestry
Low-density cholesterol	Exposure	GLGC	Trans-ancestry
Triglycerides	Exposure	GLGC	Trans-ancestry
Total cholesterol	Exposure	GLGC	Trans-ancestry
Systolic blood pressure	Exposure	ICBP	European
Diastolic blood pressure	Exposure	ICBP	European
Pulse pressure	Exposure	ICBP	European
Alkaline phosphatase	Exposure	UK Biobank	European
Alanine aminotransferase	Exposure	UK Biobank	European
Gout	Disease outcome	GUGC	European
Serum urate	Continuous outcome	GUGC	European

MAGIC, The Meta-Analyses of Glucose and Insulin-related traits Consortium; GIANT, Genetic Investigation of ANthropometric Traits consortium; ICBP, International Consortium for Blood Pressure; GLGC, Global Lipids Genetic Consortium; GUGU, Global Urate Genetics Consortium.

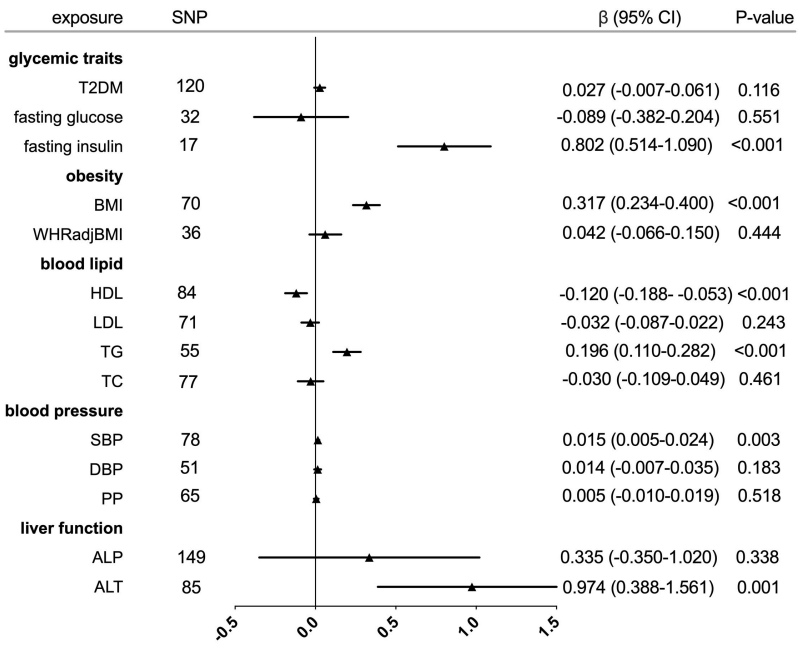

FIGURE 1
Forest plot of Mendelian randomization analyses for the genetical associations of glycemic traits, obesity, blood lipid, blood pressure, and liver function with increased serum urate. CI, confidence interval; SNP, single-nucleotide polymorphism.

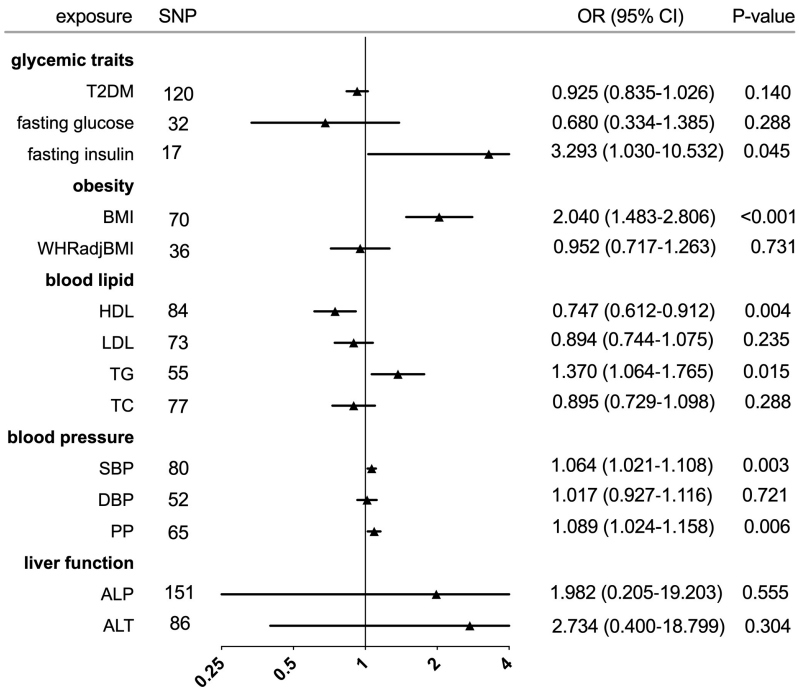

FIGURE 2
Forest plot of Mendelian randomization analyses for the genetical associations of glycemic traits, obesity, blood lipid, blood pressure, and liver function with risk of gout. CI, confidence interval; SNP, single-nucleotide polymorphism; OR, odds ratio.

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TABLE 2 Mendelian randomization estimates of glycemic traits on serum urate or gout.

Outcome	Exposure	Method	Estimate	95% CI	P-value	MR-Egger intercept (P-value)
Serum urate	T2DM	IVW MR	0.027	-0.007-0.061	0.116	0.004 (0.112)
		Weighted median	0.029	-0.001-0.059	0.058	
		MR-Egger	-0.029	-0.104-0.046	0.456	
		MR-PRESSO	0.052	0.029-0.080	<0.001	
	FBG	IVW MR	-0.089	-0.382-0.204	0.551	0.005 (0.494)
		Weighted median	-0.028	-0.170-0.115	0.702	
		MR-Egger	-0.268	-0.854-0.318	0.377	
		MR-PRESSO	0.017	-0.116-0.15	0.802	
	FI	IVW MR	0.802	0.514-1.090	<0.001	0.005 (0.723)
		Weighted median	0.891	0.603-1.179	<0.001	
		MR-Egger	0.483	-1.272-2.237	0.597	
		MR-PRESSO	0.813	0.577-1.049	<0.001	
Gout	T2DM	IVW MR	0.925	0.835-1.026	0.140	0.002 (0.816)
		Weighted median	1.001	0.836-1.199	0.988	
		MR-Egger	0.903	0.719-1.135	0.383	
		MR-PRESSO	0.943	0.855-1.040	0.240	
	FBG	IVW MR	0.680	0.334-1.385	0.288	<0.001 (0.993)
		Weighted median	1.056	0.467-2.389	0.896	
		MR-Egger	0.676	0.159-2.870	0.599	
		MR-PRESSO	0.888	0.478-1.647	0.708	
	FI	IVW MR	3.293	1.030-10.532	0.045	-0.002 (0.968)
		Weighted median	4.106	0.952-17.705	0.058	
		MR-Egger	3.803	0.789-18.324	0.716	
		MR-PRESSO	3.293	1.030-10.532	0.062	

T2DM, type 2 diabetes; FBG, fasting glucose; FI, fasting insulin.

Supplementary Figure 2). In addition, weighted median analysis suggested a causal effect of fasting insulin on increased serum urate (β = 0.891, 95% CI: 0.603–1.179, P < 0.001). The MR estimate after removing outliers identified by MR-PRESSO showed similar results (β = 0.813, 95% CI: 0.577–1.049, P < 0.001) (Table 2). To further investigate the relationship between serum urate and fasting insulin, we performed bidirectional MR analyses assessing the effects of genetically predicted serum urate on fasting insulin. We observed no causal effects of serum urate on fasting insulin (Supplementary Table 2).

The relationship between fasting glucose and serum urate was also investigated. IVW analysis revealed that fasting glucose was not associated with serum urate and gout (Figures 1, 2 and Supplementary Figure 3). There was no evidence of horizontal pleiotropy influencing the estimates in that the MR-Egger intercept was not significant (Table 2). Leave-one-variant-out analysis was conducted to identify variants with exaggerated influence on the combined effect estimate (Supplementary Table 3).

Genetic association of obesity with gout and urate

The individual instrument-exposure (BMI and WHRadjBMI) is shown in Supplementary Table 4. Using the IVW analysis, BMI was associated with an increase in the level of serum urate (β = 0.317, 95% CI: 0.234–0.400, P < 0.001) and risk of gout (OR = 2.040, 95% CI:1.483–2.806, P < 0.001) based on all SNPs in Europeans, with similar and significant results for weighted median analysis (Figures 1, 2 and Supplementary Figure 4). In the MR-Egger analysis, BMI was associated with an increase in serum urate level, with a similar but non-significant result in gout. MR-Egger did not show evidence of horizontal pleiotropy. The MR-PRESSO test showed pleiotropy in serum urate (P < 0.001), but not in gout (P = 0.874). The MR-PRESSO distortion test was significant for BMI on serum urate after removing outliers (β = 0.341, 95% CI: 0.266–0.416, P < 0.001) (Table 3). Moreover, MR analyses about the effects of genetically predicted serum urate and gout on BMI were also conducted. IVW analysis revealed that

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TABLE 3 Mendelian randomization estimates of obesity on serum urate or gout.

Outcome	Exposure	Method	Estimate	95% CI	P-value	MR-Egger intercept (P-value)
serum urate	BMI	IVW MR	0.317	0.234-0.400	<0.001	0.001 (0.805)
		weighted median	0.350	0.260-0.441	<0.001	
		MR-Egger	0.288	0.043-0.533	0.024	
		MR-PRESSO	0.341	0.266-0.416	<0.001	
	WHRadjBMI	IVW MR	0.042	-0.066-0.150	0.444	-0.002 (0.842)
		weighted median	0.026	-0.098-0.149	0.684	
		MR-Egger	0.097	-0.449-0.642	0.730	
		MR-PRESSO	0.042	-0.066-0.150	0.449	
gout	BMI	IVW MR	2.040	1.483-2.806	<0.001	0.003 (0.833)
		weighted median	2.076	1.295-3.328	0.002	
		MR-Egger	1.858	0.740-4.664	0.191	
		MR-PRESSO	2.039	1.521-2.734	<0.001	
	WHRadjBMI	IVW MR	0.952	0.717-1.263	0.731	-0.029 (0.184)
		weighted median	1.040	0.710-1.522	0.841	
		MR-Egger	2.257	0.628-8.112	0.221	
		MR-PRESSO	0.952	0.720-1.258	0.730	

BMI, Body mass index ; WHRadjBMI, Waist-to-hip ratio adjusted for body mass index.

serum urate and gout were not associated with BMI (Supplementary Table 3).

IVW MR using 36 associated instrumental SNPs indicated that WHRadjBMI was not causally associated with serum urate level (β = 0.042, 95% CI: -0.066–0.150, P = 0.444) and gout (OR = 0.952, 95% CI: 0.717–1.263, P = 0.731) (Figures 1, 2 and Supplementary Figure 5). As shown in Table 3, the MR-Egger intercepts did not provide evidence of horizontal pleiotropy in any analysis, and neither did MR-PRESSO identify outliers. For both serum urate and gout, leave-one-variant-out analysis did not identify variants with exaggerated influence on the combined effect estimate (Supplementary Table 5).

Genetic association of serum lipid with gout and urate

The individual instrument-exposure (HDL, LDL, TG, and TC) is shown in Supplementary Table 6. In the IVW analysis, HDL-associated SNPs had a causal effect on decreased serum urate (β = -0.012, 95% CI:-0.188–0.053, P < 0.001) and risk of gout (OR = 0.747, 95% CI: 0.612–0.912 P = 0.004) (Figures 1, 2 and Supplementary Figure 6). The MR-Egger test precluded the possibility of horizontal pleiotropy of instrument variables. After Bonferroni correction, the result was deemed suggestive evidence of a possible association between HDL and risk of gout (0.003 < P < 0.05). To explain this association more accurately, weighted median and MR-PRESSO analyses were conducted. Although possible outlier SNPs were identified in serum urate using the MR-PRESSO test, the effect estimate of the association between genetically predicted HDL and serum urate did not change markedly after outlier correction (β = -0.139, 95% CI: -0.184–0.095, P < 0.001) (Table 4). When setting HDL as the outcome, serum urate and gout were causally associated with HDL, as shown in Supplementary Table 3.

Using 55 associated SNPs as instrumental variables, IVW MR analysis evaluated that the level of TG had a significant effect on increased serum urate (β = 0.196, 95% CI: 0.110–0.282, P < 0.001) (Figures 1, 2 and Supplementary Figure 7). After Bonferroni correction, TG and risk of gout were not causally associated (P = 0.015). MR-Egger did not show evidence of horizontal pleiotropy. After removal of outliers, the magnitude and significance of the association between TG and serum urate remained in the MR-PRESSO analysis (Table 4). When setting serum urate as the exposure, serum urate was causally associated with TG, as shown in Supplementary Table 3. Leave-one-variant-out analysis was performed to identify variants with exaggerated influence on the combined-effect estimate (Supplementary Table 7). However, instruments for the other lipid traits, including LDL and TC, did not indicate an effect on serum urate and gout (Figures 1, 2 and Supplementary Figure 8, 9).

Genetic association of blood pressure with gout and urate

The individual instrument-exposure [systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP)] is

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TABLE 4 Mendelian randomization estimates of blood lipid on serum urate or gout.

Outcome	Exposure	Method	Estimate	95% CI	P-value	MR-Egger intercept (P-value)
Serum urate	HDL	IVW MR	-0.120	-0.188- -0.053	<0.001	-0.007 (0.015)
		Weighted median	-0.094	-0.150- -0.038	<0.001	
		MR-Egger	0.013	-0.111-0.136	0.841	
		MR-PRESSO	-0.139	-0.184- -0.095	<0.001	
	LDL	IVW MR	-0.032	-0.087-0.022	0.243	-0.004 (0.125)
		Weighted median	-0.009	-0.055-0.036	0.691	
		MR-Egger	0.020	-0.065-0.105	0.654	
		MR-PRESSO	-0.012	-0.050-0.027	0.555	
	TG	IVW MR	0.196	0.110-0.282	<0.001	0.009 (0.376)
		Weighted median	0.095	0.031-0.159	0.024	
		MR-Egger	0.162	0.025-0.299	0.034	
		MR-PRESSO	0.127	0.067-0.186	<0.001	
	TC	IVW MR	-0.030	-0.109-0.049	0.461	-0.005 (0.176)
		Weighted median	-0.047	-0.103-0.010	0.106	
		MR-Egger	0.068	-0.093-0.228	0.411	
		MR-PRESSO	-0.035	0.081-0.012	0.149	
Gout	HDL	IVW MR	0.747	0.612-0.912	0.004	-0.013 (0.121)
		Weighted median	0.873	0.653-1.167	0.359	
		MR-Egger	0.960	0.662-1.391	0.829	
		MR-PRESSO	0.759	0.626-0.920	0.006	
	LDL	IVW MR	0.894	0.744-1.075	0.235	-0.003 (0.710)
		Weighted median	0.971	0.753-1.253	0.821	
		MR-Egger	0.934	0.697-1.249	0.645	
		MR-PRESSO	0.888	0.742-1.062	0.198	
	TG	IVW MR	1.370	1.064-1.765	0.015	0.009(0.376)
		Weighted median	1.006	0.723-1.401	0.971	
		MR-Egger	1.189	0.795-1.778	0.404	
		MR-PRESSO	1.370	1.064-1.765	0.015	
	TC	IVW MR	0.895	0.729-1.098	0.288	-0.006 (0.532)
		Weighted median	0.809	0.609-1.073	0.143	
		MR-Egger	1.007	0.661-1.522	0.975	
		MR-PRESSO	0.882	0.728-1.069	0.206	

HDL, high-density cholesterol; LDL, low-density cholesterol; TG, triglycerides; TC, total cholesterol.

shown in Supplementary Table 8. In the IVW analysis, SBP-associated SNPs in Europeans had a causal effect on increased serum urate (β = 0.015, 95% CI: 0.005–0.024, $P = 0.003$) and risk of gout (OR = 1.064, 95% CI: 1.021–1.108, $P = 0.003$), which was also supported by weighted median analysis (Figures 1, 2 and Supplementary Figure 10). MR-Egger did not show evidence of horizontal pleiotropy. The MR-PRESSO test showed pleiotropy in serum urate ($P < 0.001$), but not in gout ($P = 0.512$). Although possible outlier SNPs were identified in serum urate using the MR-PRESSO test, the effect estimate of the association between genetically predicted SBP and serum urate did not change markedly after outlier correction (β = 0.017, 95% CI: 0.008–0.026, $P < 0.001$) (Table 5). When setting SBP as the outcome, genetically determined serum urate and gout were not associated with SBP (Supplementary Table 3). IVW MR using 65 associated instrumental SNPs indicated that PP was causally associated with gout (OR = 1.089, 95% CI: 1.024–1.158, $P = 0.006$) and the weighted-median method also produced a similar result (OR = 1.100, 95% CI: 1.005–1.204, $P = 0.039$), which were not significant after Bonferroni correction (Figures 1, 2 and

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TABLE 5 Mendelian randomization estimates of blood pressure on serum urate or gout.

Outcome	Exposure	Method	Estimate	95% CI	P-value	MR-Egger intercept (P-value)
Serum urate	SBP	IVW MR	0.015	0.005-0.024	0.003	-0.002 (0.717)
		Weighted median	0.016	0.006-0.027	0.002	
		MR-Egger	0.024	-0.029-0.076	0.376	
		MR-PRESSO	0.017	0.008-0.026	<0.001	
	DBP	IVW MR	0.014	-0.007-0.035	0.183	0.001 (0.790)
		Weighted median	0.010	-0.011-0.030	0.365	
		MR-Egger	0.005	-0.068-0.077	0.896	
		MR-PRESSO	0.007	-0.011-0.024	0.450	
	PP	IVW MR	0.005	-0.010-0.019	0.518	-0.009 (0.076)
		Weighted median	0.002	-0.014-0.018	0.773	
		MR-Egger	0.061	-0.002-0.125	0.061	
		MR-PRESSO	0.011	-0.002-0.023	0.099	
Gout	SBP	IVW MR	1.064	1.021-1.108	0.003	0.007 (0.783)
		Weighted median	1.063	1.060-1.123	0.031	
		MR-Egger	1.031	0.821-1.294	0.793	
		MR-PRESSO	1.061	1.020-1.104	0.005	
	DBP	IVW MR	1.017	0.927-1.116	0.721	-0.001 (0.963)
		Weighted median	1.018	0.910-1.138	0.759	
		MR-Egger	1.025	0.743-1.413	0.883	
		MR-PRESSO	1.001	0.943-1.063	0.966	
	PP	IVW MR	1.089	1.024-1.158	0.006	-0.020 (0.334)
		Weighted median	1.100	1.005-1.204	0.039	
		MR-Egger	1.242	0.946-1.630	0.123	
		MR-PRESSO	1.091	1.032-1.153	0.003	

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure.

Supplementary Figure 11). The MR-Egger intercepts did not provide evidence of horizontal pleiotropy, and neither did MR-PRESSO identify outliers. Instruments for the DBP did not indicate an effect on serum urate and gout (Table 5 and Supplementary Figure 12). Leave-one-variant-out analysis did not identify variants with exaggerated influence on the combined effect estimate (Supplementary Table 9).

Genetic association of liver function with gout and urate

The individual instrument-exposure [alkaline phosphatase (ALP) and alanine aminotransferase (ALT)] is shown in Supplementary Table 10. Using 85 associated SNPs as instrumental variables, IVW MR analysis evaluated that the level of ALT had a significant effect on increased serum urate (β = 0.974, 95% CI: 0.388–1.561, P = 0.001) in Europeans (Figures 1, 2 and Supplementary Figure 13). The result was similar with that in weighted median analysis (Table 6). The MR-Egger intercepts showed evidence of horizontal pleiotropy in serum urate (MR-Egger intercept = 0.009, P = 0.004). The MR estimate after removing six outliers identified by MR-PRESSO suggested a significance of the association between ALT and increased serum urate (β = 0.879, 95% CI: 0.355–1.404, P = 0.002) (Table 6). Leave-one-variant-out analysis did not identify variants with exaggerated influence on the combined effect estimate (Supplementary Table 11). To investigate the relationship between serum urate, gout, and fasting insulin, bidirectional MR analyses were conducted to indicate that genetically determined serum urate and gout were not associated with ALT (Supplementary Table 3). Instruments for the ALP did not indicate an effect on serum urate and gout (Figures 1, 2 and Supplementary Figure 14).

Discussion

We have used data from large GWASs to evaluate the causal relevance of metabolic disorders and gout or serum urate using

TABLE 6 Mendelian randomization estimates of liver function on serum urate or gout.

Outcome	Exposure	Method	Estimate	95% CI	P-value	MR-Egger intercept (P-value)
Serum urate	ALP	IVW MR	0.335	-0.350-1.020	0.338	0.002 (0.821)
		Weighted median	-0.088	-0.806-0.630	0.811	
		MR-Egger	0.147	-1.106-1.401	0.818	
		MR-PRESSO	0.304	-0.181-0.789	0.222	
	ALT	IVW MR	0.974	0.388-1.561	0.001	0.009 (0.004)
		Weighted median	0.627	0.042-1.211	0.036	
		MR-Egger	-0.757	-2.032-0.517	0.247	
		MR-PRESSO	0.879	0.355-1.404	0.002	
Gout	ALP	IVW MR	1.982	0.205-19.203	0.555	0.001 (0.727)
		Weighted median	2.917	0.081-105.098	0.558	
		MR-Egger	1.107	0.010-119.384	0.196	
		MR-PRESSO	1.985	0.249-15.807	0.835	
	ALT	IVW MR	2.743	0.400-18.799	0.304	0.001 (0.821)
		Weighted median	1.737	0.095-31.740	0.710	
		MR-Egger	1.751	0.023-132.749	0.800	
		MR-PRESSO	2.734	0.406-18.387	0.304	

ALP, alkaline phosphatase; ALT, alanine aminotransferase.

the MR method. Our analysis showed that genetically predicted fasting insulin, BMI, TG, blood pressure, and ALT were robust associated with gout or serum urate. Further research indicated that HDL and serum urate as well as gout have a bidirectional causal effect on each other.

A meta-analysis of 23 observational studies with 575,284 gout patients showed that the incidence of diabetes in gout population increased as age increased (28). A case–control study indicated that the relative risk for incident gout among diabetes patients, as compared with individuals with no diabetes, was 0.67 (29). Pan et al.'s research came to similar conclusions (30). Using different types of drugs to treat diabetes would affect the risk of gout. A cohort study showed that adults with T2DM prescribed a sodium-glucose cotransporter-2 (SGLT2) inhibitor had a lower rate of gout than those prescribed a glucagon-like peptide-1 (GLP1 agonist) (31). For T2DM, we found no evidence of a causal relationship with serum urate and gout. We speculate that T2DM may affect uric acid metabolism by affecting other metabolic indicators, rather than directly increasing serum urate *via* genetic variants. In addition, we also studied the other glycemic traits, including fasting glucose and fasting insulin. A study conducted in economically developing regions of northwest China found that participants with higher fasting blood glucose had higher levels of serum uric acid (9). Furthermore, urinary uric acid clearance appears to decrease in proportion to increases in insulin resistance in normal volunteers, leading to an increase in serum uric acid concentration (32). Our results confirm that the associations between fasting insulin and serum urate are causal, but not gout. Gout is caused by long-standing hyperuricemia, and although fasting insulin can genetically elevate in serum urate, the concentration or duration of the elevated serum urate is not sufficient to cause gout.

Previous epidemiological studies have suggested that general obesity measured by BMI may be a risk factor for gout and serum urate (33, 34). Zhou et al. reported that once the BMI was higher than 19.12 kg/m^2 for men or 21.3 kg/m^2 for women, each 1-kg/m^2 increase in BMI was related to a 5.10-fold increment for men and a 3.93-fold increment for women in serum urate levels (35). Moreover, a prospective study showed that women with general obesity were more likely to progress from hyperuricemia to gout (11). In the current study, we found a positive association with gout or serum urate for genetically predicted BMI in European-ancestry individuals but not for abdominal adiposity as measured by WHR adjusted for BMI. Our results are consistent with a previous Mendelian analysis which is a trans-ancestry study, while we only studied the European population, suggesting that BMI may be related to gout in different ancestries (36).

In terms of serum lipid, we observed decreased HDL as a causal risk factor of gout and increased serum urate. This is similar to the results reported in observational studies (11, 37). Studies have shown that patients with gout have elevated blood lipid levels, and Mendelian analysis found that high serum urate levels were associated with increased risk of hypercholesterolemia (11, 38). Compared with MR analysis published previously, MR-PRESSO was conducted in our research to evaluate pleiotropy and identify outlier variants based on their observed distance from the regression line and estimate results after correction of outliers.

It may exclude the pleiotropy of an instrumental variable to a greater extent. Our analysis showed that HDL and serum urate as well as gout have a bidirectional causal effect on each other good management of one is potentially beneficial to the other. However, in this article we did not discover the relationship of causality between LDL, TC, and serum urate as well as gout.

Also, the causal relationship between blood pressure, liver function, and gout or serum urate was investigated. A prospective cohort study including 6,424 hyperuricemia-free participants proved positive relationships between hypertension and hyperuricemia (39). A meta-analysis including 10 articles showed that hypertensive individuals were more likely to develop gout compared with normotensive individuals (33). In terms of blood pressure, epidemiological studies indicated that patients with hyperuricemia and gout had higher SBP, DBP, and PP (40, 41). In the current study, we found that higher SBP was causally associated with risk of gout or serum urate concentrations. Increased evidence indicates that hyperuricemia and gout are associated with increased prevalence and disease severity of non-alcoholic fatty liver disease (NAFLD), and NAFLD can predict hyperuricemia as well (42). Although there are few studies about the effect of liver dysfunction on gout and serum urate, we found that elevated circulating ALT was related with increased serum urate through MR analysis.

There were also some limitations in our study. Although this article mainly selected the European population as the object for study, the GWAS on lipid included a part of the non-European population. Moreover, we used MR-Egger and MR-PRESSO to control pleiotropy. However, the possibility that pleiotropy may have influenced the results cannot entirely be ruled out as in any Mendelian randomization study. MR analysis assumes a linear relation between each genetic instrument and the risk factor of interest, as well as a log-linear association between the risk factors and outcomes. The estimated effects may not be representative of the effects of the traits in the extremes of their distributions.

Conclusions

In conclusion, our findings from the genetic study provide support that metabolic factors including BMI, HDL, and SBP are causally associated with serum urate levels and gout risk. Among the above metabolic factors, HDL and serum urate as well as gout have a bidirectional causal effect. In addition, our study demonstrates that variations in fasting insulin, TG, and ALT only have a positive causal effect on serum urate concentrations.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YY and WX designed the study. YY, DW, and SH analyzed the data, made the figures, and drafted the manuscript. YL, and HX reviewed and supervised the manuscript. All authors reviewed the manuscript and approved the final version of manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.917056/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The relationship between type 2 diabetes mellitus (T2DM)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with T2DM and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with T2DM and their risk on gout.

SUPPLEMENTARY FIGURE 2
The relationship between fasting insulin-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach,

MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with fasting insulin and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with fasting insulin and their risk on gout.

SUPPLEMENTARY FIGURE 3

The relationship between fasting glucose-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with fasting glucose and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with fasting glucose and their risk on gout.

SUPPLEMENTARY FIGURE 4

The relationship between body mass index (BMI)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with BMI and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with BMI and their risk on gout.

SUPPLEMENTARY FIGURE 5

The relationship between waist-to-hip ratio adjusted for body mass index (WHRadjBM)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with WHRadjBM and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with WHRadjBM and their risk on gout.

SUPPLEMENTARY FIGURE 6

The relationship between high-density lipoprotein cholesterol (HDL)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with HDL and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with HDL and their risk on gout.

SUPPLEMENTARY FIGURE 7

The relationship between triglycerides (TG)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with TG and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with TG and their risk on gout.

SUPPLEMENTARY FIGURE 8

The relationship between low-density lipoprotein cholesterol (LDL)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with LDL and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with LDL and their risk on gout.

SUPPLEMENTARY FIGURE 9

The relationship between total cholesterol (TC)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with TC and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with TC and their risk on gout.

SUPPLEMENTARY FIGURE 10

The relationship between systolic blood pressure (SBP)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with SBP and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with SBP and their risk on gout.

SUPPLEMENTARY FIGURE 11

The relationship between pulse pressure (PP)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with PP and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with PP and their risk on gout.

SUPPLEMENTARY FIGURE 12

The relationship between diastolic blood pressure (DBP)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with DBP and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with DBP and their risk on gout.

SUPPLEMENTARY FIGURE 13

The relationship between alanine aminotransferase (ALT)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with ALT and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with ALT and their risk on gout.

SUPPLEMENTARY FIGURE 14

The relationship between alkaline phosphatase (ALP)-associated single nucleotide polymorphisms (SNPs) and risk of increased serum urate and gout. Three different methods [inverse variance weighted (IVW) approach, MR-Egger, and weighted median] were used. **(A)** The scattered plot of SNPs associated with ALP and their risk on increased serum urate. **(B)** The scattered plot of SNPs associated with ALP and their risk on gout.

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HDAC11, an emerging therapeutic target for metabolic disorders

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Histone deacetylase 11 (HDAC11) is the only member of the class IV HDAC, and the latest member identified. It is highly expressed in brain, heart, kidney and some other organs, and located in mitochondria, cytoplasm and nuclei, depending on the tissue and cell types. Although studies in HDAC11 total knockout mice suggest its dispensable features for tissue development and life, it participates in diverse pathophysiological processes, such as DNA replication, tumor growth, immune regulation, oxidant stress injury and neurological function of cocaine. Recent studies have shown that HDAC11 is also critically involved in the pathogenesis of some metabolic diseases, including obesity, diabetes and complications of diabetes. In this review, we summarize the recent progress on the role and mechanism of HDAC11 in the regulation of metabolic disorders, with the focus on its regulation on adipogenesis, lipid metabolism, metabolic inflammation, glucose tolerance, immune responses and energy consumption. We also discuss the property and selectivity of HDAC11 inhibitors and their applications in a variety of *in vitro* and *in vivo* models of metabolic disorders. Given that pharmacological and genetic inhibition of HDAC11 exerts a beneficial effect on various metabolic disorders, HDAC11 may be a potential therapeutic target to treat chronic metabolic diseases.

KEYWORDS

HDAC11, metabolic disorders, obesity, diabetic complications, diabetes

Abbreviations: HDAC11, Histone deacetylase 11; HDACs, Histone deacetylases; NAD+, nicotinamide adenine dinucleotide; SIRT, sirtuins; AMD, age-related macular degeneration; MS, multiple sclerosis; PPAR, peroxisome proliferator-activated receptor; β-ARs, β-adrenergic receptors; WAT, white adipose tissue; BAT, brown adipose tissue; UCP1, uncoupling protein 1; WT, wild type; KO, knockout; AMPK, AMP-activated protein kinase; HFD, high fat diet; DN, Diabetic nephropathy; PAI-1, Plasminogen agonist inhibitor type 1; AP-2α, activator protein 2α; KLF15, Kruppel-like factor 15; LPL, Lipoprotein lipase; TG, triglyceride; HUVECs, human umbilical vein endothelial cells; AS, atherosclerosis; APCs, antigen-presenting cells; TSA, trichostatin A; TpxA, trapoxin A; SAHA, Suberoylanilide hydroxamic acid; i.p., intraperitoneal; IC50, half maximal inhibitory concentration; PFS, progression-free survival; AZA, 5-azacytidine; PTCL, Peripheral T-cell lymphomas; PFS, progression-free survival.

Introduction

The removal of acetyl groups from e-lysine residues in proteins (1) connected to condensed chromatin structures that inhibit gene transcription (2) is catalyzed by a class of enzymes called histone deacetylases (HDACs). Mammals currently contain 18 HDACs that are classified into two families: the Zn2+- dependent or classical HDACs, and the nicotinamide adenine dinucleotide (NAD+)-dependent HDACs or sirtuins (SIRT). According to the homology of their catalytic domains, classical HDACs are further split into three classes: class I, class II, and class IV HDACs. Class I HDACs include HDAC1, HDAC2, HDAC3, and HDAC8, whereas class II HDACs include HDAC4, HDAC5, HDAC6, HDAC8, HDAC9, and HDAC10, and class IV HDACs include HDAC11 (1).

HDAC11, the solitary member of class IV HDAC, contains an open reading frame encoding a 347-residue protein and shares sequence homology with both class I and class II HDAC proteins in the catalytic core regions. HDAC11 is highly conserved, even in invertebrates and plants as the most recently identified (3–5) and combines with other HDACs to form functional complexes (6–8). Although HDAC11 structure has still not been discovered, it has been effectively modeled from HDAC8 structure (4, 9). HDAC11 can be degraded by the proteasome system and has an unstable half-life at around four hours (10). While most class I-III HDACs are involved in deacetylating their substrates (reviewed in (11)), HDAC11 has defattyacylase activity in addition to its deacetylase activity. In fact, as the only HDAC member that has a clear predilection for the removal of long-acyl instead of acetyl groups (12, 13), HDAC11 is the family's most effective fatty deacetylase (9). It has been reported that the efficiency of HDAC11 defattyacylase activity is greater than 10,000 times its deacetylase activity (13). The activation of HDAC11 can be triggered by physiologic levels of free fatty acids and their metabolites (9).

HDAC11 is expressed in multiple organs and distributed in diverse organelles. It is primarily expressed in heart, kidney, smooth muscle (3), skeletal muscle (14–16), brain (3, 15, 17–20), testis (14, 21) and gall bladder (22). At cellular level, HDAC11 is abundant in the mitochondria of skeletal muscle cells, brain synapses (15), and the centrosomes of neurons from the dentate gyrus (19). But it locates predominantly in the cytoplasm of embryonic astrocyte precursors and mature cells (23), and the nucleus of activated astrocytes and oligodendrocytes (24). HDAC11 can be found both in the cytoplasm and the nucleus of newly isolated and unstimulated Treg cells (24), immature astrocytes and oligodendrocytes (23), retinal ganglion cells (25) and preadipocytes (26). In addition, HDAC11 is highly expressed in the rat brain, and pancreatic β cells (27).

Emerging evidence has indicated that HDAC11 is critically involved in physiological and pathological processes. HDAC11 has a variety of physiological functions, including immunomodulation (24, 28–36), genomic stability (21, 37–39), cell cycle progression (21, 40, 41), and nervous system development (42). Pathologically, HDAC11 plays a role in epithelial barrier dysfunction (43–45) and ischemic injury (46–48) and requried for the growth of several tumors (49–56), such as hepatic carcinoma (57–61), and lung cancer (62, 63). Moreover, it contributes to the development of some other diseases (56, 64), including hepatitis B (65–67) and age-related macular degeneration (68).

In the past two decades, HDACs have been revealed to be implicated in the regulation of multiple metabolic processes and pathogenesis of some metabolic disorders. For example, most class I HDAC members are associated with insulin resistance, energy metabolism and glucose homeostasis, and contribute to the pathogenesis of diabetes and its associated complications (69, 70), and obesity (71). Class II HDACs are required for regulating the transcription of genes associated with glucose homeostasis and hepatic gluconeogenesis (72). Moreover, HDACs are involved in the regulation of several events related to the pathogenesis of diabetes (i.e. oxidative stress, inflammation and fibrosis) and its associated complications (70, 73). Very recently, HDAC11 has been linked to the pathogenesis of obesity (74), diabetes, and diabetic complications (64, 75). Given that global deletion of HDAC11 in mice does not affect their development and health (24), pharmacological inhibition of HDAC11 could be a potential therapeutic approach for the treatment of metabolic disorders. In this review article, we summarize the role and possible mechanisms of HDAC11 in metabolic disorders, including obesity, metabolic inflammation, and diabetes and its complications, and provide detailed information about HDAC11 inhibitors developed so far.

HDAC11 in obesity

Obesity is an excessive fat gain due to unbalanced energy intake and consumption (76), and its prevalence rises yearly in children and adults (77). HDAC11 is related to obesity in multiple ways.

HDAC11 participates in the regulation of adipogenesis. The differentiation of adipocytes is strictly controlled. Mature adipocytes are differentiated from mesenchymal precursor cells. Several essential adipose transcription factors, such as peroxisome proliferator-activated receptor γ (PPARγ), CCAAT-enhancer-binding protein β, and sterol regulatory element-binding proteins regulate this process (78–80). It has also been reported that various HDACs, in particular, HDAC11 are critically involved in adipogenic differentiation (81–83). Silencing the HDAC11 gene by small interfering RNA results in reduced perilipin, adipoq, and PPARγ2 expression, and decreased formation of intracellular lipid droplets (84). By the use of HDAC11-KO mice and adipocytes from WT and HDAC11 KO mice exposed to FT895, it was also found that HDAC11 binds to a nearby gravin-α region and demyristoylates

those spots. Gravin-α lysine myristoylation in brown and white adipocytes is necessary for the signal through β2- and β3-adrenergic receptors (β-ARs). Gravin-α lysine myristoylation induces the expression of protective thermogenic genes by directing β-ARs to lipid raft membrane microdomains and stimulating activation of PKA and its downstream signaling. These results establish reversible lysine myristoylation as a pattern of GPCR signaling regulation and emphasize the importance of HDAC11in regulating adipocyte phenotypes (85).

HDAC11 is essential for regulating the balance of brown adipose tissue (BAT) and white adipose tissue (WAT) (86). The WAT is the body's greatest energy storage tissue, and can secretes cytokines and adipokines as part of its endocrine function; BAT is imperative in maintaining body temperature in newborns' nonshivering thermogenesis (87–90). A role for HDAC11 in regulating adipose tissue and thermogenic capability has been suggested by the fact that HDAC11 is more expressed in WAT than BAT and that deletion of HDAC11 in mice enhances the development of BAT and "browning" of WAT (26). These are essential changes as WAT contributes to obesity by storing extra energy as fat in the body, while BAT is capable of turning fat into energy (90). Meanwhile, In HDAC11-knockout (KO) mice, the histological study of BAT reveals a compacted tissue size with noticeably smaller lipid droplets (75). Mouse hepatic cell line AML12 with HDAC11 knockdown exhibits enhanced metabolic activity and oxygen consumption due to improved lipid oxidation capability (75). which is consistent with previous observations in skeletal muscle tissue (14).

Mechanistically, uncoupling protein 1 (UCP1), a mitochondrial long-chain fatty acid/H+ symporter, and PGC1-α, a primary regulator of mitochondrial biogenesis, are both downregulated by HDAC11 to inhibit the BAT transcriptional program (26). HDAC11 deletion increases metabolic pool clearance, thermogenic capability, UCP1 expression in BAT, and energy expenditure. Through its physical interaction with BRD2(an enhancer regulating Ucp1 gene) (26), HDAC11 inhibits the thermogenic gene program. HDAC11 inhibition increases oxygen consumption and boosts adiponectin, a hormone that controls fatty acid oxidation, blood glucose levels, and stimulates lipid metabolism by activating the adiponectin-AdipoR-AMPK pathway (75).

Recent studies have also shown that HDAC11 is a critical regulator of the body's overall metabolism. HDAC11 KO mice exhibit higher body temperatures than wild type (WT) controls both at room temperature (22°C) and during a 24-hour cold challenge (4°C), which is correlated with higher metabolic rate and oxygen consumption (26, 75). Importantly, HDAC11-deficient mice show alleviated hypercholesterolemia, hepatic steatosis and liver damage (26, 75).

Altogether, these results suggests that HDAC11 is a new metabolic regulator, lowering its levels might improve cells' ability to adapt to an elevated energy requirement under stressful circumstances. Furthermore, as a result of the considerable rise in metabolic rate and oxygen consumption caused by HDAC11 inhibition, there is an increase in lipid oxidation and energy expenditure. Therefore, HDAC11 would be a prospective therapeutic target for obesity and the related metabolic effects.

HDAC11 in diabetes

Diabetes

Diabetes is a metabolic, chronic, multisystem disease and chronic exposure to hyperglycemia eventually leads to multiple complications, such as diabetic nephropathy, cardiovascular disease, retinopathy and neuropathy with considerable impact on the quality of life and overall life expectancy.

HDAC11 is essential for preserving insulin sensitivity and glucose homeostasis. In mice fed with high fat diet (HFD), HDAC11 deletion significantly decreases blood insulin levels, stabilizes blood glucose, and greatly reduces blood glucose levels after insulin challenge, thereby enhancing glucose tolerance and ameliorating diabetes (75). In addition, adiponectin significantly increases in HDAC11 KO mice (91). By uisng adiponectin-knockout mice fed on a HFD or either regular chow, it has been demonstrated that adipoR2-peroxisome proliferator-activated receptor α (PPARα) and adipoR1-AMP-activated protein kinase (AMPK) pathways play a major role in adiponectin signaling in the liver (91). The vital energy sensor AMPK has been linked to the control of the hepatic metabolic processes, such as gluconeogenesis. Increased energy expenditure, improved glucose tolerance, and lower plasma cholesterol levels all result from AdipoR2 KO. Lysophospholipids are one of adiponectin's targets, and they are upregulated by a high-fat diet (HFD) and tend to cause hypertriglyceridemia, decreased glucose tolerance, and insulin resistance (91).

Diabetic nephropathy

Diabetic nephropathy (DN) is a serious complication of diabetes. It presents as localized kidney inflammation and fibrosis that lead to structural remodeling (92–94).

Although there is no report about the role of HDAC11 in DN so far, HDAC11 is vital in the response to renal inflammation and fibrosis. Plasminogen agonist inhibitor type 1, a physiological inhibitor of fibrinolysis (PAI-1), is evelvated in DN (95). Excess PAI-1 lead to the accumulation of extracellular matrix proteins, whereas PAI-1 deficiency protected the kidney from injury-induced fibrosis (96). In a murine model of renal ischemia/reperfusion (I/R), increased testosterone can decrease the ability of HDAC11 to bind to PAI-1 promoter, leading to increased histone 3 acetylation and PAI-1 expression and accelerated I/R-induced renal injury (46, 47). Moreover, HDAC11 expression are increased in the kidneys in animal

models of renal fibrosis induced by unilateral ureteral obstruction and angiotensin II by suppressing Kruppel-like factor 15, an anti-fibrogenic factor (97). Since renal inflammation and fibrosis contribute to the pathogenesis of DN, it is speculated that HDAC11 would also play a role in the development of DN. Further studies are needed to address this issue.

Diabetic cardiopathy

Type 2 diabetes and cardiovascular diseases are predisposed to by obesity (98). Increased body weight can, in fact, cause metabolic changes in cardiomyocytes that switch them from processing fatty acids to sugar, which adds to lipid storage in the pericardium and, as a severe consequence of type 2 diabetes, causes myocardial infarction (99). Interestingly, inhibition of HDAC11 activity could prevent or ameliorate diabetic cardiomyopathy. In apoE mice fed with HFD, atherosclerosis and blood lipid levels have recently been shown to be alleviated by HDAC11-AS1. HDAC11-AS1 improves lipoprotein lipase (LPL), a crucial rate-limiting enzyme involved in triglyceride (TG) hydrolysis, *via* controlling adropin histone deacetylation both *in vitro* and *in vivo* (100). Another study shows that suppression of HDAC11 enhances the prevention of pyroptosis in human umbilical vein endothelial cells (HUVECs) triggered by TNF-α, indicating that vascular endothelial pyroptosis might be prevented through downregulation of HDAC11 related signaling pathways in atherosclerosis (AS) (101). In addition, a fructose injury-induced mouse model of diabetic heart failure that lacks HDAC11 had lower levels of apoptosis, dyslipidemia, inflammation, and oxidative stress (102). HDAC11 has also been suggested to be an essential regulator in heart failure (103). Therefore, HDAC11 contributes to the pathogenesis of diabetic Cardiopathy.

HDAC11 in metabolic inflammation

Metabolic disorders are closely associated with chronic mild inflammation (104–106). Most obese people develop inflammation in their adipose tissue, like chronically damaged tissue, along with immune cell remodeling and infiltration. During the early phases of adipose swelling and the progression of chronic obesity, inflammation is induced, and the immune system is irreversibly changed into a proinflammatory phenotype (107). Changes in adipose tissue function are related to obesity, and the loss of adipocytes also contributes to chronic mild inflammation (104). The regulating function of HDAC11 in metabolic inflammation is crucial.

HDAC11 regulates metabolic inflammation primarily through the control of the IL-10 released by antigen-presenting cells (APCs) (28). Inhibition of HDAC11 causes macrophages to express more IL-10, whereas overexpression of HDAC11 reduces IL-10 expression (108, 109). In addtion, HDAC11 overexpression in APCs is efficient in reactivating tolerant T cell responses and CD4+ T cells specific for antigens. And APC had the reverse result when HDAC11 expression was absent (33). Conversely, suppression of HDAC11 resulted in impaired antigen-specific expression, increased IL-10 expression, downregulated IL-12 expression and immune cell expression (such as myeloid-derived suppressor cells, neutrophils, and T cells), leading to immune tolerance (110, 111). In addition, muted HDAC11 transcripts boosted the synethesis of IL-17 and TNF in the supernatants of HL cells (112). Moreover, liver immune tolerance is regulated by HDAC11 through TNF-α, interferon-γ, IL-2, and IL-4 (80, 90, 113–120).

HDAC11 inhibitors

Most HDACi are pan-HDACi that target multiple HDACs with different nanomolar potency. Zinc-dependent catalytic processes are shared by Classes I, II, and IV HDACs. Many pan-HDACi have been synthesized, including Aes-135 (121), AR-42, belinostat (PXD101,PX105684), fimepinostat (CUDC-907) (9), FT895 (122), M344(D237, MS344), Panobinostat (LBH589, NVP-LBH589), pracinostat (SB939), dacinostat, quisinostat (JNJ-26481585), trichostatin A (TSA), vorinostat (34) (SAHA, MK0683), mocetinostat (123)(MGCD0103), tucidinostat (Chimade, HBI-8000, CS055), trapoxin A (124) (TpxA, C34H42N4O6), garcinol (125), romidepsin (126). Recently, Compound 8, a newly designed novel HDAC6 selective inhibitor with 2-mercaptoquinazolinone as the Cap Moiety, has displayed stronger inhibition activity against HDAC11 than Belinostat (127). The toxicity caused by general inhibition of HDACs restricts their potential utility. Among the pan-HDAC inhibitors, garcinol shows more HDAC11 selectivity and efficiency than other HDACs (125). The deacetylase and demyristoylase activities of HDAC11 are also suggested to be effectively inhibited by Fimepinostat (9). At concentrations of 0.02, 0.2, and 2μM, respectively, Suberoylanilide hydroxamic acid (SAHA) could suppress 10, 50, and 90% of HDAC11 activity (34). Additionally, it has been noted that trichostatin A (TSA) and romidepsin have a nanomolar potency toward HDAC11 (126). However, pracinostat, dacinostat, mocetinostat, quisinostat, trapoxin A, and trichostatin A have been found not as efficient in inhibiting HDAC11 deacetylation activity as reported before (9). Unexpectedly, butyrate, valproate, SAHA, and TSA could trigger myeloid cells to express HDAC11 (128). And low doses of MS275 have been found to show agonistic actions (129).

Elevenostat (JB3-22) (21, 24), SIS7, SIS17 (130), and FT895 (122) are selective HDAC11 inhibitors. Nevertheless, the

inhibitory capacity of elevenostat (JB3-22) on myristoylated and acetylated peptidic derivatives is extremely poor (9). To date, the HDAC11 inhibitors that are considered to be the most potent and selective are SIS17 and FT895. SIS17 is better than FT895 and SIS7 in terms of its cell permeability and metabolic stability (60), while FT895, SIS17, SIS7 can all inhibit HDAC11's demyristoylase activity (130).

Though several HDAC11 inhibitors have been developed, only FT895 (85, 122), romidepsin (131), and quisinostat (97, 131, 132) have been reported to be utilized in animal studies. To explore the pharmacokinetic properties of FT895, it was injected to male Balb/c nude mice *via* i.v. at 1mg/kg) or i.p. at 5 mg/kg. After i.v. dosing, with a t1/2 of 9.4 hours, FT895 exhibits a high volume of distribution and a moderate clearance (42 mL/min/kg). In comparison, FT895 dosed intravenously has enhanced exposure, a similar half-life (10.2 h), a bioavailability of 81%, and sustains free drug levels above the cellular half-maximal inhibitory concentration (IC50) for up to 4 hours (122). Quisinostat (10 mg/kg Monday, Wednesday, and Friday) and romidepsin (0.3 mg/kg, 1 mg/kg, or 3 mg/kg Monday, Friday) were administered intraperitoneally (i.p.) for one week to tumor-bearing athymic NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice. Romidepsin has unacceptable toxicity at 3 mg/kg; anemia and aspartate aminotransferase elevations are a result of 1 mg/kg dosing; Without causing considerable weight loss (>20%) or neurotoxicity, both 0.3 mg/kg and 1 mg/kg are tolerated. Treatment with quisinostat (10 mg/kg Monday, Wednesday, Friday) shows no systemic toxicity (131). Similarly, in BALB/c nude mice and NOD/SCID mice, quisinostat (3 and 10 mg/kg/day, i.p) has been used to treat tongue and esophageal squamous cell carcinoma (132, 133) and malaria (134). Romidepsin is also used in C57BL/6 (0.03mg/kg twice a week) (135, 136) and BALB/C(1mg/kg/2days) (137) mice for cancer treatment. Therefore, FT895, quisinostat and romidepsin are tolerable and safe *in vivo*.

In addition, quisinostat and romidepsin have been tested in clinical trials. The maximum tolerable dose of quisinostat for the treatment of cancer in patients is 10 mg administered orally three times per week along with bortezomib and dexamethasone, with median progression-free survival (PFS) 8.2 months and median duration of response 9.4 months (138). Combined with 5-azacytidine (AZA), romidepsin (14 mg/m^2, day 8,15,22, per 35 days, IV) is used to treat peripheral T-cell lymphomas (PTCL) with the overall survival not met (at a median follow-up of 13.5 months), and the median progression-free survival (PFS) 8.0 months, duration of response 20.3 months (139). Romidepsin has also been reported to treat HIV-1-infected patients with a 5 mg/m^2 dosage as a 4 hour infusion (140).

Thus, taking effectiveness, selectivity, toxicity, half-life, tolerance and survivability *in vivo* into consideration, FT895 exhibits pharmacokinetic properties that are reasonable *in vivo* research, and the most significant potential to advance into clinical trials.

Conclusion and perspectives

The incidence of metabolic disorders is increasing worldwide, ranging from obesity to type 2 diabetes, leading to complications in the heart, kidney, retina, bone, skin and foot.

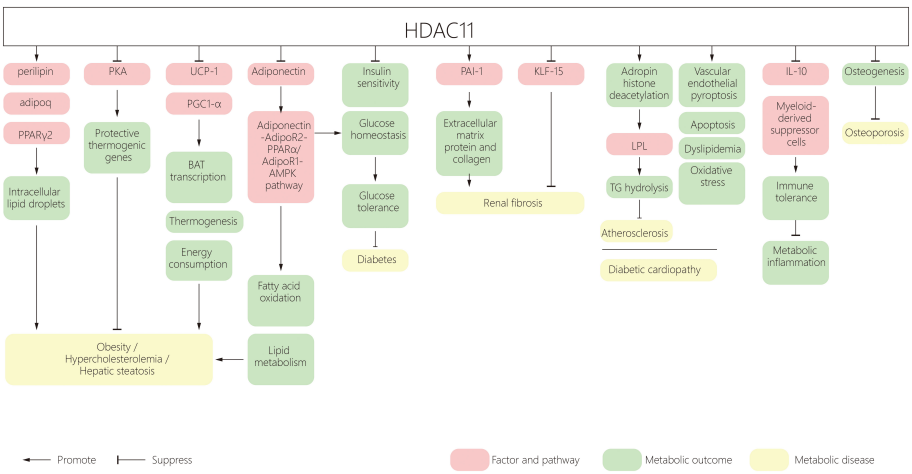

FIGURE 1
A schematic diagram of HDAC11 and its effect on metabolic disorders. HDAC11, Histone deacetylase 11; PPAR, peroxisome proliferator-activated receptor; BAT, brown adipose tissue; UCP1, uncoupling protein 1; AMPK, AMP-activated protein kinase; PAI-1, Plasminogen agonist inhibitor type 1; AP-2α, activator protein 2α; KLF15, Kruppel-like factor 15; LPL, Lipoprotein lipase; TG, triglyceride.

TABLE 1 The role of HDAC11 in metabolic disorders.

Related metabolic diseases	Animal model	Approach (HDAC11 inhibitor/ KO)	Main findings	Reference
Obesity	/	KO	HDAC11 deficiency improves muscle function, fatigue resistance and muscle strength with enhanced mitochondrial content and oxidative myofibers by lowering acylcarnitine levels, activating the AMP-activated protein kinase-acetyl-CoA carboxylase pathway and stimulating a glycolytic-to-oxidative muscle fiber switch.	(14)
	Cold challenge HFD	KO	HDAC11 deficiency increases BAT abundance and function, metabolism, and glucose tolerance resultant from acute high fat feeding.	(26)
	HFD	KO	HDAC11 deficiency enhances glucose tolerance and insulin sensitivity, attenuates liver damage, hepatosteatosis and hypercholesterolemia by boosting energy expenditure through promoting thermogenic capacity.	(75)
	/	KO	HDAC11 demyristoylates gravin-α in adipocytes, leading to protective thermogenic gene expression.	(85)
Diabetic nephropathy	Renal fibrosis	Inhibitor (quisinostat)	Inhibition of HDAC11 attenuates renal fibrosis, blocks the pro-fibrogenic response induced by Ang II through interaction with activator protein 2 to activate KLF15 transcription.	(97)
Diabetic/ obesity related cardiopathy	Atherosclerosis	Inhibitor (HDAC11) antisense	HDAC11-AS1 reduces blood lipid levels and atherosclerosis of apoE-/- mice fed with HFD by enhancing LPL and TG metabolism	(100)
	Cardiac dyslipidemia	KO	HDAC11 depletion elevates blood pressure, reduces the inguinal fat-pad mass and body weight, with improved cardiac function, dyslipidemia, enhanced SOD activity.	(102)
Metabolic inflammation	Rat orthotopic liver transplantation	Inhibitor (HADC11-shRNA)	HDAC11 inhibition promotes the expression of IL-4 and IL-10, reduces IFN-γ, TNF-α, and IL-2 levels, and induces tolerance.	(113)

HDAC11, histone deacetylase 11; WAT, white adipose tissue; BAT, brown adipose tissue; UCP1, uncoupling protein 1; KO, knockout; HFD, high fat diet; KLF15, Kruppel-like factor 15.

HDAC11 participates in many aspects of metabolic diseases. HDAC11 mediates obesity and metabolic syndrome by regulating adipogenesis, increasing energy consumption and promoting lipid metabolism. It also contributes to adipose tissue inflammation by regulating immune responses and insulin resistance. HDAC11 was shown to have inhibitory roles in the development of diabetic cardiovascular disease. (Figure 1) In addition, a recent study shows that HDAC11 contributes to osteoporosis susceptibility and reduced peak bone mass through a mechanism of 11β-HSD2's low-functional programming. This is triggered by corticosterone through GR/HDAC11 signaling, which amplifies the effect of corticosterone on inhibiting the function of BMSCs in osteogenesis (141, 142). However, studies on diabetic osteoporosis, lipoid nephrosis, fatty liver disease and obesity cardiomyopathy are still lacking. As such, further research on the effect of HDAC11 on metabolic diseases is required.

Studies listed in Table 1 have shown the well-tolerated HDAC11 global deletion in mice, suggesting that its inhibition or depletion is without apparent side effects. Currently, toxicity and safe doses of HDAC11 inhibitors are far from clear and none of the inhibitors have been used in patients with metabolic disorders. Thus, more studies on the safe dosage and toxicity of HDAC11 inhibitors in animal models are needed before advancing them to human clinical trials. In addition, development of more effective HDAC11 inhibitors with enhanced selectivity is worth investigating.

Author contributions

HC drafted the article. The manuscript was edited by SZ, CX, and QC. After reviewing the manuscript, all authors gave their approval for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Genetics, epigenetics and transgenerational transmission of obesity in children

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Sedentary lifestyle and consumption of high-calorie foods have caused a relentless increase of overweight and obesity prevalence at all ages. Its presently epidemic proportion is disquieting due to the tight relationship of obesity with metabolic syndrome and several other comorbidities which do call for urgent workarounds. The usual ineffectiveness of present therapies and failure of prevention campaigns triggered overtime a number of research studies which have unveiled some relevant aspects of obesity genetic and epigenetic inheritable profiles. These findings are revealing extremely precious mainly to serve as a likely extra arrow to allow the clinician's bow to achieve still hitherto unmet preventive goals. Evidence now exists that maternal obesity/overnutrition during pregnancy and lactation convincingly appears associated with several disorders in the offspring independently of the transmission of a purely genetic predisposition. Even the pre-conception direct exposure of either father or mother gametes to environmental factors can reprogram the epigenetic architecture of cells. Such phenomena lie behind the transfer of the obesity susceptibility to future generations through a mechanism of epigenetic inheritance. Moreover, a growing number of studies suggests that several environmental factors such as maternal malnutrition, hypoxia, and exposure to excess hormones and endocrine disruptors during pregnancy and the early postnatal period may play critical roles in programming childhood adipose tissue and obesity. A deeper understanding of how inherited genetics and epigenetics may generate an obesogenic environment at pediatric age might strengthen our knowledge about pathogenetic mechanisms and improve the clinical management of patients. Therefore, in this narrative review, we attempt to provide a general overview of the contribution of heritable genetic and epigenetic patterns to the obesity susceptibility in children, placing a particular emphasis on the mother-child dyad.

KEYWORDS

obesity, pregnancy, gestation, transgenerational transmission, genetics, epigenetics, polymorphisms, methylation

Introduction

The steadily increasing prevalence of obesity in the general population it has now become a significant burden for human health (1). Worldwide estimates revealed more than 7% of children and adolescents had obesity in 2016 compared with less than 1% in 1975 (2). According to global health estimates provided by World Health Organization (WHO) 38.2 million of children under the age of 5 years were overweight or obese in 2019. Data also indicates that the prevalence of overweight and obesity in children and adolescents (from 5 to 19 years) has increased from approximately 4% in 1975 to more than 18% in 2016 noting that no significant differences emerge between girls and boys (3).

Obesity is a multifaceted disease widely regarded by now as the main risk condition for developing the metabolic syndrome in both children and adults (4, 5). It is precisely for this aspect that obesity is closely associated with a series of pathological manifestations such as insulin resistance, Type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD) and sleep-related breathing disorder such as obstructive sleep apnea (OSA) (6, 7). In particular, over the past two decades, the mechanistic exploration of the nexus between obesity levels and metabolic diseases has highlighted a close relationship between nutrient excess, genetics, epigenetics, oxidative stress, and inflammatory signals (8, 9). It is widely accepted as a fact that the epidemic proportion reached by the obesity in pediatric population is mostly a consequence of the sedentary lifestyle and consumption of high-calorie junk foods, clearly indicating the crucial role of the current obesogenic environment in favoring obesity's and comorbidities' epidemics (10). However, the well-documented individual variation in body weight, central adiposity, and particularly the differences in the onset and severity of the obesity-associated metabolic diseases, has forced the researchers to search other driving causes, such as the genetic and epigenetic inheritable profiles, even if these mechanisms have not yet been fully explored (11). It is known that direct exposures of ancestral germline (sperm and eggs) to environmental factors and altered nutrition can reprogram the epigenome of the cells, thus transmitting the susceptibility for the obesity to future generations through a mechanism of epigenetic transgenerational inheritance (12). Moreover, maternal obesity is currently being studied to clarify the links between maternal overnutrition during gestation and breastfeeding period, and the occurrence of hepatic/metabolic/cardiovascular disorders in the resultant offspring independently of genetic predisposition. According to an increasing number of studies, environmental factors such as maternal malnutrition, hypoxia and exposure to excess hormones and endocrine disruptors, during gestation and early postnatal period, play a decisive role in the programming of adipose tissue and obesity in the childhood (13).

In this review, we provide an overview of contribution of heritable genetic and epigenetic causes in development of obesity in childhood and focus on the role of epigenetic transgenerational inheritance on the obesity susceptibility.

Methods

Here, we have summarized the existing literature that could be relevant to the purpose of our narrative review. Since we focused on the impact of heritable genetics and epigenetics factors on pediatric obesity we structured the article by two main subjects, including the role of gene mutations and epigenetic modifications. For these topics, literature search was performed in the PubMed and Scopus Databases with the following search terms: "polygenic obesity" OR "monogenic obesity", AND "children"; "epigenetic", AND "obesity", AND "children". We also reported the effects of parental and *in utero* epigenetic mechanisms on the obese phenotype and its comorbidities by extrapolating from the main search on transgenerational transmission of obesity. We searched for clinical studies, as well as reviews, published in the English language, until June of 2022. Eligible papers for our narrative review included studies that reported convincing data and theories supporting the hypothesis of genetics and epigenetics inheritance of obesity.

Genetics of pediatric obesity

Obesity is a multifactorial disease where environmental factors and several genes play important pathogenetic roles (14). Indeed, excessive fat accumulation due to an excess intake of sugar-sweetened beverages, high-calorie foods, sedentary lifestyle and poor sleeping is a well-known leading cause to obesity (15). In addition, genetics can predispose people to obesity by affecting appetite regulation, body mass index (BMI), metabolism, body-fat distribution and by influencing food preferences and response to exercise (16). Regarding the diagnostic accuracy of BMI to diagnose obesity in children, it has been questioned as it may underestimate the prevalence of adolescents with overweight and obesity (17). Indeed, although obese children and adolescents are around five times more likely to be obese in adulthood than those who were not obese, a large proportion of obese adults (about 70%) were not classed as being obese in childhood or adolescence (17). This effect may be interpreted as due to the effects related to environmental factors linked to developmental origins of health and disease (DOHaD) (18).

Several studies were focused on the identification of genes associated with obesity (19). Indeed, genetic causes of obesity

can be classified in different ways including monogenetic, polygenetic, or syndromic obesity (11).

Monogenic obesity

Monogenetic obesity is rare and involves single-gene mutations or chromosomal deletions and the subjects usually exhibited a severe early-onset obesity associated with hyperphagia and endocrine disorders (20). Most of the gene mutations linked to monogenic obesity have been discovered by experimental studies on transgenic mice (21). Gene mutation are principally located in regions encoding for proteins belonging to the leptin-melanocortin pathway, which is responsible for maintaining energy balance through food consumption and energy expenditure (22) and follows a Mendelian pattern (23). The genetic determinants of monogenic obesity in children have been reported in Table 1. In particular, leptin was identified as the product of the obese (ob) gene following the characterization of monogenic obesity (ob/ob) mice model (24). Leptin is an anorexigenic hormone synthesized and secreted by white adipocytes that regulates the food intake and energy balance (25). Concentration of circulating leptin decreases during fasting (26), but increases during refeeding or overfeeding (27). Human congenital leptin deficiency was identified, for the first time, in two obese first-degree cousins from a Pakistani family, where a homozygous frame-shift mutation (c.398delG), leading to a non-secreted truncated leptin protein was genetically characterized (28). However, studies demonstrated that severe obesity develops from rare genetic mutations that affect genes of both leptin and its receptor (29), and lead to congenital leptin deficiency or leptin resistance (30). Other loci which have been implicated in severe childhood monogenic obesity include genes of the proopiomelanocortin (POMC), the melanocortin receptor 4 (MC4R), the protein convertase 1/3 (PC 1/3), the single-minded homolog 1 (SIM1), and the brain-derived neurotrophic factor (BDNF) (31). Obesity caused by mutations in POMC gene shows autosomal recessive inheritance, whereas those in MC4R are autosomal dominant (32). POMC deficiency results in the absence of its cleavage products, and inactivation of MC4R, thus causing hyperphagia, severe obesity, and red hair (33). Mutations in MC4R represent the most common cause of monogenic childhood obesity (34), and more than 150 different nonsynonymous variants of this gene have been described (35). Indeed, rs17782313 and rs12970134 in MC4R gene polymorphisms were associated with obesity in both European and Asian adults and children (36, 37). Also, the mutation in PC 1/3 gene has been correlated with severe early-onset obesity, endocrine disorders, intestinal dysfunctions, and impairment of glucose homeostasis (38, 39). SIM1 gene encode for proteins that play a key role in neuronal development and function of the paraventricular nucleus of hypothalamus which is a crucial region for food intake and control of energy homeostasis (40). Inactivating mutations in this gene have been associated with monogenic obesity in humans and early-onset obesity, although its contribution has only been investigated in a few populations (41, 42). Moreover, few point mutations in SIM1 have been recognized as a cause of monogenic obesity (43, 44). A novel SIM1 variant, p.D134N, potentially linked to monogenic pediatric obesity has been recently identified by Stanikova et al, in a cohort of children and adolescents (45). In addition, dominant forms of non-syndromic monogenic obesity in humans have been associated with mutations in BDNF gene, encoding for the protein involved in proliferation and survival of hypothalamic neurons (46). BDNF is essential for regulating energy balance and food intake and it has been found crucial in

TABLE 1 Genetic determinants of monogenic obesity in children.

GENE NAME	GENE'S PRODUCT FUNCTION	MUTATION	PHENOTYPE	REFERENCE
Leptin (LEP)	Food intake and metabolic homeostasis	Homozygous frame-shift mutation; non-secreted truncated leptin protein	Obesity in two Pakistani origin children	[Montague CT, 1997]
Melanocortin receptor 4 (MC4R)	Energy homeostasis, food intake and body weight regulator	Heterozygous mutations; Variants: rs17782313, rs12970134	Severe obesity on Dutch children Overweight and obesity in Indian children	[van den Berg L, 2011] [Dwivedi OP, 2013]
Protein convertase 1/3 (PC 1/3)	Proteolytic activation of polypeptide hormones and neuropeptides precursors	Missense mutation	Early-onset of severe obesity in an African 6-yr-old boy	[Ranadive SA 2008]
Single-minded homolog 1 (SIM1)	Neuronal development and function, food intake and energy homeostasis control	Deletion/inactivating mutation	Early-onset obesity in an American 21-month-old boy	[Gonsalves R, 2020]
Brain-derived neurotrophic factor (BDNF)	Proliferation and survival of hypothalamic neurons; energy balance and food intake control	Chromosomal inversion loss-of-function Single-nucleotide *de novo* genetic variants (p.Ile231Val and p.Cys141Gly) p.Thr2Ile and p.Arg209Gln and missense variant	Severe obesity, impaired cognitive function in a 8-yr-old girl. Early-onset of severe obesity in two cases from a Spanish population Severe early-onset obesity in 35-year-old and 46-year-old female Brazilian patient	[Gray J, 2006] [Serra-Juhé C, 2020] [da Fonseca ACP, 2021]

leptin down-stream signaling (47). The disruption of one copy of the BDNF gene as consequence of chromosomal rearrangement was described in a child affected by severe obesity and several cognitive alterations (48). While a full functional loss of one BDNF allele seems to cause severe obesity in humans, there is no consistent evidence about the pathogenic variants of this gene. Recent data showed the link of two rare single-nucleotide genetic variants (p.Ile231Val and p.Cys141Gly), to non-syndromic severe early-onset obesity (49). Very recently, BDNF p.Thr2Ile and p.Arg209Gln missense variants were found associated with severe obesity developed during childhood in a 35-year-old and a 46-year-old female patient respectively, thus suggesting a potential obesogenic role of this gene variant (50).

Polygenic obesity

It is widely accepted that obesity epidemic cannot be traced back to a single gene disorder, but rather to the interaction of a complex genetic background in combination with environment, where single nucleotide polymorphisms (SNPs) may contribute to development of the obese phenotype if they interact with epigenetic inputs. Indeed, polygenic obesity is the most common form of the childhood disease, which results from hundreds of SNPs and observes a pattern of heritability similar to that of other complex traits and diseases (51). Evidence shows that the expression of mutations causing monogenic obesity may, at least in part, be influenced by the individual's polygenic susceptibility to obesity (52). A genetic study from 573 individuals with severe early onset obesity found 40 very rare predicted functional variants in 13 genes encoding Sema3s (Semaphorin 3 Ligands) and their receptors in severely obese cases compared to controls. Since Sema3 signaling is crucial for gonadotropin-releasing hormone development and function, the study highlighted that the critical perturbations in the development of hypothalamic melanocortin neurons are also crucial for body weight and/or fat mass control (53).

Genome-wide association studies (GWASs) are particularly effective for the identification and analysis of novel genetic variants associated with weight gain (54). The genetic determinants of polygenic obesity in children have been reported in Table 2.

Among the obesity-related genetic variants, those located in the fat mass and obesity associated (FTO) gene locus, were the first ones that showed a strong association with BMI. The FTO gene, expressed in the hypothalamus, is located on chromosome 16 and encodes a demethylation enzyme implicated in the control of energy homeostasis, food intake and energy expenditure (55). In particular, two studies demonstrated that the variants rs9939609 and rs9930506 in the first intron of the FTO gene are significantly associated with BMI, thus suggesting that the presence of these variants may increase the risk of obesity in both adults and children (56, 57). Accordingly, the rs9939609 variant was found to be associated with increased fat mass and BMI in Scottish children (58) and to obesity phenotype in Dutch children (59). A further study reported that the rs9939609 allele may predispose to pediatric obesity risk by acting on satiety responsiveness (60). An interesting study by

TABLE 2 Genetic determinants of polygenic obesity in children.

GENE NAME	GENE'S PRODUCT FUNCTION	MUTATION	PHENOTYPE	REFERENCE
Fat-mass and obesity-associated gene (FTO)	RNA demethylase that mediates oxidative RNA demethylation that acts as a regulator of fat mass and energy homeostasis	Variants: - rs9939609 (intronic variant) - rs9930506 (intronic variant) - rs1421085 (intronic variant) - rs8050136 (intronic variant)	Overweight and severe obese phenotype in different pediatric populations	[Frayling TM, 2007; Wardle J, 2008; Cecil JE, 2008; Rutters F, 2011; Jacobsson JA, 2008; Mangge H, 2011; XI et al., 2010; Todendi PF, 2020; Reuter ÉM, 2021] Scuteri A, 2007 [Grant SF, 2008; Mejía-Benítez A, 2013, Albuquerque D 2013; Inandiklioğlu N and Yaşar A, 2021]
Melanocortin receptor 4 (MC4R)	Energy homeostasis, food intake and body weight regulator	rs17782313	Obesity traits and metabolic phenotypes in Portuguese school children	[Almeida SM, 2018]
Tumor necrosis factor (TNF)-α	Pleiotropic cytokine, important mediator of inflammation	rs1800629	Males Portuguese obese adolescents Normal weight Romanian children	[Nascimento H, 2016; Mărginean CO, 2019].
Interleukin (IL)-6	Pleiotropic cytokine important in regulating immunological and inflammatory responses	rs1800795	Obesity in Egyptian children	[Ibrahim OM, 2017]
Long noncoding (LncOb) RNA	Regulator of white adipogenesis	rs10487505	BMI and leptin levels in Italian children	[Manco M, 2022]

Jacobsson et al. highlighted the rs9939609 variant was associated with obesity and gender in Swedish children (61). Moreover, in European adolescents with obesity the homozygosity for the rs9939609 FTO SNP was critically associated with trunk-weighted (62). Besides in the Caucasian population, the presence of the common risk allele of rs9939609 polymorphism has been significantly associated with BMI and obesity in large population-based survey performed in Chinese children, where the polymorphism was associated with weight, BMI, waist circumference, waist-to-height ratio and fat mass percentage (63). A meta-analysis collected and analyzed all these studies suggesting that the rs9939609 has to be considered as the major FTO gene variant associated with overweight/obesity across multiple pediatric populations (64). However, studies on the impact of rs9939609 variant were performed also in Brazilian children and adolescents (65, 66). Todendi et al. reported a correlation between AA genotype for the rs9939609 variant and alteration of waist circumference and overweight/obesity in both children and their parents, thus corroborating the parental contribution to obesity onset (65). A more recent longitudinal study by Reuter et al. have confirmed the association of rs9939609 variant and abdominal fat in Brazilian school children between 7 and 15 years old (66).

Of note, a line of evidence showed no association between FTO rs9939609 and BMI, thus highlighting that the ethnicity may be pivotal to determine the role of this variant on obesity risk (67–69). Other two FTO gene variants, the rs8050136 and the rs1421085, were found associated to obesity traits in different children population studies alone or in combination to the best investigated rs9939609 variant (67, 70–72). Although, the true role of these additional loci remain unknown, deserving further investigations in the next future, a very recent meta-analysis reported this and other variants in FTO genes and their association with pediatric obesity overall (73). Often FTO variants have been investigated with variants in the MC4R of which mutations can be positioned between monogenic obesity and the polygenic obesity (36, 73, 74).

Since there are several published studies reporting the critical pathogenic role of pro-inflammatory cytokines in obesity, further genetic association studies were focused on the influence of variants in genes encoding for these molecules in obesity (75). In obesity, increased production and secretion of a wide range of inflammatory molecules, including tumor necrosis factor (TNF)-α and interleukin-6 (IL-6) was reported in white adipose tissue (76). TNF-α was found to be involved in lipid metabolism leading to hypertriglyceridemia as a result of decreasing lipoprotein lipase activity and increasing hepatic *de novo* synthesis of fatty acids, while IL-6 is mainly associated with liver and adipose tissue inflammation (77). The rs1800629 variant in the TNF-α gene, associated with increased expression of the cytokine in adipose tissue, was found to be more frequent in obese children than in lean subjects (78, 79). However, to date the role of TNF-α rs1800629 on childhood obesity is not clear. A study on Portuguese obese adolescents revealed that only in males subjects, the circulating TNF-α levels were modulated by TNF-α rs1800629 (80). Instead, a study on Romanian children (aged 5–18 years), revealed that TNF-α rs1800629 variant was more frequent in normal weight than in overweight children (81). Few studies demonstrated that also a variant in IL-6 gene may correlate with the obese phenotype (82). In particular, the IL6 polymorphisms designated as rs1800795 as well, were associated with the risk of developing obesity in Egyptian children (83).

The mentioned SNPs only explain 6% of BMI elevation, therefore Khera et al., by incorporating the analysis from 2.1 million SNPs, proposed a polygenic risk score (PRS) that increased the prediction of BMI alteration up to 23%. Interestingly, this PRS was associated with adult BMI, but it showed a consistency in children too (84).

Recently, a GWAS identified nineteen significant genome regions associated with body fatness (85). From this study emerged, for the first time, the association of BDNF, Fragile Histidine Triad Diadenosine Triphosphatase (FHIT), and protein kinase D1 (PRKD1) gene regions with BMI in children.

Growing evidence showed that genetic variation (typically SNPs) can also affect epigenetic profiles independently of or in contribution with environmental factors. In particular, have been identified several variants in methylation quantitative trait loci (meQTLs) that are differentially distributed across the genome and are involved in obesity and metabolic traits (86). A meta-analysis showed that the rs17782313 variant may regulate the expression of MC4R gene by affecting its methylation levels, thus contributing to childhood obesity (87). Further studies by using the analysis of meQTLs with GWAS may help in understanding how genetic predisposition and environmental factors interact with each other to determine methylation status that predispose to develop an obese phenotype in children. Moreover, future research should attempt to verify whether methylome signals of prenatal exposure can be regulated by genetic variation/specific single polymorphisms in metabolic genes correlated also with children and adolescents' obesity thus confirming the possibility of both a passive and active role of DNA methylation to regulatory interactions influencing gene expression (86, 88, 89).

Finally, very recently a group of studies have reported the relevance of rs10487505 variant of the region encoding for the long non-coding RNA lncOb, which was correlated with leptin levels and BMI in adults and children (90, 91).

Pediatric obesity epigenetics

Epigenetic changes in the human genome are defined as heritable regulatory mechanisms of gene expression overlaying the information enclosed in the DNA sequence. Epigenetic changes (also referred to as epigenetic marks) include mainly

DNA methylation, post-translational modifications of histones, and ncRNAs (92, 93). DNA methylation, which is the most studied epigenetic mark, is a process occurring on the fifth carbon of cytosines mostly located in regions with symmetrical CpG dinucleotides, where the addition of a methyl group in the cytosine may cause transcriptional repression. This process is sustained by the activity of three types of DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B (92). The term of post-translational modifications of histones comprises of different chemical changes, such as histone acetylation, histone methylation, histone phosphorylation, and histone ubiquitination. These modifications may be regulated by different enzymes, such as histone acetyltransferases, histone deacetylases, histone methyltransferases and demethylases, and may be associated with both transcriptional activation and repression (93). Commonly, ncRNAs are a group of RNAs that are classified according to their length in long ncRNAs (lncRNAs), and small ncRNAs including miRNAs, small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), PIWI-interacting RNAs (piRNAs), and others, which may regulate gene expression at transcriptional, post-transcriptional, translational, and post-translational level in a variety of ways (94, 95).

There are several environmental and dietary factors (i.e. overnutrition) that may influence epigenetics, thus acting as regulators of the environment-gene interaction over the life course (96). A recent updated systematic review emphasized the association between epigenetic mechanisms and obesity in children (97). This systematic review describes studies reporting strong evidence of an epigenetic signature assessed by Epigenome-Wide Association Study (EWAS) in childhood obesity. Indeed, a recent EWAS by Robinson et al, based on the UK Avon Longitudinal study of Parents and Children (ALSPAC) cohort was able to identify for the first time the association between a differential DNA methylation profiles of blood samples in children with early life rapid weight gain. The candidate gene analysis on ALSPAC and in the replication cohort found a positive association between rapid growth gain and DNA methylation at the locus near the checkpoint with forkhead and ring finger domains (CHFR) (cg11531579) gene encoding for E3 ubiquitin- protein ligase, thus increasing the risk of subsequent pediatric overweight/obesity (98).

Xu et al. showed a substantial number of differentially methylated CpG sites between obese and non-obese adolescents (14–20 years old) and associated to gene previously linked to obesity and T2D, such as FTO, glucokinase (GCK), hepatocyte nuclear factor-1 α and β (HNF1A and HNF1B), peroxisome proliferator-activated receptor gamma (PPARγ), phosphatase and tensin homolog (PTEN), and transcription factor 7-like 2 (TCF7L2) (99). Subsequently, numerous studies have associated DNA methylation patterns with obesity, based on BMI or waist circumference (WC) in both adults and children (100, 101). Moreover, increasing evidence demonstrated that transgenerational inheritance of environmental exposures may be passed down to generations of offspring never exposed to the parental stimuli (12). All these findings support the emerging evidence that pre-pregnancy paternal exposure, and/or exposure during pregnancy to both maternal weight gain and obesogens could explain most of transgenerational epigenetic programming that explain the hereditary nature of obesity and its related comorbidities in children (102–104).

In the next paragraphs, we will report the literature evidence of transgenerational inheritance of epigenetics due to parental overnutrition or *in utero* exposure to factors that may promote an obese phenotype in children.

Transgenerational inheritance of epigenetics due to pre-pregnancy parental factors

Increasing evidence links parental nutritional status to epigenetic programming in offspring explaining the hereditary nature of metabolic diseases such as obesity, thus supporting the emerging concept of DOHaD that emphasizes the role of environmental factors during the embryonic period in determining the development of lifestyle-related disorders in adulthood (105).

In the transgenerational inheritance the insult may alter the germline epigenomic signature during preconception and transmit the epimutations to the F2 generation without any exposure as shown in Figure 1 (106). One of the first studies that reported evidence on the role of human pre-conception parent nutritional status on epigenetic inheritance examined the Dutch famine birth cohort and its offspring. In particular, the study demonstrated that offspring of exposed F1 fathers were heavier and had a higher BMI than offspring of unexposed F1 fathers (107). Recent studies especially in animal models have demonstrated that both obesity and diabetes changed levels of DNA and histone methylation, histone acetylation, and ncRNAs, such as miRNAs in oocytes and sperm (108). In particular, it is well known that DNA methylation reprogramming is a crucial event that takes place immediately post-fertilization and during gametogenesis (109). DNA methylation was analyzed at imprinted regions which include genes with monoallelic expression and for this reason susceptible to the paternal or maternal modulation in the progeny. Insulin like growth factor 2 (IGF2) gene and H19 lncRNA are well-described imprinted genes associated with childhood obesity which are subject to epigenetic regulation. The IGF-2 gene is a paternally imprinted gene encoding the protein IGF-2 that presents a high affinity with the insulin receptor and is related to mitogenesis and mitochondrial biogenesis. On the other hand, H-19 is a maternally imprinted gene that also is related to muscle insulin sensitivity (110, 111).

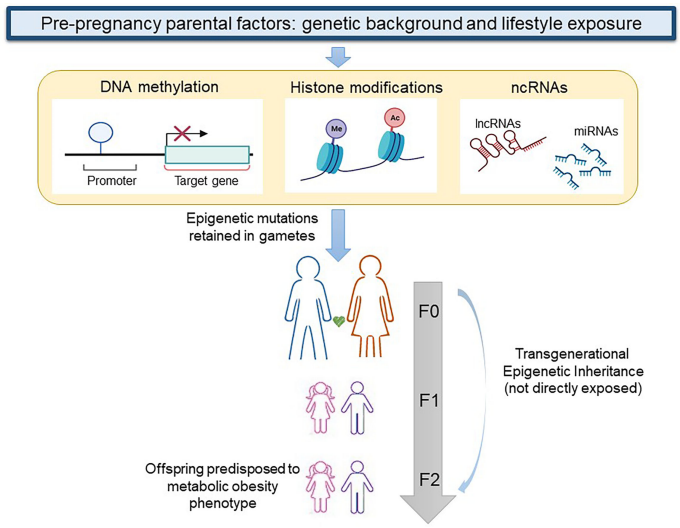

FIGURE 1
Transgenerational epigenetic inheritance of obesity. When genetic background and lifestyle exposure cause epigenetic modifications (DNA methylation, histone modifications, and changes in non-coding RNAs) in gametes of a woman or a man at F0, the first generation that exhibits a transgenerational epigenetic inheritance is the F2. *lncRNA, long non coding RNA; ncRNA, non coding RNA; miRNA, micro RNA.* Figure partially created with BioRender.com.

Unfortunately, the effect of epigenetic changes occurring in germline that may influence progeny obesity are poorly investigated because human sperms and oocytes are not available for research studies. Therefore, the negative effect on oocytes, and the link between obesity and epigenetics, have been performed mainly in animal models as reviewed by Ou et al. (108). In particular, animal models of diet, such as high-fat diet (HFD), or genetic-induced (i.e. ob/ob mice) obesity were found characterized by altered levels of DNA methylation in the oocytes (112, 113). HFD obese and ob/ob mice also exhibited a reduction of expression of STELLA protein (i.e., a maternal gene required for oogenesis and early embryogenesis) in oocytes that affected the levels of DNA methylation (114). Moreover, in the same experimental model Hou et al. reported modifications in the methylation levels of histone H3 lysine 9 and lysine 27 (113). Studies on ncRNAs and oocytes from individuals with obesity are still lacking.

Experimental studies by animal models also demonstrated a link between paternal obesity and changes in sperm cells DNA methylation profiles (112, 115). However, the interest for epigenetic inheritance of chronic metabolic disorders through the human paternal germ line is recently growing (116, 117). A previous human study demonstrated that babies born to fathers with obesity have altered DNA methylation at several regulatory regions of imprinted genes compared with children born to parents without obesity. The analysis of DNA methylation percentages at the differentially methylated regions (DMRs) from 92 umbilical cord blood leukocyte samples of newborns revealed that paternal obesity was significantly associated with lower methylation levels at the mesoderm specific transcript (MEST), paternally expressed 3 (PEG3), and neuronatin (NNAT) genes important for normal growth and development, suggesting the susceptibility for the next generation to develop chronic diseases in adulthood (118). Interestingly, a human study which compared sperm DNA methylation percentages, at 12 DMRs in 23 overweight/obese and 44 normal weight men showed that the obesity status is traceable in the sperm epigenome (119). In particular, the study reported a significant reduction of methylation levels in maternally expressed 3 (MEG3), necdin (NDN), small nuclear ribonucleoprotein polypeptide N (SNRPN), and epsilon sarcoglycan (SGCE)/paternally expressed 10 (PEG10) genes and a slightly increased of DNA methylation at the intergenic MEG3 and imprinted maternally expressed transcript (H19) DMRs in overweight/obese subjects compared to normal weight men (119). Recently, the impact of male obesity on DNA methylation reprogramming in sperm was also investigated by Keyhan et al. The author identified 3264 CpG sites in human sperm that are significantly associated with BMI in mature spermatozoa, then remarking the potential heritability of these DNA methylation alterations to the next generations (120).

Current research on how spermatozoa transmit acquired epigenetic changes affecting the metabolic health of the next generation revealed that both high-fat diets and low-protein diets may cause changes in spermatozoal content of ncRNA subtypes. In particular, altered sperm levels of snRNA due to high-fat diets in particular were associated with an altered phenotype in the offspring, with the offspring showing insulin

resistance, altered body weight, and glucose intolerance (121). In men with obesity it was also observed an altered expression level of specific miRNAs, piRNAs, tRFs, and small nuclear RNA (snRNA) fragments in the spermatozoa with respect to lean men (122). Moreover, the authors reported also changes in DNA methylation patterns between obese and lean men in some genes, including neuropeptide Y (NPY), cannabinoid receptor type 1 (CR1), cocaine- and amphetamine-regulated transcript (CART), FTO, and carbohydrate sulfotransferase 8 (CHST8). Interestingly, the sperm pattern of DNA methylation was modulated by bariatric surgery, highlighting the relevance of the impact of weight loss and nutritional intake on the epigenome of human sperm (122).

Tissue and circulating miRNAs were extensively studied in the context of pediatric obesity (123, 124) but there are no studies on their pre-pregnancy modulation and effects on the obese phenotype on offspring. A work by López and colleagues showed that two miRNAs associated with inflammation and iron homeostasis (miR-155 and miR-122) were upregulated in sperm cells of obese subjects compared to subjects with normal weight (125). These findings support the hypothesis that differentially regulated microRNAs in sperms may potentially act upon DNA methylation post fertilization.

Epigenetic effects of *in utero* exposure to maternal obesity/overnutrition and environment

The interface between mother and fetus is the placenta, which regulates the flow of nutrients, oxygen, and hormonal supply. It follows that the placenta is involved in any abnormalities of fetal growth and of the resulting hepatometabolic and cardiovascular diseases in the offspring (126, 127). As depicted in Figure 2A, the placenta of pregnant women with obesity is subjected to increased inflammation and oxidative stress (128). Several data suggest that the chronic low-grade systemic inflammation in obese pregnancy and the accumulation of macrophages in the placenta cause uncontrolled local inflammation due to the production of pro-inflammatory cytokines that may alter maternal nutrient transport (129). A nonhuman primate model of excess nutrition in pregnancy provides evidence that a HFD decreases uterine blood flow and causes placental ischemia (130).

In 2015 Sharp et al. analyzed the Avon Longitudinal Study of Parents and Children including a general population pregnancy cohort found that maternal pre-pregnancy BMI was positively associated with cord blood methylation at two CpG sites, at regions encoding for coiled-coil domain-containing 112 (CCDC112) and mucolipin TRP cation channel 3 (MCOLN3). Moreover, offspring of women with obesity had 28 CpG sites identified that were differentially methylated compared with offspring of women with normal weight. Four CpG sites in children peripheral blood were associated to maternal obesity. The study also reported a stronger association between offspring methylation and maternal obesity compared to paternal obesity, supporting an intrauterine mechanism (131).

The DNA methylation profile changes in the fetus can be influenced by the amount in the maternal diet of methyl donors from which depends the one carbon metabolism, including folate, methionine, betaine, or choline, thus leading to adverse metabolic effects during infancy and childhood. Maternal choline and betaine deficiencies also alter the methylation status of genes that participate in growth (Insulin-Like Growth Factor 2 Receptor, IGRF2, corticotropin releasing hormone,

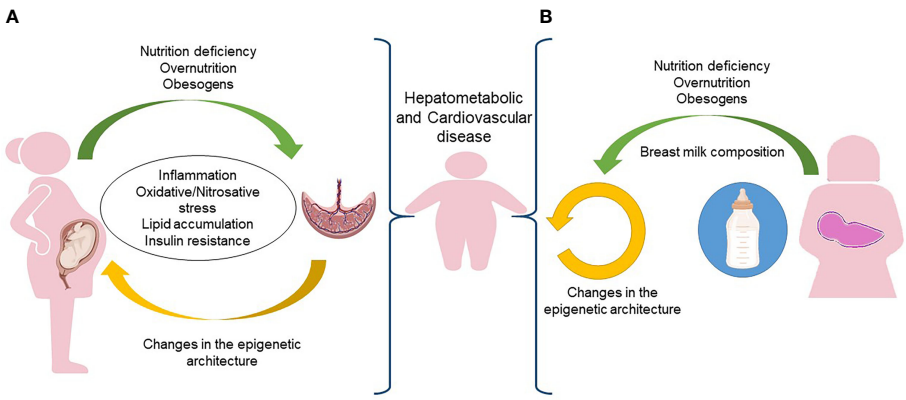

FIGURE 2
In utero and postnatal predisposition to obesity. **(A)** During pregnancy, the placenta of a woman exposed to nutrition deficiency, overnutrition or obesogens, is subjected to inflammation, oxidative/nitrosative stress, lipid accumulation and insulin resistance that increases the risk of obesity and its comorbidities in the newborn. **(B)** The same risk of obesity and comorbidities early in the life could be associated to epigenetic changes occurring in the breast milk. Figure partially created with BioRender.com.

CRH, and nuclear receptor subfamily 3 group C member 1 (NR3C1) or vascular function (vascular endothelial growth factor C,VEFGC, and angiopoietin 2, ANGPT2), resulting in developmental abnormalities, blood vessel malformation, or increased predisposition for developing steatohepatitis in the offspring (132).

In the last ten years EWAS provided a useful and successful tool for longitudinal study designs of childhood obesity. Several studies highlighted the presence of a significant association between DNA methylation, obesity and some risk factors as birthweight and maternal BMI, supporting the emerging consensus on the heritability of BMI (133, 134).

Methylation status of leptin and its receptor (LEPR) gene promoters is one of the first evidence supporting the idea that epigenetic variations linked with intrauterine exposures and DNA methylation may influence childhood obesity (135). A further study demonstrated that a maternal poor weight gain during pregnancy was associated with increased methylation status of LEPR thus affecting the expression of the protein in umbilical vein of newborns (136).

A recent systematic review reported data from EWAS which investigated the DNA methylation levels, in peripheral blood or cord blood samples of children with or without obesity and/or overweight. Overall, 19 studies out of 23 found an association between childhood anthropometric parameters with at least one methylated CpG site or region. In four genes [histone deacetylase 4 (HDAC4), prolactin-releasing hormone receptor (PRLHR), tenascin XB (TNXB) genes, and PR domain containing 16 (PRDM16)] it was found the association of a DNA methylation profile with childhood obesity (97).

A meta-analysis study concerning data from EWAS of seven cohorts participating in the Pregnancy and Childhood Epigenetics (PACE) consortium found that maternal gestational diabetes mellitus (GDM) was associated with lower cord blood methylation levels within two regions. One region covering the promoter of OR2L13, a gene associated with autism spectrum disorder, and the other one covering the cytochrome P450 family 2 subfamily E member 1 (CYP2E1) gene, which is upregulated in type 1 and T2D (137).

A recent EWAS using maternal blood and cord blood samples from FinnGeDi (Finnish Gestational Diabetes) study cohort (298 mother-offspring pairs with GDM and 238 without) aimed to explore offspring-specific epigenetic effects in response to GDM exposure and maternal methylation levels (138). By a linear regression analysis, the authors found significant association of methylation at the transcription factor CP2 (TFCP2) gene in offspring of GDM exposure and maternal methylation. Furthermore, using the false discovery rate, the authors found seven significant DNA methylation differences positions between the mother's methylation and GDM status. These loci included: DLG-Associated Protein 2 (DLGAP2) gene, H3 clustered histone 6 (H3C6) gene, family with sequence similarity 13 member A (FAM13A) gene, ubiquitin protein ligase E3C (UBE3C) gene, LOC127841, and two loci within intergenic regions. Interestingly, DLGAP2 and FAM13A, have previously been identified to have a role associated with diabetes and maternal insulin sensitivity in pregnancy, thus suggesting a possible function of their methylation in the future risk of obesity and T2D in offspring (139). Moreover, methylation changes in both FAM13A and DLGAP2 genes in newborns were also associated with environmental changes during pregnancy, including air pollution and smoking in pregnancy, respectively (140, 141).

The role of fetal programming in early infant weight gain due to maternal obesity has been recently further suggested by a study comparing the miRNA profile in peripheral blood mononuclear cell cultures of children from mothers who are normal weight or those who are overweight/obesity. The miRNA profile in mononuclear cells showed a downregulation of miR-155, miR-221, and miR-1301, as well as upregulation of miR-146a in the circulation of newborns from overweight and obese mothers. Moreover, the study showed that in the same samples from newborns, the RNA profile changes induced by overweight mothers and obesity were also associated to alteration in PPAR-γ gene expression, a master regulator of adipogenesis and altered cytokine pro-inflammatory levels *in vitro* blood mononuclear cell response to metabolic stimuli (142).

Also epigenetic regulation of intergenic regions appears to be important for obesity etiology. Indeed, Sasaki et al. (143) have recently shown that genomic loci associated with DMRs localized to intergenic regions and gene bodies most likely influence gene regulation. In particular, pathway analysis revealed that males, had a significant overrepresentation of genes involved in endocytosis and pathways in cancer, including IGF1R, which was previously shown to respond to diet-induced metabolic stress in animal models and in lymphocytes in the context of childhood obesity.

Parental epigenetic effects on obesity-related comorbidities

DNA methylation, considered as the key epigenetic mechanism by which maternal and paternal exposure to different dietary patterns, toxins or stressors causing phenotypical effects in unexposed future generations, may also explain some of the obesity-related comorbidities (132, 144). Studies reported that maternal obesity or overnutrition during pregnancy and/or lactation can modulate DNA methylation of different genes involved in energy metabolism, glucose homeostasis, insulin signaling and fat deposition, which support the role of DNA methylation in maternal obesity-induced risk of offspring NAFLD, obesity, and diabetes (145).

Several animal studies have shown that maternal nutrients and caloric regimen can impact fetal adiposity, interfering with long term metabolic homeostasis (146, 147). In murine models a

HFD during pregnancy causes quite variable effects on fetal birth weight (130). These disparities in results are consistent with human observational studies showing increased rates of either large or small-for-gestational-age infants of mothers with obesity, although still both of these neonatal conditions are associated with risks of early onset childhood metabolic disease (148, 149) and histologically proven NAFLD as well (150, 151). Since obesity related NAFLD in pregnancy is independently associated also with hypertensive complications, postpartum hemorrhage and preterm birth, it should be considered a high-risk obstetric condition, with clinical implications for pre-conception counseling and pregnancy care (152).

In a mouse model, female offspring of mothers with obesity exposed to HFD was affected by obesity with high levels of inflammation in the adipose tissue in association with hypomethylation at certain inflammatory genes not only to the immediate offspring but also over three generations (153). Human studies suggested that social, environmental (e.g. education or cigarette smoking, respectively) and metabolic factors playing a role in the health of grandparents could influence the birth weight, the cardiovascular health, or even the neurodevelopmental outcomes of the grandchildren (154). This potential transgenerational inheritance is therefore now considered a relevant contributing factor to the epidemic levels of obesity and related comorbidities.

De Jesus et al. investigated the impact of paternal and/or maternal metabolic syndrome on the epigenetic reprogramming of metabolic homeostasis in offspring by using a non-dietary, genetically liver-specific insulin receptor deficient mice model of metabolic syndrome phenotype, including hyperglycemia, insulin resistance, and dyslipidemia. The experimental study found that parental metabolic syndrome induces downregulation of growth differentiation factor 15 gene in offspring by modulating the DNA methylation levels at the promoter region of the gene and boosting hepatic lipid accumulation in offspring (155). A study, investigating DMR in nondiabetic Pima Indians who were offspring of diabetic or nondiabetic mothers, supported the hypothesis that epigenetic changes may increase the risk of T2D in the offspring of mothers with diabetes during pregnancy (156). Moreover, a human study involving 128 offspring born at term to mothers with well-controlled GDM highlighted that this condition is associated with changes of DNA methylation at the alternative transcription start site zinc finger protein 696 (ZNF696) in cord blood cells, and changes of plasma glucose levels in newborns at 1 h after birth (157).

All in all, it emerges that the heritable epigenome, such as DNA methylation, histone modifications, and the expression of ncRNAs, could also predispose individuals to metabolic disorders and obesity phenotype. The epigenetic changes could be a target to control antepartum hyperglycemia, prevent gestational diabetes, and avoid excessive weight gain during pregnancy reducing childhood obesity and its related comorbidities. This proposed link between increased maternal insulin resistance during pregnancy and the development of T2D in the offspring should be taken into consideration for actively promoting changes in lifestyle before and during pregnancy in order to prevent the risk of developing obesity and obesity related metabolic diseases (157, 158).

Effect of maternal nutrition intake during pregnancy

Maternal serum free fatty acids reach also the placenta which represents a site of inflammatory and hypoxic changes. The unfolded protein response/endoplasmic reticulum stress (ERS) is, in fact, a source of reactive oxygen species. Upon disruption of mitochondria-associated membranes integrity, miscommunication directly or indirectly disturbs Ca^2+ homeostasis and increases ERS and oxidative stress. Obesity-related maternal ERS and oxidative stress reaching the placenta may trigger inflammatory cytokines production and release. These molecules with maternal lipids may reach, through the umbilical cord vein and fetal portal vein, the fetal liver where they start to trigger hepatic lipogenesis and hepatocellular damage (159). In this scenario, the excess of maternal fatty acids could result in the accumulation of fetal ectopic fat. As the fetus subcutaneous adipose tissue (SAT) depot is not yet functioning, the accelerated adipogenesis during the perinatal window of SAT development underlies hepatometabolic and cardiovascular risk in offspring born to dams that are metabolically compromised (Figure 2). The exact mechanisms whereby these gestational events exacerbate the pathogenesis of fetal liver damage and also to cardiovascular risk are still poorly understood (150). Animal model studies showed that maternal obesity and HFD have a lasting cardiometabolic impact, and contribute to an increase in fetal liver inflammation and fatty liver in offspring (160–163).

In adult liver it appears quite well-established that NAFLD initiation and progression depend on several events. Among others, up-regulation of sterol regulatory element-binding protein-1c, a transcriptional activator of lipogenic enzymes such as stearoyl coenzyme-A desaturase1 and fatty acid synthase, which contributes to lipogenesis with an increased *de novo* synthesis of fatty acids, represent strong candidates for being involved also in fetal NAFLD (164, 165). NAFLD progression is also reflected by dysregulation of monocyte to macrophage differentiation-associated 2, the death decoy receptor TR6/DcR3 inhibiting T cell chemotaxis *in vitro* and *in vivo*, and chronic inhibition of nitric oxide synthase (166).

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Effect of chemical obesogens during pregnancy

In addition to dietary composition, the exposure to chemical obesogens during pregnancy and early life is another mechanism that can strongly influence the subsequent predisposition to obesity. Obesogens are chemicals that inappropriately stimulate directly or indirectly adipogenesis and fat storage. According to animal models, the epigenetic effects of the exposure may persist on exposed animals and their offspring at least until generation F4 (102).

Exposure to mixtures of phthalates, parabens and other phenols is very common due to their ubiquitous prevalence in personal care products and plastics with also a risk of prenatal exposure. In a longitudinal study of mothers' prenatal urinary concentration of phthalates and phenols was correlated with the z-score of BMI of the respective children (167).

Also the exposure to plastic derived endocrine disruptors EDCs (e.g. bisphenol) may affect the fetal growth and contribute to epigenetic and angiogenic changes, thus promoting the development of adult chronic diseases. Of note, these compounds may control the processes of epigenetic transgenerational inheritance of adult-onset diseases by modulating DNA methylation and epimutations in reproductive cells leading to the development of obesity and impaired glucose metabolism in the F2 generation (168). The endocrine disrupting effects of obesogens may directly or indirectly promote adipogenesis and obesity/obesity related metabolic conditions that drive metabolic syndrome through disturbance of several processes. Studies show that these effects are seen not only in individuals but also in their offspring because of their ability to epigenetically reprogram genetically inherited set points for the control of body weight and body composition at some critical stages of development, such as fetus, early life, and puberty (169, 170).

Epigenetics as a target for prevention and therapy of obesity and its related comorbidities

Promoting healthy early programming of adipose tissue is essential to implement new strategies that can control the spread of obesity and other metabolic disease (171, 172). However, the same obesogenic environment may have different effects on different individuals, highlighting an underlying individual susceptibility to obesity and fat distribution (173). In this scenario, the reversal of epigenetic changes could be used as an early and virtuous clinical intervention for reducing the risk of obesity in offspring (171). This goal is achievable through the adoption of healthy eating habits before and during critical periods of development, such as pregnancy and lactation (174). The particularly sensitive period capable of implementing epigenetic reprogramming strategies does not include only pregnancy, neonatal and infant life but encompasses a longer period defined the period of 1000 days, starting from conception and lasting the first 2 years of life (175). The milk of each mother is specific to her own baby, and the maternal-fetal dyad may affect the composition of breastfeeding and the baby's tolerance to biologic breast milk (176). The identification of factors inducing epigenetic changes in breast milk, have led to the hypothesis of the possibility of transferring epigenetic information directly through breast milk (Figure 2B). Currently, studies in humans discovered more than 2000 miRNAs that can regulate collectively one third of the genes in the genome. The human milk provides abundant amounts of miRNA-148a, miR-152, miR-29b, and miR-21, which all target DNA methyl transferases (177, 178). The concept of milk siblings has been known for years (179, 180) and on this basis a study on rats showed that adoptive siblings, who had taken the same milk, had acquired the same hereditary epigenetic heritage (miRNAs). As in the consanguineous marriages, the children born from the marriage of these "milk siblings" may develop more serious pathologies (e.g., differentially expressed miRNAs were associated with pathways regulating metabolism, survival, and cancer development insulin signaling pathways).

Interpreting these results, we can deduce that the changes and strategies (nutrition, physical activity, and no-smoking) adopted during pregnancy and lactation can reverse the trend of a previous metabolic risk in the offspring. The lack of effective drugs easily available for the treatment of obesity and its comorbidities, such as obesity related-NAFLD, requires approaches with very timely changes in lifestyle (i.e. less sedentarism, more sleeping hours and exercise) and better eating habits. It has been shown that a normal pre-pregnancy maternal BMI and long duration of exclusive breastfeeding are favorable conditions not only for a direct effect on weight control, but also to prevent the development of NAFLD later in life (175).

Due to the lack of convincing data of efficacy and safety for both pregnant women with obesity and their offspring after pharmacological interventions during gestation with available medications (i.e. metformin) (181–184), the epigenetic modifiers may represent a promising approach (145, 185). Interestingly, maternal diet supplemented with methyl donors during lactation has been shown to prevent the development of an obese phenotype and fatty liver of offspring in mice (186). Similarly, recent studies in humans found that both paternal and maternal diet supplemented with choline, betaine, folate, and methionine, can affect offspring's DNA methylation of genes involved in the regulation of metabolism, growth, and appetite (187, 188).

Although these findings suggest that a substantial component of obesity and metabolic disease risk has a prenatal developmental basis, the question remains unsolved because its complexity. In fact, in human studies it appears that there is no simple correlation between maternal intake of methyl group donors, higher or lower offspring DNA methylation and subsequent anthropometric outcomes (188). These effects could depend on the different

tested developmental ages (170, 188). Alternatively, it has been suggested that the involvement of adjacent or nearby CpGs within the same promoter showing different strengths of association with child's adiposity may imply highly specific changes in the transcriptional regulation of these genes induced by the developmental environment, rather than generalized changes in promoter methylation (170, 188). All in all, it appears that epigenetic reprogramming may represent an adaptable response through which the developing offspring modify their physiology to the postnatal environment (189).

Discussion and conclusions

The growing number of studies in the last few decades makes it clear that obesity is influenced by complex interactions between genetic, developmental, and environmental pressures. All these factors in turn create a network of relationships which are the basis for the concept of the multifactorial pathogenesis of obesity and its comorbidities.

It is obesogens evident that genetic factors play a relevant role, even though it has to be well dissected.

Purely monogenic forms of obesity presenting with severe early-onset obesity associated with hyperphagia and endocrine disruption in fact are so rare that the obesity epidemic cannot depend on single gene disorders. Therefore, the interaction of a more complex (polygenic) genetic background with the environment, where hundreds of SNPs exhibiting a heritability pattern similar to other complex traits and diseases can contribute to the development of the obese phenotype, is a more plausible explanation (20, 51).

Moreover, since environmental influences may cause epigenetic changes already in the gamete cell stage of both parents, but also during pregnancy and the early postnatal period including lactation with a lasting effect on the metabolism of the offspring, some inherent considerations follow. First, it appears now paramount the need of implementing the lifestyle and health status of both parents very early, even before conception (160, 190). Second, specific supplementation of the maternal diet with methyl donors during early gestation and during breastfeeding could prevent the development of an obese phenotype and fatty liver of offspring (186, 188). Further studies in this regard are however necessary to clarify relevant issues such as timing and dosage of methyl donors supplementation in pregnant and breastfeeding women (188, 191, 192).

However, the entire scenario of how parental diet and/or exposure to hormones and other environmental factors (e.g, endocrine disruptors) act transgenerationally on the epigenetic landscape and consequently on development of the offspring's human adipose tissue and its plasticity over the life course requires further investigation (13).

Currently, epigenetic changes appear to be correctable by targeted interventions such as body weight reduction through virtuous dietary changes, exercise, or even metabolic surgery, all of them regularly affecting DNA methylation. However, targeted interventions with specific epigenetic modifiers to reprogram epigenetic changes is an extremely interesting future playground which has already started to be explored in some other fields. Cancer studies have in fact shown that a number of natural phytochemical substances (e.g. polyphenols) can target the epigenetic machinery with potential benefit due to reverting effects in the epigenetic modifications of relevant genes and restoring their protein expression levels (193). Similar new frontiers studies of cells reprogramming will likely prove meaningful if applicable to adipocytes also in obesity and related conditions as well.

In conclusion, although more evidence is needed, the reported literature strongly suggests that the analysis of the epigenetic architecture at the interface between gene expression and the epigenetic environment could be relevant for a better understanding of obesity and its associated comorbidities. Moreover, further studies addressing the potential intertwining between genetic and epigenetics in childhood obesity could provide the hint to improve prevention and therapeutic management.

Author contributions

NP, CM, AA, and PV contributed to the conception and design of the manuscript. All authors wrote sections of the manuscript and contributed to design of Figures and Tables. All authors approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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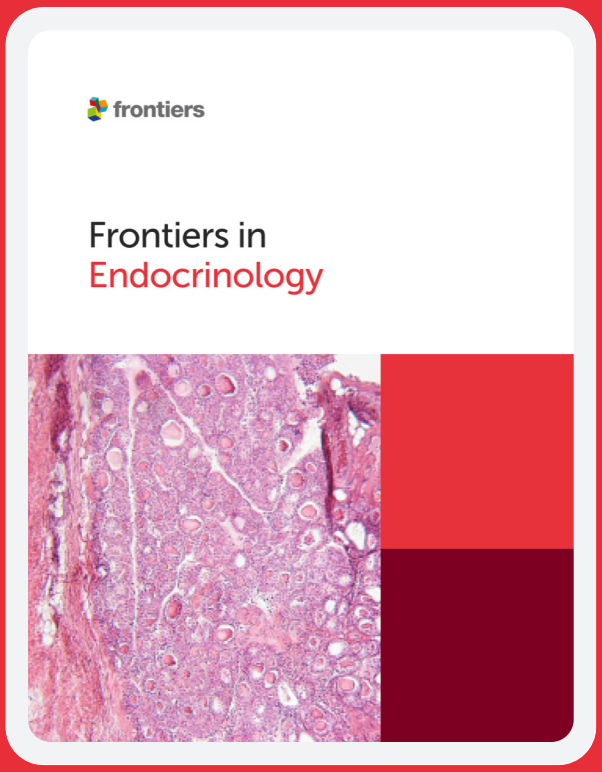

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