Research Article

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The role of immune cells in mediating the effect of hepato-ovarian axis: a mendelian randomization study on ovarian aging and NAFLD.

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ABSTRACT

Observational studies have emphasized the association of female reproductive factors with non-alcoholic fatty liver disease (NAFLD), however, the causal relationship between them still needs further investigation. The aim of this study was to investigate the genetic correlation between reproductive factors and NAFLD. First, two-sample Mendelian randomization (MR) was performed to estimate the causal associations between age at menarche (AAM), age at natural menopause (ANM), age at first birth (AFB), and NAFLD by using pooled data from the Genome-Wide Association Study (GWAS) from the Genetic Consortium. Causality was tested by a variety of methods including inverse variance weighting (IVW), MR-Egger regression, and weighted median method. Additionally, horizontal pleiotropy was verified using the MR-PRESSO metho. The Univariate MR analysis provided evidence that AAM (OR = 0.82, 95% CI: 0.74-0.91, P < 0.0001, PFDR = 0.0002), AFB (OR = 0.91, 95% CI: 0.83-0.99, P = 0.0286, PFDR = 0.0465) were causally associated with NAFLD risk. In contrast, a causal effect of ANM with NAFLD (OR = 1.01, 95% CI: 0.98-1.04, P = 0.539, PFDR = 0.564) has not been confirmed. Mediation analysis showed that the effect of AAM on NAFLD may be mediated through immune cells (CD25 on switched memory B cell and CD127 on CD28+ CD4-CD8- T cell), with mediation effects accounting for 6.23%, 6.68%, respectively. Our MR analysis suggested a causal effect of reproductive factors on NAFLD development, influenced by AAM, AFB. Early AAM and early AFB increase the risk of NAFLD in women.

Keywords : age at menarche; non-alcoholic fatty liver disease; immune cells; Mendelian randomization

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a disease of the liver that primarily characterized by excessive fat deposition in hepatocytes due to non-alcohol and other well-defined factors damaging the liver. The prevalence of NAFLD has increased steadily in recent years, with a global prevalence of approximately 25% in the general population(1). Even more alarmingly, a meta-analysis in 2022 showed that the global prevalence of NAFLD was higher than previously estimated and growing at an alarming rate(2). NAFLD is one of the most important causes of chronic liver disease, which can progress from nonalcoholic simple fatty liver to nonalcoholic steatohepatitis, hepatic fibrosis, and finally to cirrhosis and hepatocellular carcinoma (3). A new hypothesis, the "hepato-ovarian axis", has recently been proposed as a result of the growing evidence of a strong association between the liver and the female ovaries(4). Complex lipids are strongly associated with aging, for example, bis(monoacylglycero) phosphate (BMP), a characteristic lipid of aging, increases significantly with the aging process, and a short-term healthy lifestyle can reduce BMP levels in postmenopausal women (5). Genetic studies based on multi-omics data have found a decrease in healthy cardiolipin (CL) and an increase in unhealthy CL in NAFLD. Perhaps, changes in CL levels may be one of the underlying causes of hepatic steatosis and may even be a biomarker of NAFLD disease (6). Animal experiments have demonstrated that ovarian hormones in rats play a role in regulating lipid accumulation in the liver(7). Compared with sham oophorectomized female rats, true-oophorectomized rats not only had increased systemic fat weight and peripheral

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insulin resistance, but also developed severe hepatic triglyceride accumulation and were more susceptible to inducing hepatic steatosis(8). Ovarian estrogen levels in the liver are not only involved in lipid metabolism, but also have anti-inflammatory, anti-fibrotic, and anti-steatosis (9, 10). Lipid regulation disorders can directly affect ovarian hormone levels, accelerating female ovarian aging(11).

First menarche is a sign of the onset of puberty and indicates the establishment of positive feedback from estrogen to pituitary gonadotropins(12). Natural menopause, suggesting the decline of ovarian function, marks the end of female reproductive function. During the female reproductive course, hormone levels fluctuate and change with the menstrual cycle, and reproductive events. Female reproductive factors (including age at menarche, age at natural menopause, age at first birth, menopause, etc.) are closely related to women's health status. Female reproductive factors have a significant impact on cardiovascular disease, diabetes, osteoporosis and other diseases(13). Earlier age at menarche has been associated in previous Coronary Artery Risk Development in Young Adults (CARDIA) study not only with an increased risk of NAFLD in adults, but also with visceral and subcutaneous abdominal ectopic fat depots in mid-adulthood(14). Menopause is associated with progression and severity of NAFLD. In a cross sectional study involving 488 menopausal women with NAFLD(15), both premature menopause and time since menopause were associated with an increased risk of NAFLD fibrosis. Premature menopause, and time after menopause were both correlated with an increased risk of more severe liver fibrosis. Evidence from the previous NHANES study suggests that women with AFB <26 years are at greater risk of developing NAFLD compared to women with a first birth at 30-32 years old(16). These findings show a strong association between female reproductive factors with NAFLD. However, there are no clinical studies investigating the causal relationship between female reproductive factors and NAFLD.

The liver is an essential metabolic and immune organ of the human body(17). Immune cells account for 10-20% of total liver cells(18). T-lymphocyte subsets have an important role in immunomodulation. Peripheral CD8 cells, natural killer cells, and CD4 T cells are biased toward activation in NAFLD patients compared to healthy populations(19). Macrophages are involved in steatosis, inflammation and fibrosis in NAFLD(20). The number of immune cells in the female genital tract varies with the menstrual cycle, pregnancy and menopause(21). Immune cells are involved in the continuous remodeling of the endometrium and the regulation of the uterine microenvironment during the more than 400 months between the onset of menstruation and menopause(22). From the above studies, we have revealed the specific causality of the hepato-ovarian axis through two diseases and have elicited a "hepato-ovarian axis" related to aging. Immune cells have a major role in the liver and ovary. Therefore, immune cells may be the "mediators" of communication between the liver and ovarian organs.

Mendelian randomization (MR) is a method of causal inference in epidemiology based on genetic variation, whereby GWAS (genome-wide association studies) are analyzed to find SNPs with genetic variants associated with biological factors, and then the genetic variants are used as instrumental variables (IVs) to derive causal associations between the exposures and the outcomes. Therefore, we applied MR to explore the potential causal relationship between AAM, ANM, AFB and NAFLD to determine their underlying mechanisms. In addition, we propose the hypothesis that female reproductive factors may lead to alterations in certain immune cells, which in turn mediate female reproductive factor induced NAFLD. In this study, we utilized data from 731 immunophenotypes in the GWAS database(23), using mediation analysis to identify potential immune cells. The results of the study may shed light on the causal relationship between female reproductive factors and NAFLD and the association in terms of potential immune cells, and contribute to the development of clinical screening and prevention strategies for NAFLD.

METHODS

Study design

The two-sample MR study examined the causal relationship between AAM, AMP, AFB and NAFLD in two sample MR designs based on different GWAS data, including three assumptions(24): (1) robust correlations between instrumental variables (IVs) and exposures (associations); (2) IVs are not associated with confounders (independence); (3) the effects of IVs on outcomes can only be realized through exposures (exclusion restriction criteria). See **Fig 1**.

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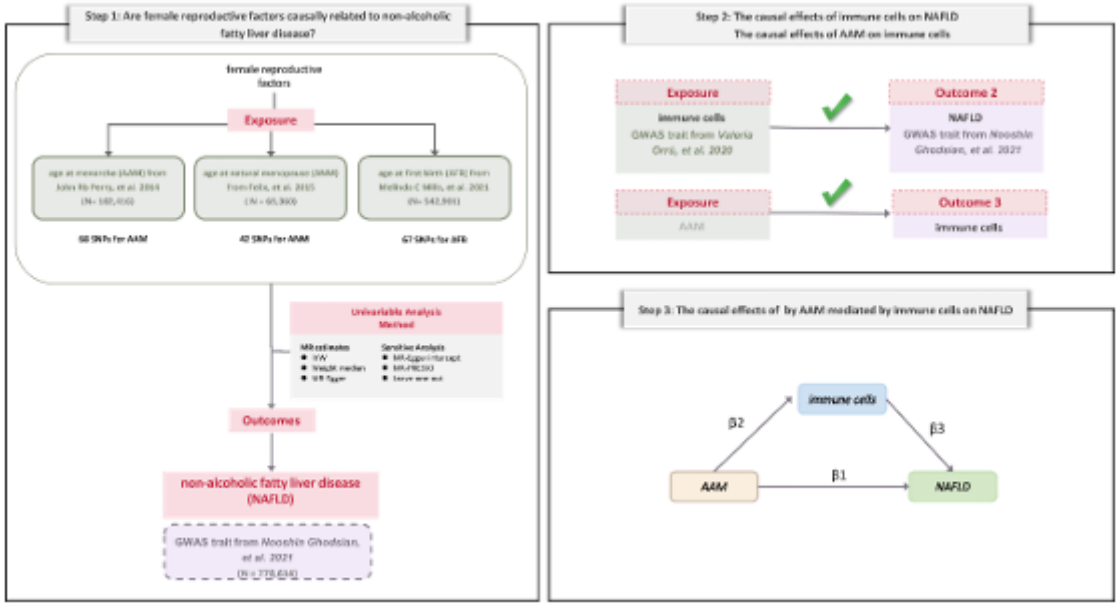
Figure 1 : MR analysis flow chart



Note: The total effect of AAM on NAFLD, β1, was derived by univariate MR (i.e., genetically predicted AAM as exposure and NAFLD as outcome). A two-step MR design was used (where β2 is the effect of AAM on immune cells and β23 is the effect of immune cells on NAFLD (adjusted for exposure)). Mediated effect (β2*β3). Mediated ratio is indirect effect divided by total effect (β2 × β3/β1). Abbreviations: AAM = age at menarche; ANM = age at natural menopause; AFB = age at first birth, NAFLD = non-alcoholic fatty liver disease; IVW = inverse variance weighted; SNP = single nucleotide polymorphism; GWAS = genome-wide association study.

Datasets

A detailed overview of all data sources is presented in **Table1**. We obtained publicly available summary statistics from GWAS for MR analysis. Data on AAM were extracted from a GWAS study that included 182,416 European women from 57 studies, incorporating 2,441,816 single nucleotide polymorphisms (SNPs)(12). The ANM summary data included 69,360 European women and 2,418,696 associated SNPs(25). A recent GWAS examined 542,901 female cases of AFB containing 9,702,772 associated SNPs(26). The NAFLD data were derived from a large GWAS-aggregated dataset with a sample size of 778,614 cases (8,434 cases and 770,180 controls)(27). Genetic data on immune cells were derived from a population of European ancestry and contained 731 immunophenotypes (GCST0001391 GCST0002121)(23). These 731 immunophenotypes consisted of four types: morphological parameters (MP), absolute cell count (AC) relative cell count (RC), median fluorescence intensity (MFI) of surface antigens, and, the numbers of which ranged from small to large were 32, 118, 192, and 389, respectively.

Table 1 : The GWAS data source details in our study

Phenotype	Consortium	Participants	Population	PMID	Type of variables
AAM	ReproGen	182,416	European	25231870	Exposure
ANM	ReproGen	69,360	European	26414677	Exposure
AFB	NA	542,901	European	34211149	Exposure
NAFLD	NA	778,614	European	34841290	Outcome
Triglycerides	UK Biobank	441,016	European	32203549	Confounder
Hypertension	UK Biobank	461,880	European	NA	Confounder
BMI	UK Biobank	454,884	European	NA	Confounder

Selection of IVs

The number of single nucleotide polymorphisms (SNPs) were screened to fulfill the following conditions : 1)SNPS: $P < 5\times10-8$, 2) removed linkage disequilibrium(LD) ($r2 < 0.001$, distance threshold = 10000kb), 3) The Phenosanner database was queried

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to remove confounders, 4) Removed palindromic SNPs with intermediate allele frequencies, 5) The F-statistic data of the selected SNPs exceeds 10 (F = beta2/se2)(28). F > 10 suggests that the relationship between IV and exposure is robust and that the results of MR analysis are not influenced by weak instrumental bias. Finally, the data from the database were extracted, organized, and merged. The effect alleles were subsequently compared to correspond to the same effect allele for both the exposure and outcome effect values. Based on prior studies(29), the IV significance threshold with each immune trait was set at 1 × 10-5 and then r2 < 0.001, with a distance threshold = 10,000 kb, to ensure that no linkage disequilibrium (LD) correlations existed.

Mendelian randomization analysis

MR analyses were conducted with Inverse Variance Weighting (IVW), MR Egger, Weighted Median, Simple Mode and Weighted Mode. We used IVW estimators with multiplicative random effects for our main analyses (if they were not heterogeneous, fixed effects IVW were applied). IVW was utilized as the primary outcome to assess the causality between exposure and results, with statistical significance set at a p-value <0.05. Since the result was binary, we translated it into a ratio-ratio (OR). In the absence of pleiotropy, IVW accurately appraises each valid IV and provides reliable causal estimates by incorporating the Wald ratio. The weighted median method calculates the causal estimate as the median estimate of the ratio of each genetic variant, weighted by the inverse of its variance. The weighted model assigns causal estimates to each genetic variant by the inverse of its variance. The simple model considers each genetic variant individually to estimate causal effects. We conducted sensitivity analyses with several methods robust to horizontal pleiotropy, which include MR Egger, weighted mode, weighted median, and MR pleiotropy residual sum and outlier test (MR-PRESSO). Different assumptions were made about the validity of IV using each method. If half of the IVs are invalid, perform the estimation of the median weights. We employed sensitivity analysis to verify the plausibility and stability of the MR results. The slope of the MR Egger regression provides an estimate of the causal relationship between exposure and outcome. In addition, the MR-Egger intercept represents the average pleiotropic effect of genetic variation, with an intercept P < 0.05 indicating the presence of pleiotropy. Leave-one-out analysis was performed by eliminating each SNP one by one to test whether the results were affected by any individual SNP. We utilized the Benjamini-Hochberg method for calibration and calculated corrected p values for false discovery rate (FDR). A strongly causal relationship was considered when the raw P < 0.05 and PFDR < 0.05. If raw P-value < 0.05 and PFDR > 0.05, it indicates that there may be a potential causal relationship between the exposure and the outcome.

We utilized a two-step MR design for mediation analyses to investigate whether immune cells mediate causality from female reproductive factors to NAFLD. The direct effect of exposure on outcome (to obtain the coefficient β1) and the reverse causality of outcome on exposure were determined in step 1. In Step 2, the effect of exposure on immune cells (to obtain the coefficient β2). Step 3, the effect of immune cells on the outcome (to obtain the coefficient β3). Finally, the mediating effect (β2*β3) was calculated. We calculated the percentage mediated by the mediating effect by dividing the indirect effect by the total effect. Finally, 95% confidence intervals were calculated using the delta method.

Finally, the results of the MR analysis were visualized utilizing scatter plots and forest plots. All MR analyses were conducted using R software packages "Two-sample MR" and "MRPRESSO". All data were analyzed by R (version 3.1.5).

RESULTS

Two-sample MR analysis

We utilized IVs that were significantly associated with MR exposure in both samples. Furthermore, we excluded SNPs with strong correlations with BMI and type 2 diabetes, and then calculated F-statistics for the remaining SNPs (See **Supplementary Tables S1-3**). The F-statistics for each instrument exceeded 10. The Univariate MR analyses showed that AAM, ANM, AFB and NAFLD had 56, 37, and 43 SNPs. Genetically determined AAM and AFB showed a possible causal effect on NAFLD, as validated by the Benjamini-Hochberg method. AAM is strongly associated with NAFLD (OR = 0.82, 95% CI: 0.74-0.91, P < 0.0001, PFDR = 0.0002). AFB has a protective effect on NAFLD (OR = 0.91, 95% CI: 0.83-0.99, P = 0.0286, PFDR = 0.0465). However, there is no causal relationship between ANM and NAFLD (OR = 1.01, 95% CI: 0.98-1.04, P = 0.539, PFDR = 0.564). The inverse MR of NAFLD with AAM demonstrated no causal association between both (OR = 0.99, 95% CI: 0.92-1.16, P = 0.75). See **Fig 2**. With the exception of ANM, for which there was horizontal pleiotropy (MR-Egger P<0.05), the P-values of the intercepts in the MR-Egger were greater than 0.05, indicating no horizontal pleiotropic effect. MR PRESSO did not detect any outliers for SNP effects, and the Cochran Q-values indicated no heterogeneity (see **Table 2**). The "Leave-one-out plot" showed that none of the SNPs had a dominant effect on the estimated causal relationship between AAM, AFB and NAFLD. See **Supplementary Fig 2**.

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Table 2: Heterogeneity and pleiotropy of AAM, ANM, AFB and NAFLD

Exposure	Heterogeneity tests				Pleiotropic test		
	MR egger		IVW		Egger intercept	se	P
	Q	P	Q	P			
AAM	51.46434	0.572782	52.11228	0.585664	-0.00746	0.009272	0.42438
ANM	36.22883	0.411088	42.68432	0.205764	0.018092	0.007245	0.017367
AFB	37.00325	0.648868	37.20587	0.681141	-0.00541	0.012008	0.654983

Additional file 1: Table S1: Characteristics of the Instrumental SNPs associated with SNP beta.exposure se.exposurepval. exposure F statistic

SNP	effect_allele. exposure	other_allele. exposure	beta.exposure	se.exposurepval. exposure	F	statistic
rs2344508	A	G	0.034	0.005747	3.30E-09	34.99828
rs466639	C	T	0.075	0.008706	7.00E-18	74.21662
rs618678	T	C	-0.034	0.006148	3.20E-08	30.58231
rs633715	C	T	-0.051	0.00725	2.00E-12	49.48395
rs6694738	A	C	-0.044	0.007885	2.40E-08	31.14062
rs6747380	A	G	0.065	0.007658	2.10E-17	72.04821
rs6758290	C	T	-0.04	0.006322	2.50E-10	40.0307
rs1518080	G	C	-0.051	0.006004	2.00E-17	72.14463
rs4369815	G	T	-0.08	0.011888	1.70E-11	45.28888
rs895526	C	T	0.044	0.007553	5.70E-09	33.93447
rs2947411	G	A	-0.052	0.007655	1.10E-11	46.1417
rs2687729	G	A	0.044	0.006575	2.20E-11	44.78416
rs6770162	A	G	0.036	0.005733	3.40E-10	39.4302
rs3870341	G	A	-0.043	0.006449	2.60E-11	44.45699
rs11715566	T	C	0.052	0.005631	2.60E-20	85.27228
rs3914188	C	G	0.044	0.006702	5.20E-11	43.10049
rs7642134	G	A	0.038	0.005912	1.30E-10	41.30851
rs17351680	G	C	0.044	0.007557	5.80E-09	33.90062
rs1482853	A	C	-0.038	0.006043	3.20E-10	39.54859
rs10938397	G	A	-0.038	0.005923	1.40E-10	41.16371
rs3733632	G	A	0.049	0.007862	4.60E-10	38.84004
rs9997604	C	A	0.039	0.008427	4.80E-08	29.79595
rs740077	C	A	-0.046	0.007045	6.60E-11	42.63401
rs13179411	T	G	0.06	0.007813	1.60E-14	58.97123
rs3115627	G	A	0.038	0.006453	3.90E-09	34.673
rs13215865	T	C	-0.042	0.002077	1.00E-200	1373.869

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rs4840086	G	A	-0.036	0.00562	1.50E-10	41.02883
rs11756454	A	T	0.034	0.005703	2.50E-09	35.53902
rs2179786	T	G	-0.039	0.00572	9.20E-12	46.49183
rs9373571	A	T	0.034	0.005926	9.60E-09	32.92064
rs2184968	C	T	-0.036	0.005672	2.20E-10	40.28045
rs6933660	A	C	-0.036	0.008444	1.50E-17	72.71226
rs11767400	A	C	0.035	0.006295	2.70E-08	30.91197
rs7821178	A	C	-0.045	0.006196	3.80E-13	52.74396
rs888345	A	G	-0.044	0.007315	1.80E-09	36.179
rs2617056	T	A	-0.036	0.005933	1.30E-09	36.81333
rs4242496	A	T	-0.033	0.005696	6.90E-09	33.56274
rs1516883	A	G	-0.091	0.002455	1.00E-200	1373.877
rs913588	A	G	-0.034	0.005683	2.20E-09	35.78795
rs12003641	T	C	0.082	0.010555	7.90E-15	60.36044
rs7853970	C	T	-0.037	0.00626	3.40E-09	34.9401
rs1874984	C	G	0.037	0.006005	7.20E-10	37.96574
rs7103411	T	C	-0.043	0.007056	1.10E-09	37.13905
rs7944630	A	G	0.047	0.005728	2.30E-16	67.32707
rs10840031	A	G	0.038	0.006406	3.00E-09	35.18383
rs12291726	G	A	0.057	0.008333	7.90E-12	46.79035
rs11022756	C	A	-0.048	0.006481	1.30E-13	54.8514
rs16938437	T	C	-0.067	0.010348	9.50E-11	41.9215
rs7119712	A	G	-0.041	0.006445	2.00E-10	40.46668
rs9565073	C	T	0.034	0.005881	7.40E-09	33.42673
rs9555810	G	C	0.047	0.006492	4.50E-13	52.41183
rs10144321	G	A	-0.042	0.006618	2.20E-10	40.28039
rs10483727	C	T	-0.037	0.005785	1.60E-10	40.90267
rs12148769	A	G	-0.055	0.009752	1.70E-08	31.81021
rs3743266	C	T	-0.045	0.006233	5.20E-13	52.12785
rs12915845	T	C	-0.035	0.005729	1.00E-09	37.32486
rs1659127	A	G	0.044	0.006372	5.00E-12	47.68702
rs12598642	G	A	0.044	0.005681	9.50E-15	59.99695
rs9939609	A	T	-0.042	0.00572	2.10E-13	53.90896
rs9635759	A	G	0.058	0.006362	7.70E-20	83.12575
rs1398217	C	G	0.046	0.005767	1.50E-15	63.63162
rs2303100	T	C	0.038	0.005684	2.30E-11	44.69706
rs4801589	G	C	0.032	0.005628	1.30E-08	32.33138
rs852069	G	A	0.036	0.005907	1.10E-09	37.13897
rs2836950	G	C	-0.035	0.006206	1.70E-08	31.8101

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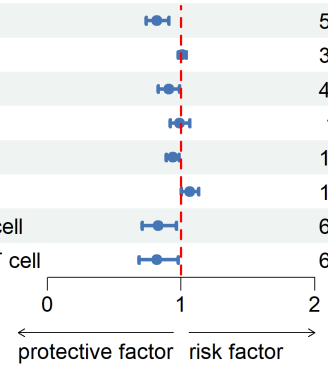
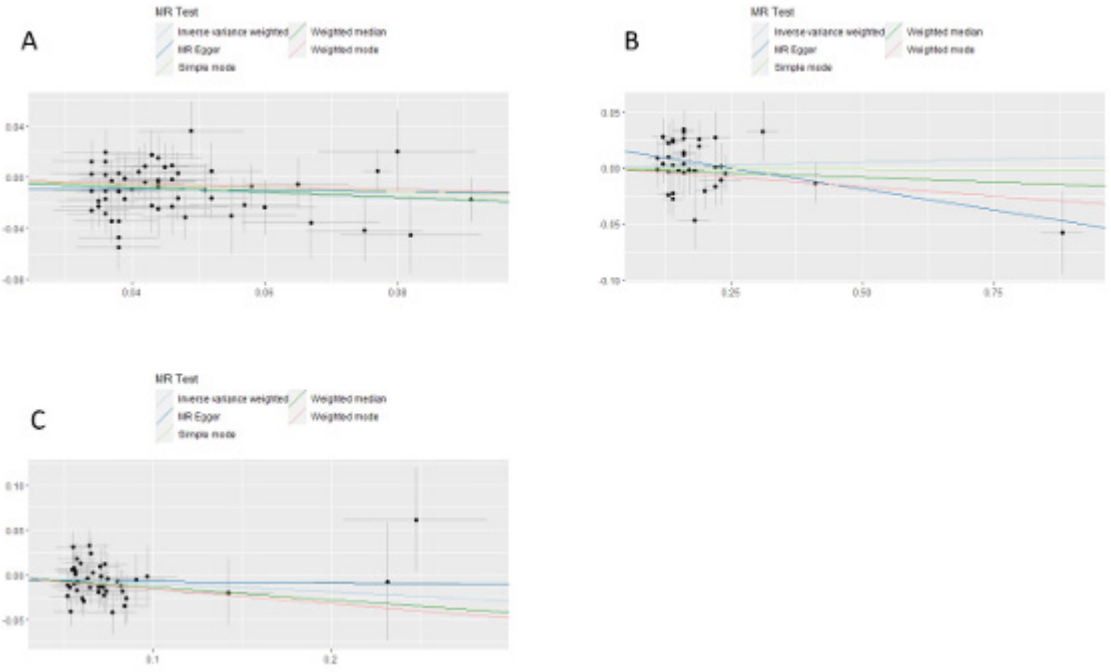
Figure 2 : Univariate MR analysis

Exposure	Outcome	Nsnp	P	OR(95%CI)
AAM	NAFLD	56	0.0001	0.82(0.74 to 0.91)
ANM	NAFLD	37	0.5385	1.01(0.98 to 1.04)
AFB	NAFLD	43	0.0286	0.91(0.83 to 0.99)
NAFLD	AAM	1	0.7500	0.99(0.92 to 1.06)
CD25 on switched memory B cell	NAFLD	19	0.0125	0.94(0.89 to 0.99)
CD127 on CD28+ CD4-CD8- T cell	NAFLD	18	0.0458	1.06(1.00 to 1.13)
AAM	CD25 on switched memory B cell	61	0.0164	0.83(0.71 to 0.97)
AAM	CD127 on CD28+ CD4-CD8- T cell	61	0.0281	0.82(0.68 to 0.98)



Supplementary materials

Supplementary Figure 1 : Scatter plots showing the effect of AAM, ANM, AFB on the risk of NAFLD A: AAM; B: ANM; C: AFB.



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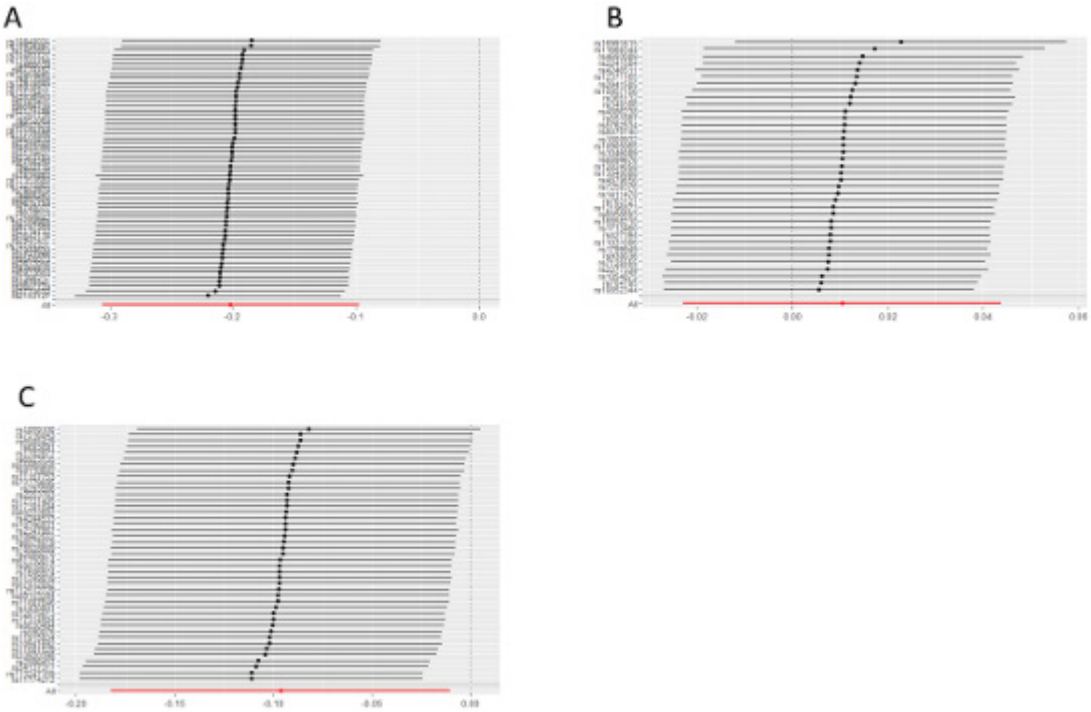
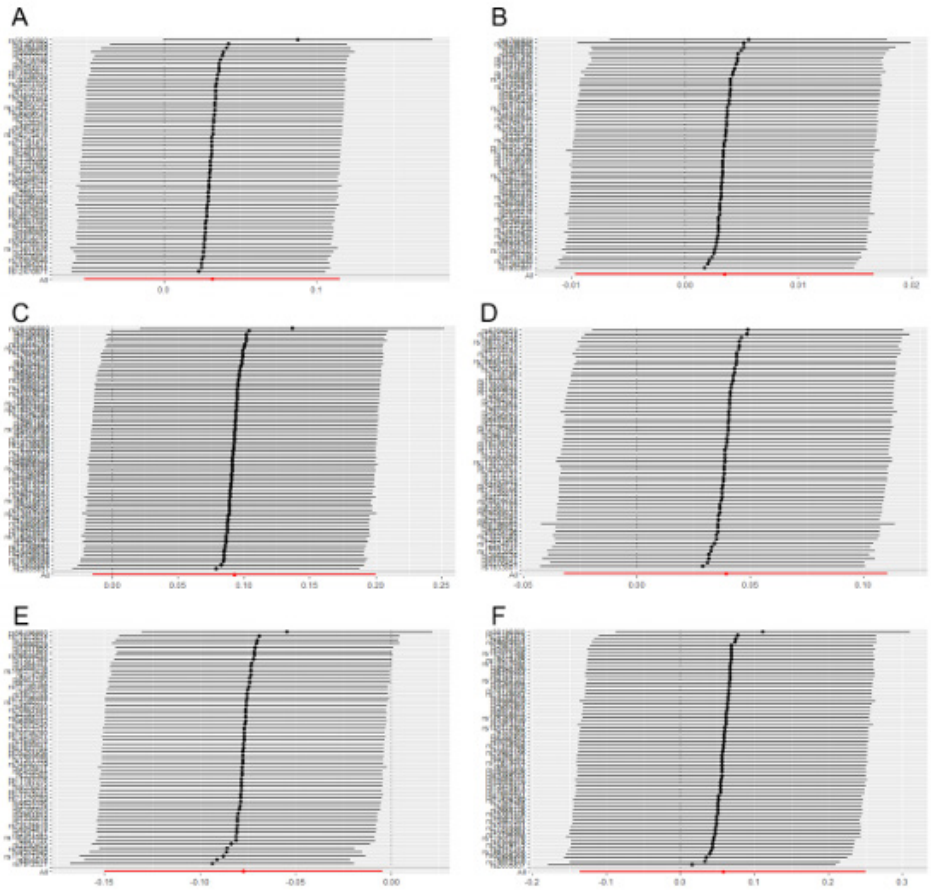
Supplementary Fig 2 : Leave-one-out plots which detect outliers SNPs in AAM, ANM, AFB on the risk of NAFLD A: AAM; B: ANM; C: AFB.



Supplementary Fig.3 Leave-one-out plots which detect outliers SNPs in male BT and CVD A: coronary artery disease; B: hypertension; C: myocardial infarction; D: heart failure; E: atrial fibrillation; F: peripheral atherosclerosis



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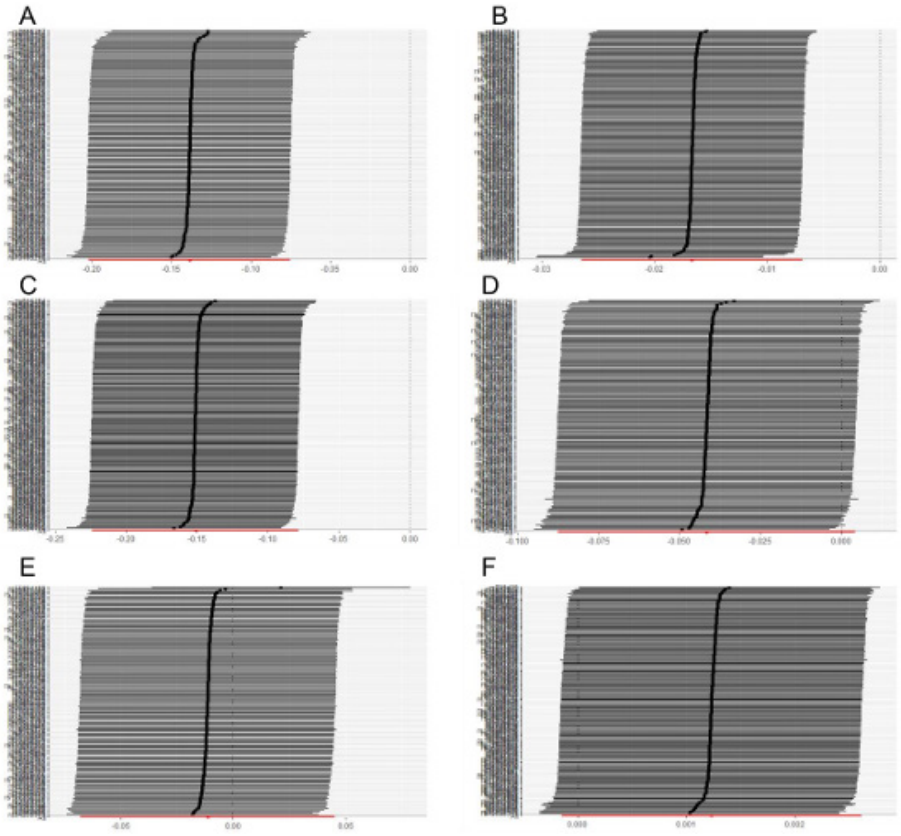
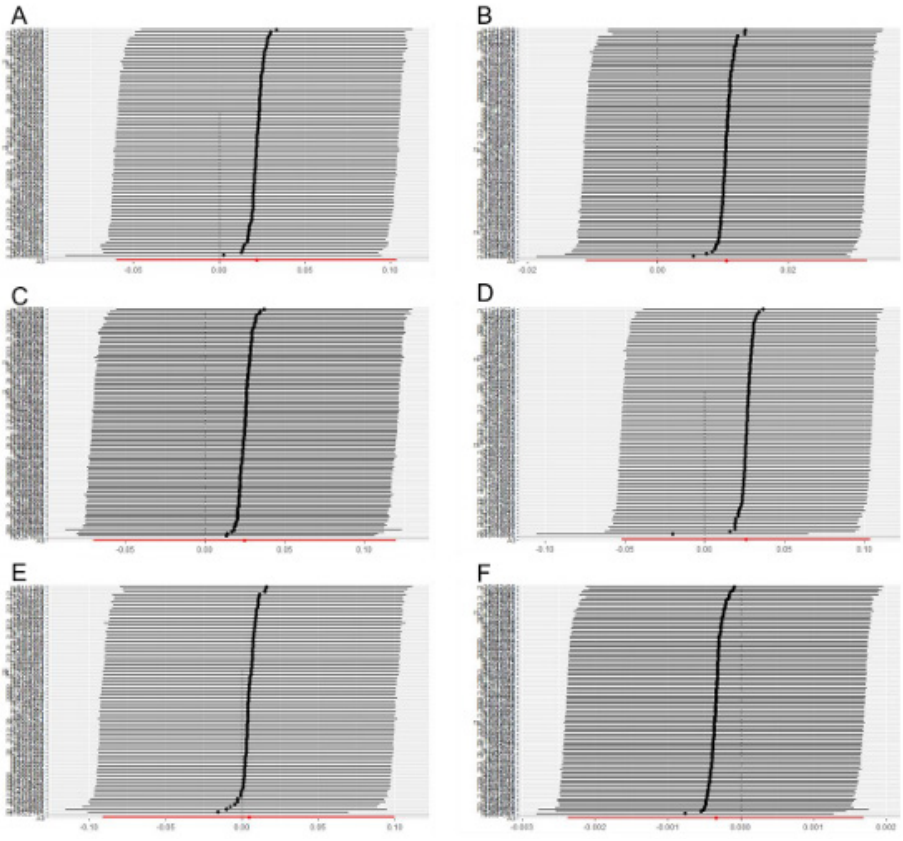
Supplementary Figure 4



Supplementary Figure.4 Leave-one-out plots which detect outliers SNPs in male SHBG and CVD A: coronary artery disease; B: hypertension; C: myocardial infarction; D: heart failure; E: atrial fibrillation; F: peripheral atherosclerosis

Supplementary Figure .5



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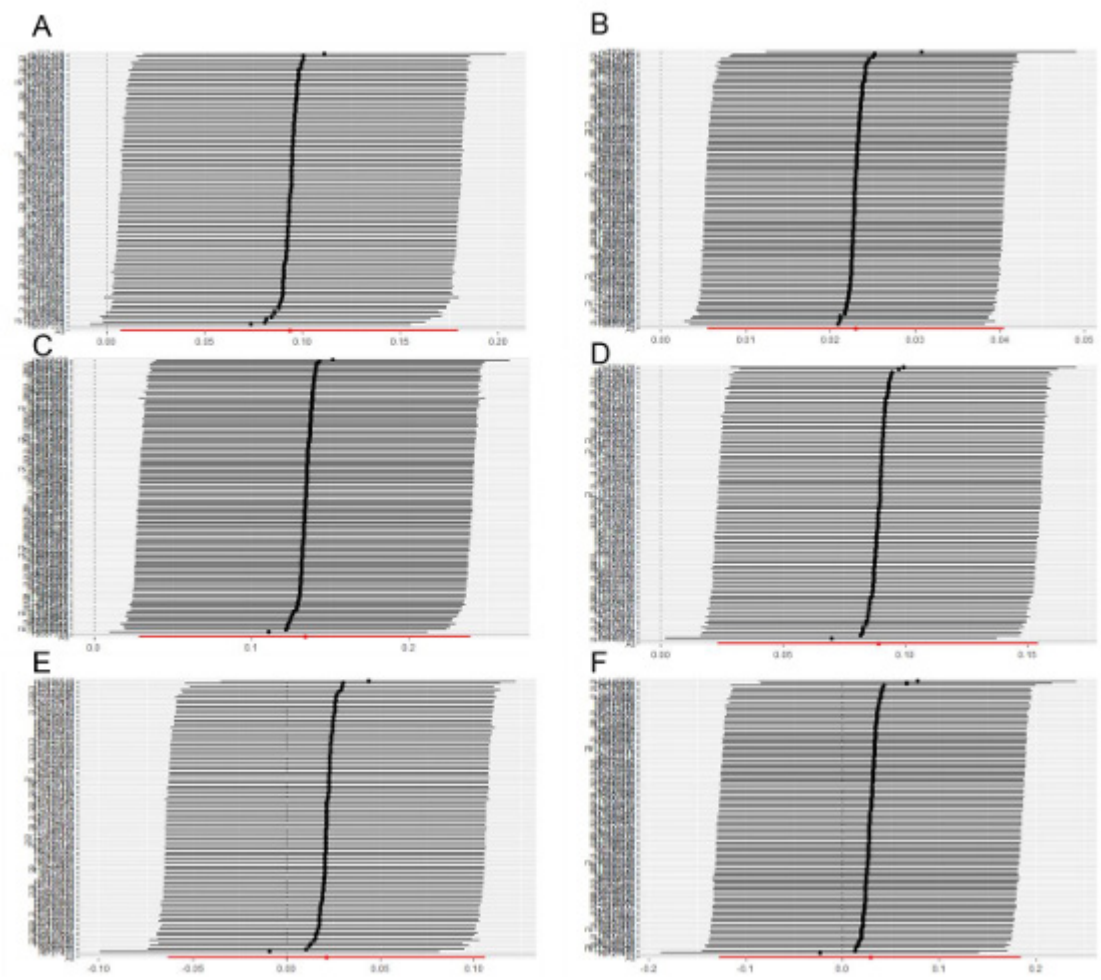
Supplementary Fig.5 Leave-one-out plots which detect outliers SNPs in female TT and CVD A: coronary artery disease; B: hypertension; C: myocardial infarction; D: heart failure; E: atrial fibrillation; F: peripheral atherosclerosis

Supplementary Figure 6 :



Supplementary Fig.6 Leave-one-out plots which detect outliers SNPs in female BT and CVD A: coronary artery disease; B: hypertension; C: myocardial infarction; D: heart failure; E: atrial fibrillation; F: peripheral atherosclerosis

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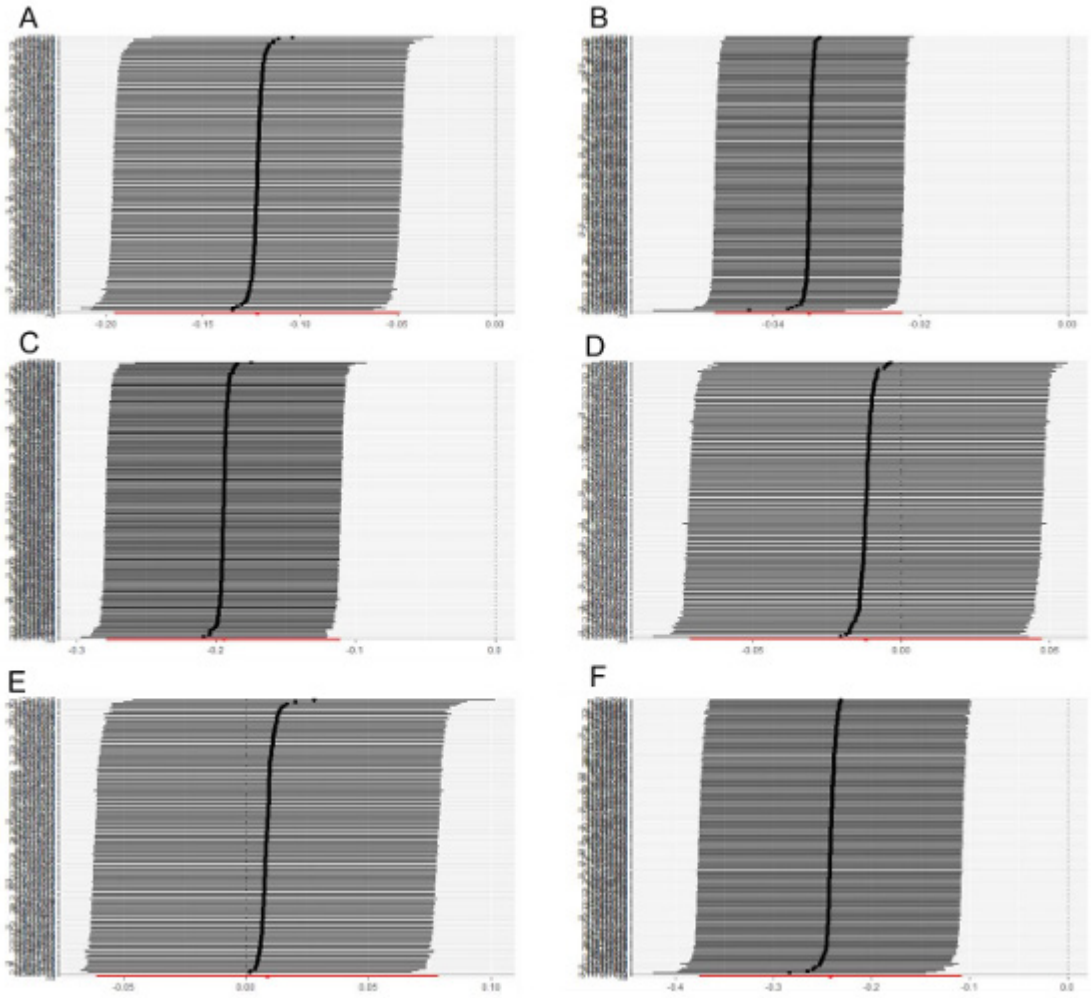
Supplementary Fig.7



Supplementary Figure 7 Leave-one-out plots which detect outliers SNPs in female SHBG and CVD A: coronary artery disease; B: hypertension; C: myocardial infarction; D: heart failure; E: atrial fibrillation; F: peripheral atherosclerosis

Mediation analysis

We performed a two-step MR analysis to estimate the mediating role of immune cells between AAM and NAFLD. AAM was causally associated with CD25 on switched memory B cell (OR = 0.83, 95% CI: 0.71-0.97, P = 0.016) and CD127 on CD28+ CD4-CD8- T cell (OR = 0.82, 95% CI: 0.68-0.98, P = 0.028. When immune cells were the exposure and NAFLD was the outcome, we found that CD25 on switched memory B cell was protective against NAFLD (OR = 0.94, 95% CI: 0.89-0.99, P = 0.012). However, CD127 on CD28+ CD4-CD8- T cell increased the risk of NAFLD (OR = 1.06, 95% CI: 1.00-1.13, P = 0.045). See **Fig 2.** The results revealed that CD25 on switched memory B cell had a mediating effect on AAM and NAFLD (β = 0.01231), with a mediating effect percentage of 6.23%. CD127 on CD28+ CD4 CD8- T cell might mediate the transition from AAM to NAFLD (β = -0.0125), with a mediating effect percentage of 6.68%.

DISCUSSION

We investigated potential causal relationships between female reproductive factors and NAFLD risk using Two-sample MR analysis. Univariate MR findings revealed a significant negative causal association between AAM, AFB and NAFLD, suggesting that early AAM, AFB may increase the risk of NAFLD. However, we did not find a clear association between ANM and NAFLD. These results provide valuable insights into the underlying biological mechanisms of NAFLD and suggest that AAM, AFB may be a potential risk factor for NAFLD. Furthermore, immune cells may mediate the effects of AAM on NAFLD. Our findings on the causal relationship between AAM, AFB, and NAFLD confirm previous epidemiologic studies. A paired cohort study including a multiethnic population showed that age at menarche was associated with the risk of NAFLD; women with age at menarche >11 years had a lower risk of NAFLD compared with women with age at menarche <11 years(30). A retrospective cohort study

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in the United States found that the odds of hepatic steatosis were significantly lower when women had an AFB >35 years, which may be related to waist circumference(31). Dividing women by age revealed that women with AFB <18, 18-20, 21-23, and 24-26 years of age had a 54%, 40%, 60%, and 33% increased risk of NAFLD, respectively, compared with participants with AFB aged 30-32 years(16)$_{o}$Relevant previous studies have shown that the prevalence of NAFLD in women becomes larger with age, with 3.9% in the 21-39 year old group; 7.6% in the 40-49 year old group; and a gradual increase in prevalence after age 50 (about 14%), peaking at age 60-69 years (about 18.9%), and a gradual increase in prevalence after age 60-69 years (about 18.9%) and decreases after the age of 70 years(32). Unlike past studies, our MR study did not find a correlation between age at menopause and NAFLD.

Early menarche can have negative effects on women's health, such as psychiatric disorders, metabolic syndrome, and cardiovascular disease(33, 34). The age at menarche has a significant impact on the body's metabolic function. Early menarche is a risk factor for metabolic syndrome. When a woman's age at menarche decreases by one year, the risk of metabolic syndrome increases by 8%(35). NAFLD involves multi-system disease and is not only a predictor of metabolism but is also strongly associated with metabolic syndrome(36). It has also been suggested that the negative association between AAM and NAFLD is mediated by obesity(37). A meta-analysis of genome-wide association studies found that several novel loci for age at menarche overlap with genetic loci related to weight regulation, BMI. In addition, studies of the majority of alleles associated with higher BMI and waist-to-hip ratio also showed an association with early menarche(38). Obesity leads to the release of large amounts of inflammatory adipokines and excess free fatty acids in the body, which promote hepatic steatosis and lipotoxicity(39). In female rats with obesity due to overfeeding, not only do they show precocious puberty, but also lead to increased levels of synthesized ceramides in the hypothalamus(40). Ceramides have an important role in the onset and progression of NAFLD. Ceramides are a class of bioactive sphingolipids composed of fatty acid chains that act as signaling molecules that are not only involved in cell proliferation, apoptosis, and senescence, and modulate stress responses, but also cause lipids to accumulate in the liver(41). The continuous accumulation of ceramides in the liver induces insulin resistance, mediates apoptosis, increases oxidative stress and inflammation, and ultimately leads to lipid deposition, inflammation and fibrosis(42).

Early AFB causes metabolic disturbances in the body, which increase the risk of obesity, hypertension, and metabolic diseases(43). These risks are closely associated with NAFLD. The immature physical development of adolescents, changes in hormone levels during pregnancy, and increased insulin resistance induced by hyperglycemia may be detrimental to normal organ development(44). Evidence from previous cross-sectional studies suggests that overweight adolescents are associated with an increased risk of NAFLD in multivariate regression models, with the prevalence of NAFLD in overweight adolescents as high as 34.2%. In addition, from a socioeconomic perspective, women with early AFB are more likely to have low levels of education and mental immaturity, which is frequently accompanied by health-hazardous behaviors such as smoking, violence, and alcohol abuse(45).

Estrogen inhibits glucagon secretion, regulates lipogenesis and fatty acid oxidation, and intervenes in reverse cholesterol output(46–48). Therefore, estrogen is considered a protective factor in NAFLD. After menopause, estrogen levels decline severely in women. Numerous studies have shown that the risk of NAFLD is significantly increased in menopausal women (15, 32). Our study did not find an association between ANM and NAFLD, which may be related to hormone replacement therapy (HRT). The results of a randomized controlled trial on HRT showed that low doses of HRT were effective in reducing serum liver function enzyme concentrations. Results of a randomized controlled trial of HRT showed that low doses of HRT were effective in reducing serum liver function enzyme concentrations(49). Another reported that waist circumference, HOMA-IR index, ferritin levels, and glutamyltransferase were all lower in postmenopausal women on HRT compared to postmenopausal women who did not use HRT(50). Liver histology using abdominal ultrasound found that The incidence of NAFLD was lower in women taking hormone replacement therapy than in women not taking hormone replacement therapy(51).

We observed an association between CD25 on switched memory B cell and a reduced risk of NAFLD. CD127 on CD28+ CD4-CD8- T cell increases the risk of NAFLD. These two immune cells may mediate the effects of AAM on NAFLD. Memory B cells are a specialized subtype of B cells that differentiate to counteract their original recognition function after the first infection, and play an important role in generating an accelerated and more potent antibody-mediated immune response (also known as the secondary immune response) in the event of re-infection(52). T cells are lymphocytes that mature in the thymus. Conventional T cells can be broadly categorized into CD8 and CD4 T cells Regulation of CD8 T cell abundance, activity, or function has been associated with the evolution of nonalcoholic steatohepatitis (NASH)(53). CD4 T cells are recruited to the liver through integrin-ligand interactions, exacerbating hepatic inflammation and fibrosis and ultimately accelerating the development of NAFLD(54). For women with AAM, valuing immune cells can help better research and treatment of NAFDL. The rate of NAFLD has been increasing yearly, causing enormous harm to public health and socio economics. Therefore, the identification and screening of people with high risk of NAFLD has become

particularly important. In this MR study, we found a significant association between AAM, AFB and NAFLD. More attention from multidisciplinary physicians and educators is necessary for women with early AAM, AFB. Understand the dangers associated with early menarche and premature childbearing with special attention to active monitoring and management of NAFLD in this group. Finally, further research is required on the link between female reproductive factors and NAFLD in order to provide solutions for policy makers to attack NAFLD.

Strengths and Limitations

Our findings show a causal relationship between AAM, AFB and NAFLD and that women's first menstrual period as well as young age at first child birth may increase the risk of NAFLD. MR has the advantage of controlling for confounding factors compared to traditional observational studies. Using recently published large-scale GWAS data, we carried out several sensitivity analyses and F-corrections to adjust for confounders to assess the robustness of our results. There are several limitations of this study to note. First, we selected a population of European ancestry, which reduces the influence of population stratification bias on the results but also limits the applicability of our results to other ethnic populations. Second, we used NAFLD as the only outcome indicator without delving into the associated mechanisms of action and the impact of AAM, ANM, and AFB on other liver diseases. Finally, our study design was not stratified by age, and thus we were unable to obtain the effect of reproductive factors on the risk of NAFLD in women at different ages. Despite revealing a causal relationship between AAM, AFB and NAFLD in women, the underlying mechanisms are unknown.

CONCLUSION

In conclusion, our findings indicated that reproductive factors (AAM, AFB) play an important role in NAFLD risk. Clinical trials and observational studies are still needed to discover more information about the mechanisms involved.

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Disclosures

The authors have no financial conflicts of interest.

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