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PUFA and intrahepatic cholestasis of pregnancy: a two-sample Mendelian randomisation analysis

Qiong Li[1†], Xinchun Xu[2†], Chenyang Zhao[1†], Yonghong Wang[1], Xiaohu Chen[3], Miao Liu[1*] and Chaoyan Yue[3*]

[1]*Department of Obstetrics and Gynecology, The First People's Hospital of Chenzhou, Chenzhou, People's Republic of China*
[2]*Department of Ultrasound, Zhangjiagang Hospital Affiliated to Soochow University, Suzhou, People's Republic of China*
[3]*Obstetrics and Gynecology Hospital of Fudan University, Shanghai, People's Republic of China*

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Abstract

This study aimed to explore the potential causal association between PUFA and the risk of intrahepatic cholestasis of pregnancy (ICP) using Mendelian randomisation (MR) analysis. A two-sample MR analysis was conducted utilising large-scale European-based genome-wide association studies summary databases. The primary MR analysis was carried out using the inverse variance-weighted (IVW) method, complemented by other methods such as MR-egger, weighted-median and weighted mode. Sensitivity analysis was also performed to validate the robustness of the findings. Results indicated a 31 % reduced risk of ICP for every 1 standard deviation (SD) increase in *n*-3 fatty acids levels (OR = 0·69, 95 % CI: 0·54, 0·89, $P = 0·004$) and in the ratio of *n*-3 fatty acids to total fatty acids (OR = 0·69, 95 % CI: 0·53, 0·91, $P = 0·008$). Conversely, there was a 51 % increased risk of ICP for every 1 SD increase in the ratio of *n*-6 fatty acids to *n*-3 fatty acids (OR = 1·51, 95 % CI: 1·20, 1·91, $P < 0·001$) and a 138 % increased risk for every 1 SD increase in the ratio of linoleic fatty acids to total fatty acids (OR = 2·38, 95 % CI: 1·55, 3·66, $P < 0·001$). The findings suggest that *n*-3 fatty acids may have a protective effect against the risk of ICP, while *n*-6 fatty acids and linoleic fatty acids could be potential risk factors for ICP. The supplementation of *n*-3 fatty acids, as opposed to *n*-6 fatty acids, could be a promising strategy for the prevention and management of ICP.

Keywords: **PUFA: Intrahepatic cholestasis of pregnancy: Mendelian randomisation analysis**

Intrahepatic cholestasis of pregnancy (ICP) is a serious hepatic disease that usually occurs during the second or third trimester of pregnancy. It is characterised by symptoms such as pruritus, elevated serum transaminases and high total serum bile acid levels[1]. While it is not harmful to pregnant women, it can have adverse effects on the fetus, including an increased risk of preterm birth, meconium-stained amniotic fluid, neonatal depression, respiratory distress syndrome and even stillbirth. Therefore, early prediction and diagnosis are important for the prevention and treatment of ICP. However, the exact aetiology and pathogenesis of ICP remain unclear. Several studies have suggested that inflammation, oxidative stress, lipid metabolism, cell growth and the immune response play a role in the development of ICP[2]. In addition, a recent research has shown that metabolic abnormalities such as impaired glucose tolerance and dyslipidaemia may also be associated with ICP[3].

Polyunsaturated fatty acids (PUFA) are essential dietary nutrients that have been linked to various diseases, including metabolic diseases, cardiovascular diseases, malignancies and neuropsychosis. *n*-3 and *n*-6 fatty acids are two important families of PUFA that have been extensively studied in relation to human health[4]. *n*-3 fatty acids have been found to have hypolipidemic and metabolic protective effects in humans, including preserving insulin sensitivity, lowering triglycerides and reducing inflammatory mediators[5,6]. In contrast, *n*-6 fatty acids, particularly linoleic acid, have biological functions that often oppose those of *n*-3 fatty acids. For example, *n*-3 PUFA can be converted into docosahexaenoic acid (DHA), eicosapentaenoic acid and prostaglandin[7], while *n*-6 PUFA can be converted into some biologically active derivatives, including PG, thromboxane, hydroxyeicosatetraenoic acids and LT[8]. It has been reported that dietary supplementation with eicosapentaenoic acid and DHA can decrease low-density lipoprotein cholesterol synthesis, while dietary supplementation with AA and thromboxane may increase serum cholesterol[9]. An imbalance in the ratio of *n*-6 to *n*-3 fatty acids has been associated with prothrombotic and pro-inflammatory conditions, contributing to the prevalence of obesity and diabetes[10]. Given the

Abbreviations: ICP, intrahepatic cholestasis of pregnanc; PUFA, polyunsaturated fatty acid; IVW, inverse variance weighted; MR, Mendelian randomisation.

* **Corresponding authors:** Miao Liu, email liumiao3567@163.com; Chaoyan Yue, email 20111250007@fudan.edu.cn

† These authors contribute equally to the research.

associations between PUFA and metabolic diseases, it is hypothesised that PUFA may also have an impact on ICP. Some studies have suggested that *n*-3 fatty acid supplementation can reduce liver fat and improve hepatic enzyme parameters[11]. Another study reported that the overexpression of ACOX1, a gene involved in fatty acid metabolism, contributes to the development of ICP[12]. However, a prospective controlled study suggested that the formation of oxidative products from *n*-3 PUFA may cause lipid peroxidation damage in ICP[13]. So far, there are no observational studies that directly investigate the relationship between PUFA and ICP.

Therefore, it is necessary to investigate the causal effect of PUFA on ICP. Mendelian randomisation (MR) is an emerging epidemiological method that uses genetic variation as an instrumental variable to assess the causal association between exposures and outcomes[14]. MR exploits genetic variants as instrumental variables to proxy the exposures of interest and evaluates the causal estimates of the outcomes. Compared to traditional observational studies, MR has several advantages in terms of minimising confounding and reverse causality biases. Furthermore, MR is particularly suitable for investigating deleterious factors and biological measurements, which may be challenging to manage in prospective cohorts. In this study, we employed a two-sample MR method and utilised two large-scale metabolic profiling genome-wide association study datasets to provide novel genetic evidence supporting the causal association between several PUFA and ICP.

Methods

Study design

In order to investigate the potential causal relationships between PUFA and the risk of ICP, a two-sample MR study was conducted utilising summary genetic associations from multiple genome-wide association studies. The study focused on *n*-3 fatty acids levels, ratio of *n*-3 fatty acids to total fatty acids, ratio of *n*-6 fatty acids to *n*-3 fatty acids, ratio of linoleic acids to total fatty acids and ICP. Various MR analysis methods, including the inverse-variance-weighted (IVW) model, MR-Egger regression, weighted-median estimator and weighted mode, were utilised to examine the associations between genetically determined PUFA and ICP. Sensitivity analyses, including Cochran's Q test, MR-Egger intercept and MR-PRESSO globlal test were conducted to assess the robustness of the MR analysis.

Data sources

Genetic instruments associated with *n*-3 fatty acids levels, ratio of *n*-3 fatty acids to total fatty acids, ratio of *n*-6 fatty acids to *n*-3 fatty acids, ratio of linoleic acids to total fatty acids were obtained from the UK Biobank which contained 115 006 samples and 11 590 399 SNP[15]. The genome-wide association study-aggregated data for ICP was obtained from the FinnGen project, a global research project in Europe, available at https://pubmed.ncbi.nlm.nih.gov/35977952/.

To initiate our MR analysis, we selected SNP that were significantly associated with *n*-3 fatty acids levels, ratio of *n*-3 fatty acids to total fatty acids, ratio of *n*-6 fatty acids to *n*-3 fatty acids, ratio of linoleic acids to total fatty acids in relation to ICP. First, we identified SNP reaching genome-wide significance ($P < 5 \times 10^{-8}$) in the UK Biobank. Next, we excluded SNP in linkage disequilibrium using the PLINK algorithm with an r^2 threshold < 0·001 and a window size greater than 10 000 kb. To ensure the effects of the SNP on exposures and outcomes corresponded to the same effect allele, we harmonised the direction of the SNP effects. Finally, we calculated the F statistic for each SNP to determine its statistical strength. SNP with an F statistic greater than 10 were deemed robust enough to avoid weak instrument bias. Ultimately, we selected a set of thirty-six common and uncorrelated SNP as genetic instruments for *n*-3 fatty acid levels, twenty-five SNP for the ratio of *n*-3 fatty acids to total fatty acids, twenty-three SNP for the ratio of *n*-6 fatty acids to *n*-3 fatty acids and twenty-eight SNP for the ratio of linoleic acid to total fatty acids, respectively.

Statistical analyses

The IVW method served as our primary statistical model to estimate the associations between the exposures and the outcome. It is important to note that the IVW method provides reliable causal estimates when there is an absence of horizontal pleiotropy[16]. In addition to the IVW method, we utilised MR-Egger, weighted median and weighted mode as supplementary methods to ensure the robustness of our findings. MR-Egger corrects for and detects horizontal pleiotropy, providing valuable estimates, although its assessment is less effective than the IVW method[17]. Weighted median uses a median-based approach combined with rate estimation from genetic instruments, offering reliable estimates even when up to 50 % of the instruments are invalid[18]. Weighted mode is utilised for MR analysis when multiple instrumental variables are included to ascertain the direction and strength of causality.

Furthermore, we performed a series of sensitivity tests to further validate our MR results. Heterogeneity among genetic variations was assessed using Cochran's Q statistic, with heterogeneity considered present when $P < 0{\cdot}05$[18]. Additionally, the MR-Egger intercept was employed to test for the presence of horizontal pleiotropy in the analyses, with a *P*-value greater than 0·05 indicating the absence of significant horizontal pleiotropy in the study. The MR-PRESSO global test was employed to detect outliers and provide a refined causal estimate after the removal of corresponding outliers. Lastly, we used the leave-one-out method to assess the potential effect of specific SNP on the observed causal effects by systematically removing individual SNP. All analyses were carried out using R software (version 4.3.0) from the R Foundation for Statistical Computing, Vienna, Austria.

Results

Results of the Mendelian randomisation study testing causal association

Scatter plots to visualise the causal effects of SNP on the incidence of ICP are displayed in Fig. 1. Although the effects of the individual SNP varied, the overall impact is notable. The IVW

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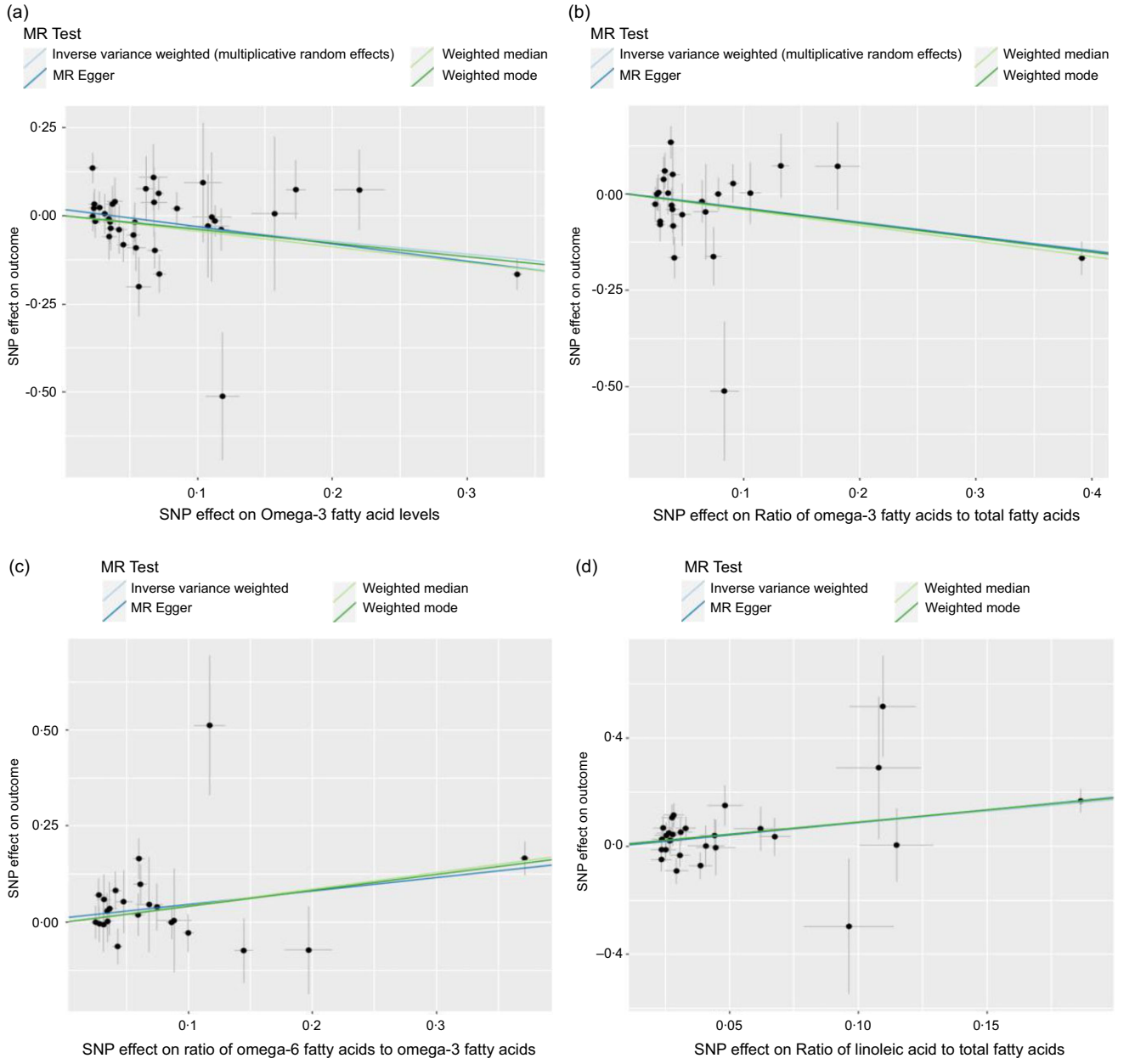

Fig. 1. Scatter plots in which the SNP outcome associations with ICP. (a) The associations of *n*-3 fatty acid level on ICP; (b) The associations of *n*-3 fatty acids to total fatty acids with ICP; (c) The associations of ratio of *n*-6 fatty acid to *n*-3 fatty acid with ICP; (d). The associations of ratio of linoleic acid to total fatty acid with ICP. ICP, intrahepatic cholestasis of pregnancy; MR, Mendelian randomisation.

method indicated a 31 % reduction in the risk of ICP for each 1 standard deviation (SD) increase in *n*-3 fatty acids levels (OR = 0·69, 95 % CI: 0·54, 0·89, $P = 0·004$), highlighting a positive effect of *n*-3 fatty acids on ICP. Similar findings were observed for the impact of the ratio of *n*-3 fatty acids to total fatty acids on ICP (OR = 0·69, 95 % CI: 0·53, 0·91, $P = 0·008$). Conversely, the risk of ICP increased by 51 % with every 1 SD rise in the ratio of *n*-6 fatty acids to *n*-3 fatty acids (OR = 1·51, 95 % CI: 1·20, 1·91, $P < 0·001$), and by 138 % with each 1 SD increase in the ratio of linoleic acid to total fatty acids (OR = 2·38, 95 % CI: 1·55, 3·66, $P < 0·001$), indicating a negative impact of the ratios of *n*-6 fatty acids to *n*-3 fatty acids and linoleic acid to total fatty acids on ICP (Table 1).

Analysis of heterogeneity and horizontal pleiotropy

The Cochrane's Q test within the IVW analysis revealed no significant heterogeneity among SNPs related to *n*-6 fatty acids to *n*-3 fatty acids ($P = 0·11$) and the ratio of linoleic acids to total fatty acids ($P = 0·10$). However, heterogeneity was observed among SNP associated with *n*-3 fatty acids levels ($P = 0·02$) and the ratio of *n*-3 fatty acids to total fatty acids ($P < 0·001$). No evidence of directional pleiotropy was found among the four types of PUFA and ICP. The *P*-values for MR-Egger intercept and MR-PRESSO in the sensitivity analysis of *n*-3 fatty acid levels, *n*-3 fatty acids to total fatty acids, the ratio of *n*-6 fatty acids to *n*-3

Table 1. MR analysis of causal associations of PUFA with ICP (OR and 95 % CI)

	IVW			MR-Egger			Weighted Median			Weighted Mode		
PUFA	OR	95 % CI	*P*	OR	95 % CI	*P*	OR	95 % CI	*P*	OR	95 % CI	*P*
n-3 levels	0·69	0·54, 0·89	0·004	0·61	0·44, 0·86	0·007	0·64	0·50, 0·82	< 0·001	0·68	0·53, 0·86	0·004
n-3/total fatty acids ratio	0·69	0·53, 0·91	0·008	0·69	0·48, 0·98	0·049	0·67	0·53, 0·83	< 0·001	0·68	0·55, 0·86	0·003
n-6/*n*-3 ratio	1·51	1·20, 1·01	< 0·001	1·42	1·03, 1·94	0·041	1·54	1·23, 1·93	< 0·001	1·51	1·21, 1·89	0·002
Linoleic acid /total fatty acids	2·38	1·55, 3·66	< 0·001	2·55	1·34, 4·83	0·008	2·46	1·58, 3·82	< 0·001	2·45	1·58, 3·80	< 0·001

MR, Mendelian randomisation; ICP, intrahepatic cholestasis of pregnancy; IVW, inverse-variance weighted.

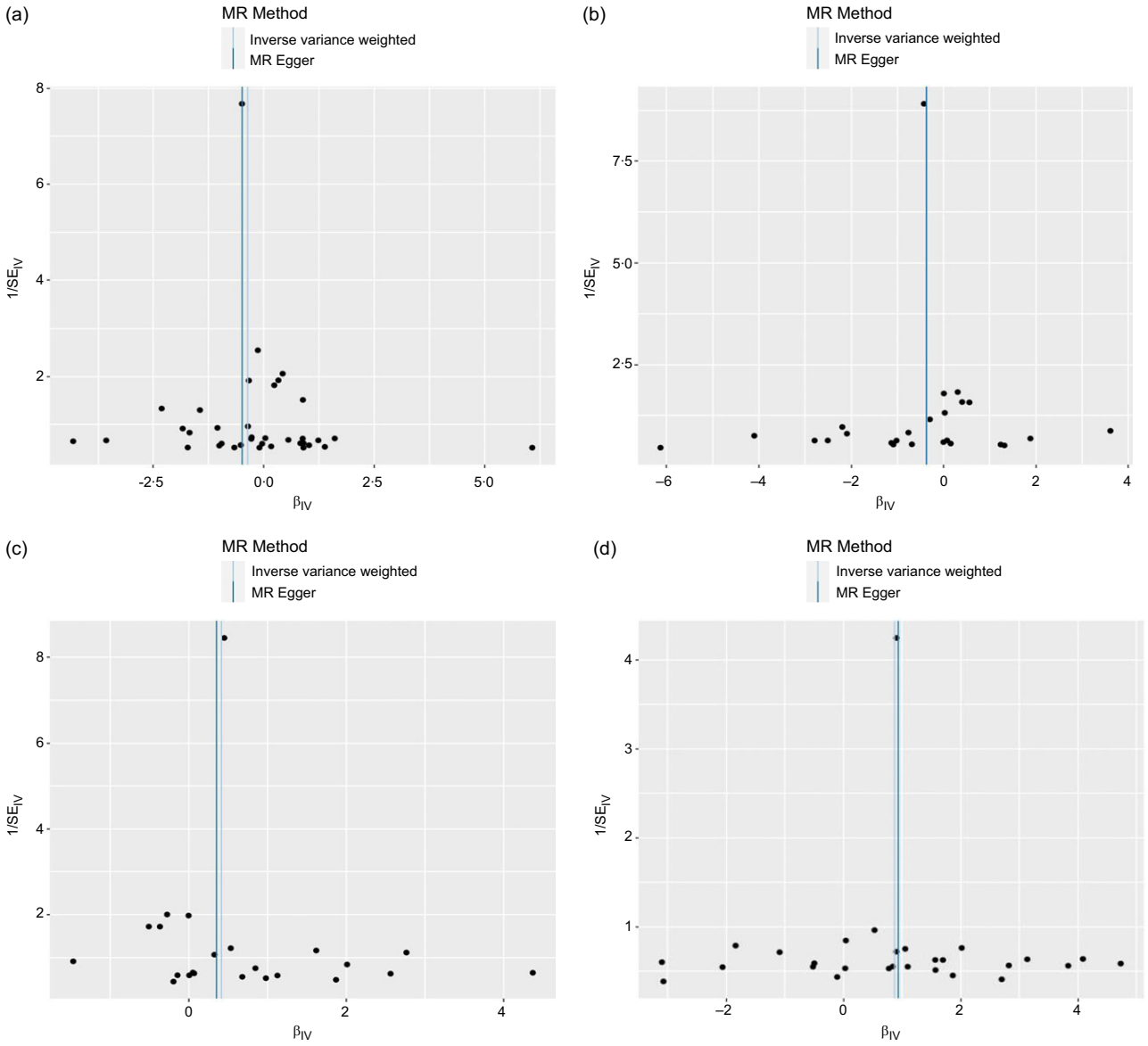

Fig. 2. Funnel plots to visualise overall heterogeneity of MR estimates for the effect of PUFA on the risk of ICP. (a) The associations of *n*-3 fatty acid level on ICP; (b) The associations of *n*-3 fatty acids to total fatty acids with ICP; (c) The associations of ratio of *n*-6 fatty acid to *n*-3 fatty acid with ICP; (d) The associations of ratio of linoleic acid to total fatty acid with ICP. MR, Mendelian randomisation; ICP, intrahepatic cholestasis of pregnancy.

fatty acids, and the ratio of linoleic acid to total fatty acids with respect to ICP were 0·29, 0·95, 0·55, 0·78 and 0·06, 0·09, 0·25, 0·17, respectively, all satisfying the condition $P > 0·05$ (Fig. 2 and Table 2). Moreover, leave-one-out analyses suggested that the causal estimates remained robust against the influence of individual SNP (Fig. 3).

Table 2. Association of PUFA with ICP in sensitivity analysis

	Heterogeneity		Pleiotropy	
PUFA	Q of IVW	*P* of IVW	*P* value of Egger Intercept	*P* value of MR-PRESSO
n-3 levels	53·59	0·02	0·29	0·06
n-3/total fatty acids ratio	47·14	< 0·001	0·95	0·09
n-6/*n*-3 ratio	30·19	0·11	0·55	0·25
Linoleic acid/total fatty acids	36·86	0·10	0·78	0·17

ICP, intrahepatic cholestasis of pregnancy; IVW, inverse-variance weighted; MR, Mendelian randomisation.

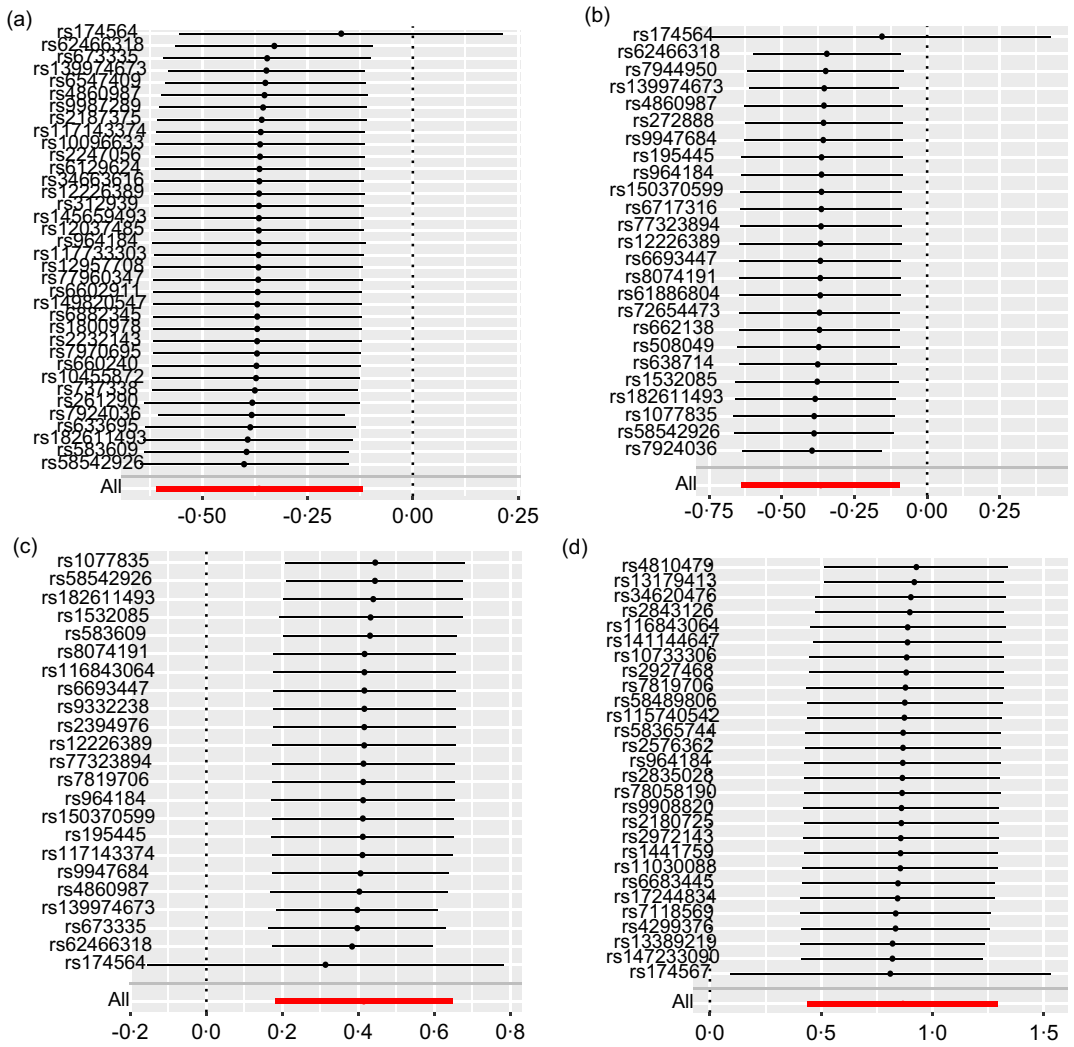

Fig. 3. Leave-one-out inverse-variance weighted MR analyses of PUFA on the risk of ICP. (a) The associations of *n*-3 fatty acid level on ICP; (b) The associations of *n*-3 fatty acids to total fatty acids with ICP; (c) The associations of ratio of *n*-6 fatty acid to *n*-3 fatty acid with ICP; (d) The associations of ratio of linoleic acid to total fatty acid with ICP. MR, Mendelian randomisation; ICP, intrahepatic cholestasis of pregnancy.

Discussion

In this study, we used a two-sample MR analysis to explore causal effect of several PUFA on ICP. Our principal findings revealed a significant decrease in the risk of ICP by 31 % for every 1 SD increase in *n*-3 fatty acids levels. Similarly, the ratio of *n*-3 fatty acids to total fatty acids also exhibited a positive effect on ICP. Conversely, the risk of ICP increased by 51 % for every 1 SD increase in the ratio of *n*-6 fatty acids to *n*-3 fatty acids, and by 138 % for every 1 SD increase in the ratio of linoleic acid to total fatty acids. These results highlight the potential benefits of *n*-3 fatty acids supplementation and suggest avoiding the use of *n*-6 or linoleic fatty acids for preventing ICP. This underscores the importance of nutritional interventions in primary ICP prevention.

While previous observational studies did not provide direct evidence of the relationship between PUFA and ICP, the protective role of high *n*-3 fatty acid levels in various metabolic disorders is well-established. Indeed, meta-analyses and randomised controlled trials have highlighted the potential benefits of *n*-3 fatty acids in conditions such as cholelithiasis, cholecystitis, obesity[19] and type 2 diabetes mellitus. For instance, *n*-3 fatty acids have been reported to improve glycaemic control, insulin secretion, lipid profiles and endothelial function in individuals with type 2 diabetes mellitus[20]. Our findings align with this existing evidence, demonstrating the protective effects of *n*-3 fatty acids on ICP. The mechanisms underlying the beneficial effects of *n*-3 fatty acids on ICP may involve multiple pathways. Firstly, metabolic studies have suggested that an increased intake of *n*-3 fatty acids may improve insulin sensitivity by changing the fatty acids composition of the adipocyte plasma membrane[21]. A classic case of highly aggravated dysregulation of the bile acid transport and thereby the bile acid metabolism was also observed in a combined state of dyslipidemia and diabetes in rats, which was reregulated on the administration of *n*-3 fatty acids[22]. Previous studies have also speculated that insulin resistance may participate in the pathogenesis of ICP by promoting the release of proinflammatory cytokines. Secondly, *n*-3 fatty acid can inhibit the occurrence of ICP by regulating serum triglyceride levels. Cholesterol can be converted into bile acid, so higher level of cholesterol produces more bile acid. A population-based study showed that TAG in the second trimester is significantly associated with the incidence of ICP, and triglyceridaemia can be considered as an important predicting biomarker of ICP. In addition, the risk of ICP also increased for every 1 mmol/l increase in serum TAG concentration in women during the third trimester[23]. A pilot study showed that concentrations of total cholesterol, LDL-C and triglyceride decreased following *n*-3 fatty acids administration[24]. In addition, *n*-3 fatty acids play critical role in systemic processes such as inflammation in addition to their roles in hepatic bile acid regulation and fat metabolism. Studies have shown that *n*-3 fatty acids served as immunomodulators and mediators of inflammation by virtue of activating several enzymes, including cytochrome P450, 5-LOX, 15-LOX and COX-2. These enzymes are dramatically up-regulated in the presence of inflammation[25,26]. The specialised pro-resolving mediators in turn block the production of pro-inflammatory leukotrienes, prostaglandins and interleukins (including LTB4, PGE2, IL6 and IL8)[25,27]. Dysregulation of inflammation and immunity in the body are mechanisms of ICP. Investigation of the *n*-3 fatty acid effect on macrophage reverse cholesterol transport in hamsters revealed that *n*-3 fatty acids, when supplemented with high-fat-diet, activated genes involved in macrophage to faeces cholesterol transport, causing higher fecal cholesterol and bile expulsion[28]. This is the direct evidence of positive effect of *n*-3 fatty acid on decreasing bile acid.

Our MR study found adverse association between the ratio *n*-6 to *n*-3, ratio of linoleic acid to total fatty acids and ICP, aligning with findings from previous studies. *n*-3 fatty acids mainly include alpha-linolenic acid (ALA), eicosapentaenoic acid and DHA which exert anti-inflammatory function, while *n*-6 fatty acids mainly include linoleic acid, gamma-linolenic acid (GLA), arachidonic acid, AdA and DPAn-6, which are pro-inflammatory. Accumulating evidence revealed that *n*-6 PUFA exert adverse effects on liver diseases, including induction of oxidative stress[29] and enhancement of inflammation[30]. Furthermore, a balanced ratio of *n*-6 and *n*-3 is important for good health and normal development. The western diet is heavily reliant on *n*-6 PUFA-rich sources (such as corn oil and soybean oil) at the expense of *n*-3 PUFA-rich food (such as oily fish), leading to an increase in the ratio of *n*-6 to *n*-3 fatty acids, which contributes to numerous deleterious health effects[31] and the development of various liver diseases[32]. Increased amounts of *n*-6 fatty acids lead to large formation of the eicosanoid metabolic products than those formed from *n*-3 fatty acids, such as prostaglandins, thromboxanes, leukotrienes, hydroxy fatty acids and lipoxins, which contribute to the formation of allergic and inflammatory disorders. Thus, a diet rich in *n*-6 fatty acids shifts the physiological state to one that is risky with some forms of atherosclerotic disease and diabetes. Researches have showed that diet rich in *n*-6 fatty acids resulted in intestinal inflammation[33] and gut microbiota alterations which lead to disorder of bile acid enterohepatic circulation. A recent study showed that decreasing ratio of *n*-6 to *n*-3 fatty acids leads to ethanol-induced alterations in intestinal homeostasis, followed by specific changes in intestinal homeostasis, the gut microbiota, bile acid metabolism and the plasma metabolome that coordinate to reduce intestinal inflammation and associated liver damage[34]. Our results are in agreement with the study. On the other hand, an unbalance of *n*-6 and *n*-3 fatty acids in the peripheral blood causes an overproduction of pro-inflammatory cytokines such as interferon γ (IFNγ), TNFα, IL-6 and IL-10. Oxidative stress and inflammation are involved in the pathophysiology of ICP[35]. Additionally, oxidative stress has been identified as a key factor associated with metabolic disorders such as cardiovascular diseases or type 2 diabetes mellitus, which can be triggered by a high-level consumption of several macronutrients: glucose or *n*-6 PUFA inducing inflammation through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated pathways[36]. A study showed that ICP is an inflammatory disorder in which the levels of inflammatory cytokines such as IL-6, TNF-α and neopterin and neopterin, TOS increased[37]. They may involve the adverse outcomes of pregnancy including ICP, by activating the acute-phase reaction, B-cell differentiation (immunoglobulin release), stimulating growth in differentiated B-cells and activating acute-phase hepatic reaction[38]. Our study suggested that an unbalanced ratio of *n*-6 *n*-3 fatty acids and high LA level had negative association with ICP, providing evidence of early nutritional intervention and management for ICP.

ICP is a pregnancy-specific condition associated with an increased risk of adverse perinatal outcomes. Despite ongoing investigations into its underlying mechanisms, effective preventive and therapeutic strategies remain a critical need. Our study has uncovered a significant association between PUFA and ICP, underscoring the importance of nutritional interventions during pregnancy. Our findings suggest that preemptive supplementation of *n*-3 fatty acids and maintaining an appropriate *n*-6 to *n*-3 ratio before conception or during early pregnancy may help reduce the risk of ICP and associated adverse outcomes. Additionally, our data emphasise the

importance of achieving a proper balance of *n*-3 and *n*-6 fatty acids relative to total fatty acid intake in the context of ICP development. These results have important clinical implications, suggesting that dietary adjustments and targeted nutritional interventions could play a crucial role in the prevention and management of ICP. Healthcare providers should consider recommending *n*-3 supplementation and dietary modifications to pregnant women, particularly those at higher risk for developing ICP, as part of a comprehensive prenatal care plan.

However, our study had certain limitations. Heterogeneity was observed in the Cochrane's Q test used for the evaluation of *n*-3 fatty acid levels and the ratio of *n*-3 to total fatty acids. We attribute this heterogeneity to several reasons. Firstly, the presence of outliers may indicate non-linear relationships, potentially leading to diverse effect sizes or directions, thus contributing to result heterogeneity even after outlier removal. Secondly, the small sample size may not completely eliminate result heterogeneity, even with the removal of outliers, due to insufficient statistical power. Thirdly, outliers may arise from measurement errors in other variables, which, even after removal, can lead to result heterogeneity. Lastly, outliers may represent distinct pathogenic mechanisms. Even if some outliers are removed, others may persist, signifying different biological processes and contributing to result heterogeneity. Nevertheless, the absence of pleiotropy in the MR-Egger test suggested balanced pleiotropy, minimising the potential bias from outliers. Additionally, the study participants were of European ancestry, limiting the generalisability of the findings to other populations and regions. Moreover, our research did not elucidate the detailed mechanisms underlying the association between PUFA and ICP, warranting further investigation. Lastly, although MR analysis can minimise the influence of confounding factors to a great extent, we have to admit that genes are not the sole determinant of outcomes. Environmental and lifestyle confounders also play a significant role in influencing the outcomes. A more in-depth investigation is needed to explore the combined impact of genetics, environment and lifestyle on the outcomes.

Conclusion

In conclusion, our data for the first time indicate the beneficial role of *n*-3 fatty acids and the adverse role of *n*-6 fatty acids in the development of ICP. An imbalanced ratio of *n*-6 to *n*-3 fatty acids and high LA levels significantly elevate the risk of ICP. The findings suggest that supplementing *n*-3 fatty acids, rather than *n*-6 fatty acids, may represent a promising strategy for the prevention and treatment of ICP. However, further exploration is needed to fully elucidate the mechanisms underlying the association between PUFA and ICP.

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