

Gut microbial-related metabolite trimethylamine-N-oxide and its precursor L-carnitine are associated with spontaneous preterm birth: a nested case-control study

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Research

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Abstract

Background: Gut microbiota has been proven to disease susceptibility and may lead to increased risk of preterm birth. To date, the link of gut microbial-related metabolite trimethylamine-N-oxide (TMAO), L-carnitine, and betaine, with spontaneous preterm birth (sPTB) has not been established. This study aimed to investigate the association of TMAO, L-carnitine and betaine, with sPTB risk.

Methods: A nested case-control study was designed including 129 sPTB cases and 258 controls based on Guangxi Birth Cohort Study. TMAO, L-carnitine, and betaine level in maternal serum were determined by liquid chromatography with mass spectrometry. Conditional logistic regression analyses were used to examine the association between maternal serum metabolites and sPTB. Stratified analyses were further conducted according to BMI and preterm prelabor rupture of membranes. Spline analyses were performed to explore the dose-response relationship between the metabolites and sPTB.

Results: Statistically significant association with decreased sPTB risk was observed for the highest L-carnitine (OR: 0.47; 95% CI: 0.23, 0.95). In risk analyses stratified by BMI, similar results were observed in normal weight gravida (BMI: 18.5~23.9 kg/cm²). The significant subtype-specific association with TMAO (OR: 0.43; 95% CI: 0.20, 0.93) and L-carnitine (OR: 0.45; 95% CI: 0.21, 0.97) were observed for preterm labor but not PPRM. Spline regression analysis indicated non-linear associations with TMAO and sPTB risk (*P* for nonlinearity: 0.057). Significant associations of TMAO with sPTB were observed in normal weight gravida (*P* = 0.028) and preterm labor subtype (*P* = 0.025). No statistically significant associations with sPTB risk were observed for betaine (*P* > 0.05).

Conclusions: TMAO and L-carnitine levels in maternal serum are inversely linked with sPTB risk. Discovery of the association between gut-microbiota initiated TMAO metabolism and sPTB may open new avenues for diagnose and therapy.

Background

Spontaneous preterm birth (sPTB), delivered at less than 37 weeks' gestation age, remains most common complication in obstetrics and gynecology [1, 2]. sPTB is the leading perinatal morbidity and mortality worldwide [3]. Preterm infants have been verified to face quite complicated issues such as low birth weight, feeding difficult, high quality of neonatal intensive care, and an increased risk of neurodevelopmental impairments, respiratory and gastrointestinal complications in adulthood compared with their term counterparts [3]. Hence, effective prediction of sPTB is useful to allow us to initiate appropriate risk-specific treatment and reduce the risk as far as possible. However, the pathogenesis of sPTB remained elusive [1, 2]. Population and genetic characteristics, individual behaviors (diabetes, smoking, pressure and drinking), environmental exposures, pathological status (hypertension, obesity), and physical markers (cervical length) appeared to low efficiency and limited accuracy to identify sPTB [2, 4, 5]. Biomarkers found in body fluids, such as cytokines (cervical IL-6), genes, proteins (serum C-reactive protein, fetal fibronectin, β -hCG, placental α -microglobulin), nutritional status have been identified as preterm risk factor, but few biomarkers have shown sufficient sensitivity and specificity in clinical application [2].

Recently, gut microbiota metabolites trimethylamine N-oxide (TMAO) has been received increasing attentions. The growing recognition of a contributory role of gut microbiota to health and disease promises that great efforts have devoted to define the cause-effect between gut microbiota and preterm birth [6–9]. Moreover, experimental and clinical studies have demonstration that TMAO synthesized by the intestinal microbiota is involved in the progression of atherosclerosis, cardiovascular disease, type 2 diabetes, vitamin D deficiency, chronic kidney disease, non-alcoholic fatty liver disease [10–12]. Those adverse impacts induced by microbial-related metabolite TMAO may directly affect the physical condition of pregnant women and birth outcomes [13, 14]. Meanwhile, TMAO is a gut microbiota-dependent metabolite of L-carnitine [15]. L-carnitine participates in energy metabolism and is essential to fetal growth and organ development [16]. L-carnitine, similar to glycine betaine (betaine) in structure, belongs to the “betaines” group [17, 18]. Betaine serves as an osmolyte to protect cell and protein from environmental stress, and a methyl group donor to homocysteine for synthesis of methionine [19, 20]. Betaine insufficiency is attributable to the high risk factors of the metabolic abnormalities, obesity, diabetes, and vascular disease [18].

Despite gut-microbiota dependent L-carnitine metabolism has been linked to maternal health and fetus developments, the association of TMAO and its precursor (L-carnitine, betaine), with sPTB risk has not yet been reported. Herein, this study conducted a nested case-control study to investigate the association of TMAO, L-carnitine and betaine levels in maternal serum, with sPTB risk in a large Chinese population.

Materials And Methods

Study Design and Population

Our nested case-control study was embedded in the Guangxi Birth Cohort Study (GBXCS), an ongoing multicenter prospective cohort study in Guangxi, South China, which aims to investigate pregnancy outcome and offspring short- and long-term health consequences of hereditary, environmental and lifestyles factors in the fast-paced society. The baseline design of the GBCS has been previously reported [21]. Flow chart of the study population was shown in Figure 1. 6,203 volunteers had been recruited at the baseline from July to September in 2015 in seven maternal & child health hospitals in Guangxi when they joined prenatal examination.

In 2016, after first follow-up survey, 5,541 (89.33%) mother-infant pairs had been observed. 206 pregnant women (delivery week < 37 weeks) were selected excluding abortion, stillbirth and birth defects, multiple pregnancies, medically premature delivery, full-term low birth weight and Macrosomia. To minimize the effect of the erratic menstrual cycle on gestation age, we carried out 129 participants delivers at < 36 weeks' gestation age as cases in this work. 258 term deliveries (delivery week \geq 37 weeks) were selected randomly according to maternal age, enrollment hospital, enrollment gestational age at baseline blood draw and infant gender as controls. This study was approved by Medical Ethics Committee of First Affiliated Hospital of Guangxi Medical University (ID: 2015(028)). All patients consented to participate in the research, and written informed consent was obtained from each patient.

Analytic measurements

The standards consisted of Trimethylamine oxide (TMAO, Sigma–Aldrich, USA), betaine (UPS Reference Standard, USA), and L-carnitine (UPS Reference Standard, USA). d_9 -TMAO, L-carnitine- d_9 , and d_{11} -betaine were obtained from Cambridge Isotope Laboratories, Inc. (USA) and used as internal standards. Serum were collected and stored at $-80\text{ }^{\circ}\text{C}$ until analysis. Frozen serum samples were thawed and centrifuged at $4\text{ }^{\circ}\text{C}$ for 5 min. Serum concentrations of TMAO, L-carnitine and betaine were quantified using stable isotope internal standards method on an Agilent 6460 triple quadrupole mass spectrometer in positive multiple reaction monitoring (MRM) mode according to the literature method with slight modifications [15, 22]. Agilent ZORBAX HILIC plus column ($2.1 \times 50\text{ mm}$, $3.5\text{ }\mu\text{m}$, Agilent, USA) was used. $20\text{ }\mu\text{L}$ plasma was aliquoted into 1.5 mL tubes and mixed with $980\text{ }\mu\text{L}$ protein precipitate solution (methanol/acetonitrile 10: 90) containing internal standards. Proteins in samples were precipitated by vortexing for 1 min, and centrifuged ($13,000\text{ rpm}$ for 10 min). Filter the solution through $0.22\text{ }\mu\text{m}$ filters, and keep it at $4\text{ }^{\circ}\text{C}$. $3\text{ }\mu\text{L}$ supernatant was injected for HPLC-MS analysis. Two solvents were used for gradient elution: (A) 5 mM ammonium acetate, (B) methanol. The flow rate was 0.4 mL/min , and the column temperature was maintained at $25\text{ }^{\circ}\text{C}$. The blind duplicate samples ($N = 20$) were interspersed to evaluate quality control. The intraclass correlation coefficients for each metabolite were as follows: TMAO, 0.912; L-carnitine, 0.998; betaine, 0.983.

Statistical Analyses

The baseline characteristics of cases and controls were performed using descriptive analysis. Continuous variables were compared using t-tests and categorical variables compared using chi-square tests. Conditional logistic regression analyses were used to examine the association between maternal serum metabolites tertile concentrations and preterm delivery by calculating odds ratios (ORs) and their 95% confidence intervals (CIs), which were adjusted by maternal age, ethnic, education levels, smoking, and drinking status during pregnancy. Stratified analyses were further conducted according to BMI (< 18.5 , $18.5 - 23.9$, $\geq 24\text{ kg/m}^2$), and PPRM (yes, no) by using logistic regression. Linear trend P -values were derived by modeling the median value of each tertile as a continuous variable in adjusted models. A spline analysis to explore the dose-response relationship of the selected biomarkers and the risk of sPTB was performed. Knots were placed at the 25th, 50th, and 75th percentiles of the serum TMAO distribution, and the reference value was set at the 25th percentile. Adjustment factors were age, pre-pregnancy BMI (not for BMI stratified model), ethnic, education, smoking, and drinking. SPSS version 22.0 (IBM) and SAS (version 9.4; SAS Institute Inc.) were applied in statistical analysis. Two-sided $P < 0.05$ was considered as statistical significance.

Results

Baseline characteristics of sPTB cases ($n = 129$) and controls ($n = 258$) are shown in Table 1. Compared with the controls, the cases involved in a higher BMI ($P = 0.02$), lower infant weight ($P < 0.001$) and shorter infant height ($P < 0.001$). No significant differences were observed in maternal age, ethnic, family income, educational levels, drinking, smoking, gestational age at sample collection, infant gender, history of gravid and parity, and infant gender (all $P > 0.05$). The sPTB group had a higher percentage of preterm labor ($n = 63$, 48.8%). As well, the percentage of PPRM was 43.4% ($n = 56$), and 10 cases were unknown (7.8%). Serum concentrations of TMAO, L-carnitine, and betaine did not differ between cases and controls.

Associations between maternal serum TMAO, L-carnitine and betaine level and sPTB risk are shown in Figure 2. Statistically significant associations with decreased sPTB risk were observed for the highest categories of L-carnitine. Multivariable-adjusted for age, pre-pregnancy BMI, ethnic, education, smoking, and drinking, women in highest tertile of L-carnitine were 53% lower sPTB risk than those in lowest tertile (OR= 0.47; 95% CI: 0.23, 0.95; P_{trend} 0.035). However, no statistically significant associations with sPTB risk were observed for TMAO and betaine (all $P > 0.05$). Spline regression analysis indicated marginally significant non-linear associations with TMAO and sPTB risk (Figure 3A, P for overall = 0.070, P for nonlinearity = 0.057), with a sharp decrease in slope for concentrations above 1.63 $\mu\text{mol/L}$. Other metabolites had no dose-relationship with sPTB (P for overall > 0.05).

In risk analyses stratified by pre-pregnancy BMI (Figure 2B - 2D), statistically significant associations with decreased sPTB risk were observed only for L-carnitine categories in subset with BMI between 18.5 and 23.9 (Figure 2C, OR= 0.46; 95% CI: 0.23, 0.93; P_{Trend} = 0.030). Of Note, Spline regression analysis indicated significant non-linear association for sPTB risk and TMAO in the same BMI subset (Figure 3B, P for overall = 0.028, P for nonlinearity = 0.011). No significant associations with sPTB risk were observed for the other metabolites (all $P > 0.05$).

The associations with serum metabolites through analyses of common sPTB subtypes PPRM and preterm labor were further explored (Figure 2E, 2F). The significant subtype-specific association with decreased TMAO (OR = 0.43; 95% CI: 0.20, 0.93, P_{Trend} = 0.033) and L-carnitine (OR = 0.45; 95% CI: 0.21, 0.97; P_{Trend} = 0.041) were observed for preterm labor. A linear relationship of TMAO and sPTB risk was found only for preterm labor cases in the spline analysis (Figure 3C, P for overall = 0.025, P for nonlinearity = 0.507). Analyses of the other metabolites did not suggest clear associations with specific sPTB subtypes.

Discussion

As far as we know, this is the first study to assess the associations of gut microbial-related metabolite TMAO and its precursor L-carnitine with sPTB risk. A nonlinear association between serum TMAO and sPTB risk were observed. According to pre-pregnancy BMI, the nonlinear association between the serum TMAO and sPTB risk were observed in normal BMI. Meanwhile, preterm birth subtypes appeared to modify the association between serum TMAO and sPTB risk with linear association observed in the preterm labor, but not in PPRM. L-carnitine levels in maternal serum were inversely associated with sPTB. The significant subtype-specific associations with L-carnitine were observed in normal BMI and preterm labor. As such, the association of maternal serum TMAO and L-carnitine with sPTB risk appears to be dependent on BMI and preterm labor availability. Additionally, no correlation was found between betaine and sPTB.

Nutrition metabolism in the context of pregnancy is crucial to maternal health and fetus development from the pre-implantation embryo to infancy [23, 24]. The gut microbiome has been identified to change during gestation [7]. Shiozaki et al. demonstrated that disturbance of intestinal microbiota would result in preterm delivery in a prospective and cross-sectional study [8]. Dahl et al. provided further evidences for the impact of the low gut diversity and low relative abundance of bifidobacterium and streptococcus on sPTB [9]. The

changes of gut microbiome diversity and evenness may alter bioactivation of nutrients and vitamins, normal physiology of host, and at same times, also affect immune system [25–27].

In this study, our data have uncovered increased sPTB risk was observed for the low categories of TMAO and L-carnitine. These findings revealed that the gut-microbiota initiated TMAO metabolism may represent a key factor contributing to sPTB. Gut microbial-related metabolite TMAO depend on the dietary sources from fish or endogenous synthesis from L-carnitine present in eggs, milk and red meat.[15] L-carnitine can be metabolized to TMA by the anaerobic intestinal bacteria, then further oxidized to TMAO [15]. L-carnitine participates in energy metabolism as the transfer of activated long-chain fatty acids into the mitochondrial matrix, regulates coenzyme A (CoA)/acylCoA ratio and maintains energy storage in the form of acetylcarnitine [16, 28]. Maternal plasma carnitine levels on delivery decrease to half of the concentrations in non-pregnant women, comparable to those found in known carnitine deficiency [29]. L-carnitine deficiency has been implicated in the pathogenesis of cardiomyopathy, skeletal weakness, diabetes mellitus, hyper-ammonaemia, and other disorders [15, 28, 30]. L-carnitine supplementation in pregnancy doses avoids insulin resistance and gestational diabetes mellitus [31]. Because the low activity of the butyrobetaine hydroxylase was to restrict the endogenous synthesis of carnitine in fetal tissue, fetal L-carnitine level mainly depended on the maternal carnitine via transplacental transfer [32, 33]. L-carnitine supplementation in pregnancy is highly demand to pregnant women and their fetuses. Therefore, low TMAO and carnitine status of pregnant women may lead to malnutrition, organ dysfunction, and embryonic development restriction. In addition, TMAO synthesized by the intestinal microbiota has been demonstrated as early predictors of metabolic syndrome, and strong linked with atherosclerosis, type 2 diabetes, vitamin D deficiency and chronic kidney disease [10–12, 34]. The metabolic syndrome induced by TMAO caused increased sPTB risk [13, 14]. Collectively, the nonlinear relationship between TMAO level and sPTB has been uncovered, with a significant positive association for the low level and an inverse association with high TMAO concentration.

In the stratified analysis, the effect of serum TMAO and L-carnitine levels on sPTB depended on the level of pre-pregnancy BMI. The adverse association of serum TMAO and L-carnitine, with sPTB risk became insignificant among the low or High BMI, and PPRM. Schugar et al. found that levels of the TMAO-producing enzyme flavin-containing monooxygenase (FMO3) is linked to obesity [35]. The effect of BMI on FMO3/TMAO metabolism pathway could partially explain the improved predictive ability with BMI stratification. In addition, a growing body of evidence suggests that utero infection and vaginal microorganisms have been causally associated to PPRM [36, 37]. The effect caused by the inflammatory activation on sPTB was much greater than those of TMAO and L-carnitine, which may cover the risk effect of TMAO and L-carnitine on sPTB in PPRM.

In this study, null associations between betaine and sPTB risk were noted. Betaine serves as an osmolyte. The betaine concentration in tissue is much higher than that in serum, while serum betaine is presumably mainly influenced by liver betaine level [18, 38]. Thus, the serum betaine could not reflect the whole level of the pregnant women, which may weaken the effect of serum betaine on sPTB.

Key strengths of this study are that this is the first study to investigate the association of maternal serum TMAO, L-carnitine and betaine, with sPTB risk in humans. Furthermore, in this nested case-control study,

cases and controls were enrolled with comparable age, ethnic, family incomes, education, drinking, smoking, gestational age at sample collection, gravid, parity and infant gender in order to minimize recall bias. Thirdly, to reduce multiple confounding factors, well-established factors (i.e., age, pre-pregnancy BMI, ethnic, education, smoking, and drinking) were included in the analyses. The main limitation of this study is that although cases and controls were recruited with complete matching by gestational age at sample collection, the concentrations of serum TMAO, L-carnitine, and betaine have changes during gestation, one sample collection may not exactly give an accurate representation of the metabolites levels. Secondly, although we have considered some confounding factors in the analyses, there is a potential for residual confounding factors that were not recorded. Thirdly, the sample size might be relatively small, limiting the statistical power to detect associations of stratified analyses. More prospective studies are required to verify the causality between TMAO, L-carnitine and sPTB.

Conclusion

TMAO and L-carnitine were inversely linked with sPTB, while betaine level is not associated with sPTB risk. If possible, these results should be confirmed by large prospective studies in the future. It is worthwhile to make further study of the mechanism of the effect of gut-microbiota initiated L-carnitine/TMA/TMAO metabolism path way on the sPTB. Discovery of a relationship between gut-microbiota initiated nutrition metabolism and sPTB provides opportunities for the development of new diagnostic procedures and therapeutic strategy for sPTB.

Declarations

Acknowledgments

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Ethics approval and consent to participate

The study was reviewed and approved by the Guangxi Medical University Medical Ethics Committee (ID: 2015(028), Approved 16 July 2015). All patients consented to participate in the research, and written informed consent was obtained from each patient.

Consent for publication

All authors have read and approved the submission of the manuscript. The manuscript has not been published and is not being considered for publication elsewhere.

Availability of data and materials

The data used to support the findings of this study are included within the article or available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

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Author Contributions

XBY, ZNM: designed the research protocol; XC, NH, XJZ, DHC, JW, JY: conducted the research; YC and MJL participated in the study conception. XC, NH, CQL, LLH, XJZ, DHC, QZH, XBY: analyzed the data; XC, NH, CQL, XBY: drafted the manuscript; XBY: had primary responsibility for final content. All authors critically revised and approved the final version of the manuscript.

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Table

Table 1. Baseline characteristics of spontaneous preterm birth cases and controls in a nested case-control study with in GBCS¹ study.

Characteristics	Cases (n=129)	Controls (n=258)	<i>p</i> 5
	n (%) or mean ± SD 5	n (%) or mean ± SD 5	
Age (years)	28.5±5.5	28.3±5.0	0.69
Ethnic, n (%)			0.93
Han	103 (79.8)	205 (79.5)	
other	26 (20.2)	53 (20.5)	
Pre-pregnancy BMI, n (%)			0.02
Underweight (<18.5)	34 (26.6)	59 (23.0)	
Normal (18.5-23.9)	73 (57.0)	178 (69.3)	
Overweight (≥24)	21 (16.4)	20 (7.8)	
Unclear	1	1	
Family income, n (%)			0.48
<50,000 (¥)	56 (43.4)	123 (47.7)	
≥50,000 (¥)	50 (38.8)	84 (32.6)	
Unclear	23 (17.8)	51 (19.8)	
Education, n (%)			0.12
High school or less	81 (62.8)	182 (70.5)	
College or more	48 (37.2)	76 (29.5)	
Drinking, n (%)			0.68
Yes	30 (23.3)	65 (25.2)	
No	99 (76.7)	193 (74.8)	
Smoking, n (%)			0.22
Yes	2 (1.6)	1 (0.4)	
No	127 (98.4)	257 (99.6)	
Gestational age at sample collection, n (%)			1.00
≤13 weeks	46 (35.7)	93 (36.0)	
14-27 weeks	74 (57.4)	147 (57.0)	
≥28 weeks	9 (7.0)	18 (7.0)	
Preterm birth			
< 34 weeks	29 (22.5)		
≥34 weeks	100 (77.5)		
Gravid, n (%)			0.79
Primigravid	39 (29.7)	80 (31.0)	
Multigravid	90 (70.3)	178 (69.0)	
Parity, n (%)			0.61
Primiparity	79 (61.2)	151 (58.5)	
Multiparity	50 (38.8)	107 (41.5)	
sPTB subtype 2, n (%)			
PPROM 3	56 (43.4)		
Preterm labor	63 (48.8)		
Missing	10 (7.8)		
Infant gender, n (%)			1.00
Male	72 (55.8)	144 (55.8)	
Female	57 (44.2)	114 (44.2)	
Infant weight (g)	2362.2 ± 485.5	3223.3 ± 355.1	< 0.001
Infant height (cm)	46.5 ± 3.4	50.4 ± 1.5	< 0.001
TMAO 4 (µmol/L)	1.90 (1.84)	2.18 (1.87)	0.119
L-carnitine (µmol/L)	24.29 (8.09)	25.27 (10.11)	0.130
Betaine (µmol/L)	16.08 (8.39)	16.69 (8.37)	0.574

GBCS, Guangxi Birth Cohort Study.

sPTB, spontaneous preterm birth.

PPROM, preterm prelabor rupture of membranes.

TMAO, Trimethylamine-N-Oxide.

Normally distributed variables were shown as mean \pm SD and *P* value was derived from t-test. Non-normally distributed variables were shown as median (IQR) and *P* value was derived from U-test. Categorical variables were shown as numbers (percentage) and *P* value was derived from Chi-square test.

Figures



Figure 1

Flow chart of the study population.

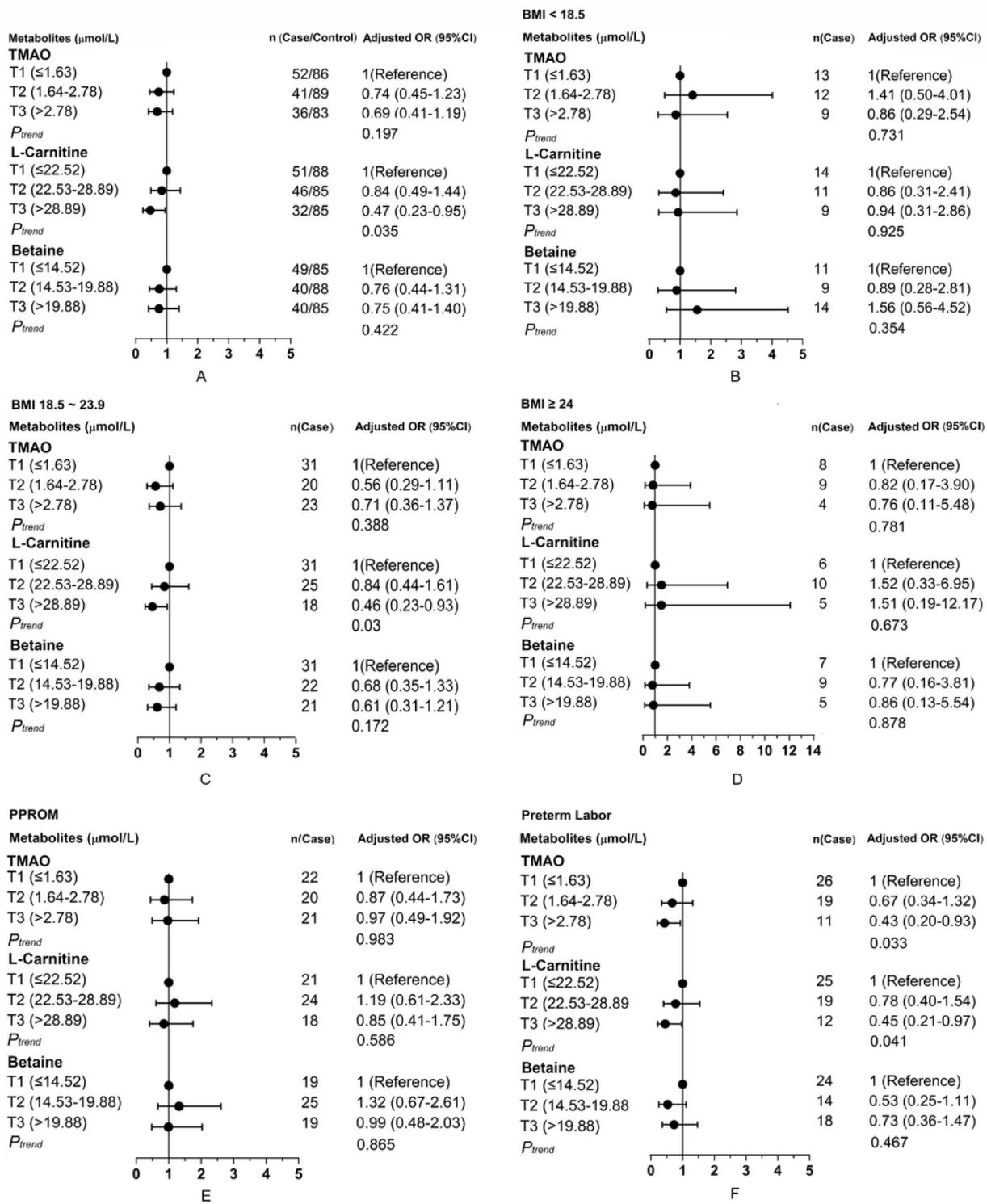


Figure 2

Associations between tertiles of serum TMAO, L-carnitine, and betaine levels and sPTB risk (A); stratified analyses by pre-pregnancy BMI: Individuals with BMI < 18.5 kg/m² (B), Individuals with 18.5 \leq BMI \leq 23.9 kg/m² (C), Individuals with BMI \geq 24 kg/m² (D); stratified analyses by preterm prelabor rupture of membranes (PPROM): Individuals with PPRM (E), Individuals with preterm labor (F). Computed using conditional logistic regression modeling, adjusting for age, pre-pregnancy BMI (not for BMI stratified model), ethnic, education, smoking, and drinking.

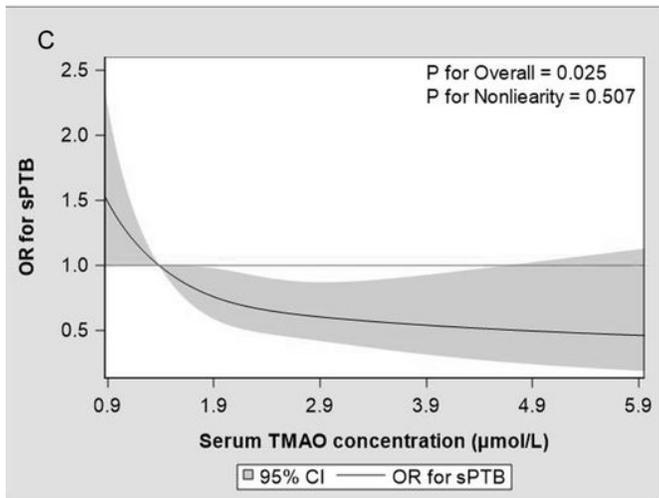
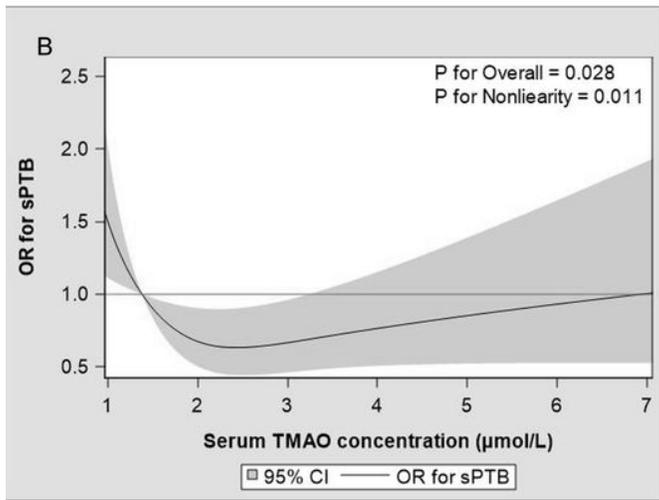
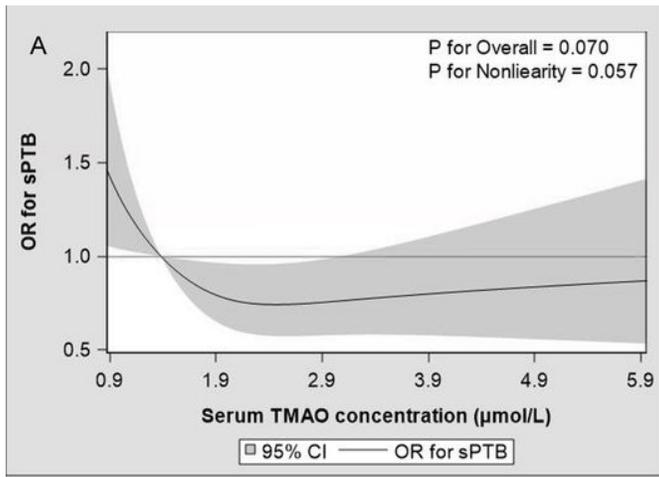


Figure 3

The restricted cubic spline for the association between maternal serum TMAO and sPTB in different models. The lines represent adjusted odds ratios based on restricted cubic splines for the levels of serum TMAO in the logistic regression model. (A) All individuals. (B) Individuals with $18.5 \leq \text{BMI} \leq 23.9$ kg/m². (C) Individuals with preterm labor.