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Association between serum cotinine concentrations on red blood cell folate concentrations in pregnant women and the mediating role of lymphocytes: an NHANES Study

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Abstract

Background Folate is essential for DNA synthesis and cell division, particularly during pregnancy, where insufficient levels can lead to adverse outcomes like neural tube defects and preterm birth. Tobacco smoke exposure, indicated by serum cotinine levels, is a known risk factor for reduced folate levels. However, the mechanisms underlying this relationship, especially the role of lymphocytes, are not well understood.

Objectives This study evaluates the relationship between serum cotinine levels and RBC folate concentrations in pregnant women, explores the mediating role of lymphocyte count, and identifies susceptibility factors that could guide targeted interventions.

Methods We conducted a cross-sectional analysis using NHANES data from 1999 to 2018, including 1,021 pregnant women. Serum cotinine levels were used as a biomarker for tobacco exposure, while RBC folate levels indicated long-term folate status. Linear regression, restricted cubic spline, and mediation analyses were performed to assess these relationships.

Results Serum cotinine levels were significantly negatively correlated with RBC folate concentrations ($P < 0.001$). A nonlinear relationship revealed more pronounced folate depletion at higher cotinine levels. Mediation analysis

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showed that elevated lymphocyte count mediated 19.3% of the cotinine-folate association. Factors such as smoking history, advanced maternal age, and heavy alcohol consumption exacerbated this negative effect.

Conclusion Tobacco exposure(as reflected by elevated cotinine levels) significantly reduces folate levels in pregnant women, with lymphocyte count playing a mediating role. These findings underscore the need for targeted public health interventions to mitigate tobacco-related risks during pregnancy.

Keywords Cotinine, RBC folate, Tobacco exposure, Pregnancy, Mediation, NHANES study

Text box 1. Contributions to the literature

- Tobacco smoke exposure is a known risk factor for folate depletion in pregnant women, yet the mechanisms remain unclear.
- This study identifies the mediating role of lymphocyte count in the relationship between serum cotinine levels and RBC folate concentrations.
- A nonlinear association between tobacco exposure(as reflected by elevated cotinine levels) and folate depletion was observed, with more severe effects at higher cotinine levels.
- Key susceptibility factors, such as smoking history, advanced maternal age, and alcohol consumption, were found to worsen the impact of tobacco exposure.
- These findings highlight the need for targeted public health interventions to protect vulnerable populations.

Introduction

Folate, also known as vitamin B9, is a water-soluble member of the B vitamin family that is essential for normal DNA synthesis and cellular division [1]. Low levels of folate can contribute to the incidence of macrocytic anemia and a range of adverse health outcomes including certain forms of cancer, cardiovascular disease, and infectious disease [2–6]. Levels of folate that are too low during pregnancy are particularly concerning, as they can contribute to negative perinatal outcomes including placental abruption, preeclampsia, severe neural tube defects, preterm birth, stillbirth, spontaneous abortion, and low birth weight [6, 7]. While global efforts aimed at reinforcing folate levels in pregnant women have been implemented and led to overall improvements in folate status among the general public, a subset of pregnant women continue to suffer from folate levels that are not sufficient [8–11]. Folate levels can be influenced by a range of variables that can range from polymorphisms in specific genes (e.g., *MTHFR*), to pathological comorbidities such as malaria, physiological conditions such as pregnancy or lactation, socioeconomic status, and specific biological factors including the levels of vitamins B6 and B12 [11–13].

Cigarette smoke exposure has been firmly established as a risk factor for many diseases, such as chronic obstructive pulmonary disease, cardiovascular disease, and cancer. This risk relationship is attributable to the various toxic chemicals present in tobacco, including polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines, aromatic amines, alkaloids (nicotine and cotinine, its primary metabolite), and a variety of heavy metals (nickel, cadmium, chromium, and arsenic) [14–16]. These chemicals can adversely impact human health by inducing DNA damage, inflammation, endothelial dysfunction, oxidative stress, and dysregulated immune activity [17]. In early studies, smoking was linked to a reduction in levels of folate in pregnant women [18–20]. Cigarette smoke can cause toxic effects that are particularly damaging to the developing fetus, as its capacity for detoxification is quite limited [21]. Despite these well-documented risks, an estimated 14% of women in the USA continue to smoke while pregnant, and ~30% are exposed to secondhand smoke (SHS) prior to or during pregnancy [22, 23]. As the primary nicotine metabolite, cotinine levels can be reliably measured to gauge an individual's passive and active tobacco smoke exposure [24, 25]. Even though there have been several reports documenting a negative correlation between levels of cotinine and folate status, the mechanistic basis for this relationship remains to be clarified [26–28].

Lymphocytes are white blood cells that are integral to the adaptive immune response, facilitating the detection and elimination of pathogens as well as damaged or infected cells, and helping to preserve homeostatic balance through the appropriate control of the immune response [29]. Cigarette smoke exposure has been suggested to primarily affect the numbers and functionality of lymphocytes through the modulation of immune signaling activity [30–37]. As folate is both an important antioxidant with anti-inflammatory potential and a key substrate for the synthesis of DNA, lymphocyte activation and proliferation may contribute to rapid folate consumption [38–42]. These factors provide support for the hypothesis that lymphocytes may serve as significant mediators of the effects that cotinine can have on folate levels [43, 44].

As tobacco exposure can adversely affect maternal and fetal health, there is a pressing need to identify factors linked to this susceptibility so that more effective targeted approaches to preventing and treating disease can be devised, thereby supporting the optimal allocation of healthcare resources. The present study was developed with the aim of leveraging the National Health and Nutrition Examination Survey (NHANES) database to

clarify whether there are linear or nonlinear relationships between serum cotinine concentrations and RBC folate levels among women who are pregnant. In addition, the potential mediating effects of lymphocyte count on the interplay between cotinine levels and folate status was explored, with subgroup analyses providing detailed insight into how this relationship is influenced by clinical, demographic, and lifestyle factors.

Methods

Study design and methods

Study population

This was a cross-sectional analysis utilizing data from the NHANES study performed by the Centers for Disease Control and Prevention (CDC) branch known as the National Center for Health Statistics (NCHS). The original NHANES study was formulated with the goal of surveying the nutritional status and overall health of non-institutionalized adults in the USA through a series of demographic surveys, dietary analyses, questionnaires, laboratory tests, and other exams. The NCHS Research Ethics Review Board approved this study and the study personnel obtained written informed consent from all participants, so no additional ethical approval was required (https://www.cdc.gov/nchs/nhanes/irba98.htm). Appropriate guidelines and regulations were adhered to for all methods in this study.

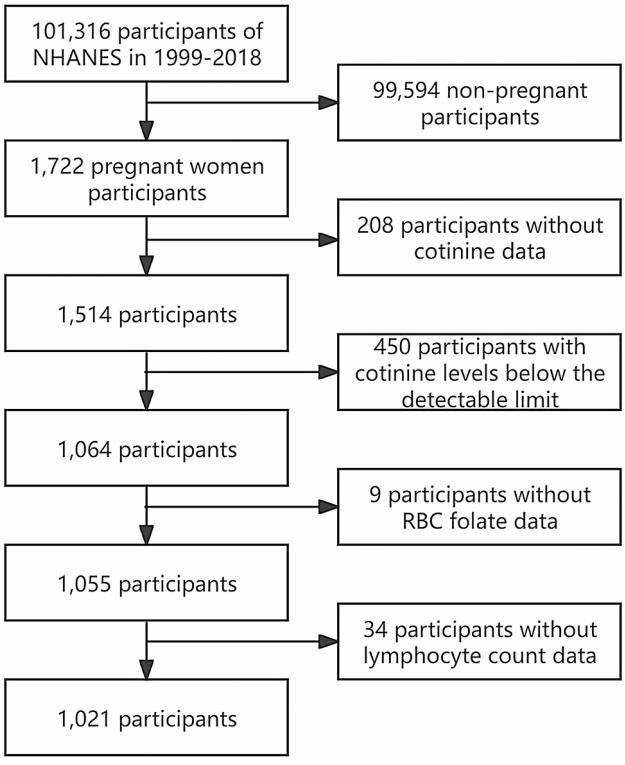

Fig. 1 Flow diagram of the study design

For the present study, 101,316 subjects from the 1999–2018 NHANES cycles were selected for analysis, and corresponding data was downloaded from the NHANES database (https://www.cdc.gov/nchs/nhanes/index.htm). In total, this study focused on a population of 1,722 pregnant women who were identified based on either their self-identified pregnancy status or by a positive urine pregnancy test. Of these individuals, 208 lacking cotinine data, 450 for whom cotinine levels were below the limit of detection (as per NHANES guidelines, these values were excluded due to their unreliable quantification for statistical analysis), 9 for whom RBC folate data were missing, and 34 for whom lymphocyte data were missing were excluded. The remaining 1,021 pregnant women were retained for analysis (Fig. 1).

Analyses of tobacco smoke exposure

Prior epidemiological studies have primarily assessed the smoking status and frequency of subjects through the use of questionnaires, introducing the potential for subjective bias, particularly when trying to account for exposure to SHS [18–20, 45]. Individuals also exhibit marked variability in their absorption of nicotine and other harmful tobacco smoke-derived compounds, leading to health risk levels that can be difficult to predict. As the primary nicotine metabolite, cotinine has a half-life of 15–17 h and can be reliably analyzed as a biomarker to gauge cumulative active and passive tobacco smoke exposure levels [24, 25]. For this study, the tobacco smoke exposure of pregnant women was thus estimated based on their serum cotinine levels [46].

Blood and serum that had been collected from appropriate NHANES participants were analyzed in a central CDC laboratory in Atlanta, GA. An isotope dilution-high performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry (ID HPLC-APCI MS/MS) approach was used for the measurement of serum cotinine concentrations, as this method has been internationally validated [47]. The internal standard used for these analyses was Methyl-D3-cotinine, which was added to alkalized samples of serum that were subsequently transferred to a supported liquid extraction (SLE) plate. Isopropanol/dichloromethane was then used for extraction, followed by sample concentration and application to a C18 HPLC column. APCI-MS/MS monitoring of sample eluents was implemented, allowing for the identification of the m/z 177 precursor ion and the m/z 80 product ion corresponding to cotinine. This assay had a 0.015 ng/mL limit of detection (LOD), with all values below this limit having been reported as LOD/$\sqrt{2}$. Over 65% of samples were retained for analysis, while those below the LOD were excluded. For details regarding laboratory testing and quality control procedures, see the corresponding documentation

available online (https://wwwn.cdc.gov/Nchs/Nhanes/2017-2018/COT_J.htm). As cotinine level distributions were not normally distributed, natural log-transformed (ln-transformed) values were used for all analyses [48].

Folate status analysis

Serum, plasma, urine, or RBC levels can all be analyzed to gauge the folate status of a given individual. Levels of folate in the serum generally reflect the recent intake of folate, and a single serum measurement cannot readily distinguish between chronic folate deficiency and short-term reductions in dietary folate intake. RBC folate levels, however, can offer more reliable insight into folate status given that RBCs have an average lifespan of 120 days and the fat that folate accumulation only occurs during erythropoiesis, leading to slower responses to shifts in the intake of folate [49]. RBC folate levels were thus utilized as a biomarker of folate status among the pregnant women in this study.

Between 1999 and 2006, the Bio-Rad (BR) Quanta Phase II radioassay was used to analyze RBC folate levels (https://wwwn.cdc.gov/Nchs/Nhanes/2005-2006/FOLATE_D.htm), whereas between 2007 and 2018 these concentrations were instead measured based on whole blood folate analyzed with a microbiological assay (MA) utilizing *Lactobacillus rhamnosus*/*Lactobacillus casei*, following adjustment for hematocrit and serum folate (https://wwwn.cdc.gov/Nchs/Nhanes/2017-2018/FOLATE_J.htm). Owing to these differences in measurement techniques, the MA method tended to yield RBC folate levels significantly higher than those derived from the BR method. To ensure that these two datasets could reliably be compared, the following steps were used for the conversion of these data, as recommended by the NHANES guidelines:

$$\mathrm{WBF} = (\mathrm{RBF} \times \mathrm{HCT}/100) + \mathrm{FOL} \times [1.0 - (\mathrm{HCT}/100)] \quad (1)$$

$$\mathrm{WBF\,adjusted} = 10\text{^}\,[0.2204 + 1.017 \times \log 10\,(\mathrm{WBF})] \quad (2)$$

$$\begin{aligned}\mathrm{FOL\,adjusted} = {} & 10\text{^}[0.0188 \times (\log 10(\mathrm{FOL}))\text{^}3 \\ & - 2.7109 \times (\log 10(\mathrm{FOL}))^{(-1/2)} + 3.8276]\end{aligned} \quad (3)$$

$$\begin{aligned}\mathrm{RBF\,adjusted} = {} & (\mathrm{WBF\,adjusted} - \mathrm{FOL\,adjusted} \\ & \times [1.0 - (\mathrm{HCT}/100)] / (\mathrm{HCT}/100)\end{aligned} \quad (4)$$

RBC folate levels were natural log-transformed (ln-transformed), as they were not normally distributed [48].

Lymphocyte measurements

Automated dilution and mixing devices were used to process samples. A Beckman Coulter MAXM from 1999 to 2006, the Beckman Coulter HMX device was used to measure lymphocyte counts from 2007 to 2012, while from 2013 to 2018 the Beckman Coulter DXH 800 device was instead used. Whole blood cell counts were quantified, classifying cells based on their volume, conductivity, and scatter (https://wwwn.cdc.gov/Nchs/Nhanes/2017-2018/CBC_J.htm).

Covariates

In light of prior reports, covariates related to the outcome or to both the exposure and the outcome were selected [26–28], including age, race/ethnicity, family poverty income ratio (PIR), education level, marital status, body mass index (BMI), mean corpuscular hemoglobin concentration (MCHC), triglyceride (TG) levels, HbA1c, and total dietary folate intake.

Questionnaires administered during household interviews were used to collect all participant covariate data. Race/ethnicity was classified as Mexican American, Other Hispanic, Non-Hispanic Black, Non-Hispanic White, or Other. Marital status categories included married, widowed/divorced, separated, never married, or living with a partner. Education levels included < 9th grade, grades 9–11, high school graduate/GED or equivalent, some college or AA degree, or college graduate and above. PIR levels were treated as a continuous variable and used to stratify subjects into three subsets: ≤1.0, 1.1-3.0, or > 3.0 (https://www.govinfo.gov/app/details/FR-2024-01-17/2024-00796). The smoking status of these subjects was assessed based on their response to the following: "Have you smoked at least 100 cigarettes in your lifetime?" Alcohol consumption was determined by the NIAA definition of moderate alcohol consumption for women based on the response of participants to the following: "During the past 12 months, on those days when you drank alcohol, on average, how many drinks did you have per day?" Their responses were used to categorize these subjects as non-drinkers, light/moderate drinkers (< 1 drink/day), or heavy drinkers (1 + drinks/day) (https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/moderate-binge-drinking).

NHANES 24-hour dietary recall results were used to assess the intake of nutrients, protein, and dietary energy. Total dietary folate intake was calculated based on the reported consumption of folate from food sources, including both natural folate and folic acid supplements. This is because folic acid supplementation during pregnancy can affect circulating folic acid levels [50, 51]. For these surveys, participants were asked by a trained staff member about the food types and quantities thereof consumed in a given day, with the results having been recorded in the NHANES Computer-Assisted Dietary Interview system. Nutrient intake estimates for each food component were made with the University of Texas Food Intake Analysis System and the USDA Survey Nutrient Database. The mobile examination center was the site of

BMI (kg/m²) measurements for all subjects, while HbA1c (%), TG (mg/dL), MCHC (g/dL), and other parameters were measured in a remote laboratory. For further details regarding the collection of blood and corresponding analyses, see the NHANES Laboratory/Medical Technicians Procedures Manual (https://wwwn.cdc.gov/nchs/nhanes/tutorials/default.aspx).

Statistical analysis

To ensure that the results were nationally representative, the sampling approach, data clustering, and sample weights from the NHANES dataset were leveraged when performing all regression analyses, testing, and model development. If sample sizes were insufficient for full weighting, the results have been annotated accordingly. R (v 4.3.2) was used for all statistical analyses.

To account for any missing covariate data, the R MICE package was used for multiple imputation by chained equations (MICE), with the classification and regression trees (CART) method having been used to impute categorical variables whereas the predictive mean matching (PMM) method was used for continuous variables to ensure the data were complete and robust [52].

Continuous variables are reported as means and standard deviations, whereas categorical variables are frequencies with percentages. Cotinine concentrations were subjected to log transformation for the overall and quartile-stratified analyses, In the overall analysis, linear relationships between cotinine levels and continuous variables were assessed with multiple linear regression models. Relationships between these log-transformed cotinine levels and categorical variables were also assessed with t-tests or ANOVAs. For quartile-stratified analyses, the median level of cotinine in each quartile (log-transformed) was computed, and this quartile-based categorical variable was then used as a continuous variable. Continuous variables were analyzed with multiple linear regression analyses, whereas linear trends for categorical variables were assessed through a logistic regression approach. Multivariate linear regression models were adjusted for potential confounding factors such as age, race, education level, marital status, BMI, hemoglobin, HbA1c, TG, MCHC, and dietary intake of folate. A backward stepwise method was used to select the final model according to the Akaike information criterion (AIC), with only significant predictors ($p < 0.05$) having been retained.

A restricted cubic spline (RCS) model was also used to analyze the nonlinear nature of the relationship between cotinine and RBC folate levels with the R spline and rms packages [53]. Inflection point values were confirmed based on the first derivative of the RCS model predictions, after which the dataset was segmented and covariate adjustment was performed in a multivariate linear regression analysis.

Next, the R mediation and boot packages were employed to conduct a mediation analysis exploring the ability of lymphocyte count to serve as a mediator of the association between cotinine and RBC folate levels. Two models were established, including one corresponding to the effect of the exposure variable on the mediator and one corresponding to the effect of the exposure and mediator variables on the outcome variable. The bootstrap method was applied in the causal mediation analysis framework for the estimation of the average causal mediation effect (ACME), average direct effect (ADE), total effect, and mediation proportion together with appropriate 95% confidence intervals (95% CIs), thereby ensuring that these estimates were robust [54].

To identify factors that may impact the association between cotinine and RBC folate levels, subgroup analyses were conducted in which subjects were stratified according to specific demographic, clinical, and lifestyle parameters. Multivariate linear regression models were then employed to analyze the relationship between cotinine levels and RBC folate levels in these different groups. Two model types were constructed, including a full interaction model with all of the potential interaction terms as well as a reduced interaction model excluding the interaction terms. The significance of interactions between susceptibility factors and the link between cotinine and RBC folate levels was evaluated with a likelihood ratio test (LRT).

Lastly, the robustness of the above findings was assessed through sensitivity analyses. Given that the BR and MA methods were separately employed when analyzing RBC folate levels during the respective 1999–2006 and 2007–2018 year intervals as part of the NHANES study, sensitivity analyses were separately performed for the 1999–2006 and 2007–2018 datasets, even though the data had been converted as per the guidelines published by the NHANES group to ensure consistency. Moreover, the RBC folate concentration threshold considered optimal by the World Health Organization in 2015 for the prevention of neural tube defects (NTDs) was 906 nmol/L for RBC folate concentration as optimal for preventing NTDs [55]. Based on previous research, sensitivity analyses were performed in which RBC folate levels were treated as a binary value according to NTDs risk level as follows: < 748 nmol/L (at risk for NTDs) and ≥ 748 nmol/L [56]. This selected threshold breakpoint was based on the measurement methods employed in the NHANES study, and is consistent with a 906 nmol/L threshold when employing the Molloy measurement method [57].

Results

Baseline participant characteristics

The baseline characteristics of the 1,021 subjects in this study from the NHANES 1999–2018 cohort are presented in Table 1. Significant differences were observed among ln-transformed cotinine quartiles (Q1-Q4) in terms of race/ethnicity, education level, marital status, lifestyle factors, and clinical characteristics. Q4, for example, included the highest frequency of non-Hispanic White individuals (55.70%) as well as the highest frequencies of subjects with a history of smoking and household smoke exposure (72.52% and 68.75%, respectively). Q4 also presented with the highest lymphocyte count ($2.19 \pm 0.05 \times 10^3$ cells/μL) and the lowest RBC folate levels (995.01 ± 47.96 nmol/L). A significant negative correlation was observed between ln-transformed cotinine and ln-transformed RBC folate levels, whereas ln-transformed cotinine levels were positively correlated with lymphocyte count ($p < 0.001$).

The relationship between ln-transformed cotinine and ln-transformed RBC folate

Regression coefficients and corresponding 95% CIs pertaining to the effects of ln-transformed cotinine levels on the levels of ln-transformed RBC folate are presented in Table 2. Under the unadjusted Model 1, a significant negative association was observed between ln-transformed RBC folate levels and ln-transformed cotinine levels [-0.064 (-0.082, -0.046); $p < 0.001$]. This negative relationship remained intact under the adjusted Model 2 [-0.054 (-0.080, -0.027); $p < 0.001$] and Model 3 [-0.048 (-0.071, -0.024); $p < 0.001$]. Similar findings were also observed when median ln-transformed cotinine levels in each quartile were selected as a continuous variable, with a significant negative association between ln-transformed RBC folate levels and ln-transformed cotinine levels [-0.059 (-0.077, -0.041); p-trend < 0.001]. This relationship again maintained intact in Model 2 [-0.048 (-0.073, -0.023); p-trend < 0.001] and Model 3 [-0.040 (-0.062, -0.017); p-trend < 0.001].

Evaluation of the nonlinear relationship between ln-transformed cotinine and ln-transformed RBC folate levels

The nonlinear nature of the relationship between levels of ln-transformed cotinine and ln-transformed RBC folate levels is presented in Fig. 2. In the overall analysis, increasing ln-transformed cotinine concentrations were associated with a decrease in ln-transformed RBC folate levels ($p < 0.001$). While RCS analyses suggested that a nonlinear trend may exist, the test for nonlinearity failed to achieve statistical significance (nonlinearity $p = 0.068$). These analyses led to the identification of three inflection points at ln-transformed cotinine levels of -1.69 ng/mL, 1.24 ng/mL, and 2.91 ng/mL. As shown in Table 3, a segmented analysis was performed. Below the first of these inflection points (ln-transformed cotinine < -1.69 ng/mL), ln-transformed RBC folate and ln-transformed cotinine levels were not significantly related [0.035 (-0.085, 0.155); $p = 0.558$], nor were they related between this inflection point and the second inflection point (-1.69 to 1.24 ng/mL) [0.017 (-0.131, 0.164); $p = 0.809$]. The relationship began approaching significance between the second and third inflection points (1.24 to 2.91 ng/mL) [-0.238 (-0.555, 0.079); $p = 0.134$], ultimately achieving significance following the third inflection point (ln-transformed cotinine ≥ 2.91 ng/mL), with a significant decrease in ln-transformed RBC folate levels [-0.198 (-0.303, -0.093); $p = 0.002$].

Analysis of the Mediating effects of Lymphocyte Count

The relationship between lymphocyte count and the link between ln-transformed cotinine and ln-transformed RBC folate levels was next evaluated (Supplementary Table 2). Following adjustment for covariates, a significant positive association was observed between ln-transformed cotinine levels and lymphocyte count [0.045 (0.023, 0.068); $p < 0.001$]. Lymphocyte count was also significantly negatively related to ln-transformed RBC folate levels [-0.177 (-0.296, -0.059); $p = 0.004$]. The mediating role that lymphocyte count plays as a regulator of the relationship between ln-transformed cotinine and ln-transformed RBC folate is shown in Fig. 3. Increased lymphocyte counts were found to significantly mediate this relationship, as evidenced by an indirect effect of -0.0136 [(-0.0127, 0.0004); $p = 0.018$] and a mediation proportion of 19.27% (1.04, 29.70)]

Subgroup analyses

Subgroup analyses for subjects stratified according to different demographic, lifestyle, and clinical parameters are presented in Supplementary Table 3, Fig. 4, and Supplementary Fig. 1, offering further insight into the link between ln-transformed cotinine and ln-transformed RBC folate levels. In general, consistent relationships between ln-transformed cotinine and ln-transformed RBC folate levels were detected across these subgroups, with significant interactions being evident in certain subgroups. Specifically, a stronger negative association between ln-transformed cotinine and ln-transformed RBC folate levels was detected among individuals with advanced maternal age (> 36 years), a history of smoking, and heavy alcohol consumption, as evidenced by the following regression coefficients, CIs, and p-values for interaction: [-0.078 (-0.139, -0.017); $p = 0.004$; p for interaction = 0.013], [-0.049 (-0.087, -0.011); $p < 0.001$; p for interaction = 0.012], and [-0.046 (-0.077, -0.015); $p < 0.001$; p for interaction = 0.004].

Table 1 Baseline demographic and clinical characteristics of subjects in the NHANES 1999–2018 cohort

Variables	Total	*P*	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-trend
	(*N* = 1,021)		(*N* = 366)	(*N* = 145)	(*N* = 255)	(*N* = 255)	
Age (years)	27.48 ± 6.41	0.177	29.56 ± 0.52	27.30 ± 0.75	25.38 ± 0.52	26.80 ± 0.81	0.054
Race/Ethnicity, N (%)		< 0.001					0.002
Mexican American	240(12.06%)		126(17.56%)	45(16.26%)	48(8.16%)	21(6.36%)	
Other Hispanic	62(5.97%)		25(8.20%)	12(6.89%)	13(6.04%)	12(2.78%)	
Non-Hispanic White	412(51.99%)		149(51.37%)	48(57.25%)	83(44.56%)	132(55.70%)	
Non-Hispanic Black	232(19.77%)		37(10.18%)	27(14.81%)	95(30.21%)	73(25.56%)	
Other Race - Including Multi-Racial	75(10.20%)		29(12.69%)	13(4.79%)	16(11.04%)	17(9.59%)	
Education level, N (%)		< 0.001					0.416
Less than 9th grade	74(4.71%)		33(3. 46%)	15(7.34%)	11(2.61%)	15(6.40%)	
9-11th grade (Includes 12th grade with no diploma)	222(18.00%)		54(8.72%)	24(13.99%)	67(23.19%)	77(27.07%)	
High school graduate/GED or equivalent	255(22.40%)		73(17.12%)	32(18.42%)	73(25.07%)	77(28.72%)	
Some college or AA degree	290(29.98%)		101(32.10%)	40(24.68%)	80(34.59%)	69(26.75%)	
College graduate or above	180(24.91%)		105(38.60%)	34(35.56%)	24(14.54%)	17(11.06%)	
Marital status, N (%)		< 0.001					< 0.001
Married	508(51.81%)		255(72.21%)	87(60.18%)	97(44.85%)	69(28.60%)	
Widowed/Divorced	32(2.86%)		9(2.25%)	1(0.69%)	9(1.38%)	13(5.95%)	
Separated	30(2.76%)		8(2.07%)	4(1.80%)	5(1.34%)	13(5.23%)	
Never married	284(26.09%)		52(13.27%)	22(16.78%)	102(36.72%)	108(37.96%)	
Living with partner	167(16.48%)		42(10.19%)	31(20.56%)	42(15.71%)	52(22.25%)	
(PIR), N (%)		< 0. 001					< 0.001
0–1.0	355(29.32%)		97(19.21%)	42(18.54%)	101(33.89%)	115(43.59%)	
1.1–3.0	387(36.48%)		128(35.55%)	60(36.57%)	100(35.96%)	99(37.95%)	
> 3.0	279(34.20%)		141(45.24%)	43(44.89%)	54(30.15%)	41(18.46%)	
Smoking, N (%)		< 0.001					< 0.001
< 100 cigarettes	609(55.53%)		286(72.34%)	105(63.07%)	149(60.60%)	69(27.48%)	
≥ 100 cigarettes	412(44.47%)		80(27.66%)	40(36.93%)	106(39.40%)	186(72.52%)	
Family Smoking, N (%)		< 0.001					< 0.001
Yes	252(27.25%)		12(3.80%)	10(11.39%)	69(20.86%)	161(68.75%)	
No	769(72.75%)		354(96.20%)	135(88.61%)	186(79.14%)	94(31.25%)	
Drinking, N (%)		0.055					0.149
Never	276(22.98%)		111(27.84%)	33(18.37%)	69(23.43%)	63(19.42%)	
Low to moderate	224(24.27%)		101(29.59%)	33(27.30%)	50(19.88%)	40(19.78%)	
Heavy	521(52.76%)		154(42.57%)	79(54.33%)	136(56.69%)	152(60.80%)	
Cancer, N (%)		0.531					0.169
Yes	20(2.20%)		5(1.86%)	2(1.07%)	6(1.33%)	7(3.93%)	
No	1001(97.80%)		361(98.14%)	143(98.93%)	249(98.67%)	248(96.07%)	
Diabetes, N (%)		< 0.001					0.078
Yes	13(1.23%)		6(2.66%)	1(0.19%)	4(1.06%)	2(0.25%)	
No	1008(98.77%)		360(97.34%)	144(99.81%)	251(98.94%)	253(99.75%)	
Hypertension, N (%)		0.437					0.398
Yes	108(9.79%)		39(10.50%)	8(6.19%)	29(8.28%)	32(12.13%)	
No	913(90.21%)		327(89.50%)	137(93.81%)	226(91.72%)	223(87.87%)	
Chronic Disease		0.694					0.545
Yes	131(12.51%)		48(14.71%)	9(7.17%)	36(10.30%)	38(14.60%)	
No	890(87.49%)		318(85.29%)	136(92.83%)	219(89.70%)	217(85.40%)	
BMI (kg/m2)	29.26 ± 7. 63	0.396	28.94 ± 0.65	28.04 ± 0.85	29.14 ± 0.66	30.41 ± 0.86	0.098
Cotinine (ng/mL)	26.56 ± 65.83	NA	0.03 ± 0.00	0.06 ± 0.00	0.38 ± 0.04	93.30 ± 9.38	< 0.001
RBC Folate(nmol/L)	1295.54 ± 715.32	< 0.001	1539.72 ± 78.30	1432.19 ± 91.25	1215.75 ± 69.76	995.01 ± 47.96	< 0.001
Cotinine (ln, ng/mL)	-0.89 ± 3.18	NA	-3.59 ± 0.03	-2.87 ± 0.03	-1.34 ± 0.11	3.76 ± 0.15	< 0.001
RBC Folate(ln, nmol/L)	7.01 ± 0.59	< 0.001	7.20 ± 0.06	7.14 ± 0.07	6.97 ± 0.05	6.73 ± 0.06	< 0.001
WBC (10^3cells/uL)	9.55 ± 2.57	0.196	9.58 ± 0.19	9.16 ± 0.37	9.40 ± 0.24	9.86 ± 0.32	0.259

Table 1 (continued)

Variables	Total	*P*	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-trend
	(*N* = 1,021)		(*N* = 366)	(*N* = 145)	(*N* = 255)	(*N* = 255)	
LYM (10^3cells/uL)	2.00 ± 0.56	< 0.001	1.88 ± 0.04	1.94 ± 0.06	1.97 ± 0.06	2.19 ± 0.05	< 0.001
NEU (10^3cells/uL)	6.69 ± 2.30	0.836	6.86 ± 0.17	6.39 ± 0.34	6.57 ± 0.22	6.76 ± 0.26	0.875
MONO (10^3cells/uL)	0.64 ± 0.22	0.263	0.65 ± 0.02	0.62 ± 0.03	0.64 ± 0.03	0.66 ± 0.02	0.426
EOS (10^3cells/uL)	0.18 ± 0.13	0.015	0.15 ± 0.01	0.17 ± 0.02	0.18 ± 0.01	0.20 ± 0.02	0.029
BOS (10^3cells/uL)	0.05 ± 0.05	0.152	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.099

Data are presented as means ± SD or n (%). The counts n in n (%) are unweighted, while the percentages % are weighted. P: After log-transforming cotinine levels, linear regression was used to assess single-factor associations for numerical variables, t-tests for binary variables, and ANOVA for categorical variables with more than two levels. P-trend: For trend analysis, the median of log-transformed cotinine within each quartile was first calculated, converting the quartile categorical variable into a continuous variable. Linear regression was then applied to numerical variables, while logistic regression was used to evaluate linear trends for categorical variables. P values: P values were adjusted for the study sampling scheme, data clustering, and sample weights to ensure national representativeness in the NHANES data. Abbreviations: PIR, Poverty-to-Income Ratio; BMI, Body Mass Index; RBC Folate, Red Blood Cell Folate; RBC Folate (ln), Log-transformed Red Blood Cell Folate; WBC, White Blood Cell; LYM, Lymphocyte; NEU, Neutrophil; MONO, Monocyte; EOS, Eosinophil; BOS, Basophil

Table 2 The associations between cotinine and RBC folate

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-trend	Linear Model	*P*
	Coefficient (95% CI)	Coefficient (95% CI)	Coefficient (95% CI)	Coefficient (95% CI)		Coefficient (95% CI)	
Model 1	Ref.	-0.057 (-0.230, 0.115)	-0.226 (-0.390, -0.062)	-0.473 (-0.628, -0.318)	< 0.001	-0.064 (-0.082, -0.046)	< 0.001
Model 2	Ref.	-0.039 (-0.201, 0.123)	-0.158 (-0.324, 0.007)	-0.381 (-0.590, -0.173)	< 0.001	-0.054 (-0.080, -0.027)	< 0.001
Model 3	Ref.	-0.061 (-0.220, 0.098)	-0.136 (-0.304, 0.032)	-0.326 (-0.524, -0.127)	< 0.001	-0.048 (-0.071, -0.024)	< 0.001

Associations between cotinine and RBC folate were measured using regression coefficients and 95% confidence intervals(95%CI). P-trend: The median of each quartile (with Quartile 1 serving as the reference group) was included as a continuous variable in the multiple linear regression model to assess trend significance. Linear Model: Concentrations of cotinine and RBC folate were log-transformed and included in the multiple linear regression model. P: The P value from the linear model. Model 1: Unadjusted. Model 2: Adjusted for race/ethnicity, education, PIR, and marital status. Model 3: Further adjusted for age, BMI, Mean Corpuscular Hemoglobin Concentration (MCHC), Glycated Hemoglobin (HbA1c), triglycerides (TG), and total dietary folate intake. All statistical estimates accounted for the study sampling scheme, data clustering, and sample weights in NHANES to ensure national representativeness

Sensitivity analyses

Two different strategies were used to perform sensitivity analyses. First, the data from 1996 to 2006 and 2007–2018 were separately analyzed. Then, RBC folate levels were analyzed as a binary variable based on the predefined NTDs risk categories. These sensitivity analyses ultimately confirmed that the above results were robust (Supplementary Table 4).

Discussion

For this study, the NHANES database was leveraged to explore relationships between serum cotinine and RBC folate levels among pregnant women and to begin probing the underlying mechanisms linking the two. Initially, ln-transformed cotinine levels and ln-transformed RBC folate levels were found to be significantly negatively correlated in pregnant women such that higher levels of cotinine exposure corresponded with a lower in folate status. RCS analyses also indicated that the negative associations of cotinine on RBC folate were most apparent and significant above the threshold value of 2.91 ng/mL. A significantly mediating effect of increased lymphocyte count on this relationship between cotinine and RBC folate levels was also detected. In subgroup analyses, advanced maternal age and other lifestyle factors were found to contribute to a stronger negative relationship between cotinine and RBC folate levels. While prior studies have probed the relationship between tobacco exposure and folate biomarker levels to varying degrees, this is the first systematic effort to evaluate the nonlinear nature of this relationship, related mediation effects, and susceptibility-related factors using two reliable biomarkers with a high degree of specificity. Together, these analyses offer unprecedented insight into how cotinine can impact folate status among pregnant women.

The relationship between serum cotinine and RBC Folate levels

The key finding from the present study was that serum cotinine levels were significantly negatively correlated with RBC folate levels among pregnant women. In past reports, smoking while pregnant has been linked to a reduction in folate concentrations [18–20, 45]. As cotinine is firmly established as a biomarker that can be assessed to reliably evaluate tobacco exposure [24, 25], in subsequent studies, cotinine concentrations and folate levels have been found to be negatively correlated with one another, although most research to date has centered around serum folate levels, which primarily reflect short-term folate status [26, 27]. RBC folate levels, in contrast, can more reliably offer insight into the storage of folate over longer periods of time [49]. Just one study to date has leveraged both of these reliable and specific biomarkers. In that analysis, Yusuf et al. assessed 496 women in the early to middle stages of pregnancy who received prenatal care in Florida from 2011 to 2015, ultimately

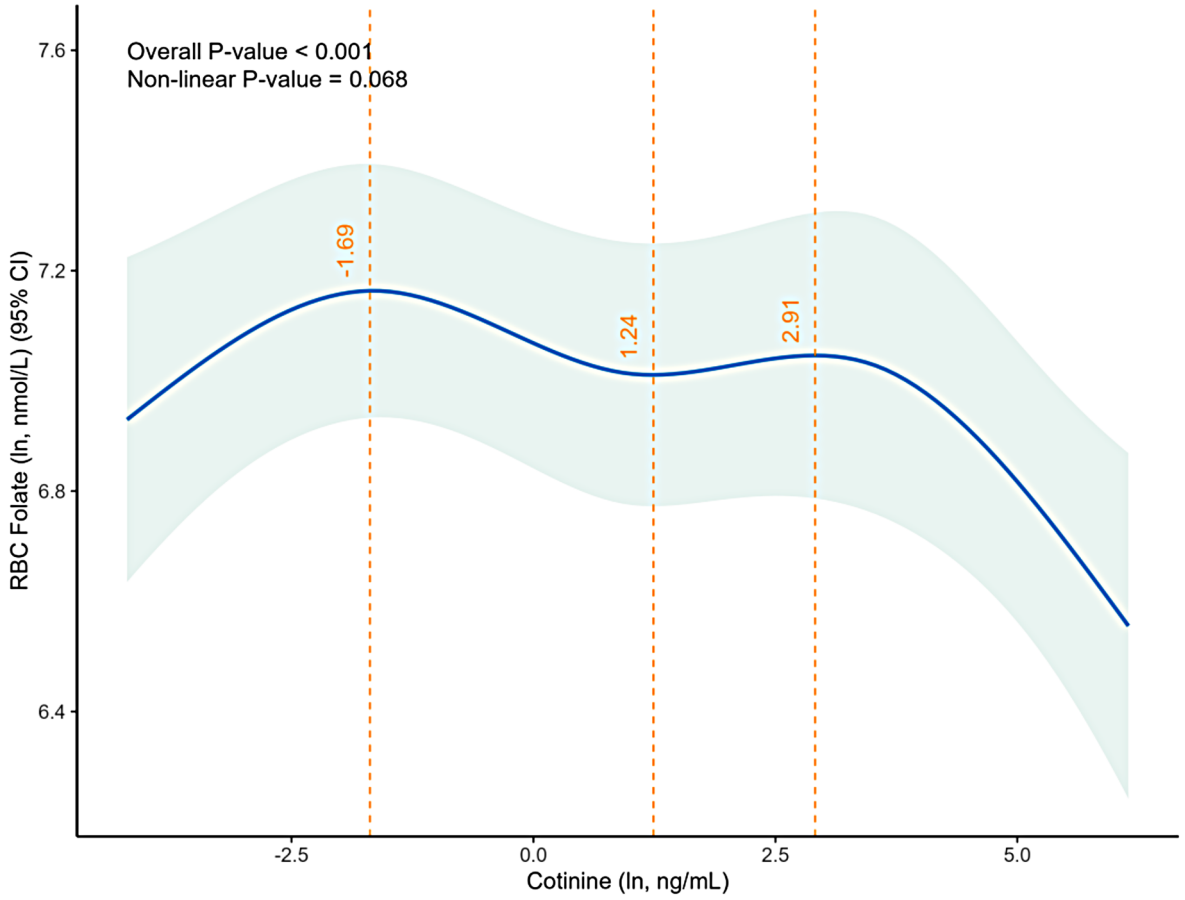

Fig. 2 Restricted cubic splines (RCS) for associations of cotinine with RBC folate. Adjusted for ethnicity, education, PIR, marital status, age, BMI, MCHC, HbA1c, TG, and total dietary folate intake. All statistical estimates accounted for the study sampling scheme, data clustering, and sample weights in NHANES to ensure national representativeness

Table 3 Threshold effect analysis of cotinine on RBC folate using piecewise linear regression

Segments	Coefficient (95% CI)	*P*
cotinine(ln) ≤ -1.69	0.035 (-0.085, 0.155)	0.558
-1.69 < cotinine(ln) ≤ 1.24	0.017 (-0.131, 0.164)	0.809
1.24 < cotinine(ln) ≤ 2.91	-0.238 (-0.555, 0.079)	0.134[a]
cotinine(ln) > 2.91	-0.198 (-0.303, -0.093)	0.002

Associations between cotinine and RBC folate were measured using regression coefficients and 95% confidence intervals (95% CI). Adjusted for race/ethnicity, education, PIR, marital status, age, BMI, MCHC, HbA1c, TG, and total dietary folate intake. Statistical estimates generally accounted for the study sampling scheme, data clustering, and sample weights in NHANES to ensure national representativeness. However, for segments marked with 'a', only sample weights were considered, without sampling scheme and clustering due to insufficient sample size

leading to the identification of a significant association between salivary levels of cotinine and a reduction in RBC folate levels [28]. In contrast with the present study, cotinine levels in that study were analyzed based on saliva biosamples, and there were no in-depth analyses of how these levels were related to RBC folate concentrations.

RCS analyses in this study indicated that cotinine and RBC folate levels differed in their relationship as a function of exposure level. At ln-transformed cotinine concentrations below − 1.24 ng/mL, the two were not significantly related, potentially because these cotinine levels may have been too low to impact folate metabolism or due to the overriding influence of dietary intake or other compensatory mechanisms in this range. A non-significant trend towards a relationship between serum ln-transformed cotinine levels in the 1.24–2.91 ng/mL range and ln-transformed RBC folate levels was detected [-0.238 (-0.555, 0.079); $p = 0.134$], suggesting that other factors may play a predominant role in modulating this relationship within this range. At ln-transformed cotinine levels of ≥ 2.91 ng/mL, significant reductions in ln-transformed RBC folate levels became evident [-0.198 (-0.303, -0.093); $p = 0.002$]. This suggests that when cotinine concentrations are sufficiently high, they may directly inhibit folate metabolism or indirectly affect folate metabolism through processes such as the induction of inflammation, oxidative stress, and DNA methylation. There are several mechanisms that may underlie these effects. For one, key enzymes involved in folate metabolism like dihydrofolate reductase (DHFR) may be inhibited by tobacco

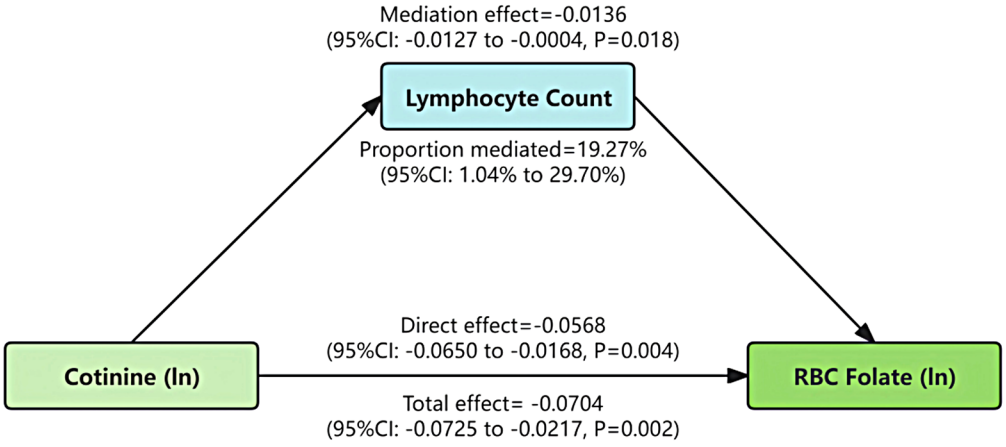

Fig. 3 Associations of cotinine with RBC folate and mediation effect of lymphocyte. Adjusted for ethnicity, education, PIR, marital status, age, BMI, MCHC, HbA1c, TG, and total dietary folate intake. All statistical estimates accounted for the study sampling scheme, data clustering, and sample weights in NHANES to ensure national representativeness

smoke-derived chemicals, leading to a reduction in folate bioavailability [58]. Secondly, smoking has been demonstrated to give rise to pronounced epigenetic changes, including altered patterns of DNA methylation, leading to long-term tissue damage and the onset of cancers and other chronic diseases [59, 60]. DNA methylation, however, is primarily dependent on carbon units provided by folate for S-adenosylmethionine (SAM) synthesis. Smoking-induced increases in methylation may lead to a corresponding rise in the consumption of folate [61]. Moreover, exposure to tobacco can trigger a range of inflammatory and oxidative stress responses. For example, cigarette smoke-derived chemicals can trigger increased expression of a range of pro-inflammatory cytokines including IL-6, IL-8, IL-17 A, and IFN-γ through MAPK and NF-κB pathway activation [62]. In a cigarette smoke-exposed mouse model, reductions in SIRT3 levels, for instance, were linked to superoxide dismutase 2 hyperacetylation and the consequent development of mitochondrial oxidative stress [63]. Folate is a key antioxidant, and it can thus alleviate inflammation through the elimination of ROS and homocysteine (Hcy) through NF-κB pathway inhibition [39]. When cotinine levels reach a particular threshold, their cumulative effects of persistent inflammation may result in significantly reduced RBC folate levels [42, 64]. Moreover, maternal folate deficiency is not only associated with the prevention of NTDs but also linked to a range of other folate-sensitive congenital anomalies in offspring, including congenital heart defects and orofacial clefts [65, 66]. In this context, the threshold effect of RBC folate level decline has critical implications for public health interventions, underscoring the importance of prioritizing pregnant women with high levels of tobacco exposure.

Mediating role of lymphocytes

As the key mediators of immune activity, lymphocytes can be readily affected by toxic compounds. In some instances, they can modulate the immune response to help mitigate the deleterious effects of these compounds. When chronic inflammatory damage develops, however, this can lead to injury at the cellular and tissue levels, culminating in greater disease risk [67–69]. Here, lymphocyte count was identified as a mediator of the relationship between serum levels of cotinine and RBC folate levels among pregnant women. Specifically, lymphocyte count was positively correlated with cotinine levels but negatively correlated with RBC folate levels. This is consistent with a prior report of positive correlations between cotinine levels and lymphocyte count in the general public [33]. This effect may be attributable to the induction of lymphocyte proliferation and activity by nicotine or metabolites thereof through the modulation of adaptive immunity-related signaling pathways [70]. Maishan et al., for instance, found that mice who had been exposed to e-cigarette aerosols (VG/PG/Nic) presented with higher pulmonary levels of the inflammatory cytokines CXCL1, MCP-1, and IL-17 A when infected with influenza as compared to air- or vehicle (VG/PG)-exposed mice [34]. Innate and immune signaling-related pathways were also upregulated, including the enhancement of B cell, T cell, and macrophage responses to infection, as well as the upregulation of B cell receptor signaling, antigen receptor-mediated regulation, lymphocyte chemotaxis, and T cell migration-related genes. Skok et al. determined that nicotine is capable of promoting the proliferation of B cells via the upregulation of the α4 and α7 nicotinic acetylcholine receptor subunits while suppressing the production of antibodies [71]. In population studies, active smokers have been found to present with higher frequencies of CD8 + T cells relative

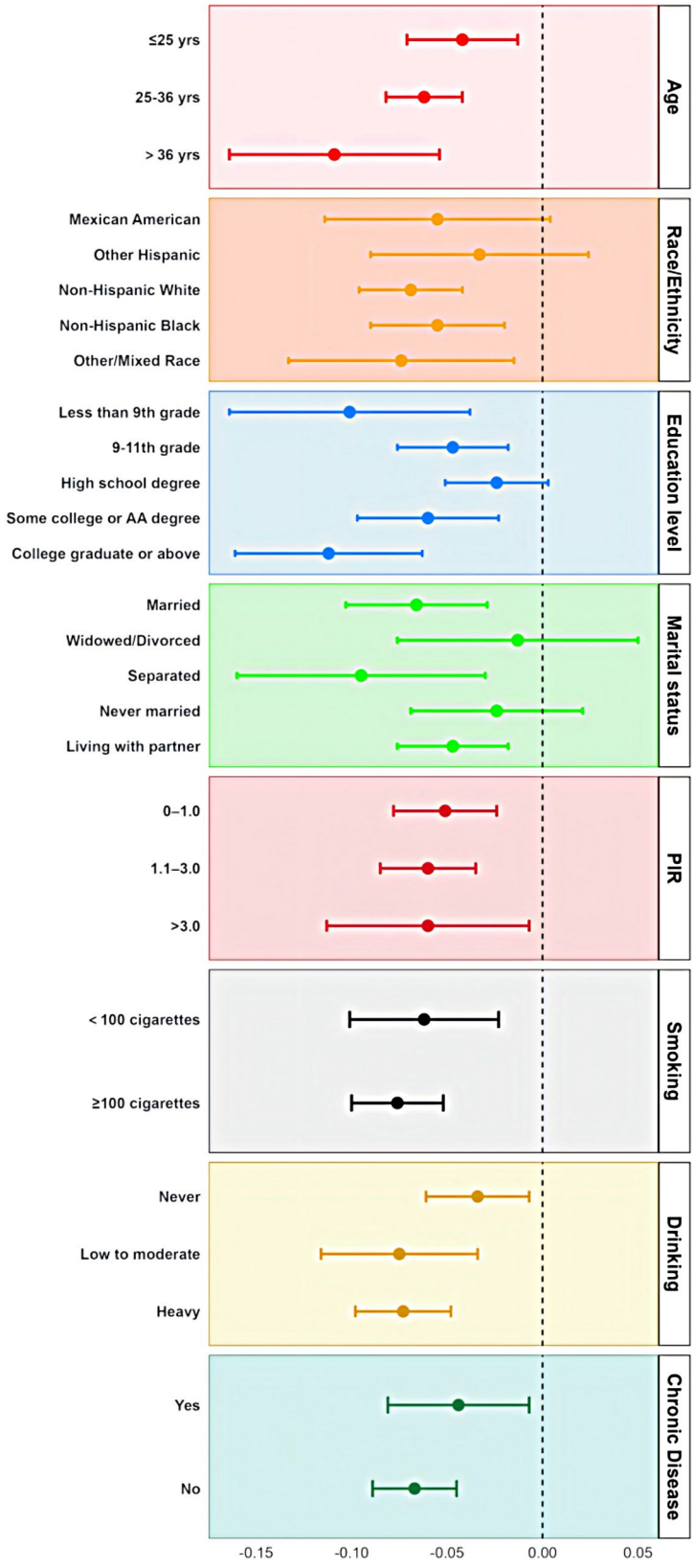

Fig. 4 Associations of cotinine with RBC folate stratified by participant characteristics. Adjusted for race/ethnicity, education, PIR, marital status, age, BMI, MCHC, HbA1c, TG, and total dietary folate intake. All statistical estimates accounted for the study sampling scheme, data clustering, and sample weights in NHANES to ensure national representativeness

to non-smokers. Passive smoking also exhibits a positive correlation with increased naïve CD3 + T cell frequency, whereas active smoking coincides with an increase in the number of peripheral blood memory B cells [31, 35, 37]. When exploring the link between inflammation and cotinine levels among younger African-American smokers, Andersen et al. determined that the frequency of GPR15 + helper T cells was higher among individuals who were cotinine-positive, and these individuals also exhibited an elevated pro-inflammatory to anti-inflammatory cytokine ratio [30]. A separate randomized single-blind crossover study performed by Flouris et al. revealed parallel increases in serum and urine cotinine levels and the levels of inflammatory cytokines (IL-4, IL-5, IL-6, TNF-α, and IFN-γ) among 8 male and 8 female non-smoking adults within a 1–3 h period after exposure to SHS, and this increase in inflammatory factor levels was also negatively associated with lung function [72]. Such reports have not been universal, however, with some studies documenting negative or nonsignificant correlations between smoking and lymphocyte count [73, 74]. Qiu et al. provided a potential explanation for these discrepant results, suggesting that smoking may impact immune homeostasis in disparate ways such that it can both exacerbate pathological immune activity while also impairing normal immunological defenses [70]. The complex variety of compounds in cigarette smoke, their multifarious biological effects, and differences in the environmental exposures and health status of individuals in this study may also account for these inconsistencies [73, 75]. In this study, cotinine levels were significantly positively correlated with lymphocyte count in this particular cohort of pregnant women.

Folate is important in the regulation of carbon metabolism, and is of particular importance for pyrimidine and purine synthesis within lymphocytes [41]. Tobacco exposure can trigger the activation and proliferation of lymphocytes, potentially triggering a demand for even higher levels of folate, exacerbating its depletion [40]. When activated, lymphocytes also produce large volumes of pro-inflammatory cytokines (IL-4, IL-5, IL-10, IL-17, TNF-α, and IFN-γ), and the antioxidant properties of folate may lead to its further consumption in an effort to counteract the resultant inflammation and oxidative stress [38, 39, 42, 76]. Furman et al. posited that inflammation may trigger localized or systemic neuroendocrine and metabolic changes aimed at conserving metabolic energy and ensuring the sufficient allocation of resources to the immune system, indirectly impacting folate bioavailability [67, 77, 78]. The mediation effect analysis performed in this study revealed that increased lymphocyte count was responsible for 19.3% of the mediating effect of the relationship between serum cotinine and RBC folate levels among pregnant women. As this mediating effect is of clear public health significance, these findings emphasize the need to further probe both the direct and indirect effects of lymphocytes in this context. However, excessive folic acid intake has also been reported to influence lymphocyte counts. For instance, Ali et al. emphasized that high levels of folic acid supplementation may modulate lymphocyte activity through alterations in immune responses [79]. Additional studies will be needed to probe the biological mechanisms that underlie interactions between folate metabolism and the immune response, thereby offering greater insight into the complex health effects attributable to tobacco exposure.

Subgroup analyses

Stratified analyses revealed that advanced maternal age (> 36 years), smoking history, and heavy alcohol consumption were all associated with exacerbated negative associations of cotinine on RBC folate levels, indicating that these are susceptibility risk factors. As a tobacco metabolite, cotinine can inhibit the folate metabolism pathway or indirectly impact the utilization of folate and its metabolism through increases in oxidative stress and inflammatory response activity [58, 63]. In combination with negative pregnancy or lifestyle-related factors, cotinine exposure may result in more severe stress and damage [39, 42, 80, 81].

Among pregnant women over 36 years of age, a stronger negative correlation between ln-transformed cotinine and ln-transformed RBC folate levels was noted, potentially owing to the physiological and metabolic changes that accompany advanced maternal age. The metabolism of cotinine primarily takes place in the liver, and involves the enzymatic activity of CYP2A6 [82]. Increasing age can coincide with declining liver function, contributing to the slower metabolism of cotinine such that it exhibits a longer half-life [83, 84]. Cotinine was also more significantly associated with lower RBC folate levels in pregnant women with a history of smoking, potentially because active smokers can be exposed to larger numbers of harmful compounds relative to passive smokers [85–87]. Even when individuals quit smoking, the cumulative toxic effects of prolonged smoking may have persistent adverse effects on folate metabolism. The gradual accumulation of these toxins triggers chronic oxidative stress and inflammation [88, 89]. Heavy alcohol consumption also worsened the negative associations of cotinine on RBC folate levels among pregnant women. The chronic abuse of alcohol is related to folate deficiency through several mechanisms [90], including mitochondrial folate transport inhibition that leads to decreased folate bioavailability [91]. Long-term intake of alcohol can also reduce intestinal proton-coupled folate transporter (Pcft) and reduced folate carrier (Rfc) levels, with both of these factors being vital for the absorption of folate [92, 93].

In conclusion, advanced maternal age and negative lifestyle factors can interact with exposure to tobacco to lead to the worsening of the folate status of pregnant women. However, further studies will be essential to clarify the mechanistic basis for these findings and to determine whether specific interventions can mitigate the negative associations of cotinine on the health of pregnant women.

Strengths and limitations

There are several strengths to this study. For one, this is the first systematic effort to explore the nonlinear nature of the relationship between tobacco exposure and folate-related biomarkers while also assessing mediating effects and the moderating role of susceptibility-related factors through the use of two robust, specific biomarkers. Together, these analyses offer detailed insight into how cotinine impacts folate status among pregnant women. Secondly, this study was based on the NHANES (1999–2018) dataset, which includes a representative sample of the population of the USA. Stratified and sensitivity analyses were also implemented to ensure that the results of this study would be stable.

Even so, this study is subject to some limitations. For one, this study only focused on adults in the USA and the results may therefore not be applicable to other populations. Secondly, these analyses were based on single-point measurements, potentially impacting the accuracy of efforts to evaluate long-term cotinine exposure. Lastly, as this was a cross-sectional study, the results may have been impacted by confounding factors that were not analyzed, despite efforts to control for many confounding factors. These analyses are also unable to clarify the causal or temporal nature of relationships among different variables. Prospective longitudinal studies will be essential to validate the present results, offering stronger evidence for the observed relationships.

Conclusion

This study based on NHANES data highlights the significant negative correlative relationship that exists between serum levels of cotinine and RBC folate levels among pregnant women, with this association being more pronounced when cotinine levels are elevated. Increased lymphocyte counts were also found to mediate this relationship. The negative impact of cotinine on RBC folate levels was additionally exacerbated by advanced maternal age, a history of smoking, and heavy alcohol consumption. Additional studies will be essential to fully understand how cotinine and RBC folate levels are related among pregnant women, thereby providing a basis for developing more effective public health interventions to prevent folate deficiency-related congenital anomalies. Nonetheless, the preventive role of folate supplementation should not be overlooked, as it has been well-established, particularly during the periconceptional period and the first 12 weeks of pregnancy. For general preventive purposes, daily folate supplementation typically ranges from 0.4 mg to 0.8 mg, while higher doses (4 mg/day) are recommended for women with a history of pregnancies affected by NTDs [94, 95].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13690-025-01533-3.

Supplementary Material 1

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Author contributions

All authors have made substantial contributions to the conception and design of the work. YB and CH (Cheng Huang): Conceptualization, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. PQW, JY, SZX, and CH (Chen Huang): Conceptualization, methodology, Writing – review & editing. QHL and MHW: Conceptualization, Writing – review & editing, Supervision. All authors have read and approved the final manuscript.

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Data availability

All information can be obtained on the NHANES official website (https://www.cdc.gov/nchs/nhanes/Default.aspx).

Declarations

Ethics approval and consent to participate

Not applicable. This study uses only secondary data analyses without any personal information identified using statistical data from NHANES website; The program was approved by the National Centre for Health Statistics (NCHS) Research Ethics Review Committee and informed consent was obtained from the participants.

Consent to publish

The authors declare that they agree with the publication of this paper in this journal.

Competing interests

The authors declare no competing interests.

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