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Urinary tobacco-specific nitrosamines and cotinine are associated with early childhood caries

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Abstract

Urinary cotinine is a well-established biomarker of nicotine exposure and has been associated with an increased risk of early childhood caries (ECC). However, the role of tobacco-specific nitrosamines remains unclear. We concurrently measured urinary cotinine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and N′-nitrosonornicotine (NNN) among 167 Indian preschoolers (mean age: 4.08 ± 0.5 years) in the Household Air Pollution Intervention Network cohort. Exposure was stratified using the NEG–TEG–MEG framework to capture gradations of environmental tobacco smoke intensity. Dental caries were assessed clinically, and biomarkers were measured via liquid chromatography–tandem mass spectrometry. Negative binomial hurdle models examined associations between biomarker concentrations and ECC. Each log-unit increase in cotinine (IRR:1.15; 95% CI:1.02–1.30) and NNN (IRR:1.11; 95% CI:1.01–1.23) levels corresponded to higher ECC incidence in the count component. Children in the highest quartile of cotinine and NNN exhibited 49% (β: 0.40) and 39% (β: 0.33) greater caries incidence, respectively. In zero model, children in the second quartile of NNN had 71% lower odds of being caries-free, suggesting that even moderate exposure increases ECC susceptibility. The NNAL/cotinine ratio was suggestive of potential thirdhand smoke exposure. Significant associations between tobacco-derived toxicants and ECC highlight the need for targeted interventions to reduce secondhand and thirdhand smoke exposure in early childhood.

Keywords Cotinine · 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol · N′-nitrosonornicotine · Environmental tobacco smoke · Secondhand smoke · Thirdhand smoke · Dental caries · Children

Introduction

Environmental tobacco smoke (ETS), a pervasive and preventable indoor air pollutant[1], encompasses secondhand smoke (SHS) and thirdhand smoke (THS)[2]. Despite global tobacco control initiatives, such as the WHO Framework Convention on Tobacco Control (FCTC), substantial populations remain unprotected from ETS

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exposure[3,4]. While public smoking bans have led to measurable reductions in exposure, evidence of corresponding household declines is still limited. Globally, over 40% of children reside in households with at least one smoker[5]. In India, 23–39% of the population is exposed to ETS, with children, adolescents, and rural residents disproportionately affected[6,7].

SHS comprises sidestream smoke from the burning end of tobacco products and exhaled mainstream smoke, leading to involuntary inhalation[8]. While ventilation may transiently dilute airborne SHS, its semi-volatile constituents adhere to indoor surfaces, accumulate in dust, and infiltrate porous materials such as carpets, upholstery, and wallboard[9,10]. Over time, these residues react with oxidants, forming secondary toxicants that can re-volatilize or resuspend as fine particles. This aged persistent fraction constitutes thirdhand smoke (THS), characterized by the "four Rs": remain, react, re-emit, and resuspend[11]. Such residues persist in homes, vehicles, rental spaces, and hotel rooms previously occupied by smokers[12–15]. Smokers also act as vectors, carrying residues on clothing, skin, and hair, thereby exposing others in ostensibly smoke-free environments[16,17]. Consequently, THS exposure occurs via inhalation, dermal absorption, and ingestion, even without concurrent smoking[9]. Young children are particularly susceptible to SHS and THS due to their developing physiology and exploratory behaviors[5,9].

Among the myriad toxicants in ETS, tobacco-specific nitrosamines (TSNAs), potent carcinogens and established biomarkers of both SHS and THS, offer insight into long-term exposure. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) are generated during tobacco curing and combustion. NNK is metabolized to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which has a half-life of 40–45 days, in contrast to ~ 16 h for cotinine[18]. Urinary NNAL levels correlate with cotinine and rise with greater ETS exposure[19].

A clear gradient in NNAL concentrations has been observed: highest among children in homes where smoking occurs indoors (SHS + THS proxy), intermediate in those whose household smokers abstain indoors (predominant THS), and lowest among children in non-smoking homes (no exposure)[20,21]. Although NNAL reflects cumulative exposure, differentiating THS from SHS remains challenging; no validated biomarker currently separates the two. To overcome this, proposed approaches include self-report, THS-specific metabolites, and the NNAL/cotinine ratio[11]. This ratio tends to be lower with SHS and higher in THS exposure, indicative of dermal and ingestion pathways. Children exhibit higher ratios than adults, consistent with behavioral differences such as floor play and hand-to-mouth activity[22,23]. Although SHS exposure has been conclusively linked to pediatric morbidities—including sudden infant death syndrome, neurodevelopmental disorders, asthma, childhood cancers, oral clefts, and dental caries[8], the role of THS remains poorly defined.

Early childhood caries (ECC), defined as the presence of one or more decayed, missing, or filled primary tooth surfaces in children under six years of age, is a major public health concern[24]. Affecting over half a billion children worldwide, ECC impairs growth, cognition, and quality of life[24,25]. It represents a dysbiotic shift in the supragingival biofilm, arising from imbalances between protective and disease-promoting factors[26]. In India, prevalence estimates range from 42% to 63%[27], mirroring disparities in limited access to dental services, financial barriers, workforce shortages, and low oral health literacy[24]. While diet and oral hygiene are major drivers, mounting evidence implicates tobacco smoke exposure. Fetal and early-life ETS exposure, even at low levels, has been associated with increased ECC risk[28–32]. However, most prior studies rely on caregiver reports or short-lived biomarkers such as cotinine, which capture only recent exposure and may misclassify chronic exposure. Mechanisms linking ETS to ECC include oxidative stress, immune suppression, salivary dysfunction and altered microbial colonization, each contributing to enamel hypoplasia.

Despite this biological plausibility, few studies have evaluated ETS-derived TSNAs in relation to ECC. Mahabee-Gittens et al. (2022) reported population-level associations between THS exposure and caries among children aged 1–11 years; both exposure and dental outcomes were caregiver-reported, and the age range extended beyond the early childhood period central to our investigation[33]. Our previous CARES-HAPIN analysis comprehensively characterized longitudinal SHS exposure from pregnancy through early childhood using repeated urinary nicotine metabolites (cotinine and 3-hydroxycotinine)[34]. Building on this work, the present study examines a distinct

toxicological dimension of tobacco smoke exposure through the simultaneous quantification of urinary cotinine, NNAL, and NNN, integrated with caregiver reported ETS information to explore potential thirdhand smoke and evaluate their associations with ECC.

Methods

Study population

The CAries Risk from exposure to Environmental Tobacco Smoke (CARES) study is nested within the Indian cohort of the multi-country Household Air Pollution Intervention Network (HAPIN) trial. The parent study design and participant details have been described previously[35,36]. Briefly, the trial enrolled approximately 800 pregnant women per site, aged 18 to < 35 years, who relied primarily on biomass for cooking and had a confirmed viable singleton pregnancy between 9 and < 20 weeks' gestation. Women were excluded if they smoked tobacco, planned to relocate within 12 months, or already used (planned to switch to) clean-fuel cookstoves.

For this analysis, 167 children aged 3–5 years were selected from the 712 followed up between April and September 2023, using a stratified multistage cluster sampling design with stratification based on district and caregiver-reported ETS exposure. Within each stratum, clusters—defined as villages or administrative blocks—were randomly drawn to ensure representation across the ETS exposure spectrum. Although this subsample was not intended to be statistically representative of the parent cohort, it was designed to capture variation in demographic and environmental diversity of the larger cohort, thereby maximizing exposure heterogeneity for biomarker analyses (Additional Table 1).

Trained field staff obtained written informed consent from parents or legal guardians on behalf of their children after explaining the study objectives and procedures. The protocol was approved by the institutional review boards of Emory University (00089799) and Sri Ramachandra Institute of Higher Education and Research (IEC-N1/16/JUL/54/49) and adhered to principles of the Declaration of Helsinki.

Baseline variables (sociodemographic characteristics, educational, occupational, lifestyle, health information) were abstracted from the HAPIN database. Additional data on child's dietary habits (snacking frequency), oral hygiene behaviors (age at brushing initiation, daily frequency, parental supervision, fluoride toothpaste use), and dental care utilization were also collected.

Environmental tobacco smoke exposure assessment

Parents/caregivers were asked whether the child currently lived with household members who smoked cigarettes, bidis, or other tobacco products. Children from non-smoking households were categorized into the no/minimal exposure group (NEG), with SHS exposure additionally assessed in other environments (e.g. restaurant, other homes, and public indoor areas).

For children living with one or more smokers, two follow-up questions were asked: (1) how many household members smoked inside the home? (0, 1, or ≥ 2); and (2) on how many of the past seven days did smoking occur indoors? (range 0–7). Children living with smokers who did not smoke indoors (0 smokers, 0 days) were classified as the predominant THS exposure group (TEG), while those with one or more smokers who smoked indoors on ≥ 1 day comprised the mixed SHS and THS exposure group (MEG). This approach was derived from Park (2020) and Merianos et al. (2024)[20,21].

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Table 1 Distribution according to caries incidence (Mean ± SD or n (%)) in CARES-HAPIN children (2023–2025, Tamil Nadu, India).

Characteristic	Overall (*N* = 167)	Children with no carious lesions (*N* = 47)	Children with non-cavitated lesions (*N* = 19)	Children with cavitated lesions (*N* = 101)	*P* value
Children's demographic information					
Age, years	4.08 (0.5)	4 (0.47)	3.89 (0.38)	4.15 (0.48)	**0.039**
Sex					
Boy	96 (57.5)	31 (66)	12 (63.2)	53 (52.5)	0.263
Girl	71 (42.5)	16 (34)	7 (36.8)	48 (47.5)	
BMI	13.13 (2.06)	13.44 (1.99)	12.19 (2.86)	13.15 (1.88)	0.190
Parent's demographic information					
Mother's Education					
No formal education	59 (35.3)	21 (44.7)	5 (26.3)	33 (32.7)	0.541
Primary school completed	51 (30.5)	11 (23.4)	7 (36.8)	33 (32.7)	
Secondary school or college completed	57 (34.1)	15 (31.9)	7 (36.8)	35 (34.7)	
Father's Education					
Don't know	1 (0.6)	1 (2.1)	0 (0.0)	0 (0.0)	0.827
No formal education	66 (39.5)	18 (38.3)	7 (36.8)	41 (40.6)	
Primary school completed	60 (35.9)	16 (34.0)	7 (36.8)	37 (36.6)	
Secondary school or college completed	40 (24.0)	12 (25.5)	5 (26.3)	23 (22.8)	
Household food insecurity					
Secure	139 (83.2)	37 (78.7)	13 (68.4)	87 (86.1)	0.185
Mild insecurity	19 (11.4)	8 (17.0)	5 (26.3)	8 (7.9)	
Moderate or severe insecurity	9 (5.4)	2 (4.3)	1 (5.3)	6 (5.9)	
Dietary diversity					
Low	135 (80.8)	40 (85.1)	15 (78.9)	82 (81.2)	0.732
Medium	28 (16.8)	7 (14.9)	3 (15.8)	16 (15.8)	
High	4 (2.4)	0 (0.0)	1 (5.3)	3 (3.0)	
Children's dietary and oral hygiene habits					
First sugar intake					
<12 months	94 (56.3)	13 (27.7)	9 (47.4)	72 (71.3)	**<0.001**
≥12 months	73 (43.7)	34 (72.3)	10 (52.6)	29 (28.7)	
Frequency of snacking					
Once a day	105 (62.9)	35 (74.5)	11 (57.9)	59 (58.4)	0.166
Twice a day	61 (36.5)	12 (25.5)	7 (36.8)	42 (41.6)	
Fed after brushing					
No	85 (50.9)	30 (63.8)	12 (63.2)	43 (42.6)	**0.029**
Yes	82 (49.1)	17 (36.2)	7 (36.8)	58 (57.4)	
Frequency of child brushing					
Once a day	78 (46.7)	28 (59.6)	14 (73.7)	36 (35.6)	**0.001**
Twice a day	89 (53.3)	19 (40.4)	5 (26.3)	65 (64.4)	
Type of toothpaste					
No toothpaste	5 (3.0)	0 (0.0)	0 (0.0)	5 (5.0)	**0.002**
Nonfluoridated toothpaste	74 (44.3)	32 (68.1)	10 (52.6)	32 (31.7)	
Fluoridated toothpaste	81 (48.5)	13 (27.7)	8 (42.1)	60 (59.4)	
Uncertain	7 (4.2)	2 (4.3)	1 (5.3)	4 (4.0)	

P values were calculated using the Kruskal–Wallis test for continuous variables and Pearson's χ^2 test for categorical variables.

Urinary biomarker measurement

Spot urine samples were collected by trained nurses and stored at -80 °C until analysis. Urinary cotinine, NNAL, NNN were simultaneously quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS), following a protocol adapted from Nikam et al.[37].

In brief, 2 mL of urine was enzymatically hydrolyzed in a buffered solution, and subjected to solid-phase extraction with isotopically labeled internal standards (13C6-NNN, 13C6-NNAL, [D3] cotinine). Analytes were separated on a Zorbax SB-C18 column and detected on an AB Sciex QTRAP-4500 mass spectrometer in multiple reaction monitoring mode. The limits of quantification (LOQ, determined at signal-to-noise ratio of 5) were 0.05 pmol/mL (10.5 pg/mL) for total NNAL, 0.01 pmol/mL for total NNN (1.7 pg/mL), and 0.05 nmol/mL (8.81 ng/mL) for cotinine. LOQs were determined before creatinine adjustment, with all subsequent concentrations normalized to urinary creatinine (ng/g Cr) to account for urine dilution[38]. Daily calibration standards and quality control duplicates (CV < 10%) ensured analytical precision. Creatinine was determined photometrically using the Jaffé method[39]. Detailed extraction and chromatographic conditions are provided in the Supplementary Methods.

Oral assessment

A calibrated pediatric dentist assessed caries using the WHO standardized surface-related decayed-missing-filled (dmfs) index for primary teeth[40]. The decayed (d) component adhered to the International Caries Detection and Assessment System (ICDAS) II criteria, where code 0 indicates caries free (CF), codes 1 to 2 represent non-cavitated lesions (NCL), and codes 3 to 6 correspond to cavitated lesions (CL). When multiple lesions were present on a single surface, the most severe code was recorded.

Statistical analyses

All analyses were performed in R (version 4.4.1, 2024). Participant characteristics were summarized as means ± SD for continuous variables and counts (percentages) or medians for categorical variables. Urinary metabolites were right-skewed and thus analyzed on natural log-transformed values. Concentrations below the LOQ were replaced with LOQ/$\sqrt{2}$ for the primary analyses. Correlations between biomarkers were assessed using Pearson's correlation coefficients. Among analyzed samples, 29.9% ($n=50$) of cotinine, 46.7% ($n=78$) of NNAL, and 21.1% ($n=35$) of NNN concentrations were below LOQ.

Given the over dispersed and zero-inflated distribution of dmfs scores (mean = 15.7, SD = 15.7), associations between tobacco smoke exposure and ECC were examined using a negative binomial hurdle (NBH) model[41](Additional Fig. 1, Additional Table 2). The model consists of: (i) a logistic regression component ("zero model") predicting the probability (π) of having no caries: $\text{logit}(\pi) = \delta_0 + \delta_1 x_i + \delta' w_i$ (ii) a negative binomial regression ("count model") estimating caries count among affected children (μ): $\log(\mu) = \lambda_0 + \lambda_1 x_i + \lambda' w_i$, where x_i is tobacco smoke exposure, w_i is a vector of covariates, and δ, λ are parameter estimates. The zero part yields odds ratios (ORs), while the count estimates incidence rate ratios (IRRs).

Covariates were selected a priori based on known or plausible associations with both exposure and ECC, as well as independent determinants of ECC (the latter to reduce model error and improve the precision of exposure effect estimates). A directed acyclic graph (DAG) was constructed to delineate relationships among key variables and distinguish confounders from causal intermediates (Additional Fig. 2). Variables on the causal pathway were excluded from the adjustment set to avoid overadjustment. All models included the same set of potential confounders or outcome predictors: maternal education (no formal education, primary school completion, and secondary school or college completion), field site (categorical), child's age (continuous), body mass index (continuous), food insecurity (categorical), timing of first sugar intake (categorical), type of toothpaste (categorical), and feeding after brushing (yes or no). To balance model parsimony, a backward stepwise elimination procedure

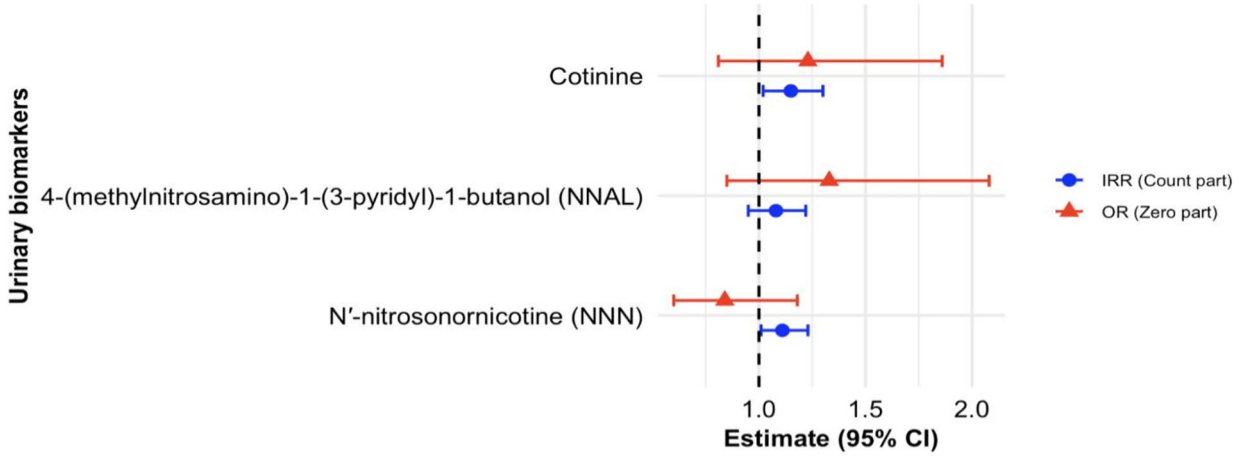


Fig. 1 Forest plot for cotinine (COT), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and N′-nitrosonornicotine (NNN) concentrations with ECC in CARES-HAPIN children. Incident rate ratios (IRR, blue) and odds ratio (OR, red) per log-unit increase in creatinine-adjusted levels are shown with error bars representing 95% confidence intervals (CIs), with the dashed line at 1 indicating no association. Estimates are adjusted for maternal education, field site, child's age at dental evaluation, body mass index, food insecurity, timing of first sugar intake, use of fluoridated toothpaste, and feeding after brushing.

Table 2 Negative binomial hurdle model estimates for ECC risk across quartiles of tobacco biomarker exposure in CARES-HAPIN Children (2023–2025, Tamil Nadu, India).

	N	Count part		Zero part	
		Coefficient (SE)	*p*-value	Coefficient (SE)	*p*-value
Cotinine[a]					
Log Linear	167	0.14 (0.06)	**0.018**	0.20 (0.21)	0.355
Quartile 2[b]	42	0.27 (0.18)	0.142	-0.54 (0.59)	0.355
Quartile 3[b]	41	0.19 (0.18)	0.289	-0.38 (0.59)	0.525
Quartile 4[b]	42	0.40 (0.17)	**0.015**	0.81 (0.64)	0.210
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol[a]					
Log Linear	167	0.07 (0.07)	0.264	0.28 (0.23)	0.213
Quartile 2[b]	39	0.28 (0.18)	0.114	0.34 (0.57)	0.550
Quartile 3[b]	41	0.28 (0.18)	0.112	-0.56 (0.57)	0.325
Quartile 4[b]	42	0.26 (0.17)	0.123	1.02 (0.63)	0.102
N′-nitrosonornicotine[a]					
Log Linear	166	0.11 (0.05)	**0.035**	-0.18 (0.17)	0.308
Quartile 2[b]	41	0.10 (0.18)	0.598	-1.23 (0.60)	**0.042**
Quartile 3[b]	41	0.18 (0.17)	0.289	-0.87 (0.64)	0.174
Quartile 4[b]	42	0.33 (0.18)	0.060	-0.75 (0.63)	0.236

[a]Adjusted for maternal education, field site, child's age at dental evaluation, body mass index, food insecurity, timing of first sugar intake, use of fluoridated toothpaste, and feeding after brushing.

[b]Quartile 1 is the referent.

guided by Akaike's Information Criterion (AIC) was applied, with variables considered eligible for removal only if they exhibited non-significance ($p \geq 0.10$) in both the count and zero components of the hurdle model.

Results

Demographic and analytical characteristics

Among the 167 children (mean ± SD = 4.08 ± 0.5 years), 57.5% were boys. Caries prevalence was high: 60.5% had cavitated lesions, 11.4% non-cavitated lesions, and 28.1% were caries-free. Although none of the mothers reported active smoking, consistent with HAPIN eligibility criteria, 52.7% of children lived with at least one

smoker (most often the father or grandfather). 40% of mothers reported SHS exposure during pregnancy, and 47.3% reported their child was exposed in the first year of life. Approximately 5.4% of households experienced moderate to severe food insecurity, while 82% reported low dietary diversity. Participant characteristics are summarized in Table 1.

The analytical method demonstrated linearity across the tested concentration ranges for cotinine (0.05–8.03 nmol/mL), NNAL (0.03–1.44 pmol/mL), and NNN (0.01–0.88 pmol/mL). Total NNAL correlated positively with cotinine ($r=0.52$, $p<0.001$) and modestly with NNN ($r=0.37$, $p=0.001$). Cotinine and NNN were also modestly correlated ($r=0.37$, $p=0.002$) (Additional Fig. 3). Maternal reports of SHS exposure were weakly but significantly correlated with urinary cotinine ($r=0.27$, $p=0.004$), but not with NNAL ($r=0.20$, $p=0.07$) or NNN ($r=0.17$, $p=0.06$).

Not all children exposed to ETS exhibited detectable biomarker levels. Boys showed slightly lower cotinine concentrations (48.8 ng/g Cr, 95% CI: 39.9, 59.7) than girls (57.1 ng/g Cr, 95% CI: 43.9, 74.3), whereas NNAL and NNN levels did not differ by sex. Children whose fathers had higher education showed lower cotinine levels (46.2 ng/g Cr, 95% CI: 36.1, 59.3), than those whose fathers with no formal education (55.7 ng/g Cr, 95% CI: 43.8, 70.8). Biomarker geometric means are presented in Additional Table 3.

Biomarker concentrations varied by exposure group (Additional Table 4). Mean cotinine levels were lowest among children with non-smoking parents (NEG: 34.3 ng/g Cr, 95% CI: 28.2, 41.6), intermediate in those with ≥1 smoking parent but no reported SHS at home (TEG: 61.5 ng/g Cr, 95% CI: 45.9, 82.2), and highest in those with both a smoking parent and reported SHS at home (MEG: 72.2 ng/g Cr, 95% CI: 52.8, 98.9). NNAL followed a similar gradient, while NNN levels remained uniformly low. The NNAL/cotinine ratio showed minimal variation.

Exposure patterns and caries prevalence across child exposure groups

Overall, 37.7% ($n=63$) of children were classified as NEG, 26.9% ($n=45$) as TEG and 35.3% ($n=59$) as MEG. Thirty-four children (20%) reported being exposed to SHS in public places during the preceding week (Additional Table 5). After adjusting for age, sex and SHS exposure in public places, ANCOVA followed by Tukey's post-hoc tests showed that NNAL concentrations were significantly higher in MEG than in NEG ($\Delta=0.46$, $p=0.02$) whereas other group comparisons were not statistically significant (Additional Table 6; Fig. 4).

ECC prevalence was higher among children from households with at least one smoker (76.2%) compared to non-smoking households (58.7%) (OR: 2.25, 95% CI:1.11, 4.57). Children whose fathers smoked 1–10 cigarettes, bidis, or other tobacco products per day showed elevated ECC prevalence (77.3%) relative to those whose fathers did not smoke (OR: 2.38, 95% CI:1.17, 4.84). Estimates for households with higher exposure levels (e.g., two smokers or ≥11 cigarettes, bidis, or other tobacco products/day) should be interpreted cautiously given small sample sizes. Compared with NEG, both TEG (OR: 2.45, 95% CI: 1.03, 5.83) and MEG (OR: 2.75, 95% CI: 1.22, 6.17) children had significantly higher odds of ECC (Additional Table 7).

Associations between urinary cotinine, NNAL, and NNN and ECC

In the fully adjusted model (Additional Table 8; Fig. 1), each one-unit increase in cotinine levels was significantly associated with increased dental caries risk (IRR, 1.15; 95% CI: 1.02, 1.30) in the count model. However, this association did not extend to the zero model (OR, 1.23; 95% CI: 0.81, 1.86), where it was not statistically significant, although the trend remained in the same direction. NNAL exhibited no significant association in either model. In contrast, one-unit increment in NNN levels coincided with greater ECC incidence (IRR, 1.11; 95% CI: 1.01, 1.23), though no discernible effect emerged in the zero models (OR, 0.84; 95% CI: 0.60, 1.18). Results remained consistent across alternative covariate specifications, supporting the robustness of the overall findings (Additional Tables 9 and 10).

Categorical analyses

While quartile estimates were not strictly monotonic, continuous exposure models showed positive linear associations (Table 2). In the count model, children in the highest quartile of cotinine (Q4 vs. Q1) exhibited a 49% higher caries incidence (β: 0.40; SE: 0.17), a statistically significant association. A similar pattern though not significant emerged for NNAL where Q4 exposure corresponded to a 30% (β: 0.26; SE: 0.17) increase in caries risk. Caries risk was exacerbated by 39% (β: 0.33; SE: 0.18) among children in the fourth quartile of NNN, though this finding was borderline significant. Intermediate quartiles showed attenuated associations, suggesting a possible threshold effect at higher exposures. In the zero models, children in the second quartile of NNN had 71% lower odds of being caries-free (β: -1.23; SE: 0.60), indicating that even moderate levels may increase the likelihood of developing ECC. No significant associations were found for cotinine or NNAL.

Discussion

This study is the first to concurrently quantify cotinine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, and N′-nitrosonornicotine levels to assess ETS exposure and its association with ECC. The NEG-TEG-MEG framework revealed discrete exposure strata that paralleled biomarker gradients and caries burden, establishing a biologically coherent continuum of exposure. Cotinine and NNAL levels increased incrementally from NEG to TEG and MEG groups, confirming a progressive exposure gradient in accordance with prior research[20,21,33]. This pattern suggests that partial smoke-free policies inadequately mitigate exposure, as evidenced by elevated NNAL concentrations even in children from homes purportedly free of smoking. These results show concordance with previous studies applying the NEG-TEG-MEG framework, reinforcing its utility as a marker of exposure intensity in pediatric populations.

Even in the absence of maternal smoking, children residing with smokers had substantially higher biomarker levels, with cotinine concentrations nearly double those observed in non-smoking households. Both cotinine and NNAL were measurable in all children, despite one-third and one-half of measurements, respectively, falling below the LOQ. Their moderate correlation ($r = 0.52$; $p < 0.001$) aligns with earlier studies[23,42], reinforcing their complementary temporal windows: cotinine reflects recent ETS uptake and NNAL captures cumulative exposure.

The geometric mean NNAL concentration (0.04 ng/g Cr) was lower than previously published ranges (5.1–48 ng/g Cr, assuming 1 g/L Cr)[23,42–46]. Such variation may be attributable to differences in age, exposure intensity, or analytical sensitivity. Notably, NNAL was detected in 11 children whose cotinine levels were below the LOQ (GM: 0.09 ng/g Cr), suggesting protracted retention or non canonical exposure pathways.

The NNAL/cotinine ratio (GM: 1.27×10^{-3}; 95% CI: 1.16×10^{-3}, 1.39×10^{-3}) mirrored prior pediatric findings[43,45–47], yet remained markedly lower than values observed in adults or heavily exposed children[23,44]. This may indicate accelerated cotinine clearance or greater dermal and ingestion-based uptake, consistent with THS exposure.

Detectable cotinine, NNAL, and NNN among children from self-reported smoke-free homes evince the discordance between subjective reports and biomarker-based exposure, concurring with previous investigations[23,42–47]. Cotinine concentrations in our "unexposed" group (0.058 nmol/mL) exceeded those documented in cohorts from Moldova, Sweden, and the United States, potentially reflecting higher community-level exposure to tobacco smoke, infiltration from nearby smokers, or other ambient nicotine sources[45,46,48]. The detection of NNN (0.04 ng/g Cr) constitutes novel evidence of low-level TSNA exposure even among nominally unexposed children, emphasizing that complete cessation is imperative for effective protection, as advocated by the WHO. In India, empirical evidence on THS remains sparse, with published work largely limited to knowledge, attitude, and practice surveys and a single biomarker study[37].

NNN accounted for 47.5% of total NNAL, and their modest correlation ($r = 0.37$; $p = 0.002$) suggests overlapping yet nonidentical exposure pathways, implying distinct metabolic or alternative routes that merit further

exploration. The use of ammonium sulfamate as a nitrosation inhibitor minimized artifactual NNN formation, ensuring analytical fidelity[49–51].

The absence of a strictly monotonic quartile gradient does not preclude an underlying exposure–response relationship. Such patterns are well recognized in epidemiologic studies and may reflect exposure heterogeneity, misclassification, residual confounding, or co-varying risk factors across exposure strata. Children in the second quartile of NNN had significantly lower odds of being caries-free, suggesting that even moderate TSNA exposure may initiate disease. A similar directional pattern for NNAL further supports its role as a robust marker of chronic exposure and a potential indicator of ECC risk. Consistent with emerging evidence integrating internal biomarkers (urinary cotinine) and external metrics (hand wipe nicotine)[52], future studies incorporating multi-matrix approaches, including surface nicotine, settled dust, and hand wipes, could enhance differentiation between SHS and THS pathways, thereby improving predictive modeling of exposure gradients.

ETS exposure frequently co-occurs with social and behavioral determinants of ECC such as lower parental education, reduced income, poor diet, and inadequate oral hygiene. Nicotine and cotinine promote the growth and virulence of Streptococcus mutans by upregulating glucosyltransferase and glucan-binding proteins, while suppressing immune responses, reducing salivary secretory IgA, buffering capacity, and antioxidant levels such as vitamin C—mechanisms that predispose to caries development[53–56]. ETS also introduces metals like cadmium, which accelerates early lesion formation[57].

NNN and NNK undergo metabolic activation via cytochrome P450 pathways, yielding intermediates that covalently bind nuclear and mitochondrial DNA to form promutagenic adducts[58,59]. These adducts can induce oxidative stress and mitochondrial dysfunction, disrupting energy metabolism in developing odontogenic cells. Excess reactive oxygen species can further impair ameloblast differentiation and secretion, leading to enamel hypoplasia and structural fragility. Contemporaneously, TSNA-induced activation of nicotinic-acetylcholine and β-adrenergic receptors triggers downstream AKT (protein kinase B) and protein kinase A signaling, amplifying inflammatory and proliferative responses[58,60]. These signaling disruptions may further interfere with epithelial–mesenchymal interactions within the developing tooth germ, regulated by Wnt, BMP, FGF, Shh (Sonic Hedgehog), and Eda (ectodysplasin) pathways[61], thereby disturbing molecular cues required for morphogenesis. These processes provide a cogent biological rationale for ETS-related vulnerability to ECC.

Although smoking in public places is prohibited under India's Cigarettes and Other Tobacco Products Act (COTPA) of 2003, smoke-free policies targeting public venues may inadvertently shift smoking into private spaces, such as homes and vehicles, where regulatory protections remain limited. Addressing these gaps requires complementary strategies, including expanding smoke-free protections in private settings, public education campaigns, and enhanced smoking cessation support. Families may benefit from targeted health communication, including information on the risks of SHS and THS exposure, and smoking cessation support. For individuals who smoke and are not yet ready or able to quit, access to a designated outdoor smoking area separated from residential buildings may facilitate adherence to smoke-free rules. Clinicians, particularly pediatricians, and dental professionals can reinforce these recommendations - integrating tobacco-smoke avoidance counseling into antenatal care and early childhood oral health programs may represent a pragmatic and cost-effective approach in resource-limited settings. In November 2025, an expert group convened under the WHO Framework Convention on Tobacco Control (FCTC) recommended, for the first time, expanding protection from secondhand smoke exposure to private and semiprivate environments[4]. Although these recommendations are not legally binding, they signal an important shift in global tobacco control; protection from SHS and THS is increasingly understood as a right that should extend to private spaces where people live, sleep, and receive care.

While causality cannot be inferred from this cross-sectional design, the direction and coherence of associations lend weight to a credible link between ETS-derived TSNAs and ECC. Limitations include small sample size, reliance on spot urine samples, and possible residual confounding. Nonetheless, the integrated biomarker approach, coupled with the NEG-TEG-MEG stratification framework, enhances the inferential strength of our findings by delineating graded exposure profiles rather than binary classifications. Analytical robustness was enhanced through the use of a negative binomial hurdle model, which effectively addressed the over-dispersed distribution

of ECC counts. Future studies with larger, more diverse populations, and longitudinal biomarker assessments are needed to clarify exposure–response dynamics and inform preventive strategies.

In conclusion, this study provides biomarker-based evidence of substantial and often unrecognized exposure to tobacco-specific nitrosamines among preschool children. Elevated NNAL and NNN levels, coupled with dose-response associations with ECC, bolster the rationale for targeted preventive strategies aimed at reducing tobacco smoke exposure in young children's environments.

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Author contributions S.S.P., N.P., and A.C. conceptualized the study. N.P., D.B.B., V.G. and S.S.P. developed the methodology. S.S.P. performed the formal analyses and drafted the manuscript. A.P. and K.S. assisted with data analysis and review. S.A. conducted the laboratory analyses. S.S.Pa. managed project administration and curated data. S.J. and L.A.W. also contributed to data curation. J.L.P., T.F.C., D.B.B., S.S.P., and N.P. contributed to funding acquisition. All authors read and approved the final manuscript.

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Data availability Datasets generated and analyzed will be made available upon reasonable request and institutional review board approval. Requests should be directed to Naveen Puttaswamy (naveen@ehe.org.in) and Sneha S. Patil (drsnehapatil@outlook.com).

Declarations

Competing interests The authors declare no competing interests.

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