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Smoke exposure, airway symptoms and exhaled nitric oxide in infants: the Generation R study

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ABSTRACT: The effect of pre- and post-natal smoke exposure on exhaled nitric oxide fraction (*F*eNO) in infants was evaluated and the association between respiratory symptoms and *F*eNO in the first 2 months of life was investigated. The Generation R study is a population-based, prenatally recruited birth cohort.

Exposures were assessed by means of questionnaires prospectively administered during pregnancy and after birth. Successful off-line *F*eNO measurements during tidal breathing were obtained in 187 infants (median age 6.9 weeks). The association between possible determinants and log *F*eNO was investigated with multiple linear regression analysis.

Infants exposed pre- and post-natally to smoke showed lower *F*eNO than infants exposed only after birth (geometric mean difference (95% confidence interval) 1.5 (1.0–2.1) ppb) and never-exposed infants (1.4 (1.0–1.8) ppb). *F*eNO was reduced in infants with severe upper respiratory symptoms compared with infants with nonsevere symptoms (1.6 (1.0–2.4) ppb). Infants with symptoms of the lower respiratory tract had lower *F*eNO than asymptomatic infants (1.2 (1.0–1.50) ppb).

In conclusion, the nature of the association between smoke exposure and exhaled nitric oxide fraction is dependent on timing and intensity of exposure. The occurrence and the severity of respiratory symptoms in the first 2 months of life are associated with lower exhaled nitric oxide fraction.

KEYWORDS: Early respiratory morbidity, exhaled nitric oxide, post-natal exposures, prenatal exposures, prospective birth cohort

Exhaled nitric oxide fraction (*F*eNO) is increased in asthmatic adults [1], children [2], and infants with eczema [3] and recurrent wheezing [4], and has been proposed as a noninvasive marker of eosinophilic airway inflammation. Compared with healthy infants, lower *F*eNO levels have been found in infants with virus-associated acute, wheezing bronchitis [5] and in infants with upper respiratory symptoms (URS), such as rhinorrhoea [6]. Several pre- and post-natal factors have been shown to influence the levels of *F*eNO in infants, such as tobacco smoke exposure [7–9], coffee consumption during pregnancy [8], maternal atopic disease [8, 10], birth weight [11], gestational age [11, 12], sex [8] and infections [13]. However, the influence of risk factors for respiratory morbidity on *F*eNO in infancy is not clear. Previous studies investigating the association between smoke exposure, one of the best known risk factors for respiratory morbidity in infants, and *F*eNO have given conflicting results. HALL *et al.* [9] found lower *F*eNO in infants exposed to smoking during pregnancy than in unexposed infants. In a subsequent report by FREY *et al.* [8], this difference was only significant in infants of mothers without atopic disease. In addition, the role of post-natal exposure to tobacco smoke in infants has been investigated but the results are not consistent. In a recent study, FRANKLIN *et al.* [7] reported higher *F*eNO in infants exposed to post-natal tobacco smoking, whereas previous studies did not show such an effect, or a lower *F*eNO in exposed asthmatics and healthy subjects [14, 15].

Previous studies that sought to investigate the effect of different determinants of *F*eNO levels in early infancy retrospectively assessed prenatal exposure variables after birth, rather than prospectively

For editorial comments see page 252.

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during pregnancy. Therefore, the temporality or succession of events was not documented and the exposure assessment was more prone to recall bias. The aim of the present prospective birth cohort study was to evaluate whether, and to what extent, prenatal and early post-natal exposures influence F_{ENO} in early infancy. The effect of URS and lower respiratory symptoms (LRS) on F_{ENO} in the first 2 months of life was also investigated.

SUBJECTS AND METHODS

The Generation R study is a prospective, population-based, prenatally recruited birth cohort study undertaken in Rotterdam (the Netherlands). A randomly selected group of 1,232 pregnant Dutch females and their children were enrolled in the Generation R focus study. In the focus study, more detailed assessments of foetal and post-natal growth and development were performed [16, 17]. Females were enrolled during pregnancy. Sociodemographic factors and exposure to risk factors for respiratory diseases were assessed by means of questionnaires administered to the mother during early (gestational age $<$18 weeks), mid (gestational age 18–25 weeks) and late (gestational age $\geqslant$25 weeks) pregnancy and to the partner at 20 weeks. Information was gathered on the following exposure variables for both the mother and the partner: sociodemographic factors, smoking habits, atopy and siblings. A questionnaire was administered to the parents when the child was 2 months old and exposure variables were again assessed, together with onset, occurrence and severity of URS and LRS.

Between November 2004 and September 2005, F_{ENO} measurements were attempted in 225 infants participating in the focus study at a median (range) age of 6.7 (3.7–16.9) weeks. Mixed oral/nasal F_{ENO} was measured off-line during tidal breathing with a face mask covering the nose and mouth without the use of sedation as previously described [18]. An F_{ENO} measurement was considered successful if: exhaled air was sampled during quiet tidal breathing; the face mask was tightly fitted to the nose and mouth during the whole procedure; and at least five breaths were obtained. All F_{ENO} measurements were conducted with the infants awake. Ambient nitric oxide (NO) was measured before each F_{ENO} measurement (Sievers 280 B; Sievers Inc., Boulder, CO, USA). In case of an ambient NO concentration $>$10 ppb, the infant inhaled $\geqslant$2 tidal breaths of NO-free air from an NO-inert 750-mL balloon in order to permit washout of the dead space of the lungs [19]. However, as F_{ENO} showed a positive significant association with ambient NO levels, ambient NO was always included in the multivariable models.

All infants were free of respiratory symptoms and had no clinical evidence of airways infection at the time of the measurement. The Medical Ethical Committee of the Erasmus Medical Center (Rotterdam) approved the study. Mothers and their partners received written and verbal information about the study and gave written informed consent.

Definition of variables considered in the analysis

The education level of the mother was divided into three categories (lower, intermediate or higher vocational training) according to the Dutch standard classification of education [20]. Parental atopy was defined as self-reported or doctor-diagnosed allergy or atopic disease (allergic asthma, hay fever, eczema). Prenatal maternal smoking was assessed in the first questionnaire by asking the mother whether she had smoked during the pregnancy (no, smoked until the pregnancy was known, or continued smoking after the pregnancy was known). In the second and third questionnaires, the mother was asked whether she had smoked in the past 2 months (no or yes). If the answer was positive at least at two time-points, the infant was classified as prenatally exposed. Exposure to passive tobacco smoking was also assessed by asking whether people regularly smoked in the house or in the working environment of the mother during pregnancy. Gestational age, birthweight and birth length were obtained from the midwife and hospital registries.

Post-natal exposure to tobacco smoke was assessed by asking whether the infant had been exposed to smoke by the mother or by any of the members of the household at least once a week. In addition, parents were asked whether their child had had a runny and/or blocked nose (URS), breathlessness, a whistling noise when breathing, wheezing, panting, difficulty breathing and/or cough (LRS) in the past 2 months. Symptoms were considered severe if they required a visit to a doctor, as reported by the parents.

Statistical analysis

F_{ENO} values were log-normally distributed. Univariable analyses using an unpaired t-test and simple linear regression were used to determine associations between log F_{ENO} and the following explanatory variables: ambient NO; birth weight; gestational age; sex; breastfeeding; maternal educational level; maternal and paternal atopy; pre- and post-natal tobacco smoke exposure; siblings; weight; length at the study date; age at the study date; URS; and LRS. Factors that had a significance level $\leqslant$0.1 from univariate analyses were included in the multiple linear regression models in order to evaluate the relationship between log F_{ENO} (dependent variable) and pre and post-natal exposures while controlling for other relevant factors. Although not directly associated with F_{ENO} in the current study population, atopic status of the mother, birth weight, sex and age at the study date were included in all regression models as covariates, as these have been shown to influence F_{ENO} or the occurrence of respiratory symptoms [4, 8]. Effect modification by maternal atopy and sex was investigated by adding interaction terms in the final models.

F_{ENO} values were back-transformed after the analysis and are reported as geometric mean (95% confidence interval; CI) in ppb. Comparisons of F_{ENO} between groups are presented as geometric mean of the difference (95% CI). A two-tailed p-value $<$0.05 was considered significant.

Due to the paucity of data in the literature, no power calculation could be performed in order to evaluate the size of the study needed to detect a difference in F_{ENO} values between groups of infants.

RESULTS

F_{ENO} measurements were attempted in 225 infants and were successful in 187 (83%) infants. During the study, 38 measurements were excluded because a quiet tidal breathing pattern was not maintained during the whole procedure (n=31) or because $<$5 breaths were collected in the sampling balloon (n=7).

TABLE 1 Baseline characteristics and anthropometrics in the 187 infants studied

Characteristics	Median (range)
Mother's age yrs	32.5 (18.5–42.9)
Father's age yrs	33.8 (16–58.2)
Gestational age at enrolment weeks	13 (8.5–23.2)
Gestational age at birth weeks	40.3 (34.6–43.0)
Birthweight g	3520 (1958–5170)
Age at study date weeks	6.9 (3.7–16.9)
Weight at study date g	4890 (3350–8230)
Length at study date cm	56.9 (50.6–66.3)

Excluded infants had younger mothers (median (range) age 30.3 (18.5–40) yrs) and fathers (32.8 (25.3–39.6) yrs) than infants with successful F_{ENO} measurements (p=0.003 and p=0.017, respectively; unpaired t-test), but the other baseline characteristics and anthropometrics at the study date did not differ between the two groups (table 1).

Pre- and post-natal exposures and F_{ENO}

In univariable analysis, anthropometrics were not related to F_{ENO} (table 2), whereas prenatal maternal smoking affected F_{ENO}, with lower levels in exposed infants (p=0.047; table 3). Paternal smoking and maternal passive tobacco smoke exposure during pregnancy did not affect F_{ENO}; therefore, an infant was considered prenatally exposed if the mother smoked during pregnancy, independent of other sources of smoke. With regard to prenatal smoke, 44 infants were exposed (prenatally only: n=23; pre- and post-natally: n=21), whereas 51 infants were exposed to environmental smoke after birth by the mother or by other members of the household (post-natally only: n=30; pre- and post-natally: n=21). Sex, parental atopic status and maternal asthma were not related to F_{ENO} (table 3). In order to compare the different smoke exposure categories directly, one variable was created from the combination of pre- and post-natal smoke exposure, with four mutually exclusive categories: never-exposed, prenatal exposure only, post-natal exposure only, and continuous exposure both pre- and post-natally. The association between the combined variable ''smoke exposure'' and F_{ENO} was significant in the univariable analysis (table 3) and also in the multivariable regression model (table 4). Infants exposed pre- and post-natally to smoke showed lower F_{ENO} than infants exposed only after birth (geometric mean difference (95% CI) 1.5 (1.0–2.1) ppb; p=0.042) and never-exposed infants (1.4 (1.0–1.8) ppb; p=0.052; fig. 1). This association was independent of respiratory symptoms and not modified by sex (p for interaction=0.69) or maternal atopy (p for interaction=0.46). However, among the 60 infants of atopic mothers, only 22 were exposed to smoke (prenatally: n=6; post-natally: n=10; pre- and post-natally: n=6). The association between ambient NO and F_{ENO} also remained significant (p<0.001) in the multivariable model.

TABLE 2 Univariable analyses of exhaled nitric oxide fraction (F_{ENO}), ambient nitric oxide (NO) and anthropometrics in the study population

Variables	β coefficient (95% CI)
Ambient NO ppb	0.0069*** (0.004–0.01)
Gestational age weeks	0.0145 (-0.016–0.045)
Birth weight kg	-0.0388 (-0.12–0.042)
Age at study date weeks	0.0025 (-0.017–0.022)
Weight at study date kg	-0.0054 (-0.06–0049)
Length at study date cm	0.0054 (-0.011–0.021)

β coefficients were estimated by linear regression analysis and should be judged as the change of log F_{ENO} per unit change in the variables. CI: confidence interval. ***: p<0.001.

Respiratory symptoms and F_{ENO}

LRS were reported for 83 infants (63 nonsevere and 20 severe), whereas 130 infants had URS (120 nonsevere and 10 severe). In the multivariable analysis, F_{ENO} values were lower in infants with LRS (n=83) than in asymptomatic (n=104) infants (geometric mean difference (95% CI) 1.2 (1.0–1.5) ppb; p=0.046; table 4), but no association was found between the severity of LRS and F_{ENO}. Sex and atopy did not modify this association (p for interaction=0.68 and 0.88, respectively). Such differences were not found for URS in univariate analysis (p=0.3), or when URS was added into the multivariable model as a binary outcome (p=0.9). However, considering the severity of the symptoms (0=no symptoms; 1=nonsevere; 2=severe), infants with a URS that required a doctor's visit (n=10) had lower F_{ENO} compared with infants with nonsevere symptoms (geometric mean difference (95% CI) 1.6 (1.0–2.4) ppb; p=0.047) and tended to have lower F_{ENO} than asymptomatic infants (1.5 (0.9–1.2) ppb; p=0.075; fig. 2). This effect was independent of smoke exposure and LRS, but still significantly associated with F_{ENO} (table 4). Tobacco smoke exposure or parental atopy, as well as the other investigated determinants, were not associated with occurrence or severity of respiratory symptoms.

DISCUSSION

In the present birth cohort study, an association was found between tobacco smoke exposure and F_{ENO} values in infants. Infants continuously exposed to smoke both *in utero* and after birth had lower F_{ENO} than never-exposed infants and infants exposed only post-natally. None of the other investigated risk factors for respiratory morbidity affected F_{ENO}. The association between respiratory symptoms and lower F_{ENO} was significant in infants with severe URS and LRS.

Few studies have addressed the separate effects of pre- and post-natal tobacco smoke exposure in infants [7–9, 14]. FREY *et al.* [8] measured F_{ENO} in a selected group of healthy infants and assessed pre- and post-natal environmental tobacco smoke exposure after birth. FREY *et al*. [8] found that maternal smoking in pregnancy was associated with lower F_{ENO}, but only in infants of mothers without asthma, whereas the same exposure in mothers with atopic disease was associated with higher levels. In univariable analysis, it was found that F_{ENO} was lower in infants of mothers who smoked during pregnancy, whereas in the multivariable analysis infants exposed to smoke prenatally had lower F_{ENO} than never-exposed infants only if

TABLE 3 Univariable analyses of pre- and post-natal variables and exhaled nitric oxide fraction (F_{ENO})

Variables	Subjects n	Geometric mean F_{ENO} (95% CI) ppb
Sex		
Males	95	10.6 (9.2–12.2)
Females	92	11.2 (9.7–12.9)
Exclusive breastfeeding		
No	123	10.5 (9.2–11.8)
Yes	64	11.7 (9.9–14.0)
Maternal education		
Low	5	9.8 (5.2–18.3)
Intermediate	72	10.9 (9.3–12.8)
High	110	10.9 (9.6–12.5)
Maternal atopy		
No	127	10.8 (9.6–12.2)
Yes	60	11.0 (9.2–13.1)
Maternal asthma		
No	175	10.8 (9.7–12.0)
Yes	12	12.1 (8.1–18.2)
Paternal atopy		
No	143	10.9 (9.7–12.2)
Yes	44	10.8 (8.8–13.3)
Paternal smoking		
No	130	10.6 (9.4–11.9)
Yes	57	11.6 (9.7–13.9)
Maternal passive smoke exposure during pregnancy		
No	69	11.1 (9.4–13.1)
Yes	118	10.8 (9.5–12.2)
Maternal smoking during pregnancy		
No	143	11.5 (10.3–12.9)
Yes	44	9.1 (7.4–11.1)[¶]
Post-natal environmental smoke exposure		
No	136	11.1 (9.9–12.5)
Yes	51	10.4 (8.6–12.6)
Smoke exposure[#]		
Never	113	11.3 (9.9–12.8)
Prenatal only	23	10.1 (7.6–13.4)
Post-natal only	30	12.3 (9.6–15.8)
Pre- and post-natal	21	8.1 (6.0–10.9)[+]
Siblings		
No	121	11.1 (9.8–12.6)
Yes	66	10.4 (8.8–12.4)
Upper respiratory symptoms		
No	57	11.7 (9.8–14.0)
Yes	130	10.5 (9.4–11.9)
Lower respiratory symptoms		
No	104	11.7 (10.3–13.4)
Yes	83	9.9 (8.5–11.5)[§]

Log F_{ENO} values were compared using an unpaired t-test. CI: confidence interval. [#]: combination of maternal smoking during pregnancy and post-natal environmental smoke exposure; [¶]: p=0.047 compared with never-exposed; [+]: p=0.042 compared with never-exposed and p=0.033 compared with post-natal exposure only; [§]: p=0.09 compared with asymptomatic infants.

TABLE 4 Multivariable linear regression model with log exhaled nitric oxide fraction (F_{ENO}) as dependent variable

Variables	Subjects n	Geometric mean F_{ENO} (95% CI) ppb
Smoke exposure		
Never	113	9.8 (8.2–11.8)
Prenatal only	23	9.6 (7.2–12.7)
Post-natal only	30	10.5 (8.0–13.9)
Pre- and post-natal	21	7.2 (5.3–9.8)[#]
Lower respiratory symptoms		
No	104	10.2 (8.5–12.2)
Yes	83	8.3 (6.7–10.2)[¶]
Upper respiratory symptoms		
No	57	10.4 (8.6–12.7)
Yes, did not visit doctor	120	10.8 (9.3–12.5)
Yes, did visit doctor	10	6.9 (4.6–10.4)[+]

The F_{ENO} values were adjusted in the regression model for all the listed factors and for sex, birthweight, maternal atopy, age at study date and ambient nitric oxide. CI: confidence interval. [#]: p=0.052 compared with never-exposed and p=0.042 compared with exposed only post-natally; [¶]: p=0.046 compared with asymptomatic infants; [+]: p=0.047 compared with nonsevere symptoms.

smoke exposure was protracted after birth. In the present study, neither parental atopy nor sex modified this association, confirming the earlier findings of HALL *et al.* [9]. However, due to the small numbers of infants per group, there was insufficient power to adequately investigate such interactions. The current study is embedded in a larger population-based birth cohort, and infants were not selected according to their health status. Furthermore, the repeated assessment of exposure variables during pregnancy provided an opportunity to study prenatal smoke exposure in greater detail and reduced the likelihood of recall bias, strengthening the validity of the current findings. In a recent study, FRANKLIN *et al.* [7] found increased F_{ENO} in infants exposed to parental smoking and evidence of a dose–response relationship. In the present study, a clear effect of post-natal smoke exposure on F_{ENO} could not be demonstrated, as it was found that F_{ENO} was higher in infants exposed to smoke only post-natally than in infants exposed both pre- and post-natally, but it was not different from never-exposed infants. The mechanisms for increased F_{ENO} in infants exposed to post-natal maternal smoking, as observed previously and herein, are not clear. A possible explanation may be a direct irritant effect of smoke on the airways [7].

The lower F_{ENO} found in infants exposed to prenatal tobacco smoking would support the hypothesis that smoke exposure during pregnancy inhibits inducible NO synthase [21]. Possible implications of such suppression are hypothetical, but as NO may serve important functions in local defence and in the maintenance of normal vaso- and bronchomotor tone, any factor that modifies baseline NO generation in the airways of young infants should be a reason for concern and further study.

The occurrence and severity of respiratory symptoms were associated with lower F_{ENO} in infants. FRANKLIN *et al.* [6] also

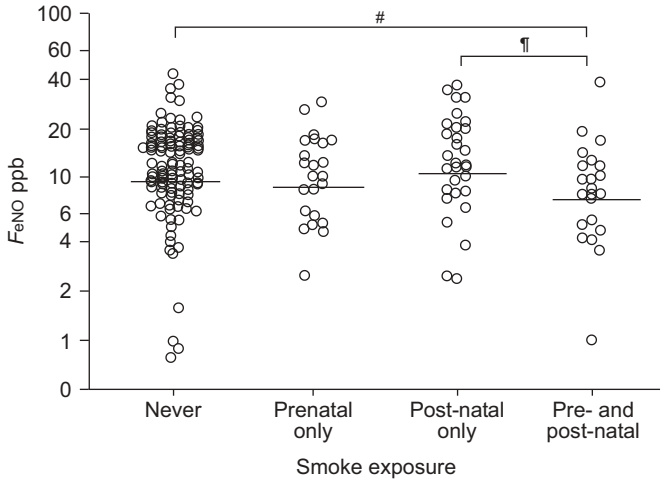

FIGURE 1. Pre- and post-natal maternal smoking exposure and exhaled nitric oxide fraction (*F*eNO) values in infants. Never-exposed: n=113; prenatal exposure only: n=23; post-natal exposure only: n=30; exposed pre- and post-natally: n=21. Horizontal lines represent geometric mean *F*eNO estimated with multivariable linear regression and adjusted for sex, birth weight, maternal atopy, age at study date, ambient nitric oxide, lower respiratory symptoms and upper respiratory symptoms. #: p=0.052; ¶: p=0.042.

found low *F*eNO in infants with ongoing rhinorrhoea, but *F*eNO increased 4–12 weeks after the initial assessment, when symptoms had resolved. Although children in the present study were free of respiratory symptoms and had no evidence of respiratory infection at the time of testing, they might have had symptoms in the weeks preceding the *F*eNO measurement. Therefore, the current results should be interpreted with caution, as the age at which symptoms occurred and the timing of symptoms in relation to the *F*eNO measurements might have influenced the findings. It cannot be excluded that a reduced *F*eNO in infants with respiratory symptoms is related to a delayed effect of acute symptoms on the NO generation or diffusion through the airways. Another reason for caution, when interpreting the present results, is that this association was shown when *F*eNO was compared between the relatively small group of infants with severe URS and infants with nonsevere symptoms. A child may be taken to the doctor for many reasons, such as symptom severity, but parental anxiety could also have influenced the decision, and this might have led to misclassification. However, if such misclassification occurred, this is not likely to bias the direction of the current results as this would mean that the effect size was underestimated.

Lower *F*eNO was found in infants who had had LRS, but no association was found between wheezing and *F*eNO. Previous studies found an association between wheezing and *F*eNO in selected populations of infants with a high risk of developing atopic disease or with recurrent wheeze [4, 22]. In addition, in a prospective study [10], higher *F*eNO at 1 month of age was predictive of the development of respiratory symptoms in the first year in infants of atopic mothers only, who are at higher risk of developing asthma. In contrast, in the same study, a trend towards a negative association between *F*eNO and severe respiratory symptoms in infants of nonatopic mothers was found. Infants in the present study were from an unselected population and maternal atopy did not modify any of the associations found, which may explain the discrepancies. Although there is evidence that most asthma starts early in life [23], respiratory morbidity in the preschool child is mostly related to neutrophilic airway inflammation [24] and there is very little evidence of chronic eosinophilic bronchial inflammation in the first months of life [25].

It could be argued that measuring mixed oral/nasal *F*eNO without controlling for expiratory flow might introduce variability [26], especially in infants exposed to tobacco smoke who may have abnormal airway mechanics. It has previously been demonstrated that *F*eNO measured with variable flow was reproducible [18] and allowed differentiation between infants with different respiratory diseases in a similar way to more sophisticated techniques, taking into account lung function parameters and breathing pattern [4]. However, differences between groups may still be due to differences in tidal flows, particularly when comparing groups with potentially different tidal breathing patterns. Indeed, differences in tidal flows might introduce variability and explain some of the overlap and the relatively small differences between the groups in the present study.

The current authors found a positive correlation between *F*eNO and ambient NO, in agreement with a previous study by PIJNENBURG *et al.* [27]. Although the correlation was also significant in the multivariable model, the current authors showed that it did not affect the results of the study, as the associations found were independent of ambient NO concentrations. In infants, the influence of ambient NO on *F*eNO could be

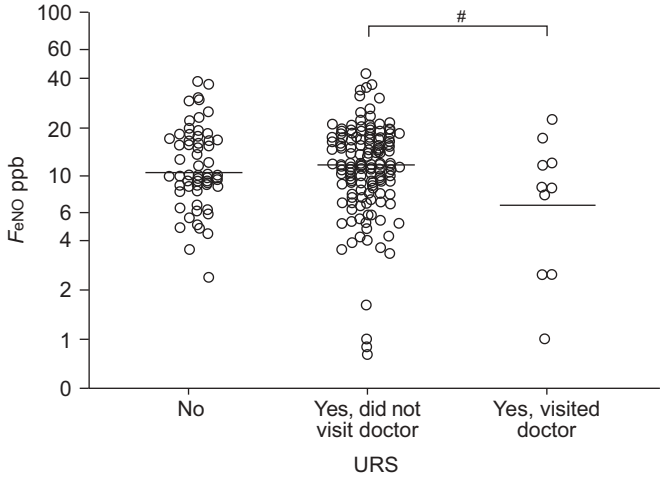

FIGURE 2. Upper respiratory symptoms (URS) and exhaled nitric oxide fraction (*F*eNO) values in infants. No symptoms: n=57; URS and did not visit a doctor: n=120; URS and did visit a doctor: n=10. Horizontal lines represent geometric mean *F*eNO estimated with multivariable linear regression and adjusted for sex, birthweight, maternal atopy, age at study date, ambient nitric oxide, smoke exposure and lower respiratory symptoms. #: p=0.047.

reduced by always using NO-free air when measuring *F*eNO [28]. However, such recommendations were not available when the present study commenced, and NO-free air was used only if room concentrations were >10 ppb, in agreement with previously published guidelines [19]. The current findings suggest that infants should inhale >2 tidal breaths of NO-free air, as this would reduce the contamination by ambient NO; however, this might reduce the success rate of the measurements, as awake infants might not tolerate the face mask for a longer period of time.

A possible limitation to the present study is that smoke exposure was assessed by means of questionnaires and not confirmed with the measurement of specific biomarkers. Although a good agreement between parental report of smoking and air nicotine concentration has been shown [29], some misclassification might have occurred, as parents would under-report smoking. If this was the case, the effect of smoke on *F*eNO would be underestimated, as smoking parents would be classified in the group of nonsmokers. Therefore, the current authors hypothesise that the size of this effect could be greater than reported.

In conclusion, pre- and post-natal tobacco smoke exposure are associated with mixed oral/nasal exhaled nitric oxide fraction in early infancy, with lower exhaled nitric oxide fraction in prenatally exposed infants and higher exhaled nitric oxide fraction in case of post-natal exposure. Reported airway symptoms, depending on the frequency and severity, were associated with lower exhaled nitric oxide fraction in the first 2 months of life. The meaning of changes in exhaled nitric oxide fraction for respiratory health in infancy needs to be further elucidated.

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