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Exhaled nitric oxide and the risk of wheezing in infancy: the Generation R Study

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ABSTRACT: We assessed whether exhaled nitric oxide fraction (*F*eNO), a marker of eosinophilic airway inflammation, at 6 months was associated with the risk of wheezing during the first 2 yrs of life.

In the Generation R birth cohort, pre- and post-natal risk factors for respiratory morbidity and respiratory symptoms were assessed by questionnaires at 6 and 24 months. In 428 infants, off-line mixed oral/nasal *F*eNO was successfully measured during tidal breathing at 6 months. Complete data on *F*eNO and respiratory symptoms within the first 6 months of life were available for 294 infants.

***F*eNO was higher in males, was positively associated with age and was negatively associated with upper and lower respiratory symptoms within the first 6 months. Logistic regression analysis showed that for every ppb increase of *F*eNO measured at 6 months, infants had a 1.06 (95% confidence interval 1.01–1.11)-fold increased risk of wheezing in the second year of life. High *F*eNO (>17.5 ppb) showed a limited added value in predicting wheezing in the second year.**

We conclude that *F*eNO at 6 months is positively associated with the risk of wheezing, but has limited added value in predicting wheezing in the second year of life in individual children.

KEYWORDS: Birth cohort, exhaled nitric oxide, infants, prospective study, wheezing

In the last decade, there has been a growing interest in measuring the fraction of nitric oxide in exhaled air (*F*eNO), a biomarker of eosinophilic airway inflammation, in young children. Increased *F*eNO levels have been found in asthmatic adults and older children [1, 2]. Studies in infants found that *F*eNO was positively associated with recurrent wheezing [3–5], whereas lower *F*eNO was associated with virus-associated wheezing [6] and with upper and lower respiratory symptoms [7, 8]. Indeed, respiratory morbidity in infants is mostly related to neutrophilic airway inflammation, which does not increase *F*eNO [9], and not to eosinophilic inflammation [10, 11]. Previous studies in young children are difficult to compare as different inclusion criteria and methods of measuring *F*eNO have been used. Furthermore, it is important to take possible confounders into account, such as pre- and post-natal smoke exposure [8, 12], parental atopy [13], sex [13], birth weight [14] and anthropometrics [4], which have been shown to influence *F*eNO in infants. In a prospective study, LATZIN *et al*. [15] measured *F*eNO on-line in 1-month-old infants and found that an increased *F*eNO was associated with the development of severe respiratory symptoms in the first year of life only if the mother had an atopic disease or had been smoking during pregnancy. These findings suggested that *F*eNO is already increased in the first months of life in children at high risk of developing asthma. In the present prospective birth cohort study, we assessed determinants of *F*eNO in infants at the age of 6 months and investigated whether *F*eNO measured at 6 months was associated with the risk of wheezing in the second year of life.

METHODS

Study population

The Generation R Study is a pre-natally recruited population-based birth cohort in Rotterdam, the Netherlands [16, 17]. In total, 9,778 females with a delivery date between April 2002 and January 2006 were enrolled in the study. Detailed assessments of fetal and post-natal growth and development were conducted in a subgroup of 1,232 Dutch children and their parents. The Medical Ethical Committee of the Erasmus Medical Center Rotterdam approved the study. Subjects and their

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partners received written and verbal information about the study and gave written informed consent.

Pre- and post-natal exposure variables

Maternal atopy/atopic disease (self-reported or doctor-diagnosed allergy and/or asthma, hayfever and eczema) and pre-natal exposure to tobacco smoke were assessed prospectively by means of questionnaires administered to the female in early (<18 weeks), mid (18–25 weeks) and late (>25 weeks) pregnancy, and to the partner at 20 weeks. Gestational age, birth weight and birth length were obtained from midwife and hospital registries. Post-natal factors, including exposure to tobacco smoke, eczema and upper respiratory symptoms (URS) within the first 6 months of life were assessed with a questionnaire administered at 6 months. Subjects were also asked to report the occurrence of respiratory tract infections (RTI), such as symptoms of influenza, ear or throat infection and respiratory syncytial virus (RSV)-bronchiolitis (defined as parental report of doctor-diagnosed RSV infection in their infants) within the first 6 months of life.

Lower respiratory symptoms

The occurrence of wheezing was assessed at 6 and 24 months by questionnaires adapted from the International Study of Asthma and Allergies in Childhood (ISAAC) core questionnaires [18]. At 6 months, wheezing was defined as parental report of at least one episode of wheezing within the first 6 months of life. At 24 months, wheezing was defined as wheezing at least once in the previous 12 months.

A combined variable ''lower respiratory symptoms'' (LRS) within the first 6 months of life was also computed and included parental report of wheezing, cough and/or breathlessness.

F_{ENO} measurements

F_{ENO} was measured off-line in awake infants during tidal breathing [8]. A facemask covering nose and mouth was connected to a two-way non-rebreathing valve (Hans Rudolph Inc., Kansas City, MO, USA) with the expiratory port attached to a 150-mL Mylar balloon. The nitric oxide (NO) concentration in the sampling balloon was measured by chemiluminescence (Sievers 280 B, Boulder, CO, USA). Ambient NO was determined before each F_{ENO} measurement. In agreement with guidelines available at the time of designing the study [19], a 750-mL NO-free air balloon was connected to the inspiratory port of the valve if ambient NO was >10 ppb, and infants inhaled two breaths of NO-free air. We previously showed good within-subject short-term reproducibility of these F_{ENO} measurements [20]. Results were excluded if a quiet tidal breathing pattern was not maintained during the whole procedure, if the mask was not tightly fitted to the infant's face and if fewer than five breaths were collected in the sampling balloon. All infants were free of respiratory symptoms for at least 1 week before the measurements and had no clinical evidence of airways infection at the time of testing.

Statistical analysis

Continuous variables were normally distributed, with the exception of gestational age and age at the study date. F_{ENO} values were log-10 transformed in order to achieve a normal distribution. Log F_{ENO} values were backtransformed after the analyses and presented as geometric mean and 95% confidence interval (CI). Unpaired t-test and Chi-squared test were used to assess differences in baseline characteristics between infants with and without complete data at 6 months. Ambient NO was significantly associated with log F_{ENO} (log F_{ENO}=0.928+0.0066 × ambient NO; $p<0.001$); hence, it was included in all the regression models. As infants inhaled NO-free air if ambient NO exceeded 10 ppb, a variable ''NO-free air'' (no/yes) was added into the models. Also, separate analyses were performed for the groups of infants who did and did not inhale NO-free air. We and others have previously shown an association between F_{ENO} and tobacco smoke exposure [8, 13, 21], maternal atopy and/or atopic disease [4, 13, 15], birth weight [14], RTIs [6], URS [7, 8] and wheezing [4]; hence, these variables were added in the regression models. Results of the linear regression models are reported as β coefficient (95% CI). Logistic regression analysis was used to investigate whether F_{ENO} was associated with the risk of wheezing in the second year of life, controlling for pre- and post-natal variables included in the linear regression model. The risk estimates of logistic regression analyses are reported as crude odds ratio (OR) and adjusted OR with 95% CI. Effect modification by maternal atopy/atopic disease and smoke exposure was evaluated by adding interaction terms into the final models [13, 15]. None of the investigated interactions was statistically significant. The area under the receiver operating characteristic (ROC) curve and sensitivity, specificity, positive and negative predictive value (PPV and NPV) were calculated to evaluate the accuracy of known risk factors for wheezing and of high F_{ENO} (F_{ENO} values >17.5 ppb, *i.e.* >75th percentile) in differentiating infants with and without wheezing in the second year. To examine the added value of F_{ENO} as a predictor of wheeze, it was also tested whether a composite index including high F_{ENO}, wheezing within the first 6 months of life and eczema of the child or maternal atopy/atopic disease could better predict wheezing in the second year of life, as compared with a clinical index (including wheezing within the first 6 months, eczema of the child or maternal atopy/atopic disease, but not F_{ENO}). For all statistical tests, two-tailed p-values <0.05 were considered significant. Data analyses were performed using the Statistical Package of Social Sciences version 15 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Study population F_{ENO} measurements were attempted in 511 infants (53% males) at a mean (range) age of 27.7 (22–48) weeks, and were successful in 428 infants. 56 measurements were excluded because tidal breathing was not maintained during the whole procedure and 27 because fewer than five breaths were collected in the sampling balloon. As the 6-month questionnaire was implemented after the start of the F_{ENO} measurements, data on respiratory symptoms within the first 6 months of life were available for 294 infants. Compared with infants with missing data at 6 months, infants with available data were less exposed to pre-natal maternal smoke (16.3% and 24.6%, respectively; p=0.04), while other characteristics did not differ. Also, there was no difference between infants with and without available data at 6 months with regard to the prevalence of wheezing in the second year. General characteristics of the study population are presented in table 1.

TABLE 1 General characteristics of the study population[#]

Gestational age weeks	40.3 (37.4–42.1)[f]
Birth weight kg	3.5 (1.3–5.2)
Age at the study date weeks	26.7 (24–33.2)[f]
Weight at the study date kg	7.9 (5.7–11.4)
Length at the study date cm	68.8 (60–79)
***F*eNO ppb**	10.3 (9.3–11.3)[##]
Males %	53
Pre-natal smoke exposure % yes	16
Post-natal smoke exposure % yes	28
Smoke exposure[¶] %	
Never	65
Pre-natal only	7
Post-natal only	19
Pre- and post-natal	9
Maternal atopy/atopic disease % yes	34
Eczema within the first 6 months % yes	20
RTI within the first 6 months[+] % yes	37
URS within the first 6 months % yes	94
LRS within the first 6 months	
Cough % yes	72
Wheezing % yes	20
Breathlessness % yes	8
Wheezing in the second year % yes[§]	17

Data are presented as mean (range), unless otherwise stated. *F*eNO: exhaled nitric oxide fraction; RTI: respiratory tract infections; URS: upper respiratory symptoms; LRS: lower respiratory symptoms. [#]: n=294; [¶]: combination of pre- and post-natal smoke exposure; [+]: n=291; [§]: n=276; [f]: median (5th–95th percentile); [##]: geometric mean (95% CI).

Pre- and post-natal factors and FeNO at 6 months

Males had a higher geometric mean (95% CI) *F*eNO than females (11.2 (9.9–12.7) ppb and 9.3 (8.1–10.6) ppb, respectively; p=0.04). *F*eNO was positively associated with weight (β coefficient (95% CI) 0.048 (0.001–0.09)), length (0.016 (0.001–0.032)) and age (0.019 (0.004–0.033)) at the study date. In order to avoid colinearity, age, Z-score for weight (weight for age) and Z-score for length (length for age) were included in the multiple linear regression models (table 2). Wheezing within the first 6 months was reported for 60 (20.4%) out of 294 infants and was not associated with *F*eNO, independent of the use of NO-free air (β coefficient (95% CI) for NO-free air not used/used 0.017 (-0.1–0.14) and 0.12 (-0.08–0.31), respectively). The model including LRS instead of wheezing within the first 6 months showed that *F*eNO was lower in infants with LRS (table 2).

FeNO at 6 months and wheezing in the first 2 yrs

Complete data on wheezing both at 6 and 24 months was available for 276 infants. At 24 months, wheezing in the past 12 months was reported for 46 (16.7%) infants and 20 of them had also wheezed in the first 6 months. *F*eNO values in relation to wheezing during the first 2 yrs of life are presented in figure 1. Ambient NO, sex, age, Z-score for weight, Z-score for length, smoke exposure, maternal atopy/atopic disease, birth weight, RTI, URS and wheezing within the first 6 months were included as covariates in the logistic regression model used to evaluate whether *F*eNO at 6 months was associated with the risk of wheezing in the second year (table 3). The regression model showed that for every ppb increase of *F*eNO at 6 months, the risk of wheezing in the second year of life increased by 6% (adjusted OR (95% CI) 1.06 (1.01–1.11)) (table 3). The results were similar in the groups of infants with or without NO-free air (table S1). As an example, a 6-month-old male born weighing 3 kg with an atopic mother who smoked both pre- and post-natally, with at least one episode of wheezing within the first 6 months and a *F*eNO at 6 months of 10 ppb, has a low risk of wheezing in the second year of life (OR=1.05). However, if the same infant had a *F*eNO at 6 months of 20 ppb or 30 ppb, the risk increases substantially (OR=1.9 and 3.4, respectively). Sensitivity analyses showed that the associations between *F*eNO and wheezing in the second year of life remained unchanged if RTI or URS were excluded from the model (adjusted OR (95% CI) 1.06 (1.01–1.10) or 1.05 (1.01–1.10), respectively) and if LRS instead of wheezing within the first 6 months was used (table 3).

TABLE 2 Multiple linear regression models fitted on log exhaled nitric oxide fraction (*F*eNO)[#]

	β coefficient (95% CI)[¶]	p-value	β coefficient (95% CI)[¶]	p-value
Ambient NO	0.006 (0.004–0.009)	<0.001	0.007 (0.004–0.009)	<0.001
NO-free air	0.03 (-0.11–0.17)	0.7	0.01 (-0.13–0.15)	0.9
Male	-0.09 (-0.17– -0.01)	0.03	-0.09 (-0.17– -0.01)	0.03
Age at study date	0.01 (0–0.03)	0.04	0.01 (0–0.03)	0.05
Weight for age Z-score	0.01 (-0.05–0.07)	0.7	0.02 (-0.05–0.08)	0.6
Length for age Z-score	0.01 (-0.04–0.06)	0.7	0.07 (-0.05–0.06)	0.8
RTI within the first 6 months	-0.07 (-0.16–0.02)	0.1	-0.04 (-0.12–0.05)	0.4
URS within the first 6 months	-0.18 (-0.36– -0.01)	0.04	-0.14 (-0.32–0.04)	0.09
Wheezing within the first 6 months	0.04 (-0.07–0.14)	0.5	[+]	
LRS within the first 6 months	[+]		-0.11 (-0.21– -0.02)	0.02

NO: nitric oxide; RTI: respiratory tract infections; URS: upper respiratory symptoms; LRS: lower respiratory symptoms; [#]: n=291, as RTI within the first 6 months had three missing values. [¶]: this should be judged as the change of log *F*eNO per unit change in the variables; values have been adjusted for the factors listed as well as for smoke exposure, maternal atopy/atopic disease and birth weight. [+]: variable not included in the model.

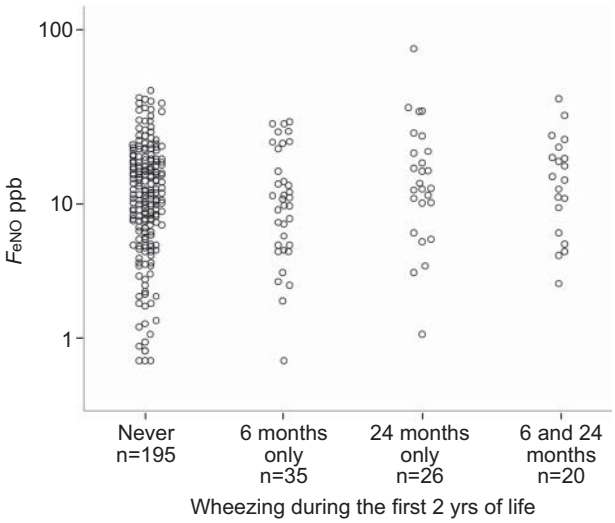

FIGURE 1. Individual exhaled nitric oxide fraction (*F*eNO) values (represented on a log-scale) and wheezing during the first 2 yrs of life. n=276.

Predictors of wheezing in the second year of life

Wheezing within the first 6 months performed better than high *F*eNO (>17.5 ppb) at 6 months in predicting wheezing in the second year (area under the ROC curve 0.64, p=0.002 and 0.53, p=0.4, respectively) (table 4). The clinical index, including wheezing and eczema of the child or maternal atopy/atopic disease, was positive in 32 infants and the composite index, which also included high *F*eNO, was positive in 40 infants. The area under the ROC curves were 0.61 (p=0.02) and 0.62 (p=0.01), respectively. Both indices showed a higher specificity and PPV and a similar NPV for wheeze in the second year of life as compared with *F*eNO alone (table 4).

DISCUSSION

In our prospective birth cohort study, *F*eNO at the age of 6 months was positively associated with the risk of wheeze in the second year of life. The presence of wheezing within the first 6 months and eczema of the child or maternal atopy/atopic disease could predict wheezing in the second year better than *F*eNO alone, and the added value of *F*eNO for the prediction of wheezing in an individual child was limited.

Only a few studies have investigated the association between *F*eNO and respiratory symptoms in infants, and most of these had a cross-sectional design and compared selected infants with respiratory diseases [4, 7, 22]. In a recent prospective study, LATZIN *et al*. [15] found that *F*eNO levels after birth were associated with increased risk of subsequent respiratory symptoms, but only in infants of atopic mothers, in infants of mothers who smoked during pregnancy, or in case of both risk factors. The study by LATZIN *et al*. [15] focused on more severe symptoms, such as awakening because of airway symptoms or general practitioner consultations, in order to identify those children most likely to develop asthma [23, 24].

It is not yet clear how *F*eNO in infancy should be interpreted, and it is especially unclear whether *F*eNO in infants indeed reflects eosinophilic airway inflammation [25]. Studies using bronchoalveolar lavage [26] and endobronchial biopsies [11] have failed to demonstrate significant airway eosinophilia in highly selected infants. *F*eNO as measured in our study derives from both the upper and lower airways, and may for this reason not straightforwardly be compared with *F*eNO in older subjects, where nasal contamination can effectively be avoided. However, our results indirectly support a role of NO metabolism in the pathophysiology of wheezing in infancy. Lower *F*eNO at 6 months has been associated with airway symptoms [6] that are most likely due to viral infection with predominant neutrophilic inflammation, which downregulates NO metabolism [9]. Conversely, the positive association between *F*eNO at 6 months and an increased risk of wheezing in the second year could reflect early activation of NO metabolism that may be related to the later development of eosinophilic bronchial inflammation. These mechanisms may both be present in a given infant, as in the first 2 yrs of life asthma-like symptoms represent a heterogeneous group of different phenotypes, and this complicates the interpretation of *F*eNO in infants.

We showed that a history of wheezing and eczema in the first 6 months and maternal atopy/atopic disease could predict

TABLE 3 Risk of wheezing in the second year of life[#]

	Crude OR (95% CI)	Adjusted OR (95% CI)[¶]	Adjusted OR (95% CI)[¶]
***F*eNO**	1.03 (1.00–1.06)	1.06 (1.01–1.11)	1.06 (1.02–1.11)
Birth weight	0.79 (0.45–1.42)	0.59 (0.27–1.3)	0.6 (0.27–1.34)
Smoke exposure			
Pre-natal only	1.00 (0.27–3.65)	0.56 (0.10–3.07)	0.61 (0.12–3.14)
Post-natal only	1.19 (0.52–2.71)	0.92 (0.36–2.37)	0.9 (0.36–2.24)
Pre- and post-natal	2.33 (0.88–6.15)	3.13 (1.07–9.12)	2.9 (0.97–8.59)
RTI within the first 6 months	1.33 (0.69–2.54)	0.96 (0.44–2.08)	1.1 (0.53–2.28)
URS within the first 6 months	3.14 (0.40–24.4)	4.22 (0.46–38.5)	3.47 (0.37–32.6)
Wheezing within the first 6 months	4.29 (2.16–8.50)	5.87 (2.62–13.15)	[+]
LRS within the first 6 months	6.27 (1.88–20.9)	[+]	7.29 (2.05–25.9)

*F*eNO: exhaled nitric oxide (NO) fraction; RTI: respiratory tract infections; URS: upper respiratory symptoms; LRS: lower respiratory symptoms; [#]: n=276; [¶]: n=273, as RTI within the first 6 months had three missing values; adjusted for the factors listed as well as for sex, maternal atopy/atopic disease, age, Z-score for length, Z-score for weight, NO-free air use and ambient NO. [+]: variable not included in the model.

TABLE 4 Predictors of wheezing in the second year of life

	Subjects	OR	Sensitivity	Specificity	PPV	NPV
Wheezing within the first 6 months	276	4.3 (2.2–8.5)	43.5 (29.2–58.8)	84.8 (79.3–89)	36.4 (24.1–50.5)	88.2 (83.1–92)
Maternal atopy/atopic disease	392	1.2 (0.67–1.9)	37.3 (26–50.1)	65.8 (60.4–70.9)	18.4 (12.5–26.1)	83.6 (78.4–87.8)
Eczema within the first 6 months	276	0.92 (0.36–2.4)	13.0 (5.4–27)	86.0 (80.7–90.1)	15.8 (6.6–31.9)	83.1 (77.6–87.5)
High *F*eNO (>17.5 ppb)	392	1.3 (0.72–2.34)	28.4 (18.3–40.9)	76.6 (71.6–81)	20.0 (12.8–29.7)	83.8 (79–87.7)
Clinical index#	276	5.3 (2.3–11.9)	28.2 (16.5–43.7)	93.0 (88.7–95.8)	44.8 (26.9–64)	86.6 (81.6–90.5)
Composite index¶	276	4.6 (2.2–9.8)	32.6 (19.9–48.1)	90.4 (85.7–93.8)	40.5 (25.2–57.8)	87.0 (81.9–90.9)

Data are presented as n or value (95% CI). PPV: positive predictive value; NPV: negative predictive value; *F*eNO: exhaled nitric oxide fraction. #: wheezing within the first 6 months plus eczema or maternal atopy/atopic disease; ¶: wheezing within the first 6 months plus eczema or maternal atopy/atopic disease plus *F*eNO > 7.5 ppb.

which infants are at risk of subsequent wheezing with better accuracy than *F*eNO alone. However, there was a limited added value of *F*eNO on top of parameters from medical history for the prediction of wheeze in the second year of life. As the current study was conducted in an unselected cohort of infants, a limited number will develop symptoms and it can *a priori* be expected that any test would have a relatively low PPV. Further long-term follow-up can show whether *F*eNO at an early age might identify infants at increased risk of developing asthma later [27].

Several methodological aspects should be considered. The 6-month questionnaire data were not available for all infants, due to a delayed implementation of this questionnaire. This was not related to the exposures or to the outcomes of interest, but reduced the power of our analyses. We consider it unlikely that any selection bias has occurred as a result of this. Another issue is the possible effect of ambient NO and inspiration of NO-free air at a cut-off value of 10 ppb. We tried to overcome any bias as a result of this procedure by adjusting the regression models for ambient NO, in agreement with previous studies [8, 28]. Furthermore, we showed that the associations between *F*eNO and wheezing were not different between infants who did or did not inhale NO-free air, which confirmed the consistency of our findings. Indeed, the group of infants using NO-free air was small, which might have reduced the possibility of evaluating to what extent ambient NO contamination could have influenced the results. Sensitivity analyses showed that the results of the models remained unchanged when RTI and URS were excluded from the regression models, suggesting that the association between *F*eNO and wheezing in the second year of life was robust and not mediated by respiratory infections or by symptoms of the upper respiratory tract.

We measured *F*eNO off-line during tidal breathing and previously found good within-subject short-term reproducibility of this method [20]. However, this method does not control for tidal flow or breathing frequency, which have been shown to influence *F*eNO [21, 29]. Hence, we cannot exclude that differences in expiratory flow between infants with and without respiratory symptoms might have influenced our findings. However, in that case, symptomatic infants at 6 months would have a low *F*eNO, and we found the opposite. Therefore, differences in respiratory flow as a result of airway symptoms within the first 6 months cannot explain our finding of a positive association between *F*eNO and the subsequent development or persistence of wheezing.

What are the clinical implications of our findings? Wheezing is very common in infancy, but only a minority of wheezy infants will develop asthma. A test that could predict the development of symptoms would clearly be of great value, and our present analyses focused on *F*eNO as such a predictive test. Unfortunately, the measurement of *F*eNO at 6 months predicted wheezing only to a very limited extent, and added little to the predictive value of readily available clinical characteristics. The children will be reassessed as they grow up in order to have a better definition of their wheezing phenotype [30], and to be able to investigate the relationship between *F*eNO in infancy and later asthma.

We conclude that *F*eNO at 6 months is positively associated with the risk of wheezing, but has limited added value on top of clinical characteristics in predicting wheezing in the second year of life in individual children.

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STATEMENT OF INTEREST

A statement of interest for J.C. de Jongste can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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