Eur Respir J 2007; 29: 251–258
DOI: 10.1183/09031936.00016106

Alterations of exhaled nitric oxide in pre-term infants with chronic lung disease

H.L. Roiha*, C.E. Kuehni#, M. Zanolari*, M. Zwahlen#, D.N. Baldwin*, C. Casaulta*, M. Nelle* and U. Frey*

ABSTRACT: Animal models suggest that reduced nitric oxide (NO) synthase activity results in lower values of exhaled NO (eNO) present at birth in those individuals who are going to develop chronic lung disease of infancy (CLDI).

Online tidal eNO was measured in 39 unsedated pre-term infants with CLDI (mean gestational age (GA) 27.3 weeks) in comparison with 23 healthy pre-term (31.6 weeks) and 127 term infants (39.9 weeks) at 44 weeks post-conceptional age, thus after the main inflammatory response. NO output (NO output (V'_{NO})=eNO × flow) was calculated to account for tidal- flow-related changes. Sex, maternal atopic disease and environmental factors (smoking, caffeine) were controlled for.

The mean eNO was not different (14.9 ppb in all groups) but V'_{NO} was lower in CLDI compared with healthy term infants (0.52 *versus* 0.63 nL·s^{-1}). Values for healthy pre-term infants were between these two groups (0.58 nL·s^{-1}). Within all pre-term infants (n=62), V'_{NO} was reduced in infants with low GA, high clinical risk index for babies scores and longer duration of oxygen therapy but not associated with post-natal factors, such as ventilation or corticosteroid treatment.

After accounting for flow, the lower nitric oxide output in premature infants with chronic lung disease of infancy is consistent with the hypothesis of nitric oxide metabolism being involved in chronic lung disease of infancy.

KEYWORDS: Chronic lung disease, infants, lung function, nitric oxide, prematurity

Recent evidence supports the hypothesis that chronic lung disease of infancy (CLDI) is the result of arrested or disturbed alveolar and vascular development [1]. The insult leading to this disturbed lung development may occur due to intra-uterine and post-natal infections, prematurity with surfactant deficiency or as a consequence of ventilation, hyperoxia (oxidative stress) or other factors. An inflammatory response with a peak at the age of ~10 days followed by a progressive decline [2] has been observed in this condition; however, little is known about the ongoing effects of these mechanisms after the acute phase during the first month of life.

Various inflammatory processes, with a dominance of neutrophilic inflammation and oxidative stress, are involved in the pathogenesis of CLDI. Premature infants exhibit an immature response to oxidative stress [3], immature interleukin-10-mediated inflammatory responses [4, 5], persistence of neutrophilic inflammation and an imbalance of α_1-protease inhibitor activity [6]. Nitric oxide (NO) metabolism plays a crucial role in many of these inflammatory processes. Exhaled NO (eNO) is altered during both oxidative stress [7] and neutrophilic inflammation [8]. There is also increasing evidence that NO is involved in lung development and growth, as well as in angiogenesis [9, 10]. Recently, it has been demonstrated that premature baboons, in which CLDI subsequently develops, show pre-existing alterations of NO metabolism at birth [11]. These animals have decreased levels of constitutive forms of NO synthase (endothelial nitric oxide synthase (NOS) and neuronal NOS) in comparison with premature animals that do not develop CLDI, whereas the inducible form of the enzyme (iNOS) is higher. However, the relative contribution of iNOS at birth is so small that the overall eNO is lower than in normal animals. These data suggest that NO metabolism may be crucially involved in the pathophysiology of CLDI.

In accordance with these animal data, a follow-up study of survivors of CLDI showed lower eNO at school age when compared with age-matched healthy children [12]. In contrast, eNO was found to be elevated in a small group of human infants with CLDI at a post-conceptional age (PCA) of 36 weeks [13]. However, this analysis did not adjust for different breathing patterns in infants with and without CLDI. The present authors

AFFILIATIONS
*Swiss Paediatric Respiratory Research Group, Depts of Paediatrics and
#Swiss Paediatric Respiratory Research Group, Social and Preventive Medicine, University of Berne, Berne, Switzerland.

CORRESPONDENCE
U. Frey
Swiss Paediatric Respiratory Research Group
Dept of Paediatrics
University Hospital of Berne
Inselspital CH-3010 Berne
Switzerland
Fax: 41 316329484
E-mail: urs.frey@insel.ch

Received:
February 02 2006
Accepted after revision:
October 10 2006

SUPPORT STATEMENT
The work presented in this paper was funded by the Swiss National Science Foundation (SNF; grant 32-69025.02). C.E. Kuehni was funded by the SNF (PROSPER grant 3233-069348 and 3200-069349). D. Baldwin was supported by a Swiss Government Grant 2002-0041 and European Respiratory Society Long-Term Research Fellowship 2002-032. The authors declare that they have no competing interests.

STATEMENT OF INTEREST
None declared.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

have recently shown that eNO in infants depends strongly on expiratory flow and breathing pattern [14, 15]. These factors should therefore be accounted for when comparing infants with CLDI and healthy controls.

Based on the hypothesis raised by AFSHAR *et al*. [11], the aim of the present observational study was to determine whether eNO is decreased in premature human infants suffering from CLDI. Therefore, eNO was measured in a large population of premature infants with and without CLDI in the post-acute phase after resolution of the main inflammatory response at 44 weeks PCA [2]. These data were then compared with measurements obtained from healthy age-matched term infants, whilst accounting for potential confounding factors [15]. Furthermore, the present authors investigated whether known clinical risk factors for CLDI were associated with the eNO levels in these infants.

METHODS

Study design and subjects

In an unmatched case-control study, eNO was measured in 39 pre-term infants with CLDI, 23 healthy pre-term infants and 127 healthy unselected term infants at a PCA of ~44 weeks (table 1). Former pre-term infants were recruited from the neonatal unit of the University Maternity Hospital (Berne, Switzerland) between January 1999 and December 2004. The parents of the 62 pre-term infants, of which 23 were classified as healthy, 12 as having mild and 27 as having moderate CLDI according to the definition of the National Institute of Child Health and Human Development/National Heart, Lung and Blood Institute/Office of Rare Diseases Workshop [16], agreed to participate. The clinical characteristics of all the pre-term infants were obtained from the patient medical record. The healthy term infants were recruited antenatally from two maternity hospitals in the Berne region as part of a prospective birth cohort study during the period 1999–2004 [14, 15].

Environmental and maternal exposures did not differ significantly between the three groups, with the exception of maternal smoking in pregnancy. This was more common in the CLDI group than in the healthy term group, and it was absent in the healthy pre-term group. Maternal coffee drinking was more common in mothers of healthy pre-term infants compared with healthy term and CLDI infants. CLDI infants had more cardiac malformations (persistent ductus arteriosus (PDA) and atrium or ventricle septum defect), were more likely to have a history of chorioamnionitis and received more post-natal corticosteroids than healthy pre-term infants.

Information on demographics, family history of atopic disease, clinical symptoms and environmental risk factors, including pre- and post-natal tobacco exposure and maternal caffeine intake, were obtained from the mothers by standardised interview. The presence of chorioamnionitis was determined based on placental histology.

Exclusion criteria were as follows: ethnicity other than white, major birth defects, treatment with caffeine and anti-inflammatory treatment or respiratory tract infection within the preceding 3 weeks. Additional exclusion criteria for the healthy controls were: pre-term delivery (<38 weeks), respiratory distress with need for oxygen for >30 min after birth or other significant perinatal disease.

The ethics committee of the university hospital and the State of Berne approved the study. Parental written informed consent was obtained prior to study commencement. Parents were generally present during the measurements.

Measurements of online eNO

All the infants were studied during unsedated quiet sleep, in a supine position with the head in the midline. Heart rate and arterial oxygen saturation (Biox 3700; Datex-Ohmeda, Helsinki, Finland) were monitored throughout the study. A compliant

TABLE 1 Family history, environmental and clinical factors of the study children

	CLDI	Healthy pre-term	Healthy term
Subjects	39	23	127
Males	24 (62)	11 (48)	73 (58)
Family history			
Maternal atopic disease[#]	9 (23)	4 (17)	53 (42)
Maternal asthma	2 (5)	0 (0)	14 (11)
Environment			
Maternal smoking in pregnancy	10 (26)	0 (0)	17 (13)
Maternal smoking post-natally	5 (13)	0 (0)	21 (17)
Maternal smoking only post-natally	0 (0)	0 (0)	4 (3)
Maternal coffee consumption in pregnancy	15 (38)	19 (83)	83 (65)
Clinical factors			
PDA	15 (38)	3 (13)	
ASD/VSD	3 (8)	0 (0)	
Pre-natal corticosteroids	32 (82)	19 (83)	
Post-natal corticosteroids	12 (31)	0 (0)	
Chorioamnionitis	19 (49)	1 (0)	

Data are presented as n (%), unless otherwise stated. CLDI: chronic lung disease of infancy; PDA: persistent ductus arteriosus; ASD: atrium septum defect; VSD: ventricle septum defect. [#]: atopic disease was defined as the presence of doctor-diagnosed asthma, hay fever or eczema.

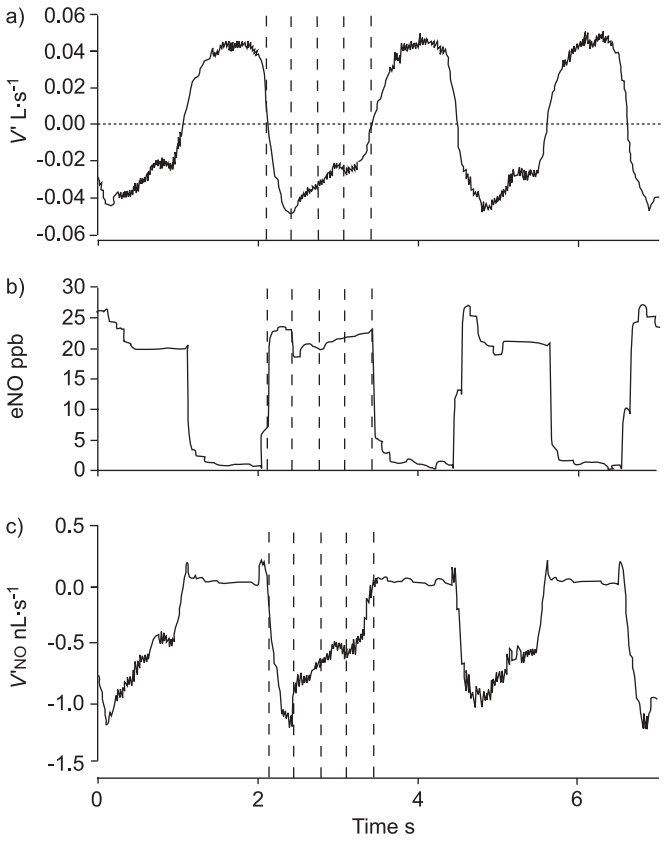

FIGURE 1. Representative example of a) online flow (V'), b) exhaled nitric oxide (eNO) and c) NO output (V'_{NO}). The levels of eNO show a steep increase at the beginning of expiration and then approximate a plateau towards the end of expiration. By multiplying V' and eNO, V'_{NO} can be calculated ($V'_{NO}=eNO\times V'$). V' rapidly approximates zero at the end of expiration.

silicon mask (Infant mask, size 1; Homedica, Cham, Switzerland) was placed over the nose and mouth, and flow–volume loops were inspected for leaks prior to commencing the measurement. An NO filter ensured that all infants inhaled NO-free air. Tidal gas flow (V'), volume (V), and eNO and CO_2 levels were measured using commercially available prototype infant lung function equipment (Exhalyser; EcoMedics, Duernten, Switzerland) as previously validated and described in detail elsewhere [14]. Briefly, a representative example of online V', eNO and NO output (V'_{NO}) shows a steep increase of eNO at the beginning of expiration, which achieves a plateau towards the end of this phase (fig. 1b). During inspiration, eNO rapidly returns to zero, indicating negligible re-breathing of NO from the equipment dead space. V'_{NO} is calculated by multiplying V' by eNO ($V'_{NO}=eNO\times V'$; fig. 1c). Flow (fig. 1a) rapidly approximates to zero at the end of expiration. These rapid flow changes result in variable V'_{NO} during the fourth quartile of the breath duration. eNO and V'_{NO} have, therefore, been measured in the third quartile of expiration, since this shows the lowest breath-to-breath variability [14] and corresponds approximately to the phase III slope [17–20]. In order to ensure consistency of analysis between infants, only the first 100 breaths in the tidal breathing data were analysed. Since eNO is strongly flow dependent and the CLDI and healthy groups were significantly different in expiratory flow (table 2), it is mandatory to calculate not only eNO but also V'_{NO} [14].

Statistical analysis

All continuous variables were normally distributed with the exception of eNO, which was therefore transformed for further analysis.

The comparison of eNO between healthy and sick children was difficult because eNO concentration is influenced by physiological, maternal and environmental factors [15] (fig. 2), some of which are also related to prematurity and disease. In view of these complexities, the present authors undertook a pragmatic approach and systematically analysed the two outcomes using two different statistical models as follows: 1) a simple unadjusted comparison of means using unpaired t-tests; and 2) a multivariable regression model adjusting for the most influential physiological covariates (expiratory time (t_E) and minute ventilation (V'_E)) and also for other factors associated with eNO and V'_{NO} in healthy term infants, including sex of the infant, maternal atopic disease, pre- and post-natal maternal smoking and caffeine exposure [15]. For the regression models, continuous variables (t_E, V'_E) were centred and categorical variables were entered as indicator variables. From these regressions, parameter estimates and their 95% confidence intervals (CI) were reported together with p-values for the null hypothesis, which presumes that these estimates equal zero. In pre-term children, the gestational age (GA) and CLDI were so closely correlated that it was not possible to separate their association with NO by simultaneous inclusion in a multivariate model. Therefore, two series of models were elaborated. In the first instance, the children were categorised according to presence or absence of CLDI (table 3) and then according to their GA, as 24–27 weeks, 28–31 weeks, 32–35 weeks and $\geqslant$38 weeks (table 4).

Within pre-term infants, it was then tested systematically using an unadjusted model whether NO was associated with any of the following clinical risk factors for CLDI: GA; clinical risk index for babies (CRIB) score; duration of oxygen supplementation; PDA; pre- and post-natal steroid treatment; administration of surfactant; and chorioamnionitis. A second test of these indices was then performed using a multivariable model adjusted for t_E and V'_E.

RESULTS

Clinical, physiological and environmental characteristics of the participating infants

The clinical, physiological and environmental characteristics of the infants are summarised in tables 1 and 2. Each of the three groups were of similar PCA; however, weight and length at study date were highest in healthy term infants. These anthropometric indices were progressively smaller at the time of study for healthy pre-term and CLDI infants. Tidal breathing parameters also differed considerably between healthy term infants, healthy pre-term infants and pre-term infants with CLDI, with the noteworthy exception of V'_E, where the group mean was similar in all three groups (table 2).

TABLE 2 Biometric, tidal breathing and clinical data of the study children[#]

	CLDI		Healthy pre-term		Healthy term	
Anthropometric data						
Age weeks	44.2±2.1	44.1 (40.1–49.6)	44.2±1.5	44.1 (40.6–46.9)	45.1±1.5	45.1 (41.4–49.7)
Weight g	3581±547	3600 (2650–4830)	3789±558	3680 (3000–5190)	4451±539	4350 (3400–6300)
Length cm	50.2±3.1	50.4 (43.0–55.1)	52.6±2.4	53.0 (48.0–57.0)	55.5±2.2	55.8 (49.0–61.5)
GA weeks	27.3±1.7	27.1 (24.0–31.6)	31.6±1.9	31.7 (27.1–34.7)	39.9±1.2	40.1 (36.1–42.3)
Weight at birth g	879±243	870 (420–1520)	1534±588	1385 (625–2840)	3443±457	3440 (2170–4915)
Length at birth cm	35.0±3.2	35 (27.0–42.0)	40.5±4.5	39.8 (32–50)	49.5±1.9	50.0 (44.0–54.0)
Tidal breathing data						
f_R breaths·min^{-1}	54.4±13.1	51.8 (36.1–90.2)	45.7±7.9	45.7 (29.1–59.1)	43.7±11.7	41.4 (24.6–75.8)
t_E s	0.66±0.18	0.66 (0.35–1.02)	0.77±0.18	0.73 (0.57–1.18)	0.85±0.28	0.81 (0.43–1.69)
V_T mL	24.5±6.1	23.1 (13.4–37.1)	30.1±7.3	31.5 (19.7–44.2)	31.3±5.8	31.3 (20.2–46.5)
PEF mL·s^{-1}	61.5±16.1	63.2 (26.2–88.9)	61.7±18.2	57.9 (39.8–110.3)	58.3±15.7	55.7 (21.1–107.2)
V'_E mL·min^{-1}	1285±265	1309 (678–1798)	1343±317	1285 (891–1901)	1312±300	1294 (590–2265)
Clinical data						
CRIB score	5.7±4.0	5 (0–13)	2.2±3.1	1 (0–12)		
Duration of intubation days	4.5±10.8	1 (0–63)	1.2±1.9	0 (0–5)		
Duration of CPAP days	36.6±17.2	38 (2–68)	5.2±6.9	3 (0–28)		
Duration of O_2 supply days	71.1±22.2	67 (31–131)	5.0±5.6	4 (0–22)		
Max F_{I,O_2}	0.55±0.28	0.48 (0.23–1.00)	0.44±0.32	0.30 (0.21–1.00)		

Data are presented as mean±SD and median (range). CLDI: chronic lung disease of infancy; GA: gestational age; f_R: breathing frequency; t_E: expiratory time; V_T: tidal volume; PEF: peak expiratory flow; V'_E: minute ventilation; CRIB: clinical risk index for babies; CPAP: continuous positive airway pressure; Max: maximum; F_{I,O_2}: fractional inspired oxygen. [#]: analysis was carried out among 39 pre-term infants with CLDI, 23 healthy pre-term infants and 127 healthy term infants.

NO measurements in pre-term infants with and without CLDI: comparisons with healthy term infants

Mean tidal eNO was 15.2 ppb in healthy term infants and this value was not different to those obtained from healthy pre-term or CLDI infants when examined with either of the models (table 3). V'_{NO} was 0.63 nL·s^{-1} in healthy term infants and 0.58 nL·s^{-1} in infants with CLDI. After adjustment for tidal breathing parameters, sex, maternal and environmental factors, the differences between healthy term and CLDI infants increased (model II: term infants 0.63 nL·s^{-1}, CLDI infants 0.52 nL·s^{-1}, p=0.024). Values for healthy pre-term infants were between the two other groups (0.58 nL·s^{-1}).

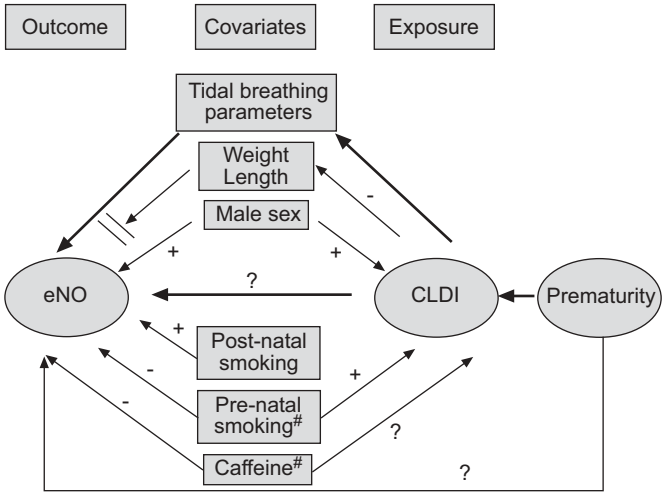

FIGURE 2. Causal pathway of concomitant factors of exhaled nitric oxide (eNO) and interactions between these factors. CLDI: chronic lung disease of infancy [#]: only in infants of atopic mothers.

NO in pre-term infants according to GA compared with healthy term infants

Categorisation of pre-term children according to GA ranges instead of lung disease revealed lower V'_{NO} in infants with a GA <28 weeks (table 4). No differences between other GA groups were identified using the two models.

Clinical factors associated with NO outcome parameters within the pre-term group

Within the group of pre-term children, there was no association between eNO and any of the potential clinical confounding factors examined. When adjusted for t_E and V'_E, mean V'_{NO} for all pre-term infants was 0.59 nL·s^{-1}. V'_{NO} decreased by 0.02 nL·s^{-1} per week decrease of GA (p=0.02) and per step increase of CRIB score (p=0.02). An increase in duration of oxygen supplementation of 1 week resulted in a decrease in V'_{NO} of 0.01 nL·s^{-1} (p=0.06). Post-natal treatment with corticosteroids was associated with a decreased V'_{NO} (-0.15 nL·s^{-1}, p=0.04). Within the CLDI group, V'_{NO} values in infants with moderate CLDI were 0.03 nL·s^{-1} lower

TABLE 3 Exhaled nitric oxide (eNO) and NO output (V'_{NO}) in infants with chronic lung disease of infancy (CLDI) and healthy pre-term infants in comparison with healthy term infants

	Model I[#]		Model II[¶]	
	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value
eNO ppb				
Healthy term	15.22 (14.10–16.38)		14.92 (12.61–17.43)	
ΔeNO				
Healthy pre-term	0.00 (-0.12–0.15)	0.897	0.00 (-0.14–0.11)	0.886
CLDI	-0.01 (-0.08–0.10)	0.902	0.00 (-0.14–0.08)	0.774
V'_{NO} nL·s^{-1}				
Healthy term	0.63 (0.59–0.67)		0.63 (0.54–0.72)	
ΔV'_{NO}				
Healthy pre-term	0.00 (-0.10–0.09)	0.946	-0.05 (-0.15–0.05)	0.342
CLDI	-0.05 (-0.13–0.02)	0.178	-0.11 (-0.20– -0.01)	0.024

CI: confidence interval; Δ: change. The parameter estimates indicate the change of the NO outcome parameters (eNO, V'_{NO}) in non-CLDI pre-term infants (n=23) and pre-term infants with CLDI (n=39) in comparison with healthy term infants (n=127). For example, using model II, V'_{NO} was 0.63–0.11=0.52 nL·s^{-1} in CLDI infants (value for healthy term infant–ΔV'_{NO} for CLDI=value for CLDI infant). [#]: univariate linear regression analysis; [¶]: model adjusted for centred values of expiratory time (t_E) and minute ventilation (V'_E) as well as for sex, maternal atopic disease, maternal smoking in pregnancy, maternal smoking both pre- and post-natally and maternal caffeine consumption. The estimated constant represents the value of a healthy female infant with average t_E and V'_E and no exposure to tobacco or caffeine and no history of maternal atopic disease.

than in infants with mild CLDI, although this finding did not reach statistical significance (p=0.34).

Both eNO and V'_{NO} were independent of the duration of ventilation, the presence of PDA, pre-natal corticosteroid therapy and surfactant treatment. V'_{NO} was 0.09 nL·s^{-1} lower in the group of pre-term infants with chorioamnionitis compared with those without (p=0.11). However, when additional adjustments were performed for GA, the presence of chorioamnionitis and all other clinical risk factors disappeared, since all of them were closely associated with low GA.

DISCUSSION

Prematurity leads to arrest of alveolar, airway and pulmonary arterial development and therefore plays a key role in the pathophysiology of CLDI. Inflammatory processes induced by various external and internal factors can also influence lung development and induce remodelling fibrosis, thus contributing to the development of this condition. This process may have particular relevance since there is evidence of an immature anti-inflammatory and anti-oxidative response in premature infants. NO metabolism plays an important part in many of these inflammatory processes. Thus, eNO was measured in unsedated infants with CLDI in comparison with healthy term and pre-term infants in the post-acute phase of the disease at a PCA of 44 weeks. The findings are complex and must be interpreted carefully. In unsedated infants, mean eNO (group mean (95% CI)) was 15.2 ppb (15.1–15.3) in CLDI, 15.2 ppb (14.1–16.4) in healthy term and 15.2 ppb (15.1–15.4) in healthy pre-term infants. Sparse data are available pertaining to tidal eNO values in healthy children. BARALDI *et al.* [21] measured mixed tidal eNO in infants and young children using a collection reservoir. The reported values of eNO from that study were 14.1±1.8 ppb in acutely wheezy subjects and lower values of 5.6±0.5 ppb in healthy controls. NO values obtained during tidal breathing in the present study, as well as those observed in previous studies using the same method [14, 15], are higher than those previously reported in healthy infants and children. These differences are most likely explained by the significantly lower tidal flows found in young infants, a fact which highlights the importance of recording and correcting for flow during tidal eNO measurements. Several other possible mechanisms may explain the observed variation in results obtained between these studies. A significant proportion of the total eNO is produced by the nasal mucosa. Measurements obtained from nose or mouth alone [21] will therefore be expected to demonstrate different NO levels than values obtained from a combined oro-nasal mask apparatus, as used in the present study. The predominance of nasal breathing in the infant population may impact further on this discrepancy. The aim of the present study was to quantify overall eNO and thus no attempt was made to separate relative contributions from nasal and lower airway passages in either of these groups. Whilst it is possible that CLDI and healthy infants may differ in the anatomical locations at which NO is produced, this was not the focus of the current work. As such, a simple technique was chosen for acquisition of tidal breathing data that is well established in the present authors' lung function laboratory. Respiratory tract infection represents a cause of elevated eNO levels. However, these children were excluded from the current study. Univariate analysis revealed similar eNO concentrations between healthy and premature infants. However, eNO was strongly correlated with both flow and tidal breathing indices.

Although the group mean V'_E was not different, individual infants with CLDI demonstrated differences in respiratory frequency, tidal volume t_E and tidal flows compared with healthy term and pre-term infants. Furthermore, flow dependency of V'_{NO} was altered in CLDI in comparison with healthy infants. Previous studies in healthy infants [14, 15] suggested that these covariates must be taken into account during statistical analysis. The median V'_{NO} averaged over 100 breaths was found to be similar in infants with CLDI in comparison with healthy term infants in a univariate (model I) but lower in multivariate analysis including such covariates (model II). Thus, the first conclusion is purely methodological. When comparing healthy subjects and those with lung disease, the measurement of eNO concentration alone may not be sufficient, since changes to airway mechanics in the presence of lung disease will influence flow dependence of eNO. Conversely, alterations in lung mechanics in disease might affect NO clearance from the lung and thus mechanical factors might dominate the eNO concentration or V'_{NO}.

TABLE 4 Exhaled nitric oxide (eNO) and NO output (V'_{NO}) in pre-term infants of different gestational age (GA) compared with healthy term infants

	Model I[#]		Model II[¶]	
	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value
eNO ppb				
GA 38–42 weeks	15.22 (14.12–16.35)		14.75 (12.39–17.30)	
ΔeNO				
GA 32–35 weeks	-0.01 (-0.41–0.18)	0.685	-0.04 (-0.53–0.12)	0.490
GA 28–31 weeks	0.01 (-0.06–0.23)	0.544	0.00 (-0.10–0.15)	0.852
GA 24–27 weeks	0.00 (-0.12–0.10)	0.962	0.00 (-0.17–0.10)	0.808
V'_{NO} nL·s^{-1}				
GA 38–42 weeks	0.63 (0.59–0.67)		0.64 (0.55–0.73)	
ΔV'_{NO}				
GA 32–35 weeks	0.00 (-0.14–0.14)	0.999	-0.06 (-0.21–0.10)	0.481
GA 28–31 weeks	0.00 (-0.09–0.10)	0.950	-0.03 (-0.13–0.07)	0.557
GA 24–27 weeks	-0.08 (-0.17–0.01)	0.085	-0.15 (-0.26– -0.05)	0.005

CI: confidence interval; Δ: change. The parameter estimates indicate the change of the NO outcome parameters (eNO, V'_{NO}) per decrease in GA. GA was divided into four groups: term infants (n=127), pre-term infants with 24–27 weeks GA (n=10), pre-term infants with 28–31 weeks GA (n=23) and pre-term infants with 32–35 weeks GA (n=29). For example, using model II, V'_{NO} was 0.63–0.15 =0.48 nL·s^{-1} in infants born at 24–27 weeks of gestation (value for healthy term infant–ΔV'_{NO} for GA 24–27 weeks=value for infant born at 24–27 weeks GA). [#]: univariate linear regression analysis; [¶]: model adjusted for centred values of expiratory time (t_E) and minute ventilation (V'_E) as well as for sex, maternal atopic disease, maternal smoking in pregnancy, maternal smoking both pre- and post-natally and maternal caffeine consumption. The estimated constant represents the value of a healthy female infant with average t_E and V'_E and no exposure to tobacco or caffeine and no history of maternal atopic disease.

Choosing the correct statistical model and reference group

The current analysis illustrates how difficult it is to define the correct statistical model and reference group in complex multifactorial disease processes such as CLDI. Some biometric factors, such as weight and length, are strongly associated with disease but not with the outcome measures (fig. 2). Adjusting for these factors may mask differences between healthy and pre-term infants. Lung functional parameters, such as tidal breathing indices and flow, are strongly related to disease but also demonstrate a relationship with the level of eNO. Pre-term infants with very low birth weight are more likely to have CLDI, thus healthy pre-term infants are more likely to be of more advanced GA than pre-term infants with CLDI. Environmental factors, such as tobacco or caffeine exposure, and maternal factors, such as atopic disease, significantly influence eNO even in healthy offspring [15]. All infants were measured at the same PCA of 44–45 weeks and undertook age adjustment for any remaining differences in PCA between the groups. GA, however, was so strongly correlated with the presence and severity of CLDI that it was unavoidable that the reference groups differed from the CLDI infants regarding this feature. This fact resulted in the inability to statistically disentangle the effects of CLDI and prematurity on eNO and V'_{NO}. Recruitment of equivalent numbers of healthy pre-term infants with the same very low GA as that observed in the CLDI infants is likely to represent an extremely challenging task and was not feasible in the context of the current study.

Clearance of eNO is influenced by changes in lung mechanical function and the present authors were able to adjust for the effects of altered mechanics using a stepwise approach. In the first instance, flow was accounted for on an individual basis by calculating V'_{NO}. Additionally, further adjustments were made for t_E and V'_E in a stepwise manner and for genetic, maternal and environmental factors (sex, maternal atopic disease, tobacco, caffeine) known to influence NO in the healthy offspring in a more complex multivariate regression model (model II). All model approaches produced fundamentally similar results. This consistency supported the robustness of the present findings.

Effects of severity and known risk factors for CLDI on eNO

Associations between risk factors and V'_{NO} were investigated using model II. Stratifying disease severity according to recently published guidelines [16], a stepwise decrease in V'_{NO} was found from pre-term infants without CLDI through mild and on to moderate CLDI disease severity. V'_{NO} was lowest in infants with low GA, high CRIB scores, long duration of oxygen therapy and in infants who had suffered from chorioamnionitis, although this was not statistically significant for the latter two factors. The relationships observed between these factors are clearly not independent of each other, since infants with low GA usually required longer oxygen therapy, suffered more often from chorioamnionitis and had higher CRIB scores. It was therefore not possible to separate the individual effects of these components in a group of 62 infants. There was no association between V'_{NO} and CLDI risk factors, such as duration of ventilation, post-natal infections or the presence of PDA. Similarly, treatment with corticosteroids or surfactant pre- or post-natally was not related to V'_{NO}.

Interpretation of the findings

Following adjustment for breathing pattern, flow and known maternal and environmental factors, V'_{NO} was found to be decreased in premature infants with CLDI in comparison with

healthy term infants. The present results require careful interpretation since there are likely to be interactions between various factors.

The present data do not support the assumption that increased eNO is purely a marker of airway inflammation in CLDI, as is the case in asthmatics. Other groups have found eNO concentration to be elevated in a small group of younger infants at the PCA of 36 weeks, with a decrease observed following corticosteroid treatment [13, 22]. Observed variations between these studies and the present findings might reflect methodological or age differences. It is possible that, in this more acute phase, the induction of iNOS during the inflammatory response is more dominant, potentially leading to an increase of eNO. The present findings are more in accordance with those of BARALDI *et al.* [12], who found eNO to be low in survivors of CLDI at school age.

Studies in baboons suggest that there might be differences in the development of NOS in premature offspring who subsequently develop CLDI. Developmental changes occurring in foetal baboon lungs during the third trimester may increase NOS expression and activity and, therefore, NO production [23]. Further work from that group showed that NOS expression was attenuated in a foetal baboon model of CLDI [11]. The group speculated that since NO is thought to play an important role in airway and parenchymal function in the immediate post-natal period, the alterations in NOS expression seen in CLDI baboons may contribute to the pathogenesis of the disease. These findings are supported by eNO measurements of newborn pre-term and term infants that demonstrate very low to absent eNO in pre-term infants compared with term infants in the first 6 h of life, suggestive of a more difficult perinatal adaptation at low GAs [24]. The present findings in human infants with CLDI are consistent with this hypothesis [11].

Alterations to NO metabolism occurring in addition to developmental differences in NOS also require consideration. Post-natal inflammatory response dominated by persistent neutrophil activity [25] and the presence of oxidative stress, may play a major role in this process since both are known to alter eNO [7, 8]. Neutrophil activity and oxidative stress both promote the oxygenation of NO to soluble peroxy-nitrites, nitrites and nitrates. Oxidative metabolites of NO are increased in both bronchoalveolar lavage (BAL) fluids [26] and plasma [27] during the first 28 days of life. However, nothing is known about the persistence of oxidative stress or neutrophilic inflammation during the late post-acute phase of 44 weeks PCA. Data from BAL samples suggest that neutrophil activity decreases following the peak inflammatory response seen in the first month of life [25, 28].

A final possible explanation for the decrease in V'_{NO} observed in CLDI infants also requires consideration. Airway epithelial remodelling may lead to epithelial dysfunction and therefore an alteration of NO flux across the airway surface.

Conclusions

Human infants suffering from mild-to-moderate CLDI demonstrate no difference in eNO in comparison to healthy term and pre-term infants when variations in flows and t_{ES} between these groups are not taken into account. Accommodation for these differences, through the calculation of exhaled NO output, reveals a small but significant decrease in NO output in infants suffering from chronic lung disease of infancy. Furthermore, less mature infants with more severe chronic lung disease of infancy were observed to have a correspondingly lower NO output in the post-acute phase of their illness. These findings indicate that NO metabolism might be involved in chronic lung disease of infancy even in a very late phase when the acute inflammatory processes are thought to have resolved. Whether NO metabolism plays a causative role in the pathophysiology of chronic lung disease of infancy remains unclear. A decrease in NO output may be the result of inborn developmental differences of NOS expression as suggested by SHAUL *et al.* [23], or the result of an altered balance between NO production and increased oxygenation due to persistent neutrophilic inflammation, persistent oxidative stress or airway epithelial dysfunction. The lack of association between post-natal interventions (ventilation, anti-inflammatory drugs) suggests that NO output may be determined very early (*e.g.* pre-natally or during the early post-natal period). The present observational studies in human infants cannot distinguish whether the lower NO output is related to developmental differences or to persistent inflammation or oxidative stress in these infants. The relationship between low gestational age with higher disease severity and low NO output, however, supports evidence that NO output is related to the degree of prematurity in infants with chronic lung disease of infancy, the most relevant risk factor for the disease. Although healthy premature infants tended to have lower NO output than term infants, in fact, low NO output was mainly seen in infants born at <28 weeks. The present data indicate that longitudinal bronchoalveolar lavage or biopsy studies commencing at birth may provide important information regarding the role of NO metabolism in the pathophysiology of chronic lung disease of infancy. The present findings also suggest that the post-acute phase of chronic lung disease of infancy is probably more important than initially anticipated and is still prone to the disturbance of airway and alveolar lung development [29–31].

ACKNOWLEDGEMENTS

The authors would like to thank: all the parents for their participation in the study; the staff of the Paediatric Respiratory Department (University of Berne, Berne, Switzerland) for their help in obtaining the data presented in this study, in particular H. Straub, C. Becher, M. Graf, G. Wirz, H. Gehr and R. Stampfli; and P. P. Wyder for technical assistance with the manuscript.

REFERENCES

1 Jobe AJ. The new BPD: an arrest of lung development. *Pediatr Res* 1999; 46: 641–643.
2 Kotecha S, Wilson L, Wangoo A, Silverman M, Shaw RJ. Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity. *Pediatr Res* 1996; 40: 250–256.
3 Saugstad OD. Bronchopulmonary dysplasia – oxidative stress and antioxidants. *Semin Neonatol* 2003; 8: 39–49.

4 Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. *Am J Respir Crit Care Med* 1999; 160: 1333–1346.
5 Oei J, Lui K, Wang H, Henry R. Decreased interleukin-10 in tracheal aspirates from preterm infants developing chronic lung disease. *Acta Paediatr* 2002; 91: 1194–1199.
6 Watterberg KL, Carmichael DF, Gerdes JS, Werner S, Backstrom C, Murphy S. Secretory leukocyte protease inhibitor and lung inflammation in developing bronchopulmonary dysplasia. *J Pediatr* 1994; 125: 264–269.
7 Ricciardolo FL, Sterk PJ, Gaston B, Folkerts G. Nitric oxide in health and disease of the respiratory system. *Physiol Rev* 2004; 84: 731–765.
8 Jones KL, Bryan TW, Jinkins PA, *et al.* Superoxide released from neutrophils causes a reduction in nitric oxide gas. *Am J Physiol* 1998; 275: 1120–1126.
9 Kawai N, Bloch DB, Filippov G, *et al.* Constitutive endothelial nitric oxide synthase gene expression is regulated during lung development. *Am J Physiol* 1995; 268: 589–595.
10 Xue C, Reynolds PR, Johns RA. Developmental expression of NOS isoforms in fetal rat lung: implications for transitional circulation and pulmonary angiogenesis. *Am J Physiol* 1996; 270: 88–100.
11 Afshar S, Gibson LL, Yuhanna IS, *et al.* Pulmonary NO synthase expression is attenuated in a fetal baboon model of chronic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2003; 284: 749–758.
12 Baraldi E, Bonetto G, Zacchello F, Filippone M. Low exhaled nitric oxide in school-age children with bronchopulmonary dysplasia and airflow limitation. *Am J Respir Crit Care Med* 2005; 171: 68–72.
13 Leipala JA, Williams O, Sreekumar S, *et al.* Exhaled nitric oxide levels in infants with chronic lung disease. *Eur J Pediatr* 2004; 163: 555–558.
14 Hall GL, Reinmann B, Wildhaber JH, Frey U. Tidal exhaled nitric oxide in healthy, unsedated newborn infants with prenatal tobacco exposure. *J Appl Physiol* 2002; 92: 59–66.
15 Frey U, Kuehni C, Roiha H, *et al.* Maternal atopic disease modifies effects of prenatal risk factors on exhaled nitric oxide in infants. *Am J Respir Crit Care Med* 2004; 170: 260–265.
16 Jobe AH, Bancalari E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2001; 163: 1723–1729.
17 Silkoff PE, McClean PA, Slutsky AS, *et al.* Marked flow-dependence of exhaled nitric oxide using a new technique to exclude nasal nitric oxide. *Am J Respir Crit Care Med* 1997; 155: 260–267.
18 Condorelli P, Shin HW, George SC. Characterizing airway and alveolar nitric oxide exchange during tidal breathing using a three-compartment model. *J Appl Physiol* 2004; 96: 1832–1842.
19 Tsoukias NM, George SC. A two-compartment model of pulmonary nitric oxide exchange dynamics. *J Appl Physiol* 1998; 85: 653–666.
20 George SC, Hogman M, Permutt S, Silkoff PE. Modeling pulmonary nitric oxide exchange. *J Appl Physiol* 2004; 96: 831–839.
21 Baraldi E, Azzolin NM, Cracco A, Zacchello F. Reference values of exhaled nitric oxide for healthy children 6–15 years old. *Pediatr Pulmonol* 1999; 27: 54–58.
22 Williams O, Bhat RY, Cheeseman P, Rafferty GF, Hannam S, Greenough A. Exhaled nitric oxide in chronically ventilated preterm infants. *Arch Dis Child Fetal Neonatal Ed* 2004; 89: 88–89.
23 Shaul PW, Afshar S, Gibson LL, *et al.* Developmental changes in nitric oxide synthase isoform expression and nitric oxide production in fetal baboon lung. *Am J Physiol Lung Cell Mol Physiol* 2002; 283: 1192–1199.
24 Colnaghi M, Condo V, Pugni L, Fumagalli M, Mosca F. Endogenous nitric oxide production in the airways of preterm and term infants. *Biol Neonate* 2003; 83: 113–116.
25 Kotecha S, Mildner RJ, Prince LR, *et al.* The role of neutrophil apoptosis in the resolution of acute lung injury in newborn infants. *Thorax* 2003; 58: 961–967.
26 Vyas JR, Currie AE, Shuker DE, Field DJ, Kotecha S. Concentration of nitric oxide products in bronchoalveolar fluid obtained from infants who develop chronic lung disease of prematurity. *Arch Dis Child Fetal Neonatal Ed* 1999; 81: 217–220.
27 Banks BA, Ischiropoulos H, McClelland M, Ballard PL, Ballard RA. Plasma 3-nitrotyrosine is elevated in premature infants who develop bronchopulmonary dysplasia. *Pediatrics* 1998; 101: 870–874.
28 Speer CP. Inflammation and bronchopulmonary dysplasia. *Semin Neonatol* 2003; 8: 29–38.
29 Hoo AF, Dezateux C, Henschen M, Costeloe K, Stocks J. Development of airway function in infancy after preterm delivery. *J Pediatr* 2002; 141: 652–658.
30 Hofhuis W, Huysman MW, van der Wiel EC, *et al.* Worsening of V'maxFRC in infants with chronic lung disease in the first year of life: a more favorable outcome after high-frequency oscillation ventilation. *Am J Respir Crit Care Med* 2002; 166: 1539–1543.
31 Gappa M, Stocks J, Merkus P. Lung growth and development after preterm birth: further evidence. *Am J Respir Crit Care Med* 2003; 168: 399.