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Alterations of exhaled nitric oxide in preterm infants with chronic lung disease

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ABSTRACT

Animal models suggest that reduced nitric oxide synthase (NOS) 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).

We measured on-line tidal eNO in 39 unsedated preterm infants with CLDI (mean gestational age GA 27.3 weeks) in comparison to 23 healthy preterm (31.6 weeks) and 127 term infants (39.9 weeks) at 44 weeks postconceptional age, thus after the main inflammatory response. We calculated NO output (V'NO=eNO*flow) to account for tidal flow related changes. We controlled for sex, maternal atopic disease and environmental factors (smoking, caffeine).

Mean eNO was not different (14.9 ppb in all groups), but V'NO was lower in CLDI compared to healthy term infants (0.52 vs. 0.63 nL/s, p=0.024). Values for healthy preterms were between these two groups (0.58 nL/s). Within all preterms (n=62), V'NO was reduced in infants with low GA, high CRIB scores and longer duration of oxygen therapy, but not associated with postnatal factors such as ventilation or corticosteroids.

After accounting for flow, the lower NO output in premature infants with CLDI is consistent with the hypothesis of NO metabolism being involved in CLDI.

INTRODUCTION

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 intrauterine and postnatal 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 IL-10 mediated inflammatory responses [4,5], persistence of neutrophilic inflammation and an imbalance of α1-protease inhibitor activity [6]. 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 eNOS, neuronal nitric oxide synthase nNOS) in comparison to 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 to age-matched healthy children [12]. In contrast, eNO was found to be elevated in a small group of human infants with CLDI at a postconceptional age (PCA) of 36 weeks [13]. However, this analysis did not adjust for different breathing pattern in infants with and without CLDI. We 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 and coauthors [11], we aimed to determine in this observational study whether exhaled NO is decreased in premature human infants suffering from CLDI. Therefore, we measured eNO 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], and compared these data with measurements obtained from healthy age-matched term infants, whilst accounting for potential confounding factors [15]. Furthermore, we 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 preterm infants with CLDI, 23 healthy preterm infants, and 127 healthy unselected term infants at a PCA of ~ 44 weeks (Table 1). Former preterm infants were recruited from the neonatal unit of the University maternity hospital in Berne, Switzerland between January 1999 and December 2004. Parents of 62 preterm infants agreed to participate, of which 23 were classified as healthy, 12 as having mild and 27 as having moderate CLDI according to the definition of the NICHD/NHLBI/ORD Workshop [16]. Clinical characteristics of all preterm 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].

For both groups, information on demographics, family history of atopic disease, clinical symptoms and environmental risk factors including pre- and postnatal tobacco exposure and maternal caffeine intake were obtained from the mothers by standardized interview. The presence of chorioamnionitis was determined based on placental histology.

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

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

Measurements of online eNO

All infants were studied during unsedated quiet sleep, in a supine position with the head in the midline. Heart rate and SaO_2 (Biox 3700: Datex-Ohmeda, Helsinki, Finland) were monitored

throughout the study. A compliant silicon mask (Infant mask, Size 1: Homedica, Switzerland) was placed over the nose and mouth and flow-volume loops were inspected for leak prior to commencing measurement. A NO filter ensured that all infants inhaled NO free air. Tidal V', volume (V), eNO and CO_2 were measured using commercially available infant lung function equipment (Exhalyser: EcoMedics, Duernten, Switzerland) as previously validated and described in detail [14]. Briefly, a representative example of online V', eNO and V'NO shows a steep increase of exhaled NO at the beginning of expiration which achieves a plateau towards the end of this phase (Figure 1, mid panel). During inspiration, eNO rapidly returns to zero indicating negligible rebreathing of NO from the equipment dead space. Calculation of NO output (V'NO) is performed by multiplying V' by eNO (V'NO=FeNO*V', Figure 1 lower panel). Flow (upper panel) rapidly approximates zero at the end of expiration. These rapid flow changes result in variable V'NO during the 4^{th} quartile of the breath duration. We have therefore measured eNO and V'NO in the third quartile of expiration, since this shows the lowest breath-to-breath variability [14] and corresponds approximately to the phase III slope [17,18,19,20]. 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

Data analyses were performed using STATA, version 8.0 for Windows (STATA Corporation, Texas, USA). With the exception of eNO, all continuous variables were normally distributed. eNO was therefore transformed for further analysis.

Comparison of eNO between healthy and sick children was difficult because eNO concentration is influenced by physiological, maternal and environmental factors [15, Figure 2], some of which are also related to prematurity and disease. In view of these complexities, we undertook a pragmatic approach and systematically analysed the two outcomes using two different statistical models: 1) a simple unadjusted comparison of means using t-tests; 2) a multivariable regression

model adjusting for the most influential physiological covariates (expiratory time and minute ventilation) and also for all other factors which were associated with eNO and V`NO in healthy term infants, including sex of the infant, maternal atopic disease, prenatal and postnatal maternal smoking and caffeine exposure [15]. For the regression models, continuous variables (expiratory time, minute ventilation) were centred (as described in the footnotes of the corresponding tables) and categorical variables were entered as indicator variables. From these regressions we report parameter estimates and their 95% confidence intervals (95 % CI) together with p-values for the null-hypothesis, that these estimates equal zero. In preterm children, gestational age and CLDI were so closely correlated, that it was not possible to separate their association with NO by simultaneous inclusion in a multivariate model. We therefore made two series of models. In the first instance we categorised the children according to presence or absence of CLDI (Table 3). Next, we categorised them according to gestational age, as 24-27 weeks, 28-31 weeks, 32-35 weeks and ≥38 weeks (Table 4).

Within preterm infants, we then tested systematically if NO was associated with any of the following clinical risk factors for CLDI: gestational age, clinical risk index for babies (CRIB) score, duration of oxygen supplementation, persistent ductus arteriosus (PDA), prenatal and postnatal steroids, administration of surfactant and chorioamnionitis, using an unadjusted model. A second test of these indices was then performed using a multivariable model adjusted for expiratory time and minute ventilation.

RESULTS

Clinical, physiological and environmental characteristics of the participating infants

Clinical, physiological and environmental characteristics of the infants are summarized 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 time of study for healthy preterm and CLDI infants. Tidal breathing parameters also differed considerably between healthy term infants, healthy preterms and preterm infants with CLDI, with the noteworthy exception of minute ventilation (MV), where the group mean was similar in all three groups (Table 2).

Nitric oxide measurements in preterm 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 preterm or CLDI infants when examined with either of the models (Table 3). NO output (V'NO) was 0.63 nL/s in healthy term infants and 0.58 nL/s 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, CLDI infants 0.52 nL/s, p=0.024). Values for healthy preterm infants were between the two other groups (0.58 nL/s).

Nitric oxide in preterm infants according to gestational age, compared to healthy term infants

Categorisation of preterm children according to gestational age ranges instead of lung disease revealed lower V'NO in infants with a gestational age less than 28 weeks (Table 4). No differences between other gestational age groups were identified using the two models.

Clinical factors associated with NO outcome parameters within the preterm group

Within the group of preterm children, there was no association between eNO and any of the potential clinical confounding factors examined. When adjusted for Te and MV, mean V'NO for all preterm infants was 0.59 nL/s. V´NO decreased by 0.02 nL/s 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 one week resulted in a decrease in V'NO of 0.01 nL/s (p=0.06), and postnatal treatment with corticosteroids was associated with a decreased V'NO (-0.15nL/s, p=0.04). Within the CLDI group, V'NO values in infants with moderate CLDI were 0.03 nL/s lower 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 duration of ventilation, the presence of PDA, prenatal corticosteroid therapy and surfactant treatment. V`NO was 0.09 nL/s lower in the group of preterm infants with chorioamnionitis, compared to those without (p=0.11). However, when we adjusted additionally for gestational age, the presence of chorioamnionitis and all other clinical risk factors disappeared as all of them were closely associated with low gestational age.

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. We thus measured eNO in unsedated infants with CLDI in comparison to healthy term and preterm 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 was 15.2 (15.1 - 15.3) ppb (group mean (95% CI)) in CLDI, 15.2 (14.1 – 16.4) ppb in healthy term and 15.4 (15.1 – 15.4) ppb in healthy preterm 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. 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 and highlight the importance of recording and correcting for flow during tidal eNO measurements. Several other possible mechanisms may explain the observed variations in results obtained between these studies. A significant proportion of the total exhaled NO is produced by the nasal mucosa. Measurements obtained from nose or mouth alone (as for example in [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 this study was to quantify overall exhaled NO and thus we did not attempt 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 we chose a simple technique for acquisition of tidal breathing data that is well established in our lung function laboratory. Respiratory tract infection represents a cause of elevated eNO levels, however, we were careful to exclude these children 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 minute ventilation was not different, individual infants with CLDI demonstrated differences in respiratory frequency, tidal volume expiratory time and tidal flows compared to healthy term and preterm infants. Furthermore, flow dependency of V'NO was altered in CLDI in comparison to healthy infants. Previous studies in healthy infants (14,15) suggested that these covariates must be taken into account during statistical analysis. We found the median V'NO averaged over 100 breaths to be similar in infants with CLDI in comparison to healthy term infants in a univariate (model I) but lower in multivariate analysis including such covariates (model II). Thus, our first conclusion is purely methodological. When comparing healthy subjects and those with lung disease, 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 exhaled NO. On the other hand, alterations in lung mechanics in disease might affect NO clearance from the lung, and thus mechanical factors might dominate the exhaled NO concentration or NO output.

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 multi-factorial disease processes such as CLDI. Some biometric factors such as weight and length are strongly associated with disease but not with the outcome measures (Figure 2). Adjusting for these factors may mask differences between healthy and preterm 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 exhaled nitric oxide. Preterm infants with very low birth weight are more likely to have CLDI, thus 'healthy' preterm

infants are more likely to be of more advanced gestational age than preterm infants with CLDI. Environmental factors such as tobacco or caffeine exposure and maternal factors such as atopic disease significantly influence exhaled NO even in healthy offspring [15]. We measured all infants at the same postconceptional age of 44-45 weeks, and undertook age adjustment for any remaining differences in postconceptional age between groups. Gestational age however, was so strongly correlated with 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 our inability to statistically disentangle the effects of CLDI and prematurity on eNO and V'NO. Recruitment of equivalent numbers of healthy preterm infants with the same very low gestational age 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 we 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, we adjusted for expiratory time and minute ventilation stepwise manner and also for all 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 findings.

Effects of severity and known risk factors for CLDI on exhaled NO

Associations between risk factors and V'NO were investigated using model II. Stratifying disease severity according to recently published guidelines [16], we found a stepwise decrease in V'NO from preterm infants without CLDI through mild and on to moderate CLDI disease severity. V'NO was lowest in infants with low gestational age, 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, postnatal infections or the presence of PDA. Similarly, treatment with pre- or postnatal corticosteroids or surfactant was not related to V'NO.

Interpretation of the findings

Following adjustment for breathing pattern, flow and known maternal and environmental factors, we found NO output to be decreased in premature infants with CLDI in comparison to healthy term infants. Our results require careful interpretation since there are likely to be interactions between various factors.

Our 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 postconceptional age of 36 weeks, with a decrease observed following corticosteroid treatment [13,22]. Observed variations between these studies and our own 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. Our findings are more in accordance with the findings 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 fetal baboon lungs during the third trimester may increase NOS expression and activity and therefore NO production [23]. Further work from this group showed that NOS expression was attenuated in a fetal baboon model of CLDI [11]. They speculate that since NO is thought to play an important role in airway and parenchymal function in the immediate postnatal 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 preterm and term infants that demonstrate very low to absent eNO in preterm infants compared to term infants in

the first 6 hours of life, suggestive of a more difficult perinatal adaptation at low gestational ages [24]. Our findings in human infants with CLDI are consistent with their hypothesis [11].

Alterations to NO metabolism occurring in addition to developmental differences in NO synthase also require consideration. Postnatal 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 exhaled nitric oxide [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 wks PCA. Data from BAL samples suggest that neutrophil activity decreases following the peak inflammatory response seen in the first month of life [25].

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 preterm infants when variations in flows and expiratory times 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 V'NO in CLDI infants. Furthermore, less mature infants with more severe CLDI were observed to have correspondingly lower NO output in the post-acute phase of their illness. These findings indicate that NO metabolism might be involved in CLDI 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 CLDI remains unclear. A decrease in NO output may be the result of inborn developmental differences of NOS expression as suggested by Shaul [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 postnatal interventions (ventilation, anti-inflammatory drugs) suggests that NO output may be determined very early (e.g. prenatally or during the early postnatal period). Our 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 relation between low GA with higher disease severity and low NO output, however, supports evidence that NO output is related to the degree of prematurity in infants with CLDI, the most relevant risk factor for CLDI. Although healthy premature infants tended to have lower V'NO than term infants, in fact, low NO output was mainly seen in infants born below 28 weeks. Our data indicate that longitudinal BAL or biopsy studies commencing at birth may provide important information regarding the role of NO metabolism in the pathophysiology of CLDI. Our findings also suggest that the post-acute phase of CLDI is probably more important than initially anticipated, and still prone to disturbance of airway and alveolar lung development [29-31].

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Ethics approval

The ethics committee of the university hospital and the State of Berne approved the study.

Competing interests

The authors declare that they have no competing interests.

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Legends

Figure 1 - A representative example of online flow (V'), eNO and V'NO showing a steep increase of exhaled NO at the beginning of expiration then approximating a plateau towards the end of expiration (middle panel). By multiplying V' and eNO, NO output (V'NO) can be calculated (V'NO=FeNO*V', lower panel). V' (upper panel) rapidly approximates zero at the end of expiration.

Definition of abbreviations: V´ = flow (L/s), eNO = exhaled nitric oxide (ppb), V´NO = nitric oxide output (nL/s).

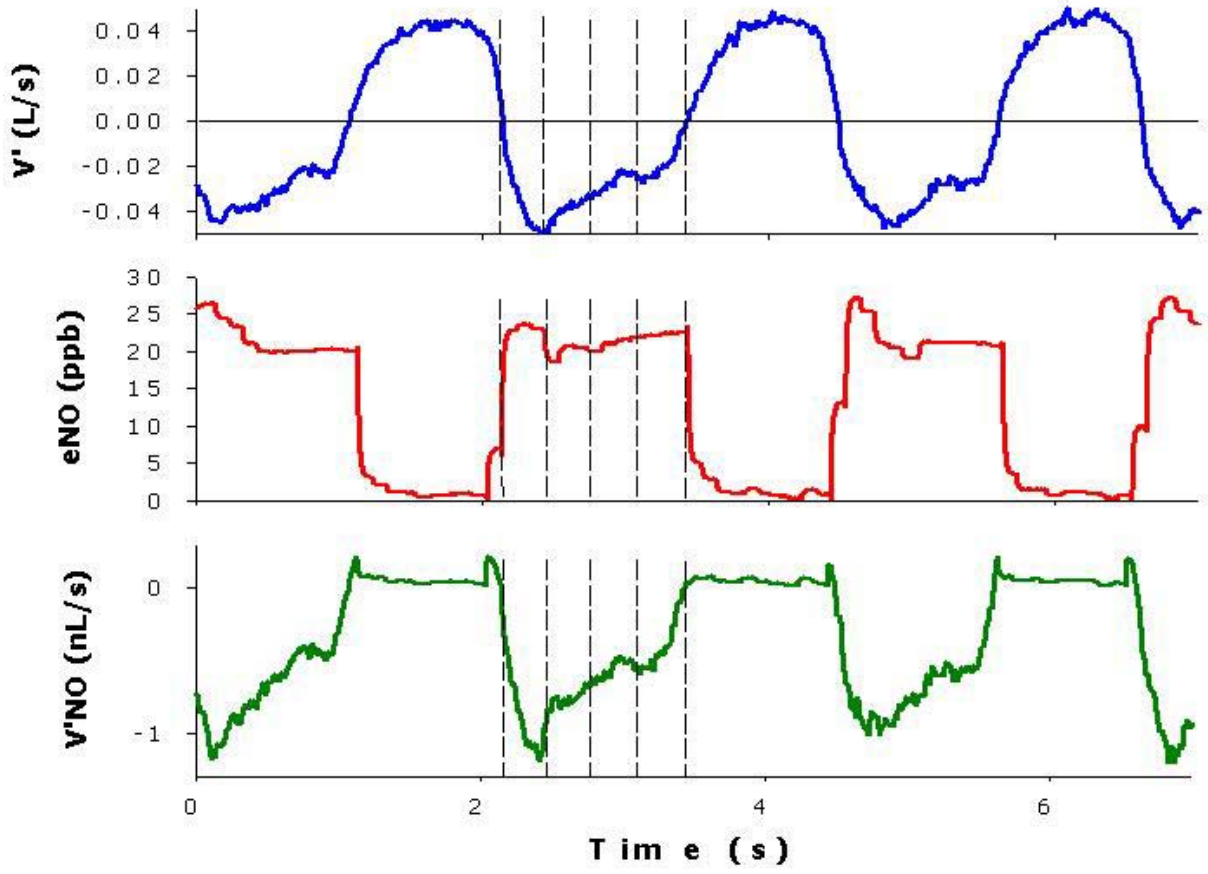

Figure 2 - Causal pathway of concomitant factors of exhaled NO and interactions between these factors.

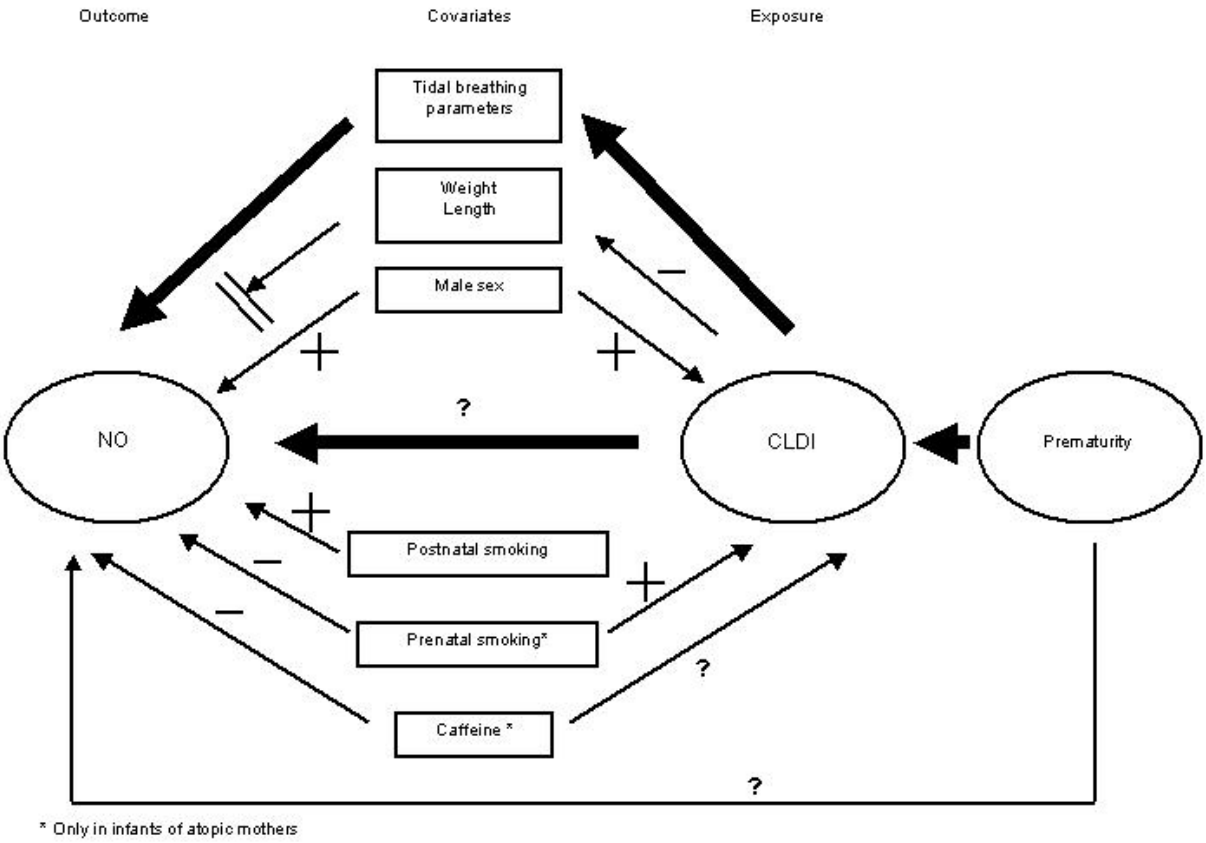
Outcome
Covariates
Exposure
Tidal breathing parameters
Weight
Length
Male sex
NO
CLDI
Prematurity
?
Postnatal smoking
Prenatal smoking*
Caffeine *
?
?
* Only in infants of atopic mothers

TABLE 1 - FAMILY HISTORY, ENVIRONMENTAL AND CLINICAL FACTORS OF THE STUDY CHILDREN.

	CLDI (39)	Healthy preterm (23)	Healthy term (127)
	N (%)	**n (%)**	**n (%)**
Male sex	24 (62)	11 (48)	73 (58)
Family history			
Maternal atopic disease*	9 (27)	4 (17)	53 (42)
Maternal asthma	2 (6)	0 (0)	14 (11)
Environment			
Maternal smoking in pregnancy	10 (30)	0 (0)	17 (13)
Maternal smoking postnaltally	5 (15)	0 (0)	21 (17)
Maternal smoking only postnatally	0 (0)	0 (0)	4 (3)
Maternal coffee consumption in pregnancy	15 (47)	19 (83)	83 (65)
Clinical factors			
PDA	15 (38)	3 (13)	
ASD / VSD	3 (8)	0 (0)	
Prenatal corticosteroids	32 (82)	19 (83)	
Postnatal corticosteroids	12 (31)	0 (0)	
Chorioamnionitis	19 (49)	1 (0)	

Definition of abbreviations: PDA = persistent ductus arteriosus, ASD = atrium septum defect, VSD = ventricle septum defect.

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 absent in the healthy preterm group. Maternal coffee drinking was more common in mothers of healthy preterm infants compared to healthy term and CLDI infants. CLDI infants had more cardiac malformations (PDA, ASD / VSD), had more often a history of chorioamnionitis and received more postnatal corticosteroids than healthy preterm infants.

** Atopic disease was defined as the presence of doctor-diagnosed asthma, hay fever or eczema (any of them).*

TABLE 2 - BIOMETRIC, TIDAL BREATHING AND CLINICAL DATA OF THE STUDY CHILDREN.

	CLDI (39)				Healthy preterm (23)				Healthy term (127)			
	Mean	**Median**	**SD**	**Range**	**Mean**	**Median**	**SD**	**Range**	**Mean**	**Median**	**SD**	**Range**
Anthropometric data												
Age at study (wk)	44.2	44.1	2.1	40.1-49.6	44.2	44.1	1.5	40.6-46.9	45.1	45.1	1.5	41.4-49.7
Weight at study (g)	3581	3600	547	2650-4830	3789	3680	558	3000-5190	4451	4350	539	3400-6300
Length at study (cm)	50.2	50.4	3.1	43.0-55.1	52.6	53	2.4	48-57	55.5	55.8	2.2	49.0-61.5
Gestational age (wk)	27.3	27.1	1.7	24.0-31.6	31.6	31.7	1.9	27.1-34.7	39.9	40.1	1.2	36.1-42.3
Birth weight (g)	879	870	243	420-1520	1534	1385	588	625-2840	3443	3440	457	2170-4915
Birth length (cm)	35.0	35	3.2	27.0-42.0	40.5	39.8	4.5	32-50	49.5	50	1.9	44.0-54.0
Tidal breathing data												
f (breaths/min)	54.4	51.8	13.1	36.1-90.2	45.7	45.7	7.9	29.1-59.1	43.7	41.4	11.7	24.6-75.8
Te (s)	0.66	0.66	0.18	0.35-1.02	0.77	0.73	0.18	0.57-1.18	0.85	0.81	0.28	0.43-1.69
Vt (ml)	24.5	23.1	6.1	13.4-37.1	30.1	31.5	7.3	19.7-44.2	31.3	31.3	5.8	20.2-46.5
PEF (mL/s)	61.5	63.2	16.1	26.2-88.9	61.7	57.9	18.2	39.8-110.3	58.3	55.7	15.7	21.1-107.2
VE (mL/min)	1285	1309	265	678-1798	1343	1285	317	891-1901	1312	1294	300	590-2265
Clinical data												
CRIB score	5.7	5	4.0	0-13	2.2	1	3.1	0-12				
Duration of intubation (d)	4.5	1	10.8	0-63	1.2	0	1.9	0-5				
Duration of CPAP (d)	36.6	38	17.2	2-68	5.2	3	6.9	0-28				
Duration of O2 supply (d)	71.1	67	22.2	31-131	5.0	4	5.6	0-22				
Max. FiO2	0.55	0.48	0.28	0.23-1.00	0.44	0.3	0.32	0.21-1.00				

Definition of abbreviations: f = breathing frequency, Te = expiratory time, Vt = tidal volume, PEF = maximal tidal expiratory flow, VE = minute ventilation, CRIB score = clinical risk index for babies score, CPAP = continuous positive airway pressure. FiO2 = fractional inspired oxygen.

TABLE 3 - ENO AND V`NO IN INFANTS WITH CLDI AND HEALTHY PRETERM INFANTS IN COMPARISON TO HEALTHY TERM INFANTS.

	Model I (crude)*			Model II (complex)†		
	Parameter Est.	**95% CI**	**p-value**	**Parameter Est.**	**95% CI**	**p-value**
ENO (ppb)						
Healthy term	15.22	14.10 – 16.38		14.92	12.61 – 17.43	
ΔeNO in						
Healthy preterm	+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)						
Healthy term	0.63	0.59 – 0.67		0.63	0.54 – 0.72	
ΔV'NO in						
Healthy preterm	-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

Definition of abbreviations: eNO = exhaled nitric oxide, V'NO = nitric oxide output. 95% CI = 95% Confidence Interval. The parameter estimates (Parameter Est.) indicate the change of the NO outcome parameter (eNO, V'NO) in non-CLDI preterm infants (n=23) and preterm infants with CLDI (n=39) in comparison to healthy term infants (n=127). E.g., using Model II, V'NO was 0.63 nL/s– 0.11 nL/s = 0.52 nL/s in CLDI infants (value of healthy term infant – ΔV'NO for CLDI = value of CLDI infant).

** Crude Model: Univariate linear regression analysis.*

† Complex Model: Adjusted for centred values of Te and MV as well as for sex, maternal atopic disease, maternal smoking in pregnancy, maternal smoking both pre- and postnatally and maternal caffeine consumption. The estimated constant represents the value of a healthy female infant with average Te and MV and no exposure to tobacco or caffeine and no history of maternal atopic disease.

TABLE 4 - ENO AND V´NO IN PRETERM INFANTS OF DIFFERENT GESTATIONAL AGE, COMPARED TO HEALTHY TERM INFANTS.

	Model I (crude)*			Model II (complex) †		
	Parameter Est.	**95% CI**	**p-value**	**Parameter Est.**	**95% CI**	**p-value**
ENO (ppb)						
GA 38 - 42 weeks	15.22	14.12 – 16.35		14.75	12.39 – 17.30	
ΔeNO in						
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)						
GA 38 - 42 weeks	0.63	0.59 – 0.67		0.64	0.55 – 0.73	
ΔV'NO in						
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

Definition of abbreviations: eNO = exhaled nitric oxide, V'NO = nitric oxide output. 95% CI = 95% Confidence Interval, GA = gestational age. The parameter estimates (Parameter Est.) indicate the change of the NO outcome parameter (eNO, V'NO) per decrease in gestational age. Gestational age was divided into 4 groups: term infants (n=127), preterm infants with 24 – 27 weeks of GA (n=10), preterm infants with 28-31 weeks of GA (n=23) and preterm infants with 32-35 weeks of GA (n=29). E.g., using Model II, V'NO was 0.63 nL/s– 0.15 nL/s = 0.48 nL/s in infants born at 24 – 27 weeks of gestation (value of healthy term infant – ΔV'NO for GA 24-27 weeks = value of infant born at 24-27 weeks of gestation).

** Crude Model: Univariate linear regression analysis.*

† Complex Model: Adjusted for centred values of Te and MV as well as for sex, maternal atopic disease, maternal smoking in pregnancy, maternal smoking both pre- and postnatally and maternal caffeine consumption. The estimated constant represents the value of a healthy female infant with average Te and MV and no exposure to tobacco or caffeine and no history of maternal atopic disease.