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Impact of chorioamnionitis on exhaled nitric oxide and endotracheal aspirate levels of nitrites-nitrates and interleukin-8 in mechanically ventilated preterm neonates

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SUMMARY

Objectives: To assess the influence of maternal chorioamnionitis on early exhaled nitric oxide (NO) and levels of nitrites-nitrates and interleukin (IL) IL-8 in endotracheal aspirate fluid in mechanically ventilated preterm neonates.

Study Design: Cross-sectional study.

Patient-Subject Selection: Between September 2007 and August 2009, 54 mechanically ventilated preterm neonates were included. Patients were divided into two groups according to the presence or absence of maternal chorioamnionitis, and those without chorioamnionitis (controls) were further stratified into two subgroups by birth weight \leq or $>$ 2000 g.

Methodology: The ventilator used was a Babylog 8000. The NO level assessed was the plateau value given by the software of the Sievers NOA apparatus. Collection of endotracheal aspirate fluid samples was performed coinciding with routine aspirations and using the dry technique.

Results: The two groups of control neonates showed statistically significant differences in exhaled NO expressed as nL/min and normalized exhaled NO expressed as either nL/min or nL/min/kg, so they are not homogeneous and cannot be used in clinical practice. Serum C-reactive protein and endotracheal aspirate levels of nitrites-nitrates were significantly higher in the chorioamnionitis group than in controls (3.6 vs 1.07 $\mu\text{mol/L}$; $p=0.035$). Nitrites-nitrates levels were positively correlated with exhaled NO in ppb ($\rho=0.367$; $p=0.006$). Minute exhaled endogenous NO was significantly higher in the chorioamnionitis group (0.48 vs 0.27 nL/min/kg; $p=0.021$).

Conclusions: In mechanically ventilated preterm infants weighing less than 2000 g, maternal chorioamnionitis was associated with an increase of early exhaled NO (nL/min/kg) and serum levels of C-reactive protein and levels of nitrites-nitrates in endotracheal aspirate fluid.

INTRODUCTION

Maternal chorioamnionitis may cause preterm delivery and be associated with an adverse perinatal outcome, including early-onset sepsis, necrotizing enterocolitis, and lung disease as well as long-term sequelae in the infant.¹⁻⁴ Histologic chorioamnionitis is associated with an increased incidence of bronchopulmonary dysplasia,⁵ a chronic lung disease that prolongs hospital stay and causes greater morbidity and mortality of respiratory origin.⁶⁻⁹

Chorioamnionitis may injure the fetal lung through the activation of alveolar neutrophils and macrophages. Due to this activation, greater expression of inducible nitric oxide (NO) synthase is to be expected, with elevation of nitrites and nitrates in endotracheal aspirates and of minute exhaled NO. Exhaled NO is raised in adults and children with asthma,^{10,11} and has also been detected among the gases exhaled in mechanically ventilated neonates.¹²⁻¹⁷ Different results of exhaled NO in neonates with bronchopulmonary dysplasia have been reported, including lower values in infants breathing spontaneously¹⁸ and higher exhaled NO peak levels in ventilated infants.¹⁹ Differences in postnatal age¹⁶⁻¹⁹ and in the methods for assessing exhaled NO (breathing spontaneously or on assisted ventilation,^{6,18} output or minute ventilation obtained using plateau NO values,¹⁸ or peak or maximum values of exhaled NO¹⁹) may account for the conflicting results. Although recommendations concerning standardized techniques for measurement of exhaled NO have been reported,²⁰ data for analysis of exhaled NO in mechanically ventilated preterm infants are lacking. Williams and associates²¹ studied the influence of ventilatory settings on measurements of exhaled NO and found that increasing peak inflating pressure as well as the inspired oxygen concentration resulted in a decrease in the peak NO levels. The authors emphasized that the conditions of measurement must be standardized in infants receiving respiratory support. In a previous study of our group,²² the following points were suggested when collecting gas samples from ventilated premature infants to obtain exhaled NO: (1) it would suffice to collect exhaled gas during 1 minute, although it is recommended to repeat collection 3 times;¹⁵ (2) for each sample collection, the parameters to record should be FiO₂, PIP, PEEP, RF, I/E, volume per minute and percentage of leakage (the two latter ones, every 15 sec during collection); and (3) the results should be expressed as exhaled NO (ppb

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3 plateau value), “minute exhaled NO per kg after correction for leakage” (exhaled NO x volume
4 per minute/weight in kg = nL or ppb/min/kg) and “normalized minute exhaled NO per kg after
5 correction for leakage” (after applying the normalization equation).²²
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7

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9
10 The cellular composition of tracheal aspirate is the gold standard for defining lung
11 inflammation.²³ Interleukin-8 (IL-8) is produced by alveolar macrophages, fibroblasts, type II
12 pneumocytes, and endothelial cells. Hypoxia, hyperoxia, and endotoxins stimulate the synthesis
13 of IL-8, which is a major neutrophil chemotactic factor in the lung. Histologic chorioamnionitis
14 is associated with an increase of IL-8 in endotracheal aspirate fluid along the first day of life (\geq
15 917 pg/mL) and this cutpoint may be used for diagnosis.²⁴ Preterm infants suffering from
16 bronchopulmonary dysplasia can present increased levels of tumor necrosis alpha (TNF- α),²⁵
17 IL-6,²⁵ IL-8,²⁶ and IL-10²⁶ in endotracheal aspirates.
18
19

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21 The present study was designed to assess changes of early exhaled NO and the
22 concentration of nitrites-nitrates and IL-8 in endotracheal aspirate fluid in mechanically
23 ventilated preterm neonates with and without history of maternal chorioamnionitis.
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26

27 28 29 30 31 32 33 34 35 **MATERIALS AND METHODS**

36 37 **Design and Patient Population**

38
39 A cross-sectional study of mechanically ventilated preterm infants was conducted to assess the
40 impact of chorioamnionitis on exhaled NO and the concentration of nitrites-nitrates and IL-8 in
41 endotracheal aspirates. Between September 2007 and August 2009, 54 mechanically ventilated
42 preterm neonates treated in the Neonatology Service of a IIIB level neonatal intensive care unit
43 were eligible. Inclusion criteria were gestational age between 25 weeks 0 days and 36 weeks 6
44 days, less than 36 hours of age, presence of mechanical ventilation for the treatment of
45 respiratory distress syndrome (RDS), hemodynamic stability, and written informed consent
46 obtained from the parents or legal guardians. Newborns with pulmonary hypoplasia or other
47 respiratory conditions other than RDS, major congenital malformations, chromosomal
48 abnormalities, sepsis, or treatment with NO donor drugs were excluded. Patients were divided
49 into two groups according to the presence or absence of maternal chorioamnionitis, and those
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3 without history of chorioamnionitis (controls) were further stratified into two subgroups
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5 according to birth weight < 2000 g or \geq 2000 g. The study protocol was approved by the Ethics
6
7 Committee of Hospital Clínic of Barcelona, Spain. The parents or legal guardians were
8
9 informed about the study and provided consent because no blood sampling was needed and
10
11 procedures performed were not painful and included in the care of the patients (tracheal
12
13 aspirates).
14
15

16 17 18 **Definitions and Procedures**

19
20 The diagnosis of clinical chorioamnionitis in the pregnant woman who presented with
21
22 premature labor or premature rupture of the membranes was made in the presence of some
23
24 combination of uterine pain, fever ($\geq 38^{\circ}\text{C}$), leukocytosis ($> 12,000$ cells/ mm^3), and increased
25
26 serum concentrations of C-reactive protein (> 30 mg/L) without any clear focus of infection. In
27
28 these cases, amniotic fluid and placenta were microbiologically cultured, and placenta and
29
30 umbilical cord histologically analyzed. Chorioamnionitis was diagnosed when clinical data were
31
32 accompanied by a positive culture from amniotic fluid and placenta (fetal and maternal sites)
33
34 and histological findings of chorioamnionitis. Polymorphonuclear infiltration in the placenta
35
36 and umbilical cord was required to establish a diagnosis of histological chorioamnionitis. Mild
37
38 chorioamnionitis had a few neutrophils (5-10 neutrophils per high-power field) scattered in the
39
40 subchorionic space and adjacent chorion; Moderate chorioamnionitis had many neutrophils (11-
41
42 30 neutrophils per high-power field) in the lower half of the chorionic plate and the
43
44 subchorionic space; and severe chorioamnionitis had dense infiltrates of neutrophils (30 per
45
46 high-power field) throughout the chorionic plate into the amnion. Mild chorioamnionitis was
47
48 recorded in 7 cases, moderate in 3, and severe in 5.
49
50
51

52
53 RDS was defined in the presence of a typical clinical course and chest radiographic
54
55 findings, with clinical improvement when exogenous surfactant was administered. RDS was
56
57 considered non-severe if only one dose of exogenous surfactant was needed and severe if two or
58
59 more doses of exogenous surfactant or high frequency oscillatory ventilation were needed as
60

1
2
3 well as in the presence of air leak or if RDS was the cause of death. Bronchopulmonary
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5 dysplasia was defined according to diagnostic criteria of Jobe and Bancalari²⁷
6
7

8 At all times, neonates received appropriate medical care according to his/her clinical
9
10 condition. Special attention was placed to maintain the inspired gas temperature at 37°C and
11
12 maximum humidity of the circuit (100%) using a Fisher-Paykel humidifier. Serial
13
14 measurements of heart rate, respiratory frequency (RF), noninvasive blood pressure, and arterial
15
16 blood gases were performed. Ventilatory parameters included peak inspiratory pressure (PIP),
17
18 positive end expiratory pressure (PEEP), inspiratory time (Ti), effective respiratory frequency
19
20 (RF), tidal volume (Vt), minute volume (Vm), and compliance (A). The Clinical Risk Index for
21
22 Babies (CRIB) score (in infants weighing < 1500 g) and the Score for Neonatal Acute
23
24 Physiology and SNAP Perinatal Extension (SNAPPE II) score were assessed to check
25
26 homogeneity of the patients' severity of illness.
27
28

29 All infants were mechanically ventilated using a constant flow, time cycled, pressure
30
31 limited device (Babylog 8000 Plus, Dräger Medical AG & Co. KGaA, Lübeck, Germany),
32
33 which measures all ventilatory parameters displayed in the screen of the device during the
34
35 expiratory time. Oxygenation was continuously monitored by pulse oximetry to maintain
36
37 arterial blood oxygen saturation (SaO₂) between 88% and 93% and arterial partial pressure
38
39 (PaO₂) between 50 and 80 mm Hg. For ventilation, we tried to achieve carbon dioxide partial
40
41 pressure (PaCO₂) in the range of 45–55 mm Hg and pH > 7.20 during the first 96 hours; then,
42
43 high PaCO₂ levels were allowed while maintaining pH > 7.20. In order to avoid stress-
44
45 induced release of NO from vascular endothelium,²⁸ gentle ventilation was used.
46
47
48

49 All newborns were intubated with a double-lumen endotracheal tube (inner diameter 2.7
50
51 and 3.2 mm in infants weighing 600–900 g and 1000–1500 g, respectively) if a deep
52
53 resuscitation was needed in the delivery room, or when a fraction of inspiratory oxygen (FiO)
54
55 greater than 0.30 was required, or in the presence of moderate, severe, or progressive respiratory
56
57 difficulty in spite of receiving continuous positive airway pressure (CPAP) of 6 cm water. All
58
59 intubated infants received surfactant (Survanta, Ross Laboratories, Columbus, OH) at 4 mL/kg
60
(100 mg/kg of phospholipids per kg), which was repeated if needed. Initial ventilatory setting

1
2
3 included flow 5–8 L/min, PEEP 5 cm H₂O, RF 60 breaths/min, and FiO₂ to keep SaO₂ in the
4
5 range of 88–93%. Thereafter, PIP and RF were adjusted to reach the aforementioned pH and
6
7 PaCO₂ levels. Sedation was not routinely used during the study.
8
9

10 11 12 **NO Sampling**

13
14 The sample of gas for NO analysis was obtained taking into account that no exogenous
15
16 surfactant was administered in the previous 6 hours and in the presence of correct
17
18 humidification of the circuit, correct calibration of the NO apparatus (chemiluminescence
19
20 technique), and low proportion of NO (< 40 ppb) in the compressed wall air. NO sampling was
21
22 performed as previously described²² and trying to standardize the ventilation parameters²¹ (FiO₂
23
24 = 0.21, RF = 50 respirations/min, Ti = 0.4 sec, PIP = 20 cm water, PEER = 5 cm water) during
25
26 the 3 min of NO sampling. This change in the ventilation parameters was well tolerated and in
27
28 only 12 cases we had to increase FiO₂ to less than 0.35. The sample of gas in which NO was
29
30 analyzed was obtained through a “6 French” arterial catheter introduced into the endotracheal
31
32 tube to its tip. This catheter is the narrowest that permits adequate aspiration of the gas sample
33
34 without generating an alarm in the Sievers NOA apparatus. Furthermore it occupies very little
35
36 of the endotracheal cross-sectional area, and hence does not affect ventilation of the lung. Three
37
38 samples of gas during 1 minute each were obtained. The mean intrasubject coefficient of
39
40 variability of exhaled NO levels (in ppb) was calculated from the mean of three 1-min sampling
41
42 periods in each of the 54 infants. The coefficient of variation was 12.7%.
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45
46
47
48

49 **Influence of Air Contamination**

50
51 The absence of environmental air contamination in exhaled NO samples was confirmed by the
52
53 lack of correlation between ambient NO and exhaled NO in ppb ($n = 54$, $\rho = -0.35$, $P = 0.802$)
54
55 as well as between inhaled NO and exhaled NO in ppb ($n = 54$, $\rho = 0.176$, $P = 0.203$). The mean
56
57 (standard deviation) inspired NO level was 1.27 (0.91) ppb (range 0–4.1).
58
59
60

Calculation of Plateau NO and Minute Exhaled NO

The NO level assessed is not the highest peak, but rather the plateau value given by the software of the Sievers NOA apparatus. This plateau value is the most reliable equivalent of the NO exhaled during expiration (corresponding to the average NO detected between 1/2 and 7/8 of the duration of expiration). Sievers NOA 280i is able to detect from 0.5 ppb to 500 ppm of NO, with a sensitivity of 0.5 ppb, aspirating a gas sample of 10-200 mL/min (100 mL/min in our study) and with a lag time of 1 second and an electronics response time of 0.067 seconds (sampling rate of 15 Hz) to 90% full scale. This high speed means the apparatus can differentiate between inspired gas (contaminated with compressed air) and expired gas, which adopts the form of a plateau. Minute exhaled NO or minute excretion of NO was calculated using the formula:

$$V'_{\text{NO}} \text{ (nL/min)} = \text{Plateau NO (ppb = nL/L)} \times V_{\text{E}} \text{ (minute ventilation in L/min)}$$

V_{E} has been previously corrected by the percentage of leakage given by the software of the Babylog 8000 ventilator using the formula:

$$V_{\text{E}} \text{ (L/min)} = \text{Volume per minute (L/min)} / ((100 - \text{leakage})/100)$$

Finally, minute exhaled NO was divided by the weight in kilograms of the newborn to obtain the minute exhaled NO per kg (nL/min/kg). To convert nL to nmoL, it should be remembered that 1 mol = 24.3 liters, when environmental temperature is 23°C.

The results can be normalized by applying the formula developed in a previous report²² as follows: Normalized minute exhaled NO = minute exhaled NO without leakage – minute exhaled NO deviation, being Minute exhaled NO deviation = $-173.668 \times (\text{exhaled NO}/100)^3 + 49.513 \times (\text{exhaled NO}/100) + 12.486 \times (\text{VM}/100)^3 + 24.075 \times (\text{PIP}/100) + 12.371 \times (\text{RF}/100)^3 - 8.126$ – average minute exhaled NO.

Collection of Endotracheal Aspirate Fluid Samples

Collection of endotracheal aspirate fluid samples was performed before the administration of exogenous surfactant or at least 6 hours later²⁹ coinciding with routine aspirations and according to the dry technique, which for research purposes is equally valid than the bronchoalveolar lavage.³⁰ To obtain the mucus aspirated inside the catheter, the catheter was cleaned with 1 mL

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2
3 of normal saline into a tube and finally purged with air. The entire procedure was repeated to
4
5 have a 2 mL sample, 1 mL of which was placed in another tube for nitrites-nitrates analysis.
6
7 Moreover, 1 mL of the same normal saline was placed in another tube as it may be
8
9 contaminated by different amounts of nitrites-nitrates. Likewise, 0.2 mL for analysis of IL-8
10
11 analysis and 0.2 mL for analysis of proteins were also kept. The diluted endotracheal aspirate
12
13 fluid samples were centrifuged and the supernatant was recovered and frozen at -80°C , as the
14
15 tube with normal saline only. Samples were delivered to the laboratory on ice.
16
17

20 **Measurement of Nitrites-Nitrates, IL-8, and Protein Levels in Endotracheal Aspirates**

22 The concentration of nitrites-nitrates in endotracheal aspirates was measured by a modification
23
24 of the fluorimetric technique based on the reaction between 2,3-diamino-naphthalene and
25
26 nitrites in acid medium. Previous filtration in Ultrafee column (UFC4 LGC, Millipore Corp.,
27
28 Bedford, MA) is needed to avoid interferences with serum components. The detection limit of
29
30 the technique is 0.01–50 $\mu\text{mol/L}$. Results were expressed in $\mu\text{mol/L}$ and in relation to the
31
32 concentration of proteins. Levels of IL-8 were measured by a highly sensitive ELISA technique
33
34 (R&D Systems) and results were expressed as pg/mL and in relation to the concentration of
35
36 proteins. Proteins were measured by an automated method to normalize nitrites-nitrates and IL-
37
38 8 concentrations per gram of proteins in the endotracheal aspirate fluid, so that errors related to
39
40 differences in the amount of collected samples were avoided.
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42
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46 **Variables**

48 In all infants and before the 36 hours of life, the following variables were recorded: gestational
49
50 age, sex, birth weight, hours of life, history of maternal chorioamnionitis, maternal smoking
51
52 during pregnancy, cesarean section, severity of illness (CRIB and SNAPPE-II score),
53
54 concomitant diseases, such as severe RDS, air leak, patent ductus arteriosus, severe peri-
55
56 intraventricular hemorrhage (grade 3–4), early sepsis, early exhaled NO (minute exhaled NO
57
58 per kg [expressed as nL/min/kg], actual and normalized value),²² and concentrations of nitrites-
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60

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3 nitrates ($\mu\text{mol/L}$ and $\mu\text{mol/g}$ proteins) and IL-8 (pg/mL and pg/g proteins) in the endotracheal
4
5 aspirate fluid.
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7

8 9 10 **Statistical Analysis**

11
12 Continuous variables were presented as mean \pm standard deviation (SD) if normally distributed
13
14 and with homogeneous variances or as median and interquartile range (IQR: 25th–75th
15
16 percentiles) if not normally distributed or without homogeneous variances. These latter
17
18 variables were birth weight; C-reactive protein levels; nitrites-nitrates, IL-8, and protein levels
19
20 in endotracheal aspirates; duration of supplemental oxygen therapy; and all exhaled NO
21
22 measurements. The influence of environmental NO or NO in compressed air on exhaled NO
23
24 was analyzed by means of Spearman correlation, as well as other correlations between exhaled
25
26 NO and other variables, particularly levels of nitrites-nitrates and IL-8 in endotracheal aspirates,
27
28 and days of supplemental oxygen therapy. The Student's *t* test was used for the comparison of
29
30 parametric variables and the Mann-Whitney *U*-test for the comparison of non-parametric
31
32 variables among the different groups according to “maternal chorioamnionitis” (yes/no). Infants
33
34 with and without history of maternal chorioamnionitis were matched by birth weight.
35
36 Categorical variables were compared with the chi-square (χ^2) test or the Fisher's exact test. To
37
38 determine the most useful cut-point of exhaled NO as a marker of history of chorioamnionitis,
39
40 the value with the best relation sensitivity-specificity and the highest positive likelihood ratio
41
42 was chosen. Statistical significance was set at $P < 0.05$.
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48

49 **RESULTS**

50 51 **Control Group**

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53 As shown in Table 1, the two groups of neonates without history of maternal chorioamnionitis
54
55 showed statistically significant differences in the use of antenatal steroids, gestational age, birth
56
57 weight, and Apgar score at 5 minutes after birth. In addition (Table 2), there were significant
58
59 differences in exhaled NO expressed as nL/min and normalized exhaled NO expressed as either
60
nL/min or nL/min/kg. Therefore, the two subgroups were not homogeneous, and the most

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2
3 similar to the study group was the subgroup with <2000 g of birth weight. This subgroup will be
4
5 considered the control group.
6
7

8 9 **Comparison of Infants with and Without History of Maternal Chorioamnionitis**

10 Clinical findings and morbidity according to history of maternal chorioamnionitis are shown in
11
12 Table 1. Preterm newborns in the chorioamnionitis group compared with controls weighing <
13
14 2000 g were less frequently born by cesarean section and mothers were older. Moreover, a
15
16 significantly higher percentage of infants with history of maternal chorioamnionitis needed
17
18 resuscitation and endotracheal intubation more frequently and presented with patent ductus
19
20 arteriosus or severe intraventricular hemorrhage. The only newborn who died from the
21
22 chorioamnionitis group was a 25 weeks' preterm infant with a birth weight of 810 g and a CRIB
23
24 score of 4, who was intubated only during the first day of life after receiving two doses of
25
26 surfactant. Seven days after birth he suffered from necrotizing enterocolitis with intestinal
27
28 perforation and died. The only newborn who died from the control group was a 25 weeks'
29
30 preterm infant with a birth weight of 740 g and a CRIB score of 4, who developed a very severe
31
32 bronchopulmonary dysplasia and died 49 days after birth.
33
34
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37

38 The values of inflammatory markers and exhaled NO according to history of maternal
39
40 chorioamnionitis are shown in Table 2. C-reactive protein was higher in the chorioamnionitis
41
42 group. IL-8 levels, either in absolute values or divided by the protein content of the endotracheal
43
44 aspirate were also higher in this group, although statistically significant differences were not
45
46 reached. However, the levels of nitrites-nitrates were significantly higher than in controls ($P =$
47
48 0.035) and were positively associated with exhaled NO in ppb ($n = 54$, $\rho = 0.367$, $P = 0.006$).
49
50

51 Minute exhaled endogenous NO, expressed in nL/min/kg, was significantly higher in
52
53 the chorioamnionitis group. The most useful cutoff for exhaled NO as a marker of history of
54
55 maternal chorioamnionitis was 0.285 nL/min/kg, with a sensitivity of 73.3%, specificity of
56
57 57.7%, positive predictive value of 50.0%, negative predictive value of 78.9%, accuracy of
58
59 63.4%, positive likelihood ratio of 1.733, negative likelihood ratio of 0.462, and area under the
60
curve of 0.718 ($P = 0.021$).

1
2
3 Peak values of exhaled NO were significantly higher in the chorioamnionitis group than
4
5 in controls ($P = 0.032$) and also showed a significant correlation with nitrites-nitrates levels in
6
7 the endotracheal aspirate fluid ($n = 54$, $\rho = 0.322$, $P = 0.018$).
8
9

10 11 12 **DISCUSSION**

13
14 In the healthy newborn, exhaled NO is not affected by the gestational age,^{13,16,29,31,32} birth
15
16 weight,³³ or mode of delivery,²⁹ although there is an increase in exhaled NO during the first
17
18 week of life.^{16,29} Exhaled NO is not related either with serum levels of nitrites and nitrates,^{13,24}
19
20 but there may be a relationship with nitrites and nitrates that are present in the low respiratory
21
22 tract.³³ In addition, there are two trends in the publications of research groups working on
23
24 expired or exhaled NO in newborns: those authors who work only with concentrations (ppb)^{6,12–}
25
26 ^{14,16,17,34} and those as our group who prefer to adapt the methodologies to present the results as
27
28 “total excretion of exhaled NO” (nL/min/kg).^{17,29,31,32,35,36} Olsen et al.,¹⁵ in 2003, measured
29
30 exhaled NO in eight preterm infants with RDS and showed a decline of median minute
31
32 excretion of NO by 48–72 hours. Williams et al.¹⁶ measured nasal and lower airway NO level in
33
34 very immature infants (median gestational age 27 weeks) and found that nasal and lower airway
35
36 NO levels did not correlate significantly with gestational age, but lower airway NO levels
37
38 correlated with postnatal age.
39
40
41

42
43 As far as we are aware, the relationship between maternal chorioamnionitis and possible
44
45 increases in nitrites-nitrates levels in endotracheal aspirates and exhaled NO during the first
46
47 days of life have not been previously investigated. In the present study, these parameters were
48
49 examined exclusively in mechanically ventilated preterm infants with RDS. The increase of
50
51 plateau and peak exhaled NO and their positive correlation with endotracheal nitrites-nitrates
52
53 suggest that both factors are related and due to the same inflammatory process. It has been
54
55 shown that nitrate concentration in bronchoalveolar lavage fluid is elevated in infants who
56
57 developed chronic lung disease of prematurity.³³ In the present study, mean IL-8 values were
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59 higher in the chorioamnionitis group than in the control group (1161 vs 497 pg/mL), although
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differences were not statistically significant ($P = 0.277$), probably because of the high dispersion

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3 of values. It is likely that changes in IL-8 would have been more apparent with a larger sample
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5 size.
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8 The birth weight cutoff point of 2000 g was empirically chosen in an attempt to
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10 homogenize the sample in order to analyze the results of exhaled NO in different forms,
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12 including ppb, nL/min, nL/min/kg and normalized values). It was observed that plateau exhaled
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14 NO expressed in nL/min was significantly different between the two control subgroups;
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16 therefore, this measurement is not useful in clinical practice. The same findings were observed
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18 for normalized plateau exhaled NO expressed as either nL/min or nL/min/kg. A comparison of
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20 the chorioamnionitis group with the control subgroup of < 2000 g seems reasonable as both
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22 groups were homogeneous regarding gestational age and birth weight. However, if comparisons
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24 would have been performed with the 15 infants in the chorioamnionitis group and all 39
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26 controls, heterogeneity of controls would probably account for the disappearance of statistically
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28 significant differences for nitrites-nitrates ($\mu\text{mol/L}$) and peak of exhaled NO (ppb) between
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30 infants in the chorioamnionitis and controls of < 2000 g. In fact, a positive aspect of the present
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32 study was the stratification of infants in the control group according to birth weight, which
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34 allowed comparing infants in the chorioamnionitis group with only those preterm babies
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36 without history of chorioamnionitis and similar features of immaturity and birth weight. This
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38 stratification also revealed that in controls, the expression of exhaled NO in nL/min, both in
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40 actual and normalized values was not homogeneous. When exhaled NO was expressed
41
42 according to the birth weight, actual values of exhaled NO appeared homogeneous, in contrast
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44 with normalized values which are even spread out more. Therefore, the attempt to normalize
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46 exhaled NO reported in a previous study,²² unfortunately is not useful in clinical practice. In this
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48 respect, plateau of exhaled NO in ppb and nL/min/kg and peak of exhaled NO in ppb seem to be
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50 the only useful parameters in clinical practice. Another positive aspect was the absence of
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52 subclinical chorioamnionitis in the control group, because in that group the histology of the
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54 placenta had to be normal.
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59 The present findings should be interpreted taking into account some limitations of the
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study, particularly the exclusion of perinatal sepsis; in this case, marked increases in

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3 endogenous NO with secondary increase of exhaled NO have been reported.³⁷ In addition, the
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5 study was restricted to the first 36 hours of life, and may be thereafter other results would be
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7 obtained
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10 One difficulty when attempting to analyze exhaled NO of bronchoalveolar origin is the
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12 risk that the determination could be invalidated due to NO contained in the gas inhaled. This is
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14 not the case when using the “breath program” software which accompanies the Sievers Nitric
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16 Oxide Analyzer (NOA 280i) in the mode designated “use pressure for breath detection mode”,
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18 and with “on-line sampling”. It is considered that the gas in the region between 1/2 and 7/8 of
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20 the duration of expiration is equivalent to the third and fourth quartiles of the expiration time,
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22 when the gas obtained is exclusively bronchoalveolar,³⁸ with no possibility of external
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24 contamination. The manufacturers of the NOA 280i only recommend the use of medicinal air
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26 (NO-free) when environmental NO levels exceed 40–50 ppb. Our compressed air NO levels
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28 never reached these values (range 0–4.1 ppb). For repeated measurements of exhaled NO in
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30 each expiration, it should be available a chemiluminescence analyzer of rapid response (≤ 0.2
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32 sec) and at a flow rate of > 1.7 mL/sec (Sievers NO analyzer [NOA 280] with a response time
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34 of 0.2 sec and a minimum sample of 100–300 mL/min).
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38 In our previous study,²² it was shown that the variable volume per minute was highly
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40 influenced by the percentage of leakage given by the ventilator. Although the leakage of gas
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42 around the endotracheal tube is low, the sample of gas aspirated by the analyzer is considered as
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44 a leak by the ventilator. This leakage can be corrected mathematically, and indeed this should
45
46 always be done. Therefore, in the present study minute exhaled NO was obtained multiplying
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48 the plateau NO value (plateau between 1/2 and 7/8 of expiration) by the volume per minute, as
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50 Olsen et al.¹⁵ have previously reported, but volume per minute was previously corrected by the
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52 percentage of leakage.
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56 In the present study, exhaled NO obtained at the tracheobronchial level and during the
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58 expiratory phase was expressed as total exhaled NO in nL/min/kg, and this value was also
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60 normalized according to setting of ventilation parameters.²² Median values of exhaled NO were
0.48 and 0.27 nL/min/kg in infants with and without history of maternal chorioamnionitis,

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2
3 respectively. These figures are similar to those reported by Olsen et al.¹⁵ who showed that
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5 minute excretion of NO (divided by the weight) in premature infants with respiratory distress
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7 syndrome and mechanically ventilated at 24 hours of life was 0.405 nL/min/kg. In our group of
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9 chorioamnionitis, higher values of exhaled NO in nL/min/kg were found. A value of exhaled
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11 NO greater than 0.285 nL/min/kg would be suggestive of history of maternal chorioamnionitis
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13 with a 50% positive predictive value. This low figure and the fact that exhaled NO should be
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15 measured in the postnatal period preclude the clinical use of this parameter as a diagnostic
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17 criterion of chorioamnionitis. The increase in exhaled NO in nL/min/kg and in nitrites-nitrates
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19 levels in the endotracheal aspirate fluid, and the positive correlation between both parameters,
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21 would support the common origin of NO in the lower respiratory airway as a result of the
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23 inflammatory process.
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27 On the other hand, a significant higher incidence of bronchopulmonary dysplasia in the
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29 chorioamnionitis groups was not observed, which may be explained by the use of antibiotic
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31 treatment and antenatal corticoids for fetal maturation, all of which attenuates the inflammatory
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33 process.³⁹ Lahra et al.⁴⁰ even suggested that fetal inflammatory response during
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35 chorioamnionitis is a protective factor for chronic lung disease. May et al.⁴¹ reported the peak
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37 exhaled NO values obtained on days 3, 5, 7, 14, 21 and 28 after birth in 80 preterm infants of
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39 less than 32 weeks of gestation, depending on the appearance of bronchopulmonary dysplasia
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41 (BPD). In the 20 preterm infants who did not develop bronchopulmonary dysplasia, mean (SD)
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43 peak exhaled NO level at 3 days was 4.8 (2.5) ppb. In our study, mean peak exhaled NO level in
44
45 the chorioamnionitis group was 2.14 ppb (range 1.62–4.29), which is somewhat lower. This
46
47 may be due to age differences at the time of sampling (mean 17.1 hours in our study and 3 days
48
49 in the study of May et al.⁴¹).
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53 We conclude that maternal chorioamnionitis is associated with an increase of early
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55 exhaled NO (nL/min/kg) in mechanically ventilated preterm infants weighing less than 2000 g.
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57 Chorioamnionitis is also associated in the newborn with an increase of C-reactive protein and of
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59 endotracheal nitrites-nitrates levels but not of early IL-8. Although an early single measurement
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of exhaled NO has little clinical applicability as a marker of lung inflammation, repeated

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3 measurements during the first days or weeks of life when the infants is intubated and
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5 mechanically ventilated showing increasing values may be useful to suspect bronchopulmonary
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7 dysplasia. May et al.⁴¹ have shown that an increase exhaled NO along the first 28 days of life
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9 was related to bronchopulmonary dysplasia, and that there was a linear trend in exhaled NO
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11 results as severity of bronchopulmonary dysplasia increased. In these circumstances, treatment
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13 could be prescribed to improve the prognosis of this chronic lung disease.
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TABLE 1. Characteristics of the Study Groups with and without Chorioamnionitis

Variable	Maternal chorioamnionitis			<i>P</i> value (groups 1 versus 2)	<i>P</i> value (groups 2 versus 3)
	Absent		Present (group 3)		
	≥ 2000 g (group 1)	< 2000 g (group 2)			
Total infants	13	26	15		
Mother and labor					
Age, years, mean (SD)	32.8 (3.9)	30.0 (3.9)	33.8 (4.8)	0.185	0.039
Smoking during pregnancy	0	6 (23.0)	0	0.158	0.120
Antibiotic treatment	9 (69.2)	15 (62.5)	13 (92.9)	0.734	0.059
Antenatal steroids	4 (30.8)	22 (84.6)	14 (93.3)	0.003	0.411
Inborn	8 (61.5)	22 (84.6)	15 (100)	0.129	0.278
Cesarean section	8 (61.5)	19 (73.1)	5 (33.3)	0.486	0.012
Multiple gestation	3 (23.1)	11 (42.3)	7 (46.7)	0.409	0.786
Newborns					
Hours of life, mean (SD)	21.8 (8.6)	21.5 (7.3)	17.1 (7.9)	0.997	0.224
Male	10 (76.9)	13 (50)	8 (53.3)	0.112	0.839
Gestational age, weeks, mean (SD)	34.1 (1.6)	29.2 (2.4)	28.1 (2.7)	0.000	0.386
Birth weight, median (IQR)	2200 (2160–2610)	1020 (970–1400)	1100 (850–1360)	0.000	0.968
Intrauterine growth restriction	0	3 (11.5)	0	0.524	0.457
Resuscitation (endotracheal tube)	0	7 (26.9)	9 (60)	0.105	0.037
Apgar score at 5 min, mean (SD)	9.8 (0.4)	8.0 (1.7)	8.1 (1.5)	0.003	0.959
CRIB score,* mean (SD)		2.5 (2.3)	2.8 (1.8)		0.746
SNAPPE II score, mean (SD)	11.8 (9.1)	21.5 (13.4)	23.7 (15.9)	0.110	0.885
Morbidity					
Severe respiratory distress syndrome	9 (69.2)	15 (57.7)	11 (73.3)	0.491	0.323
Pneumothorax	0	1 (3.8)	2 (13.3)	0.999	0.543
Patent ductus arteriosus	3 (23.1)	8 (30.8)	10 (66.7)	0.719	0.028
Intraventricular hemorrhage (grades 3–4)	0	0	4 (26.7)		0.013
Early-onset sepsis	0	0	2 (13.3)		0.128
Late-onset sepsis	0	2 (7.7)	3 (20)	0.544	0.336
Necrotizing enterocolitis	0	2 (7.7)	1 (6.7)	0.544	0.999
Supplemental oxygen, days, median (IQR)	2 (1–4)	3 (1–15)	3 (1.5–8.5)	0.208	0.883
Bronchopulmonary dysplasia	0	6 (23.1)	3 (20)	0.081	0.999
Retinopathy of prematurity	0	4 (15.4)	4 (26.7)	0.281	0.434
Deaths	0	1 (3.8)	1 (6.7)	0.999	0.999

Data as absolute numbers and percentages in parenthesis unless otherwise stated; IQR = interquartile range (25th–75th percentile).

*In infants weighing < 1500 g.

TABLE 2. Inflammatory Markers in Endotracheal Aspirate Fluid and Exhaled NO in the Study Groups with and without Chorioamnionitis

Variable	Maternal chorioamnionitis			P value (groups 1 versus 2)	P value (groups 2 versus 3)
	Absent		Present (group 3)		
	≥ 2000 g (group 1)	≤ 2000 g (group 2)			
Total infants	13	26	15		
Inflammatory markers					
C-reactive protein < 24 h life, mg/L	0 (0–0)	0 (0–1)	1 (0–10.5)	0.508	0.035
C-reactive protein < 48 h life, mg/L	3 (1–13)	2 (1–3)	9 (1–25.5)	0.738	0.140
Nitrites-nitrates, μmol/L	2.4 (0.75–4.0)	1.1 (0.6–2.2)	3.6 (1.1–5.1)	0.178	0.035
IL-8, pg/mL	477 (135–5660)	497 (191–1181)	1161 (241–2485)	0.627	0.277
Proteins, mg/dL	0.25 (0.08–0.46)	0.21 (0.11–0.39)	0.29 (0.19–0.44)	0.879	0.192
Nitrites-nitrates, μmol/L/g proteins	17.9 (4.6–31.5)	6.1 (1.6–15.4)	11.8 (8.0–17.5)	0.178	0.222
IL-8, pg/mL/g proteins	2769 (1871–25381)	3032 (351–10964)	5918(1116–12148)	0.259	0.619
Exhaled NO, expressed as					
ppb	1.48 (0.90–1.96)	1.03 (0.56–1.78)	1.33 (1.01–2.89)	0.308	0.056
nL/min	0.71 (0.38–0.91)	0.32 (0.18–0.47)	0.46 (0.28–0.92)	0.007	Not calculated*
nL/min/kg	0.30 (0.17–0.41)	0.27 (0.16–0.48)	0.48 (0.27–0.77)	0.670	0.021
Normalized exhaled NO, expressed as					
nL/min	1.23 (0.71–1.41)	1.48 (1.26–1.55)	1.55 (1.52–1.62)	0.038	Not calculated*
nL/min/kg	0.45 (0.33–0.57)	1.22 (0.92–1.54)	1.54 (1.13–1.79)	0.000	Not calculated*
Peak of exhaled NO, ppb	2.24 (1.69–2.50)	1.72 (1.07–2.41)	2.14 (1.62–4.29)	0.159	0.032

Data as median and interquartile range (IQR, 25th–75th percentile).

*Because of lack of homogeneity of the control group (i.e., statistically significant differences between groups 1 and 2).

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Impact of chorioamnionitis on exhaled nitric oxide and endotracheal aspirate levels of nitrites-nitrates and interleukin-8 in mechanically ventilated preterm neonates

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SUMMARY

Objectives: To assess the influence of maternal chorioamnionitis on early exhaled nitric oxide (NO) and levels of nitrites-nitrates and interleukin (IL) IL-8 in endotracheal aspirate fluid in mechanically ventilated preterm neonates.

Study Design: Cross-sectional study.

Patient-Subject Selection: Between September 2007 and August 2009, 54 mechanically ventilated preterm neonates were included. Patients were divided into two groups according to the presence or absence of maternal chorioamnionitis, and those without chorioamnionitis (controls) were further stratified into two subgroups by birth weight \leq or $>$ 2000 g.

Methodology: The ventilator used was a Babylog 8000. The NO level assessed was the plateau value given by the software of the Sievers NOA apparatus. Collection of endotracheal aspirate fluid samples was performed coinciding with routine aspirations and using the dry technique.

Results: The two groups of control neonates showed statistically significant differences in exhaled NO expressed as nL/min and normalized exhaled NO expressed as either nL/min or nL/min/kg, so they are not homogeneous and cannot be used in clinical practice. Serum C-reactive protein and endotracheal aspirate levels of nitrites-nitrates were significantly higher in the chorioamnionitis group than in controls (3.6 vs 1.07 $\mu\text{mol/L}$; $p=0.035$). Nitrites-nitrates levels were positively correlated with exhaled NO in ppb ($\rho=0.367$; $p=0.006$). Minute exhaled endogenous NO was significantly higher in the chorioamnionitis group (0.48 vs 0.27 nL/min/kg; $p=0.021$).

Conclusions: In mechanically ventilated preterm infants weighing less than 2000 g, maternal chorioamnionitis was associated with an increase of early exhaled NO (nL/min/kg) and serum levels of C-reactive protein and levels of nitrites-nitrates in endotracheal aspirate fluid.

INTRODUCTION

Maternal chorioamnionitis may cause preterm delivery and be associated with an adverse perinatal outcome, including early-onset sepsis, necrotizing enterocolitis, and lung disease as well as long-term sequelae in the infant.¹⁻⁴ Histologic chorioamnionitis is associated with an increased incidence of bronchopulmonary dysplasia,⁵ a chronic lung disease that prolongs hospital stay and causes greater morbidity and mortality of respiratory origin.⁶⁻⁹

Chorioamnionitis may injure the fetal lung through the activation of alveolar neutrophils and macrophages. Due to this activation, greater expression of inducible nitric oxide (NO) synthase is to be expected, with elevation of nitrites and nitrates in endotracheal aspirates and of minute exhaled NO. Exhaled NO is raised in adults and children with asthma,^{10,11} and has also been detected among the gases exhaled in mechanically ventilated neonates.¹²⁻¹⁷ Different results of exhaled NO in neonates with bronchopulmonary dysplasia have been reported, including lower values in infants breathing spontaneously¹⁸ and higher exhaled NO peak levels in ventilated infants.¹⁹ Differences in postnatal age¹⁶⁻¹⁹ and in the methods for assessing exhaled NO (breathing spontaneously or on assisted ventilation,^{6,18} output or minute ventilation obtained using plateau NO values,¹⁸ or peak or maximum values of exhaled NO¹⁹) may account for the conflicting results. Although recommendations concerning standardized techniques for measurement of exhaled NO have been reported,²⁰ data for analysis of exhaled NO in mechanically ventilated preterm infants are lacking. Williams and associates²¹ studied the influence of ventilatory settings on measurements of exhaled NO and found that increasing peak inflating pressure as well as the inspired oxygen concentration resulted in a decrease in the peak NO levels. The authors emphasized that the conditions of measurement must be standardized in infants receiving respiratory support. In a previous study of our group,²² the following points were suggested when collecting gas samples from ventilated premature infants to obtain exhaled NO: (1) it would suffice to collect exhaled gas during 1 minute, although it is recommended to repeat collection 3 times;¹⁵ (2) for each sample collection, the parameters to record should be FiO₂, PIP, PEEP, RF, I/E, volume per minute and percentage of leakage (the two latter ones, every 15 sec during collection); and (3) the results should be expressed as exhaled NO (ppb

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3 plateau value), “minute exhaled NO per kg after correction for leakage” (exhaled NO x volume
4 per minute/weight in kg = nL or ppb/min/kg) and “normalized minute exhaled NO per kg after
5 correction for leakage” (after applying the normalization equation).²²
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9
10 The cellular composition of tracheal aspirate is the gold standard for defining lung
11 inflammation.²³ Interleukin-8 (IL-8) is produced by alveolar macrophages, fibroblasts, type II
12 pneumocytes, and endothelial cells. Hypoxia, hyperoxia, and endotoxins stimulate the synthesis
13 of IL-8, which is a major neutrophil chemotactic factor in the lung. Histologic chorioamnionitis
14 is associated with an increase of IL-8 in endotracheal aspirate fluid along the first day of life (\geq
15 917 pg/mL) and this cutpoint may be used for diagnosis.²⁴ Preterm infants suffering from
16 bronchopulmonary dysplasia can present increased levels of tumor necrosis alpha (TNF- α),²⁵
17 IL-6,²⁵ IL-8,²⁶ and IL-10²⁶ in endotracheal aspirates.
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27 The present study was designed to assess changes of early exhaled NO and the
28 concentration of nitrites-nitrates and IL-8 in endotracheal aspirate fluid in mechanically
29 ventilated preterm neonates with and without history of maternal chorioamnionitis.
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34 35 36 **MATERIALS AND METHODS**

37 38 **Design and Patient Population**

39 A cross-sectional study of mechanically ventilated preterm infants was conducted to assess the
40 impact of chorioamnionitis on exhaled NO and the concentration of nitrites-nitrates and IL-8 in
41 endotracheal aspirates. Between September 2007 and August 2009, 54 mechanically ventilated
42 preterm neonates treated in the Neonatology Service of a IIIB level neonatal intensive care unit
43 were eligible. Inclusion criteria were gestational age between 25 weeks 0 days and 36 weeks 6
44 days, less than 36 hours of age, presence of mechanical ventilation for the treatment of
45 respiratory distress syndrome (RDS), hemodynamic stability, and written informed consent
46 obtained from the parents or legal guardians. Newborns with pulmonary hypoplasia or other
47 respiratory conditions other than RDS, major congenital malformations, chromosomal
48 abnormalities, sepsis, or treatment with NO donor drugs were excluded. Patients were divided
49 into two groups according to the presence or absence of maternal chorioamnionitis, and those
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3 without history of chorioamnionitis (controls) were further stratified into two subgroups
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5 according to birth weight < 2000 g or \geq 2000 g. The study protocol was approved by the Ethics
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7 Committee of Hospital Clínic of Barcelona, Spain. The parents or legal guardians were
8
9 informed about the study and provided consent because no blood sampling was needed and
10
11 procedures performed were not painful and included in the care of the patients (tracheal
12
13 aspirates).
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18 **Definitions and Procedures**

19
20 The diagnosis of clinical chorioamnionitis in the pregnant woman who presented with
21
22 premature labor or premature rupture of the membranes was made in the presence of some
23
24 combination of uterine pain, fever (\geq 38°C), leukocytosis ($>$ 12,000 cells/mm³), and increased
25
26 serum concentrations of C-reactive protein ($>$ 30 mg/L) without any clear focus of infection. In
27
28 these cases, amniotic fluid and placenta were microbiologically cultured, and placenta and
29
30 umbilical cord histologically analyzed. Chorioamnionitis was diagnosed when clinical data were
31
32 accompanied by a positive culture from amniotic fluid and placenta (fetal and maternal sites)
33
34 and histological findings of chorioamnionitis. Polymorphonuclear infiltration in the placenta
35
36 and umbilical cord was required to establish a diagnosis of histological chorioamnionitis. Mild
37
38 chorioamnionitis had a few neutrophils (5-10 neutrophils per high-power field) scattered in the
39
40 subchorionic space and adjacent chorion; Moderate chorioamnionitis had many neutrophils (11-
41
42 30 neutrophils per high-power field) in the lower half of the chorionic plate and the
43
44 subchorionic space; and severe chorioamnionitis had dense infiltrates of neutrophils (30 per
45
46 high-power field) throughout the chorionic plate into the amnion. Mild chorioamnionitis was
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48 recorded in 7 cases, moderate in 3, and severe in 5.
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53 RDS was defined in the presence of a typical clinical course and chest radiographic
54
55 findings, with clinical improvement when exogenous surfactant was administered. RDS was
56
57 considered non-severe if only one dose of exogenous surfactant was needed and severe if two or
58
59 more doses of exogenous surfactant or high frequency oscillatory ventilation were needed as
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1
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3 well as in the presence of air leak or if RDS was the cause of death. Bronchopulmonary
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5 dysplasia was defined according to diagnostic criteria of Jobe and Bancalari²⁷
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7
8 At all times, neonates received appropriate medical care according to his/her clinical
9
10 condition. Special attention was placed to maintain the inspired gas temperature at 37°C and
11
12 maximum humidity of the circuit (100%) using a Fisher-Paykel humidifier. Serial
13
14 measurements of heart rate, respiratory frequency (RF), noninvasive blood pressure, and arterial
15
16 blood gases were performed. Ventilatory parameters included peak inspiratory pressure (PIP),
17
18 positive end expiratory pressure (PEEP), inspiratory time (Ti), effective respiratory frequency
19
20 (RF), tidal volume (Vt), minute volume (Vm), and compliance (A). The Clinical Risk Index for
21
22 Babies (CRIB) score (in infants weighing < 1500 g) and the Score for Neonatal Acute
23
24 Physiology and SNAP Perinatal Extension (SNAPPE II) score were assessed to check
25
26 homogeneity of the patients' severity of illness.
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28

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30 All infants were mechanically ventilated using a constant flow, time cycled, pressure
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32 limited device (Babylog 8000 Plus, Dräger Medical AG & Co. KGaA, Lübeck, Germany),
33
34 which measures all ventilatory parameters displayed in the screen of the device during the
35
36 expiratory time. Oxygenation was continuously monitored by pulse oximetry to maintain
37
38 arterial blood oxygen saturation (SaO₂) between 88% and 93% and arterial partial pressure
39
40 (PaO₂) between 50 and 80 mm Hg. For ventilation, we tried to achieve carbon dioxide partial
41
42 pressure (PaCO₂) in the range of 45–55 mm Hg and pH > 7.20 during the first 96 hours; then,
43
44 high PaCO₂ levels were allowed while maintaining pH > 7.20. In order to avoid stress-
45
46 induced release of NO from vascular endothelium,²⁸ gentle ventilation was used.
47
48

49
50 All newborns were intubated with a double-lumen endotracheal tube (inner diameter 2.7
51
52 and 3.2 mm in infants weighing 600–900 g and 1000–1500 g, respectively) if a deep
53
54 resuscitation was needed in the delivery room, or when a fraction of inspiratory oxygen (FiO)
55
56 greater than 0.30 was required, or in the presence of moderate, severe, or progressive respiratory
57
58 difficulty in spite of receiving continuous positive airway pressure (CPAP) of 6 cm water. All
59
60 intubated infants received surfactant (Survanta, Ross Laboratories, Columbus, OH) at 4 mL/kg
(100 mg/kg of phospholipids per kg), which was repeated if needed. Initial ventilatory setting

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3 included flow 5–8 L/min, PEEP 5 cm H₂O, RF 60 breaths/min, and FiO₂ to keep SaO₂ in the
4
5 range of 88–93%. Thereafter, PIP and RF were adjusted to reach the aforementioned pH and
6
7 PaCO₂ levels. Sedation was not routinely used during the study.
8
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10 11 12 **NO Sampling**

13
14 The sample of gas for NO analysis was obtained taking into account that no exogenous
15
16 surfactant was administered in the previous 6 hours and in the presence of correct
17
18 humidification of the circuit, correct calibration of the NO apparatus (chemiluminescence
19
20 technique), and low proportion of NO (< 40 ppb) in the compressed wall air. NO sampling was
21
22 performed as previously described²² and trying to standardize the ventilation parameters²¹ (FiO₂
23
24 = 0.21, RF = 50 respirations/min, Ti = 0.4 sec, PIP = 20 cm water, PEER = 5 cm water) during
25
26 the 3 min of NO sampling. This change in the ventilation parameters was well tolerated and in
27
28 only 12 cases we had to increase FiO₂ to less than 0.35. The sample of gas in which NO was
29
30 analyzed was obtained through a “6 French” arterial catheter introduced into the endotracheal
31
32 tube to its tip. This catheter is the narrowest that permits adequate aspiration of the gas sample
33
34 without generating an alarm in the Sievers NOA apparatus. Furthermore it occupies very little
35
36 of the endotracheal cross-sectional area, and hence does not affect ventilation of the lung. Three
37
38 samples of gas during 1 minute each were obtained. The mean intrasubject coefficient of
39
40 variability of exhaled NO levels (in ppb) was calculated from the mean of three 1-min sampling
41
42 periods in each of the 54 infants. The coefficient of variation was 12.7%.
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49 **Influence of Air Contamination**

50
51 The absence of environmental air contamination in exhaled NO samples was confirmed by the
52
53 lack of correlation between ambient NO and exhaled NO in ppb ($n = 54$, $\rho = -0.35$, $P = 0.802$)
54
55 as well as between inhaled NO and exhaled NO in ppb ($n = 54$, $\rho = 0.176$, $P = 0.203$). The mean
56
57 (standard deviation) inspired NO level was 1.27 (0.91) ppb (range 0–4.1).
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Calculation of Plateau NO and Minute Exhaled NO

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3 The NO level assessed is not the highest peak, but rather the plateau value given by the software
4 of the Sievers NOA apparatus. This plateau value is the most reliable equivalent of the NO
5 exhaled during expiration (corresponding to the average NO detected between 1/2 and 7/8 of the
6 duration of expiration). Sievers NOA 280i is able to detect from 0.5 ppb to 500 ppm of NO,
7 with a sensitivity of 0.5 ppb, aspirating a gas sample of 10-200 mL/min (100 mL/min in our
8 study) and with a lag time of 1 second and an electronics response time of 0.067 seconds
9 (sampling rate of 15 Hz) to 90% full scale. This high speed means the apparatus can
10 differentiate between inspired gas (contaminated with compressed air) and expired gas, which
11 adopts the form of a plateau. Minute exhaled NO or minute excretion of NO was calculated
12 using the formula:
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$$V'_{\text{NO}} (\text{nL/min}) = \text{Plateau NO (ppb = nL/L)} \times V_E (\text{minute ventilation in L/min})$$

24
25
26
27 V_E has been previously corrected by the percentage of leakage given by the software of the
28 Babylog 8000 ventilator using the formula:
29
30

$$V_E (\text{L/min}) = \text{Volume per minute (L/min)} / ((100 - \text{leakage})/100)$$

31
32
33 Finally, minute exhaled NO was divided by the weight in kilograms of the newborn to obtain
34 the minute exhaled NO per kg (nL/min/kg). To convert nL to nmoL, it should be remembered
35 that 1 mol = 24.3 liters, when environmental temperature is 23°C.
36
37
38

39
40 The results can be normalized by applying the formula developed in a previous report²²
41 as follows: Normalized minute exhaled NO = minute exhaled NO without leakage – minute
42 exhaled NO deviation, being Minute exhaled NO deviation = $-173.668 \times (\text{exhaled NO}/100)^3 +$
43 $49.513 \times (\text{exhaled NO}/100) + 12.486 \times (\text{VM}/100)^3 + 24.075 \times (\text{PIP}/100) + 12.371 \times (\text{RF}/100)^3 -$
44 $8.126 - \text{average minute exhaled NO}.$
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53 **Collection of Endotracheal Aspirate Fluid Samples**

54
55 Collection of endotracheal aspirate fluid samples was performed before the administration of
56 exogenous surfactant or at least 6 hours later²⁹ coinciding with routine aspirations and according
57 to the dry technique, which for research purposes is equally valid than the bronchoalveolar
58 lavage.³⁰ To obtain the mucus aspirated inside the catheter, the catheter was cleaned with 1 mL
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3 of normal saline into a tube and finally purged with air. The entire procedure was repeated to
4
5 have a 2 mL sample, 1 mL of which was placed in another tube for nitrites-nitrates analysis.
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7 Moreover, 1 mL of the same normal saline was placed in another tube as it may be
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9 contaminated by different amounts of nitrites-nitrates. Likewise, 0.2 mL for analysis of IL-8
10
11 analysis and 0.2 mL for analysis of proteins were also kept. The diluted endotracheal aspirate
12
13 fluid samples were centrifuged and the supernatant was recovered and frozen at -80°C , as the
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15 tube with normal saline only. Samples were delivered to the laboratory on ice.
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20 **Measurement of Nitrites-Nitrates, IL-8, and Protein Levels in Endotracheal Aspirates**

21
22 The concentration of nitrites-nitrates in endotracheal aspirates was measured by a modification
23
24 of the fluorimetric technique based on the reaction between 2,3-diamino-naphthalene and
25
26 nitrites in acid medium. Previous filtration in Ultrafee column (UFC4 LGC, Millipore Corp.,
27
28 Bedford, MA) is needed to avoid interferences with serum components. The detection limit of
29
30 the technique is 0.01–50 $\mu\text{mol/L}$. Results were expressed in $\mu\text{mol/L}$ and in relation to the
31
32 concentration of proteins. Levels of IL-8 were measured by a highly sensitive ELISA technique
33
34 (R&D Systems) and results were expressed as pg/mL and in relation to the concentration of
35
36 proteins. Proteins were measured by an automated method to normalize nitrites-nitrates and IL-
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38 8 concentrations per gram of proteins in the endotracheal aspirate fluid, so that errors related to
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40 differences in the amount of collected samples were avoided.
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48 **Variables**

49 In all infants and before the 36 hours of life, the following variables were recorded: gestational
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51 age, sex, birth weight, hours of life, history of maternal chorioamnionitis, maternal smoking
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53 during pregnancy, cesarean section, severity of illness (CRIB and SNAPPE-II score),
54
55 concomitant diseases, such as severe RDS, air leak, patent ductus arteriosus, severe peri-
56
57 intraventricular hemorrhage (grade 3–4), early sepsis, early exhaled NO (minute exhaled NO
58
59 per kg [expressed as nL/min/kg], actual and normalized value),²² and concentrations of nitrites-
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3 nitrates ($\mu\text{mol/L}$ and $\mu\text{mol/g}$ proteins) and IL-8 (pg/mL and pg/g proteins) in the endotracheal
4
5 aspirate fluid.
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8 9 10 **Statistical Analysis**

11
12 Continuous variables were presented as mean \pm standard deviation (SD) if normally distributed
13
14 and with homogeneous variances or as median and interquartile range (IQR: 25th–75th
15
16 percentiles) if not normally distributed or without homogeneous variances. These latter
17
18 variables were birth weight; C-reactive protein levels; nitrites-nitrates, IL-8, and protein levels
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20 in endotracheal aspirates; duration of supplemental oxygen therapy; and all exhaled NO
21
22 measurements. The influence of environmental NO or NO in compressed air on exhaled NO
23
24 was analyzed by means of Spearman correlation, as well as other correlations between exhaled
25
26 NO and other variables, particularly levels of nitrites-nitrates and IL-8 in endotracheal aspirates,
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28 and days of supplemental oxygen therapy. The Student's *t* test was used for the comparison of
29
30 parametric variables and the Mann-Whitney *U*-test for the comparison of non-parametric
31
32 variables among the different groups according to “maternal chorioamnionitis” (yes/no). Infants
33
34 with and without history of maternal chorioamnionitis were matched by birth weight.
35
36 Categorical variables were compared with the chi-square (χ^2) test or the Fisher's exact test. To
37
38 determine the most useful cut-point of exhaled NO as a marker of history of chorioamnionitis,
39
40 the value with the best relation sensitivity-specificity and the highest positive likelihood ratio
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42 was chosen. Statistical significance was set at $P < 0.05$.
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49 **RESULTS**

50 51 **Control Group**

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53 As shown in Table 1, the two groups of neonates without history of maternal chorioamnionitis
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55 showed statistically significant differences in the use of antenatal steroids, gestational age, birth
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57 weight, and Apgar score at 5 minutes after birth. In addition (Table 2), there were significant
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59 differences in exhaled NO expressed as nL/min and normalized exhaled NO expressed as either
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nL/min or nL/min/kg. Therefore, the two subgroups were not homogeneous, and the most

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3 similar to the study group was the subgroup with <2000 g of birth weight. This subgroup will be
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5 considered the control group.
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10 **Comparison of Infants with and Without History of Maternal Chorioamnionitis**

11 Clinical findings and morbidity according to history of maternal chorioamnionitis are shown in
12 Table 1. Preterm newborns in the chorioamnionitis group compared with controls weighing <
13 2000 g were less frequently born by cesarean section and mothers were older. Moreover, a
14 significantly higher percentage of infants with history of maternal chorioamnionitis needed
15 resuscitation and endotracheal intubation more frequently and presented with patent ductus
16 arteriosus or severe intraventricular hemorrhage. The only newborn who died from the
17 chorioamnionitis group was a 25 weeks' preterm infant with a birth weight of 810 g and a CRIB
18 score of 4, who was intubated only during the first day of life after receiving two doses of
19 surfactant. Seven days after birth he suffered from necrotizing enterocolitis with intestinal
20 perforation and died. The only newborn who died from the control group was a 25 weeks'
21 preterm infant with a birth weight of 740 g and a CRIB score of 4, who developed a very severe
22 bronchopulmonary dysplasia and died 49 days after birth.
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38 The values of inflammatory markers and exhaled NO according to history of maternal
39 chorioamnionitis are shown in Table 2. C-reactive protein was higher in the chorioamnionitis
40 group. IL-8 levels, either in absolute values or divided by the protein content of the endotracheal
41 aspirate were also higher in this group, although statistically significant differences were not
42 reached. However, the levels of nitrites-nitrates were significantly higher than in controls ($P =$
43 0.035) and were positively associated with exhaled NO in ppb ($n = 54$, $\rho = 0.367$, $P = 0.006$).
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51 Minute exhaled endogenous NO, expressed in nL/min/kg, was significantly higher in
52 the chorioamnionitis group. The most useful cutoff for exhaled NO as a marker of history of
53 maternal chorioamnionitis was 0.285 nL/min/kg, with a sensitivity of 73.3%, specificity of
54 57.7%, positive predictive value of 50.0%, negative predictive value of 78.9%, accuracy of
55 63.4%, positive likelihood ratio of 1.733, negative likelihood ratio of 0.462, and area under the
56 curve of 0.718 ($P = 0.021$).
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3 Peak values of exhaled NO were significantly higher in the chorioamnionitis group than
4
5 in controls ($P = 0.032$) and also showed a significant correlation with nitrites-nitrates levels in
6
7 the endotracheal aspirate fluid ($n = 54$, $\rho = 0.322$, $P = 0.018$).
8
9

10 11 12 **DISCUSSION**

13
14 In the healthy newborn, exhaled NO is not affected by the gestational age,^{13,16,29,31,32} birth
15
16 weight,³³ or mode of delivery,²⁹ although there is an increase in exhaled NO during the first
17
18 week of life.^{16,29} Exhaled NO is not related either with serum levels of nitrites and nitrates,^{13,24}
19
20 but there may be a relationship with nitrites and nitrates that are present in the low respiratory
21
22 tract.³³ In addition, there are two trends in the publications of research groups working on
23
24 expired or exhaled NO in newborns: those authors who work only with concentrations (ppb)^{6,12–}
25
26 ^{14,16,17,34} and those as our group who prefer to adapt the methodologies to present the results as
27
28 “total excretion of exhaled NO” (nL/min/kg).^{17,29,31,32,35,36} Olsen et al.,¹⁵ in 2003, measured
29
30 exhaled NO in eight preterm infants with RDS and showed a decline of median minute
31
32 excretion of NO by 48–72 hours. Williams et al.¹⁶ measured nasal and lower airway NO level in
33
34 very immature infants (median gestational age 27 weeks) and found that nasal and lower airway
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36 NO levels did not correlate significantly with gestational age, but lower airway NO levels
37
38 correlated with postnatal age.
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42
43 As far as we are aware, the relationship between maternal chorioamnionitis and possible
44
45 increases in nitrites-nitrates levels in endotracheal aspirates and exhaled NO during the first
46
47 days of life have not been previously investigated. In the present study, these parameters were
48
49 examined exclusively in mechanically ventilated preterm infants with RDS. The increase of
50
51 plateau and peak exhaled NO and their positive correlation with endotracheal nitrites-nitrates
52
53 suggest that both factors are related and due to the same inflammatory process. It has been
54
55 shown that nitrate concentration in bronchoalveolar lavage fluid is elevated in infants who
56
57 developed chronic lung disease of prematurity.³³ In the present study, mean IL-8 values were
58
59 higher in the chorioamnionitis group than in the control group (1161 vs 497 pg/mL), although
60
differences were not statistically significant ($P = 0.277$), probably because of the high dispersion

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3 of values. It is likely that changes in IL-8 would have been more apparent with a larger sample
4
5 size.
6

7
8 The **birth weight** cutoff point of 2000 g was empirically chosen in an attempt to
9
10 homogenize the sample in order to analyze the results of exhaled NO in different forms,
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12 including ppb, nL/min, nL/min/kg and normalized values). It was observed that plateau exhaled
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14 NO expressed in nL/min was significantly different between the two control subgroups;
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16 therefore, this measurement is not useful in clinical practice. The same findings were observed
17
18 for normalized plateau exhaled NO expressed as either nL/min or nL/min/kg. A comparison of
19
20 the chorioamnionitis group with the control subgroup of < 2000 g seems reasonable as both
21
22 groups were homogeneous regarding gestational age and birth weight. However, if comparisons
23
24 would have been performed with the 15 infants in the chorioamnionitis group and all 39
25
26 controls, heterogeneity of controls would probably account for the disappearance of statistically
27
28 significant differences for nitrites-nitrates ($\mu\text{mol/L}$) and peak of exhaled NO (ppb) between
29
30 infants in the chorioamnionitis and controls of < 2000 g. In fact, a positive aspect of the present
31
32 study was the stratification of infants in the control group according to birth weight, which
33
34 allowed comparing infants in the chorioamnionitis group with only those preterm babies
35
36 without history of chorioamnionitis and similar features of immaturity and birth weight. This
37
38 stratification also revealed that in controls, the expression of exhaled NO in nL/min, both in
39
40 actual and normalized values was not homogeneous. When exhaled NO was expressed
41
42 according to the birth weight, actual values of exhaled NO appeared homogeneous, in contrast
43
44 with normalized values which are even spread out more. Therefore, the attempt to normalize
45
46 exhaled NO reported in a previous study,²² unfortunately is not useful in clinical practice. In this
47
48 respect, plateau of exhaled NO in ppb and nL/min/kg and peak of exhaled NO in ppb seem to be
49
50 the only useful parameters in clinical practice. Another positive aspect was the absence of
51
52 subclinical chorioamnionitis in the control group, because in that group the histology of the
53
54 placenta had to be normal.
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59
60 The present findings should be interpreted taking into account some limitations of the
study, particularly the exclusion of perinatal sepsis; in this case, marked increases in

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2
3 endogenous NO with secondary increase of exhaled NO have been reported.³⁷ In addition, the
4
5 study was restricted to the first 36 hours of life, and may be thereafter other results would be
6
7 obtained
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9
10 One difficulty when attempting to analyze exhaled NO of bronchoalveolar origin is the
11
12 risk that the determination could be invalidated due to NO contained in the gas inhaled. This is
13
14 not the case when using the “breath program” software which accompanies the Sievers Nitric
15
16 Oxide Analyzer (NOA 280i) in the mode designated “use pressure for breath detection mode”,
17
18 and with “on-line sampling”. It is considered that the gas in the region between 1/2 and 7/8 of
19
20 the duration of expiration is equivalent to the third and fourth quartiles of the expiration time,
21
22 when the gas obtained is exclusively bronchoalveolar,³⁸ with no possibility of external
23
24 contamination. The manufacturers of the NOA 280i only recommend the use of medicinal air
25
26 (NO-free) when environmental NO levels exceed 40–50 ppb. Our compressed air NO levels
27
28 never reached these values (range 0–4.1 ppb). For repeated measurements of exhaled NO in
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30 each expiration, it should be available a chemiluminescence analyzer of rapid response (≤ 0.2
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32 sec) and at a flow rate of > 1.7 mL/sec (Sievers NO analyzer [NOA 280] with a response time
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34 of 0.2 sec and a minimum sample of 100–300 mL/min).
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38 In our previous study,²² it was shown that the variable volume per minute was highly
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40 influenced by the percentage of leakage given by the ventilator. Although the leakage of gas
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42 around the endotracheal tube is low, the sample of gas aspirated by the analyzer is considered as
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44 a leak by the ventilator. This leakage can be corrected mathematically, and indeed this should
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46 always be done. Therefore, in the present study minute exhaled NO was obtained multiplying
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48 the plateau NO value (plateau between 1/2 and 7/8 of expiration) by the volume per minute, as
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50 Olsen et al.¹⁵ have previously reported, but volume per minute was previously corrected by the
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52 percentage of leakage.
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56 In the present study, exhaled NO obtained at the tracheobronchial level and during the
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58 expiratory phase was expressed as total exhaled NO in nL/min/kg, and this value was also
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60 normalized according to setting of ventilation parameters.²² Median values of exhaled NO were
0.48 and 0.27 nL/min/kg in infants with and without history of maternal chorioamnionitis,

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2
3 respectively. These figures are similar to those reported by Olsen et al.¹⁵ who showed that
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5 minute excretion of NO (divided by the weight) in premature infants with respiratory distress
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7 syndrome and mechanically ventilated at 24 hours of life was 0.405 nL/min/kg. In our group of
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9 chorioamnionitis, higher values of exhaled NO in nL/min/kg were found. **A value of exhaled**
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11 **NO greater than 0.285 nL/min/kg would be suggestive of history of maternal chorioamnionitis**
12
13 **with a 50% positive predictive value. This low figure and the fact that exhaled NO should be**
14
15 **measured in the postnatal period preclude the clinical use of this parameter as a diagnostic**
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17 **criterion of chorioamnionitis.** The increase in exhaled NO in nL/min/kg and in nitrites-nitrates
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19 levels in the endotracheal aspirate fluid, and the positive correlation between both parameters,
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21 would support the common origin of NO in the lower respiratory airway as a result of the
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23 inflammatory process.
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27 On the other hand, a significant higher incidence of bronchopulmonary dysplasia in the
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29 chorioamnionitis groups was not observed, which may be explained by the use of antibiotic
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31 treatment and antenatal corticoids for fetal maturation, all of which attenuates the inflammatory
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33 process.³⁹ Lahra et al.⁴⁰ even suggested that fetal inflammatory response during
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35 chorioamnionitis is a protective factor for chronic lung disease. May et al.⁴¹ reported the peak
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37 exhaled NO values obtained on days 3, 5, 7, 14, 21 and 28 after birth in 80 preterm infants of
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39 less than 32 weeks of gestation, depending on the appearance of bronchopulmonary dysplasia
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41 (BPD). In the 20 preterm infants who did not develop bronchopulmonary dysplasia, mean (SD)
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43 peak exhaled NO level at 3 days was 4.8 (2.5) ppb. In our study, mean peak exhaled NO level in
44
45 the chorioamnionitis group was 2.14 ppb (range 1.62–4.29), which is somewhat lower. This
46
47 may be due to age differences at the time of sampling (mean 17.1 hours in our study and 3 days
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49 in the study of May et al.⁴¹).
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53 We conclude that maternal chorioamnionitis is associated with an increase of early
54
55 exhaled NO (nL/min/kg) in mechanically ventilated preterm infants weighing less than 2000 g.
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57 Chorioamnionitis is also associated in the newborn with an increase of C-reactive protein and of
58
59 endotracheal nitrites-nitrates levels but not of early IL-8. **Although an early single measurement**
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of exhaled NO has little clinical applicability as a marker of lung inflammation, repeated

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3 measurements during the first days or weeks of life when the infants is intubated and
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5 mechanically ventilated showing increasing values may be useful to suspect bronchopulmonary
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7 dysplasia. May et al.⁴¹ have shown that an increase exhaled NO along the first 28 days of life
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9 was related to bronchopulmonary dysplasia, and that there was a linear trend in exhaled NO
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11 results as severity of bronchopulmonary dysplasia increased. In these circumstances, treatment
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13 could be prescribed to improve the prognosis of this chronic lung disease.
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TABLE 1. Characteristics of the Study Groups with and without Chorioamnionitis

Variable	Maternal chorioamnionitis			<i>P</i> value (groups 1 versus 2)	<i>P</i> value (groups 2 versus 3)
	Absent		Present (group 3)		
	≥ 2000 g (group 1)	< 2000 g (group 2)			
Total infants	13	26	15		
Mother and labor					
Age, years, mean (SD)	32.8 (3.9)	30.0 (3.9)	33.8 (4.8)	0.185	0.039
Smoking during pregnancy	0	6 (23.0)	0	0.158	0.120
Antibiotic treatment	9 (69.2)	15 (62.5)	13 (92.9)	0.734	0.059
Antenatal steroids	4 (30.8)	22 (84.6)	14 (93.3)	0.003	0.411
Inborn	8 (61.5)	22 (84.6)	15 (100)	0.129	0.278
Cesarean section	8 (61.5)	19 (73.1)	5 (33.3)	0.486	0.012
Multiple gestation	3 (23.1)	11 (42.3)	7 (46.7)	0.409	0.786
Newborns					
Hours of life, mean (SD)	21.8 (8.6)	21.5 (7.3)	17.1 (7.9)	0.997	0.224
Male	10 (76.9)	13 (50)	8 (53.3)	0.112	0.839
Gestational age, weeks, mean (SD)	34.1 (1.6)	29.2 (2.4)	28.1 (2.7)	0.000	0.386
Birth weight, median (IQR)	2200 (2160–2610)	1020 (970–1400)	1100 (850–1360)	0.000	0.968
Intrauterine growth restriction	0	3 (11.5)	0	0.524	0.457
Resuscitation (endotracheal tube)	0	7 (26.9)	9 (60)	0.105	0.037
Apgar score at 5 min, mean (SD)	9.8 (0.4)	8.0 (1.7)	8.1 (1.5)	0.003	0.959
CRIB score,* mean (SD)		2.5 (2.3)	2.8 (1.8)		0.746
SNAPPE II score, mean (SD)	11.8 (9.1)	21.5 (13.4)	23.7 (15.9)	0.110	0.885
Morbidity					
Severe respiratory distress syndrome	9 (69.2)	15 (57.7)	11 (73.3)	0.491	0.323
Pneumothorax	0	1 (3.8)	2 (13.3)	0.999	0.543
Patent ductus arteriosus	3 (23.1)	8 (30.8)	10 (66.7)	0.719	0.028
Intraventricular hemorrhage (grades 3–4)	0	0	4 (26.7)		0.013
Early-onset sepsis	0	0	2 (13.3)		0.128
Late-onset sepsis	0	2 (7.7)	3 (20)	0.544	0.336
Necrotizing enterocolitis	0	2 (7.7)	1 (6.7)	0.544	0.999
Supplemental oxygen, days, median (IQR)	2 (1–4)	3 (1–15)	3 (1.5–8.5)	0.208	0.883
Bronchopulmonary dysplasia	0	6 (23.1)	3 (20)	0.081	0.999
Retinopathy of prematurity	0	4 (15.4)	4 (26.7)	0.281	0.434
Deaths	0	1 (3.8)	1 (6.7)	0.999	0.999

Data as absolute numbers and percentages in parenthesis unless otherwise stated; IQR = interquartile range (25th–75th percentile).

*In infants weighing < 1500 g.

TABLE 2. Inflammatory Markers in Endotracheal Aspirate Fluid and Exhaled NO in the Study Groups with and without Chorioamnionitis

Variable	Maternal chorioamnionitis			P value (groups 1 versus 2)	P value (groups 2 versus 3)
	Absent		Present (group 3)		
	≥ 2000 g (group 1)	≤ 2000 g (group 2)			
Total infants	13	26	15		
Inflammatory markers					
C-reactive protein < 24 h life, mg/L	0 (0–0)	0 (0–1)	1 (0–10.5)	0.508	0.035
C-reactive protein < 48 h life, mg/L	3 (1–13)	2 (1–3)	9 (1–25.5)	0.738	0.140
Nitrites-nitrates, μmol/L	2.4 (0.75–4.0)	1.1 (0.6–2.2)	3.6 (1.1–5.1)	0.178	0.035
IL-8, pg/mL	477 (135–5660)	497 (191–1181)	1161 (241–2485)	0.627	0.277
Proteins, mg/dL	0.25 (0.08–0.46)	0.21 (0.11–0.39)	0.29 (0.19–0.44)	0.879	0.192
Nitrites-nitrates, μmol/L/g proteins	17.9 (4.6–31.5)	6.1 (1.6–15.4)	11.8 (8.0–17.5)	0.178	0.222
IL-8, pg/mL/g proteins	2769 (1871–25381)	3032 (351–10964)	5918(1116–12148)	0.259	0.619
Exhaled NO, expressed as					
ppb	1.48 (0.90–1.96)	1.03 (0.56–1.78)	1.33 (1.01–2.89)	0.308	0.056
nL/min	0.71 (0.38–0.91)	0.32 (0.18–0.47)	0.46 (0.28–0.92)	0.007	Not calculated*
nL/min/kg	0.30 (0.17–0.41)	0.27 (0.16–0.48)	0.48 (0.27–0.77)	0.670	0.021
Normalized exhaled NO, expressed as					
nL/min	1.23 (0.71–1.41)	1.48 (1.26–1.55)	1.55 (1.52–1.62)	0.038	Not calculated*
nL/min/kg	0.45 (0.33–0.57)	1.22 (0.92–1.54)	1.54 (1.13–1.79)	0.000	Not calculated*
Peak of exhaled NO, ppb	2.24 (1.69–2.50)	1.72 (1.07–2.41)	2.14 (1.62–4.29)	0.159	0.032

Data as median and interquartile range (IQR, 25th–75th percentile).

*Because of lack of homogeneity of the control group (i.e., statistically significant differences between groups 1 and 2).