EVIDENCE OF UNEXPECTED OXIDATIVE STRESS IN AIRWAYS OF ADOLESCENTS BORN VERY PRETERM

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Abstract

Prematurity and its main respiratory complication, bronchopulmonary dysplasia (BPD), are potentially associated with lifelong respiratory morbidities and/or lung function abnormalities. The mechanisms behind these long-term respiratory problems are still unclear.

We assessed airway oxidative stress in adolescents born very preterm (≤ 32 gestational weeks) by measuring 8-isoprostane concentration in the exhaled breath condensate (EBC). In addition, the study protocol included spirometry and measuring nitric oxide in the exhaled air (FE\textsubscript{NO}).

The study groups included 34 ex-preterm adolescents with BPD, 18 ex-preterm adolescents without BPD, and 34 healthy controls born at term.

Regardless of a history of BPD, the ex-premature adolescents had higher EBC 8-isoprostane levels [BPD: 9.5(7.3-12.2); preterm non-BPD: 10(8.1-16) pg/mL] than the controls [3.2(1.9-6.5) pg/mL] (p<0.001). FE\textsubscript{V}\textsubscript{1} was lower in the BPD group [Z-score:-2.1(1.58)] than in the preterm non-BPD individuals [-1.13(1.15)], who showed in turn significantly lower values than the controls [0.18(0.83); p<0.001]. FE\textsubscript{NO} was similar in the 3 groups (p=0.55).

Our data show that, after premature birth, evidence of oxidative stress in the airways may be detected into adolescence, suggesting that long-term respiratory abnormalities after preterm birth may be associated with an ongoing airway disease and not just a stabilized structural lung damage.
Keywords:
Bronchopulmonary dysplasia, exhaled breath condensate, 8-isoprostane, prematurity.

Abbreviations:
BPD, Bronchopulmonary dysplasia
FE\textsubscript{NO}, Fractional exhaled nitric oxide
FEF\textsubscript{25-75%}, Mean forced expiratory flow between the 25% and 75% of the forced vital capacity
FEV\textsubscript{1}, Forced expiratory volume in the first second of expiration
FVC, Forced vital capacity
INTRODUCTION

High rates of premature birth are common in most high-income countries and impose significant health problems and a heavy economic burden on society[1]. Prematurity is associated with a broad spectrum of respiratory symptoms and lung function abnormalities, starting early in life and possibly lasting into adult age[2]. The most severe forms of pulmonary involvement affect individuals with a diagnosis of bronchopulmonary dysplasia (BPD), the main respiratory complication of prematurity[3,4]. However, considerable long-term respiratory morbidities have also been reported in formerly premature individuals who developed no BPD[5,6]. Several studies have documented considerable respiratory symptoms and airflow limitation persisting into young adulthood[6,7,8] in these subjects raising concern that they may be at increased risk of developing a COPD-like phenotype with aging [4,9,10]. Unfortunately, no pathological data are available to elucidate which structural and pathophysiological changes underlie the clinical and functional pulmonary abnormalities seen at long-term in some prematurely delivered individuals. A relevant question is whether the long-term pulmonary consequences of prematurity and BPD depend essentially on a non-progressive reduction in airway caliber, due to stabilized early airway remodeling processes and disrupted pulmonary growth, or whether they also reflect an ongoing, active airway disease. The presence of an ongoing airway disease would point to a greater risk of anticipated or accelerated lung function decline with aging in prematurely born individuals, as seen in adults with COPD. However, no established markers of airway disease activity currently exist, despite massive research efforts focusing on asthma and COPD[11].

A potentially relevant indicator of ongoing airway disease is oxidative stress[12], which derives from an increased production of reactive oxygen species and/or decreased antioxidant defenses. Markers of oxidative stress are often overexpressed in the airways in several chronic lung disorders, and they are linked to ongoing airway inflammation and remodeling[12]. Oxidative stress has also been consistently associated with lung injury early in life, in infants who progress towards BPD[13,16], and there is increasing evidence that links early exposure to oxidative stress with potentially lifelong consequences[14,17]. Oxygen radicals have many complex effects on the body’s homeostasis, being involved in normal organ development and contributing to several physiological functions[13]. Recent findings indicate that reactive oxygen species act as second messengers for transcription factor activation and control gene expression [15], and therefore modulate cell growth, apoptosis and inflammation. Any impairment of the oxidative/antioxidative balance may durably harm the newborn, and several studies recommend limiting exposure to oxygen early in life as much as possible[16]. Despite increasing interest in its
involvement in lung injury early in life, oxidative stress has yet to be assessed in long-term survivors of premature birth or BPD.

With a view to assessing airway oxidative stress at long-term after preterm birth, we studied a group of adolescents born prematurely, with and without a history of BPD, by measuring exhaled breath condensate (EBC) concentrations of 8-isoprostane, one of the most reliable biomarkers of oxidative stress in vivo[17]. EBC affords a non-invasive means for sampling the airways and measuring biomarkers of airway inflammation and oxidative stress[17,19].
METHODS

Population and study design

We recruited a group of survivors of BPD and ex-premature subjects with no history of BPD admitted to the Neonatal Intensive Care Unit at the Pediatrics Department in Padova between January 1987 and December 1994. We only considered those delivered at a gestational age \( \leq 32 \) weeks for participation in this study.

According to the definition used at the time[21], the BPD group consisted of subjects who had persistent oxygen dependence, clinical signs of respiratory impairment, and chest radiograph abnormalities at 28 days old. To evaluate the possible influences of BPD severity on study results, patients who were oxygen dependent for at least 28 days were also classified as cases of mild, moderate or severe BPD according to their need for supplemental oxygen at 36 postmenstrual weeks[3].

The non-BPD premature group included only children who were ventilator-dependent for less than 7 days and oxygen-dependent for less than 10 days after birth. These enrollment criteria were designed so as to evaluate the impact of prematurity itself on long-term respiratory function and EBC composition, minimizing the effect of prolonged mechanical ventilation or otherwise complicated clinical courses.

A group of healthy adolescents born at term with no history of atopic or respiratory diseases was enrolled as a control group.

For lung function assessment and EBC collection, the subjects had to have been in stable clinical conditions, with no upper or lower respiratory tract infections and no need for inhaled or systemic steroids, \( \beta_2 \) agonists, or leukotriene receptor antagonists, during the previous 4 weeks. All measurements were obtained in the afternoon.

Personal medical history, personal cigarette consumption, and occurrence of wheeze, cough, and exercise-associated symptoms were recorded. Wheeze was considered when diagnosed by a doctor any time in the previous 2 years, as evaluated by the patients’ personal medical booklet. Cough was diagnosed if self-reported and occurred on a regular basis either during the day or night in the previous 2 years. Exercise-associated symptoms included self reported cough, wheeze, shortness of breath, or chest tightness associated with physical activity, or regular use of inhaled albuterol before exercising.

All subjects underwent a complete physical examination, fractional exhaled nitric oxide (FE\textsubscript{NO}) measurement, spirometry, exhaled breath condensate (EBC) collection, and a skin prick test. They were adequately instructed and trained before proceeding with the procedures.

The study was reviewed and approved by the Ethics Committee of our Hospital and all participants and their parents gave their written informed consent.
Exhaled breath condensate (EBC) collection and 8-isoprostane assay

EBC was collected and processed according to the ATS/ERS recommendations[19]. It was collected using the TURBO-DECCS (Italchill, Parma, Italy), as explained elsewhere[21,22]. The temperature is kept constant during the collection (we used a collection temperature of –4°C). The device is supplied with a disposable respiratory system that consists of a mouthpiece equipped with a one-way valve and a reliable saliva trap, connected to a collecting vial (50 ml) by means of a tube[22]. The participants breathed tidally through the mouth for 15 minutes, wearing a nose-clip and sitting comfortably. They kept their mouth dry by periodically swallowing excess saliva. EBC samples were stored at –80°C in polypropylene tubes until assay, that was performed concurrently for the 3 groups, within 8 months from samples collection. 8-isoprostane was assayed using an enzyme-linked immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI), and its concentrations in EBC were measured by plotting the values identified in the sample with the 8-iso-PGF2α standard calibration curve (0–500 pg/mL). The lowest detection limit was 3.7 pg/mL. For samples below said lowest detection limit, data were arbitrarily expressed as half the detection limit[23]. This assay was previously validated using a reference analytical method (gas chromatographic/negative ion chemical ionization mass spectrometry)[23]. The repeatability of the EIA 8-isoprostane measurements in EBC on 2 different days had previously been tested at our laboratory, showing a coefficient of repeatability of 3.2 pg/mL. The inter-assay variability was <15%[24].

Lung function test

Lung function was assessed using a 10-liter bell spirometer (Biomedin, Padova, Italy) according to international recommendations[25]. Forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and forced expiratory flow rate between 25% and 75% of the forced vital capacity (FEF25-75%) were measured and expressed as Z-scores, calculated using the reference ranges by Stanojevic et al.[26-27]

Spirometry was also performed after administering 400 μg of inhaled salbutamol using a metered-dose inhaler with a spacer device (Aerochamber, Trudell, Canada). Reversibility to β2-agonists was defined as a more than 12% increase in FEV1 after salbutamol inhalation.

Fractional exhaled nitric oxide (FE\textsubscript{NO}) measurement

FE\textsubscript{NO} was measured with the NIOX system (Aerocrine, Stockholm, Sweden), using a single-breath online method according to the ATS/ERS guidelines for measuring FE\textsubscript{NO} in children[27]. In brief, subjects inhaled NO-free air to total lung capacity and exhaled through a dynamic flow restrictor with a target flow of 50 ml/sec for 10 seconds.

Skin prick tests

All subjects underwent skin prick testing with a panel of common inhalant allergens, i.e. mixed grass pollen, Parietaria, Artemisia vulgaris, Dermatophagoides pteronissinus and farinae, Alternaria, dog and cat (Lofarma, Milan, Italy). The skin tests were considered positive if they resulted in a wheal reaction greater than 3 mm.

Statistical analysis

Results were expressed as means (standard deviation) or medians and interquartile ranges (IQR) depending on the data distribution. Kruskall-Wallis test, followed by Dunn’s test for pairwise comparisons, was used to determine differences between the study groups. Correlations were
drawn with Pearson’s and Spearman’s tests for normally and not-normally distributed data, respectively. Power calculations revealed that the sample size of the study enables to detect with the one-way ANOVA an effect size of at least 0.24 in EBC 8-isoprostane with a power greater than 90% at a two-sided $\alpha$ level of 0.05 (nQuery Advisor 6.01). The statistical analysis was performed using the software SAS version 9.1.3 for Windows (SAS Institute Inc, Cary, North Carolina).

RESULTS

Thirty-four BPD survivors and 18 non-BPD premature adolescents met the inclusion criteria and agreed to take part in the study. Thirty-four healthy children born at term formed a control group. The participants’ anthropometric characteristics and relevant details of their neonatal history are given in the Table. None of the participants were cigarette smokers. Among BPD survivors, at 36 postmenstrual weeks 25 individuals were still on supplemental oxygen (moderate-severe BPD), 9 were breathing room air (mild BPD).

Respiratory symptoms.

Seventeen of the 34 survivors of BPD (50%) and 8 of the 18 non-BPD premature participants (44%) had recurrent wheezing in the first 5 years of age. Ten individuals (29%) in the BPD group did not refer any respiratory disturbance in the past 2 years. Among the remaining 24 subjects of the BPD group, 5 referred occurrence of cough, 13 exercise-associated symptoms, 17 past or current use of inhaled steroids and/or beta2 agonists. Ten (55%) of the 18 premature non-BPD individuals did not mention any respiratory problem in the past 2 years, but 6 reported exercise-associated symptoms, 1 cough, 1 wheeze and 2 use of inhaled medications.

Exhaled breath condensate (EBC) 8-isoprostane

The 8-isoprostane concentrations in EBC were similar in the two preterm-born groups, with and without a history of BPD [9.5(7.3-12.2) pg/mL and 10(8.1-16) pg/mL, respectively], and significantly higher than in the healthy control group [3.2(1.9-6.5) pg/mL, $p<0.01$] (Figure 1). Among the preterm-born participants, with and without a history of BPD, the 8-isoprostane levels were unrelated to age ($p=0.32$), height ($p=0.58$) and weight ($p=0.52$) at study participation, to
gestational age ($r=0.006$, $p=0.96$), gestational age-adjusted birth weight z-score ($r=0.09$, $p=0.58$) time on mechanical ventilation ($r=0.036$, $p=0.82$) or oxygen dependence ($r=0.099$, $p=0.52$). No correlation was found in these subjects between EBC 8-isoprostane concentrations and the spirometric variables ($FEV_1$: $p=0.075$, $FEV_1/FVC$: $p=0.15$, $FEF_{25-75%}$: $p=0.065$), or the percentage increase in $FEV_1$ after inhaled albuterol administration ($p=0.17$), or $FENO$ ($p=0.64$). EBC 8-isoprostane levels were similar in mild and moderate-severe survivors of BPD [9.8(7.6-11.9) and 9.2(7.1-13.1) pg/mL respectively, $p=0.89$], in atopic and non-atopic participants ($p=0.36$), and in subjects with and without respiratory symptoms in the past 2 years ($p=0.27$).

**Spirometry**

Significant differences emerged between groups by comparison of all spirometric parameters (Figure 2). A marked airflow limitation was found in the BPD group, showing significantly lower results for all the spirometric variables ($p<0.01$) than the healthy controls (Figure 2). $FEV_1$ and $FEF_{25-75%}$ were also significantly reduced in the BPD survivors as compared to the preterm non-BPD group ($p<0.05$). In turn, $FEV_1$ and $FEF_{25-75%}$ were significantly lower in the preterm non-BPD adolescents than in the control group (Figure 2).

Airway obstruction was reversible in 35% of the cases in the BPD survivor group (12/34) and in 17% of the preterm non-BPD cases (3/18).

**$FENO$**

No differences in $FENO$ levels emerged between the three groups [BPD: 11.4(8.4-15.7) ppb, Preterm non-BPD: 14.1(8.4-17.1) ppb, Controls: 12.7(7.9-19.2) ppb, $p=0.552$]. As atopy is known to affect $FENO$ levels, a subgroup analysis was performed on the individuals in the three groups with negative skin prick test results, which again revealed no differences in $FENO$ values.
Skin prick tests

The skin prick tests for common aeroallergens were positive in 6/34 (18%) in the BPD cases, 3/18 (17%) in the preterm non-BPD cases and 4/34 (12%) in the healthy controls.
DISCUSSION

This study provides evidence of an increased oxidative stress in the airways of adolescents born prematurely, with and without a history of BPD, as reflected by significantly higher EBC 8-isoprostane concentrations than in healthy controls born at term. This finding prompts the hypothesis that the long-term chronic respiratory disease following premature birth not only stems from a stabilized structural damage[28], but may also be associated with an ongoing airway disease.

Lung function abnormalities are detectable early in life after premature birth[4,29], and carry a substantial risk of long-term respiratory disease[30], because a poor lung function in the first months of life is known to track over time with a greater risk of airway obstruction in childhood[31] and young adulthood[32].

The lung function limitations and respiratory symptoms seen in long-term survivors of premature birth and BPD have no clear pathological background to refer to because no biopsy studies have been conducted to elucidate lung morphology in the medium and long term after premature delivery. The airflow obstruction seen in the childhood and adolescence of individuals born preterm has often been interpreted as the result of stabilized, not progressive structural damage to the airways and/or poor alveolar growth[28].

Data from longitudinal studies on lung function at multiple time points in adolescents with a history of BPD indicate that lung function may deteriorate over time in some patients[7,33], suggesting that their respiratory disease may be progressive in nature. In addition, the persistence of significantly more frequent respiratory symptoms in adults born even only slightly preterm[5,6] arouses the clinical suspicion of active airway disease in these patients. Unfortunately, given the lack of morphological information and randomized therapeutic trials, the pathogenesis of such long-term symptoms remains elusive and their treatment empirical.

For this reason it is important to seek indirect information on long-term airway status after premature birth. Analyzing EBC may be a useful study tool, being non-invasive, simple to perform, and effort-independent. Despite some technical limitations, relating mainly to EBC dilution issues[34], recent literature supports its use as an indirect method for sampling the composition of the airway lining fluid[17,19,18,20,35].

We focused on 8-isoprostane, a prostaglandin-F2α isomer formed by arachidonic acid peroxidation catalysed by free radicals. The isoprostanes are among the most reliable biomarkers of lipid peroxidation and oxidative stress because of their chemical stability, specificity for lipid peroxidation, in vivo production, and relative abundance in biological fluids[17,18]. 8-isoprostane is an important component of the isoprostanes pathway and has been found to have powerful biological effects; it may therefore be a mediator and marker of oxygen radical injury[18], a mechanism involved in the pathogenesis of several chronic lung diseases[36], including COPD[12].
The higher concentrations of 8-isoprostane found in the EBC of adolescents born preterm indicate a three-fold lipid peroxidation rate in their airways by comparison with healthy controls born at term (Figure 1). It is worth noting that no differences emerged between the 8-isoprostane concentrations found in BPD survivors and the adolescents born preterm with no history of BPD (Figure 1), suggesting that long-term airway oxidative stress is a feature of prematurity *per se*, regardless of any development of BPD or exposure to prolonged mechanical ventilation and oxygen therapy in neonatal age.

Premature birth in itself poses a considerable oxidative challenge, as preterm infants abruptly leaving the normally hypoxic intrauterine environment face a sharp increase in oxidative load at a time when they still lack adequate specific defenses[13]. Indeed, extremely premature infants show evidence of oxidative stress persisting several weeks after a brief exposure to high concentrations of inspired oxygen at birth[16].

The 8-isoprostane levels in the EBC of our prematurely-delivered participants did not correlate with any of the spirometric variables reflecting airflow, which were significantly worse (as expected) in survivors of BPD. The 8-isoprostane levels in EBC were much the same in the subjects with and without respiratory symptoms in the previous 2 years. This suggests that lung underdevelopment and early airway injury may have a central role in determining the extent of airway obstruction. We then believe that the measurement of oxidative stress provides complementary biological information to lung function in evaluating the pathophysiological processes underlying the long-term pulmonary sequelae of preterm birth. Whether increased values of 8-isoprostane imply a risk of more severe lung function deterioration later in life remains to be seen.

The FENO values recorded in the preterm-born individuals in our series were normal, suggesting that there is no predominant eosinophil-mediated inflammatory pathway in these subjects’ airways[37], whereas increased 8-isoprostane levels in preterm-born cases point to active oxidative stress. The association of an increased oxidative stress with normal FENO values might represent an apparent paradox. NO in the lung is a signalling molecule in a wide variety of biological and homeostatic processes including airway and vascular smooth muscle relaxation and neurotransmission. However, there are reports of an increased oxidative stress with normal-to-low values of exhaled NO in other chronic respiratory diseases, such as cystic fibrosis and COPD[38]. In these diseases a neutrophilic cellular pathway is predominant in the lung and this can explain the paradox of active oxidative stress with normal FENO levels. Likewise, it could be speculated that a similar neutrophilic inflammation may develop after premature birth and BPD. There are no pathological data to support this hypothesis, however, due to a lack of information on the presence and nature of airway inflammation beyond infancy in subjects born prematurely.

Whatever its origin and nature, the long-term presence of oxidative stress in the airways after premature birth may be reminiscent of findings consistently reported in the airways of patients with
COPD[12]. Oxidative stress plays a crucial part in many of the pathogenic mechanisms behind COPD, and it is associated with protease/antiprotease imbalance and matrix injury, triggering pro-inflammatory activity and alveolar cell apoptosis[12]. Recent hypotheses suggest that cigarette smoking, the principal causative factor of COPD, mainly plays as the initial trigger of the disease, that is afterwards maintained through chronic self-supporting injury mechanisms[39,40]. We likewise hypothesize that harmful exposures early in life (including oxidative stress) trigger an adaptive disorder in the premature lung that may be maintained, or easily elicited, after the initial injury. The evidence of increased oxidative stress in our prematurely born adolescents might therefore be interpreted as a marker of ongoing airway disease, which may have an impact on these individuals’ respiratory health with aging. The design of the present study prevents any conclusions from being drawn on this issue, however, for which longitudinal data are needed on oxidative stress markers and how they correlate with lung function over time in individuals born prematurely. Further research focusing on other markers of airway oxidative stress, inflammation or remodeling in long-term survivors of prematurity and BPD may help to clarify the natural history of the chronic lung disease that follows premature birth. As well, systemic markers of oxidative stress could be targeted for further research, to evaluate the possible persistence at long term of the “oxygen radical disease” described in the newborn[13].

In conclusion, our finding of a greater oxidative stress in the airways of adolescents born very preterm suggests the presence of an ongoing airway disease, not just the stabilized structural lung damage assumed until now. This alteration is present regardless of the development of BPD. This issue warrants further investigations into the nature and evolution of the airway disease seen in survivors of prematurity and BPD as they grow older.
Table. Neonatal data and anthropometric characteristics of participants.

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<tr>
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<th>BPD (n. 34)</th>
<th>Preterm non-BPD (n. 18)</th>
<th>Full-term controls (n. 34)</th>
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<td>20/14</td>
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<td>28 (26-30)</td>
<td>39 (38-40)</td>
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<td>940 (840-990)</td>
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<td>5 (28%)</td>
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<td>1 (1-3.2)</td>
<td>--</td>
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<td>2 (1-5.5)</td>
<td>-- ANOVA</td>
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<td>58.2(11.9)</td>
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</table>

* = p<0.01 as compared with Full-term control group

FIGURE LEGENDS

Figure 1. Levels of 8-isoprostane in the EBC of adolescents born very preterm, with and without a history of BPD, and of a group of healthy controls born at full term. Boxes represent median values and interquartile ranges; bars represent the 10th and the 90th centiles of the measured concentrations.
Figure 2. Lung function results (FVC, FEV₁, FEV₁/FVC and FEF₂₅-₇₅%) in the BPD, preterm non-BPD and control groups. Data are expressed as Z-scores. Boxes represent median values and interquartile ranges; bars represent the 10th and the 90th centiles of the measured values.
REFERENCES


