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# Correlation of urinary inflammatory and oxidative stress markers in very low birth weight infants with subsequent development of bronchopulmonary dysplasia

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**Abstract**

Currently, bronchopulmonary dysplasia (BPD) occurs almost exclusively in pre-term infants. In addition to prematurity, other factors like oxygen toxicity and inflammation can contribute to the pathogenesis. This study aimed to compare urinary inflammatory and oxidative stress markers between the no/mild BPD group and moderate/severe BPD group and between BPD cases with significant early lung disease like respiratory distress syndrome (RDS) ('classic' BPD) and with minimal early lung disease ('atypical' BPD). A total of 60 patients who were a gestational age < 30 weeks or a birth weight < 1250 g were included. Urine samples were obtained on the 1st, 3rd and 7th day of life and measured the levels of leukotriene $E_4$ ($LTE_4$) and 8-hydroxydeoxyguanosine (8-OHdG). The 8-OHdG values on the 3rd day showed significant correlation to duration of mechanical ventilation. The 8-OHdG levels on the 7th day were the independent risk factor for developing moderate/severe BPD. In 'classic' BPD, the 8-OHdG values on the 3rd day were higher than those of 'atypical' BPD. In 'atypical' BPD, the $LTE_4$ values on the 7th day were higher than the values in 'classic' BPD. These results suggest that oxidative DNA damage could be the crucial mechanism in the pathogenesis of current BPD and the ongoing inflammatory process could be an important mechanism in 'atypical' BPD.

**Keywords:** *Chronic lung disease, Leukotrienes, 8-hydroxydeoxyguanosine, pre-term infants, systematic inflammatory response*

**Abbreviations:** *VLBWI, very low birth weight infant; BPD, bronchopulmonary dysplasia; RDS, respiratory distress syndrome; cCAM, clinical chorioamnionitis; hCAM, histological chorioamnionitis; $LTE_4$, leukotriene $E_4$; 8-OHdG, 8-hydroxydeoxyguanosine.*

## Introduction

The clinical presentation of bronchopulmonary dysplasia (BPD), the most common chronic lung disease in newborns, has changed from its original description by Northway et al. [1]; according to that paper, BPD, which is preceded by respiratory distress syndrome (RDS), manifests as severe fibrosis or atelectasis with cystic changes and requires subsequent mechanical ventilation. BPD still occurs despite the strategies of gentle mechanical ventilation and the decreased incidence of severe RDS in this era of surfactant and antenatal steroids. Several reports have shown the heterogeneity of current BPD according to the patterns of oxygen dependency; BPD that is characterized by minimal early lung disease, which has recently been referred to as the 'atypical' or 'new' form of BPD [2–4], is increasing in the population of very low birth weight infants (VLBWI) as compared to the classical type of BPD that is marked by initially significant lung disease [5].

There are several studies that have reported that the pathogenesis of current BPD is related to pre-natal

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injuries, such as the intra-uterine inflammatory response and maternal chorioamnionitis [6–8] and dysregulation of pro- and anti-inflammatory cytokines [9–12]. We also demonstrated in a previous study that the levels of plasma KL-6, which is a specific lung injury marker, are increased in umbilical cord blood from infants with BPD with minimal early lung disease, suggesting that lung injury occurs during the prenatal period in infants with subsequent development of current BPD [13]. In addition, oxidative metabolites have been reported to play an important role in the pathogenesis of BPD because pre-term infants are especially vulnerable to reactive oxygen species due to an under-developed antioxidant system [14,15]. We recently reported both inflammatory response and oxidative stress were playing the key roles in pathogenesis of BPD using the 'double-hit' animal model [16]. However, the exact mechanisms and the impact of each factor on the pathogenesis of current BPD in the post-surfactant era are still under debate.

In this study, we measured the urinary markers Leukotriene $E_4$ ($LTE_4$) and 8-hydroxydeoxyguanosine (8-OHdG), which, as mentioned above, reflect the two mechanisms of inflammation and oxidative damage, respectively, in relation to the subsequent development of current BPD. $LTE_4$, which is a known byproduct of the degradation of the phospholipid bilayer of the cell membrane, is an inflammatory marker that is correlated with the severity of bronchial asthma and can be detected in urine [17,18]. The molecule 8-OHdG is a marker of oxidative DNA damage; during DNA synthesis under conditions of oxidative stress, oxidized 8-hydroxyguanine (8-OHGua), instead of a normal guanine base, is incorporated into DNA and is degraded by endonuclease to produce 8-OHdG, which can be detected in the urine [19].

We also aimed to determine whether there are different underlying mechanisms in the pathogenesis of BPD according to their clinical presentations; BPD with significant early lung disease ('classic' BPD) as compared to BPD with minimal early lung disease ('atypical' BPD), by measuring these urinary biomarkers in the first week of life.

## Materials and methods

Pre-term newborns with a gestational age $<30$ weeks or a birth weight $<1250$ g who were admitted to the neonatal intensive care unit of Seoul National University Children's Hospital between July 2005 and June 2007 were included. Infants with chromosomal anomalies or major congenital malformations and infants who died before reaching a corrected age of 36 weeks were excluded.

The clinical characteristics of the patients and their mothers were reviewed in the medical records by a single reviewer. The study protocol was approved by our institutional research ethics committee and informed consent was obtained from the parents prior to the collection of urine samples.

### *Method of diagnosis*

Bronchopulmonary dysplasia and its severity were defined according to the definitions and criteria of the National Institute of Child Health Workshop (NICHD) [20]. The BPD group was sub-classified into two groups according to the criteria proposed by Chararafeddine et al. [3]: 'classic' BPD was defined in cases with respiratory distress syndrome (RDS) that did not resolve and required continuous oxygen supplementation for at least 28 days; 'atypical' BPD was diagnosed in BPD cases without RDS or in BPD cases that were preceded by initial RDS that resolved within 10 days and required no oxygen supplementation for at least 72 h, in addition to the 28-day oxygen requirement. RDS was diagnosed in cases of acute respiratory distress with increasing oxygen requirement and radiologic findings compatible with RDS. Every infant with RDS received surfactant replacement therapy (Surfacten®, Mitsubishi Pharma. Co., Osaka, Japan) of up to three doses until they showed clinical improvement.

Sepsis was diagnosed when clinical signs, such as hypotension or left-shifted white blood cell counts with immature to total WBC ratios greater than 0.2, occurred with at least one positive blood culture result. Diagnoses of patent ductus arteriosus (PDA) were restricted with symptomatic PDA diagnosed by echocardiography. Clinical chorioamnionitis (cCAM) was defined as a body temperature of 37.8°C or above and at least two of the following findings: uterine tenderness, malodorous vaginal discharge, maternal leukocytosis ($>15\ 000/mm^3$), maternal tachycardia ($>100$/min) or foetal tachycardia ($>160$/min) [21]. Histologic chorioamnionitis (hCAM) was defined as the presence of acute inflammatory changes in the membrane and/or the placental chorionic plate [6]. Necrotizing enterocolitis (NEC) was diagnosed as stages equal to or higher than II according to the modified Bell's criteria [22]. Intraventricular haemorrhage (IVH) was defined as a germinal matrix/intraventricular haemorrhage with grades equal to or higher than grade II confirmed by ultrasonographic diagnosis according to Volpe [23].

Total amount of oxygen supplementation provided for the first 3 days and 6 days of life were calculated by the following equation: total extra oxygen supplementation = supplemented extra oxygen concentration (%) (fraction of inspired oxygen – 21) × duration (h) [24].

### *Urinary LTE4 analysis and 8-OHdG*

The urine within 24 h after birth (day 1) and morning spot urine samples on day 3 and 7 were stored

at −20°C in polypropylene tubes until they were assayed. After thawing, the samples were centrifuged at 10 000 g for 5 min at 4°C. Urinary $LTE_4$ levels were measured using an ELISA kit (Cayman Chemical; Ann Arbor, MI) and 8-OHdG levels were measured using a highly sensitive ELISA kit (Japan Institute for the Control of Aging; Fukuroi City, Japan). To standardize the results, urine creatinine (Cr) was assayed by ELISA (Cayman Chemical; Ann Arbor, MI) and the results were expressed as picograms of $LTE_4$ per milligram of Cr and nanograms of 8-OHdG per milligram of Cr. All samples were analysed in duplicate.

*Statistics*

Statistical analyses were performed using SPSS version 12.0 (SPSS Inc, Chicago, IL). Comparisons between the BPD groups according to severity and between the classic and atypical BPD sub-groups were performed with the Mann-Whitney U-test, which was also used for continuous variables, whereas the Fisher's exact tests were used for categorical variables. Logistic regression analysis using the backward step-wise (Wald) method including factors that showed a $p$-value $< 0.1$ on univariate analysis was performed to determine the independent BPD-related factors. The correlation between urinary 8-OHdG levels and the duration of mechanical ventilation were analysed by correlation analysis and expressed as Spearman's rho correlation coefficients. $p$-values lower than 0.05 were considered statistically significant.

## Results

*Clinical characteristics*

During the study period, 76 patients who were less than 30 weeks old or weighed less than 1250 g at birth

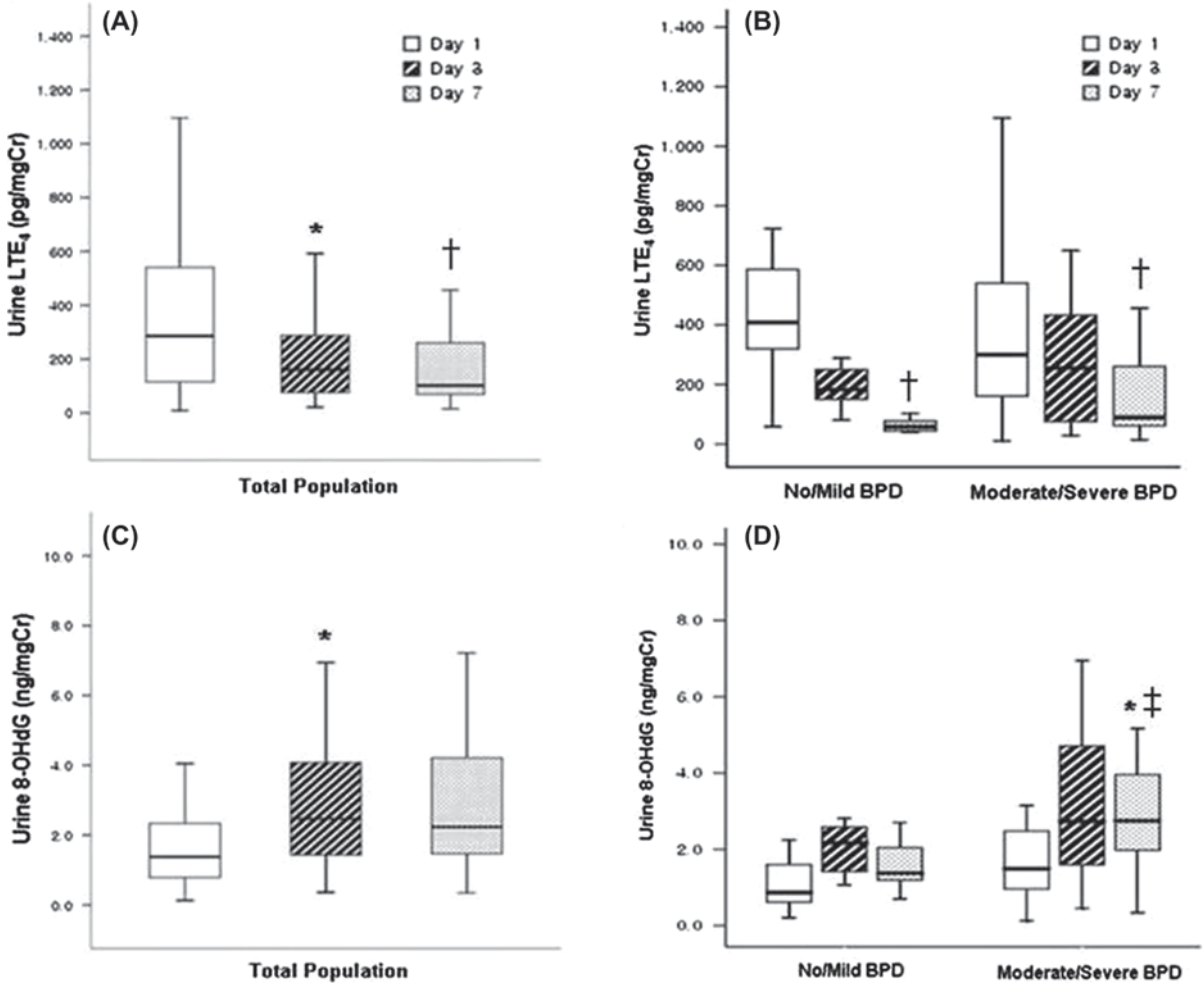


Figure 1. Urinary $LTE_4$ and 8-OHdG levels in the study population and grouped by severity of BPD. Urinary $LTE_4$ levels on 1st, 3rd, 7th day of life in (A) total study population and (B) in the groups of no/mild BPD and moderate/severe BPD. 8-OHdG levels on 1st, 3rd, 7th day of life in (C) total study population and (D) in the groups of no/mild BPD and moderate/severe BPD. The box plots show the 25th and 75th percentiles, the median (horizontal line within the box) and the 10th and 90th percentiles (bars). The definition of BPD and its severity were according to the criteria of the National Institutes of Health consensus definition [20]. *$< 0.05$ compared to Day 1 within the group by the Wilcoxon signed rank test; †$< 0.05$ compared to Day 3 within the group by the Wilcoxon signed rank test; ‡$< 0.05$ between no/mild BPD and moderate/severe BPD groups by the Mann-Whitney U-test.

passed initial inclusion criteria. Among these infants, five patients died before 36 weeks of corrected age and one patient with Down syndrome were excluded. Of the remaining 70 patients, 60 had at least one sample of three time points (on the 1st, 3rd and 7th day of life) that were analysed. The mean gestational age was $27.5 \pm 2.3$ weeks and the mean birth weight was $907.7 \pm 238.4$ g. The mean duration of hospital stay was $83.0 \pm 37.4$ days. Of the 60 patients with analysed samples, 42 (70%) were diagnosed with BPD: 'classic' BPD was diagnosed in 20 patients (47.6%) and 'atypical' BPD was diagnosed in 22 patients (52.4%). Mild BPD was diagnosed in six patients (14.6%), moderate BPD was diagnosed in 23 patients (54.8%) and severe BPD was diagnosed in 13 patients (30.9%).

### *$LTE_4$ and 8-OHdG levels in the study population*

The median $LTE_4$ levels in the study population on days 1, 3 and 7 were 286.1, 160.4 and 102.1 pg/mg Cr, respectively, which represents a statistically significant decreasing trend (Figure 1A). The median 8-OHdG levels were 1.4, 2.5 and 2.2 ng/mg Cr on days 1, 3 and 7, respectively. The 8-OHdG level on day 3 was significantly higher than the level on day 1 (Figure 1C).

### *Comparison between the no/mild BPD and moderate/severe BPD groups*

The median gestational age and birth weight in the no/mild BPD group ($n = 24$) were significantly higher than those of the moderate/severe BPD group ($n = 36$). The 5-min Apgar scores were higher and the frequency of neonatal sepsis and NEC were lower in the no/mild BPD group. The duration of oxygen therapy, mechanical ventilation and hospital stay were significantly longer in the moderate/severe BPD group (Table I).

The median $LTE_4$ values on days 1, 3 and 7 in the no/mild BPD group were 424.4, 160.4 and 96.9 pg/mg Cr, respectively, and those in the moderate/severe BPD group were 299.0, 212.4 and 140.0 pg/mg Cr, respectively. In both groups, the level of $LTE_4$ decreased during the first week of life (Figure 1B). The median 8-OHdG values on days 1, 3 and 7 were 1.1, 2.1 and 1.6 ng/mg Cr in the no/mild BPD group and 1.5, 2.6 and 2.8 ng/mg Cr in the moderate/severe BPD group, respectively. The difference between the two groups was statistically significant on day 7 ($p = 0.002$) (Figure 1D). The logistic regression analysis included variables that showed differences with a $p$-value $< 0.1$ between the moderate/severe BPD group and the non/mild BPD group; these include gestational age, birth weight, 5-min Apgar score, sepsis, duration of mechanical ventilation and necrotizing enterocolitis. The adjusted odds ratio ($\beta$ coefficient) was 3.9 for an increase in the 8-OHdG concentration of 1 ng/mg Cr ($p = 0.048$). The sensitivity of 8-OHdG on the7th day of life for predicting moderate/severe BPD using Receiver Operating Curve analysis, is 85.7%, specificity is 61.1% with cut-off value of 1.76 pg/mgCr (Area under curve: 0.770, SE 0.073, $p = 0.02$).

Table I. Clinical characteristics and neonatal outcomes in the no/mild BPD and moderate/severe BPD groups.

| | Moderate/severe BPD ($n = 36$) | No/mild BPD ($n = 24$) |
|---|---|---|
| Maternal age (y) | 32.0 [24.0–41.0] | 32.5 [27.0–40.0] |
| Gestational age (wk)* | $26^{+0}$ [$24^{+0}$–$31^{+2}$] | $29^{+0}$ [$25^{+3}$–$35^{+5}$] |
| Birth weight (g)* | 750 [430–1250] | 1125 [780–1380] |
| 1-min Apgar score | 4 [0–7] | 5 [0–8] |
| 5-min Apgar score* | 5 [1–8] | 7 [2–9] |
| Caesarean section, *n* (%) | 27 (75.0) | 19 (79.2) |
| PROM > 18 h, *n* (%) | 12 (33.3) | 6 (25.0) |
| cCAM, *n* (%) | 3 (8.3) | 0 (0.0) |
| hCAM, *n* (%) | 17 (47.2) | 10 (41.7) |
| Antenatal steroid, *n* (%) | 34 (94.4) | 19 (79.2) |
| Postnatal steroid, *n* (%) | 5 (13.9) | 0 (0.0) |
| RDS, *n* (%) | 22 (69.1) | 6 (25.0) |
| Duration of $O_2$ (d)* | 81.0 [31–231] | 9.0 [0–60] |
| Duration of MV (d)* | 16.5 [0–122] | 2.0 [0–24] |
| PDA, *n* (%) | 31 (86.1) | 18 (75.0) |
| Sepsis, *n* (%)* | 17 (47.2) | 4 (16.7) |
| NEC, *n* (%)* | 7 (19.4) | 0 (0.0) |
| IVH ≥ Gr2, *n* (%) | 5 (13.9) | 0 (0.0) |
| ROP, *n* (%) | 21 (58.3) | 4 (16.7) |
| Hospital stay (d)* | 94.0 [49.0–231.0] | 57.0 [28.0–81.0] |

Values are expressed as medians [ranges], $^*p < 0.05$.

Differences between the two groups were assessed by Mann-Whitney U-test for continuous variables or by Fisher exact test for categorical data.

BPD, bronchopulmonary dysplasia; PROM, premature rupture of membrane; CCAM, clinical chorioamniionitis; HCAM, histological chorioamnionitis; MV, mechanical ventilation; RDS, respiratory distress syndrome; PDA, patent ductus arteriosus; NEC, necrotizing enterocolitis; IVH, intraventricular haemorrhage; ROP, retinopathy or prematurity.

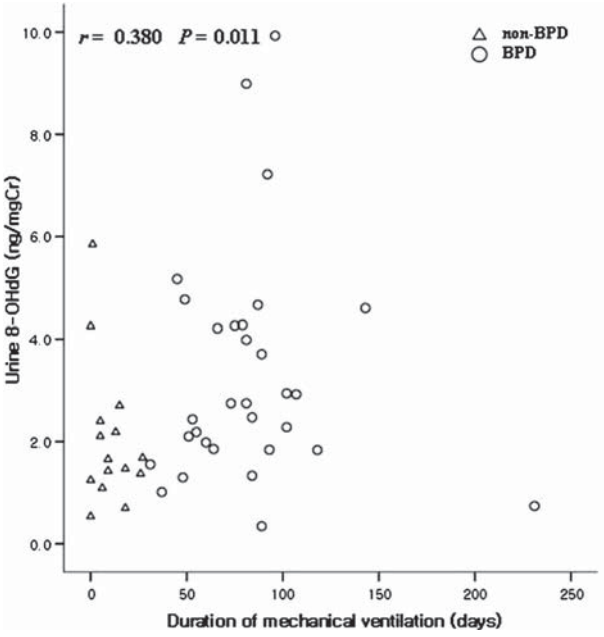


Figure 2. Correlation between the duration of mechanical ventilation and urinary concentrations of 8-OHdG on the 3rd day. The 8-OHdG levels on the 3rd day were positively correlated with the duration of mechanical ventilation (Spearman's rho correlation coefficient, $r = 0.380$, $p = 0.011$).

We studied the correlation between urinary markers levels and the duration of mechanical ventilation as a parameter of clinical severity of BPD. The 8-OHdG level on day 3 was positively correlated (Spearman's rho correlation coefficient: 0.38) with the duration of mechanical ventilation ($p = 0.011$, Figure 2 and Table II) in total study population, but this correlation was not found for urinary $LTE_4$ (Table II).

*Comparison between 'classic' BPD and 'atypical' BPD*

There was no significant difference between the 'classic' BPD and 'atypical' BPD groups in terms of median gestational age, birth weight and 1- and 5-min Apgar scores. The median value of cord blood IgM in atypical BPD group was higher but not statistically significant and CRP didn't reveal any differences (Table III). However, the duration of mechanical ventilation was longer in 'classic' BPD ($p = 0.047$). RDS was associated with 100% of 'classic' BPD cases as previously defined and with 9% of 'atypical' BPD cases ($p < 0.001$, Table III) and total amount of oxygen supplementation for the first 3 days and 6 days of life were also higher in the classic BPD group ($p = 0.001$, Table III).

The median $LTE_4$ values were 400.9, 254.8 and 85.8 pg/mg Cr on days 1, 3 and 7, respectively in 'classic' BPD, which decreased with time, and the level on day 7 was significantly lower as compared to that on day 3 ($p = 0.011$). In atypical BPD, the median $LTE_4$ values were 265.8 pg/mg Cr on day 1 and 153.0 pg/mg Cr on day 3 and increased again to 260.7 pg/mg Cr on day 7, showing a different pattern from that of 'classic' BPD. The urinary $LTE_4$ values on day 7 were significantly higher in 'atypical' BPD than in 'classic' BPD ($p = 0.006$) (Figure 3A).

In 'classic' BPD, the urinary 8-OHdG level was 1.1 ng/mg Cr on day 1, significantly increased to 2.8 ng/mg Cr on day 3 ($p = 0.023$) and to 2.5 ng/mg Cr on day 7. In contrast, in 'atypical' BPD, the urinary 8-OHdG levels on days 1, 3 and 7 were 1.68, 1.81 and 2.74 ng/mg Cr, respectively, showing an incremental change over time that was not statistically significant. The 8-OHdG levels on day 3 were significantly higher in 'classic' BPD than in 'atypical' BPD ($p = 0.038$) (Figure 3B). The sensitivity, specificity for 8-OHdG on the 3rd day of life for predicting 'classic BPD' over atypical BPD was also significant, cut-off value of 2.10 pg/mg Cr showed sensitivity of 82.4%, specificity of 62.9% and receiver operating characteristic curve analysis of urinary 8-OHdG on the 3rd day of life in the identification of classic BPD (area under curve 0.709, SE 0.89, $p = 0.037$).

Table II. Correlation between the duration of mechanical ventilation and marker level.

| Time point | Correlation coefficient |
|---|---|
| Day 1 $LTE_4$ | 0.198 |
| Day 3 $LTE_4$ | 0.007 |
| Day 7 $LTE_4$ | 0.082 |
| Day 1 8-OHdG | 0.133 |
| Day 3 8-OHdG | 0.380* |
| Day 7 8-OHdG | 0.282 |

The correlation between urinary $LT_4$ and 8-OHdG levels and the duration of mechanical ventilation were analysed and expressed as Spearman's rho correlation coefficients. Only the 8-OHdG levels on the 3rd day were positively correlated with the duration of mechanical ventilation.

*$P$-values lower than 0.05 were considered statistically significant.

## Discussion

Numerous factors, such as infection, oxidative stress and baro/volutrauma can cause injury to immature lungs that results in the subsequent development of BPD. Therefore, many biomarkers in blood, tracheal aspirate (TA) and even urine have been studied for early detection and to increase our understanding of the pathogenesis of BPD in infants. Although the measurement of biomarkers in the TA may be suitable for direct evaluation of immature lung pathophysiology, TA is obtained by an invasive procedure and has the disadvantages of difficulties with normalization and universal sampling. Urinary sampling is non-invasive and has no risk of anaemia caused by over-sampling of blood. Since recent study has reported that increased levels of urinary bombesin-like peptide are correlated with increased risk of developing BPD [25], urinary analysis of biomarkers in pre-term infants with various conditions has

Table III. Clinical characteristics and neonatal outcomes in the classic and atypical BPD groups.

| | Classic BPD ($n = 20$) | Atypical BPD ($n = 22$) |
|---|---|---|
| Maternal age (y) | 33.0 [24.0–41.0] | 32 [24.0–38.0] |
| Gestational age (wk) | $26^{+2}$ [$24^{+1}$–$29^{+2}$] | $26^{+6}$ [$24^{+0}$–$31^{+2}$] |
| Birth weight (g) | 745.0 [430.0–1120.0] | 830.0 [480.0–1250.0] |
| 1-min Apgar score, *n* (%) | 3 [0–6] | 4 [0–7] |
| 5-min Apgar score, *n* (%) | 5 [2–7] | 6 [1–8] |
| Caesarean section, *n* (%) | 15 (75.0) | 15 (68.1 ) |
| PROM > 18 h, *n* (%) | 5 (25.0) | 10 (45.5) |
| CCAM, *n* (%) | 1 (5.0) | 2 (9.1) |
| HCAM, *n* (%) | 10 (50.0) | 12 (54.5) |
| Antenatal steroid, *n* (%) | 18 (90.0) | 22 (100) |
| Postnatal steroid, *n* (%) | 3 (15.0) | 2 (9.0) |
| RDS, *n* (%)* | 20 (100) | 2 (9.0) |
| Duration of $O_2$ (d) | 81 [48–31] | 57 [8–118] |
| Duration of MV (d)* | 24 [2–122] | 7 [0–65] |
| Total extra $O_2$ supplementation | | |
| Within 3 days of life* | 699.8 [114.3–2270.9] | 231.2 [0.0–456.4] |
| Within 6 days of life* | 973.6 [202.0–4013.9] | 356.4 [0.0–695.9] |
| PDA, *n* (%) | 18 (90.0) | 18 (81.9) |
| Sepsis, *n* (%) | 11 (55.0) | 10 (50.0) |
| Cord IgM (mg/dL) | 8.5 [3–16] | 10 [3–49] |
| Cord CRP (mg/dL) | 0.01 [0.01–0.64] | 0.01 [0.01–0.1] |
| Day 7 CRP (mg/dL) | 0.06 [0.01–0.79] | 0.05 [0.01–2.44] |
| Hospital stay (d) | 101.0 [64.0–231.0] | 84 [49.0–157.0] |

Values are expressed as medians [ranges], $^*p < 0.05$.

Differences between the two groups were assessed by Mann-Whitney U-test for continuous variables or by Fisher exact test for categorical data. BPD, bronchopulmonary dysplasia; PROM, premature rupture of membrane; CCAM, clinical chorioamniionitis; HCAM, histological chorioamnionitis; MV, mechanical ventilation; RDS, respiratory distress syndrome; PDA, patent ductus arteriosus; NEC, necrotizing enterocolitis; IVH, intraventricular haemorrhage; ROP, retinopathy or prematurity; IgM, Immunogloblin M; CRP, C-reactive protein.



become more common [26,27], but relatively few studies on this topic have been published. In the present study, two urinary markers, 8-OHdG and $LTE_4$, that reflect the mechanisms of oxidative damage and inflammation, respectively, were measured in the context of subsequent development of BPD. Further we studied whether there are different underlying mechanisms in the pathogenesis of BPD according to their clinical presentations. To date, the universal concept of sub-classification of BPD according to its clinical presentation or pathogenesis has not been established yet. However, a few studies suggested a possibility that heterogeneity of pathogenesis could make certain differences in the clinical course of BPD [13,28]. Thus, we compared urinary markers levels between BPD with significant early lung disease ('classic' BPD) and that with minimal early lung disease ('atypical' BPD).

In our study population, the incidence of BPD (70%) was similar to our previous data (67%), which included pre-term infants who were < 32 weeks of gestational age (13) and was a little higher than other reports (≈ 51%) [3–5], because our study population had relative smaller gestational age and birth weight than previous studies. The median gestational age and birth weight in the moderate/severe BPD group were significantly lower than those of the no/mild BPD group. Because lower gestational age and birth weight are the most significant risk factors for BPD development, we employed logistic regression analysis including variables such as gestational age, birth weight and 5-min Apgar scores to adjust for these known risk factors to compare the groups.

Since the original description by Northway, exposure to high oxygen concentrations has been identified as an independent risk factor in the pathogenesis of BPD. Evidence regarding the role of oxidative stress in the development of BPD based on measurements of oxidative stress markers has been previously reviewed [15]. Currently, the term 'oxidative stress' has a broader application to not only supplementation with high oxygen concentrations but also to an imbalance between oxidant and antioxidant forces. Among the many oxidative stress markers, we chose urinary 8-OHdG because it reflects oxidative DNA damage and can be obtained by non-invasive sampling procedures. To date, there have only been a few studies investigating the urinary levels of 8-OHdG in pre-term populations. Matsubasa et al. [29] reported significantly increased levels of 8-OHdG in VLBW infants as compared to term controls, and pre-term infants prior to 36 weeks of corrected age had higher 8-OHdG levels as compared to those who were older than 36 weeks of corrected age. In the present study, we first measured urinary 8-OHdG levels in the context of subsequent development of BPD and showed that the level of 8-OHdG on day 7 is an independent risk factor for developing moderate/severe BPD (Figure 1D); The adjusted odds ratio ($\beta$ coefficient) was 3.9 for an increase in the 8-OHdG concentration of 1 ng/mg Cr

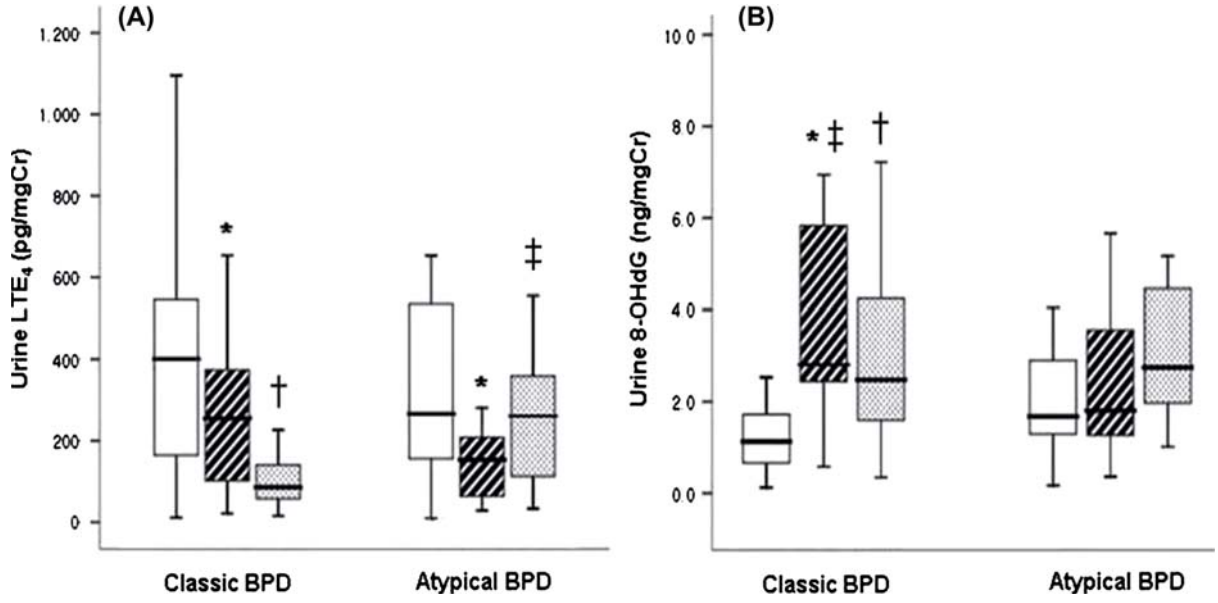


Figure 3. Urinary $LTE_4$ and 8-OHdG concentrations grouped by classic and atypical BPD. Urinary $LTE_4$ (A) and 8-OHdG (B) levels on 1st, 3rd, 7th day of life in the groups divided according to its clinical presentation (see Methods section). The box plots show the 25th and 75th percentiles, the median (horizontal line within the box) and the 10th and 90th percentiles (bars). $^{*}<0.05$ compared to Day 1 within the group by the Wilcoxon signed rank test; $^{\dagger}<0.05$ compared to Day 3 within the group by the Wilcoxon signed rank test; $^{\ddagger}<0.05$ between the classic and atypical BPD groups by the Mann-Whitney U-test.

($p = 0.048$). Our study is also the first to report that the oxidative stress measured by 8-OHdG on the 3rd day of life is related to the duration of dependency on mechanical ventilators which reflects to clinical severity of BPD (Figure 2, Table II). These results suggest that urinary 8-OHdG is a possible effective indicator of the subsequent development of relatively severe BPD and oxidative DNA damage is a crucial risk factor even in current BPD in the post-surfactant era. A recent study has also revealed that the levels of 8-OHdG in leukocytes and urine are increased in preterm infants with retinopathy of prematurity (ROP) as compared to those without the disease [30]. ROP and BPD are known to be oxidative stress diseases in pre-term infants and DNA damage caused by oxidative stress may result in mal-development of organs in very pre-term infants. Furthermore, we studied the dynamic pattern of urinary 8-OHdG levels in 'classic' and 'atypical' BPD groups during the first week of life. While the 8-OHdG level on day 3 was significantly increased as compared to that of day 1 in 'classic' BPD, there was no significant change during first week of life in the 'atypical' BPD group. This might have occurred because the 'classic' BPD group, which had significant early lung disease such as RDS, received more aggressive respiratory therapy. In addition, the results suggest the possibility that a mechanism other than oxidative stress might play a role in the development of 'atypical' BPD.

$LTE_4$ is known as a byproduct of the degradation of the phospholipid bilayer of the cell membrane and has been used as an inflammatory marker. Although normal levels of $LTE_4$ have not been established in the neonatal population, normal values for the adult population are reported to be less than 100 pg/mg Cr [18]. In this study, the level of urinary $LTE_4$ on day 1 was greater than the normal value for adults in both the no/mild BPD and moderate/severe BPD groups. Interestingly, urinary levels of $LTE_4$ decreased significantly during the first week in the entire study population and even in the moderate/severe BPD group. This implies that the pre-natal inflammatory process may play a role in pre-term delivery *per se*. An increased systematic inflammatory response has been reported as a specific risk factor for pre-term delivery and levels of intra-amniotic $LTC_4$, which is a precursor of $LTE_4$, were found to be elevated in studies on Rhesus monkeys in labour; therefore, the elevated levels in the present study may be caused by maternal uterine contractions and separation of the placenta [31]. In the evaluation of urinary $LTE_4$ in paediatric patients who were born as pre-term infants, Evans et al. [17] revealed that these children had elevated urinary $LTE_4$ levels and an increased incidence of bronchial asthma. Halvorsen et al. [32] noted increased $LTE_4$ levels and an increased tendency to develop airway hyper-responsiveness (AHR) in a population of young people who were born weighing less than 1000 g or before 28 weeks of gestational age. In correlation with subsequent BPD, Davidson et al. [33] reported elevated levels of urinary $LTE_4$ in a CLD group on the 30th day of life in pre-term infants who had a birth weight of less than 1000 g; however, another study by Sheikh et al. [34] reported no differences in the concentration of $LTE_4$ on the 2nd, 7th or 28th day of life according to the presence of BPD after correction for confounding factors. In the present study, although there was no significant difference

between the no/mild and the moderate/severe BPD groups, when we compared 'classic' and 'atypical' BPD groups, there was a significant increase in urinary $LTE_4$ levels on day 7 in the 'atypical' BPD group. We also found that the levels of $LTE_4$ on day 7 were significantly higher in the severe BPD group than in the mild/moderate BPD group when we divided the 'atypical' BPD group according to the severity of BPD (data not shown). These findings lead us to speculate that there is a possible sustained inflammatory insult in this group during the first week of life, despite minimal early lung disease and less mechanical and oxidative lung injury. However, the incidence of sepsis, NEC and maternal CAM showed no significant differences between the 'atypical' and 'classic' groups, so the underling mechanism of the sustained inflammatory process remains unclear. Further study is necessary to elucidate the role of the inflammatory process in the development of 'atypical' BPD.

Previous studies have revealed that CAM is correlated with the development of BPD [7,9]; however, a few reports have shown negative correlations between hCAM and BPD [8] or no correlations at all [6,13]. In the present study, there were no significant differences in the frequency of cCAM and hCAM between the no/mild BPD and moderate/severe BPD groups, nor between the 'classic' and 'atypical' BPD groups. However, increased levels of 8-OHdG on the 1st day of life were observed with marginal significance ($p = 0.052$) in the population with maternal hCAM as compared to those without hCAM (data not shown). This suggests the possibility of increased susceptibility to oxidative stress in infants with maternal hCAM.

## Conclusions

The 8-OHdG levels, which reflect the extent of oxidative stress, on day 7 is an independent risk factor for developing moderate/severe BPD and the levels of 8-OHdG on the 3rd day showed significant correlation to the duration of mechanical ventilation. These results suggest that urinary 8-OHdG is a possible effective indicator of the subsequent development of BPD and its severity and oxidative DNA damage is a crucial risk factor even in current BPD despite minimal respiratory therapy. On the other hand, sustained increased levels of urinary $LTE_4$ on the 7th day that was shown only in 'atypical' BPD, suggest a persistent inflammatory process despite the absence of RDS and minimal mechanical ventilation in the early neonatal period in this group. These results may imply heterogeneity in pathogenesis and clinical course of BPD in the current era.

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## Declaration of Interest

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