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To cite this article: Ozge Yilmaz, Ahmet Turkeli, Ece Onur, Sema Bilge & Hasan Yuksel (2017): Secondhand tobacco smoke and severity in wheezing children: Nasal oxidant stress and inflammation, Journal of Asthma, DOI: [10.1080/02770903.2017.1350970](https://doi.org/10.1080/02770903.2017.1350970)

To link to this article: <http://dx.doi.org/10.1080/02770903.2017.1350970>



Published online: 07 Sep 2017.



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## Secondhand tobacco smoke and severity in wheezing children: Nasal oxidant stress and inflammation

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### ABSTRACT

**Objectives:** Prenatal and postnatal smoke exposures are associated with many lung diseases in children due to impaired lung function, increased inflammation, and oxidative stress. We aimed to determine the influence of secondhand tobacco smoke exposure on the levels of nasal glutathione, IL-8, IL-17, MMP-9, and TIMP-1, as well as serum surfactant protein-D (SP-D) in wheezy children. **Methods:** We enrolled 150 children with recurrent wheezing and recorded wheezing characteristics at enrollment. We measured the levels of serum cotinine, SP-D, nasal glutathione, IL-8, IL-17, MMP-9, and TIMP-1. Serum cotinine levels between 3 and 12 ng/mL, and above 12 ng/mL were defined as lower and higher level secondhand tobacco smoke exposure, respectively. The ANOVA test, Pearson's correlation analysis and multivariate analysis with a linear regression test were used for the statistical analysis. **Results:** Ninety-one children had been exposed to lower level secondhand tobacco smoke, while 24 children were exposed to higher level secondhand tobacco smoke. Thirty-five children were not exposed to cigarette smoke. Wheezing symptom scores were higher in exposed children ( $p = 0.03$ ). Levels of other biomarkers showed no significant difference. **Conclusions:** Secondhand tobacco smoke exposure is associated with more severe respiratory symptoms in wheezing children. However, levels of nasal or serum inflammatory markers fail to explain this association, either because of different mechanical factors in the process or due to low levels of the biomarkers especially in nasal secretions.

### ARTICLE HISTORY

Received 5 February 2017

Revised 20 June 2017

Accepted 1 July 2017

### KEYWORDS

Glutathione; neutrophilic inflammation; secondhand tobacco smoke exposure; surfactant protein-D; wheezing

### Introduction

Secondhand tobacco smoke exposure is associated with an increased risk of pulmonary problems, such as asthma, in children, especially if their parents are smokers. This is not restricted to younger ages but continues later into childhood [1, 2]. Maternal smoking increases the risk of wheezing in children by 28–75% [2]. Moreover, secondhand tobacco smoke exposure increases the detrimental effects from other environmental pollution factors, while prenatal and infantile smoke exposures increase the influence of traffic-related pollution on the wheezing risk [3, 4]. These detrimental effects have many underlying mechanisms such as impaired lung function, increased inflammation, and oxidative stress [5].

Much previous research has epidemiologically investigated the relationship between wheezing and secondhand tobacco smoke exposures in children [2]. However, further information on pathogenic mechanisms will help us to better understand this relationship and improve the standard treatment for these cases. While increased oxidative stress is one of the pathogenic mechanisms associated with wheezing in children exposed to secondhand

smoke, there are other mechanisms that also need to be investigated, such as neutrophilic inflammation, systemic inflammation, and inflammatory damage [6, 7].

Therefore, this study investigated the association of nasal levels of antioxidant glutathione, tissue damage markers such as matrix metalloproteinase (MMP)-9 and the tissue inhibitor matrix metalloproteinase (TIMP)-1 and neutrophilic-inflammation-linked cytokines IL-8 and IL-17, as well as serum levels of inflammatory markers like surfactant protein-D (SP-D) in wheezing children exposed to secondhand tobacco smoke. These markers were investigated considering that they have a contribution to pathogenesis of adult lung diseases like chronic obstructive pulmonary disease (COPD).

### Methods and materials

#### Study population

We consecutively enrolled wheezing children aged—one to three years who were presented to the Pediatric Allergy and Pulmonology Department between January 2012 and

January 2013. We enrolled only children who resided in the city to minimize the variation in air pollution exposure among the group. Recurrent wheezing was defined as the presence of three or more doctor-diagnosed wheezing episodes.

Exclusion criteria included the presence of any other established pulmonary disease, such as cystic fibrosis, tuberculosis, congenital anatomic airway anomaly, or diagnosis of immunodeficiency. Moreover, we excluded children who had an upper respiratory tract infection during the previous month to avoid infection-related changes in nasal inflammation. We also excluded children who had used anti-inflammatory treatments such as inhaled or systemic steroids during the previous three-month period because these may influence the levels of the nasal biomarkers.

### **Study design and ethical approval**

The ethics committee of Celal Bayar University hospital approved this cross-sectional study. The parents of the children enrolled provided informed consent.

### **Study procedures**

At the beginning of the study, we recorded sociodemographic characteristics of the children enrolled such as age, gender, family history of pulmonary diseases, and maternal smoking during pregnancy. This information was recorded through the use of questionnaire that is administered by the researchers to the mothers. Maternal smoking during pregnancy was recorded according to the mothers' declaration to the question. We assessed wheezing symptoms and scored them by considering respiratory rate, retractions, and wheezing using the method suggested by Gajdos et al. [8]. We also took a blood sample to measure IgE, SP-D, and cotinine levels, as well as anasal lavage sample to measure glutathione, IL-8, IL-17, MMP-9, and TIMP-1. Nasal lavage samples were stored below  $-80^{\circ}\text{C}$  until measurements were complete.

### **Nasal lavage**

We performed nasal lavage by instilling 4 mL of saline into each nostril using a pediatric feeding catheter with the patient in a seated position. We then collected the saline aspirated from each nostril and stored it under  $-80^{\circ}\text{C}$  until we measured it for biomarkers [9].

### **Blood sampling and assay**

We sampled venous blood from an antecubital vein, inoculated it into non-anti-coagulated tubes, and protected it

from light. We then centrifuged the blood samples at 800 g for 10 minutes at  $4^{\circ}\text{C}$ , and then stored the serum samples at  $-70^{\circ}\text{C}$  until use.

### **Determination of smoke exposure**

We used the level of serum cotinine, measured by enzyme-linked immunosorbent assay (ELISA), to classify children as unexposed to secondhand smoke or exposed to low- or high-level secondhand tobacco smoke. In this study, we defined children with serum cotinine levels between 3 and 12 ng/mL as suffering from low-level secondhand tobacco smoke exposure and the ones with levels above 12 ng/mL as suffering from high-level secondhand smoke exposure as suggested in the literature [10].

### **Biochemical measurement**

In serum samples, we twice assayed cotinine concentrations using an ELISA kit from Bioquant (Catalog Number: BQ 096D) with the mean of the two measurements giving the final result. The analytic sensitivity of the cotinine kit was 1 ng/mL.

In nasal lavage samples, we assayed IL-8 concentrations using an ELISA kit from Human, the IL-8/NAP-1 Platinum ELISA (eBioscience, Bender MedSystems GmbH Campus Vienna Biocenter 2 1030 Vienna, Austria). Its analytical sensitivity was 2 pg/mL with intraassay coefficients of variation of 5% for 184.5 pg/mL and 5.4% for 11 pg/mL. The interassay coefficient of variation was 7.5% for 272 pg/mL. We assayed IL-17 concentrations using an ELISA kit from Human, the IL-17/ELISA (eBioscience, Bender MedSystems GmbH Campus Vienna Biocenter 2 1030 Vienna, Austria). The analytical sensitivity of IL-17 was 0.5 pg/mL with an intraassay coefficient of variation of 6.6% for 18.5 pg/mL. The interassay coefficient of variation was 8.9% for 58.3 pg/mL. We assayed MMP-9 concentrations using an ELISA kit from Human, the MMP-9/Platinum ELISA (eBioscience, Bender MedSystems GmbH Campus Vienna Biocenter 2 1030 Vienna, Austria). The analytical sensitivity of the MMP-9 was 0.005 ng/mL with an intraassay coefficient of variation of 7.7% for 132.2 ng/mL. The interassay coefficient of variation was 12.2% for 110.2 ng/mL. We assayed TIMP-1 concentrations using an ELISA kit from Human, the TIMP-1/Platinum ELISA (eBioscience, Bender MedSystems GmbH Campus Vienna Biocenter 2 1030 Vienna, Austria). The analytical sensitivity of the TIMP-1 was 10 pg/mL with an intraassay coefficient of variation of 4% for 202 pg/mL. Nasal IL-17 and MMP-9 levels were undetectable; therefore, they were not included in the final analysis.

**Table 1.** General characteristics of the study groups\*.

|                                          | Low-level tobacco smoke exposure (+) N = 24 | High-level tobacco smoke exposure (+) N = 91 | Tobacco smoke exposure (–) N = 35 | p**   |
|------------------------------------------|---------------------------------------------|----------------------------------------------|-----------------------------------|-------|
| Male sex                                 | 19(79.2)                                    | 63(70.8)                                     | 16(45.7)                          | 0.01  |
| Maternal smoking during pregnancy (+)    | 9 (37.5)                                    | 7(7.8)                                       | 5(14.3)                           | 0.001 |
| Family history of pulmonary diseases (+) | 9(37.5)                                     | 44(48.4)                                     | 23(65.7)                          | 0.08  |
| Reported in-house smoking (+)            | 16 (66.7)                                   | 37 (40.7)                                    | 15(42.9)                          | 0.07  |
| Reported outside smoking (+)             | 17(70.8)                                    | 38(41.8)                                     | 15(42.9)                          | 0.03  |

Notes.\*Values are expressed as n (% among the group).

\*\*The Chi-square test was used in the statistical analysis.

We performed all measurements of biomarkers in nasal lavage twice and took the mean of the two measurements for the final result.

We assessed nasal glutathione levels with the HPLC method, using the Agilent Technologies (Waldbronn Germany) HPLC system and Chrom systems Diagnostics kit (Kit code: AV 6600 Glutathione E, Martinsried-Germany) to measure reduced glutathione.

In serum samples, we assayed human surfactant D concentrations using an ELISA kit from BioVendor (Cat No.: RD194059100). The analytical sensitivity of surfactant D was 0.2 ng/mL with an intraassay coefficient of variation of 3.7% for 256 ng/mL. The interassay coefficient of variation was 7.5% for 129 ng/mL.

### Statistical analysis

To calculate the sample size, we used glutathione as the primary outcome with an effect size of 0.2, standard deviation of 0.4, power of 0.8, and alpha of 0.05 [11, 12]. Our estimation of the rate of secondhand tobacco smoke exposure in the study population was one-fourth.

The statistical analysis used SPSS 15.0 (Chicago, IL) with statistical significance defined as  $p < 0.05$ . The ANOVA test compared age, wheezing score, and biomarker levels between the two study groups, while Pearson's correlation analysis assessed correlations. The multivariate analysis with a linear regression test taking age, gender, maternal smoking history during pregnancy and family history of pulmonary disease as confounders was performed for each of the biomarkers as dependent variables.

## Results

### Sociodemographic characteristics

Of the 150 children enrolled in the study, 35 children were not exposed to secondhand tobacco smoke, while 91 and 34 children were exposed to low- and high-level secondhand tobacco smoke, respectively. 38% among the girls and 16.3% among the boys were not exposed ( $p = 0.01$ ). Maternal smoking during pregnancy was reported in 37.5% of the high-level secondhand tobacco smoke exposed group and 14.3% of the non-exposed group ( $p = 0.001$ ) (Table 1). Reported frequency of in-house smoking was not significantly different between the groups (42.9% vs 40.7% and 66.7% in non-exposed vs low- and high-level secondhand tobacco exposed groups, respectively,  $p = 0.07$ ). However, the presence of a family member who smokes outside the house was significantly higher for children with high-level secondhand tobacco exposure (70.8% in the high-level exposed group vs 42.9 and 41.8% in none and low-level exposed groups,  $p = 0.03$ ). Mean age of the children was  $20.0 \pm 10.0$  months in the non-exposed group and  $19.0 \pm 9.0$  and  $18 \pm 8$  months in the low- and high-level unexposed groups ( $p = 0.47$ ) (Table 2).

### Disease characteristics in secondhand smoke exposed and unexposed groups

Age at first wheezing was not significantly different between the two groups ( $6.4 \pm 4.9$  in the non-exposed vs  $7.3 \pm 5.6$  and  $6.5 \pm 4.4$  in the low- and high-level exposed

**Table 2.** Comparison of symptom score, IgE, and cotinine levels among children with and without secondhand tobacco smoke exposure\*.

|                          | Low-level tobacco smoke exposure (+) N = 24 | High-level tobacco smoke exposure (+) N = 91 | Tobacco smoke exposure (–) N = 35 | F**   | p**    |
|--------------------------|---------------------------------------------|----------------------------------------------|-----------------------------------|-------|--------|
| Age***                   | 19 ± 9                                      | 18 ± 8                                       | 20 ± 10                           | 0.75  | 0.47   |
| Age at first wheezing*** | 7.3 ± 5.6                                   | 6.5 ± 4.4                                    | 6.4 ± 4.9                         | 0.48  | 0.62   |
| Symptom score            | 1.1 ± 0.9                                   | 1.5 ± 0.8                                    | 0.8 ± 0.9                         | 3.42  | 0.03   |
| IgE                      | 74.8 ± 95.6                                 | 58.9 ± 97.9                                  | 52.1 ± 79.2                       | 0.71  | 0.49   |
| Cotinine                 | 6.5 ± 2.5                                   | 25.9 ± 14.7                                  | 2.3 ± 0.6                         | 120.3 | <0.001 |

Notes.\*Values are expressed as mean ± standard deviation.

\*\*ANOVA was used in the statistical analysis.

\*\*\*Expressed in months.

**Table 3.** Comparison of cytokine and glutathione levels in nasal lavage and SP-D levels in blood samples from children with and without secondhand tobacco smoke exposure\*\*\*.

|             | Low-level tobacco smoke exposure (+) <i>N</i> = 24 | High-level tobacco smoke exposure (+) <i>N</i> = 91 | Tobacco smoke exposure (–) <i>N</i> = 35 | <i>F</i> ** | <i>p</i> ** |
|-------------|----------------------------------------------------|-----------------------------------------------------|------------------------------------------|-------------|-------------|
| Glutathione | 1.3 ± 0.6                                          | 1.6 ± 0.8                                           | 1.4 ± 0.6                                | 0.79        | 0.46        |
| SPD         | 45.1 ± 24.6                                        | 43.1 ± 21.4                                         | 50.5 ± 25.4                              | 0.81        | 0.45        |
| TIMP-1      | 493.8 ± 559.4                                      | 447.3 ± 627.0                                       | 607.7 ± 699.1                            | 0.47        | 0.63        |
| IL-8        | 50.9 ± 81.8                                        | 33.9 ± 43.3                                         | 35.2 ± 36.3                              | 0.79        | 0.45        |

Notes. \*Values are expressed as mean ± standard deviation.

\*\*ANOVA was used for the statistical analysis.

groups, respectively,  $p = 0.62$ ). Similarly, serum IgE levels were not significantly different among the groups ( $p = 0.49$ ). On the other hand, the mean wheezing symptom score was significantly higher in low- and high-level secondhand smoke exposed children than unexposed ones ( $1.1 \pm 0.9$  and  $1.5 \pm 0.8$  vs  $0.8 \pm 0.9$ , respectively,  $p = 0.03$ ) (Table 2).

### Comparison of biomarkers between secondhand smoke exposed and unexposed groups

Nasal lavage glutathione, IL-8 and TIMP-1 levels were not significantly different among the groups ( $p = 0.46$ ,  $p = 0.45$ ,  $0.63$ , respectively). Similarly, the serum inflammatory marker SP-D was not significantly different between the groups ( $p = 0.45$ ) (Table 3).

The multivariate analysis with the linear regression test taking age, gender, maternal smoking history during pregnancy and family history of pulmonary disease as confounders revealed that the difference of SP-D levels between the two groups was not significant ( $B = -5.8$ ,  $p = 0.08$ ). Similarly, glutathione was not significantly different ( $B = -0.02$ ,  $p = 0.83$ ). For TIMP-1 levels, the presence of secondhand tobacco exposure was not a factor ( $B = -9.2$ ,  $p = 0.92$ ); however, the family history of pulmonary diseases was indeed a factor ( $B = 301.8$ ,  $p = 0.008$ ). The multiple linear regression analysis failed to demonstrate a significant difference of IL-8 levels between the groups either ( $B = 2.4$ ,  $p = 0.82$ ).

### Correlation of symptom score and biomarkers in the groups

Among the whole cohort, serum cotinine and nasal lavage glutathione levels correlated with the wheezing symptom score ( $r = 0.29$ ,  $p < 0.001$  and  $r = 0.21$ ,  $p = 0.001$ , respectively). IL-8 levels correlated with TIMP-1 levels ( $r = 0.43$ ,  $p < 0.001$ ).

The analysis of the non-exposed group showed that cotinine levels correlated with nasal lavage IL-8 levels ( $r = -0.69$ ,  $p < 0.001$ ). There was no significant correlation between any other variables. Among the low-level

secondhand tobacco smoke exposed group, cotinine levels were significantly correlated with the symptom score ( $r = 0.48$ ,  $p < 0.001$ ), glutathione levels were correlated with the SP-D levels ( $r = 0.32$ ,  $p = 0.006$ ) and IL-8 levels were correlated with the TIMP-1 levels ( $r = 0.41$ ,  $p < 0.001$ ). However, in the high-level secondhand tobacco smoke exposed group, the only correlation demonstrated to be significant was between IL-8 and TIMP-1 levels ( $r = 0.83$ ,  $p < 0.001$ ).

### Discussion

We investigated the association of nasal levels of antioxidant glutathione, TIMP-1, IL-8 and serum levels of SP-D in wheezing children exposed to secondhand tobacco smoke. The results of this study show that secondhand tobacco smoke exposure is associated with increased symptom severity in wheezy children. However, neither increased systemic inflammation nor nasal lavage cytokine levels seemed influenced by secondhand tobacco smoke exposure in this group. Moreover, serum cotinine levels correlated with the symptom score in the low-level secondhand tobacco smoke exposed group but not in the non-exposed group, possibly indicating a threshold effect. In addition, the correlation of IL-8 with TIMP-1 in the secondhand smoke exposed group but not in the non-exposed group indicates a possible role of neutrophilic inflammation and tissue damage in exposed groups. Similarly, a significant correlation of SP-D with glutathione in the low-level exposed group may indicate a correlation between systemic inflammation and nasal antioxidant level with a threshold effect.

Secondhand tobacco smoke exposure is associated with pulmonary diseases, as has been documented in different sub-populations, including children [1, 2]. Our results demonstrate that the clinical severity of wheezing is higher in wheezing children exposed to low or high levels of secondhand tobacco smoke. However, none of the antioxidant or inflammatory markers which we investigated demonstrated a correlation with the symptom score indicating that this increased severity of wheezing in secondhand tobacco smoke exposed children is related to other mechanisms.

Endogenous and exogenous oxidant stresses cause damage to DNA, proteins, and lipids; bronchial epithelium is especially vulnerable to these effects. Glutathione is one of the antioxidant mechanisms that protect the airways from oxidant damage [13]. A study that explored the influence of diesel exhaust exposure on lung epithelial lining fluid and nasal lavage revealed that glutathione levels decreased significantly, implying that these thiol antioxidants play a protective role by moving onto the surface of lung [14]. On the other hand, the ozone exposure in air pollution has been demonstrated to decrease total glutathione levels in nasal lavage samples obtained from asthmatic children [11]. We failed to demonstrate a significant change in nasal lavage glutathione levels in children exposed to secondhand smoke. This might be attributable to the difference in age of the study populations and the combined nature of oxidants in cigarette smoke as well as the use of nasal lavage samples.

The activation of neutrophils and overexpression are both associated with pathophysiologic airway changes in many diseases such as asthma and COPD [11, 15]. Neutrophils are the mainstay cells in cigarette-smoke-associated lung injury and their levels are higher in the epithelium of smokers. Furthermore, an increase in neutrophils correlates with the lung function decline in these patients [11]. Neutrophil-associated markers, such as IL-8, MMP-9, and TIMP-1, have been found to be greater in the sputum of children with asthma compared to non-asthmatics [16]. Therefore, we assessed the levels of IL-8 as a neutrophil chemotactic cytokine in the nasal lavage fluid of wheezing children and examined its relationship with secondhand tobacco smoke exposure. We failed, however, to demonstrate a significant difference between wheezing children with secondhand tobacco smoke exposure and those without. This is an unexpected finding, considering the previous research on the issue; however, it may be explained by the indirect nature of nasal lavage as an indicator of lung inflammation and by the common role of neutrophils in both groups, considering the presence of wheezing. Wheezing itself may be associated with an increased inflammation, which is neutrophilic in nature, and this might have masked the influence of secondhand smoke [17]. In concordance with the lack of difference found in nasal IL-8 levels, TIMP-1 was not significantly different between the two groups of wheezy children in our study. Indicating their neutrophilic background, these biomarkers significantly correlated with each other in groups both exposed and unexposed to secondhand smoke. However, IL-8 was found to be correlated with TIMP-1 levels as expected.

SP-D, which is mainly synthesized in the lung tissue, is elevated in serum in lung pathologies, most probably due to lung epithelial cell injury [22, 23]. Serum levels of SP-D

have been shown to be a marker of inflammation correlated with disease severity in bronchiolitis and COPD [22, 23]. Considering the inflammatory influence of secondhand smoke, we compared serum SP-D levels in wheezy children exposed and unexposed to secondhand smoke but did not detect a significant difference. This is similar to another study on adolescent smokers which did not detect a significant difference in serum SP-D with smoking despite decreased exhaled breath nitric oxide [24]. The study attributed this to early influences of smoking on some markers but a lack of influence on deep-lung epithelium may be the case in our study too [24].

The limitations of our study include the limited potential of nasal lavage fluid to indicate lower airway changes. However, obtaining bronchoalveolar lavage, a more direct indicator of the lower airways, was not considered ethical. Moreover, previous research has shown that the levels of cytokines measured in nasal lavage correlate with those in bronchoalveolar lavage, so it may be considered as an indirect method of lower airway assessment [25, 26]. Therefore, we chose to obtain nasal lavage fluid samples and make our inferences accordingly. Another limitation was that nasal lavage levels of the biomarkers are generally low, which leads to values close to the lower limit of detection of the ELISA kits. This decreased the sensitivity of the ELISA measurements.

Many previous studies have demonstrated that parental smoking is associated with serum cotinine levels in children and that secondhand smoke exposure is related to increased respiratory morbidity in these children [27, 28]. Our study adds to this knowledge by enrolling wheezing children who are already under the risk for adulthood respiratory morbidity and by the incorporation of different inflammatory and tissue damage mediators with a potential influence on the respiratory system such as SP-D and the glutathione levels.

In conclusion, secondhand tobacco smoke exposure in wheezing children is associated with increased wheezing symptom scores and this increase is correlated with cotinine levels. However, neither serum inflammatory marker SP-D nor nasal antioxidant levels seem to explain this relationship indicating the complex nature of the pathogenesis of the well-known hazardous effects of secondhand tobacco smoke exposure in wheezing children. Therefore, these results should be followed up in a special group of wheezing children with different samples or methods to measure inflammation and oxidant stress.

## Acknowledgements

The authors thank the MECOR faculty, especially Prof. Sonia Buist and Dr. Chris Goss for their generous help during the project preparation.

The findings of the study were presented at the Turkish Thoracic Society and American Thoracic Society Conferences.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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