Journal of Pediatric Surgery 55 (2020) 1516–1521

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

The relationship between oxidative stress markers in exhaled breath condensate and respiratory problems in patients with repaired esophageal atresia

Tutku Soyer [a,*], Özlem Boybeyi Türer [a], Esra Birben [b], Melike Kahveci [b], Gökçen Dilşa Tuğcu [c], Özge Uysal Soyer [b], Ebru Yalçın [c], Deniz Doğru [c], Uğur Özçelik [c], Nural Kiper [c], Bülent Şekerel [b], Feridun Cahit Tanyel [a]

[a] Hacettepe University Faculty of Medicine, Department of Pediatric Surgery, Ankara, Turkey
[b] Hacettepe University Faculty of Medicine, Department of Pediatric Allergy, Ankara, Turkey
[c] Hacettepe University Faculty of Medicine, Department of Pediatric Pulmonology, Ankara, Turkey

ARTICLE INFO

Article history:
Received 26 April 2019
Received in revised form 29 May 2019
Accepted 4 June 2019

Key words:
Esophageal atresia
Exhaled breath condensate
Gastroesophageal reflux
Children
Tracheoesophageal fistula
Oxidative stress

ABSTRACT

Aim: To evaluate the relationship between respiratory problems and oxidative stress markers in exhaled breath condensate (EBC) of patients with esophageal atresia (EA).
Methods: EA cases with respiratory problems were evaluated retrospectively for age, gender, the type of atresia, surgical treatment, outcome and respiratory symptoms. The results of gastroesophageal reflux (GER) treatment including the use of proton pump inhibitor (PPI) and fundoplication were also documented. EBC samples of 500–1000 μl were obtained by Ecoscreen machine in all cases. The levels of Glutathione (Glut), 8-isoprostane (8-iso), cysteinyl-leukotriene (Cys-LT) were measured with ELISA. Results were compared with healthy control subjects (CG, n = 26) and the relationship between oxidative stress markers and respiratory symptoms was evaluated. The results of GER treatment and oxidative stress markers in EBC were also correlated.
Results: Twenty-nine patients with a mean age of 8.8 years (3–14 years) were included. The male/female ratio was 16:13. The EA presented with distal fistula in 27 cases. While no fistula was observed in 1 case, both proximal and distal fistulae were present in another single case. Associated anomalies, most of which were cardiovascular anomalies, were observed in 65.5% (n = 19) of cases. The median Glut level was 1.03 mM/ml (0.93–1.15), iso-8 was 38.8 pg/mL (32.03–76.2) and Cys-LT was 0.44 pg/mL (20.17–61.3) in patients with EA. The median levels of oxidative markers in CG were 1.23 mM/mL (1.13–1.36), 66.3 pg/mL (33.5–106.7), and 56.9 pg/mL (27.4–80.1), respectively. Glut levels were significantly lower in EA cases compared to CG ($p = 0.01$). There was no significant difference between the groups regarding 8-iso and CYS-LT levels ($p = 0.9$, $p = 1.0$). Cys-LT levels were significantly lower in patients with PPI treatment [21.7 pg/mL (18.6–48.1)], when compared to patients without PPI treatment [41.1 pg/mL (22.5–83.1)] ($p = 0.04$) and healthy subjects [56.9 pg/mL (27.4–80.1)] ($p = 0.017$). The 8-iso levels were significantly decreased in cases with fundoplication compared to the patients without fundoplication ($p = 0.02$).
Conclusion: Glut – an antioxidant agent – levels were significantly lower in EBC of EA cases. The decrease in Cys-LT levels in cases with PPI treatment and in 8-iso levels in patients with fundoplication suggests that the oxidative damage in EBC of EA cases may be correlated with GER and its management.
Type of study: Case control study
Level of evidence: Level III

Esophageal atresia (EA) is a rare congenital anomaly caused by the abnormal development of esophagus and trachea [1]. The survival rates exceed 90% with the advances in the surgical techniques and neonatal care [1–3]. Therefore, long-term complications have been the focus of interest and several studies have been performed to improve the quality of life. The most common long-term complications are upper gastrointestinal (UGI) and respiratory problems, including gastroesophageal reflux (GER), dysphagia, recurrent respiratory tract infections, wheezing, dyspnea, and persistent cough [2,3]. Although survival rates are improving, the quality of life of EA patients is likely to worsen owing to these complications.

Despite improvement in deglutition functions with age, respiratory problems may cause persistent pulmonary damage and chronic lung disease [2]. In one series, 46% of the cases had respiratory complications

* Corresponding author at: Hacettepe University Faculty of Medicine, Department of Pediatric Surgery, Sıhhiye, Ankara, Turkey. Tel.: +90 532 665 19 60.
E-mail address: soyer.tutku@gmail.com (T. Soyer).

https://doi.org/10.1016/j.jpedsurg.2019.06.007
0022-3468/

and 19% of patients had recurrent pneumonia [3]. Respiratory problems are because of GER in 74% of the cases. The recurrent tracheoesophageal fistula (TEF) (13%), tracheomalacia (13%) and **esophageal strictures** (10%) are among the other causes [3,4]. Besides having the same embryological origin, the worsening of respiratory symptoms by GER, esophageal strictures and esophageal dysmotility suggest a cause-and-effect relationship between gastrointestinal and respiratory complications [2,5]. Therefore, a multidisciplinary approach is needed to evaluate the long-term complications with a focus on the need for new and less invasive diagnostic methods.

The exhaled breath condensate (EBC) has been proposed as a noninvasive evaluation method of airways, being suitable especially for children [5]. EBC is collected while the patient breaths in tidal volume. The exhaled air travels through a cooling condensation system, providing the collection of exhaled air in liquid form that contains both volatile and nonvolatile compounds [6–8]. **The concentration of these compounds is very small in EBC; however, the alteration in their concentration may indicate an acute oxidative stress or a chronic inflammatory disease of respiratory tract. Main pathways altering the inflammatory state of airways are the formation of nitrogen reactive species and arachidonic acid metabolites. Therefore, measuring the concentration of those metabolites provides evaluation of respiratory tract inflammation and airway injury. The most common metabolites measured in EBC are nitrogen reactive species, oxidative stress markers, metabolites and proteins [7]. For instance, nitrate levels are increased in several respiratory diseases such as asthma and cystic fibrosis. On the other hand; glutathione levels decrease in the inflammatory disease, since it is an endogenous antioxidant. A sufficient level of glutathione is crucial to protect airways from oxidative injury which are exposed to exogenous oxidants [7]. After exposure to allergens or inflammatory triggers, leukotrienes are released from inflammatory cells causing bronchial constriction and increased mucus secretion. In addition, increased oxidative stress leads to lipid peroxidation and increased isoprostane formation [7]. While levels of leukotrienes and isoprostane are low in healthy subjects, their levels increase in the presence of oxidative and inflammatory injury in airways [7].**

Although the measurement of all these metabolites in EBC has been widely used in patients with asthma, chronic obstructive respiratory disease and GER disease [6–8], it has not yet been evaluated for EA patients with respiratory problems. Therefore, our study was performed to evaluate the relationship between oxidative stress markers in EBC in EA patients and respiratory problems.

1. Materials and methods

The patients between 2 and 15 years of age who were operated for EA and admitted to the departments of pediatric pulmonology, allergy and asthma were included in this study. The children between 2 and 15 years of age, who did not have any systemic disease, did not use any medicine, did not have asthma or any other respiratory disease were included in the study as healthy controls. The patients below 2 years of age, who had asthma attack or respiratory tract infection in the last 4 weeks before admission, were excluded from the study.

The medical records of EA patients were reviewed and demographic features, associated anomalies, type of atresia, surgical treatment and outcome of the cases were retrospectively documented. The results of (GER) treatment, including use of proton pump inhibitor (PPI) and fundoplication were also noted.

From all cases and controls, 500–1000 μl of EBC samples was collected by Ecoscreen machine. In EBC samples, Glutathione (Glut), 8-isoprostane (8-iso), cysteinyl-leukotriene (Cys-LT) levels were measured. The results of EA cases (EA group, n = 29) were compared with the healthy control subjects (CG, n = 26). The relationship between oxidative markers and respiratory symptoms was evaluated. The oxidative markers in EBC were compared between patients on and without PPI treatment. Also, same markers were compared for the EA cases with and without fundoplication.

This study was approved by the Local Institutional Ethical Committee (GO 18/110-05) and performed under the recommendations of the Helsinki Declaration of human rights. All patients and their families were informed about the study and informed consent approvals were collected from all participants.

1.1. Collection of EBC

The EBC was collected with EcoScreen system (Jaeger, Hoechberg, Germany), as defined in the literature [9]. The members of the study group were asked to breathe in normal tidal volume for 10 min into the chamber, where the exhaled air is separately collected and saliva filtered. The exhaled air travels through a lamellar condenser, which is surrounded by a cooling coverage with temperature of −15 °C or lower. The collected EBC was preserved at −80 °C.

1.2. Measurement of glutathione levels in EBC

Glutathione (GSH) is an antioxidant that prevents damage from reactive oxygen species and free oxygen radicals [6,7]. The GSH level in EBC was measured by the Glutathione Assay (Cayman Chemical, Ann Arbor, Michigan, USA), as recommended by the manufacturer. The sulfhydryl regions of GSH in EBC samples were reacted with the Ellman's reagent and were converted to 5-thio-2-nitrobenzoic acid (TNB). The absorbance of the yellow colored TNB was measured at 405 nM and the results were given in mM. 50 μL of each EBC sample was used for the assay.

1.3. Measurement of 8-isoprostane (8-iso) levels in EBC

Isoprostanes are metabolites of lipid peroxidation in vivo. The increased level of isoprostanes reflects increased oxidative stress with elevated lipid peroxidation, leading to membrane degeneration [7,8]. The level of 8-iso in EBC was measured with a specific enzyme-immunoassay (EIA) (Cayman Chemical, Ann Arbor, Michigan, USA). 50 μL EBC was used for the measurements and the detection limit of the assay is 2.7 pg/ml.

1.4. Measurement of cysteinyl-leukotriene (Cys-LT) levels in EBC

Leukotrienes are synthesized by the oxidation of arachidonic acid by 5-lipoxygenase in leukocytes. The release of leukotrienes is increased

Table 1
The symptoms, long-term respiratory complications and diagnosis and treatment of GER in patients with EA.

	N	%
Symptoms		
Vomiting	25	86
Chronic cough, wheezing	9	31
Hyperreactive airway	22	75.8
Tracheomalacia	4	13.7
Recurrent respiratory tract infection	20	69
Other GER symptoms (regurgitation, heart burn, etc.)	8	27.5
Long-term surgical complications		
Anastomotic stricture	19	65
Recurrent fistula	4	14
Resistant stricture (requiring more than 3 dilatation)	5	17
GER diagnosis		
Upper GI study	15	52
24 h pH-monitoring (RI ≫ 4%)	7	24
GER management		
Medical (PPI)	15	52
Surgical (Fundoplication)	9	31

Table 2
The median values of oxidative stress marker levels in EBC of CG and patients with EA (interquartile ranges are given in parentheses).

	EA (n = 29)	CG (n = 26)	
Glut (mM/ml)	1.03 (0.93–1.15)	1.23 (1.13–1.36)	**p = 0.01**
8-iso (pg/ml)	38.8 (32.03–76.2)	66.3 (33.5–106.7)	p ≫ 0.05
Cys-LT (pg/ml)	30.4 (20.17–61.3)	56.9 (27.4–80.1)	p ≫ 0.05

Glut: Glutathione, **8-iso**: 8-isoprostane, **Cys-LT**: cysteinyl-leukotriene.

during inflammation [7]. The cysteinyl-leukotriene (Cys-LT) is a leukotriene containing a cysteine molecule [7]. The level of Cys-LT in EBC was measured by using a competitive EIA (Cayman Chemical, Ann Arbor, Michigan, USA). Note that the detection limit of this assay is 34 pg/ml and the lowest standard is 7.8 pg/ml.

1.5. Statistical analysis

The statistical analysis was done by the application of the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM, USA). The descriptive values were calculated as means and medians within the 95% confidence interval. The comparison of oxidative stress markers between groups was evaluated with nonparametric tests. The p values ≪0.05 were considered as statistically significant.

2. Results

Twenty-nine patients with EA and 26 healthy controls (CG) were included in this study. The mean age of cases was 8.8 years (3–14 years) in EA and 9.6 years (5–16 years) in CG groups (p ≫ 0.05). The male to female ratio was 16:13 and 14:12, respectively.

All cases with EA were diagnosed during the first 48 h of life and underwent surgical treatment within the first 72 h. The types of the anomalies were as follows: EA with distal fistula in 27 cases (93%), isolated EA in 1 case, and EA with both proximal and distal fistula (n = 1). Associated anomalies were seen in 65.5% of the cases (n = 19), in which cardiovascular anomalies were the most common. Primary EA repair with fistula ligation was performed in all patients except in one case. The patient with isolated EA underwent **tube gastrostomy** in neonatal period. Since the primary anastomosis was not possible at the end of waiting period, the patient underwent esophagostomy and esophageal replacement with the right colon at the end of **one year.**

The mean postoperative follow-up of the patients was 8 years (5 years–11 years) and a wide range of gastrointestinal and respiratory complications was documented (Table 1). When we evaluated the surgical complications, anastomotic strictures were seen in 65% (n = 19) of the cases. Anastomotic strictures were managed by esophageal dilatations with a median number of 3 dilatations (min: 1, max: 25). Recurrent TEF was observed in 4 cases (14%) and surgical fistula ligation was performed in all cases. As a cause of respiratory problems, concomitant GER was investigated in all patients. Contrast UGI series (n = 15) and 24-h pH monitoring (n = 13) were used to evaluate GER disease. In 24% (n = 7) of patients, 24-h pH monitoring revealed the reflux index to be higher than 4%.

GER-related symptoms including recurrent respiratory tract infections (n = 20), resistant anastomotic strictures (n = 5), and vomiting (n = 25) were observed in 86% (n = 25) of the cases (Table 1). Proton pump inhibitor (PPI) was given to all patients during infancy and the treatment continued for patients with GER after one year of age. Patients, who did respond to the PPI treatment for 6 months and manifested ongoing GER complications such as strictures, esophagitis and recurrent pulmonary infections requiring hospitalization, underwent fundoplication. **The patients who underwent fundoplication did not continue to use PPI after the surgery**. Nine of the cases (31%) had Nissen fundoplication. The respiratory symptoms including wheezing and chronic cough were observed in 8 cases that are still under follow-up. **At the time of EBC collection, none of the patients were using PPI for at least 6 months. But all patients received PPI until 1 year of age and 9 cases underwent Nissen fundoplication at least 6 months before the EBC collection time.**

The median values of the oxidative stress marker levels are given in Table 2. The median values of Glut, 8-iso and Cys-LT levels in EA cases were 1.03 (0.93–1.15), 38.8 (32.03–76.2), and 30.44 (20.17–61.3), respectively. The median values of Glut, 8-iso and Cys-LT levels in CG, on the other hand, were 1.23 (1.13–1.36), 66.3 (33.5–106.7), and 56.9 (27.4–80.1), respectively. The comparison of oxidative stress marker levels between EA cases and CG revealed that the Glut levels were significantly lower in EA cases compared to CG (p = 0.01) (Fig. 1). There was no significant difference between the study group and healthy subjects, regarding the 8-iso and Cys-LT levels (p = 0,9, p = 1,0, respectively).

When we compared the median values of oxidative stress marker levels in EA in regard to the PPI treatment, a significant difference was detected in Cys-LT levels between patients under PPI treatment and ones without the treatment (Table 3). Patients with PPI had decreased

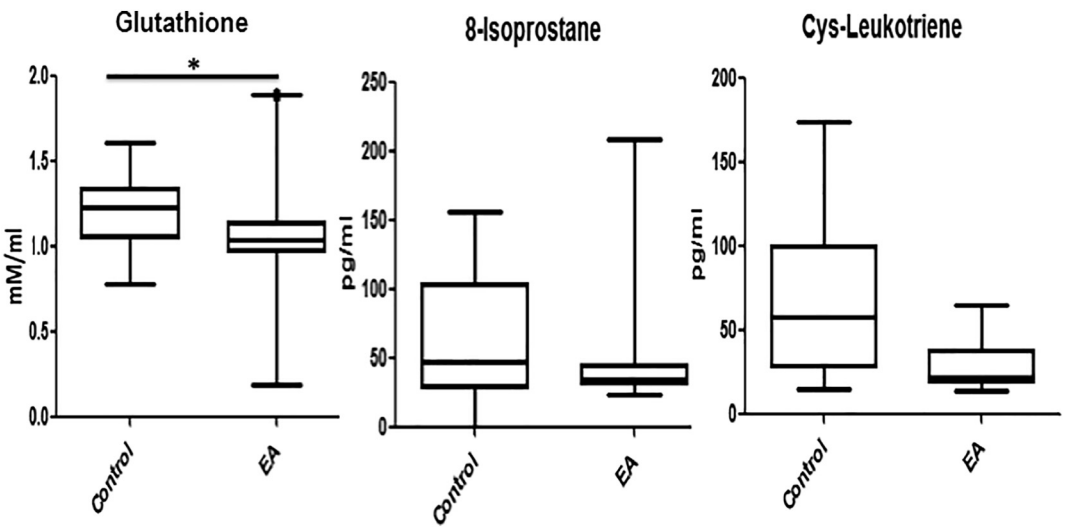

Fig. 1. The comparison of median levels oxidative stress markers (Glutathione, 8-isoprostane and cysteinyl-leukotriene) in EBC of CG and cases with EA (*: p ≪ 0.05).

Table 3
The median values of oxidative stress marker levels in EBC of CG, patients under PPI treatment [PPI (+) EA] and without PPI treatment EA patients [PPI (−) EA) (interquartile ranges given in parenthesis).

	PPI (+) EA	PPI (−) EA	CG
	(n = 15)	(n = 14)	(n = 26)
Glut (mM/ml)	1.04 (0.97–1.13) **α**	1.01 (0.7–1.19) **μ**	1.23 (1.13–1.36) **α, μ**
8-iso (pg/ml)	41.8 (31.4-83.8)	34.1 (32.2-63.1)	66.3 (33.5–106.7)
Cys-LT (pg/ml)	21.7 (18.6–48.1) **β***	41.1 (22.5–83.1) *	56.9 (27.4–80.1) **β**

Glut: Glutathione, **8-iso**: 8-isoprostane, **Cys-LT**: cysteinyl-leukotriene.
P values; *: 0.04, **β:** 0.017, **μ:** 0.037, **α:** 0.002.

Table 4
The median values of oxidative stress marker levels in EBC of EA patients with (EA + F) and without fundoplication (EA-F) (interquartile ranges given in parentheses).

	EA + F (n = 9)	EA-F (n = 20)	
Glut (mM/ml)	1.08 (1.02–1.15)	1 (0.68–1.15)	p ≫ 0.05
8-iso (pg/ml)	34.1 (29.9–47.2)	59.5 (42.2–86.3)	**p = 0.02**
CLT (pg/ml)	21.9 (19.6–52.9)	34.6 (20.2–70.9)	p ≫ 0.05

Glut: Glutathione, **8-iso**: 8-isoprostane, **Cys-LT**: cysteinyl-leukotriene.

levels of Cys-LT when compared to patients without PPI (p = 0.04), and the control group (p = 0.007) (Fig. 2).

The comparison of the median values of the oxidative stress marker levels in EA cases with and without fundoplication (F) is given in Table 4. The comparison results revealed that the 8-iso levels were significantly lower in cases with fundoplication (F), compared to the cases without F (p = 0.02) (Fig. 3).

When patients with versus without long-term respiratory complications were compared, patients with chronic cough had increased median levels of Cys-LT than the patients without chronic respiratory disease [70.71 (37.2–131.6) and 59.75 (13.8–554), respectively, (p = 0.002)]. There was no difference between these groups for Glu and iso-8 levels (p > 0.05) **(**Table 5**).**

3. Discussion

The patients with EA have gastrointestinal and respiratory problems not only in the neonatal period but also during infancy, adolescence and adulthood. The increased life expectancy of these patients increased the prevalence of these long-term problems that have become the focus of interest for the last years [2]. Among these complications, respiratory problems are the most influential factors for the quality of life of the patients with EA. The most commonly reported respiratory problems are chronic cough, wheezing, recurrent bronchitis, and dyspnea [2]. The most common gastrointestinal long-term problems including GER, dysphagia, esophageal stricture, and esophageal dysmotility problems contribute to and worsen the respiratory symptoms of EA patients [2]. Therefore, we aimed to evaluate the oxidative injury in the airways of children with EA and its relation to the respiratory symptoms.

To our knowledge, this is the first study investigating the oxidative stress marker levels in EBC in relation to the respiratory symptoms in EA patients. EBC is a noninvasive method to investigate the diseases of airways. The collection of the airway medium is simple, and enables to access the mediators of the oxidative burden in airways in children [10]. Since the alveoli are exposed to endogenous and exogenous oxidants, the presence of Glut in sufficient amounts in the airways is needed to prevent oxidative injury in airways [7]. The decreased level of Glut in EBC was reported in asthmatic and chronic obstructive respiratory disease [2,6,7]. In this study, the antioxidant **Glut** levels were significantly decreased in EA patients compared to the healthy subjects. The decreased Glut levels in the airways of EA patients may be because of either the chronic respiratory complications or congenital predisposition, which results in the lack / failure of an antioxidant mechanism. Several factors including GER, airway aspiration and surgical complications may lead to decreased antioxidant capacity in EA patients. Interestingly, Melek et al. reported that infants with EA had decreased activity of antioxidant enzymes [11]. When we compared the Glut levels in EBC with the other parameters such as GER treatment (both PPI use and fundoplication) and chronic respiratory complications, no statistical difference was detected. Therefore, we suggest that the decreased antioxidant capacity in airways of EA patients is not the result of a chronic airway disease, but may rather be one of the risk factors for long-term complications.

In this study, no statistical difference was detected between the EA cases and healthy controls in terms of 8-iso and Cys-LT levels. The measurable biomarkers in EBC are pH, nitrogen reactive species, oxidative stress markers, metabolites, proteins and amino acids [6–8,12,13]. The mean Cys-LT and 8-iso levels in EBC are affected by age and therefore, adjustment of different age groups is necessary for accurate comparison [14]. As the mean age of groups in our study was identical, our results are devoid of any age-related inconsistency. Since our findings did not reveal increased oxidative markers in EBC, other oxidative markers, different from 8-iso and Cys-LT, should be evaluated to have a firm conclusion about the oxidative injury in airways of patients with EA.

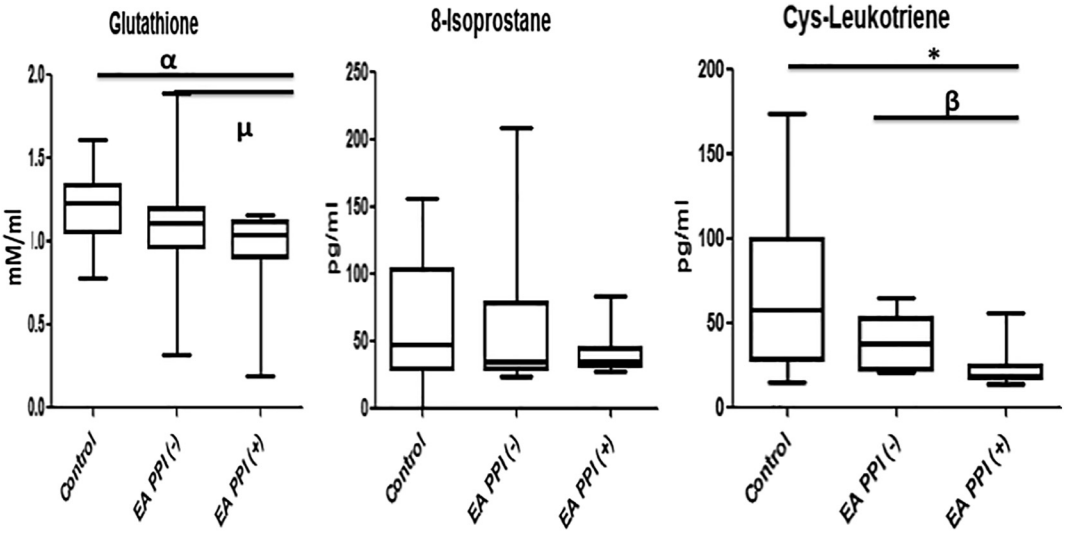

Fig. 2. The comparison of median levels of oxidative stress markers (Glutathione, 8-isoprostane and cysteinyl-leukotriene) in EBC of EA patients on PPI treatment and without PPI treatment (p values; ***:** 0.04, **β:** 0.017, **μ:** 0.037, **α:** 0.002).

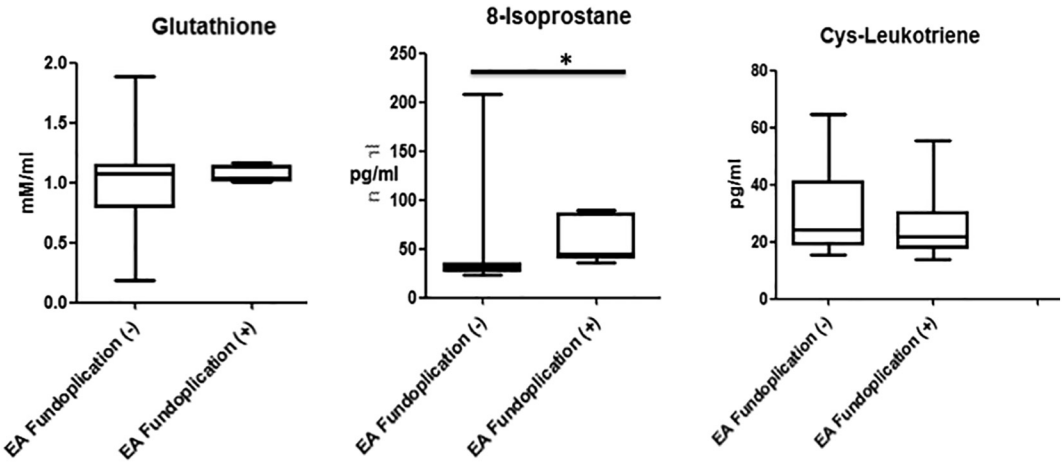

Fig. 3. The comparison of median levels of oxidative stress markers (Glutathione, 8-isoprostane and cysteinyl-leukotriene) in EBC of EA patients with and without fundoplication (*: $p \ll 0.05$).

GER is extremely common in children with EA with a frequency of 35% to 58% of patients [15]. It appears to be because of an intrinsic motor dysfunction of esophagus and/or shortened intraabdominal segment of esophagus owing to anastomotic tension [4]. GER may lead to esophageal strictures, aspiration, pneumonia, bronchial hyperreactivity, and permanent lung or airway parenchymal damage. Fifty-six percent of patients responded to medical treatment of GER and the rest underwent fundoplication [4]. The European Society of Pediatric Gastroenterology, Hepatology and Nutrition (**ESPGHAN**) guideline recommends a long-term antiacid treatment in all EA patients [2,13–16]. In 74% of EA cases, respiratory symptoms are related with GER. EBC represents the epithelial lining fluid of airways and can be used as a diagnostic tool for both respiratory and nonrespiratory diseases, such as GER, inflammatory bowel disease and cystic fibrosis [12,14,17]. The presence of pepsin in EBC was reported to suggest a possible relationship with GER disease in favor of microaspiration theory [14,18,19]. On the other hand, the detection of neurokinin A and Substance P in EBC supported the vagal reflex theory in the pathogenesis of respiratory symptoms in GER [16,20].

In this study, iso-8 and Cys-LT levels in EBC did not show statistical difference between the EA cases and healthy controls. However, our results suggest decreased oxidative markers in EA cases with GER treatment. The 8-iso levels are significantly decreased in EA cases with fundoplication. **The 8-isoprostane levels are reliable marker for lipid peroxidation and show significant increase in severe oxidative damage. The cases that underwent fundoplication were the ones who did not get better with PPI treatment and they probably had more severe inflammatory injury in airways associated with cell damage. Since severity of cell damage was different in patients with and without fundoplication, surgical treatment of GERD had significantly decreased 8-isoprostane levels. However, cases with PPI might have less severe injury. That might be the reason of the decreased levels of Cys-LT instead of 8-isoprostane levels in cases with PPI treatment**. The level of 8-iso was reported to be affected by the presence of GER in asthmatic patients and to be decreased at the end of 2 months of PPI treatment [21]. Additionally, we detected decreased levels of Cys-LT in children on PPI treatment. Our results confirm that medical and/or surgical treatment of GER causes decreased oxidative injury in airways of patients with EA. In addition to these results, EA patients with chronic respiratory problems had increased levels of Cys-LT, when compared to patients without respiratory complications. Similar to other chronic airway diseases, our results confirm that increased oxidative stress in EBC is because of chronic respiratory complications in EA patients.

Table 5
The median values of oxidative stress marker levels in EBC of patients with (CRC +) and without chronic respiratory complications (CRC−) (interquartile ranges given in parentheses).

	CRC + (n = 9)	CRC− (n = 20)	
Glut (mM/ml)	1.14 (0.32–1.89)	1.06 (0.19–3.04)	$p \gg 0.05$
8-iso (pg/ml)	60.6 (32.2–280.5)	59.5 (23.2–89.5)	$p \gg 0.05$
Cys-LT (pg/ml)	45.3 (37.2–131.65)	21.8 (13.8–584)	**$p = 0.002$**

Glut: Glutathione, **8-iso**: 8-isoprostane, **Cys-LT**: cysteinyl-leukotriene.

The most important limitation of our study is its retrospective design and the lack of EBC measurements prior to the PPI treatment and fundoplication. Although EBC examination is a common diagnostic approach in airway diseases, there is no standardization in dilution and sampling. Besides, the information on the normal levels of biomarkers in EBC is not available to consider as a reference. Despite these limitations, we report the first study that thoroughly investigates the oxidative stress marker levels in EBC in EA patients. Our results suggest decreased antioxidant mechanism in EA cases and that the oxidative marker levels decrease when GER is controlled by either PPI treatment or fundoplication.

In conclusion, Glut – an antioxidant agent – levels were significantly lower in EBC of EA cases. **This result makes us think that EA patients have decreased antioxidant defense activity which might be congenital in origin**. Given the observed decrease in Cys-LT levels in cases with PPI treatment and that of 8-iso levels in patients with fundoplication, our results strongly suggest that the oxidative damage in EBC of EA cases may be **related** to GER and its management. **Therefore, we suggest managing GER more comprehensively in all EA patients to prevent worsening of their respiratory problems.**

Acknowledgment

This study was presented at the Annual Congress of Turkish Pediatric Surgery Association in Izmir, Turkey, 2018.

This study was awarded with Prof. Akgün Hiçsönmez Best Scientific Research in Annual Congress of the Turkish Pediatric Surgery Association in Izmir, Turkey, 2018.

This study was funded through the scientific foundation of Turkish Academy of Sciences Member Prof. F.C. Tanyel.

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