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Original Article

Dynamic thiol/disulfide homeostasis in children with community-acquired pneumonia

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Abstract

***Background*:** Alteration in thiol level under oxidative stress may contribute to community-acquired pneumonia (CAP). The goal of this study was to determine whether there are changes in thiol/disulfide homeostasis and nitric oxide (NO) in children with CAP.

***Methods*:** In total, 130 participants were involved in the study. Of these, 65 had been diagnosed with CAP on admission, and the remaining 65 were healthy individuals. Serum total thiol and native thiol were measured in each participant using a novel automated spectrophotometric method. The amount of dynamic disulfide bonds and related ratios were calculated from these values. Serum NO was measured on chemiluminescence assay.

***Results*:** Average native thiol, total thiol, and disulfide in the CAP group were significantly lower than in the healthy individuals ($P < 0.0001$, $P < 0.0001$, $P = 0.0126$, respectively). In addition, disulfide/native thiol ($P = 0.0002$), and disulfide/total thiol ratios ($P = 0.0004$) were significantly higher, whereas the native thiol/total thiol ratio ($P = 0.0004$) was lower in the CAP group. High serum NO was noted in the CAP group ($P = 0.0003$), but there was no marked correlation between thiol/disulfide and NO.

***Conclusion*:** The changes in endogenous thiol levels under oxidative stress may be associated with the pathogenesis of CAP in pediatric patients.

Key words disulfide, nitric oxide, oxidative stress, pneumonia, thiol.

Community-acquired pneumonia (CAP) particularly refers to clinical signs and symptoms of pneumonia acquired outside a hospital setting.[1] Pneumonia remains one of the most common serious infections in childhood, accounting for 16% of all deaths in children <5 years of age.[2] The pathophysiology of CAP includes various mechanisms such as microorganism invasion, airway damage and activation of immune defense systems. During CAP an extensive influx of activated phagocytes into the lower airways is observed, which represents the first line of defense against invading microorganisms. The production of reactive oxygen species (ROS) and nitrogen species from neutrophils and macrophages is an important component of the host defense system for the process of pathogen clearance and modulation of immunological response.[3,4] This pulmonary increase in ROS level and proteolytic enzymes may be reflected at the systemic level as an increase in the oxidative stress in these patients.[5,6] Although oxidative stress is a crucial part of the host innate immune response to foreign pathogens, excessive oxidative stress can cause cellular damage and a systemic inflammatory response.[7,8] Chen *et al.* reported that patients with CAP had increased ROS and DNA damage in peripheral blood mononuclear cells.[9] Antioxidant vitamin C, vitamin E and glutathione (GSH) have also been shown to be diminished in children with acute pneumonia compared with controls.[10] A clinical trial also supports the relationship between GSH and CAP severity.[11] Collectively, these findings show that oxidant/antioxidant imbalance is present in CAP.

Thiol is an organic compound, and it contains an −SH group, which plays an important role in the suppression of oxidative stress in cells. The plasma thiol pool is mostly formed by albumin and protein thiols and, to a lesser extent, by low-molecular-weight thiols such as homocysteine, cysteine, cysteinylglycine, γ-glutamylcysteine, and GSH.[12] In the presence of oxidative stress, thiol groups are converted into disulfides. These disulfide bonds, however, are reversible, and can subsequently turn back into thiols. This cycle continues in a balanced state, and is referred to as dynamic thiol–disulfide homeostasis.[12–14] In disease processes associated with oxidative stress, an imbalance of dynamic thiol/disulfide homeostasis has been reported.[12,14] Currently, only one clinical study has established the association of thiol/disulfide homeostasis with CAP.[15] That study, however, was performed in adults, and there is no published research in children. Thus, the

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purpose of this study was to determine the possible role of thiol/disulfide homeostasis in children with CAP. We also investigated the contribution of serum nitric oxide (NO) to the pathophysiology of CAP, and compared the levels between children with CAP and controls.

Methods

Subjects

A total of 65 children with CAP (mean age, 2.8 ± 2.2 years; 29 boys, 36 girls) evaluated at pediatrics departments were recruited into this study. Moreover, 65 age- and sex-matched healthy control subjects (mean age, 3.0 ± 2.1 years; 31 boys, 34 girls) selected from the hospital's outpatient clinic, were also enrolled, for comparison. Children with hospital-acquired pneumonia, active tuberculosis, diabetes mellitus, malignancy, and those with underlying immunological or pulmonary disease were excluded from the study. Individuals with renal, gastrointestinal, endocrine or cardiac disease and hypertension, and those taking medication such as antioxidants or antibiotics were also excluded from the study. Diagnosis of CAP was based on the presence of clinical lower respiratory tract signs and symptoms, systemic manifestations and a new pulmonary infiltrate on chest radiography. Clinical diagnosis of CAP was supported by at least two of the following signs and symptoms: cough with purulent or mucopurulent sputum, dyspnea, chest pain, auscultation (rales and/or sign of consolidation), fever >38°C, or leukocytosis (white blood cells [WBC] $>10 \times 10^3/mm^3$). Bacterial and viral pathogens were detected using conventional methods. Microbiological tests such as blood culture, Gram stain, microscopy and culture of expectorated sputum or nasopharyngeal aspirates were performed to define the bacterial etiology of pneumonia, while serological tests were directed to atypical pathogens (*Mycoplasma pneumoniae, Chlamydia pneumoniae,* or *Legionella pneumophila*) or viruses. Local clinical ethics committee assessed and approved the study, and parental informed consent was obtained in each case. The present research was conducted according to the principles described in the Declaration of Helsinki.

Blood samples

In patients, blood sampling was done on admission. Biochemistry analysis was done ≤30 min after blood collection. For thiol/disulfide and NO analysis, serum was separated by centrifugation at 1,500 *g* for 10 min, placed into plain tubes and stored at −80°C until analysis.

Thiol/disulfide measurements

Native thiol (−SH) and total thiol (−SH + −S−S−) content in the serum samples was measured using the reagent kits from Rel Assay Diagnostics (Gaziantep, Turkey). These spectrophotometric methods, developed by Erel and Neselioglu,[14] were carried out using an auto-analyzer. The reducible disulfide bonds were first reduced to free-form functional thiol groups. The unused reductant, sodium borohydride, was consumed and removed with formaldehyde, and all of the thiol groups, including the reduced and native ones, were measured after reacting with 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB). Half of the difference between the total and native thiols yielded the dynamic disulfide (−S−S) content.

NO analysis

Nitrate and nitrite are the primary oxidation products of NO, and the stable nitrate/nitrite levels in plasma are generally used as an indicator of NO formation. The technique (NO/ozone chemiluminescence) used in the present study converts nitrate/nitrite back to NO and measures the NO level in a gaseous form. Serum NO was measured as described previously.[16] The serum samples were deproteinized with absolute ethanol at 0°C in a 1:2 v/v mix, incubated for 30 min at 0°C followed by centrifugation at 21 000 *g* for 5 min. The pellets were discarded and the supernatant was used to measure NO on the NO/ozone chemiluminescence technique (Model 280i NOA, Sievers Instruments, Boulder, CO, USA). Samples and standards were injected into the purge vessel to react with the reducing agent (vanadium III chloride dissolved in 1 mol/L HCl at 95°C), which converted nitrite, nitrate, and S-nitroso compounds to NO. A continuous stream of pure nitrogen purged the resultant NO from the reaction vessel to the chemiluminescence chamber. NO concentration of the sample was determined by comparison with the standard curve (obtained with sodium nitrate). NOAnalysis™ version 3.21 (Sievers) was used for data collection and analysis.

Statistical analysis

All data are given as mean ± SD or percentage. The distribution of all variables was checked using the Kolmogorov–Smirnov test. Mann–Whitney *U*-test was used for data with non-normal distribution. Otherwise, the differences between means of two groups were analyzed using unpaired Student's *t*-test. Gender of the two groups was analyzed using the chi-squared test. Pearson's test was used to assess the correlations, but Spearman correlation analysis was used for non-normally distributed data. GraphPad Instat version 3.05 (GraphPad Software, San Diego, CA, USA) was used. Statistical significance was accepted at the level of 0.05.

Results

Subject demographic and laboratory data are listed in Table 1. Compared with the controls, the average age, gender, weight, hemoglobin, platelet count, platelet distribution width, mean platelet volume, and neutrophils/lymphocytes ratio in the CAP group were similar. WBC, neutrophils, lymphocytes, NO, disulfide/native thiol ratio, and disulfide/total thiol ratio were all higher in the CAP group, and native thiol, total thiol,

disulfide, and native thiol/total thiol ratio were lower in the CAP group (Table 1).

On microbiology and serology, bacterial pneumonia was confirmed in 73.8% (48/65), atypical pneumonia in 10.8% (7/65), and viral pneumonia in 15.4% (10/65). Multiple pathogens were found in 27 patients (41.5%). The most common viruses were respiratory syncytial virus and influenza, and the most frequently detected bacteria were *Streptococcus pneumoniae* and *Haemophilus influenzae*.

Figure 1 shows the distribution of the native thiol, total thiol, and disulfide levels between the groups. These values were markedly low in the CAP group ($P < 0.0001$ for native thiol and total thiol; $P = 0.0126$ for disulfide). Although the disulfide/native thiol ($P = 0.0002$), and disulfide/total thiol ratios ($P = 0.0004$) were high, native thiol/total thiol ratio ($P = 0.0004$) was low in the CAP group (Fig. 2). Elevated NO was noted in the CAP group ($P = 0.0003$, Fig. 3).

On correlation analysis there were marked positive correlations between native thiol and total thiol, total thiol and disulfide, and disulfide and WBC (Table 2). Negative correlations were observed between native thiol and neutrophils, total thiol and neutrophils, and between total thiol and C-reactive protein (CRP). No significant correlations were noted between thiol and NO or CRP.

Discussion

The present study is the first study to assess dynamic thiol/disulfide homeostasis in the serum of children with CAP using a novel automated spectrophotometric method. We observed marked reductions in native thiol and total thiol in the CAP group. Consequently, disulfide was also low in the CAP group, but increased WBC, neutrophils, lymphocyte counts, augmented disulfide/native thiol and disulfide/total thiol ratios, and elevated CRP and NO were also detected. This suggests that endogenous thiols were depleted, and dynamic thiol/disulfide homeostasis is affected by CAP.

In principle, the airway epithelium carries an effective antioxidant defense system, including enzymatic antioxidants (e.g. catalase and superoxide dismutase), GSH, and enzymes associated with GSH.[17] Despite these protective mechanisms, oxidative stress in the airway epithelium is known to cause events such as DNA methylation, cell membrane damage, and lipid peroxidation, which result in widespread cellular and tissue damage contributing significantly to the worsening of lung infections.[18] GSH is well known as the most efficient antioxidant in the lung, and its depletion is associated with oxidative stress in many other diseases.[19] Oxidative stress is also important in the pathogenesis of CAP. Accumulating evidence suggests that serum total antioxidant status is decreased, and oxidative stress parameters are increased in patients with CAP.[5,10,15,20] Blood samples from patients with pneumonia demonstrated low levels and activities of antioxidants such as glutathione peroxide, superoxide dismutase, and GSH, compared with healthy controls, suggesting a high burden of oxidants.[10] Chen *et al.* also showed that severe CAP was associated with a significant increase in oxidative stress and pro-inflammatory mediators (tumor necrosis factor-α and interleukin-6) in peripheral blood and lung.[9] Recently, Parlak *et al.* found that total thiol and native thiol were significantly lower,

Table 1 Subject characteristics vs presence of CAP

	Control ($n = 65$) Mean ± SD or n (%)	CAP ($n = 65$) Mean ± SD or n (%)	P-value
Age (years)	3.0 ± 2.1	2.8 ± 2.2	0.5969
Gender			
Male	31 (47.7)	29 (44.6)	0.8603
Female	34 (52.3)	36 (55.4)	
Weight (kg)	14.3 ± 1.5	13.9 ± 1.8	0.1711
Hemoglobin (g/dL)	13.5 ± 2.4	13.1 ± 1.6	0.2656
White blood cells ($\times 10^3/mm^3$)	6.9 ± 3.0	12.1 ± 6.5	<0.0001
Platelet count ($\times 10^3/mm^3$)	261.8 ± 73.1	257.4 ± 77.3	0.7394
MPV (fL)	9.3 ± 0.7	9.1 ± 0.8	0.1318
PDW (%)	10.6 ± 1.9	10.1 ± 1.5	0.0983
Monocytes ($\times 10^3/mm^3$)	0.8 ± 0.3	1.2 ± 0.7	<0.0001
Neutrophils ($\times 10^3/mm^3$)	5.8 ± 2.6	6.7 ± 2.5	0.0464
Lymphocytes ($\times 10^3/mm^3$)	3.1 ± 1.7	3.7 ± 1.5	0.0348
CRP (mg/dL)	0.3 ± 0.1	11.9 ± 10.9	<0.0001
NO (μmol/L)	68.2 ± 42.5	106.1 ± 64.6	0.0003
Native thiol (μmol/L)	400.5 ± 105.8	227.7 ± 53.8	<0.0001
Total thiol (μmol/L)	448.4 ± 111.7	266.3 ± 58.9	<0.0001
Disulfide (μmol/L)	24.0 ± 11.5	19.3 ± 9.6	0.0126
Disulfide/Native thiol (%)	6.3 ± 3.1	8.9 ± 4.6	0.0002
Disulfide/Total thiol (%)	5.5 ± 2.4	7.3 ± 3.3	0.0004
Native thiol/Total thiol (%)	89.1 ± 4.8	85.4 ± 6.6	0.0004

CAP, community-acquired pneumonia; CRP, C-reactive protein; MPV, mean platelet volume; NO, nitric oxide; PDW, platelet distribution width.

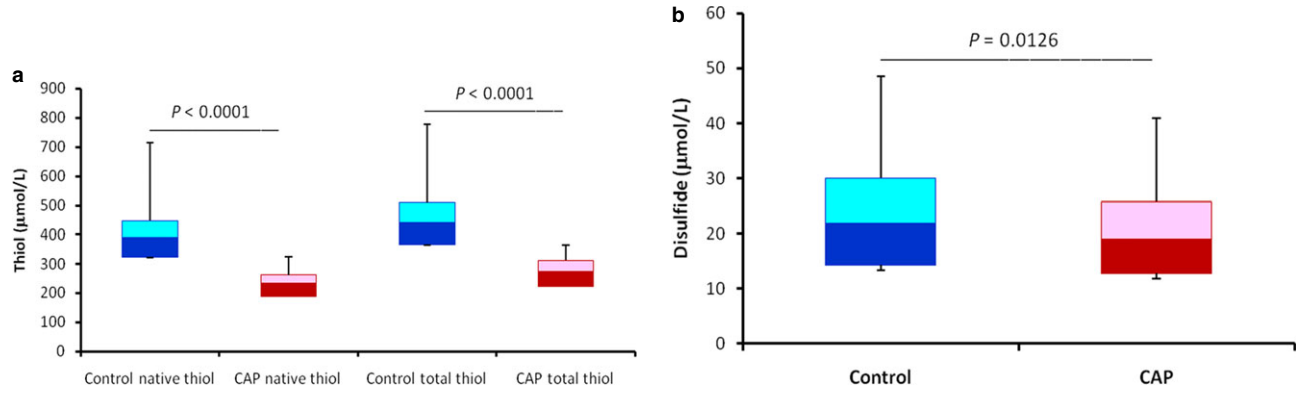

Fig. 1 (a) Native thiol, total thiol and (b) disulfide according to the presence of community-acquired pneumonia (CAP) in children (n = 65 in each group). Top and bottom ends of each box, 25th and 75th percentiles; horizontal line in the middle of the boxes, median; whiskers, lowest and highest values.

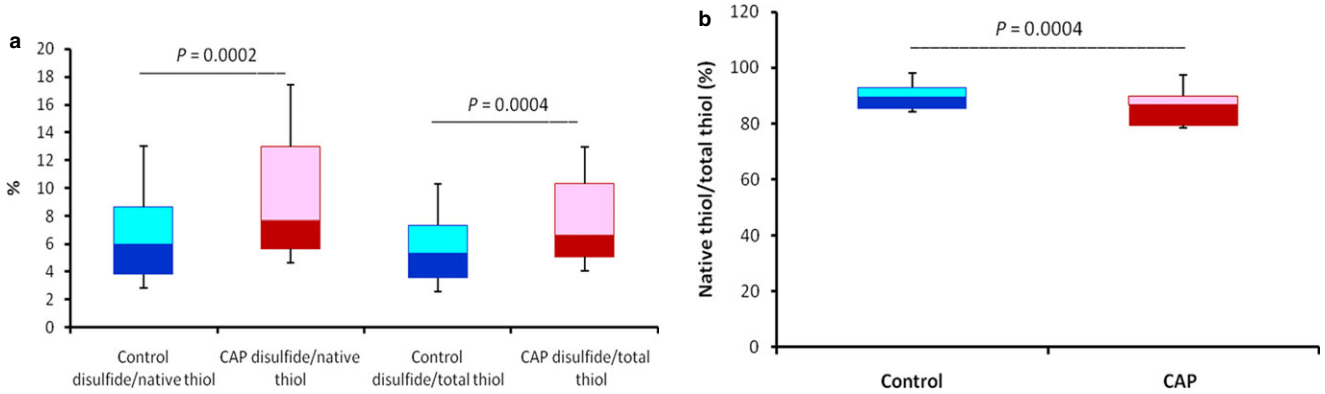

Fig. 2 (a) Disulfide/native thiol, disulfide/total thiol ratios and (b) native thiol/total thiol ratio according to the presence of community-acquired pneumonia (CAP) in children (n = 65 in each group). Top and bottom ends of each box, 25th and 75th percentiles; horizontal line in the middle of the boxes, median; whiskers, lowest and highest values.

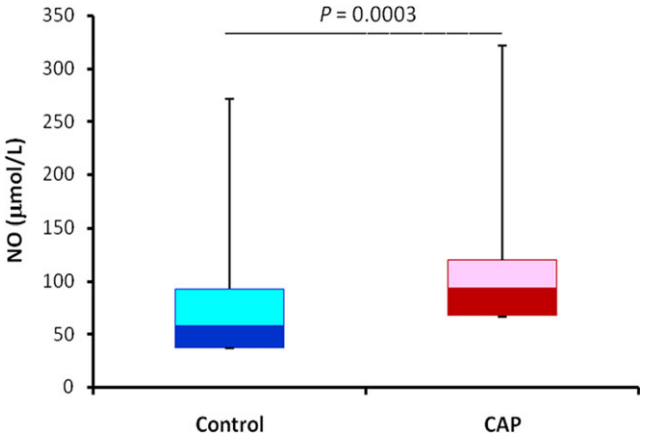

Fig. 3 Nitric oxide (NO) according to the presence of community-acquired pneumonia (CAP) in children (n = 65 in each group). Top and bottom ends of each box, 25th and 75th percentiles; horizontal line in the middle of the boxes, median; whiskers, lowest and highest values.

and disulfide/total thiol ratio was significantly higher in the CAP group compared with the controls, but that the disulfide levels were similar between the two groups.[15] The present results are in part consistent with that report, but we found that disulfide was significantly lower in the CAP group. Additionally, increased disulfide/native thiol ratio and diminished native thiol/total thiol ratio were noted in the present study. These ratios were not studied by Parlak *et al.*[15] Increased disulfide/total thiol and disulfide/native thiol ratios may indicate that the thiol/disulfide ratio had shifted towards disulfide bond formation, and that oxidative stress was augmented in CAP.

We found negative correlations between the neutrophils and thiol levels (both native and total thiols). This suggests that the ROS released from the high number of neutrophils in CAP patients may decrease the thiol levels by oxidizing the thiols. This is also in agreement with Parlak *et al.*, who noted similar correlations.[15] We also observed a marked positive correlation between disulfide and WBC count. Thus, it can be proposed that elevated ROS release from an increased number of leukocytes generates a high disulfide level.

Nitric oxide is part of the innate inflammatory response and its levels are expected to rise in acute lung infection.[3] Conflicting data on NO levels in pneumonia patients, however, have been reported. In exhaled breath analysis, the concentration of

Table 2 Correlations between thiol and disulfide in children with CAP

Parameters	Correlation coefficient (r)	Coefficient of determination (r^2)	P-value
Native thiol ↔ Neutrophils	−0.3558	0.1266	0.0104
Total thiol ↔ Neutrophils	−0.2851	0.0813	0.0425
Disulfide ↔ WBC	0.2960	0.0876	0.0349
Total thiol ↔ CRP	−0.2786	0.0776	0.0477

CAP, community-acquired pneumonia; CRP, C-reactive protein; WBC, white blood cells.

nitrate, a stable oxidative end product of NO metabolism, in patients with CAP was found to be higher than that in controls.[21] In another study, no difference in exhaled NO level was found between children with CAP and healthy controls.[22] We observed a marked increased in serum NO in children with CAP. In agreement with the present results, augmented NO production has been shown in the broncho-alveolar lavage of children with infective pneumonia.[23] This increase probably derives from an elevated lung NO production. NO is synthesized by the epithelium of the respiratory tract together with inflammatory cells.[3] During pneumonia, an extensive influx of activated phagocytes into the lower airways is observed.[24] We have detected increased leukocytes and monocytes. Thus, these cells may contribute to the increased NO level seen in the present study. NO rapidly interacts with superoxide to form peroxynitrite, which can oxidize a wide variety of biomolecules including thiols.[25] Therefore, NO can contribute to thiol/disulfide homeostasis in CAP.

In conclusion, elevated oxidative stress and impaired thiol/disulfide homeostasis play a substantial role in the etiology of CAP in children. Increased ROS generation in CAP can lead to the depletion of endogenous thiol.

Disclosure

The authors declare no conflict of interest.

Author contributions

M.T.T. and A.T.D. designed the study; M.T.T., L.T. and M.E.C. enrolled patients and collected data; S.D., A.S., N.E., E.B. and A.M. performed experiments; S.D. and A.T.D. analyzed the data and wrote the manuscript; M.T.T., S.D. and A.T.D. interpreted the data and reviewed the manuscript; A.T.D. supervised the whole study process. All authors read and approved the final manuscript.

References

1 Harris M, Clark J, Coote N *et al.* British Thoracic Society guidelines for the management of community acquired pneumonia in children: Update 2011. *Thorax* 2011; **66** (Suppl. 2): ii1–23.
2 World Health Organization. Pneumonia. Fact sheet, 7 November 2016. [Cited 17 June 2018.] Available from: https://www.who.int/news-room/fact-sheets/detail/pneumonia
3 Delclaux C, Azoulay E. Inflammatory response to infectious pulmonary injury. *Eur. Respir. J. Suppl.* 2003; **42**: 10s–4s.
4 Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radic. Biol. Med.* 2007; **42**: 153–64.
5 Katsoulis K, Kontakiotis T, Baltopoulos G, Kotsovili A, Legakis IN. Total antioxidant status and severity of community-acquired pneumonia: Are they correlated? *Respiration* 2005; **72**: 381–7.
6 Laskaj R, Dodig S, Cepelak I, Kuzman I. Gamma-glutamyltransferase activity and total antioxidant status in serum and platelets of patients with community-acquired pneumonia. *Arch. Med. Res.* 2007; **38**: 424–31.
7 Manda-Handzlik A, Demkow U. Neutrophils: The role of oxidative and nitrosative stress in health and disease. *Adv. Exp. Med. Biol.* 2015; **857**: 51–60.
8 Pruchniak MP, Araźna M, Demkow U. Biochemistry of oxidative stress. *Adv. Exp. Med. Biol.* 2016; **878**: 9–19.
9 Chen Y, Luo G, Yuan J *et al.* Vitamin C mitigates oxidative stress and tumor necrosis factor-alpha in severe community-acquired pneumonia and LPS-induced macrophages. *Mediators Inflamm.* 2014; **2014**: 426740.
10 Cemek M, Caksen H, Bayiroğlu F, Cemek F, Dede S. Oxidative stress and enzymic-non-enzymic antioxidant responses in children with acute pneumonia. *Cell Biochem. Funct.* 2006; **24**: 269–73.
11 Lai KY, Ng WY, Osburga Chan PK, Wong KF, Cheng F. High-dose N-acetylcysteine therapy for novel H_1N_1 influenza pneumonia. *Ann. Intern. Med.* 2010; **152**: 687–8.
12 Turell L, Radi R, Alvarez B. The thiol pool in human plasma: The central contribution of albumin to redox processes. *Free Radic. Biol. Med.* 2013; **65**: 244–53.
13 Jones DP, Liang Y. Measuring the poise of thiol/disulfide couples *in vivo*. *Free Radic. Biol. Med.* 2009; **47**: 1329–38.
14 Erel O, Neselioglu S. A novel and automated assay for thiol/disulphide homeostasis. *Clin. Biochem.* 2014; **47**: 326–32.
15 Parlak ES, Alisik M, Hezer H, Karalezli A, Hasanoglu HC, Erel O. Evaluation of dynamic thiol/disulfide redox state in community-acquired pneumonia. *Saudi Med. J.* 2018; **39**: 495–9.
16 Alasehirli B, Demiryürek S, Arica E, Gürsoy S, Demiryürek AT. No evidence for an association between the Glu298Asp polymorphism of the endothelial nitric oxide synthase gene and fibromyalgia syndrome. *Rheumatol. Int.* 2007; **27**: 275–80.
17 Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2002; **283**: L246–55.
18 Park HS, Kim SR, Lee YC. Impact of oxidative stress on lung diseases. *Respirology* 2009; **14**: 27–38.
19 Ghezzi P. Role of glutathione in immunity and inflammation in the lung. *Int. J. Gen. Med.* 2011; **4**: 105–13.
20 Nowak D, Zieba M, Zawiasa D, Roznіecki J, Król M. Changes of serum concentration of lipid peroxidation products

in patients with pneumonia. *Monaldi Arch. Chest Dis.* 1996; **51**: 188–93.
21 Corradi M, Pesci A, Casana R *et al.* Nitrate in exhaled breath condensate of patients with different airway diseases. *Nitric Oxide* 2003; **8**: 26–30.
22 Carraro S, Andreola B, Alinovi R *et al.* Exhaled leukotriene B4 in children with community acquired pneumonia. *Pediatr. Pulmonol.* 2008; **43**: 982–6.
23 Grasemann H, Ioannidis I, de Groot H, Ratjen F. Metabolites of nitric oxide in the lower respiratory tract of children. *Eur. J. Pediatr.* 1997; **156**: 575–8.
24 Moldoveanu B, Otmishi P, Jani P *et al.* Inflammatory mechanisms in the lung. *J. Inflamm. Res.* 2009; **2**: 1–11.
25 Demiryürek AT, Cakici I, Kanzik I. Peroxynitrite: A putative cytotoxin. *Pharmacol. Toxicol.* 1998; **82**: 113–7.