

DNA damage in children with asthma bronchiale and its association with oxidative and antioxidative measurements

Zeyrek D, Cakmak A, Atas A, Kocyigit A, Erel O. DNA damage in children with asthma bronchiale and its association with oxidative and antioxidative measurements.

Pediatr Allergy Immunol 2008.

© 2008 The Authors

Journal compilation © 2008 Blackwell Munksgaard

Increased production of reactive oxygen species leading to an imbalance between the oxidative forces and the antioxidant defense systems favoring an oxidative injury has been implicated in the pathogenesis of asthma. The aim of the study was to investigate the peripheral DNA damage, and its association with oxidative and antioxidative measurements in children with asthma bronchiale. The study population contained 42 children with asthma bronchiale and 32 healthy controls. DNA damage was assessed by alkaline comet assay in peripheral lymphocytes. Plasma levels of total antioxidant status (TAS), total peroxide concentration (LOOHs), and total oxidant status (TOS) were determined. In asthma bronchiale patients, DNA damage was significantly higher than in controls (17.9 ± 11.8 AU vs. 1.2 ± 2.0 AU, $p < 0.001$). Plasma TOS and LOOHs were higher in patients than in healthy controls (13.4 ± 7.0 vs. 9.0 ± 3.5 , $p = 0.002$; 9.9 ± 3.4 vs. 4.4 ± 1.5 , $p < 0.001$, respectively). Plasma TAS level in patients was higher than in healthy controls (5.5 ± 2.5 vs. 1.0 ± 0.6 , $p < 0.001$). DNA damage was correlated with TOS ($r = 0.616$, $p < 0.001$). The findings indicated that lymphocyte DNA damage level increases in children with asthma bronchiale. Elevated DNA damage may be related to increased oxidative stress. However, the mechanism of this association, and whether it is direct or indirect, remains to be explored.

Dost Zeyrek¹, Alpay Cakmak², Ali Atas², Abdurrahim Kocyigit³ and Ozcan Erel³

¹Department of Pediatrics, Division of Allergy and Pulmonology, Harran University School of Medicine, Sanliurfa, Turkey, Departments of ²Pediatrics and ³Biochemistry Harran University School of Medicine, Sanliurfa, Turkey

Key words: DNA damage; oxidative stress; asthma bronchiale; children

Dost Zeyrek, Department of Pediatrics, Division of Allergy and Pulmonology, Harran University School of Medicine, TR-63100, Sanliurfa, Turkey
Tel.: +90-414-3141170
Fax: +90-414-3151181
E-mail: dostzeyrek@yahoo.com

Accepted 11 June 2008

Asthma is a chronic inflammatory pulmonary disease related to increased oxidative stress (1). The association between chronic inflammation and oxidative stress is well documented. Elevated levels of reactive oxygen species (ROS), such as hydroxyl radicals, superoxides, and peroxides in inflammatory conditions have been reported previously (2). The cells infiltrating the bronchial mucosa in patients with asthma produce a variety of mediators including ROS (3). Increased production of ROS leading to an imbalance between the oxidative forces and the antioxidant defense systems favoring an oxidative injury has been implicated in the pathogenesis of asthma (4). ROS are highly reactive transient chemical

species, such as nitric oxide, superoxide, and hydroxyl radical anion. Normal cellular metabolism appears to be a primary source for endogenous ROS. Release of ROS from these cellular processes and their evasion from antioxidant pathways (e.g. glutathione peroxidase, vitamin E) result in the background levels of modification of cellular molecules, including deoxyribonucleic acid (DNA), which can be detected in normal tissue. However, when the production of damaging ROS exceeds the capacity of the body's antioxidant defenses to detoxify them, a condition known as oxidative stress occurs (5). Oxidative stress occurs in many allergic and immunologic disorders (6, 7).

DNA is a particular target for oxidation as damage may lead to important alterations. It is known that in cystic fibrosis and atopic dermatitis DNA damage is there (8, 9). Many oxidative footprints are thought to be the result of non-enzymatic reactions between reactive oxygen species and organic molecules, such as proteins, lipids, or DNA. It has been proposed that DNA damage induced by ROS may contribute to increased mutation rates, genome instability, apoptosis and associated tissue regeneration and cell proliferation (10). Therefore, we compared oxidative status, total antioxidant capacity and values of DNA damage in peripheral blood lymphocytes in patients with asthma bronchiale and healthy controls.

Methods

Study groups

Parents of all patients signed informed consent forms and the Ethics Committee of the Hospital approved the study. A total of 42 subjects between 6 and 15 yr of age who had been attending the Pediatric Allergy Unit of the Medical Faculty of Harran University for at least 1 yr were included in this study. The clinical severity of the asthma was determined using the criteria defined in the Global Initiative for Asthma guidelines (GINA) (11). Family history of atopy was considered positive if atopy was present in parents and/or siblings (bronchial asthma, allergic rhinitis, and atopic dermatitis). In all patients, allergen sensitivity was performed with specific IgE and skin prick test (SPT) to aeroallergens. Patients with clinical signs of asthma who had a positive sIgE in addition to sensitivity against at least one aeroallergen on the SPT were included in the atopic asthma groups. Immunocompromized patients, patients with a history of chronic inflammation/rheumatological disorder, and patients with autoimmune diseases were excluded. Asthma patients were not receiving any controller medication and had not had any symptoms of lower or upper respiratory tract infection or asthma exacerbation within the previous 4 wk.

The control group consisted of 32 age-matched healthy children (6–16 yr). Healthy children were chosen from those referred to a pediatric outpatient clinic in Harran University Hospital, where all children periodically undergo check-ups for their growth and development. Control patients were evaluated with regard to chronic and/or severe infections, rheumatological and autoimmune disorders, and familial and personal

history of atopy, and also by laboratory tests. Children were included in the control group if they had no personal and familial history of atopy and no signs of atopic disorder, and if they were negative for sIgE and SPT.

As it is known to have an effect on DNA damage and the patients came from non-smoking households, the controls were also selected from non-smoking households.

All the patients in the study were weighed and measured, then a calculation was made according to the Body Mass Index Standard Deviation Score (BMI SDS) (12).

Study measurements

Specific IgE levels. Serum sIgE measurements against inhaled allergens (house dust mite, yeasts, animal dander, grass pollen, trees, and wild grass) performed using CAP FEIA method (Pharmacia, Uppsala, Sweden). The result was considered positive if the measured value was greater than 0.35 kU/l.

Skin prick test. Prick test for aeroallergen sensitivity was performed using lancets (Stallerpoint, Paris, France) providing a standard puncture of 1 mm. Commercial allergen solutions manufactured by Allergopharma (Joachim Ganzer KG, Reinbeck, Germany) were used for the skin test. A total of 44 different allergens consisting of housedust mite, grass, wild grass, tree pollens, fungi, animal dander, and insects were tested. A positive SPT was that with at least a wheal of maximum diameter 3 mm, once the negative value had been subtracted.

Blood samples. After overnight fasting, venous blood was withdrawn into heparinized tubes and citrated tubes. One milliliter of heparinized blood was pipetted into another tube immediately to measure lymphocyte DNA damage. The remaining blood was centrifuged at 1300 g for 10 min to separate the plasma. The plasma samples were stored at -80°C until analysis of total antioxidant status (TAS), total oxidant status (TOS), and total peroxide concentration of plasma (LOOHs).

Lymphocyte separation. An amount of 1 ml heparinized blood was carefully layered over 1 ml Lymphoprep (Sigma and Aldrich, Oslo, Norway) and centrifuged for 35 min at 500 g and 25°C . The interface band containing lymphocyte was washed with phosphate-buffered saline (PBS) and then collected by 15 min centrifugation at 400 g. The resulting pellets were resuspended in PBS.

Membrane integrity was assessed by means of Trypan Blue exclusion method.

Measurement of lymphocyte DNA damage. Endogenous lymphocytes DNA damage was analyzed by alkaline comet assay according to Singh et al. (13) with minor modifications. Ten microliters of fresh lymphocyte cell suspension (around 20,000 cells) was mixed with 80 μ l of 0.7% low-melting-point agarose (LMA) (Sigma) in PBS at 37°C. Subsequently, 80 μ l of this mixture was layered onto slides that had previously been coated with 1.0% hot (60°C) normal melting point agarose (NMA) and covered with a coverslip at 4°C for at least 5 min to allow the agarose to solidify. After removing the coverslips, the slides were submersed in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na; 10 mM Tris-HCl, pH 10–10.5; 1% Triton X-100; and 10% DMSO added just before use) for at least 1 h. Slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/l NaOH and 1 mmol/l Na₂EDTA, pH > 13) at 4°C for unwinding (40 min) and then electrophoresed (25 V/300 mA, 25 min). All of the above steps were conducted under red light or without direct light in order to prevent additional DNA damage. After electrophoresis, the slides were stained with ethidium bromide (2 μ g/ml in distilled; 70 μ l/slide), covered with a coverslip and analyzed using a fluorescence microscope (Nikon, Tokyo, Japan) vided with epi-flourescence and equipped with rhodamine filter (excitation wavelength, 546 nm; barrier filter, 580 nm). The images of 100 randomly chosen nuclei (50 cells from each of two replicate slides) were analyzed visually from each subject, as described elsewhere (14). Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of either of 0, 1, 2, 3, or 4 (from undamaged class 0 to maximally damaged class 4), so that the total scores of the slides could be between 0 and 400 arbitrary units (AU). All procedures were completed by the same biochemistry staff, and DNA damage was detected by a single observer who was not aware of the subject's diagnosis. The blood samples from both patient and control cases were examined under the same conditions at the same time.

Measurement of total antioxidant status. Plasma TAS levels were determined using a novel automated measurement method, developed by Erel (15). In this method, hydroxyl radical, which is the most potent radical, is produced via Fenton reaction. In the classical Fenton reaction, the

hydroxyl radical is produced by mixing ferrous ion solution and hydrogen peroxide solution. In the most recently developed assay by Erel, the same reaction is used. In the assay, ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. The sequential produced radicals such as brown colored dianisidiny radical cation, produced by the hydroxyl radical, are also potent radicals. In this assay, the antioxidative effect of the sample against the potent-free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay has excellent precision values, of lower than 3%. The results are expressed as mmol Trolox equiv/l.

Measurement of total oxidant status. Plasma TOS levels were determined using a novel automated measurement method, developed by Erel (16). In this method, oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μ mol H₂O₂ equiv/l).

Measurement of total peroxide concentration of plasma (LOOHs). Total peroxide concentrations of plasma samples were determined using the 'FOX2' method with minor modifications (17). The FOX2 test system is based on oxidation of ferrous ion to ferric ion by various types of peroxides contained within the plasma samples, to produce a colored ferric-xylenol orange complex whose absorbance can be measured. The FOX2 reagent was prepared by dissolving ammonium ferrous sulphate (9.8 mg) in 250 mM H₂SO₄ (10 ml) to give a final concentration of 250 μ M ferrous ion in acid. This solution was then added to 90 ml of HPLC-grade methanol containing 79.2 mg butylated hydroxytoluene (BHT). Finally, 7.6 mg xylenol orange was added and stirred to make the final working reagent (250 μ M ammonium ferrous sulphate, 100 μ M xylenol orange, 25 mM H₂SO₄, and 4 mM BHT in 90% vol/vol methanol in a final volume of 100 ml). The blank working reagent contained all the components of the previous reagent except ferrous sulphate. Aliquots (200 μ l) of plasma were mixed with 1800 μ l FOX2

reagent. After incubation at room temperature for 30 min, the vials were centrifuged at 12,000 *g* for 10 min. Absorbance of the supernatant was then determined at 560 nm. The total peroxide content of the plasma samples was determined as a function of the absorbance difference between test and blank tubes using a solution of H₂O₂ as standard.

Statistical analyses

Data were analyzed using the SPSS® for Windows computing program (Version 11.0). The values are expressed as mean $\pm$ s.d. for the asthma patients and the healthy population, separately. When comparing the two groups, the unpaired *t*-test or Mann–Whitney *U*-test was used and correlation analyses were performed using Pearson's correlation test. Statistical significance was defined for *p* values of less than 0.05.

Results

Demographic and clinical data of patients with asthma bronchiale and controls are shown in Table 1. The mean ages of the patients and controls were 9.0 ± 2.8 and 9.4 ± 3.8 , respectively. We observed that all of our patients were in the mild-intermittent group according to GINA. There were no significant differences between the two groups with respect to age, gender, and BMI SDS. As can be seen in Fig. 1, the value of DNA damage was significantly higher in the asthma bronchiale subjects than in the healthy controls (17.9 ± 11.8 AU vs. 1.2 ± 2.0 AU, $p < 0.001$). Plasma TOS, and LOOHs were higher in patients than in healthy controls (13.4 ± 7.0 vs. 9.0 ± 3.5 , $p = 0.002$; 9.9 ± 3.4 vs. 4.4 ± 1.5 , $p < 0.001$, respectively) (Figs 2 and 3). Plasma TAS level in patients was higher than in healthy controls (5.5 ± 2.5 vs. 1.0 ± 0.6 , $p < 0.001$) (Fig. 4). As can be seen in Table 2, the value of DNA damage showed significantly positive correlations with TOS (Fig. 5) but LOOHs, and TAS had no correla-

Table 1. Demographic data of asthma bronchiale patients and healthy subjects

	Patients (n = 42)	Controls (n = 32)	OR (95% CI)	<i>p</i> *
Age (years)	9.0 ± 2.8	9.4 ± 3.8		0.60
Gender (M/F)	20/22	10/22	0.50 (0.17–1.45)	0.15
BMI SDS	0.77 ± 0.25	0.61 ± 0.20		0.14
Atopy in family (%)	17(40.4)	–		

The values represent the mean $\pm$ s.d.

*Significance was defined as $p < 0.05$.

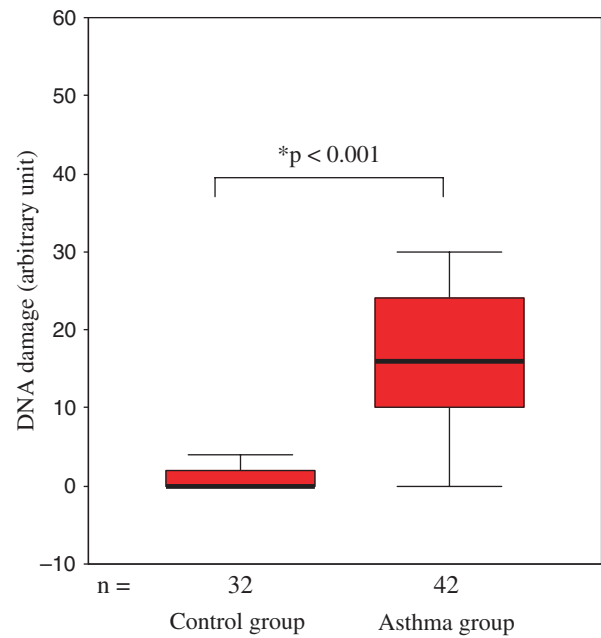


Fig. 1. DNA damage levels of asthma patients and healthy controls (mean $\pm$ s.d.). Difference between the two groups was compared using Mann–Whitney *U*-test.

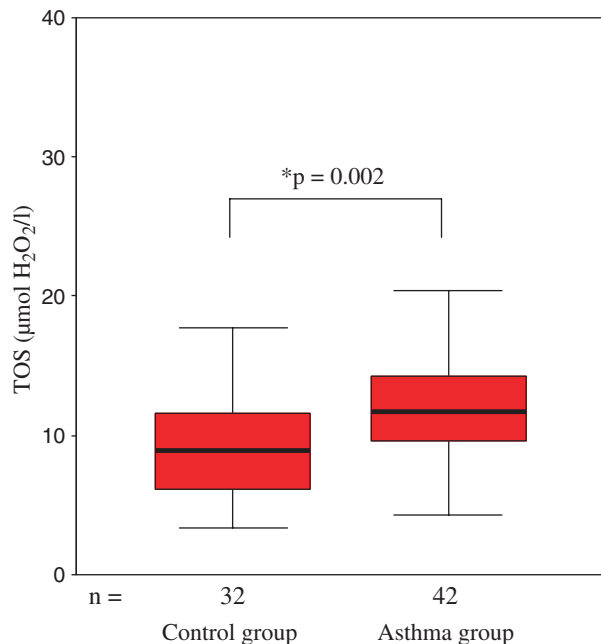


Fig. 2. TOS levels of asthma patients and healthy controls (mean $\pm$ s.d.). Difference between the two groups was compared using Mann–Whitney *U*-test.

tion ($r = 0.616$, $p < 0.001$, $r = 0.042$, $p = 0.78$, $r = -0.089$, $p = 0.57$, respectively).

Discussion

The results of the present study showed that DNA damage in peripheral blood lymphocytes

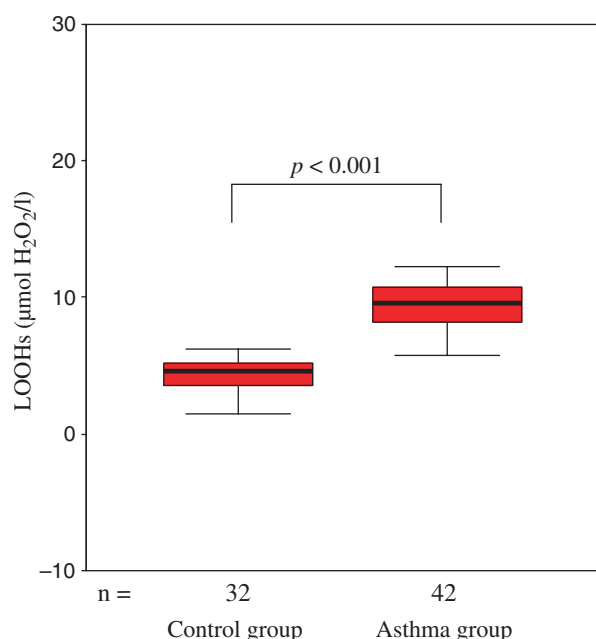


Fig. 3. LOOHs levels of asthma patients and healthy controls (mean $\pm$ s.d.). Difference between the two groups was compared using Mann–Whitney *U*-test.

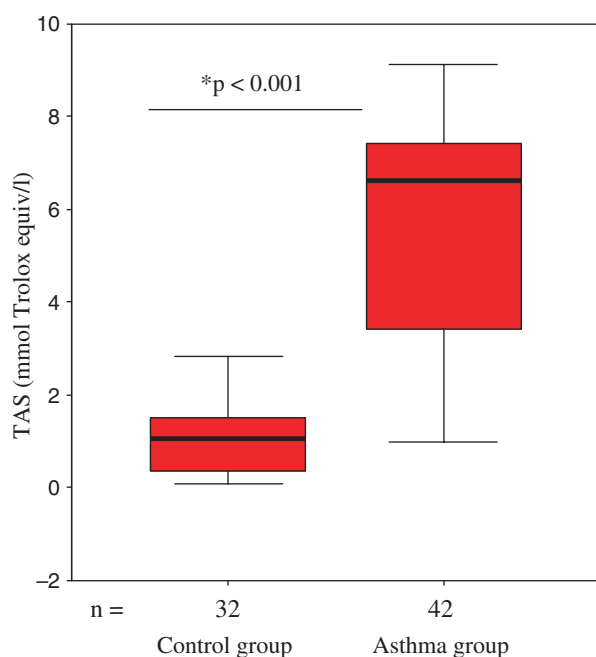


Fig. 4. TAS levels of asthma patients and healthy controls (mean $\pm$ s.d.). Difference between the two groups was compared using Mann–Whitney *U*-test.

Table 2. Correlations between DNA damage, TOS, LOOHs and TAS in patients

Parameters	<i>r</i>	<i>p</i>
DNA damage–TOS	0.616	<0.001
DNA damage–LOOHs	0.042	0.78
DNA damage–TAS	–0.089	0.57

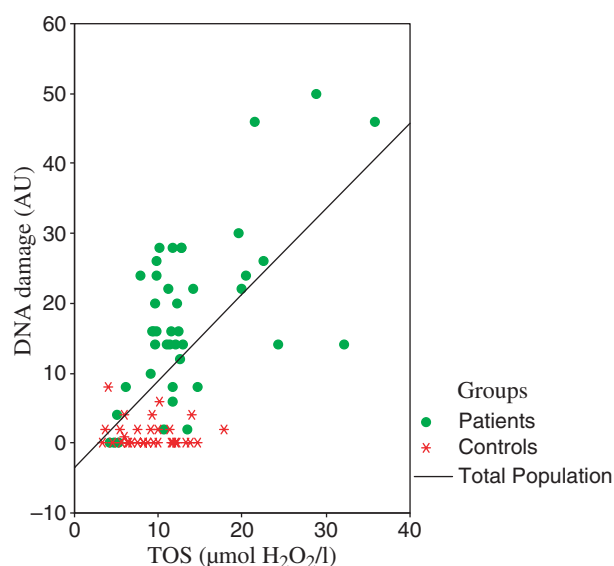


Fig. 5. Correlation between levels of DNA damage and total oxidant capacity values in subjects with asthma patients ($n = 42$, $r = 0.616$; $p < 0.001$), in subjects with controls ($n = 32$, $r = -0.108$; $p = 0.55$) and line of in all subjects ($n = 74$, $r = 0.622$; $p < 0.001$).

was significantly higher in patients with asthma bronchiale than in healthy subjects. Oxidative damage to DNA has been associated with a number of pathologies including neoplastic, neurodegenerative, cardiovascular, and autoimmune diseases (8). Many observations suggest that oxidative stress plays an important role in the pathogenesis of asthma. Airway inflammatory cells are the likely source of these increases (18).

It has been demonstrated that oxidative stress in chronic inflammation can lead to DNA damage, including DNA adducts, strand breaks and other lesions (18). The molecular basis for the increased risk is thought to be twofold: generation by inflammatory macrophages of ROS leads to DNA damage in the surrounding epithelial cells and enhanced proliferative signals mediated by cytokines released by inflammatory cells increase the number of cells at risk for mutations.

In the present study, we have shown that patients with asthma have increased TOS levels, LOOHs levels, TAS levels, and DNA damage in plasma compared with that seen in healthy subjects.

Many observations suggest that levels of oxidative stress are increased in children and in adults with asthma, not only in the lungs but also in the circulation. Excessive production of oxidants occurs spontaneously or after stimulation in blood leukocytes of stable asthmatic patients

compared with normal subjects (19). Several published studies have put forward the idea that when the oxidant system increases, there is a decrease in the antioxidant system. (20, 21). In our study group, it was determined that TOS and TAS increased together. In a study similar to ours, Nadeem et al. (19) recorded that when there was an increase in the oxidant system there was also an increase in the antioxidant system.

They also showed increased superoxide dismutase activity in red blood cells and increased total blood glutathione. Oxidative stress is a key component of inflammation and inflammatory disorders. Host antioxidant systems are generally activated in response to the oxidant attack, but individuals have different capacities of antioxidant defense, which are in part genetically determined (22). A study by Ercan et al. (23) showed that there were genetic differences in the antioxidant response. Various other studies have shown that as oxidative stress increases so the antioxidant capacity increases as a protective mechanism (24, 25).

Due to the socioeconomic conditions in the region (low incomes, insufficient health, and education services) our cases were diagnosed with asthma at a late stage and were not having regular check-ups. In this situation, they are vulnerable to long-term oxidative stress and so the development of the antioxidant system could be in response to that. However, in our patients, increased antioxidants indicate precautions against DNA damage but this occurs despite the increase in the antioxidant system. As the increased antioxidant system is not able to bring the oxidative stress level back to normal, the DNA damage is not prevented.

Several studies reveal that antioxidant dietary supplementation with vitamins, such as ascorbate and vitamin E to have been of no benefit. (26, 27). However, this is a controversial point as there are also studies which demonstrate that supplementation has been beneficial (28, 29).

The inflammation of the airway varies according to the severity of the asthma (1). Altindag et al. (30) reported an association between DNA damage, oxidative stress, and disease activity in rheumatoid arthritis patients. Our study aimed to evaluate the relationship between DNA damage, oxidative stress, and the disease activity. However, as all our patients were mild intermittent asthma sufferers, we were unable to make an evaluation. We believe that it is important to carry out this comparison from the aspect of understanding the relationship between asthma and DNA damage. This can be seen as a deficit of our study.

In conclusion, to the best of our knowledge, this is the first report which demonstrates the association between DNA damage and oxidative stress in children with asthma bronchiale. Our observations suggest that there is a link between the increased levels of oxidative stress and DNA damage seen in patients with asthma bronchiale, when compared with those seen in healthy controls. However, the nature of this link, and whether it is direct or indirect, remains to be explored.

Therefore, we are of the opinion that a comparison of mild and severe asthma patients with early and late diagnosis, undertaking regular and irregular treatment, and a comparison of their prognoses, would contribute to the understanding of the role played by DNA damage in asthma.

References

1. VIGNOLA AM, CHANEZ P, CAMPBELL AM, et al. Airway inflammation in mild intermittent and in persistent asthma. *Am J Respir Crit Care Med* 1998; 157: 403–9.
2. WISEMAN H, HALLIWELL B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J* 1996; 313: 17–29.
3. ANDREADIS AA, HAZEN SL, COMHAIR SA, ERZURUM SC. Oxidative and nitrosative events in asthma. *Free Radic Biol Med* 2003; 35: 213–5.
4. OWEN S, PEARSON D, SUAREZ-MENDEZ V, O'DRISCOLL R, WOODCOCK A. Evidence of free-radical activity in asthma. *N Engl J Med* 1991; 325: 586–7.
5. SARBAN S, KOCYIGIT A, YAZAR M, ISIKAN UE. Plasma total antioxidant capacity, lipid peroxidation, and erythrocyte antioxidant enzyme activities in patients with rheumatoid arthritis and osteoarthritis. *Clin Biochem* 2005; 38: 981–6.
6. OGASAWARA H, YOSHIMURA S, KUMOI T. Hydrogen peroxide generation by eosinophils in allergic rhinitis. *Auris Nasus Larynx* 1991; 18: 133–43.
7. POLLA BS, EZEKOWITZ RA, LEUNG DY. Monocytes from patients with atopic dermatitis are primed for superoxide production. *J Allergy Clin Immunol* 1992; 89: 545–51.
8. COOKE MS, OLINSKI R, EVANS MD. Does measurement of oxidative damage to DNA have clinical significance? *Clin Chim Acta* 2006; 365: 30–49.
9. TSUBOI H, KOUHA K, TAKEUCHI H, et al. 8-hydroxydeoxyguanosine in urine as an index of oxidative damage to DNA in the evaluation of atopic dermatitis. *Br J Dermatol* 1998; 138: 1033–5.
10. SAWA T, OHSHIMA H. Nitrate DNA damage in inflammation and its possible role in carcinogenesis. *Nitric Oxide* 2006; 14: 91–100.
11. GLOBAL INITIATIVE FOR ASTHMA. Global Strategy for Asthma Management and Prevention, revised edn. Bethesda, MD: National Institutes of Health, National Heart Lung and Blood Institute, 2002.
12. Available at: <http://cdc.gov/nchs/about/major/nhanes/growthcharts/datafiles.htm>.
13. SINGH NP, MCCOY MT, TICE RR, SCHNEIDER EL. A simple technique for quantitation of low levels of DNA

- damage in individual cells. *Exp Cell Res* 1988; 175: 184–91.
14. LAMPIDS TJ, KURTOGLU M, MAHER JC, et al. Efficacy of 2-halogen substituted D-glucose analogs in blocking glycolysis and killing “hypoxic tumor cells”. *Cancer Chemother Pharmacol* 2006; 58: 725–34.
15. EREL O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* 2004; 37: 277–85.
16. EREL O. A new automated colorimetric method for measuring total oxidant status. *Clin Biochem* 2005; 38: 1103–11.
17. MIYAZAWA T. Determination of phospholipids hydroperoxides in human blood plasma by a chemiluminescence’s-HPLC assay. *Free Radic Biol Med* 1989; 7: 209–17.
18. SANDERS SP, ZWEIER JL, HARRISON SJ, TRUSH MA, REMBISH SJ, LIU MC. Spontaneous oxygen radical production at sites of antigen challenge in allergic subjects. *Am J Respir Crit Care Med* 1995; 151: 1725–33.
19. NADEEM A, CHHABRA SK, MASOOD A, et al. Increased oxidative stress and altered levels of antioxidants in asthma. *J Allergy Clin Immunol* 2003; 111: 72–8.
20. LIAO MF, CHEN CC, HSU MH. Evaluation of the serum antioxidant status in asthmatic children. *Acta Paediatr Taiwan* 2004; 45: 213–7.
21. HANTA S, KULECI S, CANACANKATAN N, et al. The oxidant-antioxidant balance in mild asthmatic patients. *Lung* 2003; 181: 347–52.
22. BARNES PJ. Reactive oxygen species and airway inflammation. *Free Radic Biol Med* 1990; 9: 235–43.
23. ERCAN H, BIRBEN E, DIZDAR EA, et al. Oxidative stress and genetic and epidemiologic determinants of oxidant injury in childhood asthma. *J Allergy Clin Immunol* 2006; 118: 1097–104.
24. JENKINS RR. Free radical chemistry: relationship to exercise. *Sports Med* 1988; 5: 156–70.
25. SEN CK. Oxidants and antioxidants in exercise. *J Appl Physiol* 1995; 79: 675–86.
26. TING S, MANSFIELD LE, YARBROUGH J. Effects of ascorbic acid on pulmonary functions in mild asthma. *J Asthma* 1983; 20: 39–42.
27. PEARSON PJ, LEWIS SA, BRITTON J, FOGARTY A. Vitamin E supplements in asthma: a parallel group randomised placebo controlled trial. *Thorax* 2004; 59: 652–6.
28. ROMIEU I, SIENRA-MONGE JJ, RAMÍREZ-AGUILAR M, et al. Antioxidant supplementation and lung functions among children with asthma exposed to high levels of air pollutants. *Am J Respir Crit Care Med* 2002; 166: 703–9.
29. SIENRA-MONGE JJ, RAMIREZ-AGUILAR M, MORENO-MACIAS H, et al. Antioxidant supplementation and nasal inflammatory responses among young asthmatics exposed to high levels of ozone. *Clin Exp Immunol* 2004; 138: 317–22.
30. ALTINDAG O, KARAKOC M, KOCYIGIT A, CELIK H, SORAN N. Increased DNA damage and oxidative stress in patients with rheumatoid arthritis. *Clin Biochem* 2007; 40: 167–71.