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ORIGINAL ARTICLE

Alteration in systemic markers of oxidative and antioxidative status in Tunisian patients with asthma: relationships with clinical severity and airflow limitation

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Abstract

Objective: This study aims to determine the systemic oxidant-antioxidant status in Tunisian patients with asthma. **Methods:** We evaluated the levels of malondialdehyde (MDA) as thiobarbituric acid complexes, total protein carbonyls (PCs) and advanced oxidation protein products (AOPP). The levels of total thiols, protein sulfhydryls, glutathione (GSH), together with hydrogen peroxide, ascorbic acid, iron and total antioxidant status (TAS) were colorimetrically estimated. Glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) activities were assessed in plasma and erythrocytes by spectrophotometry. We also determined the levels of nitric oxide (NO) and peroxynitrite in plasma from asthmatic patients and healthy controls. The volume of fractionated exhaled NO (FeNO) was evaluated by the Medisoft HypAir method. Estimation of DNA damage was determined using the comet assay. **Results:** Asthmatic patients showed increased levels of MDA in comparison to healthy controls ($p < 0.001$), while no significant difference was found in protein carbonyls ($p = 0.79$) and AOPP ($p = 0.98$). Patients with asthma also had significantly lower levels of total thiols (355.9 ± 15.72 versus 667.9 ± 22.65 , $p < 0.001$), protein sulfhydryls (333.99 ± 16.41 versus 591.95 ± 24.28 , $p < 0.001$) and glutathione ($p < 0.001$). They also showed decreased GSH-Px activity ($p < 0.001$), whereas no significant differences in measurements of catalase and SOD enzyme activities were observed between the two groups (respectively, $p = 0.06$ and $p = 0.55$). In addition, ascorbic acid and nitric oxide levels were decreased in asthmatics in comparison to controls ($p < 0.01$). **Conclusions:** Our findings highlight that oxidative stress and defective anti-oxidative status are major alterations in Tunisian patients with asthma.

Keywords

Asthma, airflow limitation, antioxidants, glutathione, oxidative stress

History

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Introduction

Asthma is a heterogeneous chronic airway disease that affects more than 600 million people worldwide. It is characterized by chronic inflammation of the airways involving variable airflow obstruction and increased responsiveness to a variety of stimuli [1]. It is reported that inflammatory cells found in the airways of asthmatic patients can generate large amounts of oxidants that can overwhelm antioxidant defense systems of the lungs [2]. Increased oxidative stress in asthma is, also, thought to affect mucus hypersecretion and alter capillary endothelium, which may cause a leak of reactive oxygen species (ROS) into systemic circulation [3,4]. Free radicals

can react with cell membrane fatty acids and produce lipid peroxides, which can alter membrane fluidity and affect membrane-bound receptors and enzymes. ROS can, also, affect amino-acid side chains and induce protein-protein cross-linkages resulting in fragmentation and generation of protein oxidation products [5].

Previous studies have shown that oxidative stress markers are altered in the air spaces, fluids and sputum of patients with asthma [3]. Reports on the relationships between the oxidant-antioxidant imbalance and pulmonary function, as well as disease progression showed, however, contradictory findings. As an example, studies examining plasmatic levels of NO in asthmatic patients have not always produced uniform results [6,7]. In addition, investigations on the relationships between several oxidant markers and pulmonary function were inconsistent [8]. Data reported so far seem, also, to be contradictory or inconclusive regarding the

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activities of SOD, CAT and GSH-Px in patients with asthma [9,10].

To the best of our knowledge, no previous studies have investigated the impact of oxidative stress in the development of asthma in Tunisians. In the current report, we, therefore measured simultaneous changes in 16 parameters of the oxidant-antioxidant status and related these to disease progression and rate of decline of lung function. The extent of oxidative damage was estimated on the basis of its foremost biomarkers: lipid peroxidation products, protein carbonyls, advanced oxidation products, non-enzymatic antioxidants (GSH, total thiols, protein sulfhydryls) and antioxidant defense enzymes (GSH-Px, catalase, SOD). Another aim of the study was to compare the levels of Fe_{NO}, NO and peroxynitrite in asthmatic patients and to evaluate their impact on disease stage and airflow obstruction.

Patients and methods

Study subjects

Patients with bronchial asthma ($N=151$, 45% males) and healthy controls ($N=178$, 49% males) are Tunisians and were selected from the same population living in the middle coast of Tunisia. All participants gave written informed consent, and the study was approved by the local ethical committee. Adult asthmatic patients (mean age, 43.5 years) were recruited from the Services of Pneumology and Physiology at the Farhat Hached Hospital (Sousse). Exclusion criteria were (a) evidence of respiratory diseases other than asthma, (b) symptoms of lower and upper respiratory tract infection, (c) systemic disease, (d) regular intake of vitamin C or any other kind of therapy with a potential antioxidant effect. All asthmatic patients were examined in a stable phase of the disease, with no acute exacerbation in the previous two months. The diagnosis was based on recurrent symptoms of breathlessness and wheezing and improvement of greater than 200 ml and 12% in forced expiratory volume in 1 s (FEV1) after inhalation of 200 µg of salbutamol. Pulmonary function parameters were measured using body plethysmography (ZAN 500 Body II, Messeggerate, Oberthulba, Germany). Atopy was based on skin-prick tests (SPT) and determination of circulating allergen-specific immunoglobulin E (IgE) (IU/ml). SPT tests were carried out with 10 common allergens, and positive (histamine dihydrochloride) and negative (solvent) control solutions from the Stallerpoint® (Antony, France). The allergens included mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*), *Blattella germanica*, dog hair, cat hair, molds (including *Alternaria* and *Cladosporium*), tree mix and grass from *Dactylis* and latex. A skin prick reaction was considered positive if the wheal mean diameter was 3 mm or greater. Clinical severity of asthma was determined using criteria defined in global initiative for asthma guidelines (GINA, www.ginasthma.org). Patients were classified according to a combination of clinical asthma symptoms, lung function (FEV1%) and medication usage into four categories of asthma severity (intermittent, mild, moderate and severe persistent asthma). Each group was defined when the following criteria were satisfied: (a) intermittent: asthma include symptoms that

occur less than once a week, forced expiratory volume in one second (FEV1) $\geq 80\%$ of predicted and FEV1 variability $< 20\%$. These patients used short-acting β_2 -agonists as a primary treatment including salbutamol to relieve symptoms; (b) mild persistent: asthma is characterized by episodes of symptoms that occur more than twice a week but less than once a day, nocturnal symptoms more than twice a month, FEV1 $\geq 80\%$ pred and FEV1 variability $< 20\text{--}30\%$. The treatment of patients with mild persistent asthma consisted of short-acting β_2 -agonist for control of symptoms and low-doses of inhaled corticosteroids; (c) moderate persistent: asthma is characterized by daily symptoms and FEV1 between 60 and 80% of predicted and FEV1 variability $> 30\%$. These patients generally used inhaled short-acting β_2 -agonist on a daily basis and moderate doses of inhaled corticosteroids; (d) severe persistent: patients presented frequent asthma symptoms, FEV1 $\leq 60\%$ of predicted and FEV1 variability $> 30\%$. Patients with severe asthma received high doses of inhaled corticosteroids together with short-acting β_2 -agonists. In the current study, asthmatic patients (with intermittent or mild persistent disease severities) were newly recruited cases and were not taking any regular medication (inhaled corticosteroids and long acting beta 2-agonists) at their first entry to the Pneumology and Physiology departments at Farhat Hached Hospital (Sousse). For these patients, pulmonary function testing was not performed before their visit. After evaluation of lung function and level of disease severity according to international guideline recommendations (GINA), these patients were subsequently treated with short acting β -adrenergic agonists and low doses of inhaled corticosteroids, as required. Patients with moderate persistent and severe persistent severity controlled regularly their asthma symptoms by taking inhaled corticosteroids (beclomethasone dipropionate) and short-acting β -adrenergic agonists, as needed. None of the patients were taking any antioxidant supplements, theophyllin, long acting β -adrenergic agonists or oral corticosteroids. Each patient was requested to stop treatment for a period of 24 h before measurements and blood sample collections were made. Healthy subjects (mean age, 41.7 years) without clinical symptoms of any atopic diseases and with normal lung function were recruited from the National Blood Transfusion Center (Farhat Hached Hospital) as control subjects. Anthropometric measurements, comprising weight and height for each subject were determined. Body mass index (BMI) was determined as weight (kg)/height (m²). Pack years were calculated using the following formula (Number of cigarettes per day $\times$ number of years smoked $\div$ 20).

Spirometric and Fe_{NO} measurements

The fractional exhaled nitric oxide Fe_{NO} (ppb) was evaluated by Medisoft HypAir Fe_{NO} method using an electrochemical analyzer (Medisoft, Sorinnes (Dinant), Belgium). The Fe_{NO} measurements were performed according to the ATS guidelines. Three acceptable measurements were made within a 15-min period at the recommended flow rate of 50 ml/s and the mean of the three values was used. Pulmonary function was measured using a body plethysmography (ZAN 500 Body II, Messeggerate, Germany).

Blood sample collection and storage

Blood samples (10 ml) from both healthy volunteers and asthmatic patients were obtained by venipuncture and drawn into heparinized tubes. To obtain plasma, tubes were centrifuged at 3500 rpm for 15 min. Aliquots of erythrocytes and plasma were immediately frozen at -80°C until used.

Thiobarbituric acid-reactive substances

Malondialdehyde determination as an index of lipid peroxidation was determined by using a spectrophotometric method based on the reaction between MDA and thiobarbituric acid [11]. The concentration of MDA (determined at 535 nm) was expressed in micromole per liter of plasma using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Protein carbonyls and advanced oxidation protein products

Protein carbonyls were determined spectrophotometrically using the method of Reznick and Packer [12]. The method is based on the reaction of 2,4-dinitrophenylhydrazine with carbonyl groups. The absorbance of the reaction was determined at 370 nm and the results were expressed as micromole per liter of carbonyl groups using a molar extinction coefficient of $21\,000 \text{ M}^{-1} \text{ cm}^{-1}$. Advanced oxidation protein products (AOPP) were measured as previously described [13]. The concentration ($\mu\text{mol/l}$) was determined using a standard Chloramine T solution.

Total thiols, protein sulfhydryls and reduced glutathione

Total thiols were measured spectrophotometrically at 412 nm based on a method that uses 5-5'-dithiobis-2-nitrobenzoic acid (DTNB) as the thiol reagent. Results were expressed as nanomole per milliliter using a molar absorption coefficient of $13\,600 \text{ M}^{-1} \text{ cm}^{-1}$. The protein sulfhydryls were calculated by subtracting the GSH from total thiols [14]. Assay of GSH was determined by the method of Jollow and coworkers [15]. Formation of the yellow color derivative was measured at 412 nm and the result was expressed as nanomole per milliliter of plasma.

Glutathione peroxidase, catalase and superoxide dismutase

Glutathione peroxidase (GSH-Px) activity was determined according to the method of Rotruck and colleagues and expressed as micromolar of GSH consumed/minute/milliliter using a molar extinction coefficient of $13\,600 \text{ M}^{-1} \text{ cm}^{-1}$ [16]. Catalase activity in erythrocyte hemolysates was determined spectrophotometrically as previously described [17]. Results were expressed as millimolar of H_2O_2 degraded/minute/milligram of protein using a molar extinction coefficient of $43.6 \text{ mM}^{-1} \text{ cm}^{-1}$. Superoxide dismutase activity in erythrocyte hemolysates was determined spectrophotometrically and expressed as units per milliliter as previously described [18].

Hydroperoxide and iron

The hydroperoxide (H_2O_2) concentration was measured spectrophotometrically according to the method of ferrous

oxidation-xylene orange (FOX1 reagent) [19]. The absorbance of the samples was read at 560 nm and concentration ($\mu\text{mol/l}$) determined using a standard peroxide solution. Iron was measured spectrophotometrically using the xylene orange complex as previously described [20]. The absorbance of the samples was read at 560 nm and the concentration ($\mu\text{mol/l}$) determined using a standard FeSO_4 solution.

Ascorbic acid

Determination of ascorbic acid was performed with the Folin-Ciocalteu reagent as substrate according to Jagota and Dani [21]. Absorbance was measured spectrophotometrically at 760 nm and concentration ($\mu\text{g/ml}$) was determined against an ascorbic acid standard.

Nitric oxide and peroxynitrite

Plasma was immediately processed for NO and peroxynitrite measurements after centrifugation of the blood samples at 3500 rpm. Nitric oxide was assayed colorimetrically using the Griess reaction as previously described [22]. Nitric oxide levels, expressed as picomole/milligram of protein, were determined using a standard NaNO_2 solution. Peroxynitrite was measured according to Vanuffelen and colleagues [23]. The absorbance of the samples was read at 412 nm and concentration of peroxynitrite expressed in micromole/milliliter of plasma using a molar extinction coefficient of $4400 \text{ M}^{-1} \text{ cm}^{-1}$.

Total antioxidant status and oxidative stress index

Assay of plasma antioxidant status (TAS) was measured by the method of Re and colleagues with slight modifications [24]. It is based on the principle of generation of the radical of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+) by oxidation of ABTS solution. A standard curve was obtained by using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) standard solution at various concentrations (ranging from 0 to $20 \mu\text{M}$) in ethanol. The absorbance of the reaction samples was compared to that of the Trolox standard and the results were expressed in terms of Trolox equivalents (mmol trolox equivalent/l). The ratio of total peroxide to plasma antioxidant status (TAS) was used to estimate the oxidative stress index as an indicator of the degree of oxidative stress.

Protein determination

The amount of total protein was determined with the Bio-Rad protein assay. Bovine serum albumin was used as a standard.

Comet assay

The comet assay was carried out as described previously [25]. A total of 100 comets on each slide were scored according to the relative intensity of fluorescence. Quantification was performed by using the automated scoring of the OpenComet (www.openComet.org) software macro (version 1.3). The Tail moment and tail DNA % values generated by OpenComet were analyzed as a measure of DNA damage.

Statistical analysis

Statistical analyses were performed using SPSS, version 17.0 (Chicago, IL) and GraphPad Prism (v.6.04, GraphPad software, La Jolla, CA). Normally distributed variables are reported as mean $\pm$ standard deviation, and non-normal variables as median and interquartile ranges. Comparisons of quantitative variables were carried out using an unpaired *t*-test or the Mann–Whitney U-test as appropriate. A *p* value <0.05 was considered statistically significant. For assessment of reproducibility, intraclass correlation coefficients for relative reliability and coefficients of variation were determined between different repetitions. The coefficients of variation for inter- and intra-batch assessment were $<5\%$. Biochemical parameters were compared among patients with different asthma severity and smoking habits using one-way ANOVA analysis of variance or the Kruskal–Wallis test as appropriate. Correlations were analyzed with Spearman or Pearson rank correlation after Bonferroni correction. Discriminant analyses were performed with the Wilks' method using 15 parameters as discriminating variables and controls/asthmatics groups as dependent variables. The method extracts *n*–1 discriminant functions, *n* being the number of groups to discriminate among, which are linear combinations of the original quantitative variables selected. The model parameters are Wilks' Lambda; eigenvalues and canonical correlations. Principal components analysis (PCA) was performed to examine the correlational structure of the data. For the optimal solution, we used varimax rotation with Kaiser normalization. Only components with eigenvalues above 1.0 were retained in the solution.

Results

Subject characteristics

The demographic and clinical characteristics of the patients and healthy controls are shown in Tables 1 and 2. Among the asthmatic group, 72 patients were classified as intermittent stage, 16 mild persistent, 20 moderate persistent and 12 patients severe persistent. FEV1%, FEV1/FVC and FEV1/FVC% were all significantly lower in patients with severe persistent asthma compared to patients with moderate persistent, mild persistent and intermittent (Table 2).

Evaluation of oxidant-antioxidant status

As indicated in Table 3, lipid peroxidation levels measured as MDA-TBARS products were increased in asthmatic patients as compared with controls ($p < 0.001$). Scatter-plots representing median values and interquartile ranges for TBARS levels are presented in Figure 1(A). The levels of PC and AOPP were also investigated but no difference in the mean values of the explored parameters between different groups was found (Table 3). In the present study, total thiols evaluated as an indicator of redox balance were significantly lower in asthmatics than in controls (Table 3, Figure 1B). Additionally, patients with asthma had a significant decrease in protein sulfhydryl and GSH levels when compared with healthy controls (Table 3, Figure 1C and D). Interestingly, discriminant analysis revealed glutathione as the most important parameter that differentiates asthmatics from

Table 1. Basic characteristics of the control subjects and patients with asthma.

Characteristics	Controls (<i>n</i> = 178)	Asthmatic patients (<i>n</i> = 151)
Age (years)	41.71 $\pm$ 0.7	43.55 $\pm$ 0.9
BMI	27.05 $\pm$ 0.31	28.85 $\pm$ 0.44
Smoking status		
Never	92 (51.7%)	120 (79.4%)
Current-smoker	73 (41%)	14 (9.3%)
Ex-smoker	13 (7.3%)	17 (11.3%)
Pack-years	22.33 $\pm$ 1.92	20.4 $\pm$ 3.02
Light ≤ 10	17 (9.6%)	10 (6.6%)
Moderate [11–20]	25 (14%)	7 (4.6%)
Heavy [21–30]	19 (10.7%)	5 (3.3%)
Very heavy > 30	17 (9.6%)	7 (4.6%)
Asthma severity according to GINA guidelines		
Intermittent%	–	57%
Mild persistent%	–	13.9%
Moderate persistent%	–	19.2%
Severe persistent%	–	9.9%
SPT+ (%)	0%	11.3%
SPTQ	0	2 [1–4]
IgE, IU/ml	NA	198 (63.8–369)
ICS treated (last 3 months) %	0%	29.1%

Mean $\pm$ standard error of the mean (SEM) values are indicated for normally distributed variables, and median (interquartile range) values are given for non-normal variables.

BMI = Body mass index, weight (kg)/(height (m))²; Pack years: (number of cigarettes smoked per day $\times$ number of years smoked)/20. For pack years the percentages do not add to the sum because of some missing values. Skin Prick Test positivity (SPT+) was defined by a mean wheal diameter ≥ 3 mm than the negative control for at least one of 10 allergens. SPTQ: number of positive test. IgE: immunoglobulin E, NA: not available.

healthy controls (Wilks' Lambda = 0.525, $F = 19.03$, $p < 0.0001$, data not shown). In the current study, ascorbic acid levels and GSH-Px activity were significantly decreased in patients with asthma than in control subjects (Table 3, Figure 2). On the contrary, catalase (CAT) and superoxide dismutase (SOD) activities in erythrocytes were not significantly different between asthmatics and healthy controls (26.9 $\pm$ 2.1 versus 33.3 $\pm$ 2.7 and 42.7 $\pm$ 1.8 versus 41.2 $\pm$ 1.5, respectively). The level of H₂O₂ and iron were also determined but no difference was observed between the two groups (Table 3). Interestingly, in the current study, we have found a notable decrease in the plasma level of NO in asthmatics in comparison to controls (158.9 $\pm$ 6.8 versus 254.9 $\pm$ 11.6, $p < 0.001$, respectively), while an increased level of exhaled nitric oxide (Fe_{NO}) was observed ($p < 0.001$). There was, however, no correlation between Fe_{NO} and plasmatic NO levels. Peroxynitrite levels were also determined but no difference was found among the different groups ($p = 0.38$). As indicated in Table 3, a significant decrease of total antioxidant status (TAS) was also found in asthmatic patients, as compared to healthy controls (0.7 $\pm$ 0.016 versus 0.8 $\pm$ 0.017, respectively). Besides, the oxidative stress index was increased in asthmatic patients, but the difference was not significant (500.2 $\pm$ 20 versus 461.2 $\pm$ 17.8, respectively). In the current report, significant positive correlations were observed in asthmatics, between AOPP and the other studied parameters including total thiols ($r = 0.25$, $p = 0.001$) and peroxynitrite ($r = 0.3$, $p = 0.001$). In addition, a negative correlation was found between both AOPP and H₂O₂ and

Table 2. Clinical and demographic characteristics of the patients with different asthma severities.

	Asthmatic patients	Asthma Stage				F	p
		Intermittent (57%)	Mild persistent (13.9%)	Moderate persistent (19.2%)	Severe Persistent (9.9%)		
Age (years)	43.55 ± 0.9	43.84 ± 1.13	38.86 ± 1.9	43.03 ± 2.45	49.47 ± 3.65	2.63	0.052
BMI	28.85 ± 0.44	29.18 ± 0.58	27.97 ± 0.93	28.64 ± 1.22	28.59 ± 1.26	0.31	0.81
Smoking status							
Never	120 (79.4%)	72	16	20	12		
Current-smoker	14 (9.3%)	7	4	3	0		
Ex-smoker	17 (11.3%)	7	1	6	3		
Pack-years	20.4 ± 3.02	23.5 ± 5.35	23.8 ± 9.16	16.7 ± 4.29	14 ± 5.75	0.49	0.68
Light ≤10	10 (6.6%)	4	1	4	1		
Moderate [11–20]	7 (4.6%)	3	1	1	2		
Heavy [21–30]	5 (3.3%)	2	1	2	0		
Very heavy >30	7 (4.6%)	4	1	2	0		
Spirometry							
FEV1	2.1 ± 0.05	2.4 ± 0.06	2.1 ± 0.14	1.8 ± 0.11	1.1 ± 0.09	24.2	0.00
FEV1 (% pred)	87.2 ± 1.68	97.8 ± 1.26	93.5 ± 3.23	70.1 ± 1.35	49.7 ± 1.99	103.6	0.00
FVC	2.9 ± 0.07	3.1 ± 0.08	3 ± 0.2	2.7 ± 0.15	1.9 ± 0.17	11.3	0.00
FVC (% pred)	96.1 ± 1.58	103.2 ± 1.9	101.6 ± 2.46	84.2 ± 1.81	66.9 ± 2.94	31	0.00
FEV1/FVC	75.9 ± 0.87	79.5 ± 0.91	77.9 ± 1.6	70.8 ± 1.89	62.3 ± 3.17	19	0.00
FEV1/FVC(% pred)	94.2 ± 1.2	98.4 ± 1.04	91.1 ± 4.36	88.6 ± 2.51	79.8 ± 5.17	8.7	0.00

Results are expressed as mean ± S.E.M; FEV1 = forced expiratory volume in 1 s; %pred = percent of predicted value; FVC = forced vital capacity. Patients with asthma are presented as intermittent, mild persistent, moderate persistent and severe persistent. For pack years the percentages do not add to the sum because of some missing values. Comparisons were made using one-way ANOVA. Values in bold cases are significant (p was considered significant if <0.05).

Table 3. Comparison of indices of oxidant-antioxidant status among healthy controls and patients with asthma.

Study parameters	Controls	Patients	p	Controls non-smokers	Patients non-smokers	p
TBARS (μmol/l)	0.83 (0.5–1.41)	1.16 (0.75–1.83)	0.000	0.95 (0.56–1.49)	1.12 (0.75–1.91)	0.007
PC (μmol/l)	0.07 ± 0.002	0.07 ± 0.002	0.79	0.07 ± 0.002	0.07 ± 0.002	0.38
AOPP (μmol/l)	14.02 ± 0.15	14.03 ± 0.05	0.98	13.82 ± 0.2	14.03 ± 0.05	0.28
Total thiols (nmol/ml)	667.9 ± 22.65	355.9 ± 15.72	0.000	615.4 ± 28.67	338.8 ± 16.75	0.000
Proteinsulphhydryl (nmol/ml)	591.95 ± 24.28	333.99 ± 16.41	0.000	539.24 ± 31.16	320.13 ± 17.59	0.000
GSH (nmol/ml)	85.35 (54.3–132.7)	33.6 (18.6–58.95)	0.000	84.6 (43.4–135.4)	30.6 (17.5–57.15)	0.000
GSH-Px (μM/min.ml)	105.7 (42.6–142.6)	52.59 (37.7–75.8)	0.000	125.9 (49.05–145.52)	54.77 (39.79–76.3)	0.000
CAT (mM/min.mg)	33.3 ± 2.7	26.9 ± 2.1	0.06	32.95 ± 3.48	27.63 ± 2.3	0.21
SOD (U/ml)	41.2 ± 1.5	42.7 ± 1.8	0.55	39.1 ± 1.75	42.37 ± 2.13	0.24
H ₂ O ₂ (μmol/l)	356.45 ± 11.2	349.18 ± 11.2	0.65	406.6 ± 17.7	352 ± 12.7	0.01
Ascorbic acid (μg/ml)	12.8 ± 0.6	10 ± 0.5	0.001	12.93 ± 0.92	10.1 ± 0.63	0.01
Iron (μmol/l)	10.82 (8.51–13.11)	10.4 (8.68–13)	0.55	10.7 (8.25–13.3)	10.4 (8.70–12.95)	0.77
Fe _{NO} (ppb)	11 (8.25–12)	28 (22–39.5)	0.000	11 (8–12)	27 (22–38)	0.000
NO (pmol/mg)	254.9 ± 11.67	158.95 ± 6.8	0.000	250 ± 15.94	156.4 ± 7.77	0.000
Peroxynitrite (μmol/ml)	1.2 ± 0.05	1.1 ± 0.05	0.38	1.1 ± 0.06	1.1 ± 0.05	0.54
TAS (mmol trolox equivalent/l)	0.8 ± 0.017	0.7 ± 0.016	0.01	0.8 ± 0.02	0.7 ± 0.02	0.01
Oxidative stress index	461.2 ± 17.8	500.2 ± 20	0.14	507.90 ± 26.65	508.10 ± 23.82	0.99

Mean ± standard error of the mean (SEM) values are indicated for normally distributed variables, and median (interquartile range) values are given for non-normal variables. TBARS = thiobarbituric acid-reactive substances; PC = protein carbonyls; AOPP = advanced oxidation protein products; GSH = reduced glutathione; GSH-Px = glutathione peroxidase; CAT = catalase; SOD = superoxide dismutase; Fe_{NO} = fraction of exhaled nitric oxide; NO = nitric oxide; TAS = total antioxidant status. Comparisons were made using an unpaired t -test or Mann-Whitney test. p Values in bold cases remain significant after Bonferroni's correction.

nitric oxide levels ($r = -0.24$, $p = 0.01$; $r = -0.21$, $p = 0.01$, respectively). Increased levels of H₂O₂ were found to correlate positively with both SOD activity and peroxynitrite levels ($r = 0.32$, $p = 0.01$ and $r = 0.37$, $p = 0.001$, respectively). A positive relationship was also retrieved between both protein carbonylation products (PCO) and peroxynitrite and total thiol levels ($r = 0.18$, $p = 0.03$; $r = 0.51$, $p = 0.001$, respectively).

Considering each group separately, we have also evaluated the impact of smoking on the different parameters

investigated (data not shown). We have found that among controls, GSH-Px activity was significantly decreased in smokers in comparison to non-smokers [95.38 (39.78–138.98) versus 125.9 (49.05–145.52), $p = 0.006$], whereas among asthmatics, current smokers had higher protein carbonyls and peroxynitrite levels (0.08 ± 0.004 versus 0.07 ± 0.002 , $p = 0.028$ and 1.35 ± 0.1 versus 1.05 ± 0.05 , $p = 0.06$, respectively). No significant difference was observed with the other studied parameters and this might be due to the small number of smokers among asthmatics.

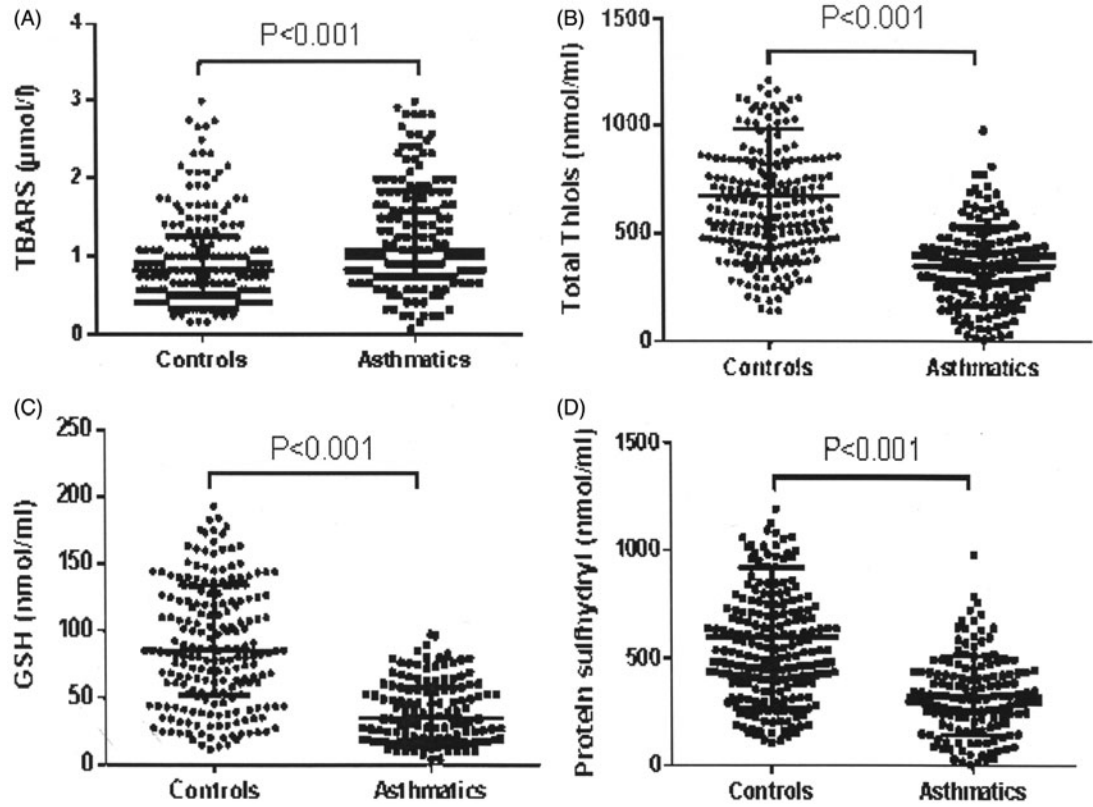


Figure 1. Scatter plots representing median $\pm$ interquartile ranges (A, C) or mean $\pm$ SD (B, D) of TBARS (A), total thiols (B), glutathione (C) and protein sulfhydryls (D) levels in controls and asthmatics. TBARS = thiobarbituric acid-reactive substances. A p value < 0.05 is significant.

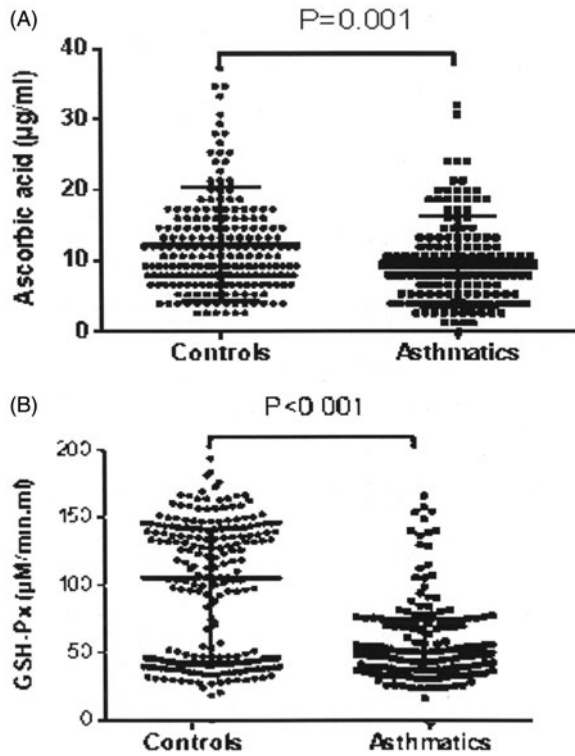


Figure 2. Scatter plots representing mean $\pm$ SD (A) or median $\pm$ interquartile ranges (B) of ascorbic acid (A) and GSH-Px (B) in controls and asthmatics. GSH-Px = glutathione peroxidase. A p value < 0.05 is significant.

Correlations with disease severity and lung function

The different biochemical parameters investigated were also compared among patients grouped according to the severity of asthma (Supplementary file 1). This study revealed an increased level of ascorbic acid in advanced disease stages, but the difference was not significant. No significant difference was observed with the other studied parameters among the different asthma groups. The correlations between the different parameters and the degree of airflow obstruction were also determined. As shown in Figure 3, ascorbic acid was found to correlate negatively with FEV1/FVC (%), ($r = -0.26$, $p = 0.004$). A similar finding was obtained between both FEV1 (%) and FVC (%) and ascorbic acid levels ($r = -0.2$; $p = 0.001$; $r = -0.37$, $p < 0.001$, respectively, data not shown). Linear regression analyses also revealed that ascorbic acid behaves as an independent factor that affects FEV1/FVC% in asthmatic patients (Table 4, $p = 0.04$). Additionally, a positive correlation was observed between reduced glutathione and FEV1/FVC (%) ($r = 0.23$, $p = 0.02$), but not with FEV1 (%).

Comet Assay

We also determined DNA strand breaks in peripheral blood mononuclear leukocytes by using the Comet assay. Figure 4 shows that the level of DNA damage in asthmatic patients with different disease stages was significantly higher than in healthy controls ($p < 0.001$).

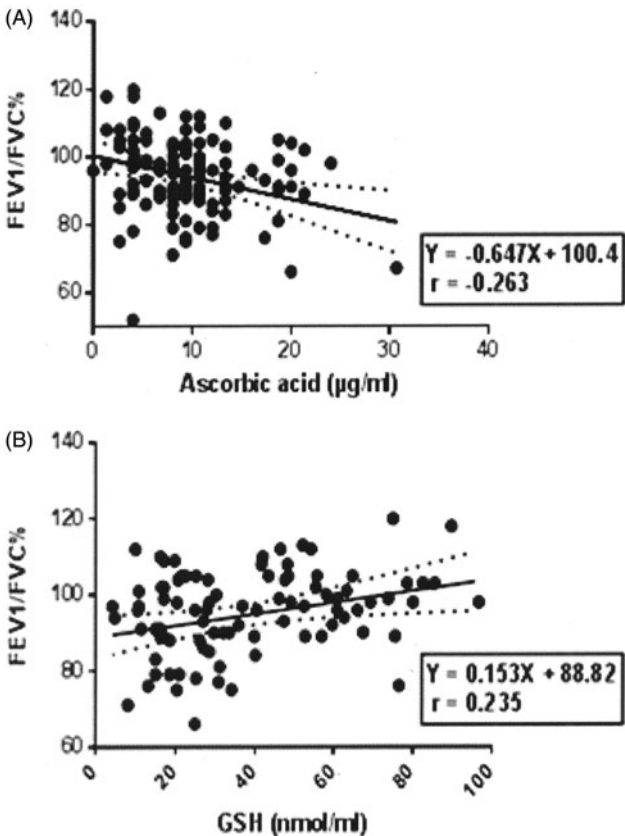


Figure 3. Relationships between FEV1/FVC (% predicted) and levels of ascorbic acid (A) and GSH (B) in asthmatic patients. Correlation coefficient is denoted by “r” and line corresponds to the fitted linear regression equation.

Table 4. Multiple linear regression analyses using FEV1/FVC (% predicted) as a dependent variable in asthmatic patients.

	Unstandardized		Standardized		<i>t</i>	<i>p</i>
	Beta	S.E.	Beta			
PC (μmol/l)	74.24	130.18	0.09	0.11	0.6 ^{ns}	
AOPP (μmol/l)	2.79	5.11	0.10	0.54	0.6 ^{ns}	
Total thiols (nmol/ml)	0.08	0.13	0.83	0.66	0.50 ^{ns}	
Proteinsulphydryl (nmol/ml)	−0.96	0.12	−0.97	−0.77	0.44 ^{ns}	
SOD (U/ml)	−0.08	0.21	−0.07	−0.37	0.71 ^{ns}	
H ₂ O ₂ (μmol/l)	−0.01	0.09	−0.1	−0.13	0.86 ^{ns}	
Ascorbic acid (μg/ml)	−1.26	0.62	−0.35	−2	0.04*	
NO (pmol/mg)	0.01	0.03	0.08	0.42	0.68 ^{ns}	
Peroxynitrite (μmol/ml)	−1.05	6.93	−0.037	−0.15	0.9 ^{ns}	
TAS (mmoltrolox equivalent/l)	−13	31.36	−0.16	−0.41	0.68 ^{ns}	
Oxidative stress index	0.02	0.06	0.27	0.34	0.74 ^{ns}	

PC = protein carbonyls; AOPP = advanced oxidation protein products; SOD = superoxide dismutase; NO = nitric oxide; TAS = total antioxidant status; %pred = percent of predicted value; S.E. = Standard error; *means significant <0.05; ns: not significant (*p* > 0.05).

Principal component assay

Principal component analysis was performed to display the maximum amount of variation in our data within a few principal components. Seven variables were extracted in the

PCA (Kaiser-Meyer-Olkin measure of sampling adequacy 0.7, Bartlett’s Test of sphericity <0.001). Two components with an eigenvalue of 1.0 or greater were identified. As indicated in Figure 5(A), the analysis revealed that the first component (PC1) includes the parameters TAS, GSH-Px, NO and MDA-TBARS. Total thiols, reduced glutathione, and ascorbic acid, which represent the reducing power of plasma separated mainly along the second principal component (PC2). Further, the analysis was performed using the two PCs and the difference between individual samples (controls and asthmatic patients) was clearly visible in the biplot (Figure 5B). The projection of the factor scores of the control group (closed circles) is on the highest values for TAS, GSH-Px, NO, total thiols, GSH and ascorbic acid. Patients with asthma (open circles) present lower factor scores for the two principal components demonstrating that these patients had lower antioxidant status measures compared with healthy controls.

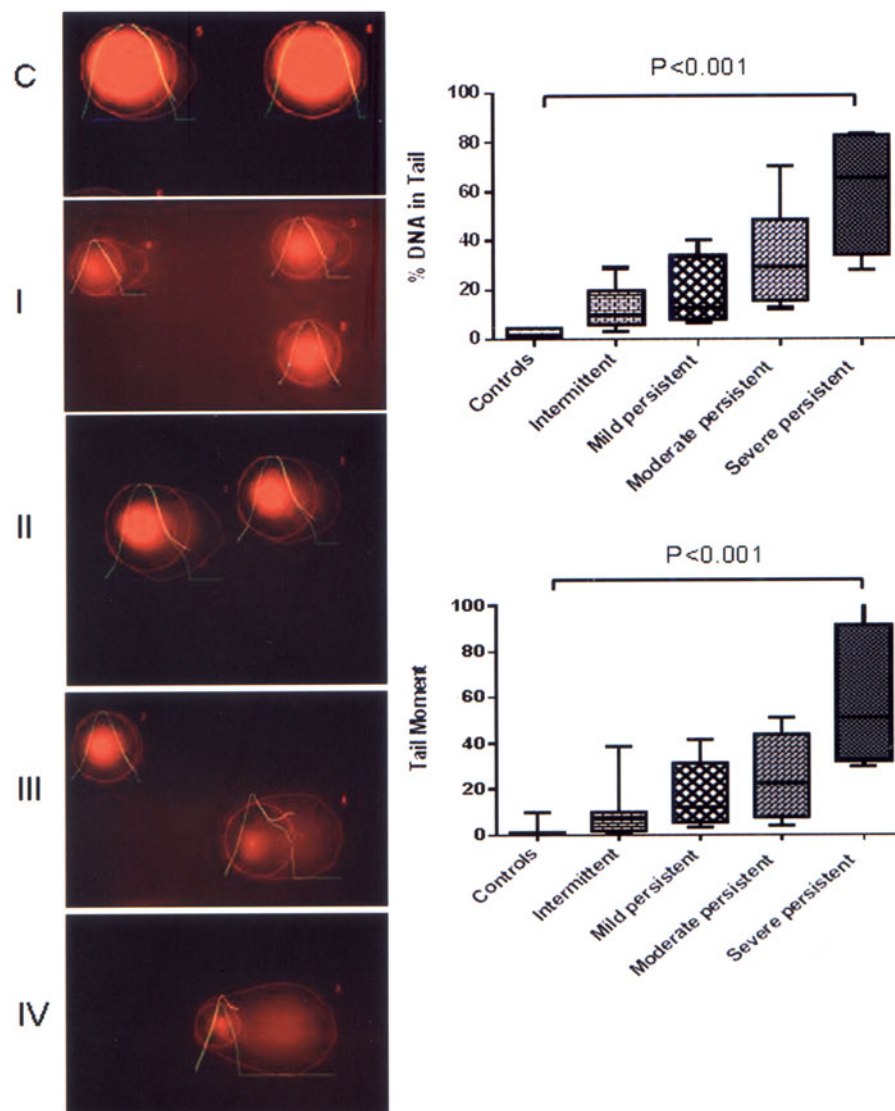
Discussion

In this study, we evaluated the alterations in 16 parameters related to the oxidant-antioxidant status of Tunisian patients suffering from asthma, as well as their relationships with airflow obstruction and disease severity. Our results revealed that subjects with asthma have increased levels of MDA supporting the hypothesis that oxidative stress is associated with the pathophysiology of this disease. In accordance with the report of Ahmad et al. [9], in Indian asthmatics, our data may likely reflect an extensive production of ROS leading to the uncontrolled peroxidative damage of lipids and membranes. Comparison between clinical stages of severity among asthmatics shows, however, no significant correlations between MDA-TBARS and disease progression. These findings are inconsistent with a previous report showing a significant difference in TBARS levels, while comparing disease stages of Indian asthmatic patients [8]. Additionally, Ruprai et al. [26] revealed an inverse correlation between lipid peroxidation and FVC (% predicted) suggesting that higher MDA-TBARS associate with chronic airflow limitation and disease progression. Overall, the obtained results may support the use of thiobarbituric acid-reactive substances as a non-invasive method to explore oxidative stress in asthma and highlight the need for the administration of antioxidative therapies for the treatment and prevention of this disease.

Protein carbonylation (PC) is another useful biomarker of protein oxidation and inflammation in several human diseases. However, in the current report, no significant difference in PC levels and advanced oxidation protein products (AOPP) was found between healthy controls and asthmatics. These results corroborate those of García-Larsen et al. [27] showing that the levels of protein carbonyls were unrelated to asthma development or lung function.

Under normal conditions, oxidants generated in biological fluids are efficiently scavenged by a variety of antioxidants that protect lung/blood from any oxidative burden. Interestingly, our results highlight a significant decrease in the levels of total thiols and protein sulphydryls in asthmatics, which is in agreement with the findings of two previous investigations [8,9]. Depletion of these compounds in combination with a low antioxidant status provides additional

Figure 4. Level of DNA damage in peripheral blood mononuclear cells determined by the comet assay. Left: Photomicrographs showing examples of comets in controls (C) and asthmatics (disease stages I–IV). Right: Scatter plots representing median values and interquartile ranges of the percentage of DNA in the tail and tail moment in controls and asthmatics with different disease stages. Kruskal-Wallis test was used to determine the level of significance. A p value <0.05 is considered significant.



evidence of enhanced oxidative stress in asthmatic patients. According to the literature, insufficient levels of these compounds in plasma may, also, increase airway T-helper type 2 responses, as well as early asthmatic symptoms and airway hyper-responsiveness [28]. In the current report, GSH levels were also decreased in asthmatics in comparison with healthy controls. This finding agrees with results obtained by Al-Afaleg and colleagues [7] and may likely reflect an insufficient protection against increased oxidant burden in asthmatic patients. Besides, we have found a significant correlation between GSH and FEV1/FVC (%) suggesting that decreased GSH levels may be associated to a decline in the lung function in asthmatics. From these findings, it could be suggested that the use of thiol compounds (e.g. N-acetyl-L-cysteine, carbocysteine) could be a promising approach to treat asthma and inflammatory lung diseases that are caused and/or aggravated by oxidative stress [29].

Increased oxidative burden in Tunisian asthmatics is, also, accompanied by a significant decrease in glutathione peroxidase (GSH-Px) activity, which might arise because of reduced levels of GSH, as well as increased ROS and MDA levels [30,31]. These results could be related to the clinical features of the disease, indicating the presence of higher lipid

peroxidation products (current findings) and enhanced levels of H_2O_2 in breath condensate of exhaled air from asthmatic patients [32]. Since GSH-Px is a major regulator of the lipoxygenase pathway of arachidonic acid metabolism, our data could, also, be a major prerequisite for the clinical amplification and perpetuation of the inflammatory response in asthma [8]. In the current study, no significant relationship was found; however, with disease progression, which proves the involvement of GSH-Px in mechanisms related to the pathogenesis rather than to severity of asthma. From these findings, one can speculate that strategies aiming at increasing GSH-Px levels through GSH-Px mimetics could be a promising approach to a more effective therapy of asthma [33]. In line with this, a number of GSH-Px mimetics have been reported to scavenge free radicals and to inhibit airway inflammation by reducing neutrophil recruitment and chemokine expression [29].

Antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) could also play an important role in protecting cells against oxidative damage and are, therefore, expected to increase in asthmatic patients. Unfortunately, in the current study, we failed to find significant differences between the different groups, which may highlight that

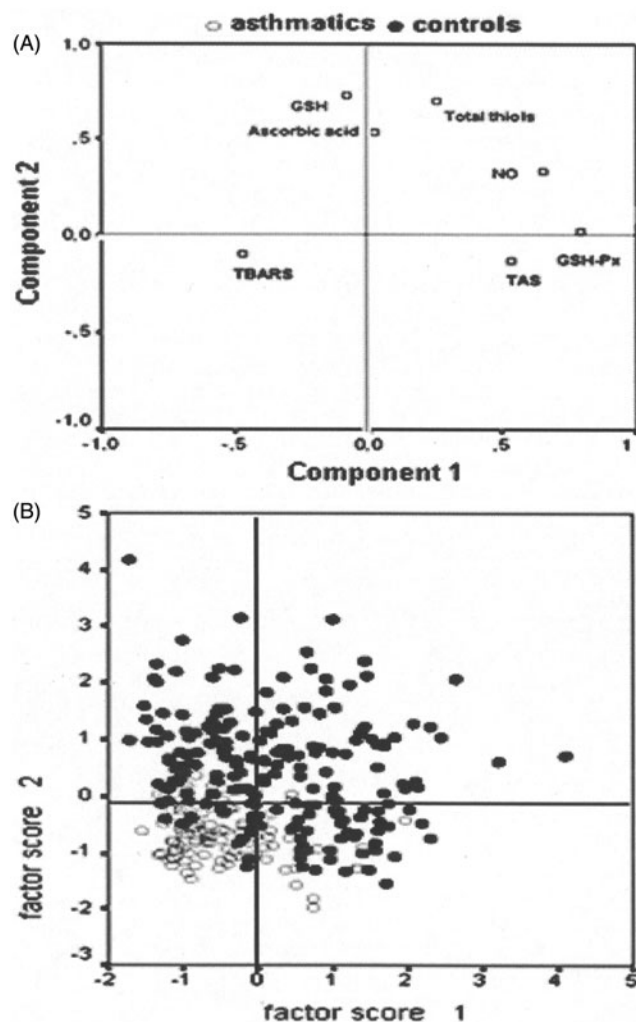


Figure 5. Results obtained by principal component analysis. (A) Loading plot diagram with seven variables contributing to the two main components. GSH-Px, NO, TAS, TBARS loaded primarily on factor score 1, while GSH, total thiols, ascorbic acid loaded on factor score 2. (B) Score plot of patients and controls in a two-dimensional space derived from the principal component analysis. Factor scores 1 and 2 have a tendency toward lower values in asthmatic patients.

various enzymatic systems differ substantially in their responses to increased oxidative stress. Similarly, none of the measured activities had a significant correlation with the degree of airway limitation. Up to now, studies on the relationships between anti-oxidative defense proteins and asthma development seem to be contradictory or inconclusive. For example, Comhair et al. [34] found no differences in GSH-Px activity between patients and controls, while others revealed increased expression of this enzyme in asthma [35]. Besides, Ahmad and colleagues [9] observed lower SOD and CAT activities in asthmatics; meanwhile, others reported enhanced expression of these anti-oxidative proteins [10]. Although more investigations are required, this discrepancy may be ascribed to the different populations investigated, as well as to the differences in disease severity and design of the different studies.

Ascorbic acid is another antioxidant that prevents lipid peroxidation and down-regulates inflammatory cytokines that are associated with immune responses. In the current investigation, ascorbic acid levels were significantly lower

in asthmatics, which highlight an increased oxidative burden in these patients. These results corroborate those of a previous study showing a lower level of ascorbic acid in plasma from Indian asthmatic patients [26]. Additionally, ascorbic acid was found to correlate negatively with severity of airway obstruction and was independently associated with lung function. These findings contrast with those reported by Ochs-Balcom et al. [36] but they are in keeping with a recent report revealing a negative association of another antioxidant with FEV1 (%) among patients with intermittent to severe asthma [9]. Consistent with our data, *in vitro* studies have, also, shown that prolonged oxidative stress conditions (i.e. moderate persistent-severe persistent) cause a transient depletion of antioxidants followed later by a significant elevation of some of these compounds [37]. Though the reason for the increased levels of ascorbic acid was not investigated in the current study, our findings may likely result from the enhanced accumulation of this compound in activated inflammatory cells from advanced asthmatic stages [38]. A higher accumulation of ascorbic acid could, also, be an adaptive response to the oxidant stress to counteract infections in advanced stages, as well as to increase intracellular antioxidant status.

The obtained results, also, highlight a decreased level of NO in asthmatics compared with healthy controls. This finding may have a clinical impact in asthma since decreased levels of NO are thought to reduce bronchoprotection and further contribute to the emergence of cardiovascular diseases [39]. No significant correlation was found, however, between NO levels and Fe_{NO} measurements, which indicates that Fe_{NO} is not an index of the endogenous production of systemic nitric oxide in asthma. Although further studies are required, the reduced bioavailability of NO in asthmatics could be related to the enhanced oxidative stress and decreased levels of glutathione in plasma [40]. Dysfunction of endothelial nitric oxide synthases by environmental chemicals and after repeated allergen challenge may also reduce NO levels and contribute to the development of hypertension in pulmonary diseases [39]. Altered levels of NO might also arise as a result of sleep-related breathing disorders and intermittent nocturnal hypoxemia. Interestingly, asthma has been recently associated with an increased risk of new-onset obstructive sleep apnea that correlates with decreased plasmatic NO levels [41,42].

The limitations of the current study should be considered. First, the measures of oxidative stress and antioxidative status did not differ significantly between patients with different disease severity. In previous studies showing differences related to severity, asthmatic patients with acute exacerbations were taken into consideration [43]. In the current report, we did not include such patients, so that we give a limited spectrum of severity among Tunisian asthmatics. Second, patients included in the current report with advanced disease stages were regularly receiving treatment with inhaled corticosteroids (ICs). Although this might be considered as a limitation of this study, these patients had airway obstruction and their asthma was active. According to the literature, systemic parameters linked to the oxidant-antioxidant balance tend to revert toward normal values after a treatment with anti-asthma drugs [44]. Additionally, inhaled corticosteroids were shown not to have any systemic effect on antioxidant

enzymes [32,45]. Accordingly, we can expect that the observed changes in Tunisian asthmatics might have been even more pronounced, had the patients not been receiving ICs treatment.

Conclusion

The current investigation is the first to examine the alterations in several measures of oxidative stress and anti-oxidant status in a large group of Tunisian patients. This study provides a strong evidence of increased oxidative stress in the plasma and erythrocytes from patients with asthma. This is accompanied by alterations in several parameters including decreased levels of total thiols, glutathione, ascorbic acid and glutathione peroxidase and increased amounts of TBARS. Interestingly, by using discriminant analysis, glutathione emerged as the most important parameter that distinguishes asthmatics from healthy controls. These findings point out the relevance of systemic markers to assess the magnitude of oxidative stress in asthma and support the need for the administration of antioxidative therapies for the treatment and prevention of this disease.

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Declaration of interest

The authors declare that they have no competing interests.

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