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A Comparative Study of Oxidative Stress Biomarker Levels in Asthmatic and Non-Asthmatic Children

Marek Biesiadecki [1], Sabina Galiniak [1,*], Marta Rachel [1,2], Agnieszka Bożek [2], David Aebisher [1,*]

[1] Medical College of Rzeszów University, Warzywna 1a, 35-310 Rzeszów, Poland; mbiesiadecki@ur.edu.pl (M.B.); sgaliniak@ur.edu.pl (S.G.); rachel@popia.pl (M.R.); daebisher@ur.edu.pl (D.A.);
[2] Allergology Department, Provincial Hospital No 2, Lwowska 60, 35-301 Rzeszów, Poland; agnieszkabozek@op.pl (A.B.);
* Correspondence: sgaliniak@ur.edu.pl (S.G.); daebisher@ur.edu.pl (D.A.);

Scopus Author ID 55865208300

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Abstract: Asthma is the most common chronic inflammatory disease of the airways. The interplay between internal genetic and external environmental factors is of key importance in the pathogenesis of this disease. Many studies have provided evidence that oxidative stress is also involved in this disease's progress. Our objective was to evaluate levels of selected oxidative stress biomarkers in children with controlled asthma compared to healthy subjects. Additionally, we attempted to uncover correlations between the selected biomarkers and the clinical parameters of the patients. To accomplish our objective, we evaluated biomarkers for oxidative damage to proteins and lipids in the serum of 30 children aged 10-17 years who have had the disease for 2 to 4 years. We measured a reduced concentration of thiol groups ($p<0.05$), tryptophan content ($p<0.01$), and total antioxidant capacity ($p<0.001$) in the serum of asthmatics. None of the other biomarkers for oxidative stress measured in this study were significantly different compared to the control group. We found a significant positive correlation between the content of N′-formylokynurenine and BMI z-score and a negative correlation between tryptophan content with patient age and total IgE. In conclusion, biomarkers of oxidative stress in asthmatic patients were detected, even in children with well-controlled asthma.

Keywords: asthma; oxidative stress; protein oxidation; malondialdehyde; total antioxidant capacity.

1. Introduction

Asthma is when airways are hyper-reactive to irritants and allergens, leading to uncontrolled airway constriction, which may result in hypoxia and death [1]. The prevalence of asthma and associated morbidity and mortality has increased in recent decades [2, 3]. Asthma is one of many diseases which elicit oxidative stress [4]. Oxidative stress is an imbalance between oxidant and antioxidant processes, leading to excessive production of reactive oxygen species (ROS) and damaging biomolecules [5]. Asthmatic lungs unbalanced by the antioxidant defense system exposed to endogenous and environmental oxidants can result in oxidative stress and pulmonary dysfunction [4, 6]. Higher levels of molecules involved in enhanced oxidative stress have been found in biological samples such as exhaled breath condensate (EBC), serum/plasma, and bronchoalveolar lavage fluid from asthmatic patients compared to levels in healthy control subjects [7–9]. It is known that the intensification of oxidative stress is also related to disease severity [10]. Our study aimed to compare oxidative stress biomarkers in children with well-controlled asthma and healthy children. Additionally,

to the best of our knowledge, this is the first report to address 3-nitrotyrosine levels and the ability of albumin to bind cobalt ions in the serum of patients. Moreover, we tried to uncover correlations between oxidative stress biomarkers and the patients' clinical parameters.

2. Materials and Methods

2.1. Ethics.

The study protocol was approved by the Bioethics Committee of the University of Rzeszów, Poland (7/10/2018). Participants and their legal guardians were acquainted with the conditions of participation and signed a consent form for participation in this study. All experiments were performed in accordance with relevant guidelines and regulations.

2.2. Study group.

A single-center study was carried out on a sample of 30 asthmatic patients aged 10-17 years and 30 control subjects. Participants were recruited from the Outpatient Department of Allergology and Cystic Fibrosis, Provincial Hospital No. 2, Rzeszów, Poland, from January 2020 to October 2020. The diagnosis of asthma was made based on GINA criteria, including the presence of respiratory symptoms in time and intensity, and with the Polish version of the validated Asthma Control Test (ACT) questionnaire [11]. Spirometry was used to confirm airway obstruction. Asthma control was estimated according to GINA principles before patients had respiratory tests. The GINA criteria for assessing asthma control were as follows: weekly frequency of asthma symptoms, awakening at night, reduction of daily patient activity due to asthma, and weekly medication administration for symptom relief. All patients were classified as having controlled asthma when they had none of the above-listed criteria for 4 weeks [12]. According to GINA recommendations, patients received inhaled corticosteroids with or without combination with short or long-acting β2-agonists. In the case of atopic dermatitis, treatment included topical steroids and emollients. The patients did not take other medications other than those prescribed. Exclusion criteria were also as follows: asthma exacerbations, acute respiratory infections, the presence of other diseases (cystic fibrosis, primary ciliary dyskinesia, tonsil hypertrophy, congenital disabilities, craniofacial injuries, foreign body, immunodeficiency, polyps, reflux, coagulopathy, nasal and paranasal tumors), systemic corticosteroid treatment, lack of cooperation with devices used for respiratory tests, refusal to participate in the study, smoking, forced expiratory volume in the first second (FEV_1) lower than 70% of the predicted value, and hospitalizations 1 month before screening. Healthy pediatric subjects were recruited concomitantly from the local pediatric clinic. The control group consisted of physically examined sex-matched participants without any disease in their medical history. Inclusion criteria for healthy subjects included no family history of respiratory or dermatological disease, no clinically significant abnormalities in blood chemistry assessments, and hematologic assessments that included complete blood count and no use of any antioxidant vitamins. The volunteers had also not taken any medications for 30 days before the study. All participants in the control group had normal pulmonary function tests. All pediatric patients had allergy tests with inhaled allergens (Skin prick tests – BASIC SET inhaled Allergopharma, Germany). The skin prick test was defined as positive by a mean wheel diameter 3 mm greater than the negative control for at least one of 10 allergens.

2.3. Materials.

All reagents were purchased from Sigma-Aldrich (Poznan, Poland) unless indicated otherwise. A 3-nitrotyrosine (3-NT) enzyme-linked immunosorbent assay kit was supplied by Immunodiagnostik AG (K7829, Bensheim, Germany). Absorptiometric measurements were performed on a Tecan Infinite 200 PRO multimode reader (Tecan Group Ltd.; Männedorf, Switzerland). All reagents used were of analytical reagent grade. Measurements were made in triplicate.

2.4. Spirometry.

Spirometry tests on all subjects were performed using a standard spirometry device (Lungtest 1000 MES SJ, Poland) according to recommendations [13].

2.5. Blood sampling.

Blood samples were obtained between 8 am, and 10 am after fasting overnight and placed into blood collection tubes. The blood samples were centrifuged (1500×g, 10 min, 4°C), and the serum obtained was aliquoted and frozen at −80°C until further analysis. The samples were stored for no longer than 2 months and thawed to room temperature only once during the analysis.

2.6. Blood counts and serum analysis.

Blood counts were obtained using a hematology analyzer (Siemens Healthineers, Germany). Total IgE was assayed using the enzyme immunoassay (VIDAS bioMérieux SA, France) according to the manufacturer's instructions.

2.7. Biochemical procedures.

2.7.1. Protein assay.

The protein concentration was estimated using the method of Lowry *et al*. [14]. Briefly, 250 µL of Lowry reagent (30 mL of 2% Na_2CO_3 in 0.1 M NaOH, 0.6 mL of 5% $C_4H_4O_6KNa{\cdot}4H_2O$, and 0.6 mL of 2% $CuSO_4$) was applied to a 96-well plate. Then, 50 µL of diluted serum was applied to each well, mixed, and incubated at room temperature for 10 min. Finally, 25 µL of Folin-Ciocalteu reagent was added, mixed, and incubated at room temperature for 30 min. Absorbance was measured at a wavelength of 750 nm.

2.7.2. Fluorescence assessment of protein glycoxidative modifications.

The contents of glycophore, dityrosine, N′-formylkynurenine, kynurenine, and tryptophan were estimated on the basis of their characteristic fluorescence at wavelengths of 325/440 nm, 330/415 nm, 325/434 nm, 365/480 nm, and 295/340 nm, respectively [15, 16].

2.7.3. AOPP assay.

Advanced oxidation protein products (AOPP) were estimated by the method of Witko-Sarsat *et al.* [17]. 200 µL of serum diluted with phosphate-buffered saline (PBS) was applied to wells of a 96-well plate, and 20 µL of glacial acetic acid was added to each well. Absorbance was measured at 340 nm against a blank containing 200 µL PBS, 20 µL of glacial acetic acid,

and 10 µL of 1.16 M potassium iodide. A calibration curve was prepared using chloramine-T at concentrations of 0–100 µmol/L by applying 200 µL chloramine-T, 20 µL glacial acetic acid, and 10 µL of 1.16 M potassium iodide to wells of the plate. AOPP concentration is expressed in nmol chloramine-T equivalents/mg protein.

2.7.4. Thiol group.

The content of thiol groups was estimated by the method of Ellman [18]. In each well of a 96-well plate, 20 µL of serum and 2 µL of 5,5′-dithiobis-(2-nitrobenzoic acid) (10 mg/mL of 0.1 M phosphate buffer, pH 8.0) were added to 100 µl 0.L M phosphate buffer at pH 8.0. The samples were incubated in the dark at 37°C for 1 h, and the absorbance was measured at 412 nm against a blank. The thiol group content was calculated based on a standard curve using glutathione as a standard.

2.7.5. 3-Nitrotyrosine.

3-nitrotyrosine concentration was assessed with the 3-nitrotyrosine ELISA kit (Immundiagnostik AG), according to the manufacturer's instructions.

2.7.6. Total antioxidant capacity with ABTS.

2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was formed by the reaction of 7 mM of ABTS solution with 2.45 mM of potassium persulfate, incubated for 24 hours at room temperature with protection from light. Briefly, appropriate amounts of serum samples were added to an ABTS solution and diluted such that 200 µL of the solution had an absorbance of 1.0±0.04 per well. The absorbance reading was measured at 734 nm after six minutes of reaction at room temperature. The results were expressed in Trolox equivalents (µmol TE/L) [19].

2.7.7. Malondialdehyde (MDA).

Serum samples (50 µL serum plus 50 µL PBS or 100 µL PBS blank) were mixed with 200 µL of a mixture (1:1) of 0.37% thiobarbituric acid (TBA) and 15% trichloroacetic acid in 0.25 M HCl to precipitate protein. The reaction was carried out at pH 2-3 at 100°C for 40 min. The precipitate was pelleted by centrifugation at 3000×g at 4°C for 10 min. The absorption of the supernatants was read at a wavelength of 532 nm. The majority of TBA-reactive substances (TBARS) is MDA; therefore, the concentration of MDA in blood serum was expressed as µM MDA. The results were calculated using an absorption coefficient for MDA of $1.56\times10^{5}M^{-1}$ cm^{-1} [20].

2.7.8. Albumin cobalt binding (ACB).

ACB levels were measured by spectrophotometry using Bar-Or's method [21]. The assay method involved adding 25 µL of 0.1% cobalt chloride to 100 µL of serum, gently mixing, and waiting 10 min for adequate cobalt-albumin binding. 25 µL of dithiothreitol (1.5 mg/mL) was added as a colorizing agent, and the reaction was stopped 2 min later by adding 0.5 mL of 0.9% NaCl. Using a spectrophotometer at 470 nm, color development with DTT was compared to a serum-cobalt blank without DTT and reported in absorbance units (ABSU).

2.8. Statistical analysis.

Data are presented as the arithmetic mean values and standard deviations or median and interquartile ranges. Statistical significance was evaluated using the Mann-Whitney U test for non-normally distributed data. If a comparison of multiple groups showed a significant difference, a post hoc analysis was then used for pair-wise comparison of post hoc ANOVA with the Mann–Whitney U test as a nonparametric test. Spearman's rank correlation coefficient analysis was used to estimate the relationships between estimated markers and other factors, assuming linear dependence. Statistical data analysis was performed using the STATISTICA software package (version 13.1, StatSoft Inc. 2016, Tulsa, OK, USA, www.statsoft.com).

3. Results

Seventeen female and 13 male asthmatic subjects were enrolled in this study. At the same time, 15 healthy females and 15 males were recruited into the control group. Baseline characteristics, clinical laboratory values, and lung function indices for asthmatics and healthy controls are presented in Table 1.

Table 1. General characteristics of studied patients.

		Healthy controls	**Asthmatic patients**	**p**
n (F/M)		30 (15/15)	30 (17/13)	
Age	mean±SD	14.37±2.3	13.20±2.17	0.340
	range	10 – 17	10 – 17	
BMI z-score	mean±SD	0.41±0.68	0.55±0.81	0.379
BMI z – score	range	– 0.93 – 1.67	– 2.12 – 1.91	
%WBC	mean±SD	7.49±2.11	8.22±2.18	0.308
	range	4.28 – 10.14	5.05 – 15.61	
FEV_1	mean±SD	101.30±5.22	98.03±6.98	**0.01**
	range	97 – 116	87 – 120	
Total IgE (kU/l)	mean±SD	20.41±10.24	187.4±153.65	**<0.001**
	range	8.6 – 48	10.87 – 556.80	
ACT	mean±SD	-	24.22±0.8	
	range	-	23 – 25	
Atopic dermatitis, n (%)		0	13 (43%)	
Sensitization, confirmed, n (%)	House dust mites	0	8 (27%)	
	Grass and cereals pollen	0	6 (20%)	
	Trees pollen	0	5 (17%)	
	Animal danders	0	2 (7%)	

There were no differences in age and BMI z-score. Clinical hematological results were similar between the asthmatic group and healthy subjects. As measured by spirometry, lung function was significantly worse in asthmatic children than in healthy participants (p=0.01). Asthmatic participants had statistically significantly elevated levels of total IgE compared to the control group (p<0.001).

Table 2. Oxidative stress markers of the patients studied.

		Healthy controls	**Asthmatic patients**	**p**
AOPP (nmol/mg protein)	mean±SD	170.1 ± 44.5	178.75±38.52	0.178
	range	122.9 – 294.25	132.09 – 289.18	
3-Nitrotyrosine (nmol/mg protein)	mean±SD	0.12±0.011	0.123±0.012	0.525
	range	0.101 – 0.143	0.102 – 0.146	
Glycophore content (a.u./mg protein)	mean±SD	3.32±0.24	3.37±0.69	0.554
	range	2.74 – 3.87	2.56 – 6.20	
Dityrosine content (a.u./mg protein)	mean±SD	2.16±0.30	2.37±0.45	0.135

		Healthy controls	Asthmatic patients	p
	range	1.58 – 2.63	1.78 – 3.38	
N′-formylkynurenine content (a.u./mg protein)	mean±SD	2.57±0.35	2.69±0.43	0.953
	range	1.90 – 3.04	2.13 – 3.83	
Kynurenine content (a.u./mg protein)	mean±SD	4.11 ± 0.48	4.06 ± 0.49	0.367
	range	3.28 – 4.97	3.35 – 6.48	
MDA (μmol/L)	mean±SD	3.39± 0.33	3.48±0.43	0.506
	range	2.92 – 4.25	2.73 – 4.42	
ACB (ABSU)	mean±SD	0.295±0.023	0.298±0.038	0.906
	range	0.245 – 0.34	0.228 – 0.401	

Controlled asthma was determined by a score of 23 and 25 points on the ACT. Atopic dermatitis was confirmed in 13 children with asthma. Among the most common allergens, the predominant allergy was mite (27% of patients), followed by grass and cereal pollen (20% of patients). Increased levels of oxidative stress biomarkers in serum were found in patients with well-controlled asthma. Statistically, significant differences are presented in Figures 1-3, and differences with no statistical significance are shown in Table 2.

We did not find any differences in the concentration of AOPP and 3-nitrotyrosine. Thiol group concentration in blood serum was decreased in asthmatic patients (p=0.023, Figure 1).

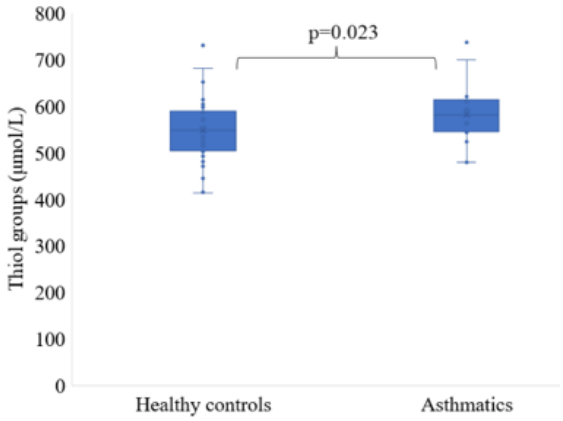

Figure 1. The concentration of thiol groups in the serum of healthy controls and children with controlled asthma.

There were no differences in the level of fluorescence characteristic for glycophore, dityrosine, N′-formylokynurenine, and kynurenine in patients with asthma in comparison to healthy participants. Tryptophan content was decreased in asthmatic patients (p=0.008, Figure 2).

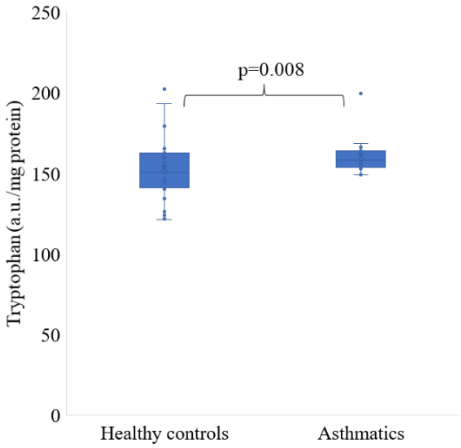

Figure 2. Content of tryptophan in the serum of healthy controls and children with controlled asthma.

Similarly, serum total antioxidant capacity measured by ABTS was significantly reduced in children with asthma as compared with healthy controls (p<0.001, Figure 3).

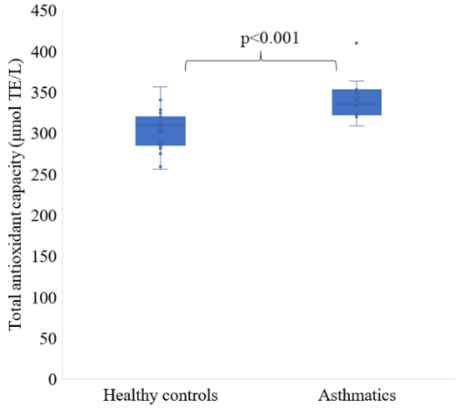

Figure 3. Serum total antioxidant capacity of healthy controls and children with controlled asthma.

There were no differences in concentration of MDA (p=0.506) as well as ACB (p=0.906) between the studied groups of children. Table 3 presents correlations between the studied markers of oxidative stress and the parameters characterizing the patients.

Table 3. Correlation coefficients between oxidative stress parameters and characteristics of asthmatic patients.

Parameter		**Age**	**BMI z-score**	**%WBC**	**FEV_1**	**Total IgE**
AOPP	*R*	0.048	-0.343	0.033	-0.269	0.112
	p	0.802	0.064	0.862	0.15	0.453
3-Nitrotyrosine	*R*	-0.228	0.087	0.107	-0.002	-0.191
	p	0.226	0.647	0.575	0.99	0.313
Thiol groups	*R*	0.32	0.21	0.216	0.209	-0.167
	p	0.865	0.285	0.252	0.267	0.378
Glycophore	*R*	0.752	0.216	0.076	0.02	0.251
	p	0.06	0.252	0.689	0.915	0.181
Dityrosine	*R*	0.184	0.186	0.218	0.872	-0.199
	p	0.249	0.326	0.247	0.031	0.293
N'-formylokynurenine	*R*	0.119	**0.396**	0.093	0.029	-0.015
	p	0.291	**0.03**	0.626	0.881	0.939
Kynurenine	*R*	0.045	0.276	0.241	0.118	0.279
	p	0.814	0.14	0.199	0.534	0.136
Tryptophan	*R*	**-0.475**	0.202	0.157	0.331	**-0.445**
	p	**0.008**	0.285	0.406	0.074	**0.014**
Total antioxidant capacity with ABTS	*R*	0.116	0.189	0.101	0.319	-0.169
	p	0.54	0.317	0.956	0.086	0.372
MDA	*R*	-0.017	-0.035	0.26	0.284	0.186
	p	0.929	0.855	0.166	0.128	0.325
ACB	*R*	-0.026	-0.415	0.124	-0.176	-0.073
	p	0.891	0.053	0.513	0.352	0.701

A positive correlation between N′-formylkynurenine content and BMI z-score was found in asthmatic patients. Moreover, tryptophan content was significantly negatively correlated with the age (R = -0.475, p=0.008) of the patients as well as with total IgE (R = -0.445, p=0.014). Nevertheless, there were no other correlations between the studied parameters.

4. Discussion

Epidemiological and clinical data support the relationship between the increased production of ROS and the pathogenesis of bronchial asthma, and the development of asthmatic pathology [22]. According to recent studies, asthmatic patients have decreased Nrf2 levels, a

stress-activated transcription factor highly responsive to oxidative stress. It suggests that antioxidant and anti-inflammatory pathways may be inhibited, contributing to the dysregulation of sinonasal epithelial cell barrier function and disease progression [23]. Moreover, a study in asthmatic mice indicated that sirtuin expression is decreased in the bronchial tissues related to apoptosis of epithelial cells and elevated expression of pro-inflammatory cytokines in bronchoalveolar lavage fluid, which promotes oxidative stress [24]. Furthermore, the genetic variants of oxidative stress-responsive kinase I was associated with the asthma exacerbation rate, indicating that asthmatics with common alleles may be vulnerable to asthma exacerbations [25]. Hence, it was shown that oxidative damage of biomolecules is strongly connected to asthmatic inflammation [26]. An increased concentration of protein carbonyls was found in bronchoalveolar lavage fluid and serum of asthmatics [8, 27]. Similar to our results, no differences in AOPP concentration (0.18±0.01 vs. 0.18±0.02 nmol/mg of protein) were found in subjects with controlled asthma [7]. The thiol groups of cellular proteins also have a protective function against oxidative cell damage, and thiol groups in the serum of asthmatics have been reported to be significantly lower than in healthy controls (365.5±65.9 vs. 561.4±119.8 μmol/L, $p<0.001$) [28]. Likewise, in our study, the concentration of thiol groups was lower in children with well-controlled asthma than in healthy children. Moreover, newly diagnosed asthmatics have a decreased plasma concentration of thiol compared to control groups, while patients who had been undertaking montelukast monotherapy had similar levels [29]. We found no difference in the parameters measured by fluorescence, apart from a decreased concentration of tryptophan in patients with asthma. Contrary to our results, tryptophan and kynurenine levels measured by high-performance liquid chromatography were found to be significantly higher in asthmatic children than in healthy controls ($p<0.01$) in a study by Licari *et al.* [30]. Systemic tryptophan and its catabolites were also markedly higher in patients with allergic asthma [31]. The amino acid tyrosine is particularly susceptible to oxidation and nitration; hence the oxidized compound 3-nitrotyrosine is a marker of oxidative stress and protein oxidation damage [32]. 3-Nitrotyrosine was higher in the EBC of asthmatic children and may be considered a noninvasive marker of nitrosative events in the airways [33]. Increased levels of 3-nitrotyrosine in breath condensate may be explained by an interaction between airway epithelial inducible nitric oxide synthase, dual oxidase-2, and thyroid peroxidase, which contribute to airway nitro-oxidative regulation [34]. So far, such a relationship has not been described in the serum of patients. Hence there may be no difference in the concentration of 3-nitrotyrosine between asthmatic and healthy patients. Similar to our results, TAC of plasma was significantly lower in asthmatic patients with controlled asthma in a study by Karadogan *et al*. [10]. Furthermore, asthmatic children had significantly reduced plasma or serum concentrations of TAC ($p<0.01$) [35, 36]. Nevertheless, no differences in TAC assayed with ABTS were found in children aged 5-15 years with well-controlled asthma compared to healthy controls (360.02±34.10 vs. 362.45±12.45 μM, $p=0.496$) [37]. In contrast, significantly higher concentrations of MDA (0.12±0.02 vs. 0.07±0.01 nmol/mg of protein, $p<0.001$) were observed in adult participants with controlled asthma [7]. Similarly, MDA was elevated in the plasma of adult asthmatics with totally controlled asthma (3.18±0.13 vs. 2.31±0.06 nmol/mL, $p<0.05$) [10]. MDA levels were found to be significantly higher in asthmatic children's EBC than in healthy controls [38, 39]. The lack of difference in the ACB test between asthmatic and healthy children suggested that albumin does not undergo oxidative modifications at the cobalt ion binding site. Nevertheless, it is worth noting that the albumin concentration in asthma patients may be reduced [40]. This study found few correlations

between oxidative stress markers and clinical parameters. This may be due to the small number of patients and the homogeneous population. However, no correlations between spirometry indices and concentration of AOPP, MDA, and glutathione were also reported by Ammar *et al*. [7]. Nonetheless, plasma MDA levels positively correlated with the reactive C-protein in asthmatics [27]. According to recent reports, ROS may promote corticosteroid insensitivity by disrupting intracellular signaling, leading to the sustained activation of pro-inflammatory pathways in immune and airway structural cells [41]. Moreover, it is worth emphasizing that asthma patients have compromised antioxidant mechanisms. Serum selenium levels, essential for the proper functioning of antioxidant enzymes, were found to be twofold lower in asthma patients as compared to the controls [42]. Lower activity of glutathione peroxidase in serum or plasma was also observed in asthmatic subjects [7, 43]. Additionally, plasma levels of micronutrients and antioxidants were significantly reduced in children with asthma [35, 44, 45].

5. Conclusions

Our study has shown a reduced concentration of thiol groups, tryptophan content, and total antioxidant capacity in children with well-controlled asthma serum. No elevated protein and lipid oxidation products concentration was observed, which may be related to the short duration of the study.

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Conflicts of Interest

The authors declare no conflict of interest.

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