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The relationship of plasma aluminum to oxidant–antioxidant and inflammation status in asthma patients

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ABSTRACT

Aluminum (Al) is a non-essential mineral which human beings are exposed to on day-to-day life. The purpose of this study was to assess the concentrations of plasma Al and the relationship of those levels with risk factors for asthma. In total, 27 allergic asthmatics and 30 healthy volunteers were enrolled. Plasma Al and selected blood parameters were measured, and a pulmonary function test was performed. Higher Al concentrations were found in the asthmatics than the healthy controls. Increased immunoglobulin E, high-sensitivity C-reactive protein, lipid peroxidation products, and pro-inflammatory cytokines (tumor necrosis factor- α and interleukin [IL]-4) were observed, but IL-10 and overall antioxidant and enzyme activities were lower. Associations between oxidative-antioxidant status and inflammatory markers with plasma Al levels in asthmatics were noted. Al status was also linked to cytokine concentrations and pulmonary function. In conclusion, abnormal Al distribution may further precipitate oxidative stress and inflammation, alter Th1/Th2 lymphocyte balance, and therefore contribute to the development of asthma.

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1. Introduction

Aluminum (Al) is a non-essential and toxic metal which humans are frequently exposed to. Exposure to Al commonly results from foods that contain artificial additives, colorings, baking powder, and irregular dehydration agents, drinking water and utensils, and occupational-, cosmetic-, and iatrogenic-sources (Greger, 1992; Soni et al., 2001). It has been documented that high blood Al-induced oxidative stress and the associated inflammatory responses are related to disease conditions such as diabetes, end-stage renal

dysfunction, infection, liver disease, some types of cancer, and neuro-toxicity (Levine et al., 1990; Exley et al., 2007; Kaptanoğlu et al., 2007; Guo et al., 2011). In the authors' previous observation, plasma Al is frequently relatively high in asthma patients even though the contamination of inhalators, drug products and environmental factors are excluded. Some food products in local food markets reportedly have excessively high levels of Al and Al exposure is raising again as an important public health concern (Lee, 2012).

Al stimulates the secretion of inflammatory cytokines by human alveolar macrophages *in vitro* (Braydich-Stolle et al., 2010). The occurrence of asthma-like condition among

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workers in Al smelter pot-rooms is associated with increased expression of high-affinity immunoglobulin(Ig) E receptor and tumor necrosis factor (TNF- α) (Arnaiz et al., 2003). Oral Al also induces T helper (Th)-2 immune responses and increases in interleukin (IL)-4 and -5 productions in a mice model (Brunner et al., 2007, 2009). Systemic Al level (responsible for Al exposure) may play a role in the induction of asthma; however, there is limited information regarding the distribution of plasma Al in asthma patients.

Allergic asthma is a chronic inflammatory disorder of the airways that is characterized by variable bronchial obstruction, increased vascular permeability, and hyper-responsiveness (Wood et al., 2003; Verstraelen et al., 2008). An infiltration of the walls of the airway by a variety of inflammatory cells is involved in the inflammatory process, which has been demonstrated to significantly correlate with asthma severity (Sugita et al., 2003). T lymphocytes (T cells) are one of the critical mediators of the airway inflammation in asthma. Activated CD4⁺ Th2-type lymphocytes can exacerbate airway inflammation and hyper-responsiveness associated with asthma (Lee et al., 2001).

Immune cells are sensitive to oxidative stimulation, which can lead to increases in the production of pro-inflammatory cytokines and oxidants (Barnes, 2008; Park et al., 2009). Oxidative stress further induces inflammatory responses, which are related to features of asthma (Shanmugasundaram et al., 2001), and oxidant–antioxidant imbalance can lead to a shift in the Th1/Th2 lymphocyte balance (Groen et al., 1997; Koike et al., 2007). Increased blood markers of inflammation including TNF- α , IL-2, IgE, and eosinophil cationic protein (ECP), have also been shown to be associated with the pathogenesis of airway hyper-responsiveness (Cardinale et al., 2005; Johnson et al., 2007). Reportedly, the development and exacerbation of asthma involves increased levels of oxidative stress and chronic inflammation.

High systemic Al may be an allergic stimulus that activates inflammatory mediators and enhances antibody production. It is therefore possible that chronic exposure to Al can induce several biological effects implicated in asthma. The purpose of the present study was to compare plasma Al with both the oxidative-antioxidant status and inflammatory status of asthma patients and healthy controls.

2. Materials and methods

2.1. Patients

Between August and December 2006, 27 patients with mild to moderate allergic asthma were enrolled in the current study. These patients had been diagnosed in the otolaryngology unit of Cheng-Ching Hospital (Taichung, Taiwan) according to the criteria recommended by the American Thoracic Society. All participants came from Taichung City and the surrounding area. None of the subjects reported being a smoker or had a respiratory tract infection or exacerbation of asthma. Subjects with heart disease, ulcer complications, diabetes mellitus, liver and kidney diseases, cancer, systemic inflammatory disorders, and or who had received immune suppressant drugs

and antacids, and supplementation with antioxidants, vitamins/minerals, and *n* – 3 fatty acids, were also excluded.

Healthy subjects (*n* = 30) of similar age, gender, and body mass index (BMI) were volunteered as controls. All subjects signed informed consent statements. The study protocol was approved by the Ethics in Human Research Committee of the Cheng-Ching Hospital.

2.2. Determination of pulmonary function

Lung function tests included forced vital capacity (FVC), and forced expiratory volume in one-second (FEV1) measurements in asthma patients. FVC is defined as the amount of air, which can be forcibly exhaled from the lungs after taking the deepest breath possible. FEV1 is the volume of air that can be expelled following maximum inspiration in the first second. Baseline values of FVC and FEV1 were determined using a Jaeger MasterScreen (VIASYS Healthcare GmbH; Hoechberg, FRG).

2.3. Biochemical analysis

Blood samples were drawn in the morning after an overnight fast of 12 h. The concentrations of total serum IgE and ECP were estimated using routine laboratory techniques.

2.4. Determination of plasma Al

Plasma samples were digested in a H₂O₂/HNO₃ mixture in a START D microwave-assisted digestion system (Milestone Microwave Labstation ETHOSD). Al level was then determined using an atomic absorption spectrophotometer (932AA, GBC, Melbourne, Australia) with graphite furnace. The instrument was adjusted to a wavelength of 309.3 nm, with a slit of 0.5 nm, and used a hollow cathode Al lamp. The lamp current was 8.0 mA, the integration time was 1 s, and double-beam and D2-background correction were used. Accuracy of this method was confirmed by comparing to serum reference materials (level 2, NO0371, Seronorm, Nycomed, Oslo, Norway).

2.5. Measurement of oxidative stress

Lipid peroxidation was measured indirectly by the formation of thiobarbituric acid reactive substances (TBARS). Plasma samples were mixed with 3% sodium dodecyl sulfate, 0.1 N HCl, 10% phosphotungstic acid, and 0.7% thiobarbituric acid, and then incubated at 95 °C for 60 min. The TBARS was extracted into *n*-butanol and the fluorescence of the *n*-butanol layer was measured by a spectrofluorometer (PerkinElmer, Turku, Finland) with excitation at 485 nm and emission at 530 nm (Guo et al., 2011).

The isoprostanes are a family of eicosanoids of non-enzymatic origin produced by the random oxidation of phospholipids. Plasma 8-iso-PGF₂ α , one of isoprostanes, after purification by use of an 8-isoprostane-affinity column and affinity sorbent, was further analyzed in duplicate using an enzyme immunoassay kit (Cayman, Cat. No. 516351); absorbance was measured at a wavelength of 412 nm.

2.6. Determination of antioxidants

The levels of vitamin E were measured by reversed-phase HPLC (HPLC Pumps, Systems & Accessories, LabAlliance, USA) (Borel et al., 1998). Briefly, plasma samples were extracted with *n*-hexane and ethanol containing α -tocopheryl acetate as an internal standard. The *n*-hexane layer was separated and evaporated to dryness. The residue was re-dissolved in a mobile phase with a mixture of methanol, acetonitrile, and tetrahydrofuran, and then loaded on a Gemini C18 column (250 mm $\times$ 4.6 mm, 5 μ m) (Phenomenex, Inc., USA). Peaks were detected at wavelengths of 295 nm.

Glutathione (GSH) was assessed using Ellmans reagent as described by Beutler et al. (1963). In brief, DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) and GSH react to generate the yellow products, 2-nitro-5-thio-benzoic acid and glutathione disulfide (GSSG), the GSH concentrations in plasma sample solutions, were determined by measuring absorbance at 412 nm.

2.7. Determination of antioxidant enzymes

Plasma activity of catalase was estimated with a commercially EIA kit (cat #707002; Cayman Chemical Co., Ann Arbor, MI). This enzyme catalyzes the conversion of two molecules of hydroperoxide to oxygen and water; one unit of activity is defined as the amount of enzyme that caused the formation of 1.0 nmol of formaldehyde spectro-photometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen at 25 °C. In addition, glutathione peroxidase (GPx) activity was measured with a kit from Cayman Chemical (cat #703102). Oxidized glutathione, produced upon reduction of an organic hydroperoxide by GPx, was recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm; it was directly proportional to the GPx activity.

2.8. Measurement of inflammatory parameters

Plasma concentrations of high sensitivity C-reactive protein (hs-CRP) were measured using the human CRP ELISA kit (E-80CRP, Immunology Consultants Laboratory, Inc, Newberg, OR, USA). The intra- and inter-assay CVs (coefficient of variation) were <3% and <5%, respectively. The absorbance at 450 nm was measured, and readings were interpolated into the standard curve.

Plasma concentrations of tumor necrosis factor- α (TNF- α) (#88-7346, human ELISA Ready-SET-Go, eBioscience, San Diego, CA, USA) and interleukin-2, -4, -10 (IL-2, -4, -10) (#88-7026, -7046, -7106, human ELISA Kit, eBioscience) were also measured. Briefly, plasma samples were added to the wells and incubated with anti-TNF- α or IL-2, -4, -10 antibodies for overnight. Combined avidin-HRP and substrate solution was added to each well and was incubated at room temperature. Absorbance at 450 nm was measured, and readings were interpolated into the standard curve.

2.9. Statistical analysis

Each value was expressed as the mean $\pm$ SD or medians (inter-quartile range), depending on the normality of data distribution (Shapiro–Wilk test). The comparisons of different variables between patients and controls were made by Student's *t*-test (normality) or Mann–Whitney test (non-normality). A two-tailed *p* value less than 0.05 was considered statistically significant. Pearson's correlation or Spearman's correlation coefficients depend on the normality of residuals were performed to identify relationships of the blood variables.

3. Results

3.1. Clinical characteristics

A total of 30 asthma patients were initially included; however, 3 patients were excluded due to insufficient sample. All data were collected for the remaining 27 asthma patients. There were no significant differences in age, sex, and BMI between asthma patients and healthy controls (*p* > 0.05) (Table 1).

Most patients have not received asthma medications; whereas seven (25.9%) subjects treated with inhaler therapy (such as pulmicort turbuhaler, seretide accuhaler) alone, or combined with oral bronchodilator (theophylline and uniphyllin) in the past three months. Compared with healthy subjects, these asthmatics had significantly higher concentrations of IgE and hs-CRP.

Table 1 – Clinical characteristics of subjects.^{a,b,c}

	Asthmatics (n = 27)	Controls (n = 30)
Age (years)	40 $\pm$ 5	37 $\pm$ 6
Gender (M/F)	7/20	9/21
Body mass index (kg/m ²)	23 $\pm$ 3	24 $\pm$ 2
Pulmonary function		
FEV1/FVC %	72.3 $\pm$ 7.0	–
FEV1 (L)	2.2 $\pm$ 0.5	–
FVC (L)	3.0 $\pm$ 0.7	–
IgE (KU/L)	337.0 (87.3–552.0) [#]	38.4 (27.4–60.5)
ECP (μ g/L)	17.4 $\pm$ 8.0	–
hs-CRP (ng/mL)	4.2 (2.1–6.4) [#]	0.9 (0.4–1.4)
Plasma antioxidant enzymes		
Catalase (nmol/min/mL)	22.5 $\pm$ 3.0 [#]	26.8 $\pm$ 3.4
GPx (nmol/min/mL)	24.6 $\pm$ 7.9 [#]	35.3 $\pm$ 7.4
Cytokines		
TNF- α (pg/mL)	50.6 $\pm$ 17.7 [#]	19.1 $\pm$ 12.5
IL-2 (pg/mL)	41.1 $\pm$ 18.0	34.0 $\pm$ 10.1
IL-4 (pg/mL)	68.6 (51.3–91.1) [#]	39.5 (29.2–47.5)
IL-10 (pg/mL)	7.1 (4.3–14.5) [#]	10.9 (8.3–15.4)

^a Values are mean $\pm$ SD or medians (inter-quartile range).

^b [#]*p* < 0.05 vs controls.

^c FEV1=forced expiratory volume in one second; FVC=forced vital capacity; IgE=Immuno-globulin E; ECP=Eosinophil cationic protein; hs-CRP=high-sensitivity C-reactive protein; GPx=glutathione peroxidase; TNF- α =tumor necrosis factor- α ; IL-2=interleukin-2.

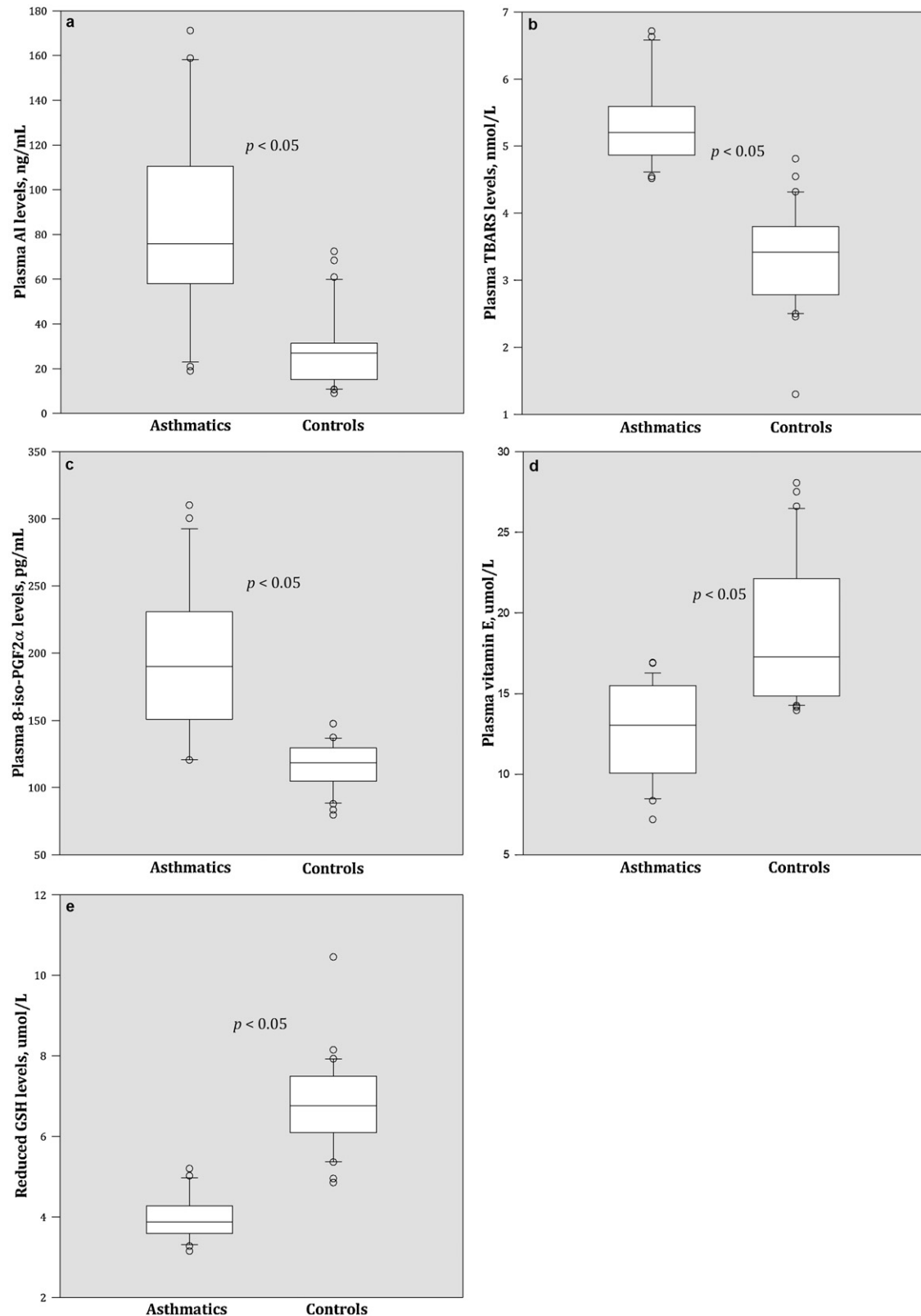


Fig. 1 – Distribution and comparison of plasma (a) Al, (b) TBARS, (c) 8-iso-PGF 2α , (d) vitamin E, and (e) reduced GSH concentrations in study participants. The bars represent medians (IQR). Al = aluminum; TBARS = thiobarbituric acid reactive substances; 8-iso-PGF 2α = 8-iso-prostaglandin (PG)F 2α ; GSH = glutathione. A p -value < 0.05 was considered statistically significant.

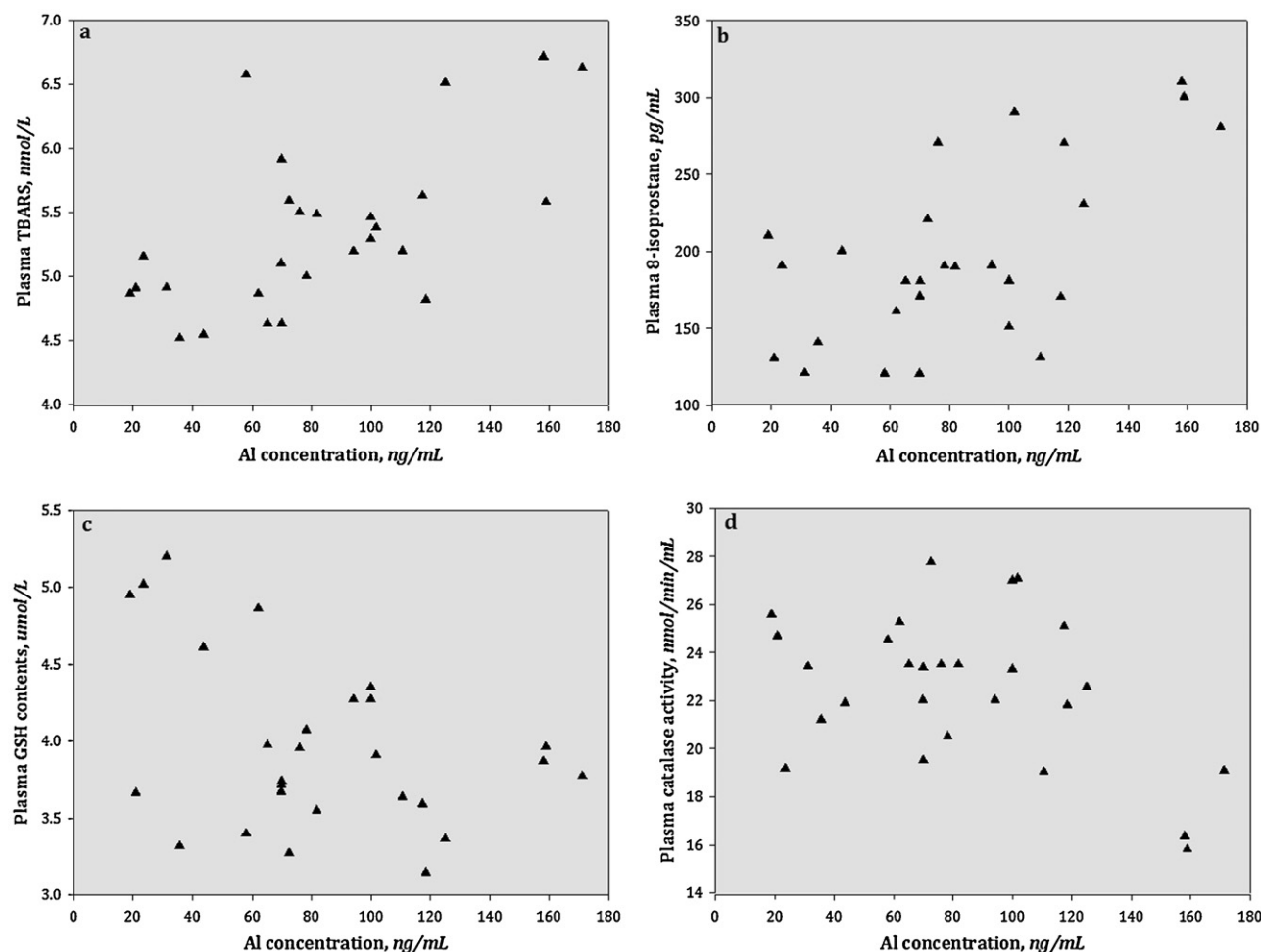


Fig. 2 – Correlations between (a) TBARS ($r = 0.586$, $p = 0.001$), (b) 8-iso-PGF 2α ($r = 0.629$, $p < 0.001$), (c) GSH ($r = -0.394$, $p = 0.042$), and (d) catalase ($r = -0.416$, $p = 0.031$) with plasma Al of asthma patients. Al = aluminum; TBARS = thiobarbituric acid reactive substances; 8-iso-PGF 2α = 8-iso-prostaglandin (PG)F 2α ; GSH = glutathione.

3.2. Plasma Al and oxidative-antioxidants status

The concentrations of plasma Al were significantly higher in asthma patients compared to healthy subjects. The concentrations of oxidative stress markers, TBARS, and 8-iso-PGF 2α , were increased in asthma patients compared with healthy subjects (Fig. 1). Asthmatics had significantly lower plasma antioxidant enzymes activity, including catalase and GPx (Table 1). Also, these patients had significantly reduced plasma concentrations of vitamins E and GSH, respectively (Fig. 1).

3.3. Cytokine profiles

There was non-significantly difference in IL-2 concentration between asthma patients and healthy controls. However, significantly higher TNF- α and IL-4, and lower IL-10 concentrations in asthma patients than healthy controls were observed (Table 1).

3.4. Correlations between Al and other parameters in asthmatics

For these asthma patients, plasma Al concentrations were statistically significantly correlated with TBARS ($r = 0.586$), and 8-iso-PGF 2α ($r = 0.629$) (Fig. 2). Plasma Al concentrations were also inversely associated with GSH concentrations ($r = -0.394$) and catalase activity ($r = -0.416$). Positive correlations between hs-CRP ($r = 0.463$), IgE ($r = 0.493$), IL-4 ($r = 0.430$), and TNF- α ($r = 0.554$) with plasma Al concentrations were demonstrated (Fig. 3). Plasma Al concentrations were also correlated with FEV1 ($r = -0.532$).

4. Discussion

In the present study, asthma patients had higher plasma Al concentrations, disturbances in oxidant-antioxidant status, and an increased inflammatory responses compared with healthy controls. In these patients, abnormal plasma

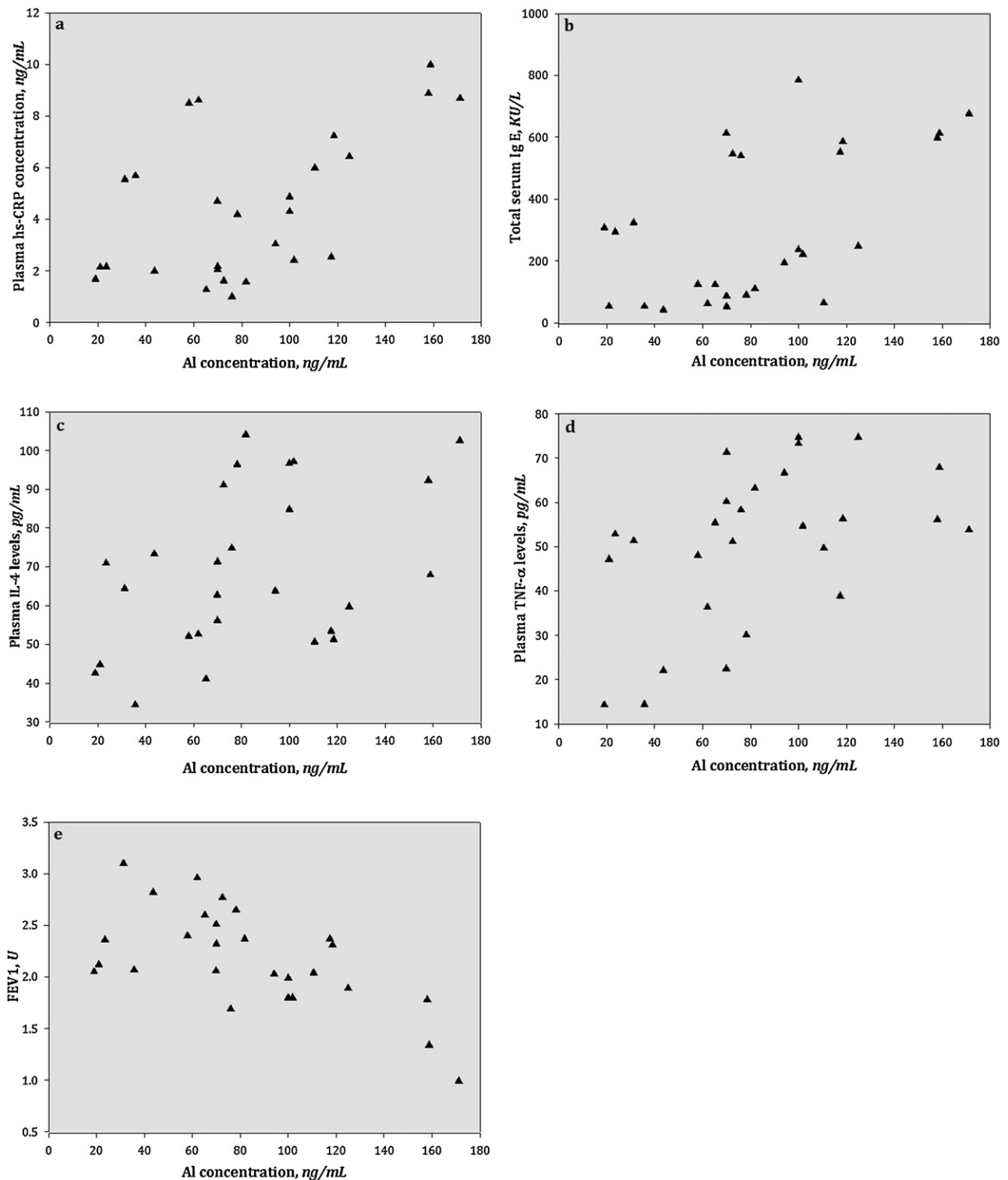


Fig. 3 – Correlations between (a) hs-CRP ($r = 0.463$, $p = 0.015$), (b) IgE ($r = 0.493$, $p = 0.009$), (c) IL-4 ($r = 0.430$, $p = 0.025$), (d) TNF- α ($r = 0.554$, $p = 0.003$), and (e) FEV₁ ($r = -0.532$, $p = 0.004$) with plasma Al of asthma patients. Al = aluminum; hs-CRP = high-sensitivity C-reactive protein; IgE = immunoglobulin E; IL-4 = interleukin-4; TNF- α = tumor necrosis factor- α ; FEV₁ = forced expiratory volume in one second.

Al content was significantly associated with oxidative stress, inflammation status, and pulmonary function.

Our results support previous studies that the asthma patients had significantly higher markers of oxidative stress (Sackesen et al., 2008; Al-Afaleg et al., 2011), although oxidant–antioxidant balance was not found to be significantly different between asthmatics and controls (Hanta et al., 2003; Koskela et al., 2012). Development of asthma is accompanied by increased oxidative stress by-products as measured by TBARS and 8-iso-PGF $_{2\alpha}$, and decreased levels of vitamin E and GSH, potent antioxidants. For example, airway inflammation has been shown to be induced by increased oxidative stress (Voynow and Kummarapurugu, 2011). Insufficient GSH status may also increase airway Th2 responses through increased NF- κ B expression, which is involved in the early asthmatic reaction and airway hyper-responsiveness (Kloek et al., 2010; Fitzpatrick et al., 2011). In addition, vitamin E deficiency has been shown to increase airway hyper-responsiveness, airway inflammation, and Th2 response including IL-4, IL-5, and IgE, and lipid peroxidation, in the lung (Mabalirajan et al., 2009; Hoskins et al., 2012). Al has no redox capacity; whereas exposure to Al has been shown to significantly decrease antioxidant enzyme activities, vitamin E, and GSH levels under certain conditions (Khanna and Nehru, 2007; Guo and Wang, 2011). The present study showed that oxidative stress-related parameters were correlated with plasma Al concentration in asthma patients. Thus, the pathophysiology of asthma is related to oxidative stress, and systemic Al is a particularly important variable in activating oxidative stress.

Furthermore, higher plasma Al concentrations are associated with increased hs-CRP and IgE status in these asthma patients. Increased hs-CRP status is associated with airflow obstruction and airway inflammation, and can serve as a marker of airway inflammation and asthma severity (Takemura et al., 2006). Moreover, IgE plays an important role in mediating the allergic inflammation and asthma (Borish et al., 2005). Increased serum IgE level is associated with low respiratory function, chronic airflow limitation, and bronchial hyper-responsiveness in asthma patients (Oryszczyn et al., 1991; Mojtaba et al., 2011). Potential cause of inflammation in asthma patients is the delayed programmed cell death of neutrophils due to increased IgE levels (Saffar et al., 2007). Recent evidence demonstrates that Al may induce NF- κ B activation, increased IL-1 β and cyclooxygenase-2 (COX-2) expression in cell culture (Alexandrov et al., 2005; Lukiw et al., 2005). There was a positive correlation between fibrosis due to Al deposits and lung inflammation (Schwartz and Weiss, 1990). IgE levels were also positively correlated with increased plasma Al in a murine model of asthma (Zhu et al., 2011). Taken together, an increased concentration of plasma Al may be a potential inducer of the inflammatory responses in these asthma patients.

Asthma is a complex and heterogeneous disease in which various cytokines contribute to airway inflammation. TNF- α is a pro-inflammatory cytokine that has been implicated in many aspects of the pathology associated with asthma (Berry et al., 2007). TNF- α blockade has reduced eosinophilia, IL-5, and IgE production in animal models of asthma (Hutchison et al., 2008). The present study reports increased TNF- α level suggesting plasma higher Al may increase pro-inflammatory

cytokines in asthma patients. Stimulation of peripheral blood mononuclear cells with Al *in vitro* induced significantly increased expression of TNF- α mRNA (Ulanova et al., 2001). Prolonged exposure with Al has also induced COX-2, TNF- α and IL-6 in macrophages (Nishanth et al., 2011).

The present investigation reports that asthma patients had significantly higher IL-4 and lower IL-10 concentrations. IL-4 is required for secretion of IgE from activated B lymphocytes. IL-4 showed synergistic effect with TNF- α or IL-5 on eosinophil activation (Luttmann et al., 1999). Moreover, IL-4 enhanced eotaxin release from fibroblasts may contribute to lung remodeling in patients with asthma (Steinke and Borish, 2001). Al has also been shown to activate CD4 $^{+}$ T cells secretion of IL-4, IL-5, and IFN- γ in mouse dendritic cells (Hsing et al., 2007; Sokolovska et al., 2007). IL-10, on the other hand, is decreased in alveolar macrophages and peripheral blood mononuclear cells of patients with asthma (Lim et al., 1998). IL-10 is derived from CD4 $^{+}$ T cells and is considered to be inhibitors of monocyte and macrophage function; as well as suppressors of pro-inflammatory cytokines in activated macrophages, T cells, and polymorphonuclear leucocytes (Chung, 2001). We report an association between plasma Al concentrations with TNF- α and IL-4 in asthma patients. Thus, the alterations of Th1/Th2-related cytokines may be partially due to higher plasma Al status.

In conclusion, the preliminary investigation found that asthma patients had significantly higher plasma Al contents, presumably related to their oxidative stress status. Furthermore, a disruption of the Al status may be responsible for the inflammatory response and Th1/Th2 lymphocyte imbalance, thereby having an adverse impact in development of asthma. Clinical observation does not rule out a causal relationship; whereas potential long-term exposure to Al, suggesting health hazard. The sources of Al-contamination should be avoided in order to prevent blood high Al levels associated with asthma. Further large-scale studies will be needed to clarify the Al effects on the development of asthma.

“Conflict of interest statement”

The authors do not have any conflicts of interest to declare.

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