ORIGINAL ARTICLE

Association of the Toll-like receptor 4 and NOX4 gene and protein levels in asthmatic patients with metabolic syndrome: A case–control study

Sevda Ghoushouni[1], Akbar Sharifi[1], Venus Zafari[2], Habib Zarredar[1], Ensiyeh Seyedrezazadeh[1]

[1]Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran, [2]Department of Basic Oncology, Health Institute of Ege University, Izmir, Turkey

Background: Understanding the contributing of influence inflammatory biomarkers in asthmatic patients with metabolic syndrome is more important. Whereby, the present study considering the important association of NADPH oxidase4 (NOX4) and Toll- like receptor4 (TLR4) in the respiratory inflammatory responses in asthmatic patients with metabolic syndrome (AS-MetS) and asthmatic (AS) patients. **Materials and Methods:** In this case-control study, 30 AS and 34 AS-MetS patients were enrolled. The Peripheral blood mononuclear cells (PBMCs) mRNA and protein levels of TLR4 and NOX4 were measured by qRT-PCR and western blot, respectively. Then their correlation was evaluated. **Results:** The significant down-regulation of mRNA and protein PBMCs expression levels of TLR4 were observed in the AS-MetS group in comparison to AS one (P=0.03), but the NOX4 expression was non-significant. Additionally, the significant correlation was exhibited between mRNA expression levels of NOX4 and TLR4 in both AS-MetS (r= 0.440, P=0.009) and AS groups (r=0.909, P=0.0001). The association between TLR4 mRNA level and triglyceride in AS-MetS group (r=0.454, P=0.008,) and also white blood cells (WBC) in AS group (r= -0.507, P=0.006,) were significant. **Conclusion:** The metabolic syndrome can significantly influence the expressions of TLR4 in AS-MetS. This study indicated that TLR4 and NOX4 altogether may provide valuable molecular knowledge of their relation with metabolic syndrome criteria for finding major pathways in different phenotype of asthma.

Key words: Asthma, metabolic syndrome, nicotinamide adenine dinucleotide phosphate oxidase, phenotype, Toll-like receptor 4

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INTRODUCTION

Metabolic syndrome as a global lifestyle problem that appears with pathologic conditions including abdominal obesity, insulin resistance, hypertension, and hyperlipidemia, is in line with World Health Organization defined.[1] The recent epidemiological studies indicated that metabolic syndrome factors are autonomously correlated with increased risk of asthma, the intensity of respiratory problems, and lung function failure. Moreover, changes of some pro-inflammatory mediators in obese asthmatics exhibited a critical function of metabolic factors in asthma pathogenesis.[2] Asthma is a heterogeneous disorder that emerged through an interaction of genetic and environmental factors. In such circumstances, complex disorders of chronic inflammatory components result in airway hyperresponsiveness, airway inner layer swollen and sensitization, and consequently, mucus production and its trigger.[3] Despite adequate treatment with anti-inflammatory medications, most of the asthmatic patients (AS) represent symptomatic conditions that are attributed to subphenotype properties with airway inflammatory responses that prompt lung tissue injury and remodeling.[3] The activation of pro-inflammatory biomarkers has been correlated with the etiology and development of chronic disorders including metabolic

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Address for correspondence: Dr. Ensiyeh Seyedrezazadeh, Tuberculosis and Lung Disease Research Center, Pashmineh Research Complex, Daneshgah Street, P.O. Box: 5142954481, Tabriz, Iran.
E-mail: e.s.rezazadeh@tbzmed.ac.ir

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syndrome.[4] Recurrent synthesis of such biomarkers persists in inflammatory cell changes and simultaneously, a vicious circle of tissues repairs that result in their remodeling. In such ways, innate immune receptor activations, such as toll-like receptors (TLRs), may act as a part of the chronic pro-inflammatory process in metabolic syndrome.[4,5] Metabolic syndrome features are autonomously correlated with increased risk of asthma, the intensity of respiratory problems, and lung function failure which is observed in about 60% of obese individuals who manifested metabolic syndrome criteria.[2,6]

One of the main pathological processes of asthma is the epithelial–mesenchymal transition (EMT) mediated by transforming growth factor β (TGF-β1).[7] Its role in fibroblast differentiation and airway remodeling is promoted through reactive oxygen species (ROS) production that is mainly generated by nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4), one of the seven members of NOX family (NOX1, NOX2, NOX3, NOX4, NOX5, Duox 1, and Duox 2) that across biological membranes transports electrons to generation intracellular superoxide in the endoplasmic reticulum. Notably, NOX4 has the leading function on stress-induced oxidative stress in airway smooth muscle in asthma.[8]

TLRs with 10 human isoforms as a pattern recognition receptor expressed in respiratory epithelial cells. They place on the cell surface or on the membrane of intracellular organelles and identify a range of different pathogen-associated molecular patterns.[9] It has been indicated that TLR4 mediated the differentiation of naïve T-cells to T-helper 2 in bronchial epithelial cells. It was demonstrated that TLR4 stimulates multiple epithelial-derived cytokines that consist of thymic stromal lymphopoietin, which its signaling has a major role for Th2 cell response for inducing airway remodeling. Moreover, TLR4 induced airway remodeling through the high-mobility group box 1 (HMGB1)/TLR4/nuclear factor kappa B (NF-Kb) pathway, which releases inflammatory cytokines including interleukin 1 beta (IL-β1), IL-10, TNF-α, andTGF-β1.[10]

Considering the recent viewpoint which is believed that the specific biomarker mechanisms affect various asthma phenotypes and promote airway remodeling, we planned to appraise the role and relation of concomitant metabolic syndrome on NOX4 and TLR4 expressions in the peripheral blood mononuclear cells (PBMCs).

SUBJECTS AND METHODS

Study subjects

In this case–control study, 64 asthmatic adult patients were included and their asthma was diagnosed on the base of ATS/ERS guidelines by respiratory physicians.[11] Patients who had other lung diseases or respiratory tract infections or/and concomitant asthma-associated disorders were excluded from the study. First, written informed consent was obtained. Patients were classified and included into two groups: (1) AS with at least three items of metabolic syndrome criteria as cases (AS-MetS) and (2) AS without metabolic syndrome criteria as controls (AS). The National Cholesterol Education Program Adult Treatment Panel III report criteria were used for MetS cases selection which has been described previously.[12] The criteria consist of five items are as follows: (1) waist circumference ≥102 cm for men and ≥88 cm for women, (2) thyroglobulin (TG) ≥150 mg/dl or drug treatment for elevated triglycerides as an alternate indicator, and (3) high-density lipoprotein (HDL-C) values of <40 mg/dl for men and <50 mg/dl for women. Moreover, (4) hypertension was defined as systolic blood pressure (BP) ≥130 mmHg and/or diastolic ≥85 mmHg or the use of antihypertensive medication, and (5) elevated fasting glucose was considered to be ≥100mg/dl or the use of glucose-lowering medication. Patients who had at least three of these five criteria considered as MetS and were eligible to including to this study as cases.

Sampling

After the participant's recruitment, the medical history including asthma history, smoking status, and other comorbidity diseases were recorded. In addition, following 12 h of overnight fasting, 5 ml of blood sample was obtained and separately collected into two tubes for PBMCs extraction or blood lipoprotein profiles and glucose measurement.

The Ethical Committee of Tabriz University of Medical Sciences has confirmed the study ethics (Code: IR.TBZMED.REC.1398.1110).

Metabolic syndrome criteria assessments

The height and weight were taken. Moreover, the mean BP was measured through twice a day measurements using an automated instrument. Quantitatively, measurements of serum fetal bovine serum (FBS), TG, and HDL were accomplished using enzymatic methods with ZellBio ELISA kit (Cat NO: ZB-10080c-H9648, GmbH, Germany) according to the manufacturer's protocols.

Peripheral blood mononuclear cells extraction

Blood samples were collected in heparinized tubes and immediately transferred to the molecular biology laboratory of the Tuberculosis and Lung Diseases Research Center and the PBMCs were extracted. An identical volume of phosphate-buffered saline (PBS) was added to each sample and added the equal volume of Ficoll solution. Thereafter, the sample was centrifuged at a rate of 2000 rpm at 4°C (20 min). After discarding the upper layer, the mid-layer

was moved to a sterile tube and three times washed by PBS. Finally, the total extracted PBMCs were stored at − 80°C until the mRNA extraction process.

RNA extraction and reverse transcription

For RNA extraction, the frozen PBMCs solution was melted at room temperature. The samples were centrifuged at 13,000 rpm and 4°C for 5 min, the upper layer was discharged and the RNA was extracted applying RiboEx reagent (GeneAll, Korea) according to the manufacturer's instruction. The RNA quality and quantity were assessed by utilizing the gel electrophoresis and Thermo Scientific™ Nano Drop™ One Spectrophotometer (Waltham, USA), and the ratio of 260/280 OD which is equal to 1.8–2 is considered as a suitable ratio. A cDNA synthesis kit (BioFACT TM, Korea) was utilized for cDNA synthesis according to the manufacturer's instruction.

mRNA expression analysis

The specific primers for *NOX4* and *TLR4* were designed by Gene Runner and NCBI-BLAST programs. The sequences of primers for *TLR4* are forward 5′-GGTGATTGTTGTGGTGTCCCA-3′ and reverse 5′-AGTGTTCCTGCTGAGAAGGCG-3′; for *NOX4*, forward 5′-GCTTGAGCACAGGGTACTTTA-3′ and reverse 5′-AGCTTGGAATCTGGGCTCTT-3′; and for *GAPDH*, forward 5′-CATGGCCTCCAAGGAGTAAG-3′ and reverse 5′-GCTTGAGCACAGGGTACTTTA-3′. *GAPDH* gene primers, an internal control, were obtained from a previous report.[13] The real-time polymerase chain reaction experiments were performed in triplicate using a 48 well Step One™ System and Master Mix kit (Ampliqon, Denmark) with the following conditions: 95°C for 10 min, 40 amplification cycles consisting of 95°C for 15 sec, the annealing 57°C for 30 sec, and 72°C for 35 s for the extension. The expression levels of the genes were analyzed based on the cycle threshold (Ct) and used the $2^{-\Delta\Delta Ct}$ methods for relative expression ratio calculation.

Western-blot analysis

The total protein concentrations in the blood supernatants were measured following the Bradford protocol, separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride or polyvinylidene difluoride membranes. The membranes were blocked with 2% low-fat milk in Tris-buffered saline, incubated for 16–18 h at 4°C with the commercially available β-Actin (C4: sc-47778), NOX4 (3H2G11: sc-517188), and TLR4 (25: sc-293072) primary antibodies. After treating the membrane by washing buffer, they incubated with the appropriate HRP-conjugated secondary antibody (m-IgGκ BP-HRP: sc-516102) and bands were visualized by enhanced chemiluminescence. Densitometric analysis was carried out using NIH Image/ImageJ software. We bought all antibodies from Santa Cruz Biotechnology Inc Company.

Statistics analysis

For assessment of normality, Kolmogorov–Smirnov with kurtosis and skewness indices tests were used. To compare the mean expression levels between groups, we used an unpaired *t*-test. In addition, Pearson's coefficient test was used to estimate relationships between studies factors. Furthermore, a partial correlation test was used for analyzing the association of the expression of NOX4 and TLR4 with controlling for age and sex. The statistical significance levels were considered $P < 0.05$. All analyses were done using the SPSS software, version 16 (Chicago, SPSS Inc; 2007).

RESULTS

The primitive features of AS are displayed in Table 1. As shown in Table 1, there was a significant distinction in body mass index (BMI) between the two groups. The analysis data are shown in Table 2. The results indicated significant differences in serum levels of FBS, TG, and HDL-c between the two groups ($P = 0.032$, $P < 0.0001$, and $P = 0.001$, respectively). Nonsignificant differences were observed between groups for pulmonary function tests [Table 3].

The *TLR4* mRNA expression levels are significantly lower in AS-MetS group compared to AS group ($P = 0.027$) [Figure 1a]. Moreover, the positive correlation was observed between *TLR4* gene expression and TG in AS-MetS ($r = 0.454$, $P = 0.008$) and a negative association with white blood cells (WBC) in AS group ($r = -0.507$, $P = 0.006$).

The analysis displayed nonsignificant differences of *NOX4* gene expression level between the two groups ($P = 0.881$) [Figure 1b]. However, we found a significant negative correlation between the *NOX4* mRNA level with waist circumference ($r = -0.433$, $P = 0.019$) and WBC ($r = -0.440$, $P = 0.019$) in AS group.

Moreover, a significant association was found between mRNA expression of *NOX4* and *TLR4* in both AS-MetS ($r = 0.440$, $P = 0.009$) and AS groups ($r = 0.909$, $P = 0.0001$).

Both TLR4 and NOX4 protein levels significantly decreased in AS-MetS group in comparison to AS group ($P = 0.013$) [Figure 2]. Moreover, a positive but nonsignificant relation was shown between protein expression of NOX4 and TLR4 in both AS-MetS ($r = 0.725$, $P = 0.275$) and AS groups ($r = 0.741$, $P = 0.259$).

DISCUSSION

Our study shows that the PBMCs mRNA expression levels of TLR4 and NOX4 are higher in (AS) than the asthmatic ones with metabolic syndrome (AS-MetS), although the NOX4 levels are nonsignificant. In this way, both TLR4 and

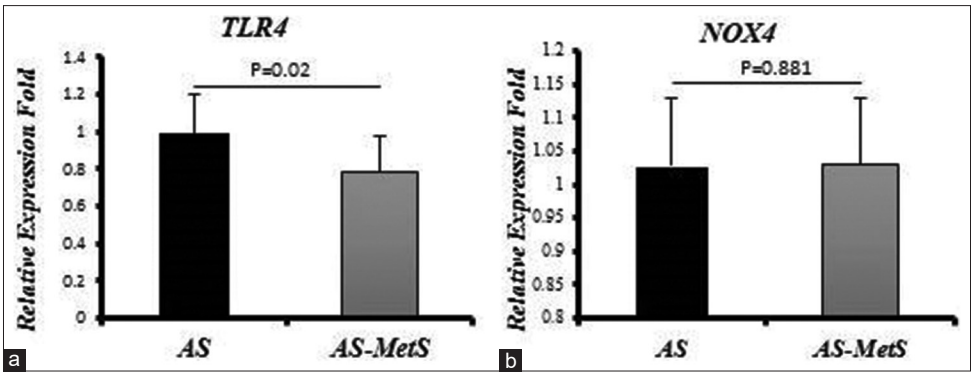

Figure 1: Relative mRNA expression ratio of NOX4 (a) and TLR4 (b) AS: Asthmatic patients; AS-MetS: Asthmatic patient with metabolic syndrome

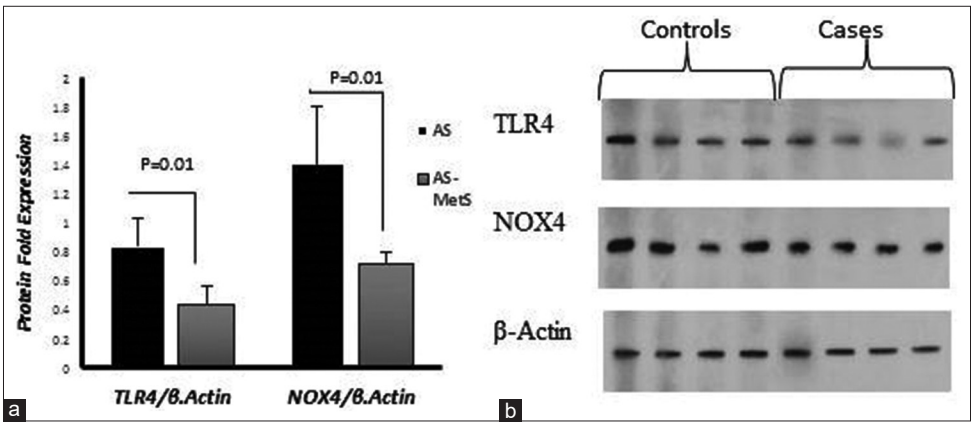

Figure 2: Total PBMC protein/β-actin ratio of NOX4 and TLR4 (a). Total protein extracted of NOX4 and TLR4 from PBMC (b). AS: Asthmatic patients; AS-MetS: Asthmatic patient with metabolic syndrome

Table 1: The baseline characteristics of studied patients

Features	Asthmatic patients (*n*=30)	Asthmatic patients with MetS (*n*=34)	*P*
Age (years)*	45.25±10.27	54.06±10.35	0.004†
Sex (%)			
Female	64.3	69.4	0.572‡
Male	35.7	30.6	
Weight (kg)	77.43±13.54	83.33±18.18	0.102†
BMI (kg/m^2)*	29.65±4.60	33.10±6.31	0.026†
Waist circumference (cm)*	107.68±13.31	113±10.54	0.109†
Smoking status (%)			
Nonsmoking	85.7	94.4	0.547‡
Active smoking	7.1	2.8	
Quit smoking	7.2	2.8	
Asthma severity (%)*			
Intermittent	-	2.8	0.098‡
Mild persistent	39.3	52.8	
Moderate persistent	42.9	27.8	
Severe persistent	14.3	16.7	
Duration of asthma (years)*	6.07±6.60	8.61±11.2	0.412†
Family history of asthma (%)	40	44.1	-

†Based on *t*-test, ‡Based on Chi-square test. Data as (mean±SD). SD=Standard deviation; Mets=Metabolic syndrome; BMI=Body mass index

NOX4 protein expressions were significantly higher in AS. We also found a notable relation between mRNA expression of *TLR4* and *NOX4* in both AS and AS-MetS groups. In addition, the correlation between *TLR4* mRNA expression and TG in AS-MetS was positive; and the relation between the mRNA level of *NOX4* with waist circumference was negative in AS group.

Recently, several airway inflammatory biomarkers have emerged as potential new valuable tools in the diagnosis

Table 2: Results of metabolic syndrome laboratory test and complete blood count test data of the studied groups

Parameters	Mean (95% CI)		*P*
	AS patients	AS-MetS patients	
FBS (mg/dl)	101.37 (95.07-107.66)	116.19 (104.84-127.54)	0.032
TG (mg/dl)	105.96 (89.01-122.90)	200.22 (171.58-228.87)	0.0001
HDLc (mg/dl)	62.81 (56.15-69.47)	50.48 (46.96-53.99)	0.001
Hct (%)	43.06 (40.66-45.47)	42.27 (40.67-43.88)	0.749
Hgb (g/dl)	14 (13.16-14.83)	13.86 (13.33-14.38)	0.948
Total WBC count (10^3/μl)	6.91 (6.17-7.64)	7.84 (7.13-8.55)	0.215
Neu. (10^3/μl)	55.83 (51.96-59.70)	55.78 (53.10-58.46)	0.867
Lym.(10^3/μl)	30.73 (28.16-33.30)	31.02 (28.25-33.78)	0.970
Eos. (10^3/μl)	5.20 (3.35-7.05)	4.29 (3.29-5.29)	0.459
Bas. (10^3/μl)	0.76 (0.60-0.92)	0.80 (0.63-0.96)	0.358
Mon. (10^3/μl)	5.16 (4.59-5.72)	5.49 (4.98-6)	0.340
RBC (10^3/μl)	4.79 (4.41-5.16)	4.98 (4.81-5.14)	0.287
MCV (fl)	87.30 (84.35-90.24)	84.88 (82.92-86.85)	0.253
MCH (pg)	28.36 (27.27-29.45)	27.70 (26.99-28.41)	0.527
MCHC (g/dl)	32.46 (32.13-32.80)	32.63 (32.34-32.93)	0.179

CI=Confidence interval; AS=Asthmatic; AS-MetS=AS-metabolic syndrome; FBS=Fasting blood sugar; TG=Triglyceride; HDLc=High-density lipoprotein; HCT=Hematocrit; Hgb=Hemoglobin; Neu=Neutrophils; Lym=Lymphocyte; Eos.=Eosinophil; Bas. Basophil; Mon.=Monocyte; RBC=Red blood cells; MCV=Mean corpuscular volume; MCH=Mean corpuscular hemoglobin; MCHC=Mean corpuscular hemoglobin concentration; and WBC=White blood cell

Table 3: Respiratory function of the studied groups

Pulmonary function	Mean (95% CI)		*P*
	AS patients	AS-MetS patients	
FVC (L)	3.33 (2.88-3.78)	2.86 (2.38-3.25)	0.264
FEV1-FEV6 (%)	68.28 (63.57-72.99)	72.30 (68.17-76.42)	0.343
FEV1 (L)	2.24 (1.94-2.53)	2.06 (1.74-2.38)	0.676
FEV1-FVC (%)	66.49 (62.06-70.91)	74.95 (69.80-80.09)	0.119
FVC.pred (L)	90.26 (79.24-101.27)	74.79 (61.41-88.16)	0.282
FEV1.pred (L)	71.47 (60.98-81.96)	64.40 (52.66-76.15)	0.769
PEF (L/S)	4.84 (4.08-5.59)	4.93 (4.21-5.65)	0.693
MEF_{75} (L/S)	0.95 (0.62-1.28)	1.43 (0.80-2.06)	0.615
MEF_{50} (L/S)	2.13 (1.67-2.59)	2.42 (1.99-2.85)	0.693
MEF_{25} (L/S)	3.52 (2.88-4.16)	3.60 (2.86-4.33)	0.776
MVV (L/m)	81.10 (70.02-92.18)	81.39 (69.34-93.44)	0.982

FVC=Forced vital capacity; FEV=Forced expiratory volume; FVC pred.=FVC predicted; PEF=Peak expiratory flow; MEF=Maximum expiratory flow; MVV=Maximal voluntary ventilation; CI=Confidence interval; AS=Asthmatic; AS-MetS=AS-metabolic syndrome; FEV1=FEV in 1 s FEV6=FEV in 6 s

and management of asthma. The analysis of biomarkers obtained noninvasively from a variety of sources such as exhaled breath, urine, or blood has great advantages in asthma diagnosis over conventional techniques. Plus, recent epidemiological findings suggest an association between metabolic syndrome and susceptibility to asthma, which etiologic causes could be linked by systemic and airway inflammation, insulin resistance, and oxidative stress.[14] Symptoms of metabolic syndrome such as high-waist circumference, BMI, and hyperlipidemia can be the major risk factors for asthma.[14] Overweight AS may respond better to leukotriene modifiers therapy than inhaled corticosteroids, which may reflect the different responses in airway inflammation of obese in comparison to nonobese patients.[15]

One of the processes that has a prominent role in various diseases is apoptosis. It is actively studied in the pathogenesis of asthma and hypertension, and it has been suggested that the deregulation of apoptosis in activated T-cells and eosinophils is involved in the development of airway inflammation in asthma. About hypertension, there is evidence of increased apoptosis in whole organs. On the other hand, TLR4 stimulation also induces apoptosis and can mediate neuronal apoptosis. Moreover, TLR4 is necessary for the immunological mechanism of apoptosis.[16] Hence, it can be one of the important factors to investigate in both asthma and metabolic syndromes and also simultaneously. In addition, the upregulated level of NOX4 has the main role in oxidative stress through the production of ROS. The evidence indicates that the induction of NOX4 expression leads to alveolar epithelial cells apoptosis, airway smooth muscle remodeling, and expands pulmonary dysfunction.[17] Increasing evidence suggests a potential role for NOX4 in the TGF-β1-mediated mechanism of epithelial changes, EMT, and airway remodeling in severe asthma and lung

fibrosis.[18,19] Furthermore, NOX4 is found in various cardiovascular cells and tissues and is co-expressed in endothelial cells, vascular smooth muscle cells, adventitial fibroblasts, and cardiomyocytes. In addition, NOX4 is found in cardiac fibroblasts. Numerous works argue in favor of the contribution of some NOX isoforms, such as NOX1 in hypertension, but the contribution of NOX4 is less clear.[20] Al-Azzam *et al*. have shown the upregulated levels of NOX4 in response to TGF-β1 in asthmatic mice cells. On the contrary, they found NOX4-specific siRNA significantly suppressed α-SMA and fibronectin protein *in vitro*.[18] A study on a high-fat-diet mouse model has been exhibited upregulation of NOX4 expression through mediated ROS production-induced metabolic syndrome featuring insulin resistance.[21] TLRs may have impressible effects on the pathogenesis of respiratory diseases including asthma and chronic obstructive pulmonary disease.[22] TLR4 can promote a pro-inflammatory response that could be recognized by the various endogenous ligands, including saturated fatty acids, modified LDLs, HMGB1, and advanced glycation end-products. It appeared that TLR4 which is activated by fatty acids through inducing insulin resistance, manifested a major role in the MetS pathogenesis, increasing the NF-κB levels, releasing monocyte chemotactic protein-1, IL-1β, IL-6, and TNF-α.[23]

Some studies have reported a relation between NOXs-derived ROS signaling pathways and different types of inflammatory responses of TLRs. Nadeem *et al*.'s study on asthmatic mice has indicated that MyD88 as an essential signal transducer in TLR4 signaling pathways mediates the increased level of NOX-4 during TLR-7 activation.[22] As well, induction of TLR4 signal inhibitors genetically or pharmacologically suppressed NOX4 expression and reduced oxidative/nitrative stress after cerebral ischemia in the murine model of asthma.[22] In the present study, TLR4 and NOX4 PBMC protein levels were significantly lower in AS-MetS patients versus AS. The reduction level of TLR4 expression in the AS-MetS group may be attributed to the metabolic syndrome condition. Moreover, AS-MetS individuals in the present study probably have taken metabolic disorder-related medication which influences TLR4 expression.[24] Elseways, it seems that the oxidative condition in two groups brought increased NOX4 condition. TLR4 level indicated a significant direct correlation with TG levels of AS-MetS individuals that consist of Jialal *et al*.'s study results.[24] According to our knowledge, although there is no report about the relation between TLR4 and lipid profile including TG, Liu *et al*. found upregulated levels of TLR4 liver tissue and serum TG in nonalcoholic fatty liver disease rat model in comparison to controls.[26] Some limitations should be brought up in the present study. First, with the lack of healthy and MetS groups, we lost some comparison detailed outcomes. Second, the medication of patients with metabolic syndrome for related diseases may affect the patterns of expression of inflammatory factors, including TLR4 and NOX4.

In summary, lower expression levels were found for mRNA and protein levels of TLR4 and NOX4 in AS with metabolic syndrome (AS-MetS) in comparison to asthmatic ones which is significant for TLR4 levels. In addition, a marked correlation was detected between TLR4 and NOX4 in the two study groups. Moreover, a positive association was observed between TLR4 mRNA expression and TG in AS-MetS patients. Further studies are suggested with more participants and also additional groups including MetS and healthy controls.

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Source of support and ethical number

This study was approved by the Tuberculosis and Lung Disease Center, Tabriz University of Medical Sciences (No. 63969), and conducted with the Ethics Committee approval number IR. TBZMED. REC.1398.1110 of Tabriz University of Medical Sciences.

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Conflicts of interest

There are no conflicts of interest.

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