

## Original Article:

Correlation of  $\alpha$ 1-Antitrypsin (A1AT), Complement Component C5a and Secretory Immunoglobulin A (sIgA) With Pulmonary Complications; 20 Years After Sulfur Mustard Exposure, Sardasht-Iran Cohort Study

Maryam Nikoonejad<sup>1</sup> , Mohammad Ebrahim Yarmohammadi<sup>1,2</sup> , Shahryar Pourfarzam<sup>1,3</sup>, Faramarz Falahi<sup>1</sup> , Elham Faghihzaheh<sup>1</sup>, Tooba Ghazanfari<sup>1\*</sup>

1. Immunoregulation Research Center, Shahed University, Tehran, Iran.

2. Department of Otolaryngology, Shahed University, Tehran, Iran.

3. Department of Internal Medicine, Shahed University, Tehran, Iran.



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**ABSTRACT**

**Background:** Little is known about pulmonary complications induced by Sulfur Mustard (SM), as a chemical warfare agent, especially considering its long term effects. The present study was carried out to investigate the association of  $\alpha$ 1-Antitrypsin (A1AT), Complement component C5a, and Secretory Immunoglobulin A (sIgA) with long-term pulmonary complications on SM-exposed individuals, 20 years after the exposure.

**Materials and Methods:** Sardasht-Iran Cohort Study (SICS) is a historical cohort study on 372 SM-exposed individuals and 128 age-matched unexposed participants. The clinical evaluations and Spirometry were performed on all the participants according to American Thoracic Society Criteria. Also, we assessed the salivary levels of A1AT, C5a, and sIgA using ELISA assay.

**Results:** The results indicated significant associations between salivary levels of A1AT, C5a, and sIgA with chronic cough in the exposed victims. Also, there were associations between C5a and sIgA with dyspnea in SM-exposed victims with chronic cough compared with those in the exposed victims without chronic cough. The salivary levels of C5a significantly decreased in severe pulmonary involvement. Moreover, a significant relationship was observed between the salivary levels of C5a and pulmonary function tests.

**Conclusion:** According to the findings, the salivary level of C5a might reflect the severity of pulmonary involvements following SM exposure. A1AT, as a protective agent, has correlations with cough and dyspnea. Although no strong correlation was found between the salivary levels of these factors with persistence of pulmonary complications, they can be effective in the development and progression of pathological changes in the lungs of the SM-exposed victims.

**\* Corresponding Author:**

Tooba Ghazanfari, PhD.

**Address:** Immunoregulation Research Center, Shahed University, Tehran, Iran.

**Phone:** +98 (21) 88964792

**E-mail:** tghazanfari@yahoo.com

## Introduction

**S**ulfur Mustard (SM) was used extensively as a chemical warfare agent during the Iran-Iraq war (1983-1988). Even at a low dose, SM causes serious damages to the vital organs, including: skin, eyes, and respiratory system. One of the main targets of SM is the respiratory system where it induces extensive injuries, but the underlying mechanism(s) by which SM causes chronic lung damages is not well known. However, about 45,000 of Iranian populations are still suffering from late respiratory complications of SM exposure [1]. Pulmonary injuries following SM exposure are often lethal in a short time after exposure and in survivors it can lead to long term complications such as Chronic Obstructive Pulmonary Disease (COPD) and Bronchiolitis Obliterans (BO), which cause serious health problems. The characteristics of mustard lung disorders, i.e. COPD and BO, due to SM exposure is different from COPD and BO induced by other factors [2].

The present study was conducted in a civilian SM-exposed population in Sardasht-Iran, henceforth Sardasht-Iran Cohort Study (SICS). In SICS patients, dyspnea is the most common symptom, and chronic cough, sputum, hemoptysis, and chest pain are also common pulmonary findings in SICS population. Previous studies have also shown that the serum levels of pro-inflammatory cytokines, including IL-1a, IL-1b, IL-1Ra, and TNF, are significantly lower in the SM-exposed victims. However, the serum level of MCP1 (CCL2) is significantly higher. But the levels of MMP9 and fractalkine (CX3CL1) are not shown to change in the serum of SM-exposed persons. In general, studies on SM-exposed individuals indicated that inflammatory mediators play a crucial role in chronic pulmonary complications [3].

The results of the studies performed on COPD caused by factors other than SM-exposure indicated a strong correlation between immunologic modulators such as  $\alpha$ -1-Antitrypsin (A1AT) and sIgA with pulmonary complications. Deficiency of  $\alpha$ -1-Antitrypsin (A1AT), and sIgA causes inflammation and airway obstruction [4, 5].  $\alpha$ -1-Antitrypsin (A1AT), an acute-phase reactant, is a serine protease inhibitor (serpin) that inhibits leukocyte proteases, attenuating tissue injury and inflammation [6]. A1AT is mainly produced by hepatocytes, but inflammatory cells like monocytes, macrophages, pulmonary alveolar, and epithelial cells also produce A1AT, as a response to the presence of inflammatory cytokines (e.g. IL-6, IL-1 and Q10 TNF- $\alpha$ ) [7].

A1AT inhibits the activity of proteases involved in lung matrix fragmentation (MMP9) and apoptosis of endothelial cells (caspase 1 and 3), especially in alveolar epithelial cells, and prevents the activation of inflammatory cells, such as macrophages and neutrophils, and reduces the pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1 and IL-8 [6-9].

A1AT deficiency showed as a definite genetic risk factor for the development of COPD in smokers [10]. A reduced serum A1AT activity was observed as a long-term effect of SM exposure, which contributes to the development of respiratory complications [11]. Secretory immunoglobulin A (sIgA), composed of IgA, Secretory Component (SC), and J chain, is the major mucosal immunoglobulin. sIgA, as the first line of defense in the respiratory tract level increases in order to protect against infection after injury [8]. Studies indicated that lack of sIgA in bronchial mucosa of COPD patients causes neutrophil infiltration and airflow obstruction [4, 5].

Complement component C5a is a powerful inflammatory mediator produced in the course of complement cascade activation. C5a induces chemotactic migration, increases cell adhesion, stimulates the oxidative burst, and releases various inflammatory mediators such as histamine and Cytokines by binding to C5a Specific Receptor (C5aR). The amount of C3a, C4a, and C5a in the saliva of normal individual is low [11]. C5a has a role in the pathogenesis of COPD and inflammatory states like vasculitis with vessel formation [12, 13]. Studies on laboratory animals showed an increase in the serum level of IL-8 shortly after SM exposure. IL-8 induces production of C5a, which in turn causes the chemotactic activity of Polymorph Nuclear cells (PMNs), Mononuclear cells (MN), and fibroblasts which are the main sources of chemotaxes produced by SM exposure in short-term toxicities [11].

In the previous studies, a significant increase in the amount of salivary A1AT and sIgA in SM-exposed victims was reported as compared to that in the unexposed control group, but there was no significant difference in the levels of salivary C5a in SM-exposed cases. The current study describes the correlation between the salivary levels of A1AT, sIgA, and C5a with SM induced pulmonary clinical complications 20 years after exposure.

## Materials and Methods

### Study design and participants

Details of the study design and methodology of SICS was previously explained [14]. Briefly, 500 volunteer

participants, including 372 SM-exposed cases and 128 controls were included in the study. SM-exposed victims were classified into two groups; hospitalized and non-hospitalized, based on the history of hospitalization after SM exposure and severity of the clinical problems. Hospitalization was used as an index of the severity of pulmonary disorders after SM exposure. The age range of the participants was 20–60 years (Mean of  $44.3 \pm 9.8$ ), hospitalized =  $46.9 \pm 7.6$  and non-hospitalized =  $41.7 \pm 11.3$ ; no significant difference was observed between the two groups (Chi square = 0.111).

Written informed consent was obtained from the volunteers. There was no significant difference in terms of age, body mass index, marital status, and smoking habits between the SM-exposed and unexposed control groups. All the individuals with known oral cavity diseases, periodontal diseases, and systemic diseases such as Sjogren syndrome and those who received anticholinergic drugs or antibiotics were excluded from the study.

### Ethical considerations

The study was approved by the ethical committee of the Board of Research Ethics of Janbazan Medical and Engineering Research Center (JMERC), the Board of the Research of the Ministry of Health, and Shahed University. Participants who agreed to sign the informed consent were recruited.

### Clinical evaluation

The volunteer participants were physically examined using a medical questionnaire including pulmonary symptoms (e.g. chronic cough, sputum, hemoptysis, and dyspnea), pulmonary findings (fine crackles, coarse crackles, and wheezing) completed for each volunteer. Chronic cough was defined as persistent cough for more than 3 weeks. Three subsequent spirometry measurements (using Chest 801 Spirometry) were performed on all the participants according to the American Thoracic Society Criteria under the supervision of a trained nurse. The suitable measurement was selected for data collection and the classification of the severity of pulmonary involvement was done according to the Global initiative for chronic Obstructive Lung Disease (GOLD).

### Saliva preparation

The saliva was collected using DRG Sali-Tubes 100 (SLA-4158). The procedure for the collection of saliva specimens entailed brushing at least 2 hours in advance and keeping the participants nil per os. The flow

of saliva was stimulated by chewing a piece of parafilm. Afterwards, three ml of saliva was collected in DRG Sali-Tubes. The samples were centrifuged and clear supernatants were collected, aliquoted, and stored at  $-70^{\circ}\text{C}$ .

### Salivary $\alpha$ 1-antitrypsin, C5a, and S-IgA measurement

Human saliva immune diagnostic ELISA kit (R&D system) was used to measure A1AT and sIgA. Levels of salivary C5a were titrated using the DRG Enzyme immunoassay kit which caters for the quantitative determination of the anaphylatoxin C5a in human saliva.

### Statistical analysis

Levels of salivary A1AT, C5a, and sIgA of SM-exposed and the unexposed control group were compared as hospitalized vs non-hospitalized exposed participants using t-test and Mann–Whitney test. The spearman rank correlation coefficient was used for calculating correlations between the factors.

## Results

### Comparison of the salivary levels of A1AT, C5a and sIgA in groups

Our previous publication has shown a significant increase of salivary sIgA and alpha antitrypsin compared to that of the control group ( $P=0.06$ ,  $P=0.018$ ). But there was no considerable difference in the salivary level of C5a in SM-exposed victims in comparison to the unexposed group [9] (Table 1).

### Association of the salivary levels of A1AT, C5a, and sIgA with pulmonary signs and symptoms

The results in Table 2 show that the salivary level of A1AT has increased in the SM-exposed victims with chronic cough a higher ( $174.5\mu\text{g/ml}$ ) compared to victims without chronic cough ( $P=0.001$ ). But there was no difference between the salivary level of A1AT in SM-exposed victims with sputum, hemoptysis, dyspnea, and chest pain as compared with those of the victims without these symptoms.

The salivary level of C5a in SM-exposed victims who had chronic cough and dyspnea were significantly higher than that in the victims without these symptoms ( $P=0.001$ ,  $P=0.05$ ). But, there was no association between wheezing and crackle and other pulmonary signs with C5a in the exposed victims.

**Table 1.** The comparison of the salivary levels of A1AT, C5a, and sIgA in the SM exposed victims with and without pulmonary symptoms

Parameters ( $\mu\text{g/ml}$ )	Have				Not Have				P	
	N	Median	Q1	Q3	N	Median	Q1	Q3		
A1AT ( $\mu\text{g/ml}$ )	Chronic cough	316	174.50	48.75	262.60	22	39.30	12.70	170.20	0.001
	Sputum	303	171.80	41.40	257.40	35	94.00	29.10	216.20	0.122
	Hemoptesi	114	171.65	47.10	262.20	224	165.40	34.15	247.35	0.408
	Dyspnea	321	169.90	42.60	256.90	17	60.80	22.80	190.20	0.086
	Chest pain	234	165.85	42.10	245.80	104	178.40	36.80	285.15	0.486
C5a ( $\mu\text{g/ml}$ )	Chronic cough	313	0.60	0.10	1.70	21	0.00	0.00	0.50	0.001
	Sputum	300	0.50	0.10	1.60	34	0.50	0.10	1.70	0.985
	Hemoptesi	111	0.60	0.20	1.50	223	0.50	0.00	1.70	0.426
	Dyspnea	318	0.55	0.10	1.60	16	0.20	0.00	0.60	0.059
	Chest Pain	232	0.50	0.10	1.65	102	0.50	0.00	1.50	0.432
sIgA ( $\mu\text{g/ml}$ )	Chronic cough	316	609.20	356.85	889.85	21	437.70	264.70	528.40	0.003
	Sputum	303	604.60	351.00	877.70	34	481.15	317.40	812.40	0.149
	Hemoptesi	113	608.00	350.10	894.10	224	574.00	350.40	844.45	0.407
	Dyspnea	321	604.60	356.10	877.70	16	379.60	233.20	531.85	0.013
	Chest pain	233	598.00	383.10	892.30	104	549.00	291.95	799.05	0.119

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A1AT, C5a and Saliva levels of sIgA were assessed in the SM exposed victims and a comparison of the salivary levels of these markers was undertaken between participants who had and who did not have any pulmonary symptoms (chronic cough, sputum, hemoptysis, dyspnea, and chest pain). Data presented as median (first and third Quartile).

A1AT:  $\alpha$ -1-Antitrypsin, C5a: Complement component C5a, SIgA: Secretory Immunoglobulin A,  $\mu\text{g/ml}$ : microgram per milliliter, p-value: Comparison between symptomatic and asymptomatic

The salivary level of sIgA increased in victims with chronic cough and dyspnea compared to that in victims without these symptoms ( $p=0.003$ ,  $p=0.013$ ). There was no significant association between other pulmonary symptoms including sputum, hemoptysis, and chest pain and pulmonary signs in the SM-exposed victims with salivary level of sIgA.

#### Correlation of the salivary levels of A1AT, C5a, and sIgA with spirometry parameters

According to Table 3, which shows the results of the functional pulmonary tests, there was no significant correlation between the values of spirometry parameters with salivary level of sIgA and A1AT in the exposed victims. However, a correlation can be observed between salivary C5a with FVE 1/ FVC and PEF in

the exposed victims compared to that of the unexposed group ( $P=0.005$ ,  $P=0.033$ , respectively).

#### Association of salivary levels of A1AT, C5a, and sIgA with pulmonary involvement severity

No correlation was observed between the salivary level of A1AT and sIgA with pulmonary complication's severity according to GOLD classification (Table 4). Also, a negative correlation was observed between the salivary level of C5a in SM-exposed victims with GOLD classification stages and there was a significant correlation between salivary level of C5a with stage III of severe pulmonary complications according to GOLD classification compared to that with other stages ( $P=0.004$ ) (Table 4). Moreover, the C5a level in the SM-exposed victims with pulmonary problems showed a decrease in comparison

**Table 2.** The comparison of the salivary levels of A1AT, C5a, and sIgA in the SM exposed victims with pulmonary signs

	Pulmonary Sign	N.	Median	Q1	Q3	P
A1AT (µg/ml)	Normal	261	171.80	38.00	251.20	
	Rale	16	205.15	112.35	293.15	0.228
	Wheezing	54	167.05	33.90	262.20	0.909
	Crackle	7	34.00	28.50	140.50	0.058*
C5a (µg/ml)	Normal	259	0.50	0.10	1.70	
	Rale	14	0.20	0.00	1.20	0.298
	Wheezing	54	0.50	0.20	1.30	0.884
	Crackle	7	0.50	0.00	2.80	0.958
sIgA (µg/ml)	Normal	260	584.15	351.95	830.90	
	Rale	16	687.95	306.50	954.05	0.690
	Wheezing	54	670.40	349.80	944.40	0.427
	Crackle	7	431.80	246.20	598.00	0.173

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P-value: a comparison of the salivary levels of sIgA, C5a and A1AT was undertaken between participants who had pulmonary sign and those with normal auscultation in the SM exposed victims. A1AT: α-1-Antitrypsin, C5a: Complement component C5a, SIgA: Secretory Immunoglobulin A, µg/ml: microgram per milliliter

to that of the SM-exposed victims without pulmonary problems (P=0.003) (Table 5).

#### 4. Discussion

SM, as an inhaled toxic, causes acute and chronic pulmonary complication. The most common long-term complications of SM on the respiratory system include dyspnea, irritant cough, and frequent respiratory infections [15]. These complications could lead to the development of various lung diseases such as COPD, airway hyper-reactivity, obstructive pulmonary disease, chronic bronchitis, asthma, bronchiolitis, and bronchiectasis [15, 16]. COPD, as a late pulmonary sequel of SM exposure, is usually associated with immune disorder(s) [16].

Previous studies on SM-exposed victims showed a significant decrease in pro-inflammatory cytokines, such as IL-1α, IL-1β, TNF-β, IL-6, and IL-8 [17, 18], and a significant increase in anti-inflammatory cytokines, namely MCP-1 and IL-10, in SM-exposed individuals as compared to those of the control group [19, 20]. In our previous study, it was shown that salivary levels of sIgA and A1AT in SM-exposed victims, as compared to those of the unexposed control group, was higher but there was no significant increase in the salivary level of C5a in SM-exposed victims in comparison to that of the unexposed individuals [11].

A1AT, a serine protease inhibitor (serpin), showed an inhibitory effect on neutrophil elastase activities. It increases during inflammation as an acute phase protein and causes a reduction of pro-inflammatory cytokine level such as IL-6, IL-8, and IL-1β [10, 21]. Serum level of A1AT also showed to be associated with development of emphysema [22]. Emphysema is a common complication of mustard lung [2] which might be due to the lower anti-protease activity of A1AT. The salivary level of A1AT in SM-exposed patients 20 years after exposure increased when compared with that in unexposed control group [11]. The results of the current study indicated a correlation between the salivary level of A1AT and chronic cough. The cough and sputum production are the features of airway's involvement and impairment of the airway's clearance [23]. The common long time symptoms of victims after SM-exposure, such as chronic cough, dyspnea, and sputum, can be caused by respiratory disorders [15, 24].

Increased level of A1AT in saliva leads to the enhancement of mucus secretion and causes chronic cough as a consequence. The results of the present study indicated that there is a correlation between salivary level of A1AT and chronic cough, but there is no significant association between the salivary level of A1AT and other lung symptoms and lung-related functional assays.



**Table 3.** Correlation of the salivary levels of A1AT, C5a, and sIgA with pulmonary function parameters in exposed victims

Parameters (%)		A1AT ( $\mu\text{g/ml}$ )	C5a ( $\mu\text{g/ml}$ )	sIgA ( $\mu\text{g/ml}$ )
FVC	r	-0.038	0.030	-0.045
	P	0.482	0.591	0.411
	N	338	334	337
FEV1	r	-0.006	0.093	0.591
	P	0.912	0.089	336
	N	337	333	-0.007
FEV1/FVC	r	0.055	0.195	0.916
	P	0.427	0.005*	208
	N	210	209	-0.114
MMEF	r	-0.027	0.082	0.148
	P	0.729	0.301	162
	N	165	163	0.056
PEF	r	0.058	0.126	0.346
	P	0.323	0.033*	289
	N	291	286	0.591

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The salivary levels of the inflammatory mediators and the pulmonary function parameters (FVC, FEV1, FEV1/FVC, MMEF, and PEF) were assessed. The correlation between the inflammatory mediators and pulmonary function parameters was undertaken in the SM exposed victims.

r: Spearman's correlation coefficient, A1AT:  $\alpha$ -1-Antitrypsin, C5a: Complement component C5a, sIgA: Secretory Immunoglobulin A,  $\mu\text{g/ml}$ : microgram per milliliter, FVC: forced vital capacity, FEV1: forced expiratory volume in 1s, MMEF: maximum midexpiratory flow, PEF: peak expiratory flow

Secretory form of IgA releases into mucosal secretions, such as saliva, and mucosal surfaces, like respiratory tract. Different environmental agents cause the activation of mucosal immunity in upper respiratory tract and induce the production of sIgA in various secretions, including saliva [25].

Decreased expression of sIgA in airway's epithelium is associated with structural abnormalities of epithelium, such as airflow obstruction and neutrophil infiltration in lung diseases like COPD. Impaired mucosal immunity may lead to persistent airway inflammation and progressive airway remodeling in COPD [4, 26]. So, sIgA deficiency, together with lung injury, can cause lung complications. Our previous study, as a part of SICS, showed that the salivary level of sIgA has increased in the SM-exposed victims compared to that of the unexposed control participants. In the current study, we demonstrated the correlation of salivary sIgA level with chronic cough and dyspnea. Also, a strong association was observed between sIgA with other lung symptoms and functional pulmonary lung tests. Moreover, sIgA increases in vic-

tims with mild and moderate injuries and decreases in victims with severe damages, thus the results proved that the amount of sIgA in saliva may be considered as an indication of lung's microenvironment.

The complement system not only plays a role in innate immunity against pathogens, but it also involves in a wide range of acute and chronic inflammatory diseases such as COPD and asthma [27]. C5a, the activated fragment of C5, is a potent chemotactic factor for many inflammatory cells (e.g. neutrophils, mast cells, basophils, eosinophils, and lymphocytes). C5a exacerbates inflammation through different mechanisms and are able to elevate degranulation of eosinophils, mast cells, and basophils and release cytokines from macrophages [28]. Moreover, alveolar type II cells can be a supplier source of C5 [29]. C5aR and Fc $\gamma$ Rs are expressed on Alveolar Macrophages. C5aR upregulates Fc $\gamma$ RIII and suppresses Fc $\gamma$ RII. Complement activation (C5a) at the site of inflammation can control Fc $\gamma$ Rs expression and links humoral arm of the immune system to the cellular one.

**Table 4.** The comparison of the salivary levels of A1AT, C5a, and sIgA in SM exposed victims with different pulmonary disease severity (GOLD classification)

Parameters (µg/ml)	N	Median (µg/ml)	Q1	Q3	P	
A1AT	Normal	283	169.30	34.30	262.20	
	Stage I	6	75.70	20.30	211.30	0.294
	Stage II	29	169.90	37.60	226.80	0.506
	Stage III	20	143.35	42.15	237.50	0.540
C5a	Normal	281	0.60	0.10	1.70	
	Stage I	6	0.35	0.00	2.20	0.577
	Stage II	30	0.25	0.00	0.80	0.079
	Stage III	17	0.20	0.00	0.50	0.004*
sIgA	Normal	282	585.05	351.00	861.60	
	Stage I	6	462.75	246.20	745.60	0.247
	Stage II	30	646.05	437.70	903.80	0.472
	Stage III	19	473.20	228.30	769.10	0.158

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Data presented as median (first and third quartile). P-value: comparisons of the salivary levels of inflammatory markers in SM exposed participants who had pulmonary impairment (stages I, II and III) with those SM exposed participants who had normal condition based on GOLD classification.

A1AT: α-1-Antitrypsin, C5a: Complement component C5a, sIgA: Secretory Immunoglobulin A, µg/ml: microgram per milliliter, GOLD: Global Initiative for chronic Obstructive Lung Disease, µg/ml: microgram per milliliter.

The regulatory role of C5a may enable immune system to respond efficiently to potentially harmful immune complexes [28]. Continuous elevations of intrapulmonary C5a level may account for emphysematous changes in patients with COPD due to the maintenance of chronic inflammation and subsequent release of cytokines and elastolytic enzymes from activated inflammatory cells [13].

According to the results of the current study, a significant decrease was observed in salivary level of C5a in victims with severe lung problems, classified by GOLD. This can be due to the damage to the cells that produce C5a in the lungs of these victims [29]. The results also indicated a relationship between salivary level of C5a with chronic cough and dyspnea in SM-exposed victims, who had pulmonary com-

**Table 5.** The comparison of the salivary levels of A1AT, C5a, and sIgA in SM exposed victims with lung problems according to GOLD classification

Parameters (µg/ml)	Lung Problem	N	Median (µg/ml)	Q1	Q3	P
A1AT	Without	283	169.30	34.30	262.20	
	With	55	130.40	33.90	232.40	0.256
C5a	Without	281	0.60	0.10	1.70	
	With	53	0.20	0.00	0.80	0.003
sIgA	Without	282	585.05	351.00	861.60	
	With	55	538.90	316.20	828.00	0.544

P-value: Comparison between symptomatic and asymptomatic

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plications, as compared to those of the SM-exposed participants who did not have these complications.

In a study, as a part of SCIS, it was indicated that C5a levels in airways were negatively correlated with forced expiratory volume in the first second (FEV1)% predicted and diffusing capacity of the lung (TLCO) [12]. This is inconsistent with the results obtained in the present study, which indicated a significant negative correlation between salivary C5a, FEV1/FVC %, and PEF % in the SM-exposed participants.

In conclusion, the salivary level of A1AT and sIgA, as two main protective factors in lung, did not show a strong correlation with long term pulmonary complications, except for chronic cough and dyspnea, in contrast to their increase in SM-exposed individuals in our previous study. Salivary level of A1AT and sIgA increased in victims with mild and moderate lung complications and decreased in victims with severe pulmonary involvements, as classified by GOLD criteria. This may indicate deficiency of A1AT and sIgA in long-term pulmonary complications induced by SM. Salivary C5a induces injuries to the lung during the chronic infection because of its role as an Anaphylatoxin, although we did not observe remarkable differences in the salivary level of C5a in SM-exposed victims compared to that of the unexposed individuals. But C5a revealed to have a significant association with cough and dyspnea. Since there was a correlation between the salivary level of C5a and functional pulmonary tests, these results may be suggestive of the side effects of C5a in inducing long term pulmonary complications in SM-exposed victims.

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### Conflict of interest

The authors of the present study report no conflict of interest.

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