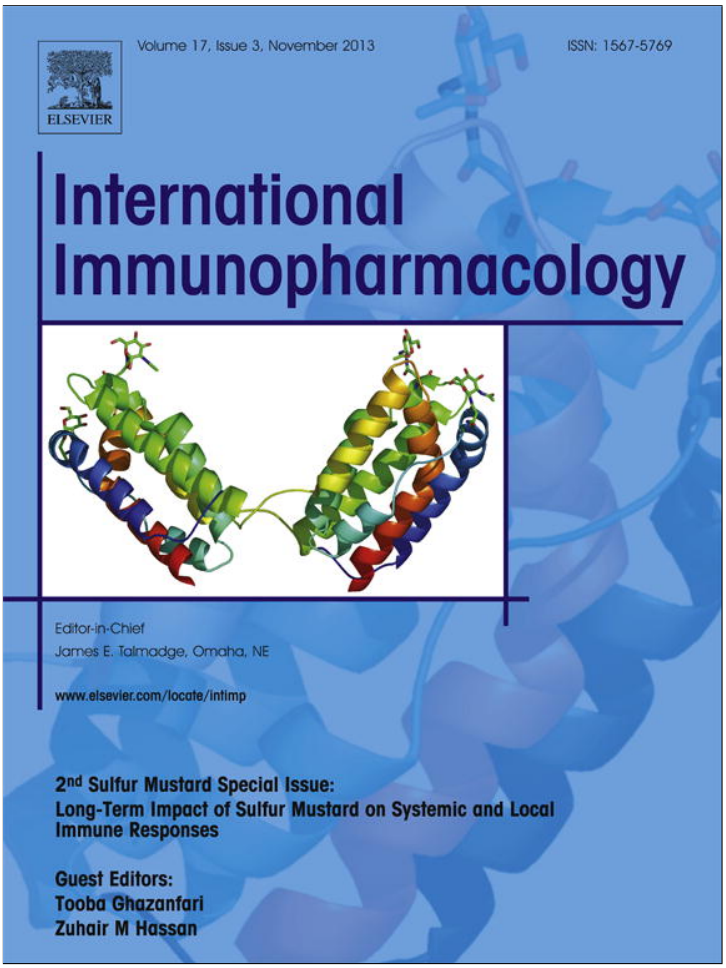

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Salivary levels of secretary IgA, C5a and alpha 1-antitrypsin in sulfur mustard exposed patients 20 years after the exposure, Sardasht-Iran Cohort Study (SICS)

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ABSTRACT

Sulfur mustard (SM) is a strong toxic agent that causes acute and chronic health effects on a myriad of organs following exposure. Although the primary targets of inhaled mustard gas are the epithelia of the upper respiratory tract, the lower respiratory tract is the focus of the current study, and upper tract complications remain obscure. To our knowledge there is no study addressing the secretory IgA (S-IgA), C5a, alpha 1 antitrypsin (A1AT) in the saliva of SM-exposed victims. In this study, as many as 500 volunteers, including 372 SM-exposed cases and 128 control volunteers were recruited. A 3 ml sample of saliva was collected from each volunteer, and the level of secretory IgA, C5a, and alpha 1 antitrypsin in the samples were compared between the two groups. The SM-exposed group showed a significantly higher amount of salivary alpha 1 antitrypsin and secretary IgA compared to the control group ($p<.006$ and $p<.018$ respectively). The two groups showed no significant difference ($p=0.192$) in the level of C5a. The results also showed that the level of salivary A1AT is more than that of IgA in severely injured cases. The findings presented here provide valuable insight for both researchers and practitioners dealing with victims of the chemical warfare agent, sulfur mustard. This research indicates that certain branches of the inflammatory processes mandate serious attention in therapeutic interventions.

1. Introduction

Chemical agents such as ammonia, chlorine, vinyl chloride, phosgene, sulfur dioxide, sulfur mustard, nitrogen dioxide, tear gas, and zinc chloride primarily induce injuries in the upper respiratory tract [1]. Sulfur mustard (SM) is one of the most dangerous organochlorine vesicant agents [2]. SM is a strong alkylating agent that causes acute and chronic effects on different organs following exposure [3]. SM is a blistering chemical agent possessing mutagenic properties [4]. Even in a low dose, SM induces potential damage on multiple organs especially in the skin, eyes, as well as the respiratory tract. The damage may cause many complications which persist for the lifespan of the exposed subject [5]. SM has short- and long-term toxicities against various organs including the respiratory system [6]. Immunologic factors of saliva play a prominent role in oral cavity immunity.

Secretory IgA (S-IgA) protects the mucosa against microorganisms and various toxins. In contrast to this, serum IgA contains IgA mainly in the monomeric form and in the IgA1 subclass, secretions contain S-IgA, which mainly belongs to IgA2 subclass, with unique structural features. Approximately 90% of S-IgA occurs in the polymeric form (dimers and tetramers), and is associated with the J chain and the secretory component (SC), which is acquired during the transepithelial transport [7]. S-IgA of the saliva is produced by plasma cells in the lamina propria adjacent to the mucosal surfaces [8]. It is thought that IgA-deficient subjects are susceptible to periodontal diseases. The

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level of serum IgA shortly after SM exposure does not show a significant alteration [9], but there is no report about the alterations in salivary IgA.

Complement component C5a is a powerful inflammatory mediator when bound to its specific receptor. C5a, which is produced in the course of complement cascade activation via the classical or alternative pathway, induces chemotactic migration, increases cell adhesion, stimulates the oxidative burst, and releases various inflammatory mediators such as histamine and cytokines [10]. The saliva of normal subjects contains a low amount of C3a, C4a and C5a [11–13]. C5a is involved in the pathogenesis of the chronic obstructive pulmonary disease and in inflammation and vacuities with vessel formation [13–15]. Studies on the laboratory animals showed that IL-8 is increased shortly after SM exposure. IL-8 induces the production of C5a, which in turn causes chemotactic activity of polymorphonuclear cells (PMN), mononuclear cells (MN) and fibroblasts. These cells are the major sources of the chemotaxins produced by SM exposure in short-term toxicities [16].

Alfa 1-antitrypsin (A1AT) is the prototypical member of the serine protease inhibitor (SERPIN) family of protease inhibitors [17]. This acute-phase reactant is induced mainly by hepatocytes and also many inflammatory and epithelial cells in response to the inflammatory cytokines (IL-6, IL-1 and TNF-α) and endotoxins [18]. There is evidence demonstrating that A1AT inhibits several proteases including neutrophil elastase and proteinase 3 (PR3), kallikreins 7 and 14 and caspase-3 [19,20]. A1AT deficiency is a genetic disorder associated with lung emphysema [21]. Proteases present in oral fluid effectively modulate the structure and function of some salivary proteins and have been implicated in tissue destruction in oral diseases. Regulation of oral fluid proteolysis is highly important, given that an imbalance in such activities is correlated to a variety of pathological conditions including oral cancer [22]. Alpha 1-antitrypsin deficiency is identified as a definite genetic risk factor for the development of COPD in smokers [23]. A reduced serum A1AT activity was observed long-term after SM exposure which contributes to the development of respiratory diseases [24].

To our knowledge through search on the internet there is no study that addresses secretory IgA, C5a, and alpha 1 antitrypsin in the saliva of SM exposed victims. The easy collection, accessibility, and availability of saliva, as well as the non-invasive manner of sample collection, are attractions for using saliva as a diagnostic specimen. The Sardasht-Iran Cohort Study (SICS) is a comprehensive historical cohort study on the SM-exposed population of civilians, designed to explore the long-term complications of sulfur mustard exposure and the basic and molecular mechanism(s) underlying the clinical manifestations. As part of the cohort study, this work is designed to evaluate the level of secretory IgA, C5a, and A1AT in the saliva of SM exposed individuals 20 years after exposure and compare with the healthy control group.

2. Materials and methods

2.1. Study design and participants

Details of the study design and methods of the SICS are explained previously [25]. Briefly, 500 participants including 372 SM-exposed cases and 128 control subjects were included [25]. The study was approved by the Shahed University Research Committee, the Ministry of Health and Medical Education, and the Board of Research Ethics of the Janbazan Medical and Engineering Research Center. Written informed consent was obtained from every volunteer participant. There was no significant difference in terms of age, body mass index, marital status, and smoking habits between the SM exposed and the control groups. All subjects with known oral cavity diseases, periodontal diseases, and systemic diseases such as Sjogren syndrome and subjects who received anticholinergic drugs or antibiotics were excluded from the study.

2.2. Saliva preparation

Saliva was obtained by using DRG Sali-Tubes 100 (SLA-4158). The procedure for the collection of saliva specimens entailed brushing at least 2 h in advance and keeping the participants nil per os (NPO). The flow of saliva was stimulated by chewing a piece of Parafilm. Afterwards, 3 ml of saliva was collected in DRG Sali-Tubes. The samples were centrifuged and clear supernatants were collected, aliquated and stored at −70 °C until use.

2.3. Serum IgA, S-IgA, C5a, and alpha 1-antitrypsin measurement

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

2.4. Statistical analysis

Levels of A1AT, C5a, and sIgA in the saliva of SM exposed and the control group were titrated and compared in the hospitalized *vs* non-hospitalized exposed cases using the *t*-test and Mann–Whitney test. Spearman rank correlation coefficient was used for correlations between the factors.

3. Results

3.1. Salivary sIgA

Salivary sIgA was assessed in all participants including the SM exposed and the control groups. SM exposed patients were categorized on hospitalization into two groups of non-hospitalized or hospitalized at the time of SM exposure. A comparison was undertaken between the exposed and the control group. Data was presented as Median (Q1–Q3). As it is presented in Table 1, a significant higher salivary sIgA is seen in SM-exposed patients compared with the control group ($p<0.018$). There was no significant difference between the amount of sIgA in the hospitalized and non-hospitalized cases.

Table 1
Comparison of salivary sIgA in SM exposed and the control groups.

Study groups	sIgA (ng/ml)						p-value[1]	p-value[2]
	N	Median	Q1	Q3	Mean	SD		
Control	122	497.85	311.10	711.10	548.00	298.67		
SM exposed	353	585.10	345.30	874.40	643.53	357.22	**0.018**	
Non-hospitalized	190	559.55	320.70	874.40	635.66	370.28	0.093	0.420
Hospitalized	163	590.20	386.20	876.30	652.70	342.27	**0.009**	

Bold data shows significant differences with p-value <0.05.
Salivary sIgA was assessed in all participants including the SM exposed and the control groups. Exposed group was categorized based on hospitalization at exactly after exposure into hospitalized and non-hospitalized. A comparison was undertaken between each of the exposed groups with the control group. Data was presented as median (Q1–Q3). p-value[1]: comparison of the exposed, non-hospitalized and hospitalized group with the control group (Mann–Whitney). p-value[2]: comparison of hospitalized and non-hospitalized group (Mann–Whitney).
SM: Sulfur mustard.

Table 2
Comparison of salivary C5a in SM exposed and the control groups.

Study groups	Salivary C5a (μ g/ml)						p-value[1]	p-value[2]
	N	Median	Q1	Q3	Mean	SD		
Control	122	0.75	0.10	2.00	1.39	1.70		
SM exposed	348	0.50	0.10	1.65	1.25	1.85	0.192	
Non hospitalized	189	0.50	0.00	1.60	1.14	1.54	0.138	0.429
Hospitalized	159	0.60	0.10	1.70	1.38	2.16	0.429	

Salivary C5a was assessed in all participants including the SM exposed and the control groups. Exposed group was categorized based on hospitalization at exactly after exposure into hospitalized and non-hospitalized. A comparison was undertaken between each of the exposed groups with the control group. Data was presented as median (Q1–Q3). p-value[1]: comparison of the exposed, non-hospitalized and hospitalized group with the control group (Mann–Whitney). p-value[2]: comparison of hospitalized and non-hospitalized group (Mann–Whitney).
SM: Sulfur mustard.

3.2. Salivary C5a

Salivary C5a was assessed in all participants including the SM exposed and the control groups. SM exposed patients were categorized on hospitalization into two groups of non-hospitalized or hospitalized at the time of SM exposure. A comparison was undertaken between the exposed and the control group. Data was presented as median (Q1–Q3). The results in Table 2 show that there was no significant difference in the level of salivary C5a between the exposed and control groups.

3.3. Salivary A1AT

Salivary alpha 1-antitrypsin was assessed in all participants including the SM exposed and the control groups. SM exposed patients were categorized on hospitalization into two groups of non-hospitalized or hospitalized at the time of SM exposure. A comparison was undertaken between the exposed and the control groups. Data was presented as median (Q1–Q3). The levels of salivary A1AT are presented in Table 3. As it is shown, there is a significantly higher level of salivary A1AT in the SM-exposed group compared with the control group ($p<0.006$), and the level of A1AT in the hospitalized exposed cases was significantly higher than non-hospitalized cases ($p<0.022$).

3.4. Correlations between salivary sIgA, A1AT, C5a

As the results in Tables 4 and 5 show there is a positive correlation in sIgA, A1AT, and C5a levels in the exposed and control groups. The most noticeable correlation was between A1AT and C5a ($r=0.664$, $p<0.000$) in the control group and between A1AT and sIgA in the exposed group($r=0.528$, $p<0.000$). Fig. 1 shows scatterplots of salivary sIgA, C5a, and alpha 1-antitrypsin pairwise correlation in control or SM exposed groups.

Table 4
Pairwise Spearman rank correlation coefficients for salivary sIgA, C5a, and alpha 1-antitrypsin in control group.

Control N = 125		Salivary sIgA	Salivary C5a	Salivary alpha 1-antitrypsin
Salivary sIgA	r	1.000	0.266 (*)	0.481 (*)
	P		0.003	0.000
Salivary C5a	r	0.266 (*)	1.000	0.664 (*)
	P	0.003		0.000
Salivary alpha 1-anitrypsin	r	0.481 (*)	0.664 (*)	1.000
	P	0.000	0.000	

Salivary sIgA, C5a, and alpha 1-antitrypsin were assessed and their correlation with each other was undertaken within control group using Spearman's rank correlations.
* Denotes a significant correlation with $p<0.001$.

Table 5
Pairwise Spearman rank correlation coefficients for salivary sIgA, C5a, and alpha 1-antitrypsin in SM expose group.

Exposed N = 320		Salivary sIgA	Salivary C5a	Salivary alpha 1-antitrypsin
Salivary sIgA	r	1.000	0.416 (*)	0.528 (*)
	P		0.000	0.000
Salivary C5a	r	0.416 (*)	1.000	0.481 (*)
	P	0.000		0.000
Salivary alpha 1-anitrypsin	r	0.528 (*)	0.481 (*)	1.000
	P	0.000	0.000	

Salivary sIgA, C5a, and alpha 1-antitrypsin were assessed and their correlation with each other was undertaken within exposed group using Spearman's rank correlations.
SM: Sulfur mustard.
* Denotes a significant correlation with $p<0.001$.

3.5. Correlations between salivary IgA, A1AT,C5a and serum CRP

The level of serum IgA was not significantly different between the SM-exposed (hospitalized and non-hospitalized) and the control groups (Table 6)

Data presented in Table 7 shows a positive correlation between sIgA and serum IgA in the control group. There was no significant correlation between serum CRP and S-IgA, C5a and A1AT in the saliva of the control group.

As shown in Table 8 there is a positive correlation between serum CRP and S-IgA, and C5a levels in the exposed group. No correlation was seen between serum IgA and sIgA in the exposed group.

4. Discussion

The aim of this study was to compare the salivary levels of A1AT, C5a, and sIgA 20 years after SM exposure to possibly explore the relationship between SM exposure and the inflammatory/anti-inflammatory markers. The findings showed that the levels of sIgA and A1AT in the saliva of SM-exposed victims were significantly higher than the control

Table 3
Comparison of salivary alpha1-antitrypsin in SM exposed and the control groups.

Study groups	Salivary alpha 1-antitrypsin						p-value[1]	p-value[2]
	N	Median	Q1	Q3	Mean	SD		
Control	122	114.70	26.40	195.70	128.97	109.07		
SM exposed	352	167.75	36.80	249.60	161.40	114.59	**0.006**	
Non hospitalized	190	132.80	27.10	242.10	149.16	117.19	0.171	**0.022**
Hospitalized	162	181.70	71.60	263.00	175.75	110.10	<**0.001**	

Bold data shows significant differences with p-value <0.05.
Salivary alpha 1-antitrypsin was assessed all participants including the SM exposed and the control groups. Exposed group was categorized based on hospitalization at exactly after exposure into hospitalized and non-hospitalized. A comparison was undertaken between each of the exposed groups with the control group. Data was presented as Median (Q1–Q3). p-value[1]: comparison of the exposed, non-hospitalized and hospitalized group with the control group (Mann–Whitney). p-value[2]: comparison of hospitalized and non-hospitalized group (Mann–Whitney).
SM: Sulfur mustard.

subjects, but the level of salivary C5a was not significantly different. Upper respiratory tract exposure to different environmental agents results in the activation of mucosal immunity and the production of S-IgA in various secretions including saliva [26]. In areas with different atmospheric pollutants, the most sensitive tests to evaluate the difference in the saliva are the levels of salivary A1AT and IgE, although the level of S-IgA was less pronounced [27]. A higher level of salivary A1AT and a significantly lower level of sIgA were seen in toluene gas toxicity [28]. To date, in the domain of environmental gas toxicity and its effects on mucosal immunity, the present study has been the first to address salivary immunity in SM-exposed subjects. In the toluene gas toxicity study, the levels of sIgA and A1AT were measured 1 month after exposure [28]. In the present study, these factors were quantified 20 years after SM exposure. A1AT is an acute-phase reactant and its elevation in the acute phase of exposure seems logical. The local elevation of A1AT 20 years after exposure to SM may suggest a possible role for anti-inflammatory factors which might be due to anti-protease activity. Authors propose A1AT may prevent tissue destruction in the chronic phase of SM exposure.

It is also showed that the level of sIgA in the SM-exposed group is significantly higher than that in the control group. sIgA is the main salivary immunoglobulin which is produced by plasma cells in the

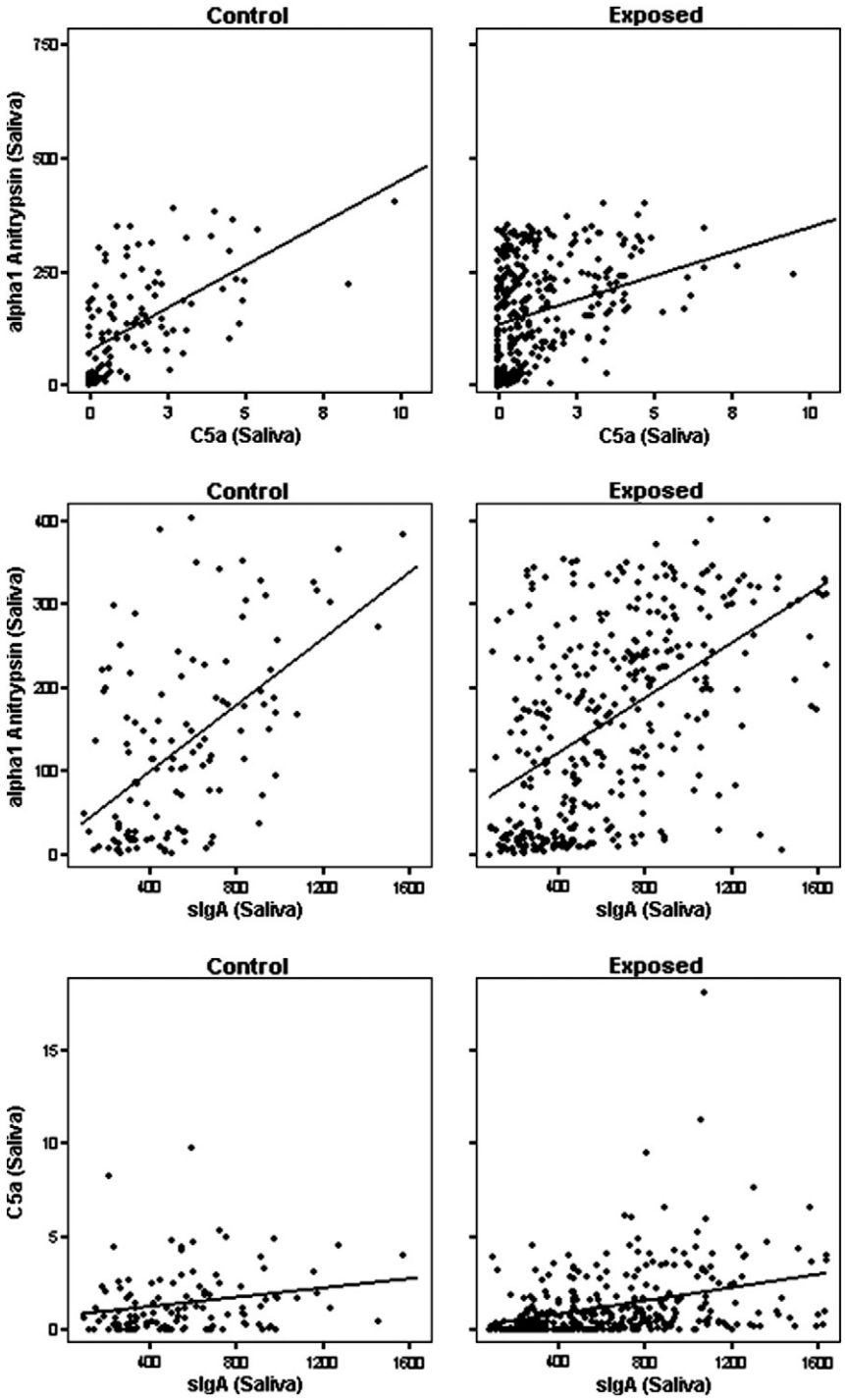

Fig. 1. Scatterplots of Salivary sIgA, C5a, and alpha 1-antitrypsin pairwise correlation in SM exposed and the control groups. Data are presented in detail in Tables 4 and 5.

Table 6
Comparison of IgA (serum) (mg/ml) between study groups.

Study groups	IgA			p-value
	N	Mean	SD	
Control	124	3.769	0.991	
SM exposed	364	3.775	1.220	0.962
Non hospitalized	199	3.879	1.128	0.374
Hospitalized	165	3.650	1.314	0.396

Serum levels of IgA were assessed in all participants including the SM exposed and the control groups. Exposed group was categorized based on hospitalization at exactly after exposure into hospitalized and non-hospitalized. A comparison was undertaken between each of the exposed groups with the control group. Data was presented as mean ± SD.
p-value1: comparison of the SM exposed, non-hospitalized and hospitalized group with the control group (*t*-test).
SM: Sulfur mustard.

lamina propria adjacent to mucosal surfaces. sIgA is generally considered to be a non-inflammatory antibody, as does not trigger inflammatory processes when binding to the antigens [29]. It is shown that in the acute phase of toluene exposure, salivary IgA, and in the acute phase of SM exposure, blood IgA levels were constant [9,28]. The elevation of sIgA in the saliva of SM-exposed patients 20 years after exposure may suggest a possible anti-inflammatory mechanism of this immunoglobulin to reduce tissue injury. The presence of positive correlation between salivary A1AT and sIgA and the absence of long-term correlation between sIgA and serum IgA after SM exposure may provide some evidence for speculation. These findings are in agreement with our previous reports concerning the overall down-regulation of pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-β, IL-6 and IL-8 [5,30] and the significant elevation of anti-inflammatory cytokines MCP-1 [6] and IL-10 [31] in this population. On the other hand, the elevation of secretory IgA may in part be due to the protective role of A1AT against salivary proteases. Although because of sIgA's considerable resistance to proteases, this suggestion may not seem correct.

The current finding may also imply a local mucosal change elsewhere; so measuring other local secretory IgA levels such as sputum IgA and gastric secretory IgA may help to find the source of sIgA secretion. Many systemic diseases impair salivary flow rate and composition and therefore incite oral pathological processes. In a study performed in the patients with myocardial infarction, the level of A1AT was increased not only in the plasma, but also in the saliva of the patients [32].

Our findings regarding the level of sIgA in the SM-exposed group are in accordance with sIgA levels in other models. In a study which analyzed the composition of the whole saliva in patients diagnosed with celiac disease, the levels of IgA and IgM were lower than those in the healthy controls [33]. In patients with primary Sjogren's syndrome (SS) the levels of IgA, IgG, and IgM in salivary fluid were significantly higher [34]. Patients with SS had higher values for salivary IgA and IgM than individuals without SS [3,35]. The results indicated that the elevation in salivary IgA in Sjogren patients is due to secretory IgA [36].

Table 7
Correlation of salivary sIgA, C5a, and alpha 1-antitrypsin vs. serum CRP and IgA levels in control group.

Control N = 125		sIgA	C5a	Alpha 1 antitrypsin
Serum CRP	r	0.023	0.030	0.057
	P	0.807	0.756	0.548
Serum IgA	r	0.211 (*)	−0.014	0.141
	P	0.021	0.879	0.124

Correlations of salivary sIgA, C5a, and alpha 1-antitrypsin with serum levels of IgA and CRP in the control group were assessed using Spearman's rank correlations. CRP: C-reactive protein.
* Denotes a significant correlation with $p<0.05$.

Table 8
Correlation of salivary sIgA, C5a, alpha 1-antitrypsin vs. serum CRP and IgA in SM Exposed group.

SM Exposed N = 320		sIgA	C5a	Alpha 1 antitrypsin
Serum CRP	r	0.121 (*)	0.124 (*)	0.005
	P	0.024	0.021	0.924
Serum IgA	r	0.089	0.041	0.043
	P	0.099	0.447	0.428

Correlations of salivary sIgA, C5a, and alpha 1-antitrypsin with serum levels of IgA and CRP in the SM exposed group were assessed using Spearman's rank correlations. SM: sulfur mustard, CRP: C-reactive protein.
* Denotes a significant correlation with $p<0.05$.

In a study carried out in dental caries and gingivitis in patients with thalassemia major showed that dental caries experience was significantly higher in the thalassemia major group, however, the median saliva concentrations of IgA was significantly lower in the patients than in the controls [37]. The mean salivary secretory IgA level in diabetes mellitus patients is significantly higher than that in normal control subjects [38]. Whole saliva samples from diabetic patients showed a significantly higher amount of IgA than the controls [39]. In systemic diseases with immunologic etiology such as diabetes mellitus and Sjogren's syndrome secretory IgA elevation was observed.

In other studies it is shown that IgA and IgM concentrations were significantly higher in older patients [40]. The salivary IgA of the elderly were significantly higher in comparison to the healthy young controls [41] which are also in agreement with our findings.

Serum levels of secretory IgA in patients with ankylosing spondylitis were elevated compared to the controls and a positive correlation between total serum IgA and sIgA was shown [42]. In our study, salivary sIgA is also elevated, but this elevation is not correlated with that of serum IgA.

Studies indicated that the activation of C5a plays a role in the neovascularization and pathogenesis of systemic autoimmune diseases such as vasculitis [43]. Inhibiting C5a receptor expression attenuates these responses [44]. Animal laboratory studies showed that IL-8 level was increased during SM exposure; as IL-8 induces the complement fragment C5a, [16] in the acute phase of SM exposure C5a serum elevation was observed in the animals; however, based on the previous report of the same group [30], systemic level of IL-8 is decreased at a delayed time after SM exposure which might explain the reason for absence of C5a elevation in these patients [30].

Further studies in regard to chemokine and complement levels and functions are necessary to explore a more precise picture of the long term clinical and pathological consequences of SM exposure.

In conclusion, the findings presented here may provide a valuable insight for researchers and practitioners dealing with victims of the chemical warfare agents especially sulfur mustard. This research indicates that certain branches of the inflammatory processes mandate serious attention in therapeutic interventions.

Declaration of interest

The authors report no conflict of interest in this study.

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