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Cigarette smoking differentially affects immunoglobulin class levels in serum and saliva: an investigation and review.

Running title: **Cigarette smoking affects immunoglobulin class levels**

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Abbreviations

BSA: Bovine serum albumin; COPD: Chronic obstructive pulmonary disease; CS: Cigarette smoke; CV: Coefficient of variation; ELISA: Enzyme linked immunosorbent assay; HRP: Horse-radish peroxidase; Ig: Immunoglobulin; PBS: Phosphate-buffered saline.

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Conflict of Interest:
The authors declare no conflict of interest.

Abstract

The aim of the present study was to compare concentrations of IgG, IgA, IgM and IgD in both serum and saliva samples from smoking and non-smoking subjects using a protein microarray assay. The findings were also compared to previous studies. Serum and saliva were collected from 48 smoking male subjects and 48 age-matched never-smoker male subjects. The protein microarray assays for detection of human IgG, IgM, IgA and IgD were established and optimized using Ig class-specific affinity purified goat anti-human Ig-Fc capture antibodies and horseradish peroxidase (HRP)-conjugated goat anti-human Ig-Fc detection antibodies. The Ig class specificity of the microarray assays was verified and the optimal dilutions of serum and saliva samples was determined for quantification of Ig levels against standard curves. We found that smoking is associated with reduced IgG concentrations and enhanced IgA concentrations in both serum and saliva. By contrast, smoking differentially affected IgM concentrations – causing increased concentrations in serum, but decreased concentrations in saliva. Smoking was associated with decreased IgD concentrations in serum, and did not have a significant effect on the very low IgD concentrations in saliva. Thus, cigarette smoking differentially affects the levels of Ig classes systemically and in the oral mucosa. Although there is variation between the results of different published studies, there is a consensus that smokers have significantly reduced levels of IgG in both serum and saliva. A functional antibody deficiency associated with smoking may compromise the body's response to infection and result in a predisposition to the development of autoimmunity.

Keywords: cigarette smoke; immunoglobulin; IgG; IgA; IgM; IgD; serum; saliva; protein microarray.

Introduction

Cigarette smoke (CS) has numerous toxic chemical constituents, which have cytotoxic, mutagenic, carcinogenic, and/or antigenic properties (1, 2). The immune system is affected in a variety of ways by CS, ranging from immunosuppression and increased susceptibility to infection to promotion of inflammation and immunopathology (3). With regard to the latter, CS is the major cause of chronic obstructive pulmonary disease (COPD) (4), and smoking is a recognised risk factor for the occurrence of autoimmune diseases (5).

An important facet of these effects is that CS causes dysregulation and impairment of B lymphocytes (6-10); nicotinic receptors are expressed by B cells (11), and long-term exposure to nicotine can suppress B cell development, proliferation and immune functions (9, 12-14). Numerous studies have investigated the effects of tobacco smoking on immunoglobulin (Ig) levels using a variety of assays. In serum samples from smokers and non-smokers, Igs have been quantified by ELISA (15), immunodiffusion assay (16), nephelometry (17-19), radioimmunoassay (20) and turbidimetry (21, 22). Investigations of Ig levels in the saliva of smokers and non-smokers have been carried out using mainly ELISA (23-29), although immunodiffusion assays (30-32) or turbidimetry (33) have also been used. These studies have generated varying results concerning the effects of smoking on the concentrations of different Ig classes (IgG, IgA, IgM) in serum or saliva, reporting that smoking is associated with increased, decreased or unchanged Ig class concentrations compared to non-smoking controls. However, these studies have not investigated simultaneously the effects of smoking on Ig class concentrations in *both* serum and saliva in the same subjects. In addition, few of these studies have investigated the effects of smoking on levels of IgD (20, 22): this is expressed particularly in the upper aerodigestive tract in humans (34), which is heavily exposed to CS in smokers.

The aim of the present study was therefore the compare concentrations of IgD, as well as IgG, IgA and IgM, in both serum and saliva samples from smoking and non-smoking subjects. Furthermore, this was undertaken using a protein microarray assay to quantify the Ig class levels – this relatively recent technique was not used in any of the previous studies referred to above (15-33). The protein microarray used was essentially a miniaturised version of a sandwich/capture ELISA. However, in comparison to ELISAs, microarrays require smaller sample volumes, are more

sensitive and have a greater dynamic range. These factors make microarrays a cheaper and potentially more sensitive alternative to ELISAs for the large-scale detection of known proteins, including Igs (35).

Materials and Methods

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (36).

Subjects. Serum and saliva were collected from 48 smoking male subjects (median age = 30 years; age range 19-51) and 48 age-matched never-smoker male subjects (median age = 32.5 years; age range 20-56), all of whom were blood bank volunteers at King Abdulaziz Hospital, Jeddah, Saudi Arabia. As healthy blood bank volunteer donors at the time of sample collection, all the subjects fulfilled the following criteria: No history or evidence of significant illness/disease, including lung/respiratory tract, heart/chest, liver, kidney, nervous system, diabetes, cancer. No infectious diseases or recent vaccinations, fever, flu-like illness, sore throat or antibiotic treatment. No recent dental extractions. No recent surgery, blood transfusion or organ transplantation. No recent anaemia, high blood pressure or abnormal pulse.

The serum and saliva samples were aliquoted and stored at -20^{o}C. They were transported on ice to Queen's Medical Centre, Nottingham, UK, and then stored frozen until assayed. Ethical approval was granted by The Biomedical Research Ethics Committee, Ministry of Higher Education, King Abdulaziz University, Faculty of Medicine. The smoking subjects smoked a median of 20 cigarettes/day (range 1-40), and had smoked for a median of 10 years (range 2-33).

Donors ceased from eating and drinking (and smoking) about 1 hour prior to donation. Smoker and non-smoker donors were requested to provide 10 ml of blood and 5 ml of unstimulated saliva after signing an informed-consent form. Blood samples were collected in plain serum separation tubes (BD Vacutainer™ Venous Blood Collection Tubes: SST™ Serum Separation Tubes: Hemogard). The tubes were centrifuged at 2000*g* for 10 minutes. Saliva was collected over a period of 5-10 minutes. After cleaning the mouth with mouthwash and rinsing with water over 5 min, donors provided saliva into plain separation tubes (Thermo Fisher Scientific) and the tubes

kept on ice. Then, the saliva samples were centrifuged at 2600*g* for 15 minutes at 4°C. Samples were aliquoted and immediately frozen at −20°C.

Human Ig class microarrays. Affinity purified goat anti-human Ig-Fc capture antibodies and horseradish peroxidase (HRP)-conjugated goat anti-human Ig-Fc detection antibodies provided in the Bethyl Laboratories Human IgG/IgA/IgM/IgD ELISA Quantitation Sets (obtained from Cambridge Bioscience) were used to established the microarray assay for quantifying human IgG, IgA, IgM and IgD, respectively. As accurate quantification of each Ig class requires the capture and detection antibodies to be highly specific for the relevant class, this was initially confirmed by running the Ig class-specific ELISAs according to the manufacturer's instructions and demonstrating that each capture/detection antibody pair bound only the relevant class of purified Ig (obtained from Athenas Research Technology); for example, the anti-IgG-Fc capture and detection antibodies detected human IgG, but not IgA, IgM or IgD (Figure 1).

To transpose the assay to the microarray platform, optimisation studies were undertaken to determine the optimal printing buffer, blocking buffer, and concentrations of each capture antibody and detection antibody. The optimised microarray assays for detection of human IgG, IgA, IgM and IgD were then performed as follows:

Capture antibody goat anti-human Ig-Fc (100µg/ml in PBS-Trehalose (50mM)) was loaded onto a 384 well plate (Genetix), and printed in double quadriplicate in a 16 x16 array format onto a poly-l-lysine-coated glass slide (Thermofisher, UK) using a Biorobotics Microgrid II arrayer (Microgrid) and a silicon contact pin (Parallel Synthesis Technologies, USA). During printing, the array chamber was set to 20^0C and 60% humidity, and the spot diameter was 315µm. Slides were held under a vacuum overnight. On the next day, the slides were blocked with BSA blocking buffer with shaking for 1 h at room temperature. After incubation, the slides were washed 3 times with PBS containing 0.05 % Tween-20 on the shaker for 3 min each wash. Ig standards, serum and saliva samples were added and incubated for 1 h. Following incubation, the slides were washed as described above. Then, 100 µl of the HRP-conjugated detection antibody was added and the slides were incubated for 1 h at room temperature on a shaker. The slides were washed, as described above, and

100 µl biotinylated-tyramide amplification reagent was applied to the slides and incubated for 10 min at room temperature. After the incubation, the slides were washed as described above, and 100 µl Cy5-conjugated streptavidin (1:1000 in 3 % BSA) was applied to each block. The slides were incubated for 15 min on the shaker in the dark. Finally, the slides were released from the holder, washed in ultra-pure water for 5 min, and centrifuged for 3 min. After processing, slides were immediately scanned in a GenePix 4200AL microarray scanner, to measure the fluorescence (Cy5 Fluor 635).

The microarray assays had intra-assay coefficient of variation (CV) <10%, and inter-assay CV <15%. The Ig class specificity demonstrated in the ELISA format was maintained in the microarray assays, as exemplified for detection of IgG (Figure 2).

Statistical Analysis. The data were not normally distributed and therefore comparisons between groups were made using the Kruskal-Wallis test for non-paired data. Statistical analyses were performed using GraphPad Prism 7.

Results

Determining optimal sample dilutions for Ig class quantification.

The microarray assays for detection of human IgG, IgM, IgA and IgD were established and optimized as described in the Material & Methods section. Experiments were undertaken to determine the optimal dilutions of human serum for accurate quantification of each Ig class. As shown in figure 3, the serum dilutions that generated signals corresponding to the sensitive regions of the standard curves were 1:40,000 for IgG and IgA, and 1:200 for IgM and IgD. A saliva dilution of 1:2 was suitable for detection of salivary Igs (data not shown).

Serum Ig class concentrations in smokers and non-smokers.

The serum Ig concentrations for the smokers and non-smokers are shown in figure 4a-d. The IgG levels in smokers were very significantly lower than those in non-smokers ($p<0.0001$; figure 4a); conversely, the serum IgM levels were very significantly higher in smokers than non-smokers ($p<0.0001$; figure 4b). The serum IgA levels were also significantly higher in smokers ($p<0.05$; figure 4c), but the smokers' IgD concentrations were significantly lower than in the non-smokers ($p<0.05$; figure 4d).

Salivary Ig class concentrations in smokers and non-smokers.

Figure 4e-h shows that, as in serum, IgG concentrations in the saliva of smokers were significantly lower than in non-smokers ($p<0.05$; figure 4e), and salivary IgA concentrations were significantly higher in smokers ($p<0.05$; figure 4g). By contrast, IgM levels were very significantly *lower* in the saliva of the smokers compared to the non-smokers ($p<0.0001$, figure 4f), which is the opposite of the findings for the serum levels of IgM described above (figure 4b). There was no significant difference in the very low concentrations of salivary IgD between smokers and non-smokers (figure 4h).

There were no statistically significant correlations between serum and salivary Ig levels in the same subjects (either smokers or non-smokers) for any of the four Ig classes measured (data not shown). Also, there were no significant correlations between smoking pack-years and Ig class levels in either serum or saliva samples.

Discussion

Our findings indicate that smoking is associated with reduced IgG concentrations in both serum and saliva, and enhanced IgA concentrations in both serum and saliva. By contrast, smoking differentially affected IgM concentrations – causing increased concentrations in serum, but decreased concentrations in saliva. We found smoking to be associated with decreased IgD concentrations in serum, and not to have a significant effect on the very low IgD concentrations in saliva.

Tables 1 and 2 summarize the findings in the present study, together with the results reported in previous studies (quoted in the Introduction) that investigated the effects of smoking on levels of IgG, IgM, IgA and IgD in serum (Table 1) or in saliva (Table 2). As mentioned in the Introduction, unlike the present study, these previous studies did not investigate Ig concentrations in *both* serum and saliva; few determined IgD concentrations (two for serum and none for saliva); and none used microarray technology. The advantages of the microarray platform include its sensitivity and broad dynamic range (0 to 65,000 fluorescence units, compared to the 0 to 4 optical density units in ELISA), and the increased number of replicates that can readily be performed

within an assay together with detailed feature analysis enabling poorly-performing replicates to be legitimately excluded, hence reducing replicate variation.

Considering all the studies summarized in Tables 1 and 2 that investigated the effects of smoking on IgG levels, 6/7 (including the present study) reported a significant decrease in IgG concentrations in the serum of smokers; also 2/3 studies (including the present study) reported a significant reduction in IgG levels in the saliva of smokers. Thus, there is a strong consensus of evidence that smoking is associated with reduced IgG levels both systemically and in the oral mucosa. It would be of interest to examine the effects of smoking on IgG subclasses, especially as these are known to be affected in COPD (37).

By contrast, there is no clear consensus for the effects of smoking on IgA levels, with different studies reporting an increase, a decrease, or no effect of smoking on IgA concentrations in either serum (Table 1) or saliva (Table 2). The reason for this variation are unclear: it does not appear to be related to different types of assays being used (e.g. nephelometry versus turbidimetry versus ELISA, etc.) since, for example, amongst the seven studies that used ELISA to determine salivary IgA levels, one reported an increase, three reported no change and three reported a decrease in smokers compared to non-smokers. Other possible factors leading to the variations could include ethnicity, amount of smoking and relative detection of IgA1 versus IgA2.

Five studies listed in Table 1 reported no significant effects of smoking on serum levels of IgM, whereas a very significant increase in smokers was observed in the present study. This may be because, in the present study, several smokers had particularly high serum IgM concentrations, plus very little variation was found in the serum IgM levels of non-smokers (figure 4b). On the other hand, the present study agrees with 2/3 other studies in finding reduced levels of IgM in the saliva of smokers compared to non-smokers. Overall, the present study agrees with the consensus view of other studies that smoking has different effects on IgM levels in serum and saliva.

The concentrations of IgD are very low in serum and extremely low in saliva, with an insufficient number of studies for a consensus view on the effects of smoking to be drawn. There is, therefore, clear scope for further studies of the effects of smoking on IgD levels in both serum and saliva. The importance of IgD in the upper aerodigestive tract in humans is indicated by the high numbers of IgD-producing B cells in tonsils, adenoids, salivary and lachrymal glands, and nasal mucosa; whereas IgD producing B cells are much less frequent in peripheral lymph nodes, spleen, bone

marrow and intestinal mucosa (34). Furthermore, IgD recognizes a range of pathogenic microbes often found in the upper aerodigestive tract of humans, consistent with a protective role for IgD in this location (34). A better understanding of the effects of smoking on IgD is therefore important.

Although the effects of tobacco smoking on IgE levels were not the subject of the present study, this is clearly of importance, not least since smoking exacerbates allergic conditions. Numerous previous studies have shown that tobacco smoking is associated with elevated levels of serum IgE (38-43); it would also be of interest to determine the effects of smoking on salivary IgE.

The lack of correlation between smoking pack years and Ig class levels in serum and saliva that we observed is broadly consistent with the findings of others. One study reported a significant inverse correlation between salivary IgA concentrations and current level of smoking, but no correlation between smoking and salivary IgG or IgM concentrations (23). By contrast, another study reported there to be no correlation between salivary IgA levels and frequency of duration of smoking (33). With regard to serum Igs, no significant correlation was found between IgD levels and duration of smoking (20). Another study reported a significant correlation between serum IgG concentrations and the number of cigarettes smoked per day; however, these authors also pointed out that there was a clearly significant difference in serum IgG levels between non-smokers and those a relatively low number of cigarettes (≤10 per day) (17). They also reported a low significance for the correlation between IgM concentrations and level of smoking, but no significance for the correlation of IgA levels and smoking (17). Overall, our findings, and these previous studies, are consistent with the proposal that any level of cigarette smoking affects Ig levels in serum and saliva, with smoking *per se* being the main factor, regardless of whether the level of smoking is low, moderate or high.

A variety of mechanisms could be involved in the differential effects of smoking on the concentrations of Ig classes in serum and saliva: these might include direct effects on B cells and indirect effects on T cells and antigen presenting cells, which could affect Ig class switching and/or differential survival of naïve B cells or memory B cells (3). In this regard, we have preliminary evidence (see online Supporting Information) that the *in vitro* production of IgG, IgM and IgA by human peripheral blood mononuclear cells (PBMCs) or purified B cells stimulated with anti-CD40 plus IL-4 and pokeweed mitogen is inhibited by cigarette smoke extract or nicotine. It is

interesting that pure nicotine appears to suppress Ig production to the same extent as CSE, suggesting that nicotine is an important contributor to the Ig-suppressive effect of whole CSE. Furthermore, these observations are consistent with the investigations of Skok et al. in murine models into the effects of nicotinic acetylcholine receptors (nAChRs) expressed by immature and mature B cells (12-14). They deduced that the activity of nAChR promotes the survival and development of immature B cells in the bone marrow, but suppresses B cell activation in response to antigenic challenge in secondary lymphoid tissues like the spleen (12, 13). In this regard, wild-type mice (expressing nAChR) were found to produce higher levels of natural antibodies that nAChR-knockout mice, but wild-type mice produced lower levels of antigen-specific IgG antibodies than the nAChR-knockout mice when challenged (12). This may help to explain our observation that smokers, compared to non-smokers, have very significantly lower levels of serum IgG (most of which is derived from antigen-stimulated B cells), but very significantly higher levels of serum IgM (that may include a higher proportion of natural antibodies). By contrast, different processes are likely to determine salivary Ig, where we found both IgG and IgM to be decreased in smokers.

It should also be noted, however, that the suppression by CSE and nicotine of IgA and IgM production by polyclonally-stimulated PBMCs and B cells (see online Supporting Information) is in contrast to our findings that IgA levels were increased in the sera and saliva of smokers, as were the levels of IgM in smokers' sera. This suggests that the *in vitro* findings cannot fully explain the effects of CSE and nicotine in smokers *in vivo*, which are likely to be much more complex. For example, the increased occurrence of infections in the upper respiratory tract of smokers (44) is likely to stimulate the production of IgA as a major mucosal Ig. This complexity is also reflected in the effects of cigarette smoking on antibodies specific to particular antigens, as distinct from total Ig class levels. For example, smokers were reported to have raised levels of anti-pneumococcal IgG antibodies, presumably due to an increased occurrence of pneumococcal infections of the respiratory tract (45). By contrast, smokers and non-smokers were found to have the same prevalence and titres of antibodies to E.coli and C. albicans, possibly because these infections are more associated with the gut than the respiratory tract (16). At another level, smoking was reported to reduce the avidity but not the quantity of IgG anti-human papillomavirus (HPV) antibodies generated in HPV vaccinated female subjects (46). Thus, numerous direct and indirect effects of cigarette

smoking are likely to contribute to the changes in total Ig and specific antibody levels in smokers.

In addition to the qualitative effects of smoking on Ig class levels, the quantitative changes in Ig concentrations associated with smoking are an important consideration in relation to the possible functional consequences. IgG constitutes most of the Ig in blood, with a concentration about four times higher than IgA and ten times higher than IgM. Thus, the 25-30% reduction in serum IgG concentrations associated with smoking observed in the present study could be particularly biologically significant. Similarly, the reduced levels of IgG and IgM in the saliva of smokers could be significant despite the slight rise in salivary IgA, even though IgA has the highest concentrations in saliva. The concentrations of IgD in both serum and saliva are too low to affect the overall Ig concentrations significantly.

Overall, the reduction in Ig levels associated with smoking might reasonably be considered as a form of moderate secondary immunodeficiency (47, 48). Similarly, the associations between smoking and autoimmune diseases (5) might result from smoking inducing a degree of immunodeficiency (including reduced Ig levels) that causes inadequate control of infective agents which, in turn, promote the development of autoimmunity (3, 49). This model provides a plausible link between smoking and infections as two environmental factors both known to be associated with autoimmune diseases (5, 49).

In addition to its association with autoimmune diseases, cigarette smoke is the main risk factor for COPD (4). In this context, others have shown that about 25% of COPD patients have total serum IgG levels below the lower limit of normal (50), consistent with our findings in smokers without COPD. This is associated with an increased incidence of exacerbations and hospitalizations (usually because of respiratory tract infections) and this is mainly in patients with deficiencies in IgG1 and/or IgG2 subclasses (37). This is consistent with reports that intra-venous Ig (IVIG) replacement therapy reduces respiratory infections and exacerbations in COPD (51, 52). Administration of IVIG is also beneficial in a range of autoimmune diseases (53), and substantial evidence exists for the occurrence of autoimmunity in COPD – particularly the production of autoantibodies (54, 55); thus, IVIG may help to reduce inflammation as well as infection in COPD.

To conclude, our data show reduced levels of IgG, and raised levels of IgA, in both the serum and saliva of smokers compared to never-smokers. By contrast, IgM

and IgD levels were differentially affected in serum and saliva by smoking. The clear evidence for reduced IgG levels associated with smoking in this and other studies may relate to the link between smoking and both autoimmune diseases and COPD.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Table 1. The effect of cigarette smoking on immunoglobulin levels in serum.

Study [ref]	**Date**	**Method**	**IgG**	**IgA**	**IgM**	**IgD**
Tarbiah et al [this paper]	2018	Microarray	v	^	^	v
Calapai et al [15]	2009	ELISA	v	–	–	
Andersen et al [16]	1982	Immunodiffusion	v	v	–	
McMillan et al [17]	1997	Nephelometry	v	–	–	
Gonzalez-Q. et al [18]	2008	Nephelometry	v	–	–	
Prajapati et al [19]	2016	Nephelometry	^	^		
Bahna et al [20]	1983	RIA				^
Al-Ghamdi et al [21]	2007	Turbidimetry	v	v	–	
Carballo et al [22]	2017	Turbidimetry				^
Total relevant studies			7	7	6	3
Increased by smoking			**1**	**2**	**1**	**2**
No effect of smoking			**0**	**3**	**5**	**0**
Decreased by smoking			**6**	**2**	**0**	**1**

Table2. The effect of cigarette smoking on immunoglobulin levels in saliva.

Study	Date	Method	IgG	IgA	IgM	IgD
Tarbiah et al [this paper]	2018	Microarray	v	^	v	–
Barton et al [23]	1990	ELISA		v	^	
Norhagen E. et al [24]	1998	ELISA		^		
Lie et al [25]	2002	ELISA		–		
Olayanju et al [26]	2012	ELISA	–	–	v	
Golpasand Hagh [27]	2013	ELISA		v		
Giuca et al [28]	2014	ELISA	v	v	v	
Nakonieczna-R. et al [29]	2016	ELISA		–		
Bennet et al [30]	1982	Immunodiffusion		v		
Doni et al [31]	2013	Immunodiffusion		v		
Koss et al [32]	2016	Immunodiffusion		–		
Shilpashree et al [33]	2012	Turbidimetry		v		
Total relevant studies			3	12	4	1
Increased by smoking			**0**	**2**	**1**	**0**
No effect of smoking			**1**	**4**	**0**	**1**
Decreased by smoking			**2**	**6**	**3**	**0**

FIGURE LEGENDS

Fig. 1. Investigation by ELISA of cross-reactivity between anti-Ig capture and detection antibodies of different Ig class specificities with Ig standards of different classes. (a) IgG standard curves with high specificity; the capture and detection antibodies measured only IgG, but not IgM, IgA, or IgD. (b) IgM standard curves with high specificity; the capture and detection antibodies measured only IgM, but not IgG, IgA, or IgD. (c) IgA standard curves with high specificity; the capture and detection antibodies measured only IgA, but not IgG, IgM, or IgD. (d) IgD standard curves with high specificity; the capture and detection antibodies measured only IgD, but not IgG, IgM, or IgA.

Fig. 2. Investigation by microarray of cross-reactivity between IgM, IgA and IgD standards with anti-IgG. Anti-human IgG was used as the capture antibody, and an HRP-conjugated goat anti-human IgG-Fc antibody served as the detection antibody. No cross-reactivity was observed between the IgM, IgA or IgD standards with an anti-human IgG capture antibody and an HRP-conjugated goat anti-human IgG antibody as the detection antibody. IgG standard curves were generated on three different occasions. The values for IgD were completely undetectable and therefore do not appear on this figure.

Fig. 3. Determination of a suitable serum dilutions. The interpolated samples with a dilution of 1:40,000 fit in the middle of the standard curves for IgG and IgA, and a 1:200 dilution was used to measure IgM and IgD.

Fig. 4. Comparing the Ig concentrations in serum samples and saliva samples from smokers and non-smoker subjects using a Mann–Whitney test revealed significant differences. (a) Serum IgG concentrations were lower in smokers than in non-smoker subjects ($p < 0.0001$). (b) Seruem IgM concentrations were higher in smokers than in non-smoker subjects ($P < 0.0001$). (c) Serum IgA concentrations were higher in smokers than in non-smoker subjects ($P < 0.05$). (d) Serum IgD concentrations were lower in smokers than in non-smoker subjects ($P < 0.05$). (e) Salivary IgG

concentrations were lower in smokers than in non-smokers ($P < 0.05$). (f) Salivary IgM concentrations were lower in smokers than in non-smokers ($P < 0.0001$). (g) Salivary IgA concentrations were higher in smokers than in non-smokers ($P < 0.05$). (h) Salivary IgD concentrations were not significantly different between smokers and non-smokers.

Figure 1.

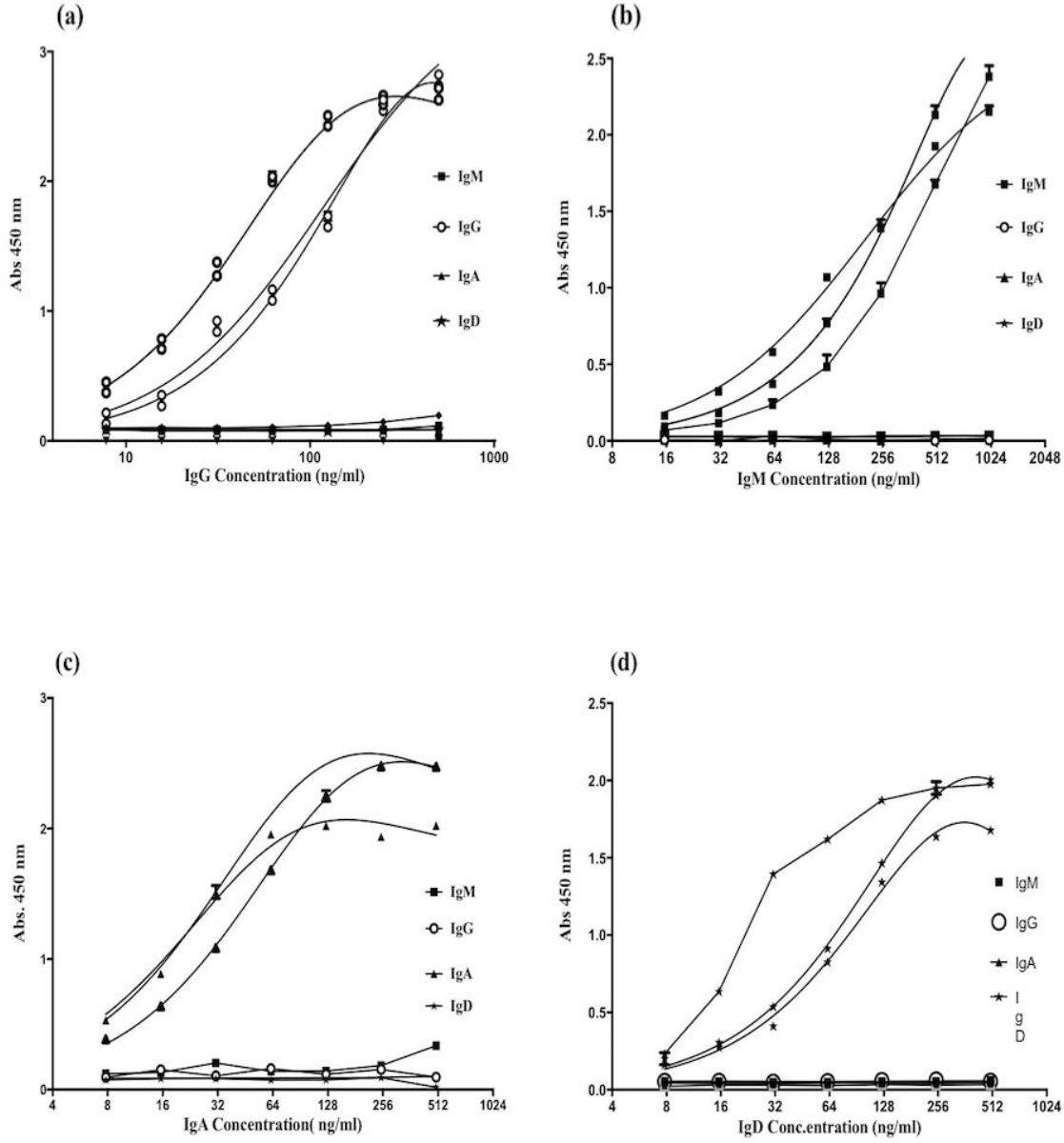

(a)
Abs 450 nm
IgG Concentration (ng/ml)
IgM
IgG
IgA
IgD
(b)
Abs 450 nm
IgM Concentration (ng/ml)
IgM
IgG
IgA
IgD
(c)
Abs. 450 nm
IgA Concentration(ng/ml)
IgM
IgG
IgA
IgD
(d)
Abs 450 nm
IgD Conc.entration (ng/ml)
IgM
IgG
IgA
I
g
D

Figure 2.

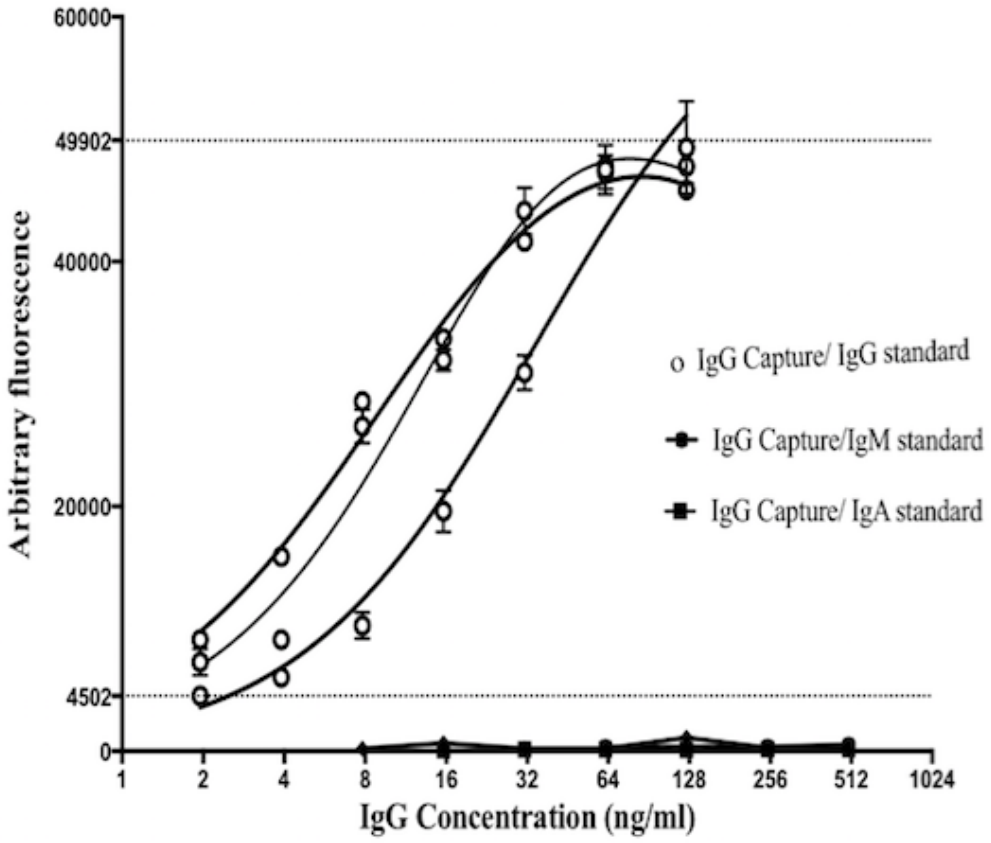

60000
49902
40000
20000
4502
0
Arbitrary fluorescence
1
2
4
8
16
32
64
128
256
512
1024
IgG Concentration (ng/ml)
IgG Capture/ IgG standard
IgG Capture/IgM standard
IgG Capture/ IgA standard

Figure 3.

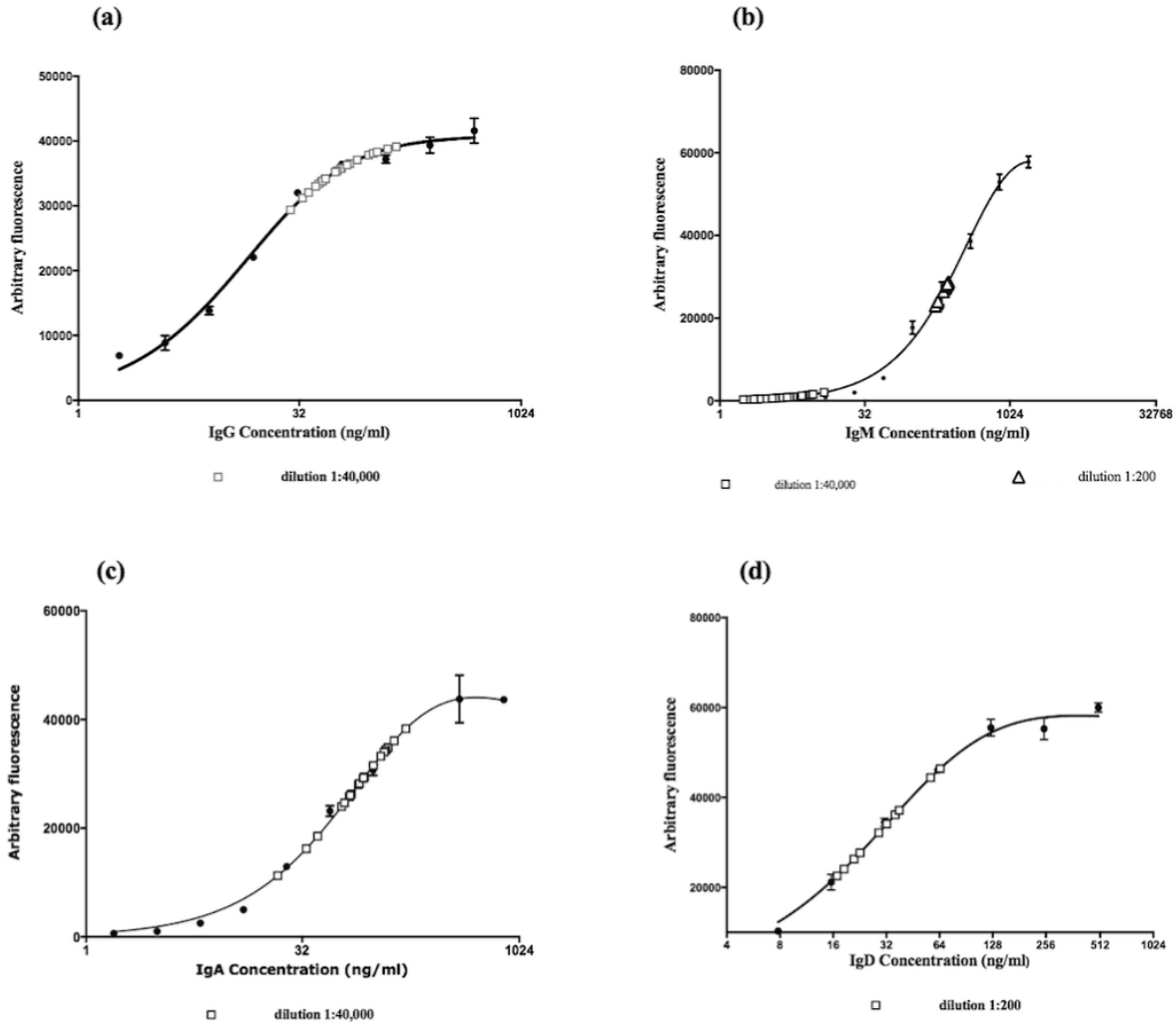
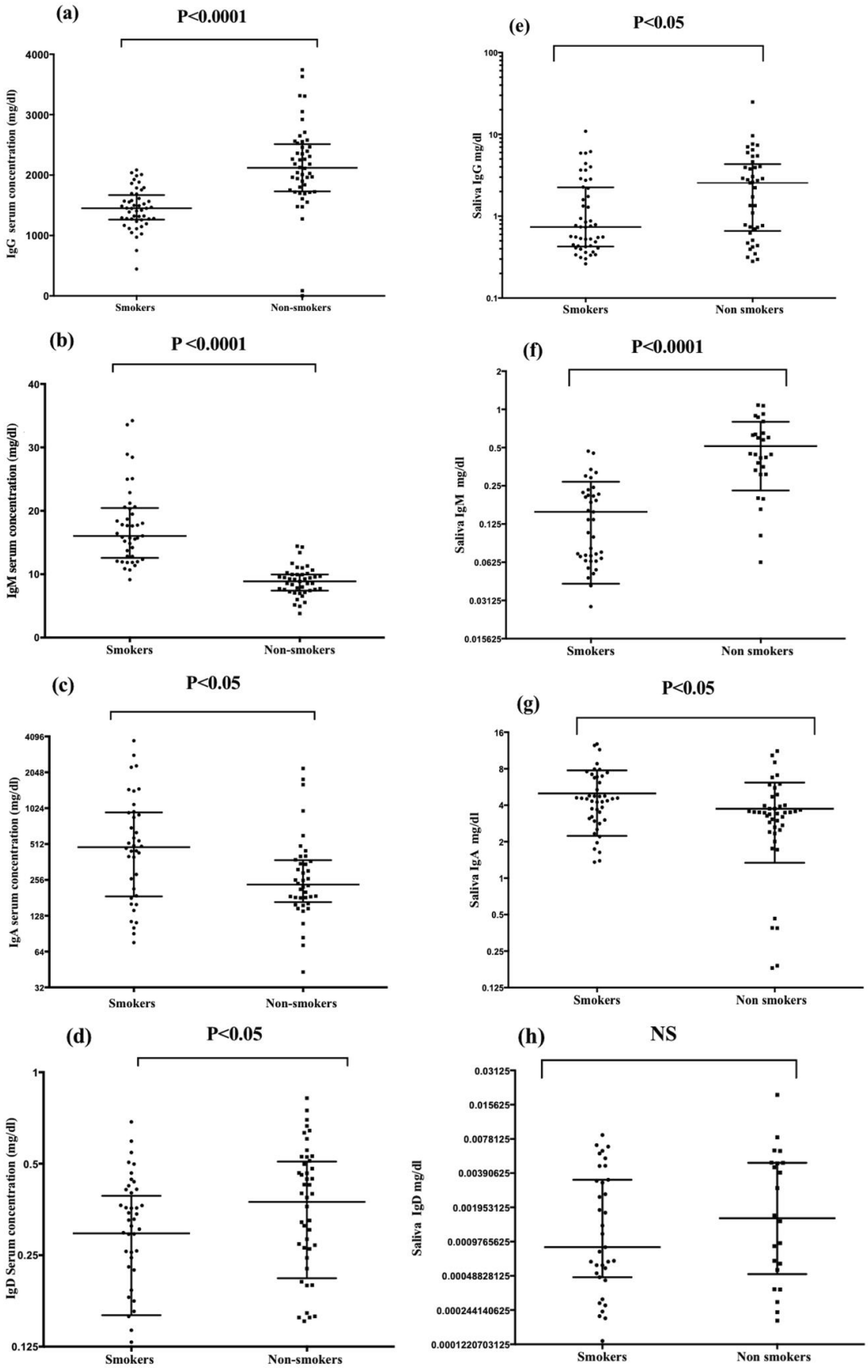

(a)
Arbitrary fluorescence
50000
40000
30000
20000
10000
0
1
32
1024
IgG Concentration (ng/ml)
dilution 1:40,000
(b)
Arbitrary fluorescence
80000
60000
40000
20000
0
1
32
1024
32768
IgM Concentration (ng/ml)
dilution 1:40,000
dilution 1:200
(c)
Arbitrary fluorescence
60000
40000
20000
0
1
32
1024
IgA Concentration (ng/ml)
dilution 1:40,000
(d)
Arbitrary fluorescence
80000
60000
40000
20000
4
8
16
32
64
128
256
512
1024
IgD Concentration (ng/ml)
dilution 1:200

Figure 4.


(a)
P<0.0001
IgG serum concentration (mg/dl)
4000
3000
2000
1000
0
Smokers
Non-smokers
(e)
P<0.05
Saliva IgG mg/dl
100
10
1
0.1
Smokers
Non smokers
(b)
P <0.0001
IgM serum concentration (mg/dl)
40
30
20
10
0
Smokers
Non-smokers
(f)
P<0.0001
Saliva IgM mg/dl
2
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
Smokers
Non smokers
(c)
P<0.05
IgA serum concentration (mg/dl)
4096
2048
1024
512
256
128
64
32
Smokers
Non-smokers
(g)
P<0.05
Saliva IgA mg/dl
16
8
4
2
1
0.5
0.25
0.125
Smokers
Non smokers
(d)
P<0.05
IgD Serum concentration (mg/dl)
1
0.5
0.25
0.125
Smokers
Non-smokers
(h)
NS
Saliva IgD mg/dl
0.03125
0.015625
0.0078125
0.00390625
0.001953125
0.0009765625
0.00048828125
0.000244140625
0.0001220703125
Smokers
Non smokers

Cigarette smoking differentially affects immunoglobulin class levels in serum and saliva: an investigation and review.

Running title: **Cigarette smoking affects immunoglobulin class levels**

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Abbreviations

BSA: Bovine serum albumin; COPD: Chronic obstructive pulmonary disease; CS: Cigarette smoke; CV: Coefficient of variation; ELISA: Enzyme linked immunosorbent assay; HRP: Horse-radish peroxidase; Ig: Immunoglobulin; PBS: Phosphate-buffered saline.

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Conflict of Interest:
The authors declare no conflict of interest.

Abstract

The aim of the present study was to compare concentrations of IgG, IgA, IgM and IgD in both serum and saliva samples from smoking and non-smoking subjects using a protein microarray assay. The findings were also compared to previous studies. Serum and saliva were collected from 48 smoking male subjects and 48 age-matched never-smoker male subjects. The protein microarray assays for detection of human IgG, IgM, IgA and IgD were established and optimized using Ig class-specific affinity purified goat anti-human Ig-Fc capture antibodies and horseradish peroxidase (HRP)-conjugated goat anti-human Ig-Fc detection antibodies. The Ig class specificity of the microarray assays was verified and the optimal dilutions of serum and saliva samples was determined for quantification of Ig levels against standard curves. We found that smoking is associated with reduced IgG concentrations and enhanced IgA concentrations in both serum and saliva. By contrast, smoking differentially affected IgM concentrations – causing increased concentrations in serum, but decreased concentrations in saliva. Smoking was associated with decreased IgD concentrations in serum, and did not have a significant effect on the very low IgD concentrations in saliva. Thus, cigarette smoking differentially affects the levels of Ig classes systemically and in the oral mucosa. Although there is variation between the results of different published studies, there is a consensus that smokers have significantly reduced levels of IgG in both serum and saliva. A functional antibody deficiency associated with smoking may compromise the body's response to infection and result in a predisposition to the development of autoimmunity.

Keywords: cigarette smoke; immunoglobulin; IgG; IgA; IgM; IgD; serum; saliva; protein microarray.

Introduction

Cigarette smoke (CS) has numerous toxic chemical constituents, which have cytotoxic, mutagenic, carcinogenic, and/or antigenic properties (1, 2). The immune system is affected in a variety of ways by CS, ranging from immunosuppression and increased susceptibility to infection to promotion of inflammation and immunopathology (3). With regard to the latter, CS is the major cause of chronic obstructive pulmonary disease (COPD) (4), and smoking is a recognised risk factor for the occurrence of autoimmune diseases (5).

An important facet of these effects is that CS causes dysregulation and impairment of B lymphocytes (6-10); nicotinic receptors are expressed by B cells (11), and long-term exposure to nicotine can suppress B cell development, proliferation and immune functions (9, 12-14). Numerous studies have investigated the effects of tobacco smoking on immunoglobulin (Ig) levels using a variety of assays. In serum samples from smokers and non-smokers, Igs have been quantified by ELISA (15), immunodiffusion assay (16), nephelometry (17-19), radioimmunoassay (20) and turbidimetry (21, 22). Investigations of Ig levels in the saliva of smokers and non-smokers have been carried out using mainly ELISA (23-29), although immunodiffusion assays (30-32) or turbidimetry (33) have also been used. These studies have generated varying results concerning the effects of smoking on the concentrations of different Ig classes (IgG, IgA, IgM) in serum or saliva, reporting that smoking is associated with increased, decreased or unchanged Ig class concentrations compared to non-smoking controls. However, these studies have not investigated simultaneously the effects of smoking on Ig class concentrations in *both* serum and saliva in the same subjects. In addition, few of these studies have investigated the effects of smoking on levels of IgD (20, 22): this is expressed particularly in the upper aerodigestive tract in humans (34), which is heavily exposed to CS in smokers.

The aim of the present study was therefore the compare concentrations of IgD, as well as IgG, IgA and IgM, in both serum and saliva samples from smoking and non-smoking subjects. Furthermore, this was undertaken using a protein microarray assay to quantify the Ig class levels – this relatively recent technique was not used in any of the previous studies referred to above (15-33). The protein microarray used was essentially a miniaturised version of a sandwich/capture ELISA. However, in comparison to ELISAs, microarrays require smaller sample volumes, are more

sensitive and have a greater dynamic range. These factors make microarrays a cheaper and potentially more sensitive alternative to ELISAs for the large-scale detection of known proteins, including Igs (35).

Materials and Methods

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (36).

Subjects. Serum and saliva were collected from 48 smoking male subjects (median age = 30 years; age range 19-51) and 48 age-matched never-smoker male subjects (median age = 32.5 years; age range 20-56), all of whom were blood bank volunteers at King Abdulaziz Hospital, Jeddah, Saudi Arabia. As healthy blood bank volunteer donors at the time of sample collection, all the subjects fulfilled the following criteria: No history or evidence of significant illness/disease, including lung/respiratory tract, heart/chest, liver, kidney, nervous system, diabetes, cancer. No infectious diseases or recent vaccinations, fever, flu-like illness, sore throat or antibiotic treatment. No recent dental extractions. No recent surgery, blood transfusion or organ transplantation. No recent anaemia, high blood pressure or abnormal pulse.

The serum and saliva samples were aliquoted and stored at -20^{o}C. They were transported on ice to Queen's Medical Centre, Nottingham, UK, and then stored frozen until assayed. Ethical approval was granted by The Biomedical Research Ethics Committee, Ministry of Higher Education, King Abdulaziz University, Faculty of Medicine. The smoking subjects smoked a median of 20 cigarettes/day (range 1-40), and had smoked for a median of 10 years (range 2-33).

Donors ceased from eating and drinking (and smoking) about 1 hour prior to donation. Smoker and non-smoker donors were requested to provide 10 ml of blood and 5 ml of unstimulated saliva after signing an informed-consent form. Blood samples were collected in plain serum separation tubes (BD Vacutainer™ Venous Blood Collection Tubes: SST™ Serum Separation Tubes: Hemogard). The tubes were centrifuged at 2000*g* for 10 minutes. Saliva was collected over a period of 5-10 minutes. After cleaning the mouth with mouthwash and rinsing with water over 5 min, donors provided saliva into plain separation tubes (Thermo Fisher Scientific) and the tubes

kept on ice. Then, the saliva samples were centrifuged at 2600*g* for 15 minutes at 4°C. Samples were aliquoted and immediately frozen at −20°C.

Human Ig class microarrays. Affinity purified goat anti-human Ig-Fc capture antibodies and horseradish peroxidase (HRP)-conjugated goat anti-human Ig-Fc detection antibodies provided in the Bethyl Laboratories Human IgG/IgA/IgM/IgD ELISA Quantitation Sets (obtained from Cambridge Bioscience) were used to established the microarray assay for quantifying human IgG, IgA, IgM and IgD, respectively. As accurate quantification of each Ig class requires the capture and detection antibodies to be highly specific for the relevant class, this was initially confirmed by running the Ig class-specific ELISAs according to the manufacturer's instructions and demonstrating that each capture/detection antibody pair bound only the relevant class of purified Ig (obtained from Athenas Research Technology); for example, the anti-IgG-Fc capture and detection antibodies detected human IgG, but not IgA, IgM or IgD (Figure 1).

To transpose the assay to the microarray platform, optimisation studies were undertaken to determine the optimal printing buffer, blocking buffer, and concentrations of each capture antibody and detection antibody. The optimised microarray assays for detection of human IgG, IgA, IgM and IgD were then performed as follows:

Capture antibody goat anti-human Ig-Fc (100µg/ml in PBS-Trehalose (50mM)) was loaded onto a 384 well plate (Genetix), and printed in double quadriplicate in a 16 x16 array format onto a poly-l-lysine-coated glass slide (Thermofisher, UK) using a Biorobotics Microgrid II arrayer (Microgrid) and a silicon contact pin (Parallel Synthesis Technologies, USA). During printing, the array chamber was set to 20^0C and 60% humidity, and the spot diameter was 315µm. Slides were held under a vacuum overnight. On the next day, the slides were blocked with BSA blocking buffer with shaking for 1 h at room temperature. After incubation, the slides were washed 3 times with PBS containing 0.05 % Tween-20 on the shaker for 3 min each wash. Ig standards, serum and saliva samples were added and incubated for 1 h. Following incubation, the slides were washed as described above. Then, 100 µl of the HRP-conjugated detection antibody was added and the slides were incubated for 1 h at room temperature on a shaker. The slides were washed, as described above, and

100 µl biotinylated-tyramide amplification reagent was applied to the slides and incubated for 10 min at room temperature. After the incubation, the slides were washed as described above, and 100 µl Cy5-conjugated streptavidin (1:1000 in 3 % BSA) was applied to each block. The slides were incubated for 15 min on the shaker in the dark. Finally, the slides were released from the holder, washed in ultra-pure water for 5 min, and centrifuged for 3 min. After processing, slides were immediately scanned in a GenePix 4200AL microarray scanner, to measure the fluorescence (Cy5 Fluor 635).

The microarray assays had intra-assay coefficient of variation (CV) <10%, and inter-assay CV <15%. The Ig class specificity demonstrated in the ELISA format was maintained in the microarray assays, as exemplified for detection of IgG (Figure 2).

Statistical Analysis. The data were not normally distributed and therefore comparisons between groups were made using the Kruskal-Wallis test for non-paired data. Statistical analyses were performed using GraphPad Prism 7.

Results

Determining optimal sample dilutions for Ig class quantification.

The microarray assays for detection of human IgG, IgM, IgA and IgD were established and optimized as described in the Material & Methods section. Experiments were undertaken to determine the optimal dilutions of human serum for accurate quantification of each Ig class. As shown in figure 3, the serum dilutions that generated signals corresponding to the sensitive regions of the standard curves were 1:40,000 for IgG and IgA, and 1:200 for IgM and IgD. A saliva dilution of 1:2 was suitable for detection of salivary Igs (data not shown).

Serum Ig class concentrations in smokers and non-smokers.

The serum Ig concentrations for the smokers and non-smokers are shown in figure 4a-d. The IgG levels in smokers were very significantly lower than those in non-smokers ($p<0.0001$; figure 4a); conversely, the serum IgM levels were very significantly higher in smokers than non-smokers ($p<0.0001$; figure 4b). The serum IgA levels were also significantly higher in smokers ($p<0.05$; figure 4c), but the smokers' IgD concentrations were significantly lower than in the non-smokers ($p<0.05$; figure 4d).

Salivary Ig class concentrations in smokers and non-smokers.

Figure 4e-h shows that, as in serum, IgG concentrations in the saliva of smokers were significantly lower than in non-smokers ($p<0.05$; figure 4e), and salivary IgA concentrations were significantly higher in smokers ($p<0.05$; figure 4g). By contrast, IgM levels were very significantly *lower* in the saliva of the smokers compared to the non-smokers ($p<0.0001$, figure 4f), which is the opposite of the findings for the serum levels of IgM described above (figure 4b). There was no significant difference in the very low concentrations of salivary IgD between smokers and non-smokers (figure 4h).

There were no statistically significant correlations between serum and salivary Ig levels in the same subjects (either smokers or non-smokers) for any of the four Ig classes measured (data not shown). Also, there were no significant correlations between smoking pack-years and Ig class levels in either serum or saliva samples.

Discussion

Our findings indicate that smoking is associated with reduced IgG concentrations in both serum and saliva, and enhanced IgA concentrations in both serum and saliva. By contrast, smoking differentially affected IgM concentrations – causing increased concentrations in serum, but decreased concentrations in saliva. We found smoking to be associated with decreased IgD concentrations in serum, and not to have a significant effect on the very low IgD concentrations in saliva.

Tables 1 and 2 summarize the findings in the present study, together with the results reported in previous studies (quoted in the Introduction) that investigated the effects of smoking on levels of IgG, IgM, IgA and IgD in serum (Table 1) or in saliva (Table 2). As mentioned in the Introduction, unlike the present study, these previous studies did not investigate Ig concentrations in *both* serum and saliva; few determined IgD concentrations (two for serum and none for saliva); and none used microarray technology. The advantages of the microarray platform include its sensitivity and broad dynamic range (0 to 65,000 fluorescence units, compared to the 0 to 4 optical density units in ELISA), and the increased number of replicates that can readily be performed

within an assay together with detailed feature analysis enabling poorly-performing replicates to be legitimately excluded, hence reducing replicate variation.

Considering all the studies summarized in Tables 1 and 2 that investigated the effects of smoking on IgG levels, 6/7 (including the present study) reported a significant decrease in IgG concentrations in the serum of smokers; also 2/3 studies (including the present study) reported a significant reduction in IgG levels in the saliva of smokers. Thus, there is a strong consensus of evidence that smoking is associated with reduced IgG levels both systemically and in the oral mucosa. It would be of interest to examine the effects of smoking on IgG subclasses, especially as these are known to be affected in COPD (37).

By contrast, there is no clear consensus for the effects of smoking on IgA levels, with different studies reporting an increase, a decrease, or no effect of smoking on IgA concentrations in either serum (Table 1) or saliva (Table 2). The reason for this variation are unclear: it does not appear to be related to different types of assays being used (e.g. nephelometry versus turbidimetry versus ELISA, etc.) since, for example, amongst the seven studies that used ELISA to determine salivary IgA levels, one reported an increase, three reported no change and three reported a decrease in smokers compared to non-smokers. Other possible factors leading to the variations could include ethnicity, amount of smoking and relative detection of IgA1 versus IgA2.

Five studies listed in Table 1 reported no significant effects of smoking on serum levels of IgM, whereas a very significant increase in smokers was observed in the present study. This may be because, in the present study, several smokers had particularly high serum IgM concentrations, plus very little variation was found in the serum IgM levels of non-smokers (figure 4b). On the other hand, the present study agrees with 2/3 other studies in finding reduced levels of IgM in the saliva of smokers compared to non-smokers. Overall, the present study agrees with the consensus view of other studies that smoking has different effects on IgM levels in serum and saliva.

The concentrations of IgD are very low in serum and extremely low in saliva, with an insufficient number of studies for a consensus view on the effects of smoking to be drawn. There is, therefore, clear scope for further studies of the effects of smoking on IgD levels in both serum and saliva. The importance of IgD in the upper aerodigestive tract in humans is indicated by the high numbers of IgD-producing B cells in tonsils, adenoids, salivary and lachrymal glands, and nasal mucosa; whereas IgD producing B cells are much less frequent in peripheral lymph nodes, spleen, bone

marrow and intestinal mucosa (34). Furthermore, IgD recognizes a range of pathogenic microbes often found in the upper aerodigestive tract of humans, consistent with a protective role for IgD in this location (34). A better understanding of the effects of smoking on IgD is therefore important.

Although the effects of tobacco smoking on IgE levels were not the subject of the present study, this is clearly of importance, not least since smoking exacerbates allergic conditions. Numerous previous studies have shown that tobacco smoking is associated with elevated levels of serum IgE (38-43); it would also be of interest to determine the effects of smoking on salivary IgE.

The lack of correlation between smoking pack years and Ig class levels in serum and saliva that we observed is broadly consistent with the findings of others. One study reported a significant inverse correlation between salivary IgA concentrations and current level of smoking, but no correlation between smoking and salivary IgG or IgM concentrations (23). By contrast, another study reported there to be no correlation between salivary IgA levels and frequency of duration of smoking (33). With regard to serum Igs, no significant correlation was found between IgD levels and duration of smoking (20). Another study reported a significant correlation between serum IgG concentrations and the number of cigarettes smoked per day; however, these authors also pointed out that there was a clearly significant difference in serum IgG levels between non-smokers and those a relatively low number of cigarettes ($\leq$10 per day) (17). They also reported a low significance for the correlation between IgM concentrations and level of smoking, but no significance for the correlation of IgA levels and smoking (17). Overall, our findings, and these previous studies, are consistent with the proposal that any level of cigarette smoking affects Ig levels in serum and saliva, with smoking *per se* being the main factor, regardless of whether the level of smoking is low, moderate or high.

A variety of mechanisms could be involved in the differential effects of smoking on the concentrations of Ig classes in serum and saliva: these might include direct effects on B cells and indirect effects on T cells and antigen presenting cells, which could affect Ig class switching and/or differential survival of naïve B cells or memory B cells (3). In this regard, we have preliminary evidence (see online Supporting Information) that the *in vitro* production of IgG, IgM and IgA by human peripheral blood mononuclear cells (PBMCs) or purified B cells stimulated with anti-CD40 plus IL-4 and pokeweed mitogen is inhibited by cigarette smoke extract or nicotine. It is

interesting that pure nicotine appears to suppress Ig production to the same extent as CSE, suggesting that nicotine is an important contributor to the Ig-suppressive effect of whole CSE. Furthermore, these observations are consistent with the investigations of Skok et al. in murine models into the effects of nicotinic acetylcholine receptors (nAChRs) expressed by immature and mature B cells (12-14). They deduced that the activity of nAChR promotes the survival and development of immature B cells in the bone marrow, but suppresses B cell activation in response to antigenic challenge in secondary lymphoid tissues like the spleen (12, 13). In this regard, wild-type mice (expressing nAChR) were found to produce higher levels of natural antibodies that nAChR-knockout mice, but wild-type mice produced lower levels of antigen-specific IgG antibodies than the nAChR-knockout mice when challenged (12). This may help to explain our observation that smokers, compared to non-smokers, have very significantly lower levels of serum IgG (most of which is derived from antigen-stimulated B cells), but very significantly higher levels of serum IgM (that may include a higher proportion of natural antibodies). By contrast, different processes are likely to determine salivary Ig, where we found both IgG and IgM to be decreased in smokers.

It should also be noted, however, that the suppression by CSE and nicotine of IgA and IgM production by polyclonally-stimulated PBMCs and B cells (see online Supporting Information) is in contrast to our findings that IgA levels were increased in the sera and saliva of smokers, as were the levels of IgM in smokers' sera. This suggests that the *in vitro* findings cannot fully explain the effects of CSE and nicotine in smokers *in vivo*, which are likely to be much more complex. For example, the increased occurrence of infections in the upper respiratory tract of smokers (44) is likely to stimulate the production of IgA as a major mucosal Ig. This complexity is also reflected in the effects of cigarette smoking on antibodies specific to particular antigens, as distinct from total Ig class levels. For example, smokers were reported to have raised levels of anti-pneumococcal IgG antibodies, presumably due to an increased occurrence of pneumococcal infections of the respiratory tract (45). By contrast, smokers and non-smokers were found to have the same prevalence and titres of antibodies to E.coli and C. albicans, possibly because these infections are more associated with the gut than the respiratory tract (16). At another level, smoking was reported to reduce the avidity but not the quantity of IgG anti-human papillomavirus (HPV) antibodies generated in HPV vaccinated female subjects (46). Thus, numerous direct and indirect effects of cigarette

smoking are likely to contribute to the changes in total Ig and specific antibody levels in smokers.

In addition to the qualitative effects of smoking on Ig class levels, the quantitative changes in Ig concentrations associated with smoking are an important consideration in relation to the possible functional consequences. IgG constitutes most of the Ig in blood, with a concentration about four times higher than IgA and ten times higher than IgM. Thus, the 25-30% reduction in serum IgG concentrations associated with smoking observed in the present study could be particularly biologically significant. Similarly, the reduced levels of IgG and IgM in the saliva of smokers could be significant despite the slight rise in salivary IgA, even though IgA has the highest concentrations in saliva. The concentrations of IgD in both serum and saliva are too low to affect the overall Ig concentrations significantly.

Overall, the reduction in Ig levels associated with smoking might reasonably be considered as a form of moderate secondary immunodeficiency (47, 48). Similarly, the associations between smoking and autoimmune diseases (5) might result from smoking inducing a degree of immunodeficiency (including reduced Ig levels) that causes inadequate control of infective agents which, in turn, promote the development of autoimmunity (3, 49). This model provides a plausible link between smoking and infections as two environmental factors both known to be associated with autoimmune diseases (5, 49).

In addition to its association with autoimmune diseases, cigarette smoke is the main risk factor for COPD (4). In this context, others have shown that about 25% of COPD patients have total serum IgG levels below the lower limit of normal (50), consistent with our findings in smokers without COPD. This is associated with an increased incidence of exacerbations and hospitalizations (usually because of respiratory tract infections) and this is mainly in patients with deficiencies in IgG1 and/or IgG2 subclasses (37). This is consistent with reports that intra-venous Ig (IVIG) replacement therapy reduces respiratory infections and exacerbations in COPD (51, 52). Administration of IVIG is also beneficial in a range of autoimmune diseases (53), and substantial evidence exists for the occurrence of autoimmunity in COPD – particularly the production of autoantibodies (54, 55); thus, IVIG may help to reduce inflammation as well as infection in COPD.

To conclude, our data show reduced levels of IgG, and raised levels of IgA, in both the serum and saliva of smokers compared to never-smokers. By contrast, IgM

and IgD levels were differentially affected in serum and saliva by smoking. The clear evidence for reduced IgG levels associated with smoking in this and other studies may relate to the link between smoking and both autoimmune diseases and COPD.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Table 1. The effect of cigarette smoking on immunoglobulin levels in serum.

Study [ref]	**Date**	**Method**	**IgG**	**IgA**	**IgM**	**IgD**
Tarbiah et al [this paper]	2018	Microarray	v	^	^	v
Calapai et al [15]	2009	ELISA	v	–	–	
Andersen et al [16]	1982	Immunodiffusion	v	v	–	
McMillan et al [17]	1997	Nephelometry	v	–	–	
Gonzalez-Q. et al [18]	2008	Nephelometry	v	–	–	
Prajapati et al [19]	2016	Nephelometry	^	^		
Bahna et al [20]	1983	RIA				^
Al-Ghamdi et al [21]	2007	Turbidimetry	v	v	–	
Carballo et al [22]	2017	Turbidimetry				^
Total relevant studies			7	7	6	3
Increased by smoking			**1**	**2**	**1**	**2**
No effect of smoking			**0**	**3**	**5**	**0**
Decreased by smoking			**6**	**2**	**0**	**1**

Table2. The effect of cigarette smoking on immunoglobulin levels in saliva.

Study	Date	Method	IgG	IgA	IgM	IgD
Tarbiah et al [this paper]	2018	Microarray	v	^	v	–
Barton et al [23]	1990	ELISA		v	^	
Norhagen E. et al [24]	1998	ELISA		^		
Lie et al [25]	2002	ELISA		–		
Olayanju et al [26]	2012	ELISA	–	–	v	
Golpasand Hagh [27]	2013	ELISA		v		
Giuca et al [28]	2014	ELISA	v	v	v	
Nakonieczna-R. et al [29]	2016	ELISA		–		
Bennet et al [30]	1982	Immunodiffusion		v		
Doni et al [31]	2013	Immunodiffusion		v		
Koss et al [32]	2016	Immunodiffusion		–		
Shilpashree et al [33]	2012	Turbidimetry		v		
Total relevant studies			3	12	4	1
Increased by smoking			**0**	**2**	**1**	**0**
No effect of smoking			**1**	**4**	**0**	**1**
Decreased by smoking			**2**	**6**	**3**	**0**

FIGURE LEGENDS

Fig. 1. Investigation by ELISA of cross-reactivity between anti-Ig capture and detection antibodies of different Ig class specificities with Ig standards of different classes. (a) IgG standard curves with high specificity; the capture and detection antibodies measured only IgG, but not IgM, IgA, or IgD. (b) IgM standard curves with high specificity; the capture and detection antibodies measured only IgM, but not IgG, IgA, or IgD. (c) IgA standard curves with high specificity; the capture and detection antibodies measured only IgA, but not IgG, IgM, or IgD. (d) IgD standard curves with high specificity; the capture and detection antibodies measured only IgD, but not IgG, IgM, or IgA.

Fig. 2. Investigation by microarray of cross-reactivity between IgM, IgA and IgD standards with anti-IgG. Anti-human IgG was used as the capture antibody, and an HRP-conjugated goat anti-human IgG-Fc antibody served as the detection antibody. No cross-reactivity was observed between the IgM, IgA or IgD standards with an anti-human IgG capture antibody and an HRP-conjugated goat anti-human IgG antibody as the detection antibody. IgG standard curves were generated on three different occasions. The values for IgD were completely undetectable and therefore do not appear on this figure.

Fig. 3. Determination of a suitable serum dilutions. The interpolated samples with a dilution of 1:40,000 fit in the middle of the standard curves for IgG and IgA, and a 1:200 dilution was used to measure IgM and IgD.

Fig. 4. Comparing the Ig concentrations in serum samples and saliva samples from smokers and non-smoker subjects using a Mann–Whitney test revealed significant differences. (a) Serum IgG concentrations were lower in smokers than in non-smoker subjects ($p < 0.0001$). (b) Seruem IgM concentrations were higher in smokers than in non-smoker subjects ($P < 0.0001$). (c) Serum IgA concentrations were higher in smokers than in non-smoker subjects ($P < 0.05$). (d) Serum IgD concentrations were lower in smokers than in non-smoker subjects ($P < 0.05$). (e) Salivary IgG

concentrations were lower in smokers than in non-smokers ($P < 0.05$). (f) Salivary IgM concentrations were lower in smokers than in non-smokers ($P < 0.0001$). (g) Salivary IgA concentrations were higher in smokers than in non-smokers ($P < 0.05$). (h) Salivary IgD concentrations were not significantly different between smokers and non-smokers.

Figure 1.

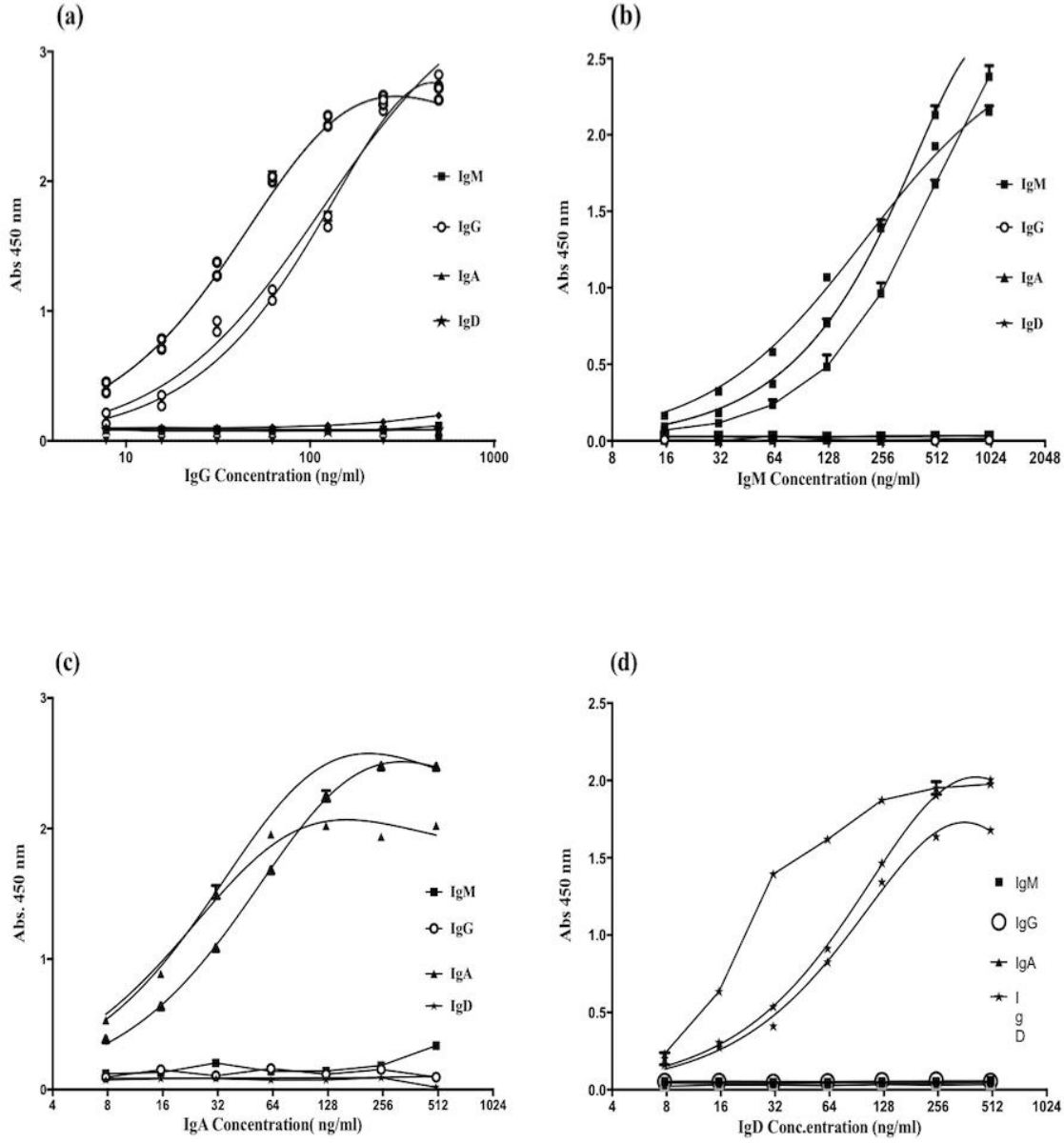

(a)
Abs 450 nm
IgG Concentration (ng/ml)
IgM
IgG
IgA
IgD
(b)
Abs 450 nm
IgM Concentration (ng/ml)
IgM
IgG
IgA
IgD
(c)
Abs. 450 nm
IgA Concentration(ng/ml)
IgM
IgG
IgA
IgD
(d)
Abs 450 nm
IgD Conc.entration (ng/ml)
IgM
IgG
IgA
I
g
D

Figure 2.

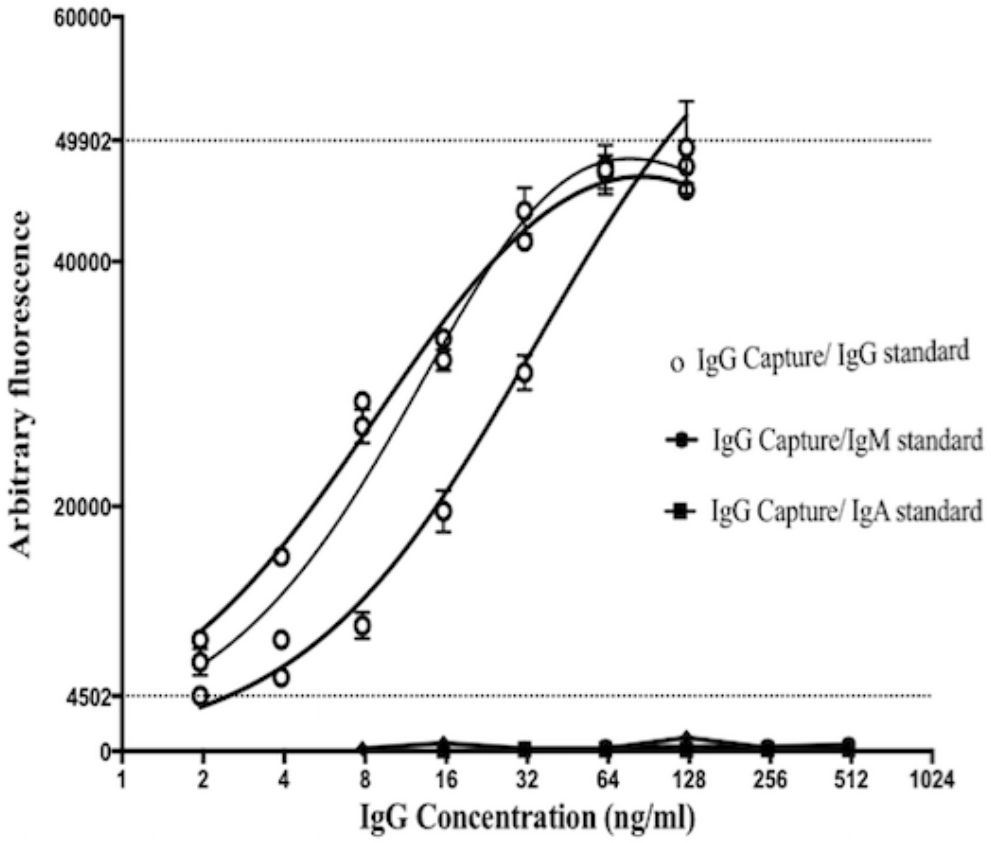

60000
49902
40000
20000
4502
0
Arbitrary fluorescence
1
2
4
8
16
32
64
128
256
512
1024
IgG Concentration (ng/ml)
IgG Capture/ IgG standard
IgG Capture/IgM standard
IgG Capture/ IgA standard

Figure 3.

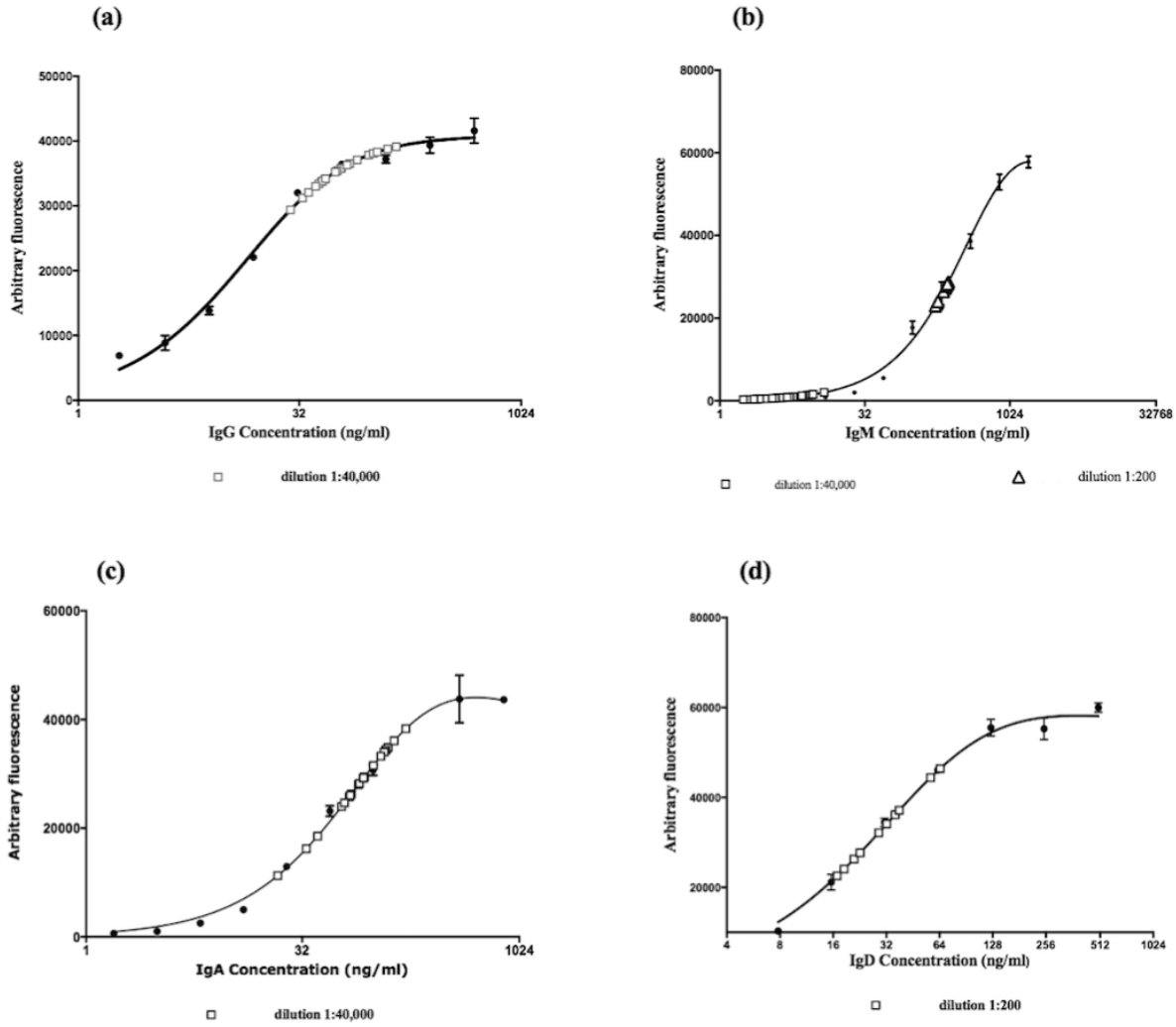
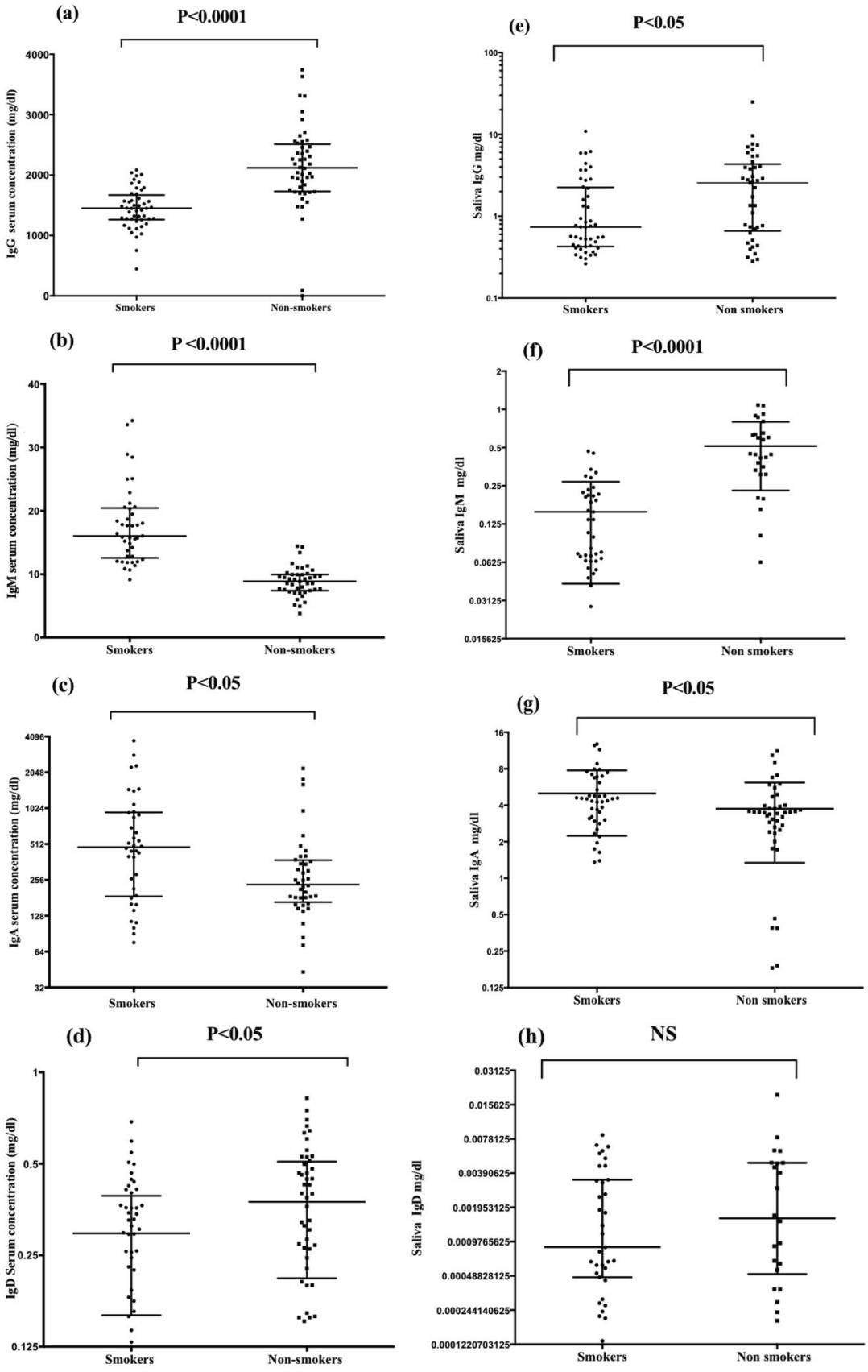

(a)
Arbitrary fluorescence
50000
40000
30000
20000
10000
0
1
32
1024
IgG Concentration (ng/ml)
dilution 1:40,000
(b)
Arbitrary fluorescence
80000
60000
40000
20000
0
1
32
1024
32768
IgM Concentration (ng/ml)
dilution 1:40,000
dilution 1:200
(c)
Arbitrary fluorescence
60000
40000
20000
0
1
32
1024
IgA Concentration (ng/ml)
dilution 1:40,000
(d)
Arbitrary fluorescence
80000
60000
40000
20000
4
8
16
32
64
128
256
512
1024
IgD Concentration (ng/ml)
dilution 1:200

Figure 4.


(a)
P<0.0001
IgG serum concentration (mg/dl)
4000
3000
2000
1000
0
Smokers
Non-smokers
(e)
P<0.05
Saliva IgG mg/dl
100
10
1
0.1
Smokers
Non smokers
(b)
P <0.0001
IgM serum concentration (mg/dl)
40
30
20
10
0
Smokers
Non-smokers
(f)
P<0.0001
Saliva IgM mg/dl
2
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
Smokers
Non smokers
(c)
P<0.05
IgA serum concentration (mg/dl)
4096
2048
1024
512
256
128
64
32
Smokers
Non-smokers
(g)
P<0.05
Saliva IgA mg/dl
16
8
4
2
1
0.5
0.25
0.125
Smokers
Non smokers
(d)
P<0.05
IgD Serum concentration (mg/dl)
1
0.5
0.25
0.125
Smokers
Non-smokers
(h)
NS
Saliva IgD mg/dl
0.03125
0.015625
0.0078125
0.00390625
0.001953125
0.0009765625
0.00048828125
0.000244140625
0.0001220703125
Smokers
Non smokers

To the Editors,

Thank you very much for considering this manuscript for publication in Basic and Clinical Pharmacology and Toxicology. We are very pleased that the manuscript is deemed acceptable, subject to an appropriate response to the reviewers' comments.

We are very grateful to the reviewers for their careful reading of the manuscript and for their helpful and constructive comments. Our responses to these comments are as follows:

Reviewer 1:

This comment in the discussion: "Thus, the 25-30% reduction in serum IgG concentrations associated with smoking observed in the present study would not be fully 'compensated' by the increase in serum IgA and IgM concentrations observed in smokers. " I believe should be omitted since different classes of Igs serve different functions and have different distributions, they cannot really compensate each other.
Response: Thank you for drawing attention to this – we agree that this statement is misleading. We have therefore modified the sentence to read as follows: "Thus, the 25-30% reduction in serum IgG concentrations associated with smoking observed in the present study could be particularly biologically significant."

This other comment; "Overall, the reduction in Ig levels associated with smoking might reasonably be considered as a form of hypogammaglobulinaemia which, in the context of primary antibody deficiencies (e.g. common variable immunodeficiency), is known to be associated with autoimmune disorders (44, 45)." I would suggest, "Overall, the reduction in Ig levels associated with smoking might reasonably be considered as a form of moderate secondary immunodeficiency (44, 45)." This is because common variable immunodeficiency is a primary immunodeficiency and goes with more markedly reduction of Igs levels and requires the demonstration of a functional impairment of specific antibody response. Secondary immunodeficiencies do not associate with autoimmunity (or nor clearly).
Response: We agree with this helpful comment and have changed the sentence as proposed.

Reviewer 2:

This is an interesting paper that falls within the scope of the journal.
It covers describes the development of a novel assay for measuring Ig concentrations, and the application of the assay to compare the levels different Ig classes in serum and saliva of smokers and non-smokers. The paper also includes supplemental data on the effects of smoke extract and nicotine on Ig secretion in vitro and a review of previously published data.
The work is clearly presented and well-described, and the data make a worthwhile contribution to the important field of the effects of smoking on immunity. The main part of the study, measuring Ig, is well-designed and appropriate conclusions are drawn. The inclusion of preliminary results on Ig production in vitro is also

helpful, and although it would have strengthened the conclusions had more samples been tested, the work is appropriate for supplemental data.
Response: Thank you for these positive and supportive comments.

There are discussion points that could be addressed or amplified. The question of the correlation (or lack) between Ig levels for heavy and light smoking merits more consideration…..
Response: Thank you for drawing attention to this important point. We have added the following paragraph to the discussion to address this: "The lack of correlation between smoking pack years and Ig class levels in serum and saliva that we observed is broadly consistent with the findings of others. One study reported a significant inverse correlation between salivary IgA concentrations and current level of smoking, but no correlation between smoking and salivary IgG or IgM concentrations (23). By contrast, another study reported there to be no correlation between salivary IgA levels and frequency of duration of smoking (33). With regard to serum Igs, no significant correlation was found between IgD levels and duration of smoking (20). Another study reported a significant correlation between serum IgG concentrations and the number of cigarettes smoked per day; however, these authors also pointed out that there was a clearly significant difference in serum IgG levels between non-smokers and those a relatively low number of cigarettes (≤10 per day) (17). They also reported a low significance for the correlation between IgM concentrations and level of smoking, but no significance for the correlation of IgA levels and smoking (17). Overall, our findings, and these previous studies, are consistent with the proposal that any level of cigarette smoking affects Ig levels in serum and saliva, with smoking *per se* being the main factor, regardless of whether the level of smoking is low, moderate or high."

….. as does the observation that smokers have higher IgA levels in serum and saliva, whilst smoke extract/nicotine suppresses IgA production in vitro. More detailed consideration as to whether specific antibody responses might be reflected by the differences in Ig class would be helpful.
Response: These are also both very good points, thank you. We have added the following paragraph to the Discussion that is intended to address these points: "It should also be noted, however, that the suppression by CSE and nicotine of IgA and IgM production by polyclonally-stimulated PBMCs and B cells (see online Supporting Information) is in contrast to our findings that IgA levels were increased in the sera and saliva of smokers, as were the levels of IgM in smokers' sera. This suggests that the *in vitro* findings cannot fully explain the effects of CSE and nicotine in smokers *in vivo*, which are likely to be much more complex. For example, the increased occurrence of infections in the upper respiratory tract of smokers (44) is likely to stimulate the production of IgA as a major mucosal Ig. This complexity is also reflected in the effects of cigarette smoking on antibodies specific to particular antigens, as distinct from total Ig class levels. For example, smokers were reported to have raised levels of anti-pneumococcal IgG antibodies, presumably due to an increased occurrence of pneumococcal infections of the respiratory tract (45). By contrast, smokers and non-smokers were found to have the same prevalence and titres of antibodies to E.coli and C. albicans, possibly because these infections are more associated with the gut than the respiratory tract (16). At another level, smoking was reported to reduce the avidity but not the quantity of IgG anti-human papillomavirus (HPV) antibodies generated in HPV vaccinated female subjects (46). Thus, numerous direct and indirect effects of cigarette

smoking are likely to contribute to the changes in total Ig and specific antibody levels in smokers."

We hope that, with these modifications, the manuscript will now be fully acceptable for publication in Basic and Clinical Pharmacology and Toxicology.

Yours faithfully,
Lucy Fairclough

Tarbiah et al. – Supplementary Information.

Cigarette smoking differentially affects immunoglobulin class levels in serum and saliva: an investigation and review.

Isolation of PBMCs

Buffy coats were obtained from anonymous blood donations via the Regional Blood Transfusion Service (Sheffield, UK), who provided ethical approval, within 24h of collection. Peripheral blood mononuclear cells (PBMCs) were isolated from 50ml buffy coat samples diluted into 150ml PBS. The diluted blood samples were transferred into 6 sterile 50ml centrifuge tubes each containing 10–15 ml Histopaque (Histopaque-1077; Sigma-Aldrich, Irvine, UK) and at 800*g* for 30 min. The white blood cell layer was collected with a sterile Pasteur pipette and placed into new sterile tubes, and 25 ml Hank's Balanced Salt Solution was used to wash the cells (Hanks 123K2368; Sigma-Aldrich, Irvine, UK). The samples were centrifuged again at 800*g* for 10 min, and the supernatant was discarded. Then, the samples were washed twice in PBS with centrifugation at 300*g* for 10 min, after which they were counted.

Magnetic separation of B cells

To separate B cells, isolated PBMCs were subjected to immunomagnetic negative selection of B-lymphocytes, using a Negative B Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; Catalogue No. 130-091-151). Briefly, the cell pellet from the previous step was resuspended in cold MACS buffer (50ml PBS, 200µl EDTA, and 830µl of 30 % BSA; Sigma-Aldrich, Irvine, UK) at a ratio of 40µl/10^7 cells. Then, Biotin-Antibody Cocktail (cocktail of biotin-conjugated monoclonal antibodies against CD2, CD14, CD16, CD36, CD43, and CD235a) was added at a ratio of 10µl/10^7 total cells. Next, the samples were incubated for 5 min in a refrigerator (2–8^{o}C). Subsequently, 30µl MACS buffer and 20µl anti-Biotin Micro Beads were added/10^7 total cells, and the suspension was incubated for an additional 10 min in the refrigerator (2–8^{o}C). Then, the cell suspension was resuspended in 1 ml MACS buffer and loaded onto a cold LS separation column (Catalogue No. 120-000-472; Miltenyi Biotec). Magnetic cell sorting was initiated after setting the magnet on the holder and the column on the cold magnet. A 3 ml LS column was equilibrated

with MACS buffer, and the cells were then passed through the column. The column was rinsed 3 times with 3 ml LS column MACS buffer to elute the B cells. After the third wash, the column was removed from the magnet and placed in a new sterile tube, and 3 ml MACS buffer/LS column was added and plunged through to remove the remaining leukocytes. The B cell samples were then centrifuged at 300*g* for 5 min to remove the MACS buffer. Finally, the B cells were resuspended at a concentration of 10^6/ml in RPMI 1640 complete medium (Catalogue No. RNBB7148; Sigma-Aldrich, Irvine, UK) supplemented with 10 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Sigma-Aldrich, Irvine, UK), and 10 % fetal bovine serum (FBS). Normally, the B cell preparation was 95 % pure ($CD19^+$), with less than 0.5 % $CD3^+$ T cells and $CD14^+$ monocytes.

Treatment of cell cultures with CSE or nicotine

Unfractionated PBMCs and purified B cells were suspended in complete RPMI 1640 medium and cultured in 24-well tissue culture plates (Costar, High Wycombe, UK). Two ml of cell suspension was added to each well (2 x 10^6 cells/well). Cells were stimulated with a mixture of IL-4 (100 U/ml), anti-CD40 mAb (1 mg/ ml), and pokeweed mitogen. Some wells were treated with a 3% concentration of CSE, prepared as described previously (1). Nicotine (N3876, Sigma-Aldrich, Irvine, UK) was added at final concentration of 75µg/ml to assess the effects of this important constituent of cigarettes on B cells, and some cells were incubated with an equal amount of complete medium alone as a negative control. Cultures were routinely maintained at 37^{o}C in a 5% CO_2 humidified atmosphere for up to 8 days. Then, the supernatants were harvested by centrifugation at 400*g* for 10 min at 4^{o}C. Cell-free supernatants was stored at -20^{o}C until aliquots were used for IgG, IgM, IgA, and IgD determinations.

Determination of Ig concentrations in culture supernatants

Cell-free supernatants from PBMC and B cell cultures were diluted 1:10 in sample diluent and used to measure the levels of IgG, IgM, IgA, and IgD by capture ELISA using Bethyl Laboratories Human IgG/IgA/IgM/IgD ELISA Quantitation Sets (obtained from Cambridge Bioscience).

Results

As shown in Figures S1 and S2, when human PBMCs or purified B cells were stimulated with a combination of IL-4, anti-CD40 and pokeweed mitogen, there was a marked increase in production/secretion of IgG, IgM and IgA compared to unstimulated cells; IgD was undetectable (data not shown). However, the addition of 3% CSE or 75µg/ml nicotine to the cultures at the same time as the stimulators suppressed the production/secretion of IgG, IgM and IgA to levels similar to those seen in the unstimulated cultures. Statistical analysis was not performed due to the low number of independent experimental repeats (n=3 or n=2, respectively).

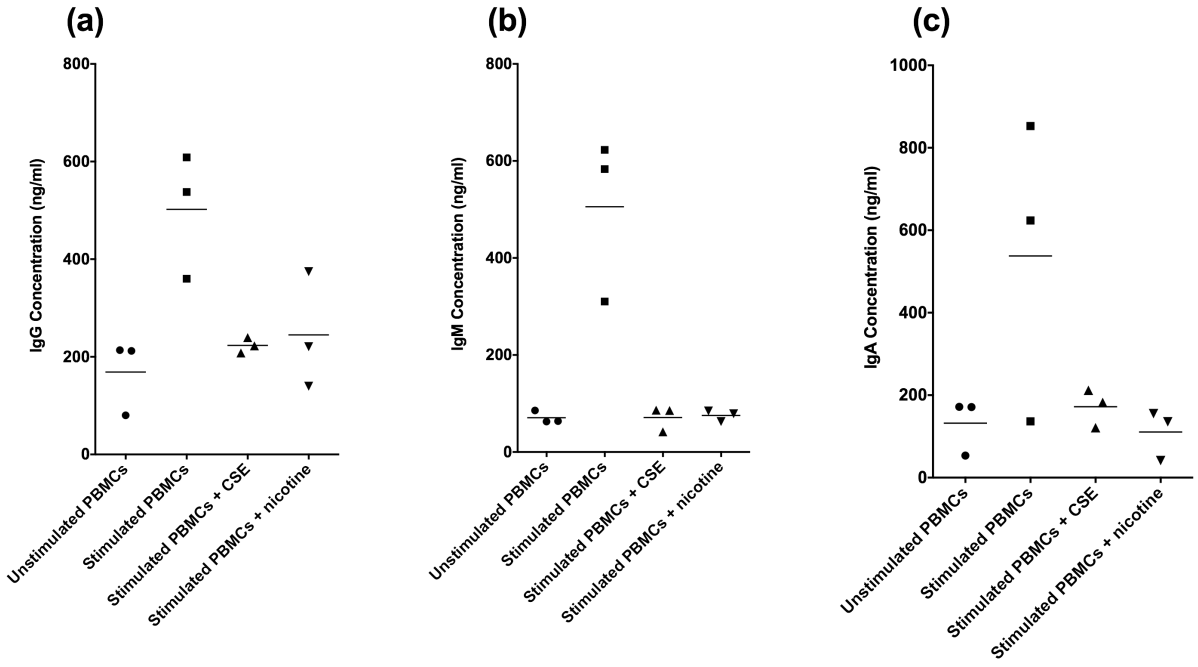


Fig. S1. Stimulated human PBMCs were incubated for 8 days in the presence of anti-CD40, IL-4, PWM, 3% CSE, or 75µg/ml nicotine. IgG, IgM, and IgA were detected in the supernatants by ELISA. a) IgG levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. b) IgM levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. c) IgA levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. Data from three independent donors is shown.

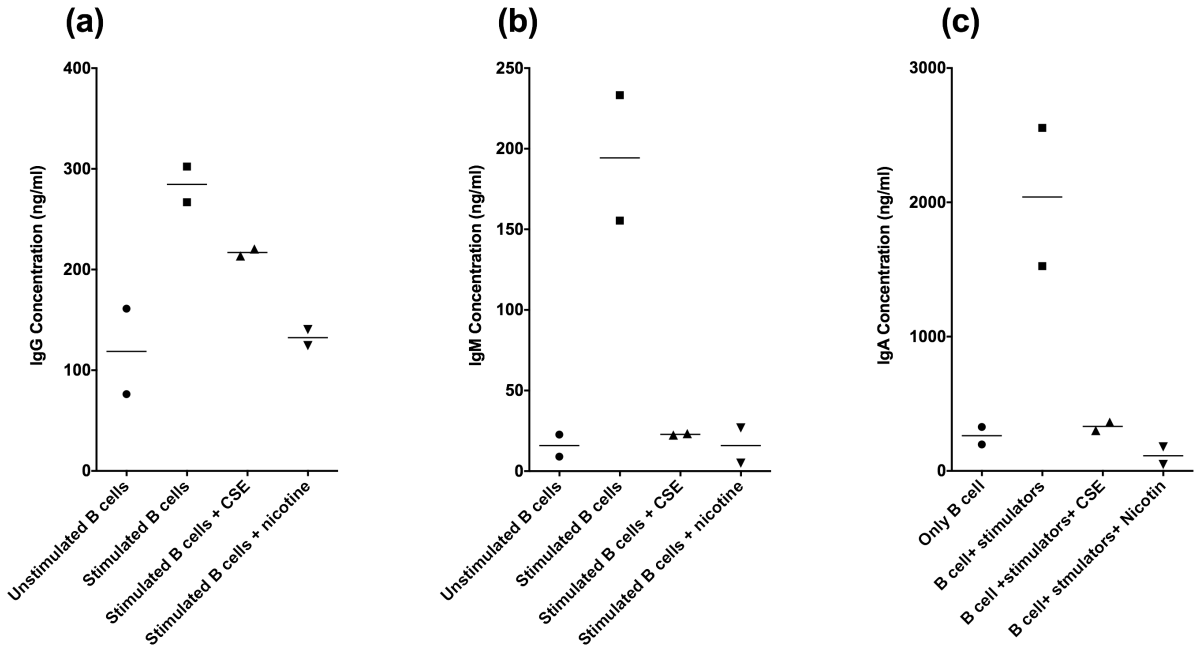


Fig. S2. Purified human B cells were incubated for 8 days in the presence of anti-CD40, IL-4, PWM, 3% CSE, or 75µg/ml nicotine. IgG, IgM, and IgA were detected in the supernatants by ELISA. a) IgG levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. b) IgM levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. c) IgA levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. Data from two independent donors is shown.

1. Alkhattabi N, Todd I, Negm O, Tighe PJ, Fairclough LC. Tobacco smoke and nicotine suppress expression of activating signaling molecules in human dendritic cells. Toxicol Lett. 2018;299:40-6.