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Altered antigenic profiling and infectivity of *Porphyromonas gingivalis* in smokers and non-smokers with periodontitis

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Abstract

Background—Cigarette smokers are more susceptible to periodontal diseases and are more likely to be infected with *Porphyromonas gingivalis* than non-smokers. Furthermore, smoking is known to alter the expression of *P. gingivalis* surface components and to compromise IgG generation. The aim of this study was to evaluate whether IgG response to *P. gingivalis* is suppressed in smokers in vivo and whether previously established in vitro tobacco-induced phenotypic *P. gingivalis* changes would be reflected in vivo.

Methods—We examined the humoral response to several *P. gingivalis* strains as well as specific tobacco-regulated outer membrane proteins (FimA and RagB) by ELISA in biochemically-validated (salivary cotinine) smokers and non-smokers with chronic (CP, $n = 13$) or aggressive (AP, $n = 20$) periodontitis. We also monitored the local and systemic presence of *P. gingivalis* DNA by PCR.

Results—Smoking was associated with decreased total IgG responses against clinical (10512, 5607, and 10208C; all $p < 0.05$) but not laboratory (ATCC 33277, W83) *P. gingivalis* strains. Smoking did not influence IgG produced against specific cell surface proteins, although a non-significant pattern towards increased total FimA-specific IgG in CP subjects, but not AP subjects, was observed. Seropositive smokers were more likely to be infected orally and systemically with *P. gingivalis* ($p < 0.001$), as determined by 16S RNA analysis.

Conclusions—Smoking alters the humoral response against *P. gingivalis*, strengthening the evidence that mechanisms of periodontal disease progression in smokers may differ from non-smokers with the same disease classification.

Keywords

Antigen-Antibody reactions; periodontitis; Porphyromonas gingivalis; smoking; vaccines

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Smoking alters the humoral response against *P. gingivalis*, strengthening the evidence that mechanisms of periodontal disease progression in smokers may differ from non-smokers with the same disease classification.

Introduction

Cigarette use increases susceptibility to infection with multiple bacteria, including the anaerobic, assacharolyic, Gram negative *Porphyromonas gingivalis* and the development of more severe and recalcitrant periodontal diseases [1-2]. Tobacco smoking has also been shown to lead to a generalized suppression of the antibody response to pathogenic bacteria [2].

P. gingivalis is clearly antigenic in humans [3-4], with differences antibody titers detectable, e.g., upon treatment or in smokers, even in small numbers of subjects [4-6]. Indeed, of all tested oral bacterial species, only *P. gingivalis*-specific antibodies have been associated with disease severity [7-8]. In vitro, cigarette smoke extract (CSE) represents an environmental stressor to *P. gingivalis* [9]. Phenotypically, CSE exposure leads to loss of capsule and increased major fimbrial protein (FimA) but not minor fimbrial antigen (Mfa1) expression [10]; augmented expression of the outer membrane virulence factors, RagA and RagB [9]; promotion of biofilm development; and a suppressed innate response against *P. gingivalis* [9-11].

We hypothesized that tobacco smoking would lead to a suppression in the overall IgG response to *P. gingivalis* and that previously reported CSE-induced phenotypic *P. gingivalis* changes [9-10] would be reflected in vivo. Therefore, we aimed to examine the humoral response to specific tobacco-regulated outer membrane proteins and whole *P. gingivalis* in biochemically validated human smokers and non-smokers with chronic or aggressive periodontitis and to check for local and systemic *P. gingivalis* DNA. As large variations in antibody titers against different strains of *P. gingivalis* are known to occur [12], several isolates were tested. *P. gingivalis* ATCC 33277, the type strain, and W83 represent the workhorses of molecular oral biology. 10208C, 10512 and 5607 are low passage clinical strains.

Materials and Methods

Study Population

The study was conducted in full accordance with the World Medical Association's Declaration of Helsinki of 1975, as revised in 2000, and was approved by the Institutional Review Board of the University of Louisville and Ege University. Forty-two informed and consenting individuals who sought dental treatment at Ege University were recruited between September 2011 and August 2012; 24 otherwise healthy, according to medical history recorded on recruitment, untreated patients with aggressive periodontitis (AP) and 18 otherwise healthy, untreated individuals with chronic periodontitis (CP). The higher number of AP cases reflects the patient referrals to Ege University. Medical and dental histories, including smoking histories were obtained. Those with antibiotic or periodontal treatment in the last 6 months were excluded. AP and CP was diagnosed using World Workshop in Periodontitis criteria [13] and current smokers (≥10 cigarettes/day; >5 years) and non-smokers included. Inclusion criteria for the AP group was presence of at least six permanent teeth, including incisors and/or first molars, with at least one site with PD and CAL ≥5 mm and six teeth other than first molars and incisors with similar PD and CAL measurements, familial aggregation (all individuals were asked if they had any family member with current or

history of severe periodontal disease) and radiographic bone loss of ≥ 30% of root length affecting ≥ 3 permanent teeth other than first molars and incisors. All AP and CP patients were categorized as "generalized." Those smoking for <5 years and or <10 cigarettes/day for >5 years were excluded.

Saliva and Serum Sampling

Whole, unstimulated saliva samples were obtained by expectoration into polypropylene tubes prior to any clinical measurement or periodontal intervention in the morning following an overnight fast during which subjects were requested not to drink (except water) or chew gum, essentially as reported by Navazesh[14]. The saliva samples were clarified by centrifugation (800 × *g*) for 10 min at room temperature and 500 μL amounts were placed in polypropylene tubes and immediately lyophilized. Venous blood (5 ml) was taken from the antecubital vein by standard venipuncture. Serum was separated by centrifugation at 1500 × *g* for 10 min and immediately frozen at −40°C. Samples were shipped to the University of Louisville for biochemical analysis.

Clinical Periodontal Measurements

Subsequent to saliva and serum sampling, clinical periodontal recordings were performed at 6 sites on each tooth present, except the third molars: plaque index (PI, [0-4]), probing depth (PD, mm), clinical attachment level (CAL, mm) and bleeding on probing (BOP, +/−), as previously described [15].

Quantification of salivary cotinine

Salivary cotinine levels were measured by immunoassay kit*, according to the manufacturer's instructions. Aliquots of frozen saliva samples were thawed, vortexed and centrifuged at 1500 × *g* for 15 minutes to pellet precipitated mucins. Samples from self-reported smokers were diluted tenfold. Self-reported non-smokers were not diluted. Cotinine standards (0.8 ng ml^{-1} to 200 ng ml^{-1}), high and low cotinine controls, and samples were tested in triplicates. Plates were measured using a microplate reader†. The reported sensitivity of the assay is 0.15 ng ml^{-1} and a pre-determined cut-off point of ≥15 ng ml^{-1} was selected to classify active smokers [16].

Growth of *P. gingivalis*

P. gingivalis strains W83, 33277, 10208C, 10512 and 5607‡ were grown to mid-to-late exponential phase (OD_{600} = 1.0) in Gifu Anaerobe Medium (GAM)§ under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37°C in an anaerobic chamber k. Purity was determined by Gram staining.

*High sensitivity cotinine immunoassays were obtained from Salimetrics, State College, PA.
†Emax Precision Microplate Reader, Molecular Devices, Sunnyvale, CA.
‡ATCC, Manassas, VA (*P. gingivalis* W83 and 33277). Laboratory collection (10208C, 10512 and 5607).
§Nissui Pharmaceutical, Tokyo, Japan.
kCoy Laboratories, Grass Lake, MI.

Preparation of *P. gingivalis* antigens

P. gingivalis cells were harvested by centrifugation (1900 × ***g***, 30 min, 4°C) and washed 3x with sterile PBS. Pellets at an OD_{600} of 1.0 in sterile water were ultrasonically disrupted[¶]. Protein concentrations in debris-free sonicates were determined by a bicinchoninic acid (BCA) protein assay [§], according to the manufacturer's instructions. rFimA and rMfa1 were expressed in and purified from *E. coli*, as we have described previously [10]. rRagB was also expressed in and purified from *E. coli* TOP10 using a plasmid system[#] and W83 DNA as a template with *ragB* amplified using the following primers[**]; Fwd: *CACCGAGCTTGACCGCGACCCC* and Rev: *TATCGGCCAGTTCTTTATTAACTGCGG*.

Quantification of antibody response to *P. gingivalis* antigens

Sonicates (10 μg/ml) and recombinant proteins (2.5 μg/ml) were used (100 μl) to coat flat-bottomed 96-well microplates overnight at 4°C which were washed 5x with PBS containing 0.05% Tween-20 (PBS-T), blocked with 200 μl of 1x diluent for 1 h, RT and washed again 5x with PBS-T. 100 μl samples diluted in 1x diluent (1:250 for total IgG Fc; 1:100 for IgG subclasses and IgA1 assays, respectively) were added to each well for 2 h at RT. Uncoated wells served as controls. Plates were washed 10 x with PBS-T, and incubated with 100 μl of biotinconjugated detection antibodies[‡‡] diluted in 1x ELISA diluent (1:5000 for total IgG; 1:1000 for IgG subclasses; and 1:2500 for IgA_1, respectively) for 1 h at RT and washed 10x with PBS-T. 100 μl Avidin-HRP (1:1000 in 1x diluent) was added to each well, 30 min, RT and the plates washed 10x with PBS-T. 100 μl TMB substrate was added and development stopped within 10 mins with 50μl of 2N H_2SO_4. Absorbances were read at 450 nm using a microplate reader[†]. Antibody titers are presented as relative absorbance at O.D. 450 nm.

Detection of local and systemic *P. gingivalis*

The presence or absence of *P. gingivalis* DNA was established in saliva and plasma samples, essentially as described previously by Matto et al [17] using primers first reported by Slots et al [18]. However, samples were centrifuged at 13,000 × *g*, 6 min, alternate nucleic acids[#] were employed, and there were 40 amplification cycles. Additionally, 10 ng *P. gingivalis* W83 DNA served as a positive control. 10ng *A. actinomycetemcomitans* DNA and water each served as negative controls. Amplicons were separated in 2% agarose/ ethidium bromide gels and documented using an imaging system[§§].

Statistical analyses

Similar sample sizes have previously been shown to be adequate to determine statistically significant differences in antibody titres between smokers and non-smokers [19-23]. Differences in cotinine levels between groups were determined by the Kruskal-Wallace test. Differences in *P. gingivalis* infection rates were determined using the Fischer's Exact Test.

[¶]Vibra-Cell Sonicator, Sonics and Materials, Danbury, CT.
[§]Thermo Scientific, Waltham, MA.
[#]pBAD202/D–TOPO and PCR Supermix, Invitrogen, Carlsbad, CA.
[**]Primers came from BioSynthesis, Lewisville, TX.
[‡‡]Sigma Aldrich, St. Louis, MO (anti-human IgG_{total}- and IgG_{1-4}-biotin, Fc-specific monoclonal antibodies [clones HP-6017, 8c/ 6-39, HP-6014, HP-6050 and HP-6025]). Abcam, Cambridge, MA (anti-human IgA_1-biotin antibodies [clone B3506B4]).
[§§]BioDoc-It, UVP, Upland, CA.

All remaining data were assessed by t-test or Mann-Whitney test, depending on distribution[KK].

Results

Smoking alters clinical parameters in subjects with chronic or aggressive periodontitis

Epidemiological and disease parameters of all subjects whose smoking status was confirmed biochemically, who were seropositive in any assay for *P. gingivalis* by ELISA and with uncompromised samples and, thus, included in the final data analyses (AP, $n = 20$; CP, $n = 13$), are presented in *Table 1*. Four subjects were excluded from the AP group and 5 from the CP group (smoking status: n = 1 and 1; compromised samples: n = 3 and 4, respectively). As expected, BOP was suppressed in CP smokers relative to CP non-smokers ($p < 0.05$). CAL was more severe in the subjects with AP who smoked, compared to those who did not ($p < 0.05$). Otherwise, there were no significant differences in clinical parameters between smokers and non-smokers within periodontal disease groups. As shown in *Figure 1*, salivary cotinine levels, indicating smoke intake, were elevated in the smokers of both disease groups compared to non-smokers (both $p < 0.001$).

Smoking suppresses the systemic IgG response to *P. gingivalis*

Total IgG titers against two laboratory strains and three recent clinical isolates of *P. gingivalis*, measured in the serum of individuals with CP or AP are presented in *Figure 2*. Smoking was associated with decreased total IgG production against low passage clinical isolates (10512, 5607, and 10208C) and all strains combined in subjects with CP (*Figure 2*), but not in the commonly employed laboratory strains (ATCC 33277 and W83). There were no statistically significant differences in the relative IgG_1, IgG_2, IgG_3, IgG_4 and IgA_1 antibody titers against W83 or the five combined strains measured in the serum of individuals with CP or AP (*data not shown*), with the mean relative antibody titer always higher in non-smokers than in smokers.

Smoking does not influence the systemic antibody response to specific outer membrane proteins (FimA, Mfa1 and RagB)

While smoking status exerted important effects on the overall IgG response to *P. gingivalis* cells, smoking did not induce significant differences in antibody production against three major outer membrane proteins of *P. gingivalis* - FimA and RagB, which are upregulated by CSE in vitro, and Mfa1, which is unaffected [9-11] (*Figure 3*). Nevertheless, in the CP subjects, the non-significant trend was towards an increased FimA response in smokers ($p > 0.05$), in contrast to all other comparisons.

Local and systemic *P. gingivalis* DNA detection

P. gingivalis-specific 16S DNA was detected in most CP saliva samples; 6/7 (86%) and 5/6 (83%) for smokers and non-smokers, alike ($p > 0.05$). In AP subjects, saliva samples from smokers (5/7, 86%) were more likely to contain *P. gingivalis* DNA, than non-smoking AP

[KK]InStat v3.06, Graphpad Software Inc., La Jolla, CA.

subjects (7/13, 54%, $p < 0.001$) while CP smokers (1/7, 14%) where more likely than non-smoking CP (0/6, 0%) patients to have serum detectable *P. gingivalis* DNA ($p < 0.001$). For AP smokers and non-smokers, systemic infection rates were 1/7 (14%) and 3/13 (23%), respectively ($p > 0.05$).

Discussion

Smoking is known to alter antibody production [2, 24], but few studies have addressed the influence of smoking on oral pathogens specifically. The great majority [5-6, 22, 25-26], but not all [7], have shown that smoking reduces the antibody response to *P. gingivalis*, particularly IgG. We show that the total IgG response mounted against low passage clinical isolates (5607, 10208C and 1052), but not laboratory strains, of *P. gingivalis* (33277 and W83) is significantly suppressed in smokers with CP. Thus, while clearly facilitating impressive molecular microbiology advances, research strains of *P. gingivalis* may not always be the most relevant to in vivo situations. There were no statistically significant differences in the intensity of the humoral response to *P. gingivalis* between current smokers and non-smoking subjects with AP, in keeping with the findings of Hayman *et al*, who have recently reported that correlations between pathogen and commensal antibody ratios in individuals with periodontitis disappear in those with aggressive disease [7]. This may be partly explained by our finding that current smokers with AP are more likely to be currently infected with *P. gingivalis* than non-smokers with AP.

The mechanisms of a generalized suppression of the IgG response in human smokers are not clear. Cigarette smoke inhibits T cell-dependent and -independent antibody responses to a number of antigens in animals [27-28]. Furthermore tobacco-induced suppression of the normal inflammatory response could impair leukocyte infiltration into the gingival tissues, negatively influencing the presentation of *P. gingivalis* antigens. While nicotine-induced periodontal vasoconstriction is often cited, the evidence for this in humans is unprofound [2, 29]. Rather, tobacco inhibits plaque-induced angiogenesis [2, 30] in a manner reversible upon cessation [31]. Indeed, the key marker of periodontal inflammation, BOP, was significantly lower in smoking CP subjects compared to non-smokers. Thus, angiogenic suppression could also contribute to a reduced humoral response to periodontal pathogens.

Antibody responses to *P. gingivalis*, particularly IgG_2, may vary significantly by race and type of periodontal disease [7, 25-26, 32]. All recruits herein were Caucasian and no tobacco-related differences in IgG subtype titers could be ascribed. It is unfortunate that 21% (9/42) of the recruits were excluded from the final analyses. However, smokers often provide false statements on their smoking status, therefore, biochemical detection of cotinine levels is specifically employed to avoid such false reports and to calculate more objectively the real smoking status of the patient [16]. Furthermore, while *P. gingivalis* is clearly a key a key pathogen, not all subjects with periodontitis harbor this particular species [33], thus not all seropositive individuals were positive for oral *P. gingivalis* DNA.

We had hypothesized that CSE-upregulation of RagB and FimA would be reflected in an increased antibody titer to these surface antigens in smokers. However, this was not validated and the suppressive overall influence of tobacco use on antibody production may

have been sufficiently strong to mask any increased antigen specific antibody production in current smokers. Additionally, those who ceased smoking close to the study onset may have passed our cotinine screen. Indeed, ex-smokers are the most likely to be deceitful by claiming to have never smoked [16]. Furthermore, it is possible that expression of *P. gingivalis* proteins in *E. coli* may have led to alternate post-translational processing that rendered them less efficient antigens. It is interesting, however, that the sole exception to the trend of suppressed antibody production in smokers was total IgG against FimA in CP smokers, which was increased relative to non-smokers. *P. gingivalis* fimbriae are the bacterial structures first to engage the host. FimA promotes colonization through strong interactions with several host proteins, including collagen and fibronectin, oral epithelia and other plaque bacteria, such as *Streptococcus spp.* [34-37]. More importantly, FimA induces TLR2-specific innate tolerance in an IκBα- and IRAK-1-dependent manner [10] with reduced antibody production one possible downstream consequence of FimA-mediated immune suppression.

Fascinatingly, saliva and serum from smokers were more likely than non-smokers to contain *P. gingivalis* 16S RNA gene sequences, presumably reflecting current or recent oral infection with this bacterium. Indeed, this report is the first to correlate antibody responses specific to variant *P. gingivalis* strains and antigens with local and systemic infection in biochemically validated subjects with two types of destructive periodontal disease. However, due to the relatively low number of recruits, these data require validation. Potential variation in the sensitivity of the immunoglobulin assays and uneven distribution of participants also limit the study.

In summary, smoking suppresses total IgG production to *P. gingivalis* in smokers with CP compared to those who are not current smokers. This association is true for clinical isolates only and not the workhorse strains, ATCC 33277 and W83. All antibody assessments performed herein, and the literature in general, show depressed IgG antibody production in smokers. An exception appears to be a non-significant trend towards an increase in IgG specific for the major fimbrial antigen, FimA, in CP smokers. This may be reflective of the significantly increased FimA expression known to occur in vitro upon exposure to CSE. IgG is considered particularly important in clearing plaque bacteria. Tobacco-induced suppression of IgG production in response to *P. gingivalis* infection may promote *P. gingivalis* survival, potentially contributing to increased susceptibility to CP. Furthermore, current oral and systemic infection with *P. gingivalis* may be more likely in smokers than in non-smokers, helping to explain the differential antibody responses in these two disease classifications and shedding light on systemic sequelae of periodontal diseases that share smoking as a major risk factor.

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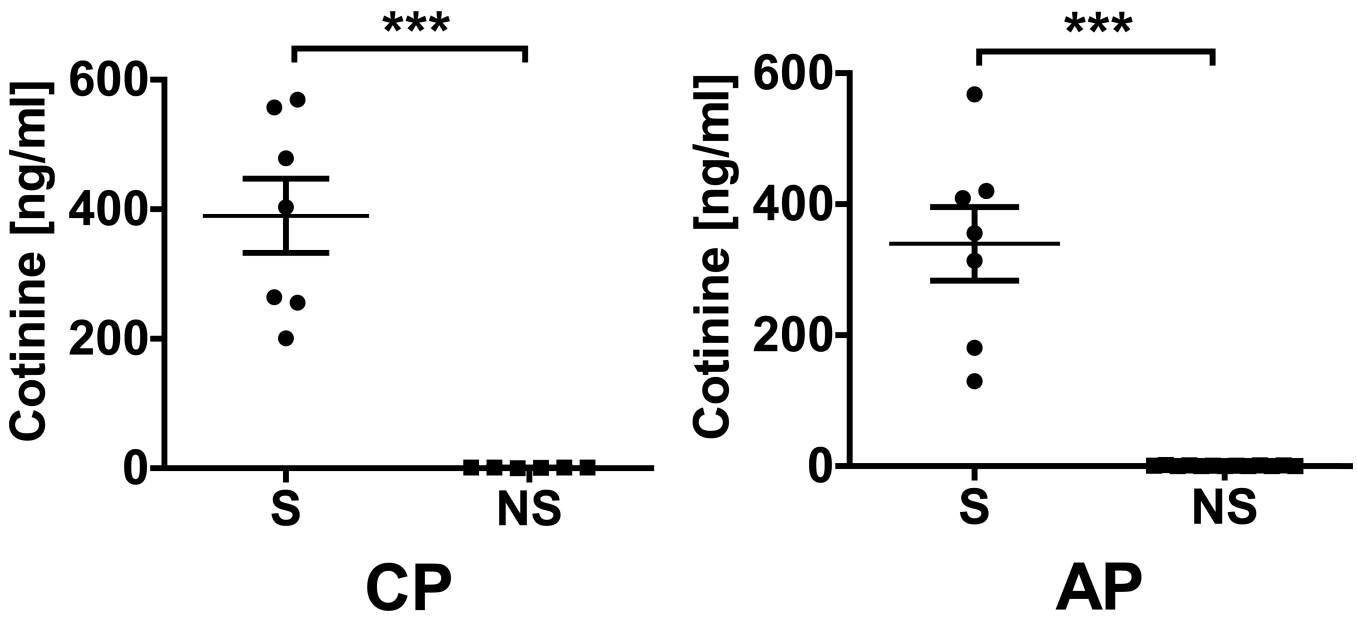
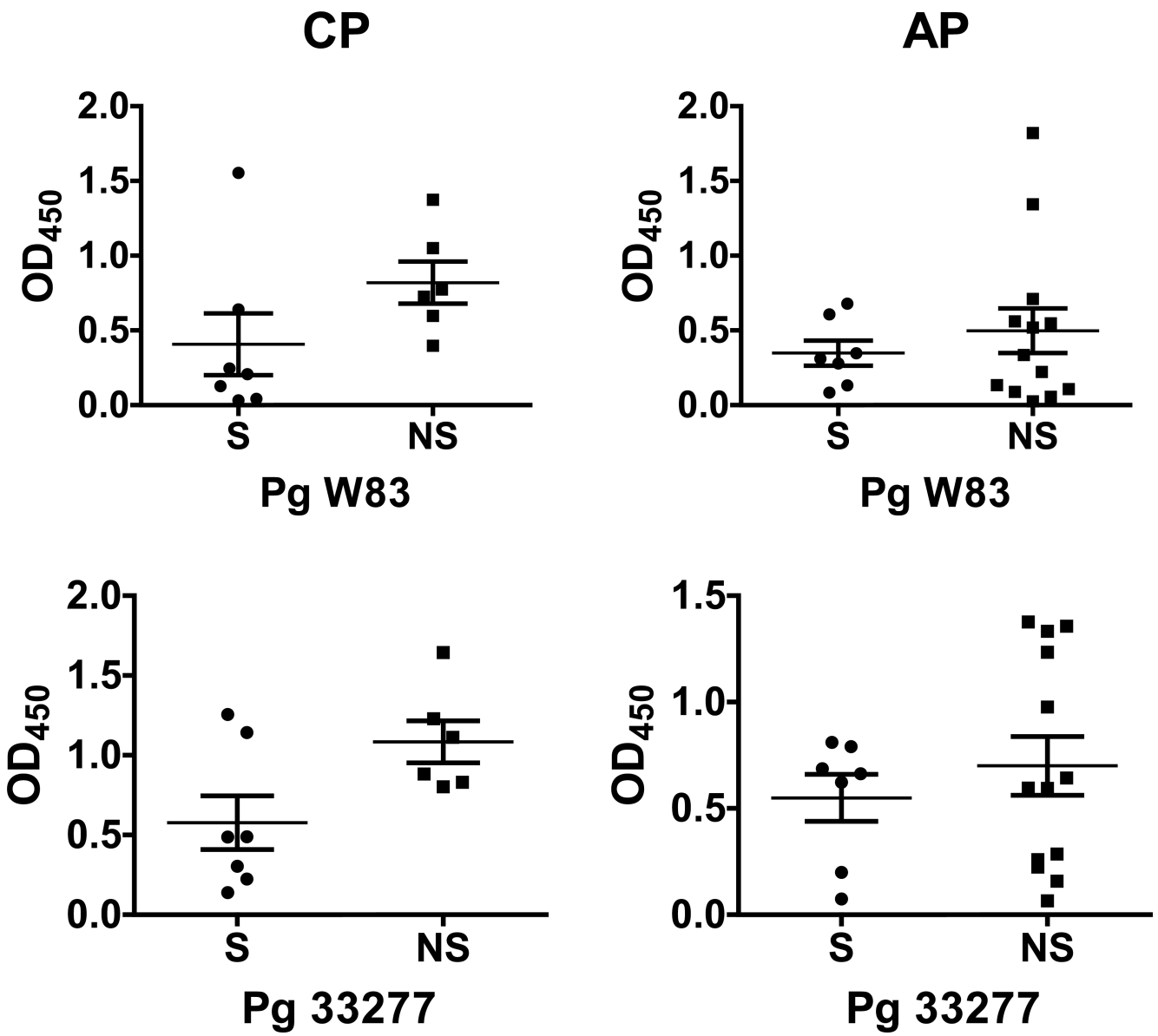


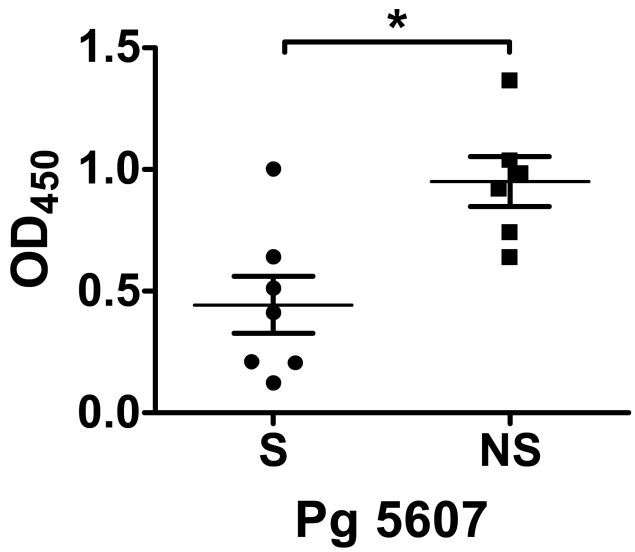
Figure 1.

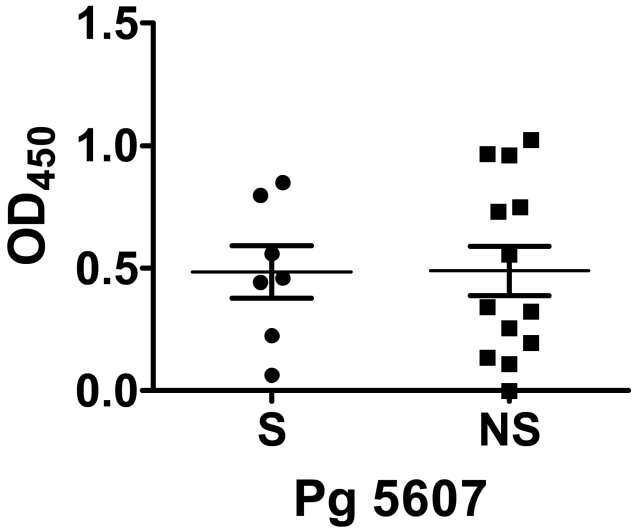
Salivary cotinine concentrations (mean ± S.D.) in validated current smokers and current non-smokers with chronic periodontitis and aggressive periodontitis.

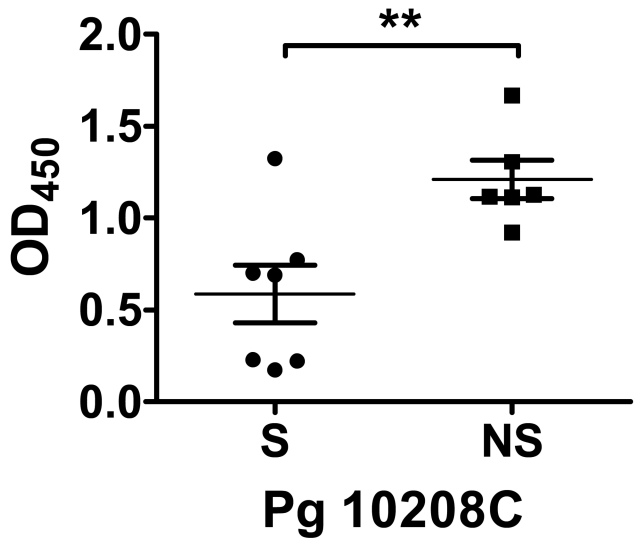
Non-detectable cotinine concentrations were entered as 0.0 ng ml^{-1}.

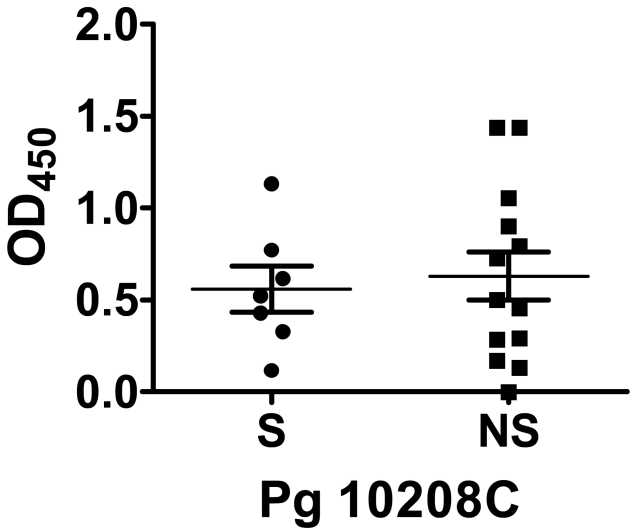
*** $p < 0.001$.











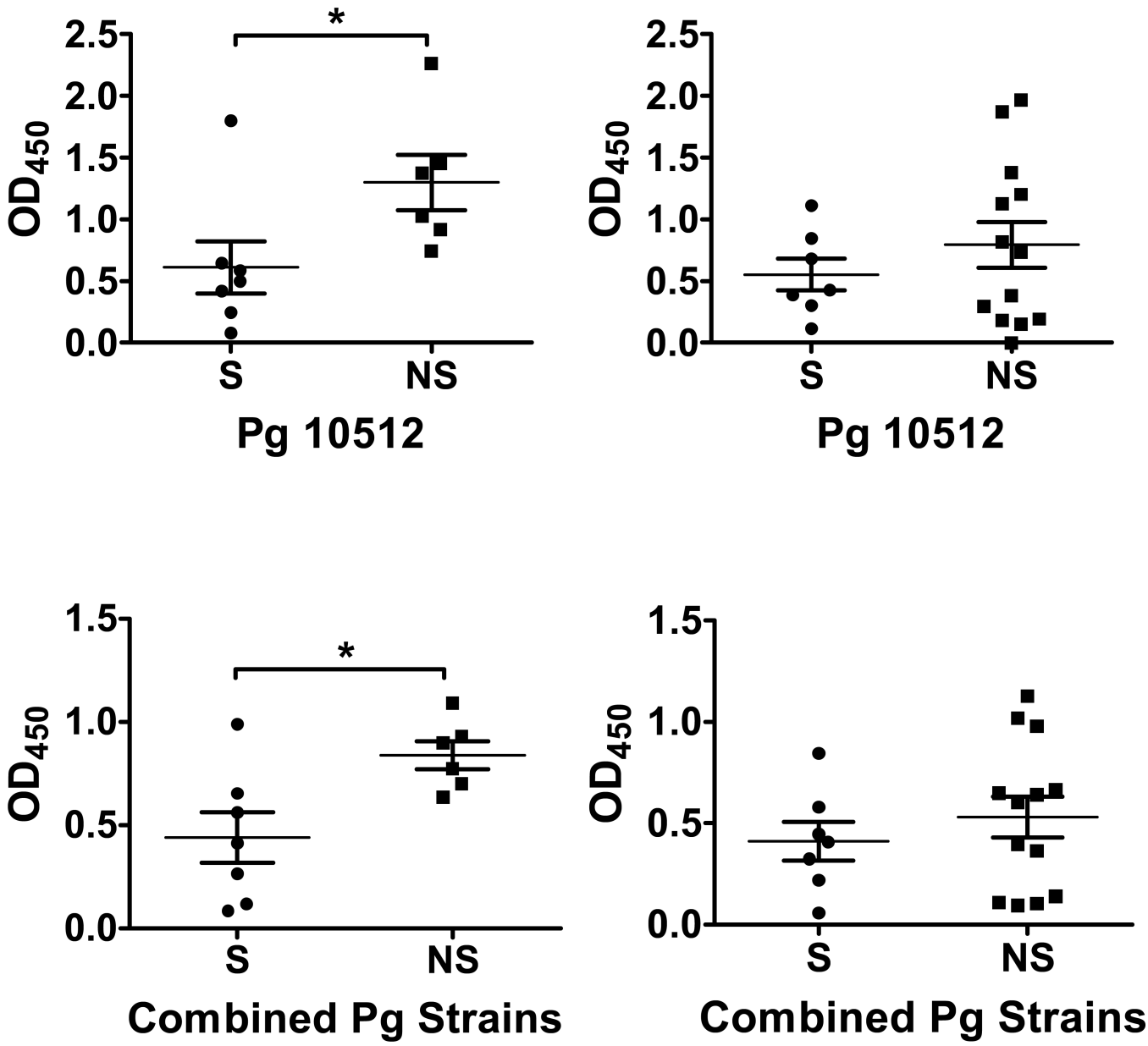
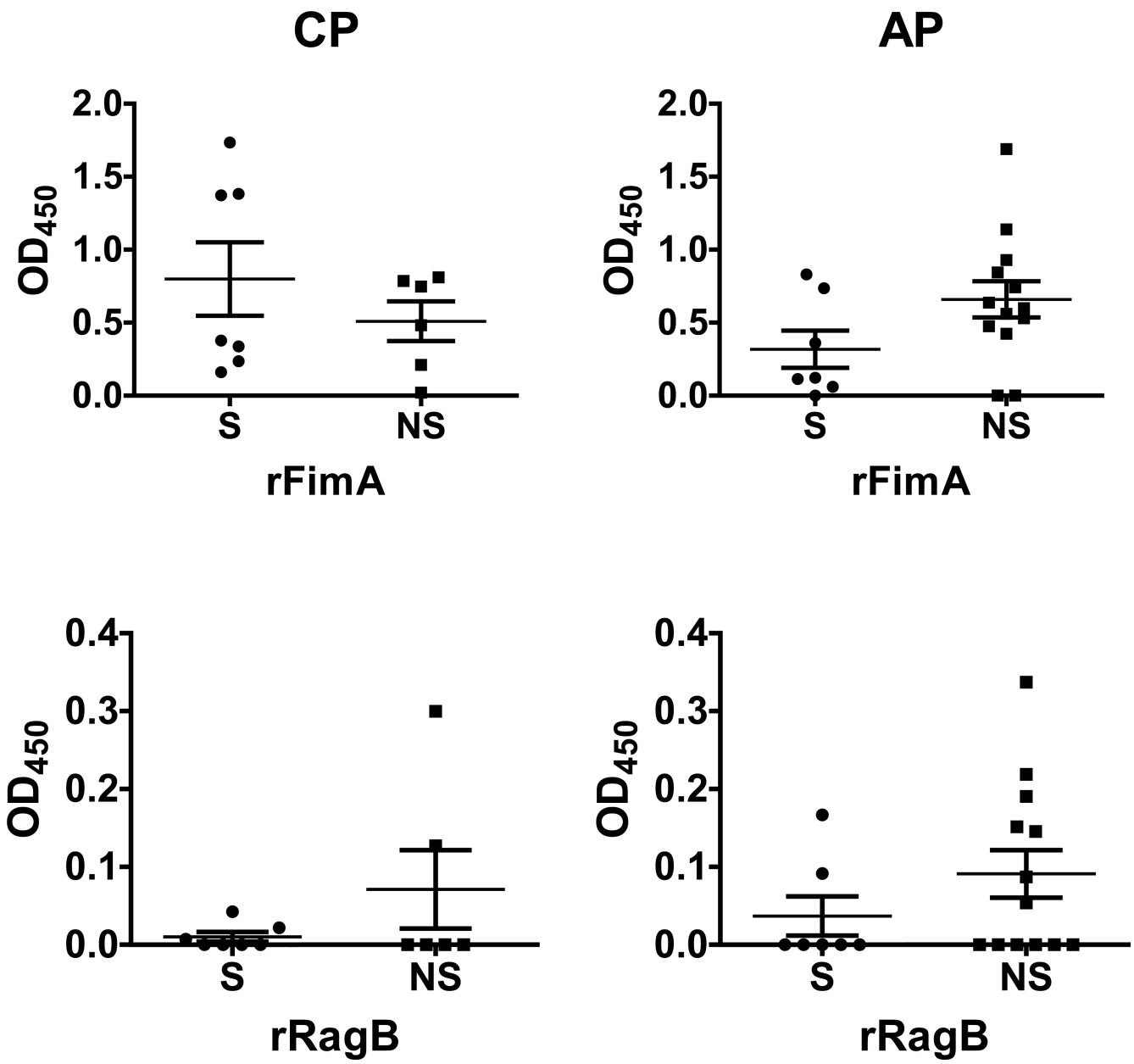


Figure 2.

Total IgG titers (mean ± S.D.) against laboratory and clinical strains and *P. gingivalis* strains in smokers and current non-smokers with chronic periodontitis and aggressive periodontitis. * $p < 0.05$; ** $p < 0.01$.



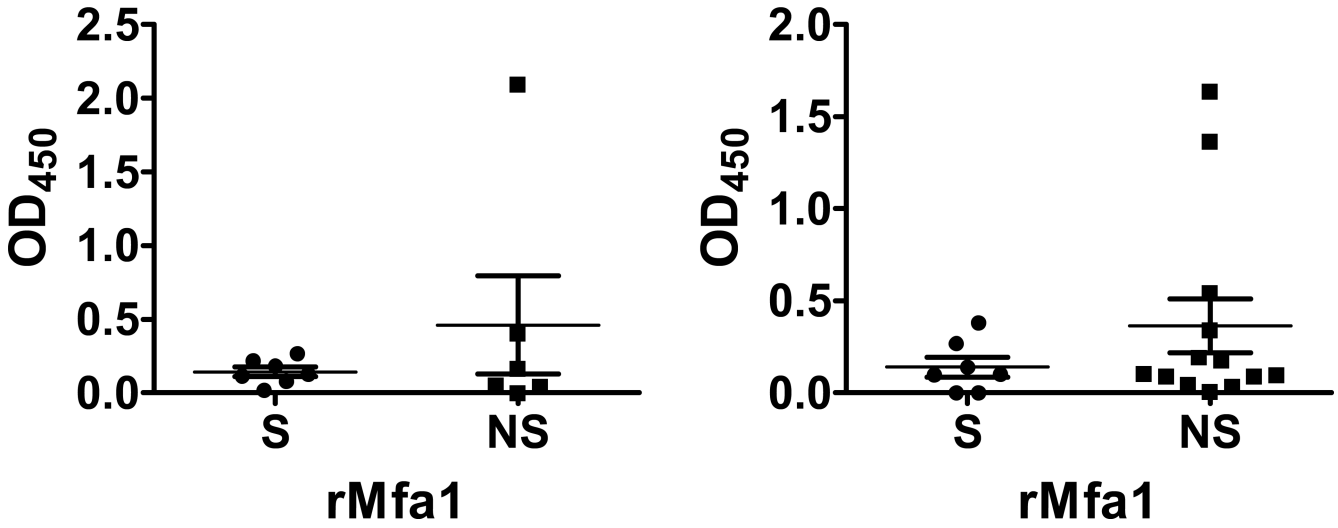


Figure 3.

Total IgG titers (mean ± S.D.) against *P. gingivalis* surface proteins in smokers and current non-smokers with chronic periodontitis and aggressive periodontitis.

Table 1

Clinical data of current smokers and current non-smokers with aggressive or chronic periodontitis.

	CP, S (n = 7)	CP, NS (n = 6)	AP, S (n = 7)	AP, NS (n = 13)
Age	51.9 (5.5)	48.7 (6.3)	35.0 (3.1)	38.1 (4.2)
PD (mm)	4.9 (1.0)	5.5 (1.0)	5.4 (0.9)	4.5 (1.0)
CAL (mm)	6.0 (0.6)	6.2 (1.4)	**6.1 (1.2)***	4.9 (1.1)*
Plaque Score (0-4)	2.7 (0.5)	2.8 (0.4)	2.0 (0.6)	1.8 (0.7)
BOP (%)	**74 (20)***	92 (12)*	83 (13)	80 (13)

Data represents the mean (s.d.)

CP, chronic periodontitis; AP, aggressive periodontitis; S, smoker; NS, non-smoker.

* $p < 0.05$, smokers vs non-smokers.