The prevalence of BANA-hydrolyzing periodontopathic bacteria in smokers


Abstract. Smoking has been identified as a risk factor for development of periodontal disease and a strong indicator for treatment failure in periodontal patients. This study examined 172 patients categorized as current smokers (n=55), previous smokers (n=38) or individuals that had never smoked (n=79). A total of 670 interproximal plaques collected with a wooden toothpick were analyzed for hydrolysis of the synthetic trypsin substrate benzoyl-DL-arginine naphthylamide (BANA). About 95% of the BANA hydrolysis by plaque is due to the presence of one or more of the periodontopathogens, P. gingivalis, T. denticola or B. forsythus. Gingival health was measured using the papillary bleeding score (PBS). Current smokers had less gingival bleeding than previous smokers or those who had never smoked (20% versus 41% and 25%, respectively). Plaque removed from non-bleeding sites in current smokers were 11× more likely to have a positive BANA reaction when compared to plaque removed from non-bleeding sites in individuals who never smoked. A significant positive relationship exists between smoking and colonization by the BANA periodontopathogens. Smoking may select for these periodontopathic species in the plaque and may be one reason why smoking is a risk factor in periodontal disease development.

Key words: BANA; smoking; T. denticola; B. forsythus; P. gingivalis; tobacco; periodontal pathogens

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Many studies have shown that smokers have higher levels of periodontal disease than non-smokers of the same age and gender (Feldman et al. 1983, Ismail et al. 1983, Jette et al. 1993, Grossi et al. 1994, Bergstrom & Preber 1994, Haber et al. 1993), indicating that smoking may be a risk factor for periodontal disease. Other studies indicate that there are specific bacterial risk indicators associated with periodontal disease (Grossi et al. 1994, Loesche 1990, Loesche 1987, Moore & Moore 1994, Haffajee & Socransky 1994). Several investigators have sought to establish a link between smoking and the overgrowth in the plaque of several of these putative periodontopathic organisms, but the results have been equivocal. Stoltenberg et al. were unable, using polyclonal antibodies to Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Prevotella intermedia, Fusobacterium nucleatum and Eikenella corrodens, to demonstrate an increased prevalence of these species in the plaques of smokers compared to non-smokers (Stoltenberg et al. 1993). Similarly, Preber et al. (1992) using DNA probes to A. actinomycetemcomitans, P. gingivalis and P. intermedia, were unable to find differences in plaque colonization by these bacteria in smokers versus non-smokers.

This inability to associate putative periodontal pathogens with smoking may be due to the exclusion of two important putative periodontal pathogens, Bacteroides forsythus and Treponema denticola, from the analyses. These organisms have been associated with advanced forms of periodontal disease using cultural methods, immunological reagents, and DNA probes (Loesche et al. 1992, Simonson et al. 1992, Socransky et al. 1998, Ashimoto 1996). In a large cross-sectional survey of over 1300 adults, smoking, increasing age, and the presence of B. forsythus and P. gingivalis were suggested to be risk indicators for attachment and bone loss in periodontal disease (Grossi et al. 1994, Grossi et al. 1995). In these subjects, Zambon et al. (1996), using immunological reagents, identified Bacteroides forsythus, and possibly P. gingivalis as being elevated in smokers, and concluded that cigarette smoking increased the likelihood of subgingival colonization with these periodontal pathogens.

P. gingivalis, B. forsythus and T. denticola are the plaque species most frequently associated with periodontal disease (Loesche et al. 1992, Simonson et al. 1992, Socransky et al. 1998, Ashimoto 1996, Grossi et al. 1995, Haffajee et al. 1998). Each possesses an enzyme which hydrolyzes the synthetic peptide,
benzoyl-DL-arginine naphthylamide (BANA), whereas 59 other cultivable plaque species do not, and 4 others are weakly or variably BANA positive (Loesche et al. 1990). The detection of *P. gingivalis*, *B. forsythus* and *T. denticola* in plaque samples using DNA probes and/or immunological reagents, is highly correlated with the hydrolysis of BANA by plaque samples (Loesche et al. 1992). In this study, we sought to monitor for the presence of one or more of these bacteria in the plaque of smokers and non-smokers, taking advantage of the fact that BANA hydrolysis by plaque bacteria can be measured by a simple and rapid chairside test (Loesche et al. 1990).

**Material and Methods**

Subjects were examined in the Patient Admitting and Emergency Services Clinic at the University of Michigan School of Dentistry. There is a short turnover time for patients in this clinic as they are usually referred to other clinics, given emergency treatment and dismissed, or directly assigned to student or faculty care providers. The advantage of this clinic, for the purposes of our study, was the high number of patients that could be seen in a day, and their similarity to the type of patient seeking treatment in a private practice situation. After signing an informed consent, patients were asked to complete a short questionnaire that surveyed age (date of birth), gender, and smoking status (current smoker, previous smoker or never smoked).

We limited our clinical contact with the patient to about two to three minutes so as to mimic the amount of chair-time that a clinician might devote to the collection of additional data relative to the periodontal health of his/her patients. This decision precluded any detailed periodontal examination of the patients. Since periodontal disease frequently begins subgingivally at the interdental papillae, periodontal health was determined at interproximal sites using the Papillary Bleeding Score (PBS) (Loesche 1979). The PBS has been shown to be the most reliable of several gingival bleeding indices that have been described (Marks et al. 1993). Proximal plaque was sampled from between the first molar and second premolar in each quadrant using a wooden toothpick (Stim-u-dent®, Johnson and Johnson Windsor, NJ). This site was chosen as first molars are among the teeth most likely to show periodontal involvement (Lee et al. 1978). If one site was absent, an adjacent interproximal area in the same quadrant was sampled. 2-4 samples from each of 172 patients were collected, yielding a total of 670 plaque samples.

The PBS is a 6 level scale with a score of 0 reflecting health, 1 indicating some degree of inflammation in the absence of bleeding, 2 indicating spotting of blood in the papilla, and PBS from 3 to 5 corresponding to various degrees of bleeding. The same investigator (CK) collected all plaque samples and judged each PBS.

The same toothpick used to determine the PBS was used for the BANA test. A separate toothpick was used for each site. Thus after withdrawing the toothpick from the interproximal site, the adherent plaque on it was wiped from the toothpick onto the BANA-imregnated strip found at the lower edge of a four-sample BANA reagent card. An upper reagent strip containing Evan's black dye was then activated through dampening with distilled water and the two strips were folded to contact each other. After folding, the card was inserted into a heating block and incubated for 5 min at 35°C, a time and temperature protocol which gives the best balance of sensitivity and specificity for the BANA test when screening patients of unknown periodontal status (Loesche et al. 1997). Naphthylamide released due to the presence of any one of the BANA-hydrolyzing bacterial species diffused into the upper reagent strip where it reacted with the Evan's black dye to form a permanent blue-black color.

Scores were assigned based on the amount of blue color visible on the upper reagent strip after incubation. A score of 1 (negative) was given when no blue color was visible, a score of 2 (weak positive) when a faint blue color was noted and a score of 3 (positive) when distinct blue color was observed. For statistical analysis, scores of 2 and 3 were grouped as indicative of a positive response.

Statistical analyses consisted of contingency tables and regression modeling. The bivariate contingency table analyses were used to examine the distributions of PBS and BANA scores and to assess how smoking status was reflected in the sites' periodontal and microbiological status as measured by the PBS and the BANA test, respectively. Four comparisons of gingival health were used: PBS=0 versus PBS=1–5, which compared healthy papillae with any type of gingivitis in the papilla; PBS=0–1 versus PBS=2–5, which compared healthy and non-bleeding papillae with any type of bleeding papilla; PBS=0–2 versus PBS=3–5, which compared healthy papillae and mild gingivitis with papillae that bled with a flow; and, because of the prominence of the PBS=1 score, a 3 level stratification using PBS=0 versus PBS=1 versus PBS=2–5. We also tested the 6 category original specification of PBS.

A series of regression models were evaluated using each of the specifications of gingival health (the 5 specifications of PBS described previously), 2 specifications of smoking status, and age (as a continuous variable) to evaluate the outcome, positive BANA score. The 1st smoking status variable was a dichotomous specification contrasting sites in those who had "ever smoked" (combining sites in subjects who were current smokers and who had quit smoking) with those who had never smoked. The 2nd was a 3-category specification for those who had never smoked, were previous (but not current) smokers, and current smokers. Regression modelling first used ordinary logistic regression to estimate the odds ratio for sites having a positive BANA score and to determine the specifications of smoking status and gingival health that created the best fitting model (as determined by the likelihood ratio test). 2 additional, separate regression models were also used to test the association between smoking and the BANA test, excluding PBS as a covariate. One model was limited to sites that did not bleed, i.e., PBS of 0 or 1, and the other included only sites that bled, i.e., a BANA test score >1. By eliminating bleeding sites from the analysis, the model including only sites with PBS of 0 or 1 provided an alternative method to control for the influence of gingival bleeding selecting BANA positive plaque species. This restricted model controlled for any potential confounding by gingival bleeding on the association between smoking and positive BANA score. Finally, to control for non-independence of observations using sites and analytic units, the regression parameter estimates were also obtained using generalized estimating
The results showed significant associations were obtained with the PBS specifications of from the sites with either healthy or bleeding papillae. As shown in Table 1, the distribution of plaque BANA scores as a function of the smoking status of the subject is shown in Fig. 2. Only 18% (121/670) of the plaque samples were BANA-negative and the majority of these, i.e., 81% (98/121), were in subjects who never smoked. Even so, 68% of the plaques removed from the never or non-smokers were BANA-positive with over half (37%) giving a strong positive reaction (Fig. 2). In the quit smoking and current smoking subjects, 91 and 96% of the plaques were BANA-positive, with about 70% being strong positives (Fig. 2).

The BANA reactions were stratified by PBS score and smoking status as shown in Fig. 3. The % BANA-positive sites, (weak plus strong scores), significantly increased as both a function of the PBS and the smoking status (Cochran Mantel Haenzel statistic = 30.2, \( p < 0.001 \)). Almost all plaques removed from current smokers were BANA-positive, ranging from 75% of the plaques removed from healthy sites, to 94 to 98% of the plaques removed from sites where the PBS was 1 and 2–5 respectively (Fig. 3). Similar high values were seen in the quit smoking subjects. In the “never” smoked subjects, 55% of the sites with PBS = 0 were BANA-negative, whereas 69% of the sites with PBS = 1 and 81% of the sites with PBS = 2–5 were BANA-positive.

A series of multivariable regression models were performed to determine how smoking status, PBS categories, and age were associated with the BANA scores of the individual. The final GEE regression models evaluating how smoking status, PBS and age were associated with the BANA scores, are pre-
sent in Tables 2-4. Table 2 shows the model evaluating plaques from current smokers with those from individuals who had either never smoked or who had quit smoking, when the bleeding status of the papilla and the age of the subject were controlled. Plaques from individuals who had quit smoking were 4.1× more likely to be BANA-positive and plaques from current smokers were 10.1× more likely to be BANA-positive than plaques removed from sites in individuals who had never smoked. Higher values of PBS for a site were significantly associated with BANA positive sites, whereas the age of the subject had little effect in the BANA status of the plaque.

The BANA positive species could be selected for in the plaque by nutrients made available when there is gingival bleeding, which could account for some of the observed increases in BANA positive scores in the smokers. This possibility was addressed by performing a separate GEE regression analysis on sites in which there were no bleeding, PBS <1, and again on sites in which the PBS was >1. Plaques removed from sites without any papillary bleeding, in individuals who had quit smoking, were 4.26× more likely to have a positive BANA reaction compared to plaques removed from non-bleeding sites in individuals who never smoked. Plaque removed from non-bleeding sites in current smokers, however, were 11.19× more likely to have a positive BANA reaction compared to plaques removed from non-bleeding sites in individuals who never smoked (Table 3). When a GEE regression analysis was performed on sites which bleed, i.e., PBS >1, plaques removed from previous and current smokers were 12 times more likely to yield a positive BANA score than plaques removed from bleeding sites in non-smokers (Table 4).

Smokers have an increased risk of developing periodontitis (Feldman et al. 1983, Haber et al. 1993, Bergstrom & Preber 1994). Bleeding and gingivitis are early signs of periodontal pathology, but as seen in Fig. 1, smokers, especially current smokers, have a PBS profile similar to that of nonsmokers. Only former smokers tended to have a higher PBS than nonsmokers. The reduced prevalence of bleeding in smokers has been a consistent observation of others (Feldman et al. 1983, Bergstrom 1990, Preber & Bergstrom 1985, Bergstrom & Flederus-Myrhed 1983, Bergstrom et al. 1988), and has led to the conclusion "that the validity of gingival bleeding as a sign and a symptom of inflammatory periodontal disease may be reduced as a consequence of smoking" (Bergstrom & Flederus-Myrhed 1983). When the experimental gingivitis model was studied in smokers and non-smokers, the intensity of the vascular reactions in smokers was only 50% of that observed in nonsmokers, despite the fact that plaque accumulations were equal (Bergstrom et al. 1988).

The reason for the low bleeding tendency in smokers has been attributed to a nicotine effect, in that chronic low doses of nicotine can cause a sustained peripheral vasoconstriction and decreased peripheral blood supply (Bergstrom & Preber 1994, Haber et al. 1993, Harvey & Champe 1992, Loesche 1993). Due to this sustained peripheral vasoconstriction, smokers should have a decreased blood flow to the tissues of the periodontium which might manifest clinically as inflammation without bleeding. Thus the PBS system, or any other scoring system dependent upon bleeding as an index of gingival health, would be an insensitive method of evaluating gingival inflammation in individuals who smoke (Bergstrom & Flederus-Myrhed 1983). As such, some of the early warning signs of periodontal disease related to gingival bleeding are masked in smokers, so that periodontal disease may go unnoticed until clinical attachment loss, or radiographic bone loss, are detected (Feldman et al. 1983, Ismail et al. 1983, Jette et al. 1993, Grossi et al. 1994, Bergstrom & Preber 1994, Haber et al. 1993, Bergstrom 1990, Preber & Bergstrom 1985, Bergstrom & Flederus-Myrhed 1983).

This scenario would indicate that

The BANA test could provide a means by which the clinician could document the presence of bacterial risk factors in the plaques of smokers in the absence of gingival bleeding. Both the bivariate and multivariable analysis indicate that plaques removed from interproximal sites in smokers, especially current smokers, are significantly more likely to give a BANA-positive reaction, even in the absence of bleeding (Tables 2-4). Thus, plaque samples taken from sites that did not bleed in individuals who were current smokers were 11 times more likely to yield a BANA positive reaction than those plaques removed from non-bleeding sites in non-smokers (Table 3). Plaques removed from former smokers were 4X more likely to be BANA positive than plaques from non-smokers, indicating that current smoking was a primary reason for the high prevalence of BANA positive plaques removed from sites where there was no papillary bleeding. This was not the case in sites that bled, as current and former smokers' plaques were both 12X more likely to be BANA positive when compared to plaques removed from bleeding sites in non-smokers (Table 4). This indicates that individuals who "ever" smoked have plaques that are almost universally colonized by the BANA-positive species.

It is estimated that approximately 95% of the BANA hydrolysis by a subgingival plaque sample is due to the presence of one or more of the following species: *P. gingivalis*, *B. forsythus* and *T. denticola* (Loesche et al. 1992), so that the present findings can be interpreted as showing that these anaerobic periodontopathogens are ubiquitous in plaques removed from interproximal sites in smokers. The presence of BANA species in plaques is an ominous sign since they have a significantly higher prevalence and incidence at diseased sites than at healthy sites (Loesche et al. 1992, Haifajee & Socransky 1994, Ashimoto et al. 1996, Papapanou et al. 1997, Socransky et al. 1998). However, there is the possibility that other plaque species could be contributing to the BANA-positive reaction observed in these plaque samples as a small number of Capnocytophaga and Bacteroides species are weakly and variably BANA-positive in vitro (Loesche et al. 1990).

These results confirm the finding from a large epidemiological study of Erie County New York residents, which showed that smokers harbored significantly higher levels and were at significantly greater risk of infection with *B. forsythus* than non-smokers, and that after adjusting for disease severity, *P. gingivalis* was more likely to be present in plaques of smokers than in non-smokers (Zambon et al. 1997). The third BANA-positive species, *T. denticola*, was not evaluated in that study. Van Winkelhoff & Winkel (1998) have found that the proportions of *B. forsythus*, but not *P. gingivalis*, to be higher in current smokers than in non-smokers. Perhaps the failure of other investigators to associate puta-

### Table 2. GEE model of the effects of current smoking versus quit smoking versus never smoked on BANA reaction; N=670 sites

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>SE</th>
<th>p-value</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept referent</td>
<td>0.728</td>
<td>0.531</td>
<td>~</td>
<td>~</td>
<td>1.0</td>
</tr>
<tr>
<td>never smoked</td>
<td>-0.728</td>
<td>0.531</td>
<td>~</td>
<td>~</td>
<td>1.0</td>
</tr>
<tr>
<td>quit smoking</td>
<td>1.420</td>
<td>0.421</td>
<td>0.001</td>
<td>4.14</td>
<td>1.81-9.45</td>
</tr>
<tr>
<td>age (years)</td>
<td>-0.009</td>
<td>0.008</td>
<td>0.231</td>
<td>0.99</td>
<td>0.98-1.01</td>
</tr>
</tbody>
</table>

### Table 3. GEE model of the effect of smoking on BANA scores when there is no bleeding associated with the sampled site, i.e., PBS=0 or 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>SE</th>
<th>p-value</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept referent</td>
<td>0.9725</td>
<td>0.412</td>
<td>0.018</td>
<td>~</td>
<td>1.71-10.62</td>
</tr>
<tr>
<td>never smoked</td>
<td>0.9725</td>
<td>0.412</td>
<td>0.018</td>
<td>~</td>
<td>1.71-10.62</td>
</tr>
<tr>
<td>quit smoking</td>
<td>1.449</td>
<td>0.460</td>
<td>0.002</td>
<td>4.26</td>
<td>1.70-10.62</td>
</tr>
<tr>
<td>age (years)</td>
<td>-0.008</td>
<td>0.009</td>
<td>0.37</td>
<td>0.99</td>
<td>0.98-1.01</td>
</tr>
</tbody>
</table>

### Table 4. GEE model of the effect of smoking on BANA scores when there is bleeding associated with the sampled site, i.e., PBS=2, 3, 4, or 5

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>SE</th>
<th>p-value</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept referent</td>
<td>0.77</td>
<td>0.636</td>
<td>0.226</td>
<td>~</td>
<td>1.19-3.72</td>
</tr>
<tr>
<td>never smoked</td>
<td>0.77</td>
<td>0.636</td>
<td>0.226</td>
<td>~</td>
<td>1.19-3.72</td>
</tr>
<tr>
<td>quit smoking</td>
<td>2.56</td>
<td>0.972</td>
<td>0.008</td>
<td>12.97</td>
<td>1.92-87.23</td>
</tr>
<tr>
<td>age (years)</td>
<td>0.02</td>
<td>0.016</td>
<td>0.23</td>
<td>1.02</td>
<td>0.99-1.05</td>
</tr>
</tbody>
</table>
tive periodontal pathogens with smoking reflected that their choice of bacterial species to monitor did not include either *B. forsythus* or *T. denticola*, i.e., *A. actinomycetemcomitans, P. intermedia,* and *P. gingivalis* (Preber et al. 1992), and *A. actinomycetemcomitans, P. intermedia,* and *P. gingivalis, E. corrodens* and *F. nucleatum* (Stoltenberg et al. 1993). The New York study included some of these species and found that the prevalence of *P. intermedia,* *T. denticola,* and *A. actinomycetemcomitans* could not be significantly associated with smoking. Thus by not looking for *B. forsythus* and *T. denticola* along with *P. gingivalis,* these investigators were not searching for those species most likely to be associated with periodontal disease.

The BANA testing procedure for 4 representative interproximal plaque samples takes only a few minutes and the results are known after a 5-min incubation period. The toothpick sampling method assures that a standard amount of plaque is removed from each site allowing standardized comparisons between sites and individuals. The sampled plaque would be a mixture of both supragingival and subgingival plaque, so that at first approximation it is surprising that this type of sample, reflecting the bacterial flora about the gingival margin, would yield useful bacteriological information on the level of infection with periodontal pathogens. However, there are reports indicating that the putative periodontal pathogens can be detected in plaques associated with gingival health and with gingivitis in periodontally diseased individuals (Amalfitano et al. 1993, Riviere et al. 1996, 1998, Tanner et al. 1998), and that these plaques reflect the flora found in periodontal pockets.

Our finding that smokers, both current and previous, are almost universally colonized/infected with one or more of the BANA-positive species, i.e., *P. gingivalis, T. denticola* and *B. forsythus,* suggests that the BANA test may be useful in evaluating the periodontal status of smokers. The presence of three or four BANA-positive plaques out of the four samples taken could alert the clinician of the presence of these bacterial risk factors for periodontal disease, which in turn could lead to a more thorough periodontal examination and possibly to the use of interceptive treatments. The emerging epidemiological data linking periodontal disease with cardiovascular disease (Mattila et al. 1995, DeStefano et al. 1993, Beck et al. 1996, Grau et al. 1997) suggests that the extent of periodontal disease in cardiovascular patients should be determined. In this regard a positive BANA test and an increased PBS were independent risk indicators for a medical diagnosis of coronary heart disease among older United States veterans (Loesche et al. 1998). This suggests that the BANA test and the PBS could be simple means of identifying cardiovascular patients with inflammatory periodontal disease.

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**Zusammenfassung**


Résumé

Prévalence des bactéries pathogènes pour le parodonte hydrolysatrices *BANA* chez les fumeurs

Le fait de fumer a été identifié comme un facteur de risque de développement de maladie parodontale et un puissant indicateur d'échecs du traitement parodontal. La présente étude concerne 172 patients classés de fumant actuellement (current, n=55), ayant fumé antérieurement (quit, n=38) ou n'ayant jamais fumé (never, n=79). Une analyse de l'hydrolyse du substrat de trypsine synthétique, la benzoyl-DL-arginine naphthylamide (BANA), a été faite pour en tout 670 prélèvements interproximaux de plaque, recueillis avec un cure-dent en bois. Environ 95% de l'hydrolyse de BANA sur la plaque sont dus à la présence d'un ou de plusieurs des pathogènes parodontaux, *P. gingivalis, T. denticola* ou *B. forsythus.* La santé gingivale a été mesurée à l'aide du score de saignement papillaire (PBS). Les patients fumant actuellement avaient moins de saignement gingival que ceux ayant fumé antérieurement ou ceux n'ayant jamais fumé (20% versus respectivement 41% et 25%). La probabilité que les échantillons de plaque recueillis dans les sites sans saignement chez les patients fumant actuellement donnent une réaction BANA positive était 11 fois plus grande que pour les échantillons recueillis dans les sites sans saignement chez les sujets n'ayant jamais fumé. Une relation positive significative existe entre le fait de fumer et la colonisation par les pathogènes parodontaux hydrolysant BANA. Le fait de fumer peut effectuer dans la plaque une sélection de ces espèces pathogènes pour le parodonte, ce qui peut expliquer pourquoi il représente un facteur de risque dans le développement de la maladie parodontale.

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