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ORIGINAL ARTICLE

Multivariate multilevel models for repeated measures in the study of smoking effects on the association between plaque and gingival bleeding

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Abstract Multivariate multilevel modeling was applied to analyze repeated measures data on the influence of heavy smoking on the association between the amount of supragingival plaque and gingival bleeding on probing (BOP) in a steady-state plaque environment. Data acquired in 65 systemically healthy young adults with mild plaque-induced gingivitis were analyzed. 33 heavy smokers consumed at least 20 cigarettes per day while 32 were non-smokers. Periodontal examinations at the outset consisted of periodontal probing depth, clinical attachment level, BOP, plaque index, and presence of calculus at 6 sites of every tooth present. They were repeated 3 times every 8 weeks. A multivariate 4-level variance component model revealed that the odds of BOP was twice as high in smokers. In addition, females had a lower likelihood for BOP but, with increasing bleeding scores during the course of the study, this effect attenuated. Low biserial correlations for BOP at the site level of between 0.11 and 0.2 were found. At the tooth level, correlations were moderate (0.2–0.5), and highest at the subject level (0.8–0.9). Variations at subject and tooth levels were very large at the outset but notably attenuated in the course of the study. Plaque consistently influenced the tendency for BOP with an odds ratio of about 1.7–1.8 for each increase in score in both smokers and non-smokers. The present study did not reveal evidence for attenuation of the plaque/gingival bleeding relationship in heavy smokers.

Keywords Binary response · Bleeding on probing · Multilevel models · Repeated measures · Smoking

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Introduction

Bleeding upon mechanical manipulation with, e.g., a periodontal probe is regarded a rather reliable sign of gingival and periodontal inflammation induced by dental plaque. Different methods for provoking inflammatory gingival bleeding have recently been reviewed [16]. While bleeding may be delayed in certain subjects [17], a dampened inflammatory response to the same amount of microbial dental plaque has been documented in smokers [8]. It has been demonstrated that the number of bleeding sites increased after smoking cessation although plaque levels did not change [15]. These observations may point to an attenuated association between the amount of supragingival plaque and gingival inflammation in smokers.

In a recent article [14], some longitudinal data collected in a steady-state plaque environment were presented indicating that (i) plaque levels were consistently higher in young adult heavily smoking soldiers with plaque-induced gingivitis than in their non smoking companions and, (ii) at the end of the 6 months time period, gingival bleeding on probing (BOP) was more prevalent in smokers. Furthermore, (iii) a trend of weaker associations between presence of supragingival plaque and BOP in smokers was reported. And finally, (iv) a marginal, multivariable logistic model adjusted for plaque and periodontal probing depth was presented indicating that the first transition of a non-bleeding site to a bleeding site was influenced by smoking status pointing to smoking as an independent factor [14]. Marginal models employing, e.g., Generalized Estimating Equation methods, are frequently used in analyzing highly correlated data such as site-specific periodontal findings in a certain individual, particularly if the correlation structure itself is considered a nuisance. On the other hand, the

plenty of information in typical dental, hierarchical data are preferably dealt with by multilevel modeling which provides correct estimates of standard errors and allows the correlation of responses at each level to be modeled. Not at least, it gives a direct way of considering the influence of subjects' unobserved characteristics on the response variable and hence a direct measure of the effects which we are assessing [6]. Repeated measures binary response data were recently analyzed by a multivariate approach [7, 20]. Thus, the aim of the present study was to re-analyze the data acquired in that particular longitudinal study [14] in order to further assess BOP in a steady-state plaque environment in smokers and non-smokers with plaque-induced gingivitis.

Materials and methods

The study was approved by the respective Commander of the mission. Study population, exclusion criteria, clinical examinations, and follow-up had been described in detail elsewhere [14]. In brief, participants were medically fit soldiers of the German Armed Forces serving in the 5th German Contingent of the Stabilization Force (GCONS FOR) stationed for a 6-month mission at Rajlovac, Bosnia-Hercegowina during December 1999 and May 2000. A total of 65 volunteers gave written informed consent to be examined every 8 weeks during the following 6 months after having briefed on risks and benefits of the study. They were between 19 and 30 years old and presented with mild plaque-induced gingivitis. 23 males and 10 females were heavy smokers smoking at least 20 cigarettes per day with an average number of packs consumed per day times years of 11 (standard deviation 10). The remaining 28 males and 4 females had never smoked. Clinical periodontal examination was carried out at 6 sites per tooth. Periodontal probing depth and clinical attachment level were measured using a simple pressure-controlled probe (ClickProbe 1395, KerrHawe, Bioggio, Switzerland) to the nearest mm. Since the probe tip diameter is 0.5 mm and probing force, according to the manufacturer, 0.25 N, an approximate probing pressure of 1.27 MPa may be calculated when the probe bends with a palpable click. BOP was assessed about 20 s after probing. Site-specific presence of supragingival calculus was scored and the amount of plaque assessed after disclosing with erythrosine by applying criteria of the plaque index [18]. Examinations were repeated every 8 weeks for 3 times while participants were exhorted not to alter their oral hygiene habit. During the study period they were provided with only 2 fluoridated toothpastes both not containing triclosan. A prophylaxis session was offered after termination of the study.

Statistical analysis

Summary measures of periodontal conditions were analyzed by repeated measures analysis of variance (ANOVA). The primary outcome variable was the *association* between plaque and gingival BOP in a steady-state plaque environment. A 4-level multivariate variance components model without further explanatory variables may be written as

$$\text{logit}(\pi) = \sum \beta_0 + \Omega_4 + \Omega_3 + \Omega_2.$$

The fixed part of the model estimates proportions of sites bleeding on probing at different time intervals by the β_0 coefficients. It can be extended by including explanatory variables in order to assess their respective associations with bleeding on probing. The random part consists of the variance-covariance structures Ω_4, Ω_3, and Ω_2 of subject, tooth, and site levels, respectively. There is no level 1 (occasion) variation, as at level 2 (site) binomial variates among occasions are allowed to covary within sites. At this level, a covariance structure is estimated in which diagonal terms are constrained to have binomial variance and off-diagonal terms are estimated [20]. Thus, the dependence of observations at this level is fully accounted. We may also unconstrain level 2 variance to assess the assumption of conditionally independent Bernoulli trials [7]. Further model assumptions were confirmed through analysis of residuals generated by the software (*MLwiN* 2.0, Centre for Multilevel Modeling, Bristol University, Bristol, UK).

Results

Overall observations

A total of 61 volunteers (94%) completed the final examination, 31 smokers and 30 non-smokers. Distributions of summary measures of clinical variables BOP, amount of plaque, and calculus are presented in box-and-whisker plots in Figs. 1, 2 and 3. The proportions of bleeding sites during the study period are shown in Fig. 1. Repeated measures ANOVA revealed that average bleeding tendency, which was very low at the outset, slightly increased during the study in both groups ($p<0.001$). An accelerated pace was observed in smokers ($p<0.01$), who had consistently higher proportions of bleeding sites ($p<0.01$). As shown in Fig. 2, average plaque index scores also increased in both groups ($p<0.001$). Smokers had higher average plaque index scores ($p<0.01$), but levels slightly increased in both groups during the study at an identical pace. A similar trend was seen for mean calculus scores (Fig. 3). Smokers had slightly more calculus ($p=0.052$) and developed calculus at a faster pace than

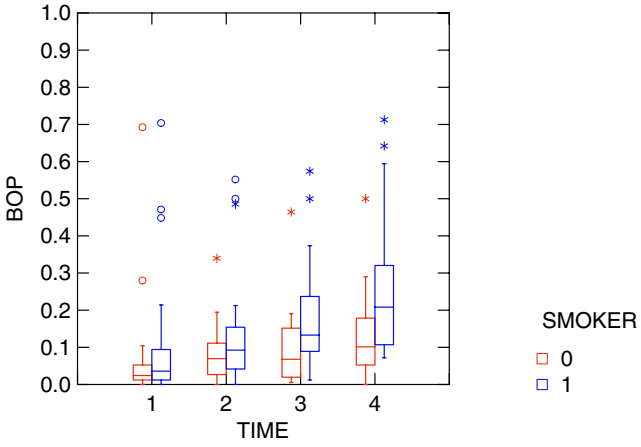

Fig. 1 Box-and-whisker plots showing proportions of sites bleeding on probing (BOP) in smokers and non-smokers at different time intervals

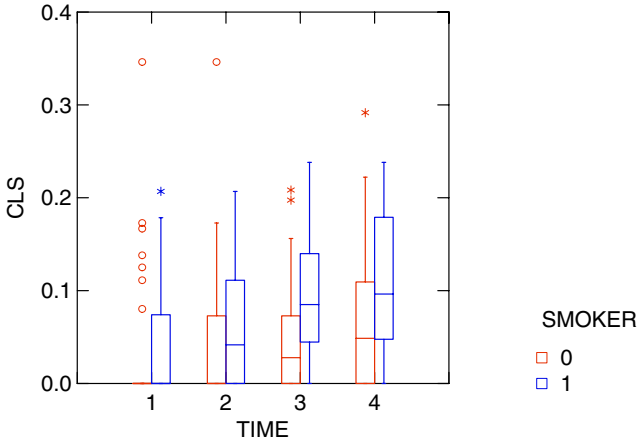

Fig. 3 Distribution of calculus (CLS) in smokers and non-smokers at different time intervals

non-smokers ($p<0.05$). While mean periodontal probing depths slightly decreased during the study from 2.22 to 2.06 mm (standard deviation 0.35 mm), a slight average loss of clinical attachment of 0.1 mm was noted. No differences between smokers and non-smokers were observed.

Multilevel models

Estimates of a variance components, multivariate, 4-level, model of BOP (with further subject-related covariates smoking and gender) are shown in Table 1. According to intercepts β_0, the predicted proportions of BOP during the 4 examinations increased from 6.0% (*antilogit* of −2.76) to 14.9%. Throughout the study, smokers had about twice the odds (derived by the *exp* of 0.63 through 0.73) of BOP. This trend accelerated at the end of the study (odds ratio 2.1, 95% confidence interval 1.3–3.5). Numerical data from the model largely confirm overall results displayed in Fig. 1. In addition, females had a lower likelihood of BOP, but, with increasing bleeding scores during the course of the study, this effect attenuated.

The random part of the model in Table 1 revealed low biserial covariances for BOP at the site level of between 0.08 and 0.15, corresponding to correlations of between 0.11 and 0.2. At the tooth level, correlations were moderate, and highest at the subject level (0.8–0.9). Hence, individual tendency of bleeding did not substantially change during the study, while bleeding at the site level was largely unpredictable. Variations at both the subject and tooth levels were very large at the outset but notably attenuated in the course of the study. At the site level, estimated extrabinomial parameters for the first binary response considerably differed from 1, which points to underdispersion of the response variable, since BOP rarely occurred at that examination.

The main question of the present study was whether the association between BOP and the amount of supragingival plaque is attenuated in smokers as compared to non-smokers. Estimates describing this association are displayed in Table 2 and were derived from a multivariate 4-level model of BOP with site-specific plaque index as a covariate. With a notable exception at the outset examination when bleeding infrequently occurred, plaque consistently influenced the tendency for BOP with an odds ratio of about 1.7–1.8 for each increase in score in both smokers and non-smokers.

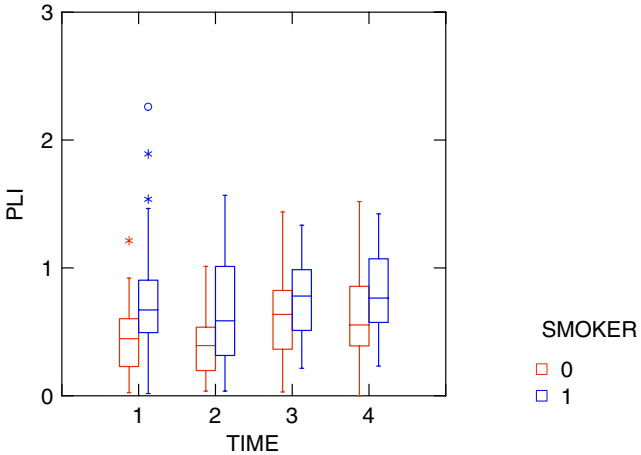

Fig. 2 Distribution of average plaque index (PII) in smokers and non-smokers at different time intervals

Discussion

Although average PLI scores slightly, albeit ‘significantly’ varied over time, the present study model can be regarded a typical steady-state plaque environment. No intervention was executed. As is shown in Fig. 2, highest median of

Table 1 Multivariate 4-level variance components model for BOP

	Parameter	Baseline (t=1)	Week 8 (t=2)	Week 16 (t=3)	Week 24 (t=4)
Fixed:	$\beta_{0,t}$	−2.76 (0.34)	−2.40 (0.19)	−2.09 (0.20)	−1.74 (0.19)
	Smoking[a]	0.69 (0.48)	0.63 (0.26)	0.71 (0.28)	0.73 (0.26)
	Gender[b]	−0.87 (0.58)	−0.63 (0.32)	−0.62 (0.34)	−0.30 (0.32)
Random: Level 4 (subject)	$\sigma^2_{f_t}$	3.41 (0.62)	0.97 (0.19)	1.14 (0.21)	1.01 (0.19)
	$\sigma_{f_{t,t+1}}$	1.44 (0.30) *0.79*	0.84 (0.18) *0.80*	1.01 (0.19) *0.94*	
	$\sigma_{f_{t,t+2}}$	1.76 (0.34) *0.90*	0.83 (0.17) *0.85*		
	$\sigma_{f_{t,t+3}}$	1.65 (0.32) *0.89*			
Level 3 (tooth)	$\sigma^2_{v_t}$	2.22 (0.11)	1.25 (0.08)	0.67 (0.06)	0.51 (0.05)
	$\sigma_{v_{t,t+1}}$	0.56 (0.07) *0.34*	0.34 (0.05) *0.37*	0.29 (0.04) *0.49*	
	$\sigma_{v_{t,t+2}}$	0.54 (0.06) *0.45*	0.24 (0.05) *0.30*		
	$\sigma_{v_{t,t+3}}$	0.24 (0.05) *0.22*			
Level 2 (site)	$\sigma^2_{e_t}$	0.66 (0.01)	0.83 (0.01)	0.79 (0.01)	0.79 (0.01)
	$\sigma_{e_{t,t+1}}$	0.09 (0.01) *0.11*	0.14 (0.01) *0.17*	0.15 (0.011) *0.20*	
	$\sigma_{e_{t,t+2}}$	0.09 (0.01) *0.12*	0.12 (0.01) *0.14*		
	$\sigma_{e_{t,t+3}}$	0.08 (0.01) *0.12*			

[a]reference non-smoking; [b]reference male
Standard errors are given in brackets and correlations in italics

average plaque indices was not seen at the end of the observation period but at 4 months in both groups, while the lowest median of average plaque indices was not found in the beginning but at 2 months (conceivably due to the well-known Hawthorne effect).

Immediately after having arrived in the camp, smokers had significantly stronger associations between supragingival plaque and BOP. The very low plaque levels at the outset in particular in non-smokers may be responsible for this observation. After a short orientation phase when the soldiers had to get used to the new, indeed, war situation smoking did no longer change the 'causal' (site-specific) relationship between plaque and gingival bleeding. In both groups, the odds of bleeding was increased by about 75% with each increase in plaque index score. That smoking did not alter the plaque/bleeding relationship is apparently in contrast to a previous report on the same data set [14]. There we found that, according to heterogeneity tests, 95% of individual associations were more or less consistent over time, and thus combined the evidence from respective fourfold tables by calculating a Mantel-Haenszel overall estimate R_{MH}. We observed a trend (p<0.07) for weaker associations in smokers as compared to nonsmokers with a median R_{MH} of 1.91 versus 2.89, respectively. Apart from very circumstantial combination of individual fourfold tables, another explanation for the discrepant result in the former analysis [14] is certainly that only presence or absence of plaque was considered, while in the present reanalysis plaque was entered as a covariate into the repeated measures, multivariate multilevel model as originally scored on a 0–3 scale.

Certain new observations of our present analysis have particularly to be addressed. First, variances of the outcome at both subject and tooth levels were large immediately after having arrived at the camp (3.4 and 2.2, respectively) but considerably attenuated (to 1 and 0.5, respectively; see random part of Table 1) thereafter. Far-reaching standardization and even synchronization of the daily course in the camp is a most probable explanation for this striking finding made by multilevel modeling, which gives a direct way of looking at the influence of subjects' unobserved characteristics on the response variable. Although this question could not be addressed in the present study, it is conceivable that differences in coping with considerable stress at the beginning of the mission might have been present. Pro-inflammatory cytokines in gingival crevice fluid have been shown to increase during stressful situations in both experimental gingivitis sites and sites

Table 2 Odds ratios (and 95% confidence intervals) for PLI derived from a multivariate 4-level model adjusted for gender

	Baseline	Week 8	Week 16	Week 24
Smoker	2.09 (1.79–2.44)***	1.76 (1.56–1.98)	1.71 (1.55–1.88)	1.71 (1.57–1.86)
Non-smoker	1.29 (1.10–1.51)	1.75 (1.51–2.03)	1.68 (1.50–1.87)	1.79 (1.63–1.96)

Dependent variable BOP
***p<0.001

with perfect oral hygiene [3]. Stress frequently leads to neglecting oral hygiene [4]. While average oral hygiene slightly impaired during the course of the present study the range of individual mean plaque index scores and proportion of bleeding sites decreased. Another possible reason for attenuation of subject level variance of bleeding scores may be limitation of availability of only two toothpaste brands in the camp both not containing, for example, anti-inflammatory triclosan. The actual effect of different toothpastes on the overall variation of bleeding scores is under current investigation.

Multilevel modeling also allows the correlation of responses at each level to be considered by analysis of the covariance structure. At the site level, biserial correlations of gingival bleeding between subsequent occasions were very low as compared to the situation at the outset (0.11–0.12), but increased during the study and reached, for example, 0.2 when comparing the 2 last examinations. The low biserial associations derived from univariate Mantel-Haenszel tests which were reported in a previous attempt of characterizing gingival bleeding tendency in this population [13] could now be confirmed and more accurately described in the multivariate repeated measures model. While biserial correlations of the response variable were moderate at the tooth level, they were strong at the subject level, pointing to considerable consistency of an individual's tendency of bleeding gingiva after probing in this steady-state plaque environment.

In previous gingivitis experiments [19] or a steady-state plaque environment [11, 14] considerable individual differences in the inflammatory response to dental plaque have been reported. They have traditionally been ascribed to a large array of intrinsic and extrinsic factors such as sex hormone fluctuations, stress, systemic non-steroidal drugs, smoking, and genetic make-up [9]. In the present study smokers had consistently higher numbers of gingival units which bled on probing, and this corresponded to larger amounts of supragingival plaque at both the site and the subject levels. In several recent studies certain older observations of a decreased inflammatory response to sub- and supragingival plaque (for review, see [14]) have been confirmed. For example, the correlation between mean proportions of gingival units bleeding on probing and tooth surfaces covered with plaque is lower in smoking periodontitis patients or dental hospital admissions [2], and quitting smoking may reverse this effect [15]. In a representative sample, heavy smoking had a strong suppressive effect on gingival bleeding [5], however, information on plaque levels was not collected in that study. Our findings do not corroborate these observations. A possible explanation may be the time factor for assessing gingival bleeding after probing which might be crucial since bleeding can be largely delayed in particular in smokers [17]. Whether vasoconstrictive properties of nicotine may contribute to an observed delayed gingival bleeding upon mechanical manipulation is still a matter of debate. Whereas smoking cessation led to an increase in average gingival blood flow [11], in a recent smoking experiment a modest, bimodal, hyperaemic response in gingiva has been reported [10].

Previously, it has been argued that assumptions for a univariate multilevel, repeated measures, logistic model, which seems to provide a natural way to model these kind of data, are untenable and must lead to biases [1]. The bias is assumed to arise from the existence of a mixture distribution with certain sites having a constant response (for example, consistently not bleeding) and others having responses which vary from occasion to occasion. In contrast, the multivariate multilevel model allows for strong dependence between successive outcomes to the same unit but does not require long series of repeated measures [20]. The dependence is modeled by the covariance structure at the lowest level (the site), in which the diagonal terms are assumed to have binomial variance and off-diagonal terms, i.e., biserial covariances are estimated. The unconstrained binomial variance parameters for the outcome were generally less than 1, pointing to a certain degree of underdispersion. Constraining the model to fit binomial variation at the lowest level mainly attenuated random parameter estimates at the tooth level (not shown).

In conclusion, application of multivariate multilevel modeling for repeated measures showed that the causal relationship between BOP and amount of supragingival plaque is not affected by heavy smoking in a steady-state environment. At the site level, the odds of gingival bleeding was increased by about 70–80% with each increase in plaque index scores by one unit. Standardization and even synchronization of life style in the present population may be reflected by considerable attenuation of random parameters at the subject and tooth level which deserves further study of the steady-state plaque situation.

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