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ORIGINAL ARTICLE

Multilevel modeling of gingival bleeding on probing in young adult carriers of non-JP2-like strains of *Aggregatibacter actinomycetemcomitans*

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Abstract The influence of *Aggregatibacter actinomycetemcomitans* on inflammation in subjects with gingivitis has not been studied in great detail. Seventeen healthy young adults with plaque-induced gingivitis or localized mild chronic periodontitis harboring cultivable numbers of *A. actinomycetemcomitans* were thoroughly examined. Samples of subgingival plaque were obtained from mesial surfaces of all teeth present. In addition, 12 oral mucosal surfaces and unstimulated saliva were sampled. Species identity, presence of the leukotoxin gene, and absence of a specific 530 b deletion in the leukotoxin promoter region indicating non-JP2-like strains were assessed by polymerase chain reaction. Based on a multilevel random intercept model adjusted for probing depth, age, and smoking status, the odds of bleeding on probing was increased by a factor of 1.89 (1.09–3.29, $p=0.024$) if, in addition to plaque, *A. actinomycetemcomitans* could be recovered from the site. At a site without visible supragingival plaque but with cultivable numbers of subgingival *A. actinomycetemcomitans* the odds ratio of bleeding on probing was 3.37 (0.86–13.2, $p=0.081$). Simulating variance partition coefficients revealed that between 1–2% (a clean, shallow site without *A. actinomycetemcomitans*; a deep site covered by plaque containing *A. actinomycetemcomitans*) and 6–7% (a moderately deep site with neither visible plaque nor cultivable *A. actinomycetemcomitans*) of the residual variance was attributable to differences between subjects. The present cross-sectional study indicates that non-JP2-like strains of *A. actinomycetemcomitans* may enhance gingival bleeding tendency even in the absence of clinically visible supragingival plaque.

Keywords Plaque-induced gingivitis · *Aggregatibacter actinomycetemcomitans* · Bleeding on probing · Plaque · Multilevel modeling · Site-specific

Introduction

Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans* is a gram-negative, capnophilic coccobacillus which has long been associated with several oral and nonoral diseases [4]. While especially a highly leukotoxic clone, JP2, had been shown to cause destructive periodontitis in subjects infected with the organism [18], non-JP2 clones of *A. actinomyctemcomitans* may increase the risk for development of the disease as well [5]. The topographical distribution of the organism in cases of chronic periodontitis has been described in some detail [25, 28]. It could be shown, for instance, that the presence of *A. actinomycetemcomitans* is strongly related to deeper pockets. Apart from supra- and subgingival plaque, *A. actinomycetemcomitans* may colonize oral mucosal surfaces as well [33].

In most gingivitis experiments, *A. actinomycetemcomitans* was not considered or rarely detected in subgingival plaque samples [21, 22], since subjects were usually not specifically screened for the organism. Very few studies have been dedicated to the topographical distribution of this and other organisms in gingivitis and early stages of periodontal disease [29], which would be crucial for getting deeper insights into the potential effects of the bacteria at the site of interest, the gingival unit, during infection.

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The clinical characteristics of plaque-induced gingivitis or mild periodontitis were recently described in some detail in young adults with a wide distribution of *A. actinomycetemcomitans* in subgingival plaque samples and compared with those of subjects where the organism was rarely found [30]. A striking observation was that the (univariate) site-specific association between gingival bleeding on probing and presence of supragingival plaque was significantly weaker in subjects where *A. actinomycetemcomitans* was recovered from more than, say, 20% of subgingival plaque samples. Conceivable reasons for this observation may include microbial antagonism with plaque-forming bacteria [39] and certain immune-suppressive actions of *A. actinomycetemcomitans* [6].

In the present reanalysis of the data, more complex, multivariable, multilevel models [9] of the binary response, gingival bleeding on probing, were employed to further illustrate the effects of heavy intraoral load with *A. actinomycetemcomitans* on the clinical expression of plaque-induced gingival disease.

Material and methods

Screening for subjects harboring cultivable numbers of oral *A. actinomycetemcomitans* was done among 102 soldiers of the German Armed Forces with plaque-induced gingivitis or localized mild periodontitis [32] and has been described in detail elsewhere [29]. All had signed an informed consent form which had been approved by the Ethical Committee of the Medical Faculty of Heidelberg University, Heidelberg, Germany. Immediately before the dental examination, five microbiological samples were obtained in each soldier and placed in separate vials with 1 ml sterile saline each: about 0.1 ml unstimulated saliva, swab samples of left and right cheek mucosa, a sample from the dorsum of the tongue, and a pooled sample from the mesiobuccal surface of each first molar or the adjacent tooth if missing. Samples were stored at 4°C and processed (see below) within 24 h. Clinical periodontal conditions were assessed at six sites of every tooth present and consisted of measurements of the periodontal probing depth and clinical attachment level, bleeding on probing after about 20 s, and presence of supragingival plaque after disclosing with a 3% solution of erythrocin.

A. actinomycetemcomitans was recovered in at least one subgingival sample of 17 soldiers (16.7%). They were between 20 and 27 years of age. Sixteen were male and of Northern European heritage, one female had an Afro-American father. Nine were current smokers with a mean of 4.3 pack-years (range 0.9–9). Systematic resampling for presence of cultivable numbers of *A. actinomycetemcomitans* was carried out about 1 week after the periodontal examination and had previously been described in detail [29]. In brief, in each case 12 oral mucosal samples from left and right cheek mucosa, left and right side of the tongue, dorsum of the tongue, left and right palatal tonsillar region, the palatal masticatory mucosa, and four buccal gingival samples from each of the quadrants were obtained with wetted (sterile saline) cotton swabs and transferred to vials containing 1 ml sterile saline. In addition, 0.1 ml unstimulated saliva was sampled. Subgingival samples were obtained from mesial sites of each tooth with curettes after any supragingival plaque had been removed with scalers. Plaque samples were transferred to 1 ml vials with the above transport medium. Samples were stored in the refrigerator and processed within 24 h.

Cultivation, presumptive identification, and genetic species identity of *A. actinomycetemcomitans* has been described previously [29]. Briefly, undiluted and diluted aliquots of samples were streaked on freshly prepared TSBV agar [34] for selective recovery of *A. actinomycetemcomitans*. The limit for detection was about 15–30 CFU ml^{-1} transport fluid with 95% confidence. Plates were incubated for 3–5 days in air supplemented with 10% CO_2 at 36°C. Presumptive identification of *A. actinomycetemcomitans* was based on colony morphology and positive catalase test. Suspect colonies were counted. From each plate at least one colony was picked and subcultivated. Isolates were stored at −70°C. Species identity and presence of the *ltxA* gene were confirmed by multiplex polymerase chain reaction (PCR). Primers and conditions for amplification have been reported previously [29]. Another set of primers [40] was used to identify a specific 530 bp deletion in the promoter region of the leukotoxin gene, characteristic for the highly leukotoxic clone JP2. Positive and negative controls were laboratory strains of *A. actinomycetemcomitans* including JP2, and *Escherichia coli*.

Data analysis

The primary outcome in the present reanalysis of the data was the binary variable gingival bleeding on probing. Adjusted for periodontal probing depth, its relations to the presence of supragingival plaque and presence of *A. actinomycetemcomitans* were of major interest. The previous observation of an attenuated association between plaque and bleeding on probing in subjects with a wide distribution of *A. actinomycetemcomitans* as compared to those where the organism was infrequently found [30] was further explored on a site-specific basis considering the hierarchical structure of the wealth of the collected data. Data were analyzed using the multilevel software package *MLwiN* (version 2.10 Beta, Center for Multilevel Modeling, Bristol University, Bristol). A 2-level (sites indexed by *i* within subjects indexed by *j*), random coefficient, logistic

regression model of the binary response of bleeding on probing, y_{ij}, may be written as:

$$\left.\begin{array}{l} y_{ij} \sim \text{Binomial}\left(1, \pi_{ij}\right) \\ \log it\left(\pi_{ij}\right) = \beta_{0j} + \beta_{1j}x_{1ij} + u_{0j} + u_{1j} \\ u_j \sim \text{N}(0, \Omega_u) \\ \text{var}\left(y_{ij}\middle|\pi_{ij}\right) = \alpha\pi_{ij}\left(1 - \pi_{ij}\right) \end{array}\right\}$$

where x_{1ij} is, for example, a site characteristic, say, periodontal probing depth randomly varying at the subject level. Random effects at the subject level are assumed to follow a normal distribution with mean 0 and covariance matrix Ω_u. Penalized quasi-likelihood estimation with second order Taylor series approximation [8] and reweighted iterative generalized least squares led to convergence of the model in all cases. Possible problems with extra-binomial variation were addressed by unconstraining the level 1 variance, introducing a scale factor α. The model was then extended by including further site-specific explanatory variables such as presence of supragingival plaque and presence of *A. actinomycetemcomitans*, as well as subject covariates age and smoking status. In order to establish relative odds (the main focus of this study), interactions of covariates supragingival plaque and *A. actinomycetemcomitans* were included in the model. All explanatory variables were centered [20]: quasi-continuous variables age and periodontal probing depth at the mean, binary variables such as plaque and *A. actinomycetemcomitans* by adding 0.5 to 0 (absence) and 1 (presence). A simulation method [10] was applied to compute variance partitioning coefficients.

Results

Species identity, presence of the leukotoxin gene, and its intact promoter region (signaling non-JP2-like strains) were confirmed by PCR for all examined isolates of *A. actinomycetemcomitans*. Extent and severity of periodontal disease in the screened population at large, as well as in the 17 subjects harboring cultivable numbers of *A. actinomycetemcomitans* were very similar and had been reported elsewhere [29]. Subjects presented with either mild to moderate gingivitis or localized mild periodontitis.

Figure 1 indicates quantile plots of log-transformed counts of colony forming units of *A. actinomycetemcomitans* in samples of subgingival plaque (Fig. 1a) and oral mucosa/saliva (Fig. 1b) at different cut-offs. While most samples contained the organism at low numbers of 10^2 CFU ml^{-1} transport medium or less, more than 50% of the subjects had at least one subgingival sample containing *A. actinomycetemcomitans* at or in excess of 10^4 CFU ml^{-1}, with more than 20% sites examined above this threshold in one subject (Fig. 1a). The %s of subgingival and mucosa/saliva samples positive for the organism at more than 10^2 CFU were significantly correlated (Spearman's rho of 0.73, $p<0.05$).

In total, 477 sites were sampled for *A. actinomycetemcomitans* in 17 subjects. While 171 bled on probing (35.8%), 161 (33.8%) harbored cultivable numbers of the organism. The results of a 2-level (site, subject), random intercept, logistic regression model of bleeding on probing are presented in Tables 1 and 2. Only one subject was female, so gender was not considered as a covariate. Both age and smoking, either as smoking status or as lifetime exposure (pack-years, not shown), had a very small, non-significant influence on bleeding on probing. There was a strong influence of periodontal probing depth on the bleeding tendency. For instance, for each mm increase in probing depth, the odds of gingival bleeding increased by a factor of $exp(0.951)=2.59$ (95% confidence interval, 1.79; 3.74, $p<0.001$). With a site without visible supragingival plaque and no cultivable *A. actinomycetemcomitans* as

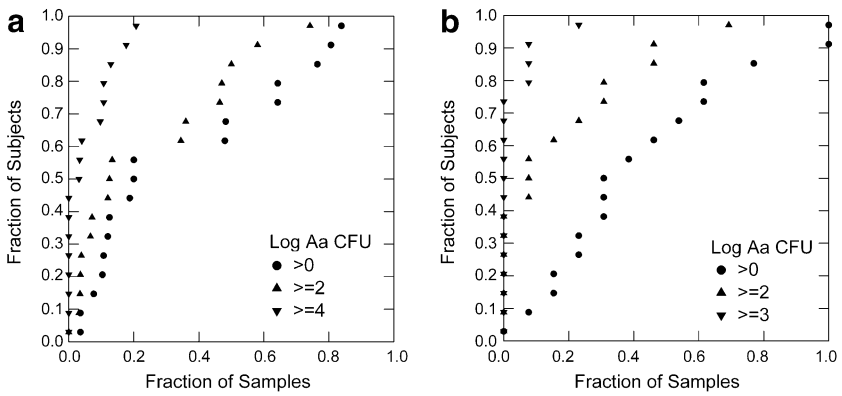

Fig. 1 Quantile plots (cumulative frequency plots) of the distribution of sites with different levels of *A. actinomycetemcomitans* at different cut-offs as expressed as log-transformed counts of colony forming units (Log Aa CFU) in samples obtained in 17 subjects harboring the organism. (**a**) Subgingival plaque samples. Note that in 50% of the subjects 20% or more subgingival samples were culture positive. While in all subjects at least one sample was culture-positive for *A. actinomycetemcomitans*, in one subject the organism was found in more than 80% samples. High numbers of CFU of 10^4 or more were found only in 55% of subjects in always less than 20% sites. (**b**) Oral mucosa/saliva samples

Table 1 Estimates (with standard errors in parentheses) of a 2-level (site, subject), random intercept, logistic regression model of bleeding on probing

	Estimate (standard error)
β_{0j}	−2.732 (0.803)
Age (year)	0.033 (0.091)
Smoking status (0, 1)	−0.065 (0.346)
PPD (mm)	0.951 (0.187)
Visible plaque (0, 1)	1.307 (0.529)
A. actinomycetemcomitans (0, 1)	1.215 (0.696)
Visible plaque×*A. actinomycetemcomitans*	−0.577 (0.517)
u_{0j}	0.307 (0.170)
Extra-binomial parameter	0.992 (0.065)

Covariates centered

reference, a plaque-covered site without *A. actinomycetemcomitans* would have an odds ratio of 3.70 (1.31; 10.4, p=0.013) for bleeding on probing. If, in addition, *A. actinomycetemcomitans* could be recovered, the odds ratio would be $exp(1.307+1.215-0.577)=6.99$ (1.78; 27.4, p=0.005). Thus, the odds of bleeding on probing was increased by a factor of 1.89 (1.09; 3.29, p=0.024) if, in addition to visible plaque, *A. actinomycetemcomitans* could be recovered from the site. At a site without visible supragingival plaque but with cultivable numbers of *A. actinomycetemcomitans* the odds of bleeding on probing was increased by 3.37 (0.86; 13.2, p=0.081). The unexplained variance at the subject level amounted to 0.307 (standard error 0.170). The extrabinomial parameter α was close to 1 confirming the assumption of binomial distribution of the outcome. Variance partition was simulated and revealed that between about 1–2% (for example, a shallow site without visible plaque and cultivable numbers of *A. actinomycetemcomitans*; or a deep site covered by plaque containing subgingival *A. actinomycetemcomitans*) and about 6–7% (a moderately deep site with neither visible plaque nor cultivable *A. actinomycetemcomitans*) of the residual variance was attributable to differences between subjects.

Clinical conditions were recorded at 6 sites of each tooth present while microbiological data were collected only from one site per tooth (and 12 mucosal surfaces and saliva). In previous analyses [30], subjects with a wide distribution of *A. actinomycetemcomitans* (recovery from ≥20% subgingival sites, n=8) were differentiated from subjects where the organism was infrequently found (<20% subgingival sites affected, n=9). In a 2-level model of gingival bleeding on probing considering all clinical data, wide distribution of *A. actinomycetemcomitans* was entered as a subject-related covariate. Moreover, probing depth was allowed to vary randomly among subjects (see equation). Thus, a random coefficient model was built (not shown). The strong influence of periodontal probing depth on the bleeding tendency after probing did not significantly vary among the subjects (variance $\sigma^2_{u1} = 0.010$, standard error 0.024). The positive covariance between the intercept and the subject periodontal probing depth (σ_{u01}=0.016, standard error 0.030) was not significant either. Therefore, in the final model (Table 3) periodontal probing depth was entered as a fixed effect only. With a site without visible supragingival plaque in a subject where *A. actinomycetemcomitans* was infrequently found as reference, a plaque-covered site in a subject with wide distribution of the organism had an odds ratio of 5.25 (2.48; 11.1, p<0.001) for bleeding upon probing. In a subject with low frequency of the organism the respective odds ratio was 5.12 (3.22; 8.14, p<0.001). In a subject with wide distribution of *A. actinomycetemcomitans* even sites without visible plaque tended to bleed more frequently with an odds ratio of 2.44 (1.19; 5.00, p=0.015). With 1.024 (0.027), the extrabinomial parameter was slightly greater than 1 pointing to some over-dispersion. According to simulated variance partition coefficients for different situations 2–5% of the residual variance was attributable to differences between subjects.

In several models, adjusted for plaque, periodontal probing depth, age, and smoking, the influence of increasing numbers of colony-forming units of *A. actinomycetemcomitans* in subgingival samples on the odds of bleeding on probing (as compared to a culture-negative sample) was determined. The results derived from the fixed parts of the models are shown in Fig. 2. While presence of the organism in general increased the bleeding tendency after probing by a factor of 1.64 (1.00, 2.67), odds ratios increased at higher counts of $>10^3$ or $>10^4$ to 2 to 3, regardless of probing depth or presence of plaque.

Table 2 Odds ratios and 95% confidence intervals as derived from 2-level model of bleeding on probing in Table 1

	Reference		
	No plaque/no *Aa*	No plaque/*Aa*	Plaque/no *Aa*
No plaque/*Aa*	3.37 (0.86–13.2)		
Plaque/no *Aa*	3.70 (1.31–10.4)	12.5 (1.26–123)	
Plaque/*Aa*	6.99 (1.78–27.4)	2.08 (1.24–3.47)	1.89 (1.09–3.29)

Sites with and without visible supragingival plaque and subgingival *A. actinomycetemcomitans* (*Aa*) were considered

Table 3 Odds ratios (and 95% confidence intervals in parentheses) as derived from 2-level (site, subject) model of bleeding on probing, adjusted for periodontal probing depth, smoking, and age

	Reference		
	No plaque/low *Aa*	No plaque/wide *Aa*	Plaque/low *Aa*
No plaque/wide *Aa*	2.44 (1.19–5.00)		
Plaque/low *Aa*	5.12 (3.22–8.14)	12.5 (4.28–36.4)	
Plaque/wide *Aa*	5.25 (2.48–11.1)	5.12 (3.22–8.14)	1.03 (0.61–1.73)

All sites were considered. Subjects with >20% subgingival sites with *A. actinomycetemcomitans* (*Aa*) had a 'wide' distribution of the organism as compared to those with <20% ('low'). Note that no plaque refers to a site without visible supragingival plaque

Discussion

The primary aim of this project had been to describe the microbial ecology of *A. actinomycetemcomitans* in young adults with plaque-induced gingival disease or localized mild chronic periodontitis [29]. For that purpose, 102 soldiers of the German Armed Forces had been screened for cultivable numbers of the organism. Screening had been done in a similar way as in a previous survey of 201 male German soldiers in the age range of 18–25 years, where a 27% prevalence of *A. actinomycetemcomitans* had been reported [27]. Thus, mucosal samples of the cheeks and tongue, a saliva sample, and a pooled sample of subgingival plaque were obtained and selectively cultivated for the organism by standard methods [34]. Here, about 17% of the screened subjects (95% confidence interval 10–26%) harbored cultivable amounts of *A. actinomycetemcomitans* when a very low detection limit of 15–30 CFU ml^{-1} transport fluid was established. This corresponds well with recently reported prevalence data for healthy subjects and subjects with early periodontitis in a similar age range [35], when DNA probes were employed.

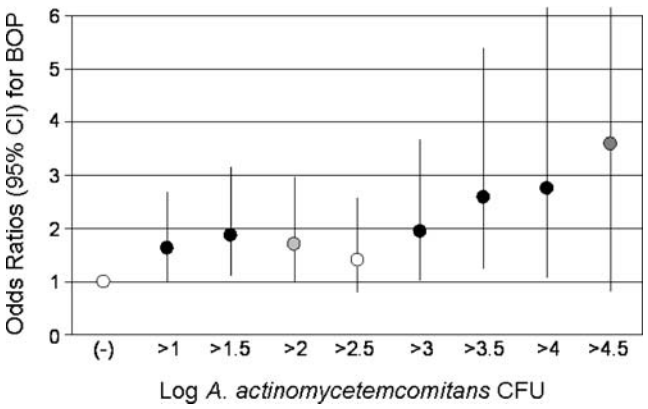

Fig. 2 Results of several multilevel random intercept models (adjusted for periodontal probing depth, plaque, age, smoking) illustrating odds ratios (and 95% confidence intervals) for bleeding on probing in sites with increasing log-transformed numbers of colony forming units (CFU) of *A. actinomycetemcomitans*. Reference was, in each case, a culture-negative sample. *Black dots*, $p<0.05$; *grey dots*, $p<0.1$

When the culture-positive volunteers were re-sampled after about 1 week, the organism could be recovered from all. On the other hand, it is very likely that some of the 85 seemingly culture-negative subjects in fact harbored *A. actinomycetemcomitans* at the time of screening at very low numbers. When the results of the screening procedures and the detailed re-examinations were utilized for assessing the reproducibility of selective cultivation of *A. actinomycetemcomitans* [30], the overall proportion of agreement with regard to presence or absence was 59%, but kappa was very low, 0.18 (standard error 0.11). A recent larger-scale study on immediately repeated subgingival curette samples [38] revealed rapidly decreasing counts of periodontal pathogens in particular from healthy and gingivitis sites. These results cannot really be compared with the present figures which were obtained after about 1 week. Also, the detection limit was set, in that study, at DNA of about 10^5 cells which might have rendered almost all of our culture-positive samples false-negative. In the present study, oral mucosal samples contributed significantly to the identification of *A. actinomycetemcomitans*-positive subjects. Sampling oral mucosal surfaces had been shown to be particularly valuable for identification of minimally diseased subjects colonized with the organism [3].

JP2-like, i.e. highly leukotoxic, strains were not detected in the present material. A strong racial tropism had been reported for the specific 530-bp promoter deletion of the leukotoxin gene of *A. actinomycetemcomitans* [16, 23] which had been associated with enhanced leukotoxin production [1]. Thus, Africans and African Americans seem to be primarily infected [15] and may then develop aggressive forms of periodontal disease [17, 18]. Among the present study population, one female soldier had an African American mother. However, all isolates tested had the intact promoter region of the leukotoxin gene. While it has been shown in recent longitudinal studies that the highly leukotoxic clone of *A. actinomycetemcomitans* may almost inevitably *cause* aggressive periodontitis in infected subjects [17, 18], non-JP2-like strains are associated with localized or generalized forms of the disease as well [14] and were recently also causally related to disease initiation

in teenagers [5]. Thus, the present finding of a rather wide intraoral distribution of *A. actinomycetemcomitans* in some of the young adults in the present study is certainly of importance. In a previous paper describing the present material [30], univariate analyses indicated a weaker association between presence of supragingival plaque and bleeding on probing in subjects with a heavy intra-oral load of *A. actinomycetemcomitans* as compared to subjects where the organism was infrequently found. The cut-off was set at the median percentage of subgingival sites found to be positive for *A. actinomycetemcomitans* (i.e., 20%). However, in the eight subjects with 'wide' distribution of *A. actinomycetemcomitans* in fact 62% samples (subgingival and mucosal/salivary) were positive, on average, but only 15% in the group of nine subjects with infrequent recovery (see Fig. 1). With regard to average (overall) periodontal probing depth, attachment level, bleeding on probing and plaque, the two groups did not differ in any respect [30]. In the present reanalysis, several important findings were made by employing multilevel models, which were adjusted for other important covariates at the site and subject levels. First, at sites visibly covered with supragingival plaque, the odds of bleeding on probing was almost twice as high if *A. actinomycetemcomitans* could be detected in subgingival samples. Secondly, there was a trend of increased bleeding tendency at sites without visible plaque if *A. actinomycetemcomitans* was present. This was essentially confirmed in a model considering all periodontal sites where wide or infrequent occurrence of *A. actinomycetemcomitans* was entered as a covariate. In subjects with a higher load, the odds of bleeding at sites without plaque was more than twice as high than in subjects with infrequent detection of *A. actinomycetemcomitans*. All models were adjusted for periodontal probing depth, age, and smoking status. Variance partition coefficients revealed that only between 1% and 7% of the unexplained variance could be attributed to subject differences. In particular in shallow sites without visible plaque and cultivable *A. actinomycetemcomitans*, or rather deep sites covered by supragingival plaque and harboring the organism, subject differences seemed to play a very low role for the probability of bleeding on probing. That sites without visible supragingival plaque but subgingival *A. actinomycetemcomitans* bled at a higher rate in subjects with a wide distribution of the organism corresponds with the observation [30] of a weaker overall association between bleeding on probing and supragingival plaque.

In further adjusted multilevel models, various thresholds of counts of *A. actinomycetemcomitans* were considered for calculating odds ratios for bleeding on probing when comparing the situation of culture-negative sites. Higher counts of 10^3 or 10^4 CFU of *A. actinomycetemcomitans* per 1 ml transport medium increased the odds of bleeding on probing by factors two and more than three, respectively. Notably, counts in excess of 10^4 cells in samples of subgingival plaque (in total, 25 in the present study) have been reported to considerably increase the risk of new attachment loss [12].

The present report seems to be the first describing aggravating effects of *A. actinomycetemcomitans* on gingival inflammation in subjects with gingivitis or localized mild periodontitis. There are several possible factors which might have obscured the presently described relationship in the past. First, *A. actinomycetemcomitans* is rather infrequently found in young and otherwise healthy adults [13, 19, 27, 36]. Moreover, although mainly subjects in their early twenties are invited to participate in gingivitis experiments, they are usually not screened for certain bacteria before entering the study. Some recent studies on the microbiology of gingivitis used DNA probes and checkerboard DNA–DNA hybridization with a rather high limit of detection [37].

Another aspect is the application of insensitive and in certain cases even misleading statistical analyses that may actually prevent deeper insights into pathogenetic mechanisms at the level of interest, the gingival unit. There is no doubt that observations made in an oral cavity must never be considered independent. A still popular strategy is, therefore, aggregating data at the subject level with at least two undesired consequences. Firstly, most of the valuable site-specific information is immediately lost. Secondly, making correct inferences with regard to observed associations seen at the higher level to those at the lower level (which might be 'causal') may be difficult if not impossible. Misleading conclusions are known by the term 'ecological fallacy', i.e., cross-level bias in estimating site-effects from aggregate data, and typical and revealing examples are given by Greenland [11]. When observing the hierarchical structure of the data in the present study multilevel modeling provided correct fixed estimates for calculating associations of several covariates at the site and subject levels with gingival bleeding on probing. Moreover, the random part of the models revealed interesting new aspects, for example, the low amount of unexplained variance attributable to subject differences.

There are several shortcomings of this study which have to be addressed. The main objective of the project had been the description of the topographical distribution of *A. actinomycetemcomitans* in young adults, and the respective results have been published [29, 30]. Clinical data were primarily collected in order to document the rather healthy conditions of the study population. Clinical examinations were only done at the time of screening for *A. actinomycetemcomitans* whereas the detailed microbiological re-examination took place about 1 week later. It was not expected that the periodontal situation would have

changed in the meantime in any respect. However, while the steady-state plaque environment has been described as rather stable over prolonged periods of time [31], bleeding on probing usually shows considerable variation even if repeated within 24 h [26]. A bleeding site at the time of screening for *A. actinomycetemcomitans* might well have reverted to a nonbleeding site at the time of microbiological re-examination 1 week later, and vice versa. In turn, probing itself may have distributed *A. actinomycetemcomitans* within the oral cavity [2]. This shortcoming of not being able to accurately sample clinical and microbiological information at the same time applies to most studies on periodontal microbiology. Some authors tried to circumvent the problem when reporting bleeding of gingiva upon sampling subgingival material [24] which is certainly not the same as bleeding on probing with a quite standardized probing procedure. The uncertainty here is, on the other hand, mainly relevant to the model described in Table 1 where site-specific clinical and microbial data were entered which were collected about 1 week apart. The model described in Table 3 provides very similar estimates but considers the *intraoral load* of *A. actinomycetemcomitans* at the subject level, i.e., a wide distribution of the organism as compared to infrequent occurrence, which is not expected to change within a week in the absence of any therapeutic intervention. Another drawback is that the present data set is only cross-sectional. The here observed strong association of bleeding on probing with an established periodontal pathogen, *A. actinomycetemcomitans*, has certainly to be confirmed in longitudinal studies and/or gingivitis experiments. Of even greater significance would be modeling site-specific disease initiation and progression by including microbiological data collected at the gingival unit. Recently, fluctuations in clinical attachment levels in a similar group of young adults over a 30-month period have been modeled in random coefficient time series models [7], yielding the certainly revealing observation of cyclic changes. Including 'causal' covariates in these models, as, for example, presence of certain pathogens or groups of bacteria, would allow deeper insights into disease-related mechanisms.

In conclusion, the present report seems to be the first pointing at an enhanced site-specific inflammatory response to an established periodontal pathogen, *A. actinomycetemcomitans*, in carriers with plaque-induced gingival or mild periodontal disease. This observation and the finding of a load-dependent relationship was made after adjusting for periodontal probing depth, age, and smoking habit by applying multilevel modeling. In future studies on the microbiota of early periodontitis, the great advantages of multilevel modeling for obtaining unbiased estimates of covariates and random effects (variances/covariances at site, tooth, and subject levels, for example) should be utilized. Disregarding the hierarchical structure of the observations in an oral cavity and summarizing data at the subject level should no longer be regarded good practice.

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Conflict of interest The author declares that he has no conflict of interest.

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