Java project on periodontal disease. Periodontal condition in relation to vitamin C, systemic conditions and tooth loss

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Chapter 3

Java Project on Periodontal Diseases.
Periodontal bone loss in relation to environmental and systemic conditions

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Abstract

Objective: To assess in a population deprived from regular dental care the relationship between alveolar bone loss (ABL) and environmental/systemic conditions.

Material & Methods: The study population consisted of subjects from the Purbasari tea estate on West Java, Indonesia. A full set of dental radiographs was obtained of each subject and amount of ABL was assessed. In addition, the following parameters were evaluated: plasma vitamin C, vitamin D_3, HbA1c and CRP, the haptoglobin phenotype, the presence of putative periodontopathic bacteria and viruses, dietary habits, smoking and anthropometrics.

Results: In this population 45% showed vitamin C depletion/deficiency, 82% had vitamin D_3 insufficiency/deficiency, 70% were in a pre-diabetic state, 6% had untreated diabetes, 21% had high CRP values ranging from 3.1-16.1mg/l. Results of the regression analysis, including all above mentioned parameters, showed four significant predictors, explaining 19.8% of the variance of ABL. Number of *P. gingivalis* cells and CRP values showed a positive relationship with ABL whereas BMI and number of guava fruit servings were negatively related.

Conclusion: Results suggest that elevated levels of *P. gingivalis* may be indicative for periodontitis progression whereas increased consumption of guava fruit may play a protective role in periodontitis of a malnourished population.

Introduction

Periodontitis is a multifactorial disease caused by an imbalance between environmental factors and the host defense. The environmental factors include, apart from microorganisms, life style factors and living conditions. In addition, systemic conditions may play a role in the development of the disease.

Bacteria that have been implicated in periodontal disease include *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Fusobacterium nucleatum, Parvimonas micra* and *Treponema denticola* (Slots & Ting 1999, Socransky et al. 2002, Van Winkelhoff et al. 2002). In addition, viruses like Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Herpes Simplex virus (HSV) have been reported to be related to periodontitis (Slots 2010).

One of the most important life style factors related to the development and severity of periodontal disease is smoking. Depending on the definition of disease and the exposure to smoking, the risk to develop destructive periodontal disease is 5- to 20-fold elevated for a smoker compared to a never-smoker (Bergström 2004).

Nutrition is another important life style factor related to periodontal disease (Van der Velden et al. 2011). The importance of vitamin C for periodontal health has been known for quite some time. Insufficient consumption of vegetables and fruits, the two major sources of vitamin C, can lead to depletion or deficiency states for the vitamin (Taylor et al. 2000, Wrieden et al. 2000). Evidence shows that plasma vitamin C levels are inversely related to the severity of periodontitis (Amarasena et al. 2005, Amaliya et al. 2007, Chapple et al. 2007). *In vivo* vitamin C is prone to oxidation which is to some extent prevented by the plasma protein haptoglobin (Hp). This protein has been associated with a genetic polymorphism resulting in three major phenotypes (Hp 1-1, Hp1-2 and Hp 2-2) with functional differences. It was found that the stability of vitamin C in serum is lowest in Hp 2-2 individuals (Langlois et al. 1997). Thus Hp phenotype may also be important in relation to periodontal disease.

It has been shown that, in addition to vitamin C, vitamin D may be linked to periodontal disease (Dietrich et al. 2005, Alshouibi et al. 2013). Vitamin D can be obtained from food or from endogenous production in the skin when exposed to adequate sunlight (Holick, 2008). With aging there is a dramatic decline of the skin to produce vitamin D (MacLaughlin & Holick 1985). Thus it may be expected that especially in individuals deprived from sunlight and in older age groups, vitamin D deficiency may develop.

Another aspect of diet is its systemic effects. Overconsumption of carbohydrates and fats may result in overweight or obesity and the latter is a risk indicator for periodontal disease (Van der Velden et al. 2011, Suvan et al. 2011). It has been shown that diets with a high glycemic index increase the risk of chronic life-style related diseases like diabetes type 2 (Barcley et al. 2008), and higher HbA1c levels increase the risk for periodontitis (Morita et al. 2012). Elevated CRP levels are a strong independent risk factor for the development of diabetes type 2 (Pradhan et al. 2001, Hu et al. 2004) and have been related to periodontitis (Paraskevas et al. 2008). Also, periapical lesions may contribute to elevated CRP levels since surgical treatment reduced CRP levels (Marton & Kiss 1992). Thus, HbA1c and CRP levels are important indicators for general and dental health.

In 2005 plasma vitamin C levels were assessed in a population deprived from regular dental care that previously participated in a study on the natural development of periodontitis (Amaliya et al. 2007). The results of that study showed an inverse relationship between plasma vitamin C levels and periodontal breakdown. As discussed above it is suggested that, in addition to vitamin C, the amount of periodontal destruction in this population as well as the presence of periapical lesions may have been influenced by other environmental factors and systemic conditions. Therefore, the purpose of the present study was to investigate in this population whether the amount of alveolar and periapical bone loss was related to the levels of plasma vitamin C, vitamin D, HbA1c and hs-CRP, the Hp phenotype, the presence of putative periodontopathic bacteria and viruses, dietary habits and anthropometrics.

Material and Methods

In 2005 plasma vitamin C levels were assessed in 123 subjects of the original Indonesian study population (Amaliya et al. 2007). In 2011, the same 123 subjects were asked to participate in the present study. Prior to the start of the study, subjects were informed in detail about the objectives of the investigation and those willing to participate were requested to sign an informed consent form. The study was approved by the Ethics Committee of the Hasan Sadikin Hospital Bandung-West Java, Indonesia.

Clinical procedures

After signing the informed consent form, various variables were assessed including: age, gender, smoking habits and education level. Non-smokers included 3 former smokers who stopped smoking several years ago. Current smokers were asked about the estimated number of cigarettes/kreteks they smoke daily and the number of years they had smoked.

Dietary evaluation

The dietary assessment was conducted by a trained examiner using a structured interview employed previously (Amaliya et al. 2007). The dietary habits were recorded by evaluating the food frequency, taken in the last month. For each food item the number of servings of vitamin C containing food was assessed. The level of vitamin C content of the various food products was based on the values provided by the National Nutrient Database for Standard Reference (USDA 2010) and Woot-Tsuen et al. (1968). Food products were categorized as high, fair, low or no vitamin C when they provided >60 mg, 31–60 mg, 2–30 mg and <2 mg vitamin C/100 g respectively (Kuzmanova et al. 2012).

Blood sampling

Fasting venous blood samples were collected into (i) a lithium heparin tube for vitamin C and 25-hydroxyvitamin D_3 assessment (further referred to as vitamin D_3), (ii) an EDTA tube for HbA1c assessment and (iii) a plain tube for hs-CRP analysis, seropositivity of cytomegalovirus (CMV) and Epstein Barr virus (EBV) and haptoglobin (Hp) phenotyping. Tubes were kept in 4^{o}C until analysis in Hasan Sadikin Hospital Bandung-West Java. Plasma for vitamin C analysis was prepared immediately after sampling in order to minimize the oxidation of vitamin C.

Microbiological sampling

Subgingival microbiological samples were taken from the 4 sites that had also been previously sampled i.e. the deepest bleeding pocket with the greatest amount of attachment loss per quadrant (Timmerman et al. 2001). After careful removal of the supragingival plaque by means of a curette, subgingival plaque samples were taken using 2 sterile paper points per pocket. One paper point for bacteriological- and the other for viral evaluation. Paper points were transferred into a vial with 1ml lysis buffer (Biomerieux, NucliSens® Lysis Buffer, Marcy l'Etoile, France) resulting in 2 vials per subject with a pooled sample. The samples were kept in 4^{o}C at the Hasan Sadikin Hospital Bandung-West Java until further processing.

Radiographic examination

A full set of dental radiographs was obtained of each subject using a long cone paralleling technique (Gnatus Timex 70 X-Ray Mobile Column, Brazil). Radiographs needed to meet the criterion that the landmarks used had to be visible i.e. the cemento-enamel junction, the

alveolar crest and the apices of the teeth. Radiographs were scanned using a commercially available, high-resolution device (Epson Perfection 4870 Photo, Seiko Epson Corporation, Suwa, Nagano, Japan) at a standard setting of 360 dpi. Scans were entered in the dental patient management software (Visiquick V3 3.0.1.611, Thomas Monitor Systems, Amsterdam, the Netherlands) for further analysis. Alveolar bone loss (ABL) was assessed mesially and distally of all teeth, except for M3, and the percentage of ABL relative to the root length was determined. For each individual, the mean ABL percentage was calculated by dividing the sum of the percentages of ABL mesially and distally of all teeth by the total number of surfaces. Furthermore, the number of teeth showing mesially or distally ABL≥30% or ABL≥50% was determined. In addition to ABL, periapical bone loss was also evaluated at all teeth by assessing presence or absence of periapical radiolucencies in terms of a widened periodontal ligament space (minimal periapical lesion) and an evident periapical radiolucency (evident periapical lesion). Intra-examiner repeatability of ABL and periapical radiolucencies was satisfying (Spearman's correlation coefficient 0.96;$p<0.001$ and Kappa 0.86; $p<0.001$ respectively).

Anthropometric evaluation of distribution of body dimensions

Weight and height were recorded while subjects were wearing light clothing and without shoes. Body mass index (BMI) was calculated as the ratio of weight (in kg) to the square of height (in m). Subjects were classified as obese (BMI≥25kg/m2) according to the Asia-Pacific perspective redefining obesity in adult Asian (WHO 2000). Waist circumference (WC) was measured just below the lowest rib according to Bosy-Westphal et al. (2010). Hip circumference (HC) was determined at the widest point. The Waist to Hip Ratio (WHR) was defined as WC divided by HC.

Laboratory procedures

Vitamin C and D analysis

Within 10 min after sampling tubes were centrifuged with a low-speed centrifuge (Shanghai Surgical Instrument Factory, Shanghai, China) at 1559xg for 4 min. to separate plasma from blood cells. For vitamin C and vitamin D_3 analysis, plasma was subsequently prepared according to the manufacturer's instructions (Chromsystems, Vitamin C and Vitamin 25-OH-D3/D2 Diagnostics Kits by HPLC, Munich, Germany). Plasma vitamin C levels were categorized as follows: normal (≥4.0mg/l), depletion (2 – 3.9mg/l) and deficiency (<2mg/l)

(Hampl et al. 2004). The used reference ranges of plasma vitamin D_3 levels were: normal (50-200nmol/l), insufficiency (20-50nmol/l) and deficiency (<20nmol/l) (Haq et al. 2007).

HbA1c analysis

Hba1c analysis was performed by means of Cobas c 501 instrument (Turbidimetric-Inhibition Immunoassay, Roche Diagnostics GmBH, Mannheim, Germany). The anticoagulated whole-blood samples were hemolyzed automatically on the Cobas c 501analyzer with Cobas c Hemolyzing Reagent Gen.2. Measuring range of HbA1c assessment was 2.3-18.9%, with the lower detection limit of 0.8%. The criteria for HbA1c levels were as follows: normal (≤5.6%), pre-diabetes (5.7 – 6.4%), and diabetes (≥6.5%), (American Diabetes Association 2012).

hs-CRP analysis

hsCRP measurement was performed from sera with Cobas® c501 autoanalyzer (Roche Ltd, Mannheim, Germany). Reagents were purchased from the same vendor and the tests were performed according to the recommendation of the manufacturer. Measuring range was 0.5-75 mg/L with the lower detection limit of 0.1 mg/L. The classification for CRP levels with regard to risk of cardiovascular disease was as follows: low (< 1 mg/l), intermediate (1-3 mg/l), high (> 3 mg/l) (Pearson et al. 2003).

Haptoglobin phenotyping

The Hp phenotype (Hp 1-1, Hp 2-1 and Hp 2-2) of the subjects was determined in serum using a chemiluminescence detection method for the rapid detection of Hp-phenotype after non-denaturating polyacrylamide gel electrophoresis as described previously (Huang et al. 2004).

Seropositivity of CMV and EBV.

Seropositivity of CMV was measured with the electrochemiluminescence immunoassay ECLIA for the determination of IgG antibodies to CMV in human sera by means of a Cobas c501 immunoassay analyzers (Roche Ltd, Mannheim, Germany). Reagents were purchased from the same vendor and the tests were performed according to the recommendation of the manufacturer. Measuring range was 0.25 – 500 u/ml. Results obtained were interpreted as follows: <6.0 AU/ml = non-reactive and ≥6.0 AU/ml = reactive.

Seropositivity of EBV was also assessed by means of an Enzyme Linked Immunosorbent Assay (ELISA) using human antibodies of the IgG against EBV in human serum. Photometric measurement of the colour intensity was made at a wavelength of 450 nm and a reference wavelength of between 620 nm and 650 nm within 30 min. of adding the stop solution. The interpreting results recommended by EuroImmun are as follows: <16 RU/ml = negative, ≥16 to <22 RU/ml = borderline, ≥22 RU/ml = positive (EUROIMMUN AG, Luebeck, Germany).

Quantitative polymerase chain reaction (qPCR) for bacterial and viral detection

Bacterial DNA was extracted and purified using a column system (Spin Protocol, Qiagen, Germany) according to the manufacturer's instructions. Isolated DNA was kept in -80^{o}C until use. Previously published primer/probe sequences and protocol for the bacterial species were used (Bizzarro et al. 2013). In short, quantitative PCR analysis of *A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythia, P. micra, F. nucleatum* and *T. denticola*, was carried out with LightCycler®480II (Roche Molecular Diagnostics, Germany).

Viral DNA was isolated using CMV and EBV isolation kit QIAmp DSP Virus and QIAmp DNA Mini Kit (Qiagen Ltd., Hilden, Germany) according to the manufacturer's instructions. Viral DNA were analyzed by means of Light Cycler 2.0^{TM} (Roche Ltd, Penzberg, Germany) with specific reagent for Real-Time PCR artus$^{®}$ Herpes Virus LC-PCR Kits (Qiagen Ltd., Hilden, Germany). PCR conditions were set according the manufacturer's instructions.

Mean values of bacterial cells and viral copy counts were calculated by dividing the sum of bacterial cells or viral copy counts by the number of subjects that was positive.

Statistical analysis

Descriptive statistics and data analyses were performed with statistical software from SPSS (version 19.0; IBM SPSS Inc., Chicago, IL, USA). Differences between males and females were assessed by means of Chi-square and Mann-Whitney U tests where appropriate including Bonferroni correction for multiple comparisons. For assessment of relationships between the dependent variable mean ABL and the predictor variables, data of continuous variables were first analyzed whether they showed normal distributions (Shapiro-Wilk test $p<0.05$). Because after log transformation all variables, except mean ABL, maintained their non-normal distribution, the Box-Cox procedure was employed, finding the optimal normalizing transformation for each individual variable (Osborne 2010). This procedure

resulted in normal distributions of the dependent variable mean ABL and the predictor variables plasma vitamin C, vitamin D and CRP, as well as BMI, WC, WHR, *P. gingivalis, T. denticola, P. micra* and number of guava fruit servings. All remaining variables were dichotomized on the basis of the median or variable characteristics i.e. smoker/non-smoker, elementary completed or not completed, Hp phenotype and in case of *A. actinomycetemcomitans* on presence or absence. In order to investigate the predictive value of all above mentioned variables for ABL, a forward stepwise linear regression analysis was used. A logistic regression analysis was employed to evaluate the relationship between presence/absence of periapical radiolucences and the above mentioned variables. For all analyses, p-values <0.05 were considered statistically significant.

Results

This study included 98 subjects, 53 women and 45 men with an age range from 39 to 50 years. In Table 1 background and dental characteristics are presented for the total population and for females and males separately.

Table 1. Background and dental characteristics of the total population and subpopulations according to gender.

Variable Mean (SD) or N(%)	Total population (N=98)	Female (N=53)	Male (N=45)
Age	45.6 (3.5)**[1]	44.8 (3.8)	46.5 (2.9)*
Education level			
Elementary school			
not completed	54 (55.1 %)	36 (67.9 %)	18 (40.0 %)*
completed	44 (44.9 %)	17 (32.1 %)	27 (60.0 %)
# of smokers	45 (45.9%)	7 (13.2%)	38 (84.4%)*
# smoking years	23.5 (9.6)	7.4 (8.4)	26.4 (6.2)*
# of cigarettes per day	10.0 (6.4)	2.7 (4.1)	11.3 (5.8)*
# heavy smokers > 10/day	26 (58%)	1 (14%)	25 (66%)*
# light smokers ≤ 10/day	19 (42%)	6 (86%)	31 (34%)
BMI (kg/m^2)	22.9 (3.5)	24.7 (3.5)	20.8 (1.9)
WHR	0.89 (0.08)	0.90 (0.09)	0.87 (0.07)
Dental status			
# of teeth	24.1 (3.7)	23.8 (3.7)	24.3 (3.5)
% ABL	30.1 (7.3)	29.0 (7.2)	31.5 (7.3)
# of teeth ABL ≥ 30%	9.6 (4.7)	8.7 (4.7)	10.6 (4.4)
# of teeth ABL ≥ 50%	1.9 (2.7)	1.6 (2.5)	2.4 (2.9)
# of teeth with PR			
minimal	0.6 (0.9)	0.6 (0.9)	0.5 (1.0)
evident	1.1 (1.6)	1.1 (1.5)	1.0 (1.8)

BMI, body mass index; WHR, waist to hip ratio; ABL, alveolar bone loss; PR, periapical radiolucency; N, number.
*significant dirrence between female and male $p<0.05$

Subjects of this population showed a mean of 30% ABL in their dentition, ranging from 19% to 54%. Analysis showed a significant positive relation between the mean percentage ABL and age, years of smoking, number of cigarettes that people smoke and the BMI. The population included 6 heavy smokers of which the number of cigarettes per day ranged from 15 to 24 and 39 light smokers who smoked on average 8 cigarettes per day. Females were

younger, had less education and smoked less. No significant gender differences were found regarding BMI, WHR, ABL and number of teeth with periapical radiolucencies. In 62 subjects periapical radiolucencies were found. Minimal periapical lesions were found in 36 subjects whereas 50 subjects showed presence of evident periapical lesions. The mean number of evident lesions per subject was 1.1 ranging from 1 to 8 lesions.

Table 2. Subgingival prevalence of bacteria and viruses; number of positive subjects and mean number of bacterial cells ($x10^6$) or mean number of viral copy counts per ml ($x10^3$) in positive subjects (total number of subjects is 98).

Microorganism	Number of positive subjects	Mean (SD)
A.actinomycetemcomitans	46	1.70 (3.89)
P. gingivalis	97	24.84 (32.35)
P. intermedia	98	0.32 (0.39)
T. forsythia	98	1.23 (7.65)
P. micra	98	0.75 (0.84)
F. nucleatum	98	0.10 (0.32)
T. denticola	98	1.66 (2.14)
Epstein Barr virus (EBV)*	73	20.69 (57.33)
Cytomegalovirus (CMV)*	0	0

*Note: all subjects were sero-positive for EBV and CMV.

Microbiological evaluation showed that almost all subjects were positive for *P. gingivalis*, *P. intermedia, T. forsythia*, *P. micra*, *F. nucleatum* and *T. denticola*, whereas about half of the population was positive for *A. actinomycetemcomitans* (Table 2). *P. gingivalis* showed a 10 fold higher number of bacterial cells compared to the other putative periodontal pathogenic bacteria. No differences were found between females and males. Analysis of serum showed that all subjects were sero-positive for EBV and CMV. However, none of the subjects was subgingivally positive for CMV, whereas 75% of the population was subgingivally positive for EBV.

Table 3. Vitamin C, Vitamin D_3, HbA1c and CRP values of the total sample group and variable categories including Hp phenotype

Variables assessed in plasma	Variable Categories			
Vitamin C	**Total sample group**	**Normal** (≥ 4.0 mg/l)	**Depletion** (2.0 - 3.9 mg/l)	**Deficiency** (< 2.0 mg/l)
N	98	54	33	11
Mean (SD) mg/l	5.19 (3.30)	7.34 (2.97)	2.88 (0.63)	1.48 (0.34)
Vitamin D_3	**Total sample group**	**Normal** (50-200 nmol/l)	**Insufficiency** (20-50 nmol/l)	**Deficiency** (< 20 nmol/l)
N	91	10	41	40
Mean (SD) nmol/l	26.35 (15.95)	57.73 (5.30)	32.34 (8.08)	12.35 (4.70)
HbA1c	**Total sample group**	**Normal** (≤ 5.6 %)	**Pre-diabetic** (5.7 - 6.4 %)	**Diabetes** (≥ 6.5%)
N	98	23	69	6
Mean (SD) %	5.96 (0.88)	5.42 (0.19)	5.89 (0.19)	8.68 (2.04)
CRP	**Total sample group**	**Low VRG** (< 1 mg/l)	**Intermediate VRG** (1-3 mg/l)	**High VRG** (> 3 mg/l)
N	98	45	32	21
Mean (SD) mg/l	2.16 (2.73)	0.52 (0.21)	1.69 (0.56)	6.36 (3.26)
Hp phenotype	**Total sample group**	**Hp 1-1**	**Hp2-1**	**Hp 2-2**
N	97	21 (21.6%)	48 (36.9%)	28 (21.5%)

HbA1c, glycated hemoglobin; CRP, C-reactive protein; Hp, haptoglobin; VRG, vascular risk group; N, number of subjects.

From this population, 45% showed vitamin C depletion/deficiency, 70% were in a pre-diabetic state, 6% had untreated diabetes, 21% had high CRP values ranging from 3.1-16.1mg/l and 33% showed intermediate values. Due to technical difficulties plasma vitamin D_3 could not be assessed in 7 subjects, in the remaining population 82% had vitamin D_3 insufficiency or deficiency. The majority of the subjects showed a Hp2-1 phenotype (Table 3).

Table 4. Mean number of servings during one month by food products and their vitamin C content in the population (N=98).

	Mean number of servings (SD)
High vitamin C : 61-280 mg/100 g	
Cassava leaves	3.0 (3.1)
Chili	5.8 (2.5)
Guava fruit	8.4 (7.2)
Fair vitamin C : 21-60 mg/100 g	
Kangkung	2.9 (2.8)
Sweet potato	9.9 (9.5)
Low vitamin C : 2-20 mg/100 g	
Cabbage	9.0 (6.6)
Soybean sprouts	8.9 (6.8)
Banana	5.5 (4.9)
Avocado	0.8 (3.5)
Carrot	8.8 (7.6)
Onion	6.9 (0.7)
Cassava	4.9 (6.4)
No vitamin C : < 2 mg/100 g	
Corn	3.1 (18.1)
Rice	86.0 (12.8)
Salted fish	5.1 (11.1)
Carp	0.5 (1.9)
Goldfish	3.9 (3.8)
Chicken	4.0 (3.4)
Lamb	0.1 (0.5)
Beef	1.8 (5.2)
Egg	17.2 (11.5)
Garlic	6.8 (1.2)

The various components of the diet are presented in Table 4 in terms of the mean number of servings taken during one month in relation to their vitamin C content. For the fruit products with high vitamin C content the number of servings during one month ranged between 0-16 for cassava leaves, 0-7 for chili and 0-30 for guava fruit.

The results of the regression analysis, in which levels of plasma vitamin C, vitamin D, HbA1c and hs-CRP, the Hp phenotype, the presence of putative periodontopathic bacteria and viruses, the dietary habits and anthropometrics were entered as predictive variables for ABL, are presented in table 5.

Table 5. Significant predictor variables for ABL as assessed by means of a forward stepwise linear regression analysis (N=98).

Variable	Unstandardized Coefficient *B*	SE	Correlation Coefficient β	P-value
BMI (kg/m^2)	− 0.516	0.153	− 0.335	0.001
P. gingivalis	0.157	0.070	0.210	0.028
CRP (mg/l)	1.039	0.456	0.226	0.025
Guava fruit	− 0.137	0.061	− 0.209	0.029
Model constant	3.950	0.443		< 0.0001

The model showed an explained variance (R^2) of 19.8% (adjusted R^2=0.069%);
SE, standard error.

It can be seen that the BMI, numbers of subgingival *P. gingivalis* cells, plasma CRP values and number of guava fruit servings were significant predictors, explaining 19.8% of the variance of ABL. *P. gingivalis* and CRP showed a positive relationship with ABL whereas BMI and guava fruit were negatively related. Post hoc analysis regarding the relationship between BMI and ABL showed that the lower decile of this population, having a BMI ≤ 19, had significantly more ABL than the remaining part of the population (34.6% versus 29.6% respectively, p=0.039). No relationships were found between periapical radiolucences and any of the predictor variables.

Discussion

The present study is a follow-up investigation in an Indonesian population of a cohort that previously participated in an investigation on the natural development of periodontitis (van der Velden et al. 2006) and in which the contribution of diet to the experienced periodontal breakdown was studied also (Amaliya et al. 2007). In that study, actually carried out in 2005, a significant relationship was found between plasma vitamin C values and attachment loss as assessed in 2002. Surprisingly, in the present study no relation was found between plasma vitamin C values and the amount of ABL. Analysis of the present data set and that of the same subjects of 2002, showed a highly significant correlation between the present ABL and the attachment loss in 2002 ($p<0.0001$). In contrast, no correlation was found between plasma vitamin C values of the present study and those of 2005. A major difference between the two studies is that in 2005 non-fasting blood samples were collected whereas in the present study fasting blood samples were obtained. In other studies which have reported a significant inverse relationship between plasma vitamin C levels and the severity of periodontal disease, it was not mentioned whether fasting or non-fasting blood samples were used (Väänänen et al. 1993, Amarasena et al. 2005, Panjamurthy et al. 2005, Chapple et al. 2007). Therefore, most likely non-fasting blood samples were used and it may be assumed that non-fasting blood samples reflect in a better way the relationship between vitamin C plasma values and disease. In a recent study it was suggested that, due to genetic variation of vitamin C transporter protein SVCT 1, periodontitis patients may be less capable in vitamin C uptake (Kuzmanova et al. 2012). Thus, plasma vitamin C levels of non-fasting blood samples may better reflect individual variation in the vitamin C uptake capacity of subjects. This effect may has been masked by in fasting blood samples, which reflect vitamin C levels after distribution into cells and tissues.

Since the previous study of this population found an inverse correlation between vitamin C and attachment loss (Amaliya et al. 2007), this time also the haptoglobin phenotype in this population was assessed. Haptoglobin is a circulating protein that binds free hemoglobin to prevent heme-driven oxidative damage. However, when the antioxidant function of haptoglobin is insufficient, vitamin C has been proposed to act in its place, subsequently depleting serum ascorbic acid (Delanghe et al. 2007). This applies especially to subjects with Hp2-2 phenotype in which an increased risk of vitamin C deficiency has been demonstrated (Cahill & El-Sohemy 2010). In the present study population 26% of subjects had the Hp2-2 phenotype however no relation was found with plasma vitamin C levels nor with ABL. Likewise the lack of relation between plasma vitamin C values and the amount of

ABL, the lack of relation between Hp phenotype and vitamin C plasma levels and ABL could also be explained by the fact that fasting blood samples were used.

A number of previous studies have shown an inverse relationship between dietary vitamin C intake and the severity of periodontal disease (Vogel & Wechsler 1979, Ismail et al. 1983, Nishida et al. 2000). This observation was confirmed in the present study in which an inverse relation was found between the number of guava fruits consumed during the previous month and the amount of ABL. In the previous 2005 study we also evaluated the guava intake during the previous month but were not able to confirm a relationship. Re-analysis of the old data set showed that the correlation between guava fruit intake and mean attachment loss was marginally significant (p=0.10). Probably, the nine years of disease progression and the loss of 25 subjects no longer available for examination, contributed to the finding of a significant inverse relation between guava fruit and ABL in the present study.

A poor quality of diet may be reflected in the unhealthy condition of the study population with 70% in a pre-diabetic state and 6% having undiagnosed diabetics. Most varieties of white rice are regarded as foods with a high glycemic index (Miller et al. 1992). In a large systematic review regarding prospective studies on development of chronic diseases in general, it has been shown that diets with a high glycemic index are associated with increased risk of the development of several chronic diseases including diabetes (Barclay et al. 2008). It is generally accepted that there is an association between diabetes mellitus type 2 and periodontitis, and that diabetes can be considered as a risk factor for periodontitis (Chávarry et al. 2009). In the present study no relation was found between HbA1c plasma levels and ABL possibly due to the fact that the number of subjects with HbA1c values ≥6.5% was too small. However, the poor diet may have resulted in the relatively low BMI (22.9 kg/m^2), a negative relationship between BMI and ABL and 10 subjects with a BMI ≤19 (kg/m^2). The latter subjects may be regarded as malnourished, which could explain the more severe ABL.

In the present study plasma CRP levels were related to the severity of ABL. This finding is in agreement with the results of a relatively recent systemic review showing strong evidence from cross-sectional studies that plasma CRP in periodontitis is elevated compared with controls (Paraskevas et al. 2008). It has also been suggested that CRP levels are increased in subjects with apical periodontitis compared with healthy controls (Gomes et al. 2013). However this could not be confirmed in the present study.

Since periodontitis is inextricably linked to a number of putative periodontopathic bacteria, microbiological evaluation was included from the start of the study on the natural

development of periodontitis in 1987. Results of the 15 year prospective evaluation showed that subgingival presence of *A. actinomycetemcomitans* but not *P. gingivalis* could be regarded as a risk factor for the onset of disease (Van der Velden et al. 2006). In the present study almost all subjects were positive for *P. gingivalis* and a relationship was found between the number *P. gingivalis* cells and ABL. Data of the 1987 database showed that already in 1987 86.7% of the present subjects were positive for *P. gingivalis.* These findings suggest that *P. gingivalis* load may be regarded as a risk indicator for disease progression, which is in line with the literature (Moore et al. 1991, Grossi et al. 1994). Moreover the present results confirm that *P. gingivalis* is best described as a pathobiont as suggested by Cugini et al. (2013), i.e. symbiont that is able to promote pathology only when specific genetic or environmental conditions are altered in the host (Chow & Mazmanian 2010).

It has been suggested that the vitamin D_3 status could influence the periodontal condition. The effect on gingivitis has been well established (Dietrich et al. 2005, Hiremath et al. 2013). The relationship between vitamin D_3 and periodontal breakdown is less clear. Some studies showed an inverse relationship (Dietrich et al. 2004, Miley et al. 2009, Alshouibi et al. 2013), while Millen et al. (2013) found no relationship with ABL and Liu et al. (2009) who observed higher plasma vitamin D_3 level in aggressive periodontitis patients compared to controls. In the present study with a population having low plasma vitamin D_3 levels, no relationship could be assessed between vitamin D_3 and ABL, confirming the results of the study of Millen et al. (2013).

In conclusion, results of this study suggest that elevated levels of *P. gingivalis* may be indicative for the risk of periodontitis progression whereas increased consumption of guava fruit may play a protective role in periodontitis of a malnourished population.

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