Periodontitis in twins : smoking, microbiological and immunological aspects

Torres de Heens, G.L.

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Chapter 4

Monozygotic twins are discordant for chronic periodontitis

Clinical and bacteriological findings

G. L. Torres de Heens[1], U. van der Velden[1] and B. G. Loos[1]

[1]Department of Periodontology, Academic Center for Dentistry Amsterdam, ACTA, The Netherlands

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Abstract

Objectives: The aim of this study was to assess, in monozygotic (MZ) and dizygotic (DZ) twin pairs of which the proband of the twin pair is suffering from moderate to severe chronic periodontitis, the contribution of genetics, periodontal pathogens and life style factors to the clinical phenotype.

Material and Methods: For this study 18 adult twin pairs were selected on the basis of interproximal attachment loss ≥ 5 mm in ≥ 2 non-adjacent teeth in 1 twin member. The study included 10 MZ and 8 DZ twin pairs, in which the periodontal condition, presence of periodontal pathogens, educational level, smoking behavior and Body Mass Index was evaluated.

Results: Both MZ and DZ twins were discordant regarding attachment loss and alveolar bone loss. Discordance was greater in DZ compared to MZ twins. In MZ twins the discordance could not be explained by education, smoking, Body Mass Index and periodontal pathogens. In DZ twins 45.6% of the discordance could be explained by more pack-years of the probands.

Conclusion: The results confirm a possible role of genetic factors in periodontitis. However, the magnitude of the genetic effects on disease severity may have previously been overestimated.

Introduction

Periodontitis is initiated by microbial plaque, which accumulates at the gingival crevice region and at present *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* are recognized as main periodontal pathogens (Fine et al. 2007, Van der Velden et al. 2006, van Winkelhoff et al. 2002). Although bacteria are essential for inducing an inflammatory response in the periodontal tissues, they are insufficient to cause periodontitis as sole etiological factor (Page et al. 1997). Life style factors, like smoking are also believed to be important for the severity as well as treatment of periodontitis (Palmer et al. 2005). Moreover, there is now consensus that genetic factors play a role in the susceptibility and severity to periodontitis (Loos et al. 2005, Tonetti & Claffey 2005).

There are a limited number of family studies on periodontitis, but collectively their results suggest that periodontitis aggregates in families (Loos et al. 2008). Although family studies might give a first impression of familial aggregation, they can not distinguish between the influence of genetic and shared environmental effects as an explanation for the familial clustering of periodontitis. In this respect twin studies are especially useful. For chronic periodontitis relatively few twin studies have been carried out, but the results suggest a substantial role of genetic factors in the etiology (Corey et al. 1993, Michalowicz et al. 1991a, Michalowicz et al. 2000, Mucci et al. 2005). Nevertheless the latter studies have limitations; the results of Corey et al. (1993) and Mucci et al. (2005) are based on self reported evidence of periodontal disease and the subjects in the studies of Michalowicz et al. (1991a,b, 2000) were mildly affected by periodontal breakdown. Interestingly, results of another twin study of Michalowicz et al. (1999) suggested that the presence of periodontal bacteria in subgingival plaque was not determined by host genetic factors. An earlier family study on periodontitis indicated that the main periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis,* can be transmitted between parents and their children (Petit et al. 1993). Therefore, it is plausible that susceptibility or resistance to periodontitis, as it was proposed for other infectious diseases, may be dependent on genetically controlled differences in immune responses after pathogen exposure; thus the disease is not exclusively restricted to the exposure itself, but rather by the mechanisms of the host elicited in response to the exposure (Baker et al. 2000). This concept combines both environment, life style and genetics as contributing factors to the risk of multifactorial diseases like periodontitis.

Susceptibility to periodontitis may increase due to the experienced life style factors. The effect of smoking in the development of chronic periodontitis is well documented. There is strong evidence that smoking contributes to a higher prevalence and severity of periodontitis (Albandar & Rams 2002, Baharin et al. 2006, Tomar & Asma 2000). Moreover, a twin study showed that the nicotine dependence is influenced for 75% by genetic factors, which provides evidence for a substantial impact of genetic factors on the smoking behavior (Vink et al. 2005). Another factor which may promote the progression of periodontitis is overweight. (Ylostalo et al. 2008) found in a large epidemiological study among non-diabetic, non smoking adults a significant relationship between body weight and periodontitis. For Body Mass Index (BMI) there is overwhelming evidence that variation in the population is influenced by genotype. Results from twin studies suggest that genetic factors explain 50 to 90% of the variance in BMI (Maes et al. 1997, Schousboe et al. 2003).

Furthermore, it has been suggested that measures of socioeconomic status including education are fairly good indicators for periodontitis. Groups with low education are at a higher risk of having periodontitis (Drury et al. 1999) and twin studies suggest a moderate heritability for education (Silventoinen et al. 2000). It has also been suggested that common genetic factors may affect educational attainment and body weight (Silventoinen et al. 2004). Therefore, in order to avoid oversimplification, a number of factors have to be considered for the understanding of the complexity of multifactorial diseases like periodontitis.

We hypothesized that with regard to periodontitis monozygotic (MZ) twin pairs would have a comparable periodontal phenotype whereas dizygotic twin pairs may differ to some extent. Therefore, the aim of the present study was to assess, in MZ and dizygotic (DZ) twin pairs selected on the basis of one sib of a twin pair having moderate to severe chronic periodontitis, the contribution of genetics, life style factors and periodontal pathogens to the clinical phenotype of the disease.

Material and Methods

Subjects

Twin pairs were obtained as follows. A first set of twins was recruited through the identification of patients with moderate to severe periodontal breakdown, who were part of a twin pair, and who were referred to various periodontal clinics across the Netherlands for the treatment of periodontitis (including patients referred to the clinic of the Department of Periodontology at the Academic Center for Dentistry Amsterdam [ACTA]). Another set of twins was recruited with the aid of the Dutch Association of Twins. Possible eligible subjects from this latter set of twins, underwent a preliminary periodontal clinical examination (screening for suitability) to determine whether their periodontal status met the inclusion criteria of our study.

The selection criteria for the twin subjects with moderate to severe periodontal breakdown included: 1) Caucasian descent, 2) age between 25 and 65 years, 3) diagnosis of chronic periodontitis in one member of the twin pair defined by the presence of interproximal attachment loss $\geq$ 5 mm at $\geq$ 2 non-adjacent teeth. Exclusion criteria were: 1) presence of any systemic condition that may affect the periodontal status, 2) pregnancy, and 3) use of antibiotics within the last 6 months preceding the study. The periodontal condition of the co-twin was not part of the selection procedure as the apparent phenotype of the co-twin is part of the results of the present study. The patient recruitment resulted in 25 potentially eligible pairs of twins. Of the 25 twin pairs, 18 pairs (36 subjects) volunteered to participate in the present study. Common reasons for refusal of participation were lack of agreement of both subjects of the twin pair to participate or distant household location making transportation to the research venue difficult.

The study population consisted of 18 reared-together twin pairs and prior to the clinical examination a verbal and a written informed consent were obtained from all twins. This study was approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam. Data from the twin subjects were obtained in the following order: 1) microbiological samples from buccal mucous membranes and tongue, 2) venous blood, 3) full-mouth periapical radiographs, 4) periodontal examination: attachment loss (AL), probing pocket depth (PPD), bleeding on probing (BOP) and plaque index (PI); and 5) microbiological samples of supragingival and subgingival plaque.

Clinical examination

The clinical examination was carried out at the interproximal sites of all teeth from buccal and lingual aspects. The following assessments were performed: PI according to (Silness & Löe 1964); BOP recorded as 0 = no bleeding, 1 = point bleeding within 30 seconds, 2 = immediate and overt bleeding; PPD, recorded in mm (measurements were rounded off to the nearest mm marking) and AL, again in whole mm, using the cemento-enamel junction as a reference. All clinical assessments were performed using a periodontal probe (PQW, Hu-Friedy, Chicago, IL., USA).

Radiographic examination

All participants underwent a full-mouth radiographic survey consisting of 14 periapical and 2 bitewing radiographs using the long-cone paralleling technique with a Heliodent MD digital device, setting of 70 kV, 7mA (Sirona Dental Systems, Bensheim, Germany). Images were obtained using the Emago/Advanced 5.2 program (Exan Academic Inc., Port Coqvitlam, BC, Canada) and printed on photographic paper (Drystar DT 1 B, dry medical film, 25 x 30) using the Agfa Drystar 4500 printer (Agfa, Mortsel, Belgium). All teeth were radiographically examined for interproximal bone loss at the mesial and distal sites, using cemento-enamel junction (CEJ) of the tooth and the bone crest as reference points. Using the Schei ruler technique, the percentage of bone loss at the deepest interproximal site of each tooth was measured (Schei 1959).

Microbiological procedures

Prior to any clinical measurement, samples for microbiological analysis were obtained from the buccal mucous membranes: right and left buccal mucosa, and dorsum of the tongue (from the vallate papillae to the tip of the tongue). The mucous membranes were sampled with a sterile swab and were immediately suspended in Reduced Transport Fluid (RTF). After the clinical examination, 4 sites (1 from each quadrant) were chosen for bacterial sampling according to the following criteria and in the following order: 1) the deepest pocket with the greatest amount of AL and BOP; 2) if no AL was found, the deepest pocket which showed BOP; 3) if only shallow healthy pockets were present, samples were taken mesially from the first permanent molars. The selected sites were isolated with cotton rolls and supragingival plaque samples were taken with a sterile Gracey curette. Subsequently, the remaining supragingival plaque was removed and subgingival plaque samples were obtained by inserting 1 sterile paper point per pocket during 10 s. Both pooled

supragingival and pooled subgingival plaque samples were suspended in RTF. All microbial samples were transported to the laboratory and processed within 24 h.

The presence and proportions of *A. actinomycetemcomitans* were determined by means of TSBV plates and that of *P. gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Tannerella forsythia* and *Campylobacter rectus* by means of blood agar plates. These isolates were all purified and identified to species level as described by Van Winkelhoff et al. (2002).

Zygosity testing

First information about zygosity was obtained during the questionnaire-based medical history by asking the participant if he/she was part of a MZ or DZ twin. Aware of the possibility of zygosity misclassification solely by self-report (with an agreement between zygosity diagnoses from questionnaire and DNA data of 97%), and to verify the obtained verbal report, DNA testing from each member of the twin pair was done (Middeldorp et al. 2006, Reed et al. 2005, Rietveld et al. 2000). Genomic DNA from all twin pairs (38 subjects) was extracted from EDTA venous blood samples with a commercially available DNA purification kit according to the manufacturer's instructions (Puregene DNA isolation kit, Gentra Systems, Minneapolis, MN, USA). Thereafter, zygosity was assesssed by the department of paternity testing (Sanquin Diagnostic Services, Sanquin, Amsterdam, The Netherlands) by testing 17 autosomal short tandem repeats (STR) loci. The PCR amplification was performed using the fluorescent STR multiplex system PowerPlex16 (Promega, Madison, WI, USA) and the AmpFISTR™PCR Amplification kit (Applied Biosystems, Foster City, CA, USA). The PCR products were separated by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Data analysis was performed using the GeneScan Analysis and Genotyper software (Applied Biosystems) and further statistical analysis was performed using the Kinship program (Brenner 1997).

Life style characteristics

Data on life style characteristics were obtained by means of a self-administered questionnaire. For assessment of the education level, the reported highest education level was used and classified according to 3 categories: 1. secondary education low level, 2. secondary education high level and 3. higher education. In the questionnaire, current and former smokers were asked to estimate their daily number of cigarettes usually smoked and the number of years they had

smoked. Pack-years were calculated on the basis of 20 cigarettes per pack (Grossi et al., 1994). The BMI (kg/m^2) was calculated on the basis of the self reported height and weight.

Data Analysis

Twins were considered both as individuals and as members of a pair depending on the analysis. After both subjects of each twin pair were clinically examined, members of each twin pair were classified as either the *proband* or *co-twin*. The term proband is used to define the sib showing the greatest mean AL, and the remaining brother/sister is termed the *co-twin*.

Descriptive statistics and data analysis were performed with statistical software from SPSS (version 14.0 for Windows, Chicago, IL, USA). First the data were analysed whether they showed normal distributions (Kolmogorov-Smirnov goodness-of-fit test $p<0.05$). For comparisons between probands and co-twins irrespective of zygosity, paired t-tests and Wilcoxon matched-pairs signed ranks tests were used when appropriate. A repeated measures ANOVA was employed for comparisons between MZ probands and MZ co-twins versus DZ probands and DZ co-twins followed by paired t-tests to assess difference between probands and co-twins. In case of non-normal distributions, differences between MZ twins and DZ twins were tested by means of the Mann-Whitney U test followed by Wilcoxon matched-pairs signed ranks tests for comparisons between probands and co-twins within each twin type. In DZ twins, a multivariate analysis (backward stepwise linear regression with $p\leq0.10$ to enter and $p\leq0.05$ to leave) was performed to identify factors explaining the observed variation in periodontal breakdown between DZ probands and co-twins. The predictor variables entered were smoking, education, BMI and periodontal pathogens subgingivally. p values <0.05 were considered statistically significant.

Results

Results of the zygosity testing by means of DNA analysis showed that one twin pair classified as DZ by questionnaire was typed to be MZ. Thus, the final study sample consisted of 10 MZ twin pairs (6 female and 4 male) and 8 DZ twin pairs (7 same-sexed pairs: 6 female and 1 male, and 1 opposite-sexed pair). The aid of the Dutch Association of Twins resulted in 6 MZ twin pairs and the contribution of the periodontal clinics included 4 MZ and 8 DZ twin pairs.

Descriptive characteristics of the study population regarding demographic and life style data, clinical parameters and oral microbiological parameters are presented in Table 1 for probands and co-twins, irrespective of zygosity. The mean age was 48.2 years and close to 75% of the participants were females. The majority of the subjects had completed the high level of secondary education or higher education. In this respect no difference could be assessed between probands and co-twins.

With regard to smoking, a minority of subjects were never smokers i.e. 2 and 5 out of the 18 probands and co-twins respectively. The probands included more current or former smokers compared to the co-twins. They also showed a higher number of pack-years although this failed to reach the level of statistical significance. The mean BMI was of normal weight and comparable between probands co-twins.

The number of teeth ranged between 12-32 in the probands and between 17-29 in the co-twins. Comparing probands and co-twins for their periodontal condition, analysis showed significant higher values for probing pocket depth, attachment loss, number and percentage of teeth with attachment loss $\geq$ 5 mm, percentage of teeth with $\geq$ 30% and with $\geq$ 50% bone loss in the probands compared to the co-twins (Table 1). Regarding the oral presence of periodontal bacteria the results showed that *P. gingivalis* was more prevalent in probands than in their co-twins (p=0.03). Few subjects harbored *A. actinomycetemcomitans* and *C. rectus*, whereas *F. nucleatum* was present in all twins (Table 1).

Table 1. Demographic, lifestyle, clinical and laboratory data in probands and co-twins of MZ and DZ twins combined.

Parameters	Probands (N= 18)	Co-twins (N= 18)	p-value
Age	48.2 ± 12.0	48.2 ± 12.0	nd
Gender (female)	14	13	nd
Education			
Secondary education			
low level	4	2	
high level	7	7	0.19
Higher education	7	9	
Smoking status			
Never smokers	2	5	
Former smokers	9	9	0.04
Current smokers	7	4	
Pack-years	12.2 ± 10.8	6.9 ± 7.2	0.08
Body MassIndex (kg/cm^2)	23.8 ± 2.5	23.6 ± 2.5	0.77
Clinical parameters			
No. of teeth	23.8 ± 5.2	25.4 ± 3.1	0.17
Plaque Index	0.9 ± 0.6	0.9 ± 0.3	0.98
Bleeding on probing	0.8 ± 0.6	0.8 ± 0.4	0.90
Probing pocket depth	3.4 ± 0.9	2.8 ± 0.5	0.02
Attachment loss (AL)	3.0 ± 1.4	1.4 ± 0.6	< 0.001
# of teeth AL ≥ 5 mm	9.1 ± 6.0	1.8 ± 2.2	< 0.001
% teeth AL ≥ 5 mm	39.0 ± 24.9	2.7 ± 7.1	< 0.001
% of teeth ≥30% bone loss	59.4 ± 39.4	15.7 ± 17.4	< 0.001
% of teeth ≥50% bone loss	14.4 ± 14.0	2.7 ± 0.1	0.006
Bacteriological parameters: culture positive at subject level			
A. actinomycetemcomitans	1	3	0.16
P. gingivalis	9	3	0.03
P. intermedia	8	8	1.00
T. forsythia	14	12	0.48
P. micra	15	14	0.66
F. nucleatum	18	18	1.0
C. rectus	2	1	0.32

Descriptive characteristics of periodontal data for MZ and DZ sibs separately, are presented in Table 2. It can be seen that the DZ probands showed the most severe periodontal condition in terms of attachment loss and bone loss. Both within MZ twins and within DZ twins, analysis showed that the probands had a worse periodontal condition compared to their co-twins. The differences between probands and co-twins were smaller in the MZ twins compared to the DZ twins. The periodontal condition of the MZ co-twins was very similar to that of the DZ co-twins. Both co-twin groups were suffering from periodontitis to a lesser extent since they showed either no teeth with attachment loss ≥ 5 mm or only a few.

Age and lifestyle characteristics of MZ and DZ twins are presented separately in Table 3. The mean age of the MZ and DZ twins was comparable i.e. 49.5 and 48 years respectively. No differences could be assessed between MZ sibs as well as DZ sibs for education level. However, the difference between probands and co-twins was significantly smaller in MZ twins compared to DZ twins. MZ sibs had the same education, 9 out of the 10, whereas in the DZ sibs this was 5 out of 8. Furthermore, in 2 DZ twins the probands had the lowest education level whereas their co-twins had the highest.

Evaluation of smoking behavior showed that in MZ twins 8 probands and 7 co-twins were smokers or former smokers, whereas in the DZ twins the numbers were 8 and 6 respectively (Table 3). The probands and co-twins of the MZ twins showed a comparable amount of pack-years (mean difference 0.9 ± 7.6) ranging between 0.5-28 and 2-22 pack-years respectively. In the DZ twins the probands smoked significantly more than their co-twins, 18.7 versus 5.6 pack-years respectively (mean difference 13.1 ± 12.9). The difference in pack-years between probands and co-twins was significantly smaller in MZ twins compared to DZ twins (Table 3). Analysis of the 4 groups showed that the probands of the DZ twins had the highest amount of pack-years. To further explore which factors explained significantly the observed difference in periodontal breakdown between DZ probands and co-twins a linear regression analysis was performed. For the number of teeth with AL ≥ 5 mm a final model was obtained in which only the number of pack years was retained as significant ($p = 0.004$) explaining 45.6% of the variation.

Eighteen of the 20 MZ sibs were of normal weight i.e. the BMI was below 25 kg/m^2. One MZ proband and 1 non-related co-twin were overweight: BMI values of 26.6 and 29.5 kg/m^2 respectively. In the DZ twins, 6 sibs, 4 probands and 2 co-twins, were overweight, BMI values ranging between 25.2-29.4 and 25.4-27.8 kg/m^2

respectively. The difference in BMI between the MZ sibs (1.1 ± 2.2 kg/m^2) was significantly smaller than between the DZ sibs (1.9 ± 3.2 kg/m^2) (Table 3).

Table 2. Periodontal characteristics (mean values ± SD) in monozygotic (MZ) and dizygotic (DZ) twins

	MZ (N= 10 pairs)			DZ (N= 8 pairs)			
Clinical parameters	Proband	Co-twin	p-value	Proband	Co-twin	p-value	p-value* dMZ versus dDZ
# of teeth	24.7 ± 4.1	25.0 ± 3.5	0.85	22.8 ± 6.5	26.0 ± 2.7	0.07	0.20
Plaque Index	1.1 ± 0.5	0.9 ± 0.4	0.21	0.6 ± 0.4	0.9 ± 0.2	0.015	0.05
Bleeding on probing	1.0 ± 0.5	0.9 ± 0.5	0.39	0.5 ± 0.4	0.6 ± 0.3	0.52	0.30
Probing pocket depth	3.4 ± 0.7	2.9 ± 0.5	0.09	3.4 ± 1.1	2.7 ± 0.3	0.12	0.59
Attachment loss (AL)	2.3 ± 1.3	1.6 ± 0.8	0.04	3.5 ± 1.2**	1.2 ± 0.4	<0.0001	0.01
# of teeth AL ≥ 5 mm	7.2 ± 5.2	2.2 ± 2.4	0.005	11.2 ± 6.4**	1.4 ± 2.1	0.001	0.08
% teeth AL ≥ 5 mm	30.3 ± 22.1	9.2 ± 9.7	0.005	50.0 ± 25.1**	5.4 ± 7.8	0.001	0.03
% teeth ≥ 30% bone loss	41.7 ± 29.3	15.6 ± 17.7	0.006	81.5 ± 40.1**	15.7 ± 18.1	0.001	0.01
% teeth ≥ 50% bone loss	8.4 ± 9.7	3.2 ± 8.9	0.20	21.7 ± 15.5**	2.1 ± 4.0	0.01	0.05

* p-values indicate whether the differences (d) between MZ twins are significantly different from those of DZ twins.
** values of DZ probands are significantly higher compared to MZ probands and MZ co-twins $p < 0.01$

Table 3. Age and lifestyle characteristics education, smoking and Body Mass Index (BMI) of monozygotic (MZ) and dizygotic (DZ) twins. Number of subjects and mean values (SD) for pack-years are presented.

	MZ (N= 10 pairs)			DZ (N= 8 pairs)			
Lifestyle characteristics	Proband	Co-twin	p-value	Proband	Co-twin	p-value	p-value* dMZ versus dDZ
Age	49.5 ± 13.6			48.0 ± 10.3		0.26	
Education							
Secondary education							
low level	1	1		3	1		
high level	4	5	0.19	3	2	0.10	0.03
Higher education	5	4		2	5		
Smoking status							
Never smoker	2	3		0	2		
Former smoker	5	4	0.56	4	5	0.06	0.12
Current smoker	3	3		4	1		
Smoking							
Pack-years	7.1 ± 9.8	8.0 ± 8.8	0.87	18.7 ± 8.8**	5.6 ± 4.9	0.02	0.01
Body Mass Index (kg/m^2)	22.8 ± 1.9	23.9 ± 2.4	0.94	25.0 ± 2.7	23.1 ± 2.6	0.12	0.03

* p-values indicate whether the differences (d) between MZ twins are significantly different from those of DZ twins.
** values of DZ probands are significantly higher compared to MZ probands and MZ co-twins $p < 0.05$

Table 4. Prevalence of periodontal pathogens on a subject level and at 4 oral sites in monozygotic (MZ) and dizygotic (DZ) twins. *a)* Number of positive subjects and mean proportions in positive subjects (in parenthesis) are presented, *b)* Number of positive subjects.

		MZ (N= 10 pairs)		DZ (N= 8 pairs)	
Bacteriological parameters		Proband N (%)	Co-twin N (%)	Proband N (%)	Co-twin N (%)
a) Per site					
Subgingival plaque	*A. actinomycetemcomitans*	0	2 (4.0)	1 (2.0)	1 (0.9)
	P. gingivalis	5 (18.5)	2 (14.8)	4 (16.1)	1 (56.3)
	P. intermedia	5 (3.2)	3 (0.5)	2 (1.3)	4 (1.4)
	T. forsythia	9 (3.6)	7 (1.7)	5 (5.1)	5 (3.2)
	P. micra	9 (5.1)	9 (3.6)	5 (10.0)	5 (7.5)
	F. nucleatum	10 (3.9)	10 (3.8)	8 (8.7)	8 (5.1)
	C. rectus	0	0	2 (12.5)	1 (3.0)
Supragingival plaque	*A. actinomycetemcomitans*	0	1 (0.01)	1 (0.01)	1 (0.01)
	P. gingivalis	4 (1.4)	2 (1.2)	3 (3.4)	1 (0.8)
	P. intermedia	2 (0.4)	2 (5.5)	0	2 (0.7)
	T. forsythia	6 (0.4)	1 (0.3)	3 (2.0)	2 (2.0)
	P. micra	5 (0.5)	2 (2.0)	3 (1.0)	4 (1.3)
	F. nucleatum	9 (1.8)	9 (1.8)	7 (1.0)	8 (2.3)
Tongue	*T. forsythia*	2 (0.5)	0	1 (0.04)	1 (0.01)
	P. micra	1 (2.0)	2 (0.2)	1 (1.3)	1 (0.1)
	F. nucleatum	8 (0.3)	8 (1.0)	6 (1.8)	5 (1.2)
Mucosa	*P. intermedia*	1 (0.2)	0	0	1 (0.06)
	P. micra	0	1 (0.3)	1 (0.01)	0
	F. nucleatum	3 (0.6)	4 (0.8)	5 (0.2)	6 (0.8)
b) All sites	*A. actinomycetemcomitans*	0	2	1	1
	P. gingivalis	5	2	4	1
	P. intermedia	7	3	2	5
	T. forsythia	9	7	5	5
	P. micra	10	9	5	5
	F. nucleatum	10	10	8	8
	C. rectus	0	0	2	1

No significant differences were found

In Table 4 the prevalence of the periodontal pathogens at the various oral sites is presented for MZ and DZ twins separately. It can be seen that the highest prevalence of periodontal pathogens was found in the subgingival plaque followed by the supragingival plaque. For all oral sites no significant differences were found between the probands and co-twins of both the MZ as well as the DZ twins. Also no significant differences could be assessed between the intra-pair discrepancies of MZ and DZ twins. Although the prevalence values of the periodontal pathogens were the highest in the MZ probands compared to the other 3 groups this failed to reach the level of significance. Nevertheless *P. gingivalis* was both in MZ and DZ probands present subgingivally in half of the subjects, whereas for the co-twins this was 2 out of 10 and 1 out of 8 for MZ and DZ respectively. Further analysis of the subgingival presence of *P. gingivalis* in DZ twins revealed that in the only case that the co-twin was *P. gingivalis* positive, the proband twin was positive as well. For the MZ twins it was found that in 1 twin pair both sibs were positive, in 4 twin pairs only the probands were positive and in 1 twin pair only the co-twin was *P. gingivalis* positive.

Analysis of the periodontal condition of MZ twins with regard to subgingival presence of *P. gingivalis* showed no difference between *P. gingivalis* positive and negative subjects. However, in DZ twins, when *P. gingivalis* positive and negative subjects were compared it was found that *P. gingivalis* positive subjects had more attachment loss (mean attachment loss (mm): 3.7 ± 1.6 vs 1.7 ± 0.9, $p=0.007$), a higher percentage of teeth with AL ≥ 5 mm (57.0 ± 33.5 vs 14.2 ± 14.3, $p=0.003$) and a higher percentage of teeth with bone loss $\geq 30\%$ (86.7 ± 55.7 vs 31.3 ± 28.7, $p= 0.01$).

Discussion

Historically, most studies on the heritability of periodontitis concentrate on segregation analysis of nuclear families selected on the basis of probands suffering from Juvenile Periodontitis/ Early Onset Periodontitis (EOP). The results of these studies all suggested a substantial role for genetics in the development of EOP (Loos et al. 2008). The most powerful tool to study the heritability of periodontitis is the twin model. Only one study has evaluated the periodontal condition in terms of probing depths in juvenile MZ and DZ twins and found no evidence that pocket depth was an inherited characteristic (Ciancio et al. 1969). This finding is most likely due to the inherent difficulties in finding the appropriate young twins suffering from severe periodontitis, which at that age has a very low prevalence.

For the current study it was anticipated that for chronic periodontitis twin studies may be easier to perform since the prevalence of the disease is approximately 10% (Albandar et al. 1999, Page & Eke 2007). Unfortunately, it appeared that also for chronic periodontitis it was extremely difficult to recruit moderate to severe periodontitis patients having a MZ or DZ sib as a primary selection criterion. Nevertheless a major effort was made to obtain these patients from private periodontal clinics, the clinic of the department of periodontology of ACTA and with the aid of the Dutch Association of Twins.

To date results of twin studies of chronic periodontitis show converging results suggesting a substantial role of genetics in this condition (Corey et al. 1993, Michalowicz et al. 1991b, Mucci et al. 2005). Nevertheless, these studies have some limitations. The results of the study of Corey et al. (1993) and Mucci et al. (2005) were based on questionnaires and not on clinical measurements. The studies of Michalowicz et al. (1991a,b, 2000) included study populations with relatively minor periodontal destruction. Up to now all twin studies have been based on subjects selected because of their twinship and not on the presence of moderate to severe periodontitis. Interestingly, a case report of a 40 year old MZ twin presented a proband who suffered from localized moderate to severe alveolar bone loss around several premolars and molars whereas her twin sister had shallow pockets and essentially normal bone architecture (McDaniel et al. 1999). One of the present authors (UvdV) also came across such a case in his practice. Therefore, the aim of the present study was to initiate a twin study in which twins were selected on the basis of a proband with moderate to severe periodontitis. Consequently, the patient selection for this study was based on the presence of interproximal attachment loss $\geq$ 5 mm at $\geq$ 2 non-adjacent teeth. Surprisingly, the plaque and bleeding scores were relatively low. This phenomenon was mainly due to the 12 twin pairs of which the proband was referred to the periodontal clinics. The subjects of these twins had mean plaque and bleeding score scores of 0.7 and 0.6 respectively, whereas the plaque and bleeding scores of the 6 twin pairs recruited with the aid of the Dutch Association of twins amounted to 1.26 and 1.23 respectively (p-values $<$ 0.001). Most likely, the lower plaque and bleeding scores of the referral twins is caused by previous treatment in the practice of the general practitioner before referral, resulting in improved oral hygiene and reduced inflammation at shallow pockets in the probands.

The most important result of this study is the finding that MZ twins appeared to be discordant with regard to mean attachment loss, number and percentage of teeth with AL $\geq$ 5 mm and percentage of teeth with bone loss $\geq$ 30 %. This finding is in agreement with the results of Tabrizi et al. (2007) who found in monozygotic twins discordant for coronary hart

disease that the twin patient with coronary hart disease was also discordant for periodontal breakdown. Although the number of twins included in the present study was rather small, the statistical power for the assessed differences was at or above 80%. These discrepancies can obviously not be explained by basic variations in genetic make-up, neither could it be explained by the prevalence of periodontal pathogens nor by the life style factors education, smoking and BMI, factors that are all known to be related to destructive periodontal disease (Grossi et al. 1994, Tomar & Asma 2000, van Winkelhoff et al. 2002, Ylostalo et al. 2008). As a matter of fact these life style factors were concordant and in agreement with the literature: there is consistent evidence from twin studies that genetic factors play a role in educational attainment (Silventoinen et al. 2000, Silventoinen et al. 2004), smoking (Munafo & Johnstone 2008, Vink et al. 2005) and BMI (Maes et al. 1997, Schousboe et al. 2003). Since MZ twins are discordant for the amount of periodontal breakdown, it is not surprising that the DZ twins are discordant as well. It must be noted that DZ sibs differed to a greater extent from each other than the MZ sibs, confirming that the genetic component does play a role. The DZ probands showed the worst periodontal condition compared DZ co-twins, MZ probands and MZ co-twins. This finding is in line with the studied life style factors, because this group included only 2 subjects with higher education, showed the highest percentage of smokers with the highest number of pack-years, and 4 out of 8 subjects were overweight. In the study population all subjects had attachment loss to some extent; the least in one MZ co-twin having at 3 interproximal sites 2 mm attachment loss and the most in one DZ proband having at 9 teeth (40%) 9 mm or more interproximal attachment loss. Results showed that for all parameters of periodontal breakdown used, all individual probands had more periodontal breakdown than their co-twins. The finding that MZ twins are discordant for the amount of periodontal breakdown could imply that the influence of genetics in the development of chronic periodontitis may have been overestimated although it may still play a significant role.

The subgingival microbiological profile of the probands of the present study population, consisting of subjects with moderate to severe periodontitis and having a mean age of 48 years, is in agreement with the prevalence of periodontal pathogens in periodontitis patients of that age (van Winkelhoff et al. 2002). The prevalence of periodontal pathogens on the mucous membranes seems somewhat lower than expected (Van der Velden et al. 2006). The additional sampling of the mucous membranes did not give extra information compared to supra- and subgingival sampling only. In the present study no statistical significant influence of the microbial flora could be assessed. However, it must be realized that the

number of twins in the present study is small and the statistics did not include corrections for multiple comparisons. Therefore, from one point of view the results on the basis of p-values ≥ 0.01 should be interpreted with care. However, on the other hand the small number of twins may have been also responsible for the many non significant differences, e.g. the subgingival presence of *P. gingivalis*. In the MZ twin group, 5 out of the 10 probands were positive for *P. gingivalis,* whereas 2 out of the 10 co-twins were positive for this bacterium. A larger study population of MZ twins could have shown that *P. gingivalis* plays a significant role in the etiology of periodontitis.

At present the discordant MZ twin model is regarded as the best option to study the etiology of a disease (Vaag & Poulsen 2007). Discordance between MZ twins regarding diseases has been reported for a number of disorders. For example, it has been found that the discordance of MZ twins for a complex disease like rheumatoid arthritis may amount to about 85% (Silman et al. 1993). Because MZ twins start life with identical genomes, within twin pair differences reflect exposure to an individual-specific environment and life style which may ultimately act through genetic or epigenetic modifications of gene expression (den Braber et al. 2008). Epigenetic mechanisms result in heritable modifications of the DNA, resulting in variation of expression of genes independent of basic DNA code. (Petronis 2001) suggested that epigenetic mis-regulation of genes is more consistent with features of complex diseases than the DNA sequence. Discordance of MZ twins is usually explained by the differential effect of environmental and life style factors. At present both aging, smoking and environmental factors like nutrition have been shown to be involved in epigenetic changes (Fraga et al. 2005, Zochbauer-Muller et al. 2001, Kauwell 2008) possibly explaining discordance in MZ twins. Epigenetic changes may be important for controlling the immune and inflammatory responses and thus for controlling periodontitis (Wilson 2008). DNA modifications by environmental and life style factors (epigenetics) could explain the discordance in the periodontal condition of the MZ twins in the present study.

In conclusion, since in the present study MZ sibs are discordant regarding the amount of periodontal breakdown, the role of genetics in the development of chronic periodontitis may have been overestimated although it clearly plays a role. In addition, differences in the periodontal condition of the MZ sibs could not be explained by differences in the microbial flora nor by the life style factors education, smoking and BMI. Furthermore, the factors that play an important role in the development of chronic periodontitis have yet to be determined. To this end, studies including large numbers of MZ twins selected for the presence of moderate to severe periodontitis are needed.

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