Periodontitis in twins : smoking, microbiological and immunological aspects

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Chapter 5

Monozygotic twins are discordant for chronic periodontitis

White blood cell counts and cytokine production after *ex vivo* stimulation

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Abstract

Objectives: The aim of this study was to investigate the extent of concordance in number of leukocytes and their cytokine secretion after *ex vivo* stimulation in a twin population discordant for the amount of periodontal breakdown.

Material and Methods: Venous blood was collected from 18 adult twin pairs (10 monozygotic and 8 dizygotic twins). Each twin pair consisted of a diseased twin (proband) and his/her co-twin. In venous blood, leukocytes were counted. The cytokines interleukin (IL)-1β, IL-6, IL-8, IL-10 and IL-12p40 were assessed after stimulation of monocytic cells, while IL-13 and interferon (IFN)-γ were determined after lymphocytic stimulation.

Results: In the study population as a whole probands showed higher total numbers of leukocytes and lower IL-12p40 levels compared to their co-twins. In monozygotic twins no difference was found in leukocyte counts, but probands secreted more IL-6 than their co-twins; an opposite trend was found for IL-12p40.

Conclusion: The results suggest that the observed discordance in periodontal breakdown in the studied monozygotic twin population may be related to relatively high levels of IL-6 and low levels of IL-12p40 secretion after *ex vivo* stimulation of whole blood cell cultures. This cytokine secretion profile may be regarded as a risk indicator of periodontitis.

Introduction

Periodontitis is a chronic, multifactorial, infectious disease of the supporting tissues of the teeth characterized by gradual loss of periodontal attachment and alveolar bone (Kinane & Lappin 2001). Periodontitis is initiated by microbial plaque, which accumulates in the gingival crevice region and induces an inflammatory response. Although bacteria are essential for the induction of the inflammatory response, they are insufficient to cause the disease (Page et al. 1997). In conjunction with the bacterial challenge, the host immune response plays an important role in the onset and progression of periodontitis (Ebersole & Taubman 1994). In fact, variability in host response may be a component of a genetic predisposition to periodontitis (Michalowicz et al. 1991). It is possible that genetically determined differences in immune regulation or in homeostatic bone remodeling are also important to the outcome of periodontal disease (Baker et al. 2000, Kornman et al. 1997).

In the innate and adaptive immunity, monocytes play an orchestrating role. When triggered by bacteria, monocytes produce cytokines which direct both immune responses (Seymour & Gemmell 2001). Furthermore, cytokines derived mainly from dendritic cells, monocytes and macrophages, play a pivotal role in directing lymphocytic differentiation of non-committed precursors CD4+ T cells into either T helper type 1 (Th1) or Th2 cells. Previous studies have shown that periodontitis patients display a monocytic-cytokine profile which may favor a Th2 immune response (Fokkema et al. 2002, Gemmell & Seymour 1994). Interestingly, the monocytic directional Th2 response is even more pronounced in smokers (Torres de Heens et al. 2009). It is likely that changes in cytokine profiles that modulate the Th1/Th2 balance may affect the susceptibility to or the course of the periodontal infection (Gemmell et al. 2002). Studies in infectious diseases other than periodontitis provide convincing evidence that host genetic factors are important in determining who will succumb to the pathogen and who will not (Davies & Grange 2001, Lama & Planelles 2007). Susceptibility or resistance to many infectious diseases is dependent on genetically controlled differences in inflammatory responses, cytokine secretion, or T-cell recruitment after exposure to the pathogen (Gervais et al. 1984, Skamene 1994, Stevenson & Tam 1993). Moreover, twin studies have confirmed a genetic component for cytokine production (Grainger et al. 1999, Reuss et al. 2002).

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. 1991, Michalowicz et al. 2000, Mucci et al. 2005). Nevertheless these studies have limitations. The results of Corey et al. (1993) and Mucci et al. (2005) are based

on self reported evidence of periodontal disease. Subjects in the studies of Michalowicz et al. (1991a, b, 2000) were selected based on their twin ship rather than their periodontal condition, resulting in a population with mild periodontal breakdown. Therefore a twin study was initiated that studied in monozygotic (MZ) and dizygotic (DZ) twin pairs selected on the basis of one sib of a twin pair (the proband) suffering from moderate to severe chronic periodontitis, the contribution of genetics, life style factors and periodontal pathogens to the clinical phenotype of the disease (Torres de Heens et al. 'in press'). The clinical results showed that the MZ twins were discordant regarding attachment loss and bone loss. The discordance was greater in DZ compared to MZ twins. In MZ twins the discordance could not be explained by education, smoking, Body Mass Index or presence of periodontal pathogens. In DZ twins the discordance could be explained by more cigarette smoking of the probands. The aim of the present study was to investigate the extent of concordance in number of white blood cells and monocytic and lymphocytic cytokine secretion after *ex vivo* stimulation among the previously studied twin population selected on the basis of one sib of a twin pair suffering from moderate to severe chronic periodontitis

Material and Methods

Subjects

Twin subjects were recruited as previously described (Torres de Heens et al. 'in press'). In short, subjects were recruited among patients referred to various periodontal clinics across the Netherlands for the treatment of periodontitis, and from members of the Dutch Association of Twins whose periodontal status met the inclusion criteria of our study. The selection criteria 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 ≥5mm in ≥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. Approval for this study was obtained by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam.

The study population consisted of 18 reared-together twin pairs and prior to the clinical examination a verbal and written informed consent was obtained from all twins. The clinical examination was carried out at the interproximal sites of all teeth from buccal and lingual aspects. The following assessments were performed: Plaque Index according to

(Silness & Löe 1964); bleeding on probing recorded as 0 = no bleeding, 1 = point bleeding within 30 seconds, 2 = immediate and overt bleeding; probing pocket depth, 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 Hu-Friedy® PQW probe (Chicago, Illinois, USA). In addition, the participants underwent a full-mouth radiographic survey on which all teeth were 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. By means of the Schei ruler technique, the percentage of bone loss at the deepest interproximal site of each tooth was measured (Schei et al. 1959). Reported smoking habits of the twins were recorded in pack-year according to three groups: (1) non-smokers: subjects who had never smoked, (2) former smokers: subjects that stopped smoking before entering the study, and (3) smokers: subjects who were current smokers. Zygosity was assessed by the department of paternity testing (Sanquin Diagnostic Services, Sanquin, Amsterdam, The Netherlands) by testing 17 autosomal short tandem repeats (STR) loci as previously described (Torres de Heens et al. 'in press')

Venous blood collection and differential cell counts

From each subject, venous blood was collected by venipuncture from the antecubital fossa in sterile pyrogen-free blood collection tubes. For whole blood cell cultures (WBCC) sodium heparine tubes were used (Vacuette, Greiner, Alphen a/d Rijn, The Netherlands). For differential blood cell counts EDTA (K_3)-containing tubes (Becton Dickinson Vacutainer System Europe, Meylan, France) were used and the cell counts (neutrophils, eosinophils, basophils, lymphocytes and monocytes) carried out in the clinical chemistry laboratory of the Slotervaart Hospital Amsterdam, The Netherlands, using standard automated procedures (Cell-Dyn 4000, Hematology Analyzer, Abbott Laboratories, Park, Illinois, U.S.A.).

Preparation of stimuli

Three stimuli for WBCC were used in the study:

(1) Lipo-oligosaccharide (LOS) was purified from *Neisseria meningitides* strain H44/76 (a kind gift from Dr. J. Poolman, Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven, The Netherlands (van der Pouw Kraan et al. 1995).

(2) *Porphyromonas gingivalis* (*Pg*) strain 381 was grown in brain heart infusion broth enriched with hemin (5 mg/l) and menadione (1 mg/l) in an anaerobic atmosphere (80% N_2, 10% H_2, 10% CO_2) for 48 h at 37 °C. Bacteria were harvested in the log phase, pelleted by

centrifugation (8000 *g*), washed three times in PBS, and resuspended in PBS at an optical density of 1 at 690 nm, corresponding approximately to 7 x 10^8 CFU/ml. Aliquots (500 µl) of resuspended bacteria were disrupted using a sonifier in a sonicating vessel on ice (Soniprep MSE 150, York, United Kingdom; amplitude 18, 4 min, 5 sec intervals). The degree of disruption of the bacteria was assessed by phase-contrast microscopy and with Gram-staining by light microscopy. Sonicates were stored at 4 ºC until use. Before use, *P. gingivalis* sonicates were centrifuged (8000 x *g*, 1 min) and used in WBCC as described below.

(3) Mouse monoclonal antibodies raised against human CD3 (anti-CD3, CLB-T3/4.E) and CD28 (anti-CD28 CLB-T3/4.E) were obtained from Sanquin, Amsterdam, The Netherlands (van Lier et al. 1987).

Whole Blood Cell Cultures

WBCC were performed in 96-well flat bottom microtitre plate (Nunc, Roskilde, Denmark). Heparinized venous whole blood was diluted 1/10 with Iscove's modified Dulbecco's medium (IMDM, Bio Whittaker, Verviers, Belgium), supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml) (Gibco, Merelbeke, Belgium), 0.1% endotoxin-free fetal calf serum (FCS, Bodinco, Alkmaar, The Netherlands), and 15 U/ml sodium heparin (Leo Pharmaceutical Products B.V., Weesp, The Netherlands).

Two hundred µl of the diluted blood was stimulated during 18 hours with LOS at a final concentration of 1000 pg/ml or with *Pg* sonic extract (*Pg*-SE) 1:100 dilution, in the presence of anti-CD3 at 1 µg/ml. LOS and *Pg*-SE concentrations used have been previously shown to be the most optimal concentration for these stimulation assays (Torres de Heens et al. 2009). Unstimulated diluted whole blood served as a negative control. Supernatants were harvested and stored at -20 °C until cytokine measurements (Gerards et al. 2003).

To stimulate T lymphocytes a combination of a mouse monoclonal antibody against human CD3 and CD28 was added to the 200 µl aliquot of diluted whole blood, as previously described (Gerards et al. 2003). Cultures were performed in duplicate and unstimulated diluted whole blood served as a negative control. After 72 hours of incubation with anti-CD3/anti-CD28, supernatants were harvested and stored at -20 °C until cytokine analysis was performed.

Assays for cytokines

Cytokine levels of IL-1β, IL-6, IL-8, IL-10, IL-12p40, IL-13 and IFN-γ were measured in the supernatants of WBCC using commercially available enzyme-linked

immunosorbent assay (ELISA) kits (PeliKine Compact™ human ELISA kits, Sanquin, Amsterdam, The Netherlands) as previous described (van der Pouw Kraan et al. 1997). The plates were read in an ELISA reader (Labsystems Multiskan Multisoft, Helsinki, Finland) at 450 nm, with 540 nm as a reference. Cytokine production of IL-1β, IL-6, IL-8 and IL-12p40 was adjusted for the number of monocytes and neutrophils, IL-10 only for the number of monocytes and IL-13 and IFN-γ for the number of lymphocytes.

Data Analysis

Twins were considered both as individuals and as members of a pair depending on the analysis. 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 attachment loss (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 analyzed whether they showed normal distribution (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 a 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-pair signed ranks tests for comparisons between probands and co-twins within each twin type. The significance was set at $p<0.05$.

Results

Descriptive characteristics of the twin population including demographic, life style, clinical and microbiological data have been reported before (Torres de Heens et al. 'in press'). In brief, 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 mean age was 48.2 years and the probands included more current or former smokers than their co-twins. Probands showed more attachment- and bone loss than their co-twins which was highly significant (Table 1). White blood cell counts revealed that the total number of leukocytes and lymphocytes were significantly higher in probands than in their co-twins.

Table 1. Background clinical parameters and White blood cell (WBC) data (mean values ± SD) in probands and co-twins of MZ and DZ twins combined.

Parameters	Probands (N= 18)	Co-twins (N= 18)	p-value
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 ≥30% bone loss	59.4 ± 39.4	15.7 ± 17.4	< 0.001
Smoking status			
Non- or former smoker	12	14	0.31
Current smoker	6	4	
Total Leukocytes (10^9/L)	7.41 ± 2.84	6.13 ± 1.74	0.02
Monocytes	0.53 ± 0.14	0.50 ± 0.12	0.48
Lymphocytes	2.50 ± 0.77	2.11 ± 0.61	0.04
Neutrophils	4.16 ± 2.15	3.39 ± 1.27	0.09
Basophils	0.02 ± 0.04	0.01 ± 0.03	0.41
Eosinophils	0.13 ± 0.10	0.16 ± 0.07	0.26

Table 2 presents data on cytokine secretion in the supernatants of WBCC from all the probands and their co-twins, after stimulation with LOS, *Pg* and anti-CD3/CD28. Probands showed significantly lower amounts of IL-12p40 after LOS stimulation. With regard to the other cytokines measured after LOS stimulation, no differences were found between the probands and their co-twins. After *Pg* stimulation no differences in cytokine values could be

assessed although the IL-12p40 values tended to be higher in the co-twins, but they did not reach significance (p = 0.07). IL-12p40/IL 10 ratios were calculated, but both for LOS and *Pg* stimulation no differences between probands and their co-twins were found. After anti-CD3/CD28 stimulation no significant differences in IFN-γ and IL-13 values were found between probands and their co-twins.

Table 2. Cytokine secretion (picograms/ml x 10^2): IL-1β, IL-6, IL-8, IL-12p40 adjusted for the number of monocytes and neutrophils together, IL-10 adjusted for the number of monocytes and IFN- γ and IL-13 adjusted for the number of lymphocytes, after LOS, *Pg* and anti CD3/CD28 stimulation of whole blood in monozygotic (MZ) and dizygotic (DZ) twins. Values represent mean ± SD.

Parameters	Probands (N= 18)	Co-twins (N= 18)	p-value
Cytokine production after LOS stimulation			
IL-1β	1.00 ± 0.74	1.03 ± 0.59	0.19
IL-6	6.60 ± 0.48	5.51 ± 3.82	0.10
IL-8	25.63 ± 14.44	32.06 ± 36.03	0.91
IL-10	1.64 ± 20.77	1.58 ± 2.44	0.52
IL-12p40	0.24 ± 0.16	0.34 ± 0.31	< 0.001
IL-12p40/IL10	0.01 ± 0.03	0.02 ± 0.02	0.26
Cytokine production after *Pg* stimulation			
IL-1β	1.24 ± 0.77	1.26 ± 0.83	0.61
IL-6	6.57 ± 2.81	7.06 ± 4.49	0.77
IL-8	62.56 ± 38.96	59.10 ± 38.75	0.91
IL-10	0.88 ± 1.11	1.02 ± 1.43	0.83
IL-12p40	0.29 ± 0.26	0.38 ± 0.31	0.07
IL-12p40/IL10	0.03 ± 0.06	0.02 ± 0.02	0.61
Cytokine production after CD3/CD28 stimulation			
IFN-γ	219.88 ± 28.42	216.10 ± 17.05	0.65
IL-13	13.78 ± 12.72	14.03 ± 8.53	0.40

Probands of DZ twins had the most severe attachment loss and the highest percentage of teeth with ≥ 30% bone loss. In addition, there was an absence of concordance for both these measures in both the MZ and DZ twins (Table 3). No differences in white blood cell counts were found between the MZ probands and their co-twins. Significantly higher leukocyte and lymphocyte counts were found in the DZ probands compared to their co-twins. These values were also higher than those found in the MZ probands and their co-twins. In addition, the difference in the lymphocyte counts between MZ probands and their co-twins was smaller than the difference between the DZ twin pairs.

Table 3. Background clinical parameters and white blood cell data (mean values ± SD) in monozygotic (MZ) and dizygotic (DZ) twins.

	MZ (N= 10 pairs)			DZ (N= 8 pairs)			
Parameters	Proband	Co-twin	p-value	Proband	Co-twin	p-value	p-value* dMZ versus dDZ
Clinical parameters							
# 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
% 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
Smoking status							
Non- or former smoker	7	7	1.00	5	7	0.16	0.27
Current smoker	3	3		3	1		
Total Leucocytes (10^9/L)	6.29 ± 1.54	5.85 ± 1.58	0.39	8.80 ± 3.54**	6.48 ± 1.95	0.02	0.13
Monocytes	0.49 ± 0.13	0.48 ± 0.10	0.92	0.56 ± 0.13	0.52 ± 0.15	0.35	0.43
Lymphocytes	2.09 ± 0.60	2.07 ± 0.57	0.89	3.01 ± 0.70**	2.16 ± 3.01	0.02	0.02
Neutrophils	3.59 ± 1.11	3.12 ± 1.12	0.31	4.87 ± 2.93	3.73 ± 1.43	0.18	0.77
Basophils	0.01 ± 0.03	0.02 ± 0.42	0.56	0.03 ± 0.05	0 ± 0	0.08	0.83
Eosinophils	0.09 ± 0.06	0.10 ± 0.06	0.45	0.16 ± 0.11	0.17 ± 0.06	0.43	0.72

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

In Table 4 the cytokine secretion in the supernatants of WBCC is presented for probands and their co-twins of the MZ and DZ twins. After LOS stimulation IL-6 levels in MZ twins were higher for the probands than for their co-twins. The values of the other cytokines were not significantly different between probands and their co-twins, both in MZ and DZ twins. The MZ probands as well as their co-twins had significantly higher values of IL-6 and IL-12p40 after LOS stimulation than the DZ probands and their co-twins. After *Pg* stimulation, the IL-12p40 values of the DZ twins were lower for the probands than for their co-twins. For the other cytokines after *Pg* stimulation no significant differences between probands and their co-twins were found both in MZ and DZ twins. The MZ probands as well as their co-twins had significantly higher values of IL-12p40 after *Pg* stimulation compared to DZ probands and their co-twins. With regard to the IL-12p40/IL-10 ratios both in MZ and DZ twins no differences were found between probands and their co-twins. Also after lymphocytic stimulation with anti-CD3/CD28 no significant differences between probands and their co-twins in MZ and DZ twins were found for IFN-γ and IL-13 values.

Table 4. Cytokine secretion (picograms/ml x 10^2): IL-1β, IL-6, IL-8, IL-12p40 adjusted for the number of monocytes and neutrophils together, IL-10 adjusted for the number of monocytes and IFN- γ and IL-13 adjusted for the number of lymphocytes, after LOS, *Pg* and anti CD3/CD28 stimulation of whole blood in monozygotic (MZ) and dizygotic (DZ) twins. Values represent mean ± SD.

	MZ (N= 10 pairs)			DZ (N= 8 pairs)			
Cytokine production	Proband	Co-twin	p-value	Proband	Co-twin	p-value	p-value* dMZ versus dDZ
after LOS stimulation							
IL-1β	1.08 ± 0.78	1.19 ± 0.30	0.22	0.90 ± 0.72	0.84 ± 0.37	0.57	0.75
IL-6	8.52 ± 5.67**	6.33 ± 4.65**	0.02	4.21 ± 1.85	4.48 ± 2.34	0.91	0.08
IL-8	27.15 ± 14.95	40.30 ± 46.75	0.49	23.73 ± 14.55	21.76 ± 11.16	0.81	0.49
IL-10	0.71 ± 0.77	1.11 ± 2.31	0.93	2.81 ± 2.64	2.18 ± 2.62	0.26	0.58
IL-12p40	0.29 ± 0.17**	0.46 ± 0.37**	0.11	0.18 ± 0.13	0.19 ± 0.10	0.43	0.61
IL-12p40/IL10	0.01 ± 0.01	1.78 ± 1.84	0.56	0.02 ± 0.05	0.01 ± 0.02	0.28	0.81
after Pg stimulation							
IL-1β	1.40 ± 0.88	1.52 ± 0.96	0.33	1.04 ± 0.61	0.92 ± 0.50	0.89	0.86
IL-6	7.09 ± 1.79	8.31 ± 5.41	0.83	5.90 ± 3.77	5.51 ± 2.49	0.86	0.96
IL-8	63.35 ± 43.45	66.39 ± 0.40	0.88	61.59 ± 35.45	49.10 ± 37.38	0.78	0.50
IL-10	0.42 ± 0.48	0.78 ± 1.07	0.32	1.45 ± 1.43	1.33 ± 1.82	0.51	0.24
IL-12p40	0.37 ± 0.22***	0.45 ± 0.37***	0.78	0.18 ± 0.27	0.29 ± 0.20	0.04	0.06
IL-12p40/IL10	0.04 ± 0.08	0.02 ± 0.01	0.44	0.003 ± 0.006	0.02 ± 0.03	0.19	0.11
after CD3/CD28 stimulation							
IFN-γ	300.37 ± 364.33	226.93 ± 143.71	0.64	119.27 ± 68.23	202.56 ± 208.90	0.33	0.26
IL-13	14.80 ± 12.06	12.89 ± 7.65	0.76	12.50 ± 14.24	15.45 ± 9.86	0.27	0.27

* significant p-values indicate that the differences (d) between MZ twins are significantly different from those of DZ twins.

** values of MZ probands and co-twins significantly higher compared to DZ probands and co-twins $p < 0.01$

*** values of MZ probands and co-twins significantly higher compared to DZ probands $p < 0.01$

Discussion

Periodontitis is considered to be a complex disease of which the phenotype is determined by the genetic make-up, the environmental influences and the life style of the affected individual (Loos et al. 2008). In the previous clinical analysis of our twin study the results showed that the MZ probands suffered from more severe periodontitis than their co-twins (Torres de Heens et al. 'in press'). This discrepancy between the MZ twins could not be explained by the studied life style and environmental factors, such as education, smoking and periodontal pathogens. Since the MZ probands and co-twins were significantly discordant for the amount of periodontal breakdown, it was not surprising that the DZ twins were also discordant. Analysis showed that the difference in periodontal condition between the DZ twin pairs differed to a greater extent than the differences between the MZ twin pairs, suggesting that some genetic component is at play (Torres de Heens et al. 'in press').

In order to investigate whether monocytic and lymphocytic cytokine secretion after *ex vivo* stimulation could explain the observed discordance in periodontal breakdown of MZ and DZ twins, LOS from *N. meningitidis* and a sonicate of *P. gingivalis* were used (Torres de Heens et al. 2009). In stimulation studies often LPS from *Escherichia coli* has been used, however this may be criticized since in the *in vivo* situation and during infection, *E. coli* LPS is surely not the only bacterial component interacting with immune cells (Fokkema et al. 2002). Secondly, *E. coli* is not a periodontal pathogen. In order to overcome these problems to some extent, a sonic extract of *P. gingivalis,* a major periodontal pathogen which signals through Toll-like receptor 2 (TLR2), was used. Nevertheless, it must be realized that 50% of the probands and 81% of the co-twins were culture negative for *P. gingivalis* (Torres de Heens et al. 'in press'). Therefore, in addidion to the *Pg* sonic extract, a generic stimulant (*N. meningitidis* LOS) which signals through TLR4 was used for WBCC stimulation. In our results, the amount of cytokine measured in the supernatants stimulated with LOS or *Pg* was adjusted for to the number of monocytes and/or neutrophils where appropriate. It should be noted that on a cell basis the production of IL-1β, IL-6, IL-8 and IL-12p40 from neutrophils is much less than that from monocytes, which are the principal producers of all these cytokines (Moore et al. 1993, Snijders et al. 1996, van der Pouw

Kraan et al. 1995, Wang et al. 1994). Nevertheless, there may be a significant contribution of neutrophils to the overall cytokine production during inflammation due to the quantitative predominance of these cells over the monocytes in the peripheral blood and at sites of acute inflammation. For these reasons it was decided to consider both monocytes and neutrophils as the main producing cells for the mentioned cytokine set. Levels of IL-10 were only adjusted for the number of monocytes, since neutrophils do not secrete this cytokine. IFN-γ and IL-13 production were corrected for lymphocyte cell counts because we selectively stimulated those cells with anti- CD3 and CD28 (Gerards et al. 2003, Yamada-Ohnishi et al. 2004).

Discordance of periodontal breakdown in MZ twins offers the unique possibility to disentangle the etiology of the disease. Therefore the number of leukocytes and their cytokine production after *ex vivo* stimulation were investigated. In the study population as a whole, probands showed higher total numbers of leukocytes compared to their co-twins. This discrepancy could almost completely be explained by the DZ probands having the worst periodontal condition, compared to DZ co-twins, MZ probands and MZ co-twins. The higher number of leukocytes in these subjects is in agreement with previous studies which showed that periodontitis patients have higher numbers of leukocytes compared to controls (Christan et al. 2002, Fredriksson et al. 1998, Kweider et al. 1993, Loos et al. 2000). Nevertheless, the MZ probands who suffered also from moderate to severe periodontitis, showed comparable numbers of leukocytes to their own co-twins as well as to the DZ co-twins both having a far better periodontal condition. This finding may be explained by a lower degree of severity of periodontitis in the MZ probands as compared to the DZ probands. Possibly the severity of disease of the MZ probands was not severe enough to cause a significant increase in the number of leukocytes.

Leukocytes, when triggered by whole bacteria as well as bacterial components, produce cytokines which direct both innate and adaptive immunity (Seymour & Gemmell 2001). Cytokines such as the pro-inflammatory interleukin IL-1β, IL-6, IL-8, IL-12 and anti-inflammatory IL-10 have been shown to be part of the inflammatory response in periodontitis and may determine the host susceptibility and thereby variation in periodontal destruction (Gemmell et al. 1997, Gemmell et al. 2002, Niho et al. 1998,

Seymour & Gemmell 2001). In the twin population as a whole, probands and co-twins showed no differences in cytokine secretion, except for a lower level of IL-12p40 in the probands after LOS stimulation and to a lesser extent after *Pg* stimulation. In addition, in the probands, a trend towards higher levels of IL-6 after LOS stimulation can be seen (p-value 0.10). The IL-6 data of the MZ twins show that MZ probands secrete higher levels of this cytokine than their co-twins. IL-6 is a pleiotropic cytokine and plays a major role in bone remodeling, neuro-endocrine homeostasis, haemopoiesis and immune-inflammatory response regulation. In particular, it plays a pivotal role in acute phase responses and in balancing the pro-inflammatory/anti-inflammatory pathways. As reviewed (Ershler & Keller 2000), it is suggested that elevated expression of IL-6 may contribute to (generalized) autoimmune diseases, such as rheumatoid arthritis. Since after stimulation the cells of the MZ probands secreted more IL-6 than their co-twins and the MZ probands have more periodontal breakdown an association may be suggested between hyper responsive cells secreting IL-6 and the risk for periodontal breakdown. Since the MZ twins have identical DNA sequence, other phenomena might play a role; epigenetic modifications of DNA have recently been observed (Barros & Offenbacher 2009). Whether the higher secretion level is due to epigenetic mechanisms or other than the studied environmental and life style factors education, smoking, BMI and periodontal pathogens, cannot be deduced on the basis of the present data.

The lower levels of IL-12p40 as found in the probands compared to their co-twins in the study population as a whole, is indicative for a Th2 response. This together with the greater periodontal breakdown found in the probands, is in line with the concept that considers periodontitis as a Th2-type disease. In general it is assumed that susceptible individuals may have a predominant Th2 type response, which contributes to tissue breakdown (Aoyagi et al. 1995, Bartova et al. 2000, Gemmell et al. 2007, Manhart et al. 1994, Torres de Heens et al. 2009). However in the present study this is neither supported by a lower IL-12p40/IL-10 ratio, which would strongly indicate a type 2 response, nor by the elevated IL-13 values of the probands compared to their co-twins in both MZ and DZ twins. The lack of supporting evidence may be due to the finding that the subjects in this twin population showed relatively low plaque and bleeding scores, suggestive for previous periodontal treatment in general practice before referral to the periodontal

clinics. This supposition is supported by the finding that the twins recruited via periodontal clinics have lower plaque and bleeding scores than the non referred twins recruited with the aid of the Dutch Association of Twins. The low bleeding scores, that were almost identical for probands and co-twins in both MZ and DZ twin groups, are indicative for relatively little inflammation in the periodontal pockets. Therefore, the Th2 profile of subjects with active disease may have changed into a Th1 profile associated with periodontal stability (Gemmell & Seymour 2004, Gemmell et al. 2007, Seymour et al. 1996). On the other hand the lack of supporting data of the present study towards a type 2 response is in line with the recent understanding that the Th1/Th2 paradigm cannot accurately describe periodontal disease independent of the involvement of the novel Th17 subset (Gaffen & Hajishengallis 2008). Unfortunately, IL-17 measurements were not included in the present study and therefore no real suggestions can be made. Nevertheless, in the study population as a whole, monocytes of the probands, when stimulated *ex vivo*, secreted less IL-12p40 than the co-twins. Also, in the small number of MZ twins a trend may be seen towards lower levels of IL-12p40 after LOS stimulation in the probands. If 16 instead of the present 10 MZ twins would have been included in this study, a statistical significant difference could have been assessed suggesting an association between the level of IL-12p40 secretion and disease risk. On the other hand, 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 the results on the basis of p-values ≥ 0.01 should be interpreted with care. However, the small number of twins may have been also responsible for the many non significant differences.

In the search of etiological factors for diseases in general the twin model has been used for decades and with time, epidemiologic studies included increasing numbers of twins. Traditionally, MZ and DZ twins were recruited from databases or twin registers, irrespective of disease status. However, during the last decade studies are focusing on MZ twins that are discordant for the disease in question. The discordant twin design, which may have small numbers of twins, allows the investigation of between twin differences that are specifically due to influences of environmental and life style risk factors (Martin et al. 1997). Because MZ twins start life with identical genomes, within twin pair differences reflect exposure to an individual-specific environment which may

ultimately act through genetic or epigenetic modifications of gene expression (den Braber et al. 2008). For example for the development of distinct Th cell lineages, the initial instructions are received by the naive $CD4^+$ T cells from the antigen-presenting cells. The instruction are converted by responding T cells into changes in the abundance, interactions and locations of transcription factors, which in turn lead to changes in gene expression. As suggested before (Wilson et al. 2009), more precise gene expression is achieved through epigenetic processes which facilitate heritable and stable programs of gene expression. Such mechanisms may well have been the cause for the observed differences in IL-6 and to a lesser extent IL-12p40 in the MZ twins.

In conclusion, the results of the present study suggest that the observed discordance in periodontal breakdown in the studied twin population may be related to relatively high levels of IL-6 and low levels of IL-12p40 secretion after *ex vivo* stimulation of WBCCs. This cytokine secretion profile may be regarded as a risk indicator of periodontitis.

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