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Analysis of matrix metalloproteinase (MMP-8 and MMP-2) activity in gingival crevicular fluid from children with Down's syndrome

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Yamazaki-Kubota T, Miyamoto M, Sano Y, Kusumoto M, Yonezu T, Sugita K, Okuda K, Yakushiji M, Ishihara K. Analysis of matrix metalloproteinase (MMP-8 and MMP-2) activity in gingival crevicular fluid from children with Down's syndrome. J Periodont Res 2010; 45: 170–176. © 2009 John Wiley & Sons A/S

Background and Objective: High levels of colonization by periodontopathic bacteria and a high prevalence of chronic inflammatory periodontal disease have been reported in children with Down's syndrome. Matrix metalloproteinases (MMPs) are mediators of extracellular matrix degradation and remodelling, and are deeply involved in the course of periodontal disease. To clarify the relationship between Down's syndrome and periodontitis, we investigated levels of MMP-2 and MMP-8 in gingival crevicular fluid (GCF) and detection of periodontopathic bacteria from subgingival plaque.

Material and Methods: Samples of GCF and plaque were isolated from central incisors. Levels of MMPs were evaluated by enzyme-linked immunosorbent assay, and periodontopathic bacteria were detected by polymerase chain reaction.

Results: Levels of MMP-2 and MMP-8 in Down's syndrome patients were higher than those in healthy control subjects. In the Down's syndrome group, increases in these MMPs were observed in GCF from patients with an oral hygiene index score of < 2 and in GCF from sites that were negative for bleeding on probing. The detection rate of periodontopathic bacteria in Down's syndrome patients was higher than that in the control subjects. Matrix metalloproteinase-2 levels in sites harbouring *Porphyromonas gingivalis* or *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* were lower than in those without these microorganisms.

Conclusion: These results suggest an increase in MMP-2 and MMP-8 in Down's syndrome patients, regardless of whether inflammation of periodontal tissue is present or not.

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Down's syndrome (DS) is an autosomal chromosomal anomaly resulting from trisomy of chromosome 21 (1). Several reports have described a high prevalence of chronic inflammatory periodontal disease in children with DS (2–5). In DS, the frequency of periodontitis lesions with alveolar bone loss is higher around the mandibular incisors than around the first maxillary molars (4). Several systemic deficiencies, including defective neutrophil

chemotaxis and a reduction in immunoglobulin M production have been reported in DS (2,6).

Matrix metalloproteinases (MMPs) are a group of endogenous proteinases that contribute to the degradation of the extracellular matrix and basement membrane components (7). Matrix metalloproteinases are believed to act as mediators of extracellular matrix degradation and remodelling and to be deeply involved in the course of periodontal disease (8). Human periodontal tissue cells, such as fibroblasts, keratinocytes, macrophages, polymorphonuclear leukocytes (PMNs) and endothelial cells, are all capable of expressing and releasing MMPs, and a number of studies have suggested that MMP expression and levels are good indicators for a clinical diagnosis of periodontal disease (9,10). Matrix metalloproteinase-2 is a gelatinase, which mainly cleaves type IV collagen and degrades native fibrillar interstitial collagens (11–13). Gelatinases are believed to play an important role in tissue destruction in periodontitis (14–18). Matrix metalloproteinase-8 is a PMN-type collagenase involved in periodontal tissue degradation in periodontal disease (10). The PMN-type collagenase is stored in specific granules within PMNs, and is released rapidly when PMNs are triggered. Sorsa *et al*. (19) demonstrated that the major collagenase involved in periodontitis was MMP-8.

Recent study has reported significantly higher levels of MMP-8 in saliva in DS children than in control subjects (20). Levels of MMP-2 in cultured gingival fibroblasts from Down's syndrome patients were significantly higher than in control subjects (21). In addition, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Prevotella nigrescens*, *Campylobacter rectus*, *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and *Capnocytophaga* were detected with significantly greater frequency in Down's syndrome patients (22,23). Periodontal microorganisms have been reported to induce release of MMP-8 and MMP-9 in the gingival crevice via activation of host immune response (24,25). However, the reason for the increased levels of MMPs in DS patients has not yet been clarified.

The aim of this study was to investigate the MMP-2 and MMP-8 expression in gingival crevicular fluid (GCF) in DS patients without periodontitis to clarify the factors involved in the increased levels of MMPs in DS patients.

Material and methods

Subjects

Fourteen children with DS (9 boys and 5 girls; mean age 12.7 years, range 6–18 years) and 14 healthy children (8 boys and 6 girls; mean age 12.2 years, range 7–17 years) were enrolled in this study. The DS children were selected at random from the Department of Pediatrics, Dental Faculty, Tokyo Dental College and a school for physically handicapped children at Chiba University. None of either the DS patients or the healthy children had any medication over a 3 month period prior to sampling, and they were otherwise healthy. Informed written consent was obtained from the parents of the children, and approval for the study from the Tokyo Dental College Ethical Committee.

Clinical examination of periodontal status

All clinical examination was performed after collection of GCF. Clinical examination consisted of evaluation according to the oral hygiene index (OHI; 26), probing pocket depth, and bleeding on probing (BOP). After GCF collection, probing pocket depth and BOP in all teeth was examined and existence of gingivitis evaluated. Probing depth was defined as the distance from the free gingival margin to the base of the periodontal pocket for each tooth. Bleeding on probing was scored as presence or absence of bleeding within 30 s of probing. Any patients who had a manifestation of disease at examination were excluded from the study.

Analysis of metalloproteinase production

Maxillary central incisors were chosen for sampling of GCF because of the low possibility of contamination with the saliva. The GCF was collected by means of Durapore filter membranes (pore size: 0.22 μm; Millipore Corp., Bedford, MA, USA) without touching the marginal gingiva. The GCF samples were collected first to avoid any tendency the site might have for bleeding on/after plaque sampling and probing. The site was isolated with cotton rolls, and the surface gently dried to avoid contamination by saliva. Durapore membrane was inserted 1 mm into the sulcus and left in place for 5 min. The membrane was then placed in a microcentrifuge tube containing 500 μL phosphate-buffered saline (pH 7.4) and stored at −80°C until use in determining MMP-2 and MMP-8 levels. Where there was visible contamination with blood, the strips were discarded. In the case of visible contamination, GCF samples were collected on another day.

The samples were thawed at room temperature before determination of MMP levels. Levels of MMP-2 and MMP-8 were determined using the Human Biotrack enzyme-linked immunosorbent assay (ELISA) system (GE Health Care, Tokyo, Japan) according to the manufacturer's instructions, and the results are expressed as nanograms per membrane. The detection limits for MMP-2 and MMP-8 with the kits used were given by the manufacturer as 1.5–24 and 0.25–4 ng, respectively.

Detection of bacteria from subgingival plaque

Subgingival plaque was collected from the maxillary central incisors. After careful removal of supragingival plaque deposits, sampling sites were isolated with cotton rolls, gently air-dried, and subgingival plaque was collected with two sterile paper points. Samples were isolated from the maxillary central incisors, and three to four samples were collected from each person. Paper points from all experimental sites were

Table 1. Clinical data (means ± SD) from Down's syndrome patients and control subjects

	DS patients (n = 14)	Control subjects (n = 14)	p-value
Oral hygiene index (OHI)	1.95 ± 1.1	1.68 ± 0.62	$p < 0.05$
Average probing depth (mm)	2.89 ± 0.43	2.57 ± 0.44	n.s.
No. of subjects with probing depth ≥ 5 mm	1	0	—
No. of teeth with BOP (+)/total teeth (%)	14.9 ± 16.7	3.51 ± 2.82	$p < 0.05$

pooled in 200 μL boiling buffer containing 20 mM Tris-HCl buffer (pH 8.5), 2 mM EDTA and 1% Triton X-100. The suspension was treated at 100°C for 10 min and supernatant obtained by centrifugation. Genomic DNA from the supernatant was isolated by phenol extraction. Polymerase chain reaction (PCR) was used to detect microorganisms. *P. gingivalis* and *A. actinomycetemcomitans* were detected according to the method of Ashimoto *et al.* (27). The primers used for *Treponema denticola*, (5′-GCGAACAGATATTTGACATAACTAGGGAAG-3′ and 5′-CTATTCTTTCGCTTGACCATATTATTGTCC-3′; amplicon length 155 bp) and *Campylobacter rectus* (5′-AACTTCTCTATCCGATTACCGCTTAAG-3′ and 5′-TACTAGCCAAGGCATCCACCACTTAC-3′; amplicon length 203 bp) were designed based on the 16S and 23S rRNA sequences of *T. denticola* and *C. rectus*, respectively, in GenBank at the National Center of Biotechnology Information (Bethesda, MD, USA). The specificity of these primers was confirmed against 39 oral bacterial species. Polymerase chain reaction for these microorganisms was performed as previously reported for *Fusobacterium nucleatum/periodonticum* (28). Obtained products were analysed using 2% agarose gel electrophoresis.

Statistical analysis

Quantitative differences between the DS children group and the healthy control group were determined using unpaired Student's t-test. Differences of MMPs were determined by a one-way ANOVA followed by Student–Newman–Keuls test to make multiple comparisons among groups in DS patients separated according to their oral condition and in control subjects. Detection rate of periodontal pathogens between DS patients and healthy subjects were compared by Fisher's Exact test.

Results

Clinical findings

A summary of the OHI, probing depth and BOP in both the DS and control groups is given in Table 1. In the DS group, one patient had 5 mm deep gingival pockets. No significant difference was found in probing depth between the DS patients and the control subjects. The OHI in DS patients was significantly higher ($p < 0.05$) than that in the control subjects. The level of BOP-positive sites was significantly higher ($p < 0.05$) in the DS patients than in the control subjects. The mean ± SD of probing depth of sampling sites in DS patients and control subjects were 2.9 ± 0.58 and 2.9 ± 0.56 mm, respectively. These values are similar to the mean values for all the teeth in the mouse in Table 1.

Production of MMP-2 and MMP-8

In the GCF samples, levels of MMP-2 and MMP-8 were significantly higher in the DS patients ($p < 0.01$) than in the control subjects (Fig. 1A, B). It is possible that this difference in MMP levels between the two groups was influenced by OHI and BOP values. Therefore, MMP levels were compared after dividing the DS patients based on levels of OHI and BOP. Figure 1E,F shows levels of MMP-2 and MMP-8 revealed after dividing the DS patients into high (> 2) and low OHI groups (< 2). The levels of MMP-2 and MMP-8 in DS patients with both low OHI and high OHI scores were significantly higher than those in control patients. No statistically significant difference was observed in MMPs level between high and low OHI in DS patients ($p < 0.05$). Levels of MMP-2 and MMP-8 at BOP-positive and -negative sites in DS patients were higher than those in the control patients. No statistically significant difference, however, was found in MMP-2 and MMP-8 levels between BOP-positive and -negative sites in DS patients (Fig. 1C,D; $p < 0.05$).

Bacterial prevalence in DS patients and control subjects

We compared the prevalence of subgingival bacterial species between the DS group and the control group (Table 2). For all periodontopathic bacteria tested in this study, the detection rate in DS patients was higher than that in the control group; however, statistical difference was observed in only *C. rectus*. Matrix metalloproteinase levels in DS patients with or without each periodontopathic bacterium are shown in Fig. 2. Levels of MMP-2 were significantly lower in DS patients harboring *P. gingivalis* or *A. actinomycetemcomitans* than in those who were not ($p < 0.01$).

Discussion

A high susceptibility to and prevalence of periodontitis in DS patients has been reported (29). However, the reason for this high susceptibility and prevalence remains to be clarified. In the present study, we showed an increase in levels of MMP-2 and MMP-8 in DS patients compared with those in healthy control subjects. This result agrees with that of a previous report in DS patients (20,21). Both MMP-2 and MMP-8 were reported to show an increase in chronic periodontitis and aggressive periodontitis patients (18,19,30). It is possible that the

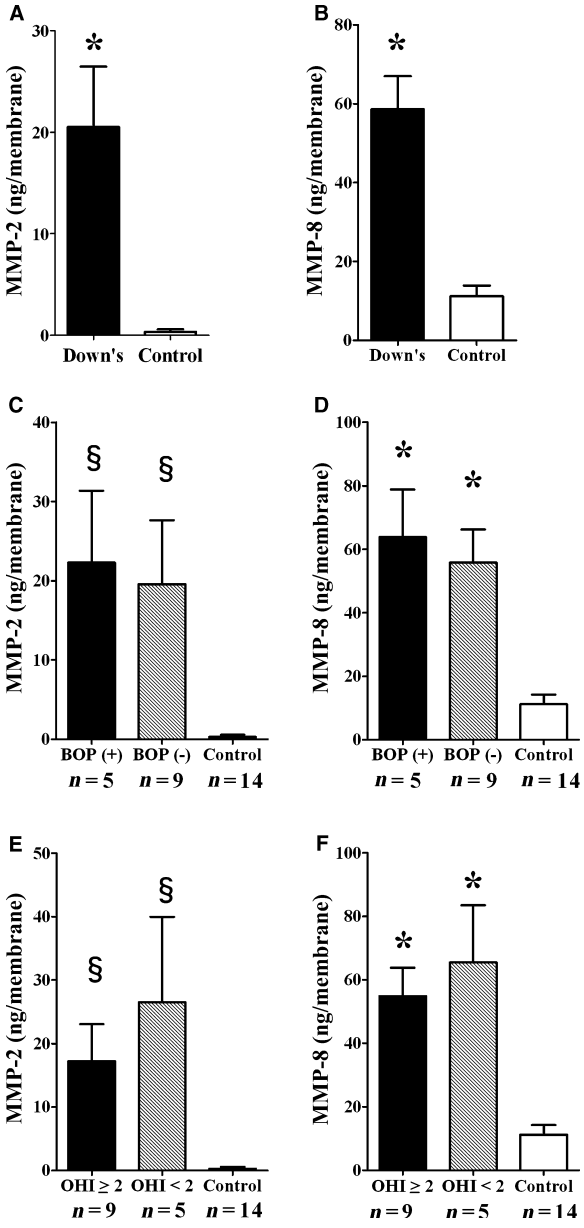

Fig. 1. Matrix metalloproteinase-2 and MMP-8 levels (nanograms per membrane ± SE) in GCF. (A,B) Matrix metalloproteinase-2 (A) and MMP-8 levels (B) in GCF from DS patients and control subjects. (C,D) Matrix metalloproteinase-2 (C) and MMP-8 levels (D) in GCF by BOP. (E,F) Matrix metalloproteinase-2 (E) and MMP-8 levels (F) in GCF by OHI. Patients were separated into two groups by the score of OHI > 2 or < 2. (A,B) Analysed by unpaired Student's *t*-test (*$p < 0.01$). (C–F) Analyzed by one-way ANOVA for repeated measurements, used for inter-group comparisons. Student–Newman–Keuls test was used for multiple comparisons (§$p < 0.05$, *$p < 0.01$ compared with the control subjects).

increase of MMPs in gingival tissue induces periodontal destruction. In the present study, we evaluated the levels of MMP-2 and MMP-8 using the membrane filter technique and expressed as nanograms per membrane. An increase in vascular permeability of the blood vessels following gingival stimulation has been reported (31), suggesting that the levels of MMPs in GCF could be influenced by stimulation in sampling. Concentration of the unstimulated GCF may reflect the level of MMPs in gingival tissue. Although collection of GCF using filter paper is frequently used (30,32,33), further study by measuring the real concentration of MMPs in unstimulated GCF is required.

In the present study, the ratio of BOP-positive sites in DS patients was somewhat higher than that in healthy control subjects, indicating gingival inflammation in DS patients, although average probing depth in DS patients was the same as that in the control subjects. Matrix metalloproteinase-8 is a neutrophil-derived collagenase. It is possible that the higher levels of MMP-2 and MMP-8 seen in DS patients in this study were a result of inflammation caused by poorer oral hygiene than that in the healthy control subjects. To eliminate the effects of the level of oral hygiene or inflammation on the increase in MMP production in DS patients, the DS patients were divided by OHI or BOP score at the sampling site. Down's syndrome patients with an OHI score of < 2 also showed significantly higher levels of MMP-2 and MMP-8 than healthy subjects. Levels of these MMPs showed no statistically significant difference between DS patients with an OHI score of < 2 and those with a score of 2 or more. Matrix metalloproteinase-2 and MMP-8 levels in DS patients at BOP-negative sites were significantly higher than those in the control subjects. No difference was found in these levels between BOP-positive and -negative sites in DS patients. These results suggest a difference in production of MMP-2 and MMP-8 in DS patients, regardless of inflammatory response. Matrix metalloproteinase-2 was reported to be constitutively expressed at low levels in the periodontium (34). The increase in MMP-2 observed in this study agrees with that seen in a previous report by Komatsu *et al.* (21). They reported an increase in MMP-2 by increase in MMP-2 mRNA expression in fibroblasts from DS patients *in vitro*. It is possible that there is a difference in MMP-2 production between DS patients and healthy control subjects. Matrix metalloproteinase-8 levels usually reflect inflammation level, since MMP-8 is a neutrophil-derived proteinase (8). The present results showed no significant difference in MMP-8

Table 2. Detection rate (%) of specific periodontal pathogens in subgingival plaque samples

Species	DS patients (n = 14)	Control subjects (n = 14)
P. gingivalis	42.9	21.4
A. actinomycetemcomitans	64.3	57.1
C. rectus	35.7*	0
T. denticola	35.7	7.1

*$p < 0.05$ by Fisher's exact test.

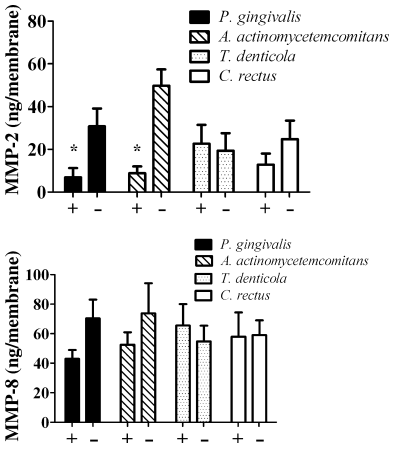

Fig. 2. Comparison of MMP-2 and MMP-8 production (nanograms per membrane ± SE) based on detection of periodontopathic bacteria (*$p < 0.01$ by unpaired Student's t-test).

levels between DS patients with an OHI score of < 2 and an OHI score of 2 or more, or between BOP-positive and BOP-negative sites in DS patients. These results suggest that factors other than migration of PMNs induced by inflammation is involved in increasing the levels of MMP-8. These observations suggest that level of oral hygiene and inflammation are not the only cause of increase in MMPs in DS patients.

Detection rates of periodontopathic bacteria in DS patients were higher than those in the control subjects. The profile was similar to that in a report by Amano *et al.* (22). Interestingly, if the DS patients were separated by detection of microorganism, levels of MMP-2 and MMP-8 were lower in DS patients harbouring *A. actinomycetemcomitans* and *P. gingivalis*. Differences in MMP-2 were statistically significant ($p < 0.01$). Lipopolysaccharide of *A. actinomycetemcomitans* was reported to increase production of MMP-2 from fibroblasts (35). *P. gingivalis* significantly up-regulated MMP-2 and MMP-9 mRNA expression by oral epithelial cells (36). The present results disagree with these reports. However, Garlet *et al.* (37) reported a correlation between an increase in MMP-1, -2 and -9 and interleukin-1β, tumor necrosis factor-α and interferon-γ in *A. actinomycetemcomitans*-infected mice. The expression of MMP-2 in their study showed a progressive increase from 24 h until 15 days of infection, with a decrease at 30 and 60 days. *P. gingivalis* and *A. actinomycetemcomitans* have factors which induce an immune response, such as lipopolysaccharide, and also immunosuppressive factors (38–40). Immune dysfunction has also been reported in Down's syndrome (41). It is possible that immunomodulation by these microorganisms or immune dysfunction in patients affected the production of MMP-2; however, further analysis with more samples are required to confirm the reduction. Levels of MMP-8 in subjects infected by periodontopathic bacteria are slightly low or the same as those without infection. Most MMP-8 in periodontal lesions is released from neutrophils, and MMP-8 has been reported to be associated with the severity of chronic periodontitis (10). Defects and dysfunction of bactericidal capacities have been described (6,42). These types of dysfunction may affect the level of MMP-8 in DS patients. In addition, colonization by periodontopathic bacteria did not affect the levels of MMPs in the present study. This also suggests that inflammation caused by periodontopathic bacterial colonization does not involve an increase in MMP-2 and MMP-8 in DS patients. Colonization by *P. gingivalis* and *A. actinomycetemcomitans* has been reported to induce an immune response in many studies (43). The discrepancy also suggests immune dysfunction. To clarify the dysfunction of the immune response in DS patients, further analysis to compare the condition of the periodontal tissue and the immune response in DS patients is required.

The present results showed an increase in MMP-2 and MMP-8 in DS patients. Recently, Bildt *et al.* reported that levels of pro-MMP-2, active MMP-2 and the MMP-2 complex were higher in patients with chronic periodontitis than in healthy subjects, suggesting the involvement of these metalloproteinases in periodontitis (44). Involvement of MMP-8 production in periodontitis has also been reported (8). However, the present results indicate an increase in MMPs, regardless of whether inflammation of gingival tissue is present or not. Taken together with the results of earlier studies, the results of the present study suggest that an increase in MMP-2 and MMP-8 levels is involved in higher susceptibility to and prevalence of periodontitis in DS patients, although further analysis with a larger population will be required for clarification.

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References

1. Desai SS. Down syndrome: a review of the literature. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1997;**84:**279–285.
2. Cohen MM, Winer RA, Schwartz S, Shklar G. Oral aspects of mongolism. I. Periodontal disease in mongolism. *Oral*

Surg Oral Med Oral Pathol 1961;**14:**92–107.
3. Barnett ML, Press KP, Friedman D, Sonnenberg EM. The prevalence of periodontitis and dental caries in a Down's syndrome population. *J Periodontol* 1986; **57:**288–293.
4. Modeer T, Barr M, Dahllof G. Periodontal disease in children with Down's syndrome. *Scand J Dent Res* 1990;**98:**228–234.
5. Meyle J, Gonzales JR. Influences of systemic diseases on periodontitis in children and adolescents. *Periodontol 2000* 2001; **26:**92–112.
6. Izumi Y, Sugiyama S, Shinozuka O, Yamazaki T, Ohyama T, Ishikawa I. Defective neutrophil chemotaxis in Down's syndrome patients and its relationship to periodontal destruction. *J Periodontol* 1989;**60:**238–242.
7. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res* 2006;**69:**562–573.
8. Sorsa T, Tjaderhane L, Konttinen YT *et al*. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med* 2006;**38:**306–321.
9. Kiili M, Cox SW, Chen HY *et al*. Collagenase-2 (MMP-8) and collagenase-3 (MMP-13) in adult periodontitis: molecular forms and levels in gingival crevicular fluid and immunolocalisation in gingival tissue. *J Clin Periodontol* 2002;**29:**224–232.
10. Sorsa T, Uitto VJ, Suomalainen K, Vauhkonen M, Lindy S. Comparison of interstitial collagenases from human gingiva, sulcular fluid and polymorphonuclear leukocytes. *J Periodontal Res* 1988; **23:**386–393.
11. Imai K, Ohta S, Matsumoto T *et al*. Expression of membrane-type 1 matrix metalloproteinase and activation of progelatinase A in human osteoarthritic cartilage. *Am J Pathol* 1997;**151:**245–256.
12. Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J Biol Chem* 1995;**270:**5872–5876.
13. Konttinen YT, Ceponis A, Takagi M *et al*. New collagenolytic enzymes/cascade identified at the pannus-hard tissue junction in rheumatoid arthritis: destruction from above. *Matrix Biol* 1998;**17:**585–601.
14. Birkedal-Hansen H, Moore WG, Bodden MK *et al*. Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 1993; **4:**197–250.
15. Ingman T, Sorsa T, Lindy O, Koski H, Konttinen YT. Multiple forms of gelatinases/type IV collagenases in saliva and gingival crevicular fluid of periodontitis patients. *J Clin Periodontol* 1994;**21:**26–31.
16. Makela M, Salo T, Uitto VJ, Larjava H. Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. *J Dent Res* 1994;**73:**1397–1406.
17. Korostoff JM, Wang JF, Sarment DP, Stewart JC, Feldman RS, Billings PC. Analysis of in situ protease activity in chronic adult periodontitis patients: expression of activated MMP-2 and a 40 kDa serine protease. *J Periodontol* 2000;**71:**353–360.
18. Ejeil AL, Igondjo-Tchen S, Ghomrasseni S, Pellat B, Godeau G, Gogly B. Expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in healthy and diseased human gingiva. *J Periodontol* 2003; **74:**188–195.
19. Sorsa T, Ding YL, Ingman T *et al*. Cellular source, activation and inhibition of dental plaque collagenase. *J Clin Periodontol* 1995;**22:**709–717.
20. Halinen S, Sorsa T, Ding Y *et al*. Characterization of matrix metalloproteinase (MMP-8 and -9) activities in the saliva and in gingival crevicular fluid of children with Down's syndrome. *J Periodontol* 1996; **67:**748–754.
21. Komatsu T, Kubota E, Sakai N. Enhancement of matrix metalloproteinase (MMP)-2 activity in gingival tissue and cultured fibroblasts from Down's syndrome patients. *Oral Dis* 2001;**7:**47–55.
22. Amano A, Kishima T, Kimura S *et al*. Periodontopathic bacteria in children with Down syndrome. *J Periodontol* 2000; **71:**249–255.
23. Barr-Agholme M, Dahllof G, Linder L, Modeer T. *Actinobacillus actinomycetemcomitans, Capnocytophaga* and *Porphyromonas gingivalis* in subgingival plaque of adolescents with Down's syndrome. *Oral Microbiol Immunol* 1992; **7:**244–248.
24. Soder B, Airila Mansson S, Soder PO, Kari K, Meurman J. Levels of matrix metalloproteinases-8 and -9 with simultaneous presence of periodontal pathogens in gingival crevicular fluid as well as matrix metalloproteinase-9 and cholesterol in blood. *J Periodontal Res* 2006; **41:**411–417.
25. Yamazaki T, Miyamoto M, Yamada S, Okuda K, Ishihara K. Surface protease of *Treponema denticola* hydrolyzes C3 and influences function of polymorphonuclear leukocytes. *Microbes Infect* 2006;**8:**1758–1763.
26. Greene JC, Vermillion JR. The oral hygiene index. A method for classifying oral hygiene status. *J Am Dent Assoc* 1960; **61:**172–179.
27. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;**11:**266–273.
28. Kobayashi N, Ishihara K, Sugihara N, Kusumoto M, Yakushiji M, Okuda K. Colonization pattern of periodontal bacteria in Japanese children and their mothers. *J Periodontal Res* 2008;**43:**156–161.
29. Reuland-Bosma W, van Dijk J. Periodontal disease in Down's syndrome: a review. *J Clin Periodontol* 1986;**13:**64–73.
30. Alfant B, Shaddox LM, Tobler J, Magnusson I, Aukhil I, Walker C. Matrix metalloproteinase levels in children with aggressive periodontitis. *J Periodontol* 2008;**79:**819–826.
31. Egelberg J. Permeability of the dentogingival blood vessels. IV. Effect of histamine on vessels in clinically healthy and chronically inflamed gingivae. *J Periodontal Res* 1966;**1:**297–302.
32. Pirhan D, Atilla G, Emingil G, Sorsa T, Tervahartiala T, Berdeli A. Effect of MMP-1 promoter polymorphisms on GCF MMP-1 levels and outcome of periodontal therapy in patients with severe chronic periodontitis. *J Clin Periodontol* 2008;**35:**862–870.
33. Grayson R, Douglas CW, Heath J, Rawlinson A, Evans GS. Activation of human matrix metalloproteinase 2 by gingival crevicular fluid and *Porphyromonas gingivalis*. *J Clin Periodontol* 2003; **30:**542–550.
34. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003;**92:**827–839.
35. Bodet C, Andrian E, Tanabe S, Grenier D. *Actinobacillus actinomycetemcomitans* lipopolysaccharide regulates matrix metalloproteinase, tissue inhibitors of matrix metalloproteinase, and plasminogen activator production by human gingival fibroblasts: a potential role in connective tissue destruction. *J Cell Physiol* 2007; **212:**189–194.
36. Andrian E, Mostefaoui Y, Rouabhia M, Grenier D. Regulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases by *Porphyromonas gingivalis* in an engineered human oral mucosa model. *J Cell Physiol* 2007; **211:**56–62.
37. Garlet GP, Cardoso CR, Silva TA *et al*. Cytokine pattern determines the progression of experimental periodontal disease induced by *Actinobacillus actinomycetemcomitans* through the modulation of MMPs, RANKL, and their physiological inhibitors. *Oral Microbiol Immunol* 2006; **21:**12–20.

38. Page RC. The role of inflammatory mediators in the pathogenesis of periodontal disease. *J Periodontal Res* 1991; **26:**230–242.
39. Shenker BJ, Slots J. Immunomodulatory effects of Bacteroides products on in vitro human lymphocyte functions. *Oral Microbiol Immunol* 1989;**4:**24–29.
40. Shenker BJ, McKay T, Datar S, Miller M, Chowhan R, Demuth D. *Actinobacillus actinomycetemcomitans* immunosuppressive protein is a member of the family of cytolethal distending toxins capable of causing a G2 arrest in human T cells. *J Immunol* 1999;**162:**4773–4780.
41. Cuadrado E, Barrena MJ. Immune dysfunction in Down's syndrome: primary immune deficiency or early senescence of the immune system? *Clin Immunol Immunopathol* 1996;**78:**209–214.
42. Gregory L, Williams R, Thompson E. Leucocyte function in Down's syndrome and acute leukaemia. *Lancet* 1972;**1:**1359–1361.
43. Ishikawa I, Nakashima K, Koseki T *et al*. Induction of the immune response to periodontopathic bacteria and its role in the pathogenesis of periodontitis. *Periodontol 2000* 1997;**14:**79–111.
44. Bildt MM, Bloemen M, Kuijpers-Jagtman AM, Von den Hoff JW. Collagenolytic fragments and active gelatinase complexes in periodontitis. *J Periodontol* 2008; **79:**1704–1711.