INFLAMMATORY RESPONSE IN PERIODONTAL TISSUE IN CHILDREN WITH DOWN SYNDROME

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"I don't really have Down's syndrome; I just have a slight case of it."

Chris Burke

Abstract

Periodontal diseases are inflammatory diseases affecting the supporting tissues of the teeth. Subjects with Down syndrome have a higher prevalence of periodontal disease compared to healthy controls. Periodontal disease in Down syndrome is considered to be multifactorial, although the aetiology is uncertain. The aim of this thesis was to study the inflammatory response in periodontal tissue in terms of cytokines, prostaglandins, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in children with Down syndrome as well as in healthy controls.

In **study I**, 18 subjects with Down syndrome and 14 controls were clinically and radiographically examined and matched for age and degree of gingival inflammation expressed as percentage of bleeding on probing (BOP%). In all subjects, gingival crevicular fluid (GCF) was collected from six sites with paper strips, and levels of prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4), and MMP-9 were analysed using RIA and ELISA kits. BOP% and volume of GCF (μL) were similar in both groups while Down syndrome patients had significantly higher ($p<0.05$) mean levels of PGE_2, LTB_4, and MMP-9 in GCF than controls.

In **study II**, PD and BOP% were clinically assessed in subjects with Down syndrome (n=24) and controls (n=29) (both groups, mean age 16.4 yr). The controls were matched for age and BOP% to subjects with Down syndrome. GCF was collected and Bio-Plex cytokine multiplex assays were used to determine levels of interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and interleukin (IL)-1β, IL-4, -6, -10, -12, and -17. GCF volume (μL) was significantly higher in subjects with Down syndrome ($p<0.001$) than controls. Mean levels of IL-1β, IL-4, IL-6, IL-10, IL-12, IFN-γ, and TNF-α in GCF were significantly ($p\leq0.005$) increased in subjects with Down syndrome compared with controls. The correlation between IFN-γ and IL-4 in GCF in subjects with Down syndrome differed significantly from controls ($p<0.01$).

In **study III**, 21 adolescents with Down syndrome exhibiting gingivitis (DS-G), 12 subjects with Down syndrome exhibiting periodontitis (DS-P), 26 controls with gingivitis (HC-G), and 8 controls with periodontitis (HC-P) were clinically and radiographically examined. All patients were between ages 11 and 20 yr. GCF was collected from each subject and the amounts of MMP-2, -3, -8, -9 and -13 and of TIMP-1, -2 and -3 were

determined with R&D multianalyte kits. The amounts of MMP-2, -3, -8, and -9 and of TIMP-2 in GCF were significantly higher (p<0.005) in the DS-G than the HC-G group. The correlation coefficient between MMP-8 and TIMP-2 also differed significantly (p<0.01) between the DS-G and HC-G groups. In contrast, the correlation coefficients between the MMPs and TIMPs did not differ significantly between the DS-P and the HC-P groups. The DS-P group, however, exhibited significantly (p<0.005) lower amounts of TIMP-2 in GCF compared to the HC-P group.

In **study IV**, children with Down syndrome (n=10) and controls (n=10) were clinically and radiographically examined during dental treatment under general anaesthesia. Peripheral blood and GCF were gathered from each patient and levels of MMP-2, -3, -8 and -9, of TIMP-1, -2 and -3 in serum, and of GCF were determined. Peripheral blood leukocytes were isolated, and the relative amounts (%) of the various cells were determined with flow cytometry. Peripheral blood cells were stimulated with lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (*Pg*) and MMP and TIMP levels were measured. Levels of MMP-3 and -8 and TIMP-1 in serum were significantly enhanced (p's<0.05) in subjects with Down syndrome compared to controls. When peripheral blood leukocytes were cultured in the presence or absence of *Porphyromonas gingivalis* lipopolysaccharide, MMP-8 levels were significantly ($p < 0.05$) higher in the Down syndrome group compared to controls. Children with Down syndrome exhibited significant positive correlations of CD8+ T cells with MMP-8 (r=0.630; p=0.050) and MMP-9 (r=0.648; p<0.05) and of CD56+ NK cells with MMP-3 (r=0.828; p<0.005) compared to controls.

Conclusions

Subjects with Down syndrome had increased levels of the arachidonic acid metabolites PGE$_2$ and LTB$_4$, the cytokines IL-1β, IL-4, IL-6, IL-10, IL-12, IFN-γ and TNF-α, and of MMP-2, -3, -8 and -9 and TIMP-2 in GCF compared to controls. In addition, the balance between pro- and anti-inflammatory cytokines and between MMPs and TIMPs was altered in subjects with Down syndrome but not in controls. Furthermore, in contrast with controls, no significant differences in MMP and TIMP levels in GCF were observed between Down syndrome patients with gingivitis and periodontitis. This finding might indicate that the inflammatory response in Down syndrome is already upregulated during early stages of periodontal disease. We also demonstrate an association between MMPs and lymphocyte subpopulations (CD8+ T-cells and CD56+ NK-cells), which may facilitate the

migration of immune cells into the periodontal tissue. This assumption is well compatible with the higher levels of MMPs in GCF found in Down syndrome subjects. These findings, may contribute to the increased periodontal inflammation demonstrated in this current cohort of Down syndrome subjects.

List of Publications

I. **Tsilingaridis G**, Yucel-Lindberg T, Modéer T. Enhanced levels of prostaglandin E_2, leukotriene B_4 and matrix metalloproteinase-9 in gingival crevicular fluid from patients with Down syndrome. Acta Odont Scand 2003; 61: 154-8.

II. **Tsilingaridis G**, Yucel-Lindberg T, Modéer T. T – helper related cytokines in gingival crevicular fluid from Down syndrome adolescents. Clin Oral Investig 2012;16: 267-73.

III. **Tsilingaridis G**, Yucel-Lindberg T, Modéer T. Altered relationship between MMP-8 and TIMP-2 in gingival crevicular fluid in adolescents with Down syndrome. J Periodontol Res 2013; Epub ahead of print : 9 January.

IV. **Tsilingaridis G**, Yücel-Lindberg T, Concha Quezada H, Modéer T. The relationship between matrix metalloproteinases (MMP-3, -8, -9) in serum and peripheral lymphocytes (CD8+, CD56+) in Down syndrome children with gingivitis. (Submitted)

Contents

List of abbreviations

AA	Arachidonic acid
Aa	*Aggregatibacter actinomycetemcomitans*
BOP	Bleeding on probing
COX	Cyclooxygenase
DMEM	Dulbecco's modified Eagle's medium
DS-G	Down syndrome group with gingivitis
DS-P	Down syndrome group with periodontitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
GCF	Gingival crevicular fluid
HC-G	Healthy controls with gingivitis
HC-P	Healthy controls with periodontitis
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LT	Leukotriene
LTB$_4$	Leukotriene B$_4$
MMP	Matrix metalloproteinase
NaH$_2$PO$_4$	Monosodium phosphate
NK	Natural killer
OPG	Osteoprotegerin
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PD	Periodontal probing depth
PG	Prostaglandin
PGE$_2$	Prostaglandin E$_2$
PGE synthase	Prostaglandin E synthase
Pg	*Porfyromonas gingivalis*
PI	Plaque index
PLA$_2$	Phospholipase A$_2$
PMN	Polymorphonuclear leukocyte
RANKL	Receptor activator of nuclear factor kappa-B ligand
RIA	Radioimmunoassay
SOD-1	Superoxide dismutase-1
TIMP	Tissue inhibitor of metalloproteinase
TGF-β	Transforming growth factor-β
Th	T-helper
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor-α
T-reg	Regulatory T cell
TX	Tromboxane
5-LO	**5-**lipoxygenase

Introduction

Down syndrome

Genetics

Paintings and frescoes from the 1400s portray children and adults with Down syndrome. The first known medical description of a person with Down syndrome was made in 1838 by the Spaniard Jean Esquirol (1). In 1866, John Langdon Down published an article in the London Medical Journal in which he described many of the characteristics and problems of Down syndrome (2). It was he who lent his name to the syndrome. In 1956, Albert Levan and his colleagues determined that humans have 46 chromosomes (3), which made it possible 3 years later, in 1959, for Jerome Lejéune to report that Down syndrome was due to an extra copy of chromosome 21 (4) (Figure 1).

Down syndrome is the result of a trisomy of chromosome 21. In 94% of persons with Down syndrome, this is due to nondisjunction, resulting in an extra chromosome 21 in all cells (5). In 4% of the cases, Down syndrome is due to translocation of all or part of chromosome 21, and in 2% of the cases, Down syndrome is the result of mosaicism where only some cells have 47 (trisomy 21) chromosomes (5).

There are two major theories for how trisomy 21 causes Down syndrome. Both theories are based on the view that if a gene exists in three copies instead of two, the level of gene expression will increase. In the first theory, the gene dosage theory, increased expression of specific trisomic genes on the distal half of the long arm, cytologically known as band 21q22, are directly responsible for specific features of Down syndrome (6). In the second theory, the amplified developmental instability theory, the number of phenotypic features associated with Down syndrome is primarily due to the elevated activity of sets of genes, which regardless of their identity, will decrease in genetic stability or homeostasis – and not to the direct contributions of specific genes on the distal half of the long arm on chromosome 21. (6, 7). So the greater the number of trisomic genes, the more susceptible the foetus will be to developmental abnormalities.
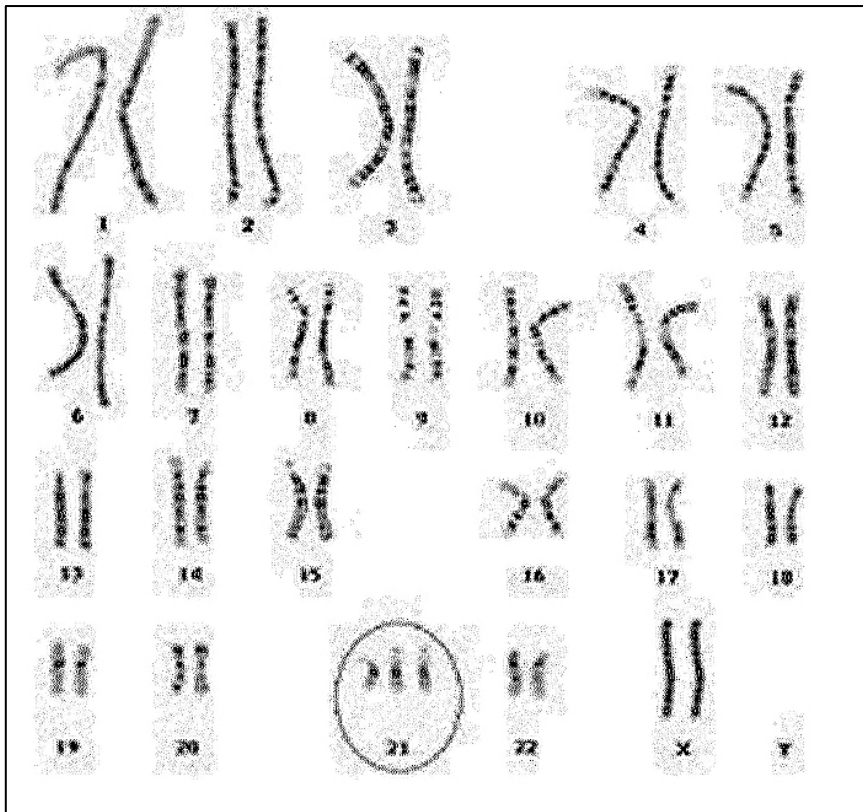
Figure 1. G-banded karyotype of a trisomy 21 female, showing three copies of human chromosome 21 (HSA21). Adapted from Antonarakis and coworkers 2004 (8).

Medical features associated with Down syndrome

Down syndrome is characterized by mental retardation and a variety of morphological characteristics that occur in varying frequency (9). In addition to the morphological characteristics, patients with Down syndrome more often suffer from heart disease, leukaemia, growth retardation, hormonal disturbances, obesity, neuropsychiatric disorders, and increased susceptibility to infection (9).

Approximately 40% of all infants with Down syndrome have congenital heart defects, the most common of which are atrial septal defects (10). Furthermore, children with Down syndrome are at a higher risk of developing acute leukaemia compared to other groups (11). Physical growth in Down syndrome is also affected, and children with the syndrome suffer from short stature and weight problems. As infants, their length is normal, but growth retardation becomes evident in the first years of life (12). Thyroid hormone deficiencies, such as hypothyroidism, are common (13, 14). And overweight and obesity (BMI>25 or >30) are a well-known problem (15). In Sweden, one in three individuals with Down syndrome is overweight by age 18 (16). Neuropsychiatric disorders such as attention deficit hyperactivity disorder and autism-spectrum disorder are overrepresented

in Down syndrome (17, 18). In addition, patients with Down syndrome diagnosed with autism-spectrum disorder have more severe learning disabilities than patients with only Down syndrome (19). Other features in the Down syndrome group include ocular disorders, gastrointestinal malformations, orthopaedic problems, and hearing and otologic disorders (20-23) (Table 1).

Table 1. Common medical conditions associated with Down syndrome with known frequencies (%).

Condition	Frequency (%)
Neuropsychiatric	
Attention-deficit-hyperactivity-disorder (18, 24)	11-44
Autism (25, 26)	1
Autism-spectrum disorder (17)	18
Gastro-intestinal	
Celiac disease (27)	1.4
Congenital heart defects (10)	42
Otolaryngology	
Hearing impairment (28)	56
Stenotic (narrow) ear canals (28)	40
Otitis media (28)	38
Obstructive sleep apnea (29, 30)	50
Hematology	
Leukemia (9)	1
Transient myeloproliferative disorder (31-33)	3-10
Ocular manifestations (9)	45-70
Nystagmus (21)	29
Strabismus (21)	26
Endocrinology	
Thyroid dysfunction (13, 14)	28-38
Growth retardation (12)	
Obesity (16)	31-36
Orthopedics	
Atlanto-axial instability (34)	15
Increased infection susceptibility (35)	

Immunological features

Cells in the innate immune system such as natural killer (NK) cells and polymorphonuclear leukocytes (PMNs) express various pattern-recognition receptors, which recognize signature molecules of pathogens known as pathogen-associated molecular patterns (36-38). To date, several classes of pattern-recognition receptors such as Toll-like receptors (TLRs) have been identified. The TLRs are considered to be key players in the detection of invading pathogens; so far, 12 members of the TLR family have been identified (39). TLRs recognize pathogens and initiate a cascade of signalling pathways that result in the production of chemokines and adhesion molecules. These, in turn, lead to the migration of neutrophils and the production of inflammatory mediators (40-43).

Several studies of the innate immune system in Down syndrome subjects have described functional defects in PMNs and monocytes that result in impaired phagocytic function, increased oxidative stress, and poorer chemotactic ability (44-46). It has been proposed that the oxidative stress in Down syndrome is due to overexpression of superoxide dismutase 1 (SOD-1), an antioxidant enzyme coded on chromosome 21 (47).

In periodontal tissue from subjects with Down syndrome it has previously been described an impaired chemotactic ability of PMNs suggesting that PMNs do not reach the infection site (48, 49). Furthermore, in a recent study by Khocht and coworkers (50) demonstrated undiminished granulocyte and monocyte phagocytic intensities in subjects with Down syndrome, although a significantly lower percentage of monocytes was actively involved in phagocytosis compared to controls. Phagocytosis is followed by an oxidative burst and production of oxygen radicals to kill encapsulated pathogens. The same research group (50, 51) recently demonstrated that the oxidative burst activity of PMNs and monocytes is higher in subjects with Down syndrome and suggested that it may contribute to periodontal tissue inflammation and destruction in the Down syndrome group.

Adaptive immunity includes antibody-mediated (humoral) immunity as well as T-cell-mediated immunity, both of which protect against infection (52).

Antibodies or immunoglobulins (Ig) are proteins that are produced by B-cells in response to antigens (53). The five classes of immunoglobulins – IgG, IgA, IgM, IgE and IgD – are characterized by differences in structure and function. Two classes have distinct subclasses: IgG has four subclasses and IgA, two (53). Interaction between the various immunoglobulins and the antigens results in either direct inactivation of the micro-

organism or activation of a variety of inflammatory mediators such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α to destroy the pathogen (52-55).

In the Down syndrome group, B-cell counts and levels of IgG, IgA, IgM and IgE antibodies are largely normal, but the patterns of serum IgG subclasses differ from in persons without Down syndrome. It has been shown that in both children and adults with Down syndrome, serum levels of IgG2 and IgG4 are low while serum levels of IgG1 and IgG3 are elevated (56).

T-cell development requires migration of precursor T cells to the thymus where they undergo differentiation into two distinct types of T cells: the CD4+ T-helper (Th) cell and the CD8+ pre-cytotoxic T cell (52). T-cytotoxic cells attack antigens directly and destroy cells that bear foreign antigens (57). Th cells play a crucial role in host response and through the influence of specific cytokines differentiated into subsets of Th1, Th2, Th17, and regulatory T (T-reg) cells, which mediate inflammation, tissue damage and autoimmunity (58-62). Recently, Kaplan (62) described a fifth group of Th cells: Th9 cells. Concerning cellular immunity, Murphy and co-workers (63, 64) reported that thymus function in the Down syndrome group is altered, with a decreased number of mature thymocytes. Several studies have reported reduced levels of CD4+ T lymphocytes and increased levels of CD8+ T lymphocytes and CD56+ NK cells (65-69). Although the absolute numbers of T lymphocytes in the Down syndrome group gradually approach those of non-Down syndrome children over time, it is doubtful whether the phenotype and function of these cells are normal in subjects with Down syndrome (70).

Orofacial features

The mid-facial region in children with Down syndrome is often underdeveloped, sometimes with malocclusions such as mandibular protrusion, open bite, and posterior cross bite as a consequence (71-73). Reduced muscle tone in the lips, tongue, and soft palate can impair the ability to suck in the neonatal period and cause difficulties in chewing, swallowing, speech, and other orofacial functions (74). The nasal airway is often narrow and partially blocked as a result of a deviated nasal septum and thickened mucosa, frequently resulting in mouth breathing (20, 75). Because the tongue is hypotonic, it often protrudes and appears to be too large for the mouth (76). Dental developmental disturbances are also common. Dental eruption is often delayed and tooth agenesis, microdontia, and short roots are common (77) . Low salivary flow rates (mL/min) and

mouth breathing often lead to a condition of dry mouth in subjects with Down syndrome (78). Despite low salivary flow rates, the prevalence of caries in persons with Down syndrome is low (79).

The increased susceptibility to infection of subjects with Down syndrome results in an elevated incidence of oral fungal infections. *Candida albicans* occurs as erythematous or pseudomembranous lesions on the tongue, palate, and cheek to a greater extent in children with Down syndrome than controls (80). Furthermore, patients with Down syndrome are more prone to periodontal disease than healthy subjects or other groups of mentally handicapped patients (79, 81) (Table 2).

Table 2. Common orofacial conditions associated with Down syndrome with known frequencies (%).

Condition	Frequency (%)
Malocclusion	
Mandibular protrusion (82)	48
Open bite (82)	36
Posterior cross-bite (83)	77
Dental developmental disturbances	
Hypodontia (84)	56
Conic teeth/Microdontia (77)	16
Canine impaction/transposition (85)	30
Delayed tooth eruption (86, 87)	28
Low salivary flow rate (78)	
Hypotonia of the orofacial musculature (74)	
Relative macroglossia (87)	63
Protruding tongue (87)	41
Increased infection susceptibility	
Candida infection (80)	69
Periodontal disease (81, 88)	35-74

Periodontal disease in Down syndrome

Gingivitis is the most common form of periodontal disease in young persons (89) and is reversible in most children. In some, however, the balance between micro-organisms and the host response is disturbed, and tooth loss and periodontitis result. The prevalence of periodontitis is about 3-5% among adolescents (90, 91).

Compared with controls, subjects with Down syndrome have more extensive gingival inflammation and earlier signs of alveolar bone loss, mainly localized around incisors in the lower front region (92, 93) (Figure 2A and 2B). The prevalence of periodontal disease in subjects with Down syndrome varies depending on whether the subjects live at home or in institutions; prevalence is higher in subjects living in institutions (94, 95). Agholme and co-workers (88) used bitewings and periapical radiographs in a longitudinal study to diagnose periodontal disease and found that one-third of Down syndrome adolescents (mean age 16.6 yr) suffered from alveolar bone loss compared to 74% at the 7-year follow-up (88). Saxén and co-workers (1977) used panoramic radiographs to evaluate the degree of periodontal disease and reported a prevalence of 69% in Down syndrome subjects between ages 9 and 39; 5 yr later, the prevalence of alveolar bone loss had increased to 75% (96, 97).
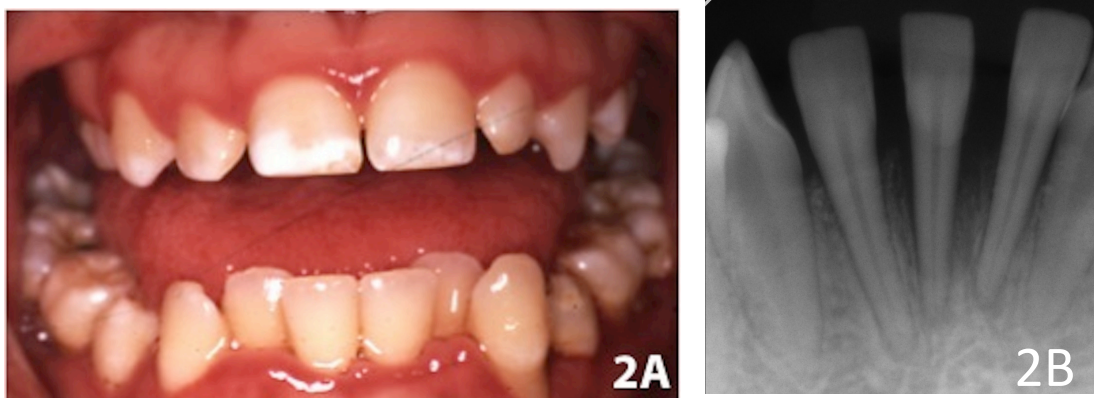

Figure 2A. **Gingival inflammation.** Gingival inflammation of the margins of the gingiva. Figure 2B. **Periodontitis** in the lower incisors in 18-year old patient with Down syndrome.

Microbiology

Oral biofilm play a key role in the aetiology of oral disease. Biofilms are microbial communities composed of numerous diverse organisms that exist in a collective state (98, 99). Today over 700 species have been identified in the human oral cavity (100). Micro-organisms in oral biofilm are widely considered to initiate gingival inflammation (101, 102). Periodontal destruction might be due to an upregulated inflammatory response to bacterial products from Gram-negative anaerobic periodontopathogens present in oral biofilm (103-105). Few studies have investigated the microbiological composition of plaque from patients with Down syndrome regarding different types of micro-organisms involved in the periopathogenic process. Barr-Agholme and co-workers (106) found higher percentages of *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Capnocytophaga* in subjects with Down syndrome compared to controls, indicating an altered microbial composition of the subgingival plaque in persons with Down syndrome. Amano and co-workers (107) found that children with Down syndrome between ages 2 and 13 more frequently exhibited micro-organisms such as *Aa, Porfyromonas gingivalis (Pg) , Bacteroidus forsythus,* and *Treponema denticola* in subgingival plaque than controls. According to the authors, these findings suggest that individuals with Down syndrome experience colonization by various micro-organisms associated with periodontal disease early in childhood and that the resulting altered composition of subgingival plaque may lead to early initiation of periodontal disease (107). Furthermore, Sakellari and co-workers (108) reported that Down syndrome adolescents more frequently present higher levels of *Aa* and *Pg* compared to age-matched healthy subjects. However, subgingival microflora in adults with Down syndrome are no different than in matched individuals without Down syndrome (109, 110).

Gingival crevicular fluid

Gingival crevicular fluid (GCF) is mainly an inflammatory exudate that is collected in the gingival crevices surrounding the teeth (111-113). GCF components have a variety of sources and contain substances originating from the host as well as from micro-organisms in subgingival and supragingival plaque. Substances from the host include molecules from the blood as well as contributions from periodontal cells and tissues. When micro-organisms in the dental biofilm initiate an inflammatory response, various inflammatory

mediators that can be detected in GCF, such as cytokines, prostaglandins, and enzymes like matrix metalloproteinases (MMPs), are produced (40-43, 103-105, 114) (Figure 3).
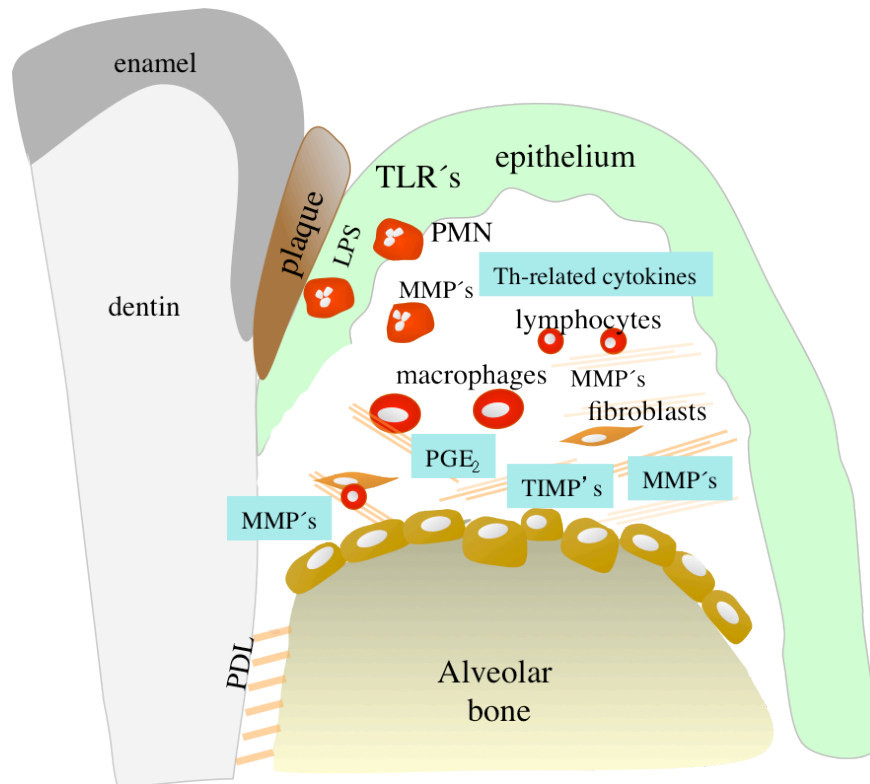

Figure 3. A schematic illustration of the **host response in periodontal disease.** The host response in periodontal disease includes complex interactions between a multitude of cell types, inflammatory mediators and tissue degrading enzymes, some of which are illustrated above. Bacterial antigens such as lipopolysaccharides (LPS) are recognized by the toll-like receptors (TLR´s) that initiate the recruitment of inflammatory cells into the periodontal tissue. Inflammatory cells and resident cells produce inflammatory mediators (cytokines and prostaglandins) as well as proteolytic enzymes (matrix metalloproteinases). Modified from Lerner 2005 (115).

The collection and analysis of GCF is a useful, non-invasive method for evaluating the host response in periodontal disease. The volume of GCF present at a given site may be directly related to tissue inflammation as well as permeability and ulceration of the crevicular epithelium. With increased inflammation, the volume of GCF increases (116). One of the methods of collecting GCF is with paper strips (Figure 4). The advantages of using a paper strip are that the method is quick and easy as well as that individual sites can be sampled. Furthermore, when used correctly, paper strips is probably the least traumatic means of collecting GCF from the gingival crevice (114).
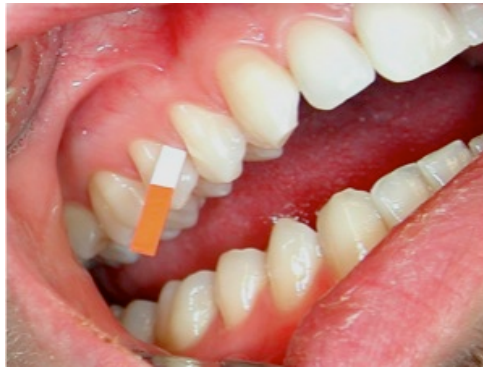
Figure 4. GCF collecting with paper strip.

Arachidonic acid metabolites

Membrane phospholipids release arachidonic acid (AA) in response to phospholipases A_2 (PLA_2) (Figure 5), a family of enzymes that are activated by mechanical injury, infection, allergens and cytokines, including IL-1β and TNF-α (117, 118). Free AA is then metabolized in the cyclooxygenase (COX) pathway, where the isoenzymes COX-1 and COX-2 convert AA to prostaglandin H_2 (PGH_2) (119). PGH_2 is subsequently metabolised to prostaglandins (PG) and thromboxanes (TX) (119, 120). The isoenzyme prostaglandin E synthase (PGE synthase) catalyse the conversion of COX-derived PGH_2 to prostaglandin E_2 (PGE_2) (121, 122). AA can also be oxidized along the lipoxygenase pathway. The central enzymes, 5-lipoxygenase (5-LO), leukotriene (LT) A_4 hydrolase, and LTC_4 synthase, produce several classes of leukotrienes and lipoxins, among them LTB_4 (123-125).
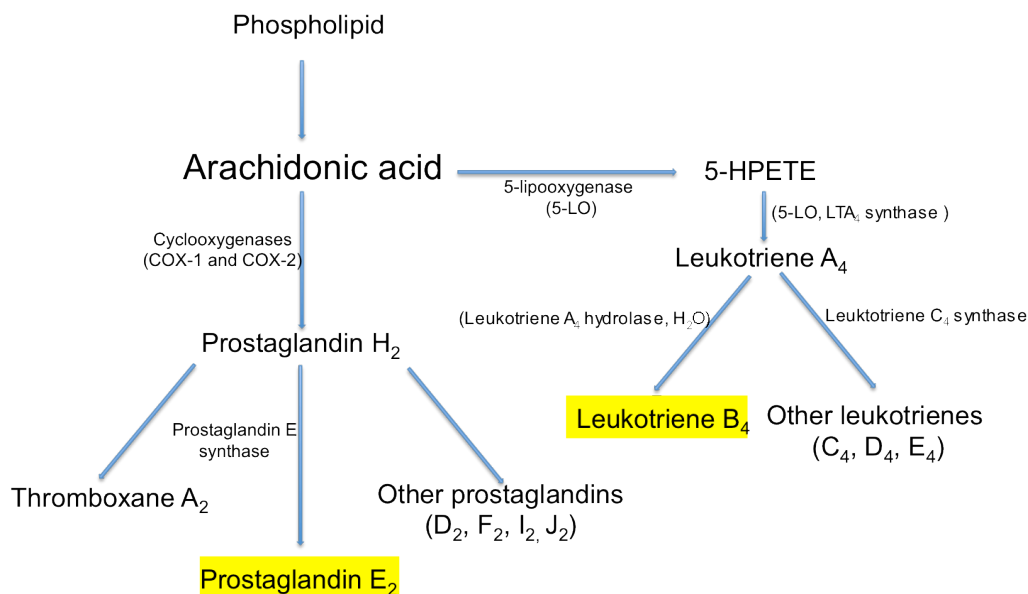

Figure 5. **Overwiew of the arachidonic acid pathway**. Arachidonic acid, derived from phospholipids can be metabolized by a range of enzymes to form prostaglandins (including PGD_2, PGE_2, PGI_2 and PGF_2) and leukkotrienes (including LTB_4, LTC_4, LTD_4 and LTE_4) as well as thromboxanes (including TXA_2).

Prostaglandin E$_2$

One of the best known and well-studied prostaglandins, PGE$_2$ (126), is involved in the pathogenesis of several chronic inflammatory diseases including periodontitis, rheumatoid arthritis, and atherosclerosis (127-129). The levels of this mediator are elevated in the gingival tissue and in the GCF of patients with periodontitis compared to periodontally healthy subjects (128, 130, 131). It is also well established that PGE$_2$ partly induces bone resorption, since PGE$_2$ stimulates osteoclast formation by increasing receptor activator of nuclear factor-κB ligand (RANKL) expression and inhibiting osteoprotegerin expression in osteoblasts (132-134). The inhibition of osteoprotegerin expression allows RANKL to interact with its receptor RANK on osteoclast lineage cells to drive differentiation to osteoclasts (135, 136). In addition, PGE$_2$ also induces the production of MMPs, which are associated with periodontal tissue destruction (137).

In Down syndrome, information on PGE$_2$ production in periodontal tissue is limited. Barr-Agholme and co-workers (138) previously reported enhanced PGE$_2$ levels in the GCF of Down syndrome patients compared with age-matched controls. Furthermore, Otsuka and co-workers (139) demonstrated that in response to *A.a* LPS treatment, gingival fibroblasts isolated from subjects with Down syndrome produced higher levels of PGE$_2$ compared to gingival fibroblasts from healthy controls.

Leukotriene B$_4$

Higher levels of the AA metabolite LTB$_4$ have been found in various inflammatory diseases such as asthma, rheumatoid arthritis, and periodontal disease (118, 140). LTB$_4$ has several functions: it promotes the aggregation and adhesion of PMNs to endothelial cells, it facilitates the migration of PMNs to the inflammation site, it activates NK cells, and it regulates IL-1 and IFN-γ production of T lymphocytes in an immunoregulatory role (141, 142). Heasman and co-workers (143) demonstrated that LTB$_4$ is enhanced in GCF collected from patients during experimental gingivitis. In addition, Pradeep and co-workers (140) demonstrated that the more severe the periodontal disease, the higher the levels of LTB$_4$ and that after periodontal treatment, LTB$_4$ levels in GCF decreased. Rodrigues Freire and co-workers (144) reported increased chemotactic activity toward neutrophils as well as increased levels of 5-LO mRNA expression in Down syndrome subjects with periodontal disease compared to controls. To our knowledge, no reports of LTB$_4$ levels in GCF from patients with Down syndrome were available at the start of this project.

Cytokines

The immune system (*e.g.,* monocytes, macrophages, and T cells) produces most cytokines, but other cells, like mast cells, fibroblasts and endothelial cells also produce cytokines. Cytokines interact in a complex way, inducing or inhibiting the production of each other. They are also involved in the initiation and development of inflammation, regulating the amplitude and duration of the response (145, 146).

There are two distinct types of T cells, the CD4+ Th cell and the CD8+ pre-cytotoxic T cell (52). Th cells were initially subdivided into two subsets, Th1 and Th2, on the basis of their pattern of cytokine production (147). In general, immune responses mediated by T cells polarized into a Th1-type phenotype are characteristically cellular and pro-inflammatory, while Th2 cells are associated with humoral immunity and present anti-inflammatory properties (147, 148). Studies have also described four other Th cell subsets: Th9, Th17, Th22 and T-reg cells (149-152). The key cytokine involved in the Th1 response, which stimulates cytotoxic T-lymphocyte responses, is IFN-γ; the key cytokine in the Th2 response, which stimulates B lymphocytes, is IL-4 (58, 153). Th17 cells, which are involved in the pathogenesis of autoimmune diseases such as inflammatory bowel disease and rheumatoid arthritis, are a Th subpopulation that is characterized by the production of its key cytokine IL-17 (153-155). The fourth group of Th cells, T-reg cells, plays a critical role regulating immune response by cytokines such as transforming growth factor (TGF)-β, IL-10, and IL-35 (156). The key cytokine involved in the Th9 response is IL-9, which promotes inflammation in a variety of models but seems to be particularly important in promoting allergic inflammation (62, 157). The sixth group of Th cells, Th22 cells are characterized by the production of IL-22 that increases the antimicrobial defense by enhancing the expression of antimicrobial peptides (152).
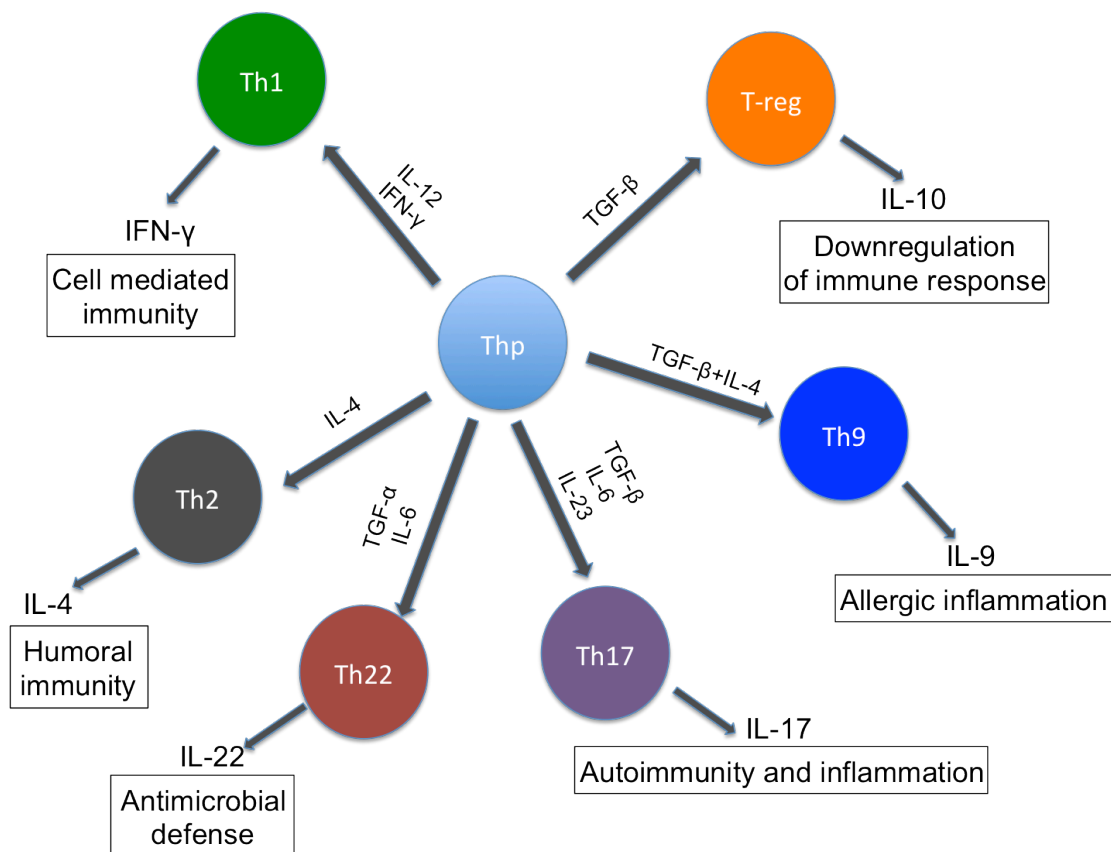
Figure 6. **Overview of Th cell differentiation.** Th precursor (Thp) cells differentiate into subsets as a result of their cytokine environment. Th1 cells are induced by IL-12 to produce IFN-γ that drive cell mediated immunity. Th2 cells are induced by IL-4 to produce IL-4 that drive humoral immunity. Th17 cells are induced by cytokines like TGF-β, IL-6 and IL-23 to produce IL-17. Th-17 cells play a vital role in inflammation and autoimmunity. T-reg cells are induced by TGF-β to produce IL-10 and is essential in the regulation of an immune response by suppressing the activation of other T-cells. Th9 cells are induced by TGF-β and IL-4 to produce IL-9 that is assumed to play an important role in allergic inflammation. Th22 cells are induced by TGF-α and IL-6 to produce IL-22 that increases the antimicrobial defense of skin keratinocytes by enhancing the expression antimicrobial peptides and plays a role in inflammatory skin disease. Modified from Kramer and Gaffen (158).

Subjects with Down syndrome demonstrate an altered cytokine production, as both TNF-α and IFN-γ are overexpressed in the thymus (63). Few studies have investigated the role of cytokines in periodontal disease in subjects with Down syndrome. Barr-Agholme and co-workers (138) previously reported unaffected levels of IL-1β in GCF from subjects with Down syndrome compared to controls. Recent studies have reported reduced expression of IL-10 as well as a reduced interferon-mediated response against microbial stimulus in the periodontal microenvironment in subjects with Down syndrome exhibiting periodontitis (159, 160). And Iwamoto and co-workers (161) reported that IFN-γ induces IL-6 in fibroblasts from Down syndrome.

Matrix metalloproteinases

MMPs, collectively known as matrixins, form a family of structurally related endopeptidases that mediate the degradation of the main components in the extracellular matrix and thus play important roles in cell migration, wound healing, and tissue remodelling (162). To date, of the 24 known MMPs, 23 are found in humans (162). MMPs are classified into groups based on substrate specificity, such as collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), stromelysin-like (MMP-7, -11, and -12) and membrane-type (MMP-14, -15, -16, and -17) (163). MMPs are secreted as latent, inactive pro-enzyme forms with cytokines like IL-1β and TNF-α as likely inducers of MMP expression (164-167).

The balance between MMP expression and synthesis is regulated by their major endogenous inhibitors, TIMP-1, -2, -3, and -4, which partly control and stabilize MMP expression (168). Almost all MMPs can be inhibited by the four TIMPs, although differences in binding affinity have been reported (168). In periodontal tissue, MMPs and TIMPs are expressed by both inflammatory cells (monocytes, macrophages, lymphocytes and PMNs) and resident cells (fibroblast, epithelial cells and endothelial cells) (169, 170).

Compared with healthy periodontal tissue, MMP levels are generally higher in inflamed periodontal tissue, in which TIMP levels exceed MMP levels; the more severe the inflammation, the higher the levels of MMPs (171). Compared with healthy controls, GCF and gingival tissue from subjects with periodontal disease exhibit significantly higher levels of MMP-1, -2, -3, -8, and-9, in contrast to significantly lower levels of TIMP-1 and-2 (172, 173). Li and co-workers (174) reported increased levels in serum of MMP-1, -3, and -9 in patients with chronic periodontitis compared to healthy controls.

Information on MMP and TIMP levels in periodontal tissue in Down syndrome is limited although Yamazaki-Kubota and coworkers (175) reported that MMP-2 and -8 levels are enhanced in the GCF of subjects with Down syndrome. In addition, Halinen and co-workers (176) demonstrated increased immunoreactivity of MMP-8 in the GCF of subjects with Down syndrome compared to healthy controls.

Subjects with Down syndrome exhibit a higher prevalence of periodontal disease (81). It has also been demonstrated that Down syndrome children exhibit much more extensive gingival inflammation than healthy controls despite similar levels of plaque between the two groups (92). Information on the early inflammatory response in terms of AA metabolites (PGE$_2$ and LTB$_4$) and pro- and anti-inflammatory cytokines as well as

information on periodontal tissue turnover in terms of MMPs and TIMPs in GCF from Down syndrome subjects is limited. Our hypothesis is that subjects with Down syndrome exhibit an altered host response in the gingival crevice during the early stages of periodontal disease compared to controls and that the altered host response possibly contributes to increased levels of MMPs in this patient group.

General aim

To study the inflammatory response in terms of cytokines, prostaglandins, and matrix metalloproteinases (MMPs) in the periodontal tissue of children with Down syndrome compared with controls.

Specific aims

Study I

To study the levels of prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and MMP-9, in gingival crevicular fluid (GCF) from Down syndrome patients.

Study II

To investigate the levels of T-helper (Th) 1-, Th2-, and Th17-related cytokines in the GCF of subjects with Down syndrome.

Study III

To study whether the relationship between MMPs and tissue inhibitors of metalloproteinases (TIMPs) in GCF from Down syndrome subjects is altered.

Study IV

To investigate the relationship between MMPs in serum and peripheral lymphocytes of Down syndrome children.

Materials and methods

T his section gives a brief overview of the methods used to obtain the results presented in this thesis. The study design was cross-sectional and the Ethics Committee at Karolinska University Hospital, Karolinska Institutet, Huddinge, Sweden, approved the study protocol, methods, and selection of subjects. The subjects and/or their parents received verbal as well as written information, and all subjects and/or their parents gave their informed consent for participation in the studies.
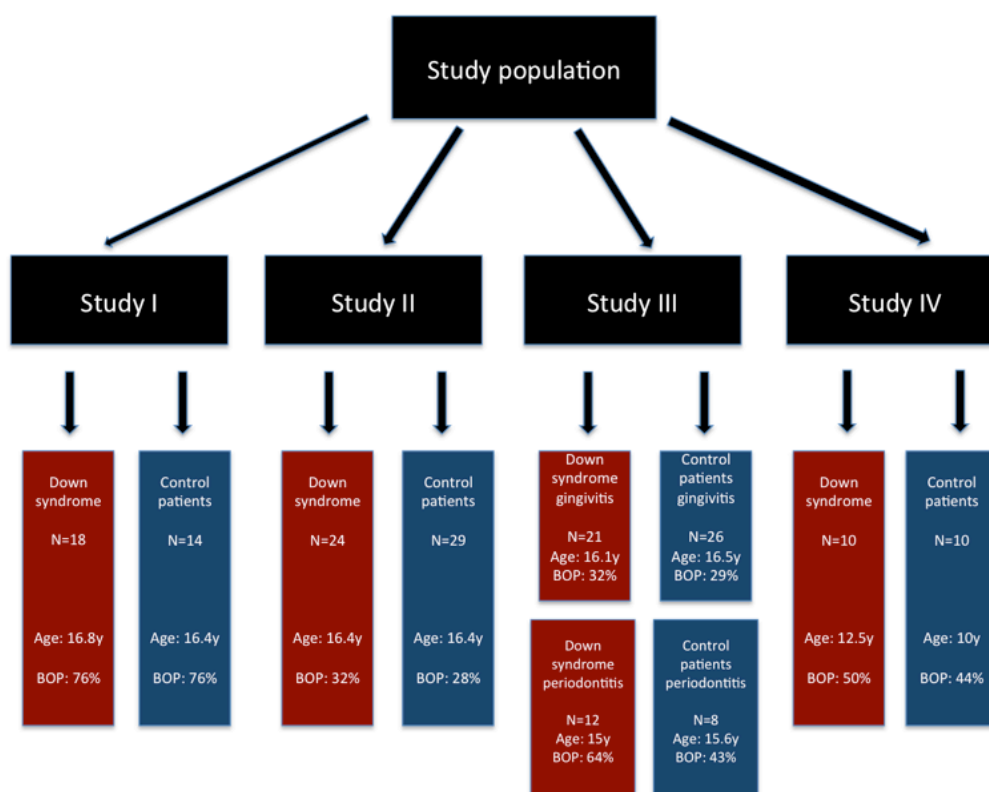
Study population

Figure 7. Overview of the study population in the four studies included in this thesis.

Study I

Twenty-six subjects with Down syndrome and 90 healthy controls were examined. Inclusion criteria were age and degree of inflammation expressed as percentage of bleeding on probing (BOP%). Exclusion criteria were one or more sites with a periodontal probing

depth (PD) > 4 mm or marginal alveolar bone loss. The final study group comprised 18 subjects with Down syndrome (mean age 16.8 yr) and 14 controls (mean age 16.4 yr) matched for age and degree of gingival inflammation. All subjects received regular dental treatment at the Department of Paediatric Dentistry, Karolinska Institutet, Huddinge, Sweden.

Study II

The study population comprised 50 subjects with Down syndrome who had been consecutively referred from the Public Dental Health Services, Stockholm, to the Department of Paediatric Dentistry at Eastmaninstitutet in Stockholm and 78 control subjects who had been randomly selected from the Public Dental Health Services, Eastmaninstitutet, with respect to age. Inclusion criteria were age (13-20 yr) and degree of gingival inflammation expressed as BOP less than 50%. Exclusion criteria were previous and on-going smoking habits, on-going orthodontic treatment, one or more sites with a PD > 3 mm, and the occurrence of marginal alveolar bone loss on radiographs. The final study group comprised 24 subjects with Down syndrome and 29 matched controls (both groups, mean age 16.4 yr).

Study III

The study population comprised 56 subjects with Down syndrome who had been consecutively referred to the Department of Paediatric Dentistry at Eastmaninstitutet and 88 control subjects with gingivitis and periodontitis. The control subjects with gingivitis were selected from the Public Dental Health Services in Stockholm and the control subjects with periodontitis had been consecutively referred to the Department of Paediatric Dentistry at Eastmaninstitutet and the Department of Periodontology, Stockholm. For all patients, inclusion criteria were age between 11 and 20 yr. The additional inclusion criterion for subjects with gingivitis was BOP < 50%. Additional inclusion criteria for subjects with periodontitis were one or more sites with a PD > 3 mm and marginal alveolar bone loss on radiographs. For all patients, exclusion criteria were recent use of antibiotics (last 3 months), previous or on-going smoking and on-going orthodontic treatment. For the controls, an additional exclusion criterion was a diagnosed chronic medical disorder. The final study group comprised 21 Down syndrome subjects with gingivitis (DS-G, mean age 16.1 yr), 12 Down syndrome subjects with periodontitis (DS-P, mean age 15.0 yr), 26

healthy control subjects with gingivitis (HC-G, mean age 16.5 yr) and 8 healthy control subjects with periodontitis (HC-P, mean age 15.6 yr).

Study IV

The study population included subjects with Down syndrome and healthy controls who had been consecutively referred to the Department of Paediatric Dentistry at Eastmaninstitutet for dental treatment under general anaesthesia due to behavioural management problems. Inclusion criteria for all subjects were fully erupted permanent first molars or incisors and presence of gingivitis. For all subjects, the exclusion criteria were presence of periodontitis, use of antibiotics in the past 3 months, previous or on-going smoking and on-going orthodontic treatment. In the control group, a diagnosed chronic medical disorder was an additional exclusion criterion.

Of all subjects with Down syndrome (n=24) who had been referred for dental treatment under general anaesthesia in 2012, 4 declined to participate in the study and 10 were excluded due to unerupted permanent first molars. Thus, the final Down syndrome group included 10 subjects (mean age 12.5 yr). The control group was selected from 144 subjects treated under general anaesthesia during 2012. Of the 144 subjects, 94 subjects had a chronic medical condition (excluding Down syndrome) and 26 had unerupted first permanent molars. Thus, 24 subjects fulfilled the inclusion criteria for the control group. Of these, 3 were excluded because they had received emergency treatment under general anaesthesia, and 11 declined to participate in the study. The final control group comprised 10 patients (mean age 10 yr).

Clinical and radiographic examinations (studies I-IV)

The author of the thesis performed all clinical and radiological examinations of the patients. All parents/subjects answered a medical history questionnaire regarding oral hygiene habits, smoking habits, medication, and the occurrence of medical disorders. An interpreter assisted when subjects did not understand the Swedish language.

Gingival inflammation

In **studies II–IV**, gingival inflammation was based on BOP% of the gingival sulcus at four sites per tooth at all teeth (wisdom teeth excluded), and in **study I**, on six sites per

tooth at all teeth. The percentage of surfaces with BOP was calculated for each individual and expressed as BOP%.

Periodontal probing depth

PD was recorded using a graded periodontal probe (Hu-Friedy, Chicago, IL, USA) and measured to the nearest mm at four sites per tooth at all teeth (wisdom teeth excluded). PD was considered pathological when the subject exhibited one or more sites with a periodontal PD > 3 mm.

Marginal alveolar bone loss

Radiographic examination generally consisted of digital and conventional bitewing and periapical radiographs. Due to lack of cooperation, some subjects with Down syndrome were examined with panoramic radiographs. Alveolar bone loss was noted when the distance from the cemento-enamel junction to the alveolar crest on the radiograph exceeded 2 mm on molars, premolars, or incisors (177).

Sampling of gingival crevicular fluid (studies I-IV)

Collection of GCF samples (studies I-IV)

Prior to the clinical examination, GCF samples were collected from each patient from the mesial surfaces of teeth 16, 26, 36, 46, and 41 and the distal surface of 11. Before GCF collection, supragingival plaque was eliminated using a cotton pellet and curette, and the tooth surface was gently dried with air. A paper strip (Periopaper; ProFlow, Inc., Amityville, NY, USA) was inserted into each sulcus and left for 15 s. Paper strips contaminated with blood during GCF sampling were discarded. GCF volume was determined by a Periotron 8000 (ProFlow, Inc.) system and calculated by interpolation from a standard curve and expressed as volume GCF (μL). The periopaper was placed in 120 μL of assay buffer containing 0.9% NaCl, 0.01 M EDTA, 0.3% bovine-globulin, 0.005% Triton-X-100, 0.05% sodium azide, 0.0255 M NaH_2PO_4, and 0.0245 M Na_2HPO_4 (pH 6.8) and kept frozen at -70°C.

Analysis of GCF samples (study I)

PGE_2, LTB_4, and MMP-9 levels in GCF were determined. Levels of AA metabolites, PGE_2 and LTB_4 were assessed using commercially available radioimmunoassay (RIA) kits (NEN

Life Science products, Belgium) with ^{125}I-PGE$_2$ as the tracer for PGE$_2$ and ^{3}H as the tracer for LTB$_4$. The levels of total MMP-9 (active MMP-9 and pro-MMP-9) were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, UK).

Analysis of GCF samples (studies II–IV)

The levels of MMP-2, -3, -8, -9, and -13 and TIMP-1, -2, and -3 were determined in GCF samples using the commercially available Human MMP/TIMP Multianalyte Kit (R&D systems Inc., MN, USA) according to the manufacturer's instructions. Briefly, a 96-well microplate was pre-wet with 100µL wash buffer/well and 50µL of diluted microparticle mixture added to each well. The diluted GCF samples (1:2 for MMP-2, -3, -13; 1:40 for MMP-8, -9; and 1:10 for TIMP-1, -2, -3) were added to each well and incubated for 2 h at room temperature on a horizontal orbital microplate shaker. After washing, 50µL of diluted antibody cocktail was added to each well and incubated for 1 h at room temperature. Finally, 50µL of diluted Streptavidin-PE was added to each well and incubated for 30 min at room temperature. After the last incubation, MMP and TIMP levels were determined using a Luminex analyzer (Bio-Rad Laboratories, CA, USA). According to the manufacturer, MMPs and TIMPs have < 0.5% cross-reactivity with other MMP and TIMP family members. MMP-2, -3, -8, -9, and -13 recognize both natural and recombinant human pro, mature as well as TIMP-1 complexed MMP-2, -3, -8, -9 and -13. TIMP-1, -2, -3, and -4 recognize both natural and recombinant human TIMPs.

The levels of IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-12, and IL-17 in GCF were determined using the commercially available Bio-Plex Cytokine Assay (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions. In brief, a 96-well microplate was pre-wet with 100µL wash buffer/well and 50µL of coupled magnetic beads were added to each well. GCF samples, 50µL, were then added to each well and incubated for 30 min at room temperature. After washing, 25µL of detection antibodies were added to each well and incubated for 30 min at room temperature. Finally, 50µL of diluted Streptavidin-PE was added to each well and incubated for 10 min at room temperature. The levels of the various cytokines were determined in a Luminex analyzer (Bio-Rad Laboratories).

Serum sampling (study IV)

During general anaesthesia, 12ml peripheral venous blood was collected in three vacutainer glass tubes containing heparin. One glass tube was immediately centrifuged and the serum was kept frozen at -70°C. The remaining blood was used for flow cytometry analysis. All serum samples were analysed for MMP-2, -3, -8, and -9 and TIMP-1, -2, and -3; levels were determined using the commercially available Human MMP/TIMP Multianalyte Kit (R&D systems, Inc.) according to the manufacturer's instructions.

Immunophenotyping of peripheral blood cells (study IV)

Peripheral blood (8ml) from the children with Down syndrome and the matched controls was lysed by adding FACSLysing solution (BD Biosciences, San Jose, CA USA) according to the manufacturer's protocol. The cells were then washed twice with phosphate-buffered saline (PBS), stained in 50μL of PBS with conjugated antibodies, counted, and analyzed using multicolour flow cytometry to determine the relative amounts (%) of the various cell phenotypes. Five- or eight-color fluorescence analyses were performed in an eight-color flow cytometer (BD FACSVerse™ 8 color flow cytometer, BD Biosciences) according to the manufacturer's recommendations. Briefly, the immunophenotypic analysis of the cells was done using the following conjugated anti-human monoclonal antibodies with the appropriate concentration of fluorochrome. After the addition of antibodies and incubation for 30 min at 4°C in the dark, the cells were washed with PBS and then analysed. Each analysis required a minimum of 10,000 cells. Data were analysed using BD FACSSuite™ software (BD Biosciences) and FlowJo (version 8.5.3; Tree Star, Inc., OR, USA).

Cell cultures of peripheral blood leukocytes (study IV)

Leukocytes were isolated from peripheral blood as previously described. The cells (3×10^6) were seeded in 60-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Life Technologies, Scotland, UK), cultured, and then incubated at 37°C in 1.2mL DMEM with or without *Pg* LPS (10μg/mL) (Sigma-Aldrich, St. Louis, MO, USA). After 16 h of incubation, the culture medium was collected and centrifuged (1500 rpm/min), after which the supernatant was removed and stored at -70°C until MMP-8

and MMP-9 could be assessed (R&D Systems Inc.). The leukocytes were stained with various antibodies as previously described and analysed using flow cytometry to determine the percentage of T-lymphocyte subpopulations (CD3+, CD4+, CD8+) and NK cells (CD56+).

Statistical analysis

In **study I**, the Student's independent *t*-test (two-tailed) was used to compare means between the groups and correlations within groups. A general linear model ANOVA test was used to test differences in correlation coefficients between Down syndrome patients and controls. All statistical calculations were done using the Statistical Package for the Social Sciences (SPSS 10.0).

In **study II**, the Student's independent *t*-test (two-tailed) was used to compare the means and the chi-square exact test was used to compare the categorical variables of the groups. Pearson's correlation was used to calculate the correlation within the group. Fisher's z-transformation was used to test the difference in correlation coefficients between subjects with Down syndrome and controls. The Bonferroni analysis was used to adjust for multiple testing. All statistical calculations were done using SPSS (version 13.0) and MedCalc (version 10.2; MedCalc Software, Mariakerke, Belgium).

In **study III**, The Mann-Whitney U test (two-tailed) was used to compare the medians and the chi-square exact test was used to compare the categorical variables of the groups. Pearson's correlation was used to calculate correlations between groups. Fisher's z-transformation was used to test the difference in correlation coefficients between subjects with Down syndrome and controls. The Bonferroni analysis was used to adjust for multiple testing. All statistical calculations were done using the SPSS (IBM SPSS Statistics for Windows, Version 20.0, 2011, Armonk, NY: IBM Corp).

In **study IV**, the Mann-Whitney U test (two-tailed) was used to compare the medians of the variables. Pearson's correlation was used to calculate correlations between groups. All statistical calculations were done using the SPSS (IBM SPSS Statistics for Windows, Version 20.0, 2011, Armonk, NY: IBM Corp).

Results and Discussion

The four studies on which this thesis is based describe the inflammatory response in terms of cytokines, prostaglandins, and MMPs in the periodontal tissue of children with Down syndrome and in controls. **Study I** deals with the levels of the AA metabolites PGE_2 and LTB_4 as well as of MMP-9 in gingival crevicular fluid from patients with gingivitis. In **study II**, the balance between pro- and anti-inflammatory cytokines in gingival crevicular fluid from patients with gingivitis was studied. **Study III** investigated homeostasis in periodontal tissue by studying the balance between MMPs and TIMPs in gingival crevicular fluid from both gingivitis and periodontitis patients. **Study IV** further explored the possible role of MMPs in periodontal disease by investigating the association between MMPs in serum and peripheral blood leukocytes from patients with gingivitis. All four studies have been submitted to peer-reviewed journals and three are published. The articles can be found in their entirety in the appendix. This section gives a brief overview of the results of these studies and a discussion of the findings in relation to current literature.

Clinical conditions of the subjects

In all studies, the subjects were clinically and radiographically examined to determine BOP%, periodontal PD, and presence of marginal alveolar bone loss. We did not use the plaque index (PI) in any study since oral hygiene in children varies from day to day, making the PI an unreliable indicator of gingival inflammation (178-180). Furthermore, cooperation in subjects with Down syndrome is often low and they easily become impatient. Thus, the validity of BOP% as an indicator of gingival inflammation in children is more reliable than PI and the reason we decided to assess BOP instead of PI (181). Underestimation of periodontal PD in subjects with Down syndrome compared to controls is also likely, due to insufficient cooperation during probe penetration of periodontal pockets.

All subjects in **studies I–IV** exhibited gingivitis. **Study III** also included patients with periodontitis.

AA metabolites (study I)

Several studies have suggested that PGE_2 and LTB_4 are involved in the pathogenesis of periodontal disease. Enhanced levels of both PGE_2 and LTB_4 have been detected in the gingival tissue and GCF of patients with periodontal diseases compared to periodontally healthy subjects (131, 182-184). In **study I**, levels of PGE_2 and LTB_4 in GCF expressed as pg/mL were significantly ($p<0.05$) higher in subjects with Down syndrome compared to controls matched for degree of gingival inflammation. An overexpression of SOD-1 in subjects with Down syndrome might explain these elevated levels. Hensley and co-workers (185) reported that astrocytes overexpressing SOD-1 *in vitro* exhibit elevated release of PGE_2 and LTB_4. Furthermore, the altered subgingival microflora in subjects with Down syndrome (106, 108) may contribute to enhanced levels of PGE_2 in GCF since previous studies have reported that various periodontal pathogens stimulate production of PGE_2 by resident cells and monocytes (186-189).

LTB_4 was significantly negatively correlated with the clinical variables BOP% and PD. In contrast, the correlation was positive in controls, which is well compatible with the view that LTB_4 plays an important role in the recruitment of neutrophils during inflammation and, thus, is strongly associated with gingival inflammation (140, 143). Although subjects with Down syndrome demonstrated higher levels of LTB_4, the negative correlation with

BOP% found in this study might be related to the impaired chemotactic ability observed in subjects with Down syndrome (35).

Relationship between pro- and anti-inflammatory cytokines in GCF (study II)

The novel findings in **study II** were higher levels of Th1-, Th2-, and Th17-related cytokines in GCF and an altered relationship between Th1 cytokine IFN-γ and Th2 cytokine IL-4 in subjects with Down syndrome compared to controls.

The relationship between IFN-γ and IL-4 in GCF differed significantly ($p < 0.05$) between subjects with Down syndrome and controls. This difference indicates an altered immune response regarding the balance between pro- and anti-inflammatory cytokines in the Down syndrome group, although it is unclear whether the inflammatory response was enhanced or decreased. Interestingly, Kalinski (126) reported that PGE_2, reduces production of Th1 cytokine IFN-γ but not the Th2 cytokine IL-4 in human CD4+ T cells. Thus, it is possible that PGE_2 promotes Th2 responses since it has been described to be involved in Th2-associated diseases such as asthma (126). However, the role of PGE_2 in the altered relationship of IFN-γ/IL-4 in GCF observed in the Down syndrome group is an important topic for future studies.

Correlations of BOP% and GCF volume (μL) with the cytokines IL-1β, -4, -6, -10, and -12, IFN-γ, and TNF-α were also investigated. In the controls, cytokine levels of IL-1β, -4, and -10, IFN-γ, and TNF-α were significantly ($p < 0.05$) positively correlated with GCF, which did not occur in the Down syndrome subjects. Lack of a significant correlation between the clinical variables and the investigated cytokines in subjects with Down syndrome further supports the concept of an altered host response regarding pro- and anti-inflammatory cytokines. However, lack of positive correlations between GCF volume and the various cytokine levels in subjects with Down syndrome could partly be a result of the greater variation in cytokine levels in GCF as well as the heterogeneity within the Down syndrome group related to differences in genotypes.

We also demonstrate significantly ($p < 0.05$) higher levels of IL-1β, -4, -6, -10, and -12, IFN-γ, and TNF-α in GCF of subjects with Down syndrome compared to controls. The higher cytokine levels may result from the enhanced number of pro-inflammatory monocytes (CD 14^{dim} CD16^{+}) observed in subjects with Down syndrome compared to

healthy controls (190). Pro-inflammatory monocytes, which represent approximately 10% of the total monocyte population, have superior antigen presenting cell activity and produce significant amounts of pro-inflammatory cytokines (191-194). Interestingly, **study IV** investigated the percentages of various peripheral blood leukocytes and found no difference regarding CD14+ monocytes in Down syndrome subjects compared to controls. However, this project did not have the opportunity to identify which periodontal tissue cells produced the cytokines that contribute to enhanced levels of cytokines in GCF.

Relationship between MMPs and TIMPs in GCF (studies I, III, IV)

A balance between tissue regeneration and tissue degradation is required to maintain periodontal tissue homeostasis. The long-term result of a disturbance of that balance may be periodontal tissue breakdown (168). Increasing evidence implicates MMPs as key mediators in the tissue destruction associated with various forms of periodontal disease, including the progression from gingivitis to periodontitis (195-197). MMP activity is controlled by the TIMPs (TIMP-1, -2, -3 and -4), which contribute to the stabilization of MMPs and thereby participate in tissue remodelling during periodontal tissue destruction (162, 168, 198).

   Studies I, III, and IV found significantly ($p<0.05$) higher levels of MMP-2, -3, -8 and -9 as well as of TIMP-2 in the GCF of patients with Down syndrome compared to controls matched for degree of gingival inflammation. In addition, in **study III**, the correlation coefficient between MMP-8 and TIMP-2 differed significantly ($p<0.01$) between the Down syndrome group with gingivitis (DS-G) and the healthy control group with gingivitis (HC-G). The enhanced amounts of MMP-8 and TIMP-2 in GCF may partly explain the altered relationship between MMP-8 and TIMP-2 in the DS-G group, but if the amount of TIMP-2 was insufficient to balance the enhanced MMP production, it might partly explain the increase in tissue breakdown (199). In addition, the enhanced GCF levels of PGE_2 in subjects with Down syndrome demonstrated in **study I** might also contribute to the increased levels of MMPs in GCF as well as the altered relationship between MMP-8 and TIMP-2 in subjects with Down syndrome. This assumption is well compatible with the fact that MMP expression can be suppressed by inhibiting PGE_2 and, in contrast, increases TIMP levels in epithelial and stromal cells *in vitro* (200).

Furthermore, **study III** found no difference in the amounts of MMPs and TIMPs when comparing the DS-G group with Down syndrome patients with periodontitis (DS-P). This was in contrast to the control groups, where the control patients with periodontitis (HC-P) exhibited significantly higher amounts of both MMPs and TIMPs compared to the HC-G group. The lack of difference between the two Down syndrome groups could indicate that the inflammatory response in Down syndrome subjects is already strongly upregulated during the period of gingivitis.

Study III also found a significant positive correlation between TIMP-3 and TNF-α in the HC-G group, which could not be demonstrated in the DS-G group. TIMP-3 has been reported to regulate inflammation by inhibiting TNF-α converting enzyme (201, 202), which is involved in the proteolytic cleavage of pro-TNF-α on the cell surface, resulting in the release of soluble TNF-α (203, 204).

The increased levels of MMPs as well as the altered relationship between MMPs and TIMPs in GCF might be a result of the higher levels of PGE_2 and the altered balance between pro- and anti-inflammatory cytokines demonstrated in subjects with Down syndrome in the current cohort.

Relationships between MMPs in serum and peripheral blood lymphocytes (study IV)

The percentages of CD3+ and CD8+ T cells and of CD56+ NK cells were significantly higher ($p<0.05$) in the Down syndrome group compared to the controls. In contrast, no significant differences were found in the percentages of CD4+ T cells, CD14+ monocytes, CD15+ granulocytes, CD19+ B cells or CD45 cells, between the Down syndrome and control groups. Furthermore, the mean CD4/CD8 ratio was significantly lower ($p<0.01$) in the Down syndrome group compared to the controls.

The increased levels of CD3+ and CD8+ T cells as well as of CD56+ NK cells in peripheral blood demonstrated in **study IV** is in accordance with earlier studies (65-69, 205, 206). Our finding regarding the enhanced percentage of CD8+ T cells is noteworthy, since activated CD8+ T cells may play an important role in the degradation of periodontal tissue (164, 207, 208). The decreased CD4+/CD8+ ratio in the subjects with Down syndrome might indicate an altered immune response since the CD4+/CD8+ ratio is considered to be an important marker of immune system functions (209-211).

Interestingly, patients with early onset periodontitis have been reported to exhibit a decreased CD4+/CD8+ ratio compared to controls (212).

Study IV also determined MMP and TIMP levels in serum collected from subjects with Down syndrome and controls. Levels of MMP-3, MMP-8, and TIMP-1 in serum were significantly ($p<0.05$) elevated in the Down syndrome group compared to the control group, which may be related to the increased number of T and NK cells that **study IV** demonstrated. However, one must take into account that MMPs are also expressed by various inflammatory cells such as monocytes, macrophages, and polymorphonuclear cells (PMN) as well as resident cells such as fibroblasts, epithelial cells, and endothelial cells (169).

Furthermore, a significant positive correlation between CD8+ T cells and MMP-8 ($r=0.630$; $p=0.050$) and between CD8+ T cells and MMP-9 ($r=0.648$; $p<0.05$) was observed in the Down syndrome group that was not seen in control subjects. A significant positive correlation between CD56+ NK cells and MMP-3 ($r=0.828$; $p<0.01$) was also observed in the Down syndrome group, in contrast to the controls. The positive relationship between CD8+ T cells and both MMP-8 and -9 in subjects with Down syndrome is well compatible with Séguier and co-workers (213), who reported a positive correlation between CD8+ T cells and MMPs in gingival tissue from patients with perio-dontitis. In addition, experimental studies indicate that MMPs partly contribute to the migration of T cells and NK cells (214). In light of these findings, the positive relationship of T-cell subpopulations and NK cells with MMPs demonstrated in subjects with Down syndrome may emphasize the significance of the MMPs in the migration of infiltrating T-cell populations and NK cells into sites with periodontal inflammation. This assumption is compatible with the increased levels of MMPs in GCF in subjects with Down syndrome demonstrated in **studies I, III, and IV**.

The positive relationship between MMPs in serum and lymphocyte subpopulations in peripheral blood **(study IV)** and the enhanced levels of inflammatory mediators PGE$_2$, LTB$_4$, and TNF-α **(studies I and II)** in the Down syndrome group is interesting, since it has been demonstrated that PGE$_2$, LTB$_4$, and TNF-α upregulate the production of MMPs by T cells and NK cells (215-221).

Production of MMPs and TIMPs in cell cultures of peripheral blood leukocytes (study IV)

In **study IV**, peripheral blood leukocytes were treated with LPS from *Pg* and its effect investigated on MMP and TIMP levels as well as the relative amounts (%) of the various lymphocyte subpopulations. MMP-8 levels in the supernatant (expressed as pg/10,000 cells) and percentages of CD8+ T cells were significantly higher ($p<0.05$) in the Down syndrome group compared to controls. In cells not treated with *Pg* LPS, MMP-8 and TIMP-1 levels as well as the percentages of CD3+ T cells and CD8+ T cells were significantly ($p<0.05$) higher in the Down syndrome subjects compared to the controls. Furthermore, no significant differences in MMP and TIMP levels or in the lymphocyte subpopulations within the two groups were observed after stimulation with *Pg* LPS. Because **study IV** subjects exhibited gingivitis, a lower cellular response to stimuli by periodontal pathogens might explain the lack of increased MMP expression after stimulation with *Pg* LPS. This assumption is well compatible with the findings of Restaino and co-workers (222), who reported an increased expression of MMP-9 in neutrophils stimulated with *Pg* LPS in periodontitis patients but not in healthy controls with gingivitis.

Strengths and weakness of the studies

Studies I-IV: Down syndrome is a heterogeneous patient group. Cooperation in dental treatment is often low and subjects with Down syndrome get easily impatient, which makes it difficult to collect clinical data such as PI, PD, BOP, and GCF. It is therefore a challenge to conduct studies in this patient group. Another strength is that the same examiner examined all children in both groups and thereby minimized the variation in periodontal diagnosis. A weakness is that the difficulties in collecting GCF in Down syndrome subjects, compared with the controls, increased the risk of contamination and might have affected the biochemical results negatively. Furthermore, behavioural management problems during dental treatment in the Down syndrome group limited the number of subjects in this cohort.

    Study I-IV: GCF collection is a sensitive technique, and both contamination and prolonged collection time can affect the GCF measurements. It is important to be careful when placing the paper strip in the periodontal pocket so that the sample is plaque free (supragingival plaque must be removed) and that there is no blood or saliva

contamination. Contamination can influence the volume of fluid that is collected leading to an increase in GCF measurements (223). Furthermore, the problem with prolonged collection times is the increase of the protein content in GCF with increasing time (224). Also the way to report the data of the measured levels of host mediators in GCF is a matter of discussion. Unlike the analysis of serum, where the sample fluid is a small part of the total fluid volume, sampling of GCF varies from tooth site to tooth site. Based on the studies by Lamster and coworkers (225, 226) who developed a system to GCF sampling that standardizes the time of collection, they recommend that GCF data should be reported as total amount in the timed sample. Another factor to consider when reporting GCF data, is also that one has to take into account the dilution factor of the buffer in which the paper strip is placed when reporting the concentration of different host mediators in GCF.

Study III included both patients with gingivitis and patients with periodontitis, which made it possible to study the differences in inflammatory response in the early stages of periodontal disease and periodontitis.

Study IV was a functional study on peripheral blood from Down syndrome subjects and controls that investigated associations between MMPs in serum and peripheral blood leukocytes. However, this study must be considered a pilot study due to the relatively low number of subjects included. In addition, it is not possible to conclude what type of cells in the peripheral blood and in the periodontal tissue produce the different MMPs studied.

The factors described above should be taken into consideration when interpreting the results of this thesis.

Future perspectives

It has been incredibly interesting to participate in the studies on which this thesis is based, and they have provided some answers to the research questions. In the future, however, it would be of great interest to investigate the production of inflammatory mediators and MMPs from different leukocytes in periodontal tissue and peripheral blood and to further clarify their role in periodontal tissue inflammation in the Down syndrome group. Furthermore, longitudinal studies on the effect of routine preventive treatment on the inflammatory response in subjects with Down syndrome as evidenced by inflammatory mediators and proteolytic enzymes in GCF would be highly interesting.

Main findings

This section lists the main findings of the present thesis on the inflammatory response in children with Down syndrome.

o Down syndrome subjects exhibited increased levels of arachidonic acid metabolites (PGE_2 and LTB_4) in gingival crevicular fluid (**study I**).

o Increased levels of T-helper-related cytokines such as pro-inflammatory (IL-1β, IL-6, IL-12, IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10) were observed in gingival crevicular fluid from subjects with Down syndrome. There was an altered relationship between the anti-inflammatory cytokine IL-4 and the pro-inflammatory cytokine IFN-γ in gingival crevicular fluid from subjects with Down syndrome (**study II**).

o Levels of MMP-2, -3, -8, and -9 and of TIMP-2 in gingival crevicular fluid are increased in subjects with Down syndrome (**study I, III, IV**). In addition, a positive correlation was observed between MMP-8 and TIMP-2 in subjects with Down syndrome (**study III**).

o Serum MMP levels (MMP-3 and -8) are increased in individuals with Down syndrome (**study IV**).

o A relationship between MMPs (MMP-3, -8, -9) and lymphocyte subpopulations (CD8+T cells and CD56+ NK cells) in peripheral blood was observed in subjects with Down syndrome (**study IV**).

Clinical implications

The results of this thesis confirm that subjects with Down syndrome in the current cohort have an altered inflammatory response in periodontal tissue compared to healthy controls. The increased levels of AA metabolites (PGE_2 and LTB_4) as well as the altered relationships between pro- and anti-inflammatory cytokines and between MMPs and TIMPs in Down syndrome subjects with gingivitis is well compatible with our hypothesis of a strongly upregulated inflammatory response in periodontal tissue.

It is important to inform patients with Down syndrome and their parents and to make them aware of the altered inflammatory response in periodontal tissue observed in this patient group. These findings suggest that it may be important to begin routine preventive treatment in patients with Down syndrome at an early age, before they develop the first signs of periodontitis, to reduce periodontal tissue breakdown and thus improve patients' quality of life.

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