

Studies on onset and lesion characteristics in periodontitis

Sara Thorbert-Mros

Department of Periodontology
Institute of Odontology
Sahlgrenska Academy
University of Gothenburg



UNIVERSITY OF GOTHENBURG

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sara.thorbert@odontologi.gu.se

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To my family

with love

Níklás,

Svante, Sígne,

Pappa, Mamma, Carolína, Erik

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ABSTRACT

Will early forms of periodontitis in childhood predict future risk for severe periodontitis? At what age may onset of periodontitis be detected in subjects with severe periodontitis? Are there differences in cell composition between lesions representing longstanding gingivitis and severe periodontitis?

In *study I*, 11 children (7–13 years) with localized aggressive periodontitis (LAP) were re-examined after 14–19 years. While bleeding on probing was a general finding in the group, only two of the subjects exhibited recurrence of disease with probing pocket depth ≥ 6 mm and 3–4 mm of bone loss at several teeth. The age of onset of disease in 42 patients, 30–45 years of age, diagnosed with severe, generalized periodontitis was assessed in *study II*. The earliest age at which a radiographic examination revealed distance between the cement-enamel junction (CEJ) and alveolar bone crest (BC) ≥ 3 mm (F3) at any site was recorded, as well as the highest patient age at which a radiographic examination revealed absence of sites with CEJ-BC ≥ 3 mm (L0). Onset of disease, i.e. the interval between L0 and F3, occurred on the average between 22 and 28 years. In *study III* and *IV* differences between lesions representing longstanding gingivitis and severe periodontitis were analyzed. Gingival biopsies were collected and prepared for histological examination and RT-qPCR analysis. Periodontitis lesions were twice as large as gingivitis and contained significantly larger proportions and higher numbers of plasma cells and macrophages than gingivitis lesions. T cells were not the dominating cell type in gingivitis lesions, as B cells together with their subset plasma cells comprised a larger number and proportion than T cells. In addition, the total number and density of IL-17 producing T cells were larger and expression of IL-17mRNA was higher in periodontitis than in gingivitis lesions.

Conclusions:

Children treated for LAP do not always exhibit recurrence of periodontitis in the absence of supportive periodontal therapy over periods of 14–19 years. Disease in the current sample of 30–45 year-old subjects with severe, generalized periodontitis, commenced mainly between 22 and 28 years of age. Large number and high density of plasma cells are the hallmarks of advanced periodontitis lesions and the most conspicuous difference in relation to longstanding gingivitis lesions. IL-17 producing T cells represent a significant feature in the detection of differences between destructive and non-destructive lesions.

Keywords: [periodontitis, onset, lesion characteristics, plasma cells, IL-17]

SAMMANFATTNING

Kan parodontit i barndomen förutsäga att man kommer drabbas av svår parodontit som vuxen? Vid vilken ålder kan man se de första tecknen på parodontit hos unga vuxna med en hög känslighet för sjukdomen? Finns det skillnader i cellsammansättning mellan lesioner vid kronisk gingivit och avancerad parodontit?

I *studie I* undersöktes en grupp på elva patienter, som vid 7-13 års ålder diagnosticerats med lokaliserad aggressiv parodontit, 14-19 år efter initial diagnos. Trots att gingival inflammation var ett vanligt fynd vid uppföljningen var det bara två individer som uppvisade recidiv av sjukdomen med sonderingsdjup på ≥ 6 mm och omfattande benförlust vid flera tänder.

Ålder för sjukdomsdebut hos 42 patienter (30-45 år gamla) med avancerad generell parodontit undersöktes retrospektivt i *studie II*. Den lägsta ålder då avståndet mellan emalj-cementgränsen och det alveolära benet kunde uppmätas till ≥ 3 mm på röntgen noterades, tillika den högsta ålder då inga ytor uppvisade detta fynd. Sjukdomsdebut räknades infalla mellan dessa åldrar och uppträdde i denna studie mellan 22 och 28 års ålder.

I *studie III* och *IV* undersöktes skillnader i cellsammansättningar i lesioner från kronisk gingivit och avancerad parodontit. ParodontitleSIONERNA var dubbelt så stora och innehöll större antal och högre %-andel plasmaceller och makrofager än gingivitleSIONERNA. Den dominerande celltypen i gingivitleSIONERNA var inte T celler utan B celler som tillsammans med plasma celler fanns i både större antal och proportioner. Andelen och antalet Th17 celler var större i parodontitleSIONERNA än i gingivitleSIONERNA. ParodontitleSIONERNA uppvisade även ett högre IL-17 mRNA uttryck.

Slutsats: Barn som behandlats för lokaliserad aggressiv parodontit uppvisar inte alltid recidiv efter 14-19 år trots brist på parodontal stödbehandling. Sjukdomsdebuten hos personer som vid 30-45 års ålder uppvisar avancerad generell parodontit inföll mellan åldrarna 22-28. Stort antal och stor andel plasmaceller kännetecknar lesioner från avancerad parodontit och är tillsammans med Th17 celler, den utmärkande skillnaden i jämförelse med gingivitleSIONER.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their roman numerals.

- I. Thorbert-Mros S & Berglundh T (2010) Aggressive periodontitis in children: a 14-19-year follow-up. *Journal of Clinical Periodontology* 37:283-287.
- II. Thorbert-Mros S, Cassel B, Berglundh T (2017) Age of onset of disease in subjects with severe periodontitis: A 9- to 34 -year retrospective study
Journal of Clinical Periodontology 44:778-783
- III. Thorbert-Mros S, Larsson L & Berglundh T (2015) Cellular composition of long-standing gingivitis and periodontitis lesions. *Journal of Periodontal Research* 50:535-544.
- IV. Thorbert-Mros S, Larsson L, Kalm J & Berglundh T (2018) IL-17 producing T-cells and IL-17 mRNA expression in periodontitis and gingivitis lesions. *Submitted*

ABBREVIATIONS

A.a	Aggregatibacter actinomycetemcomitans
ANCOVA	Analysis of Covariance
APRIL	A Proliferation-Inducing Ligand
BAFF	B cell Activating Factor
BC	Bone Crest
BoP	Bleeding on Probing
BW	Bite-Wing radiograph
CAL	Clinical Attachment Level
CD	Cluster of Differentiation
CEJ	Cemento-Enamel Junction
CPITN	Community Periodontal Index of Treatment Needs
GCF	Gingival Crevicular Fluid
ICT	Infiltrated Connective Tissue
Ig	Immunoglobulin
IHC	Immuno Histo Chemistry
IL	Interleukin
LAP	Localized aggressive periodontitis
MMP	Matrix Metallo Proteinases
mRNA	Messenger Ribonucleic Acid
OPG	Osteoprotegerin
PMN	Polymorphonuclear cell
PPD	Probing Pocket Depth
RA	Rheumatoid Arthritis
RANK	Receptor Antagonist Nuclear Factor κ B
RANKL	Receptor Antagonist Nuclear Factor κ B-ligand
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
SD	Standard Deviation
SOFAT	Secreted Osteoclastogenic Factor of Activated T cells

1 INTRODUCTION

Periodontitis is a chronic inflammatory disease caused by microorganisms and characterized by progressive destruction of the tooth supporting tissues. When the inflammation does not include destruction it is called gingivitis. Moderate forms of periodontitis are common and affect about 50% of the population around the age of 50 with an increasing prevalence by age (Eke et al. 2012; Eke et al. 2015; Hugoson et al. 2005). Severe forms of the disease are found in about 10% of the population including advanced loss of tooth supporting structures, if left untreated, even loss of teeth. Even if the disease usually is painless, advanced forms of periodontitis affect the quality of life negatively due to loss of function and aesthetics (Ferreira et al. 2017; Jansson et al. 2014; Cunha-Cruz et al. 2007; Needleman et al. 2004). A similar proportion of the population exhibit a low susceptibility to periodontitis and despite presence of gingival inflammation there is no progression into periodontitis. It is not fully understood which individual will develop periodontitis and who will be relatively resistant. Risk factors such as plaque, age, smoking, diabetes mellitus and psychological factors have been identified but do not fully explain the expression of disease (Van Dyke & Sheilesh 2005).

Genetic factors are also part of the explanation. Studies of identical twins describe that heredity can explain 50% of the variability of disease (Michalowicz et al. 2000). Genetic conditions like Papillion-Lefèvre syndrome and Heimlich-Munchs syndrome manifest with dramatic periodontal breakdown (Deas et al. 2003). Other genetic variations like single nucleotide polymorphisms have also been studied. Laine et al. (2012) stated that there is emerging evidence that polymorphisms may be associated with susceptibility to periodontitis. Lately, epigenetic factors and transcription factors have also been investigated for effects on disease susceptibility (Larsson et al. 2015).

Periodontal disease can be arrested and periodontal health may be maintained over long periods of time by infection control (Axelsson et al. 2004).

Therefore, early identification of highly susceptible patients is important to be able to intervene early and prevent irreversible loss of tissue and teeth. An

understanding of when in life onset of disease occurs is helpful in the identification of the susceptible patients.

1.1 Will early forms of periodontitis in childhood predict future risk for severe periodontitis?

Periodontitis may develop at any time point in life after the eruption of primary and permanent teeth (van der Velden 1991). It is not known, however, if early forms of periodontitis serve as good indicators for severe periodontitis.

Classification

The most common forms of periodontitis are chronic and aggressive periodontitis, according to the classification presented in the consensus report authored by Armitage et al (1999). Both forms can be found in adults as well as in children. The definition of aggressive periodontitis includes rapid attachment loss, accumulation of cases in families indicating a strong genetic predisposition and being otherwise healthy. Aggressive periodontitis can be localized or generalized. The localized form shows a predilection for incisors and first molars (Baer 1971). Chronic periodontitis can also be found in localized and generalized forms and is characterized by a slow progression and a strong association to plaque and calculus. In the previous literature periodontitis in children was called juvenile periodontitis or prepubertal periodontitis.

Prevalence

The prevalence of periodontitis in children is low. Susin et al. (2014) reported in a systematic review that aggressive forms occur in less than 0.5% of the world population with slightly higher prevalence rates in African countries. Most of the studies in the review included adolescents. Saxén (1980) examined 8096 16-year-old children in Finland and found severe periodontal disease in 0.1% of the children examined. Harley et al. (1988) found a prevalence of 0.8% of radiographic and clinical findings of periodontitis in a study on 1001 adolescents, 12-19 years of age in Nigeria. Sjödin and Matsson (1994) examined radiographs from 3900 children; 7-9 years of age in Örebro and marginal bone loss in the primary dentition was found in 2-4.5% of the children. The prevalence from other Nordic studies on the permanent

dentition was reported to be 3.5 - 4.5% (Aass et al. 1988; Källestål et al. 1991)

Data concerning older teenagers are more diverse. Juhlin et al. (2008) in a study on 696 Swedish 19-year-olds found incipient bone loss with distances between cement enamel junction (CEJ) and alveolar bone crest (BC) measured in radiographs, of ≥ 2 mm in 5.1% of the subjects. A higher prevalence was reported by Ericsson et al. (2009). They found that sites with CEJ-BC > 2 mm occurred in 32%. Morales et al. (2015) reported in a study on a large cohort of 19-year-old adolescents from South America that 33% of individuals presented clinical attachment level (CAL) of ≥ 3 mm in ≥ 1 site.

Different cut off bone levels have been used to identify disease. To low levels may overestimate the prevalence and with to high levels there might be a risk to miss early signs of disease. The importance of cut-off levels was highlighted in a study on clinical attachment levels in students, 12-21 years of age, in Chile. While 70% exhibited CAL ≥ 1 mm and 16% ≥ 2 mm, only 4.5% exhibited CAL ≥ 3 mm (López et al. 2001). Sardana et al. (2014) reported in a study on 50 children 6-8 years old that the mean distance of CEJ-BC was 1 ± 0.5 mm in primary teeth and if distances were greater than 2.5 mm they should be considered diseased. Sjödin and Matsson (1992) reported similar results. They examined the primary teeth in 128 children 7, 8 and 9 years old and found that the mean CEJ-BC was 1mm (mean). The normal range was 0.0 to 2.0 mm while distances > 2 mm was considered to have pathological bone loss. Studies on prevalence of periodontitis in children and adolescents are presented in Table 1.

Table 1. Prevalence of periodontitis in children and adolescents

References	Samples & methods	Main findings
Saxén (1980)	8069 16-year-olds Examination of radiographs 27 invited for clinical examination 8 confirmed with periodontitis.	0.1% exhibited bone-loss in radiographs and clinical signs of periodontitis
Sweeney et al. (1987)	Bite wing (bw) radiographs from 2264 children 5-11 years of age	0.8% exhibited bone loss on one or more primary molars.
Bimstein et al. (1988)	bw radiographs from 1026 children 2-18 years of age 1) Normal bone levels < 2mm 2) CEJ-BC slightly increased 2-3mm 3) CEJ-BC increased > 3mm	6.9% group 2 8.9% group 3. Periodontitis was judged to be the reason for 33% of the proximal bone loss in group 3.
Aass et al. (1988)	2767 14-year-old school children, Norway bw radiographs, CEJ- BC >2mm	4.5% exhibited CEJ- BC > 2mm
Harley et al. (1988)	1001 12-19 years old children in Nigeria	0.8% showed radiographic evidence of bone loss as well as increased probing depths.
Källestål et al. (1991)	16-year olds born 1959 and 1972 400 /group. Retrospective analysis of bw radiographs with a threshold of >2mm CEJ-BC	3.5% exhibited CEJ-BC > 2mm in both groups
Sjödin et al. (1993)	118 patients with juvenile periodontitis, 13-19 years old, 168 matched healthy controls Retrospective analysis	40% exhibited > 2mm CEJ-BC in the primary dentition
Sjödin & Matsson (1992)	128 children 7, 8 and 9 years old Analysis of radiographs	Normal range of distances between CEJ and BC was 0.0-2.0 mm, mean 1.0 mm >2 mm was considered to be diseased.
Sjödin & Matsson (1994)	8666 children 7, 8 and 9 years old in Jönköping Analysis of radiographs Threshold > 2mm CEJ-BC	CEJ-BC >2mm 7-year-olds 2.0% 8-year-olds 3.1% 9-year-olds 4.2%
Timmerman et al. (2001); Timmerman et al. (1998)	255 individuals from Java 15-25 years old were examined clinically and microbiologically	No or minor periodontitis Attachment loss (AL) : 0-2 mm: 66% Moderate periodontitis AL 3-4 mm: 26% Advanced periodontitis AL ≥ 5 mm: 8%,

References	Samples & methods	Main findings
Haubek et al. (2001)	301 adolescents 14-19 years old from Morocco	15% were diseased (≥ 3 mm attachment level) 60% harbored A.a 6% harbored A.a JP2 clone Strong association between periodontitis and A.a JP2
López et al. (2001)	9162 students, 12-21 years old from Chile.	70% exhibited attachment loss of ≥ 1 mm, 16% ≥ 2 mm and 4.5% ≥ 3 mm. Higher age, poor oral hygiene and lower socioeconomic background associated with occurrence of clinical attachment loss.
Darby et al. (2005)	Bw radiographs from 542 children 5-12 years of age in Australia were examined.	Overall prevalence of 13% : CEJ-BC ≥ 3 mm definite bone loss 26% : CEJ-BC > 2 and < 3 mm questionable bone loss
Fine et al. (2007)	<i>Cross sectional</i> 1075 students 15 ± 2.2 (11-17) years old were screened clinically and microbiologically <i>Longitudinal</i> 96 students were followed for 1-3 years and progression of > 2 mm attachment loss was registered.	<i>Cross sectional</i> 3.9% had ≥ 1 pocket with PPD > 6 mm and CAL > 2 mm 1.2% had ≥ 2 pockets PPD > 6 mm and CAL > 2 mm 14% harbored A.a <i>Longitudinal</i> 8/38 seropositive for A.a at baseline and 0/58 A.a seronegative exhibited progression .
Juhlin et al. (2008)	696 Swedish 19-year-olds were examined for incipient bone loss ≥ 2 mm CEJ-BC	Incipient bone loss was found in 5.1%
Ericsson et al. (2009)	506 19-year-olds in Västra Götaland, Sweden. Clinical and radiographic examination.	32% exhibited CEJ-BC > 2 mm 62% had a gingivitis score $\geq 50\%$
Guimarães et al. (2010)	450 Brazilian children 2-11 years old Radiographic examination	1) No Bone loss (BL) 91% CEJ- BC ≤ 2 mm 2) Questionable BL 10 % CEJ - BC > 2 and < 3 mm 3) Definite BL 0.7% CEJ -BC ≥ 3 mm
Sardana et al. (2014)	50 children 6-8 years old were examined clinically and radiographically	CEJ-BC was 1 ± 0.5 mm in primary teeth Distances greater than 2.5 mm should be considered diseased.

References	Samples & methods	Main findings
Morales et al. (2015)	Cross-sectional study of 1070 adolescents from Latin America 15-19 years old. Clinical examination at 6 sites/tooth at all teeth	CAL \geq 3mm in \geq 1 site was found in 33% and PPD \geq 4mm 60% of the subjects. Smoking, attending a public school and having a BoP \geq 25% were positively associated with having CAL \geq 3mm in \geq 1 site
Jensen et al. (2016)	Cross-sectional study in 513 children in Morocco 7-10 years old, were examined clinically for attachment loss and microbiologically on their carrier frequency of JP2 and non-JP2 genotypes in the mixed dentition.	9.0% A.a JP2 36% A.a non-JP2 55% no A. a detected 6.7% exhibited CAL \geq 3 mm at two or more sites.

What happens over time?

There is evidence that suggests that aggressive forms of periodontitis in adolescents have been preceded by bone-loss in the primary dentition (Sjödín et al. 1989). In a retrospective radiographic analysis of 118 patients with periodontitis at the age of 13-19 years Sjödín et al. (1989) found that 40% of the subjects with localized aggressive periodontitis exhibited radiographic bone-loss in the primary dentition at one or more sites. Brown et al. (1996) followed a group of initially 13-20 years old adolescents over 6 years. At the time of re-examination the severity and extent of periodontitis had increased. A further progression over time was also reported by Oliveira Costa et al. (2007) in a 52-month prospective study on 44 children, initially 8-15 year old.

In a two year prospective study on 700 healthy school-children in Morocco with a mean age of 12.5 years, Haubek et al. (2008) reported that \geq 3mm loss of attachment was strongly associated with presence of the *Aggregatibacter actinomycetemcomitans* (A.a) JP2 clone. The JP2 is a unique clonal type with a 530 base pair deletion in the leukotoxin operon implicating an enhanced leukotoxic activity.

Periodontitis in children can be treated and periodontal health can be maintained after therapy. Waerhaug (1977) reported in an 8-34 years observational study on 21 patients, 12-24 years of age, that incomplete plaque control lead to rapid bone loss and extraction. However, when plaque

control was achieved the teeth could be successfully treated and a sufficient number of teeth for good function could be retained. In a 5 years study Lindhe and Liljenberg (1984) compared surgical and non-surgical therapy, using systemic antibiotic as an adjunct to periodontal therapy in the treatment of 16 adolescents (14-18 years) with localized aggressive periodontitis. Good results were reported for both treatment modalities with bone fill and resolution of gingival inflammation. In another 5 years study similar positive results were found in the absence of systemic antibiotics (Wennström et al. 1986). Saxén et al. (1986) also reported on good results 6-12 years after periodontal therapy without antibiotics.

Although favorable results can be achieved with treatment and good infection control it seems that, if left untreated, periodontitis in children progresses over time. Since many studies included older children with observation periods of less than 10 years, there is limited evidence suggesting that periodontitis in children and adolescents will develop into aggressive periodontitis as adults over time. In fact, in the consensus report by Lang et al. (1999) it is suggested that aggressive periodontitis in children and adolescents may be self-arresting.

A longer observation period was used by Höglund Åberg et al. (2009). They examined 13 subjects initially 7-9 years old, with radiographic signs of bone loss in the primary dentition and colonized with *A.a* in the deep pockets. Sixteen years later, the subjects were reexamined. Although gingivitis was a common finding, only three out of the 13 subjects exhibited deep periodontal pockets and severe bone loss at reexamination. Studies on the development of periodontal disease over time are presented in Table 2.

Conclusion:

The clinical diagnosis of aggressive periodontitis in children is uncommon. However, radiographic signs of bone loss are commonly found and the prevalence depends on thresholds of bone levels used in the studies.

If left untreated, periodontitis in children progresses in the short term. There is limited evidence on the risk of recurrence of disease in young individuals over longer periods of time and whether or not early forms of periodontitis in childhood predict future risk for severe periodontitis.

Table 2. Development of periodontitis over time

References	Observation period years	Initial age years	Samples & methods	Main findings
Waerhaug (1977)	8-34	12-24	21 patients with periodontitis	When good infection control could be achieved teeth could be successfully treated.
Lindhe & Liljenberg (1984)	5	14-18	16 adolescents with localized juvenile periodontitis (LJP), Adult periodontitis (AP) served as control. The patients were treated with tetracycline, surgical and non-surgical infection control. Supportive periodontal treatment every 3 months	Healing was good in both groups. Healing of the lesions of LJP took place in a similar manner as AP.
Wennström et al. (1986)	5	14-19 23-29	16 adolescents + young adults Oral hygiene instruction was given and surgical/ non-surgical therapy, split mouth design performed. Year 0-2: strict maintenance with 3 month intervals Year 3-5: maintenance with regular dentist	Young individuals respond to periodontal therapy in similar pattern as adults. Good results for both surgical and non-surgical without antibiotics as long as maintenance was continued. Some deterioration of treatment result and new sites with periodontitis could be seen when maintenance was discontinued.
Löe et al. (1986)	15	14-31	Longitudinal study; Sri Lanka	Three patterns of progression were found: 8% rapid progression, CAL 0.1 - 1 mm / year 81% moderate progression 0.05 - 0.5 mm / year 11% no progression 0.01 - 0.05 mm / year
Saxén et al. (1986)	6-12	15-29	20 patients with juvenile periodontitis, treated without antibiotics	No pockets of ≥ 7 mm remained at follow up Number of pockets with PPD 4-6 mm had decreased from 237 to 46

References	Observation period years	Initial age years	Samples & methods	Main findings
Sjödin et al. (1989)	3-12	13-22	17 patients with juvenile periodontitis 17 matched controls Radiographs were available 3-12 years prior to referral to specialist clinic Retrospective analysis of radiographs from age 6-18	16/17 of juvenile periodontitis 0/17 controls exhibited at least \geq site \geq 3mm CEJ-BC in the primary dentition.
Albandar et al. (1991)	3	13	222 adolescents from Brazil. Bw radiographs from annual examinations were analyzed. Individuals displaying an arch shaped bone lesion adjacent to \geq 2 first molars were diagnosed as having juvenile periodontitis \geq 2 first molars with longitudinal bone loss were defined as high risk.	1.3% of 13-year-olds 1.8% of 16-year-olds displayed juvenile periodontitis. 3.6% of all subjects were classified as having high risk and displayed deterioration over time.
Albandar (1993)	1		502 Iraqi schoolchildren were examined and subjects displaying \geq 3mm CEJ-BC and an arch shaped lesion at \geq 2 first molars were diagnosed as having juvenile periodontitis (JP). These children and a group of non-JP children were reexamined after one year.	1.8% children exhibited Juvenile periodontitis. There was no difference in gingival inflammation between JP and non-JP children. All JP children exhibited signs of progression of disease between baseline and reexamination.
Sjödin et al. (1993)		13-19	118 patients with juvenile periodontitis (JP) and 168 matched healthy controls Retrospective analysis of bw radiographs threshold $>$ 2mm CEJ-BC	40% of JP exhibited $>$ 2mm CEJ-BC in the primary dentition
Aass et al. (1994)	8	14	2767 school children born 1970 in Norway bw radiographs examined CEJ- BC $>$ 2mm was registered A random sample was followed for 8 years. 210 young adults (22 years old) were reexamined	Prevalence of 4.5% of CEJ- BC $>$ 2mm at initial examination 7% exhibited CEJ- BC $>$ 2mm at 8 - year-examination.

References	Observation period years	Initial age years	Samples & methods	Main findings
Gunsolley et al. (1995)	3 & 4		40 patients with localized juvenile periodontitis (LJP) 48 patients with severe generalized early onset periodontitis (SP) were followed for 3 (LJP) and 4 years (SP). 42% of the SP and 53% of the LJP never attended treatment.	LJP patients that received periodontal therapy had more teeth and less bleeding and plaque than those that did not receive therapy. The difference was not so obvious in the SP cases and the therapy had very little effect.
Brown et al. (1996)	6	13-20	14,013 US adolescents were examined. 143 patients exhibited ≥ 3 mm CAL at 96 were reexamined after 6 years	Prevalence ≥ 3 mm PAL: 0.01% Molars and incisors exhibited most progression. 35% of subjects with LJP progressed to GJP
Neely et al. (2001)	20	15-31	154 subjects from Java.	Age, gingival index, calculus index and time were associated with attachment loss.
Schätzle et al. (2004)	26	16-34	Longitudinal study in Norwegian males examined 8 times.	Teeth surrounded by inflammation free tissues were maintained. Teeth consistently surrounded by inflamed tissues yielded a 46 times higher risk of being lost. Only 2/3 of these teeth were maintained at the end of the 26 years.
Thomson et al. (2006)	6	26	Longitudinal study on a birth cohort born 1972 and 73 of 882 young adults from New Zealand.	The prevalence of having CAL ≥ 4 mm increased from 19% to 22% over the 6 years. Changes in probing depth were more pronounced than recessions.
van der Velden et al. (2006)	15	15-25	255 subjects from West Java, Indonesia. 128 subjects were available for reexamination.	Number of sites with PPD ≥ 5 mm, presence of A.a at baseline and age was associated with progression of disease (55% of the subjects had A.a at baseline)

References	Observation period years	Initial age years	Samples & methods	Main findings
Oliveira Costa et al. (2007)	4.3	8-15	360 children in Brazil 44 subjects were identified with CAL \geq 4mm or bone loss in radiographs ($>$ 2mm CEJ-BC) No preventive care or treatment was offered 52 month clinical and radiographic examination	Periodontitis in adolescents increase in severity and extension if left untreated over 52 months (4.3 years)
Haubek et al. (2008)	2	12.5	700 adolescents from Morocco were classified according to A.a JP2 carriage. 428 subjects attended reexamination	17% of the subjects exhibited CAL \geq 3mm at the re-examination. The risk to develop bone loss was higher for JP2 A.a carriers (64% of the subjects were A.a positive at initial examination)
Höglund Åberg et al. (2009)	16	7-9	13 subjects exhibiting radiographic signs of bone loss at their primary teeth and colonized with A.a in the deep pockets.	At reexamination, 3 /13 subjects exhibited deep periodontal pockets, BoP, and severe bone loss.
Thomson et al. (2013)	12	26	Longitudinal study in 882 young adults from New Zealand, born 1972 and 73 at the age of 26, 32 and 38.	There was an acceleration of periodontal attachment loss from mid thirties to late thirties with a doubling of sites with attachment loss. A strong association with smoking and low socioeconomic standard.
Dahlén et al. (2014)	2	13.2	500 adolescents in Ghana were examined for CAL and for A.a JP2 carriage, longitudinal study	Compared to non A.a carriers, A.a JP2 genotype carriers and A.a non JP2 experienced a 7.3 and 3.4 odds ratio (OR), respectively, of exhibiting \geq 3mm progressive bone loss. Male gender, public school and A.a JP2 carriage were associated with increase of number of sites AL \geq 3mm

References	Observation period years	Initial age years	Samples & methods	Main findings
Merchant et al. (2014)	1	5-21	97 African American patients with localized aggressive periodontitis (LAP). Patients were treated with full mouth periodontal mechanical debridement at 3, 6 and 12 month.	Treatment outcomes were good both for permanent and primary teeth but the healing potential was greater at primary teeth
Dopico et al. (2016)	7	16-65	66 subjects treated at a specialist clinic were examined after periodontal treatment	The mean annual tooth loss rate was 0.3 teeth/year Having received surgical therapy and good interproximal cleaning reduced tooth loss while deep probing after treatment was associated with increased tooth loss rates. OR 4.4 for PPD 5-6mm OR 12 for PPD > 6mm Compared with PPD < 5mm
Bahrami et al. (2016)	10 years	20-29, 30-39, 40-49, 50+	362 randomly selected individuals in different age groups	Mean annual bone loss was 0.1 mm. The subjects that exhibited the most rapid bone loss over the 10 years were the subjects that were 20-29 in the initial examination and exhibited bone loss already at that stage.

1.2 At what age may onset of periodontitis be detected in subjects with severe periodontitis?

Periodontitis is a disease that is most frequently found in older persons because of the accumulation of cases with age and because the disease is chronic. In a global meta-analysis including 72 studies from 37 countries Kassebaum et al. (2014) investigated incidence and prevalence of severe periodontitis. The studies entailed 291 170 individuals aged 15 years or older and severe periodontitis was defined as having clinical attachment level (CAL) > 6mm, PPD > 5mm or Community Periodontal Index of Treatment Needs classified as 4 or more. The authors described an increase of cases with severe periodontitis between 30 and 40 years of age with a peak incidence at the age of 38 years. Thomson et al. (2013) reported in a longitudinal study, with examinations performed at the age of 26, 32 and 38 years that affected subjects experienced an acceleration of periodontal attachment loss from the mid-thirties to the late thirties with a doubling of the proportion of sites showing attachment loss. The pattern of progression involved both extent and severity.

The relationship between an increasing prevalence and decreasing number of new cases after the age of 50 was described in the study by Hugoson et al. (2005) it was reported that 5% exhibited bone-loss $\geq 1/3$ of the root length in the 40-year age-group. The corresponding figures for the 50-year age-group and the 70-year-olds were 21% and 33% respectively. Disease with a higher severity with loss of more than two thirds of the bone support in a majority of teeth was found in 2% in the age-group of 40 while the corresponding figure for 50 and 70 year-olds were 7%. It should be noted that no data were reported on the age at which the subjects had their first signs of periodontal breakdown.

In Sweden dentists are obliged to keep patient files for 10 years after the patient terminated treatment. There is also a high attendance to regular recalls. In the 2009-2011 period 71 % of adults in Sweden attended at least one dental examination (Swedish National Board of Health and Welfare 2013). Children and adolescents who benefit government supported dental-care, attend examinations to an even higher extent. In addition, the policy of the Public Dental Health Services, Region Västra Götaland, is that all patient records should be permanently filed at the Central Archive of the Region.

Conclusion:

Severe periodontal disease has a high incidence rate between 30 and 40 years of age but there is limited evidence on the age of onset of disease.

1.3 Are there differences in cell composition between lesions representing longstanding gingivitis and severe periodontitis?

When bacterial plaque is accumulated on the surface of the tooth a host response is triggered with a cascade of antigen recognition, activation and recruitment of immune competent cells. What differs in this process between a susceptible and resistant individual? The outcome of the inflammatory process in the susceptible individual is activation of osteoclasts and bone destruction, while such a response is rarely found in the gingival lesion of the resistant individual. Much is known about differences between healthy and diseased sites, as healthy tissue often has been used as a control. But the differences between the two inflammatory conditions longstanding gingivitis and periodontitis however, is less investigated.

Cell composition of the periodontal lesion

The cell compositions in the periodontal lesion was previously described in two reviews by Berglundh and coworkers (2005, 2011) (fig 1). The authors described that the dominant cell in the sub-epithelial infiltrated connective tissue (ICT) in periodontitis was the plasma cell followed by the B cell, both belonging to the same cell-line. The authors reported that B lineage cells represented 60% of the cells in the lesions followed by T helper cells (Th) 13% of the cells, and T cytotoxic cells (Tc) 4%. Macrophages and polymorphonuclear cells (PMN) represented 5% and 7% respectively of the lesion.

The search terms used in the meta-analysis above were periodontitis, periodontal disease, biopsy, cell phenotype, histopathology, human, IHC, inflammatory cells, inflammatory lesion and inflammation and included literature published until 2010. The search was repeated including literature from 2011-2017 and is presented in table 3.

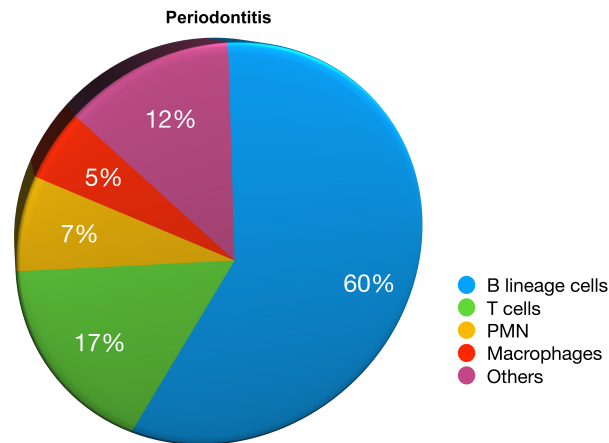


Fig 1. Adapted from Berglundh et al 2011 with permission

Age

Age seems to play a role in the composition of cells in the gingival lesion. Fransson et al. (1996) performed a 4-week experimental gingivitis study in two groups of subjects. One young group included individuals 20-25 years of age and the other group subjects 65-80 years old. The two groups formed similar amounts of plaque during the experimental period. The subjects in the older age group developed more inflammation than the younger group, and in biopsies obtained at day 7 and 21 of plaque accumulation, more inflammatory cells could be seen in the biopsies from the older subjects. In addition, the ICT was larger and contained a higher plasma cell concentration in the older subjects, while the ICT of the young group contained a larger concentration of vascular structures and lymphocytes. In a recent publication Dutzan et al. (2017) demonstrated an age-dependent, expansion of T helper cell 17 (Th17) cells with increased frequencies of IL-17⁺ cells in older individuals, in the gingival biopsies from periodontally healthy volunteers from different age groups (18-25 vs 40-50 years old). Both studies indicated the importance of considering age in studies on the composition of cells in gingival inflammation.

Severity

Another factor affecting the cell composition is severity of disease. The cellular composition of periodontitis lesions in 11 children (9.5 ± 2.0 years) with aggressive periodontitis was compared with lesions from 21 adults (48.1 ± 5.8 years) with advanced chronic periodontitis in a study by Berglundh et al. (2001). They reported that the host response in children has many features in common with adult periodontitis but the proportion of B-cells was larger in the lesions from the children, representing a more aggressive form of disease, than from the adults.

Activity

The effect of activity of disease on the cell composition was described by Zappa et al. (1991). “Active” lesions, sites with a recent history of attachment loss (≥ 2 mm change in probing attachment levels), were found to have larger proportions of plasma cells and larger total number of inflammatory cells than inactive sites. Liljenberg et al. (1994) reported on an increase in T and B cells when comparing biopsies from active sites (attachment loss of > 2 mm) with those from inactive sites. In a study on cell composition before and after treatment of periodontitis Berglundh et al. (1999) reported that, while the treatment did not affect the lymphocyte composition in blood, significant differences were found in gingival tissues. The size of the ICT was smaller after treatment and thus the total number of inflammatory cells. The proportion of B cells decreased while the proportion of T cells remained unchanged.

Cell composition of the gingivitis lesion and healthy gingiva

Yamazaki et al. (1993) compared gingival biopsies from 19 patients with moderate to advanced chronic periodontitis with biopsies from 7 subjects with gingivitis. The authors concluded that the relationship between the proportions of B cells to the proportion of T cells was (B/T) 1.3 in periodontitis and 0.25 in gingivitis. This indicated that the proportion of T cells outnumbered B cells in gingivitis. The T and B cell ratio in gingival tissues was also described by Gemmell et al. (2001, 2002). It was concluded that the B cell proportion increased with lesion size and that the proportion B cells compared to T cells was larger in periodontitis lesions than in lesions from healthy or gingivitis samples. No markers for plasma cells were included in the studies. Healthy gingiva is a clinical diagnosis. Gemmell et al.

(2001) concluded that clinically healthy gingiva usually displays histological evidence of inflammation similar to gingivitis. Dutzan et al. (2016) examined gingival biopsies from 50 periodontally healthy subjects using flow-cytometry of collagenase-digested tissue. The authors found a predominance of T cells, minimal amounts of B cells and a large presence of granulocytes/neutrophils. The difference between health and gingival disease is absence or presence of clinical signs of inflammation (BoP), but the discrimination between periodontitis and gingivitis is the destruction of connective tissue and supporting bone. Thus, it is important to further investigate the mechanisms in inflammation leading to tissue destruction in periodontitis.

Osteoimmunology

Bone is constantly being remodeled and there is an osteoclast-osteoblast coupled homeostasis in health. Osteoimmunology is the cross talk between the immune system and the bone turnover mechanisms that take place at the presence of inflammation (Takayanagi 2005). Osteoclast-precursors and monocytes from the bloodstream carry a “receptor activator of nuclear factor kappa-B” (RANK) receptor and when exposed to the ligand (RANKL) osteoclast formation and activation takes place. RANK has a soluble decoy receptor called osteoprotegerin (OPG), which blocks the RANK-RANKL interaction and serves protective to bone. OPG is produced by osteoblasts and stromal cells. In a study on gingival biopsies Kawai et al. (2010) demonstrated that less than 20% of the T and B cells in healthy tissues expressed RANKL, while 40% of T cells and 90% of B cells expressed RANKL in periodontitis. This indicated that B cells are the main cellular source of RANKL in periodontitis. The B cell marker used in the study by Kawai et al. (2010) was the CD20 marker. CD20 is lost as B cells develop into antibody secreting plasma cells. To investigate the contribution of plasma cells in the RANKL production in periodontitis, Mahanonda et al (2016) analyzed presence of intracellular and surface RANKL and reported a high expression of RANKL by plasma cells. RANKL can also be produced by osteoblasts upon activation by IL-17 (Kramer & Gaffen 2007). If imbalanced, the RANK/RANKL/OPG system can promote diseases like osteopetrosis, calcification of aorta and the kidney arteries, or on the other hand, osteoporosis and osteodegenerative diseases (Boyce & Xing 2008).

Secreted osteoclastogenic factor of activated T cells (SOFAT) is a novel cytokine that induces bone resorption in a RANKL-independent manner. Jarry et al. (2016) described that SOFAT is highly expressed in the gingival tissue in subjects with chronic periodontitis. In a sequel publication from the same group, the authors analyzed the cellular source of SOFAT in periodontitis. Gingival biopsies of 5 subjects with chronic periodontitis and 5 healthy subjects were analyzed with immunohistochemistry and immunofluorescence. SOFAT staining was associated to the lymphocytic infiltration of the periodontal tissue. T cells as well as B cells including plasma cells exhibited a strong staining for SOFAT.

B lineage cells

B lineage cells are the most prevalent cells in the established and advanced periodontal lesions (Berglundh et al. 2005, 2011) and appear in three different subgroups B-1a, B-1b and B-2 (conventional B cell). B cells develop from hematopoietic stem cells into immature B cells in the bone marrow where they mature and then leave the bone marrow. In the secondary lymphoid organs such, as the lymph nodes and the Peyer's patches, they go through a tolerance checkpoint and then circulate between spleen, bone marrow and lymph nodes. When they meet a B cell receptor specific antigen they develop into a memory B cell or plasma cell. The occurrence of different B cell subsets in periodontitis was reported in a study by Mahanonda et al (2016). They analyzed gingival biopsies and peripheral blood from 21 subjects with periodontitis, 6 subjects treated for periodontitis, 8 with gingivitis and 29 with healthy gingiva. The authors reported that the B cell population in tissue from healthy, gingivitis and treated periodontitis patients consisted to 87% of memory B cells while in the periodontitis samples 58% of the B cells were antibody secreting plasma cells. Naïve B cells were the most prevalent B cells in peripheral blood, with no differences between the groups.

The classical task for the B lineage cells is production of antibodies and the partaking in the humoral response. B cells can develop into plasma cells for a more enhanced antibody production. However, the B lineage cells are also involved in other processes in inflammation and exhibit important immunoregulatory functions like cytokine production including IL-1, IL-6, IL-10, TNF α , expression of matrix metalloproteinases and osteoclastogenic cytokines like SOFAT and RANKL (Kawai et al. 2010), thus having the

potential to partake in induction of tissue degradation of both soft and hard tissues. B cells have also found to be the main antigen-presenting cell in periodontitis (Gemmell et al. 2001). B cells internalize the antigen through binding of the antigen to the membrane-bound B cell receptor and after degradation, the antigen is attached to major histocompatibility complex molecule class II, exposed on the surface of the B cell and presented to T helper cells (Berglundh et al. 2007). B cells also serve as antigen presenting cells in autoimmune diseases (Liang et al. 2006).

IL-10

IL-10 is considered to be a strong inducer of immunoglobulin secretion and B cell survival (Moore et al. 2001; Rousset et al. 1992) and may act as an autocrine growth factor for B-1a cells (Howard & O'Garra 1992). The primary sources of IL-10 are B cells and monocytes. The auto-reactive B-1a cells are especially active in the IL-10 production (Mocellin et al. 2004) and high levels of IL-10 have been reported to be produced by B cells in autoimmune diseases like Sjögren's syndrome, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Mongan et al. 1997). Higher levels of IL-10 have been found in periodontitis compared to gingivitis tissue (Nakajima et al. 2005) and a single nucleotide polymorphism in the IL-10 promoter has been associated with periodontitis (Berglundh et al 2003)

Development and function of B1 cells

The first B cells produced in the fetus are the B-1 cells. In adults they are located primarily in the peritoneal and pleural cavities. B-1 cells are a part of the innate immune response and produce low affinity IgM antibodies recognizing bacterial and self-antigen (Porakishvili et al. 2001). B-1 cells are further divided into CD5 positive B-1a cells and CD5 negative B-1b cells. Autoantibodies produced by B-1a cells react to nuclear antigens, cell surface molecules and intracellular matrix proteins. This may affect tolerance against self-antigens and lead to a dysfunction of the immune system (Klinman & Steinberg 1987). Large amount of B-1a cells has been reported in lesions of chronic periodontitis patients and subjects with periodontitis have also been found to exhibit a larger fraction of circulating B-1a cells than periodontally healthy subjects (Berglundh et al. 2002; Sugawara et al. 1992; Aramaki et al. 1998). Subjects with chronic periodontitis subjected to non-surgical therapy did not exhibit a decrease of circulating B-1a cells after treatment and it was

suggested that the level of B-1a cells may be a marker for susceptibility rather than an indicator of the presence of disease (Berglundh et al. 1999).

Differentiation:

Classically IL-4, IL-5 and IL-6 are involved in B cell differentiation into antibody secreting plasma cells (Matsuda et al. 1989). It has been shown that IL-10, IL-21, IL-17 also partake in B cell recruitment, survival and differentiation (Havens et al. 2008). Yoon et al. (2009) reported that IL-21 could efficiently induce B cells to become plasma blasts and was a growth and differentiation factor for human B cells. However, the authors described that IL-10 was potent than IL-21 in stimulating B cells to differentiate into plasma cells.

Two novel cytokines belonging to the TNF superfamily have recently gained interest in periodontitis. The “B cell activating factor” (BAFF) and “A proliferation-inducing ligand” (APRIL) contributes to survival and maturation of B cells. BAFF is produced by activated myeloid cells (dendritic cells, neutrophils and macrophages), and APRIL by epithelial cells (Scapini et al. 2010; He et al. 2007) Upon secretion of the cytokines into the extra cellular space they interact with receptors on B cells and plasma cells. Abe et al (2015) examined the presence of APRIL and BAFF in human biopsies from 14 periodontitis subjects (mean age 58 years) and from 14 healthy subjects (mean age 50 years). They found that APRIL and BAFF, were colocalized with terminally developed antibody secreting plasma cells detected by the Ig κ L chain and found in higher concentrations in the periodontitis samples. The authors also described in the same publication that alveolar bone-loss was inhibited by antibody neutralization of APRIL and BAFF in a ligature induced periodontitis model in mice. Mahanonda et al. (2016) reported that the expression of BAFF, APRIL, IL-10 and IL-21 was higher in periodontitis tissue than in clinically healthy tissue.

Plasma cells

When B cells are activated they develop into memory cells or plasma cells. In the development to plasma cells the B cell lose most of their surface markers like CD20 and CD19 and get an enhanced antibody production. Plasma cells are the cells that increase the most in proportion as periodontal disease increase in severity or activity (Joachim et al. 1990). This is elucidating that the functions of the B lineage cell type is important for the understanding of

the pathogenesis of periodontitis. Plasma cells produce besides large quantities of antibodies also cytokines like RANKL and MMPs.

Antibodies

Antibody levels have generally been found to be elevated in periodontitis compared to healthy controls. But even though the antibodies are specific to the periodontal pathogens they seem not to be able to eliminate the bacteria effectively. This could be due to low affinity of the antibody or that the antibody cannot penetrate the bacterial biofilm. Mahanonda et al. (2016) examined antigen specificity of plasma cells in periodontal tissue and reported that IgG was more frequent than the IgA isotype and that IgG specificity to *Porphyromonas gingivalis* was present in all tissue samples (5/5). Specificity to A.a was present in 3/5 samples, while no samples exhibited specificity to the commensal *Streptococcus gordinii* or to collagen type I. In contrast to Mahonanda and coworkers several authors reported on the presence of auto-antibodies to type I collagen in periodontitis tissues (Jonsson et al. 1991; Hirsch et al. 1988; Anusaksathien et al. 1992). Higher levels of anti-collagen type I IgG antibodies were also found in GCF from periodontitis patients than in healthy subjects or peripheral blood (Sugawara et al. 1992). Other auto-reactive antibodies that have been found to be higher in periodontitis are antidesmosomal IgG (GCF) and anti-phospholipids (Govze & Herzberg 1993; Schenkein et al. 2003).

T cells

Berglundh et al (2011) reported that 13% of the cells in the lesion are T helper (Th) cells while 4% of the cells are cytotoxic cells. Th cells are traditionally divided into the subsets Th1 and Th2 and both types have been reported in periodontitis. The evidence of the presence and importance of another specific T cell subset, T helper 17 (Th17), in periodontitis has been described by several authors (Adibrad et al. 2012; Allam et al. 2011; Cardoso et al. 2009; Moutsopoulos et al. 2012; Ohyama et al. 2009; Duarte et al. 2012; Behfarnia et al. 2013). Th17 cells need TGF- β , IL-6 and IL-1b to develop but the expansion and activation of the Th17 cell is induced through IL-23 (Kramer & Gaffen 2007). Th17 cells are the main producers of IL-17. The function of IL-17 is diverse and involve both inflammation and protection against extracellular pathogens like *Candida albicans* (Hernández-Santos & Gaffen 2012; Zenobia & Hajishengallis 2015). Th17 cells and IL-

IL-17 play a potential role in autoimmune disorders like psoriasis and rheumatoid arthritis (Waite and Skokos 2012). IL-17 also stimulate osteoblasts and stromal cells to produce RANKL (Okamoto & Takayanagi 2011). Duarte et al. (2012) reported on a higher transcription of IL-17 in gingival biopsies from subjects with chronic periodontitis than in biopsies from periodontally healthy subjects. Vernal et al. (2006) examined gingival crevicular fluid (GCF) from subjects with chronic periodontitis and found higher amount of IL-17 than in GCF from healthy subjects. The control groups of the studies mentioned above were periodontally healthy subjects without gingival inflammation. In a study by Honda et al. (2008) IL-17A expression was reported to be higher in periodontitis tissue than in the controls. The control group consisted of gingivitis patients who were significantly younger (31.5 ± 8.9 years old) than the periodontitis patients (55.1 ± 8.9 years old) and had a limited amount of inflammation (BoP 22%). The authors described that also sites without clinical signs of inflammation were included. Hence, it is difficult to say if the elevated expression of IL-17 could be ascribed to periodontitis including bone-loss or to gingival inflammation in general. It remains thus to be elucidated whether the presence of IL-17 in gingival biopsies serves as a sign for inflammation or is a part of the mechanisms leading to tissue destruction in periodontitis.

The CD161 marker

Different combinations of markers have been used to detect Th17 cells in the tissue. A classic combination is CD4 and IL-17. This combination reflects the presence of the IL-17 cytokine in both intra and extracellular compartments. The combination of the markers CD161 and CD3/CD4 offers a possibility to use membrane bound surface markers. The C-type lectin like receptor CD161 is expressed on the surface of a variable portion of CD4+, CD8+, NK and NK T cells. CD161 is one of the most up-regulated genes in Th17 compared to Th1 and Th2 (Cosmi et al. 2008). It has been concluded that CD161 is good surface marker for IL-17 producing cells (Maggi et al. 2010; Fergusson et al. 2011). CD161 positive T cells were also correlated with higher disease activity in RA (Miao et al. 2013).

IL-17 / B cell associations

There are several reports on the interactions between B-lineage cells, IL-17 and Th17 cells. It has been reported that IL-17 may induce B-cell germinal

centers and follicles (Khader et al. 2011; Hsu et al. 2008). Grund et al. (2012) stimulated B cells in cell cultures with IL-17A and BAFF and concluded that IL-17 together with BAFF promotes differentiation into plasma cells and protection from apoptosis. Diseases like RA, SLE and psoriasis, in which B cells and plasma cells play an important role, have lately been described as diseases where also IL-17 and Th17 cells are important (Kramer & Gaffen 2007; Waite & Skokos 2012). RA is efficiently treated with anti-CD20 monoclonal antibodies (Edwards et al. 2004). Anti CD20 therapy in RA patients reduces the Th17 response in tissue and synovia but does not affect the Th1 or Th2 activity (van de Veerdonk et al. 2011). One reason for the reduction in IL-17 expression after B cell depletion could be that Th17 cells are dependent on IL-6 and IL-1b to differentiate and B cells account for 65-95% of the IL-6 production in a stimulated lymph node (Barr et al. 2012). Halwani et al. (2014) reported from a study in cultured cells, that IL-17 may have a direct chemotactic effect on B cells in asthma. Other reports from cell-cultures stated that B cells upon stimulation may produce IL-17 (Vazquez-Tello et al. 2012; Schlegel et al. 2013)

Neutrophils and Macrophages

Neutrophils and macrophages are found in low concentrations in periodontitis lesions (7% and 5% respectively) (Berglundh & Donati 2005). Neutrophils migrate towards chemotactic agents towards the gingival crevice and in biopsies from periodontitis lesions they are more commonly found in the pocket or in the epithelium. The presence of neutrophils in the periodontal tissue is vital for health. When neutrophils are lacking in number, for example in different forms of neutropenia, or when the neutrophils have a defect migration or function like in leucocyte adhesion deficiency or Papillon-Lefèvre syndrome, a dramatic periodontal destruction at an early age can be observed (Haritha & Jayakumar 2011). It is clear that the neutrophils have an important protective role in the first line of defense against oral pathogens. According to the Page & Schroeder (1976) model the periodontal lesion is initiated as an acute inflammation characterized by neutrophils migrating to the periodontal crevice through the junctional epithelium (Page and Schroeder 1976). Neutrophils can besides granule derived antimicrobial substances and enzymes also synthesize immunoregulatory chemokines and interleukins and induce recruitment of Th17 cells to the inflamed site (Pelletier et al. 2009). Furthermore,

neutrophils contribute to the proliferation, survival and B cells and maturation into plasma cells through the secretion of BAFF (Huard et al. 2008). Activated neutrophils can also produce RANKL and matrix metalloproteinases (MMPs), which partake in the degradation of connective tissue (Chakravarti et al. 2009). While the functions of the neutrophils may indicate a possible contribution to the periodontal breakdown, the presence and proper function of neutrophils are required to maintain periodontal health.

Macrophages also play a part in the early inflammatory reactions. They phagocytose microbes and express inflammatory molecules like the costimulatory CD86. Allam et al. (2011) reported that the CD68 positive macrophage-like cells were correlated to the amount of B-cells and IL-17 cells in the periodontal pocket. It was also reported that macrophages unlike B cells produce IL-23 that is an IL-17 stimulatory cytokine important for proliferation and activation of Th17 cells.

Conclusion:

There is convincing evidence in the literature that B lineage cells play an important role in periodontitis and that the B cell derived plasma cell is the cell type that increases the most with increased severity. The literature is limited concerning the specific differences between periodontitis and non-destructive inflammatory gingival condition from subjects in relevant age groups.

Table 3.

References	Periodontitis N, Age (years)	Control N, Age (years)	Samples & Methods	Main findings
Arabaci et al. (2010)	Chronic periodontitis N = 17 Age: 36.7 (29-46)	Healthy N = 18 Age: 30.9 (23-37)	Biopsy	NFkB was highly activated in periodontitis.
Allam et al. (2011)	Chronic periodontitis PPD >6mm N = 16 Age 56.5 ± 10.6		Biopsy: IHC Immunofluorescence, RT-qPCR Flow cytometry	B cells, macrophages and Th17 cells were predominantly found in the tissue adjacent to the bottom of the periodontal pocket.
Thomasini et al. (2011)	Chronic periodontitis Mean PPD 5.6mm Mean CAL 6.2mm N = 20 Age: 52 (39-69)	Healthy PPD+ CAL ≤ 3mm N = 12 Age: 34 (20-40)	Biopsy PCR IHC	Cytomegalovirus and herpes virus was found in higher amounts in periodontitis affected sites
Rojo-Botello et al. (2011)	Periodontal disease, diabetes - N=10 Periodontal disease, diabetes+ N=10 Age: 37-72	Healthy N = 10	Biopsies Immunofluorescence	Toll like receptors (TLR) 2, 3, 4 and 9 were increased in periodontitis compared to healthy tissues TLR 2,9 and 4 were even more enhanced in periodontitis specimen from diabetic patients
Adibrad et al. (2012)	Chronic periodontitis PPD ≥5mm, CAL ≥3mm Bop 78% N = 30 Age: 40.1 ± 8.6	Healthy Bop 14% N = 30 Age: 28.3 ± 6.9	Biopsy RT-qPCR IHC	IL-17A was significant increased in periodontitis compared to control

References	Periodontitis N, Age (years)	Control N, Age (years)	Samples & Methods	Main findings
Saraiva et al. (2012)	Aggressive periodontitis N = 45 Age: 34 ± 9 (15-53) Chronic periodontitis N=85 Age: 49±9 (31-70)	Healthy controls N=72 Age: 31 ± 10 (14-62)	Blood Oral swab	Difference in IL-17A genotype AA genotype =health GG genotype =periodontitis
Moutsopoulos et al. (2012)	Severe chronic periodontitis BoP PPD ≥6mm CAL >6mm	Gingivitis PPD CAL < 3mm	Biopsy IHC RT-qPCR Blood Cell-culture ELISA, Westernblot	mRNA expression of IL-17 was elevated in periodontitis compared to minimal inflammation. Th17 was more frequently found in periodontitis than Th1, Th2
Corrêa et al. (2012)	Chronic periodontitis N = 30	Healthy	Peripheral blood	IL-17A-197A allele was associated with increased risk for chronic periodontitis
Himani et al. (2013)	periodontitis pre and post treatment N = 18 Age: 30-39	Healthy: N = 18 Gingivitis: N = 18 Age: 30-39	GCF ELISA	Highest concentrations of IL-23 were found in untreated periodontitis patients.
Liu et al. (2013)	Moderate to severe chronic periodontitis N = 7	Healthy donors N = 6 No age or gender difference between the groups	Blood, biopsy IHC, ELISA, Western blot	Increase of cyclophyllinA (CypA) in periodontitis.

References	Periodontitis N, Age (years)	Control N, Age (years)	Samples & Methods	Main findings
Wang et al. (2013)	Untreated chronic periodontitis N = 15 Age: 44.1 ± 9.1	Healthy N = 10 Age: 23.6 ±6.5	Biopsies IHC Immunofluorescence Western blot	EMMEPRIN (Glycosylated extracellular matrix metalloproteinase inducer) influences the caveolin-1 ability to induce Matrix metallo proteinase (MMP) production. Periodontitis exhibited more highly glycosylated EMMEPRIN and more MMP-1
Parachuru et al. (2014)	Intense inflammation N=17	Minimal inflammation N=12	IHC	FOXP3+ cells were more predominant than IL-17+ cells in both groups Very few IL-17+ cells were found
Azman et al. (2014)	periodontitis ≥6 sites with PPD ≥5 mm BOP 36% N=97 Age: 47 (42–53)	Healthy PPD <2 mm, no CAL BOP 3% N=77 Age: 38 (27–49)	Serum, Saliva GCF ELISA	IL-17A was associated with increased BoP, CAL and PPD. IL-17E was a marker of health.
Lorenzi et al. (2014)	Generalized chronic periodontitis N=14 Age: 47±6 Generalized aggressive periodontitis N=10 Age: 25±5	Clinically healthy N=16 Age: 24±6 Gingivitis N=16 Age: 37±10	Biopsy, RT-PCR IHC	HtrA1 (High temperature requirement A1) cleaves fibronectin, whose fragments induce MMP production. HtrA1 expression in plasma cells could be correlated with destruction of periodontal tissue. HtrA1 was found in healthy tissues but the staining in plasma cells was increased from gingivitis to chronic and most intense in aggressive periodontitis.

References	Periodontitis N, Age (years)	Control N, Age (years)	Samples & Methods	Main findings
Mesa et al. (2014)	Chronic periodontitis ≥1 site CAL ≥3mm+PPD ≥6mm N = 16	Gingival health BoP = 0% PPD < 3mm N = 10 Gingivitis BoP > 0% PPD < 3mm N=15	IHC	COX-2 expression in plasma cells and monocytes was associated with periodontal disease
Mizutani et al. (2014)	Severe periodontitis N = 20 Age: 67(47-98)		Biopsy, blood ELISA IHC, immunofluorescence Antibody production	Plasma cells producing antibodies against <i>Porphyromonas gingivalis</i> antigens were found in 17/18 tissue specimens (94%) suggesting a local production in the gingival lesion.
Carcuac & Berglundh (2014)	Generalized severe chronic periodontitis bone loss ≥50% and PPD ≥7mm at ≥4 teeth N = 40 Age: 64±11	Peri- implantitis ≥1 implant ≥3mm peri- implant bone-loss PPD ≥7mm N=40 Age: 70 ±10	Biopsy IHC	Periimplantitis lesions were twice as large as the periodontitis lesions and the proportions of plasma cells, macrophages and neutrophils were significantly larger in periimplantitis than in periodontitis
Abe et al. (2015)	periodontitis PPD ≥5mm CAL ≥4mm BoP+ N = 14	Healthy PPD ≤3mm no BoP no CAL N = 14	Biopsy RT-qPCR IHC	The expression of APRIL and BAFF mRNA were upregulated periodontitis relative to healthy controls.

References	Periodontitis N, Age (years)	Control N, Age (years)	Samples & Methods	Main findings
Mitani et al. (2015)	Chronic periodontitis ≥6 teeth PPD ≥5mm, CAL ≥6mm N = 10 Age: 54.1(29-74)	Healthy No CAL PPD ≤ 3mm BoP <10% N = 10 Age: 39.7 (29-61)	Biopsy: RT-qPCR GCF: ELISA	IL-17 was higher in GCF and in biopsies from chronic periodontitis.
Luo et al. (2014)	Chronic periodontitis N = 30 Age: 42.4±11.0	Healthy N=25 Age: 38.5± 10.2	Peripheral blood, flow cytometry	IL-17+ cells were associated with periodontitis
Zacarias et al. (2015)	Chronic periodontitis PPD ≥5mm CAL ≥3mm BoP >25% N = 140 Age: 47.03±9	Healthy No CAL PPD <4mm BoP <25% N = 173 Age: 45.61±9	Peripheral blood Genotype determination	IL-17A AA genotype more frequent in periodontitis
Larsson et al. (2016)	Severe Chronic periodontitis N = 21 Age: 55±10	Gingivitis N = 17 Age: 60±7	Biopsy, blood IHC RT-qPCT	Larger proportion of TET2 (ten-eleven translocation 2) in periodontitis (IHC) Global methylation patterns differed between blood and tissue in periodontitis.
Chauhari et al. (2016)	Localized aggressive, N = 35, Age: 21±4.6 Chronic periodontitis N = 35 Age: 37±4.2	Healthy N = 35 Age matched	Peripheral blood Genotype determination	Polymorphism in IL-17 gene was associated with disease.
Teixeira et al. (2016)	Patients with periimplant mucositis and: I. No bone-loss N = 10 II. Periimplantitis sites N = 14 III. Periodontitis sites N = 9 Age: 61.7±7.1		IL-17 related cytokines measured in periimplant fluid (I-II) and GCF (III)	The expression of IL-17 related cytokines was similar regardless of the presence or not of bone loss around implants and teeth

References	Periodontitis N, Age (years)	Control N, Age (years)	Samples & Methods	Main findings
Cheng et al. (2016)	Chronic periodontitis >1 site PPD and CAL \geq 4mm N = 68 Age: 47.6 \pm 9.3	Healthy No CAL or PPD >3mm N = 43 Age: 40.1 \pm 10.9	Blood biopsy Flow cytometry, IHC RT-qPCR	IL-17A levels were higher in biopsy and blood in periodontitis compared to healthy samples.
de Oliveira Nóbrega et al. (2016)	Chronic periodontitis CAL and PPD \geq 4mm BoP+ N = 30	Clinically healthy gingiva N=32 Gingivitis N=28	Biopsies IHC	EMMEPRIN and MMP7 were found in higher concentrations in periodontitis and in pockets with PPD >7mm
Jarry et al. (2016)	Chronic periodontitis >30% of sites PPD and CAL \geq 4mm; biopsy site >7mm CAL+ PPD, BoP+) N = 5	Healthy CAL 0 N = 5	IHC immunofluorescence. RT-PCR	SOFAT staining was found in the infiltrated connective tissue in periodontitis specimens. Plasma cells, B cells and T-cells exhibited strong SOFAT staining. RT-PCR revealed a significant expression both in stimulated B and T cells. B-lineage cells may be an important source of SOFAT
Mahanonda et al. (2016)	periodontitis N = 21 Treated periodontitis N = 6	Gingivitis N = 8 Healthy N = 29	Biopsy Peripheral blood Flow cytometry IHC ELISPOT Assay RT-PCR	Memory B-cells (CD19+CD27+CD38-) represented the majority in the B cell population in gingivitis, healthy and treated periodontitis tissues. (87%) Antibody secreting plasma cells (CD19+CD27+CD38+) were the most common B cells in periodontitis (58%) Naïve B-cells (CD19-CD27-CD38-) were the most prevalent B cells in peripheral-blood, no differences between the groups.

2 AIM

Overall aim

To analyze the onset of disease and lesion characteristics in subjects with severe periodontitis.

Specific aims

- I. To assess recurrence of disease in subjects with a history of localized aggressive periodontitis.
- II. To retrospectively assess the age of onset of disease in a group of patients, 30-45 years of age, diagnosed with severe generalized periodontitis.
- III. To analyze differences in cell characteristics between lesions representing longstanding gingivitis and severe periodontitis.
- IV. To analyze the density and number of IL-17 producing T-cells and IL-17 mRNA expression in lesions representing either severe periodontitis or longstanding gingivitis.

3 MATERIAL AND METHODS

All the studies were approved by the regional human ethics review board of University of Gothenburg and the patients received written information and signed an informed consent form prior to enrolment. (D-nr 677-05 and D-nr 169-12)

3.1 Study population & design

Study I

The study population consisted of a group of 11 children (7 girls and 4 boys aged of 7-13 years mean 9.5 ± 2.0) who at examination exhibited signs of localized aggressive periodontitis. Ten of the children from four families had an African ethnic origin (Cap Verde Islands), while one subject (patient no. 7) was Caucasian (Sweden). The clinical and radiographic assessments included detection of sites that exhibited bleeding on probing and a distance between CEJ-BC > 2 mm. Samples from the subgingival microbiota and soft tissue biopsies were obtained from the identified sites. Results from the different examinations including the finding of A.a. were reported by Berglundh et al. (2001) and further microbiological analyses on A.a.JP2 clone were reported by Haubek et al. (1996). Patients 3, 4, 8, 9, 10 and 11 were positive to this clone in the initial examination.

Following examination, the subjects received non-surgical periodontal therapy including mechanical infection control. All affected deciduous molars and, in patient no.10, two permanent teeth were extracted. The non-surgical therapy in the remaining sites resulted in pocket closure and resolution of inflammation.

The subjects were enrolled in a regular recall program within the home clinic of the public dental services. This program consisted of annual controls with clinical and, when indicated, radiographic examinations, while no specific supportive periodontal therapy program was provided to this group of subjects.

Reexamination

At 14–19 years after the initial examination and treatment, the 11 subjects

were recalled for clinical and radiographic examinations at the Clinic of Periodontics, Public Dental Services, Gothenburg. One of the subjects (patient no. 7) did not attend the clinical examination but recently obtained radiographs were accessible. The age of the 10 remaining subjects (three males and seven females) at the follow-up examination varied between 21 and 32 years (mean 26 ± 3.2). Questionnaires and data from patient files were used to document the frequency and type of examinations and treatment performed during the 14–19-year period between the initial and the follow-up examinations. The clinical follow-up examination included assessments of PPD and BoP. All teeth were assessed at 4 sites/tooth using a graded probe. BoP was registered dichotomous as bleeding / non-bleeding and PPD was measured to the nearest millimeter. Bitewing radiographs from pre-molar and molar segments were obtained.

Study II

A total of 103 patients, 30–45 years of age, diagnosed with generalized, severe periodontitis with >30% bone loss at >30% of the teeth and treated at the Clinic of Periodontics in Gothenburg, Public Dental Health Services, Region Västra Götaland, Sweden, were identified. Seventy-four of the patients agreed to be part of the study. Data from clinical examinations performed at the Clinic of Periodontics were collected from patient files, and current radiographs were retrieved. Thus, BoP, PPD, increased tooth mobility, furcation involvement and number of remaining teeth were recorded together with data on general health. All patients were contacted by phone, and were interviewed of dental history as far back in life as they could remember. The same questions were posed in a letter to the subjects that could not be reached by phone. Requests for old patient files and radiographs were made to > 80 private and public dental clinics.

Study III & IV

Two groups of patients, representing generalized severe periodontitis or longstanding gingivitis, were recruited. The gingivitis group consisted of 28 patients on the average 59.5 ± 7.9 (41–70) years of age, with evident signs of gingival inflammation but no attachment loss. The patients of this group had

≥ 16 remaining teeth exhibiting BoP and, as assessed in radiographs, a distance of < 3 mm between CEJ and BC in $\geq 90\%$ of proximal sites. The periodontitis group consisted of 36 patients, mean 52.3 ± 9.5 (33–67) years of age. This group of patients had ≥ 16 remaining teeth presenting with PPD ≥ 6 mm, BoP and about 50% radiographic bone loss in $> 80\%$ of the proximal sites. No subjects had any known systemic disease or used drugs that could influence the periodontal conditions, including systemic antibiotics, for 6 months. The patients were recruited from the Clinics of Periodontics in Gothenburg and Mölndal, the Clinic for Undergraduate Training in Gothenburg, Public Dental Health Services, Region Västra Götaland and Institute of Odontology, The Sahlgrenska Academy at University of Gothenburg.

Biopsy procedure

From each patient a gingival biopsy was obtained from one selected diseased site. The biopsy sites in both groups demonstrated clinical signs of inflammation (BoP). In addition, the selected site in the periodontitis patients presented with PPD ≥ 7 mm and $> 50\%$ bone loss. The biopsy sites in the gingivitis group exhibited PPD ≤ 5 mm and no signs of bone loss. The biopsy procedure was carried out in conjunction with tooth extraction or periodontal surgery (periodontitis group) or crown-lengthening procedures (gingivitis group) and was previously described by Zitzmann et al. (2005). Following local anesthesia, two parallel incisions, 4 mm apart, were made through the soft tissue to the crestal bone and were connected with a perpendicular incision at distance of 4 mm from the gingival margin. The biopsy was carefully dissected, rinsed in saline and placed in a fixative (4% formaldehyde) for 24 h.

3.2 Analysis of radiographs

Interproximal sites in radiographs presenting with an identifiable CEJ and BC were included. The vertical distance between CEJ and BC was measured.

Study I

Analogue radiographs were measured using an Olympus SZH10 stereo microscope (Olympus optical co. Ltd., Tokyo, Japan) with a Leica DFC280 camera connected to a computer with a Leica QWin software (Leica Imaging

Systems Ltd., Cambridge, UK).

The finding of BoP and a distance $> 3\text{mm}$ between CEJ and BC in radiographs was considered as recurrence of disease.

Study II

Intra-oral digital and analogue radiographs were included. Digital radiographs were analyzed using the Romexis® (Planmeca, USA) viewer and PACS (Siemens, Germany) software, while analogue radiographs were analyzed using a magnifying lens (7x) with a 0.1 mm-based scale. Two thresholds were used; CEJ-BC $\geq 3\text{ mm}$ and $\geq 5\text{ mm}$. The number of interproximal sites assessed and age of patient at each radiographic examination were recorded. The lowest patient age at which a radiographic examination revealed a CEJ-BC distance of $\geq 3\text{ mm}$ and $\geq 5\text{ mm}$ at any site was recorded and termed “first 3 mm” (F3) and “first 5 mm” (F5), respectively. Similarly, the highest patient age at which a radiographic examination revealed the absence of sites with CEJ-BC $\geq 3\text{ mm}$ (“latest 0”; L0) was assessed. Double assessments of F3 were made by two examiners (ST-M and BC), and agreement was expressed by Cohen’s Kappa score.

3.3 Immunohistochemical preparation

Study III & IV

The gingival tissue samples were dehydrated, embedded in paraffin and sections, 5 μm in thickness, were produced. The sections were de-waxed and incubated in DIVA antigen retrieval solution (Biocare medical, Concord, CA) at 60°C over night. Proteinase K (DakoCytomation) was used for antigen-retrieval for the elastase antibody.

Following blocking of endogenous peroxidase the sections were incubated with the primary antibody for 30 minutes (table 4). Detection of positive cells in study III was performed using EnVision+ System-HRP, DAB (Dakocytomation, Glostrup, Denmark), including HRP-labeled polymer and DAB solution. In study IV, ABC kit (Vector Laboratories kat no. PK-7200) was used for CD3 and MACH4 (Biocare Medical kat no. M4U536) for CD161. Positive cells were detected using DAB substrate (Biocare medical) for CD3 and WARP red (Biocare medical) for detection of CD161 in study IV.

For double staining for CD5 and CD20, the sections were incubated for 60 min with CD5 antibodies followed by incubation for 30 min with MACH 2 mouse ALP (Biocare Medical). After 10 min incubation with the Ferengi blue substrate (Biocare Medical) for the detection of CD5-positive cells, the sections were incubated for 5 min with a denaturing solution (Biocare Medical). Incubation for 30 min with an antibody against CD20 was followed by a second, 30-min incubation with MACH 2 mouse ALP. CD20-positive cells were detected using the Vulcan Fast Red substrate. The sections were counterstained with haematoxylin. A rabbit IgG antibody was used as a negative control.

Histological analysis

Histological quantitative assessments were made in a microscope equipped with an image system (Leitz DM-RBE microscope and Q-500 MC® image system; Leica, Wetzlar, Germany). The area of the ICT was assessed using a mouse cursor. Identification of positive cells was made using a point counting procedure to determine the percentage of positive cells within the ICT according to methods described by (Liljenberg et al. 1994). Thus, a lattice comprising 400 points was superimposed over the tissue area at a magnification of 400. Positive cells positioned in a cross point were counted and expressed as a percentage relative to the total counts for the entire ICT.

The cell area was assessed for 10 cells for the different cell types in 10 randomly selected patients from both groups. Data on the ICT area, cell size and the % area of the ICT occupied by respective cell types were used to calculate the number of positive cells within the ICT and cell density, expressed as number of cells / mm².

Reproducibility assessments of cells positive for CD3, CD138 and CD68 in specimens representing 4 patients in each group were performed with a 3-month interval by the same examiner.

Table 4. Primary Antibody

Target	Antibodies	Clone	Dilution	Study
T cells	CD3	Rabbit polyclonal DakoCytomation Glostrup, DK	1:200	III
B cells	CD20	Rabbit polyclonal Thermo	1:1500	III
Plasma cells	CD138	Clone MI15 Mouse monoclonal DakoCytomation, Glostrup, DK	1:50	III
B-1a cells	CD5	Clone 54/F6 Mouse monoclonal DakoCytomation, Glostrup, DK	1:50	III
Neutrophil granulocytes	Elastase	Clone NP 55 Mouse monoclonal DakoCytomation, Glostrup, DK	1:100	III
Macrophages	CD68	Clone PG-M1 DakoCytomation, Glostrup, DK	1:100	III
T cells	CD3	Mouse anti human, DAKO M7254 lot. 00074382	1:50	IV
IL-17 producing T cells	CD161 KLRB1	Rabbit anti human, Sigma Aldrich kat no. HPA039113	1:100	IV
Negative control	IgG	Rabbit PP64, Millipore SAS, Molsheim, France		IV

3.4 RT-qPCR

To study the IL-17 gene expression, total RNA was extracted from formalin fixed sections and 100ng RNA per sample was reverse transcribed into cDNA and analyzed with real time PCR. The number of cycles needed to amplify the IL-17 cDNA over the detection threshold (Cycles threshold-Ct) was normalized using the Ct values of the reference genes YWHAZ and RPLP0. The $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001) was used to analyze the relative change in gene expression from real-time quantitative PCR experiments with reference genes used as internal controls and gingivitis as calibrator. TATAA Universal RNA Spike SYBR (TATAA Biocenter, Gothenburg, SE cat. no RINH250S) was used to test for inhibition of RT-reaction. The Minimum Information for Publication of RT-qPCR Experiments (MIQE) guidelines were followed for reliable interpretation of qPCR results (Bustin et al. 2009).

Table 5. RT-PCR primers

Gene	PrimePCR, Bio-Rad Laboratories AB, Solna SE
IL-17A	cat.no qHsaCID0015941
RPLP0	cat.no qHsaCED0036271
YWHAZ	cat.no qHsaCID0013897

3.5 Data analysis

The subject was used as the statistical unit in all analyzes. Mean values and standard deviations (SD) were calculated for the clinical and radiographic data in study I-IV. Cohen's kappa score was calculated for double assessments of F3 in study II. The parametric student's t-test for unpaired observations was used for statistical analysis of differences in ICT area and cell proportions between the groups. An Analysis of Covariance (ANCOVA) was performed to analyze the effect of gender, smoking and age on the results in study III and IV. The $2^{-\Delta\Delta C_T}$ method was used to analyze the relative change in gene expression from real-time quantitative PCR experiments (Livak & Schmittgen 2001). The result was presented as fold increase of the mRNA levels in periodontitis samples compared to the gingivitis samples. 95% confidence interval was calculated for the fold change.

4 RESULTS

4.1 Study I

The subjects were at reexamination between 23 and 32 years old (mean 25.7). None of the subjects were smokers. Information obtained from the subjects and the analysis of patient files revealed that the frequency of annual recall visits in their home clinics varied between 40-70% during the 14-19 year period between the two examinations. The overall % BoP varied between 19% and 59% in the subjects. All subjects but one had sites with PPD \geq 4 mm, while sites with PPD \geq 6mm were detected in 4 of the subjects. The number of premolar and molar sites with bone levels identified at > 3 mm from CEJ were found in 5 of the subjects, while only 2 subjects (patient 1 and 2) had tooth sites with bone levels of > 4 mm.

Two of the subjects (patient 1 and 2) exhibited recurrence of disease in several sites during the period between the two examinations and, in these siblings, tooth extractions were performed. In patient nr 1 the tooth 36 was extracted about 2 years after the first examination, while 16 was extracted in conjunction with the second examination. Radiographs from subject nr 1 are presented in Fig 2. Patient nr 2 was referred for periodontal therapy immediately prior to the second examination and, in addition to non-surgical and surgical periodontal therapy extractions of the teeth 26 and 36 were performed.

In the majority of the remaining group of subjects, no data indicating recurrence of disease were found despite presence of gingival inflammation. This group of subjects are represented by subject 8 in fig 3.

Subject number 9 and 10 exhibited moderate forms of disease. Both exhibited bleeding of probing and had several sites exhibiting 4-6 mm pockets. Subject nr 10 exhibited 4 sites with a moderate bone loss and distances CEJ-BC between 3.15 and 3.34 mm and subject nr 9 one site with a distance of 3.63

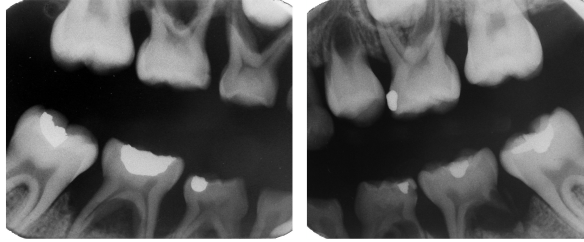
mm between CEJ and BC on the mesial surface of 36. This site had been affected already when she was 12 year old at the initial examination and the distance at that time was 2.37. Subject number 11 exhibited one site with a distance between CEJ and BC that slightly surpassed 3mm. The initial examination was performed at 12 years of age on this individual and there had been practically no change on that surface during the 13 years until re examination. BoP was low and she exhibited no pockets of 4mm or more.

Table 6. Radiographic and clinical data at reexamination.

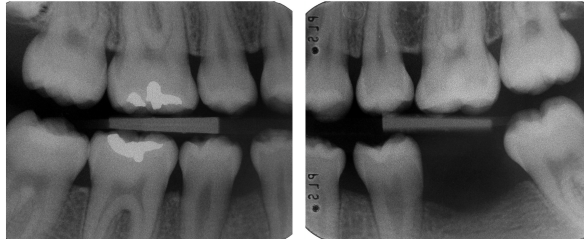
Subject	Age at 2 nd examination	BoP%	PPD \geq 3mm	CEJ-BC >3mm	CEJ-BC >4mm	CEJ-BC Mean (range) mm
1	23	25	10	3	3	7.4 (5.0-10)
2	27	57	8	11	7	6.6 (3.1-13)
3	25	53	0	0	0	
4	26	19	0	0	0	
5	23	51	0	0	0	
6	24	49	0	0	0	
7	25	Missing		0	0	
8	21	21	0	0		
9	30	50	2	1	0	3.6
10	27	59	8	4	0	3.3 (3.2-3.3)
11	32	29	0	1	0	3.2

Subject 1

Initial examination
8 years



12 years



Re-examination, 21 years

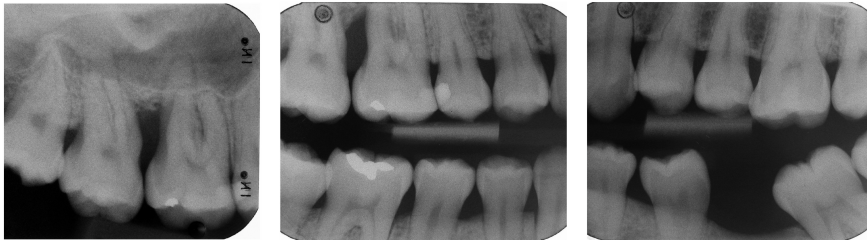
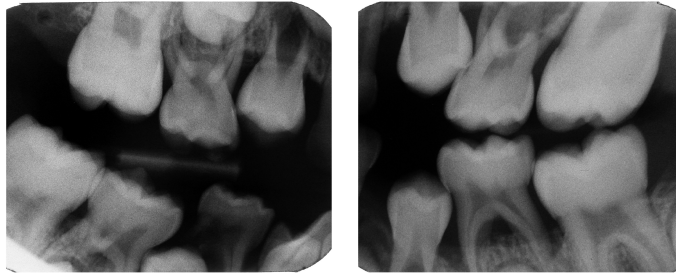


Fig 2 Subject 1 was one of the two subjects exhibiting recurrence of disease

Subject 8

Initial examination 7 years



Re-examination, 23 years

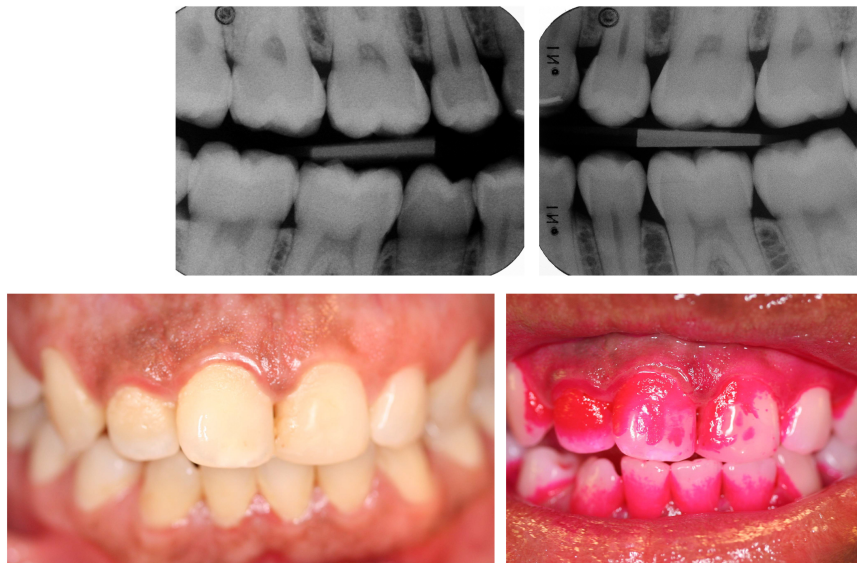


Fig.3 Subject 8 represented the majority of the subjects. Although obvious signs of disease were detectable at the initial examination, no signs of recurrence were detected at re-examination.

4.2 Study II

The 74 patients (study sample) that were included in the study were between 30 and 45 years old (mean 38.4 ± 4.0) at time of the clinical examination. While 53% of the patients were smokers, 8 % used smokeless tobacco. At the time of the clinical examination 12 patients suffered from asthma or different kinds of allergy. Two individuals had diabetes type 2 and one had recovered from breast cancer. Three patients reported on cardiovascular disease with hemorrhage in the brain, thrombosis and multiple vault-stenosis. Three patients had hypertonia and two patients presented with psoriasis. 44 of the 74 patients did not report on any general health problems.

BoP occurred in $74.2 \pm 23.9\%$ of sites and frequencies of PPD ≥ 4 mm and ≥ 6 mm were 58.2 % and 31.7%, respectively. On the average, patients presented with 3 teeth with furcation involvement degree II or III, while about 4 teeth per patient exhibited increased mobility of varying degree.

The evaluation of “final” radiographs, i.e. those obtained in conjunction with the clinical examination and justifying inclusion of patients to the study, revealed that 75% and 46% of evaluated interproximal sites presented with CEJ-BC ≥ 3 mm and CEJ-BC ≥ 5 mm, respectively. Demographic and clinical data of the 29 patients not entering the study were overall similar to those described for the 74 patients who agreed to be part of the study.

Data from patient files and radiographs representing previous examinations were obtained from 42 of the 74 patients (patient file sample). The most common reason for absence of information of previous dental history was that that long periods (>10 years) passed since the last visit. Among the 42 patients with access to previous radiographs and information in patients files, 19 patients (target sample) had documentation from periods prior to the first signs of periodontal breakdown (F3), i.e. CEJ-BC ≥ 3 mm, while 26 patients presented with documentation from periods prior to first signs of severe periodontal breakdown (F5; CEJ-BC ≥ 5 mm).

The observation time in the 19 patients was 21.6 ± 9.2 years; range 9-34 years. The patient with a 34-year documentation period contributed with 25 separate radiographic examinations from the age of 4 to 38 years. The

youngest age of documentation among the patients was 3 years. In 10 patients, radiographs were retrieved from age ≤ 11 years. A series of radiographs from one patient is presented in Fig. 4.

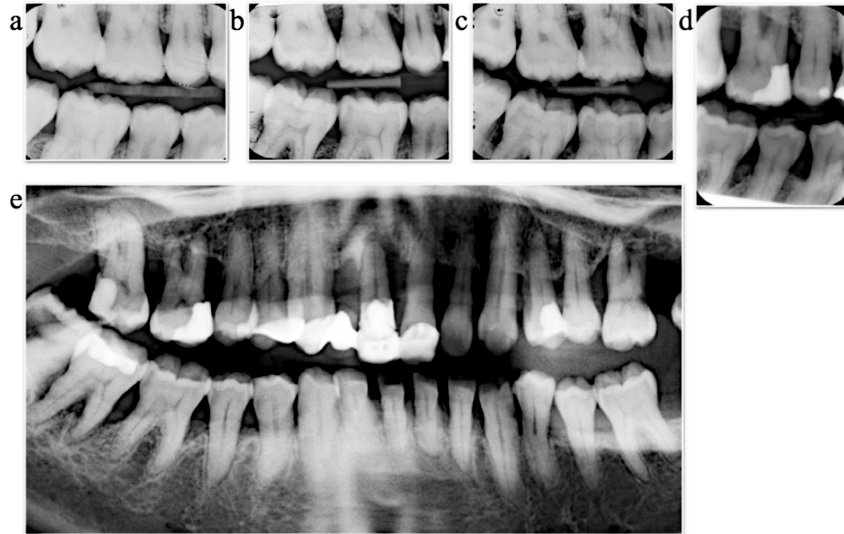


Fig 4. A male subject at a) L0 23 years, b) F3 25 years, c) F5 37 years, d+e) Clinical examination 41 years

The number of radiographs obtained at each of the 9.7 ± 6.6 examinations among the 19 patients varied between 2 and 22 and included bite-wing projections and full-mouth recordings. The number of interproximal sites assessed at each examination was on the average 24.4 ± 9.7 for bite-wings and 48.6 ± 9.5 for full-mouth protocols. Agreement between two examiners in the assessment of F3 among the 19 patients was 0.89 (Kappa score).

Onset of periodontitis, as defined by the time interval between the highest age of a radiographic examination demonstrating absence of periodontal breakdown (L0) and the lowest age of detection of $CEJ-BC \geq 3$ mm (F3), occurred between 22.3 ± 3.8 and 28.1 ± 5.3 years of age. In addition, F3 was consistently observed in multiple sites (37.3 ± 29.1 % of analyzed sites). Earliest signs of severe periodontal breakdown ($CEJ-BC \geq 5$ mm; F5) were on the average detected at an age of 32.4 ± 5.7 years. F3 was never

detected at deciduous teeth. Flow chart of the study population is illustrated in fig 5.

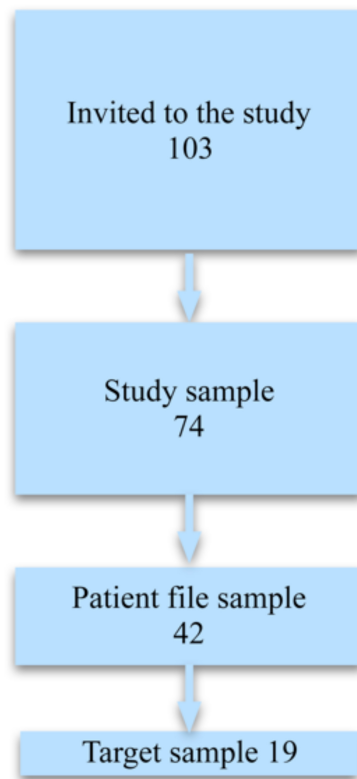


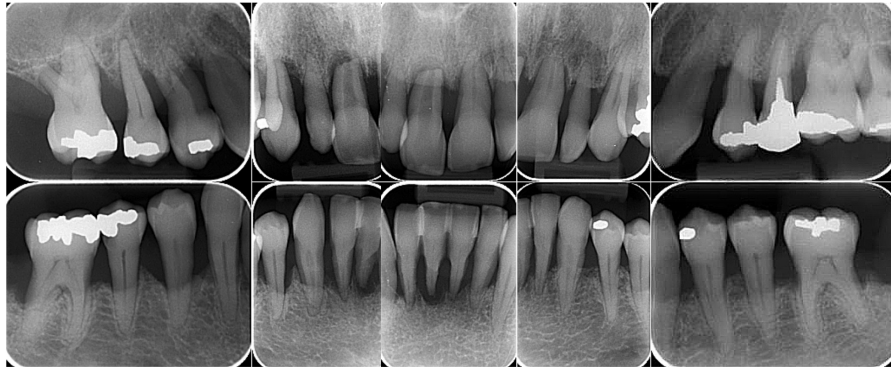
Fig 5.

4.3 Study III+ IV

There were no statistically significant differences regarding distribution of age and gender between the two groups of patient.

The proportion of smokers was larger in the periodontitis than in the gingivitis group (39% vs 18%). The PPD at the biopsy site was mean 8.93 ± 1.76 mm in the periodontitis sites and 3.41 ± 1.19 mm in the gingivitis sites.

Periodontitis group 48 years, male



Gingivitis group 67 years, male

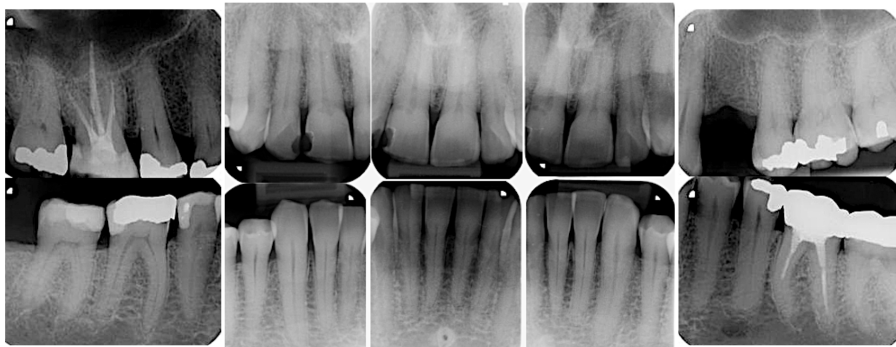


Fig 6. Radiographs from a representative patient from each group

Histological findings

Paraffin-embedded sections prepared from gingival tissue samples representing patients of each group are presented in Fig 8 and Fig 9. T cells (CD3+) and B cells (CD20+) were found predominately adjacent to the pocket epithelium, while plasma cells (CD138+) occupied large peripheral areas of the lesion, distant from the pocket epithelium. Neutrophils (Elastase+) were in relative terms few and were found close to the pocket epithelium while macrophages (CD68+) were found in both peripheral and central parts of the lesion. The lesions in the biopsies from the gingivitis group were smaller than those in the periodontitis group. Area percentages of plasma cells and macrophages were significantly larger in the periodontitis lesions than in the gingivitis lesions. No statistically significant differences were found between the other cell-types.

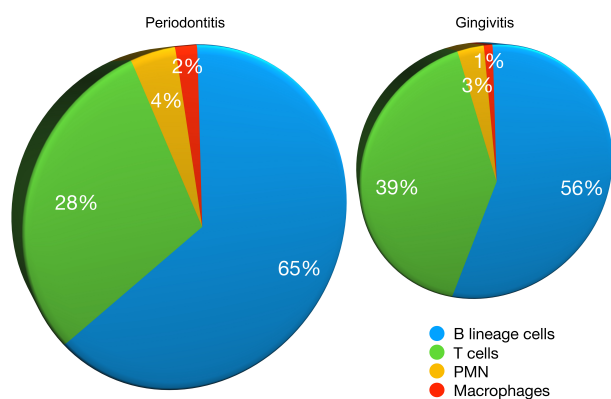


Fig 7. Proportions of cells in the ICT and relation of size of the inflammatory lesions

The percentage of B cells presenting CD5 as an additional marker was 12.4 % in the gingivitis lesions. In the periodontitis lesions the corresponding number was 19.9 % and that difference was statistically significant. Among the T cells in the periodontitis lesion 34% were also positive for CD161. The corresponding figure for the gingivitis specimens was 24%. 99% of all CD161 positive cells in both periodontitis and gingivitis sections exhibited CD3. IL-17 producing T cells (CD3+ CD161+) were significantly greater in

percentage and total number of cells in the periodontitis than in the gingivitis specimens. The results from the histological analysis are presented in Fig 7 and Table 7.

Table 7. Results from the histological analysis. Area of ICT and % area of cells of the ICT. Mean values and standard deviations

	Gingivitis	Periodontitis	<i>p-value</i>
CD3	6.4 (4.3)	6.2 (4.7)	0.89
CD3+CD161	0.9 (0.6)	1.7 (1.3)	0.01
CD20	4.9 (5.4)	4.5 (4.2)	0.70
CD5+CD20 (% of CD20)	12.4 (8.8)	19.9 (7.7)	0.02
CD138	4.3 (3.3)	9.9 (7.0)	0.0002
CD68	0.5 (0.4)	0.9 (0.5)	0.007
Elastase	0.2 (0.3)	0.5 (0.7)	0.11
ICT Area (mm ²)	0.9 (0.59)	1.79 (1.4)	0.002

Periodontitis lesions contained larger total number of cells than gingivitis lesions and significantly larger numbers of cells positive for CD3, CD138 and CD68. The number of *cells/mm²* revealed small differences between the two types of lesions for CD3 and CD20 positive cells, while the densities of CD138 and CD68 positive cells were significantly larger in periodontitis than in gingivitis. CD3 cells represented 43% of the cells in the gingivitis lesions, while the B-lineage cells (CD20 and CD138 together) made up 49% of the cells. B-lineage cells represented 58% of cells in the periodontitis lesion. Elastase and CD68 positive cells represented 5% and 2% in gingivitis lesions and 7% and 2% in periodontitis lesions, respectively.

The ANCOVA analysis revealed that differences in numbers and densities of cells positive for CD3+CD161, CD20, CD138, CD5+CD20 and CD68 between the periodontitis and gingivitis specimens remained after adjusting for smoking, age group (below or over median) and gender. In addition CD3+CD161 positive cells were found in larger proportions in males than females.

Reproducibility of histological assessments

The results from the reproducibility assessments of the point-counting procedure of cell markers in study III were expressed as standard deviations of single assessments. (Table 8)

Table 8.

	CD3	CD138	CD68
Periodontitis	0.69	0.67	0.30
Gingivitis	0.52	0.56	0.32

RT-qPCR

The RT-qPCR analysis and calculations of $2^{-\Delta\Delta C_T}$ revealed a 1.4-fold increase of IL-17 m-RNA expression in the periodontitis specimens compared with longstanding gingivitis. The 95% confidence interval for the fold increase was 1.2-1.7.

Periodontitis

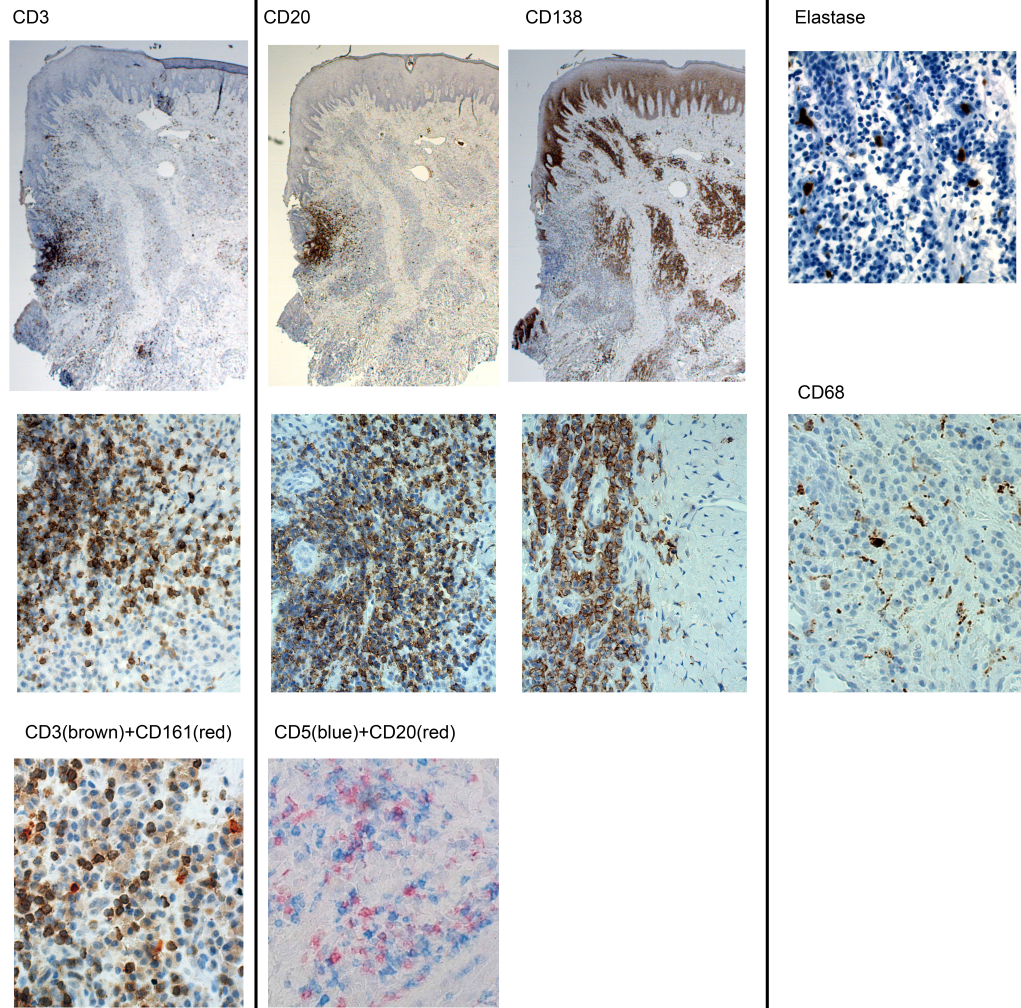
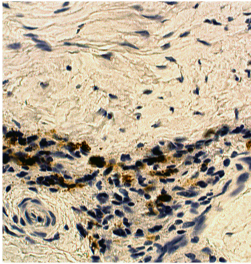
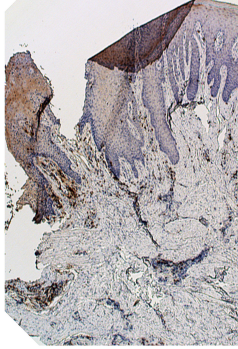


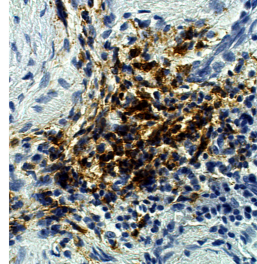
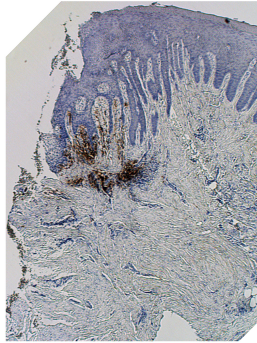
Fig 8.

Gingivitis

CD3



CD20



CD138

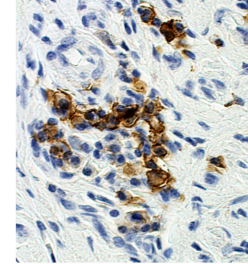
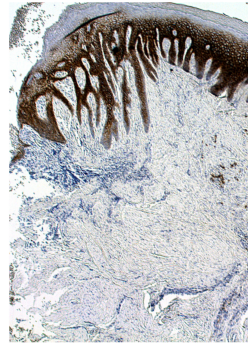


Fig 9.

5 MAIN FINDINGS

- * Two out of 11 subjects initially diagnosed with LAP, exhibited recurrence of disease with PPD \geq 6mm and bone loss of 3–4mm at several teeth at 14-19 years of follow-up.
- * Onset of disease in patients with generalized, severe periodontitis occurred on the average between 22 and 28 years of age and severe bone loss was detected at the age of about 32 years.
- * Periodontitis lesions were twice as large and contained significantly larger numbers and densities of plasma cells and macrophages than did gingivitis lesions.
- * The proportion of B-1a cells was significantly larger in periodontitis lesions than in gingivitis lesions.
- * The total number and density of IL-17 producing T-cells were larger in periodontitis than in longstanding gingivitis lesions.
- * The IL-17 mRNA expression was higher in periodontitis than in gingivitis lesions

6 CONCLUDING REMARKS

Will early forms of periodontitis in childhood predict future risk for severe periodontitis?

In *study I*, 11 children with a history of localized aggressive periodontitis initially diagnosed and treated at the age of 7-13, were reexamined 14-19 years after initial diagnosis. Although gingival inflammation was a common finding in the group only two subjects disclosed recurrence of disease with PPD \geq 6mm and bone loss 3-4 mm at several teeth. Another two subjects exhibited a few diseases sites with limited amounts of bone loss. It was concluded that children treated for localized aggressive periodontitis do not always exhibit recurrence of periodontitis in the absence of supportive periodontal therapy over periods of 14-19 years.

This finding is in accordance with the data presented by Höglund Åberg et al. (2009). They reported that only 3 out of 13 subjects exhibited deep periodontal pockets and severe bone loss at the 16-year reexamination. The study group was at initial examination 7-9 years old. The ages at initial and reexamination as well as the findings corroborates with the design and findings in *study I*. The findings presented in a 8-34 year follow-up study on treatment of juvenile periodontitis by Waerhaug and co-workers (1977) are to some extent in contrast to the data in *study I*. While similar positive results were achieved in subjects with a good infection control in the study by Waerhaug et al. (1977), progression of disease occurred consistently among subjects with incomplete plaque control. The 21 subjects in the study by Waerhaug et al. (1977), however, were considerably older (12-24 years) than the subjects in *study I* at initial examination.

Conclusion

Based on the findings from *study I* and other reports, periodontitis in childhood may not be an effective way to detect subjects at risk for severe periodontitis as adults.

At what age can the first signs of periodontitis be detected in subjects with severe periodontitis?

If children with periodontitis are not the obvious candidates to develop severe periodontitis as adults, then the question is, at what age does the first signs of disease appear? *Study II* was designed to approach this question. Previous radiographs were acquired from 42 subjects, 30-45 years of age, diagnosed with severe periodontitis, to retrospectively analyze onset of disease. In 19 subjects radiographs prior to the first sign of disease were retrieved. This material comprised documentation from the age of 11 or younger in 10 of the subjects. None of the subjects presented bone-loss in the deciduous dentition. On the basis of the findings from *study II* the conclusion was that onset of disease in patients with generalized, severe periodontitis occurred on the average between 22.3 and 28.1 years of age and severe bone loss was detected at the age of about 32.4 years.

The results in *study II* corroborates the findings reported in the meta-analysis by Kassebaum et al (2014). They reported that a start of incidence of severe periodontitis around the age of 20 years was followed by a steep increase up to the peak incidence at the age of 38. In the study by Kassebaum et al. (2014) the aim was to detect incidence of severe periodontitis while *study II* aimed at finding the first obvious radiographic signs of disease at an earlier time point in disease progression. In a study by Thomson et al (2013) on subjects 26, 32 and 38 years of age it was reported that prevalence and extent of attachment loss increased with age. The increase of attachment loss was more pronounced between 32 and 38 than between 26 and 32 years of age. The authors stated that the increase was characterized by newly detected sites rather than progression at diseased sites. This pattern was also observed in *study II* where both onset and progression of periodontitis was found in a generalized pattern at multiple sites passing the first and second threshold. The results from *study II* partly supports the finding from *study I* indicating that aggressive periodontitis in children may not be a reliable predictor of severe periodontitis as adults.

Conclusion

On the basis of this work, severe periodontitis may often commence between the ages of 20 and 30 years. This finding indicates that this age interval is

critical both for diagnostic awareness and preventive interventions.

Are there differences in the characteristics of the longstanding gingival inflammatory lesions between patients with high or low susceptibility to periodontitis?

In *study II* and *IV*, cellular compositions as well as mRNA expression for IL-17 in gingival biopsies from two groups of patients were analyzed. The recruitment of a well-defined control group was of main importance. The gingivitis patients, despite generalized gingival inflammation and a mean age of 60 years, did not exhibit bone loss, thus representing a group of individuals with a low susceptibility to periodontitis. The periodontitis patients exhibited severe generalized disease.

In *study III* and *IV* it was concluded that large numbers and high density of plasma cells are the hallmarks of advanced periodontitis lesions and the most conspicuous difference in relation to longstanding gingivitis lesions. Periodontitis lesions were twice as large and contained significantly larger proportions, numbers and densities of plasma cells and macrophages than did gingivitis lesions. The proportion of B cells that expressed the additional CD5 marker (B-1a cells) was also significantly larger in periodontitis lesions than in gingivitis lesions. The densities of T cells and B cells did not differ between periodontitis lesions and gingivitis lesions. T cells were not the dominating cell type in gingivitis lesions, as B cells together with their subset of plasma cells comprised a larger number and proportion than T cells. While the density of T cells did not differ between the two types of lesions, the total number and density of cells positive for IL-17-producing T cells were larger in periodontitis than in longstanding gingivitis lesions. About 30 % of the T cells in periodontitis lesions were also positive for CD161. The corresponding figure for gingivitis samples was 15 %. The IL-17 mRNA expression was higher in periodontitis than in gingivitis. In addition, males had larger proportions of IL-17 producing T cells than females in both groups.

The distribution of plasma cells, B cells, T cells, macrophages and PMN cells in periodontitis lesions presented in *study III* was similar to the description of the periodontitis lesion presented in the meta-analysis by Berglundh and

coworkers (2011). In a publication on differences between periodontitis and periimplantitis lesions by Carcuac & Berglundh (2014) proportions of plasma cells, B cells and T cells in the periodontitis samples also presented data which were consistent with those reported in *study III*.

In *study III* it was reported that T and B cell populations occurred in similar proportions in periodontitis and gingivitis lesions. However, when all B lineage cells were collected (B cells and plasma cells), they outnumbered the T cell population in both types of lesions. Yamazaki et al. (1993) evaluated differences in cell populations between healthy gingiva, gingivitis and periodontitis tissues and concluded that while B cells outnumbered T cells in periodontitis, the opposite was true for gingivitis. The conclusion that T cells outnumber B cells in periodontitis in gingivitis was also supported by findings reported by Gemmell et al. (2001, 2002) when comparing gingivitis and healthy tissues with periodontitis specimens. The difference in results between *study III* and the studies mentioned above might be explained by the recruitment to the gingivitis group. The control subjects in the study by Yamazaki et al. (1993) were between 14 and 33 years of age while the periodontitis patients were between 35 and 57 years. The gingivitis group in *study III* and *IV* were considerably older than the gingivitis group in the Yamazaki study.

In a study by Berglundh et al. (1999) it was described that 30% of the B cells in periodontal tissue and in peripheral blood also exhibited the CD5 marker. In *study III* the corresponding finding was 20% in periodontitis, and that was significantly higher than in gingivitis. Previous studies on B-1a cells in longstanding gingivitis lesions seem to be lacking. The role of the so-called auto-reactive B-1a cell in periodontitis is not fully understood.

In *study III* and *IV* it was reported that both macrophages and IL-17 producing T cells were found in higher proportions and numbers in periodontitis compared to longstanding gingivitis specimens. The same results from immunohistochemical analyses were reported in studies by Cheng et al. (2016) and Moutsopoulos et al. (2012) comparing periodontitis lesions to healthy and gingivitis lesions exhibiting minimal inflammation. Observations made by Parachuru et al. (2014) indicate less agreement. The authors reported that very few IL-17+ cells were found in gingival tissue

exhibiting intense or minimal inflammation. In *study IV* it was also demonstrated that the IL-17 mRNA expression was significantly higher in periodontitis than in gingivitis tissue. The results were supported by the findings reported in a study by Mitani et al. (2015) where healthy tissue was used as a control. The finding that males exhibited more IL-17 producing T cells than females in the gingival tissues were in line with the results from in vitro analysis of human blood presented by Zhang et al. (2012) and Blanco et al. (2013).

Conclusion

It is suggested that there are qualitative and quantitative differences between “destructive” and “non-destructive” lesions. B and T cells are found in similar proportions in the two types of lesions, but B cells in periodontitis seem to be driven into differentiation to plasma cells to a higher extent than in gingivitis. In the periodontal lesion, the B cells more often exhibit autoimmune traits than in gingivitis. The IL-17 producing T cells are found in higher proportions in the lesions from periodontitis, as well as a higher IL-17 mRNA expression, indicating a role in the destructive inflammatory pattern. Understanding the specific characteristics of the local host response in subjects with a high susceptibility to periodontitis may provide tools for early detection of risk individuals.

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APPENDIX

- I. Thorbert-Mros S & Berglundh T (2010) Aggressive periodontitis in children: a 14-19-year follow-up. *Journal of Clinical Periodontology* 37:283-287.
- II. Thorbert-Mros S, Cassel B, Berglundh T (2017) Age of onset of disease in subjects with severe periodontitis: A 9- to 34 -year retrospective study
Journal of Clinical Periodontology 44:778-783
- III. Thorbert-Mros S, Larsson L & Berglundh T (2015) Cellular composition of long-standing gingivitis and periodontitis lesions. *Journal of Periodontal Research* 50:535-544.
- IV. Thorbert-Mros S, Larsson L, Kalm J & Berglundh T (2018) IL-17 producing T-cells and IL-17 mRNA expression in periodontitis and gingivitis lesions. *Submitted*