Studies on Pellicle and Early Dental Plaque in Relation to Periodontal Conditions

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Gothenburg 2012

Printed in Sweden by Ineko AB, Gothenburg
ISBN 978-91-628-8550-2

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Abstract

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Background and Hypothesis: Bacterial receptors in dental pellicles may influence colonisation and subsequent plaque formation. Studies on such receptors in the dental pellicles and bacterial adherence have mostly been performed *in vitro* and focused on proteins of salivary origin. We have only a limited knowledge of the receptor functions of plasma proteins and even less on the *in vivo* situation where they could reach the pellicle via the gingival crevicular fluid. Our hypothesis was that plasma proteins in the gingival crevicular fluid affect pellicle formation and the establishment of the early dental microflora on the tooth surface.
Material and Method: In the present series of studies, plasma proteins in pellicles formed on hydroxyapatite *in vitro* and on teeth *in vivo* and the adherence of bacteria to these pellicles were examined. *In vivo* studies were performed at different periodontal conditions in periodontally healthy and diseased subjects. Samples were taken at healthy and experimentally inflamed gingival margins as well as before and after surgical pocket elimination. The samples were taken from the gingival and incisal parts of teeth and, in one study group, even from surgically exposed root surfaces. Pellicle proteins were analysed using sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotting and image analysis. Bacterial adherence *in vitro* was examined using radiolabelled bacteria and liquid scintillation. *In vivo* plaque samples were analysed by culturing and the PCR technique.
Results: Components from plasma were readily incorporated into the experimental pellicles and into natural pellicles on tooth surfaces *in vivo*. These components mediated the adherence of *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Actinomyces* spp. *in vitro* and were found to a higher extent in pellicles formed at the gingival part of the tooth surface than at the incisal part. The amount of pellicle proteins and the numbers of bacteria were higher in the presence of periodontal inflammation. In experimentally inflamed gingival margins of periodontally healthy individuals, this observation was pronouncedly seen on the incisal parts of the tooth surfaces. In the presence of periodontal pockets, higher amount of pellicle proteins and numbers of bacteria was seen on the gingival tooth surfaces when compared with the situation after surgical pocket elimination. In periodontally healthy individuals, the bacterial findings indicated a pattern of less streptococci and *Actinomyces* spp. and more bacteria associated with periodontitis in the 4-hour dental plaque formed during experimentally inflamed conditions, compared with healthy conditions. Periodontitis-associated bacteria were also more frequently found in the 4-hour plaque in the presence of periodontal pockets compared with the status after pocket elimination surgery. Pellicle and early dental plaque on surgically exposed root surfaces contained significantly more plasma proteins and total numbers of bacteria compared with the adjacent gingival enamel surfaces. *Actinomyces* spp. were found in comparably high numbers on the exposed root surfaces.
Conclusions: Plasma proteins with the ability to mediate the *in vitro* adherence of periodontitis-associated bacteria are important components of the *in vivo* pellicle, particularly in the presence of periodontal inflammation. As the gingival crevicular fluid flow increases so does the relative amounts of plasma proteins in the pellicle, thereby modifying bacterial attachment and early dental plaque composition. Surgically exposed root surfaces were found to bind significantly higher amounts of plasma proteins and total number of bacteria than the adjacent enamel surfaces. Further on, the extent of the root surface exposure significantly reduced the amount of plasma proteins binding to the adjacent enamel surface. On the basis of our observations, we suggest that the bacterial composition of early dental plaque may be governed by the presence of plasma proteins in the pellicle and the presence of exposed root surfaces.

Key words: bacterial adherence, *in vitro* pellicle, *in vivo* pellicle, periodontitis-associated bacteria, gingival crevicular fluid, plasma proteins, dental biofilm, experimental gingivitis, chronic periodontitis, periodontal pocket, exposed root surfaces

ISBN 978-91-628-8550-2

Preface

This thesis is based on the following studies, which are referred to in the text by their Roman numerals:

I. Carlén A, Rüdiger SG, Loggner I, Olsson J. Bacteria-binding plasma proteins in pellicles formed on hydroxyapatite *in vitro* and on teeth *in vivo*. *Oral Microbiology and Immunology* 2003;18:203-207.

II. Rüdiger SG, Carlén A, Meurman JH, Kari K, Olsson J. Dental biofilms at healthy and inflamed gingival margins. *Journal of Clinical Periodontology* 2002;29:524-530.

III. Rüdiger SG, Dahlén G, Carlén A. Pellicle and early dental plaque in periodontitis patients before and after surgical pocket elimination. *Acta Odontologica Scandinavica* 2012;70:615-621.

IV. Rüdiger SG, Dahlén G, Carlén A. Proteins and bacteria on root surfaces exposed after periodontal surgery. Submitted.

Permission for reprinting the papers published in *Oral Microbiology and Immunology* and *Journal of Clinical Periodontology* was given by John Wiley & Sons and permission for reprinting the paper published in *Acta Odontologica Scandinavica* was given by Informa Healthcare.

List of abbreviations

GCF	Gingival crevicular fluid
HA	Hydroxyapatite
IgA	Immunoglobulin A
IgG	Immunoglobulin G
PCR	Polymerase chain reaction
PRP	Proline-rich protein
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TVC	Total viable counts

Introduction

Dental diseases

The most commonly occurring dental diseases are caries and periodontitis, both of which result from the activity of bacteria in the dental plaque and their metabolic products (Shibly et al. 1995, Liljemark & Bloomquist 1996). Acidic substances dissolve dental hard tissues in caries. Other toxic agents, notably lipopolysaccharides, trigger endogenous processes leading to inflammatory reactions in the periodontium. The inevitable reaction is an inflammation of the gingival margins, gingivitis.

Gingivitis

Plaque-induced gingivitis occurs in dentate individuals of all ages and is the most common form of periodontal disease (Page 1985). The initial inflammatory reaction may not be noticed clinically, but an increase of the gingival crevicular fluid (GCF) flow and an inflammatory infiltrate prove a reaction to the bacterial colonisation during the first days (Payne et al. 1975, Brecx et al. 1987, Kunimatsu et al. 1995). Clinical signs, commonly bleeding, edema and erythema, develop as gingivitis progresses (Löe et al. 1965). The swelling deepens the gingival crevice and the microflora of the supragingival plaque changes composition to more anaerob gram-negative species (Theilade et al. 1966). When adequate oral hygiene is re-established, gingivitis resolves (Löe et al. 1965).

Periodontitis

In some individuals the inflammatory reactions eventually induce breakdown of the periodontal tissues (Kornman 2008). The gingival crevice deepens further, creating a new environment, the periodontal pocket, where subsequently subgingival plaque becomes established. More anaerob and proteolytic bacteria appear producing tissue degrading enzymes. Bacterial metabolic products stimulate immune responses leading to additional tissue breakdown (Amano 2010). Unlike gingivitis, which affects everybody leaving behind bacterial plaque at the gingival margin, the prevalence of severe periodontitis and tooth loss usually does not exceed 10% of an adult population (Wennström et al. 1993, Norderyd & Hugosson 1998, Norderyd et al. 1999). As the formation of dental plaque plays a key role in the etiology and pathogenesis of dental diseases, the success of treatment is dependent on the patient’s oral hygiene measures (Garmyn et al. 1998).

Our knowledge on dental plaque formation is mainly based on experimental studies examining the importance of saliva for the establishment of bacteria on the tooth surface. In the oral cavity the tooth surface is in contact with GCF, a plasma exudate constantly leaving the gingival crevice surrounding the tooth. Plasma components may therefore affect pellicle formation and the acquisition of the early dental plaque bacteria, and especially in individuals with gingivitis or periodontitis having an increased GCF flow.

Dental plaque formation

The initial step in the process leading to dental plaque is the formation of the dental pellicle, an acellular, essentially bacteria-free protein layer (Dawes et al. 1963). Bacteria must be firmly attached in order to colonise a surface. The first bacteria to colonise the tooth bind specifically by means of surface structures, adhesins, to receptors in the pellicle. Adhesins and receptors are complementary to each other in a key and lock fashion (Gibbons & Hay 1989). Early colonisers (mainly *Streptococcus* spp. and *Actinomyces* spp.) bind directly to the tooth surfaces and create eventually a closed bacterial layer. Late colonisers (often anaerobic and Gram-negative bacteria) co-adhere by binding on receptors on the early colonisers. Thus, growing plaque becomes an increasingly multi-species biofilm (Kolenbrander et al. 2010).

Dental pellicle

Within minutes after polishing a tooth surface, it is covered by a protein film (Sönju et al. 1974). Within one to 1.5 hours, the pellicle reaches a plateau in terms of protein content and thickness which remains unchanged for the first two to six hours and can attain a thickness of 1 to 3 µm (Leach & Saxton 1966, Sönju & Rölla 1973, Lie 1977, Berthold 1979, Eggen & Rölla 1983, Kuboki et al. 1987, Rykke & Sönju 1991, Skjörland et al. 1995). Using electron microscopic analysis, the internal heterogeneous structure of the pellicles has been described as having a globular, granular or fibrillar/dendritic character, which may reflect periodic protein deposition or aggregated proteins (Lie 1977, Berthold 1979, Hannig 1997, Deimling et al. 2004).

The main constituents of the pellicle are glycosylated proteins and phosphoproteins (Al-Hashimi & Levine 1989), attracted and bound to the tooth surface by van der Waal's attraction forces, and electrostatic and hydrophobic interactions (Pruitt 1977, Haynes & Norde 1994, Hannig & Hannig 2009). Pellicles had been considered to be primarily of salivary origin on the basis of comparative carbohydrate and/or amino acid analysis, and studies on

natural pellicles on extracted teeth (Armstrong 1967, Leach et al. 1967, Mayhall 1970) and on experimental *in vivo* pellicles harvested from teeth *in situ* (Sönju & Rölla 1973, Eggen & Rölla 1982, Eggen & Rölla 1983, Al-Hashimi & Levine 1989). Deviations in the protein composition of saliva and pellicles were explained by the selective nature of the adsorption process (Örstavik & Kraus 1973, Rykke et al. 1990). Similarities were also revealed between natural pellicles and *in vitro* pellicles formed from saliva on cleaned enamel slabs (Armstrong 1967, Mayhall 1970). High molecular weight proteins and salivary mucins were identified as important constituents of *in vivo* pellicles (Embery et al. 1986, Fisher et al. 1987). Furthermore, acidic proline-rich proteins (PRP), Immunglobulin A (IgA), lysozyme, amylase and Immunglobulin G (IgG) were found (Kousvelari et al. 1980, Deimling et al. 2004). Analysis of PRP in the pellicle revealed that pellicle formation is a dynamic process. The PRP content was found to increase during the first hour of pellicle formation and then slowly decrease during the following 24 hours (Bennick et al. 1983). Ultrastructural examination of *in vitro* pellicles further revealed that the proteins are not evenly distributed on the apatitic surface (Schüpbach et al. 2001).

Saliva is an important and comprehensively studied source of pellicle formation (Liljemark & Bloomquist 1996). Other potential sources (e.g. GCF, bacteria, food remnants) have been less thoroughly investigated. This may be due in part to the methods used in studies of pellicle formation. Materials like plastic films (Brecx et al. 1980), hydroxyapatite stents (Lie 1979) and restorative materials (Hannig 1997) or slabs of extracted teeth (Örstavik & Kraus 1973, Bennick et al. 1983, Fisher et al. 1987) were exposed to saliva in the oral cavity. Pellicles from natural teeth were typically collected well away from the gingival sulcus (Sönju & Rölla 1973, Al-Hashimi & Levine, 1989, Carlén et al. 1998). A tooth surface is also exposed to the GCF. Examination of enamel surfaces by means of a fluorescent antibody technique suggested the presence of serum proteins on the enamel (Kramer & Ramanathan 1966). IgG, fibrinogen and albumin were among the first serum components to be identified in dental pellicles (Örstavik & Kraus 1973, Levine et al. 1985, Li et al. 2004). Experimental pellicles formed on dentine slabs placed subgingivally for two hours contained albumin, IgA, IgG and IgM in descending order (Abbas et al. 1991). It is also known that salivary and plasma proteins may interact with each other during pellicle formation (Kajisa et al. 1990).

Bacterial adhesion

A number of salivary components have been found to mediate the adhesion of oral bacteria *in vitro* (Scannapieco 1994). Early colonising *Streptococcus sanguinis* (formerly *S. sanguis*) was reported to bind to IgA (Kilian et al.1981, Gong et al. 2000) and, like *Streptococcus gordonii,* to amylase (Scannapieco et al. 1995, Gong et al. 2000, Rogers et al. 2001). Together with *Streptococcus oralis,* these streptococci may also bind to low-molecular-weight mucin and proline-rich glycoproteins (Murray et al. 1992). The adherence of *Streptococcus mutans*, associated especially with caries initiation, is promoted by salivary agglutinins (Ericson et al. 1984, Carlén & Olsson 1995), *Actinomyces* spp. e.g. *A. oris* (formerly*: A. naeslundii* genospecies 2), *A. naeslundii* (formerly *A. naeslundii* genospecies 1) and *A. viscosus* (now regarded as an animal species) attach to the tooth surface by binding to IgA and to the phosphoproteins, PRP and statherin (Kilian et al.1981, Bratt et al. 1999).

In the adhesion of oral bacteria, plasma components have been studied less extensively than salivary proteins. The adherence of strains of streptococcal species (*S. mutans*, *S. sobrinus* and *S. rattus*) was shown to be inhibited by fibronectin (Babu & Dabbous 1986), which appeared to support the adherence of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* (Naito et al. 1993, Babu et al. 1995). Human serum and albumin also promoted the binding of *F. nucleatum* (Xie et al. 1991). The adhesion of *S. mutans* was lower to serum-coated hydroxyapatite than to saliva-coated hydroxyapatite (Nikawa et al. 1998).

Bacterial colonisation on the tooth surface starts in surface irregularities (Nyvad & Fejerskov 1987a). Morphologically, early dental plaque is characterised by gram-positive, saccharolytic coccoid and rod-shaped bacteria which appear approximately four hours after a clean surface is exposed to the oral cavity (Lie 1978, Brecx et al. 1980, Nyvad & Fejerskov 1987b). Recently, bacteria have been identified in plaque less than an hour after introduction of enamel slabs into the oral cavity, but distinct bacterial micro-colonies were identified only after two hours (Hannig et al. 2007).

During the first hours, *Streptococcus* spp. are the predominant genera, accounting for up to 70% of the total viable counts. *Actinomyces* spp. appear in proportions of up to 30%. The proportion of *Streptococcus* spp. gradually decreases and higher numbers of *Actinomyces* spp. are seen (Ritz 1967, Theilade et al. 1982, Liljemark et al. 1986, Nyvad & Kilian 1987, Diaz et al. 2006). Within 24 h, a continuous layer is formed (Nyvad & Fejerskov 1987a). When the tooth surface is covered by a layer of the initially colonising streptococci and *Actinomyces* spp., further colonisation occurs through co-adhesion, which is defined as the binding of

suspended bacteria to already-adhering bacteria of different species (Bos et al. 1996). The binding of genetically distinct bacteria to each other in suspension is called coaggregation. A whole range of coaggregation partnerships has been identified for bacteria found in dental plaque. Among coaggrating bacterial species, *F. nucleatum* excels in having the ability to participate in a broad range of coaggregations (Bolstad et al. 1996, Nagayama et al. 2001). Bacterial coaggregations preformed in planktonic phase have been shown to influence the development of a multispecies biofilm. *A. oris* and *Veillonella atypica* were seen in higher proportions in biofilms when bacterial coaggreations were present during biofilm development (Foster & Kolenbrander 2004).

Supragingival plaque

Clinically, it can take up to four days before supragingival plaque is visible to the naked eye in the whole dentition (Furuichi et al. 1992). The internal structure of dental plaque reflects the successive colonisation of different microbial species. Coccoid bacteria are seen close to the tooth surface and filamentous bacteria in the periphery (Nyvad & Fejerskov 1989). As plaque ages, so-called late colonising, anaerobic and Gram-negative bacteria occur (Theilade et al. 1966, Syed & Loesche 1978, Van Palenstein Helderman 1981, Al-Ahmad et al. 2007). Among the first late colonising bacteria to appear were *Capnocytophaga* spp., *F. nucleatum* and *Veillonella* spp. (Palmer et al. 2006, Al-Ahmad et al. 2007, Al-Ahmad et al. 2009, Uzel et al. 2011). Other species such as e.g. *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, *Prevotella* spp., *P. gingivalis* and *Tannerella forsythia* (formerly: *Bacteroides forsythus*) (Moore 1987, Socransky et al. 1998, Haffajee et al. 1998) follow and attach to the tooth surface by co-adhesion (Rickard et al. 2003, Kolenbrander et al. 2010). This process eventually leads to a complex multispecies layer of dental plaque. Traditionally, the internal structure of dental plaque has been examined by electron microscopy (Listgarten 1976, Nyvad & Fejerskov 1987b). Older dental plaque can contain bacterial coaggregation arrangements of coccoid and rod- shaped or filamentous cells, which are referred to as corn cobs, rosettes and bristle brushes (Listgarten et al. 1975, Listgarten 1976, Nyvad & Fejerskov 1987b). The fixation necessary for this technique may cause considerable distortion of the material. The technique of confocal laser scanning microscopy allows the investigation of undisturbed four-day dental plaque. An open architecture, interpreted as “circulatory” channels and voids, was found which would allow bacterial communication within the dental biofilm (Wood et al. 2000, Stoodley et al. 2002, Marsh et al. 2011). A biofilm is defined as a

community of microorganisms attached to a surface forming a three-dimensional structure with a matrix of extracellular material (Marsh & Martin 2009, Kolenbrander et al. 2010, Zijnge et al. 2010). Within the dental biofilm, the environment determining bacterial growth, e.g. pH, can shift within a microscopic space (Schlafer et al. 2011). Bacteria in dental biofilms live together and can communicate with each other. Late colonising *P. gingivalis* associated with periodontitis cannot, as a single species, grow together with early colonising *S. oralis,* but when another early coloniser, *S. gordonii*, was added to the culture, *P. gingivalis* overcome its incompatibility with *S. oralis*. Other examples of bacteria facilitating growth of other species are *F. nucleatum* in relation to *S. oralis* and *P. gingivalis* and *Veillonella* spp. in relation to *P. gingivalis* and *Prevotella* spp. (Periasamy & Kolenbrander 2009, Periasamy & Kolenbrander 2009, Periasamy et al. 2009). Although many of the interactions are beneficial for the bacteria, several inhibitory interactions have been identified. Of those, the negative association of *S. mutans* and *S. sanguinis* is well-known (Jakubovics 2010, Huang et al. 2011).

Subgingival plaque

In healthy conditions and with good oral hygiene, the microbial composition of supra- and subgingival plaque is similar with Gram-positive, saccharolytic bacteria dominating (Ximenéx-Fyvie et al. 2000a). As plaque grows, gingival inflammation develops resulting in a swelling of the gingival margins and an increase of the pocket probing depths. A general shift of the microflora to anaerob Gram-negative, proteolytic bacterial species is seen (Berezow & Darveau 2011). In periodontitis susceptible individuals, the gingival inflammation eventually results in a breakdown of the tooth-supporting tissues creating a new microbiological compartment, the periodontal pocket (Lindhe et al. 1973). The subgingival plaque within the periodontal pocket is protected from being flushed or wiped away by e.g. the saliva or the tongue. Compared with the supragingival plaque, it is thinner (< 0.2 mm) and has a less organized structure (Wærhaug 1976, Listgarten 1976). Smaller cells without any particular orientation form a layer of varying thickness adhering to the root surface. Further away from the root surface, more distinctive filamentous and rod-shaped microorganisms are observed. In the subgingival plaque facing the pocket wall, “bristle brush” or “test-tube brush” formations of bacteria are seen, consisting of Gram-negative rods or short filaments bound to large filamentous bacteria. (Listgarten 1976). In a recent reports, fluorescence *in situ* hybridization (FISH) was used for analysis of subgingival plaque. Extended

polytetrafluoroethylene membranes inserted in periodontal pocket for 3 to 6 days were covered by a thin (40 to 45 µm) layer of bacteria. The most prominent species which was morphologically identified was *Treponemes* (Wecke et al. 2000). In another study, naturally formed plaque was analysed on extracted teeth. *Actinomyces* spp. were identified in the layer closest to the pocket epithelium, followed by an intermediate layer with *F. nucleatum* and *T. forsythia* (Zijnge et al. 2010).

There is a large body of literature on the identification of bacteria in subgingival samples. Bacteria typically found in periodontitis patients are *P. gingivalis*, *Treponema denticola* and *T. forsythia*, forming the so-called “red” complex (Socransky et al. 1998, Haffejee et al. 1998, Tanner et al. 1998, Dahlén & Frandsen 2002, Diaz 2012). *T. forsythia*, together with *Campylobacter rectus* and *Selenomonas noxia*, *T. denticola* and *P. gingivalis*, were identified as species associated with sites converting from periodontal health to disease (Tanner et al. 1998, Haffejee et al 1998). The difference between sub- and supragingival plaque as well as between periodontal disease and health is characterized by less proportions of *Actinomyces* spp. and higher proportions of the “orange” complex bacteria (e.g. *P. intermedia*, *Prevotella nigrescens*, *Peptostreptococcus micros* and *Fusobacterium* spp.) (Ximénez-Fyvie et al. 2000b, Rescala et al. 2010). The grouping of subgingival bacterial species in clusters places the “orange” complex between periodontal health and disease (Socransky et al. 1998). *F. nucleatum*, a versatile coaggregating bacterium, is among the most frequently detected species in this complex. The shift to more proteolytic, anaerob bacterial species during disease development can partially be reversed by treatment. The proportion of the “red” and “orange” complex has been shown to significantly decrease from more than 40% before to around 25% of total counts after non-surgical treatment (Rosalem et al. 2011).

Plaque formation on exposed root surfaces at gingival recessions

As periodontal disease develops and the gingival margin recedes in apical direction, the root surface is gradually exposed to the oral cavity creating a so-called recession. Recessions occur frequently in periodontitis patients (Serino et al. 1994). The exposed root surfaces would mainly be a dentin surface as the root cementum usually is thin in the coronal part of the root (Bosshardt & Selvig 1997). It was found that the superficial surface of exposed root surfaces can be removed by abrasive tooth brushing (Sangens & Gjermo 1976). The composition of dental plaque in cases of gingival recession has been shown to be different from supragingival

plaque. Generally higher bacterial counts were seen on the exposed root surfaces than on the enamel surfaces. This difference was shown to be significant for *Actinomyces* spp., *Capnocytophaga* spp. and *Eikenella corrodens* (Haffajee et al. 2008).

Periodontal conditions and plaque formation

The impact of inflammation of the periodontal tissues in early dental plaque formation has long been discussed. In subjects with a high full-mouth gingival index, bacterial aggregation started within an hour after cleaning whereas it took over three hours in subjects with a very low gingival index before the first bacteria appear (Saxton 1973). In studies using an experimental gingivitis model, higher plaque accumulation and an earlier development of a morphologically complex bacterial flora was observed when the participants had abstained from oral hygiene over two to three weeks and a clinically visible gingival inflammation had developed (Hillam & Hull 1977, Quirynen et al. 1991, Ramberg et al. 1994). Even a short period without oral hygiene resulted in a notably different microflora at the gingival margin. Higher total numbers of bacteria and early occurrence of Gram-negative microorganisms and rods and filamentous organisms were observed (Brecx et al. 1980). Both the plaque thickness (Hillam & Hull 1977, Ramberg et al. 1994, Daly & Highfield 1996) and the tooth surface area covered by plaque were increased (Quirynen et al. 1991) during inflammation of the gingival margin.

In periodontitis patients, a positive correlation was found between plaque formation and gingival inflammation as well as varying pocket depths (Goh et al. 1986). Interestingly, the association between plaque formation and pocketing was no longer statistically significant as soon as non-surgical periodontal treatment (including oral hygiene instruction and root-planing) was initiated.

In concordance to what is said above, plaque formation in periodontitis patients was shown to be higher before rather than after treatment (Dahan et al. 2004, Rowshani et al. 2004). In a detailed analysis at different stages of periodontal conditions, it was demonstrated that the rate of plaque formation was lowest in periodontitis patients after comprehensive treatment and highest in the presence of untreated periodontitis. Plaque formation in treated periodontitis patients with remaining pockets and bleeding on probing and in peridontally healthy patients was approximately the same, in between the two extremes. Data available on whether the amounts of plaque accumulated during longer periods without oral hygiene differ between periodontitis patients and periodontally healthy subjects are inconsistent (van der Velden et al

1985, Brecx et al. 1991, Johnson et al. 1997, Trombelli et al. 2006). As plaque ages, its further growth may be determined by macroanatomical (tooth position in the dental arch) and mechanical factors (abrasion by tongue and food) rather than the GCF flow, the inflammatory status of the gingival margin or the subject's susceptibility to periodontitis.
The mechanisms for the observation of increased early plaque formation in the presence of periodontal inflammation are not fully understood. The inflammatory oedema of the gingivae may provide a shelter for the growing bacteria (Walsh et al. 1991, Rosin et al. 2002).
The GCF flow rate has been shown to correlate with the level of gingivitis (Löe & Holm-Pedersen 1965) and to decrease after periodontal therapy (Tsuchida & Hara 1981, Suppipat et al. 1978). It has therefore been suggested that an enhanced GCF flow during inflammation increases the supply of nutrients for plaque-forming bacteria (Loesche 1968, Rüdin et al. 1970, Saxton 1973, Mukherjee 1985, Lopatin et al. 1989, Lamster et al. 1990, Lamster & Novak 1992). As a plasma exudate, the GCF also contains plasma proteins (Holmberg & Killander 1971, Bickel et al. 1985, Marcus et al. 1985, Talonpoika et al. 1993, Adonogianaki et al. 1996). Thus, another explanation for increased bacterial adhesion and plaque formation in the presence of periodontal inflammation could be a higher exposure of plasma protein in the pellicle, which may affect initial bacterial adhesion and the bacterial composition of dental plaque.

Treatment of gingivitis and periodontitis

Gingivitis is defined as an inflammation of the gingivae without breakdown of periodontal tissues. The treatment of gingivitis aims to eliminate inflammation, which resolves as soon as oral hygiene is properly performed (Löe et al. 1965). It takes around two weeks for the clinical signs of inflammation to disappear.
The goal of periodontitis treatment is to arrest disease progression. Treatment success is evaluated by absence of bleeding on probing (Lang et al. 1986), low numbers of remaining pockets (Rams et al. 1996) and low plaque scores (Eickholz et al. 2008). Periodontitis treatment is often initiated by a cause-related, usually, non-surgical phase. In this phase, the patients are informed, motivated and instructed to perform optimal oral hygiene, and subgingival debridement, so called depuration, to remove calculus and obtain a smooth root surface, is performed. In many cases, this treatment results in healing of the periodontal lesion (Kaldahl et al. 1996). Although it has been shown that even deep pockets were reduced after non-surgical therapy it also became obvious that non-surgical therapy can leave behind

pockets with remaining pathology, i.e. bleeding on probing (Badersten et al. 1984). When bleeding on probing to the bottom of the pocket (Mühlemann & Son 1971) occurs in the absence of bleeding after running the probe briefly along the gingival margin (Löe 1967), improved plaque control will not result in further healing, nor would re-depuration when a smooth root surface is achieved. In such cases, surgical periodontal treatment can be performed. An established surgical technique is raising a soft tissue flap in order to get access to the remaining periodontal infection and inflammation at the bottom of the pocket. Inflammatory tissue is then removed and the root surfaces re-scaled if remaining calculus is detected. Osseous surgery is performed to eliminate the pockets. The flaps are apically repositioned on the level of the recontoured bone and retained in this position by sutures. The healing result is usually evaluated two to four months after the surgical intervention. Surgical periodontal therapy has been shown to result in substantial pocket reduction and, if periodontal supportive therapy is provided, in long-term stable clinical periodontal health (Lindhe & Nyman 1984). Histology has nevertheless revealed a remaining connective tissue infiltrate (Zitzmann et al. 2005).

The main reason to the present studies on subjects with different periodontal conditions were to see if and how plasma components could affect the formation of the pellicle and early dental plaque under natural conditions *in vivo.* Most of the studies on plaque formation in the literature were performed *in vitro* using saliva and experimental tooth surfaces or using the latter carried in appliances on teeth *in situ* where they were not reached by the GCF fluid. Studies examining early natural plaque formed *in vivo* are scarce.

Hypothesis & Aims

Our hypothesis was that plasma proteins in the gingival crevicular fluid affect pellicle formation and the establishment of the early dental microflora on the tooth surface.

The aims of the studies were to examine:

i) plasma proteins in pellicles formed *in vitro* on hydroxyapatite and *in vivo* on the gingival and incisal parts of the tooth surface,

ii) the adherence of parodontitis associated bacteria to experimental plasma and saliva pellicles,

iii) proteins and microorganisms in dental biofilms

- at healthy and inflamed gingival margins in periodontally healthy and diseased subjects,
- before and after periodontal surgery in subjects diagnosed with chronic periodontitis, and
- in relation to the extent of root surface exposure.

Materials & Methods

Saliva and plasma (Study I)

Plasma and saliva were collected from one healthy individual. Plasma was obtained from blood collected in a heparinised tube. Parotid and submandibular/sublingual saliva was collected by means of Lashley cups and custom-made devices, respectively, after stimulation with citric acid. Stimulated whole saliva was obtained after chewing on paraffin wax.

Experimental pellicles (Study I)

Experimental pellicles for protein analysis were formed by the 60-min incubation of 40 mg of hydroxyapatite (HA) beads with 1.0 ml portions of series of plasma and saliva dilutions. After thorough washing, proteins were desorbed from the HA by heating in electrophoresis buffer with SDS (Figure 1).

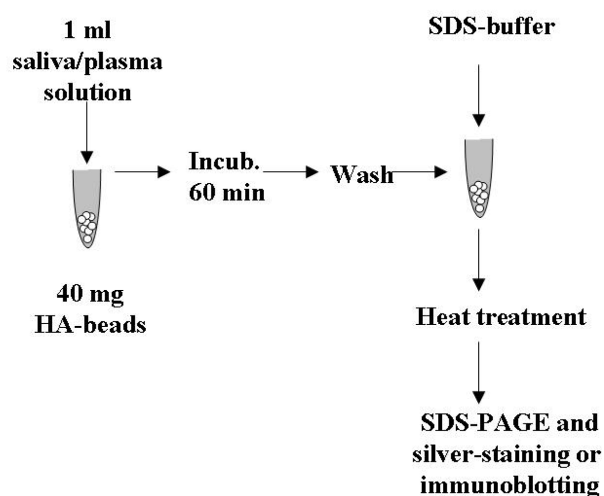


Figure I. Formation and analysis of the *in vitro* pellicles.

In vivo pellicles (Studies I, II, III & IV)

Subject recruitment

Five (Study I: aged 29 to 53 years) and eight (Study II: aged 21 to 49 years) periodontally healthy subjects were selected. At recruitment, periodontal health was clinically diagnosed by the absence of gingival redness and pathological pocketing. Eleven subjects (aged 39 to 66 years), diagnosed with advanced general chronic periodontitis, were recruited at re-evaluation after basic therapy, when surgical elimination was deemed necessary to reduce infection and

to arrest the progression of the disease (Study III). Another 11 patients (aged 46 to 58 years) with periodontitis were enrolled at completion of comprehensive periodontal therapy, including basic therapy and surgical elimination of remaining periodontal pockets (Study IV). Exclusion critera were medication or medical conditions affecting oral health. Smoking habits were registered. The aim of our studies was to find a model for studying pellicle and early plaque formation at varying GCF flow. The differences of the GCF flow at the selected periodontal conditions were considered higher than possible variation due to smoking. Therefore both smokers and non-smokers were included into the studies.

Sampling principles

In Study I, samples were taken without further intervention. In Study II, our goal was to compare healthy and experimentally inflamed conditions. It is known that clinically diagnosed gingival health can coincide with histological signs of inflammation. To reduce possible subclinical inflammation, all the teeth were professionally cleaned every second day for a two-week period before samples were taken. Experimental gingivitis was established by first instituting gingivally healthy conditions by professional tooth cleaning for a fortnight, prior to a five-day period without oral hygiene. Study III aimed to investigate the impact of three stages of periodontal inflammation on pellicle and early plaque formation, namely (i) a remaining subgingival infection in the absence of marginal inflammation, (ii) a reduced but healthy periodontium after surgical pocket elimination and (iii) an experimentally induced marginal infiltrate after surgical pocket elimination. Before sampling at the respective stages, healthy gingival conditions and experimental marginal inflammation were induced, as in Study II (see flow chart, Figure II). The objective of Study IV was to examine proteins in the dental pellicles and bacteria in early dental plaque formed on root surfaces exposed after periodontal surgery (recession depth of at least 3 mm) and to compare to the pellicles and plaque formed on the enamel surface of the treated as well as of non-treated teeth in the same patient. The surgically treated teeth had before pocket elimination approximal pockets ≥ 5 mm with bleeding on probing. The non-treated teeth had probing depths of no more than 4 mm without bleeding.

The sample collection procedure was the same in all studies. Undisturbed by food or drink, *in vivo* pellicles were allowed to form on polished buccal and lingual tooth surfaces. After 60 minutes, the teeth were rinsed and air-dried and pellicles were collected by rubbing with fibre pellets soaked with SDS.

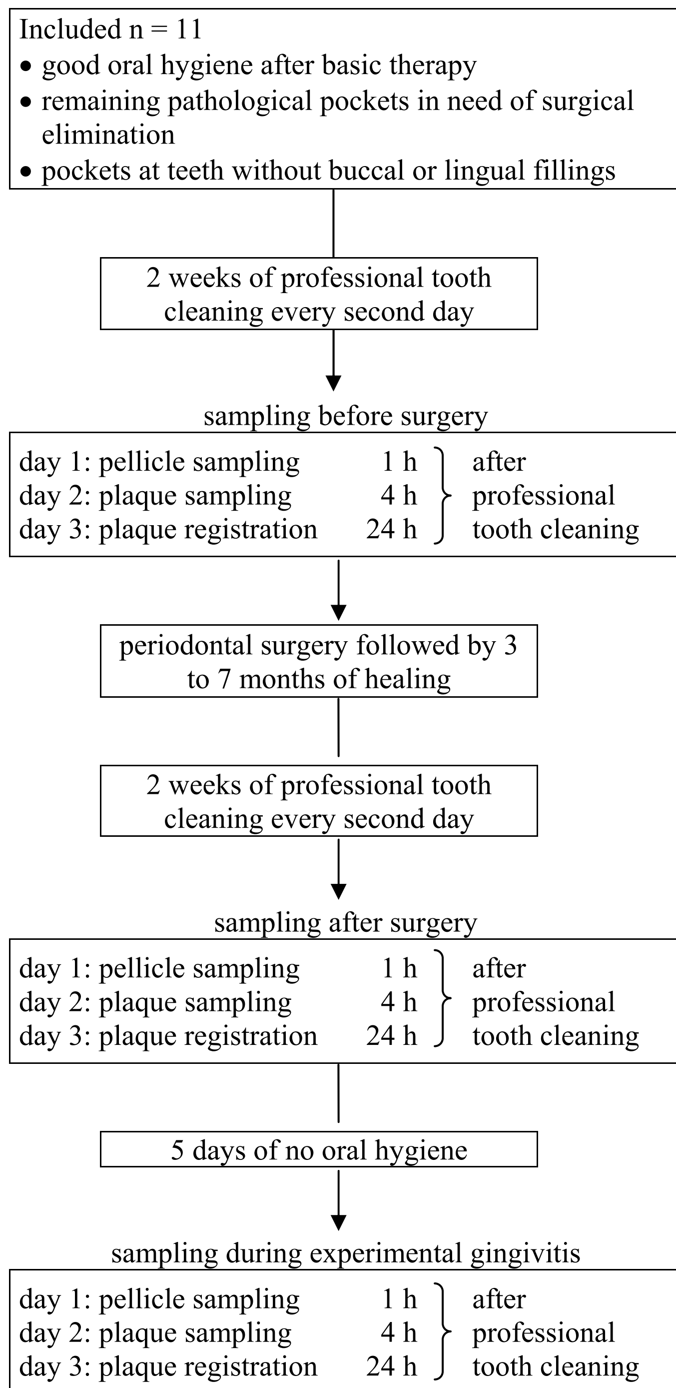
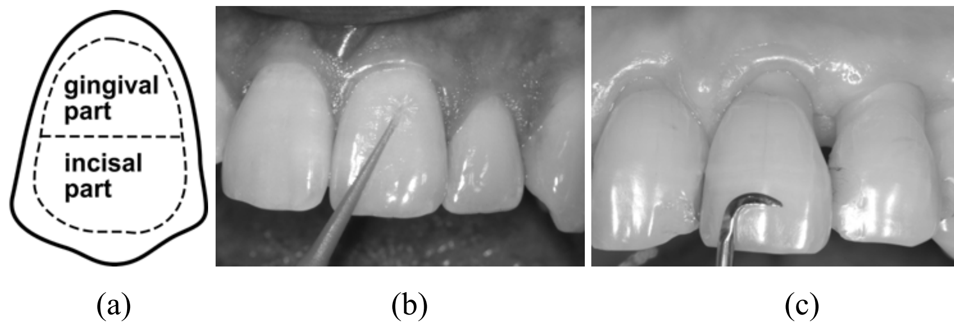


Figure II. Flow chart for the three sampling occasions before and after surgical pocket elimination, and during subsequent experimental gingivitis (Study III).

Samples were taken separately from the gingival and incisal parts of the buccal and lingual surfaces (Figure IIIa,b). The pellicle proteins were dissolved from the pellets by heating in electrophoresis buffer with SDS. In Study II, buccal and lingual samples and samples from the upper and lower jaws were all analysed separately. In Study III and IV, samples were pooled from the buccal and lingual surfaces and from upper and lower teeth into one gingival

and one incisal sample at each sampling time point. In Study IV, an additional supragingival sample was taken from the exposed root surfaces at gingival recessions of the surgically treated teeth and analysed separately.



(a) (b) (c)

Figure III. Samples were taken separately from the gingival and incisal part of the tooth surface (a). Clinical examples of pellicle sampling from the gingival part in Study I (b) and plaque sampling from the incisal part after pocket elimination in Study III (c).

Analysis of pellicle proteins (Studies I, II, II & IV)

Proteins in pellicles were analysed by electrophoresis on pre-cast 4% to 15% gradient gels, which were either silver stained or blotted onto nitrocellulose membranes. Specific proteins were analysed by immunoblotting using antibodies to a panel of saliva and plasma proteins. The detection limits for all proteins except agglutinin and PRP were determined from the staining of purified, serially diluted proteins in the immunoblots.

The amount of protein in the samples was estimated by densitometric analysis after scanning and digitising the stained gels and membranes. Pixel values from the densitometric analysis were used for comparisons of samples, which had been analysed in parallel on the same silver-stained gels or immunoblots (Figure IV).

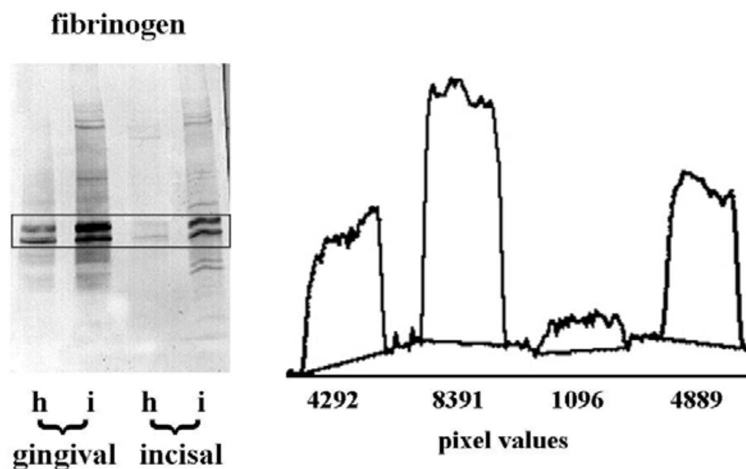


Figure IV. Semiquantification of the amount of fibrinogen in gingival and incisal pellicles formed at healthy (h) and experimentally inflamed (i) gingival margins by densitometric analysis of the bands representing fibrinogen.

Bacterial adherence (Study I)

^{35}S-labelled strains of *P. gingivalis*, *F. nucleatum*, *A. naeslundii, A. oris* and *A. viscosus* were used to test the adherence to pellicles formed from saliva, plasma and specific plasma proteins on HA beads. The number of bound bacteria was determined by scintillation counting and adherence was expressed as the number of cells bound to the HA beads as a proportion of the number of cells added to the beads.

Plaque sampling (Study II, III & IV)

The teeth were polished using a rubber cup and fine-grade pumice. After four hours without food or drink (except water), the teeth were rinsed and air-dried and plaque was collected by scraping the tooth surfaces with sterile scalers (Figure IIIa,c). In Study II, samples for culture and PCR analysis of the micro-organisms were collected from the respective contra-lateral teeth. In Study IV, also subgingival samples were obtained from the mesial aspect of each non-treated tooth and from the surgically treated approximal site of each surgically treated tooth by inserting sterile paper points into the gingival sulcus for a short time.

Microbial analysis (Study II, III & IV)

The plaque samples were cultured on enriched Brucella agar and selective agar for the enumeration of the total numbers of bacteria and the numbers of black-pigmented *Prevotella* ssp., *P. gingivalis, Capnocytophaga* spp., *C. rectus*, *F. nucleatum*, *Streptococcus* spp., *S. mutans, Actinomyces* spp., *A. actinomycetemcomitans*. Polymerase chain reaction (PCR) techniques were additionally used in Study II for the analysis of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia, P. intermedia and P. nigrescens*. The PCR analyses allow the detection of non-viable bacteria and bacteria like *T. forsythus*, which is difficult to identify on the agar plate, as well as the discrimination between *P. intermedia* and *P. nigrescens.*

Clinical variables (Study II, III & IV)

The amount of GCF was measured using Perio papers® and a Periotron 8000® device. The rate of plaque formation was measured after professional tooth cleaning and abstinence from oral hygiene for 24 h. Single-blinded registration of the presence or absence of plaque and gingival bleeding (Study II) was performed at six sites per tooth in the entire dentition.

Statistical analysis

In Study II, the sixteen pellicle and plaque samples from each subject (eight taken during healthy conditions and eight during inflamed conditions) were analysed separately. The four individual mean values from the following sites were used for the analysis of differences.

- From the gingival surfaces: (a) in healthy conditions and (b) in inflamed conditions
- From the incisal surfaces: (a) in healthy conditions and (b) in inflamed conditions

In Study III an Study IV, samples from different tooth surfaces in the same individual were pooled before analysis. Thus in Study III, two samples (one gingival and one incisal) after each sampling time point (before and after surgery as well as in the presence of experimental gingivitis) were forwarded for analysis. In Study IV, samples from the gingival and incisal surfaces of surgically treated and non-treated teeth, respectively, and from the exposed root surfaces at gingival recessions of the surgically treated teeth were pooled and, thus, five individual supragingival samples per patient (three from the surgically treated teeth and two from the non-treated) were analysed. In addition, one subgingival microbiological sample from the teeth in each category (surgically and non-treated teeth) was available. Calculations of the amounts of protein and bacteria that were detected in the majority of samples (> 60%) were performed on the basis of quantitative data (pixel values and proportion of TVC respectively). Otherwise, the detection frequencies, i.e. the proportion of samples in which the protein/bacterial species was detected, were used. An exception was made in Study IV where all pellicle proteins (even those detected in < 60% of the samples) were analysed using the pixel values, as the detection frequencies on the exposed root surfaces were much higher than on the enamel surfaces. Since normal distribution cannot always be assumed for small numbers of study samples, non-parametric tests were performed. Wilcoxon's matched pairs signed rank sum test was applied for comparison of samples. Spearman's correlation analysis was used to test the correlation between bacteria and proteins. In Study I, the number of participants was too small for analytical statistics.

Results & General Discussion

Pellicle composition

After analysis of the components recovered from *in vitro* pellicles formed from whole plasma serially diluted up to 1/200, bands of all plasma proteins tested could be clearly identified on the immunoblots (Study I, Figure 2). *In vivo*, proteins from the crevicular fluid compete with components from saliva and bacteria for sites on the tooth surface. Furthermore, proteins may form complexes (Iontcheva et al. 1997), which can affect their incorporation into the pellicle. The incorporation of a specific plasma or saliva protein in the experimental pellicles and in the *in vivo* pellicles may therefore differ. This can be illustrated by salivary amylase, which was frequently found in the *in vivo* pellicles, although it was not seen in the *in vitro* pellicles (Carlén et al. 1998).

Table I. The relative amount of proteins (based on pixel values) in gingival and incisal pellicles (gingival *vs* incisal) in different states of periodontal inflammation.

	Study II		Study III			Study IV	
	healthy	exp. inflamed	before surgery	after surgery	experimental gingivitis	non-treated teeth	surg. treated teeth
Total proteins [a]	1.3^{*}	1.0	1.9^{**}	1.3^{*}	3.2^{**}	1.1	1.2
IgG	2.0^{*}	1.0	2.5^{**}	2.9^{**}	3.8^{**}	1.9^{*}	1.8^{*}
Albumin	1.5^{*}	1.2	2.6^{**}	2.8^{**}	3.1^{**}	2.6^{**}	2.1^{**}
Amylase	0.5	1.1	0.7	1.1	1.1	0.8	0.9
IgA	1.2	1.2	1.7	1.5	2.3	0.8	1.2

[a] Total amount of proteins as determined from silver-stained gels
* $p<0.05$, **$p<0.01$ for the difference represented by the ratio

In *in vivo* pellicles (Study I to IV), the plasma proteins IgG and albumin were identified in > 60% of all samples as were fibrinogen and fibronectin in samples from periodontally healthy individuals. In periodontitis patients, fibrinogen and fibronectin were found only in around 50% of the samples. The densitometric analysis of the silver-stained gels and immunoblots revealed more total and specific proteins respectively in the gingival pellicles than in the incisal ones (Table I). The difference between gingival and incisal pellicles was clearly seen under healthy conditions both when gingival health was based on clinical inspection only (Study I, Table 1) and when gingival health was created by a fortnight of professional oral hygiene measures every second day (Table I). The total protein content and the content of plasma proteins increased significantly in the incisal pellicle under inflamed conditions

(p<0.05) (Table II). The increase was in fact so high that the difference between gingival and incisal pellicles was no longer statistically significant (Table I). These observations may indicate that saturation with plasma proteins occurred on the gingival part, while the incisal part was still able to bind proteins.

Table II. The relative amount of proteins (based on pixel values) in pellicles formed at gingival margins with relatively high and low GCF flow respectively, on gingival and incisal parts of the teeth.

	Study II Exp. gingivitis *vs* healthy gingiva		Study III Before *vs* after surgery		Study III exp. gingivitis *vs* after surgery		Study IV Surgically treated *vs* non-treated teeth	
	gingival	incisal	gingival	incisal	gingival	incisal	gingival	incisal
Total proteins[a]	1.1	1.4*	2.6*	1.8	2.0**	0.8	1.3	1.1
IgG	1.1	2.3*	2.1*	2.4*	1.5	1.1	1.4	1.5*
Albumin	1.2	1.6*	1.6*	1.7	1.3	1.1	1.3	1.6*
Amylase	2.4	1.2	0.5*	0.8	1.2	1.1	1.2	1.1
IgA	2.0	1.9	1.3	1.1	0.8	0.5	2.0	1.3

[a] Total amount of proteins as determined from silver-stained gels
* p<0.05; ** p<0.01 for the difference represented by the ratio

In periodontitis patients (Study III), total pellicle proteins and the amounts of plasma proteins IgG and albumin were statistically significant higher on the gingival than on the incisal pellicles (Table I). They decreased significantly ($p < 0.05$) on the gingival surfaces after pocket elimination (Table II). At experimental gingivitis, an increase was observed, statistically significant only for the total protein content of the gingival pellicles (Table II). The ratio between the findings of these proteins in gingival and incisal pellicles remained statistically significant (Table I).

Fibronectin and fibrinogen were less frequently detected in pellicles of periodontally diseased than of healthy subjects, although, *in vitro*, both proteins adhere to hydroxylapatite (Carlén et al. 1998, Kilpadi et al. 2001). Plasma proteins in dental pellicles derive from the GCF. Smokers are known to have a lower GCF flow (Morozumi et al. 2004) and the individuals included in Study III were, in contrast to Study II, mainly smokers. Thus, the amount of plasma proteins available for pellicle formation may differ between these two studies. Further on, gingival recessions, a frequent finding in periodontitis patients (Serino et al. 1994), increases the distance between the gingival margin and the enamel surface from which the pellicle samples were taken. On leaving the gingival crevice, fibronectin and fibrinogen, which both have high molecular weights compared with IgG and albumin, may be too large to

traverse the exposed root surface, which has a higher surface roughness than the enamel (Yurdaguven et al. 2012).
Despite the smaller sampling surfaces, fibronectin and fibrinogen were found more frequently and in higher amounts (for fibronectin $p < 0.05$) on the exposed root surfaces than on the adjacent enamel surface of the crowns (Study IV, Tables 2 and 3). In addition, we found a negative correlation (for albumin and fibronectin $p < 0.05$) between the overall average extent of the exposed root surfaces and the amount of pellicle proteins on the enamel surfaces (Study IV, Table 5). Our findings in Study IV were in support of the notion of exposed root surfaces being responsible for the lower prevalence of fibronectin and fibrinogen in dental pellicles in the periodontitis patient of Study III compared with the periodontally healthy individuals of Study II. A further comparison revealed that the prevalence of these two plasma proteins was even lower on the non-treated teeth in periodontitis patients (Study IV) than on the teeth of periodontally healthy individuals. This may be explained by the fact that gingival recessions are generally seen in periodontally diseased individuals (Serino et al. 1994) whereas they are not a frequent finding in younger periodontally healthy individuals.
When comparing the content of plasma proteins in incisal pellicles before and after surgery in Study III (Table I), the gingival pellicles always contained more plasma proteins than the incisal ones. This difference was statistically significant for IgG and albumin. Further on, the content of IgG and albumin in incisal pellicles was higher on surgically treated than on non-treated teeth (Study IV). Saturation of the pellicles on the gingival surface, as speculated for healthy individuals during experimental gingivitis (Study II), did not seem to be applicable for periodontitis patients. Exposed root surfaces, frequently seen in periodontitis patients, were found to bind a considerable portion of the plasma proteins from the GCF (Study IV). Thus, a saturation of the enamel surface, at least with plasma proteins on the gingival part of the crown, may not occur in the periodontitis patients.

In both Study III and Study IV, higher amounts of pellicle proteins were accompanied by clinical findings of greater probing depths, more bleeding on probing and higher GCF flow. An association with periodontal inflammation and higher total and plasma proteins in the dental pellicle is in agreement with the findings of Study II. Interestingly, pellicles formed at healthy gingival margins in periodontally healthy individuals (Study II) contained less plasma proteins than pellicles formed at healthy gingival margins after periodontal pocket elimination in periodontitis patients (Study III). The same difference was observed between pellicles on

surgically treated compared with non-treated teeth in Study IV. Although the aim of periodontal surgery is always the elimination of the pocket and the periodontal inflammation, such intervention can leave behind a remaining infiltrated connective tissue (Zitzmann et al. 2005). This may be responsible for elevated amounts of total and plasma protein in dental pellicles even after periodontal treatment and at non-treated teeth in the patients.

Saliva components were found less frequently in periodontally healthy individuals (Study II, Table 1) than in periodontitis patients (Study III, Table 2 and Study IV, Table 2). In the periodontally healthy individuals, the detection frequency was 59% to 84% for amylase and 50% to 69% for IgA. Agglutinin and PRP were found in 41% to 56% of the samples. In periodontitis patients, amylase and IgA were seen in the majority of the samples (82% to 100%) and the detection frequency of PRP was 36% to 73% (Study III, Table 2 and Study IV, Table 2). Agglutinin was not investigated in Study III and Study IV. In periodontally healthy individuals, the amount of amylase and the detection frequencies of IgA also increased both on the gingival and the incisal parts of the crowns in connection with experimental inflammation but without reaching statistical significance. In periodontitis patients, the amount of amylase increased significantly on gingival surfaces after pocket elimination, whereas the level of IgA was decreased, although not statistically significantly (Table II). PRP was least frequently (45.5%) seen in gingival pellicles before periodontal surgery and most frequently (82%) after pocket elimination on the same location (Study III, Table 2). Pellicles on surgically treated teeth in periodontitis patients had significantly higher amounts of amylase than pellicles on the non-treated teeth (Study IV, Table 3). Variations in the prevalence of PRP in the pellicles of non-treated and surgically treated teeth in periodontitis patient were small (Study IV, Table 2).

Higher prevalence of the salivary amylase and IgA in periodontitis patients (Study III, Table 2) than in periodontally healthy individuals (Study II Table 1) was accompanied with generally higher GCF flow in the patients. It may be speculated that salivary proteins have an affinity to plasma proteins or possibly other components in the GCF, eventually promoting their incorporation in the dental pellicle. The pellicle samples in our studies were tested only for a small number of salivary components. It may therefore further be speculated that other, unaccounted-for salivary components decreased in the presence of plasma proteins and leave space for the salivary proteins for which we tested.

Bacterial adherence *in vitro* and prevalence *in vivo*

The overall ranking order for adhesion to *in vitro* pellicles was *Actinomyces* spp. > *F. nucleatum* > *P. gingivalis* (Study I, Table 2). This order parallels the proportions normally found in supragingival dental plaque. Saliva pellicles bound the highest numbers of cells (up to 69%), which may illustrate that salivary components are of major importance for the attachment of oral bacteria, but the bacteria also adhered to plasma proteins, frequently found in the *in vivo* pellicles close to the gingiva. It is therefore reasonable to assume that plasma proteins also play a role in the adhesion of parodontitis associated bacteria to the tooth surface. This assumption was further substantiated by an increased finding of such bacteria during inflammation (Study II, Table 4) and a decreased finding after surgical elimination of pockets (Study III, Table 4).

In gingival health (periodontally healthy individuals, Study II) and generally in periodontitis patients (Study III), higher numbers of bacteria were found on the gingival part of the tooth surface than on the incisal parts (Table III). During experimental gingivitis in periodontally healthy individuals, increased values for the total bacterial numbers were seen, especially on the incisal part (Table III). In periodontitis patients after surgical pocket elimination (Table III), total bacterial counts on gingival and incisal surfaces decreased by the approximately the same magnitude. This decrease was statistically significant for the gingival surfaces. During subsequent experimental gingivitis, a slight increase of the bacterial numbers occurred (Study III, Table 4). On the exposed root surfaces of surgically treated teeth, the total bacterial counts were higher than on the adjacent enamel surface (Study IV, Table 4). Higher, though not statistically significantly higher counts were also seen on the gingival surface of non-treated compared with surgically treated teeth (Table III). This may be due to a higher binding capacity of the exposed root surfaces for the plasma proteins, which thus would not reach the enamel surface of the surgically treated teeth resulting in less bacterial receptors.

The most frequently identified species were streptococci and *Actinomyces* spp., which were identified in the majority of the samples (> 70%) in periodontally healthy individuals (Study II). In periodontitis patients, streptococci were present in almost all samples (>94%), whereas *Actinomyces* spp. were less frequently seen (Study III: 32%, Study IV: 53%).

Table III. Bacteria in early gingival and incisal plaque (mean±SD).

	Study II				Study III				Study IV			
	gingival		incisal		gingival		incisal		gingival		incisal	
	healthy	inflamed	healthy	inflamed	before	after	before	after	surgically	non-	surgically	non-
	healthy subjects		healthy subjects		surgery		surgery		treated teeth		treated teeth	
Log (TVC) [a]	1.84 ±0.39	1.92 ±0.55	1.46 ±0.4	2.08 ±0.4	5.51 ±2.18*	4.35 ±1.42	4.45 ±2.28	3.63 ±1.50	3.31 ±1.34	4.10 ±1.75	3.16 ±0.99	3.39 ±1.16
streptococci[b]	37.5 ±19.8	26.2 ±9.3	41.2 ±21.0	29.7 ±11.5	76.6 ±33.9	65.1 ±28.8	79.8 ±30.7	89.4 ±14.1	73.3 ±43.3	45.1 ±40.1	61.6 ±38.8	72.8 ±58.0
Actinomyces spp.[b, c]	15.3 ±7.2	17.9 ±11.9	27.0 ±19.0	18.1 ±10.9	36.7%	45.5%	27.7%	27.7%	27.3%	27.3%	18.2%	18.2%

[a]log (TVC)=total viable counts logarithmically transferred.
[b]percentage of TVC Study II where *Actinomyces* spp. were identified in > 70% of all samples.
[c]detection frequency in Study III and IV
* $p<0.05$ för differences before and after surgery

In accordance with previous reports (Loesche & Syed 1978, Moore et al. 1987), the numbers of streptococci decreased during experimental gingivitis (Table III). In experimental gingivitis in periodontitis patients, the number of streptococci was statistically significantly ($p<0.05$) lower than after surgery (Study III, Table 4). Recently, a significant association between the presence of *S. sanguinis* and low values of periodontal indices was found (Stingu et al. 2008). *Actinomyces* spp. bind to salivary PRP and statherin (Clark et al. 1989, Gibbons & Hay 1989), although the binding capacity can vary between species and strains. In Study I, an *A. viscosus* strain was included. This is not a typically human strain (Li et al. 1999), but it was included because it expresses type 1 fimbriae, distinguishing it from those of *A. oris* and *A. naeslundii* (Li et al. 1999). Previous studies have revealed that it adheres preferentially to PRP and statherin in saliva pellicles but, in contrast to *A. naeslundii* and *A. oris*, it does not appear to bind to glycosylated structures (Strömberg et al. 1992). We found that *A. viscosus* further differed from *A. naeslundii* and *A. oris* by not binding to typical plasma proteins (Study I). This indicates that strain-specific differences in binding capacities may be due to variations in the expression of fimbrial adhesins. This was possibly also true for the *P. gingivalis* strains, which express different fimbriae that bind to saliva and plasma proteins with different binding mechanisms (Amano et al. 1999, Nakamura et al. 1999).

In periodontitis patients, *Actinomyces* spp. were most frequently detected and at a 10^3-10^4 level on gingival enamel surfaces before surgery (Study III) and on exposed root surfaces (Study IV). In all other supragingival samples, their mean number was low (<100). In the subgingival samples, their mean number was $1.1 \pm 1.5 \times 10^3$ (SD) at the non-treated teeth and

$2.8 \pm 4.7 \times 10^3$ (SD) at the surgically treated teeth (Study IV). No obvious pattern was seen for the distribution of *Acinomyces* spp. on incisal and gingival surfaces.

In line with our finding that *A. naeslundii* and *A. oris* bound in highest numbers to saliva pellicles (Study I, Table 2), *Actinomyces* spp. was often observed in higher numbers and more frequently when the degree of inflammation was low (Table III). However, *Actinomyces* spp. were positively correlated with the presence of IgG and albumin in the *in vivo* pellicle (Study II, Table 6) which corroborates an avid binding of *A. naeslundii* and *A. oris* also to these components in the experimental pellicle (Study I, Table 2). Contrary to previous reports (Loesche & Syed 1978, Moore et al. 1987), the numbers of *Actinomyces* spp. were not affected during gingivitis and in the presence of remaining periodontal pockets. This discrepancy could be due to the fact that older plaque was sampled in the previous studies. Also, different species or strains of *Actinomyces* spp. may dominate in early and older plaque.

In previous studies, higher subgingival counts of *Actinomyces* spp. were found in periodontitis patients than in periodontally healthy individuals (Haffejee et al. 1998, Ximénez-Fyvie et al. 2000a). In Study IV, we found a similar although not statistically significant intra-individual relation between the counts in subgingival samples from surgically treated and non-treated teeth. We further observed higher counts of TVC and *Actinomyces* spp. in subgingival than in supragingival samples (Study IV), whereas the reverse was reported in an earlier study, (Ximénez-Fyvie et al. 2000b). In contrast to the earlier report, we compared an experimental four-hour-old supragingival plaque, which was retrieved from a comparatively small area (gingival surface) of the tooth, with an older, naturally formed subgingival plaque. Therefore, higher subgingival bacterial number may have been expected.

As is the case for *Actinomyces* spp., *P. gingivalis* and *Fusobacterium* spp. may bind to the salivary PRP and statherin in the pellicle (Gibbons & Hay 1989, Gillece-Castro et al. 1991, Xie et al. 1991). *P. gingivalis* may further bind to fibrinogen (Gibbons & Hay 1989) and *Fusobacterium* spp. to fibronectin and albumin (Xie et al. 1991, Babu et al. 1995). We found that the prevalences of parodontitis-associated *Prevotella* spp., *F. nucleatum* and *Capnocytophaga* spp. were correlated ($r > 0.7$; $p < 0.05$) with the presence of plasma proteins in the gingival pellicle at inflamed gingival margins (Study II, Table 6). The detection frequencies for these bacteria also increased during inflammation and decreased after surgical

pocket elimination. Strains of *F. nucleatum* tested in Study I displayed a relatively avid adherence to pellicles formed from plasma and plasma components and some adhered in high numbers to saliva pellicles (Study I, Table 2). The ability to adhere to both saliva and plasma components is in accordance with the frequent appearance of these bacteria in both supra- and subgingival dental plaques (Ximénez-Fyvie et al. 2000a). Smaller standard deviations for plasma than for saliva binding (Study I, Table 2) together with the finding of positive correlations for *F. nucleatum* with plasma components in the pellicle may indicate that binding to plasma components is a first alternative for *F. nucleatum* strains.

Since culturing techniques are sensitive to aerobic conditions during the handling and growth of the samples, a PCR method was applied in Study II to see if anaerobic bacteria could be identified more frequently. This was not the case. Black-pigmented *P. intermedia* and *P. nigrescens* were identified together by culturing, whereas PCR analysis could distinguish between these two species. *P. intermedia*, which is more closely associated with periodontal disease, was found in only one sample out of 128, whereas *P. nigrescens* could be detected in 3 to 25% of the samples (Study II). This corroborates previously reported data in which *P. nigrescens* was more frequent than *P. intermedia* in the subgingival plaque of healthy subjects (Gharbia et al. 1994) and in the older supragingival plaque of both naturally occurring gingivitis (Ximénez-Fyvie et al. 2000a) and 14-d-experimental gingivitis (Lie et al. 2001).
In periodontitis patients, Gram-negative species (*Campylobacter* spp., *Capnocytophaga* spp., *F. nucleatum or* - in Study IV only - black-pigmented *Prevotella* spp.) were more often found on gingival than on incisal surfaces and more often before than after surgical pocket elimination (Study III, Table 4). They were as often seen on exposed root surfaces as on the gingival surfaces and present in the majority of the subgingival samples (Study IV, Table 4). In no case were *A. actinomycetemcomitans* or *P. gingivalis* or *T. forsythia* detected. This might have been expected as these bacteria are usually found subgingivally in advanced periodontal disease (Zambon 1996, Socransky & Haffejee 2005). *P. gingivalis* can also appear in shallow pockets in subjects in whom oral hygiene is not optimal (Mombelli et al. 1998). *P. gingivalis* is regarded as a late coloniser which is attached to the tooth surface by co-aggregation with early colonising bacteria (Rickard et al. 2003). Previous reports on the saliva-mediated binding of *P. gingivalis* (Sweet et al. 1990) and our own observations of binding to plasma proteins in the experimental pellicle (Study I) indicate that this species has the potential to bind to the *in vivo* pellicles. Whether or not a bacterium in the *in vivo* situation

can eventually become established and colonise in the dental plaque depends on several additional factors such as pH, redox potential and nutrient supply (Marsh 1994).

Three Gram-negative bacteria (*F. nucleatum, Capnocytophaga* spp. and *C. rectus*) were identified in the periodontitis patients in both Study III and Study IV. *F. nucleatum* and to a lesser extent *Capnocytophaga* spp. have been shown to contribute to the development of a multispecies biofilm in the early stages of dental plaque formation (Kolenbrander et al. 2010). Our results suggest that also *C. rectus* may belong to the group of Gram-negative species helping to create an environment that facilitates the establishment of the "red" complex. Gram-negative species associated with periodontal disease were less frequently found in Study III and IV on periodontitis patients than in Study II on healthy subjects. High levels of oral hygiene may explain the low number in periodontitis patients who usually underwent a thorough oral hygiene training program, which healthy individuals rarely would receive. Although these species more often appear in the presence of periodontal inflammation and close to the gingival margin, they also appear on the incisal part of the crown (Study III and Study IV). Their early supragingival appearance after tooth cleaning has previously been observed (Ramberg et al. 2003, Teles et al. 2012). It is known that extra-crevicular intraoral sites can harbour Gram-negative species (Mager et al. 2003), and thus, in supragingival surroundings, find favourable conditions for adhesion to the tooth surface. This may indicate that supragingival colonisation can occur from extra-crevicular intraoral sites.
In contrast to the total bacterial counts and other bacterial species, the number of Gram-negative bacteria found on the exposed root surfaces (Study IV) did not correspond with their numbers in the subgingival plaque at the surgically treated teeth. This may indicate differences in the adhesion properties of pellicles on exposed root surfaces compared with enamel surfaces.

It is known that periodontitis-associated bacteria can metabolize plasma proteins and especially fibronectin (Wikström & Linde 1986, Wikström et al. 1983). The ability of the bacteria to bind to plasma proteins could therefore be a mechanism mainly for nutritional purposes. However, at the same time this mechanism may allow the bacteria to become established close to the source of nutrients, GCF, which further governs their growth. Salivary mucins may represent a similar mechanism for early colonizing plaque bacteria that could not only bind to mucin in the pellicle but also use it as nutrient (Scannapieco et al. 1993,

Wickström & Svensäter 2008). Furthermore, pellicle-bound amylase is a receptor for *S. gordonii* adherence and bind to the bacterial surface with retained enzymatic activity, promoting growth by providing nutrients from degraded starch (Scannapieco et al 1993).

Exposed root surfaces at recessions

Our finding of higher total bacterial counts on the exposed root surfaces than on the directly adjacent tooth surfaces (Study IV, Table 4) may be due to the fact that dentin has a higher surface roughness than enamel (Yurdaguven et al. 2012) and that higher numbers of bacteria adhere to rougher surfaces in the oral cavity (Quirynen et al. 1990, Arakawa et al. 2009). The finding of *Actinomyces* spp. in higher numbers and more frequently on the exposed root surface than on the enamel surfaces is in line with a previous report (Haffajee et al. 2008). It also corroborates our own findings of positive, albeit not statistically significant, correlations between *Actinomyces* spp. and IgG and albumin on gingival tooth surfaces in experimental gingivitis (Study II, Table 6). This was further paralleled by higher amounts of plasma proteins in the pellicle on exposed root surfaces (Study IV, Table 3). Though not as highly associated with root caries lesions as *S. mutans* and lactobacilli, *Actinomyces* spp. were found in root caries lesions (Fure et al. 1987, Emilson et al. 1993, Preza et al. 2008). The number of streptococci found on the exposed root surfaces was basically not different from the adjacent enamel surfaces (Study IV, Table 4). It may be that the positive correlation between the finding of streptococci in the subgingival samples and on the root surfaces in combination with high counts of these bacteria and *Actinomyces* spp. in early dental plaque on root surfaces, explain the frequent and, in the long run, practically inevitable development of root caries in periodontitis patients (Ravald et al. 1981, Reikler et al. 1999, Fadel et al. 2012).
A direct comparison of Studies II, III and IV reveals that the detection frequencies and amounts of fibrinogen and fibronectin in gingival pellicles within each study are higher with higher GCF flow (Table IV). When exposed root surfaces were presumed or known to be present (Study III) their findings were lower. This is in line with our hypothesis of a decisive impact of the GCF flow on the pellicle formation and supports the notion that the exposed root surface could influence pellicle formation on the enamel, which lead to Study IV. The difference of fibrinogen and fibronectin in the gingival pellicle on the non-treated and surgically treated teeth in Study IV may further suggest that a higher GCF flow could compensate for an extended exposed root surface.

Table IV. Comparison of the GCF flow and fibrinogen and fibronectin in gingival pellicles in Study II, III and IV (mean±SD).

	Study II		Study III		Study IV	
	healthy	inflamed	before	after	surgically	non-
	healthy subjects		surgery		treated teeth	
GCF µl/10s	0.046±0.036*	0.081±0.046	0.115±0.025*	0.043±0.023	0.045 ± 0.021*	0.029 ± 0.0097
Fibrinogen [a]	75.5	84.4	54.5	54.5	72.7	45.5
Fibronectin [a]	65.6	81.3	63.6	54.5	63.6	54.5
Fibrinogen [b]	3891 ± 2454	4253 ± 1995	733 ± 1022	344 ± 452	1217 ±1495	732 ± 1260
	1.1		2.1		1.7	
Fibronectin [b]	3061 ± 2550	3688 ± 2142	787 ± 891	484 ± 559	2469 ± 3655	1061 ± 1464
	1.2		1.6		2.3	

[a] detection frequency %
[b] pixel values (mean ± SD) and relative amount of proteins (based on pixel values) in pellicles formed at gingival margins with relatively high and low GCF flow, respectively
*$p<0.05$ for the differences within the respective study

Biofilms on upper and lower lingual surfaces

In addition to the analysis reported in Study II, biofilms on lingual surfaces were further investigated (Table V). Despite a GCF flow and an amount of pellicle protein of the same magnitude on upper and lower lingual surfaces, the plaque formation rate was higher on the lower lingual surfaces compared with the upper ones during healthy conditions. At inflamed margins, the GCF flow and pellicle proteins increased moderately in the upper jaw and were markedly increased in the lower jaw but the plaque formation rate increased more in the upper than in the lower one.

Table V. Biofilms on upper and lower lingual surfaces (mean ± SD)

	Healthy		Inflamed	
	Upper	Lower	Upper	Lower
Gingival crevicular fluid [a]	0.037 ± 0.043	0.036 ± 0.029	0.046 ± 0.039	0.093 ± 0.074
Silver-stained gel [b]	7216.6 ± 1289.5	7942.4 ± 1176.5	7259.0 ± 2305.5	8911.0 ± 1176.1
Plaque formation rate [c]; exp. teeth	4.3 ± 6.5	17.4 ± 20.7	16.9 ± 11.5	25.6 ± 17.3
Log(TVC) [d]	2.31 ± 1.1	2.11 ± 0.89	1.96 ± 0.64	2.46 ± 1.38

[a] µl/10s
[b] pixel values
[c] % surfaces covered by plaque
[d] log (TVC) = total viable counts logarithmically transferred

Plaque forms more rapidly on mandibular than on maxillary lingual surfaces (Axelsson 1991, Furuichi et al. 1992). This was also observed in Study II and especially under healthy conditions. More rapid plaque formation (24-h plaque) did not appear to result from higher GCF and protein concentrations and was not always preceded by higher bacterial counts in the four-hour dental plaque. It is known that salivary clearance is relatively high on lower lingual surfaces (Lecomte & Dawes 1987, Macpherson & Dawes 1994). Plaque formation might therefore possibly be favoured on lower lingual tooth surfaces by higher salivary flow providing a higher nutrient supply for bacterial growth. Furthermore, the tongue may not influence plaque formation in the lower jaw. These local factors, cannot explain the changes observed during gingival inflammation. The plaque formation rate on upper lingual tooth surfaces increased four-fold, while it only grew by 50% on lower, lingual tooth surfaces. It may be that an initially low plaque formation rate could permit a higher increase than an initially higher formation rate.

Clinical variables

In Study II, it was not possible to diagnose inflammation by inspection alone. The GCF flow has previously been shown to increase within a few days after the cessation of oral hygiene (Kunimatsu et al. 1995) and gingival inflammation coincided with increased plaque formation in a number of clinical investigations (Hillam & Hull 1977, Brecx et al. 1980, Quirynen et al. 1991, Ramberg et al. 1994, Daly & Highfield 1996). We therefore used the GCF flow and the plaque formation rate as markers for the fact that gingivitis had developed (Study II, Table 5). Both the GCF flow (Table IV) and the plaque formation rate (25±11 to 39±10% tooth surfaces covered by plaque after 24 h without hygiene; $p < 0.05$) increased during inflammation. In Study II, bleeding on probing never exceeded 5%. These observations indicate that, after five days without oral hygiene, pellicle had formed in a state of increased GCF flow but was unaffected by bleeding. Also, marginal bleeding had to be avoided since it might have interfered with pellicle formation and our intention to investigate only GCF-derived plasma components in the pellicle.

In periodontitis patients (Study III), the GCF flow was highest before surgery in the presence of periodontal pockets (Table IV). It decreased ($p<0.05$) after surgical pocket elimination. This observation was paralleled by a lower ($p<0.05$) post-operative plaque formation rate on these teeth (pre-operative 40±28% *vs* post-operative 27±20% tooth surfaces covered by plaque after 24 h without hygiene). In cases of experimental gingivitis, a slight increase was observed

for the GCF flow and the plaque formation rate (29±19%). In Study IV, the GCF flow was higher ($p < 0.05$) at surgically treated than at non-treated teeth (Table IV).
Plaque scores as registered on a single visit have a poor predictive value for future disease progression (Kaldahl et al. 1990). Consistently higher plaque scores during periodontal supportive therapy over several years were associated with a long-term deterioration of the periodontal status (Eickholz et al. 2008). Higher bacterial counts and increased plaque formation rate in the presence of periodontal pockets, as found in Study III, may be one reason for long term deterioration of sites constantly covered by plaque.

Consideration of methods

In previous studies on *in vivo* pellicle, samples were collected by scraping with a scaler (Sönju & Rölla 1973). At a later stage, a preceding step of chemical dissolution with $CaCl_2$ was introduced (Embery et al. 1986) in order better to desorb glycoproteins from the tooth surface (Rölla & Embery 1977). In our studies, as in a previous investigation (Carlén et al. 1998), the material was collected by dissolving and rubbing with fibre pellets soaked with 2% SDS, which may effectively desorb proteins from surfaces with various chemical characteristics (Källtorp et al. 2000). In addition, this method may better permit comparisons with the apatitic *in vitro* pellicle, which was dissolved in SDS-containing buffer. In a previous study, pellicles were collected well away from the gingival margin, to avoid contact with the soft tissue during the sampling procedure (Carlén et al. 1998). In the present studies, each tooth surface to be sampled was divided into an incisal and a gingival part. When taking the gingival sample and samples from the recessions, a safety distance to the gingival margin was kept to avoid contact with the soft tissue. Therefore, only exposed root surfaces at gingival recessions of ≥ 3 mm depth were selected for sampling (Study IV).

The pellicles and early dental plaque were sampled on buccal and lingual surfaces. Periodontal pockets are mainly located approximally. Therefore, it may be argued that sampling was not performed at the sites of main interest with regard to the aim of the studies. Anatomically, the periodontal sulcus can be regarded as one single circular opening and it is reasonable to anticipate that the GCF is by and large spread over the whole tooth surface following, e.g. capillary forces. From a practical point of view sampling from the approximal surface would not be possible in a closed tooth row. The risk for contamination with saliva or plaque from the surface of the neighbouring tooth would be high.

No template was used for sample collection. The differences seen between the gingival and incisal parts of the tooth surfaces might conceivably reflect differences in the size of the surface areas. Generally speaking, more protein was collected from the gingival surfaces than from the incisal surfaces, although the former were often estimated to have been somewhat smaller than the latter. Samples from the exposed root surfaces (Study IV) were taken from areas generally considered to be smaller than the respective adjacent gingival surfaces of the tooth crown. The fact that the same investigator (SR) took the samples ensured standardised sample collection. Possible small variations in surface area due to different tooth sizes and types were less critical when differences due to the state of gingival inflammation were examined, as samples from the same surfaces were compared. Staining intensity of gels and immoblots can vary. Therefore, samples to be compared were run on the same gel in all studies. Apart from the factors named above, variations between bacterial counts in the microbiological samples may depend on individual variations in plaque formation rate (Simonsson et al 1987). Small pellicle samples and minor amounts of protein in the samples did not allow the determination of total protein by available, commercial methods. Instead, the total amount of protein was estimated from image analysis of the silver-stained gels although it is known that silver staining does not reveal all proteins to the same extent and stains glycoproteins in particular only poorly (Jay et al. 1990). Based on pixel values, a relatively high level of protein, as revealed by silver staining, was accompanied by a concomitant higher reading in the protein specific immunoblot staining. This indicates that silver staining was a suitable marker for the total amount of protein in our material.

In order to test whether the finding of salivary proteins in smaller amounts and less frequently than plasma proteins could be due to the fact that the immunoblot system used was less sensitive to salivary components, detection limit analyses were performed. This may not be the only explanation, as the limits for IgA/IgG and amylase/fibrinogen respectively were of the same order. The concentrations of protein in plasma may be 10 to 1,000 times higher than those of proteins in saliva. A high plasma protein content, especially in the gingival pellicles, might therefore be expected.

It could be argued that leaking GCF was the source of plasma proteins found in our pellicle samples. A comparison of plasma protein concentrations in gingival pellicle samples and reported plasma protein concentrations in the GCF does not support this notion (Table VI). Although the albumin concentration is higher under inflamed conditions for both the pellicle

samples and the GCF, the difference between health and inflammation was much more pronounced in the pellicle samples. The fibronectin concentration increased during inflammation in our samples, although it was almost undetectable in GCF at inflamed sites. Fibrinogen was found in much higher concentrations in the pellicle samples than the other proteins, which could indicate an accumulation of fibrinogen on the tooth surface. As a result, the plasma proteins do not appear in the same quantities or in the same relative proportions in our samples as in the GCF.

Electrophoretic separation and subsequent immunoblotting were used for the analyses of pellicle proteins. This technique was used since they allowed the analyses of minor amounts of protein in small sample volumes. Even if today there are new, sensitive methods that may allow similar or even better analyses, the same method was used in all studies to facilitate the comparisons between the results obtained.

Table VI. Protein concentration (µg/ml) in the selected gingival pellicles (Study II & III) and in the GCF (values taken from the literature) at healthy and inflamed gingival margins.

	Pellicle samples (Study II)		Pellicle samples (Study III)		GCF (µg/ml; values from the literature)		
	Healthy	Inflamed	before surgery	after surgery	Healthy	Inflamed	References
IgG	5.8	14.9	12.8	7.4		15	Holmberg & Killander 1971
Albumin	1.4	9.3	14.8	2.0	16.8	23.1	Bickel et al. 1985
					23		Marcus et al. 1985
Fibronectin	0.022	0.25			0.106	0.0037	Lopatin et al. 1989
Fibrinogen	10.4	26.8			0.74		Talonpoika et al. 1993
Amylase	0.67	1.5	5.4	3.0			
IgA	-	1.3	11.1	8.0		1.6	Holmberg & Killander 1971
Lactoferrin	0.20	0.47			0.34		Adonogianaki et al. 1996

For the subgingival sampling in Study IV, the paper point method was used. Our aim was to analyse the part of the subgingival plaque that possibly could be flushed out of the pocket together with the GCF and influence the supragingival plaque formation. The curette method would even sample the part of the subgingival plaque firmly attached to the root surface and was therefore not regarded suitable, even though differences between these techniques mainly

concern a significantly higher TVC in samples harvested with curette than with paper points (Jervøe-Storm et al. 2007).

P. gingivalis, *A. actinomycetecomitans* and *T. forsythia*, associated primarily with the deep periodontal pocket, were included on generally and periodontally healthy individuals. They were included since sensitive PCR-techniques were used and it was reported that they may be present, although in low numbers, in supragingival plaque in healthy individuals and in other parts of the mouth, e.g. on the dorsum of the tongue, also in healthy individuals (Mager et al. 2003, Haffajee et al. 2008).

The participants in Study III were recruited at re-evaluation after basic therapy, when surgical elimination was deemed necessary to reduce infection and to arrest the progression of the disease. The treatment was planed according to the patients' individual needs and not part of the study. If an indication for pocket elimination is seen, surgery is planed for the whole dentition or at least per quadrant. Once the decision to perform surgery is taken all pockets with bleeding on probing will be surgically treated. It is not reasonable to plan a surgical intervention separately for each individual pocket. As a result some pockets with probing depth of less the 6 mm, a number, which sometimes as a rough clinical rule of thumb due to rare absence of bleeding on probing (Lang et al. 1986) is regarded as threshold for the indication of pocket elimination surgery, were included into Study III.

Ethical considerations

Ethical approval was granted for each of the four studies. A main ethical question in the current series of studies concerns the periods of experimental gingivitis where patients are asked to do something that may seem harmful to their oral health. The model used for experimental gingivitis is well established and has been used in many studies. Experimental gingival inflammation totally resolves within two weeks, as soon as regular oral hygiene is reinstituted (Löe et al. 1965). In periodontitis patients, longstanding gingival inflammation and bleeding on probing may increase the risk for recurrent disease (Lang et al. 1990) but short term experimental gingivitis in well-controlled periodontitis patients, who are thoroughly instructed to resume oral hygiene after the experimental period, is an accepted model in periodontal research (e.g. Johnsson et al. 1997, Zitzmann et al. 2005).

Another ethical dilemma usually not specifically addressed in applications for formal ethical approval concerns discontinuation of participation. This could jeopardize the completion of the study and, at it worst, make the efforts and sacrifices of those who fulfilled it worthless (Ludvigsson 2002). Performing clinical studies often involves asking participants to follow a research protocol, without any obvious benefit for their health. In the present studies, the treatment itself was not connected with the scientific question and, thus, the patient received therapy as was individually indicated. Participation in the study was outside their routine treatment. In these cases, it is usually advisable to invite the patients to participate and to introduce participation in a research project as something one must in principle either like or dislike (Polonsky & Waller 2010). The patient's answer to this question can identify those who in the end are willing to follow the study protocol and help avoid unmotivated patients who may later drop out.

Further perspectives - possible relevance for periodontal disease development

Several studies have shown that the quantity of plaque does not predict the inflammatory response of periodontal tissues (Jenkins et al. 1988, Lindhe et al. 1989, Baelum et al. 1997), and that the subgingival presence of periodontitis-associated bacteria is not always coincide with irreversible tissue destruction (White & Mayrand 1981, Van Oosten et al. 1988, Dahlén et al. 1989, McNabb et al. 1992, Papapanou et al. 1997, Mombelli et al. 1998, Van Winkelhoff et al. 1999, Macheleidt et al. 1999, Ximénez-Fyvie et al. 2000a). While the microbial composition of subgingival plaque at periodontally diseased sites has been extensively studied (Zambon 1996), supragingival plaque in periodontal disease has only recently become a topic of interest (Ximénez-Fyvie et al. 2000a & b, Haffajee et al. 2008, Teles et al. 2012). The composition of the supragingival plaque is different in the presence of periodontal disease (Ximénez-Fyvie et al. 2000a). Higher bacterial counts were seen in supragingival plaque at periodontal inflammation (Dahan et al. 2004, Rowsahni et al. 2004, Study III). Further on, a shift from Gram-positive, facultative anaerobic, saccharolytic bacterial species to Gram-negative, anaerobic, proteolytic species has been noted, which may start a vicious circle. The main nutrients of the Gram-positive bacteria (glycosylated salivary proteins) are displaced by the proteins from the GCF. As a result of the decomposition of GCF proteins by Gram-negative bacteria, the pH and ammonia levels raise. Thus, the supragingival plaque may play a previously underestimated role in the development of periodontal disease. It seems to be crucial to reduce the GCF flow to prevent the supply of

receptor molecules and nutrition for the bacteria. At the same time, it appears to be difficult to reduce the GCF in periodontitis to the levels of periodontal health.

Our observations indicated that increased GCF in periodontitis patients results in higher amounts of plasma proteins in the dental pellicle. It can further be speculated that variations in GCF flow and pellicle formation may characterise or rather induce periods of active disease progression. To what extent GCF flow and pellicle formation may be connected with individual susceptibility to periodontal disease in unclear. These questions merit further studies on pellicle and early dental plaque formation in a direct comparison of periodontal healthy and diseased individuals.

It has frequently been stated in the literature that the complex nature of the oral environment may not be adequately modelled by in *in vitro* systems (Tanzer et al. 2003, Hannig 2007). *In vivo* studies involving a sufficiently large number of patients for correlations analysis between pellicle proteins and bacteria in early plaque may complement experimental studies on bacterial adhesion and acquisition on the tooth surface. Studies on bacterial acquisition on exposed root surfaces in periodontitis patients may reveal further details on the impact of early plaque on the periodontal status of the gingival margin.

In our laboratory, pellicle proteins have for many years been analysed by electrophoretic separation and subsequent immunoblotting (Carlén et al. 1998). Recently, methods have been developed aiming at a complete simultaneous analysis of the proteome (Kraj & Silberring 2008). This method has been applied for pellicle (Siqueira at al. 2007) and GCF (Carneiro et al. 2012) samples. 130 different proteins, including albumin, IgG and amylase, were found in the pellicle, 89 of which were repeatedly seen in different subjects. In the GCF, 199 proteins were identified, mostly (57%) plasma proteins, but even intracellular proteins and enzymes were detected. Studies revealing the whole range of proteins incorporated into dental pellicles may further clarify the early steps of dental biofilm formation in health and disease and indiate new markers or predictors for disease.

In this series of studies, it was not possible to identify all relevant *Streptococcus* and *Actinomyces* species. Further studies are necessary to reveal whether the same species were present in all samples.

Clinical implications

Our data suggest that bacterial adhesion and plaque formation increase in the presence of periodontal inflammation. These findings may be of interest in the discussion on treatment

decisions. Even a slight subclinical periodontal inflammation can significantly increase plaque formation and thereby possibly initiate a viscous circle of disease progression. The question when to eliminate periodontal pockets surgically may be re-considered. Moderately deep periodontal pockets with practically healthy gingival margins in cooperative patients may, in an everyday clinical situation, lead to treatment choice dilemmas: either surgical pocket elimination or re-depuration and watchful waiting are the options.

The formation of dental pellicle and early dental plaque vary between teeth in the individual oral cavity due to differences in gingival recessions, history of surgical periodontal treatment and the subgingival microflora. Our findings are consistent with the established view in periodontal supportive therapy, that diseased teeth in a diseased individual may need more attention during maintenance than healthy teeth in the same individual.

Concluding Remarks

i) Plasma proteins are abundant in the dental pellicle. They are found in experimental pellicles *in vitro* on hydroxyapatite and in *in vivo* pellicles. They are identified in higher amounts in pellicles close to the gingivae compared with the incisal part of the tooth surface.

ii) Bacteria associated with periodontal disease were shown to adhere to plasma components in the *in vitro* pellicle.

iii) Proteins and microorganisms in dental biofilms

- During experimental gingivitis, the amount of plasma protein and the bacterial counts may increase on the tooth surface. The proportion of bacteria associated with periodontal disease is increased and they are found more often on the tooth surface in close proximity to their natural habitat, the gingival crevice, than on the incisal part of the tooth surface. Moreover, an increase in the plaque formation rate is observed.
- In the presence of periodontal pockets, the amount of plasma proteins, the bacterial counts and the prevalence of bacteria associated with periodontal disease may be higher than after surgical pocket elimination. Again, a higher plaque formation rate is observed.
- On exposed root surfaces at gingival recessions, the quantity of plasma proteins and the bacterial counts were higher than on the adjacent enamel surface on the other side of the cemento-enamel junction. Higher proportion and higher counts of *Actinomyces* spp. were observed.

On the basis of our observations, we suggest that the bacterial composition of early dental plaque may be governed by the presence of plasma proteins in the pellicle and the presence of exposed root surfaces.

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Acknowledgments

I would like to express my sincere thanks to all those who have helped me throughout my research with their advice, support and friendship:

Associate Professor Anette Carlén, my scientific supervisor, for her guidance, enthusiasm, never-ending patience and support throughout all my years as a research student,

Professor Gunnar Dahlén, my scientific co-supervisor, for his constant support and encouragement,

Professor Jan Olsson, my scientific co-supervisor, for his enthusiastic initiative to the project and valuable discussions,

Professor Jukka M. Meurman and Kristi Kari for open and constructive co-operation,

Ingela Loggner, Gunilla Hjort and Susanne Blomqvist for introducing me to laboratory techniques and help with the laboratory analysis and

Mauro Donati and Maj-Louise Fridh for doing the plaque registrations.

I am indebted to Dr Malamud, University of Philadelphia, USA, and Dr Bennick, University of Toronto, Canada, for providing antibodies to salivary agglutinin and PRP respectively.

The present studies were supported by the Swedish Medical Research Council (project no. 8697), Patentmedelsfonden för Odontologisk Profylaxforskning, the Colgate Research Foundation, the Lars Hiertas Minne foundation, the German Research Council (DFG Ru 754/1-1) and the County of Skåne (obj.nr. 697).

Ein besonderer Dank gilt meiner lieben Familie für alle Geduld und Unterstützung. Karin, Elias und Isak hielten während meiner zahlreichen Aufenthalte in Göteborg in Malmö die Stellung.