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DISSERTATION

Prevalence analysis of putative periodontal pathogens in patients with aggressive periodontitis and healthy elderly. A molecular study

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Exzerpt

Marginale Parodontitis, die multikausale Erkrankung des Parodonts ist in erster Linie eine Infektionskrankheit, modifiziert durch Wirtsfaktoren und äußere Einflüße. Die als pathogene Mischflora bezeichnete Kombination kommensaler Mikroorganismen, die opportunistische Infektionen und damit Immunreaktionen auslösen können, spielt die primäre Rolle in der Ätiopathogenese der Parodontitis. In der Aufstellung des Studienziels wurden einzelne, vermutlich pathogene Bakterienarten (*Tannerella forsythensis*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*, *Fusobacterium nucleatum*, *Fusobacterium* spp., *Prevotella intermedia*, *Eikenella corrodens*, *Veillonella parvula* und *Capnocytophaga ochracea*) auf Grund der Evidenz von früheren Studien ausgewählt, die eventuell als "Markerkeime" in der aggressiven Form der Parodontitis betrachtet werden können. Dazu wurde eine Kontrollgruppe (die Senioren) untersucht, die eine gesunde parodontale Flora besitzen. Die angewandte Nachweismethode basiert auf eubakterieller PCR-Amplifikation von 16S rDNA und darauffolgender dot-blot Hybridisierung mit spezifischen Oligonukleotidsonden. Die entsprechenden Sonden wurden hergestellt und evaluiert. Die Optimierung der Hybridisierungsbedingungen einzelner Sonden folgte unter Einsatz von Positiv- und Negativkontrollen - PCR-Produkte von 42 gezüchteten Ziel- bzw. phylogenetisch nahliegenden Bakterienstämmen. Für die epidemiologische Untersuchung wurde subgingivale Plaque von vier Parodontaltaschen und einer Kontrollstelle von 45 Patienten mit aggressiver Parodontitis, sowie an fünf Stellen von 21 Senioren entnommen. Die Prävalenz der einzelnen Bakterienarten wurde mit Hilfe des Chi-quadrat-Tests verglichen. Die Ergebnisse dieser Studie bewiesen die erfolgreiche Einsetzbarkeit der hergestellten Oligonukleotidsonden. Es konnte gezeigt werden, daß alle untersuchten Bakterien in beiden Gruppen vorkommen. Obgleich eine hohe interindividuelle Variabilität der Kolonisationsmuster zu beobachten war, konnten *T. forsythensis*, *P. gingivalis* und *F. nucleatum* sehr häufig in den Parodontaltaschen nachgewiesen werden. Obwohl diese Arten auch an den gesunden Stellen der parodontal Erkrankten sowie der Senioren festzustellen waren, blieb die Häufigkeit dieser Besiedlung signifikant seltener.

A. actinomycetemcomitans konnte nur bei einzelnen Patienten mit aggressiver Parodontitis festgestellt werden. Die Ergebnisse für *P. intermedia* und *E. corrodens* ließen keine eindeutige Assoziation sowohl mit der aggressiven Parodontitis als auch mit dem gesunden Parodontalzustand zu. Bei Senioren wurde *C. ochracea* besonders häufig nachgewiesen. Zusammenfassend kann man die vermutlichen Parodontalpathogene wie *T. forsythensis*, *P. gingivalis*, *F. nucleatum* und *C. rectus* als Leitkeime aggressiver Parodontitis ansehen. Bezüglich der polymikrobiellen Natur der Parodontitis würde eine umfassende Untersuchung der oralen Mikroflora und deren Zusammenspiel mit den Wirtsfaktoren zur Aufklärung der Ätiopathogenese der Parodontitis eher beitragen als der Nachweis einzelner Arten.

Schlagwörter:

Parodontalpathogene

aggressive Parodontitis

Oligonukleotidsonden

Dot-blot Hybridisierung

PCR-Amplifikation

Abstract

A multifactorial risk pattern of periodontitis has been recognized, where in addition to host and environmental factors a pathogenic microbiota plays a primary role. At present no definite answer can be given to the question of whether the expression of either aggressive etiological agents (implying infection with a virulent microbiota), or a high level of individual susceptibility to periodontal disease, or a specific combination of both is the conductive factor in the etiopathogenesis of aggressive periodontitis. The purpose of the current research was to analyze the prevalence of periodontitis-associated microorganisms in patients with aggressive periodontitis and periodontally healthy elders by using molecular-biologic detection methods like eubacterial PCR-amplification of 16S rDNA in combination with dot-blot hybridization. The oligonucleotide probes for the detection of *Tannerella forsythensis*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*, *Fusobacterium nucleatum*, *Fusobacterium* spp., *Prevotella intermedia*, *Eikenella corrodens*, *Veillonella parvula* and *Capnocytophaga ochracea* were designed and evaluated. The PCR products of 42 cultivated target and closely related bacteria were obtained for the optimization of hybridization conditions. For the epidemiological study subgingival plaque was sampled from four pockets and one healthy site of 45 aggressive periodontitis patients as well as from five sites of 21 elderly. The differences in the prevalence of bacterial species was analyzed by chi-square test. The results of the study confirmed the reliability of the oligonucleotide probes in a specific and sensitive detection of the respective oral species. The data of the epidemiological study revealed frequent colonization by *T. forsythensis*, *P. gingivalis*, *F. nucleatum* and *C. rectus* in patients with aggressive periodontitis, however individual variations were obvious. These microorganisms could be predominantly identified in periodontal pockets, but were significantly less common in the healthy sites of the periodontitis patients and in the elderly subjects. *A. actinomycetemcomitans* could be detected in only a few patients, reducing its suspected importance in the etiopathogenesis of aggressive periodontitis. No direct association for *P. intermedia* and *E. corrodens* with aggressive periodontitis or periodontal health could be seen. *C. ochracea* was highly prevalent in the well-maintained elderly, being rarely found in the diseased group. The putative pathogens *T. forsythensis*, *P. gingivalis*, *F. nucleatum* and *C. rectus* can be conclusively suggested as the key-bacteria in patients with aggressive periodontitis. However, considering that periodontitis is a polymicrobial infection, the screening of the microbial population, rather than the isolation of single members of the subgingival flora, should give a more comprehensive perspective in etiopathogenetic research of periodontitis.

periodontal pathogens

16S rRNA

oligonucleotide probes

dot-blot hybridization

aggressive periodontitis

PCR-amplification

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1 Introduction

The most common diseases of the periodontal tissues are inflammatory processes of the gingiva and attachment apparatus of the tooth. The periodontal diseases are polymicrobial infections associated with local accumulation of dental plaque, a subgingival pathogenic periodontal flora, and calculus.

Periodontitis is an important global public health problem which involves mostly the adult population over 35-40 years of age. It begins with gingivitis and without therapy leads to a progressing destructive periodontitis. The variance and severity of this disease is influenced up to 90% by age and oral hygiene (1, 2). However, in only about 10% of the population severe forms of periodontitis occur where no correlation exists between the supragingival plaque index and the severity of the disease. The proportion of these patients increases with age and reaches the highest prevalence at the age of 40-50 years (3-5).

The aggressive periodontitis, a rare form of periodontitis, which often begins in childhood, is characterized by severe, rapidly progressing tissue destruction. The prevalence of juvenile periodontitis in 13-20 year olds is 0.1-0.5% (6-8).

1.1 Classification of periodontal diseases

According to the new classification, introduced in 1999 by the World Workshop of Periodontics (9), the following forms of periodontal diseases exist:

- Gingival diseases
- Chronic periodontitis (localized, generalized)
- Aggressive periodontitis (localized, generalized
- Periodontitis as a manifestation of systemic diseases
- Periodontitis associated with hematological disorders
- Periodontitis associated with genetic disorders
- Not otherwise specified (NOS)
- Necrotizing ulcerative gingivitis (NUG)
- Necrotizing ulcerative periodontitis (NUP)
- Abscesses of the periodontium
  - Gingival abscess
  - Periodontal abscess
  - Pericoronal abscess
- Periodontitis associated with endodontic lesions
- Developmental or acquired deformities and conditions

The recent classification has united a broad panel of severe forms of periodontitis that are independent of age into a group of "aggressive periodontitis". In earlier literature this clinical

entity is referred to as "severe periodontitis in young adults" (10), "advanced destructive" (11), "postjuvenile" (12), "early-onset" (13, 14) and "rapidly progressive periodontitis (RPP)" (15).

The present study included patients with aggressive periodontitis, who according to the classification from 1989 belonged to a group of "early-onset periodontitis (EOP)" (16). The term was used as a collective designation for a group of dissimilar destructive periodontal diseases that affected young patients, i.e. prepubertal, juvenile and RPP.

1.2   Bacterial etiology of periodontal diseases

From earlier studies there is evidence for the primary role of bacteria in the etiology of destructive periodontal diseases (18-21).

It has been previously estimated that about 500 bacterial species colonize the human oral cavity (17-19). The majority of these organisms are commensals and live in complex communities forming oral biofilms on tooth surfaces. In general these microorganisms live in harmony with a host, but under certain circumstances this dynamic interaction may lead to opportunistic infections resulting in breakdown of periodontium. The evidence for the infectious nature of periodontal disease comes from several sources, including:

- Studies which correlate most forms of gingivitis and periodontitis with accumulated dental plaque.
- Treatment studies which demonstrate that elimination of plaque microorganisms can be correlated with clinical improvement.
- In vivo and in vitro studies demonstrating the relative virulence of different plaque bacteria.

It has been observed that clinically most periodontal sites in most subjects do not appear to be undergoing breakdown at any given time, even though they are continuously colonized by varying numbers and species of bacteria. This suggests that there are remarkably effective host defense mechanisms to be overcome and that only specialized uniquely talented bacterial species (with multiple virulence factors) may have the requisite set of properties to cause tissue damage. Thus, bacteria are important in the etiology of disease, but the outcome, protection or tissue damage, is affected by the nature and level of the immune response (22).

While the infectious etiology of periodontal diseases is generally accepted, there is an ongoing discussion as to the relative importance of individual bacterial species within dental plaque. This is reflected in the distinction between the non-specific and the specific plaque hypothesis.

The non-specific plaque hypothesis implicates the mere quantity of dental plaque, with any species having the possibility of causing disease. The bacterial mass causes a periodontal

disease when it accumulates to the point of exceeding host-defense mechanisms. Variability was recognized, but the true extent of differences in bacterial composition was not acknowledged. This hypothesis is supported by studies of experimental gingivitis, e.g. by Löe et al. (23) showing that the cessation of oral hygiene is associated with the development of gingivitis. Resumption of oral hygiene resolved the gingivitis.

Until as recently as the early 1970s, it was thought that any organism present in subgingival plaque contributed to periodontal destruction (23, 24).

Studies carried out over the last 20 years questioned this hypothesis and contrasted it with the specific plaque hypothesis (25). It proposes that dental plaque isolated from periodontitis lesions is qualitatively distinct from that isolated from healthy sites. It assumes that clinically different forms of periodontitis are associated with distinct species and colonization patterns. For example, the composition of subgingival plaque from lesions of juvenile periodontitis is markedly different from that found in patients with adult periodontitis (26). However, the diversity of bacterial complexes, as well as the variation in host response to bacterial species are some of the major reasons that the specific etiology of periodontal disease has not been definitely established (27, 28).

Originally the concept of "pathogen" was devised for specific microorganisms which caused specific diseases. This cannot be applied to periodontal diseases. At present, it appears unlikely that one single species is a sole agent of periodontal destruction, since no single species occurs as an important part of the flora in all cases of gingivitis or periodontitis (17). The more suitable term "pathogenic microbiota" acknowledges the fact that periodontal diseases have a polymicrobial etiology, and that a multitude of defense systems have to be mobilized by the host against these infections. However, the designation "periodontal pathogen" can be applied to those bacteria which have specific mechanisms to perturb the host defense and in that way cause an accelerated destruction of periodontal tissues. Such a pathogen will never work in isolation, it is always a member of a complex bacterial community.

A set of criteria, derived from Koch's postulates of a monoinfection, has been developed to identify these specific microorganisms in a mixed microflora and apply to an opportunistic infection (29). These criteria are as follows:

- The presence of high numbers of putative periodontal pathogens in periodontal lesions compared to either their absence or presence in low numbers in healthy or non-progressing sites
- Elimination of the microorganisms from periodontal lesions should result in clinical improvement
- Induction of active immune responses in the host
- Presence of virulence factors

- Appropriate animal models demonstrating tissue destruction in the presence of the microorganisms.

Periodontal research has attempted to define periodontopathic bacteria that induce periodontitis (20, 30-33). Relatively few, about 10-20 species, may play a causal role in the pathogenesis of destructive periodontal diseases. However, it is assumed that about 50 percent of the total oral bacterial flora is still unknown. An attempt made by Paster et al. (19) to investigate the bacterial diversity in subgingival plaque using culture-independent molecular methods revealed 215 novel phylotypes. It is likely that yet-undiscovered bacteria may play a role in the etiology of the disease.

1.2.1 Healthy flora

The dental plaque associated with periodontal health is characterized by predominantly gram-positive facultative coccoid microorganisms, such as Streptococcus and Actinomyces species (17, 18). The presence of gram-negative cocci, rods and filaments is a frequent observation; however, they are in much lower proportions as compared to gram positive flora (30, 34-38). Similar species are associated with healthy subjects in cross-sectional studies, and as "beneficial" species in inactive periodontal pockets (39).

1.2.2 Gingivitis

Without oral hygiene the dental plaque grows in thickness forming a distinct organized structure (40, 41). Subsequent mineralization gives rise to calculus. The undisturbed growth of supragingival plaque results within few days in soft tissue alterations in the adjacent gingiva. It has been hypothesized that the transition between health and gingivitis is due to an overgrowth of gram-positive species (42, 43). However, other investigators have found an increased proportion of gram-negative species as the major determinant (40, 44). In fact, the inflammatory conditions provide a relatively anaerobic environment which favors the colonization by anaerobic motile rods and spirochetes. The species associated with experimental gingivitis include Actinomyces, Streptococcus, Veillonella, *Fusobacterium* and Treponema species. Additionally *Prevotella intermedia* and Campylobacter species have been cultivated from the plaque of chronic gingivitis (23, 40, 42).

1.2.3 Periodontitis

Subgingival plaque develops by apical progression of supragingival plaque. Alterations in the integrity of the junctional epithelium allow a gradual colonization of the tooth surface leading to the formation of periodontal pocket. The culture-based studies indicate that anaerobic microbiota predominate (10, 17, 31, 45). Although the bacterial composition changes, there is still no direct evidence to conclude which bacterial species initiate the first step of pocket development.

Scanning immunoelectron microscopy showed that rods, filaments, and spirochete-shaped

bacteria formed small aggregates at the bottom of the periodontal pockets in the so-called "plaque-free zone" (46). Investigations with fluorescence in-situ hybridization assay which analyzed artificial carriers from deep periodontal pockets found that gram-negative rods and treponemes dominate in the deepest part of the pocket, forming a confluent biofilm (47). These findings suggest that bacteria in the "plaque-free zone" may be critical periodontopathogens in the frontier area of apical plaque.

Studies of the predominant subgingival flora in periodontitis lesions have revealed great microbial diversity. Different forms of periodontitis show variations in the microorganisms colonizing periodontal pockets (14, 31, 45, 48). For instance, patients with juvenile periodontitis have shown infection with *A. actinomycetemcomitans* (26, 49, 50). The frequently detected bacterial species in periodontal lesions in cases of chronic and aggressive periodontitis are listed in Table 1 (51).

Table 1. Bacterial species associated with chronic and aggressive periodontitis.

Chronic periodontitis	Aggressive periodontitis
***Treponema* spp.**	***Porphyromonas gingivalis***
Prevotella intermedia	***Tannerella forsythensis***
Porphyromonas gingivalis	***Actinobacillus actinomycetemcomitans***
Tannerella forsythensis	***Eikenella corrodens***
Peptostreptococcus micros	***Campylobacter rectus***
Campylobacter rectus	
Actinobacillus actinomycetemcomitans	
Eikenella corrodens	
***Fusobacterium* spp.**	
***Selenomonas* spp.**	
***Eubacterium* spp.**	

Although there are strong indications that certain species are likely etiological agents of periodontal diseases in distinct subject groups, it is still difficult to determine which of the likely candidates is the critical organism (or a set of bacteria) in a given subject. Recently Mombelli et al. (52) after reviewing cross-sectional and longitudinal studies by stringent criteria questioned the strict distinction of specific microbiota in patients with aggressive and chronic periodontitis. The presence or absence of *A. actinomycetemcomitans*, *P. gingivalis*,

P. intermedia, *T. forsythensis* and *C. rectus* could not discriminate between subjects with different clinical disease entity.

It is apparent that the mere presence of one or more pathogenic species in a subject is insufficient to produce significant deterioration in the periodontal status of the individual. Multiple other factors, such as host and environmental factors, as well as local bacterial interactions that can modulate the virulence of bacterial species contribute to the clinical outcome.

1.2.4 Bacterial consortia

The bacteria in subgingival plaque form an ecosystem where complex relationships exist between population members as well as between bacteria and the host. The microorganisms tend to form coaggregations where bacterial interactions play an important role in species survival (53, 54). So-called "beneficial" species can turn these interactions beneficial to the host by affecting disease progression in a number of ways:

- by "passively" occupying a niche which might otherwise be colonized by a pathogen
- by actively limiting a pathogen's ability to adhere to appropriate tissue surfaces
- by adversely affecting the vitality or growth of a pathogen
- by affecting the ability of a pathogen to produce virulence factors
- by degrading virulence factors

It has been observed that high levels of both *P. gingivalis* and *C. ochracea* diminish the risk of new attachment loss (27). In contrast, in subjects with high levels of *P. gingivalis*, but low levels of *C. ochracea* there was a tendency for disease progression. It has been shown that subjects who received an adjunctive antibiotic therapy showed a higher percentage of sites with attachment level gain and higher levels of the suspected beneficial species like *C. ochracea* and S. sanguis II post-therapy than subjects who did not get antibiotics (55). Therefore, the aim of the therapy must not be exclusively the reduction or elimination of pathogens, but also supporting colonization shift to high levels of beneficial species.
The differences in subgingival microbial constellations between patients with similar clinical signs as well as spatio-temporal variations have been acknowledged (45).
As yet it is not clear whether the individual differences in flora composition are controlled primarily by genetic disposition of the host or by environmental influences. Moore et al. found that the periodontal flora of twin children were significantly more similar than those of unrelated children of the same age, indicating that genetics as well as environment influence the composition of the flora (56). Once the oral microbiota is established in an individual, it may be more difficult to introduce new species or clonal types (57).

1.2.5 Biofilm

When supragingival hygiene is not maintained, dental plaque immediately develops by a

dynamic intraplaque interaction, which climaxes with the establishment of a well-structured community of microflora - a biofilm (58, 59). The formation of biofilm begins with attachment of planctonic cells (mostly gram-positive cocci) on the pellicle of the tooth surface. Auto-aggregation with each other or coaggregation with other planctonic cells or neighbors begins. Gradually the microenvironment of the inner community changes from aerobic/capnophilic to facultative anaerobic and to anaerobic. The community is re-organized, new ecological niches are involved by spirochetes and motile organisms.

The biological characteristics and growth rate of bacteria in a biofilm are different from their planctonic counterparts, and thus the gene expression of virulence factors may be different according to their living conditions (60). The biofilm mode of growth seems to be advantageous for microorganisms. These three-dimensional structured communities contain fluid channels for transport of substrates, waste products and signal molecules (59). Glycogalyx, the polymeric substances that make up the matrix of a biofilm, retard the diffusion of antibiotics and host-driven antimicrobial factors (58, 61). Thus, biofilms are more resistant to immune defense mechanisms, less susceptible to antibiotic therapy and even not easily controlled by mechanical means (62).

1.2.6 Virulence factors

The expression of virulence factors may be an important indicator of the potential of a species to contribute to disease progression; however, the virulence traits of individual species in vitro might bear little resemblance to their behavior in a microbial community and, indeed, in vivo (63). It is unlikely that a single virulence factor will be responsible for tissue damage. Often a series of virulence factors is expressed under coordinate regulation. Very often the pathogen's environment appears to regulate the expression of these virulence factors (53, 64). Environmental factors such as temperature, osmolarity, iron and Mg levels have been shown to affect the expression (63, 65, 66). Interbacterial relationships play an important role in species survival. Some relationships are favorable and others are antagonistic. The interspecies aggregation can enable attachment of some species. Co-aggregation has been observed between *P. gingivalis* and *F. nucleatum*, *C. ochracea* and *S. sanguis*, *P. gingivalis* and *A. viscosus*, *F. nucleatum* and *S. sanguis* (67-69). Antagonistic substances may prevent aggregation or even kill other bacteria. For instance *A. actinomycetemcomitans* produces bacteriocin which inhibits the growth of *S. sanguis*. *S. sanguis* on the other hand produces H2O2 which kills *A. actinomycetemcomitans* (70). Inhibitory microbiota may help in preventing an infection by oral pathogens.

In order to colonize subgingival sites, a species must be able to attach to available surfaces, multiply, compete against other species in this habitat, and defend itself against host defense mechanisms (53). Bacteria attach to specific receptors on the host cell or tooth pellicle by specific adhesion molecules (71). Fimbriae and other cell-associated proteins have been

identified as adhesins in several subgingival species. Attachment of *P. gingivalis* to epithelial cells, gram-positive bacteria, basement membrane, and type I and IV collagen has been demonstrated (72-76). By using scanning immunoelectron microscopy it could be demonstrated that *P. gingivalis* participates in biofilm formation in the most apical part of a pocket, in so-called "plaque-free zone" probably using its attachment ability (46). Adhesins of *E. corrodens* and *A. actinomycetemcomitans* enable these species to attach to the epithelial cells (53, 77, 78, 88). It has been shown that strains of *F. nucleatum* adhere to red blood cells, basement membrane, and type IV collagen (53, 72, 73). T. denticola adheres well to fibroblasts, fibronectin, basement membrane, as well as type I and IV collagen (79). Adherence to host cells might be the prerequisite for further invasion of deeper tissues (76, 78, 80).

Earlier the term "invasion" was taken to mean intercellular penetration, i.e. bacteria locating between the host cells. The introduction of optical sectioning by confocal scanning laser microscopy (CSLM) enabled a three-dimensional localization of the bacteria. An intracellular location of *A. actinomycetemcomitans* and *P. gingivalis* within buccal epithelial cells of healthy subjects was observed using this technique (81). The authors suggested that intracellular location may be a common ecological niche for these bacterial species in both health and disease. Intracellular invasion has also been documented for *Prevotella intermedia* (82), *Fusobacterium nucleatum* (83) and *Tannerella forsythensis* (84). However, this property is not universal, e.g. *Treponema denticola* does not invade epithelial cells (85). The closer proximity to host targets allows destructive bacterial products to cause greater havoc upon the structural integrity of the periodontal tissues (86).

Some of the suspected pathogens produce an unusually wide spectrum of proteases including those which degrade collagen (like *P. gingivalis*. *T. denticola* and *A. actinomycetemcomitans*) (73, 76, 79, 87-91), and fibronectin (like *P. gingivalis* and *P. intermedia*) (76, 91, 92). Trypsin-like activity has been demonstrated for *P. gingivalis*, *T. forsythensis*, *T. denticola* and *Capnocytophaga* spp. (79, 93-95). Also the metabolic end-products (such as volatile sulfur compounds, NH3, fatty-acids and indole), produced by *P. gingivalis* and some by *F. nucleatum*, adversely affect mammalian cells (96, 97).

It has been shown that lipopolysaccharide (LPS), the so-called endotoxin, which is an integral component of the cell wall of gram-negative bacteria, induces the production of biologically active molecules, such as IL-1, TNF- and prostaglandins from monocytes or macrophages (98-101). Besides their proinflammatory properties, these cytokines are capable of stimulating bone resorption. Recently, it was discovered that the toll-like receptors play a crucial role in transduction of the signals of LPS (102). Interestingly, LPS from *P. gingivalis* and *C. ochracea* showed antagonistic activity by not inducing human TLR4-mediated signaling (103). Antagonistic activity would be of great advantage for the microorganisms to escape from the innate immune system.

Several bacterial species possess mechanisms to overcome the defense of the host's immune system. IgG and IgA proteases of *P. gingivalis*, *P. intermedia* and *Capnocytophaga* spp. are able to specifically destroy antibodies (77, 104). A number of species have developed strategies to interfere with the killing mechanisms of the polymorphonuclear leukocytes and monocytes. These include the production of leukotoxin by *A. actinomycetemcomitans* (105, 106) and *C. rectus* (107). Additionally, leukotoxin from *A. actinomycetemcomitans* can induce apoptosis in a variety of host immune cells (108).

1.3   Pathogenesis of periodontitis

The indigenous oral microflora and the host are normally in a state of equilibrium. The interactions between the microorganisms and the host are very dynamic, thus allowing the complex interplay between host molecules and bacterial antigens (109). The exact mechanisms that allow the host to "tolerate" non-pathogenic microorganisms are largely unknown. Any disruption of the "established" state, whether by commensal bacteria, pathogenic bacteria or a compromise in the local or systemic health of the host will lead to an altered host condition, resulting in disease (110). The pathogenesis of periodontitis is thus mediated by interactions between host and microbial factors, complicated by genetic and environmental risk factors.

Mixed consortiums of microorganisms are involved in periodontal disease, which develops as a consequence of imbalances in microbial biofilm inducing an inflammation in host tissues. The environment is altered by increased flow of gingival crevicular fluid and nutrients, as well as a pH rise that favors growth of periodontopathic bacteria (111). The microorganisms increase in number and produce several bioactive end products, endotoxins and exotoxins (58). Protease-producing bacteria, such as *P. gingivalis*, *T. forsythensis* and *T. denticola*, may be involved as initiators of disease activity.

The host has several defense strategies to protect its barriers against bacterial invasion. The defense mechanisms - innate and adaptive immunity - function in a complex way. Innate immunity is responsible for initiation of the inflammation process, acting as the first line of host defense against microbial pathogens (22). Adaptive immunity, mediated by B and T lymphocytes, which carry immunoglobulins and T-cell receptors, respectively, present a more effective defense against specific bacterial species, however, several steps are required before its efficient activation (102). Recently it was discovered that toll-like receptors (TLRs) play a crucial role in recognition of invading pathogens (102, 112). There are currently 10 known TLRs, each of which recognizes a different spectrum of pathogen-associated molecular patterns (PAMPs), e.g. TLR2 recognizes bacterial peptidoglycan and lipoproteins, TLR3 recognizes double-stranded RNA, TLR4 lipopolysaccharide, TLR5 flagellin and flagellated bacteria, TLR9 procaryotic DNA (113). TLRs are known to be expressed in a number of tissues and by a variety of cell types including monocytes, neutrophils, endothelial

cells, fibroblasts, osteoblasts and dendritic cells (110). Signaling through TLRs leads to a set of innate immune response - production of proinflammatory cytokines and upregulation of costimulatory molecules, and ultimately also induction of adaptive immunity (113, 114). The molecular basis of TLR-dependent signal transduction is an extremely active area of investigation, as these findings might explain different innate immune responses to various pathogens.

One of the recent exciting discoveries was that TLRs are critical molecules in adaptive immune response as well. They are required for the upregulation of co-stimulatory molecules such as CD80/86 and major histocompatibility complex (MHC) on dendritic cells (DCs) (113). TLRs can also regulate T-cell differentiation status by producing proinflammatory cytokines such as IL-12.

Genetic variations or polymorphisms associated with TLRs might explain to some extent the species-specific response and thereby different susceptibility of host to infections (113, 115).

The primary function of the innate immune system is to provide a rapid response to bacterial pathogens. Bacterial products are chemotactic for neutrophils, activate the plasma proteinase cascade systems, trigger mast cells to release biogenic amines, and stimulate inflammatory cells and resident tissue cells to form cytokines (IL-1, tumor necrosis factor), platelet activating factor and prostanoids (e.g. prostaglandins, leukotrienes) (116-119). Polymorphonuclear leukocytes (PMNs) seem to play a central role in the pathogenesis of periodontitis (123). Specific adhesion molecules like ICAM-1, ELAM-1 promote the movement of PMNs from blood vessels into the connective tissue and sulcus, where they phagocytose the bacteria (120). The defects in vitality and function of PMNs are modifying factors for disease pattern or severity (121).

Most of the tissue destruction results from direct effect of the bacteria, together with the resulting inflammatory and immunological host responses. Reactive oxygen products from inflammatory cells injure tissue cells, and proteases from both inflammatory cells and resident tissue cells degrade components of the extracellular tissue matrix (122, 123). In the periodontal tissues prostanoids, cytokines and thrombin directly or indirectly induce degradation of the extracellular matrix, activate osteoclasts and initiate bone resorption (116, 124). An involvement of TLR4 in bone resorption was demonstrated with TLR4-deficient mice. It was observed that bone loss was significantly less in TLR4-deficient mice than in wild-type controls (125). This decrease was correlated with reduced expression of the bone resorptive cytokines IL-1 and IL-1 as well as the proinflammatory cytokine IL-12. An immunohistochemical investigation of gingival tissue of periodontitis patients showed the association of the expression of the TLR4 with severe periodontal disease (126). It becomes apparent that TLR levels influence the magnitude of inflammatory responses, underscoring the need to clarify the molecular mechanisms modulating TLR expression (113).

Only now are we beginning to appreciate the complexities of the evolutionary conserved innate immune system, and the essential role it plays in maintaining homeostasis. The disruption of an intact innate immune system is detrimental to the health of the host in either a localized or a systemic manner.

The adaptive immunity is based on specific antigen-antibody reaction, as well as specific T-cell recognition. Antigens are presented by Langerhans cells to lymph tissue, where the B-cells will be converted to plasma cells to produce antibodies. The reaction is supported by T-cells. The antibodies induce aggregation of bacteria, inhibit adhesion of bacteria to epithelium, lead to antibody-complement-mediated bactericidal activity or through opsonisation to phagocytosis by neutrophils and macrophages (127). Persons able to provide an effective antibody reaction are supposed to be more resistant to periodontitis than those with a quantitatively or qualitatively ineffective response. It has been shown that the production of IgG2 predominates over IgG1 concentration by patients with early-onset periodontitis (128). This suggests that functionally less-effective IgG2 antibodies play an important role in susceptibility and dimension of periodontal destruction in those patients. Some authors have stressed the importance of testing the titer of antibodies to putative pathogens and the avidity of antibodies in determining the status of periodontal disease (129).

The bacteria play an important role in the etiology of periodontitis, but the response of the host is the decisive factor for the susceptibility of periodontitis. Risk factors like smoking, diabetes, stress modify to a large extent the susceptibility of the host and progression of the disease (130, 131). It turns out that smoking has the highest impact on the course of the disease, modulated by all the other factors (132). Regardless of the different microbial profiles identified in smokers and non-smokers in the majority of the investigations it is unclear whether the increased presence of certain microorganisms is the cause or the consequence of a more severe disease condition. However, conflicting results have been reported about the influence of smoking on the subgingival microflora of periodontitis patients (133). It can be concluded that smoking and stress influence host-related factors, thereby modifying the microflora to be more pathogenic. Bergsröm et al. (134) proposed to regard destructive periodontal disease as a systemic disease in the same way as heart disease or lung disease. In smokers the periodontal disease is initiated and driven by smoking, where the elevated morbidity does not depend on particular microflora (134).

1.4   Clinical studies seeking evidence for etiological role of bacteria

A major limitation of many microbiological studies has resulted from the selection of appropriate patients and controls. Favored subjects most often chosen are those with the most advanced cases of periodontitis. Analysis of the complex microflora of these samples from cross-sectional studies did not reveal whether the investigated microorganisms initiated

the disease or whether they colonized later. The presence of suspected pathogens may result from, rather than cause the disease.

There are multiple forms of destructive periodontal disease that are difficult to define clinically. Combining subjects that represent two or more disease types into a single group diminishes the likelihood of discriminating the pathogens from other species.

1.4.1   Prevalence studies

A positive correlation between bacterial numbers and severity of gingivitis or periodontitis and amount of bone loss has been demonstrated in cross-sectional studies (135). A higher prevalence and increased proportions of *P. gingivalis*, *T. forsythensis*, *P. intermedia*, *Fusobacterium* spp., *Campylobacter* and *Treponema* spp. were detected in periodontitis patients as compared to periodontally healthy subjects (30, 32, 44).

1.4.2   Progression of disease

An important piece of evidence in defining periodontal pathogens comes from longitudinal studies examining the subgingival microflora in active sites undergoing attachment loss (27, 33, 39, 135, 136). Several resident putative periodontal pathogens have been reported to be responsible for the progression of attachment loss. Haffajee et al. (137) followed longitudinally the changes in pockets that subsequently lost attachment. Significantly higher levels of *P. gingivalis*, *C. rectus* and significantly lower levels of *C. ochracea* were found in active subjects prior to breakdown.

By studying the microbiota of active destructive periodontal lesions and inactive sites, species such as *T. forsythensis*, *P. gingivalis*, *P. intermedia*, *E. corrodens*, *F. nucleatum*, *Str. intermedius, P. micros*, *A. actinomycetemcomitans* were found frequently in high numbers, suggesting that they may represent causative agents. In inactive sites *S. mitis, S. sanguis*, *Actinomyces* spp., *C. ochracea* and *V. parvula* were elevated (33, 39, 49, 138 -142). Liljenberg et al. however, compared periodontitis patients with progressive and non-progressive disease in a cross-sectional study and found no differences in the subgingival microbiota between groups (143). Furthermore, even patients with progressive disease did not show differences between progressive and non-progressive sites (144).

1.4.3   Risk factor studies

Risk assessment studies were used to confirm etiological agents in periodontal diseases. A periodontal site in a carrier-state with bacterial pathogens was considered to be a future risk indication of periodontal breakdown. It has been observed that subgingival colonization with *T. forsythensis*, *P. gingivalis* and *A. actinomycetemcomitans* was associated with a risk for attachment and severe bone loss (3, 145 -147). Similarly an increase in levels of bacteria was associated with an increased risk of attachment loss, however, different threshold levels were reported for different bacterial species. Additionally, several combinations of species

were associated with an increased risk for disease progression (48). However, some authors refer to periodontal pathogens as minor risk indicators due to the fact that the odds ratios between the presence of these specific bacteria individually and periodontitis are not high enough to classify them as risk factors (148).

1.4.4 Treatment studies

Successful therapy is aimed at diminishing the level of pathogens, supporting the colonization with beneficial species and leading to an attachment level gain (55, 149-151). The prerequisite for an efficient therapy is an excellent oral hygiene. Scaling and root planing is considered the standard therapy of periodontitis. Combined with a regular maintenance program the supra- and subgingival debridement has been shown to be effective in most cases of periodontal therapy (152 -154).

Surgical intervention in the form of modified Widman flap surgery or apically repositioned flap may be needed if non-surgical therapy was not effective and deep pockets still persist. It provides better access to the roots for the debridement. Comparison between the surgical and non-surgical therapy demonstrated higher attachment gain in deep pockets after the surgery (149).

The mechanical debridement not only decreases plaque mass but also radically changes the composition of the subgingival microbiota (139, 149, 152). The disruption of biofilm is effective in altering the biofilm's composition so that the putative pathogens are eliminated or reduced to nonpathogenic levels, and bacteria associated with health are positively selected. The qualitative shift may be mediated not only through the direct effect of mechanical debridement but also indirectly through an altered immune response (155).

Patients with aggressive or refractory periodontitis often need an adjunctive systemic antibiotic therapy. Refractory periodontitis is characterized by ongoing deterioration of periodontal sites and associated with a continued presence of *T. forsythensis*, *P. gingivalis*, *C. rectus*, *P. intermedia*, *P. micros*, spirochetes, enterococci (147, 156, 157). Predictable results have been achieved with the administration of a metronidazole / amoxicillin combination (158, 159). The strictly anaerobic gram-negative species and *A. actinomycetemcomitans* are the main targets of this antibiotic combination.

Although some success has been reported due to antibiotic therapy, several limitations have become evident. Most of these limitations are due to the fact that periodontal infections result from the formation of biofilm. Therefore, disrupting the biofilm mechanically is still the basis for successful periodontal treatment (160).

The clinical stability of periodontal status means a dynamic balance between the presence of opportunistic bacteria and immune response. The maintenance therapy is thus aimed at keeping the bacterial colonization under control.

1.5   Detection methods

A variety of methods have been developed and applied for the detection and identification of microorganisms. Bacterial culture has long been regarded as the "gold standard". However, culture-based techniques suffer the limitation that they are highly delicate and time-consuming, requiring experienced personnel and strict quality assurance procedures. Many organisms will not grow on currently available culture media. Several studies showed that culture-based analyses of complex microbiota do not reflect the true composition of the microbial population as often only these species which grow easily in vitro are cultured (161). Additionally, identification based upon phenotypic characterization has been found to be unreliable. One disadvantage of culture techniques is that only small numbers of samples can be studied. More rapid unbiased techniques are required for examining large numbers of samples and reflecting more reliably the real diversity of the flora. These techniques include immunofluorescence using monoclonal or polyclonal antibodies, hybridization using either whole-genomic DNA probes or oligonucleotide probes, and PCR amplification assays.

The 16S rRNA with its altering conserved and variable domains has been found to be the most reliable and stable molecule for identification, enumeration and phylogenetic classification of procaryotes (162, 163).

Molecular biologic methods have a higher sensitivity and specificity as compared to bacterial culture hence increasing the accuracy of the analysis (161, 164). They are especially valuable for the detection of slow-growing, fastidious or yet uncultured bacteria.

In the situation where the putative pathogens belong to an indigenous flora, the detection of minute amounts of bacteria is irrelevant. In epidemiological studies, however, estimation of accurate prevalence of these bacteria in different population groups allows the assessment of their possible association with the disease.

New techniques like transmission or scanning electron microscopy, fluorescence-in-situ hybridization help to visualize, identify, localize and enumerate the microorganisms in biological samples. The development of a real-time PCR-amplification assay allows reliable quantification of bacterial species and assessment of their proportions in a total flora (165).

1.6   Aggressive periodontitis

1.6.1   Clinical diagnosis

It has become popular to speak about different periodontal disease entities, which may have different specific etiology. However, only necrotizing periodontal diseases and localized aggressive periodontitis (earlier LJP) are well-defined disease entities. Most periodontitis cases are difficult to classify clinically in the gradual range from gingivitis to more-or-less advanced or aggressive periodontitis. This makes statistical associations between the disease status and microflora problematic.

According to the new classification aggressive periodontitis is a specific form of periodontitis

with distinct clinical and laboratory characteristics (166). These include:

- Besides periodontitis subjects systemically healthy
- Rapidly progressing periodontal destruction
- Familiar aggregation

Often, but not always:

- Disproportion between dental plaque and tissue destruction
- High prevalence of *A. actinomycetemcomitans* or *P. gingivalis*
- Abnormal function of neutrophils or monocytes
- Hyperresponsive macrophage-phenotype with increased production of PGE2 and IL-1ß
- The destructive process may cease spontaneously or greatly slow down

The localized form:

- Begins in puberty
- First molars and central incisors are affected
- High level of antibodies against putative pathogens in serum

The generalized form (GAP):

- Patients younger than 30 years
- Generalized destruction of the dentition
- At least 3 teeth are affected
- Intermittent course of the disease
- Low level of antibodies against putative pathogens in serum

1.6.2 Microorganisms associated with aggressive periodontitis

There have been few studies concerning the associated pocket microflora of generalized aggressive periodontitis, but the available data implicate *P. gingivalis*, *T. forsythensis*, *A. actinomycetemcomitans*, *P. intermedia*, *E. corrodens*, *F. nucleatum*, *C. rectus*, *C. ochracea*, *V. parvula*, spirochetes, *Eubacterium* spp., *P. micros* as important suspected pathogens. They have been found in higher proportions and more frequently in aggressive periodontitis patients (10, 13, 15, 17, 167 -169, 170). However, aggressive periodontitis is considered a distinct form of periodontitis, microbiological criteria are not regarded as primary features in defining the disease entity (52).

1.7   Control group (Elderly)

A well-documented epidemiological study, the so-called "New England Elders Dental Study", revealed a high prevalence of periodontal destruction among older adults (171). Moderate pockets with pocket depth (PD) 4-6 mm were found in 66% of the study sample, and severe periodontal pockets (PD>6 mm) were observed in 21% of subjects. Only 8% of that population had no pockets. The authors drew the conclusion that age was significantly

associated with periodontal destruction within this elders' population.

The only study investigating subgingival microbiota of an elderly population and comparing it with healthy and periodontitis patients was performed by Haffajee et al. (34). The mean probing depth of the 35 elders was 2.6±0.4 mm and as few as 6% of the sites revealed PD 4-6 mm. No pocket >6mm was documented. Several subjects had periodontal treatment in the past. At the time of the study all the subjects were on regular maintenance (mean duration 14.2 years). These individuals exhibited minimal evidence of disease progression and tooth loss. Marked similarities in the subgingival microbiotas of the healthy and well-maintained elders was observed.

2 Aim of the study

Although a strong association between various bacteria and the etiology of periodontitis has been shown, etiopathogenesis is still not resolved. One way to prove the etiologic relevance of putative pathogens would be the performance of a series of epidemiological and longitudinal investigations with all population groups using a uniform detection method. If "marker species" which play an important role in initiation or exacerbation of periodontitis are existing, their identification should be made easily applicable in dental practice.

From the technical point an easier, less biased, and more specific and sensitive microbiological detection method is needed in order to investigate large number of samples and patients with different clinical conditions.

Clinically, the GAP population reveals a high risk of periodontal breakdown and tooth loss. A comprehensive microbiological and immunological investigation is necessary in order to define the critical factors responsible for disease development, and

also regarding control of patient's oral health during the treatment and maintenance phase. A comparison of the microbiological status between different diseased patient groups and healthy subjects may be of help to critically assess the importance and role of certain putative periodontal pathogens. Unfortunately several past clinical studies are missing a control group. In the present investigation the elderly subjects represent a control group, revealing microflora compatible with periodontal health in advanced age.

The aim of this study was to design, optimize and evaluate 10 oligonucleotide probes for the identification of putative periodontal pathogens, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis*, *Campylobacter rectus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Veillonella parvula*, *Capnocytophaga ochracea* and *Fusobacterium* spp. The probes were used in an epidemiological study to analyze and compare the subgingival colonization of the target species in both untreated GAP patients and elderly subjects with a well-maintained periodontium.

The current investigation is part of a larger epidemiological study comprising the identification of known as well as not-yet cultivable suspected pathogens (treponemes) in various patient populations using a uniform molecular genetic detection method.

3   Materials and methods

3.1   Probe design

The oligonucleotides were designed on the basis of the first 530 basepairs of the 16S rRNA. Hypervariable regions were identified and species-specific oligonucleotide probes were selected by alignment of the sequences. The following criteria for probe design were applied:

- minimum length of at least 15 bases
- at least one mismatch between the probe and closely related species
- no self-complementarity (checked by the program OLIGO, version 4.0, National Biosciences, Plymouth, MN)
- G/C content between 30-60%

The species-specific oligonucleotides were used as 16S rRNA/DNA directed probes. Previously published oligonucleotides (172, 173) were re-evaluated. In order to assess the specificity, the target sequences were compared with those of all entries of procaryotes at the EMBL and Genbank databases accessible (July 2002) by using the program BLASTN of the Husar program package (version 4.0; Heidelberg Unix Sequence Analysis Resources; DKFZ, Heidelberg, Germany). All probes were checked with the aid of the program OLIGO 4.0 for their practical use in a hybridization assay.

3.2   Culturing of target- and phylogenetically closely related species

To ensure the specificity of the probes 42 phylogenetically closely related and target species were cultivated as negative and positive controls, respectively. The species were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), CCUG (Culture Collection University of Göteborg) and kindly provided by R. Mutters, Marburg, Germany; G. Conrads, Aachen, Germany; C. Wyss, Zürich, Switzerland; A. Mombelli, Bern, Switzerland and E. Esdorn, Charité, Campus Virchow Clinic, Berlin, Germany.

The strain designations and the original sources used in the study are listed in Supplement, Table 1.

Positive controls for the probe for *A. actinomycetemcomitans* were 3 isolates of the species, ATCC 43718, ATCC 33384, and a serotype a. The closely related oral species *Leptotrichia buccalis* (MCCM 00448), *Pasteurella haemolytica* (ATCC 33396) and *Haemophilus paraphrophilus* (ATCC 29241) are all colonizers of the oral cavity and with at least one mismatch in the target sequence served as negative controls.

The probes for *P. gingivalis* and *P. intermedia* were checked by using the target species *P. gingivalis* (ATCC 33277) and *P. intermedia* (ATCC 25611) and related oral species such as *Porphyromonas asaccharolytica* (ATCC 25260), *Prevotella nigrescens* (NCTC 9336), *Prevotella oralis* (MCCM 00684) and *Prevotella buccalis* (ATCC 33690) with more than one

basepair difference. *P. asaccharolytica* has been implicated in pelvic inflammatory disease, endometritis, and bite wound infections (183).

Capnocytophaga ochracea (ATCC 27872) served as a positive control in optimizing the respective probe. It was checked against *Capnocytophaga sputigena* (ATCC 33612) which has 95% similarity within the 16S rRNA with the target species (172) and one mismatch in the respective probe sequence.

The specificity of the probe for *C. rectus* was tested by including the reference strain *Campylobacter rectus* (ATCC 33238) and a very closely related periodontal species *Campylobacter concisus* (ATCC 33236).

Eikenella corrodens (CCUG 2138) and the frequently detected oropharyngeal species *Kingella kingae* (ATCC 23330) which has one mismatch with the target species, enabled the optimization of the respective probe.

The strain *Veillonella parvula* (ATCC 10790) served as a positive control for the respective probe and *Veillonella dispar* (ATCC 17748) which contains one ambiguity in the target region was applied as a negative control.

Five strains of the genus *Fusobacteria* were cultivated to prove the specificity of the genus-specific probe and of the probe for *F. nucleatum*.

The bacteria were delivered either in lyophilized form or on agar plates. Lyophilized bacteria were suspended in TS-medium (Suppl. 1.5.), incubated at 37°C in anaerobic or aerobic conditions as required and after 3 days aliquots (3 µl) were plated onto the respective agar plates (pre-reduced for anaerobic strains). Columbia agar with 5% sheep blood (Becton Dickenson, Meylan Cedex, France) (Suppl. 1.2.) was used for the cultivation of aerobic bacteria. Either Columbia agar with vitamin K and hemin or ETSA - agar (Suppl. 1.4.) was used for the culturing of anaerobic bacteria. Some species were cultivated in fluid-universal-medium (FUM) (Suppl. 1.1.). Anaerobic species were incubated anaerobically at 37°C in jars within an atmosphere containing 80% nitrogen, 10% carbon dioxide, and 10% hydrogen provided by AnaerogenTM35 (Oxoid, Hampshire, England) for 3-5 days. The microaerophilic bacteria were incubated in an atmosphere with 10% CO_2 for 2-4 days. Anaerobic bacteria were grown at 37°C for 2-3 days.

The bacterial species, respective culture media and growth conditions are listed in Supplement, Table 2.

The DNA of *Tannerella forsythensis* was kindly provided by Dr. Olson, Institute of Oral Biology, Oslo University.

The identity of target bacteria and closely related species was verified by 16S rDNA sequencing or biochemical tests using the rapid ID32A system (bioMérieux, Marcy-l'Etoile, France) (Suppl., Table 1).

Sequencing was carried out by the Sanger dideoxy-mediated chain-termination method (174)

using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, England). After DNA extraction (s. 3.3.) 16S rRNA genes or parts thereof were amplified using eubacterial primers (s. 3.4.). The PCR-products were purified on a silica-matrix (2.5 g Silica, 25 ml PBS) (Suppl. 2.9.).

The following tubes were prepared:

- 16 µl 2.5% DMSO (in H2O)
- 8 µl purified DNA
- 2 µl Primer (5'-IR- labeled)

The mixture was divided into 4 tubes, 6 µl in each. The reaction buffer (2 µl) which contained sequenase, all 4 deoxynucleotides (dNTPs) and one of the dideoxynucleotide (ddATP, ddCTP, ddGTP or ddTTP) was added into each tube. The tubes were covered with 30 µl mineral oil to avoid evaporation.

The sequencing was performed in a thermal cycler (Trioblock; Biometra, Göttingen, Germany) by repeated 29 cycles of denaturation at 95°C for 30 seconds, followed by annealing of the primer at 60°C for 30 sec. and extension/termination at 70°C for 40 sec. At the end of the program stopping-buffer (5 µl) (Suppl. 2.11.) was added to each tube to stop the reaction. The tubes were held on ice prior to loading onto the gel.

Reaction products were separated by electrophoresis on a polyacrylamide gel (Suppl. 3.2.) and detected with a laser-detector in a LICOR DNA-Sequencer (Model 4000, MWG Biotech). The automated analysis of the sequences was performed by an attached computer using the IBM OS/2 Base ImageIR Software with Data Collection and Image Analysis programs. The sequences were evaluated using the Husar software program package (version 4.0; Unix Sequence Analysis Resources, DKFZ, Heidelberg, Germany).

3.3 DNA isolation

A single bacterial colony grown on agar was suspended and washed in 100 µl phosphate-buffered-saline (PBS) (Suppl. 2.9.). Alternatively a 100µl aliquot of bacterial culture from a liquid-medium was removed into a 0.5 ml Eppendorf tube. The suspension was centrifuged in a Labofuge 400 R centrifuge (Hereus, Hanau, Germany) at 13 000 x g for 10 min at 4°C. The supernatant fluid was removed and the pellet suspended in 100 µl PBS, vortex-mixed and recentrifuged under the described conditions. The resulting pellet was resuspended in 100 µl of ice-cold lysis buffer (Suppl. 2.10.), vortex-mixed and stored at -20°C. No further purification of the nucleic acids was performed.

3.4 PCR amplification of 16S rDNA

A commercial kit (AmpliTaq DNA Polymerase with GeneAmp , Perkin Elmer, Branchburg, NJ, USA) was used for the amplification. The amplification was carried out in a volume of 100 µl containing:

- 1 µl of dissolved bulk DNA

- 1.5 mM MgCl2

- 1 x PCR buffer (50 mM KCl, 10 mM Tris; pH 8.3)

- 200 µM dNTPs

- 0.2 µM each primer

- 2.5 U Taq polymerase

- finally 50 µl sterile mineral oil was added.

The eubacterial primers used for the 16S rRNA gene amplification were:

- TPU 1 (5'-AGA GTT TGA TCM TGG CTC AG-3', corresponding to positions 8 to 27 in the Escherichia coli 16S rRNA gene)
- RTU 3 (5'-GWA TTA CCG CGG CKG CTG-3', corresponding to positions 519 to 536 in E. coli 16S rDNA).

As negative control a tube containing 1 µl sterilized PCR-water instead of a sample was included in every amplification run.

Amplification (30 cycles) was performed in an automated thermal cycler (Trioblock; Biometra, Göttingen, Germany). Initial DNA denaturation was carried out for 5 min at 95°C. During this step the Taq-Polymerase was added (hot-start). Each of the following cycles consisted of three steps: denaturation for 1 min at 95°C, primer annealing for 1 min at 56°C, and strain extension for 1 min at 72°C. The 30th extension step was prolonged to 3 min.

A 3.5 µl aliquot of the PCR-amplified product was mixed with 1 µl loading dye for the electrophoresis. The correct size and amount of the amplicons was verified by 1.2% agarose gel (Suppl. 3.1.) electrophoresis at 120 V for 1 hour. The resulting DNA bands were visualized under ultraviolet light.

After denaturation of the PCR products at 95° for 5 minutes the tubes were quickly chilled on ice and aliquots of 1 µl were spotted onto nylon membranes (Hybond N; Amersham, Buckinghamshire, UK) in 80x45 mm size. The DNA was immobilized on the membrane by ultraviolet-crosslinking, done by exposing both sides of the membrane to UV light (254 nm) in "UV-Crosslinker" (MWG Biotech, Ebersberg, Germany) for 3 min.

3.5 Probe labeling

Commercially synthesized oligonucleotide probes were diluted with sterilized distilled water to a concentration of 25 pmol/µl.

The nonradioactive DIG system was used for labeling the probes (Boeringer Mannheim, Mannheim, Germany). Digoxigenin, a steroid hapten, coupled to ddUTP was incorporated by terminal transferase to the 3'-end of the oligonucleotide probe.

The labeling reaction mixture consisted of the following:

- 4 µl 5 x buffer (1 M potassiumcacodylat, 125 mM Tris-HCl, 1.25 mg/ml BSA)
- 4 µl CoCl2 (25 mM)
- 1 µl DIG-ddUTP
- 4 µl Oligonucleotide (25 pmol/µl)
- 1 µl Terminal transferase (50 U/µl)
- 20 µl distilled water

After incubation at 37° for 15 min 2 µl of stop-solution 1 µl glycogen (20 µg/ml) with 200 µl EDTA (0.2 mM) , 2.5 µl LiCl, and 75 µl ethanol (absolute) was added. The subsequent precipitation took place at -20°C for about 24 hours. Additional purification was performed by precipitation with 50 µl ethanol (absolute). The oligonucleotide was vacuum dried, diluted in 20 µl distilled water and stored at -20°C.

The probes were immunodetected with anti-digoxigenin and then visualized with the chemiluminescence substrate CSPD . The light emission was recorded on X-ray films.

3.6 Optimization of hybridization conditions

The hybridization was performed in glass tubes by continuous rotation in hybridization ovens (Micro 4, MWG-Biotech).

Hybridization consisted of the following steps:

(for the composition of reagents see Supplement, Buffers)

1. Prehybridization at 54°C (40°C) in a prehybridization solution (Suppl. 2.1.) (10 ml) for 30 min..
2. Hybridization at 54°C (40°C) with a heat denaturated labeled probe (3.6 pmol) in a hybridization solution (Suppl. 2.4.) for 2 hours.
3. Washing under stringent conditions with respective salt buffers (Suppl. 2.5.-2.7.) (10 ml) and temperatures 2 x 15 minutes
4. Blocking reaction with 1% blocking solution (Suppl. 2.2.) at 37°C for 30 minutes
5. The hybrids were detected by adding anti-digoxigenin alkaline phosphatase conjugate (75 mU/ml corresponds to v/v 1:10000 in 1% blocking solution) with binding at 37°C for 30 minutes
6. Washing at 37°C 2 x 15 min with maleic acid buffer (Suppl. 2.1.) (10 ml)
7. Equilibration in detection buffer (Suppl. 2.3.) (5 ml) at 37°C for 2-5 minutes
8. Luminescence reaction with (chemiluminescent substrate) CSPD (1:100 in detection buffer) at 37°C for 15 minutes.

The wet membrane was sealed in a plastic foil and exposed to X-ray film (Hyperfilm, Amersham Life Science, Buckinghamshire, UK) for 2 hours at 37°C. An additional film was exposed for 12 hours at 24°C. The films were developed automatically in CURIX 60 (AGFA). The membranes were washed with stripping buffer (Suppl. 2.8.) (10 ml) for 2 x 15 min at

37°C to remove the probe and rinsed thoroughly in 2 x SSC (10 ml). Identical membranes were used for multiple hybridization experiments.

3.7 Patient populations

3.7.1 GAP patients

A total of 45 GAP patients were included into the epidemiological analyses.

All patients were previously untreated and referred to the Department of Periodontology and Synoptic Dentistry, Charité, Berlin, between 1997-1998. Patients were diagnosed according to their advanced clinical and radiographic signs, considering their age and history of periodontal disease.

Patients with chronic periodontitis or those who had received anti-inflammatory or antimicrobial therapy within the previous 6 months were excluded from the study. The average age of GAP patients was 34.7 years.

Four subgingival plaque samples per patient were taken prior to any therapy from the deepest periodontal pockets (PD 4 mm) which bled on probing, preferably one from each quadrant. Additionally, one control sample from a clinically unaffected site was obtained. After supragingival plaque removal with a sterile curette and cotton pellet, three sterile paper points (ISO 35; Becht, Offenburg, Germany) were inserted into the pocket. After 10 seconds the paper points were removed and placed into 1 ml of reduced transport fluid (RTF, Suppl. 1.6.) (175), transferred to the laboratory and processed immediately.

A total of 224 subgingival plaque specimens (180 from periodontal pockets and 44 from healthy control sites) from the GAP population was investigated. The mean pocket depth of the sampled sites was 8.7±1.8 mm and the mean probing depth of the control sites was 2.7±0.8 mm.

3.7.2 Elderly subjects

The second group of the epidemiological study consisted of 21 healthy senior subjects. These individuals represented a population with a well-maintained periodontium and served as a control group for the periodontitis patients.

The inclusion criteria were as following:

- age 65 years
- presence of at least 20 teeth
- no history of severe periodontitis
- no site with PD > 5 mm

The exclusion criteria were the presence of gingivitis, severe periodontitis, chronic systemic disease or anti-inflammatory or antimicrobial therapy within the previous 6 months. The subjects did not receive regular periodontal maintenance care. Five randomly chosen

periodontal sites of elderly subjects were sampled.

3.8   Dot-blot hybridization of patient material

The bacteria were eluted from the paper points by vortex-mixing them for 30 sec. in a tube containing 1 ml RTF (Suppl. 1.6.). Aliquots (100 µl) of the plaque specimen were centrifuged at 13 000 x g for 10 min in a Labofuge 400 R centrifuge (Hereus, Hanau, Germany) for the DNA isolation. Resulting bacterial pellets were placed in 100 µl of ice-cold lysis buffer (Suppl. 2.10.), vortex-mixed and stored at -20°C until further processing. The PCR amplification with 1 µl of bulk DNA was performed as described above (3.4). The amount and correct size of the amplicons were checked by agarose-gel electrophoresis. The PCR products were denaturated and blotted onto nylon membranes (Hybond N; Amersham, Buckinghamshire, UK).

Dr. Moter kindly provided the prepared membranes with processed samples of both study groups for the dot-blot hybridizations.

The dot-blot hybridizations of patient plaque material were performed sequentially with every probe under the respective hybridization conditions. Negative and positive controls were included in each run of the assay.

The membranes were washed with stripping buffer (10 ml) for 2 x 15 min at 37°C to remove the probe and rinsed in 2 x SSC (10 ml). Identical membranes were used for multiple hybridization experiments with all of the reported probes.

Dot-blot hybridization of PCR-amplified plaque material was used to detect small amounts of target periodontal bacteria in patients. The PCR-amplification enables the detection of at least 100 bacterial cells in a sample (168). The detection limit of PCR-amplification and subsequent hybridization has been reported to be about 20 CFU/ml using a pure culture (176).

4   Statistical analysis

Statistical analyses was supported by Ms. Siebert, Institute of Medical Biometrics, Humboldt University. For all calculations SPSS software v. 10.0 was used.

All 45 GAP patients and 21 elderly subjects were included in the statistical analysis. The patient served as a statistical unit. A difference of $p < 0.05$ was considered statistically significant.

The differences in prevalence (number of positive subjects) between the two groups was computed for each species with chi-square test. A patient was considered positive when at least one sampled site was positive.

When comparing the colonization of periodontal pockets and control sites of the GAP patients, only one arbitrary chosen pocket and one shallow site per patient was analyzed and evaluated using the chi-square test.

Assessment of the prevalence of the species in periodontal pockets of the GAP patients versus sampled sites of the elderly was performed by including four sites per subject and using the chi-square test.

As only one control site was available from each GAP patient, the comparison of the presence of bacteria in similar shallow sites in elders was carried out using a single arbitrary site per elderly subject and the chi-square test.

The analysis of the presence of bacteria at different pockets depths was performed with the data of only 23 GAP patients using the chi-square test.

The non-parametric Mann-Whitney test for two independent samples was used to test the null hypothesis that the load of bacteria per patient, i.e. the number of positive sites per patient, is the same in both subject groups. Four pockets per patient from the GAP group and four sites per subject from the elderly population were analyzed.

The relationship between two bacterial species, i.e. the frequency of co-existence, was analyzed by odds ratio (OR) calculations as suggested by Socransky (177). The odds of an event are defined as the ratio of the probability of the event to the probability of its complement. For each pair of the investigated species within the GAP patient group, 2x2 contingency tables were constructed. The magnitude of the association between two species is indicated by the amount OR differs from 1.0, the indication of no association. OR<0.5 shows a negative association and OR>2 a positive one.

5 Results

5.1 Design of species-specific oligonucleotide probes

It was possible to select unique sequences about 16-30 basepair in length that discriminated each species (except *F. nucleatum*) down to the level of one mismatch when compared to the respective target sequences of all other species described so far. The *Fusobacterium nucleatum* probe also cross-hybridized with the respective sequence of *Fusobacterium periodonticum*. These species exhibit a 100% homology in the 528 initial basepairs of the 16S rRNA (178). Besides *F. nucleatum* the genus *Fusobacterium* comprises a wide range of species, such as *F. necrophorum, F. mortiferum, F. simiae, F. gonidoformans, F. alocis, F. varium, F. russi, F. ulcerans, F. periodontiticum, F. perfoetens*, all complementary to the respective probe. Most of the species are considered a part of the indigenous oral flora.

All probes are non-self-complementary and with low G-C content (checked with the program OLIGO).

The oligonucleotide probe sequences are listed in Table 1.

Table 1. Probe sequences (5'-3') with respective hybridization conditions. Washing buffers listed in Suppl., 2.5.-2.7.

PROBES 5' - 3'	T°C (Hyb)	T°C (Wash)	Washing buffer
Actinobacillus actinomycetemcomitans (A.a.) **TCC-ATA-AGA-CAG-ATT-C**	40°	47°	WP 0
Tannerella forsythensis (T.f.) **CGT-ATC-TCA-TTT-TAT-TCC-CCT-GTA**	54°	64°	WP 0
Campylobacter rectus (C.r.) **TTA-ACT-TAT-GTA-AAG-AAG**	40°	40°	WP 0
Capnocytophaga ochracea (C.o.) **TCG-GGC-TAT-CCC-CCA-GTG-AAA-GGC-AGA-T**	54°	61°	WP 2
Eikenella corrodens (E.c.) **AGT-TAT-CGG-CCG-CTC-GAA-TAA-CGC**	54°	62°	WP 1+2
Fusobacterium nucleatum/F. periodonticum (F.n.) **GCC-TCA-CAG-T(C,G)TA-GGG-ACA-ACA-T**	54°	67°	WP 1
Fusobacterium spp. (Fuso) **GAG-AGC-TTT-GCG-TCC-CAT-TAG**	54°	62°	WP 1
Porphyromonas gingivalis (P.g.) **CAA-TAC-TCG-TAT-CGC-CCG-TTA-TTC**	54°	62°	WP 1+2
Prevotella intermedia (P.i.) **CTT-TAC-TCC-CCA-ACA-AAA-GCA-GTT-TAC-AA**	54°	62°	WP 1
Veillonella parvula (V.p.) **TCT-AAC-TGT-TCG-CAA-GAA-GGC-CTT-T**	54°	56°	WP 0

In order to obtain positive and negative controls for the probe optimization and evaluation, the reference bacterial strains were cultured and their identity was checked by sequencing the PCR products or by biochemical tests (Suppl., Table 1). 16S rDNA of these strains was amplified in the PCR and the products were dotted onto the nylon membranes which were further used for the optimization and evaluation of the constructed probes.

5.2 Optimization of the hybridization conditions and evaluation of the oligonucleotide probes

Membranes were initially hybridized with eubacterial probe (EUB 338) to demonstrate successful PCR-amplification and immobilization for all samples investigated.

Oligonucleotide probes were able to distinguish between complementary and nearly complementary sequences on the basis of single mismatch achieving the expected specificity. However, this requires stringent hybridization conditions which often results in a reduction of the quantitative probe binding to the target nucleic acid (179). The intention of this study was to achieve an optimal balance between probe sensitivity and specificity by optimizing hybridization parameters, such as the washing temperature and the ionic strength of the washing buffer.

Each probe was tested against a panel of PCR products isolated from 42 reference microorganisms common to the oral cavity or extra-oral regions (Suppl., Table 1). The analysis indicated that all probes (except for *Fusobacterium nucleatum*) were specific and did not cross-hybridize with closely related species. The hybridization conditions for each probe are listed in Table 1.

Using the described assays the detection of target bacteria in multiple samples could be performed rapidly (1h DNA isolation, 4 h PCR-amplification, 5 h dot-blot hybridization, 1 h film exposure).

5.3 Epidemiological study - GAP patients and healthy elderly individuals

A total of 224 subgingival samples from 45 GAP patients and 84 samples from 21 elderly subjects were analyzed.

All investigated species were detected in the subgingival plaque of GAP patients and well-maintained elderly (for the prevalence calculation one GAP patient was excluded because of a missing control site). All GAP patients and elders were colonized by the members of genus *Fusobacterium*, however, *F. nucleatum*/*F. periodonticum* were detected in 91% of GAP patients but significantly less (57%) in elders (Fig.1). *T. forsythensis* occurred in 95.5% of GAP patients and in 85.7% of the elders showing no significant difference between both groups. The microorganism appears to be a common colonizer even in well-maintained individuals. The prevalence of *P. gingivalis* and *P. intermedia* in the GAP patients was 63.6% and 70.5%, respectively. In this study no significant difference was observed between the groups. From the GAP patients 36.4% harbored *A. actinomycetemcomitans*. In contrast, only two out of 21 elderly subjects were colonized by this species. As compared to healthy controls, significantly more GAP patients were colonized by *E. corrodens* (75%) and *C. rectus* (56.8%) than the elderly. The prevalence of *V. parvula* was moderate in both groups (GAP 25.4%, elderly 42.9%), without statistically significant difference. A highly significant difference ($p<0.0001$) was observed for *C. ochracea*. As much as 95.2% of the elders were

positive, while the prevalence in GAP population was only 15.9%. The prevalence calculations suggest the evidence of colonization with target species in both groups (Fig.1).
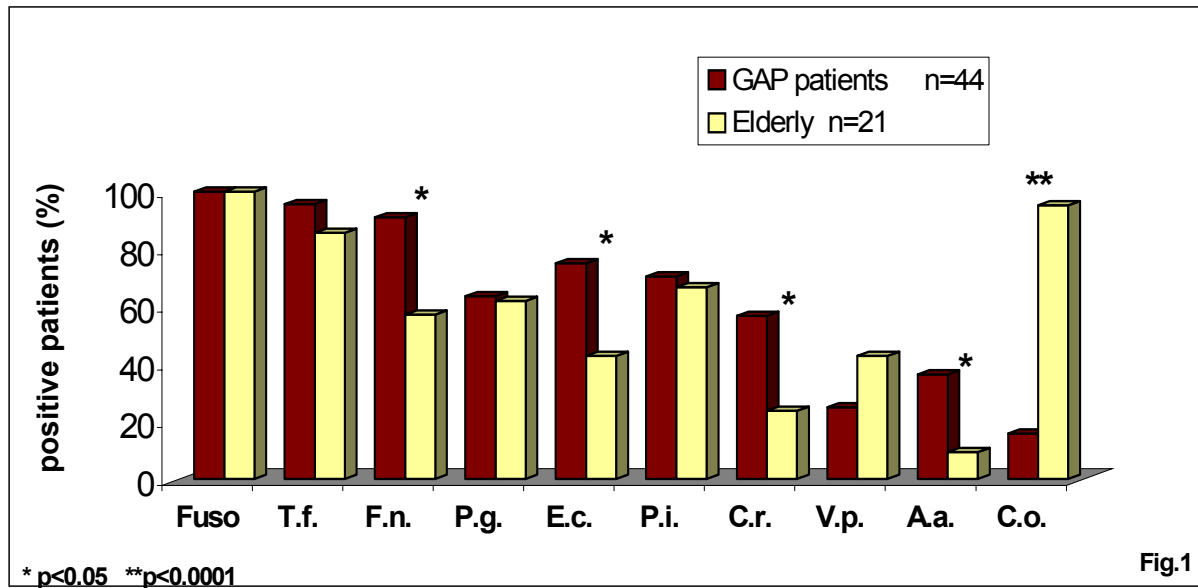

Fig. 1 Bar chart showing the prevalence of the respective species in 44 GAP patients (one GAP patient was excluded because of a missing control site) and 21 elderly subjects. A patient was regarded positive when at least one site harbored the respective species. The significance of differences between the groups was calculated using the chi-square test.

The detection frequency of the bacteria in periodontal lesions and healthy control sites of 44 GAP patients was studied (Fig. 2). One arbitrarily chosen pocket and one control site per patient was included in the analysis. The patients revealed a high number of pockets colonized by *Fusobacterium* spp. (97.7%), *F. nucleatum*/F.p. (70.5%), *T. forsythensis* (88.6%) and *P. gingivalis* (59%) (Fig. 2). *P. intermedia*, *E. corrodens* and *C. rectus* could be identified in 30%-40% of the pockets. The comparison between positive pockets and control sites demonstrated a highly significant difference ($p < 0.001$) for *Fusobacterium* spp., *F. nucleatum*, *T. forsythensis* and *P. gingivalis*. These species, as well as *C. rectus* and *P. intermedia*, were more frequently detected in diseased sites as compared to clinically healthy sites. Low detection frequencies in periodontal lesions were observed for *A. actinomycetemcomitans* (20.5%), *V. parvula* (6.8%) and *C. ochracea* (2.4%). However, all the investigated species (except *V. parvula*) could be identified in the control sites, but less frequently (Fig. 2).

Colonization of sites without clinical signs of a disease is still remarkable. *T. forsythensis* could be identified in 34%, *F. nucleatum* in 25%, *P. gingivalis* and *E. corrodens* in 22.7%, *P. intermedia* and *A. actinomycetemcomitans* in 15.9% of the control sites of GAP patients (Fig. 2).
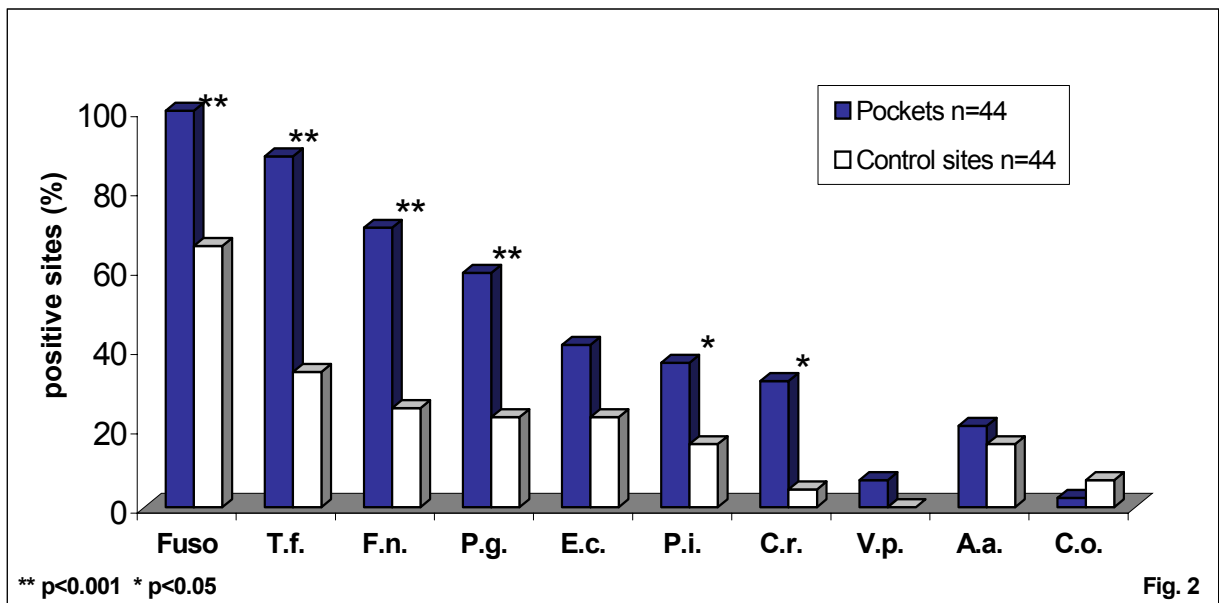
Fig. 2. Percent of positive periodontal pockets and control sites in 44 GAP patients. The significance of differences was evaluated using the chi-square test.

More detailed information about the differences in the prevalence of species between the two groups could be gained from the comparison of the number of positive pockets of GAP patients and positive sites of the elderly (Fig. 3). From every subject four sites were included (from GAP patients only pockets) into the calculations. Pronounced discrepancies were observed between the groups. All species, except *V. parvula*, were significantly more frequently detected in the pockets of GAP patients as compared to the elderly (Fig. 3). For most of the species (except *P. intermedia*) the difference was highly significant ($p<0.0001$). Interestingly, *T. forsythensis* was identified in as much as 48.8%, *P. gingivalis* in 32.1%, *F. nucleatum* in 25% and *P. intermedia* in 28.6% of the sites of elderly. The prevalence of *V. parvula* was similar in both groups (15% positive sites). In the elders *E. corrodens*, *C. rectus* and *A. actinomycetemcomitans* were detected with lowest frequency (8.3%, 9.5% and 3.6%, respectively). Only *C. ochracea* was found significantly ($p<0.0001$) more frequently in the control group (54.8% positive sites) (Fig. 3).
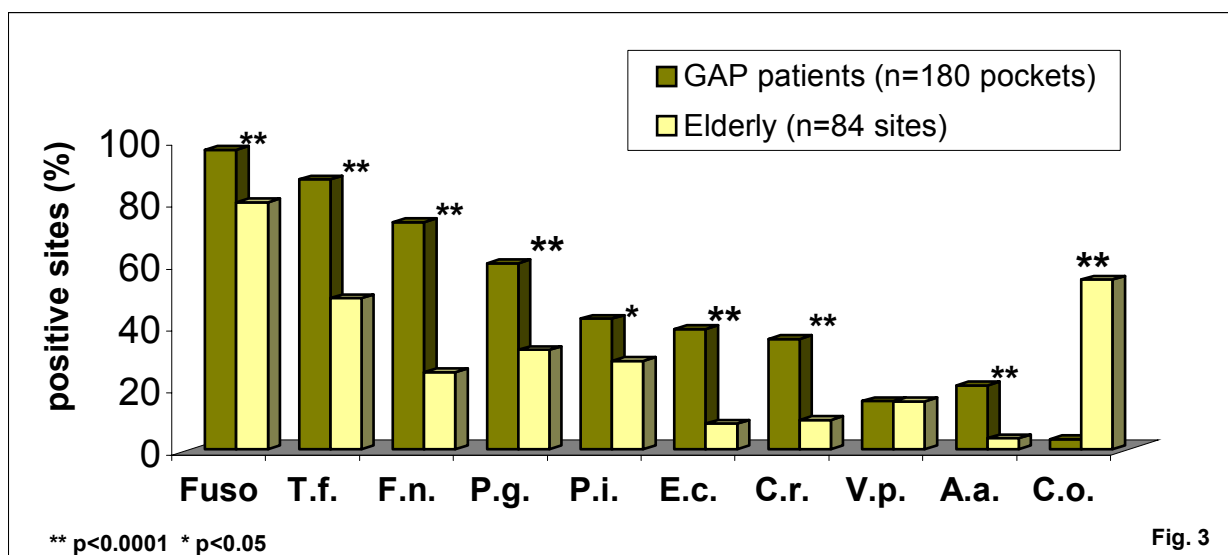
Fig. 3. Site-prevalence of the species in 45 GAP patients and 21 elderly (4 sites per subject were included). The significance of the differences between the groups was determined using the chi-square test.

The comparison of shallow sites (PD 1-3 mm) of GAP patients and the elderly revealed no significant difference for most of the species (Fig. 4).
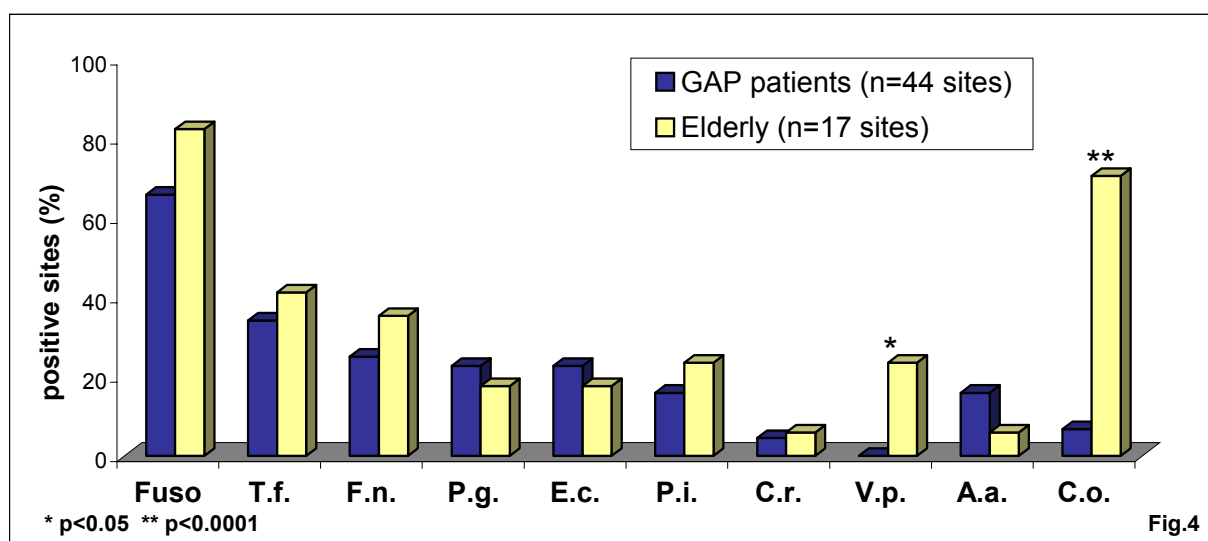
Fig. 4. Comparison between the colonization of shallow sites (PD 1-3 mm) in 44 GAP patients and 17 elderly. Only one site per subject could be included. The significance of differences was evaluated using the chi-square test.

The exception was *C. ochracea*, which was more often detected in the elderly subjects (70.6% in elderly versus 6.8% in GAP patients). The difference was highly significant (p<0.0001). *V. parvula* could be detected more frequently from the shallow sites of the elderly as well (p<0.05). However, the total number of shallow sites in the control group was very

small (n=17). As only one control site was sampled from the GAP patients only one arbitrary chosen site from the elderly subjects could be included in the statistics.

Comparison of the presence of the bacteria between sites with PD 1-3 mm and pockets with PD 4-7 mm in the GAP patients demonstrated a significant difference for most of the species. Only 23 patients with available samples from every pocket depth category could be included in the statistics. The 4-7 mm pockets of the GAP patients were colonized significantly more frequently by the investigated bacteria than the control sites, with the exception for *A. actinomycetemcomitans*, *C. ochracea*, and *V. parvula* (Fig. 5).
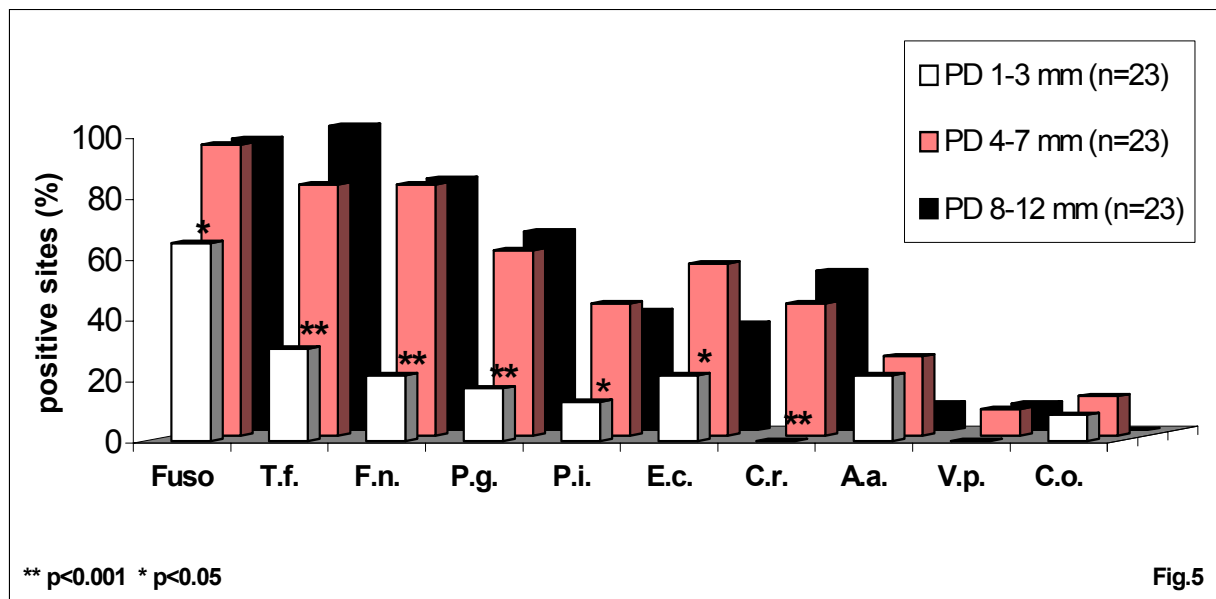
Fig. 5. Bar chart of the percent of positive sites with probing depth 1-3 mm, 4-7 mm and 8-12 mm in GAP patients (n=23). The significance of differences was determined using the chi-square test.

The presumption that the putative pathogens occur mostly in deep pockets of periodontitis patients could not be confirmed. No significant difference was observed between the number of deep and moderate lesions positive for any species in the GAP patients. *C. ochracea* was never detected in deep pockets (Fig. 5).

The bacterial load in a patient was determined by the number of positive sites. In general, fewer sites in a patient were colonized by putative periodontal pathogens in the healthy elderly than in the GAP group (Fig. 6). The data for *T. forsythensis* and *F. nucleatum* showed highly significant difference (p<0.0001).
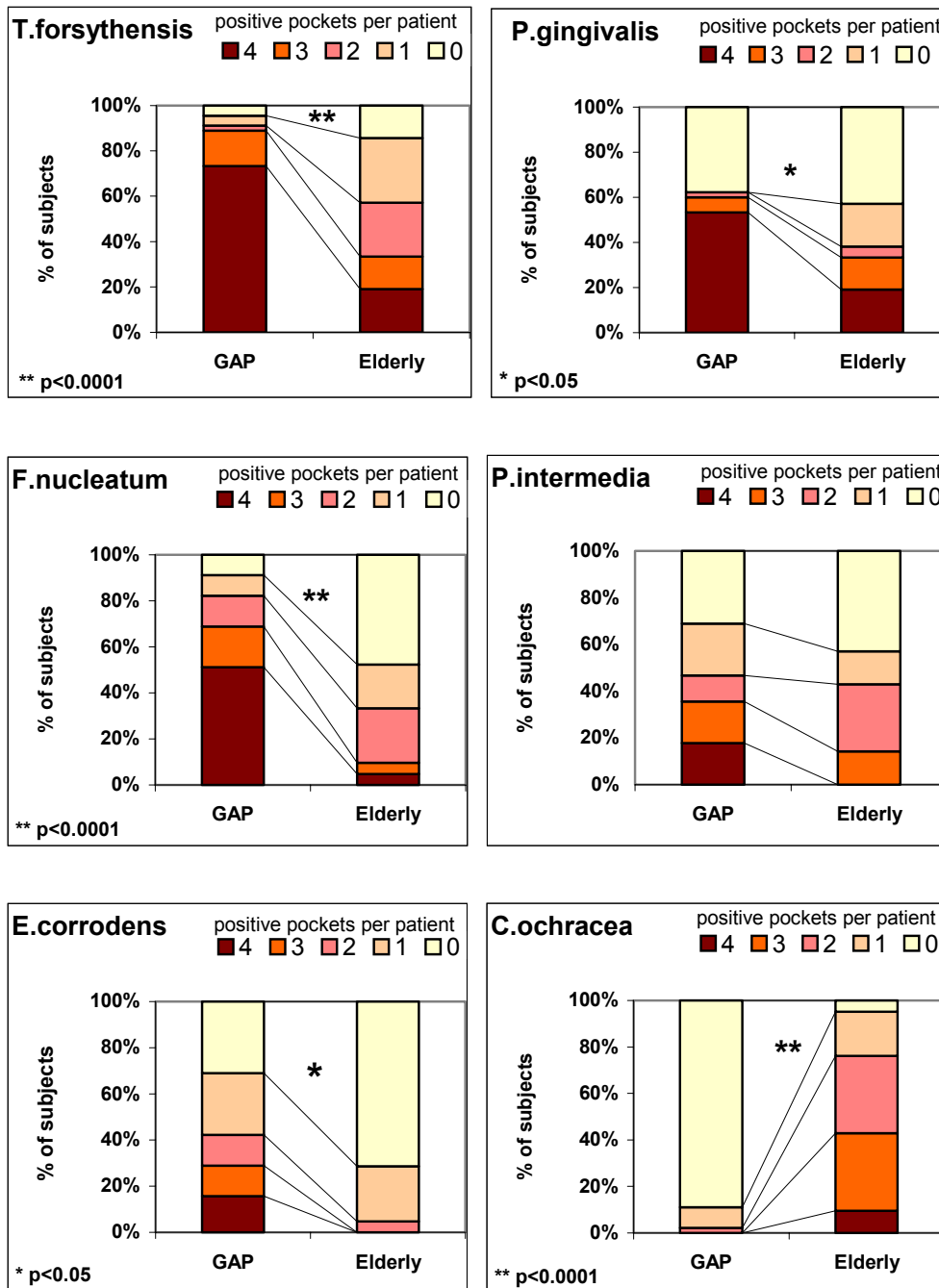
Fig.6. The bars depict the percent of subjects with either 0, 1, 2, 3 or 4 positive sites for *T. forsythensis*, *P. gingivalis*, *F. nucleatum*, *P. intermedia*, *E. corrodens* and *C. ochracea* in GAP group (n=45) and elderly (n=21). The significance of differences between the groups were determined using the Mann-Whitney test.

The load of *T. forsythensis*, *P. gingivalis* and *F. nucleatum* was high in the GAP patients. The number of patients with four positive pockets was 73.3%, 53.3% and 51.1%, respectively. The load of these species was significantly lower in the elderly. However, 37.7% of the GAP patients revealed no colonization of *P. gingivalis* in any sampled pocket. In comparison with the elderly significantly more pockets per patient in the GAP group were positive for *E. corrodens*, *C. rectus* and *A. actinomycetemcomitans* (p<0.05). Interestingly, *A.*

actinomycetemcomitans occurred in all four sampled pockets in 11% of the GAP patients. The load of *P. intermedia* and *V. parvula* showed no significant difference between the groups.

C. ochracea consistently was detectable in more than one site in the healthy subjects. The load of this species was significantly (p<0.0001) higher in the elderly subjects than in the GAP patients (Fig. 6).

5.4 Bacterial consortia

An attempt to investigate bacterial profiles in subgingival areas and assess how complexes of bacteria relate within an ecosystem was made by Socransky et al. (177). A simplified approach using the 2x2 contingency tables was applied in the current study to quantify strengths of associations between two bacterial species. Odds ratio (OR) >2 shows a positive and <0.5 a negative association.

Table. 2. Odds ratios of associations among investigated species. The value 0 is the result of the absence of one species in any site in one group.

	P.g.	P.i.	F.n.	Fuso	T.f.	C.r.	E.c.	A.a.	V.p.	C.o.
P.g.	--	--	--	--	--	--	--	--	--	--
P.i.	2,7	--	--	--	--	--	--	--	--	--
F.n.	1,5	1	--	--	--	--	--	--	--	--
Fuso	12,9	14,3	--	--	--	--	--	--	--	--
T.f.	23,5	10,7	9,1	0	--	--	--	--	--	--
C.r.	2	2,3	10,5	0	35,6	--	--	--	--	--
E.c.	0,2	0,7	6,3	3,6	1,4	0,2	--	--	--	--
A.a.	0,5	0,9	2	0,8	0,6	0,8	5,8	--	--	--
V.p.	0,8	0,7	0	0	2,3	3,5	5,7	1,6	--	--
C.o.	0,4	0,5	0	0	0,3	1,5	4	2,7	8,9	--

A strong positive association exists between *T. forsythensis* and *C. rectus* (OR 35.6), *P. gingivalis* (OR 23.5) or *P. intermedia* (OR 10.7). *Fusobacterium* spp. occurred frequently together with *P. gingivalis* (OR 12.9) and *P. intermedia* (OR 14.3). *F. nucleatum* was observed frequently with *C. rectus* (OR 10.5). *E. corrodens* and *A. actinomycetemcomitans* occurred together relatively often (OR 5.8).

A negative association was observed between *E. corrodens* and *C. rectus* (OR 0.2) or *P.*

gingivalis (OR 0.2).

6   Discussion

The oral cavity presents an ecosystem where the members of the indigenous microbiota have no adverse effects on the host as long as the host-bacterial relationship is in balance. The same flora, or some members of this flora, may cause periodontal disease if the general resistance of the host or the local resistance of the gingival tissues is reduced. Bacteria can be considered the primary etiological agents in the periodontal disease process, but the clinical extent and severity of the disease is modified by both environmental and host risk factors. Most of the tissue destruction comes from the direct effect of the bacteria, together with the resulting inflammatory and immunological host responses. Understanding these interrelations between microbial activity and host response is crucial for preventive or therapeutic measures. A number of possible etiologically relevant pathogens have been suggested based upon the strength of the evidence of their association with disease, animal pathogenicity, and virulence factors. Attempts have been made to find etiological associations of certain bacterial pathogens with clinically different forms of periodontitis. A small subset of microorganisms has been suggested as primary etiological agents in the pathogenesis of aggressive periodontitis. However, the epidemiological studies have not yet revealed any constant correlation between the different bacteriological parameters that lead to a diagnosis of aggressive periodontitis (52, 180).

The aim of the present investigation was to study the associations of the suggested pathogens with generalized aggressive periodontitis and the periodontal status of healthy elderly individuals using molecular biological methods.

6.1   Detection methods

A variety of methods with different sensitivity and specificity have been used to detect and identify microorganisms complicating the comparability of studies.

Selective bacterial culture has been the classical method used to identify and enumerate the most probable number of specific microorganisms in clinical specimens. However, this approach is hampered by the fact that it does not accurately reflect true microbial populations, as many species, especially anaerobes, cannot be grown in vitro due to their fastidious nature or that they are unable to survive the stress of sampling, dispersion, oxygen exposure, or lack of suitable nutrients in the culture media (181, 182). An additional disadvantage of the cumbersome culturing technique is the limited number of samples and subjects that can be investigated.

In earlier research an inadequate identification and taxonomy could have led to misinterpretation. For instance, the common term "black-pigmented Bacteroides species" comprises a broad category of gram-negative anaerobic rods that form black-pigmented

colonies, only some of which are species relevant in gingivitis, periodontitis, endodontic infection and odontogenic abscesses (183,184). The development in phylogenetic analysis of 16S rRNA has clarified the position of the Bacteroides subgroup and its clusters in the phylogenetic classification (185). The use of inaccurately identified and characterized type strains raises problems not only in taxonomic studies, but also for the subsequent identification, classification, and characterization of clinical isolates (186).

Culture-based techniques, though not adequate, are often used as a reference against which other tests like nucleic acid hybridization assay, PCR amplification, or immunological methods are compared and validated (161, 173, 187-189). Often the culture-independent methods showed the presence of the bacterium, but the bacterial culture was negative (11, 161, 164). It has been assumed that the culture-based method especially underestimates the presence of *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythensis* (11, 161, 173, 189-192). Conrads however, demonstrated high conformity between the bacterial culture and the hybridization assay for the detection of *P. intermedia* and *P. gingivalis* (193). The culture-independent methods are definitively superior in detecting fastidious anaerobes, as well as in identification of cultivable bacterial strains with phenotypically divergent behaviour. Because of the absence of an indisputable reference standard, no definitive conclusions may be drawn on the capability of any given method to better reflect reality. However, the use of inadequate uncontrolled detection methods has often resulted in an incorrect microbiologic analysis.

Immunoassays which use either species-specific polyclonal antiserum or monoclonal antibodies (mAb) are sensitive, and allow enumeration of bacterial cells, but need exhaustive microscopy. The disadvantage is that some members of a given species can be non-reactive with the serological agents available so far or that other bacteria express cross-reacting epitopes (181, 194).

Gmür reported about the frequent detection of *T. forsythensis* in high numbers by immunofluorescence, but the culture analysis was consistently negative (195). It was necessary to use three mAbs for distinct epitopes of *T. forsythensis* in order to rule out false negative results (182). For *P. gingivalis* and *P. intermedia* culture scores were similar or lower than those obtained with the serological technique.

Microbiology has recently entered a state of transition, changing from traditional culture-based methods towards the identification of specific nucleic acid sequences by applying more sensitive nucleic acid hybridization and in vitro amplification techniques (161, 164). Besides culturable organisms the methods enable the detection and identification of yet uncultured bacteria (168). However, false positive as well as negative reactions of the molecular genetic methods have to be considered (187, 188, 196). On the other hand, the higher sensitivity of the culture-independent assays can explain the discrepancies between

the methods (173). The specificity of the assays is obviously dependent on the accuracy and completeness of the database, as well as the selection of the target sequence. This seems to be an inherent limitation of the experimental design since probe design relies on the extent and the availability of bacterial DNA sequences (197).

The polymerase chain reaction (PCR) can detect as few as 10-50 CFU/ml in a pure culture and has, therefore, the highest sensitivity of any microbiological method (181, 198). The detection limit in artificially infected subgingival plaque is approximately 102-103 CFU/ml (199, 200). The detection of minute amounts of bacteria with ultrasensitive methods is probably clinically irrelevant. Bacterial amounts below the detection level of 102 CFU/ml are produced by transient rather than by colonizing microorganisms (201).

The PCR-amplification using species-specific primers has been broadly used for the detection of periodontal bacteria. Several targets for primer annealing have been reported, as for example *A. actinomycetemcomitans*' leukotoxin gene-directed primers (199), for *P. gingivalis* collagenase gene-directed primers (200), and for several periodontal species 16S rRNA gene (rDNA)-directed primers (11). False-positive results with the PCR method can not be excluded. Phylogenetically closely related species might have 16S rRNA genes that differ in only a few nucleotides and might not be distinguishable by 16S rRNA gene analysis. Also the conditions of PCR performance (annealing temperature, magnesium concentration) are crucial in order to avoid cross-reactivity (198, 199). Bacterial species not thoroughly studied can contain genes with some homology with the target gene. Therefore, the identity of an amplicon has to be confirmed by an additional detection assay, e.g. hybridization with a probe different from the primers or by sequencing (162). The PCR-amplification method is prone to contamination, possibly leading to false positive results.

False-negative results can emerge from PCR performance failures due to inhibitors or from an incomplete knowledge of heterogeneity of the target genes. Molecular analysis of 16S rRNA genes gained from subgingival bacterial samples revealed a diversity of clones for the investigated species (202). Only 70% of all the analyzed sequences showed a similarity of at least 99% identical to investigated periodontal pathogens.

The sequence analysis of the collagenase gene of *P. gingivalis* clinical isolates verified the genetic heterogeneity among the clinical strains (203). Furthermore, not all *P. gingivalis* strains contain the selected fragment of the collagenase gene (200). As a consequence, not all strains of a given species will be the targets of the detection assay. This can be of advantage in detecting virulent strains. However, the biological relevance still remains unclear.

Nucleic acid probe assays use a piece of DNA or RNA - either a whole-genomic probe, a cloned probe, or a synthetic oligonucleotide probe - to hybridize to a complementary nucleic acid sequences in the target microorganism.

The whole-genomic probes are widely used in the detection of periodontal species (36, 191, 204-206). Socransky et al. introduced a checkerboard DNA-DNA hybridization assay for hybridizing large numbers of DNA samples with multiple whole-genomic DNA probes on a single support membrane (207). This method requires sophisticated laboratory equipment and expertise. The disadvantage of genomic probes is the cross-reactivity of 1%, as well as their undefined composition from pooling the probes (193, 205). The simultaneous use of multiple (up to 45) probes under identical hybridization conditions in the checkerboard DNA-DNA hybridization assay leads to doubts about the accuracy of its performance. The sensitivity of the assay is not high, 103-104 bacterial cells are necessary for a positive identification (207).

In 1988 Chuba, Göbel et al. introduced the 16S rRNA-directed oligonucleotide probes, to detect *P. gingivalis*, *P. intermedia* II, *P. assacharolytica*, and *A. actinomycetemcomitans* (208). The specificity of the probes was 100% and the detection limit using isotopical labeling was less than 5x103 organisms. Dix, Moncla et al. developed species-specific 16S rRNA-directed oligonucleotide probes for further periodontal species and demonstrated their higher specificity and sensitivity upon the genomic probes (209). The oligonucleotide probes are able to distinguish between closely related species which contain homologous sequences, for example, H. aphrophilus and *A. actinomycetemcomitans*. The whole-genomic probes failed to differentiate between these species (209). An additional advantage is the high detection sensitivity because the number of rRNA target molecules is larger, being at least 100 times greater than that of bacterial DNA targets (210). However, the isolation of nucleic acids plays a crucial role for maximum sensitivity of the DNA/RNA hybridization procedure (211). The oligonucleotide probes are suitable for specific detection of bacterial species in highly heterogeneous plaque samples. The short oligonucleotide probes complementary to hypervariable regions of 16S rRNA have been used frequently (172, 173, 176, 212-214).

The culture - independent molecular genetic method used in the present study was based upon the combination of PCR-amplification and dot-blot hybridization with species-specific 16S rDNA-directed oligonucleotide probes. The PCR-amplification was performed within the conserved region of 16S rDNA of bacterial cells using eubacterial primers. The amplification step increases the sensitivity of the detection method over the conventional nucleic acid hybridization.

The issue of heterogeneity within a species was considered by the design of the probes. The appropriate target sequence had to have 100% homology to the sequences of all strains of a species deposited in a database (as of July 2002). Thorough control was considered crucial to ensure the quality of the hybridization results. Therefore, all probes were checked in dot-blot hybridization against a wide range of possible cross-reacting strains.

Only the probe for *F. nucleatum* additionally detected a related species, *F. periodonticum*. This was also reported by Dix et al. (209) and Socransky et al. (215). Sequence homology of

16S rRNA between these two species is exceedingly high, 97.3% - 99.5% (178). *F. periodonticum* has been detected infrequently in periodontitis patients and healthy individuals, thus the role of that species is unclear (34, 216).

With the described method it is possible to investigate large numbers of samples in a reasonable time. This allows comprehensive epidemiological studies of the subgingival microbiota in subjects with different clinical status. The methods used in the present study represent qualitative data. No quantification attempt has been undertaken.

In several studies the quantitative detection of microorganisms has been performed. However, quantification in fact proves nothing more than the mere presence of a microorganism. It is obvious that the mean numbers of bacteria increase as the pocket depth progresses. Therefore, it is more informative to know the proportion of the target species in a total bacterial mass. It has long been known that there is a shift in the proportions of microorganisms in the flora as a site progresses from a healthy to a diseased state (217). The culture-based method and immunoassays are not adequate for the proportional estimation. The competitive PCR method has been used for the quantification, however the amplification distorts the endpoint proportions of amplicons of target species (218). The recently developed real-time PCR assay has overcome the so-called "plateauing effect" by measuring the PCR-products throughout the reaction (165). Simultaneous PCR-amplification and hybridization by this method allows quantification of a single species and total number of bacterial cells in a sample using specific controls of known quantity. However, the exact copy number of 16S rRNA operons within each cell of the numerous species of oral bacteria has not been clarified and doubling time varies among bacterial species. This represents the major limitation to the absolute determination of bacterial numbers by real-time PCR based on the 16S rRNA gene sequence (197, 219). The requirement of expensive and sophisticated technology besides mentioned shortcomings in accuracy limits the application of this method as a routine clinical diagnostic tool.

Generally, the evaluation of quantitative aspects is complicated by the fact that amounts of bacteria depend on the method of sampling, the number of samples taken in a subject, the site selection criteria, the method used to calculate counts based on sampling volumes and sample dilution, and the way mean counts are determined (physical vs. mathematical pooling of multiple samples) (52).

Although different methods have been applied for the detection and quantification of periodontal bacteria in microbiological diagnosis, there is no single assay that has demonstrated ideal characteristics.

Nevertheless, besides methodological differences, the study design i.e. site selection, and the number of samples taken in a subject, influences the microbiological results of the study (52).

6.2 Epidemiological study

6.2.1 *Tannerella forsythensis*

Initially, *Tannerella forsythensis* was thought to be a relatively uncommon subgingival species. The studies of Gmür et al. using monoclonal antibodies to enumerate the species directly in plaque samples suggested that the species was more common than previously found in culture-based studies (182). According to the extensive literature bacterial culture is considered inadequate for the detection of *T. forsythensis* (161, 192). Therefore, studies using this method will not be discussed here.

Recently, 16S rDNA sequence analysis confirmed that *T. forsythensis* (formerly *Bacteroides forsythus*) was not a species within the genus *Bacteroides* sensu stricto. A new genus, *Tannerella*, was proposed for *B. forsythus*, with one species, *Tannerella forsythensis* (220). Interestingly, the nearest genetic neighbour, oral clone BU063, has been associated with oral health (221). The oligonucleotide probe used in the present study is not complementary with that clone. It is conceivable that earlier studies mistakenly pooled several strains of *T. forsythensis* with different pathogenic potentials.

In the current study *T. forsythensis* was one of the species with a high prevalence (96%) in patients with generalized aggressive periodontitis (Fig.1). Additionally, the patients showed high load of *T. forsythensis*, i.e. 73.3% of patients had all pockets sampled positive for this species (Fig.6). *T. forsythensis* could be detected significantly more often in periodontal pockets than in healthy control sites (88.6% and 34.1% respectively) (Fig.2). However, no significant differences in detection frequency between different pocket depths could be seen (Fig. 5). Gmür et al. using a quantitative approach could demonstrate that the levels of *T. forsythensis* were strongly related to increasing pocket depth (182). Young patients with aggressive periodontitis studied by Kamma et al. (170) revealed a similarly high prevalence of *T. forsythensis* (98.5%). In that investigation indirect immunofluorescence assay was used. The site prevalence (83% positive pockets) was in good agreement with our study. However, no control sites were sampled.

In young adults with advanced periodontitis a high prevalence of *T. forsythensis* (60-70%) has been reported by using DNA probe approach (191, 206, 212). Similar results were obtained with a PCR-assay (11, 14).

Studies with the aim of correlating clinical parameters and the presence of certain bacterial species concluded that *T. forsythensis* was positively correlated with the clinical signs of a disease and can be regarded as a risk indicator for attachment and bone loss (145, 146, 222). Persistence of *T. forsythensis* post-therapy and the subsequent deterioration of periodontal conditions suggests evidence for the species association with recurrent periodontitis (156). It has been shown that with the reduction of *T. forsythensis* below the detectable level clinical improvement was significant (151). However, clinical improvement is

never a consequence of a reduction / elimination of single species.

Choi et al. used a detection method similar to our study for the evaluation of the prevalence of *T. forsythensis* in patients with chronic periodontitis and in healthy persons (176). High prevalence (96.6%) was reported in chronic periodontitis patients. The prevalence was significantly lower in healthy persons. However, the comparison between the groups is unreliable because of incorrect statistics.

More sensitive microbiological tests have been of advantage for detecting *T. forsythensis* in healthy subjects. The current study particularly identifies *T. forsythensis* as a much more frequent member of the microflora of healthy sites than previously suspected on the basis of culture investigations. This contradicts the hypothesis that the mere presence of *T. forsythensis* may be taken as an indicator of active periodontal breakdown. *T. forsythensis* could be detected in 85.7% of the elderly (Fig.1). Almost half of the sampled sites in this patient group were positive (Fig.3). It has been reported that the colonization of supragingival plaque of healthy subjects, even in early childhood, is frequent (34, 35, 223-226). These data suggest the indigenous nature of *T. forsythensis*. However, in special periodontal / host conditions or specific proportional bacterial constellations *T. forsythensis* can induce periodontal breakdown. The presence of putative pathogens increases the risk of disease development. The presence of *T. forsythensis* has been especially correlated with attachment loss, increasing the risk by a factor of 2.45 - 5.3 (36, 145, 227). Even 8.16 times greater odds of attachment loss by presence of *T. forsythensis* has been reported in a longitudinal study (222). A significant increase in the proportions of *T. forsythensis* has been observed in sites with periodontal breakdown (182).

Little information is available on the virulence of *T. forsythensis*. Beside the LPS and the production of a trypsine-like protease (228) its ability to penetrate host cells and to induce apoptosis has received attention (84, 229). Recently, a cysteine protease gene (prtH) has been identified (226). It was shown that 85% of *T. forsythensis* isolates from diseased sites revealed a prtH genotype, however only 10% of such strains could be detected in healthy sites. There is a need for more comprehensive research of the virulence and taxonomic variability of *T. forsythensis*.

The evidence for associating *T. forsythensis* with severe periodontal disease has mainly been based on epidemiological data and evaluation of the correlation between the presence of the species and clinical conditions.

The data of the present study support the evidence of *T. forsythensis* being a periodontopathogen. The species was significantly more frequently detected in periodontal pockets than in control sites of GAP patients (Fig. 2). Also, the significantly higher prevalence and load of *T. forsythensis* in GAP patients than in healthy elders (Fig. 3, 6) indicates its strong association with aggressive periodontitis in young adults.

6.2.2   *Porphyromonas gingivalis*

Porphyromonas gingivalis is the most intensively investigated periodontal species. It has frequently been associated with severe periodontitis (10, 15, 31, 167, 230). The culture-based approach can be considered adequate for the detection of *P. gingivalis*, as was shown by Conrads through comparative analysis using DNA hybridization (193).

The prevalence of *P. gingivalis* in the generalized aggressive periodontitis group was high, 63.6% in the present study (Fig.1). Interestingly, most of these positive subjects showed colonization with *P. gingivalis* in all sampled pockets (Fig.6). The species was significantly more frequently detected in periodontal pockets than in control sites (59.1% and 22.7%, respectively) (Fig. 2). The site-prevalence was significantly lower in the elderly group than in the GAP population (Fig.3). *Porphyromonas gingivalis* could hence be associated with the development of aggressive periodontitis.

Loesche et al. recovered high proportions of *P. gingivalis* along with high proportions of spirochetes from one EOP patient group, so-called "type B" patients (13). This group resembles the GAP entity as derived from clinical descriptions. These patients had significantly higher proportions of *P. gingivalis* than in four other patient categories. Also Alabandar et al. associated *P. gingivalis* and *Treponema denticola* with more severe and progressive forms of EOP (230). Kamma et al. recovered more frequent and significantly higher proportions of *P. gingivalis* from deeper periodontal pockets as compared to shallow sites in RPP patients (15). Her recently published data obtained by using indirect immunofluorescence technique revealed higher detection frequency of *P. gingivalis* in aggressive periodontitis group than the results of our study (170). The subject prevalence was as high as 89.4%, and 80% of the sampled pockets were positive for *P. gingivalis*. *P. gingivalis* has also been strongly associated with chronic periodontitis (176, 231, 232) and recurrent periodontitis (142). Several investigators have found a significant correlation between proportions of *P. gingivalis* and attachment loss (39, 137, 182, 233).

Contradictory data exists on the presence of *P. gingivalis* in healthy periodontal conditions. In the current study, a relatively high prevalence (62%) was observed in the elderly with no significant difference to the periodontitis group (Fig.1). However, only 32% of the sampled sites of elderly were positive (Fig. 3), showing infrequent colonization of *P. gingivalis* in the control group. The difference to the periodontitis group was highly significant (p<0.0001). The species was rarely found in shallow sites (Fig.4).

Healthy persons investigated by Tanner et al. were not colonized by *P. gingivalis* (36). This was proven by bacterial culture and checkerboard hybridization methods. Healthy subjects and the elderly investigated by Haffajee et al. showed very low site-prevalence of *P. gingivalis* (4% and 5% respectively) (34). Absence of *P. gingivalis* in pre-school children and students confirmed by PCR assay demonstrates that this bacterium is usually not part of the

resident oral flora in young healthy people (201, 224). Other authors have found a prevalence of 10-30% in older healthy subjects (232, 234). It raises the question whether these individuals may carry different, possibly less-virulent strains of *P. gingivalis*, or does the host response determine the outcome?

There is extensive evidence of the pathogenic nature of *P. gingivalis* (s. 1.2.6.). However, it is complicated to prove the relevance of the reported virulence factors in vivo. Additionally, it has been proven that virulence varies among strains. *P. gingivalis* is able to invade human gingival epithelial cells in vitro (76). In a mouse model different strains of *P. gingivalis* exhibited varying levels of invasiveness (235). Proteolytic enzymes have been suggested as a possible virulence factor. However, no differences in proteolytic enzyme production between invasive and non-invasive strains could be demonstrated (236).

As yet it is unknown which virulence factor correlates with more virulent *P. gingivalis* strain. In animal studies, strains W 50 or W 83 were highly virulent. Various strain-typing approaches (RFLP, ribotyping, serotyping, multilocus enzyme electrophoresis) have been used to identify highly virulent genotypes and to correlate them with disease. As yet, there is still no convincing evidence to associate specific genetic clone clusters with periodontal disease and hence numerous *P. gingivalis* genotypes were associated with disease (234, 237). Several studies confirm that patients are usually colonized by a single, unique genotype, present in sites with different clinical status. (237, 238). Healthy subjects were likely to harbour several strains (234).

Obviously, there are various virulence factors of *P. gingivalis* playing a role in the etiopathogenesis of periodontitis. Therefore, an attempt of epidemiological studies to detect virulent *P. gingivalis* strains by targeting only one suspected virulence gene is questionable. Currently it is not possible to associate any genotype with aggressive periodontal disease. *P. gingivalis* can hence be regarded as an opportunistic pathogen. This evidence is supported by the low prevalence of *P. gingivalis* in healthy adults. Under suitable yet unknown environmental / host conditions certain strains of *P. gingivalis* can multiply and express virulence factors inducing disease development. Frequent detection of *P. gingivalis* together with *T. forsythensis* in active/deep periodontal pockets supports the evidence that certain consortia are of particular importance in progressive disease. The present epidemiological data have confirmed a strong association of *T. forsythensis* and *P. gingivalis* with generalized aggressive periodontitis.

6.2.3   *Actinobacillus actinomycetemcomitans*

Actinobacillus actinomycetemcomitans is considered to be of major etiologic relevance in the localized form of aggressive periodontitis (LAP) (14, 26, 49, 50, 239). The role of *A. actinomycetemcomitans* in generalized aggressive periodontitis is still unclear. The study presented here showed a low prevalence of this species (36.4%) in GAP patients (Fig.1).

However, in 11% of the patients *A. actinomycetemcomitans* was present in all sampled pockets (data not shown). Generally, the species was evenly distributed in periodontal pockets and shallow sites (Fig.2). There is no preferential niche in diseased sites (Fig.5). In patients with a high load of *A. actinomycetemcomitans* the species can play an important role (240). It cannot be ruled out that some of the GAP patients previously had the localized form of aggressive periodontitis.

Kamma et al. (170) found a low prevalence (25%) of *A. actinomycetemcomitans* in patients with aggressive periodontitis, similarly to our results. Tanner et al. detected high proportions of *A. actinomycetemcomitans* in young adults with advanced disease (241). Older subjects with advanced disease did not harbour this organism. Similarly, older GAP patients investigated by Loesche et al. were negative for *A. actinomycetemcomitans* (13). Slots et al. observed that the prevalence of *A. actinomycetemcomitans* decreased significantly with age. The authors reported that 74.4% of the 15-24 year-age group were colonized by the microorganism compared to 38.7% of those 25-34 years of age (242).

Van Winkelhoff et al. observed a negative association between *A. actinomycetemcomitans* and *Fusobacterium* spp. in subgingival plaque (243). The authors followed LJP patients and found that *A. actinomycetemcomitans* was not recovered after these patients reached middle-age and developed more widespread periodontal breakdown. It may be that fusobacteria possess the capability to inhibit the colonization of *A. actinomycetemcomitans* as the genus *Fusobacterium* emerges as a prominent taxon of generalized EOP (243). Similarly, in the study presented here *Fusobacterium* spp. showed a high site-prevalence (96.7%) and negative association with *A. actinomycetemcomitans* (Table 2).

There are, however, studies demonstrating a low prevalence of *A. actinomycetemcomitans* even in LJP patients (13, 244). Obviously, *A. actinomycetemcomitans* plays an important role in some cases of localized and generalized forms of aggressive periodontitis. The evidence of this association can be traced back to the virulence of the species, epidemiological data and correlation with improvement after treatment.

Single clones of exceedingly high virulence may be implicated in etiopathogenesis of periodontitis. *A. actinomycetemcomitans* strains differ in their ability to produce leukotoxin. This exotoxin lyses polymorphonuclear leukocytes and macrophages (106). Highly leukotoxic strains produce 10-20 times more leukotoxin than other strains (245). Bueno et al. found that subjects harbouring *A. actinomycetemcomitans* with a 530-bp deletion in the leukotoxin promoter region were more likely to convert to LJP than subjects who had A.a.-variants containing the full-length leukotoxin promoter region (246). In addition to the predominant colonization of *A. actinomycetemcomitans* in younger patients, it has been observed that very young patients (mean age 12.7 y.) harboured highly toxic strains (245). Young adults (mean age 25.5 y.) were colonized by minimal toxic strains. However, since the strains without the 530-bp deletion have been recovered from LJP lesions as well (245), high

leukotoxin production may not be a prerequisite for pathogenicity. No epidemiological study looking of the distribution of highly virulent strains has been performed with GAP patients. *A. actinomycetemcomitans* strains also vary in their capability to invade epithelial cells (247). The failure of non-surgical therapy to effectively control *A. actinomycetemcomitans* from subgingival sites may stem from the ability of the organism to invade gingival tissue and thereby evade the effect of mechanical debridement and periodontal healing (248). Investigation of biofilm formation in a plaque-free-zone of the bottom of a pocket demonstrated no participation of *A. actinomycetemcomitans* (46).

Studies evaluating the correlation between treatment outcome and the presence of bacteria in LJP and "severe periodontitis" found a clear association between the improvement of periodontal conditions and the elimination of *A. actinomycetemcomitans* (249, 250). The failure to eliminate the combination of *A. actinomycetemcomitans* and *T. forsythensis* resulted in attachment loss (151). These microorganisms have been detected frequently in refractory periodontal lesions (231). An exceedingly high proportion of subgingival *A. actinomycetemcomitans* in periodontal sites undergoing active breakdown gives substantial credence to the notion of it being a key bacterial pathogen in certain cases of LJP (231). In chronic periodontitis patients the microorganism was not found to be related to an increased risk of disease recurrence (137).

Healthy elderly individuals in our study showed a very low prevalence (9.5%) of *A. actinomycetemcomitans* (Fig.1, 3), demonstrating that the species is not a common colonizer in healthy periodontal conditions. This is in agreement with other reports about healthy individuals (34, 176, 231). Healthy children were shown to be negative for *A. actinomycetemcomitans* by PCR (201, 251). Even patients with chronic periodontitis are seldom colonized with this species (34, 192). Gmür et al. studied dental hygienists and observed a prevalence of 33% in supragingival plaque in spite of their better than average personal plaque control. However, the detected cell numbers were <1% of the sampled microbiota (35). *A. actinomycetemcomitans* has also been found in tongue and saliva samples, even without subgingival colonization (252).

The strains identified in healthy subjects or patients with chronic periodontitis were shown to be minimal leukotoxic strains(245).

Chronic periodontitis patients investigated within the comprehensive epidemiological study with the identical methods described here showed a site-prevalence of only 7% (Dr. Moter, personal communication). Interestingly, Choi et al. could detect this organism in almost 90% of chronic periodontitis patients in a Korean population using a similar detection method (176). The high prevalence can be explained by ethnic, immunological, nutritional factors. Apparently, *A. actinomycetemcomitans* (probably special strains) can be regarded as etiologically relevant in aggressive periodontal disease in young adults, however, the evidence is not as strong as for the localized form (14, 230, 239, 240, 253).

6.2.4 *Campylobacter rectus*

Campylobacter rectus could be detected in more than half of the GAP patients; however, only 31.8% of the pockets were positive (Fig.1,2). Still a significant difference was observed in the colonization pattern of periodontal pockets and control sites in the study population (Fig. 2). When compared to the occurrence in the healthy elderly group a highly significant difference could be seen in site-prevalence (Fig. 3). The species could be rarely detected in healthy population (prevalence 23.8%) (Fig.1).

High levels of *C. rectus*, especially when found together with *T. forsythensis* have been related to an increased risk of disease progression (31, 33, 36, 39, 137, 138, 254). The highest association among all organisms in the current study was observed for these two species (Table 2).

Kamma et al. recovered *C. rectus* exclusively in the deep pockets of GAP patients (15). The microorganism has been seen predominately in the middle and deep pocket zones forming large clumps when examined by scanning immunoelectron microscopy (46). *C. rectus* may advance to the most apical border of plaque area by the use of its motility and associate with biofilm formation in the plaque-free-zone and with disease progression.

The high prevalence of *C. rectus* in aggressive periodontitis patients has been reported in the literature in contrast to low detection in healthy populations (14, 15, 30, 170, 255). The data of Gmür et al. confirm that supragingival plaque is a natural habitat for *C. rectus* in periodontally healthy persons with good oral hygiene (35, 225). The species could be identified in 48% of the investigated subjects using an immunoassay. Between the elderly and the healthy control group no difference was seen (34). *C. rectus* was identified in less than 20% of the subgingival samples. The site-prevalence was not higher in patients with chronic periodontitis (19.3%) (Dr. Moter, personal communication).

Patients with aggressive periodontitis showed significantly higher and more frequent elevation of serum IgG antibody to this organism as compared to chronic periodontitis patients or healthy controls (256).

C. rectus may be considered as a putative pathogen that occasionally, probably in constellation with *T. forsythensis*, contributes to the development of aggressive periodontitis. It may need to exceed relatively high critical threshold values in the subgingival flora to lead to a progression of the disease (257).

6.2.5 *Fusobacterium nucleatum*

According to the literature *Fusobacterium nucleatum* is the most commonly isolated organism in subgingival samples, especially in deep periodontal pockets. It has often been recovered in high proportions in different patient groups (10, 31, 33, 241).

The data of the present study suggest a strong association of *F. nucleatum* with GAP. The species was present in 91% of the GAP patients (Fig.1) and significantly more frequently in

periodontal pockets than in shallow sites (Fig. 2). All 4 sampled pockets were positive for this organism in half of the patients (Fig.6). The load in the elderly subjects was significantly lower. The site-prevalence in the elderly was only 25% (Fig. 3). Haffajee et al. in contrast, identified *F. nucleatum* ss. polymorphum in 58% of the sites of the elderly subjects using checkerboard hybridization method (34).

It is however, difficult to interpret the role of *F. nucleatum* from our data, as the probe simultaneously detected F. periodonticum. There are reports of F. periodonticum being a frequent colonizer of the supra- and subgingival plaque of healthy individuals and periodontitis patients (30).

Furthermore, the phenotypic and genetic heterogeneity of *F. nucleatum* has led to an attempt to classify the strains into taxonomically relevant groups, such as subspecies, but these efforts have not resulted in a widely accepted taxonomy (258). Within the species *F. nucleatum*, a number of investigators have identified a range of distinct genetic clusters which have subsequently been designated as subspecies. Unfortunately the results often do not correlate with each other. Morris et al. could demonstrate that the species *F. nucleatum* consists of at least three distinct species (186). There is a need for revision of the previously designated genetic divisions as well as phenotypic characterization of *F. nucleatum* and genus *Fusobacterium* as a whole.

Therefore, no differentiation between the subspecies was undertaken in the present study. Loesche et al. did not associate *F. nucleatum* with GEOP, because the species formed only ca. 3% of the total bacterial count (13). In the study of Kamma et al. *F. nucleatum* was detected most often in the medium and deep lesions of GAP patients (15). Chronic periodontitis patients exhibited high prevalence of *F. nucleatum* (204). A prevalence of 81.3% was observed in the reported epidemiological study (Dr. Moter, personal communication). The adherence factors of *F. nucleatum* have been attributed to the potential pathogenicity of certain strains, however, no correlation could be established between any particular subspecies (259). *F. nucleatum* could not be detected in the plaque-free zone at the bottom of periodontal pockets suggesting that this microorganism does not primarily participate in the apical progression of plaque (46). The species was usually located in the middle and deep pocket zones in an unattached plaque area.

F. nucleatum has been implicated in disease progression, as significantly higher proportions were detected in active lesions than in inactive sites (39, 140).

Our data provide evidence that *F. nucleatum* might be associated with aggressive periodontitis. The species is only infrequently detected in healthy subjects (Fig.1, 3).

6.2.6 *Prevotella intermedia*

Contradictory reports exist about *Prevotella intermedia*. This microorganism has been found in high proportions in adults with moderate to severe periodontal breakdown and in EOP

patients (13, 183). At the same time, a high prevalence has been reported in treated and maintained patients, in some of them in high proportions (13). Colonization of *P. intermedia* in children has also been observed (201, 224).

Recently, strains of *Bacteroides intermedius* with similar phenotypic traits have been classified into two species, *Prevotella intermedia* and *Prevotella nigrescens*. This distinction makes earlier studies on these organisms difficult to interpret, since data from two different species may have been inadvertently pooled (260). It has been reported that *P. intermedia* is associated with periodontitis, whereas *P. nigrescens* is a natural inhabitant of the gingival sulcus and the supragingival plaque (35, 40, 201, 261).

In the present study no clear association for *P. intermedia* with GAP or periodontal health was found. There was no significant difference in subject-based prevalence and load of *P. intermedia* between the groups (Fig.1, 6). The species was more often detected in the pockets of GAP patients than in the control sites (Fig. 2), and when compared to the positive sites of elderly (Fig. 3). The difference however, was always only moderately significant (p<0.05).

Mullally et al. (14) associated *P. intermedia* with GEOP because of its high prevalence (58.8%), which is lower than in our study. However, no control group was included in the study, reducing the relevance of the results.

Kamma et al. (15) found no difference in the detection frequency of *P. intermedia* between shallow sites and deep pockets, however, the proportions were significantly higher in deep pockets.

Earlier studies using unreliable "predominant cultivable microbiota" method recovered higher proportions of *P. intermedia* in active sites when compared to inactive sites, however, without significant difference (39).

Concerning the probable differences in virulence of *P. intermedia* clones no correlation could be found since the same genotypes were found at both diseased and non-diseased sites (237).

Choi et al. reported a high prevalence of *P. intermedia* (90%) in patients with chronic periodontitis and significantly lower in healthy subjects (only 5%) in a Korean population (176).

The data of the elderly group in our study revealed frequent detection of the species in well-maintained subjects (prevalence 66.7%) (Fig.1) incriminating *P. intermedia* as a common colonizer of the oral cavity in healthy oral conditions. Conrads et al. (201) found *P. intermedia* very frequently in plaque samples from children with PCR detection assay.

However, contradictory results have been gained. Molecular genetic analysis revealed no significant difference in site-prevalence between healthy, elderly and chronic periodontitis groups (34). Interestingly, despite more frequent supra- and subgingival colonization of *P. intermedia* in periodontitis patients than in healthy persons, the quantitative assessment

showed no significant difference in proportions (30).

Although in some studies the etiologic role of *P. intermedia* in periodontitis was considered to be high (11, 13, 30, 230), a relatively low risk (1.6) for periodontal breakdown in the presence of *P. intermedia* has been reported (48). It seems that higher mean counts of *P. intermedia* than for *P. gingivalis* are needed for the progression of the disease. Rams et al. assessed the relative risk for periodontal breakdown with respect to the proportions of certain species and found that much higher proportions of *P. intermedia* are needed to reach a 2.5 relative risk for periodontitis recurrence when compared to other putative pathogens (233). Analysis of the humoral host response against *P. intermedia* has shown similar antibody levels in individuals with and without periodontal disease (262). This lack of association with periodontal disease could be explained in part by the frequent colonization of *P. intermedia* in locations other than periodontal pockets (183).

It is difficult to evaluate the role of *P. intermedia* given the variable and contradictory literature. Our data incriminates this organism as one of moderate importance in GAP patients, but it is obviously not a key putative pathogen. The majority of data implicates *P. intermedia* as a common part of oral microflora in healthy persons. According to the literature an increase of its proportions is considered critical in etiopathogenesis of periodontal disease.

6.2.7 *Eikenella corrodens*

Since *Eikenella corrodens* has been suggested in earlier research as a pathogen because of its assotiation with initiation and progression of the disease in juvenile and refractory cases (49, 241, 263, 264), it was of interest to compare its distribution in GAP patients and the elderly. *E. corrodens* was more frequently identified in GAP patients than in elderly subjects (Fig.1, 3, 6). In the majority of the elderly only one sampled site was positive for *E. corrodens* (Fig.6). Interestingly, no significant difference was found between the number of colonized pockets and control sites in the diseased group (Fig.2). *E. corrodens* tends to colonize GAP patients, probably when the environmental conditions become favorable. It is questionable whether it plays a role in disease progression.

The results are consistent with the data of Kamma et al. who could not find significant qualitative and quantitative difference between colonization of deep pockets and shallow sites by *E. corrodens* in RPP patients either (15). Albandar et al. detected this species in 91% of EOP patients and in 89% of subjects without disease (230). Similar relations between chronic periodontitis and health has been observed frequently (11, 34, 36). The level of this bacterium seems to be independent of disease classification or the rate of progression.

In conclusion, *E. corrodens* is a commensal species that does not play a significant role as a primary opportunistic periodontal pathogen.

6.2.8   *Veillonella parvula*

Veillonella parvula was an infrequently detected species in the GAP and elderly groups, with a prevalence of 25% and 43%, respectively (Fig. 1). No significant difference in detection frequency between the two groups (Fig. 1, 3) or between diseased and healthy sites in periodontitis patients could be demonstrated (Fig. 2, 5). This is in accord with the results of Kamma et al. that revealed a similar presence of this bacterium in shallow and deep sites (53% and 33%, respectively) of RPP patients, however, as comparison to our study with a much higher site-prevalence (15).

Studies of experimental gingivitis included *V. parvula* in the group of species which increased in proportion as inflammation developed (23). However, a majority of publications show only a weak association between periodontitis and this microorganism (39, 140). *V. parvula* together with *E. corrodens* and *Capnocytophaga* spp. was shown to be a common member of microbiota in subgingival sites of diseased and healthy subjects and was detected in similar proportions in both groups (36, 217). *V. parvula* has been detected more frequently in inactive sites when compared to active sites (39, 137, 140). Haffajee et al. showed the site-prevalence of *V. parvula* in the elderly (60%) being higher than in the younger healthy group (48%), however without significant difference (34). Low detection frequency of the species in our study raises the question of the accuracy of the performed identification. Low sensitivity of the oligonucleotide probe cannot be ruled out.

Results of our study suggest that *V. parvula* should not be regarded as an adequate marker of healthy flora.

6.2.9   *Capnocytophaga ochracea*

The prevalence of *Capnocytophaga ochracea* in the GAP patients of the current study was distinctly lower (16%) than of the elderly (95%) (Fig. 1). As few as 3% of the pockets of the GAP subjects were positive, in contrast 55% of the sites of the elderly demonstrated colonization by *C. ochracea* (Fig. 3). The differences were highly significant. Even the shallow sites of GAP patients were rarely colonized, while 70% of the 1-3 mm sites of the elderly were positive (Fig. 4). Interestingly, patients with aggressive periodontitis exhibited seldom *C. ochracea* in subgingival plaque.

Frequent detection of *C. ochracea* in gingival sulci of children by Conrads et al. was interpreted as a physiological condition (201).

Increased levels of the species appeared to be consistent with a decreasing risk of new attachment loss. Similarly lower levels were found prior to breakdown (36, 137, 140, 265). In several studies, contradictory to our results, *C. ochracea* tends to be a common colonizer in bacterial plaque regardless of whether the samples were obtained from supra-or subgingival plaque of diseased or healthy subjects, from active or inactive sites (15, 30, 34, 39). In some medical case reports *C. ochracea* has been made responsible for endocarditis

and cervical abscess (266). Tanner et al. in earlier research has associated *C. ochracea* with juvenile periodontitis as high proportions of the species were recovered from diseased sites in young adults (241). Our data clearly confirm the association of *C. ochracea* with periodontal health. The use of this species may be a good indicator for periodontal health.

6.2.10 Colonization of healthy sites

The comparison of the microflora of shallow sites from GAP patients with the flora from subjects with healthy periodontium can give valuable information as to whether microorganisms originate from the adjacent pockets as a consequence of a "spill over", or belong to the resident microflora. Both hypotheses are presented in literature (267). Riviere et al. tested the hypothesis that certain bacteria at healthy sites would be detected more frequently in subjects with periodontitis than in subjects without periodontitis (255). Using an immunological assay he could show statistically significant differences only for *P. gingivalis* and *Treponema* spp. The data reported by Ximenez-Fyvie et al. support Riviere's hypothesis (30). The authors obtained quantitative data using checkerboard hybridization with whole-genomic probes. Higher mean counts of periodontitis-associated bacteria were observed in shallow sites of periodontitis patients when compared to healthy subjects. Similarly Haffajee et al. using the same detection method showed that *T. forsythensis*, *P. gingivalis, T. denticola* and *Selenomonas noxia* were found more frequently, and at higher levels, in shallow pockets of periodontitis subjects than at similar sites in the healthy group (34). The data of the present study contradicts this hypothesis. We found no significant difference between the groups for any species, except for *C. ochracea*, in colonization of shallow sites (Fig. 4). The results suggest rather that the putative periodontopathogens belong to a resident flora. Thus, the mere presence of a putative pathogen has limited value as an adjunct to clinical diagnosis and treatment planning. However, the risk that the disease may occur at these sites is highly dependent on the host, as well as the variation in bacterial virulence.

6.2.11 Bacterial consortia

"Profiles" of microbial complexes have been recognized upon clustering of the detected species from distinct clinical conditions (140, 177). A high degree of association between organisms may indicate a symbiotic relationship in periodontal pockets. A pathogen may more readily colonize subgingival sites already occupied by other organisms, due to gingival inflammation or growth factors produced by other organisms. However, some organisms may occur together in periodontitis lesions merely because they both induce destructive disease without interacting with each other. Putative pathogens acting together may produce additive or even synergistic damage to the periodontal tissues. Therefore a therapeutic regimen leading to concomitant suppression or elimination of symbiotic microorganims may achieve particularly great clinical benefits.

The definition of five subgingival plaque bacterial complexes by Socransky et al. (215) was based on the analysis of the microbial community of over 13,000 plaque samples from 185 subjects (EOP patients excluded) by using whole-genomic DNA probes in checkerboard hybridization assays. 5 clusters were formed:

1. Red cluster - *P. gingivalis, T. forsythensis, T. denticola*
2. Orange cluster - *F. nucleatum, P. intermedia, P. nigrescens, Peptostreptococcus micros, Campylobacter* spp., *E. nodatum, S. constellatus*
3. Green cluster - *Capnocytophaga* spp.*, Campylobacter concisus, Eikenella corrodens, A. actinomycetemcomitans* serotype a
4. Yellow cluster - *Streptococcus* spp.
5. Purple cluster - *Actinomyces odontolyticus, Veillonella parvula*

The members of the red complex have frequently been detected together and exhibit a very strong correlation with pocket depth (215). The biological basis of the association among these species is not known. However, strong interspecies adherence has been demonstrated among these putative pathogens (268).

The co-existence of *T. forsythensis* and *P. gingivalis* has been frequently reported (11, 151, 177, 182, 192, 215, 223). Slots et al. (269) showed an odds ratio of 18.6. It has been speculated that *T. forsythensis* precedes the colonization by *P. gingivalis*, since *T. forsythensis* alone is detected more frequently (182, 191). The data of the present study are consistent with this report. *P. gingivalis* was detected alone in only 4 samples. The odds ratio of detecting these species together was 23.5. Since *T. forsythensis* and *P. gingivalis* were strongly associated with GAP, a co-infection with both microorganisms may lead to a particularly aggressive form of periodontal disease.

The red complex was shown to be closely associated with the orange cluster (215). The odds ratios of the present study show mutual relationships among the species belonging to these 2 complexes. *T. forsythensis* and *C. rectus* were most frequently detected together in periodontal pockets (OR 35.6). Colonization by these two bacterial species has often been associated with the induction of a shift from periodontal health to disease (17, 31, 33, 36, 39, 48, 144, 254). Similarly to our results Kamma et al. (170) observed the strongest positive association between *T. forsythensis* and *C. rectus* (OR 109.5).

The statistical analysis of Haffajee et al. revealed that combinations of species are better predictors of new attachment loss. Significantly higher levels of *T. forsythensis* and *C. rectus*, and significantly lower levels of *C. ochracea* were found in active subjects prior to breakdown (265).

T. forsythensis is often detected together with *F. nucleatum* (36, 223). In culture *F. nucleatum* enhances the growth of *T. forsythensis* (228). These species were frequently identified in the same pockets (OR 9.1) (Table 2). The species mentioned, together with *C. rectus*, have

often been found in sites which exhibited active disease and those which responded poorly to therapy (140).

Mullally et al. demonstrated a strong association between *P. intermedia* and *E. corrodens* in EOP patients (14). This constellation however could not be confirmed in our study. Rather *E. corrodens* was associated with *A. actinomycetemcomitans* (OR 5.8) (Table 2). According to Socransky et al. they both belong to a green cluster (215). This pair has frequently been identified in lesions of LJP (33, 49).

Ashimoto et al. found positive associations between *C. rectus* and *E. corrodens*, as well between *P. gingivalis* and *E. corrodens* when investigating heterogeneous patient groups (11). Our results and those of Socransky et al. (177) indicated a negative association between these species. The evaluation of heterogeneous population groups and the use of different detection methods may explain these discrepancies to some extent.

6.3   Additional species associated with periodontitis

The present investigation of the microbial profile of aggressive periodontitis was confined to a few putative pathogens which have earlier been associated with etiopathogenesis of the disease. However, the disease process is related to a complex microbiota, where a large, still undefined number of microorganisms might play a role.

With the advent of molecular biology, especially the utilization of 16S rRNA molecule, it has become available to study mixed bacterial communities in their entirety. Research has revealed that about 50% of the oral flora is unculturable, certainly including novel pathogens (270). The analysis of 2,522 clones obtained from the sites with various periodontal conditions by Paster et al., revealed an unexpected diversity (19). About 60% of the clones fell into known species and 215 novel phylotypes were identified. Associations with chronic periodontitis and healthy periodontium were observed for several new species and phylotypes by Kumar et al. (271). Several uncultivated phylotypes showed a very strong relationship to disease, suggesting that there may be previously unrecognized organisms that play an important role in the pathogenesis of periodontitis.

Spirochetes, an example of as yet uncultivated bacteria, have been found in subgingival plaque samples of periodontitis patients at high frequencies. Their role in the pathogenesis of periodontal diseases is less clear, due to the difficulties of culturing them in vitro. Along with dark-field microscopy results, culture-independent techniques have shown an association of treponemes with the severity of periodontal disease and provided strong evidence for a particular role in patients with aggressive disease (17, 168, 230, 290).

The investigation of subgingival samples of the RPP population (identical with the present study) has revealed great discrepancies between the prevalence of cultivable and hitherto uncultivable treponemal species (168). *Treponema denticola*, a cultivable species, was identified in only 40% of the samples, whereas the as yet uncultured group II treponemes

was present in 72% of the pockets. Group IV treponemes were found in each patient and in 97.5% of the samples. The in situ hybridization results indicated that these organisms were present in high proportions and thus are part of the predominant flora (168).

Several other microbial species and genera have been implicated to be associated with destructive periodontal diseases. These include *Micromonas micros* (formerly *Peptostreptococcus micros*), *Selenomonas* spp., *Eubacterium* spp., *Streptococcus intermedius,* enteric rods, pseudomonads (17, 18).

Recently, the role of viruses has been discovered. Various herpesviruses, human cytomegalovirus (HCMV) and Epstein-Barr virus type-1 (EBV-1) have been detected in subgingival samples from patients with aggressive periodontitis (58, 272, 273). The virus-infected inflammatory cells can reduce host defense mechanisms, giving periodontopathic bacteria the opportunity to overgrow in the subgingival area and to invade tissues and cells more efficiently (272).

Kumar et al. (271) reported about associations of new species and phylotypes with chronic periodontitis, including uncultivated clones from the *Deferribacteres* and *Bacteroidetes* phylum, *Megasphaera* clone BB166 and the named species *Eubacterium saphenum*, *Porphyromonas endodontalis, Prevotella denticola*, and *Cryptobacterium curtum*.

The microbial diversity involved in periodontitis has become more complex than previously thought. In the future, a reevaluation of the present knowledge about the composition and mutual associations of the oral microflora in disease and health may be necessary.

6.4   Host factors

Remarkable improvements in understanding the microbial / host interactions, intrinsic host defense ability, evaluation of individual immune responsiveness and susceptibility to infectious diseases have been made in recent years. It has been suggested that patients with a genetic predisposition to an altered level of inflammatory response may be less capable of tolerating the presence of putative pathogens, thereby putting certain patients at higher risk of periodontitis (148). There is evidence for specific hyper-inflammatory traits present in patients affected with severe periodontitis (274). Specific immune response results in much greater destruction in periodontal tissues. Aggressive periodontitis aggregates in families, suggesting that genetic variations in host responses play an important role in disease susceptibility (275). Also studies in twins showed that a significant part of the variance in clinical parameters of periodontitis may be attributable to genetic factors (276).

Aggressive periodontitis patients represent susceptible hosts for periodontal disease, in which various host risk factors may be amplified. A recent comprehensive study aiming to define a characteristic immunological profile of different disease entities failed to explain the pathogenesis of aggressive periodontitis based on a single host risk factor (277). A wide heterogeneity was observed even in subgroups. The association of host immunological risk

factors in patients with aggressive periodontitis is widely varied and more complex than previously thought.

Recent evidence has indicated that patients with severe periodontitis have a perturbation of their systemic inflammatory status manifested by increased local and systemic levels of IL-1, IL-6, IL-8, TNF- , C-reactive protein, fibrinogen and moderate leukocytosis when compared to unaffected control groups (133, 278, 279, 280).

Immunohistochemical and in-situ hybridization research showed enhanced accumulation of PMNs in gingival specimens of aggressive periodontitis patients, which is associated with the upregulation of IL-8, ICAM-1, IL-1 and TNF- expression (281). Beside the hyperreactivity of PMNs, dysfunctions, mainly depressed chemotaxis and phagocytosis in patients with aggressive periodontitis has been demonstrated (277).

Data on antibody responses in severe periodontitis patients are, however, inconsistent. In periodontitis patients the levels of antigen-specific serum antibodies are usually elevated (277). Alabander et al. found that patients with generalized aggressive periodontitis and very high levels of anti-LPS IgG had significantly less attachment loss than similar patients with lower levels of these antibodies (282). However, depressed antibody response against bacterial species has been observed in aggressive periodontitis cases, probably indicating high-risk subjects (277).

A major portion of antibody to periodontitis-associated bacteria form the IgG2-subclass (109). It has been shown that the production of IgG2 predominates over IgG1 concentration by patients with early-onset periodontitis (128). It suggests that the functionally less-effective IgG2 plays an important role in susceptibility and dimension of periodontal destruction in those patients.

Also, lower percentages of pan T, suppressor/cytotoxic T cells, and pan B cells have been found in some patients with aggressive periodontitis (277, 283).

Gene polymorphisms have been acknowledged as genetic mechanisms by which some individuals, if challenged by bacterial accumulations, may have a more vigorous immuno-inflammatory response leading to more severe periodontitis. Kornman et al. (284) showed the association between severity of periodontitis and carriage of a composite genotype that included allele 2 of the IL-1 -889 and IL-1 +3953. Functionally the IL-1 genotype is associated with high levels of IL-1 production. The IL-1 gene polymorphism has been regarded as a genetic marker for increased susceptibility for aggressive periodontitis (285). However, inconsistent results have been reported. A study of a Brazilian family with aggressive periodontitis showed a lack of relationship between the genetic parameter and disease susceptibility discussed above (169).

Recently a gene-environmental interaction between smoking and the IL-1 genetic polymorphism has been shown (132). Smokers bearing the genotype-positive IL-1 allele combination have an increased risk of periodontitis. The authors stated that the IL-1

genotype has no influence on non-smokers.

Genetic polymorphisms in human TLR genes have been associated with physiologically important traits. Recently, two common cosegregating missense mutations, Asp299Gly and Thr399Ile, affecting the extracellular domain of the TLR4 protein have been characterized (115). Both mutations lead to an impaired efficacy of LPS signaling and reduced capacity to elicit inflammation. Consistently, a significantly increased risk for gram-negative infections was found for individuals carrying these mutations (286). However, not all the subjects who were hyporesponsive to LPS had the polymorphisms in TLR4 gene. And as well, not everyone with the TLR4 mutations was hyporesponsive to inhaled LPS (287). This suggests high complexity of the involvement of genes in host response. Interestingly, various mutations of the TLR2 and TLR4 genes could not be associated with chronic periodontitis so far (288). Further investigations with patients with aggressive periodontitis are necessary.

Rapid development in automated high-throughput genetic assay techniques and databases allows large-scale investigation of genotypes and to determine single-nucleotide polymorphisms (SNPs). An Increasing number of possible genetic risk factors for severe periodontitis has been reported, like single nucleotide polymorphisms in genes of IL-10, Fc- , TNF-receptor-associated factor-1, CD14, etc. (110, 289). As the research is mostly based on statistical comparison of genotypes between healthy subjects and patients with severe periodontitis, the translation of these observations into reproducible genotype-phenotype associations stays limited. So far the suggested SNPs cannot be regarded as true susceptibility indicators for aggressive periodontitis.

In conclusion, several defects within the immune system could act separately or synergistically, creating a dysfunctional inflammatory response that disables the protective mechanisms of the host, increasing host susceptibility to periodontal disease. It still needs to be clarified to what extent the microbiological factors influence the progression of the disease. It is evident that bacteria are necessary to initiate the disease, but the genetic background of the host appears to have much higher modifying impact than thought before.

7   Summary

In general, periodontal destruction results from imbalances in an individual's innate and acquired immune responses to their oral microbiota. A multifactorial risk pattern of periodontitis is recognized, including bacterial challenge, smoking, age, diabetes, and socioeconomic and genetic factors. In the initial phase pathogenic microbiota rather than single periodontal pathogens seem to play an important role. Despite the improvement of microbiological detection methods the actual bacterial dimension and their mutual relationships in an oral ecosystem is still unclear. At present no definite answer can be given to the question of whether the expression of either aggressive etiological agents (implying

infection with a highly virulent microbiota), or a high level of individual susceptibility to periodontal disease, or a specific combination of both is the conducive factor in the etiopathogenesis of aggressive periodontitis.

The purpose of the current research was to analyze the prevalence of periodontitis-associated microorganisms in patients with generalized aggressive periodontitis and periodontally healthy elders using molecular-biologic detection methods like eubacterial PCR-amplification in combination with dot-blot hybridization. The oligonucleotide probes for the detection of periodontitis-associated bacteria *Tannerella forsythensis*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Fusobacterium* spp., *Campylobacter rectus*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Eikenella corrodens*, *Veillonella parvula* and *Capnocytophaga ochracea* were designed and evaluated. The results of the epidemiological study revealed frequent colonization by *T. forsythensis*, *P. gingivalis*, *F. nucleatum* and *C. rectus* in patients with aggressive periodontitis, however, individual variations were obvious. These microorganisms could predominantly be identified in periodontal pockets, but were significantly less common in healthy sites and in the elderly subjects. The putative pathogens *T. forsythensis*, *P. gingivalis*, *F. nucleatum* and *C. rectus* can hence be suggested as the key-bacteria in patients with aggressive periodontitis. *A. actinomycetemcomitans* could be detected in only a few patients, reducing its suspected importance in the etiopathogenesis of generalized aggressive periodontitis. Only a weak association for *P. intermedia* and *E. corrodens* with aggressive periodontitis or periodontal health could be seen. The results support earlier findings that generalized aggressive periodontitis is associated with a complex microbiota.

It is still controversial whether the presence of putative pathogens in periodontal sites without clinical disease increases the risk for future periodontal breakdown. The periodontitis-associated microorganisms are part of the indigenous oral microflora. In certain circumstances they are induced by environmental and host genetic factors to increase in proportions and unfold their virulent nature. The frequently observed co-infection of *T. forsythensis* and *P. gingivalis* or *T. forsythensis* and *C. rectus* in patients with aggressive periodontitis can implicate constellations of species which have the synergistic effect of enhanced virulence. Microbiological detection tests for putative periodontal pathogens are not of themselves diagnostic for periodontal disease, as periodontitis is a consequence of an opportunistic infection caused by microorganisms belonging to the resident microflora. Obviously, the influence of host immunity factors modifies the clinical outcome to a high extent. Patients with an altered inflammatory response may be less capable of tolerating the presence of specific organisms. Individuals with apparently low risk of developing destructive periodontal disease may have established a protective, so-called beneficial subgingival flora. In the present research *C. ochracea* was highly prevalent in the well-maintained elderly, being rarely found in GAP patients. This is evidence for regarding *C. ochracea* as a marker

organism for periodontal health.

Considering that periodontitis is a polymicrobial infection, the screening of the microbial population, rather than the isolation of single members of the subgingival flora, should give a more comprehensive perspective in research of etiopathogenesis of the different forms of periodontitis.

The described oligonucleotide probes allow, in combination with PCR-amplification and dot-blot hybridization, a specific and sensitive detection of the respective oral species. Subsequent epidemiological studies using identical molecular genetic detection methods, as well as the investigation of complex subgingival biofilms by applying oligonucleotide probes in a fluorescence-in-situ hybridization, are in progress.

8 Zusammenfassung

Die Ätiopathogenese der aggressiven Parodontitis beruht auf einem bislang noch nicht im Detail verstandenen Ungleichgewicht zwischen der oralen Mikroflora einerseits und Entzündungs- bzw. Immunreaktionen andererseits. Neben Wirtsfaktoren (u.a. immunorelevante Genpolymorphismen) und äußeren Einflüssen (wie z. B. Mundhygiene, Rauchen) spielt eine als pathogene Mischflora bezeichnete Kombination kommensaler Mikroorganismen, die opportunistische Infektionen und damit Immunreaktionen auslösen können, eine bedeutende Rolle. Obgleich die rasante Entwicklung der molekulargenetischen Nachweismethoden in der Mikrobiologie eine zunehmend detaillierte Charakterisierung der Besiedlungsmuster der oralen Mischflora erlaubt, fehlt bislang ein umfassendes Bild der bakteriellen Kolonisation und der Zusammenhänge im gesamten oralen Ökosystem.

Im Mittelpunkt der vorliegenden Arbeit steht die Untersuchung der subgingivalen Mischflora bei Patienten mit generalisierter aggressiver Parodontitis und bei gesunden Senioren. Dabei wurde Material aus Zahnfleischtaschen und gesunden parodontalen Abschnitten untersucht. Zur Analyse der Parodontitis-assoziierten Mikroorganismen wurden eubakterielle PCR-Amplifikationsverfahren und die dot-blot-Hybridisierung mit Oligonukleotidsonden angewendet. Die Oligonukleotidsonden für den Nachweis von *Tannerella forsythensis*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Fusobacterium* spp., *Campylobacter rectus*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, *Veillonella parvula* und *Capnocytophaga ochracea* wurden hergestellt und kontrolliert. Obgleich eine hohe interindividuelle Variabilität der oralen Kolonisation nachweisbar war, konnten bei Patienten mit aggressiver Parodontitis einige Keime (*T. forsythensis*, *P. gingivalis* und *F. nucleatum*) sehr häufig in den Zahnfleischtaschen identifiziert werden; sie sind in hohem Maße mit der aggressiven Parodontitis assoziiert und sind daher als Leitkeime für diese Erkrankung anzusehen. Obwohl diese Arten auch in den gesunden Stellen festzustellen waren, blieb die Häufigkeit der Besiedlung signifikant seltener. Außerdem hat man eine Koinfektion von *T. forsythensis* und *P. gingivalis*, sowie *T. forsythensis* und *C.*

rectus in den erkrankten Stellen beobachtet. *A. actinomycetemcomitans* konnte nur bei einzelnen Patienten mit generalisierter aggressiver Parodontitis festgestellt werden. Die Ergebnisse für *P. intermedia* und *E. corrodens* ließen als Schlußfolgerung keine eindeutige Assoziation sowohl mit der aggressiven Parodontitis als auch mit dem gesunden Parodontalzustand zu.

Bestimmte äußere und Wirtsfaktoren können die Vermehrung dieser opportunistischen Pathogene fördern; möglicherweise wird durch Stämme mit hoher Virulenz bzw. bestimmter Artzusammensetzung das Risiko einer aggressiven Parodontitis zusätzlich gesteigert.

Bei Gesunden wurde *C. ochracea* sehr häufig nachgewiesen; möglicherweise handelt es sich hier um eine Spezies einer im Hinblick auf die Entwicklung einer Parodontitis-protektiven Mundflora.

Bezüglich der polymikrobiellen Natur der Parodontitis würde eine umfassende Untersuchung der oralen Mikroflora und deren Zusammenspiel mit den Wirtsfaktoren zur Aufklärung der Ätiopathogenese der verschiedenen Formen der Parodontitis eher beitragen als der Nachweis einzelner Arten.

Die PCR-Amplifikation in Kombination mit der dot-blot Hybridisierung ermöglicht einen spezifischen und sensitiven Nachweis der untersuchten oralen Keime. Weitere epidemiologische Studien mit diesen Nachweismethoden, sowie Untersuchungen des subgingivalen Biofilms mittels Fluoreszenz-in-situ Hybridisierung, werden zur Zeit auch unter Einsatz der beschriebenen Oligonukleotidsonden durchgeführt.

9   Supplement

9.1   Tables

Table 1. Strain designations, original sources, control identification method used in the study.

1) *Actinobacillus actinomycetemcomitans*	ATCC 43718	DSM	sequencing
2) *Actinobacillus actinomycetemcomitans*	ATCC 33384	Conrads	sequencing
3) *Actinobacillus actinomycetemcomitans*	serotyp a	Mombelli	--
4) *Leptotrichia buccalis*	MCCM 00448	Mutters	sequencing
5) *Pasteurella haemolytica*	ATCC 33396	Mutters	sequencing
6) *Haemophilus influenzae*	ATCC 33391	Mutters	sequencing
7) *Haemophilus influenzae*	klin. Isolat	Conrads	--
8) *Haemophilus aphrophilus*	NCTC 55906	Gmür	sequencing
9) *Haemophilus paraphrophilus*	ATCC 29241	Mutters	sequencing
10) *Porphyromonas gingivalis*	ATCC 33277	DSM	sequencing
11) *Prevotella intermedia*	ATCC 25611	DSM	sequencing
12) *Porphyromonas asaccharolytica*	ATCC 25260	Gmür	sequencing
13) *Prevotella nigrescens*	NCTC 9336	Gmür	sequencing
14) *Prevotella oralis*	MCCM 00684	Mutters	--
15) *Prevotella buccalis*	ATCC 33690	Mutters	API
16) *Capnocytophaga ochracea*	ATCC 27872	Gmür	sequencing
17) *Capnocytophaga sputigena*	ATCC 33612	Gmür	sequencing
18) *Capnocytophaga gingivalis*	ATCC 33624	Conrads	sequencing
19) *Campylobacter rectus*	ATCC 33238	Mombelli	sequencing
20) *Campylobacter concisus*	ATCC 33236	Gmür	sequencing
21) *Tannerella forsythensis*	ATCC 43037	Olson	sequencing
22) *Bacteroides gracilis*	ATCC 33236	Gmür	sequencing
23) *Bacteroides fragilis*	ATCC 25285	Charité	--

24) *Eikenella corrodens*	CCUG 2138	Gmür	sequencing
25) *Kingella kingae*	ATCC 23330	DSM	sequencing
26) *Veillonella parvula*	ATCC 10790	Gmür	sequencing
27) *Veillonella dispar*	ATCC 17748	DSM	--
28) *Klebsiella pneumoniae*	ATCC 23357	Charité	--
29) *Fusobacterium nucleatum*	ATCC 25586	Mombelli	sequencing
30) *Fusobacterium* odoratum	MCCM 02932	Mutters	sequencing
31) *Neisseria lactamica*	ATCC 23970	Charité	--
32) *Streptococcus mutans*	ATCC 35668	Virchow	--
33) *Streptococcus intermedius*	ATCC 27335	Virchow	--
34) *Actinomyces pyogenes*	ATCC 19411	Virchow	--
35) *Actinomyces israelii*	ATCC 10048	Virchow	--
36) *Actinomyces naeslundii*	ATCC 12104	Virchow	--
37) *Actinomyces viscosus*	ATCC 15987	Virchow	--
38) *Eubacterium lentum*	ATCC 25559	Mutters	--
39) *Selenomonas* sp.	Clin. strain	Mutters	--
40) *Fusobacterium simiae*	CCUG 16798	CCUG	sequencing
41) *Fusobacterium periodonticum*	CCUG 14345	CCUG	--
42) *Fusobacterium necrophorum*	NCTC 25286	Mombelli	sequencing

Table 2. The bacterial species, respective culture media and growth conditions.

1) *Actinobacillus actinomycetemcomitans*	ATCC 43718	fac. anaerob.	TSBV
2) *Actinobacillus actinomycetemcomitans*	ATCC 33384	fac. anaerob.	TSBV
3) *Actinobacillus actinomycetemcomitans*	serotyp a	fac. anaerob.	TSBV
4) *Leptotrichia buccalis*	MCCM 00448	anaerobic	Fluid thyogl.
5) *Pasteurella haemolytica*	ATCC 33396	aerobic	Col. agar
6) *Haemophilus influenzae*	ATCC 33391	microaerophil.	anaer. Col. agar
7) *Haemophilus influenzae*	Clinical strain	microaerophil.	anaer. Col. agar
8) *Haemophilus aphrophilus*	NCTC 55906	microaerophil.	anaer. Col. agar
9) *Haemophilus paraphrophilus*	ATCC 29241	microaerophil.	anaer. Col. agar
10) *Porphyromonas gingivalis*	ATCC 33277	anaerobic	anaer. Col. agar
11) *Prevotella intermedia*	ATCC 25611	anaerobic	anaer. Col. agar
12) *Porphyromonas asaccharolytica*	ATCC 25260	anaerobic	anaer. Col. agar
13) *Prevotella nigrescens*	NCTC 9336	anaerobic	anaer. Col. agar
14) *Prevotella oralis*	MCCM 00684	anaerobic	anaer. Col. agar
15) *Prevotella buccalis*	ATCC 33690	anaerobic	anaer. Col. agar
16) *Capnocytophaga ochracea*	ATCC 27872	anaerobic	anaer. Col. agar
17) *Capnocytophaga sputigena*	ATCC 33612	anaerobic	anaer. Col. agar
18) *Capnocytophaga gingivalis*	ATCC 33624	anaerobic	anaer. Col. agar
19) *Campylobacter rectus*	ATCC 33238	anaerobic	FUM+supplem.
20) *Campylobacter concisus*	ATCC 33236	anaerobic	FUM +supplem.
21) *Bacteroides gracilis*	ATCC 33236	anaerobic	anaer. Col. agar
22) *Bacteroides fragilis*	ATCC 25285	anaerobic	anaer. Col. agar
23) *Eikenella corrodens*	CCUG 2138	anaerobic	Col. Agar
24) *Kingella kingae*	ATCC 23330	aerobic	Col. agar
25) *Veillonella parvula*	ATCC 10790	anaerobic	FUM +supplem.

26) *Veillonella dispar*	ATCC 17748	anaerobic	anaer. Col. agar
27) *Klebsiella pneumoniae*	ATCC 23357	aerobic	Col. Agar
28) *Fusobacterium nucleatum*	ATCC 25586	anaerobic	ETSA
29) *Fusobacterium odoratum*	MCCM 02932	anaerobic	anaer. Col. agar
30) *Neisseria lactamica*	ATCC 23970	microaerophil.	Col. Agar
31) *Streptococcus mutans*	ATCC 35668	aerobic	Col. Agar
32) *Streptococcus intermedius*	ATCC 27335	aerobic	Col. Agar
33) *Actinomyces pyogenes*	ATCC 19411	anaerobic	anaer. Col. agar
34) *Actinomyces israelii*	ATCC 10048	anaerobic	anaer. Col. agar
35) *Actinomyces naeslundii*	ATCC 12104	anaerobic	anaer. Col. agar
36) *Actinomyces viscosus*	ATCC 15987	anaerobic	anaer. Col. agar
37) *Eubacterium lentum*	ATCC 25559	anaerobic	anaer. Col. agar
38) *Selenomonas* sp.		anaerobic	anaer. Col. agar
39) *Fusobacterium simiae*	CCUG 16798	anaerobic	ETSA
40) *Fusobacterium periodonticum*	CCUG 14345	anaerobic	ETSA
41) *Fusobacterium necrophorum*	NCTC 25286	anaerobic	ETSA

9.2   List of used mediums and chemicals

9.2.1   Mediums

9.2.1.1   FUM - Fluid Universal Medium

(R. Gmür, B. Guggenheim, Infect. Immun. 1983; 42: 459-470)

Stock-solutions (stored at 4°C)

Hemin:	0.1 M KOH	100ml
	Ethanol conc.	50ml
	Dist. Water	50ml
	Hemin-chlorid	200mg
Menadione:	Menadione	25mg
	Ethanol conc.	50ml

Reduced transport fluid RTF 1:		K_2HPO_4	6g
		dist. water ad	1000ml
	RTF 2:	NaCl	12g
		(NH4)2SO4	12g
		KH2PO4	6g
		MgSO4 x 7 H2O	2.5g
		distilled water ad	1000ml

Solution A:		Solution B:	
Bacto Tryptone	10g	Glycose	3g
Yeast extract	5g	Cysteine-HCl	0.5g
KNO3	1g	Na2CO3	0.5g
NaCl	2g	Dist. Water	50ml
Hemin solution 1 ml 1ml		Filter-sterilized	

Autoclaved 20 min. at 120°C and stored at 4°C

Production of FUM:

RTF 1	75 ml
RTF 2	75 ml
Menadione solution	2 ml

Filter-sterilized. Pooled into Solution B and added to Solution A.

5 % heat inactivated (30 min at 56°C) horse serum (GIBCO) was added.

For *T. forsythensis* :   1% N-Acetylmuramic acid C11H19NO8 10mg in
                               1 ml dist. water

For *C. concisus*, *C. rectus* :   Fumaric acid disodiumsaline C4H2Na2O4 1 g +
                               Sodiumformiat CHNaO2 1 g in 10 ml dist. water

For *V. parvula* :   Putrescin C4H12N2 4,5 mg in 10 ml dist. water

9.2.1.2   Columbia agar

40 mg/l	Columbia agar (Becton-Dickenson)
5 mg/l	Hemin
50 µg/l	Vitamin K
5%	Sheep blood

9.2.1.3   TSBV - Trypticase-Soy-Bacitracin-Agar (J. Slots, J Clin Microbiol 1982: 15: 606)

40g/l	Trypticase soy agar
75 mg/l	Bacitracin
5 mg/l	Vancomycin
1 g/l	Yeast extract
2g/l	Glycose
10%	Horse serum

9.2.1.4   ETSA - Enriched-Trypticase-Soy-Agar

Bulk preparation A

20 ml	10% Potassium nitrate
4 ml	Sodium lactate syrup
4 ml	Hemin
Distilled water ad	2000 ml
4 g	Yeast extract
2 g	Sodium succinate
2 g	Sodium formate
Autoclaved, cooled, freezed	

Bulk preparation B

25 g	Trypticase soy broth
19 g	Agar
Dist. water ad 500 ml	

Autoclaved, cooled in 50°C water bath,
added to prep. A

Filter sterilized ingredients added:

25 ml	Dist. water
2 ml	Menadione
2 ml	Fumarate
0.4 g	Cysteine
0.1 g	Dithiothreitol
1 g	Glycose
0.4 g	Sodium carbonate
30 ml	Sheep blood

9.2.1.5   Trypticase Soy Yeast Extract Medium

30 g	Trypticase soy broth
3.0 g	Yeast extract
15.0 g	Agar

Distilled water ad 1000 ml
pH adjusted to 7.0 - 7.2

9.2.1.6   RTF

A) Standard medium 1

6 g/l	K2HPO4 autoclaved

B) Standard medium 2

12 g/l	NaCl
12 g/l	(NH4)2SO4
6 g/l	KH2PO4
2.5 g/l	MgSO4

Autoclaved

C)	38 g/l	0.1M EDTA
D)	8 g/100 ml	Na2CO3
E)	0.2 g/20 ml	1% Dithiothreitol (DTT)
F)	0.1 g/100 ml	Resazurin

Preparation:

75 ml	Standard medium 1
75 ml	Standard medium 2
10 ml	Portion C
5 ml	Portion D
20 ml	Portion E
1 ml	Portion F

25% Glycose

Distilled water ad 1000 ml, filter-sterilize

9.2.2   Buffers

9.2.2.1   Maleic acid buffer

0.1 M Maleic acid
0.15 M NaCl
Adjust pH 7.5 with solid NaOH

9.2.2.2   1% Blocking solution

Blocking reagent 1g
Maleic acid buffer 100 ml
Dilute by stirring at 65°C, autoclave, store at 4°C

9.2.2.3   Detection buffer

0.1 M Tris-HCl
0.1 M NaCl
Adjust pH 9.5 with solid NaOH

9.2.2.4   Hybridization buffer

5 x SSC
1% Blocking solution
0.1% N-lauroylsarcosine
0.02% Sodium dodecyl sulfate (SDS)

9.2.2.5   Washing buffer 0:

5 x SSC
0.2% SDS

9.2.2.6   Washing buffer 1:

2 x SSC
0.1% SDS

9.2.2.7   Washing buffer 2:

0.1x SSC
0.1% SDS

9.2.2.8   Stripping solution

0.2 M NaOH
0.1% SDS

9.2.2.9 Phosphate buffered saline (PBS)

NaCl 8 g
KCl 0.2 g
Na2HPO4 1.44 g
KH2PO4 0.24 g
ad 1 l distilled water, adjust pH 7.4

9.2.2.10 Lysis buffer

500 mM Tris-HCl pH 9.0
20 mM EDTA
10 mM NaCl
1% SDS

9.2.2.11 Stop-Solution

9,5 ml Formamid
0.4 ml EDTA (500 mM)
5.0 mg Bromphenolblau
5.0 mg Xylencyanol FF
0.1 ml Dist. water

9.2.3   Gels

9.2.3.1   1.2% Agarose gel

1.2 mg Agarose
100 ml TBE buffer
1µl Ethidiumbromid

9.2.3.2   Polyacrylamidegel

21 g Urease
6 ml 10xTBE buffer
4.5 ml Long-Ranger (40%)
Dist. water ad 50 ml
Stirred at 50°C, filter-sterilized
240 µl APS (10%)
24 µl TEMED

10 References

1. Baelum V, Fejerskov O, Karring T. Oral hygiene, gingivitis and periodontal breakdown in adult Tanzanians. J Periodont Res 1986; 21/*3:* 221-232.

2. Baelum V, Fejerskov O, Manji F.: Periodontal diseases in adult Kenyans. J Clin Periodontol 1988; 15/*7*: 445-452.

3. Beck JD, Koch GG, Rozier RG, Tudor GE. Prevalence and risk indicators for periodontal attachment loss in population of older community-dwelling blacks and whites. J Periodontol 1990; 61/*8*: 521-528.

4. Locker D, Leake JL. Periodontal attachment loss in independently living older adults in Ontario, Canada. J Public Health Dent 1993; 53/*1*: 6-11.

5. Hugoson A, Laurell L, Lundgren D. Frequency distribution of individuals aged 20-70 years according to severity of periodontal disease experience in 1973 and 1983. J Clin Periodontol 1992; 19/4: 227-232.

6. American Academy of Periodontology. Position Paper: Periodontal disease in children and adolescents. J Periodontol 1996; 67/1: 57-62.

7. Saxen L. Prevalence of juvenile periodontitis in Finland. J Clin Periodontol 1980; 7/3: 177-186.

8. Löe H, Brown LJ. Early onset periodontitis in the United States of America. J Periodontol 1991; 62/10: 608-616.

9. Armitage GC. Development of a classification system for periodontal diseases and conditions. Ann Periodontol 1999; 4/1: 1-6.

10. Moore WEC, Holdeman LV, Smibert RM, et al.. Bacteriology of severe periodontitis in young adult humans. Infect Immun 1982; 38/3: 1137-1148.

11. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 1996; 11/4: 266-273.

12. Liljenberg B, Lindhe J. Juvenile periodontitis. Some microbiological, histopathological and clinical characteristics. J Clin Periodontol 1980; 7/1: 48-61.

13. Loesche WJ, Syed SA, Schmidt E, Morrison EC. Bacterial profiles of subgingival plaques in periodontitis. J Periodontol 1985; 56/8: 447-456.

14. Mullally BH, Dace B, Coulter WA, et al.. Prevalence of periodontal pathogens in localized and generalized forms of early-onset periodontitis. J Periodont Res 2000; 35/4: 232-241.

15. Kamma JJ, Nakou M, Manti FA. Predominant microflora of severe, moderate and minimal periodontal lesions in young adults with rapidly progressive periodontitis. J Periodont Res 1995; 30/1: 66-72.

16. The American Academy of Periodontology. Proceedings of the World Workshop in Clinical Periodontics. Chicago: The American Academy of Periodontology; 1989; 1: 23-24.

17. Moore WEC, Moore LVH. The bacteria of periodontal diseases. Periodontol 2000, 1994; 5: 66-77.

18. Socransky SS, Haffajee AD. Evidence of bacterial etiology: a historical perspective. Periodontol 2000, 1994; 5: 7-25.

19. Paster BJ, Dewhirst FE, et al.. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001; 183/12: 3770-3783.

20. Socransky SS. Relationship of bacteria to the etiology of periodontal disease. J Dent Res 1970; 49/2: 203-222.

21. Socransky SS. Microbiology of periodontal disease – present status and future considerations. J Periodontol 1977; 48/9: 497-504.

22. Ebersole JL, Taubman MA. The protective nature of host responses in periodontal disease. Periodontol 2000, 1994; 5: 112-141.

23. Löe H, Theilade E, Jensen SB. Experimental gingivitis in man. J Periodontol.1965; 36/4: 177-187.

24. Matsson L. Development of gingivitis in preschool children and young adults. J Clin Periodontol 1978; 5/1: 24-34.

25. Listgarten M. Nature of periodontal diseases: pathogenic mechanisms. J Periodont Res 1987; 22/3: 172-178.

26. Gunsolley JC, Ranney RR, Schenkein HA, et al.. Actinobacillus actinomycetemcomitans in families afflicted with periodontitis. J Periodontol 1990; 61/10: 643-648.

27. Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. J Periodontol 1992; 63/Suppl.4: 322-331.

28. Socransky SS, Haffajee AD, Smith GLF, et al.. Difficulties encountered in the search for the etiologic agents of destructive periodontal diseases. J Clin Periodontol 1987; 14/10: 588-593.

29. Socransky SS. Criteria for the infectious agents in dental caries and periodontal disease. J Clin Periodontol 1979; 6/7: 16-21.

30. Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J Clin Periodontol 2000; 27/9: 648-657.

31. Slots J. The predominant cultivable microflora of advanced periodontitis. Scand J Dent Res 1977; 85/2: 114-121.

32. Dahlen G, Manji F, Fejerskov O, et al.. Putative periodontopathogens in "diseased" and "non-diseased" persons exhibiting poor oral hygiene. J Clin Periodontol 1992; 19/1: 35-42.

33. Dzink JL, Tanner ACR, Haffajee AD, Socransky SS. Gram-negative species associated

with active destructive periodontal lesions. J Clin Periodontol 1985; 12/8: 648-659.

34. Haffajee AD, Cugini MA, Socransky SS, et al.. Subgingival microbiota in healthy, well-maintained elder and periodontitis subjects. J Clin Periodontol 1998; 25/5: 346-353.

35. Gmür R, Guggenheim B. Interdental supragingival plaque – a natural habitat of Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus, and Prevotella nigrescens. J Dent Res 1994; 73/8: 1421-1428.

36. Tanner A, Maiden MFJ, Kent RL, et al. Microbiota of health, gingivitis, and initial periodontitis. J Clin Periodontol 1998; 25/2: 85-98.

37. McNabb H, Mombelli A, Lang NP, et al.. Periodontal pathogens in the shallow pockets of immigrants from developing countries. Oral Microbiol Immunol 1992; 7/5: 267-272.

38. Frisken KW, Higgins T, Palmer JM. The incidence of periodontopathic microorganisms in young children. Oral Microbiol Immunol 1990; 5/1: 43-45.

39. Dzink JL, Socransky SS, Haffajee AD. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J Clin Periodontol 1988; 15/5: 316-323.

40. Moore LVH, Moore WEC, Cato EP, et al.. Bacteriology of human gingivitis. J Dent Res 1987; 66/5: 989-995.

41. Theilade E, Theilade J, Mikkelsen L. Microbiological studies on early dentogingival plaque on teeth and molar strips in humans. J Periodont Res 1982; 17/1: 12-25.

42. Loesche WJ, Syed SA. Bacteriology of human experimental gingivitis. Effect of plaque and gingivitis score. Infect Immun 1978; 21/3: 830-839.

43. Syed SA, Loesche WJ. Bacteriology of human experimental gingivitis: effect of plaque age. Infect Immun 1978; 21/3: 821-829.

44. Tanner A, Kent R, Maiden MFJ, Taubman MA. Clinical, microbiological and immunological profile of healthy, gingivitis and putative active periodontal subjects. J Periodont Res 1996; 31/3: 195-204.

45. Moore WEC, Holdeman LV, Palcanis KG, et al.. Variation in periodontal floras. Infect Immun 1984; 46/3: 720-726.

46. Noiri Y, Ebisu S. Identification of periodontal disease-associated bacteria in the "plaque-free zone". J Periodontol 2000; 71/8: 1319-1326.

47. Wecke J, Kersten T, Bernimoulin J-P, et al.. A novel technique for monitoring the development of bacterial biofilms in human periodontal pockets. FEMS Microbiol Lett

2000; 191/*1*: 95-101.

48. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000, 1994; *5*: 78-111.

49. Mandell RL. A longitudinal microbiological investigation of Actinobacillus actinomycetemcomitans and Eikenella corrodens in juvenile periodontitis. Infect Immun 1984; 45/*3*: 778-780.

50. Slots J. The predominant cultivable organisms in juvenile periodontitis. Scand J Dent Res 1976; 84/*1*: 1-10.

51. Listgarten MA. The structure of dental plaque. Periodontol 2000, 1994; *5*: 52-65.

52. Mombelli A, Casagni F, Madianos P. Can presence or absence of periodontal pathogens distinguish between subjects with chronic and aggressive periodontitis? J Clin Periodontol 2002; 29/*Suppl.3*: 10-21.

53. Socransky SS, Haffajee AD. Microbial mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. J Periodont Res 1991; 26/*3Pt.2*: 195-212.

54. Ohta H, Kato K, Fukui K, Gottschal JC. Microbial interactions and the development of periodontal disease. J Periodont Res 1991; 26/*3Pt.2*: 255-257.

55. Socransky SS, Haffajee AD. Effect of therapy on periodontal infections. J Dent Res 1993; 64/*Suppl.8*: 754-759.

56. Moore WEC, Burmeister JA, Brooks CN, et al.. Investigation of the influences of puberty, genetics, and environment on the composition of subgingival periodontal floras. Infect Immun 1993; 61/*7*: 1891-1898.

57. Greenstein G, Lamster I. Bacterial transmission in periodontal diseases: a critical review. J Periodontol 1997; 68/*5*: 421-431.

58. Nishihara T, Koseki T. Microbial etiology of periodontitis. Periodontol 2000, 2004; *36*: 14-26.

59. Costerton JW. Introduction to biofilm. Int J Antimicrob Agents 1999; 11/*3-4:* 217-221, 237-239.

60. Forng RY, Champagne C, Genco CA et al. Environmental cues and gene expression in *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. Oral Dis 2000; 6/*6*: 351-365.

61. Brown MR, Gilbert P. Sensitivity of biofilms to antimicrobial agents. J Appl Bacteriol 1993; 74: 87S-97S.

62. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic agents. Periodontol 2000, 2002; *28*: 12-15.

63. Finlay BB, Falkow S. Common themes in microbial pathogenicity. Microbiol Rev 1989; 53/*2:* 210-230.

64. Miller JF, Mekalanos JJ, Falkow SF. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science 1989; 243/*4893*: 916-922.

65. Barua PK, Dyer DW, Neiders ME. Effect of iron limitation on *Bacteroides gingivalis*. Oral Microbiol Immunol 1990; 5/*5*: 263-268.

66. Maurelli AT. Temperature regulation of virulence genes in pathogenic bacteria: a general strategy for human pathogens? Microbial Pathogenesis 1989; 7/*1*: 1-10.

67. Kinder SA, Holt SC. Characterization of coaggregation between *Bacteroides ginigvalis* T22 and *Fusobacterium nucleatum* T18. Infect Immun 1989; 57/*11*: 3204-3209.

68. Ellen RP, Grove DA. *Bacteroides gingivalis* vesicles bind to and aggregate *Actinomyces viscosus*. Infect Immun 1989; 57/*5*: 1618-1620.

69. Kaufman J, DiRienzo JM. Isolation of a corncorb (coaggregation) receptor polypeptide from *Fusobacterium nucleatum*. Infect Immun 1989; 57/*2*: 331-337.

70. Hammond BF, Lillard SE, Stevens RH. A bacteriocin of *Actinobacillus actinomycetemcomitans*. Infect Immun 1987; 55/*3*: 686-691.

71. Gibbons RJ. Bacterial adhesion to oral tissues. A model of infectious diseases. J Dent Res 1989; 68/*5*: 750-760.

72. Winkler JR, John SR, Murray PA, et al.. Attachment of oral bacteria to a basement-membrane–like matrix and to purified matrix proteins. Infect Immun 1987; 55/*11:* 2721-2726.

73. Winkler JR, Matarese V, Murray PA, et al.. An in vitro model to study bacterial invasion of periodontal tissues. J Periodontol 1988; 59/*1*: 40-45.

74. Naito Y, Gibbons RJ. Attachment of *Bacteroides gingivalis* to collagenous substrat. J Dent Res 1988; 67/*8*: 1075-1080.

75. Isogai H, Takano K, et al.. Specific inhibition of adherence of an oral strain of *Bacteroides gingivalis* 381 to epithelial cells by monoclonal antibodies against the bacterial fimbria. Arch Oral Biol 1988; 33/*7*: 479-485.

76. Holt SC, Kesavalu L, Genco CA, et al.. Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000, 1999; 20: 168-238.

77. Slots J, Genco RJ. Microbial pathogenicity: Black-pigmented *Bacteroides* species, *Capnocytophaga* species and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonization, survival, and tissue destruction. J Dent Res 1984; 63/*3*: 412-421.

78. Meyer DH, Mintz KP, Fives-Taylor PM. Models of invasion of enteric and periodontal pathogens into epithelial cells: a comparative analysis. Crit Rev Oral Biol Medical 1997; 8/*4*: 389-409.

79. Chan EC, McLaughlin R. Taxonomy and virulence of oral spirochetes. Oral Microbiol Immunol 2000; 15/*1*: 1-9.

80. Lamont RJ, Yilmaz Ö. In or out: the invasiveness of oral bacteria. Periodontol 2000, 2002; 30: 61-69.

81. Rudney JD, Chen R, Sedgewick GJ. Intracellular *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in buccal epithelial cells collected from human subjects. Infect Immun 2001; 69/*4*: 2700-2707.

82. Dorn BR, Leung KL, Progulske-Fox A. Invasion of human oral epithelial cells by *Prevotella intermedia*. Infect Immun 1998; 66/*12*: 6054-6057.

83. Han YW, Shi W, Genco RJ. et al.. Interactions between periodontal bacteria and human oral epithelial cells. *Fusobacterium nucleatum* adheres to and invades epithelial cells. Infect Immun 2000; 68/*6*: 3140-3146.

84. Rudney JD, Chen R. Intracellular *Bacteroides forsythus* in buccal epithelial cells. J Dent Res (Ann. meeting of AADR) 2001; Abstr. 799.

85. Ellen RP. Perturbation and exploitation of host cell cytoskeleton by periodontal pathogens. Microb Infect 1999; 1/*8*: 621-632.

86. Lamont RJ, Jenkinson HF. Life below the gum line. Pathogenic mechanisms of *Porphyromonas gingivalis.* Microbiol Mol Biol Rev 1998; 62/*4*: 1244-1263.

87. Meyer DH, Sreenivasan PK, Fives-Taylor PM. Evidence for invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans*. Infect Immun 1991; 59/*8*: 2719-1726.

88. Sreenivasan PK, Meyer DH, Fives-Taylor PM. Requirements for invasion of epithelial cells by *Actinobacillus actinomycetemcomitans*. Infect Immun 1993; 61/*4*: 1239-1245.

89. Rozanis J, Slots J. Collagenolytic activity of *Actinobacillus actinomycetemcomitans* and black-pigmented *Bacteroides*. J Dent Res 1982; 61/*Spec. Issue*: 275 (Abstr. 870).

90. Suomalainen K, Sorsa T, Uitto VJ, et al.. Collagenase activity in gingival crevicular fluid of patients with juvenile periodontitis. Oral Microbiol Immunol 1991; 6/*1*: 24-29.

91. Smalley JW, Shuttleworth CA, et al.. The degradation of type I collagen and human plasma fibronectin by the trypsin-like enzyme and extracellular membrane vesicles of *Bacteroides gingivalis* W50. Arch Oral Biol 1988; 33/*5*: 323-329.

92. Lantz MS, Hook M, et al.. *Bacteroides gingivalis* and *Bacteroides intermedius* recognize different sites on human fibrinogen. J Bacteriol 1990; 172/*2*: 716-726.

93. Tanner ACR, Strzempko, McKinley GA. API-Zym and API-ANADENT reactions of fastidious gram negative species. J Clin Microbiol 1985; 22/*3*: 333-335.

94. Slots J. Detection of colonies of *Bacteroides gingivalis* by a rapid fluorescence assay for trypsin-like activity. Oral Microbiol Immunol 1987; 2/*3*: 139-141.

95. Slots J. Enzymatic characterization of some oral and nonoral gram-negative bacteria with the API ZYM system. J Clin Microbiol 1981; 14/*3*: 288-294.

96. Van Steenbergen TJ Van der Mispel LMS, De Graaff J. Effects of ammonia and volatile fatty acids produced by oral bacteria on tissue culture cells. J Dent Res 1986; 65/*6*: 909-912.

97. Stevens RH, Hammond BF. The comparative cytotoxity of periodontal bacteria. J Periodontol 1988; 59/*11:* 741-749.

98. Lindemann RA, Eilber E. Activation of human natural killer cells by lipopolysaccharide from *Actinobacillus actinomycetemcomitans*. Arch Oral Biol 1989; 34: 459-463.

99. Lindemann RA, Economou JS. *Actinobacillus actinomycetemcomitans* and *Bacteroides gingivalis* activate human peripheral monocytes to produce interleukin-1 and tumor necrosis factor. J Periodontol 1988; 59/*11*: 728-730.

100. Garrison SW, Holt SC, Nichols FC. Lipopolysaccharide-stimulated PGE2 release from human monocytes. Comparison of lipopolysaccharide prepared from suspected periodontal pathogens. J Periodontol 1988; 59/*10*: 684-687.

101. Nair BC, Chen PB, Levine MJ, Hausmann E. Biological effects of a purified lipopolysaccharide from *Bacteroides gingivalis*. J Periodont Res 1983; 18/*1*: 40-49.

102. Kaisho T, Akira S. Toll-like receptors and their signaling mechanism in innate immunity. Acta Odontol Scand 2001; 59/*3*: 124-130.

103. Yoshimura A, Hara Y, et al.. Lipopolysaccharides from periodontopathic bacteria *Porphyromonas gingivalis* and *Capnocytophaga ochracea* are antagonists for human toll-like receptor 4. Infect Immun 2002; 70/*1*: 218-225.

104. Grenier D, McBride BC, et al.. Further studies on the degradation of immunoglobulins by black-pigmented *Bacteroides*. Oral Microbiol Immunol 1989; 4/*1*: 12-18.

105. Brogan JM, Demuth DR, et al.. Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. Infect Immun 1994; 62/*2*: 501-508.

106. Rabie G, Shenker BJ, et al.. Immunosuppressive properties of *Actinobacillus actinomycetemcomitans* leukotoxin. Infect Immun 1988; 56/*1*: 122-127.

107. Gillespie J, De Nardin E, Zambon JJ, et al.. Production of an extracellular toxin by the oral pathogen *Campylobacter rectus*. Microb Pathog 1992; 12/*1*: 69-77.

108. Korostoff J, Wang JF, Lally ET, et al.. *Actinobacillus actinomycetemcomitans* leukotoxin induces apoptosis in HL-60 cells. Infect Immun 1998; 66/*9*: 4474-4483.

109. Michalek SM, Katz J, Ballovetz DF, et al.. Microbial / host interactions: mechanisms involved in host responses to microbial antigens. Immunol Res 2002; 26/*1-3*: 223-234.

110. Dixon DR, Darveau RP, et al.. Modulation of the innate immune response within the periodontium. Periodontol 2000, 2004; 35: 53-74.

111. Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res 1994; 8/*2*: 263-271.

112. Medzhitov R. Toll-like receptors and innate immunity. Nature Reviews / Immunology 2001; 1/*2*: 135-145.

113. Cook DN, Schwartz DA, et al.. Toll-like receptors and the genetics of innate immunity. Curr Opin Allergy Clin Immunol 2003; 3/*6*: 523-529.

114. Kaisho T, Akira S. Toll-like receptors as adjuvant receptors. Mol Cell Res 2002; 1589/*1*: 1-13.

115. Arbour NC, Schwartz DA, et al.. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000; 25/*2*: 187-191.

116. Dinarello CA. Multiple biological properties of recombinant human interleukin 1β. Immunology 1986; 172/*3-5*: 301-315.

117. Hanahan DJ. Platelet activating factor: a biologically active phosphoglyceride. Ann Rev Biochem 1986; 55: 483-509.

118. Old LJ. Tumor necrosis factor. Polypeptide mediator network. Nature 1987; 326/*6111*: 330-331.

119. Van Dyke TE, Levine MJ, Genko RJ. Neutrophil function and oral disease. J Oral Pathol 1985; 14/*2*: 95-120.

120. Moughal NA, Kinane DF, et al.. Endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in gingival tissue during health and experimentally induced gingivitis. J Period Res 1992; 27/6: 623-630.

121. Genco RJ, van Dyke TE, Levine MJ, et al.. Molecular factors influencing neutrophil defects in periodontal disease. J Dent Res 1986; 65/12: 1379-1391.

122. Fantone JC, Ward PA. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. Ann J Pathol 1982; 107/3: 398-418.

123. Taichman NS, Tsai CC, Boehringer H. Neutrophil interactions with oral bacteria as a pathogenic mechanism in periodontal diseases. Adv Inflam Res 1984; 8: 113-142.

124. Birkendal-Hansen H. Role of cytokines and inflammatory mediators in tissue destruction. J Periodont Res 1993; 28/6Pt2: 500-510.

125. Hou L, Stashenko P, et al.. Toll-like receptor 4-deficient mice have reduced bone destruction following mixed anaerobic infection. Infect Immun 2000; 68/8: 4681-4687.

126. Mori Y, Hara Y et al. Immunohistochemical localization of Toll-like receptors 2 and 4 in gingival tissue from patients with periodontitis. Oral Microbiol Immunol 2003: 18/1: 54-58.

127. Genco RJ, Goldman HM, Cohen DW. Contemporary Periodontics. Mosby 1990;191-193.

128. Wilson ME, Hamilton RG. Immunoglobulin G subclass response of localized juvenile periodontitis patients to *Actinobacillus actinomycetemcomitans* Y4 lipopolysaccharide. Infect Immun 1992; 60/5: 1806-1812.

129. Mooney J, Adongianaki E, Kinane DF. Relative avidity of serum antibodies to putative periodontopathogens in periodontal disease. J Period Res 1993; 28/6Pt1: 444-450.

130. Bergstrom J. Eliasson S. Noxious effect of cigarette smoking on periodontal health. J Period Res 1987; 22/6: 513-517.

131. Seppala B, Seppala M, Ainamo J. A longitudinal study on insulin-dependent diabetes mellitus and periodontal disease J Clin Periodontol 1993; 20/3: 161-165.

132. Meisel P, Kocher T, et al.. Dose-effect relation of smoking and the interleukin-1 gene polymorphism in periodontal disease. J Periodontol 2004; 75/2: 236-242.

133. Kamma JJ, Mombelli A, et al.. Cytokine profile in gingival crevicular fluid of aggressive periodontitis: influence of smoking and stress. J Clin Periodontol 2004; 31/10: 894-910.

134. Bergström J. Tobacco smoking and chronic destructive periodontal disease. Odontology 2004; 92/1: 1-8.

135. Socransky SS, Haffajee AD, Dibart S, et al.. Relation of counts of microbial species to clinical status at the sampled site. J Clin Periodontol 1991; 18/10: 766-775.

136. Goodson JM, Tanner AC, Socransky SS, et al.. Patterns of progression and regression of advanced destructive periodontal diseases. J Clin Periodontol 1982; 9/6: 472-481.

137. Haffajee AD, Socransky SS, Smith C, Dilbart S. Relation of baseline microbial parameters to future periodontal attachment loss. J Clin Periodontol 1991; 18/10: 744-750.

138. Tanner AC, Socransky SS, Goodson JM. Microbiota of periodontal pockets losing crestal alveolar bone. J Periodont Res 1984; 19/3: 279-291.

139. Haffajee AD, Socransky SS, Ebersole JL, Smith DJ. Clinical, microbiological and immunological features associated with the treatment of active periodontosis lesions. J Clin Periodontol 1984; 11/9: 600-618.

140. Socransky SS, Haffajee AD, Dzink JL. Relationship of subgingival microbial complexes to clinical features at the sampled sites. J Clin Periodontol 1988; 15/7: 440-444.

141. Umeda M, Tominaga YHT, Ishikawa I, et al.. Microbial flora in the acute phase of periodontitis and the affect of local administration of minocycline. J Periodontol 1996; 67/4: 422-427.

142. Bragd L, Dahlen G, Wikström M, Slots J. The capability of *Actinobacillus actinomycetemcomitans, Bactreoides gingivalis* and *Bacteroides intermedius* to indicate progressive periodontitis; a retrospective study. J Clin Periodontol 1987; 14/2: 95-99.

143. Liljenberg B, Lindhe J, Dahlen G, et al.. Some microbiological, histopathological and immunohistochemical characteristics of progressive periodontal disease. J Clin Periodontol 1994; 21/10: 720-727.

144. Moore WEC, Moore LH, Schenkein HA, et al.. The microflora of periodontal sites showing active destructive progression. J Clin Periodontol 1991; 18/10: 729-739.

145. Grossi SG, Zambon JJ, Genco RJ, et al.. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. J Periodontol 1994; 65/3: 260-267.

146. Grossi SG, Genco RJ, Zambon JJ, et al.. Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. J Periodontol 1995; 66/1: 23-29.

147. Listgarten MA,Slots J,Sullivan P,et al.. Incidence of periodontitis recurrence in treated

patients with and without *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromonas gingivalis*: a prospective study. J Periodontol 1991; 62/*6*: 377-386.

148. Ezzo PJ, Cutler CW. Microorganisms as risk indicators for periodontal disease. Periodontol 2000, 2003; 32: 24-35.

149. Pedrazzoli V, Kilian M, Karring T, Kirkegaard E. Effect of surgical and non-surgical periodontal treatment on periodontal status and subgingival microbiota. J Clin Periodontol 1991; 18/*8*: 598-604.

150. Simonson LG, Cohen ME, Morton HE, et al.. *Treponema denticola* and *Porphyromonas gingivalis* as prognostic markers following periodontal treatment. J Periodontol 1992; 63/*4*: 270-273.

151. Takamatsu N, Yano K, Ishikawa I, et al.. Effect of initial periodontal therapy on the frequency of detecting *Bacteroides forsythus, Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans.* J Periodontol 1999; 70/*6*: 574-580.

152. Renvert S, Wikström M, Egelberg J, et al.. Effect of root debridement on the elimination of *Actinobacillus actinomycetemcomitans* and *Bacteroides gingivalis* from periodontal pockets. J Clin Periodontol 1990; 17/*6*: 345-350.

153. Gunsolley JC, Zambon JJ, et al.. Maintenance therapy in young adults with severe generalized periodontitis. J Periodontol 1994; 65/*3*: 274-279.

154. Badersten A, Nilvéus R, Egelberg J. Four-year observations of basic periodontal therapy. J Clin Periodontol 1987; 14/*7*: 438-444.

155. Ebersole IL, Taubman MA, Smith DI, et al.. Effect of subgingival scaling on systemic antibody responses to oral microorganisms. Infect Immun 1985; 48/*2*: 534-539.

156. Listgarten MA, Lai C-H, Young V. Microbial composition and pattern of antibiotic resistance in subgingival microbial samples from patients with refractory periodontitis. J Periodontol 1993; 64/*3*: 155-161.

157. Haffajee AD, Socransky SS, Ebersole JL, et.al. Clinical, microbiological and immunological features of subjects with refractory periodontal diseases. J Clin Periodontol 1988; 15/*6*: 390-398.

158. van Winkelhoff AJ, Tijhof CJ, de Graaff J. Microbiological and clinical results of metronidazole plus amoxicillin therapy in *Actinobacillus actinomycetemcomitans*-associated periodontitis. J Periodontol 1992; 63/*1*: 52-57.

159. Pavicic MJA, van Winkelhoff AJ, et al.. Microbiological and clinical effects of

metronidazole and amoxicillin in *Actinobacillus actinomycetemcomitans* – associated periodontitis. J Clin Periodontol 1994; 21/2: 107-112.

160. Dörfer CE. Antimicrobials for the treatment of aggressive periodontitis. Oral Diseases 2003; 9/*Suppl 1*: 51-53.

161. Loesche WJ, Lopatin DE, Hujoel PP, et al.. Comparison of various detection methods for periodontopathic bacteria: can culture be considered the primary reference standard? J Clin Microbiol 1992; 30/2: 418-426.

162. Göbel UB. Wunschdenken und Realität: Möglichkeiten und Grenzen molekulargenetischer Nachweisverfahren in der medizinischen Mikrobiologie.

163. Woese CR. Bacterial evolution. Microbiol Rev 1987; 51/2: 221-271.

164. Savitt ED, Strzempko MW, French CK, et al.. Comparison of culture methods and DNA probe analysis for the detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis*, and *Bacteroides intermedius* in subgingival plaque samples. J Periodontol 1988; 59/7: 431-438.

165. Lyons SR, Griffen AL, Leys EJ. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. J Clin Microbiol 2000; 38/6: 2362-2365.

166. Armitage GC. Periodontal diagnosis and classification of periodontal diseases. Periodontol 2000, 2004; 34: 9-21.

167. Rodenburg JP, van Winkelhoff AJ, Winkel EG, et al.. Occurrence of *Bacteroides gingivalis, Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in severe periodontitis in relation to age and treatment history. J Clin Periodontol 1990; 17/6: 392-399.

168. Moter A, Hoenig C, Choi B-K, Riep B, Göbel UB. Molecular epidemiology of oral treponemes associated with periodontal disease. J Clin Microbiol 1998; 36/5: 1399-1403.

169. Trevilatto PC, Tramontina VA, Line SR, et al.. Clinical, genetic and microbiological findings in a Brazilian family with aggressive periodontitis. J Clin Periodontol 2002; 29/3: 233-239.

170. Kamma JJ, Gmür R, et al.. Microbiological profile of early onset / aggressive periodontitis patients. Oral Microbiol Immunol 2004; 19/5: 314-321.

171. Fox CH, Jette A, Douglass CW, et al.. Periodontal disease among New England elders. J Periodontol 1994; 65/7: 676-684.

172. Conrads G, Mutters R, Seyfarth I, Pelz K. DNA-probes for the differentiation of

Capnocytophaga species. Mol Cell Probes 1997; 11/*5*: 323-328.

173. Moncla BJ, Motley ST, Vermuelen MJ, et al.. Use of synthetic oligonucleotide DNA probes for identification and direct detection of *Bacteroides forsythus* in plaque samples. J Clin Microbiol 1991; 29/*10*: 2158-2162.

174. Sanger F, Nickeln S, Coulson AR. DNA sequencing with chain terminating inhibitors. Biotechnology 1992; 24: 104-108.

175. Syed S, Loesche W. Survival of human dental plaque flora in various transport media. Appl. Microbiol 1972; 24/*4*; 638-644.

176. Choi BK, Park SH, Kim CK, et al.. Detection of major putative periodontopathogens in Korean advanced adult periodontitis patients using a nucleic acid-based approach. J Periodontol 2000; 71/*9*: 1387-1394.

177. Socransky SS, Haffajee AD, Dzink JL, et al.. Associations between microbial species in subgingival plaque samples. Oral Microbiol Immunol 1988; 3/*1*: 1-7.

178. Lawson PA, Gharbia SE Collins MD, et al.. Intrageneric relationships of members of the genus *Fusobacterium* as determined by reverse transcriptase sequencing of small-subunit rRNA. Int J Sys Bacteriol 1991; 41/*3*: 347-354.

179. Amann RI, Krumholz L, Stahl DA. Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. J Bacteriol 1990; 172/*2*: 762-770.

180. Kinane DF, Attstrom R. Group A summary. J Clin Periodontol 2002; 29/*Suppl 3*: 37-38.

181. Zambon JJ, Haraszthy V. The laboratory diagnosis of periodontal infections. Periodontol 2000, 1995; 7: 69-82.

182. Gmür R, Strub JR, Guggenheim B. Prevalence of *Bacteroides forsythus* and *Bacteroides gingivalis* in subgingival plaque of prosthodontically treated patients on short recall. J Periodont Res 1989; 24/*2*: 113-120.

183. Zambon JJ, Reynolds HS, Slots J. Black pigmented *Bacteroides* spp. in the human oral cavity. Infect Immun 1981; 32/*1*: 198-203.

184. Van Winkelhoff AJ, Steenbergen TJM, de Graaf J. The role of black-pigmented *Bacteroides* in human oral infections. J Clin Periodontol 1988; 15/*3*: 145-155.

185. Paster BJ, Dewhirst FE, Olsen I, Fraser GJ. Phylogeny of *Bacteroides, Prevotella* and *Porphyromonas* spp. and related bacteria. J Bacteriol 1994; 176/*3*: 725-732.

186. Morris ML, Andrews RH, Rogers AH. Investigations of the taxonomy and systematics of

Fusobacterium nucleatum using allozyme electrophoresis. Int J Syst Bact 1997; 47/*1*: 103-110.

187. van Steenbergen TJM, Timmermann MF, de Graaff J, et al.. Discrepancy between culture and DNA probe analysis for the detection of periodontal bacteria. J Clin Periodontol 1996; 23/*10*: 955-959.

188. Melvin WL, Assad DA, York AK, et al.. Comparison of DNA probe and ELISA microbial analysis methods and their association with adult periodontitis. J Periodontol 1994; 65/*6*: 576-582.

189. Tay F, Liu YB, Flynn MJ, Slots J. Evaluation of a non-radioactive DNA probe for detecting *Porphyromonas gingivalis* in subgingival specimens. Oral Microbiol Immunol 1992; 7/*6*: 344-348.

190. Riggio MP, MacFarlane TW, Kinane D, et al.. Comparison of polymerase chain reaction and culture methods for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in subgingival plaque samples. J Periodont Res 1996; 31/*7*: 496-501.

191. Lotofo RFM, Flynn J, Chen C, Slots J. Molecular detection of *Bacteroides forsythus* in human periodontitis. Oral Microbiol Immun 1994; 9/*3*: 154-160.

192. Yano-Higuchi K, Takamatsu N, Ishikawa I, et al.. Prevalence of *Bacteroides forsythus, Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in subgingival microflora of Japanese patients with adult and rapidly progressive periodontitis. J Clin Periodontol 2000; 27/*8*: 597-602.

193. Conrads G. Nachweis von parodontopathogenen Bakterien mit Hilfe von nicht-radioaktiv markierten DNA-Sonden. Dissertation, Aachen: Mainz 1994: 15-62.

194. DeMacario ED, Macario AI. Monoclonal antibodies for bacterial identification and taxonomy. Clin Lab Med 1985; 5/*3*: 531-540.

195. Gmür R. Applicability of monoclonal antibodies to quantitatively monitor subgingival plaque for specific bacteria. Oral Microbiol Immunol 1988; 3/*4*: 187-191.

196. Siqueira JF, Rocas IN, Santos RN, et al.. Comparison of 16S rDNA-based PCR and checkerboard DNA-DNA hybridization for detection of selected endodontic pathogens. J Med Micr 2002; 51/*12*: 1090-1096.

197. Nonnenmacher C, Heeg K, et al.. Quantitative detection of periodontopathogens by real-time PCR. J Microbiol Methods 2004; 59/*1*: 117-125.

198. Conrads G, Flemmig TF, Lütticken R, et al.. Simultaneous detection of *Bacteroides*

forsythus and *Prevotella intermedia* by 16S rRNA gene – directed multiplex PCR. J Clin Microbiol 1999; 37/5: 1621-1624.

199. Flemmig TF, Rüdiger S, Karch H, et al.. Identification of *Actinobacillus actinomycetemcomitans* in subgingival plaque by PCR. J Clin Microbiol 1995; 33/12: 3102-3105.

200. Bodinka A, Schmidt H, Karch H, et al.. Polymerase chain reaction for the identification of *Porphyromonas gingivalis* collagenase genes. Oral Microbiol Immunol 1994; 9/3: 161-165.

201. Conrads G, Mutters R, Lampert F, et al.. PCR reaction and dot-blot hybridization to monitor the distribution of oral pathogens within plaque samples of periodontally healthy individuals. J Periodontol 1996; 67/10: 994-1003.

202. Hutter G, Schlagenhauf U, Vögel U, et al.. Molecular analysis of bacteria in periodontitis: evaluation of clone libraries, novel phylotypes and putative pathogens. Microbiology 2003; 149/Pt1: 67-75.

203. Teanpaisan R, Douglas CW, Walsch TF, et al.. Clonality of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella nigrescens* isolated from periodontally diseased and healthy sites. J Periodont Res 1996; 31/6: 423-32.

204. Lippke JA, Peros WJ, French CK, et al.. DNA probe detection of *Eikenella corrodens, Wolinella recta*, and *Fusobacterium nucleatum* in subgingival plaque. Oral Microbiol Immunol 1991; 6/2: 81-87.

205. Strzempko MN, Simon SL, Vaccaro KK, et al.. A cross-reactivity study of whole genomic DNA probes for *Haemophilus actinomycetemcomitans, Bacteroides intermedius*, and *Bacteroides gingivalis*. J Dent Res 1987; 66/10: 1543-1546.

206. Haffajee AD, Socransky SS, Smith C, Dibart S. The use of DNA probes to examine the distribution of subgingival species with different levels of periodontal destruction. J Clin Periodontol 1992; 19/2: 84-91.

207. Socransky SS, Smith C, Levin AE, et al.. "Checkerboard" DNA-DNA hybridization. Biotechniques 1994; 17/4: 788-792.

208. Chuba PJ, Pelz K, Krekeler G, de Isele TS, Göbel UB. Synthetic oligodeoxynucleotide probes for the rapid detection of bacteria associated with human periodontitis. J Gen Microbiol 1988; 134/7: 1931-1938.

209. Dix K, Watanabe SM, Moncla B, et al.. Species-specific oligodeoxynucleotide probes for the identification of periodontal bacteria. J Clin Microbiol 1990; 28/2: 319-323.

210. Eisenstein BI, Engleberg NC. Applied molecular genetics: new tools for microbiologists and clinicians. J Inf Dis 1986; 153/*3*: 416-430.

211. Göbel UB, Geiser A, Stanbridge EJ. Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between mycoplasma species. J Gen Microbiol 1987; 133/*7*: 1969-1974.

212. Gersdorf H, Meissner A, Pelz K, Krekeler G, Göbel UB. Identification of *Bacteroides forsythus* in subgingival plaque from patients with advanced periodontitis. J Clin Microbiol 1993; 31/*4*: 941-946.

213. Moncla BJ, Braham P, Schwartz D, et al.. Use of synthetic oligonucleotide DNA probes for the identification of *Bacteroides gingivalis*. J Clin Microbiol 1990; 28/*2*: 324-327.

214. Conrads G, Pelz K, Devine DA, et al.. Optimized oligonucleotides for the differentiation of *Prevotella intermedia* and *Prevotella nigrescens*. Oral Microbiol Immunol 1997; 12/*2*: 117-120.

215. Socransky SS, Haffajee AD, Kent RL, et al.. Microbial complexes in subgingival plaque. J Clin Periodontol 1998; 25/*2*: 134-144.

216. Slots J, Potts TV, Masimo PA. *Fusobacterium periodonticum*, a new species from the human oral cavity. J Dent Res 1983; 62/*9*: 960-963.

217. Moore WEC. Microbiology of periodontal disease. J Periodont Res 1987; 22/*5*: 335-341.

218. Rupf S, Merte K, Eschrich K. Quantification of bacteria in oral samples by competitive polymerase chain reaction. J Dent Res 1999; 78/*4*: 850-856.

219. Klappenbach IA, Dunbar JM, Schmidt TM. rRNA operon copy number reflects ecological strategies of bacteria. Appl Environ Microbiol 2000; 66/*4*: 1328-1333.

220. Sakamoto M, Suzuki M, Benno Y, et al.. Reclassification of *Bacteroides forsythus* as *Tannerella forsythensis* corrig., gen. nov., comb. nov.. Int J Syst Evol Microbiol 2002; 52/*Pt3*: 841-849.

221. Leys EJ, Lyons SR, Griffen AL, et al.. Association of *Bacteroides forsythus* and a novel *Bacteroides* phylotype with periodontitis. J Clin Microbiol 2002; 40/*3*: 821-825.

222. Hamlet S, Seymour G, et al.. Persistent colonization with *Tannerella forsythensis* and loss of attachment in adolescents. J Dent Res 2004; 83/*3*: 232-235.

223. DiMurro CD, Lopatin DE, Cattabriga M, et al.. Occurrence of *Porphyromonas gingivalis, Bacteroides forsythus*, and *Treponema denticola* in periodontally healthy and diseased subjects as determined by an ELISA technique. J Periodontol 1997; 68/*1*: 18-23.

224. Kimura S, Ooshima T, Hamada S, et al.. Periodontopathic bacterial infection in childhood. J Periodontol 2002; 73/*1*: 20-26.

225. Gmür R, Marinello M, Guggenheim B. Periodontitis associated bacteria in supragingival plaque of dental hygienists: stability of carrier state and clinical development. Eur J Oral Sci 1999; 107/*3*: 225-228.

226. Tan KS, Song KP, Ong G. *Bactroides forsythus* prtH genotype in periodontitis patients: occurrence and association with periodontal disease. J Periodont Res 2001; 36/*6*: 398-403.

227. Tran SD, Hodges JS, et al.. Persistent presence of *Bacteroides forsythus* as a risk factor for attachment loss in a population with low prevalence and severity of adult periodontitis. J Periodontol 2001; 72/*1*: 1-10.

228. Tanner ACM, Listgarten MA, Ebersole JL, Strzempko MN. *Bacteroides forsythus* sp. nov., a slow-growing fusiform *Bacteroides* sp. from the human oral cavity. Int J Sys Bacteriol 1986; 36/*4*: 213-221.

229. Arakawa S, Tsuchida N, et al.. Novel apoptosis-inducing activity in *Bacteroides forsythus*: a comparative study with three serotypes of *Actinobacillus actinomycetemcomitans*. Infect Immun 2000; 68/*8*: 4611-4615.

230. Albandar JM, Brown JL, Löe H. Putative periodontal pathogens in subginigval plaque of young adults with and without early-onset periodontitis. J Periodontol 1997; 68/*10*: 973-981.

231. Slots J, Ting M. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. Periodontol 2000, 1999; 20: 82-121.

232. van Winkelhoff AJ, Loos BG, van der Velden, et al.. *Porphyromonas gingivalis, Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. J Clin Periodontol 2002; 29/*11*: 1023-1028.

233. Rams TE, Listgarten MA, Slots J. Utility of 5 major putative periodontal pathogens and selected clinical parameters to predict periodontal breakdown in patients on maintenance care. J Clin Periodontol 1996; 23/*4*: 346-354.

234. Griffen AL, Lyons SR, Leys EJ, et al.. *Porphyromonas gingivalis* strain variability and periodontitis. J Clin Microbiol 1999; 37/*12*: 4028-4033.

235. Laine ML, van Winkelhoff AJ. Virulence of six capsular serotypes of *Porphyromonas gingivalis* in a mouse model. Oral Microbiol Immunol 1998; 13/*5*: 322-325.

236. Neiders ME, Chen PB, Genco RJ, et al.. Heterogeneity of virulence among strains of *Bacteroides gingivalis*. J Periodont Res 1989; 24/*3*: 192-198.

237. Teanpaisan R, Douglas CW, Walsch TF, et al.. Clonality of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella nigrescens* isolated from periodontally diseased and healthy sites. J Periodont Res 1996; 31/*6*: 423-432.

238. Loos BG, Mayrand D, Genco RJ, et al.. Genetic heterogeneity of *Porphyromonas gingivalis* by genomic DNA fingerprinting. J Dent Res 1990; 69/*8*: 1488-1493.

239. de Graaff J, van Winkelhoff AJ, Goene RJ. The role of *Actinobacillus actinomycetemcomitans* in periodontal disease. Infection 1989; 17/*4*: 269-271.

240. Van der Velden U, Abbas F, van Steenbergen TJ, et al.. Prevalence of periodontal breakdown in adolescents and presence of *Actinobacillus actinomycetemcomitans* in subjects with attachment loss. J Periodontol 1989; 60/*11*: 604-610.

241. Tanner ACR, Socransky SS, et al.. A study of the bacteria associated with advancing periodontitis in man. J Clin Periodontol 1979; 6/*5*: 278-307.

242. Slots J, Feik D, Rams TE. *Actinobacillus actinomycetemcomitans* and *Bacteroides intermedius* in human periodontitis: age relationship and mutual association. J Clin Periodontol 1990; 17/*9*: 659-662.

243. van Winkelhoff AJ, Rams TE, Slots J. Systemic antibiotic therapy in periodontics. Periodontol 2000, 1996; 10: 45-78.

244. Rams TE, Flynn MJ, Slots J. Subgingival microbial associations in severe human periodontitis. Clin Infect Dis 1997; 25/*Suppl2*: 224-226.

245. Zambon JJ, Demuth DR, et al.. The microbiology of early-onset periodontitis. Association of highly toxic *Actinobacillus actinomycetemcomitans* strains with localized juvenile periodontitis. J Periodontol 1996; 67/*3*: 282-290.

246. Bueno LC, Mayer MPA, DiRienzo JM. Relationship between conversion of LJP-susceptible children from health to disease and *Actinobacillus actinomycetemcomitans* leukotoxin promoter structure. J Periodontol 1998; 69/*9*: 998-1007.

247. Meyer DH, Sreenivasan PK, Fives-Taylor PM. Evidence for invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans*. Infect Immun 1991; 59/*8*: 2719-2726.

248. Slots J. Update on *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease. J Int Acad Periodontol 1999; 1/*4*: 121-126.

249. Christersson LA, Zambon JJ. Suppression of subgingival *Actinobacillus actinomycetemcomitans* in localized juvenile periodontitis by systemic tetracycline. J Clin Periodontol 1993; 20/*6*: 395-401.

250. Goene RJ, Winkel EG, de Graaff J, et al.. Microbiology in diagnosis and treatment of severe periodontitis. A report of four cases. J Periodontol 1990; 61/*1*: 61-64.

251. Nakagawa S, Machida Y, Nakagawa M, et al.. Infection by *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, and antibody responses at different ages in humans. J Periodont Res 1994; 29/*1*: 9-16.

252. Asikainen S, Alaluusua S, Saxen L. Recovery of *Actinobacillus actinomycetemcomitans* from teeth, tongue and saliva. J Periodontol 1991; 62/*3*: 203-206.

253. Zambon JJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease. J Clin Periodontol 1985; 12/*1*: 1-20.

254. Rams TE, Feik D, Slots J. *Campylobacter rectus* in human periodontitis. Oral Microbiol Immunol 1993; 8/*4*: 230-235.

255. Riviere GR, Smith KS, Adams DF, et al.. Periodontal status and detection frequency of bacteria at sites of periodontal health and gingivitis. J Periodontol 1996; 67/*2*: 109-115.

256. Ebersole JL, Taubman MA, Smith DJ. Quantitative comparison of serum IgG antibody to periodontal microorganisms. J Dent Res 1985; 64/*1*: 361-366.

257. Lai C-H, Oshima K, Slots J, Listgarten MA. *Wolinella recta* in adult gingivitis and periodontitis. J Periodont Res 1992; 27/*1*: 8-14.

258. Gharbia SE, Shah HN. Heterogeneity within *Fusobacterium nucleatum*, proposal of four subspecies. Lett Appl Microbiol 1990; 10: 105-108.

259. Roques CG, Elkaddouri S, et al.. *Fusobacterium nucleatum* involvement in adult periodontitis and possible modification of strain classification. J Periodontol 2000; 71/*7*: 1144-1150.

260. Shah HN, Gharbia SE. Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal of a new pigmented species, *Prevotella nigrescens* sp. nov.. Int J Syst Bacteriol 1992; 42/*4*: 542-546.

261. Dahlen G, Wikström M, Guggenheim B, et al.. Biochemical and serological characterization of *Bacteroides intermedius* strains isolated from the deep periodontal pocket. J Clin Microbiol 1990; 28/*10*: 2269-2274.

262. Gmür R, Saxer UP, Guggenheim B. Double-blind analysis of the relation between adult

periodontitis and systemic host response to suspected periodontal pathogens. Infect Immun 1986; 52/3: 768-776.

263. Magnusson I, Marks RG, McArthur WP, et al.. Clinical, microbiological and immunological characteristics of subjects with "refractory" periodontal disease. J Clin Periodontol 1991; 18/5: 291-299.

264. Wolff LF, Pihlstrom B, Fischer G, et al.. Natural distribution of 5 bacteria associated with periodontal disease. J Clin Periodontol 1993; 20/10: 699-706.

265. Haffajee AD, Socransky SS, Smith C, Dibart S. Microbial risk indicators for periodontal attachment loss. J Period Res 1991; 26/3Pt2: 293-296.

266. Buu-Hoi AY, Joundy S Acar JF. Endocarditis caused by *Capnocytophaga ochracea*. J Clin Microbiol 1988; 26/5: 1061-1062.

267. Tanner A. Is the specific plaque hypothesis still tenable? In: Guggenheim B. ed. Periodontology Today, Zürich: Karger Basel 1988; 123-131.

268. Yao ES, Lamont RJ, Weinberg A, et al.. Interbacterial binding among strains of pathogenic and commensal oral bacterial species. Oral Microbiol Immunol 1996;11/1: 35-41.

269. Slots J, Chen C. Detection of *Porphyromonas gingivalis* associated with human periodontitis by DNA methods. Clin Infect Dis 1993; 16/Suppl.4: 317-318.

270. Wade W. Unculturable bacteria – the uncharacterized organisms that cause oral infections. J R Soc Med 2002; 95/2: 81-83.

271. Kumar PS, Griffen AL, Leys EJ, et al.. New bacterial species associated with chronic periodontitis. J Dent Res 2003; 82/5: 338-344.

272. Slots J. Herpesviruses, the missing link between gingivitis and periodontitis? *J Int Acad periodontol* 2004; 6/4: 113-119.

273. Contreras A, Slots J. Active cytomegalovirus infection in human periodontitis. Oral Microbiol Immunol 1998; 13/4: 225-230.

274. Offenbacher S. Periodontal diseases: pathogenesis. Ann Periodontol 1996; 1/1: 821-878.

275. Schenkein HA. Finding genetic risk factors for periodontal diseases: is the climb worth the view? Periodontol 2000, 2002; 30: 79-90.

276. Michalowicz BS. Genetic and heritable risk factors in periodontal disease. J Periodontol 1994, 65/Suppl.5: 479-488.

277. Takahashi K, Ohyama H, Murayama Y, et al.. Heterogeneity of host immunological risk factors in patients with aggressive periodontitis. J Periodontol 2001; 72/4: 425-437.

278. Rawlinson A, Dalati MHN, et al.. Interleukin-1 and IL-1 receptor antagonist in gingival crevicular fluid. J Clin Periodontol 2000; 27/10: 738-743.

279. D'Aiuto F, Tonetti MS, et al.. Gene polymorphisms in pro-inflammatory cytokines are associated with systemic inflammation in patients with severe periodontal infections. Cytokine 2004; 28/1: 29-34.

280. Loos BG, Van der Velden U, et al.. Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. J Periodontol 2000; 71/10: 1528-1534.

281. Liu RK, Gao Y, et al.. Polymorphonuclear neutrophils and their mediators in gingival tissues from generalized aggressive periodontitis. J Periodontol 2001; 72/11: 1545-1553.

282. Albandar JM, Winn DM, et al.. Associations between serum antibody levels to periodontal pathogens and early-onset periodontitis. J Periodontol 2001; 72/11: 1463-1469.

283. Suarez LJ, Rodriguez A, et al.. Relative proportions of T-cell subpopulations and cytokines that mediate and regulate the adaptive immune response in patients with aggressive periodontitis. J Periodontol 2004; 75/9: 1209-1215.

284. Kornman KS, Crane A, Duff GW. The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol 1997; 24/1: 72-77.

285. Parkhill JM, Hennig BJ, Taylor JJ, et al.. Association of interleukin-1 gene polymorphisms with early-onset periodontitis. J Clin Periodontol 2000; 27/9: 682-689.

286. Agnese DM, Lowry SF, et al.. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. J Infect Dis 2002; 186/10: 1522-1555.

287. Schwartz DA. The genetics of innate immunity. Chest 2002; 121/Suppl.3: 62S-68S.

288. Folwaczny M, Folwaczny C, et al.. Toll-like receptor 2 and 4 mutations in periodontal disease. Clin Exp Immunol 2004; 135/2: 330-335.

289. Suzuki A, Kamoi K, et al.. Large-scale investigation of genomic markers for severe periodontitis. Odontology 2004; 92/1: 43-47.

290. Choi BK, Paster BJ, Dewhirst FE, Göbel UB. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect Immun 1994;

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11 Danksagung

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Eidestattliche Erklärung

Ich erkläre hiermit an Eides Statt, daß die Dissertation von mir selbst und ohne Hilfe Dritter verfasst wurde, auch in Teilen keine Kopie anderer Arbeiten darstellt und die benutzten Hilfsmittel sowie die Literatur vollständig angegeben sind.

Berlin, den 13.04.05