UMEÅ UNIVERSITY

PERIODONTITIS IN ADOLESCENTS

PRESENCE OF *AGGREGATIBACTER* SPECIES AND INTERACTIONS WITH THE HOST

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Cover: Scanning electron micrograph of *Aggregatibacter actinomycetemcomitans ompA1 ompA2* double mutant cultured on agar (front). Adapted from Lindholm M, Min Aung K, Nyunt Wai S, Oscarsson J. Role of OmpA1 and OmpA2 in *Aggregatibacter actinomycetemcomitans* and *Aggregatibacter aphrophilus* serum resistance. *Journal of Oral Microbiology. 2018 Oct 26;11(1):1536192.* Images are originally produced at Umeå Core Facility for Electron Microscopy. Atomic force micrograph of *Aggregatibacter actinomycetemcomitans* D7SS cultured on agar (back). Courtesy of Monica Persson, Dept. of Molecular Biology, Umeå University.
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To my family

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Abstract

Aggregatibacter actinomycetemcomitans and *Aggregatibacter aphrophilus* are closely related gram-negative bacteria mainly found in the oral cavity. Periodontitis in adolescents is associated with the presence of *A. actinomycetemcomitans*, but not with the presence of *A. aphrophilus*. However, both can cause extra-oral infections such as endocarditis and brain abscesses. Several unknown virulence and environmental factors could influence their prevalence in different populations. A shift in the microbiological and demographical characteristics could also influence the prevalence of periodontitis in adolescents residing in Sweden.

The aims of this PhD thesis were to study mechanisms that might influence serum resistance in *A. actinomycetemcomitans* and *A. aphrophilus*. In relation to this we wanted to study the presence of these bacterial species in two different adolescent populations (Västerbotten, Sweden and Kenya), factors influencing their prevalence, and the prevalence of periodontitis in adolescents in Sweden.

In **paper I**, serum resistance was detected in all tested strains of *A. actinomycetemcomitans* and *A. aphrophilus*. The OmpA paralogues, OmpA1 and OmpA2 were important for serum resistance in both species, i.e., inactivation of their genes, respectively resulted in significantly reduced serum resistance. In *A. actinomycetemcomitans*, *ompA1* mutant derivatives were fortuitously found with regained serum resistance as a result of increased expression of OmpA2. OmpA1 and OmpA2 from *A. actinomycetemcomitans*, and OmpA1 from *A. aphrophilus* were able to bind C4bp, an inhibitor of parts of the activating paths of the complement system, suggesting this to be one mechanism to mediate bacterial serum resistance.

In **paper II**, we investigated the effects of outer membrane vesicles (OMV) on the serum resistance of *A. actinomycetemcomitans*. The survival rate of an almost totally serum sensitive *A. actinomycetemcomitans ompA1* and *ompA2* double mutant increased 100-fold when OMVs from a wildtype strain were supplemented. This effect was similar when OMVs from the *ompA1 ompA2* double mutant were used, suggesting an OmpA-independent effect.
On the other hand, OMVs from an LPS-deficient *A. actinomycetemcomitans* strain did not contribute to serum survival,

consistent with an LPS-dependent serum resistance carried out by the vesicles. This appeared to be due to a substantial consumption of serum complement components by LPS in the OMVs.

In **paper III**, the salivary prevalence of *A. actinomycetemcomitans* and *A. aphrophilus* was high (71.8% and 99% respectively) in the adolescent population from Maasai Mara, Kenya. *A. aphrophilus* was found in higher amounts compared to *A. actinomycetemcomitans*.
Cultivation from subgingival samples of a subset of the population yielded a lower prevalence of *A. actinomycetemcomitans* (22.1%). The highly leukotoxic genotype, JP2, was discovered in one salivary sample, indicating the first JP2-genotype identified in East Africa.

In **paper IV**, screening of dental radiographs from an adolescent population born in 2001 and residing in Västerbotten County, Sweden (n=1656) was performed. This resulted in 24 individuals (1.45%) positive for marginal bone loss (>2 mm).
The dropout-rate was rather high and, eventually, thirteen of these individuals (cases) were periodontally examined, as well as 26 controls who did not display any marginal bone loss. Twelve of the cases were diagnosed with periodontitis, indicating an increase in the prevalence of periodontitis in adolescents. Moreover, this emphasized the potential of radiographs to identify small changes on marginal bone levels to diagnose periodontitis in adolescents.
The cases had significantly higher values of clinical attachment loss, pocket probing depth and bleeding on probing.
Significantly higher loads and prevalence of *A. actinomycetemcomitans*, *Porphyromonas gingivalis* and *Filifactor alocis* were generally detected in the saliva and subgingival plaque samples from the cases. In contrast, *A. aphrophilus* was more prevalent in the controls.

In conclusion, outer membrane proteins OmpA1 and OmpA2, and outer membrane vesicles display protective effects contributing to serum resistance in *A. actinomycetemcomitans* and *A. aphrophilus*. The prevalence of these bacteria varies in different populations, and they are also affected by environmental factors. Finally, the prevalence of periodontitis in adolescents in Västerbotten is suggested to have increased and is associated with distinct microbiological characteristics and geographic origin.

Abbreviations

16S rRNA	16S ribosomal RNA gene
BoP	Bleeding on probing
Bp	Basepairs
C1-C9	Complement factors
C4bp	C4b-binding protein
cagE	Cytotoxin associated gene E
CAL	Clinical attachment level/loss
cAMP	Cyclic adenosine monophosphate
Cdt	Cytolethal distending toxin
CFU	Colony forming units
CMV	Cytoplasmic membrane vesicle
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EU	Endotoxin units
HACEK	*Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, Kingella*
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
JP2 genotype	Deletion of 530 bp in the promoter region of *A. actinomycetemcomitans*
kDa	kilodalton
LppAB	Braun's lipoprotein
LPS	Lipopolysaccharide
ltx	Leukotoxin gene
LtxA	Leukotoxin
MAC	Membrane attack complex
MASP	Mannan-binding lectin serine protease
MBL	Mannose binding lectin
MMP	Matrix metalloproteinase
MV	Membrane vesicle
NaCl	Sodium chloride
NHS	Normal human serum
OIMV	Outer-inner membrane vesicle
Omp	Outer membrane protein
OMV	Outer membrane vesicle

OPG	Osteoprotegerin
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PGE2	Prostaglandin E_2
PMN	Polymorphonuclear neutrophile
PPD	Pocket probing depth
PQS	Pseudomonas Quinolone Signal
PS	Plaque score
qPCR	quantitative polymerase chain reaction
RANKL	Receptor activator of nuclear factor-κB ligand
SDS	Sodium dodecyl sulfate
TBS	Tris buffered saline
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TVC	Total viable count

Enkel sammanfattning på svenska

Parodontit är en sjukdom som innebär att tändernas fäste förstörs på grund av en inflammation som orsakas av bakterier i munhålan.
Parodontit hos ungdomar är en relativt ovanlig sjukdom i Sverige och beräknas drabba ca 0,1% av populationen. I andra delar av världen, framför allt vissa delar av Afrika, är detta en betydligt vanligare sjukdom och kan drabba upp till 12% av populationen.
Parodontitens utveckling associeras med en obalans i bakteriefloran i munnen och i området kring tandköttet och i tandköttsfickorna runt tänderna. I flera fall har man sett att bakterien *Aggregatibacter actinomycetemcomitans* finns hos dessa ungdomar.

I denna avhandling studerades olika faktorer hos denna bakterie som skulle kunna förklara dess överlevnad i humant serum, som utgör en del av blodet och liknar den vätska som finns vid inflammerade tandköttsfickor.
Genom att använda sig av ett protein (OmpA), som utgör en del av bakteriens yttermembran, kunde vi visa att detta protein interagerar med komplementsystemet i immunförsvaret. Bakterien binder upp, med hjälp av OmpA, ett av kroppens egna proteiner och skyddas på så sätt mot avdödning. Vi kunde även se liknande resultat i en nära besläktad art, *Aggregatibacter aphrophilus*.
Vi kunde även se skydd mot komplementsystemet genom att *A. actinomycetemcomitans* producerade små blåsor som avknoppas från yttermembranet, så kallade yttermembranvesiklar. Vesiklarna kunde skydda bakterien från avdödning genom att agera lockbete för komplementsystemet och på detta sätt använda upp komplementfaktorerna så att de inte nådde bakterierna.

I avhandlingen studerades också närvaron av båda bakterierna i olika populationer. I en population ungdomar från Maasai Mara i Kenya kunde vi visa att dessa bakterier fanns i hög utsträckning i saliven. Gällande *A. actinomycetemcomitans* så var detta också tydligt hos en population ungdomar från Västerbotten, dock enbart hos de som drabbats av parodontit.
I samband med detta visade det sig också att de som drabbats av parodontit hade ett annat geografiskt ursprung, vilket delvis kan förklara den ökade risken för parodontit. Denna delstudie påpekade också vikten av noggrann diagnostik och analys av röntgenbilder hos de med parodontit. Detta då

mycket små förändringar i bennivåerna runt tänderna kunde påvisa parodontit med god säkerhet.
Sammanfattningsvis kan denna avhandling påvisa olika taktiker hos *A. actinomycetemcomitans* som gör att den kan överleva i munhålan och undkomma immunförsvaret.
Resultaten tyder även på dess närvaro i olika populationer men även dess koppling till parodontit hos ungdomar.
Slutligen bör också nämnas att resultaten tyder på en ökning av parodontit hos ungdomar i Västerbotten i jämförelse med äldre studier.

Original papers

This thesis is based on four original papers, which will be referred to by their Roman numerals:

I. **Lindholm M**, Min Aung K, Nyunt Wai S, Oscarsson J. Role of OmpA1 and OmpA2 in *Aggregatibacter actinomycetemcomitans* and *Aggregatibacter aphrophilus* serum resistance.
Journal of Oral Microbiology. 2018 Oct 26;11(1):1536192.

II. **Lindholm M**, Metsäniitty M, Granström E, Oscarsson J. Outer membrane vesicle-mediated serum protection in *Aggregatibacter actinomycetemcomitans*.
Journal of Oral Microbiology. 2020 Apr 8;12(1):1747857.

III. **Lindholm M**, Claesson R, Kemoli A, Mulli T, Oscarsson J, Haubek D, Johansson A. *Aggregatibacter actinomycetemcomitans* and *Aggregatibacter aphrophilus* in a Kenyan Maasai Adolescent Population and Inhibition of Leukotoxic Activity by Herbal Plants Used as Part of Oral Hygiene Procedures.
Journal of Clinical Medicine. 2021 Nov 19;10(22):5402.

IV. **Lindholm M**, Claesson R, Chiang HM, Löf H, Oscarsson J, Johansson A, Höglund Åberg C. Radiograhic and clinical signs of periodontitis in a Swedish adolescent population.
Submitted, under peer review.

Introduction

The oral bacterial ecosystem

At present the oral cavity is estimated to be inhabited by about 700 different bacterial species with 150-200 of these species colonizing simultaneously in one individual (Lamont et al., 2019). The oral cavity is unique in that it presents both shedding and non-shedding surfaces. The shedding surfaces, such as the epithelial surfaces of the mucosa, do not harbour bacteria in large numbers because of the renewal and subsequent shedding of the epithelium. The non-shedding surfaces, the teeth, however, offer a good surface for bacteria to attach to and to form a biofilm,"plaque", on due to the inert surface (Lamont et al., 2019).
The teeth are initially coated with a thin layer termed "pellicle". The pellicle mainly consists of salivary glycoproteins and offers a good attachment for the primary bacterial colonizers, usually being streptococcal species, such as *Streptococcus mitis*, *oralis* and *gordonii*. These provide attachments for subsequent bacteria, termed late or secondary colonizers (Lamont et al., 2019).
As the biofilm matures new bacterial species will be incorporated into the biofilm by interbacterial co-adhesion, mediated by adhesins, leading to bacterial successions over time. Extracellular glucans, produced by *Streptococcus mutans*, *S. mitis* and *Actinomyces naselundii* can also facilitate interbacterial binding (Rosan & Lamont, 2000).
If the biofilm is not removed by mechanical means, it will proliferate and incorporate several bacterial species. The interbacterial relationships will result in a community that can withstand the environmental pressure from antimicrobials produced by the host as well as exogenous antibiotics. If the biofilm is still not removed, due to poor oral hygiene, it can lead to incipient dysbiosis in the biofilm, meaning a shift in the microbiota in favor of periodontitis-associated bacteria. This in combination with a susceptible individual will lead to an uncontrolled immune response that results in periodontitis (Hajishengallis & Lamont, 2021).

Periodontal disease and epidemiology

The periodontium is a term collectively describing the tooth-supporting tissues. It consists of gingiva, periodontal ligament, root cementum and alveolar bone. The dentogingival junction is the part of gingiva that is closest to the tooth and consists of epithelium (gingival, sulcular and junctional epithelium) and connective tissue. The junctional epithelium plays an important role since it seals off periodontal tissues from the oral environment, thus important for maintaining a healthy periodontium (Nanci & Bosshardt, 2006). The main function of the periodontium is to attach the tooth to the alveolar bone. This unit represents a dynamic system that undergoes changes related to age as well as alterations in the oral environment (Könönen et al., 2019; Lang, 2015).
In case of inflammation in these tissues, either gingivitis or periodontitis might develop, comprising the two major categories of periodontal disease (Nanci & Bosshardt, 2006).
Clinically, gingival health is defined as < 10% bleeding sites with probing depths ≤3 mm (for an intact periodontium and a reduced and stable periodontium) (Chapple et al., 2018). If the bleeding sites exceed 10%, the patient is diagnosed as having gingivitis. Depending on the case definition of gingivitis, epidemiological studies suggest a prevalence of up to 95% (for mild localized gingival inflammation). In the more extensive cases of gingival inflammation the prevalence is lower.

Periodontitis is defined as a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms and characterized by progressive destruction of the tooth-supporting apparatus (Papapanou et al., 2018). Clinically, interproximal clinical attachment loss (CAL) of ≥2 mm or ≥3 mm at ≥2 non-adjacent teeth is used to identify periodontitis. In order to confirm the interproximal CAL, radiographic bone loss is commonly used. Unlike gingivitis, which only affects the gingiva, periodontitis manifests as clinical attachment loss and radiographically assessed alveolar bone loss. In addition, periodontal pocketing (>3 mm) and gingival bleeding is also present in most cases.
The prevalence of severe periodontitis is high, affecting approximately 10% of the global population (Frencken et al., 2017). Severe periodontitis was here defined as CAL of more than 6 mm, a probing depth of more than 5 mm or Community Periodontal Index of Treatment Needs (CPITN) score of 4. In Sweden the prevalence of severe periodontitis is approximately the same (11%) (Wahlin et al., 2018).

At the moment there is insufficient evidence to support the fact that the prevalence of severe periodontitis has changed over time (Frencken et al., 2017).

The destruction of the periodontium, resulting from periodontitis, is usually a slow process. However, this process can progress rapidly, especially in children and adolescents where classical hallmarks of periodontitis (e.g. calculus) can be absent (Albandar, 1993; Höglund Åberg, Kwamin, et al., 2014). Usually this affects the permanent first molars and incisors but can also appear in a generalized form affecting all teeth.
According to the former periodontal classification system this was termed ”aggressive periodontitis”, in contrast to ”chronic periodontitis”, which is characterized by a slower progression rate (Armitage, 1999). In the new classification system these entities do not exist separately but are merged to simply ”periodontitis” (Papapanou et al., 2018). The classification system is now based on staging and grading of periodontitis. Whereas the stages are defined by the severity, complexity, extent and distribution of periodontitis, the grades reflect the rate of periodontitis progression and factors affecting this. Hence, the old term ”aggressive periodontitis” would fit into grade C. This stage is defined by a rapid rate of progression in the new classification, still signifying a periodontal condition that requires early diagnosis and rapid treatment.

The prevalence of periodontitis affecting young individuals (defined earlier as ”aggressive periodontitis”) varies greatly between populations and may be a significant health problem in certain populations (Haubek et al., 2008; Höglund Åberg, Kwamin, et al., 2014). In European populations the prevalence is reported to vary between 0.1-0.2% (Saxby, 1987; Saxén, 1980; Van der Velden et al., 1989). However, there are large differences based on ethnicity. While Caucasians have a disease prevalence of 0.1-0.2%, Hispanics have a prevalence of up to 1.08% and those with an African origin have up to 12.3% (Kissa et al., 2022; Susin et al., 2014).
A recent meta-analysis confirmed these results, suggesting that the highest prevalence was in Africa (4.2%), followed by South America (4.0%), Asia (1.2%), North America (0.8%) and Europe (0.1%). The global pooled prevalence was estimated to be 1.6% (Bouziane et al., 2020). However, many of the studies on the prevalence of aggressive periodontitis display a high degree of heterogeneity since the definition of aggressive periodontitis has been highly variable in these studies. This might have an

impact on the results reported in such prevalence studies and makes it difficult to compare studies (Ramírez et al., 2018).
Due to increased globalization, periodontitis among adolescents seems to increase in prevalence in Sweden, mainly due to an increase of people with different geographic backgrounds (Åberg et al., 2009). Two recent studies in Sweden indicate an increase of marginal bone loss and periodontitis among adolescents from Somalia, indicating a prevalence of up to 12% in this group (Svensson et al., 2016; Thorbert-Mros et al., 2021).

Maasai Mara, Kenya

Maasai Mara is located in the south-western part of Kenya and is contiguous with the Serengeti national park in Tanzania. The two areas are mainly famous for the great wildebeest migration involving a rich diversity of large wild animals.
Maasai Mara is also famous for the Maasai tribe living within the borders of the national reserve.
The Maasai Mara National Reserve belongs to the Narok County and Transmara administrations. It is estimated that about 340 000 people in Narok County live in poverty. The school attendance in the county is also relatively low with only 10.4% (14-17 years) attending secondary school. Access to public health care is restricted due to long distances and neonatal mortality is high. Access to clean water is restricted and only 21.4% of the households in Narok County have access to clean water (Ayiemba, 2015). Even though the Maasai tribe is affected by the modernization of Kenya many still hold on to their cultural heritage and traditions.
To conserve the Maasai Mara ecosystem, the "Maasai Mara Science and Development Initiative (MMSDI)" was launched in 2014 (https://mgmt.au.dk/maasaimarascience). This project also includes health-related subprojects which will be specified in the "Discussion" part of this thesis.
Periodontitis among adolescents in Kenya is not well-studied, but one study indicates a prevalence of 0.28% (Wagaiyu & Wagaiyu, 1992). Microbial analyses regarding periodontal bacteria in this population is lacking.

Västerbotten County, Sweden

Västerbotten County forms part of Northern Sweden with a land area of 55 190 km^2 and a population of 273 192 in 2020. The biggest cities in the county are Umeå, Skellefteå and Lycksele (*Informationsmaterial och riktlinjer*, 2022).

The dental care is provided by the county ("Folktandvården") for the majority of children (0-23 years of age) since the county has the legal responsibility for this ("Tandvårdslagen," 1985). The intervals for dental examinations for this age category are often ranging between two and three years but are dependent on the risk assessment of the individual patient (*Tandvård för barn och unga 0-23 år*, 2021). The dental examinations are performed by a dentist or dental hygienist. Frequently, radiographs are indicated for diagnosis of caries or periodontitis. If a child is at risk or exhibits a dental or oral disease, such as caries or periodontitis, they are to be offered causal treatment and preventive measures. In the cases of periodontitis in adolescents exhibiting rapid progression they are to be referred to the specialist clinic in Periodontology according to the guidelines within Folktandvården.

Periodontal conditions in Västerbotten County have been evaluated in a study from 1990 (Källestål et al., 1990). The study reported that radiographic bone loss was seen in about 1% of the 16- (n=287) and 18-year-olds (n=283) included in the study. However, periodontitis was not diagnosed in this study.

Oral microbiology

The functional role of bacteria in periodontal disease has been discussed for decades. In the end of the 19th century the non-specific plaque hypothesis was the prevailing opinion signifying the amount of plaque (total bacterial challenge) (Lang, 2015). The study by Löe et al. (1965), where the impact of bacterial load on gingivitis and its reversibility by oral hygiene measures, pioneered the way for the non-specific plaque hypothesis. This study did however note a microbial shift between health and disease. Lindhe et al. (1973) published a study that would account for the microbial involvement in periodontitis as well, this time studied in beagle dogs.

Over the years the focus shifted from the hypothesis that the amounts of plaque contribute to periodontitis to the hypothesis that certain specific bacteria are more virulent than others, hence termed the specific plaque

hypothesis (Loesche, 1979). This was mainly due to better diagnostics and new technologies involving DNA-processing and analyses leading to more specific identification of microbes involved in periodontitis. The publication by Socransky et al. (1998) utilized these new technologies and saw that they could cluster bacteria depending on their frequency and if they were found together in the biofilm.

The role of the environment on the plaque/biofilm has also been discussed and termed the ecological plaque hypothesis (Marsh, 2003). Rather than pinning certain microbes the emphasis is on the environmental conditions and how changes in these can modify the bacterial composition in the biofilm. The hypothesis is that changes in the environment can result in a disrupted homeostasis between microbes related to health and disease. The enrichment of periodontopathogenic bacteria will further aggravate the inflammatory response related to periodontal diseases.

Dysbiosis of bacterial biofilms has arisen as a hypothesis on the bacterial effect on periodontitis (Darveau et al., 2012). Instead of emphasizing the effects of the environment on the biofilm, the emphasis is on single pathogens having pathogenic effects on the commensal flora and thereby inducing dysbiosis of the biofilm leading to an inflammatory response by the host. *Porphyromonas gingivalis* has been suggested to be such a "keystone pathogen" in periodontitis since it induces changes in the commensal oral flora but also has a profound effect on the host immune response by manipulating the complement system.

The most recent model to explain periodontitis was proposed by Van Dyke et al. (2020). The model, termed IMPEDE (Inflammation-Mediated Polymicrobial-Emergence and Dysbiotic-Exacerbation) emphasizes the role of uncontrolled inflammation as a driving force for the induction of dysbiosis in the biofilm, ultimately leading to periodontitis.

Detection of oral bacteria

Historically, cultivation of oral bacteria has represented the gold standard for detection. In recent years this has been substituted by other methods, mainly methods based on DNA due to the ability to detect uncultivable bacteria (Teles et al., 2013). These methods mainly include PCR, qPCR, 16S rRNA and checkerboard DNA-hybridization (Socransky & Haffajee, 2005). New methods to analyze and profile the microbiome are arising and include methods such as transcriptomics, metabolomics and proteomics (Bostanci et al., 2019). However, cultivation still represents an important method in detection of oral bacteria and the study of their functional roles

in the pathogenesis of periodontitis. Rather than excluding one method for the detection of bacteria they should be used to complement each other (Donachie et al., 2007).

Subgingival plaque has traditionally been used to analyze microbial content in the periodontal pocket due to the relatively easy access and ability to cultivate from the samples (Christersson et al., 1991). In conjunction with the use of DNA-based methods, the cheek mucosa and saliva have arisen as alternatives to detect bacteria that are involved in periodontitis (Müller et al., 1995; Umeda et al., 1998; Zhou et al., 2015). Saliva has been proposed to be used as a substitute to subgingival plaque sampling because it is easier to sample, no trained dental professionals are needed and similar results can be obtained as with subgingival plaque sampling (Belstrøm et al., 2017). Because of its ease of sampling, saliva has also been indicated in mass screening of periodontitis (Ma et al., 2021). However, a recent meta-analysis of the red-complex bacteria (*P. gingivalis*, *T. forsythia* and *T. denticola*) in saliva and subgingival plaque revealed that detection frequencies and relative abundances of these bacteria were significantly lower in saliva than in subgingival plaque (Jiang et al., 2021). Nonetheless, the relative abundances of these bacteria in saliva displayed a significant positive correlation to subgingival samples. However, site specific microbiology is not possible to examine based on results from salivary analyses.

Aggregatibacter actinomycetemcomitans

A. actinomycetemcomitans is a gram-negative, non-motile, facultatively anaerobic, capnophilic rod (Nørskov-Lauritsen, 2014). The bacterial colonies can reach up to 2 mm in diameter and display a “rough” appearance when initially grown on agar (Figure 1) (Nørskov-Lauritsen, 2014). The “rough” appearance is associated with the presence of fimbriae, however, these can be lost by re-cultivation giving the appearance of smooth colonies (Figure 1) (Inouye et al., 1990). The loss of fimbriae is partly associated with mutations of the *flp* promoter (Wang et al., 2005).
The bacterium can further be classified according to different serotypes, based on the distinct O-polysaccharide components of their respective lipopolysaccharide (LPS) molecules (Nørskov-Lauritsen et al., 2019; Zambon et al., 1983). At the moment seven serotypes, designated a-g have been identified to exist, with serotype g being the most recent (Takada et al., 2010).

The species is present in the oral cavity of a large proportion of the human population as part of the commensal flora (Henderson et al., 2010). Nevertheless, the prevalence is highly variable depending on the population studied, but according to studies performed in the Nordic countries the prevalence is 20-25% (Könönen et al., 2007; Papapanou et al., 1993), however a Danish study indicated the prevalence to be less than 3% (Belstrøm et al., 2014). In healthy European adolescents the prevalence is also variable and ranges in large-scale studies from 13%-30% (Eick et al., 2013; Jensen et al., 2020; Müller et al., 1996; Paolantonio et al., 2000). In contrast, *A. actinomycetemcomitans* is more prevalent among adolescents from Africa, indicating a prevalence from 54-64% (Haubek et al., 2008; Åberg et al., 2012). Some of these variations could be explained by methodological differences in sampling and/or analyses.

Although *A. actinomycetemcomitans* is considered in many cases to be a part of the resident and commensal flora of the oral cavity it is still considered to be a periodontal pathogen because of its strong association to periodontitis in adolescents (Könönen & Müller, 2014). The large genetic diversity that exists within this species might contribute to explain its association to diseased as well as to healthy periodontium (Kittichotirat et al., 2016)
A. actinomycetemcomitans can also spread from the oral cavity into the blood circulation and thereby to other body sites, as evidenced by the occurrence of various severe non-oral infections (van Winkelhoff & Slots, 1999).

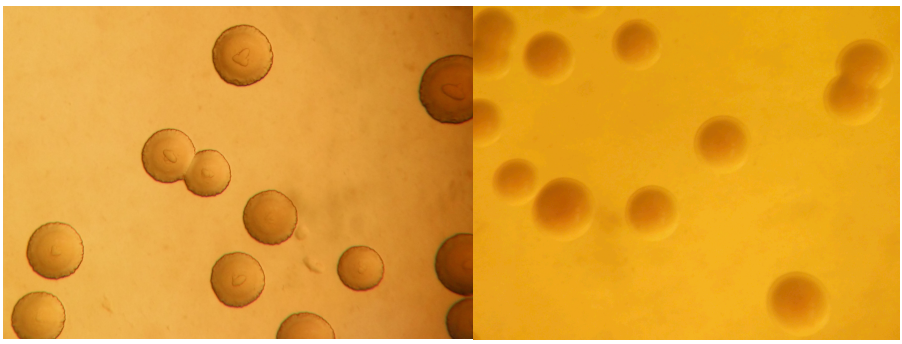

Figure 1
Colonies of *A. actinomycetemcomitans* photographed under a light microscope. Rough-colony variant, strain D7S to the left, and smooth-colony variant D7SS to the right.

Aggregatibacter aphrophilus

A. aphrophilus is a gram-negative, capnophilic, non-motile, rod-shaped bacterium, with colonies that phenotypically resemble those of *A. actinomycetemcomitans*, but *A. aphrophilus* strains often have a somewhat faster growth. *A. aphrophilus* does not produce catalase as *A. actinomycetemcomitans* does (Nørskov-Lauritsen & Kilian, 2006).

Originally named *Haemophilus aphrophilus* by Khairat in 1940 (Khairat, 1940) it was reclassified in 2006 to belong to the genus *Aggregatibacter* along with *A. actinomycetemcomitans* (then termed *Actinobacillus actinomycetemcomitans*) due to their high resemblance (Nørskov-Lauritsen & Kilian, 2006). At the same time, it was concluded that the division of *Haemophilus paraphrophilus* and *Haemophilus aphrophilus* was not justified due to their large genetic similarities, and that they should be considered as one species, namely *Aggregatibacter aphrophilus*.

Later studies have also indicated close relationships between *A. aphrophilus* and *A. actinomycetemcomitans* and both also form a part of the HACEK-group (Kittichotirat et al., 2016) (Nørskov-Lauritsen, 2014). The HACEK group is a group of gram-negative bacteria that can cause infective endocarditis. Both *A. actinomycetemcomitans* and *A. aphrophilus* can cause other non-oral systemic infections in addition to endocarditis as well (Paju et al., 2003).

A. aphrophilus is mainly found in the oral cavity as a commensal with no known association to periodontitis. In the oral cavity, it is primarily found in supragingival plaque and saliva (Tempro & Slots, 1986), however, it can also be found in the subgingival plaque (Liljemark et al., 1984). It is

suggested to be a primary colonizer of smooth surfaces on teeth (Kilian et al., 1976).

Even though *A. aphrophilus* and *A. actinomycetemcomitans* are very similar, *A. aphrophilus* is as far known not associated with periodontitis. Interestingly, *A. aphrophilus* does not encode for leukotoxin (LtxA) and cytolethal distending toxin (Cdt), two exotoxins produced by *A. actinomycetemcomitans* (Tønjum & Haas, 1993; Yamano et al., 2003). They also display differences in the composition of the LPS, which could have an impact on the differential virulence in periodontitis (Brondz & Olsen, 1989). However, their outer membrane protein profiles are rather similar (Bolstad et al., 1990).

Additional *Aggregatibacter* species

In addition to *A. actinomycetemcomitans* and *A. aphrophilus*, two other *Aggregatibacter* species exist.

Aggregatibacter segnis and *Aggregatibacter kilianii* are generally found in the oral cavity and upper respiratory tracts (Murra et al., 2018; Nørskov-Lauritsen & Kilian, 2006). Neither of these are associated with periodontitis but have been implicated in other systemic infections such as infective endocarditis, cholecystitis and pyelonephritis (Lau et al., 2004; Yang et al., 2022)

Bacterial virulence factors

Bacterial virulence factors are products contributing to virulence produced by pathogenic bacteria in order to survive/multiply within the host. The function of virulence factors is to evade or interfere with the immune system of the host but also to aid in adhesion/invasion of the bacterium to host cells (Sharma et al., 2017).

To accomplish these different functions, several types of virulence factors are used by bacteria, often simultaneously. In order to facilitate adhesion to host cells, the bacteria produce adhesins, which can be fimbriae, however, capsules and lipopolysaccharides can also be involved in adhesion (Dahlén et al., 2019). Several oral bacteria, such as *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* have the ability to invade epithelial cells providing shelter from the immune system of the host (Rudney et al., 2005). The endotoxin LPS, which is found on the cell envelope of gram-negative bacteria, exhibits immunoreactive properties

leading to activation of innate immunity and thus enhanced inflammation (Raetz & Whitfield, 2002). In contrast to endotoxin, which is a structural component of the bacterium, exotoxins are released from the bacterium to the surrounding environments.
As mentioned earlier, *A. actinomycetemcomitans* produces two different exotoxins, leukotoxin that induces killing of leukocytes, and cytolethal distending toxin that acts as a genotoxin causing cell cycle arrest (Oscarsson et al., 2019; Sugai et al., 1998; Taichman et al., 1980). The leukotoxin gene cluster *(ltxCABD*) is found in all known strains of *A. actinomycetemcomitans*, but the *cdt* gene cluster (*cdtABC*) is not (Kittichotirat et al., 2011).
The production of leukotoxin between strains is highly variable with some strains producing little to no leukotoxin at all and some producing high amounts of the toxin (Brogan et al., 1994). The strains producing enhanced amounts of leukotoxin are associated with higher disease activity in contrast to the low level-producing strains, suggesting an important clinical role of this exotoxin in periodontitis (Höglund Åberg, Haubek, et al., 2014). In contrast, the disease progression of periodontitis does not seem to be as strongly related to *cdt* and its activity (Höglund Åberg et al., 2013). However, some conflicting results regarding the association of Cdt to periodontitis exists (Wang et al., 2014).

High level production of leukotoxin can partly be explained by differences in the promoter region of *ltxCABD* in serotype b strains since, sequence variations, such as deletions and insertions, might affect binding of transcriptional regulator(s) and thus affect the leukotoxin expression. One such sequence variation, and the most well-known deletion is the 530-bp deletion, typical of the JP2-genotype, which exhibits a 10-20-fold higher production of leukotoxin compared to non-JP2 genotypes of *A. actinomycetemcomitans* (Brogan et al., 1994). Insertion sequences in the promoter region have also been shown to associated with a higher production of leukotoxin (He et al., 1999). Interestingly, two atypical promoters with different sizes of deletions in this region have also been identified here at Oral Microbiology, Odontology, in highly leukotoxic strains. However, not all sequence variations seem to affect the production of leukotoxin (Claesson et al., 2020). High leukotoxin production is not exclusively dependent on variations in the promoter region of *ltxCABD*. The presence of *cagE*, a gene encoding a ≈39 kDa protein, initially suggested to be a putative exotoxin, is also associated with an enhanced leukotoxin production (Johansson et al., 2017; Teng & Hu, 2003).

The leukotoxin affects several different host cells with the ability to lyse monocytes/macrophages, polymorphonuclear leukocytes, lymphocytes, and erythrocytes (Baehni et al., 1979; Balashova et al., 2006; Simpson et al., 1988; Taichman et al., 1980). It also activates neutrophil degranulation and a pro-inflammatory response in macrophages (Johansson et al., 2000; Kelk et al., 2022).

Clinically, when the bacteria translocate to a site in the subgingival crevices, a persistent colonization may lead to periodontal destruction and development of periodontitis in susceptible individuals.
While many bacteria that earlier have been proposed to be important in periodontitis, Dahlén and co-workers (2019) suggest that only for the JP2 genotype of *A. actinomycetemcomitans* there is clinical evidence supporting a causative role in disease progression. *A. actinomycetemcomitans* could therefore also be regarded as a keystone pathogen in periodontitis (Fine et al., 2019).
This could be seen in prospective studies performed in Morocco and Ghana (Haubek et al., 2008; Höglund Åberg, Kwamin, et al., 2014). In these studies, disease progress was highly dependent on the presence of the JP2-genotype. However, also presence of *A. actinomycetemcomitans* of non-JP2 genotype in adolescents from different populations has in prospective studies showed a significantly increased risk for the carriers to develop periodontal attachment loss (Fine et al., 2007; Haubek et al., 2008; Höglund Åberg, Kwamin, et al., 2014; Van der Velden et al., 2006).
In addition, based on subgingival plaque samples analyzed at the clinical laboratory for oral microbiology at the dental school in Umeå, it was shown that the prevalence and proportion of *A. actinomycetemcomitans* were significantly higher in samples from young individuals (≤ 35 years) compared to older individuals (> 35 years) (Claesson et al., 2017). Characterization of the isolates from the different samples showed a high prevalence of a sub-group of serotype b from the non-JP2 genotype. Interestingly, this genotype was also identified in the longitudinal study by Höglund Åberg and co-workers (2014) as a risk marker for onset and progression of periodontitis. This subgroup has later been characterized as the previously mentioned *cagE* genotype (Johansson et al., 2019; Johansson et al., 2017). Taken together, this indicates a role also of non-JP2 *A. actinomycetemcomitans* in periodontal disease, depending on the age of the colonized individual and the characteristic of the colonizing bacterium.

Interestingly, leukotoxin has been proposed to be associated with the onset of rheumatoid arthritis due to the ability of leukotoxin to cause hypercitrullination in host neutrophils (Konig et al., 2016).

Host defense in periodontitis

The host defense in the periodontal pocket is activated when the resident cells recognize pathogen associated molecular patterns (PAMPs) on the surfaces of bacteria, such as bacterial antigens (e.g. LPS). The PAMPs are recognized by Toll-like receptors (TLRs) found on dendritic cells, polymorphonuclear neutrophiles (PMNs) and macrophages. As these cells are activated, the inflammatory response is initiated in the periodontal tissues and an increase in cytokines is seen. Thus, PMNs increase in the tissues and activation of the complement system occurs.
The complement factors C3a and C5a act as anaphylatoxins and lead to vasodilatation due to release of histamine and vasoactive amines from mast cells. Tumor necrosis factor α (TNFα) is also released by the mast cells and in addition to vasodilatation the vascular permeability and expression of adhesion molecules will increase. The adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and p-selectin are expressed on the endothelial cells and hence attract more PMNs to the tissues from the circulation (Lang, 2015).
The first signs of tissue destruction can be noted at the stage of infection process described above and it is a consequence of the release of lysosomal enzymes from the PMNs. The PMNs also produce cytokines such as IL-1 and IL-17. IL-17 will consequently increase the production of IL-8 from epithelial cells. The chemokine IL-8 will attract more PMNs to the site of inflammation. At this point lymphocytes and macrophages will start to increase in the tissues and the antigen presenting cells, such as dendritic cells and B-lymphocytes will activate the T-lymphocytes. The lymphocytes now mainly consist of T-lymphocytes. Even though tissue destruction is occurring, it is confined to collagen and not bone at this stage, hence clinical signs of gingivitis are evident but not periodontitis (Lang, 2015; Seymour et al., 1988).
If this process is allowed to continue, without interventions directed at removing the bacteria by mechanical cleaning, the tissue destruction will include loss of bone, i.e., periodontitis. The bone loss occurs as the macrophages form osteoclasts that can resorb bone. At this stage the lymphocyte composition will also shift towards B-lymphocytes, in

particular plasma cells. The fibroblasts will produce matrix metalloproteinases (MMPs), leading to further degradation of the extracellular matrix, in response to IL-1, IL-6, TNFα and PGE2. These compounds will also affect the expression of receptor activator of nuclear factor-κB ligand (RANKL), that activates the osteoclasts, and osteoprotegerin (OPG) that inhibits the osteoclasts (Yucel-Lindberg & Båge, 2013).

It has been hypothesized that there would be a difference in the host response in adolescents affected by periodontitis when compared to adults (aggressive/chronic periodontitis). However, no clear evidence of this has been found (Kulkarni & Kinane, 2014) and sufficient evidence of a different pathophysiology has not been supported (Papapanou et al., 2018). Familial aggregation of this type of periodontitis seems important but neither here sufficient evidence of genetic factors seem to exist (Fine et al., 2018).

On the other hand, several systemic diseases that affect the immune system display a significant association to periodontitis. Leukocyte adhesion deficiency syndrome, Papillon-Lefèvre syndrome, Kostmann syndrome and Chediak-Higashi syndrome are examples of such diseases that display early onset periodontitis (Albandar et al., 2018; Carlsson et al., 2006).

Complement system

Function

The complement system represents the first line of defense against pathogens. It can be divided into three different pathways (classical, lectin, alternative) based on different modes of activation (Rus et al., 2005). The classical pathway is activated by antibodies recognizing antigens on the surface of microbes while the lectin pathway is activated by mannose binding lectin (MBL), that binds carbohydrate structures, and the alternative pathway is continuously activated at a low level (Merle, Church, et al., 2015). However, other activation pathways have been presented. For the classical pathway antibody-independent options exist, such as LPS, lipid A, porins on the bacterial surfaces, apoptotic cells, adiponectin, and C-reactive protein (Damgaard et al., 2015). Regarding the activation of the lectin pathway it can also be activated by ficolins (Endo et al., 2006).

The classical pathway is initiated when the complement factor C1q is activated by the above-mentioned factors. When C1q is bound to these factors the serine protease C1r is activated, that in turn activates another

serine protease, C1s. This will lead to the cleavage of the C4 and subsequently C2 and activation of the C3 convertase C4bC2a.
The lectin pathway is initially activated by MBL or ficolins bound to carbohydrate domains or acetylated compounds. This leads to activation of MBL/ficolin-activated serine proteases (MASPs) cleaving C4 and C2 in the same manner as in the classical pathway producing the same C3-convertase.
As the alternative pathway is continuously activated it does not require a specific activator, rather it is activated by the spontaneous hydrolysis of C3. This together with factor B activates factor D that will cleave factor B and form the C3 convertase (C3bBb), this is also stabilized by properdin.
The three pathways will converge and form the C3 convertase that will cleave the complement factor C3 into C3a and C3b. C3a is an anaphylatoxin (along with C5a) that induces inflammation and activates immune cells and non-myeloid cells (Merle, Noe, et al., 2015). C4a has sometimes been described as an anaphylatoxin as well but since no C4a receptor has been identified its definition as an anaphylatoxin is questionable (Barnum, 2015). C3b will bind to target surfaces (opsonization) and hence lead to increased phagocytosis of the target cell. In addition to opsonization this will also lead to activation of the adaptive immune system through formation of C3d (via Factor I) that binds complement receptor 2. This receptor can be found on B-cells, dendritic cells, and T-cells (Cherukuri et al., 2001; Fischer et al., 1991; Reynes et al., 1985).
Complement amplification will occur when C3b is bound to the surface of the target cell, meaning that factor B is recruited and leads to production of more C3-convertases and thus to cleavage of more C3. This will also lead to cleavage of C5 producing C5a and C5b. This will initiate the last part of the complement cascade when C5b associates with C3b to produce the C5-convertase. This will eventually lead to the formation of the MAC (membrane attack complex) when complement factors C6-C9 bind together with C5b. This will result in lysis of the bacterium due to differences in osmotic pressure.
The effect of the MAC is thought to be more important for lysis of gram-negative bacteria than gram-positive bacteria, probably due to the thicker peptidoglycan layer. Interestingly, gram-positive bacteria can produce inhibitors for the MAC and it has been showed that the MAC can be localized on gram-positive bacteria as well (Berends et al., 2013).
An overview of the complement system is presented in Figure 2.

Bacterial interactions with the complement system

In order to regulate the complement system, the system contains an array of inhibitors. The inhibitors present in the serum can in turn be utilized by bacteria present in periodontitis to confer serum resistance. This will lead to an increased survival of these bacteria, e.g. *A. actinomycetemcomitans* (Sundqvist & Johansson, 1982), in the subgingival pocket where gingival crevicular fluid, a fluid that resembles serum in composition (Alfano, 1974), is present.

This has been described in detail for several periodontopathogenic bacteria.

P. gingivalis has the ability to degrade C3 with gingipains, a group of cysteine proteases, which also are produced by *Prevotella intermedia* but termed interpain A (InpA)(Potempa et al., 2009; Wingrove et al., 1992). *P. intermedia* can also bind the complement inhibitor Factor I, responsible for the degradation of C3b and C4b (Malm et al., 2012). *Treponema denticola* can bind the complement factor H (McDowell et al., 2009), a regulator of the alternative pathway that inhibits the C3 convertase and use a bacterial neuraminidase to prevent the deposition of membrane attack complexes (Kurniyati et al., 2013). *Tannerella forsythia* can evade the complement by production of the metalloproteinases, karilysin and mirolysin, which inhibit all the complement pathways (Jusko et al., 2012; Jusko et al., 2015). It also produces a surface layer, termed S-layer, that inhibits the deposition of C3b onto the cell surface (Shimotahira et al., 2013). Recently, *Filifactor alocis* has also displayed inhibition of complement by the FACIN protein (*F. alocis* complement inhibitor) by binding C3 and locking the C3-convertase (in the alternative pathway) in an inactive state (Jusko et al., 2016).

P. gingivalis not only has the ability to degrade components of the complement system but also to "hijack" the system. Since *P. gingivalis* and its gingipains can cleave C5 it will lead to local generation of C5a and C5b. While C5b is proteolytically cleaved, C5a can bind to C5aR on macrophages and exploit it to induce cAMP that will inhibit the killing capacity of the macrophages (Hajishengallis et al., 2011). This induction is enhanced synergistically due to crosstalk between TLR2 and C5aR (Wang et al., 2010).

In periodontitis the complement system represents one of the first lines of defense against periodontal pathogens. As described earlier, many periodontopathogens have evolved strategies to evade the complement system. The complement system in gingival crevicular fluid is activated to

a higher degree in periodontitis-affected sites and periodontal therapy has proven effective in lowering the activity of the complement system (Niekrash & Patters, 1985). In the study mentioned earlier (Hajishengallis et al., 2011), the indirect effects of the complement system on bone loss were demonstrated, underlining the importance of the complement system in periodontitis and the effects of the commensal microbiota. Synthetic compounds blocking parts of the complement system have showed protective effects in periodontitis, suggesting a role of the complement system in periodontitis (Maekawa et al., 2016).

In several gram-negative bacteria, e.g., *Yersinia enterocolitica* and *Acinetobacter baumanii*, outer membrane proteins have an important role to protect the bacteria from antibacterial proteins and the complement system. The complement system interacts with many bacterial outer membrane proteins, which is why many bacterial species have developed mechanisms to evade the complement system through outer membrane proteins (Bliska & Falkow, 1992; Kim et al., 2009). For *A. actinomycetemcomitans*, the importance of the outer membrane protein Omp100 in serum resistance has been demonstrated. The mechanism was dependent on the binding of Factor H to Omp100, which is a regulator of the alternative pathway (Asakawa et al., 2003).

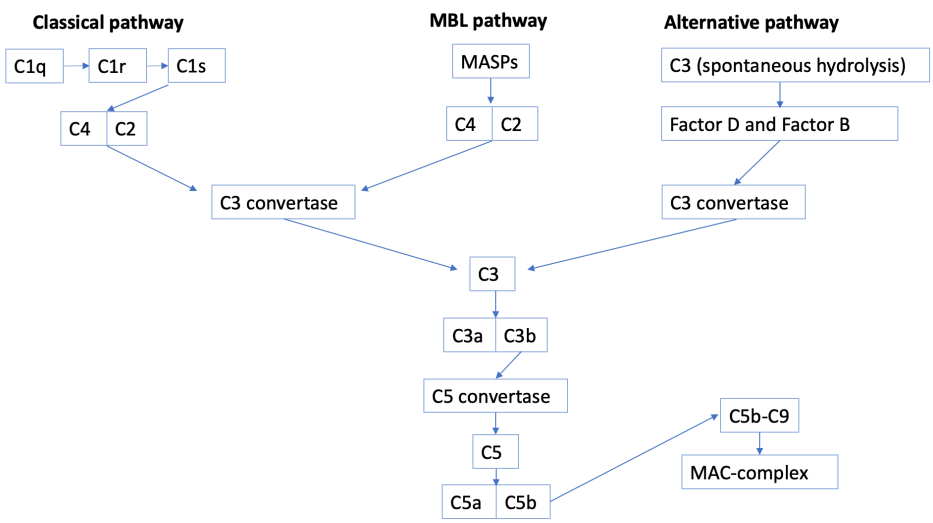

Figure 2
An overview of the complement system and its activation pathways.

Membrane vesicles (MVs)

Membrane vesicles are small (20-400 nm), round particles released from bacteria during normal growth, but also in response to environmental stress (McBroom & Kuehn, 2007; McMahon et al., 2012). The vesicles can contain a diverse range of bacterial products and substances, such as proteins (including virulence factors), lipids and nucleic acids (Kim et al., 2015). Bacterial membrane vesicles include the subgroups outer membrane vesicles (OMVs), outer-inner membrane vesicles (OIMVs) and cytoplasmic membrane vesicles (CMVs). These subtypes are defined based on type of bacterium (gram-negative/gram-positive) and what parts of the membranes are included in the vesicles (gram-negative bacteria) (Toyofuku et al., 2019).

Outer membrane vesicles, which are produced by a majority of gram-negative bacteria, can contain a similar array of outer membrane proteins as the bacterial cells from which they have been shed (Nho et al., 2015; Schwechheimer & Kuehn, 2015). In species such as *P. gingivalis*, *Moraxella catarrhalis* and *Vibrio cholerae* the role of OMVs in serum resistance has been demonstrated (Aung et al., 2016; Grenier & Belanger, 1991; Tan et al., 2007).

In periodontitis, roles of membrane vesicles in host interaction have mainly been researched regarding OMVs, even though cytoplasmic membrane vesicles are gaining more interest. However, only a few studies have looked at this and at the moment only data on *F. alocis* CMVs exist (Kim et al., 2020).

Regarding OMVs in periodontitis-associated bacteria, studies have shown that the red-complex bacteria involving *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola* produce OMVs (Friedrich et al., 2015; McKee et al., 1986; Rosen et al., 1995). Among these species *P. gingivalis* is the most prolific producer of OMVs followed by *T. forsythia* and *T. denticola*. Interestingly *P. gingivalis* is also the one that exhibits the greatest stimulatory activity on pattern recognition receptors (Cecil et al., 2016).

Periodontopathogenic bacteria outside the red-complex, such as *Fusobacterium nucleatum* (Liu et al., 2019) has also been shown to produce OMVs, as well as *A. actinomycetemcomitans* (Figure 3) and its close relative *A. aphrophilus* (Holt et al., 1980). OMVs in periodontopathogenic bacteria can deliver virulence factors such as leukotoxin, cytolethal distending toxin, gingipains and peptidylarginine deiminase to the host cells (Kato et al., 2002; Nakao et al., 2014;

Rompikuntal et al., 2012). Since many of these virulence factors are enriched in the vesicles, their potential role in periodontal disease could be of importance. Due to their small size, they can also spread more easily in comparison to the bacterial cell and have been shown to cross the blood-brain barrier in mice (Han et al., 2019).

Biogenesis of membrane vesicles

The biogenesis of membrane vesicles has not been well understood but has gained more interest during the past years. The biogenesis differs based on the type of vesicles produced.

OMVs

OMVs are usually secreted through outer membrane blebbing. The blebbing occurs due to disturbances in the cell envelope. This was speculated to occur at the site of cell division because the cell envelope exhibits changes in conjunction to this (Hoekstra et al., 1976). However, the OMVs can also be produced at other sites. This is mainly due to local differences in linkages between the inner membrane, peptidoglycan and outer membrane, which in turn can lead to remodeling and the release of vesicles (Deatherage et al., 2009). Outer membrane proteins, such as OmpA, were also shown in the same study to influence the production of OMVs. This occurred most likely due to loss of connections between the outer membrane and peptidoglycan. However, there were differences in vesicle production depending on the protein. For example, *lppAB* mutants exhibited an increased vesicle production compared to *ompA* mutants because OmpA exhibits non-covalent linkages compared to LppAB that exhibits covalent linkages between the outer membrane and peptidoglycan. Size of membrane vesicles was affected depending on if they were released from the site of cell division (large) or along the cell body (small), probably due to differences in the amounts and quality in membrane linkages in these positions. The *lppAB* mutants produced smaller vesicles while *pal*, *tolA* and *tolB* mutants produced larger vesicles in comparison to wild type.

The accumulation of phospholipids in the outer membrane can also influence the vesiculation of bacteria. This will eventually lead to an asymmetric expansion of the outer leaflet of the outer membrane and finally result in a vesicle that is pinched off. This was demonstrated in a study by Roier et al. (2016) where deletion of *vacJ* and *yrb*, which are part of an ABC-transport system involved in the transport of phospholipids

from the outer membrane to the inner membrane, resulted in an increased vesiculation. Interestingly, this had no effect on the membrane integrity.
Another model suggests that OMVs are produced in response to stress where misfolded proteins are accumulated in the outer membrane. In response to this, OMVs are produced to counteract this by releasing these proteins in vesicles (McBroom & Kuehn, 2007; Tashiro et al., 2009).
Other models exist for vesicle biogenesis; however, these are usually only studied in isolated species. One such is Pseudomonas Quinolone Signal (PQS) that has an effect on the curvature of the outer membrane and thus initiates vesicle formation (Mashburn-Warren et al., 2008). In this case PQS interacts with LPS that leads to a curvature in the outer membrane. This model has been termed the bilayer-couple model (Schertzer & Whiteley, 2012).
Recently, a model describing the association between flagellum and release of OMVs has been presented (Aschtgen et al., 2016). This seems to be associated with a sheathed flagella and the rotation of the flagellum. However, it is not clear if this leads to increased vesiculation from the whole cell body or only locally (at or near the flagellum).

OIMVs
Since OIMVs contain both parts of the inner as well as the outer membrane the biogenesis of these differ from that of OMVs. The first evidence of OIMVs was found in 1995 by Kadurugamuwa & Beveridge because DNA was contained in the vesicles of *Pseudomonas aeruginosa* and none of the earlier described models could explain this. However, it took until 2013 for the term OIMV to be coined (Pérez-Cruz et al., 2013). In this study, the OIMVs were visualized, using cryo-TEM, in the gram-negative organism *Shewanella vesiculosa* M7^{T} clearly indicating both OMVs with a single membrane and OIMVs with a double membrane. The inclusion of DNA in the OIMVs was also demonstrated and visualized by using a TEM immunolabelling technique with an antibody specific for dsDNA. The theory, earlier proposed by Kadurugamuwa & Beveridge (1995), that a breach in the peptidoglycan layer by autolysins leading to OIMVs was also confirmed. This was because vesicles produced by *S. vesiculosa* M7^{T} induced cell lysis in other gram-positive bacteria indicating the presence of hydrolyzing enzymes in the same manner as in vesicles produced by *P. aeruginosa*.
The inclusion of chromosomal DNA in vesicles can also be indicative of cell lysis, which is why explosive outer-membrane vesicles (EOMVs) also can contain DNA (Turnbull et al., 2016). EOMVs are separated from

regular OMVs due to different biogenesis. OIMVs can also be produced as a response to cell lysis and are termed explosive OIMVs (Baeza et al., 2021). In these cases, the cell lysis is thought to produce fragments of the cell envelope that consequently anneal and encapsulate free DNA. Recently, a new model was introduced, in addition to previously mentioned models. This model suggests the incorporation of plasmid DNA into OMVs occur through leakage of DNA from the cytoplasm, because of deficiencies in the peptidoglycan leading to increased inner membrane permeability (Aktar et al., 2021).
OIMVs have also been demonstrated for *A. actinomycetemcomitans* and *T. denticola* when performing proteomic analyses of OMVs (Kieselbach et al., 2015; Veith et al., 2019). No visualizations (e.g. by microscopy) were done in these studies but the proteomic analyses clearly showed the inclusion of cytoplasmic proteins and cytoplasmic membrane in the vesicles indicating OIMVs.

CMVs
Cytoplasmic membrane vesicles have not received as much attention as outer-membrane vesicles historically and only recently this type of vesicles has gained more attention. First noted in 1990 (Dorward & Garon, 1990) but not described until 2009 in *S. aureus* (Lee et al., 2009) CMVs have thereafter been found in several gram-positive organisms such as *Streptococcus mutans*, *Bacillus anthracis* and *Listeria monocytogenes* (Lee et al., 2013; Liao et al., 2014; Rivera et al., 2010). As mentioned earlier, the gram-positive periodontopathogen *F. alocis* also produces vesicles (Kim et al., 2020).
The production of CMVs is not clearly understood but is thought to occur in a similar way as for EOMVs and OIMVs, namely by bacterial cell lysis. This has been demonstrated in *Bacillus subtilis* and depended on the expression of an endolysin encoded by a defective prophage leading to vesicle formation (Toyofuku et al., 2017). In contrast to EOMVs and OIMVs, where the bacterial cells are lysed before the assembly of vesicles, the CMVs were released from intact bacterial cells initially. This was thought to be because of the thicker peptidoglycan layer that resulted in holes in the peptidoglycan where cytoplasmic membrane material could protrude through rather than lysis. Eventually the bacterial cells in this case would also die due to loss of membrane integrity.

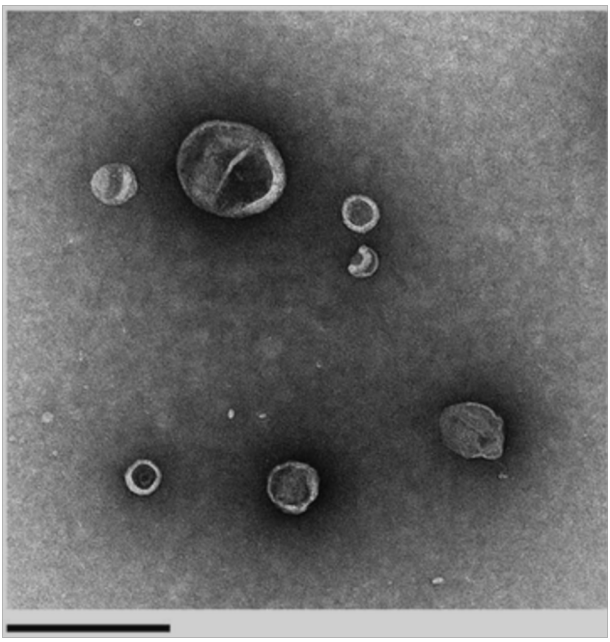

Figure 3
Transmission electron micrograph of OMVs released from *A. actinomycetemcomitans* strain D7SS (Bar = 200 nm). Adapted from Lindholm et al. 2019.

Aims

The overall aim of this thesis was to examine virulence characteristics in *A. actinomycetemcomitans* and *A. aphrophilus*, specifically related to the interaction between the complement system, outer membrane proteins and outer membrane vesicles. This also included to study these bacteria in a clinical setting and their role in periodontitis among adolescents. In conjunction with this the prevalence of periodontitis among adolescents was assessed.

The specific aims were

1. To investigate if outer membrane protein A (OmpA) plays a role in serum resistance in *A. actinomycetemcomitans* and *A. aphrophilus*.
2. To investigate if outer membrane vesicles play a role in serum resistance in *A. actinomycetemcomitans.*
3. To examine the salivary presence and levels of *A. actinomycetemcomitans* and *A. aphrophilus* in a population of adolescents in the Maasai Mara area of Kenya. The secondary aim was to assess factors that could potentially reduce the virulence of *A. actinomycetemcomitans*, such as high levels of the close relative *A. aphrophilus* or local tools used for oral hygiene.
4. To examine the presence of radiographic bone loss in an adolescent population in Västerbotten County, Sweden. Also, to compare periodontal and microbial parameters, as well as demographical patterns in this population.

Materials and Methods

Experimental studies

Bacterial collections

Paper I

A. actinomycetemcomitans

In paper I and II, *A. actinomycetemcomitans* strain D7SS was used mainly because it is a naturally genetic competent, smooth-colony derivative of D7S (serotype a), which was originally isolated from a patient with aggressive periodontal disease (Wang et al., 2002).

The reason for using mainly D7SS instead of D7S in experiments is because it does not aggregate as easily.

In paper I, mutants of *A. actinomycetemcomitans* were transformed as described below (see "Gene replacement").

A. aphrophilus

The type strains of *A. aphrophilus*, CCUG 3715 and NJ8700 were used (Di Bonaventura et al., 2009; Nørskov-Lauritsen & Kilian, 2006). The naturally genetic competent *A. aphrophilus* strains HK83 (CCUG 49494), and CCUG 11575 were originally sampled from saliva, and a brain abscess, respectively (Nørskov-Lauritsen & Kilian, 2006).

Other *A. aphrophilus* strains used are part of the collection of clinical isolates of *A. aphrophilus* in our laboratory and at the clinical laboratory, Oral Microbiology.

Paper II

Gene replacement mutants constructed in paper I are also used in paper II. In addition, bacterial strains below are used.

SA3138 is a serotype a *A. actinomycetemcomitans* wild-type strain (Kanasi et al., 2010). SA3139, which was isolated from the same patient as SA3138, carries the serotype a-specific antigen gene cluster, however, does not express the LPS O-antigen polysaccharide, rendering this strain non-serotypeable using immunoassay (Kanasi et al., 2010).

Escherichia coli C600 is a prototypical K-12 laboratory strain (Appleyard, 1954). Strain SE600 is an *ompA* mutant, generated in C600 S, which is a streptomycin-resistant derivative of C600 (Emory & Belasco, 1990).

A summary of bacterial strains used are listed in Table 1.

Table 1
Bacterial strains and their gene replacement mutant derivatives used in papers I and II

Paper I (strains)	**Paper I (mutants)**
A. actinomycetemcomitans **D7S** **D7SS**	D7SS *ompA1::spe* [Spe^r], D7S *ompA1::spe* [Spe^r], D7SS *ompA2::kan* [Km^r], D7SS *ompA1::spe*, *ompA2::kan* [Spe^r, Km^r], D7SS *omp100::kan* [Km^r], and D7SS *omp100::kan*, *ompA1::spe* [Km^r, Spe^r]
A. aphrophilus **NJ8700** **CCUG 3715** **HK83 (CCUG 49494)** **CCUG 11575**	HK83 *ompA1::spe* [Spe^r], HK83 *ompA2::kan* [Km^r], and HK83 *ompA1::spe*, *ompA2::kan* [Spe^r, Km^r]
E. coli **K-12 DH5** α	

Paper II (strains)	**Paper II (mutants)**
A. actinomycetemcomitans **D7SS** **SA3138** **SA3139**	D7SS *ompA1::spe* [Spe^r], D7SS *ompA1::spe*, *ompA2::kan* [Spe^r, Km^r], SA3138 *ompA1::spe* [Spe^r], SA3139 *ompA1::spe* [Spe^r]
E. coli **C600** **SE600**	

Cultivation of bacteria (paper I and II)

A. actinomycetemcomitans and *A. aphrophilus* strains were cultivated in air supplemented with 5% CO_2, at 37°C, on blood agar plates (5% defibrinated horse blood, 5 mg hemin/l, 10 mg Vitamin K/l, Columbia agar base).
For transformation assays, the strains were grown on Trypticase soy broth supplemented with 0.1% yeast extract, 5% heat-inactivated horse serum, and 1.5% agar (sTSB agar), and when needed, supplemented with 100 µg/ml (final concentration) spectinomycin, or kanamycin. This medium

was used due to an increased transformation frequency in comparison to blood agar (Wang et al., 2002).
E. coli K-12 laboratory strain DH5α was used for maintenance of plasmids and was cultured aerobically at 37°C in Luria-Bertani (LB) broth, or on LB broth solidified with 1.5% (w/v) agar.

Gene replacement (paper I and II)

To study the influence of the outer membrane proteins on the serum resistance of *A. actinomycetemcomitans* and *A. aphrophilus* gene replacement mutants were constructed.
The naturally competent strains *A. actinomycetemcomitans* (D7SS), and *A. aphrophilus* (HK83) were used to construct these gene replacement mutants (Table 1).

Briefly, the PCR fragments flanking the target genes were amplified. The PCR primers contained BamHI or SalI restriction sites, allowing ligation of the PCR fragments to flank either the spectinomycin resistance gene from plasmid pK-Spe (LeBlanc et al., 1991), or the kanamycin resistance gene from pUC4K (Vieira & Messing, 1982).
In order to replace the selected genes, homologous recombination of the target genes were undertaken by using recombinant DNA fragments that were inserted between two flanking regions of the target gene (Wang et al., 2002).
The ligation products were used to transform D7SS and HK83 on agar plates.

Confirmation of allelic replacements and the orientation of the inserted resistance cassette were done by DNA sequencing and PCR. Plating of the transformed bacteria onto selective media were also used to confirm the allelic replacements.

The same method was used in paper II to transform strains SA3138 and SA3139.

SDS-PAGE and Western blot (Paper I and II)

Coomassie or silver staining was used to visualize proteins after electrophoretic separation (SDS-PAGE).
Western blot was used to detect the outer membrane proteins present in *A. actinomycetemcomitans* and *A. aphrophilus* (Towbin et al., 1979).

Bacterial samples were prepared by mixing them with Laemmli-buffer and boiling the samples. Separation of proteins were made on pre-cast gels at a constant voltage of 150V.

Gels were stained using Coomassie Brilliant Blue or silver staining.
To identify proteins from selected protein bands after Coomassie- or silver-staining, they were excised from gels and subject to liquid chromatography tandem mass spectrometry (LC-MS/MS) at the Proteomics Core Facility at Chemical Biological Centre, Umeå University and Swedish University of Agricultural Sciences.

For the Western blot, the proteins were transferred onto a nitrocellulose membrane.
A rabbit polyclonal antiserum specific for *E. coli* OmpA (Henning et al., 1979) and a patient serum from a periodontitis subject, which exhibits strong reactivity to *A. actinomycetemcomitans* antigens were used (Brage et al., 2011).
As secondary antibody, anti-rabbit or anti-human horseradish peroxidase (HRP)-conjugate was used. Immunoreactive bands were then visualized.

In paper II, the procedures for Western blot were the same as for paper I. In paper II, normal human serum samples from six periodontally healthy donors were used to test if antibodies against *A. actinomycetemcomitans* OMV antigens were present.

Far Western blot analysis (Paper I)

Far western blotting is used to detect protein-protein interactions, in contrast to conventional western blotting where only proteins of interest are detected (Blackwood & Eisenman, 1991; Wu et al., 2007).

The method shares steps with the Western blot. However, instead of detecting a blotted protein it uses a bait protein that binds to the prey protein on the membrane.
In our case this was used to detect binding of the complement regulator, C4b-binding protein (bait protein) to OmpA (prey protein). The method is similar to Western blotting with some exceptions, as stated below.
Briefly, incubation of the membranes was performed with human recombinant C4bp in TBS-T with milk for 2 h at room temperature after the initial blocking step. After washing, the membrane was incubated with a rabbit polyclonal antiserum specific for human C4bp. After additional washes, anti-rabbit HRP-conjugate was used, and immune detection was performed.

Extraction of outer membrane proteins (Paper I)

Extraction of outer membrane proteins was performed in order to be used in the protein analyses.
Briefly, *A. actinomycetemcomitans* or *A. aphrophilus* cells were harvested from an average of four blood agar plates. After an initial step of centrifugation, bacterial pellets were resuspended in HEPES and subject to sonication. After centrifugation, supernatants were subject to ultracentrifugation. Pellets were resuspended in sodium-lauryl-sarcosinate in HEPES, and after sequential steps of ultracentrifugation pellets were resuspended in buffer A containing 0.5M NaCl, and finally in 20 mM sodium phosphate buffer containing SDS.

Isolation of outer membrane vesicles (paper I and II)

To assess the production and impact of OMVs in paper I and II, the vesicles produced by *A. actinomycetemcomitans* and *A. aphrophilus* were isolated.
Ultracentrifugation was the main method to isolate OMVs from *A. actinomycetemcomitans* and *A. aphrophilus* cells. These were harvested from an average of 10 blood agar plates.

Microscopy (Paper I)
Atomic Force Microscopy

Atomic force microscopy was used to detect vesicle production and to assess uniformity of OMV preparations in selected bacterial strains.
The samples were magnified through a Nanoscope V Atomic Force Microscope (Bruker AXS GmbH, Karlsruhe, Germany). These analyses were performed by Monica Persson at the Department of Molecular Biology, Umeå University.

Electron microscopy

Transmission and scanning electron microscopy were used to identify the surface structures of the bacterial cells, to assess membrane integrity and vesicle production.
These analyses were carried out at the Umeå Core Facility for Electron Microscopy (UCEM). Samples were examined with a Talos 120C transmission electron microscope (FEI, Eindhoven, The Netherlands). For scanning electron microscopy, the morphology of the samples was analyzed with a field-emission scanning electron microscope (Zeiss Merlin FESEM).

Serum sensitivity assays (paper I and II)

To test the sensitivity of the bacterial strains (wildtype and mutants) to serum, normal human serum (NHS) was used. NHS was taken from healthy volunteers. Preliminary tests indicated no difference when pooled or individual sera was used. Neither did we notice any major differences in bacterial cell viability when using different concentrations of serum, in line with previous publications (Guentsch et al., 2010) However, different strains of *A. actinomycetemcomitans* display differential activation of the complement system (Damgaard et al., 2017) and environmental conditions can also have an impact on the expression of virulence factors.

The procedures for the assays were largely followed as described previously (Asakawa et al., 2003; Aung et al., 2016).
A. actinomycetemcomitans, and *A. aphrophilus* strains were grown on agar for 2-3 days. The bacteria were harvested, and suspensions were adjusted to 1.0×10^9 cells/ml in PBS. Reaction mixtures contained NHS (50%) and

the bacterial suspension and were incubated at 37°C for 2 h. Cell survival was assessed by plating serial dilutions on agar. Heat-inactivated (56°C, 30 min) NHS (HI-NHS) was used as control. Survival rates were calculated as the ratio (%) of colony forming units (CFU) NHS/ HI-NHS.

Serum is heat-inactivated to diminish the effects of complement activity (Cowie & Chapin, 1907; Noguchi & Bronfenbrenner, 1911).
To distinguish between the classical and MBL, and the alternative pathway of complement activation, serum killing assays were performed in the presence of compounds that inhibit the pathways selectively. To only permit the alternative pathway, 5 mM $MgCl_2$, and 10 mM EGTA (Mg^{2+}/EGTA) was used (Des Prez et al., 1975). To block the MBL pathway, serum was filtered through D-mannose-agarose beads, as described previously (Aung et al., 2016). Briefly, NHS was passed through a 5-ml column of mannose-agarose beads to bind lectin and deplete the MBL pathway.

In paper II, serum sensitivity assays were conducted in the same manner as in paper I. However, to test the impact of OMVs on the survival of the bacteria, the assay was supplemented with OMVs from *A. actinomycetemcomitans*, equivalent to 20, 100, and 200 µg protein. Alternatively, 50 ng (500 EU) LPS was added to the reaction mixtures. Bacterial serum survival was determined by viable count, and the increase in serum survival upon OMV or LPS supplementation was determined relative to incubations in vesicle-free controls (50% NHS in PBS).

Isolation of LPS (paper II)

LPS was isolated to test the effect of LPS alone in the complement consumption assay (described below).
The isolation of LPS was performed according to a previous study (Ahlstrand et al., 2019) and is described briefly below.
A. actinomycetemcomitans cells were harvested from 12 blood agar plates and dissolved in PBS. The bacterial cells were subject to sonication to lyse the bacteria. Bacterial cell debris and intact bacteria were removed by centrifugation. Ultra-centrifugation was performed to harvest the bacterial cell membranes. The membranes were then solubilized in 1% sodium lauryl sarcosinate to separate the inner and outer membrane whereafter the outer membranes were collected after ultracentrifugation. LPS was

removed from the outer membrane by a buffer containing mercaptoethanol and SDS. Proteinase K was also used to digest residual proteins. LPS was precipitated with sodium acetate and ethanol. Precipitated LPS was collected by centrifugation. The step was repeated using only ethanol. LPS preparations were resuspended in 100 µl H_2O and the yield was estimated using an endotoxin kit.

Complement consumption (paper II)

To determine the consumption of complement by *A. actinomycetemcomitans* OMVs and LPS, they were analyzed with a complement consumption kit (Wieslab® Complement System Screen kit, COMPL300 (Svar Life Science AB, Malmö, Sweden)).
The kit is based on the principles of Enzyme-linked Immunsorbent Assay (ELISA). Briefly, the wells on the plate are coated with specific activators of the classical, MBL and alternative pathway. The kit includes specific diluents that block the respective parts of the complement system. The serum with OMVs or LPS were incubated with the pathway specific diluents followed by washing with a kit-specific washing solution. The formation of the MAC-complex is detected with alkaline phosphatase labelled antibody. The optical density is measured and compared with positive and negative controls to calculate the complement consumption.

Clinical studies

Study populations (paper III and IV)

Maasai Mara, Kenya (paper III)

The study population in Maasai Mara, Kenya consisted of adolescents aged 14 to 18 years. They were recruited from the four primary schools and one mixed secondary school in Mara North Conservancy (Narok County). The schools were boarding schools.
The study population was examined from January-February 2016. Out of the total of 340 individuals, 284 adolescents accepted to participate in the clinical examination and microbial sampling.

The participants were informed about the study in their native language by Kenyan researchers and provided a written consent for their participation in the study.

Information on social and economic status of the teenagers and their families was not available to the researchers. All schools in the area were considered to be at a similar standard and with similar physical and educational possibilities.
In addition to clinical examinations and microbial sampling, face-to-face interviews were also conducted regarding age, gender, general health, oral health, IOM practice and Oral Health-Related Quality of Life (OHRQoL). These results have been published and described elsewhere (Haubek et al., 2021; Kemoli et al., 2018).

Västerbotten County, Sweden (paper IV)

Initially, we identified individuals born in 2001, residing in Västerbotten County, Sweden and who had had a routine dental examination in the public dental health care (Folktandvården Västerbotten). For the year 2016, this resulted in 1656 individuals.
This corresponded to a large proportion (61%) of the population of adolescents in Västerbotten born in 2001 (total 2719 adolescents according to Statistics Sweden (Statistiska centralbyrån, 2022-02-22)).

Assessment of radiographic bone loss (paper IV)

To identify bone loss in the study population (n=1656), radiographs from the routine dental examination in 2016 were used. Since radiographs could have been taken at other time points, due to other reasons than a routine dental examination we used radiographs from 2014 and 2015 as well, and if radiographs from 2016 were inadequate. Bitewing-radiographs were primarily used for identification of proximal bone loss, however, if other types were available, such as apical radiographs, these could be used as a complement.
The limit for proximal bone loss on the radiographs was set to >2 mm (distance between cemento-enamel junction and marginal bone) since this would be indicative of periodontal disease (Hausmann et al., 1991; Sjödin et al., 1993; Stoner, 1972). If this criterion was met on any proximal site, the individual was considered as positive for bone loss (case). Since bone

loss can be present without periodontitis, as in the case of impacted third molars, tooth malposition, and deep caries/fillings, these were not considered as cases.
The screening was conducted by 3 examiners: one experienced specialist in periodontology, one dentist undergoing specialist training in periodontology, and one general dentist (ML). All examiners were calibrated by examining a random sample of the population individually (n =165). The radiographs were classified according to bone loss (positive), no bone loss (negative), borderline, or inadequate radiographs. After the first calibration, a second calibration was performed on 50 individuals. This material was then divided equally between the three examiners and individually examined. In the case of disagreements, the examiners reached a consensus regarding these samples.

Clinical examinations (paper III and IV)
Field conditions in Maasai Mara, Kenya

The oral examinations were conducted by trained dental professionals from Sweden and collaborators from Denmark. The examinations were done under field conditions in available classrooms in the schools (Figure 4). More specifically, the participant was made to lie on top of a table during the examination. A supplementary light source, a headlamp, was used to augment the natural light during the examination of the oral cavity. Using clean disposable mouth mirrors and tweezers, an oral examination was carried out to detect the status of the dentition. The methods and data from the clinical examination have also been described previously (Haubek et al., 2021; Kemoli et al., 2018). The examinations did not involve periodontal parameters.

Clinical conditions in Västerbotten County, Sweden

All individuals with radiographic bone loss (cases) and two controls per case were invited to participate in the clinical examination by one investigator (ML) using mail and/or telephone. The controls did not display any radiographic bone loss and were randomly selected from the original population and matched to the cases with regards to age, gender, and clinic.

The examinations were performed at the participants' nearest public dental health care clinic (Folktandvården Västerbotten) with access to dental chairs and equipment.
This resulted in 14 dental clinics in total. For practical reasons, group identity (case/control) was not possible to conceal.
All periodontal examinations were carried out by one general dentist (ML) after calibration with an experienced periodontist.
Prior to the clinical examination, each participant was interviewed by the examiner (ML) regarding general health, geographic origin, antibiotic use, tobacco use, oral hygiene habits, experience of previous periodontal care, and history of periodontitis in the family. If the participant had any questions regarding the questionnaire these were answered in conjunction with the interview.

In contrast to the examinations performed in Kenya, the clinical examination involved periodontal registrations such as bleeding on probing (BoP), clinical attachment level (CAL), pocket probing depth (PPD), and plaque scores (PS). Each parameter was registered on mesial and distal surfaces of all fully erupted teeth, except third molars. PS were registered on four surfaces (mesial, distal, buccal and lingual/palatal).
CAL was measured from the cemento-enamel junction to the bottom of the periodontal pocket. PPD was measured from the gingival margin to the bottom of the periodontal pocket. The recordings were made with a Hu-Friedy PCP11 periodontal probe (3-6-8-11 mm; Hu-Friedy Mfg. Co., Chicago, IL), and registered to the nearest mm. BoP and PS were assessed as dichotomized registrations. The number of surfaces positive for plaque or bleeding on probing were divided, respectively, with the total number of surfaces to give a mean percentage score.
Periodontitis was diagnosed if interdental attachment loss was detectable at ≥2 non-adjacent teeth and the observed attachment loss could not be ascribed to non-periodontitis related causes (Papapanou et al., 2018).

Figure 4
Field examination from Maasai Mara, Kenya.

Sampling of saliva (paper III and IV)

In paper III, stimulated whole saliva was sampled before the clinical examinations. The participants chewed on a piece of paraffin wax for one minute, and the stimulated whole saliva was collected in a disposable plastic cup. One ml of the saliva was mixed with an equal volume of Saliva DNA Preservation Buffer (2X) (Norgen Biotek Corporation, Thorold, ON, Canada) in a 2 mL sterile tube and stored at room temperature until the DNA was isolated. The preservation buffer was chosen since it was not possible to freeze the samples due to field conditions. According to the manufacturer it preserves DNA at room-temperature for two years.

In paper IV, the collection of saliva was similar to the procedure in paper III, however, the samples were collected after the clinical examination and the samples were placed directly on ice. The samples were transported as quickly as possible to the laboratory for freezing. The time for this could vary from 30 minutes to about 6 hours depending on the location of the dental clinic.

Sampling of subgingival plaque (paper III and IV)

In paper III, subgingival plaque was collected with sterile paper points and inserted subgingivally in four periodontal pockets (mesial periodontal pockets on the four first permanent molars). The paper points from each patient were pooled into a tube with 2 mL of VMGAIII (Dahlén et al., 1993) transport medium supplemented with Nystatin (2 mg/L).
Due to field conditions and risk for overgrowth of bacteria it was not possible to sample subgingival samples, with the last day being the exception, since the transportation time would have been too long.

In paper IV, in addition to pooled plaque samples, subgingival plaque samples were also collected from one site with attachment loss, and from one site without attachment loss, from the 13 individuals that were identified to have radiographic bone loss. Hence, none of these samples were collected from the controls.

Isolation of DNA from saliva and subgingival samples (paper III and IV)

In paper III, isolation of DNA was performed with the saliva preservation buffer present. An addition of Tris buffer with 1 mM EDTA (pH = 8.0) was done prior to isolation in the extraction instrument.
Due to changes in the DNA extraction kits from the manufacturer the pre-treatment of the DNA was necessary in paper IV. Pre-treatment was also deemed necessary since gram-positive bacteria were to be quantified. Therefore, addition of Proteinase K and Lysozyme was used when it was appropriate.

Quantitative PCR (paper III and IV)

Quantitative PCR with SYBR Green chemistry was used to quantify the loads of *A. actinomycetemcomitans* and *A. aphrophilus* from saliva in paper III. In addition to these, *P. gingivalis* and *F. alocis* were also quantified from saliva in paper IV. In paper IV, quantification was also performed for subgingival plaque samples for the four bacterial species mentioned.

Specific primers and PCR cycling conditions used were as previously described (Kirakodu et al., 2008; Lindholm et al., 2021; Siqueira & Rôças, 2003).
Briefly, the qPCR mixtures (10 µL) for the quantification, contained 5 µL Kapa Sybr Green (KK 4601) (Kapa Biosystems, Boston, MA, USA), 4 µL template, and 1 µL of the specific primer mix (0.5 µmol/L each). Each run included three negative samples (H_2O) and standard mixtures with a given concentration equivalent to 10^1-10^8 cells/mL were prepared as described for the samples.

Sero- and Genotyping of *A. actinomycetemcomitans* in Stimulated Whole Saliva and subgingival plaque samples (paper III and IV)

Serotyping of isolated *A. actinomycetemcomitans* strains/DNA-extracts was performed with oligonucleotide primers specific for serotype a-f as described previously (Kaplan et al., 2001; Suzuki et al., 2001). Leukotoxin promoter typing (JP2/non-JP2) was performed for serotype b strains and has been described elsewhere (Poulsen et al., 2003). In brief, suspensions were boiled for eight minutes and centrifuged. Supernatants were used as a template in the PCR-analysis with serotype-specific primers.
The PCR products were analyzed by agarose gel (1.2%) electrophoresis.
PCR amplicons corresponding to the size (bp) of the JP2 genotype were isolated from agarose gels and their DNA sequences determined as described previously (Claesson et al., 2015).
In paper III, the DNA band that corresponded in size to the JP2-genotype was excised from the agarose gel and sent for DNA sequencing. The sequencing confirmed that there was a 530 bp deletion, which is indicative of the JP2-genotype.

Cultivation from subgingival plaque samples (paper III and IV)

In paper III and IV, the samples were spread on blood agar (5% defibrinated horse blood, 5 mg hemin/L, 10 mg Vitamin K/L, Columbia agar base), and on trypticase–bacitracin–vancomycin (TBV) agar plates, an *A. actinomycetemcomitans*-selective medium (Slots, 1982). After incubation of the blood agar plates in an anaerobic environment at 37°C for seven days the total number of viable bacteria (total viable count; TVC) was calculated and examined for the presence of bacterial species. After

incubation of the TBV-plates in 37°C for three days in an aerobic atmosphere containing 5% CO_2, the plates were examined for presence of *A. actinomycetemcomitans* colonies. These were identified as rough, catalase-positive and having a star-shaped inner structure. The number of *A. actinomycetemcomitans* colonies were counted and the proportion of the bacterium of the TVC was subsequently calculated.

Determination of leukotoxicity

For the determination of the leukotoxicity, the cell lysis assay was used. Briefly, this assay reveals loss of membrane integrity as indicated by the activity of lactate dehydrogenase when THP-1 cells (monocytic cell line (Tsuchiya et al., 1980) are exposed to leukotoxic extract (Johansson et al., 2000).

Briefly, the isolates of *A. actinomycetemcomitans* were cultured on peptone yeast extract agar and harvested after 48 h, into 300 mM NaCl in phosphate buffered saline (PBS). The density was adjusted to OD 600 nm = 10 ($\approx 10^{10}$ cells/mL) and the cells were pelleted by centrifugation. The cell-free supernatant (5%) was added to cultures of phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells for 120 min, and cell lysis was determined by quantification of the leakage of LDH (lactate dehydrogenase) from damaged cells.

Collection and Extraction of Herbal Plants

Collection of the herbal plants in Kenya mainly relied on personal communication on-site with staff and pupils at the schools.

Plant species collected were (1) *W. ugandensis* twigs, (2) *W. ugandensis* leaves, (3) *Toddalia asiatica* twigs, (4) *T. asiatica* leaves, (5) *Eucalyptus* spp. twigs, (6) *Grewia similis* (oirii) twigs, (7) *Psidium guajava* leaves, (8) fresh extracts of *W. ugandensis* leaves, and (9) fresh extract of *W. ugandensis* bark.

The plant material was disintegrated and mixed with 70% ethanol (250 mg/ml), and supernatants (1%) were, after centrifugation, analyzed in leukotoxin neutralization assays. This was done by adding the supernatants to cultures of PMA-differentiated THP-1 cells in the presence of leukotoxin (200 ng/mL) for 120 min, and cell lysis was determined by quantification of the leakage of LDH as mentioned previously.

Statistics

Statistical analyses were performed using SPSS version 28 (IBM, Chicago, Illinois) and Excel (Microsoft, Redmond, WA, USA).

For calculation of statistical significance, a one-tailed (paper III) or two-tailed t-test (paper I, II and IV) was mainly used. The level of statistical significance was set to $P < 0.05$
In paper I and II, data was expressed with mean-values and standard errors of the means (SEM).
Power-calculations were performed (paper IV) to estimate how many patients were needed to detect any differences between cases and controls with respect to periodontal parameters and demographical differences. According to the power-calculation, 1168 individuals were needed to reach 80% power and 5% alpha-error. To minimize the risk for uncertainties in the initial assumptions regarding prevalence of periodontitis, a total of 1656 individuals were screened.

In paper IV, Mann-Whitney U test was used to determine if statistically significant differences were found between cases and controls for the variables CAL, PPD, BoP and PS, as well as concentrations of bacteria in plaque and saliva.
Fischer's exact test was used for determination of differences in prevalence of bacterial species in plaque/saliva between cases and controls.
Cohen's kappa (Cohen, 1960) was used for calculation of agreement between pooled plaque and saliva samples.
Student's t-test was used to determine significant differences for mean proportions of bacterial species in cultivation of pooled subgingival plaque samples.
Paired samples t-test was used for determination of significant differences between plaque samples (qPCR and cultivation) from sites with and without attachment loss.

Images for figures were assembled using Adobe Photoshop CS6 or Microsoft PowerPoint (paper I and II). SPSS (IBM, Chicago, Illinois) was used for figure assembly (paper III and IV).

Ethical considerations

In paper III, ethical clearance was sought and obtained from the KNH-UoN Ethics and Research Committee, Kenya (P711/11/2015). The parent/guardian and child also provided written informed consent and assent, respectively, after full disclosure of the study had been given to them. Permission to carry out the research at schools in the Mara North Conservancy was sought from the relevant authorities in Kenya.
In paper IV, the study was approved by the Regional Ethical Review Board at Umeå University (Dnr 2017-227-31M.). All procedures were conducted in accordance with the guidelines of the local ethics committee at the Medical Faculty of Umeå University, which are in accordance with the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, October 2013). Permission from Folktandvården Västerbotten was applied and approved in order to access the available radiographs from the routine dental examinations. Written consent was obtained from all individuals participating in the clinical examination.

Ethical considerations are most important when studies include persons under the age of 18. This applies to paper III and IV.
This relates to the fact that they are regarded as minors and are under the supervision of a guardian until the age of 18. In general, minors need the to provide an informed consent from their legal guardian to participate in a scientific study. However, according to the Ethical Review act ("Lag (2003:460) om etikprövning av forskning som avser människor," 2003), children over the age of 15 are allowed to participate in a study under the condition that they understand what it means to participate in a study. Since the research question was restricted to periodontitis in adolescents it was not possible to perform the study in another, older study population either. To meet the prior mentioned condition, the study participants were informed both in the invitation to participate and at the visit to the clinic. It was also ensured that they understood the information that was given to them. Written informed consent was also obtained.
Regarding paper III, it may be regarded doubtful from an ethical point of view, that the clinical examinations were performed in Kenya but most of the analyses and results were produced in Sweden and Denmark. However, great measures were taken to ensure that the results came to use for the local population as well. Both ethical approval from governmental authorities and approval from local authorities was obtained. An advantage

was also local collaborators from the University of Nairobi who participated in all parts of the study. At the end of the examination, in each school, the local collaborators gave common feedback to the school about the general impression of the oral status and what could be done to improve it. In paper III, informed consent was also a prerequisite for the adolescents to participate in the study.

In papers III and IV biological sampling and clinical examinations were performed. These were assessed not to pose any major health risks for the participants due to the low level of invasiveness.

In both paper III and IV, information was given to the participants if they exhibited any dental health related issues. This was done in order for them to seek proper treatment early.

Results

Main findings

The main findings described in this PhD thesis display the importance of bacterial virulence factors for survival of *A. actinomycetemcomitans* and *A. aphrophilus* in human serum by expression of the outer membrane proteins OmpA1 and OmpA2. It also highlights alternate modes to subvert the host immune system by production of outer membrane vesicles in *A. actinomycetemcomitans*.
A. actinomycetemcomitans and its close relative *A. aphrophilus* were commonly found in saliva in two highly diverse adolescent populations.
It also seems evident that periodontitis among adolescents in Västerbotten is increasing, suggesting more preventive measures, and especially close attention to demographical differences and microbiological characteristics.
Early diagnosis of periodontitis can be achieved by close examination of marginal bone levels on radiographs, even very minute changes.

Experimental studies (paper I and II)

In paper I we studied the effects of outer membrane proteins in *A. actinomycetemcomitans* and *A. aphrophilus* serum resistance.
All tested *A. actinomycetemcomitans* strains were completely resistant to serum killing in our assays. *A. aphrophilus* strain HK83 was moderately resistant to serum killing, however, testing of other strains displayed higher degrees of serum resistance suggesting variability among strains.
After construction of the gene replacement mutants (*ompA1*) it was evident that this influenced the serum sensitivity in both *A. actinomycetemcomitans* and *A. aphrophilus* with 16 times lower survival in *A. actinomycetemcomitans* and four times lower survival in *A. aphrophilus*. Inactivation of the gene encoding another outer membrane protein, Omp100, did not result in similarly low survival rates.
Interestingly, serum resistance of *ompA1* mutant *A. actinomycetemcomitans* was regained after further cultivation of colonies surviving in the serum sensitivity assays. This depended on the upregulation of OmpA2 expression. This OmpA1 paralogue was found to be produced at low levels in the wild type strains, as noted on Western

blots and LC-MS/LC analysis. In mutants only deficient in OmpA2 expression, serum sensitivity was not affected, whereas for the *ompA1* and *ompA2* double mutants the survival in serum was extremely low, for both bacterial species.

Inhibition of the respective pathways in the complement system demonstrated an effect on all the pathways in the killing of serum-sensitive *A. actinomycetemcomitans* and *A. aphrophilus* mutants, however no single inhibition restored the complete serum resistance. Inhibition of the classical and MBL pathway did seem to increase the survival of these mutants the most.
The mechanism for serum resistance mediated by the *Aggregatibacter* OmpA proteins appears to include an OmpA-dependent binding of C4bp.

The almost total serum sensitivity of the double mutants could also be a result of membrane instability due to lack of the stabilizing membrane proteins. This would be consistent with the observation that the double mutants produced more vesicles than the wildtypes, seen for both *A. actinomycetemcomitans* and *A. aphrophilus*.

Related to the increased vesicle production we proceeded to study this in **paper II**, where the aim was to investigate if, and how the vesicles influenced serum resistance in *A. actinomycetemcomitans*.
Addition of OMVs, originating from wildtype *A. actinomycetemcomitans* (D7SS), to the double-mutant produced in paper I (D7SS *ompA1 ompA2*) increased the survival rate in serum (approximately 100-fold). No additional increase in survival was seen for wildtype *A. actinomycetemcomitans* (D7SS) when OMVs were added.
The effect when using OMVs from the double mutant did not differ significantly, suggesting an OmpA-independent protective effect.

To investigate alternative mechanisms for vesicle mediated effects on serum resistance, we used two *A. actinomycetemcomitans* strains to compare the effects of LPS on serum resistance. Both strains belong to serotype a, and are similar but with the difference found in the LPS. Strain SA3138 has a normal LPS whereas strain SA3139 has a deficient LPS since it is lacking the O-antigen polysaccharide part.
The OMVs from SA3139 displayed a very low protective effect relative to vesicles from SA3138. The effects were similar when using LPS

preparations from these strains indicating LPS-dependent serum resistance in OMVs.
The consumption of complement indicated that all complement pathways were activated to a high degree for strains D7SS, D7SS *ompA1 ompA2* double mutant and SA3138 (OMVs). For SA3139 OMVs, the activity mostly remained for all pathways, indicating the importance of LPS in complement consumption. The assay resulted in similar results when comparing LPS from SA3138 and SA3139.
Human sera from six periodontally healthy patients were analyzed by Western blot, and this revealed that *A. actinomycetemcomitans* OmpA1 could be recognized in five of the six patients, indicating that there are likely antibodies present that normally can initiate classical complement activation.
Results from paper I and II are summarized in Figure 5.

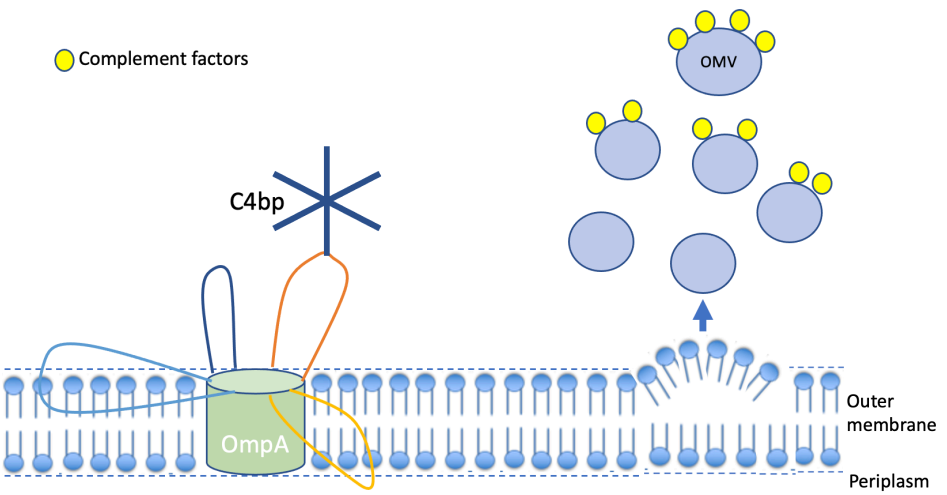

Figure 5
Schematic illustration explaining the two modes of serum resistance, characterized in the present PhD thesis work.
To the left, an illustration displaying the binding of C4bp to OmpA.
To the right, an illustration of OMVs acting as decoys and titrating the concentration of complement factors.

Clinical studies (paper III and IV)

As antigens from *A. actinomycetemcomitans* could be found in the serum of periodontally healthy individuals we also wanted to examine the salivary presence of the bacterium and *A. aphrophilus* in two distinct populations (papers III and IV).
In **paper III**, the presence of *A. actinomycetemcomitans* and *A. aphrophilus* was shown to be high in the adolescents from Maasai Mara, Kenya when analyzing saliva with qPCR, 71.8% and 99% respectively.
In **paper IV**, the prevalence in the Swedish population is similar for *A. aphrophilus* in cases and controls (100% and 96% respectively). For *A. actinomycetemcomitans*, the prevalence was higher for the cases (77%), similar to the population in Kenya, compared to controls (23%).

In **paper III**, one JP2-genotype was detected in the saliva samples, which was confirmed by sequencing of the *ltx* promoter. However, no JP2-genotype was found in paper IV.
Cultivation from pooled subgingival samples of a subset of the population was performed and indicated lower prevalence of *A. actinomycetemcomitans* (22.1%) than in saliva, and exhibited five serotype a, four serotype c and three serotype f.
In **paper IV**, cultivation resulted in a prevalence of 69% in cases and 3% for controls. Serotyping indicated two serotype a, three serotype b, seven serotype c and one serotype e.

In **paper III**, the leukotoxicity of the cultivated *A. actinomycetemcomitans* isolates indicated low and intermediate leukotoxic phenotypes. No JP2-genotype was detected using cultivation.
However, the bark extract from a local herbal plant, *Warburgia ugandensis*, displayed a dose-dependent neutralizing capacity of leukotoxin. Interestingly, this plant is used for oral hygiene by the population living in Maasai Mara.

In addition to detecting *A. actinomycetemcomitans* and *A. aphrophilus* in **paper IV**, *P. gingivalis* and *F. alocis* were also detected in saliva in cases (92% and 100% respectively) and controls (15% and 54% respectively). When using pooled subgingival plaque samples, the detection rate was 69%, 46%, 77% and 92% in cases for *A. actinomycetemcomitans*, *P. gingivalis*, *A. aphrophilus* and *F. alocis* respectively. For controls, the corresponding detection rates were 38%, 8%, 92% and 69% respectively.

In paper IV, radiographical bone loss was also assessed in addition to microbiological parameters in a population of adolescents in Västerbotten County, Sweden (n=1656). This resulted in 24 individuals positive for bone loss (1.45% of the total population).
Thirteen of these accepted to be examined clinically as well as 26 healthy controls. Eleven controls dropped out for various reasons as described in Figure 6.

Most of the adolescents were healthy and did not present any major differences in antibiotic or tobacco usage. However, 12 cases were diagnosed with periodontitis in contrast to the controls where no periodontitis was diagnosed. This highlighted the importance and accuracy of radiographs in diagnosis of periodontitis in adolescents. Also, the birth country was outside the Nordic countries for all cases, as opposed to the controls where only a minority stated their birth country to be non-Nordic. In the cases, clinical parameters (CAL, PPD and BoP) displayed significantly higher values compared to the cases, however no differences in PS were seen.

Microbiological differences were clearly seen for cases and controls as indicated earlier. Significantly higher loads and prevalence in saliva was seen for *A. actinomycetemcomitans*, *P. gingivalis* and *F. alocis*. Significantly higher loads were also noted in pooled plaque samples for these bacterial species when analyzed with qPCR. *P. gingivalis* was also significantly more prevalent in pooled plaque samples. However, *A. aphrophilus* exhibited significantly higher loads in the controls, whereas *A. aphrophilus* otherwise did not exhibit significant differences between cases and controls.
No significant differences were found when comparing loads in plaque samples from sites with and without attachment loss in the same individual.
The correlation between pooled plaque samples and saliva samples from both cases and controls indicated moderate agreement for *P. gingivalis* and *A. actinomycetemcomitans* (κ= 0.426 and κ = 0.433 respectively). For *F. alocis* fair agreement (κ =0.289) was noted and no agreement was noted for *A. aphrophilus* (κ =-0.045). Fair agreement indicates a Kappa-value between 0.21-0.40 and moderate between 0.41-0.60.

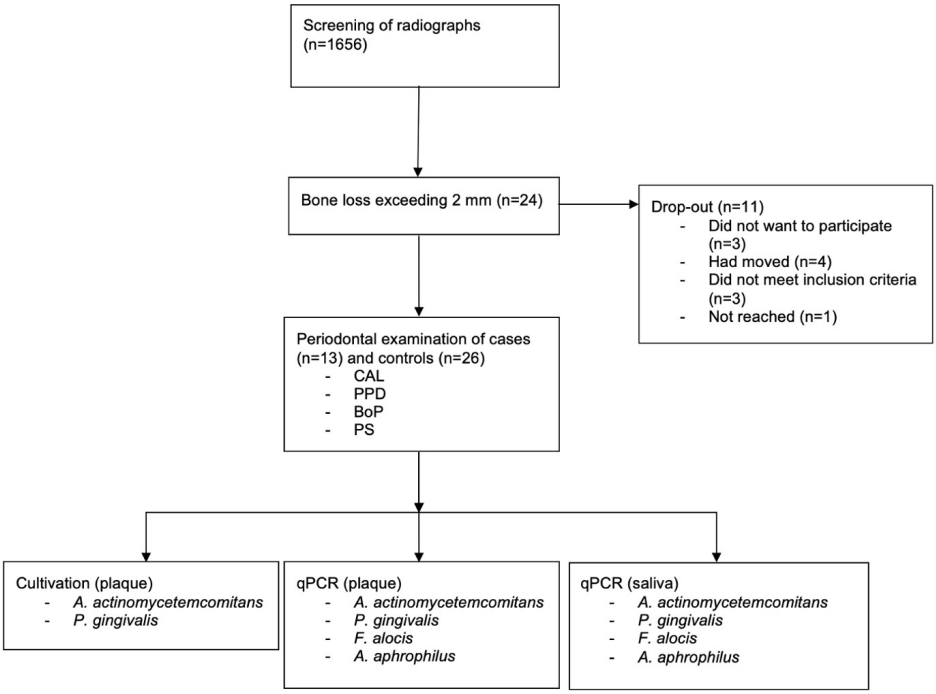

Figure 6
Flowchart describing the outline of paper IV.

Discussion

Influence of outer membrane proteins on serum survival

In paper I, we concluded that OmpA had a major role in serum survival of *A. actinomycetemcomitans* and *A. aphrophilus* and was mediated, at least partly via binding of C4bp. The importance of OmpA as virulence factor has been demonstrated in other bacterial species and demonstrated it to be a highly conserved protein among gram-negative bacterial species (Krishnan & Prasadarao, 2012). It also serves an important structural function in the outer membrane.

Whereas OmpA and OmpA-like proteins are highly conserved among gram-negative bacterial species they have not been studied extensively in relation to periodontitis. A few previous studies have studied the outer membrane proteins in *A. actinomycetemcomitans* (Komatsuzawa et al., 2002; Wilson, 1991) recognizing OmpA (referred to as Omp29/34).

A few studies have also investigated the effect of OmpA on host cells, indicating the role of OmpA in bacterial entry into gingival epithelial cells and apoptosis of the cells (Kajiya et al., 2011; Yoshimoto et al., 2016). One study has displayed the effect of outer membrane proteins on the complement system, indicating the role of Omp100 in trapping factor H, an inhibitor of C3b (Asakawa et al., 2003). However, no previous study appears to have characterized the effect of OmpA in *A. actinomycetemcomitans* and its effect on the complement system.

Interestingly, a recent report displayed the effects of OmpA1 and OmpA2 in suppressing the expression of CXCL-8, a chemokine, which may indicate a decrease in neutrophils in the periodontal pocket and thus permit initial colonization of *A. actinomycetemcomitans* (da Silva et al., 2022).

OmpA and OmpA-like proteins have also been described in *P. gingivalis* and *T. forsythia*, indicating their role in adhesion to oral epithelial cells and biofilm formation (Horie et al., 2016; Kishi et al., 2012; Naylor et al., 2017). OmpA-like proteins in *P. gingivalis* have been implicated to influence serum resistance as well (Inomata et al., 2018), and gingipains in *P. gingivalis* have the ability to bind C4bp (Potempa et al., 2008).

For the closely related bacterium, *A. aphrophilus*, no data has previously displayed the effects of outer membrane proteins on the serum resistance. Few studies in general exist regarding *A. aphrophilus*, mostly being case series describing its role in endocarditis and brain abscesses (Bieger et al.,

1978). Interestingly, a recent study detected the bacterium in freshly drawn blood donations from a patient with periodontitis (Damgaard et al., 2021). As the bacterium is mainly found in the oral cavity it suggests an oral origin in the blood samples. Hypothetically, this could partly be explained by the serum resistance of OmpA described in paper I and explain the survival of the bacterium in blood.

From a methodological point of view, one must also consider the variations of bacterial strains when studying mechanisms of virulence since the expression of virulence factors might differ. Expression of proteins is variable even when comparing rough and smooth morphology strains of *A. actinomycetemcomitans* (Fu et al., 2022). However, it was concluded that OmpA was expressed in all tested strains regardless of morphology. The complement system is also variably activated among strains of *A. actinomycetemcomitans* (Damgaard et al., 2017). Different strains also respond differently when exposed to human serum but not to horse serum, suggesting human serum-specificity (Tang-Siegel et al., 2016). A recent study suggested the effect of serum to depend on the presence of bacteriophages in *A. actinomycetemcomitans* (Tang-Siegel et al., 2022). Outer membrane proteins in bacteria are important for stabilizing the outer membrane of gram-negative bacteria (Iwami et al., 2007). Hence, one could also consider the possibility that the outer membrane stability is compromised when it lacks OmpA, which is highly abundant in the outer membrane, which could lead to increased serum sensitivity. This could explain the reduced growth rate of *ompA* mutant strains *in vitro*.

In the present work, serum resistance was ubiquitous among all tested strains of *A. actinomycetemcomitans* and *A. aphrophilus*. However, the strains displayed variability in the level of serum resistance, as some were completely serum resistant, and others had a moderate level of serum resistance.

Influence of OMVs on serum survival

As noted in paper II, the OMVs had a significant effect on survival of *A. actinomycetemcomitans* in serum. The effects were hypothesized to be dependent on OmpA as in paper I since outer membrane proteins can be found in the membranes of the vesicles (Kato et al., 2002). For OMVs produced by *A. baumannii*, OmpA has been shown to promote

pathogenesis through mitochondrial fragmentation (Tiku et al., 2021). However, it seemed that OmpA was not a key factor in protecting the bacterial cells by OMVs in *A. actinomycetemcomitans*. As LPS is also found in abundance in the membrane of OMVs and can activate the complement system efficiently (Morrison & Kline, 1977), *A. actinomycetemcomitans* strains lacking LPS O-antigen polysaccharide were utilized.
In our case the strain lacking LPS O-antigen was isolated from a patient carrying two strains of *A. actinomycetemcomitans*, and luckily the other strain had an intact LPS. As the LPS could activate and consume complement system components we hypothesized that this was because the complement components were depleted and thus protected the bacterial cells from the complement system and subsequent lysis.
However, LPS from *A. actinomycetemcomitans* exhibits other functions as well, such as induction of proinflammatory mediators and activation of matrix metalloproteinase 2 (MMP-2) (Gholizadeh et al., 2017; Tiranathanagul et al., 2004).
Contaminations of the OMV preparations and the isolated LPS should be taken into account when considering their effects on the survival of the bacterium. Nevertheless, strict protocols were followed, and meticulous handling of the samples were done to avoid any contaminations.

OmpA, OMVs and their role in periodontitis in adolescents

As OmpA and OMVs are present and generated by several oral bacteria, including *A. actinomycetemcomitans* as shown in papers I and II, their role in periodontitis in adolescents could be of importance.
As OmpA and OMVs are highly immunoreactive and OMVs can contain toxins produced by the bacterium, these components in combination can become powerful tools for the bacterium to overcome the immune system in children and adolescents. This may be aggravated by hormonal changes during puberty as well deficiencies in the host defenses (Kulkarni & Kinane, 2014; Oh et al., 2002). As the inflammatory burden increases in the periodontal tissues the bacteria would also thrive to a higher degree, partly because of the increased flow of gingival crevicular fluid that contains many nutrients for the periodontal bacteria, but also providing an environment for the serum resistant bacteria (Barros et al., 2016).
One could also speculate in the effects of OmpA and OMVs in the context of extra-oral infections. As OmpA confers serum resistance, *A.*

actinomycetemcomitans could be able to survive when entering the blood stream from a periodontal pocket, thus leading to an increased risk of endocarditis or atherosclerosis in a susceptible individual. In both conditions, associations with the presence of *A. actinomycetemcomitans* have been demonstrated (Brouqui & Raoult, 2001; Kozarov et al., 2005).
When present in the periodontal pocket or blood stream, *A. actinomycetemcomitans* could also release OMVs into the surrounding tissues. A study by Han et al. (2019) demonstrated that extracellular RNAs from OMVs, released by *A. actinomycetemcomitans*, could cross the blood-brain barrier and thus indicate this as a cause for neuroinflammatory diseases such as Alzheimer's disease.
However, as periodontitis can influence the occurrence of extra-oral infections this needs to be considered as well.

Presence of *Aggregatibacter* species in diverse populations

Papers III and IV investigated the presence of two closely related *Aggregatibacter* species, *A. actinomycetemcomitans* and *A. aphrophilus*, in two highly different populations. The presence of the bacteria revealed that *A. aphrophilus* could be found in a majority of the individuals, indicating its presence as part of the normal oral flora, and especially in saliva. Few studies have investigated the presence of this bacterium, but this result is nonetheless in line with another previous study (Tempro & Slots, 1986). While this study indicated a presence of around 50% in saliva, we discovered that it was highly abundant in saliva and recovered from a majority of the individuals. This could be explained by differences in techniques used to detect the bacterium as cultivation was used in the previous study and we used qPCR.
It seems that *A. aphrophilus* is part of the commensal oral flora when viewed in a global perspective as well, since it was highly present in the two very different populations studied in papers III and IV.
Regarding *A. actinomycetemcomitans*, there are some discrepancies in the presence of the bacterium when comparing the study populations. While being highly prevalent in the population from Maasai Mara, the prevalence was lower in the population from Västerbotten. However, when comparing the presence in the case group (paper IV) the prevalence was rather similar when comparing saliva samples. The drawback when comparing these are unfortunately the lack of periodontal measurements in paper III, excluding the possibility to draw any conclusions regarding periodontitis and

presence of *A. actinomycetemcomitans*. The presence of *A. actinomycetemcomitans* in the context of periodontitis in adolescents has been studied previously in African populations and indicated an increased risk in the presence of *A. actinomycetemcomitans* (Haubek et al., 2008; Höglund Åberg, Haubek, et al., 2014).

In paper IV, the case group had significantly higher levels of *A. actinomycetemcomitans* in saliva and subgingival plaque when compared to the control group. Interestingly, for *A. aphrophilus* this was inversed in subgingival plaque, displaying significantly higher levels in the controls. The interactions between *A. actinomycetemcomitans* and *A. aphrophilus* have not been studied but the results from paper III and IV could indicate interactions in the biofilm between these two species. This could suggest antagonistic effects between these species.

The presence of *A. actinomycetemcomitans* has been extensively studied in different populations and displays a large variability depending on the population studied (Könönen & Müller, 2014).

What this depends on remains to be determined but several factors such as socio-economy and ethnicity/geographic origin could have an impact. A study from Trollhättan, Sweden recently indicated significantly higher prevalence of *A. actinomycetemcomitans* among participants of Somali heritage in comparison to Swedish participants of non-Somali heritage (Thorbert-Mros et al., 2021). This is in line with the results from paper IV where *A. actinomycetemcomitans* was more prevalent in the case group where the geographic origin of the individuals was of non-Nordic origin as opposed to the control group.

Socioeconomic factors have also been indicated to be of importance in detection of *A. actinomycetemcomitans*, even in African countries such as Ghana. In the study by Höglund Åberg et al. (2012) the adolescents attending public schools had a higher prevalence of *A. actinomycetemcomitans* compared to adolescents attending private schools.

Presence and colonization of *A. actinomycetemcomitans* in children is also dependent on the carriage of this bacteria by the parents, as it can be vertically transmitted (Monteiro et al., 2021). It has been estimated that if one parent carries *A. actinomycetemcomitans* the child presents a 16 times higher risk to carry it, compared to parents who do not carry it (Monteiro et al., 2014).

A factor related to demographics is also the use of plants for oral hygiene. While not being too common in the Nordic countries this is relatively common in developing countries, especially the rural areas, and frequently

observed in Africa due to accessibility and cheapness, but also owing to traditional beliefs (Sofowora, 1996). A wide variety of plants have been described to be used for dental hygiene by the Maasai (Bussmann et al., 2006). In paper III, the plant species *W. ugandensis*, introduced to us by the local tribe, was not mentioned in the study by Bussmann et al. suggesting tribal differences in the usage of the plants for oral hygiene. Interestingly, *W. ugandensis*, had a profound effect on the neutralization of leukotoxin indicating this as an environmental factor that could affect the presence of *A. actinomycetemcomitans*. However, as *A. actinomycetemcomitans* was found in abundance in saliva the effect of *W. ugandensis* could more likely influence the virulence of *A. actinomycetemcomitans* rather than having a bactericidal effect. Taken together, high levels of *A. aphrophilus* as well as daily use of a leukotoxin neutralizing plant for oral hygiene might impair the virulence of *A. actinomycetemcomitans*.

Even though *A. actinomycetemcomitans* was generally found in Maasai Mara, only one JP2-genotype could be identified in the population. This could suggest that migration from the North-Western parts of Africa, where this genotype is more prevalent, has been uncommon. Nonetheless, the first JP2-genotype in Kenya could be identified even in the remote areas of Maasai Mara and in a rather homogenous population.

The genetic diversity of *A. actinomycetemcomitans* is large (Kittichotirat et al., 2022), which also might interfere with its presence and virulence in different populations (Brígido et al., 2014). In a study from the county of Västerbotten, Sweden it was shown that certain genotypes of *A. actinomycetemcomitans* were more prevalent and found in high numbers in young (< 35 years) periodontitis patients (Claesson et al., 2017).

Periodontitis in adolescents, associated factors and early diagnosis

As described in paper IV, periodontitis was diagnosed in the majority of the case-group (92%) whereas none in the control-group had signs of periodontitis. This suggests that the screening using radiographs, with a cut-off of 2 mm (distance between cemento-enamel junction and proximal bone level), proved to be very effective in finding periodontitis.
The findings of proximal bone destruction on radiographs indicate that the periodontitis has initiated prior to this event, since the host inflammatory

response has already reacted to the dysbiotic oral microbiota (Figure 7). However, as no sufficiently sensitive biomarkers predicting the onset of periodontitis still exist, radiographs displaying proximal bone levels offer an excellent diagnostic tool at present. As radiographs are readily taken during routine dental examinations in Sweden, large-scale screenings can be performed without any additional sampling strategies. However, other factors influencing the proximal bone levels such as impacted teeth, large fillings and carious lesions must be considered.

The case-group consisted of individuals with a non-Nordic geographic origin which partly might explain the increased prevalence of periodontitis in this age group since it is known that periodontitis is more prevalent in some populations (Susin et al., 2014). Poor oral hygiene could also have an impact on the disease progression in this group, a factor mentioned as a possible reason for periodontitis in the earlier mentioned study by Thorbert-Mros et al. Interestingly, in our study there were no significant differences between the plaque levels when comparing the case-group with the control-group. Hence, the amount of plaque did not seem to be the key factor for periodontitis in our study population.

One possible explanation could be the lack of earlier dental care in their home country affecting disease onset and progression. A majority of the participants in the case-group did however state that they had received some dental and/or periodontal care in Sweden.

Familial aggregation is suggested to be strongly linked to periodontitis in children and adolescents and heritability has been suggested to be up to 30% (Diehl et al., 2005; Vieira & Albandar, 2014). Indeed, in the case-group there were individuals who stated that there was a history of periodontitis in the family. However, not all stated that this was the case suggesting that environmental factors also have a role in the etiology of periodontitis. These data were retrieved using the questionnaire which is why this needs to be carefully interpreted.

In addition to the increased prevalence and higher amounts of *A. actinomycetemcomitans*, *P. gingivalis* and *F. alocis* were also found more frequently and in higher amounts in the case-group.

P. gingivalis is a well-recognized periodontitis associated bacterium that frequently is found in elderly individuals with periodontitis and more seldomly in adolescents with periodontitis (Cortelli et al., 2008). The same applies for the more recently identified periodontitis associated bacterium *F. alocis*, suggesting it is more frequent in elderly periodontitis subjects rather than in adolescents (Neelakandan et al., 2021; Schlafer et al., 2010).

This is in contrast to the results in paper IV where these bacteria were found in a majority of the individuals in the case-group.
This could depend on the study population and might suggest that the individuals in the case-group exhibited an established periodontitis with a mature biofilm. However, the results regarding *F. alocis* are not unambiguous as some studies indicate their presence in periodontitis in adolescents (Shaddox et al., 2012).
A. actinomycetemcomitans was highly prevalent in the case-group which is in line with previous studies regarding its presence in periodontitis in adolescents (Fine et al., 2007; Haubek et al., 2008). No JP2-genotype was found in the work described in paper IV, however other highly leukotoxic strains of *A. actinomycetemcomitans* could have been present (Claesson et al., 2020; Johansson et al., 2017).

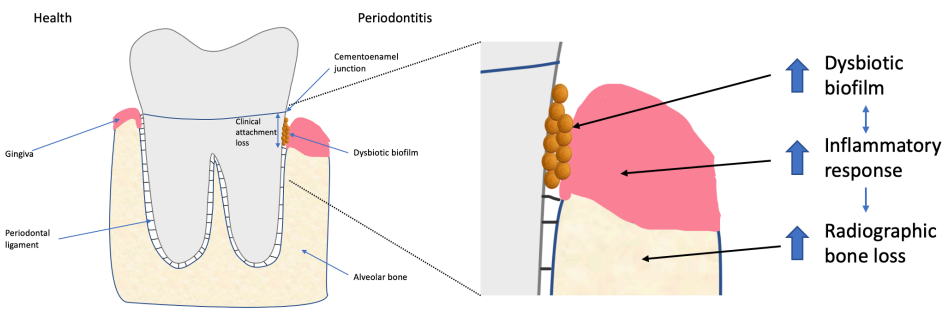

Figure 7
The figure represents the course of periodontitis, where the increasing dysbiotic biofilm leads to an increased inflammatory response and subsequent degradation of the periodontal tissues, ultimately leading to radiographic bone loss.

Saliva and subgingival plaque samples in diagnostics

Saliva and subgingival plaque samples were collected in papers III and IV to determine the presence and amounts of *A. actinomycetemcomitans*, *A. aphrophilus*, *P. gingivalis* and *F. alocis*.
Both methods are well-established, and our studies indicated rather good correlations, regarding presence of selected bacterial species, when comparing saliva and subgingival plaque. In general, studies comparing saliva and subgingival plaque suggest a good correlation between the two

methods (Belstrøm et al., 2017; Cortelli et al., 2005; Haririan et al., 2014). However, others report a low level of correlation in selected bacterial species (Umeda et al., 1998).
To be considered in these studies are relatively small populations, which applies for paper IV as well, that might skew the results depending on the study population.
One must also take into account what type of analyses are to be performed and what the goal is with the different sampling techniques.
Cultivation is possible to perform when live bacteria are present, as is the case when using subgingival plaque samples and adequate media for this purpose. This is a major advantage of cultivation from subgingival plaque since it permits antibiotic sensitivity testing, which might be of importance as antibiotics are a valuable tool in the treatment of periodontitis in adolescents (Loomer, 2004). The disadvantages are labor intensiveness and high costs as highly trained personnel is needed. Another disadvantage is also the inability to cultivate all bacteria present in the oral cavity.
Techniques for detecting DNA, such as PCR and qPCR can be used for both subgingival plaque samples and saliva. These techniques do not differentiate live or dead bacteria as cultivation does. However, the advantages are rapid processing and high sensitivity.
Regarding the sampling procedure, saliva would seem to be superior due to easiness of collection. Therefore, saliva sampling would be the method of choice if a large screening of a population is performed, and the goal is to identify the presence of bacteria. In clinical practice, subgingival plaque sampling would still be important, especially if antibiotic susceptibility testing is necessary.

Methodological considerations

Study design and systematic errors

The study designs in the clinical studies are of a cross-sectional design and in paper IV, a case-control procedure was included to determine differences between the group with radiographic bone loss and the group without radiographic bone loss.
Due to this type of study design, causality cannot be determined but can be addressed by adding randomization and matching to minimize the risks for confounding (Vandenbroucke, 2004).

Systematic errors (bias) are a disadvantage when using the cross-sectional design.
Recall bias refers to systematic differences in the information provided by a study participant (Carneiro & Howard, 2011). This type of bias is common when using questionnaires, such as in paper III and IV.
It would have been preferable to use other types of records, such as patient records, to minimize this type of bias. However, some questions could not be acquired in any other way which is why a questionnaire was necessary. The participants were also interviewed individually face-to-face, making it possible for them to ask the interviewer if they had questions regarding the questionnaire.
This procedure could unfortunately increase the risk for interviewer bias, since the interviewer was not blinded, and could thus lead to answers the interviewer seeks. Therefore, a blinded interviewer would have been preferable in these cases.

The dropout rate in the second part of paper IV was high and one must consider the risk for volunteer bias. Could the dropouts have differed in any way and affected the outcome of the study?
While being possible, the radiographic proximal bone levels did not differ significantly, suggesting that they might only have had a minor effect on the outcome and results. However, the volunteers accepting to participate could have exhibited more health awareness than the dropouts and if so, presented with a better oral health.

The clinical examinations were performed by one examiner that could have influenced the results from the clinical examinations. The examiner was not blinded due to practical reasons, since the examiner also invited the participants, and the participants were examined in conjunction with their ordinary examinations at their nearest dental clinics.
However, in this case a blinded examiner would also have been preferable to minimize the risk of bias.
To counteract errors in measurements (measurement bias) from the periodontal examinations the examiner performed calibrations with an experienced periodontist prior to the clinical examinations. The inter- and intra-rater agreement has been reported to be rather high in periodontal examinations, for CAL as well as PPD (Badersten et al., 1984). However, this can vary depending on the degree of periodontal defects, where larger defects display larger variability. The cemento-enamel junction can also

be difficult to determine in some instances, thus affecting the CAL measurements.
The registrations of clinical attachment loss and pocket probing depth was restricted to proximal surfaces in paper IV. The inclusion of registrations on buccal and lingual/palatal sites would have been positive in the aspect that it would have led to more information of the periodontal status. However, this was estimated to only have a minor impact on the results since a majority of attachment loss on buccal sites in adolescents is caused by trauma, usually by brushing, and not by periodontitis (Clerehugh et al., 1988).

When all systematic errors have been addressed the possibility of errors by chance still exist, defined as random errors (Carneiro & Howard, 2011). Random errors were addressed by including a power analysis in paper IV to have a sufficiently large sample size.

Generalizability

Taken together, the results from papers I and II would be generalizable to all so far tested strains of *A. actinomycetemcomitans* and *A. aphrophilus* regarding their intrinsic serum resistance, dependent on OmpA. Likewise, as OMVs released by these organisms normally contain LPS, they would be expected to have a general protective effect in proximity of any bacterial cells with low or lacking serum resistance. However, as mentioned earlier, inter-strain variability probably exists to some extent.

In paper III, a population from Maasai Mara was studied. This population was expected to be rather homogenous, and thus generalizability based on this population could be rather low.
However, the population would seem interesting since it might not be affected by dental care and modern oral hygiene measures. This would render the population very suitable for microbiological assessment, much in line with the study by Löe et al. (1986) where periodontitis was assessed in a population devoid of any oral hygiene measures and dental care.
It must also be highlighted that this study was performed in parallel with another study in the area, thus facilitating microbiological sampling. The parallel study was part of the "Maasai Mara Science and Development Initiative (MMSDI)" and was initiated to conserve the Maasai Mara ecosystem. This was a collaboration between the University of Nairobi, Aarhus University (Denmark), Kenya Wildlife Trust, the Mararianta

community and the Karen Blixen Camp Trust. As a part of this initiative oral health among the adolescents in Maasai Mara was evaluated. Specifically, the tradition of infant oral mutilation (IOM), also termed “ebinyo”, where germectomy of developing primary or permanent mandibular incisors or canines are performed (Girgis et al., 2016) was evaluated. As part of MMSDI the prevalence of IOM and its effects on the dentition was studied (Kemoli et al., 2018).

The study population in paper IV comprised a majority of the adolescents in Västerbotten born in 2001 (n=1656).
The radiographs were examined by three different examiners and were examined together in case of uncertainties, thereby ensuring that no proximal bone loss was missed and reducing the risk for bias. It could have been advantageous to involve a maxillofacial radiologist to further reduce the risk for missing proximal bone loss as interpretation of radiographs and marginal bone levels can be dependent on experience (Hellén-Halme et al., 2020).
The results from the radiographic screening resulted in 24 individuals positive for proximal bone loss. Because of a high dropout rate, only 13 of these cases could be examined in a clinical setting. This might have a consequence on the generalizability of the results due to a small study population. However, the initial population was large and strengthens the study.
The radiographs from the dropouts did not indicate any significant differences in proximal bone loss, possibly indicating that the outcome would not have changed if they would have been included in the clinical examinations. As periodontitis in adolescents in the Nordic countries is reportedly low (Saxén, 1980) it was expected to result in few cases in paper IV. Even so, the prevalence of periodontitis is suggested to have increased based on our results. The strength in this study is also that a clinical examination was performed in order to diagnose periodontitis, according to the most recent classification system (Papapanou et al., 2018), and not solely relying on radiographic evidence of periodontitis.

Conclusions

This PhD thesis work adds new data to the understanding of the microbiological involvement in periodontitis through the discovery of new mechanisms for survival of *Aggregatibacter* species and their interactions with the host.
It also adds new knowledge in the field of epidemiology of periodontitis in adolescents, as well as highlighting the presence of specific bacterial species in two highly different populations.

In conclusion:

- The outer membrane protein OmpA was found to be important for the survival of *A. actinomycetemcomitans* and *A. aphrophilus* in human serum. The mechanism for this was dependent on the binding of the complement system inhibitor C4bp to OmpA.

- Outer membrane vesicles from *A. actinomycetemcomitans* were able to protect a serum sensitive *A. actinomycetemcomitans ompA1 ompA2* double mutant. This effect was dependent on the presence of LPS on the OMVs.

- LPS from *A. actinomycetemcomitans* was able to efficiently consume factors in the complement system, thus titrating these factors and preventing them from interaction with the bacterial cells.

- *A. actinomycetemcomitans* and *A. aphrophilus* were highly prevalent in the population from Maasai Mara, Kenya. For the first time, the JP2-genotype was also identified in this East African population.

- Minute changes on radiographic proximal bone levels could predict periodontitis to a high degree.

- The prevalence of periodontitis in adolescents is indicated to increase in the population in Västerbotten County, Sweden. This was associated with an increase in *A. actinomycetemcomitans*, *P. gingivalis* and *F. alocis*, but also with differences in demographical patterns.

- Demographical patterns, but also environmental factors such as local plants (*W. ugandensis*) and specific bacterial species could have an impact on the microbiota associated with periodontitis in adolescents.

- Saliva and subgingival plaque could be used for detection of oral microbiota, on an individual level, with good agreement.

Clinical implications

Periodontitis in adolescents is strongly associated with the biofilm present subgingivally and especially the presence of *A. actinomycetemcomitans* (Haubek et al., 2008). As periodontitis is multifactorial, several aspects need to be considered in the etiology and pathogenesis of periodontitis (Papapanou et al., 2018).
However, as presence of certain bacteria in periodontitis affecting children and adolescents exhibits such a strong association, microbiological diagnostics should be considered in the treatment of these cases. One of the key points for microbiological diagnosis is the ability to identify periodontitis-associated bacteria, such as *A. actinomycetemcomitans*, and consider antibiotic treatment (Teughels et al., 2014). If antibiotic treatment is necessary, sensitivity testing should be done to choose the correct antibiotic and minimize the risk for antibiotic resistance, as antibiotic resistance poses a serious global health problem (Davies & Davies, 2010).
As *A. actinomycetemcomitans* and *A. aphrophilus* can be found in extra-oral infections such as endocarditis and brain abscesses, knowledge about these bacteria are not only important in the field of periodontology but also in general health care (Nørskov-Lauritsen, 2014).
To be able to counteract the increasing antibiotic resistance, experimental studies are of utmost importance to provide us with new insights in the treatment of periodontal infections. As shown in papers I and II, OmpA and OMVs are important for the survival of *A. actinomycetemcomitans*. By creating new strategies in targeting and blocking these virulence factors this could, in best case scenario, be used instead of broad-spectrum antibiotics. Vaccine-based solutions could also serve the same purpose.
Other compounds already found in nature, such as extracts from *P. guava*, have been shown to have an inhibitory effect on *A. actinomycetemcomitans* and *P. gingivalis* (Shetty et al., 2018). This is in line the results from paper III where the local plant from Maasai Mara, *W. ugandensis*, indicated an effect on the neutralization of leukotoxin. This exhibits intriguing evidence that other compounds than antibiotics could be used in the treatment of periodontitis, especially in areas where dental health care is absent. However, at the moment, many of these studies only support *in vitro* results.

Epidemiological studies on periodontitis in adolescents with the aim to identify high-risk individuals are important and could provide vital data on the determinants of the periodontal infection, and this information is

needed for the establishment of effective health promotion measures. However, there is a lack of actual and relevant information on the epidemiology and demographics of periodontitis among adolescents and young adults in our country, as well as in the other Scandinavian countries. The results from paper IV indicate an increase in the prevalence of periodontitis in adolescents in Västerbotten County. Even though the prevalence still is rather low compared to other populations (Kissa et al., 2022), these results should be highlighted since there is a risk that the disease is missed because of its low prevalence. For the affected individual this could have a major impact since early diagnosis is of utmost importance in the treatment of periodontitis, as it can rapidly progress otherwise. Even very small changes in the proximal bone levels seen on dental radiographs should be interpreted with caution since it could easily be missed. As demonstrated earlier, radiographs offer an excellent method for finding early signs of periodontitis and its use for these purposes should be emphasized.

The examiner should also be aware that periodontitis in adolescents is, as mentioned earlier, more prevalent in certain populations and that it can present as familial aggregation. Hence, other family members should be made aware of this and, if possible, be examined with regards to periodontal conditions. In these cases, microbiological testing is important, especially when systemic antibiotics are considered for treatment. The subgingival plaque samples still represent the method of choice for this, but one should also consider the possibility to use saliva for detection of periodontitis associated bacteria. This would seem very appealing if the patient was not able to visit the dental clinic in person but could do the sampling at home. In large scale screenings the use of saliva as a sampling method would also be very attractive.

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