Exotoxins of *Aggregatibacter actinomycetemcomitans* and periodontal attachment loss in adolescents

Carola Höglund Åberg
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To my beloved children Jonas and Minna!
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ABSTRACT

Aggregatibacter actinomycetemcomitans is an oral bacterium that is mainly associated with aggressive forms of periodontitis, which most often starts at an early age. Amongst the virulence factors of *A. actinomycetemcomitans*, two exotoxins, the leukotoxin (LtxA) and the cytolethal distending toxin (Cdt), are suggested to play an important role in the pathogenicity of aggressive periodontitis. There is also a genetic diversity of the different strains of *A. actinomycetemcomitans*, and a variation in the ability of different strains to express and release exotoxins has been suggested. Of the different genotypes of *A. actinomycetemcomitans*, the highly leukotoxic JP2 genotype, which is prevalent in individuals of African origin, seems to be the genotype that is most strongly associated with localized aggressive periodontitis.

This thesis is built upon studies of a West African adolescent population. The aim was to study the virulence characteristics of *A. actinomycetemcomitans* genotypes with a specific focus on the LtxA and the Cdt in relation to the progression of attachment loss (AL). The specific aim was first, to investigate cross-sectionally the presence of the JP2 and non-JP2 genotypes of *A. actinomycetemcomitans* in relation to the prevalence of AL and then prospectively to assess the progression of AL in a Ghanaian adolescent population. Second, in clinical isolates of *A. actinomycetemcomitans* obtained from the participants of the study, the serotypes and the virulence characteristics related to the two exotoxins were studied and associated with the progression of AL at the individual level.

In Paper I, based on the study population consisting of 500 adolescents (mean age 13.2 years; SD ±1.5), it was shown that the overall carrier rate of *A. actinomycetemcomitans* was high (54.4%) and that the presence of this bacterium was associated with AL ≥ 3 mm. The JP2 genotype was prevalent (8.8%) in this population. In Paper II, 397 (79.4%) of the study participants were periodontally examined again at a 2-year follow-up. The presence of the JP2 genotype of *A. actinomycetemcomitans* in subgingival plaque was strongly associated with the progression of AL. This study also provided support for an enhanced estimated risk (odds ratio, OR=3.4), though less
pronounced, for the progression of AL in individuals positive for the non-JP2 genotypes of *A. actinomycetemcomitans*.

In **Paper III**, all three *cdt* genes (*a*, *b* and *c*) were detected in 79% of the examined *A. actinomycetemcomitans* isolates, all of which expressed an active toxin. The distribution of the *cdt* genes showed a serotype-dependent pattern. In particular, the presence of the b serotypes (both JP2 and non-JP2 genotypes) was associated with the disease progression, whereas the expression of Cdt was not particularly related to the disease progression. In **Paper IV**, it was shown that the presence of *A. actinomycetemcomitans* isolates with high leukotoxicity, also those of the non-JP2 genotypes of *A. actinomycetemcomitans*, were associated with an increased risk of the progression of AL in relation to the reference group. The main proportion of the serotype b isolates was distributed in the category of highly leukotoxic isolates. The analyses of the non-JP2 genotypes of serotype b indicated a diversity linked to the level of leukotoxicity.

In conclusion, *A. actinomycetemcomitans* in general was associated with the progression of AL. Individuals with an increased risk of developing progression of AL mainly harboured isolates of *A. actinomycetemcomitans* with a high leukotoxicity, which suggests that the LtxA is an important virulence factor. Of the two exotoxins, the pathogenic potential was mainly associated with the LtxA, while the role of the Cdt is unclear.

**Key words:** *Aggregatibacter actinomycetemcomitans*, periodontal attachment loss, JP2 genotype, non-JP2 genotypes, LtxA, Cdt.
ABBREVIATIONS

\textit{A.a} \quad \textit{Aggregatibacter actinomycetemcomitans}

Aae \quad \text{outer membrane autotransporter protein}

AgP \quad \text{aggressive periodontitis}

AP PCR \quad \text{arbitrarily primed polymerase chain reaction}

ApiA \quad \text{outer membrane autotransporter protein}

ApiBC \quad \text{outer membrane autotransporter protein}

AL \quad \text{attachment loss}

Bp \quad \text{basepair}

BoP \quad \text{bleeding on probing}

BSA \quad \text{bovine serum albumin}

Cdt \quad \text{cytotoxical distending toxin}

CEJ \quad \text{cemento-enamel junction}

CI \quad \text{confidence interval}

DNA \quad \text{deoxyribonucleic acid}

ELAM-1 \quad \text{endothelial leukocyte adhesion molecule-1}

FBS \quad \text{fetal bovine serum}

Flp \quad \text{fimbrial low molecular weight protein}

GAgP \quad \text{generalized aggressive periodontitis}

GCF \quad \text{gingival crevicular fluid}

GM \quad \text{gingival margin}

Hbp-2 \quad \text{haemoglobin binding protein-2}

IAL \quad \text{incidental attachment loss}

ICAM-1 \quad \text{intercellular adhesion molecule-1}

IL \quad \text{interleukin}

IS \quad \text{insertion sequence}

IS1301 \quad \text{insertion sequence}

JHS \quad \text{junior high school}

JP2 genotype \quad \text{deletion of 530 bp in the promoter region}

KDa \quad \text{kilo Dalton}

LAgP \quad \text{localized aggressive periodontitis}

LDH \quad \text{lactate dehydrogenase}

LFA \quad \text{lymphocyte function-associated antigen}

LPS \quad \text{lipopolysaccharide}

LtxA \quad \text{leukotoxin}

Ltx \quad \text{leukotoxin gene}

ltx3 \quad \text{primer}
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<td>ltx4</td>
<td>primer</td>
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<tr>
<td>MMP-8</td>
<td>matrix metalloproteinase 8</td>
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<tr>
<td>non-JP2 genotype</td>
<td>complete leukotoxin promoter</td>
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<td>NRU</td>
<td>neutral red assay</td>
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<td>OR</td>
<td>odds ratio</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>pI</td>
<td>isoelectric point</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PMNs</td>
<td>polymorphonuclear leukocytes</td>
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<tr>
<td>PPD</td>
<td>probing pocket depth</td>
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<td>PYG</td>
<td>peptone yeast glucose broth</td>
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<tr>
<td>RR</td>
<td>relative risk</td>
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<tr>
<td>RANK</td>
<td>receptor activator of nuclear factor k-B</td>
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<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor k-B ligand</td>
</tr>
<tr>
<td>RTX</td>
<td>repeats in toxin family of toxins</td>
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<tr>
<td>THP-1 cell</td>
<td>human acute monocytic leukemia cell line</td>
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ORIGINAL PAPERS

This thesis is based on the following original Papers, which will be referred to in text by their Roman numerals:


*Papers I and III are reprinted with kind permission from the publishers.*
INTRODUCTION

Periodontal disease represents several disease entities with different clinical presentations. The diagnosis of periodontitis is based on a visible amount of tissue destruction due to the ongoing or preceding inflammatory process in the periodontal tissues.

It is well established that bacteria in the dental plaque play a key role in the initiation of periodontitis, but the contribution of bacteria to disease progression is still poorly understood. The microflora of the dental plaque is extremely complex, it differs with the nature of the anatomical site, and it changes over time. In addition, a variety of microorganisms might contribute diversely to the periodontal disease development in different populations and individuals, possibly due to host tropism, degree of host susceptibility or different virulence characteristics of the bacteria. In other terms, bacteria are necessary but not sufficient for disease activity to occur. Studies based on microbial sampling have demonstrated that there is a strong association between the type of microbial community found in the gingival crevice and the host inflammatory and immunological response (Ebersole, 2003).

Bacterial exotoxins released by bacteria are a diverse collection of proteins, which are responsible for many of the biological effects caused by pathogens during infection. Aggregatibacter actinomycetemcomitans is an opportunistic pathogen of humans that is present in the oral cavity, particularly noted for its involvement in aggressive forms of periodontitis. This thesis focuses on the virulence capacity of A. actinomycetemcomitans exotoxins, the leukotoxin (LtxA) and the cytolethal distending toxin (Cdt), both of which are released by this bacterium. The prospective cohort study is suitable for studying the role of these exotoxins as predictors for disease onset and progression.

In periodontics, the dentist is challenged with a choice of treatments rather than with a treatment of choice in managing periodontitis cases. However, the main treatment of periodontitis today is still directed towards removal of the biofilm and thereby possibly achieving control of the periodontal infection. Further progress in the therapeutic management of periodontal disease
requires a thorough understanding of the inherent virulence properties of periodontopathogenic microorganisms, their epidemiology, and how they cause disease.

**Periodontal disease**

*The periodontium*

The periodontium (peri=around, odous=tooth) is a functional system of different tissues that surround and support the teeth, and this includes the gingiva, periodontal ligament, root cement and alveolar bone (Hassell, 1993). The oral cavity consists of several habitats. These include the tooth surface, the epithelial surfaces of the gingiva and the periodontal pocket, and specialized mucosal surfaces, all of which provide ecological niches for microorganisms (Figure 1). The tooth, a hard tissue element, passes through the soft tissues with an anatomically unique interruption of the epithelial seal. The tooth has non-shedding surfaces that are highly inviting for microbial colonization (Lindhe et al., 2008).

The periodontal tissues constitute a developmental, biological and functional unit (Figure 1), which undergo morphologic changes during life related to different alterations in the oral environment. The age-related tendency to develop gingivitis, evident in adolescents around puberty, may be related to changes in the bacterial composition of the dental biofilm, the inflammatory cell response, hormonal changes, morphological differences, tooth eruption and shedding.
INTRODUCTION

Figure 1.
The healthy periodontium (left) contains **gingival connective tissue**, a supporting structure attached to the tooth and alveolar bone through fibrous attachments. The **oral epithelium** covers this supporting tissue, and a **specialized junctional epithelium** connects it to the tooth surface. The space between the epithelial surface and the tooth is called the sulcus and is filled with gingival crevicular fluid. The **sulcular epithelium** is nonkeratinized and lines the gingival sulcus. It faces the tooth without being in contact with the tooth surface.
The periodontium exists in an environment rich in various species of microorganisms. To function as a pathogen, a bacterium must colonize the appropriate host tissue site and cause destruction of the host tissues. Entry of the bacterium itself (invasion) or entry of bacterial products into the periodontal tissues may be essential in the disease process. Inherent in the successful colonization of host tissues is the ability of the bacterium to evade host defense mechanisms through secretion of bacterial substances that directly or indirectly cause degradation of the periodontal tissues. (Redrawn from Darveau 2010, Nature Reviews Microbiology. With permission).
Classification and diagnosis of periodontitis

Periodontitis forms a group of infection-induced inflammatory diseases that affect tooth-supporting soft tissues and alveolar bone. Periodontitis involves progressive loss of the alveolar bone around the teeth, and if left untreated, the disease can lead to the loosening and subsequent loss of teeth. Both inherited and acquired disorders of the periodontal tissues can be defined as periodontal disease. Periodontal diseases are classified into several disease categories, but they share common pathways of pathogenesis (Page et al., 1997, Armitage, 1999). The classification system for periodontal diseases used today was introduced by the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions (Armitage, 1999).

Gingivitis and periodontitis are the two major categories of periodontal disease. Periodontal diseases are not limited to adults. Epidemiologic surveys performed in many parts of the world indicate that a small but significant proportion of children, adolescents and young adults are affected by some form of periodontal attachment loss and bone loss (Albandar and Tinoco, 2002).

Gingivitis is an inflammatory lesion of the marginal gingiva, which is confined to the gingival sulcus with no accompanying destruction of the periodontal tissues. Mild and moderate forms of gingival inflammation are an almost universal finding in young people (Lindhe et al., 2008). The condition is recognized by gingival inflammation, and it is frequently measured as bleeding on probing (BoP). In general, the severity of gingivitis increases with the amount of microbial biofilm, while the removal of the dental plaque induces a resolution of the inflammation.

Periodontitis occurs predominantly in a slowly progressing form, which rarely interferes with tooth function before adulthood. The more severe and rapidly progressive form of periodontitis is denoted aggressive periodontitis (AgP) and has its debut at an early age in children and adolescents without clinical evidence of systemic disease (Armitage, 1999, Armitage, 2004). The localized form of aggressive periodontitis (LAgP) usually affects permanent first molars and central incisors, and the onset of this disease is often circumpubertal. AgP may become rapidly aggravated and results in tooth loosening already in adolescence, but the disease can also be self-arrested. Generalized aggressive
periodontitis (GAgP) affects individuals under 30 years of age and represents the most heterogeneous group in terms of clinical presentation with a generalized interproximal attachment loss (AL). The course of the disease is more advanced and generally affects permanent teeth other than first molars and incisors. The occurrence of AL in young individuals that does not fit the specific diagnostic criteria for AgP has been termed incidental attachment loss (IAL). This disease entity may include initial clinical signs of periodontitis. Individuals with this diagnosis should be considered to be at risk for further disease progression. IAL also includes a variety of defects. These include recessions associated with tooth brushing trauma, thin alveolar bone, tooth position and AL associated with conditions around third molars, etc. Tooth surfaces that exhibit local factors, which facilitate plaque retention such as calculus, defective dental restorations and untreated manifest caries lesions, may also be a risk for developing IAL.

**Epidemiology of periodontal diseases**

Epidemiology is the study of the pattern (distribution) and dynamics of diseases in a human population. The purpose of epidemiology is to increase the understanding of the disease process (i.e. to identify the risk factors and determinants of disease) and thereby identify methods for control and prevention (Lilienfeld, 1978). Certain populations might be at high or low risk for the specific disease under investigation.

Epidemiologic studies indicate that plaque-induced gingivitis of varying severity is nearly universal in all age groups (Jenkins and Papapanou, 2001). Estimation of the prevalence of AgP is difficult to compare due to differences in study design and diagnostic criteria (Jenkins and Papapanou, 2001, Albandar and Tinoco, 2002). Aggressive forms of periodontitis seem to exist in all ethnic groups throughout the world, although there may be variations, and the estimated prevalence rates may also vary within ethnic groups. According to population-based studies, the prevalence of LAgP varies greatly: from 0.1-0.2% reported in European populations to 5% in Hispanic populations, and up to 10% for African-Americans in the U.S. (Papapanou, 1996, Albandar and Tinoco, 2002). It has been reported that African-American teenagers are 15 times more likely to develop aggressive periodontitis than Caucasians (Löe and Brown, 1991). However, a large variation may also exist in different African populations. In Nigeria (Harley and Floyd, 1988,
Arowojolu and Nwokorie, 1997) the reported prevalence of AgP was 0.8–1.6% in Nigerian teenagers and young adults. In a population-based survey of 12-25-year-old Ugandan school attendees (Albandar et al., 2002) a prevalence of 6.5% AgP (either localized or generalized) and 22% incidental attachment loss was found. Other ethnic groups than those of African affiliation may also show a high prevalence of periodontal disease. In a recent study from Israel, AgP was found in 5.9% of the subjects (Levin et al., 2006).

**Risk factors affecting the prevalence and severity of periodontitis**

A risk factor is defined as any characteristic, behavior or association with a particular disease, and the degree of causation may be variable. A risk can also be defined as the probability that an event will occur in the future. A risk factor that cannot be modified is often referred to as a risk determinant, e.g. age, gender, race. This determinant type involves background characteristics, and is not considered to be aetiologic for the disease of interest, but might function as effect modifiers or confounders for the risk factors. A risk marker or risk predictor is known as a factor used to predict the future course of a disease.

Periodontal diseases are multi-factorial, and the manifestation and progression of the disease is influenced by a wide variety of risk factors and risk determinants, including the number and composition of bacteria in the subgingival dental plaque, subject characteristics, social and behavioural factors, systemic factors, genetic factors, tooth-level factors, and other emerging risk factors.

The strong association that exists between poor oral hygiene and periodontal disease outlines the presence of supra and/or subgingival bacterial plaque as the primary aetiologic agent. The increase in gingivitis levels from infancy to puberty may be related to hormonal factors, but it is also attributed to the increase in number of sites at risk for plaque accumulation and subsequent gingival inflammation associated with tooth exfoliation and tooth eruption. The prevalence, severity and extent of plaque-induced gingival inflammation reach a peak at puberty and then decline during adolescence.
A complex interaction between the host and environmental modifying factors (e.g. level of education, income, social circumstances, living conditions, and cigarette smoking) contribute to the clinical manifestation and course of periodontal disease.

Periodontitis is considered to be polygenic and are associated with variations in multiple genes, each having a small overall contribution to the disease progression. Several studies have indicated that cases diagnosed with AgP have a tendency to aggregate within certain families (Llorente and Griffiths, 2006, Meng et al., 2011), indicating that genetic factors may be important in the susceptibility to AgP. Besides genes of major effect that determine susceptibility to AgP, other genes might act as disease-modifying genes, which influence the disease process. In addition, polygenic differences may also modify the susceptibility for various bacterial species and their pathogenic potential (DiRienzo et al., 1994).

General aspects on living conditions and oral health status in adolescents in sub-Saharan African countries

Health related problems and infectious diseases continue to be severe, especially in the sub-Saharan Africa, despite gradual improvements in public health services (Petersen, 2009). There are several factors associated with the control of infections, such as hygiene, sanitation, safe drinking water, living conditions in families, sufficient food supplies, and all these factors are interconnected. A group of modifiable risk factors is common to many chronic diseases, and this is also important for periodontal disease, which is the most common oral disease worldwide (Lindstrand, 2006, Petersen, 2009). The ethnic background may also include differences in diet, oral hygiene habits, caries prevalence and severity, and the microbial composition of dental plaque. These are population characteristics that possibly influence the course of the periodontal destruction. The oral hygiene conditions have generally been described as poor in African populations with accumulation of plaque and calculus deposits from early ages (Baelum and Scheutz, 2002). A substantial portion of the population practices tooth cleaning. The cleaning methods include toothbrushes with or without toothpaste, but also traditional tools such as a chewing stick, chewing sponge, plantain stems with ash and use of fingers or textiles (Addo-Yobo et al., 1991, Åberg et al., 2012).
**Ghana**

This thesis is built upon studies where the data collection was performed in Ghana. Therefore I want to give a short description of the country from an historical and demographical point of view.

Already in the 11th and 12th centuries the history about the ancient Ghana tells about trade routes through the Sahara. Ghana derived power and wealth from gold mines, and goods were transported by routes that were taken by traders from North Africa. The routes ran between Ghana and Southern Morocco (Figure 2). The Portuguese were the first to arrive in the late 15th century, followed by people from other European countries, with an interest in the trading for gold, ivory, and pepper. With the opening of European plantations in the “New World” during the 1500s, which suddenly expanded the demand for slaves in America, trade in slaves soon overshadowed gold as the principal export of the area. The west coast of Africa became the principal source of slaves for the “New World” (Figure 2). Ghana was created as a parliamentary democracy in 1957 and has today a reported population of about 24 million people. The median age of Ghanaians is 30 years, and the average household size is 3.6 persons. The population of Ghana consists mainly (99%) of people of African origin. Ghana has a universal health care system, and the infant mortality is about 39 per 1000 live births. The demographics of religion of the Ghanaian citizens reflect many historical events and the influences by colonizers during centuries from the many European countries. Christianity is the most prevalent religion and predominates in southern Ghana, while Islam and traditional religions are more widespread in the northern regions (Gocking, 2005).
Figure 2.
Illustration of the geographic area of Africa and the dissemination pattern of population migrations in a worldwide perspective. These events gave rise to the transmission and spread of bacterial clones, i.e. the JP2 clone, that initially emerged as a distinct genotype in Northern Africa approximately 2,400 years ago and subsequently spread to West Africa, from which it was transferred to the American continents during the transatlantic slave trade. (Redrawn from Haubek 2010 APMIS. With permission).
Aetiology and pathogenesis of periodontal disease

Host defense in periodontal diseases

Both the innate (non-specific) and the adaptive (specific) immune responses play important roles in host defense processes. The biofilm provides a constant challenge to the innate host immune system. Innate immune mechanisms include the barrier function of oral epithelia and the vascular and cellular aspects of inflammatory reactions. The polymorphonuclear leukocytes (PMNs) are the predominant leukocytes within the periodontal pocket. Early in local inflammatory response to infection PMNs are recruited from the circulation to the periodontal pocket by the process of extravasation induced by the formation of local gradients of chemotactic compounds such as bacterial products and pro-inflammatory cytokines. The inflammatory response, a part of the innate immune system, is extremely well-coordinated and comprises the migration of PMNs, monocytes, lymphocytes and plasma cells into the affected tissues (Lindhe et al., 2008, Lamster and Novak, 1992, Kinane et al., 1999). Endothelial leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) are crucial for cellular trafficking.

A variety of functional defects of neutrophils have been reported from cases with LAgP and GAgP. These include depressed neutrophil chemotaxis and migration, abnormal antibacterial functions and dysfunctions of phagocytosis (Genco et al., 1980, Van Dyke et al., 1982, Van Dyke et al., 1988). Local inflammatory responses have been characterized as exaggerated in AgP cases by an intense recruitment of PMNs both within the connective tissues and into the periodontal pocket. It has been reported that hormonal changes during puberty may influence the endothelium and cause an increased vascular permeability, which affects the recruitment of leukocytes to the inflamed tissue. These changes may facilitate changes in the composition of the subgingival microflora as evidenced by an increase in Gram-negative species (Wojcicki et al., 1987, Nakagawa et al., 1994).

PMNs possess a variety of means to phagocytize and kill most of the microorganisms and enzymatically degrade their virulence substances. Bacteriocidal and bacteriostatic compounds, such as lysozymes, lactoferrin, defensins, cathepsin G and elastase, are released into the surrounding
environment following phagocytosis and degranulation (Van Dyke and Hoop, 1990). This underlines the importance of the PMNs in the local defense against the microbial invasion but also their potential role in the host-mediated tissue destruction. If the infection persists the concomitant tissue destruction may trigger the immune response to release an excessive amount of cytokines that are secreted by cells involved in both the innate and adaptive host responses. These molecules, which are released by the host cells into the local environment, assist the cell-to-cell communication and assist in the regulation of immune effector cells. Interleukins are important members of the cytokine group, and these are involved in communication between leukocytes and other cells, such as epithelial cells, endothelial cells and fibroblasts. All of these cells are involved in the immune and inflammatory processes in the periodontal tissues. Chemotactic cytokines play an important role in cell-mediated immune responses in recruiting defense cells (PMNs, macrophages, lymphocytes) to the area of inflammation (Graves, 1999).

Tissue macrophages, which are derived from blood monocytes, are important defense elements due to their ability to phagocytize bacteria, debris and toxic compounds (Hassell, 1993). However, activated phagocytes (PMNs and macrophages) are also responsible, directly or indirectly, for an increased production of tissue-degrading enzymes, including the matrix-metalloproteinases (Birkedal-Hansen, 1993). On the other hand, macrophages are also stimulated by cytokines or bacterial products to synthesize and release a broad range of substances that can regulate tissue turnover and the immune response, and the macrophages can also attract PMNs (Dinarello 2011). This could be of importance to maintain homeostasis in the periodontal connective tissues (Darveau, 2009).

Toll-like receptors (TLRs) play a central role in the host cell recognition and in the initiation of immune responses (both innate and T cell-adaptive responses) against microbial pathogens. TLRs are expressed predominantly in cells, which mediate first-line defense, such as neutrophils, monocytes/macrophages, and dendritic cells, as well as by epithelial cells. Bacterial lipopolysaccharide (LPS), a common antigen of Gram-negative bacteria, is specifically recognized by host receptors such as TLRs (Kawai and Akira, 2011).

The chronic infectious disease periodontitis leads to loss of connective tissue and bone. Bone resorption is mediated by osteoclasts, which are tissue-
specific bone-resorbing cells derived from the monocyte/macrophage haematopoietic lineage. The osteoclasts exhibit specific abilities to degrade organic and inorganic components of bone. An important system in osteoclast activation includes the receptor activator of nuclear factor-kappa β (RANK), a receptor expressed by osteoclast progenitor cells, the RANK ligand (RANKL) and the osteoprotegerin receptor (OPG), secreted by stromal cells/osteoblasts. In the periodontal lesion, osteoclast formation can be induced by the activation of RANKL in osteoblasts induced by infiltrating T cells and by resident cells such as fibroblasts and periodontal ligament cells (PDL) (Lerner, 2004). Osteoblasts and osteoclasts are known to express TLRs (Myneni et al., 2013). The finding that activation of TLRs also influences osteoclastogenesis suggests that TLRs may form a link between inflammation and enhanced bone resorption also in bacteria-induced inflammatory diseases such as in periodontitis (Myneni et al., 2013).

Activation of the complement cascade can be initiated from cell wall components of both Gram-positive (lipoteichoic acid) and Gram-negative bacteria (lipopolysaccharides). The activation of the complement system mediates migration of PMNs and macrophages from peripheral blood to the site of inflammation in the gingiva. Complement factors may initiate bacterial cell lysis and enhance the phagocytosis of bacteria by opsonization (Marcotte and Lavoie, 1998).

The adaptive defense system includes humoral (antibody-mediated) as well as cell-mediated responses, and it is a second line of defense (Berglundh and Donati, 2005). The humoral and cell-mediated host defense systems include T lymphocytes (T cells), which play a role in cell-mediated immunity, and B lymphocytes (B cells), which are precursors for plasma cells in the humoral immune response, and natural killer cells (NK cells). Plasma cells are the terminal cells in the developmental proliferation of B cells. This activation and differentiation of B cells is also influenced by certain cytokines such as IL-4, IL-5, and IL-6 (Gemmell et al., 1997). The systemic antibodies produced by plasma cells in the gingival lesions are mainly directed towards antigens present in the subgingival biofilm. The antibodies have been shown to function in a variety of mechanisms, and this includes an ability to aggregate bacteria, inhibit adherence and colonization, enhance phagocytosis, lyse bacteria and inhibit virulence factors (Ebersole and Taubman, 1994, Kinane et al., 1999).
Dysfunctions in the host defense system are correlated with an increased susceptibility to periodontitis. An association has been reported between a modified IgG-response and periodontitis (Lu et al., 1994). In general, conditions that affect antibody production do not necessarily lead to periodontal diseases (Lamster and Novak, 1992, Dahlén et al., 1993). In contrast, conditions with deficient functions of PMNs, such as agranulocytosis, neutropenia, Papillon-Lefevre syndrome, Kostmann syndrome, Chediak-Higashi syndrome, diabetes mellitus and leukemia, are known to result in an increased susceptibility to periodontitis (Hart et al., 1994, Carlsson et al., 2006, Schenkein, 2006).

The microbiology of periodontitis

General aspects

The bacterial biofilm is defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix (Costerton et al., 1999). The consortium of bacteria participating in the initiation and progression of periodontitis resides in the dental bacterial plaque, i.e. microbial biofilm. The microbial community forms a protective environment for the pathogens with its inherent resistance to antimicrobial agents. When supragingival hygiene is not maintained, the dental plaque accumulates and develops into a community of microflora containing many bioactive end products and bacterial cells. Subgingival plaque can be described and divided into three types. First, the attached, tooth-associated plaque is dominated by Gram-positive rods and cocci. Secondly, Gram-negative motile rods dominate in unattached plaque, which extends apically to the strictly anaerobic part of the pocket. This is a very bioactive area due to a large amount of gingival crevice fluid excreted from the inflamed periodontal tissues. The epithelium-associated plaque, mainly consisting of Gram-negative and motile rods, is especially involved in the bacterial invasion of the connective tissue and plays thereby an important role in the periodontal pathogenesis (Fives-Taylor et al., 1999).

The microorganisms associated with periodontal diseases can be characterized as opportunistic or endogenous pathogens (Socransky and Haffajee, 2005). The oral microflora contains about 700 different species/phylotypes of aerobic and anaerobic bacteria, which are capable of colonizing various sites of the mouth and can live in harmony with a healthy host. Commensal bacteria may
also induce a protective response that prevents the host from developing disease (Darveau, 2009).

Advances in the understanding of the periodontal infection have revealed inherent problems in fulfilling Koch’s postulates. The criteria for defining pathogens of periodontal diseases have been extended and modified by several investigators. A bacterium has to fulfill certain criteria in order to be classified as a periopathogen: (1) the species should be found more frequently and in higher numbers in diseased than in healthy sites, (2) elimination of the species will result in stopping disease progression, (3) the species will induce antibody production in the host, (4) the species should be associated with a unique mechanism of pathogenicity, and (5) the species should be able to cause disease in an animal model (Socransky, 1979, Socransky and Haffajee, 1992). Two of the microorganisms most commonly associated with periodontitis that meet these criteria are *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*.

The first step in the periodontal infectious process occurs when the microorganism colonizes its preferred target tissue site, the junctional or pocket epithelium. This permeable epithelial barrier with its specific cell and fluid dynamics plays an important role in preserving the epithelial continuity across the hard and soft tissue interface. When a persistent biofilm is established a series of complicated interactions take place in this local environment over a defined period of time. Extracellular components of periodontopathogenic bacteria, such as fimbrie and outer surface membrane molecules, are potent mediators of adherence and facilitate the invasion of host cells (Fives-Taylor et al., 1999). The time prior to a tissue-damaging infection varies and relates to the virulence of the organisms and the susceptibility of the host. The presence of the bacterial biofilm activates an inflammatory response in the surrounding tissues, which recruits a substantial number of immune cells from the peripheral circulation to the inflamed site. The increase in the amount of microbial plaque causes inflammatory reactions, which result in a detachment of the gingival epithelium from the tooth and the epithelial lining of the periodontal pocket migrates apically on the root-surface. An imbalance in the host response might lead to degradation of the tooth supporting tissues, bone and connective tissue, and finally to tooth loss. A large number of microbial components, such as toxins and proteases that are released from the biofilm, can affect the cellular response of the host.
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(Nishihara and Koseki, 2004). Tissue destruction may be induced directly by action of the bacterial toxins or enzymes, or indirectly as a consequence of the host inflammatory response caused by bacterial products.

Circumpubertal changes have been shown in the proportions and the frequency of periodontopathogenic bacteria, and these are distinct in different stages of physiological maturation (Mombelli et al., 1989, Nakagawa et al., 1994, Mombelli et al., 1995). Black-pigmented Bacteroides species are commonly found in gingivitis lesions, and these are closely related to the presence of gingival inflammation. *A. actinomycetemcomitans* may be frequently detected in healthy periodontal sites in the young, but it is still more often related to sites with signs of inflammation and increasing pocket depths (Bimstein and Matsson, 1999, Van der Velden et al., 2006, Fine et al., 2007). The strongest evidence regarding a bacterial aetiology of AgP comes from studies of the localized form of the disease. In general, cases with AgP show a sparse visible plaque accumulation. Early studies using culture techniques have identified a predominant flora with a limited number of Gram-negative species including *A. actinomycetemcomitans*, and bacteroides-like organisms (*Prevotella intermedia, Porphyromonas gingivalis, Capnocytophaga species, Eikenella corrodens and Campylobacter rectus*). Of these organisms *A. actinomycetemcomitans* is viewed as a key organism that is mainly associated with localized forms of AgP (Slots, 1976, Newman and Socransky, 1977, Haffajee and Socransky, 1994).

**Virulence mechanisms of bacteria**

**Virulence factors** are molecules expressed and secreted by bacteria that enable them to colonize a certain niche in the host and to evade or to interfere with the immune response (including the innate and acquired immune system) of the host (Wilson et al., 2002). Many of the interactions between a bacterium and its host are influenced by the virulence properties of the microorganism. It has been a general opinion that virulence mechanisms of bacteria comprise the production of bacterial toxins that have the capacity to “kill” host cells. However, most toxins are specific proteins that are able to modulate, to the advantage of the bacterium, particular aspects of the host cells’ actions (Los et al., 2013). Adhesion to host cells and invasion of cells are two key virulence mechanisms of bacteria. Other virulence factors, which can directly damage host tissues, include enzymes such as proteases and an immunodominant
antigen such as the LPS. However, the presence or absence of a certain bacteria acting in a complex polymicrobial community may not be the only indicator of disease initiation and progression. It is reasonable to assume that the virulence of bacterial phenotypes is enhanced by synergistic interactions with a pool of periodontopathogenic bacteria resulting in increased disease severity. Expression of virulence factors has mostly been evaluated under standard *in vitro* conditions, and little is known so far about the regulation of virulence-related genes in conditions *in vivo*.

**Aggregatibacter actinomycetemcomitans**

*General background*

*A. actinomycetemcomitans* is a Gram-negative, capnophilic, facultative anaerobic rod approximately 0.4-0.5μm x 1.0-1.5μm in size. *A. actinomycetemcomitans* was originally identified in 1912 by Klinger in human Actinomycotic lesions (Zambon, 1985). *A. actinomycetemcomitans* has been identified in several systemic infections such as bacterial endocarditis, meningitis, lung, brain and urinary tract infections (van Winkelhoff andSlots, 1999), and in all cases the source of infection was thought to be the oral cavity. *A. actinomycetemcomitans* was first implicated as a potential oral pathogen in LAgP, found in children and adolescents. The bacterium received particular attention viewed as a key microorganism in the aetiology of AgP (Slots, 1976). The most abundant evidence regarding a bacterial aetiology of AgP comes from studies of LAgP. Longitudinal studies have indicated an important role of this bacterium in the initiation of the AgP (Timmerman et al., 2000, Van der Velden et al., 2006, Fine et al., 2007, Haubek et al., 2008).

*Detection of* A. *actinomycetemcomitans*

The prevalence of *A. actinomycetemcomitans* in subgingival plaque samples is estimated by traditional cultivation methods on selective media, as well as by molecular (PCR-based) techniques. *A. actinomycetemcomitans* forms small colonies approximately 0.5-1.0 mm in diameter in primary isolates on agar plates. Freshly isolated *A. actinomycetemcomitans* have rough colony morphology with a characteristic star-shaped formation in the center. (See front cover of the thesis). Upon repeated subculture a change in colony morphology from rough to smooth-surfaced colonies occurs. The surface
ultrastructure of *A. actinomycetemcomitans* includes characteristics such as fimbriae, vesicles, and extracellular amorphous material. It is proposed that fimbriae most probably function in adherence of rough variants, whereas nonfimbrial components (such as vesicles) are probably involved in adherence of the smooth, highly invasive strains (Fives-Taylor et al., 1999).

The PCR-based method allows a detection of *A. actinomycetemcomitans* with a high sensitivity and specificity from clinical plaque samples. The PCR method used in this thesis determines only the presence or absence of *A. actinomycetemcomitans*.

**Taxonomy and genetic characterization of A. actinomycetemcomitans**

*A. actinomycetemcomitans* is most closely related to the bacterial family of *Pasteurellaceae* (Slots, 1999). Recently, the bacterium was reclassified into the new genus *Aggregatibacter* together with its close relatives *Aggregatibacter (Haemophilus) aphrophilus*, and *Aggregatibacter (Haemophilus) segnis* (Norskov-Lauritsen and Kilian, 2006). The presence of *A. actinomycetemcomitans* in plaque samples and the differentiation between genotypes of *A. actinomycetemcomitans* is done with PCR with direct amplification using primers ltx3 and ltx4 (Poulsen et al., 2003). Molecular genetics has made it possible to identify a biodiversity in genomic DNA from different isolates of *A. actinomycetemcomitans*. The bacterium has proven to be genetically heterogeneous, which means that it contains evolutionary lineages that may have different pathogenic potential (Kittichotirat et al., 2011). Comparative genomic analysis with a pan-genome microarray revealed distinctions between genotypes (JP2 and non-JP2 genotypes) of *A. actinomycetemcomitans* (Huang et al., 2013).

**Serotypes of A. actinomycetemcomitans**

Seven serotypes, a to g, have been identified in *A. actinomycetemcomitans* isolates based on surface O-polysaccharides; each serotype represents a distinct clonal lineage (Kaplan et al., 2002, Kilian et al., 2006). It has been proposed that *A. actinomycetemcomitans* can be grouped into three major phylogenetic lineages comprising: (1) serotype b strains, (2) serotype c strains, and (3) serotype a, d, e, f strains (Kaplan et al., 2002). The serotype g is newly
identified and has not yet been assigned to a phylogenetic line (Takada et al., 2010). Most individuals harbour one single serotype that remains stable in the host over time, while some individuals may harbour two or more serotypes of *A. actinomycetemcomitans*. The distribution of *A. actinomycetemcomitans* serotypes appears to vary according to the person’s ethnicity and geographical location (Kim et al., 2009).

Serotype b is usually correlated with aggressive and severe chronic periodontitis in certain populations (Zambon et al., 1983, Gunsolley et al., 1991). A particularly virulent clonal lineage of *A. actinomycetemcomitans* serotype b, termed the JP2 clone, with a characteristic 530 basepair (bp) deletion in the promoter region of the leukotoxin operon, has been identified and almost exclusively isolated from individuals of African descent (Brogan et al., 1994, Haubek et al., 2001, Haubek et al., 2004, Haubek et al., 2007, Haubek et al., 2008). The strains with an intact promoter region are called non-JP2 strains and are referred to as “low leukotoxic” or variable, based on their ability to produce LtxA in vitro (Spitznagel et al., 1991). Studies performed in Morocco reveal that the occurrence of the JP2 genotype is associated with an increased prevalence of AgP (Haubek et al., 2001).

*Acquisition and transmission of A. actinomycetemcomitans*

There is substantial evidence that transmission of *A. actinomycetemcomitans* genotypes can occur vertically between family members (Van Winkelhoff and Boutaga, 2005), and that family members usually share the same strains (Asikainen et al., 1997). Several studies have also provided evidence for an intrafamilial transmission of the JP2 genotype (Haubek et al., 1997, Bueno et al., 1998, Haubek and Westergaard, 2004). The high accumulation of the JP2 genotype in individuals of African origin has indicated a possible host tropism, but could also be the result of the strict vertical transmission pattern of this bacterium (Kilian et al., 2006, Haubek 2010). It has also been shown that horizontal transmission pathways of *A. actinomycetemcomitans* due to specific eating and drinking habits associated with inter-individual salivary contact, could possibly be associated with the presence *A. actinomycetemcomitans* infection and a higher level of AL in studied individuals (Haubek et al., 2005).
Virulence characteristics of *A. actinomycetemcomitans*

Bacterial cell surface proteins are the first to come in contact with the host cells, and these proteins may serve as a means of attachment. *A. actinomycetemcomitans* adhere to and invade a variety of cells. Several adhesins have been identified in strains of *A. actinomycetemcomitans* including both fimbrial and afimbrial proteins. The major fimbrial component is a 6.5-kDa peptide, designated Flp (fimbrial low molecular weight protein). An extracellular amorphous material, which is associated with the bacterium or released into the milieu, is also connected to adhesiveness to *A. actinomycetemcomitans*. Certain proteins (adhesion Aae) that bind to host components have a role in the invasion of *A. actinomycetemcomitans* into epithelial cells. Other proteins (ApiA, ApiBC) are involved in binding to collagen types II, III and V (Fives-Taylor et al., 1999, Fine et al., 2006). Some studies have clearly demonstrated that *A. actinomycetemcomitans* has the ability to infiltrate the oral epithelium (Saglie et al., 1982, Christersson et al., 1987). The invasion process of *A. actinomycetemcomitans* into epithelial cells is a rapid mechanism involving the formation of cell-surface craters. The bacterial cells undergo rapid multiplication followed by transcytosis of bacteria via protrusions, which may extend between neighboring epithelial cells. The protrusions are thought to mediate the cell-to-cell spread of *A. actinomycetemcomitans* (Meyer and Fives-Taylor, 1993, Meyer et al., 1996, Rompikuntal et al., 2012).

A prominent feature of *A. actinomycetemcomitans* is vesicles, which originate from and are continuous with the outer membrane. Vesicles are also released into the external environment in large numbers. These vesicles also exhibit adhesive properties and may function as delivery for transport of toxic compounds from *A. actinomycetemcomitans* (Meyer and Fives-Taylor, 1993, Rompikuntal et al., 2012).

Studies on the characterization of *A. actinomycetemcomitans* have identified several virulence mechanisms such as secreted or cell wall-associated components, which potentially influence a wide variety of cell types. *A. actinomycetemcomitans* LPS acts as an endotoxin and is a potent inducer of cytokine responses in epithelial cells, neutrophils, fibroblasts, and macrophages in periodontal tissues (Fives-Taylor et al., 1999). Biologically active components such as bacteriocins secreted by *A. actinomycetemcomitans*
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may also be considered as virulence factors since they suppress the growth of competing species or different clonal types of the same species (Fine et al., 2006).
Exotoxins of *A. actinomycetemcomitans*

Special attention has been drawn to the unique capacity of *A. actinomycetemcomitans* to express two exotoxins, the leukotoxin (LtxA) and a cytolethal distending toxin (Cdt). The most thoroughly studied effects elicited by the toxins secreted by *A. actinomycetemcomitans* upon host cells are cell death, growth arrest, and induction of inflammatory mediator responses, including bone resorption (Henderson et al., 2003).

*The leukotoxin of A. actinomycetemcomitans*

The LtxA is primate-specific, and this specificity is restricted to humans and Old World primate white blood cells (Tsai et al., 1984, Taichman et al., 1987). The LtxA is a member of the repeats in toxins (RTX) family of toxins, which includes *E. coli* α-hemolysin, *Bordetella pertussis* adenylate cyclase, and *Mannheimia haemolytica* leukotoxin (Linhartová et al., 2010). The various RTX toxins recognized in the different gram-negative bacterial species show similar gene organization and require post-translational modification to become biologically active (Linhartová et al., 2010).

*Organization of the leukotoxin operon*

The *A. actinomycetemcomitans* leukotoxin operon consists of four coding genes designated *ltxC*, *ltxA*, *ltxB* and *ltxD* and an upstream promoter (Kolodrubetz et al., 1989).

![Figure 3](image-url)

**Figure 3.** Schematic illustration of the operon organization of *A. actinomycetemcomitans* leukotoxin. The promoter is located upstream of the *ltxC*.

The first gene, *ltxC*, encodes the acyltransferase, which is responsible for the posttranslational modification of the toxin. *LtxA* is responsible for the toxin structure, while *ltxB* and *ltxD* encode proteins that are required for the secretion of the toxin across the bacterial membrane. LtxA is a large molecule
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consisting of 1055 amino acids with a molecular weight of 116 kDa (Lally et al., 1989). The toxin was first isolated from a highly toxic strain of *A. actinomycetemcomitans* (Tsai et al., 1984). The crystalline structure of the LtxA has not yet been solved.

*Regulation and secretion of the leukotoxin (LtxA)*

The LtxA, like other RTX toxins, appears in both cell-associated and secreted forms (Kachlany, 2010). It has been shown that the LtxA increases under anaerobic conditions of growth, while down regulation is proposed at high levels of oxygen, and nutrients such as fructose may signal cells to produce lower levels of LtxA (Spitznagel et al., 1991). Iron appears to play an important role in the regulation of the LtxA production independently from gene regulation (Balashova et al., 2006, Kachlany, 2010). Most studies on LtxA expression have focused on the two types of leukotoxin promoters, the JP2 genotype and the non-JP2 genotypes. Strains that produce high levels of LtxA are referred to have a JP2 promoter, which is characterized as having a 530 base-pair deletion in the promoter region. It is suggested that the lower leukotoxicity in strains of non-JP2 genotypes of *A. actinomycetemcomitans* compared with JP2 genotype strains, is due to the binding of repressor proteins to sequences that are deleted in JP2 strains (Brogan et al., 1994). The sequence differences in the complete promoter may explain the differences in the LtxA production among non-JP2 strains (Guthmiller et al., 1995, Umeda et al., 2013). Another type of a highly leukotoxic strain was found to have an insertion element (IS1301) integrated upstream from the leukotoxin operon. Disruption or uncoupling of a cis-acting negative regulator has been suggested to be an explanation for the relatively high leukotoxicity found in this particular *A. actinomycetemcomitans* strain with integration of the IS1301 (He et al., 1999).

LtxA occurs in two forms, in a secreted form and in a cell-bound form. In addition to being secreted as a soluble protein, LtxA can also be retained by cells as a cell-associated protein, and LtxA has been localized to the cell surface in the outer membrane fraction (Berthold et al., 1992, Johansson et al., 2003). The cell-associated LtxA may be more important for bacteria that are in the bloodstream or become invasive or ingested, because those stages occur when the bacteria are in direct contact with immune cells. The secreted toxin may be more important in connective tissue and in the interaction with PMNs
and macrophages, where the LtxA would have a major impact on the first line of defense against professional phagocytes. It has also been shown that the high isoelectric point (pI=8.9) of the LtxA is of importance for its interaction with the environment (Linhartová et al., 2010). By increasing the ion strength the electrostatic forces between the LtxA and nucleic acids can be broken, which results in the release of the LtxA from the bacterial surface. A similar effect can be achieved in the presence of serum, indicating that at physiological conditions the LtxA can be secreted from the bacterial cells (Johansson et al., 2003). An antagonistic effect between virulence factors of periodontitis-associated bacteria have been demonstrated in vitro (Johansson et al., 2000b). It is suggested that the LtxA production by A. actinomycetemcomitans may not always play an important pathogenic role in a periodontal lesion with a high occurrence of black-pigmented species (Johansson et al., 2000b).

**Interaction between the leukotoxin and host cells**

The leukotoxin selectively affects human cells of haematopoetic origin. The target cell receptor involved in the leukotoxin-induced cell lysis is the lymphocyte function-associated receptor 1 (LFA-1), which is recognized by a domain of the toxin (Lally et al., 1997).

![Figure 4](image-url) The interaction between *A. actinomycetemcomitans* and defense cells at the site of the periodontal infection causes a cytokine signaling. These molecules function as inflammatory mediators that enable intercellular signaling in the periodontium. Defense cells are attracted from the peripheral circulation through chemotaxis towards a gradient of molecules released from the dental plaque, as well as from activated host cells. (Redrawn from Kachlany 2010. Journal of Dental Research. With permission).
The primary toxic effects of the LtxA consist of its ability to selectively kill human polymorphonuclear leukocytes (PMNs), either by lysis or apoptosis (Baehni et al., 1979, Tsai et al., 1979, Lally et al., 1999). The LtxA causes a disruption of the cell-membrane integrity and induces a release of cell and tissue-destructive enzymes that can further dampen the host resistance (Baehni et al., 1979, Johansson, 2011). A high concentration of the toxin induces cell lysis, whereas a low concentration is reported to induce apoptosis (Korostoff et al., 1998). The interaction between the LtxA and PMNs mediates activation and release of matrix metalloproteinase 8 (MMP-8), (Claesson et al., 2002), and PMNs exposed to LtxA show an extracellular release of proteolytic enzymes from both primary and secondary granules (Johansson et al., 2000a).

Monocytes/macrophages have been found to be very sensitive to cell lysis (Kelk et al., 2003). The toxin causes an inflammatory cell death of these cells, where caspase-1 (i.e. an enzyme that proteolytically cleaves other proteins) is involved in the lytic process, and a rapid release of the cytokine IL-1β (Kelk et al., 2005, Kelk et al., 2008). Human lymphocytes and erythrocytes can also be affected, but at higher concentrations of the LtxA than those which lyse PMNs and monocytes (Mangan et al., 1991, Balashova et al., 2006). The expression of LtxA is also regulated by environmental factors, such as growth conditions and substrates (Spitznagel et al., 1991, Kachlany, 2010, Longo et al., 2013).

Osteoclastogenesis in vitro is suggested to occur via an enhanced expression of osteoclast-stimulating cytokines (TNF-alpha, IL-1β, IL-6, IL-11, IL-17) in the inflamed tissues (Souza and Lerner, 2013). Stimulation of cytokine production can be induced by a variety of bacterial surface components and virulence factors, including secreted outer membrane material related to A. actinomycetemcomitans (Henderson et al., 2003, Kelk et al., 2008).

All these mechanisms of leukotoxicity, and adherence and invasion of the bacterium, cause an imbalance in the host response in many different ways, and might also contribute to help the bacterium to survive and evade the host immune response (Baehni et al., 1979, Tsai et al., 1979, Johansson, 2011, Munksgaard et al., 2012).

**Haemolytic activity of A. actinomycetemcomitans**

Iron is an essential nutrient to most bacteria. Iron is also an important environmental signal, which influences the differential expression of a large
number of genes, some of which code for important bacterial virulence factors including the formation of biofilms. The vast majority of iron is found intracellularly bound to haemin and ferritin compounds, whereas in serum it is bound to transferrin and lactoferrin. These two latter proteins maintain levels of free extracellular iron at an extremely low level, which is far below the level needed for optimal growth of the microorganisms. Consequently, specific systems to acquire iron are developed by bacteria that colonize humans. Bacteria have responded to this nutrient limitation by expressing high-affinity iron-acquisition systems that acquire iron by either interacting directly with host-binding proteins or by obtaining iron from these proteins via the secretion and internalization of chelators known as siderophores (Kachlany, 2010). *Actinomycetemcomitans*, like other members of the *Pasteurellaceae*, does not produce siderophores (small inorganic chelators with a high affinity ferric iron). *Actinomycetemcomitans* is unable to obtain iron from human transferrin or lactoferrin (Rhodes et al., 2007). However, some iron transport function has been described in the context of an uptake system for iron on the cell surface of *Actinomycetemcomitans*. It has also been hypothesized that the LtxA plays a role in iron acquisition, or that iron could play a regulatory role in the production of LtxA (Kachlany, 2010).

Different strains of *Actinomycetemcomitans* with varying expression of LtxA show a specific pattern when cultured on blood agar plates containing fresh horse blood. The haemolytic activity was suggested to be related to a haemolysin of *Actinomycetemcomitans* (Kimizuka et al., 1996), but most likely the haemolysis should be ascribed to the effect of the LtxA (Balashova et al., 2006). The finding that the LtxA could cause red blood cell lysis raised new questions whether this haemolytic capacity represents another potential virulence factor of *Actinomycetemcomitans* (Balashova et al., 2006, Reinholdt et al., 2013).
**Table 1. A.** Virulence mechanisms elicited by the LtxA.

<table>
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<tr>
<th>Exotoxins of <em>A.a</em></th>
<th>Target cells (specificity)</th>
<th>Mode of actions</th>
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<td>Cell lysis</td>
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<td></td>
<td>Activation and release of proteolytic enzymes</td>
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<td>Activation and release of MMPs</td>
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<td></td>
<td>Protection against phagocytic killing</td>
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<td></td>
<td></td>
<td></td>
<td>Tissue degradation</td>
<td>(Brown et al., 2013)</td>
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<tr>
<td><strong>LtxA</strong></td>
<td><em>Macrophages</em></td>
<td>Interactions with the target cell membrane through LFA-1 receptor</td>
<td>Cell lysis</td>
<td>(Tsai et al., 1979)</td>
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<td></td>
<td></td>
<td>ATP release</td>
<td>Apoptosis</td>
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<td></td>
<td></td>
<td>Activation of caspase 1</td>
<td>Immune evasion</td>
<td>(Kelk et al., 2003)</td>
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<td>Activation and secretion of cytokines (IL-1β, IL-18)</td>
<td>Imbalance in the inflammatory host response</td>
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<td>Activation of osteoclast differentiation and bone resorption</td>
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<td><strong>LtxA</strong></td>
<td><em>Lymphocytes</em></td>
<td>Interactions with the target cell membrane through LFA-1 receptor</td>
<td>Cell lysis</td>
<td>(Simpson et al., 1988)</td>
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<td>Apoptosis</td>
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<td>Immune evasion</td>
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<td>Suppression of cell function</td>
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</table>
The cytolethal distending toxin (Cdt) of A. actinomycetemcomitans

Cytolethal distending toxin (Cdt) is a genotoxin produced by *A. actinomycetemcomitans*, and this is the only known oral bacterial species with the capacity to express this toxin. The Cdt has also been identified in *Haemophilus ducreyi, Shigella* species, *C. jejuni* and *E. coli* (Jinadasa et al., 2011). Approximately 85% of the isolates harbour *cdt* genes, but there is a variation in the level of toxin production (Ahmed et al., 2001, Fabris et al., 2002, Yamano et al., 2003, Ando et al., 2010). The Cdt is encoded by three linked genes, namely *cdtA, cdtB* and *cdtC*, organized in a single operon (Sugai et al., 1998). These genes encode the respective subunits of the toxin, namely CdtA, CdtB and CdtC, which combine to form the tripartite toxin complex (Lara-Tejero and Galan, 2001). It is proposed that the CdtA and CdtC facilitate the entry of the active B-unit into the cell. There is a general consensus that all three subunits are required for the intoxication of various cell types.

Inside the target cell the B unit migrates from the cytoplasm to the nucleus, where it acts as a nuclease and/or phosphatase, and causes double strand breaks in the DNA, which leads to a characteristic cell cycle arrest at the G2/M transition phase (Lara-Tejero and Galan, 2001). All Cdt-intoxicated cells become enlarged except the T-cells (Lara-Tejero and Galan, 2001). It is shown that growth-arrested T and B-cells progress to apoptotic cell death (Shenker et al., 2001, Ohara et al., 2004), while dendritic cells may become apoptotic upon Cdt intoxication (Li et al., 2002). The Cdt of *A. actinomycetemcomitans* may also have a cytokine-stimulatory capacity since the Cdt proteins have been shown to induce the synthesis of IL-1β, IL-6, and IL-8 by human peripheral blood mononuclear cells (Akifusa et al., 2001, Belibasakis et al., 2005a). The Cdt has been linked to induction of RANKL expression in human gingival fibroblasts and periodontal ligament cells (Belibasakis et al., 2005b). The interaction of RANKL with RANK on the surface of osteoclast progenitor cells mediates the maturation of bone-resorbing osteoclasts.
Table 1. B. Virulence mechanisms elicited by the Cdt.

<table>
<thead>
<tr>
<th>Exotoxins of A.a</th>
<th>Target cells (specificity)</th>
<th>Mode of actions</th>
<th>Virulence mechanisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdt</td>
<td>Mammalian cells</td>
<td>Exhibit DNase I activity</td>
<td>Cell intoxication</td>
<td>(Sugai et al., 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA damage</td>
<td>Apoptosis</td>
<td>(Lara-Tejero and Galan, 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibit cell proliferation</td>
<td>Induction of humoral immune response</td>
<td>(Akifusa et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell cycle arrest</td>
<td>Inducing the inflammatory response</td>
<td>(Belibasakis et al., 2005a,b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stimulation of cytokines (IL-1β, IL-6 and, IL-8)</td>
<td>Affecting the inflammatory response</td>
<td>(Belibasakis et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induction of RANKL expression</td>
<td>Bone resorption</td>
<td>(Mbwana et al., 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Ando et al., 2010)</td>
</tr>
</tbody>
</table>
AIMS

The overall aim of this thesis was to examine virulence characteristics of JP2 and non-JP2 genotypes of *A. actinomycetemcomitans* with specific focus on the two exotoxins: the leukotoxin (LtxA) and the cytolethal distending toxin (Cdt), in relation to the onset and the progression of attachment loss (AL).

The specific aims were to study:

- the presence of JP2 and non-JP2 genotypes of *A. actinomycetemcomitans* and to assess the prevalence of AL in relation to the carrier status of *A. actinomycetemcomitans* genotypes in a Ghanaian adolescent population (Paper I)

- the association between the presence of the JP2 and non-JP2 genotypes of *A. actinomycetemcomitans* and the initiation and progression of AL during a two-year follow-up period (Paper II)

- the presence of *cdt* genes and Cdt activity in isolates from individuals of the study population. The selected characteristics of *A. actinomycetemcomitans* were analyzed in relation to progression of AL (Paper III)

- the leukotoxic and haemolytic activity in isolates from individuals of the study population. The selected characteristics of *A. actinomycetemcomitans* were analyzed in relation to progression of AL (Paper IV)
METHODS

Study design

This thesis is based on a cross-sectional study (Paper I), a 2-year prospective cohort study (Paper II), and two experimental studies (Papers III, IV) (Table 2).

Table 2. Overview of study designs, research questions and study populations.

<table>
<thead>
<tr>
<th>Study design</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Research question</strong></td>
<td>Cross-sectional study</td>
<td>2-year prospective cohort study</td>
<td>Experimental study</td>
<td>Experimental study</td>
</tr>
<tr>
<td>Are the JP2 and non-JP2 genotypes of A.a prevalent in West African populations? Does an association exist between the presence of genotypes of A.a and AL?</td>
<td>Does the baseline presence of the JP2 and non-JP2 genotypes of A.a influence on the initiation and progression of AL?</td>
<td>Do selected characteristics of A.a such as serotypes and expression of the Cdt influence the progression of AL?</td>
<td>Do selected characteristics of A.a such as leukotoxicity and haemolytic activity influence the progression of AL?</td>
<td></td>
</tr>
<tr>
<td><strong>Data collection</strong></td>
<td>Questionnaire</td>
<td>Questionnaire</td>
<td>Isolates of A.a</td>
<td>Isolates of A.a</td>
</tr>
<tr>
<td></td>
<td>Periodontal examination</td>
<td>Periodontal examination</td>
<td>Clinical data</td>
<td>Clinical data</td>
</tr>
<tr>
<td></td>
<td>Microbiological sampling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Population</strong></td>
<td>JHS level 1 students</td>
<td>JHS level 1 and 3 students</td>
<td>JHS level 1 and 3 students</td>
<td>JHS level 1 and 3 students</td>
</tr>
<tr>
<td><strong>characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of</strong></td>
<td>500 study participants</td>
<td>397 study participants</td>
<td>148/200 (Subgroup)</td>
<td>146/200 (Subgroup)</td>
</tr>
<tr>
<td><strong>individuals</strong></td>
<td></td>
<td></td>
<td>190* (Reference group)</td>
<td>190* (Reference group)</td>
</tr>
<tr>
<td><strong>Gender m/f</strong></td>
<td>237/263</td>
<td>181/166</td>
<td>71/77</td>
<td>71/75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>84/106*</td>
<td>84/106*</td>
</tr>
<tr>
<td><strong>Mean age (yr)</strong></td>
<td>13.2 (10-19)</td>
<td>15.0 (12-20)</td>
<td>15.2 (12-20)</td>
<td>15.2 (12-20)</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td></td>
<td>14.7 (13-19)*</td>
<td>14.7(13-19)*</td>
</tr>
</tbody>
</table>

*The reference group.
Study population

The Ablekuma South Sub-Metro district in Greater Accra, Ghana, is an urban-based district, with a mixture of migrants from all parts of Ghana (Figure 5). This region is one of the most densely populated, and the people living there have a diverse socioeconomic distribution. In the Ablekuma South Sub-Metro, there are 116 public schools, some of which are grouped into clusters sharing one large compound. From a list of all schools in this sub-metro, eight public and three private schools, representing Junior High School level-one adolescents from the whole sub-metro, were chosen with the intention to cover the diversity in the population and their socioeconomic conditions. This thesis focuses on Junior High School level one adolescents around puberty, aged 10-19 years (mean age 13.2, SD ± 1.5) in Accra, Ghana.

Figure 5. The Ablekuma district of the greater Accra, located in the south-central part of the country, consisted of about 10,000 of JHS1 pupils. Out of them the selection was eight public and three private schools. In all 500 pupils were selected.
Characteristics of the study population according to age group studied

Adolescence is defined as the transitional period between puberty and adulthood in human development, and it is characterized by physical, physiological and behavioural changes. The period of adolescence is most closely associated with the teenage years. During adolescence individuals develop health behaviour patterns that might persist into adulthood. This age group studied seemed appropriate to examine for detection of the non-JP2 genotypes and the JP2 genotype at this point in time and to show the association with disease development.

Questionnaire

The participants were interviewed by local dental students using a questionnaire regarding demographic and general health characteristics, social background, possible treatment with antibiotics, earlier dental treatment and cigarette smoking habits. Regarding oral hygiene practices, information was collected about self-reported tooth brushing frequency and about the oral hygiene tools used. According to ethnic and geographic origin, all the studied individuals originated from Ghana or neighboring countries (Burkina Faso, Nigeria).

Clinical measurements

The periodontal examination was performed according to the same procedure at baseline and at follow-up and included the measurement of probing pocket depth (PPD) and the distance from the free gingival margin (GM) to the cemento-enamel junction (CEJ) recorded at the mesiobuccal aspect of the mesial and distal surfaces of all fully erupted permanent teeth (except third molars) by means of a manual periodontal probe (CP-2 Hu-Friedy Mfg. Co. Inc., Chicago, IL, USA) color coded at 2, 4, 6, 8, and 10 mm. The amount of attachment loss (AL) was identified as the difference between the distance from the GM to the CEJ and the recorded PPD measurement, according to the cut-off point for disease used. In case of a gingival recession, the GM-CEJ value was added to the recorded PPD.
**Outcome measures**

The primary outcome variables used in the studies (Papers I and II) was the presence of \( AL \geq 3 \) mm and the progression of \( AL \geq 3 \) mm. The disease status was established at the individual level using the cut-off point of proximal \( AL \geq 3 \) mm in one or more sites in the dentition. Individuals were defined as having progressing disease if they showed one or more proximal sites with a progression of \( AL \geq 3 \) mm based on data collected at the baseline and the follow-up examinations.

**Statistics**

The statistical analyses were performed using a software package of programs (SPSS v. 21.0, Chicago, IL, USA, and Stata v. 8.0).

*Kappa statistics* (Cohen’s kappa) was used to calculate the intra-examiner reliability of clinical recordings (Papers I, II).

*Descriptive statistics* were used for mean, standard deviation, range and frequency distribution. The Chi-square test was used for testing categorical data between groups (Papers I, II). The *Mann-Whitney U* test was used for testing continuous variables (differences between mean ages) between groups (Paper II). Differences between groups according to mean number of teeth with AL were measured with the *Mann-Whitney U* test (Paper III).

*Cumulative frequency distribution* was used to describe the distribution of the study population according to mean AL (Paper I) and mean AL progression (Paper II) in the groups with different carrier status of *A. actinomycetemcomitans*.

*Logistic regression analyses* were performed to estimate the relationship between the dependent variables and one or more independent variables (Papers I, II). Results were interpreted in terms of odds ratios. Odds ratios (OR) were calculated with a 95% confidence interval (95% CI).

When comparing three or more independent samples the *Kruskal-Wallis test* was used (Paper II). To evaluate the significance of changes during the follow-up period in the three exposure groups (JP2, non-JP2 and *A.
*actinomycetemcomitans* negatives) for data not normally distributed the non-parametric *Mann-Whitney U* test was used (Paper II).

Related samples were analyzed with the *Wilcoxon-signed rank test* for repeated measures of the study participants at baseline and at follow-up (Paper II).

**The relative risk (RR)** of developing AL ≥ 3 mm according to the carrier status of the JP2 and non-JP2 genotypes of *A. actinomycetemcomitans* was calculated as the ratio between the proportions of the individuals with AL ≥ 3 mm amongst the exposed individuals (JP2 and the non-JP2 genotype-positive individuals) divided by the proportion of those with AL ≥ 3 mm in the *A. actinomycetemcomitans*-negative group (non-exposed individuals), (Paper II).

In the experimental studies (Papers III and IV) the estimated risk of having a progression of AL ≥ 3 mm in selected subgroups in relation to the reference group (*A. actinomycetemcomitans* negative individuals) was calculated as *odds ratios (OR)* with 95% confidence interval (95% CI).

In all Papers, a *p* value < 0.05 was considered statistically significant.
Microbial detection methods of \textit{A. actinomycetemcomitans}

\textbf{Sampling}

When using the cultivation as detection method, subgingival plaque samples were collected from the distal proximal sites of first molars (pooled sample from four sites) from all 500 adolescents. For the polymerase chain reaction (PCR) method the following sites were sampled: the mesial crevices on each of the four permanent first molars (pooled molar sample) and from the distal crevices on each of the four permanent incisors (pooled incisor sample).

\textbf{Cultivation}

The plaque samples were placed in a viability-preserving microbiostatic, anaerobic transport medium (VMGAIII) supplemented with nystatin (2 mg/l). From the samples collected in VMGAIII tubes, aliquots (100 µL) were spread on agar plates containing a specific media for \textit{A. actinomycetemcomitans} (Slots, 1982, Holm et al., 1987). These media were slightly modified (2 mg/l nystatin and 5 mg/l neomycin were added) to inhibit growth of contaminating bacteria (mainly enterobacteria) and yeast. The plates were incubated for three to five days at 37°C in an aerobic atmosphere containing 5% CO$_2$. The detection level for cultivation was estimated to be 100 bacterial cells/ml of sample. Isolates of \textit{A. actinomycetemcomitans} were collected from these plates (one to seven per individual, for a total of 792 isolates) and stored in a freezer for later genetic characterizations (Åberg et al., 2012).

\textbf{PCR}

The samples collected in saline were centrifuged at 20,000 x g for 20 minutes at 4°C. The supernatant was removed, and a Tris buffer (10 mM [pH 8.0]) (100µl) was added. The detection level for the PCR method used corresponds to 1000 bacterial cells/ml (corresponding to 25 bacterial cells/ PCR reaction). For PCR in a volume of 25µl, containing 23µl of sample, 10 pmol of each of the two primers was added to the test tube. Both the non-JP2 and the JP2 genotypes of \textit{A. actinomycetemcomitans} could be simultaneously detected by the method used (Poulsen et al., 2003).
Figure 6. Agarose gel showing DNA fragments of the \( ltx \) region amplified in PCR with the \( ltx3 \) and \( ltx4 \) primers (Poulsen et al., 2003) used. The estimated sizes of the fragments are shown on the right side of the gel (6). As controls (1, 2) isolated DNA from HK1519 (JP2) and D7s (non-JP2) were used. Clinical isolates (3, 4, and 5) were examined by DNA preparations from three different colonies from each individual.

The **definition** of an individual according to the carrier status of *A. actinomycetemcomitans* was as follows:

Since both the cultivation and the PCR methods rendered results regarding detection of *A. actinomycetemcomitans*, the overall carrier status of *A. actinomycetemcomitans* at the individual level was based on the results from the two methods. An individual where *A. actinomycetemcomitans* could be detected irrespectively of detection method was counted as positive for *A. actinomycetemcomitans*. Furthermore, an individual who was positive for the JP2 genotype alone or positive for both the non-JP2 and the JP2 genotype, was considered as a JP2 genotype-positive individual.
Experimental studies (Papers III and IV)

**Figure 7.** Flowchart showing the experimental design of the genetic characterization of *A. actinomycetemcomitans* isolates and of the cytotoxic and biological interactions of *A. actinomycetemcomitans* isolates with target cells.
Genetic characterization of *A. actinomycetemcomitans* isolates

**PCR-based characterization**

Before the PCR-based analyses were performed, DNA was purified by the usage of the GenElute Bacterial DNA kit (Sigma-Aldrich, St. Louise, MO, USA). For preparation of the PCR mixtures, PureTaq Ready-To-Go PCR (GE Healthcare; Buckinghamshire, UK) was used. For the sequencing procedure, DNA of amplified genes was sent to Eurofins MWG Operon (Ebersberg, Germany).

**The leukotoxin promoter types of the JP2 genotype and non-JP2 genotypes of A. actinomycetemcomitans**

A PCR method was used (Poulsen et al., 2003) where the JP2 and the non-JP2 genotypes of *A. actinomycetemcomitans* could be simultaneously detected in DNA templates (Figure 6) from the 792 isolates of *A. actinomycetemcomitans* (Paper I).

**Serotyping**

The 792 isolates were serotyped by PCR (Suzuki et al., 2001, Kaplan et al., 2002) (Paper III, Figure 4).

**Cdt genotyping**

From the 792 serotyped and leukotoxin promoter-typed isolates, 249 isolates were selected for *cdt* genotyping by PCR. One isolate from each of the 200 subjects was used, but when more than one serotype and/or leukotoxin promoter type (JP2 or non-JP2) were identified in the same individual, these additional isolates were included. For the detection of the three *cdt* genes (*cdtABC*), two PCR-based methods were used (Ahmed et al., 2001, Fabris et al., 2002). One of the methods was designed for detection of all three genes (*cdtABC*) and revealed a 2105-base pair (bp) product. When discrepancy between detection of *cdtABC* and Cdt activity occurred, the individual *cdt* genes (*A, B and C*, respectively) were screened for by the second method.
(Ahmed et al., 2001). The PCR products were documented as described for serotyping (Paper III, Figure 2).

**AP PCR**

The arbitrarily-primed (AP PCR) analysis was used for the characterization of the serotype b isolates. For amplification, the random sequence oligonucleotide OPB-3 (AGTCAGCCAC) (Invitrogen, Carlsbad, CA, USA) (0.4 μM) was used. The PCR mixture also contained MgCl₂ (2.5 μM). The amplification was carried out as described by Dogan and co-workers (Dogan et al., 1999) (Paper IV).

**Sequencing of the house keeping gene hbpA-2**

By sequencing of the *hbpA-2* gene, the origin of the JP2 genotype isolates could be determined. For the amplification, the primers 5′-TATTTTGACGCAATTGCTGTTC and 5′-TAGGGCACTTATCATTTCCATC (0.4 μM each) were used (Paper IV).

**Sequencing of the leukotoxin promoter region**

By sequencing the promoter region of the leukotoxin operon, sequence differences among the serotype b isolates could be studied. For amplification, primers *ltx3* (5′-GCGGACACAACCTTTAAGTCT-3’) and primer *ltx4* (5′-GCCGATAACCAAGGCCACATAC-3’) (0.4 μM each) were used. The amplification was carried out as described in Poulsen and co-workers (Poulsen et. al 2003) (Paper IV).
Detection of biological and cytotoxic effects

Cdt activity

The 249 selected isolates were analyzed for Cdt activity in a cell culture assay (Belibasakis et al., 2004).

A suspension of OD$_{600}$ nm 2.0 was centrifuged (10,000 x g, 10 min), and the supernatant was added to a cell culture assay with HL-60 cells (human acute monocytic leukemia cell line, ATCC 16). The HL-60 cells (1 ml 5x10$^5$ cells/ml) were cultured in RPMI 1640 with 10% fetal bovine serum (Sigma-Aldrich) and transferred to each well in a 24-well cell culture plate (Nunc), and mixed with 1 µl of each of the bacterial supernatants. After 24 h of incubation the cells were transferred to a 2-ml Eppendorf tube and washed with PBS by centrifugation (500 x g, 5 min). The cell pellet was resuspended in 300 µl PBS and 900 µl ice-cold 99% ethanol and fixed for 1 h at 4°C. The cells were washed with PBS by centrifugation and treated with RNase (100 µl, 100 µg/ ml, Sigma-Aldrich) for 15 min at 37°C. After the incubation, 400 µl of propidium iodide (Molecular Probes, Eugene, OR, USA) in 3.8 mM sodium citrate in PBS was added and further incubated in darkness for 1–3 h at 4°C. Cdt activity was determined by the ability of the bacterial supernatants to inhibit proliferation and by causing the typical accumulation of the target cells in the G2/M-phase. This was examined by the increase of cells in the G2/M-phase as measured by cell cycle analysis using flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, US). Bacterial isolates that resulted in ≥ 50% of the target cell population accumulating in the G2/M-phase after 24 h incubation were classified as positive for Cdt activity (Paper III, Figure 3).

The relationship between AL progression and *A. actinomycetemcomitans* Cdt activity was calculated using an odds ratio (OR). The definition of an individual’s Cdt status as “positive” or “negative” was defined as follows:

The *A. actinomycetemcomitans* isolate of an individual characterized with the presence of Cdt activity was determined to be representative for the individual studied, looked upon as a statistical unit. If more than one serotype of *A. actinomycetemcomitans* was detected in an individual, the isolate/serotype that was Cdt activity-positive was chosen to be representative for the individual.
An "A. actinomycetemcomitans" Cdt activity-negative individual was an individual where Cdt activity was not detected in any of the examined isolates.

**Leukotoxic activity**

One or more isolates of *A. actinomycetemcomitans* from each of the 199 individuals were subcultured on blood agar plates in successive rounds to get a smooth and nonadherent phenotype (Paper IV). This procedure makes it possible to cultivate isolates of *A. actinomycetemcomitans* in broth.

THP-1 cells were cultivated in RPMI 1640 (Sigma-Aldrich, St Louis, MI, USA) with 10% fetal bovine serum (FBS) (Sigma-Aldrich) for logarithmic growth. The cell concentration was assessed in a Burker chamber, and the cells are centrifuged at 500 x g for 5 minutes. The isolated macrophages were suspended in RPMI 1640 with 10% FBS at a density of $5 \times 10^6$ cells/ml. To differentiate THP-1 cells into leukotoxin-sensitive adherent macrophages 50 nM PMA (phorbol 12- myristate 13-acetate) (from a 200 μM stock solution ([2.5 µl to 10 ml cell suspension]) was added. To each well in the 96-well culture plate 100 µl was distributed and then incubated at 37°C for 24 hours.

**Cell lysis (LDH release)**

LDH catalyzes the oxidation of NADH to NAD and causes a decrease in the absorbance at 340 nm (NADH has high absorbance while NAD has low).

\[
\text{NADH + pyruvate} \Leftrightarrow \text{NAD + lactate}
\]

Lactate dehydrogenase (LDH) is an intracellular enzyme that catalyzes the conversion of lactate to pyruvate. Cell injury is determined from the activity of lactate dehydrogenase (LDH) that is released extracellularly in the reaction mixtures. LDH is a large cytosolic molecule (140 kDa) and a marker for cytopathogenic membrane leakage. The mixtures were centrifuged and the supernatants were analyzed for LDH activity. Cells incubated in the absence of bacteria served as the negative control. The release of LDH was expressed as % of the maximal activity of the Triton-lysed control (Paper IV).
**Cell death (NRU)**

This method is based on the ability of viable cells to incorporate and bind the supra vital dye neutral red. This weakly cationic dye penetrates cell membranes by nonionic passive diffusion and concentrates in the lysosomes, where it binds by electrostatic hydrophobic bonds to anionic and/or phosphate groups of the lysosomal matrix. The dye is then extracted from the viable cells using an acidified ethanol solution, and the absorbance of the solubilized dye is quantified using a spectrophotometer.

THP-1 cells, used as target cells, were exposed to supernatants of purified LtxA from isolates of *A. actinomycetemcomitans*.

Inside the lysosomes, there is a proton gradient to maintain a pH lower than that of the cytoplasm. Thus, the dye becomes charged and is retained inside the lysosomes. When the cell dies or the pH gradient is reduced, the dye cannot be retained. Consequently, the amount of retained dye is proportional to the number of viable cells. Lysosomai integrity, with the concomitant binding of the neutral red dye, is a highly sensitive indicator of cell viability (Repetto et al., 2008). After a 2 h staining period at 37°C in 5% CO₂ with medium containing neutral red, the medium was removed and the cells were rinsed with 150 μl PBS and then dissolved in 150 μl lysis solution (1% acetic acid in 50% ethanol). The staining intensity of each well was quantified spectrophotometrically at 550 nm. Cell viability (%) was calculated in relation to the absorbance of a sample incubated without leukotoxic extract (Repetto et al., 2008). The leukotoxicity of the isolates was classified in relation to their capacity to induce cell death: low leukotoxic ≤ 30%, average leukotoxic ≥ 31-60% or highly leukotoxic ≥ 61% reduction of viable cells.

**The definition** of an isolate determined as exhibiting “high, average or low leukotoxicity” according to the two methods is as follows:

**For Cell lysis (LDH):**

PMA-differentiated THP-1 cells (0.5 x 10⁶) were exposed to LtxA extracted from 2 x 10⁹ smooth bacteria for 1 h at 37°C. Cell lysis was examined by quantification of the activity of LDH released from the damaged cells. A bacterial isolate that causes release of ≤ 30% of the total amount of the cytosolic LDH released by Triton-lysis, was classified as “low leukotoxic”.
When 31-60% of the total amount of the cytosolic LDH was released, the isolate was classified as an “average leukotoxic” isolate, and ≥ 61% indicated a”highly leukotoxic” isolate.

**For Cell death (NRU):**

PMA-differentiated THP-1 cells (0.5 x 10⁶) were exposed to LtxA extracted from 2 x 10⁹ smooth bacteria for 1 h at 37°C. The exposed cells were stained for 2 h with neutral red and the accumulation of the vital dye in the cell monolayer was quantified spectrophotometrically. A bacterial isolate that causes a reduction of viable cells by ≤ 30% was classified as a “low leukotoxic” isolate, when 31-60% reduction or ≥ 61% reduction of viable cells was seen, these isolates were classified as “average leukotoxic” and “highly leukotoxic” isolates, respectively.

To assess the risk (OR) of **progression of AL** for an individual, who harboured strains from the three LtxA categories (low, average or high), **the definition** was as follows:

The isolate of *A. actinomycetemcomitans* characterized with the highest score of leukotoxicity was determined to be representative for the individual studied, looked upon as a statistical unit. If more than one serotype of *A. actinomycetemcomitans* was detected in an individual, the isolate/serotype with the highest score of leukotoxicity was chosen to be representative for that individual.

**Haemolytic activity**

The leukotoxicity of isolates was evaluated by examination of their capacity to induce β-haemolysis on blood agar plates containing deferoxamine (Balashova et al., 2006). The bacterial isolates were cultivated on blood agar plates for 3 - 5 days. Subsequently, 5 μl OD 2 suspensions in 0.9% NaCl (approximately 4 x 10⁹ cells/ml) of the isolates were added to blood agar plates (Columbia blood agar base, Acumedia, Neogen, Lansing, Michigan, USA) containing 5% horse blood and 10 μM deferoxamine (Sigma Aldrich; St. Louise, MO, USA.). Each plate contained bacterial isolates and suspensions of a low-, an average- and a highly leukotoxic reference strain producing none or small zones (1), average-size zones (2) and large-size zones (3), respectively. After incubation of the plates for 5-7 days at 37°C in a CO₂-containing (5%) environment, the
size of the haemolytic zones for each isolate was categorized into one of the three leukotoxin groups. The haemolytic activity was evaluated based on the distribution of isolates, which were visually scored according to the size of the haemolysis zones. This screening method is considered to be a semi-quantitative method according to the haemolytic activity of the leukotoxin.

![Image of haemolytic zones](image)

**Figure 8.** Reference strains according to **haemolytic activity**: 3=high haemolytic activity (large size zone), 2=average (average size zone), and 1=low (none-small size zone), respectively. Reference strains “high” (HK1519), “average” (D7s) and “low” (ΔltxAD7s).

To assess the risk (OR) of **progression of AL** for an individual according to harbouring strains with a low, average or high haemolytic activity, the **definition** was as follows;

The isolate of *A. actinomycetemcomitans* characterized with the highest score of haemolytic activity was determined to be representative for the individual studied, looked upon as a statistical unit. If more than one serotype of *A. actinomycetemcomitans* was detected in an individual, the isolate/serotype with the highest score of haemolytic activity was chosen to be representative for the individual.
RESULTS AND DISCUSSION

Main findings

The presence of *A. actinomycetemcomitans* was significantly associated with an enhanced risk for progression of AL, which was identified after the two-year follow-up period. Individuals that harboured *A. actinomycetemcomitans* with a high leukotoxic activity showed the most pronounced risk for the progression of AL. All isolates of the JP2 genotype of *A. actinomycetemcomitans* were highly leukotoxic, while a substantial diversity was detected in the isolates of the non-JP2 genotypes of *A. actinomycetemcomitans*. The highly leukotoxic isolates of the non-JP2 genotypes of serotype b, exhibited a similar leukotoxicity to that of the JP2 genotype isolates. The role of the Cdt expression in relation to disease progression was not obvious in the studied population.

Methodological considerations

Study designs

This thesis is based upon a cross-sectional study where a screening examination for detection of AL and collection of microbiological samples was performed in a Ghanaian adolescent population (Paper I). A follow-up examination of the same cohort of adolescents was done after two years with identical procedures for the measurement of AL. Population-based cross-sectional studies often involve large populations, making it possible to collect data on a multitude of variables for assessments of potential risk factors and to include analysis of possible confounders. Performing a population-based study in Ghana, or in African countries elsewhere, may involve a weakness in the sample selection, due to the lack of reliable population demographics. A weakness in longitudinal studies might also be that the study participants leave school and are thereby lost to follow-up evaluations.
The prospective cohort study design used in the follow-up (Paper II) provides a robust form of evidence with its exposure-based study design according to the research questions raised. The rate of disease progression under investigation was studied as a function of exposure. The carrier status of *A. actinomycetemcomitans* at the individual level (*A. actinomycetemcomitans*-negative, positive for the non-JP2 genotypes or positive for the JP2 genotype of *A. actinomycetemcomitans*), was assessed at baseline, and this parameter was used as the variable of exposure, which was evaluated prospectively.

To minimize the risk of examiner bias, a blinded procedure would have been preferable. This was not possible, but at the follow-up examination the examiner was unaware of the baseline periodontal status of the actual individual to be examined.

**Generalizability**

Generalizability of results is mainly determined by characteristics of the study population. The external validity of observational studies (i.e. cohort studies) is usually not considered as robust as in randomized clinical trials. Several confounders, some of which are difficult to anticipate, may influence the interpretations of the results. The main strength of this thesis is that the different study designs used, dealing with the same study population, all indicate a clear association with the presence of *A. actinomycetemcomitans* and a progression of AL. Furthermore, the results are in line with previous studies within this research field.

**Data collection of AL measurements**

The data collection was based on clinical recordings by the measurements of AL.

**Attachment loss (AL)** that is evaluated by periodontal probing is the most sensitive screening approach currently available. We had no access to facilities to do comprehensive periodontal examinations with radiographs to establish a proper periodontal diagnosis. Examinations with radiographs to assess the marginal bone level, as a complement to the clinical registration, would have been preferable as an additional screening tool in those individuals who were in the developmental stage of having mixed dentitions. Therefore, AL is
referred to as the “biological endpoint” of loss of periodontal support in all studies included in the thesis.

In the periodontal literature (Lindhe et al., 2008), the distance of at least 2 mm between the cemento-enamel junction and the alveolar crest argues for a suspected diagnosis of periodontitis. Under healthy conditions the normal distance from the CEJ to the attachment fibers on the alveolar crest in the permanent dentition, determined histologically, has an average distance (which is age-dependent) of 1.08 mm, with a range of 0.04 to 3.36 mm (Lindhe et al., 2008). In erupting permanent teeth, the alveolar bone crest is normally slightly apical to the CEJ.

An indirect AL measurement method was chosen (Papers I and II). From a measurement error point of view it is suggested (Corraini et al., 2013) that the direct AL measurement recording method in general is preferable. However, one single examiner (CHÅ) performed all measurements of the periodontal parameters. It is a universal finding that the inter-examiner reliability of recordings is worse than the intra-examiner reliability (Corraini et al., 2013). Probing measurement errors are considered to be randomly distributed in the interval $-1$ mm to $+1$ mm, and the standard deviation of intra-examiner replicate measurements of AL are normally less than 1 mm. The cut-off point chosen for disease was the presence of one or more proximal sites with AL $\geq 3$ mm or one or more sites with progression of AL $\geq 3$ mm (López and Baelum 2003). This means that changes of AL were probably real and unlikely to be due to measurement error.

**Microbiological sampling**

Incisor and molar sites were chosen for plaque sampling since AL in most adolescents initially manifests at these teeth (Hormand and Frandsen, 1979). It has been reported that *A. actinomycetemcomitans* attach to the superficial layers of the tooth-attached biofilm. The paper point sampling method has been shown to be accurate in receiving representative samples of the loosely bound, unattached subgingival biofilm within the periodontal crevices for the detection of *A. actinomycetemcomitans* (Noiri et al., 2001, Socransky and Haffajee, 2005). To assess the overall carrier status of *A. actinomycetemcomitans*, multiple plaque samples also seem to be needed to minimize false-negative results (Haffajee and Socransky, 1992).
Results and Discussion

Papers I and II

Detection of *A. actinomycetemcomitans* and association with attachment loss (AL)

The overall detection frequency of *A. actinomycetemcomitans* (54.4%) was high in this Ghanaian population studied. The JP2 genotype of *A. actinomycetemcomitans* was prevalent (8.8%) in these adolescents (Paper I). The carrier rate of *A. actinomycetemcomitans* is considerably high, considering also the fact that we sampled both healthy and diseased sites, according to the sampling technique with pooled plaque samples. Nearly half (196/393) of the periodontally healthy adolescents (AL < 3 mm) at baseline were carriers of *A. actinomycetemcomitans* (Paper I). The relatively high prevalence of *A. actinomycetemcomitans* in the periodontally healthy individuals might eventually suggest that there is a variation in the virulence properties of *A. actinomycetemcomitans* and that all individuals are not equally susceptible to develop AL. One study in European adolescent populations (Asikainen et al., 1986) has shown a prevalence of *A. actinomycetemcomitans* in 4 out of 100 (4%) periodontally healthy adolescents. Another study (Slots et al., 1980), in which the ethnicity of the study participants was not told, showed a slightly higher prevalence of *A. actinomycetemcomitans* in healthy sites. In this study population (Slots et al., 1980), the presence of *A. actinomycetemcomitans* in subgingival plaque was generally related to the presence of gingival inflammation, also in *A. actinomycetemcomitans* carriers without yet diagnosed periodontal breakdown. This may indicate the increased risk of future periodontal breakdown due to the microbial colonization. Gingival inflammation (diagnosed as bleeding on probing [BoP]) was a common finding also in these Ghanaian adolescents, most of them having no access to dental treatment or access to proper dental hygiene tools. Future studies of the data available from this Ghanaian cohort are planned to deal with collected data as BoP and recordings of probing pocket depth (PPD) in relation to disease progression.

Qualitative analysis was only performed to show the presence of *A. actinomycetemcomitans* in subgingival plaque. We evaluated longitudinally the impact of the bacterial challenge (positive for the non-JP2 genotypes or the JP2-genotype of *A. actinomycetemcomitans* versus negative for *A. actinomycetemcomitans*) assessed at the individual level at baseline (Paper I),
in relation to the development of AL ≥ 3 mm and in relation to the progression of AL ≥ 3 mm (Paper II).

**Distribution of attachment loss (AL) in the dentition**

In total, 156 individuals in this study population were identified at the follow-up as having one or more proximal sites with AL ≥ 3 mm according to the cut-off point for disease used. At the tooth level, 46 out of 156 (29.5%) individuals exhibited teeth with localized AL with at least one molar and one incisor affected. A more generalized distribution of AL at the tooth level was seen in 24 (15.3%) of the individuals with molars, incisors, and/or premolars and canines affected. Of the remaining 86 individuals, 70 (44.9%) exhibited AL localized to single molars, 5 individuals (3.2%) to incisors only, while 11 individuals (7.1%) presented with AL localized to single molars and premolars.

The upper first molars (mean AL range 1.91-1.76 mm) had the highest mean AL, followed by the first lower molars (mean AL range 1.28-1.36), and incisors (mean AL range 0.50-0.89 mm). These teeth were the most often affected when recorded by tooth type. In some slightly older cases, also permanent teeth such as second molars were affected by proximal AL (mean AL 1.13-1.60 mm). In general, premolars and canine teeth were over all much less frequently affected. Among these 156 individuals, 31 JP2 genotype-positive individuals out of 38 (81.6%) had AL ≥ 3 mm; in the non-JP2 positive group, 88 out of 169 (52.1%) had AL ≥ 3 mm, and in the *A. actinomycetemcomitans* negative group, 37 out of 190 (19.5%) had AL ≥ 3 mm.

Adolescents with signs of AL at baseline (Paper I) acquired new lesions and existing lesions tended to progress during the two-year follow-up period, making the disease more generalized and severe (Paper II, Table 2,3, Fig. 2A). This is accordance with other observational studies dealing with teenage populations with different ethnic origins and socioeconomic distributions (Albandar et al., 1991, Clerehugh et al., 1995, Brown et al., 1996, Lopez et al., 2006).

The mean AL increased for all the three exposure groups between the baseline and the follow-up examination (Figure 9 A, B). Among the JP2 genotype-positive individuals (n= 38), the baseline mean AL was 0.34 mm (0.00 – 1.76,
SD ± 0.33), but increased the most to the mean AL 1.01 mm at the follow-up (range 0.38 – 3.14, SD ± 0.60) compared with the two other groups (Paper II, Table 3). During pairwise testing of the \textit{A. actinomycetemcomitans}-negative individuals, the individuals positive for the non-JP2 genotypes and the JP2 genotype revealed significant differences both at baseline and at the follow-up ($p < 0.05$), except between the groups of individuals positive for the non-JP2 genotypes (n=169) and those positive for the JP2 genotype (n=38) at baseline ($p = 0.67$) (Figures 9A, B). Considering the ratio of those individuals in the follow-up population (n=397) that remained periodontally healthy, the figures are 7 individuals out 38 (18.4%) in the JP2 group, 81 individuals out 169 (47.9%) in the non-JP2 group, and 153 out of 190 (80.5%) in the \textit{A. actinomycetemcomitans}-negative group. Thus, a total of 241 individuals remained healthy (AL < 3 mm) during the whole study period. These data are comparable with findings in adolescents in other population-based studies (Brown et al., 1996, Timmerman et al., 2000, Van der Velden et al., 2006), but the disease progression seems to be more pronounced in the present West African population, also considered in the light of the relatively short follow-up period.
Figure 9. The cumulative frequency distribution of the 397 participants according to mean AL at baseline (2009) (A) and at follow-up (2011) (B). Results are given according to the presence of the JP2 and non-JP2 genotypes of *A. actinomycetemcomitans* and the *A. actinomycetemcomitans*-negative individuals assessed at baseline. *Aa*; *A. actinomycetemcomitans*, AL; attachment loss.
**Prediction of attachment loss (AL) progression**

A total of 87 individuals in the study population that were periodontally healthy at baseline had developed AL ≥ 3 mm at the follow-up. The presence at baseline of the JP2 genotype substantially increased the risk of developing AL over the next two years (RR=7.3) (Table 3). The risk was enhanced, though less pronounced, in individuals positive for the non-JP2-genotypes of *A. actinomycetemcomitans* (RR=3.6) (Table 3). The lower RR for the JP2 genotype-positive individuals reported in this Ghanaian population compared with studies performed in Morocco (Haubek et al., 2008), was due to the fact that there were more diseased individuals (proximal AL ≥ 3 mm) in the *A. actinomycetemcomitans*-negative group (the reference) in the Ghanaian population studied (Paper II).

**Table 3. Relative risks (RR) for attachment loss (AL ≥ 3 mm) according to the baseline carrier status of JP2 and non-JP2 genotypes of *A. actinomycetemcomitans* (*A.a*) in individuals (n=315).**

<table>
<thead>
<tr>
<th></th>
<th>AL ≥ 3 mm</th>
<th>AL &lt; 3 mm</th>
<th>Crude RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP2</td>
<td>23</td>
<td>7</td>
<td>7.3</td>
<td>(4.5 to 11.9)</td>
</tr>
<tr>
<td>Non-JP2</td>
<td>47</td>
<td>76</td>
<td>3.6</td>
<td>(2.2 to 6.0)</td>
</tr>
<tr>
<td><em>A.a</em>-neg</td>
<td>17</td>
<td>145</td>
<td>1.0</td>
<td>Reference</td>
</tr>
</tbody>
</table>

At baseline the participants of the study were too young to make firm conclusions on the specific association between the JP2 genotype and the presence of AL (Paper I). However, in this West African population studied, our results at the follow-up (Paper II) confirmed the formerly demonstrated strong association between the presence of the JP2 genotype of *A. actinomycetemcomitans* and initiation and progression of AL in adolescents (Haubek, 2010). The presence of the non-JP2 genotypes of *A. actinomycetemcomitans* was also associated with an increased risk for the progression of AL ≥ 3 mm (Paper II). The presence of the non-JP2 genotypes as a risk factor for the onset and progression of periodontal disease is also found in other populations of young individuals where the JP2 genotype is
absent (Timmerman et al., 2000, Van der Velden et al., 2006, Fine et al., 2007, Elamin et al., 2010).

The role of *A. actinomycetemcomitans* and the association with AL in young children has been shown in a population-based study of 3896 7-9-year-old children living in Sweden (Sjödin et al., 1995). It was found that 32 children (0.8%) exhibited radiographic periodontal bone loss at ≥ 2 proximal surfaces of their deciduous teeth. In fourteen of the twenty-six (53.8%) cases, who were available for further examination, detectable levels of *A. actinomycetemcomitans* were found in samples obtained from the deepest periodontal sites at baseline. Thirteen (93.0%) of these 14 individuals were periodontally and microbiologically re-examined after 16 years (Åberg et al., 2009). Signs of localized AL were now found in three out of the 13 (23%) examined individuals and *A. actinomycetemcomitans* was recovered from six (46%) of the individuals. All these individuals had received regular dental care during the follow-up period. This indicates the role of *A. actinomycetemcomitans* in the initial phases of disease development, where all the 7-9-year-old children with AL were infected with *A. actinomycetemcomitans*. Although a diverse microflora of bacterial species was detected at the follow-up (Åberg et al., 2009), nearly half of the individuals were still colonized with *A. actinomycetemcomitans*. Not all “prepubertal periodontitis” cases develop into LAgP cases with time, as shown in this Swedish cohort, but the co-existence of the presence of *A. actinomycetemcomitans* within both these disease entities may indicate a future potential risk of periodontal disease.

**Paper III**

**Serotypes of*** A. actinomycetemcomitans**

In this Ghanaian cohort all the examined isolates were serotypable, and more than one serotype was detected in samples from about 20% of the isolates. The serotype c was most frequently detected (42% of isolates) followed by serotype a (24% of isolates) and serotype b (19% of isolates). This serotype distribution follows the pattern reported from studies of two Asian populations (Mombelli et al., 1999, Bandhaya et al., 2012) and from populations in the United States (Chen et al., 2010) and Brazil (Cortelli et al., 2012). A similar occurrence of serotypes a, b and c has been reported in studies performed in
Europe (Asikainen et al., 1995) and from a German study including both immigrants and natives (Jentsch et al., 2012).

There is convincing evidence that the occurrence of particular serotypes of \textit{A. actinomycetemcomitans} is related to geography and/or ethnic group (Kim et al., 2009). The serotype distributions of \textit{A. actinomycetemcomitans} have also been found to be related to disease status in both oral and non-oral infections (Zambon et al., 1983, Paju et al., 2000) or may be related to other members of the microbial complexes in subgingival plaque (Socransky et al., 1998, Yoshida et al., 2003).

\textit{Serotypes of A. actinomycetemcomitans and association with progression of attachment loss (AL)}

The risk of having a progression of $\text{AL} \geq 3$ mm for an individual according to harbouring the serotype a, b or c showed a significant association with disease progression for all the serotypes examined (Paper III, Table 3). However, the highest OR for disease progression was associated with the b serotype, even after exclusion of the individuals infected with the JP2 genotype. In analysis of the risk estimates of progression of AL, the individuals were excluded who harboured more than one serotype of \textit{A. actinomycetemcomitans}. Among those, who were infected with several serotypes, the serotype b was detected in 17 (38\%) of the individuals.

\textit{Presence of cdt genes and Cdt activity}

From the 792 serotyped and leukotoxin promoter-typed isolates, 249 isolates were selected for \textit{cdt}-genotyping by PCR. One isolate from each of the 200 subjects was used, but when more than one serotype and/or leukotoxin promoter type (JP2 genotype or non-JP2 genotypes) were identified in the same subject, these additional isolates were included and summed up to 249. The presence of \textit{cdt} genes and Cdt activity was detected in nearly 80\% of the isolates. All serotype b, d and f isolates were positive for \textit{cdt}-encoding genes and Cdt activity, while a total of 35 (33.7\%) of the serotype c isolates were negative for the presence of \textit{cdt} genes (Paper III). Studies using clinical isolates from various populations have shown an analogous prevalence of the \textit{cdt} genes (Ahmed et al., 2001, Fabris et al., 2002, Yamano et al., 2003, Kawamoto et al., 2009).
Presence of Cdt activity and association with progression of attachment loss (AL)

The association of the Cdt with pathogenesis of periodontitis is not fully understood, but it is shown that patients with GAgP may exhibit serum antibody responses to the Cdt (Ando et al., 2010). Furthermore, quantitative differences in Cdt production were observed in strains from a cohort of individuals (patients with chronic and aggressive periodontitis, and periodontally healthy individuals), which could possibly reflect a virulence potential of this bacterial toxin (Fabris et al., 2002, Jinadasa et al., 2011). The progression of periodontal disease in relation to Cdt activity has not earlier been studied in longitudinal population-based cohorts of adolescents. We showed that individuals colonized with Cdt-positive or Cdt-negative A. actinomyceseetemcomitans were significantly more often found amongst the individuals who showed a progression of AL ≥ 3 mm, in relation to the progression seen in the A. actinomyceseetemcomitans-negative individuals (the reference group). However, the progression of AL was concluded to be mainly associated with the overall presence of A. actinomyceseetemcomitans. Our data did not provide evidence for the presence of Cdt activity as a specific virulence factor associated with enhanced risk of periodontal disease progression (Paper III). Most studies aiming to show an association between the Cdt and periodontitis have analyzed selected patient cohorts with periodontitis (Tan et al., 2002, Leung et al., 2005, Jentsch et al., 2012). There are also variations in methodology for assessing the prevalence of cdt genes and Cdt activity in these studies, which possibly might affect the detection frequency of cdt genes, Cdt activity and the association with periodontal disease.

To further evaluate the impact of the Cdt as a key virulence factor of A. actinomyceseetemcomitans, a rat model is reported on of A. actinomyceseetemcomitans-induced periodontitis (Schreiner et al., 2013). This study indicated that the Cdt could possibly be involved to some extent in mechanisms of bone loss in vivo, but this could also be due to immunoregulatory effects due to the presence of A. actinomyceseetemcomitans infection. However, it seemed in this study that A. actinomyceseetemcomitans leukotoxin exerts a greater effect than the Cdt on bone loss. The effect of
CdtB-activity was also tested in vitro on rat lymphocytes (CD4+ T cells) using *A. actinomycetemcomitans* leukotoxin as a negative control. The ability of the CdtB to inhibit or induce proliferation of rat lymphocytes appears to depend on the dose and duration of the toxin under culture conditions. Early inhibition of the proliferation of CD4+ T cells was seen and was suggested to be a result of cell cycle arrest or apoptosis induction. However, it could not be excluded in this in vitro test that the effect of the Cdt was related to an overall immune response to the toxins present in the assay examined (Schreiner et al., 2013).

Cdt is produced by gram-negative pathogenic bacteria from the phylum **Proteobacteria**. Many of these bacteria infect humans and cause persistent infections, e.g. *Shigella dysenteriae* (causes dysentery), *Haemophilus ducreyi* (causes chancroid) and *Escherichia coli* (various infectious diseases) (Jinadasa et al., 2011). The Cdt toxins are unique in their ability to induce DNA damage, to cause cell cycle arrest, and to cause apoptosis in intoxicated cells. The Cdt mode of actions (Table 1B) and the association of the Cdt with other human infectious diseases suggests a role for this exotoxin as a putative virulence factor to be valid also for the Cdt of *A. actinomycetemcomitans*.

**Paper IV**

There are several lines of clinical evidence based on cross-sectional observations that support the association between an increased prevalence of *A. actinomycetemcomitans* in subgingival plaque and aggressive forms of periodontitis. *A. actinomycetemcomitans* is found more frequently in samples obtained from subjects with LAgP compared with samples obtained from periodontally healthy subjects or subjects with gingivitis or other forms of periodontal disease (Slots et al., 1980). It was early shown that individuals carrying leukotoxin-producing *A. actinomycetemcomitans* also exhibit significantly elevated levels of leukotoxin-neutralizing antibodies in serum (Tsai et al., 1981, Zambon, 1985, Sjödin et al., 1995, Califano et al., 1997).

**Leukotoxic activity of A. actinomycetemcomitans**

Of the various virulence factors of *A. actinomycetemcomitans*, the leukotoxin (LtxA) has been most extensively examined. Studies on the capacity of *A. actinomycetemcomitans* to express the LtxA, i.e. its leukotoxicity, have focused mainly on *ltxA* and its gene product, LtxA, the leukotoxin (Fine et al.,
Quantitative aspects of the LtxA expression are of considerable interest. The capacity of *A. actinomycetemcomitans* strains to express leukotoxin has been shown to be highly diverse (Baehni et al., 1979, Spitznagel et al., 1991). Strains isolated from patients with AgP have been shown to produce more leukotoxin than those isolated from periodontally healthy individuals or those with chronic adult periodontitis (Zambon 1985, Tervahartiala et al., 1989, Haraszthy et al., 2000). The clinical evidence that sustains the role of the LtxA as a true virulence factor comes from studies concerning the JP2 genotype of *A. actinomycetemcomitans*. Highly leukotoxic strains of *A. actinomycetemcomitans* (i.e. the JP2 genotype) are strongly associated with LAgP in young individuals mainly of African descent (Bueno et al., 1998, Haraszthy et al., 2000, Haubek et al., 2001, Haubek and Westergaard 2004, Haubek et al., 2004, Haubek et al. 2006, Haubek et al., 2008).

*The leukotoxin of A. actinomycetemcomitans influences the host in several ways*

The immune defense-evading ability of this bacterium, in which the LtxA lyses human leukocytes (PMNs), is one of the most important defense mechanisms and is considered as a strong virulence factor for this bacterium (Baehni et al., 1979, Tsai et al., 1979, Fives-Taylor et al., 1999, Johansson et al., 2000a). However, analyses of different subsets of leukocytes have shown that monocytes have a greater sensitivity to LtxA than PMNs and lymphocytes (Kelk et al., 2003). A complete release of the active LtxA from the surface of *A. actinomycetemcomitans* is likely to occur in a protein-rich environment such as in the gingival crevicular fluid (GCF) in the periodontal pocket (Johansson et al., 2003). The LtxA-induced monocyte lysis has been shown to involve activation of caspase-1, which indicates an involvement of pro-inflammatory intracellular signaling with activation and secretion of the cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Kelk et al., 2003, Dinarello, 2009, Kelk et al., 2011). IL-1β is a key component involved in acute and chronic inflammation, and it is an important regulator of bone resorption (Dinarello, 2011). This associates IL-1β to the loss of tooth supporting structures in the periodontal inflammatory process (Schett, 2011, Souza and Lerner, 2013). It has also been shown that enhanced expression of IL-1 in periodontal tissues and increased concentrations of this cytokine in gingival crevicular fluid correlate with progression of periodontal disease.
RESULTS AND DISCUSSION

(Roberts et al., 1997, McGee et al., 1998, Boch et al., 2001). Individuals carrying detectable levels of *A. actinomycetemcomitans* in the periodontal pocket have been shown to harbour an elevated serum titer with LtxA-neutralizing antibodies (Tsai et al., 1981, Sjödin et al., 1995). This indicates that all strains of this bacterium produce LtxA at sufficient levels *in vivo* to activate the humoral systemic immune response.

**Leukotoxicity in isolates of A. actinomycetemcomitans**

From a cohort consisting of 500 individuals, who originated from the study population (Paper I), we analyzed 239 isolates of *A. actinomycetemcomitans* according to leukotoxicity (Paper IV). We used a cell assay to test differences in the leukotoxic activity of clinical isolates. Leukotoxicity was determined in isolates of *A. actinomycetemcomitans* by three different methods: cell lysis (LDH), cell death (NRU), and by the haemolysis method (Paper IV, Fig. 1). This is the first population-based study among adolescents where leukotoxicity of clinical isolates of *A. actinomycetemcomitans* has been analyzed in relation to progression of AL ≥ 3 mm.

When focusing on the capacity of the serotypes a-f to produce LtxA, we found a substantial diversity both between and within the different serotypes. The main part of the examined isolates belonged to the category with a low leukotoxic activity (Paper IV, Fig. 1). The serotype b isolates were more leukotoxic than the other isolates of serotype a-f (Paper IV, Fig. 4 A, B, Fig.5). Similar results were achieved by all the three methods used. However, we consider the cell lysis method (LDH) most relevant for the leukotoxicity determination, because it documents specifically the rapid loss of membrane integrity that is a typical sign for leukotoxin-induced cell injury. All the JP2-isolates were highly leukotoxic and showed a West-African origin based on a point mutation in the *hbpA*-2 gene (Paper IV). However, all isolates with a high leukotoxic activity were not of the JP2 genotype of *A. actinomycetemcomitans*. According to the serotype distribution the main part (>75.0%) of the highly leukotoxic isolates were of serotype b (Paper IV, Fig. 5). An interesting finding was that a subgroup of the highly leukotoxic isolates of serotype b of the non-JP2 genotypes of *A. actinomycetemcomitans*, showed a similar AP PCR banding pattern as the JP2 genotype of *A. actinomycetemcomitans* (Paper IV, Fig. 6). This might indicate that there are
isolates of the serotype b of the non-JP2 genotypes of *A. actinomycetemcomitans* that may have a comparably high leukotoxic activity as the JP2 genotype. The AP PCR banding pattern was more diverse in the isolates belonging to the category with a low leukotoxic activity (Paper IV, Fig. 6). AP PCR is a technique, which does not require previous sequence information of the DNA to be typed. It is based on the use of universal primers and provides “fingerprint patterns” which may reflect the diversity and similarity of the genome within or between bacterial species or clones. In addition, sequencing of the *ltx* promoter gene showed no differences between the non-JP2 isolates of serotype b in the various LtxA categories (Paper IV). Red blood cell (erythrocyte) lysis caused by *A. actinomycetemcomitans* involves a potential interaction with the LtxA (Balashova et al., 2006). Erythrocytes lack the receptor LFA-1, a key molecule for leukotoxin-induced leukocyte lysis (Lally et al., 1999). The haemolysis requires a higher dose of the LtxA than the dose required for lysing leukocytes. It has been speculated whether the haemolytic activity constitutes a virulence mechanism of *A. actinomycetemcomitans* (Kachlany, 2010).
Association with progression of attachment loss (AL) in relation to leukotoxicity and haemolytic activity

Table 4. Odds ratio (OR) showing the association of progression of attachment loss (AL) ≥ 3 mm among individuals in relation to leukotoxicity in isolates of *A. actinomycetemcomitans*. Subgroup of individuals (n=146) positive for *A. actinomycetemcomitans*. The reference group, individuals (n=190) are negative for *A. actinomycetemcomitans*.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
<th>Total number (N)</th>
<th>N individuals (%) with progression of AL ≥ 3 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell lysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>4.1</td>
<td>2.3 - 7.3</td>
<td>&lt; 0.001</td>
<td>110</td>
<td>41 (37.3)</td>
</tr>
<tr>
<td>Average-high</td>
<td>10.9</td>
<td>4.9 - 24.1</td>
<td>&lt; 0.001</td>
<td>36</td>
<td>22 (61.1)</td>
</tr>
<tr>
<td><strong>Cell death</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-average</td>
<td>4.3</td>
<td>2.4 - 7.7</td>
<td>&lt; 0.001</td>
<td>97</td>
<td>37 (38.1)</td>
</tr>
<tr>
<td>High</td>
<td>7.8</td>
<td>3.9 - 15.8</td>
<td>&lt; 0.001</td>
<td>49</td>
<td>26 (53.1)</td>
</tr>
<tr>
<td><strong>Haemolysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>4.8</td>
<td>2.6 - 8.8</td>
<td>&lt; 0.001</td>
<td>90</td>
<td>37 (41.1)</td>
</tr>
<tr>
<td>Average</td>
<td>3.9</td>
<td>1.8 - 8.5</td>
<td>&lt; 0.001</td>
<td>39</td>
<td>14 (35.9)</td>
</tr>
<tr>
<td>High</td>
<td>16.6</td>
<td>5.4 - 51.3</td>
<td>&lt; 0.001</td>
<td>17</td>
<td>12 (70.6)</td>
</tr>
<tr>
<td><em>A.a-neg</em></td>
<td>1.0</td>
<td>reference</td>
<td></td>
<td>190</td>
<td>24 (12.6)</td>
</tr>
</tbody>
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*A.a; A. actinomycetemcomitans*, AL; attachment loss

**Cell lysis-LDH** (low=0-30%, average-high= ≥31% release of cytosolic LDH),  
**Cell death-NRU** (low-average=0-60%, high= ≥ 61% reduction of viable cells),  
**Haemolysis** (low=none to small zone, average=average sized zone, high=large sized zone).

There was a significantly increased risk (OR=10.9) of having a progression of AL ≥ 3 mm in individuals with the presence of isolates from the “high” and “average LDH” category. The risk ratio was enhanced but lower (OR=4.1) for the “low leukotoxic LDH” category (Table 4).
RESULTS AND DISCUSSION

Of the individuals harbouring isolates with a “high” haemolytic activity determined with the haemolysis method, the corresponding enhanced risk for the progression of AL was OR= 16.6.

The significantly increased risk (OR=16.6) for progression of AL ≥ 3 mm in the “High Haemolysis” category included mainly individuals with the serotype b of the JP2 genotype or the non-JP2 genotypes of *A. actinomycetemcomitans*.

While more than 75% of the serotypes, i.e. a, c, d, e and f, were distributed within the low leukotoxins categories, nearly 60% of the isolates of serotype b (27 out 46) were distributed within the “average-high” leukotoxins category (LDH method). This proportion was reduced to 55% when the JP2 genotype isolates were excluded.

Exclusion of the JP2-genotype-positive individuals resulted in the “average-high” leukotoxin LDH group with an OR= 6.9 (95% CI [2.9 – 16.2], *p* < 0.001). In the “low” LDH category the exclusion did not substantially change the estimates as shown with an OR= 3.9 (95% CI [2.1 – 7.0], *p* < 0.001). This shows that disease progression is not solely associated with the JP2 genotype-positive individuals in the “average-high” leukotoxin LDH category, a fact that could partly be due to the limited number of JP2 genotype-positive individuals included in the analysis. On the other hand, this also shows that a substantial number of the non-JP2 genotype positive individuals harboured highly leukotoxins isolates (mainly of serotype b) and that there was still a highly significant association with the progression of AL.

The impact of the LtxA as a putative virulence factor is also shown by the fact that 22 out of 36 (61.1%) of the individuals with isolates from the “high and average” LDH category were included among the individuals with a progression of AL ≥ 3 mm during the two-year follow-up period. On the other hand, 41 individuals (37.3%) in the “low” LDH category (n=110) had also a progression of AL ≥ 3 mm, indicating that the overall presence of *A. actinomycetemcomitans* in the subgingival biofilm is of significant importance for the progression of AL (Table 4).

Exclusion of the JP2-positive individuals in the “low-average” NRU category resulted in an OR= 4.1 (95% CI [2.2 – 7.6], *p* < 0.001). In the “high” NRU category the corresponding value was an OR=5.1 (95% CI [2.4 - 10.9], *p* <
RESULTS AND DISCUSSION

0.001). Exclusion of the individuals with the presence of the JP2-genotype in the “low” and “average” haemolysis categories did not change the estimates substantially as shown in “low haemolysis” category, an OR=4.2 (95% CI [2.3 – 7.8], \( p < 0.001 \)) and in the “average haemolysis” category with an OR=3.5 (95% CI [1.4 – 8.0], \( p=0.004 \)), respectively. While the same exclusion in the “high haemolysis” category resulted in an OR=11.1 (95% CI [3.3 – 36.6], \( p < 0.001 \)).

In summary, according to the risk of developing a progression of AL \( \geq 3 \) mm during the two-year follow-up, the presence of any of the different genotypes and phenotypes of \( A. \) actinomycetemcomitans studied, significantly predicted the progression of AL. Individuals with a noticeably increased risk of developing AL progression were distributed within the category of individuals harbouring isolates with an average to high leukotoxicity (LDH method). Consequently, the presence of highly leukotoxic \( A. \) actinomycetemcomitans correlated most strongly with the progression of AL. These isolates were mainly of serotype b, but other serotypes (a-f) were also involved. However, the presence of the JP2 genotype of \( A. \) actinomycetemcomitans, shown in Paper II, provided clinical evidence for its specific virulence characteristics according to the enhanced risk (RR= 7.3) for the onset and the progression of AL in the young shown in this geographic area.

Successful treatment of patients with LAgP has been related to the reduction of \( A. \) actinomycetemcomitans, whereas failure has been simply attributed to the persistence of high levels of this microorganism in the diseased lesions (Haffajee et al., 1984). One study (Cortelli et al., 2009) dealt with periodontal treatment (non-surgical and surgical treatment) of JP2 and non-JP2 genotype-positive individuals with AgP. The results indicated that non-JP2 positive patients responded more efficiently to the periodontal therapy applied in this study, than did the JP2-strain-positive patients. However, a weakness of the study was the relatively short follow-up period of 12 months.

**Methodological aspects**

Leukotoxicity of the isolates measured with two methods, the cell lysis (LDH) and the cell viability (NRU) were mainly in accordance (Paper IV, Fig. 2). However, using the NRU assay requires that the cells are exposed for a longer period of time to the LtxA. In addition, the NRU method requires a functional
proton gradient for accumulation of vital dye in the lysosomes, an event
affected early in the cell death process (Repetto et al., 2008). This renders the
method slightly more sensitive to score isolates into the category of high
leukotoxicity. However, the LDH and NRU methods frequently produced
similar results (Paper IV, Fig. 2).

A question that remains to be answered is the quantitative aspect according to
the presence of *A. actinomycetemcomitans* bacterial cells in clinical plaque
samples. Is there a need for higher amounts of *A. actinomycetemcomitans*
bacterial cells with a low leukotoxic activity to exert leukotoxicity in the *in vivo*
situation? On the other hand, can a restricted amount of *A. actinomycetemcomitans*,
but with a high leukotoxicity (i.e. the JP2 genotype),
cause tissue damage *in vivo*?

One weakness of the study is that we were not able to isolate JP2-positive
strains from the pooled molar samples analyzed by cultivation, corresponding
to the detection frequency of the JP2 genotype analyzed with PCR-based
techniques. However, the sampled sites were not the same sites according to
the two different methods for detection of *A. actinomycetemcomitans* used.
Methodological problems, such as overgrowth of agar plates by other
microorganisms due to the transportation time for plaque samples, may have
affected to varying degrees the detection frequency of *A. actinomycetemcomitans*. Due to the shortcomings related to the two detection
methods used, the combining of these two methods rendered a relevant tool
for the determination of the presence of the bacterium at the individual level.
When we did the exclusion of the individuals who were positive for the JP2
genotype of *A. actinomycetemcomitans*, we excluded also those individuals
who were JP2-genotype positive by the direct PCR-based technique only.

Identification of highly leukotoxic isolates was also performed with the
haemolysis method. The restricted number of individuals included in the
category of “High haemolytic” activity (Table 4) makes it difficult to draw
firm conclusions about the reliability of the data. However, according to the
enhanced risk for progression of AL in individuals carrying isolates with a
high haemolytic activity, the overall results might indicate the ability of this
method to function as a screening method for highly virulent *A. actinomycetemcomitans* genotypes. The haemolysis assay may also function
as a method for studying the intra-species variation in the leukotoxic activity.
CLINICAL IMPLICATIONS

Periodontitis comprise the most widespread infections of humans, and periodontal attachment loss may first manifest at a young age. In a global perspective a possible trend toward a lower prevalence of periodontitis has been suggested, and this includes all ethnic groups (Borrell et al., 2005, Hugoson and Norderyd, 2008). Epidemiological data indicate that aggressive forms of periodontitis (LAGp and GAgP) might be prevalent and severe in selected groups of susceptible individuals (Meng et al., 2011), certain families and/or ethnic groups (Brown et al., 1996, Jenkins and Papapanou, 2001).

The study of oral infections presents us with complicated microbial community interactions superimposed on complex signaling pathways in the hosts. Pathogenic bacteria use an array of virulence factors to manipulate diverse cellular processes in the host, and these virulence factors benefit the microbe but not the host (the patient). The oral bacterium A. actinomycetemcomitans possesses virulence traits that enable it to colonize and invade, to avoid the host-defense strategies, and to cause tissue destruction. The special characteristics of A. actinomycetemcomitans make the role of the bacterium most crucial in the initial stages of the periodontal disease process. Early diagnosis of the periodontal lesion is of outmost importance in these cases because intervention can then be instituted at the first signs of disease without serious consequences for the patient. Microbial diagnosis to detect the different genotypes of A. actinomycetemcomitans is advisable for aggressive periodontitis cases, where the rate of tissue destruction might progress in a rapid manner. Microbial diagnosis provides information about the nature of the involved pathogenic microorganisms and enables a targeted selection of an antibiotic regimen if indicated. The microbiological and clinical diagnosis of periodontitis is challenging due to the complexity of the disease and the episodic nature of the course of the periodontal inflammation. In the disease process the “inflammatory peak” often precedes the “degradation peak”, when signs of tissue destruction are visible and a clinical diagnosis is possible to assess (Gursoy et al., 2011). This makes it sometimes difficult to get an appropriate microbial sample that mirrors the actual disease activity.
The overall results in this thesis demonstrate a close relationship between the presence of *A. actinomycetemcomitans* in the subgingival biofilm and the initiation and progression of AL in the age group studied (Paper I, II). The biological nature of the two exotoxins studied, the Cdt and the LtxA, and their close association with other infectious diseases, further implicates *A. actinomycetemcomitans* as a disease-provoking bacterium highly associated with aggressive forms of periodontitis found in adolescents and young adults.

In general terms it is stated that the highly leukotoxic JP2 genotype of *A. actinomycetemcomitans* appears primarily to be found in young individuals of African descent. We screened an adolescent population in a geographic area not examined before. The JP2 genotype was also shown to be prevalent among the young in West Africa (Paper I). In the studied Ghanaian population a strong association was found between the presence of the JP2 genotype and disease progression, which strengthens the possible importance of the LtxA as a virulence factor in the aetiology and pathogenesis of aggressive periodontitis (Paper II). A high leukotoxicity in all isolates of the JP2 genotype of *A. actinomycetemcomitans* was confirmed (Paper IV).

Further, this thesis provides new knowledge on the diversity of the leukotoxic activity in strains of *A. actinomycetemcomitans* in relation to the progression of AL (Paper IV). Isolates of non-JP2 genotypes of *A. actinomycetemcomitans* constituted the main collection of the isolates characterized. The risk of having progression of AL was enhanced even after exclusion of the JP2-genotype positive individuals (Paper IV). The results also showed that the highly leukotoxic non-JP2 genotypes of *A. actinomycetemcomitans* (mainly of serotype b) indicated an enhanced risk for progression of AL. This makes the data applicable also to populations where the JP2 genotype is absent. However, the JP2-genotype positive individuals showed the highest rate of disease progression according to the incidence of affected individuals (AL ≥ 3 mm) during the observation period (Paper II). Compared with the non-JP2 positive and the *A. actinomycetemcomitans* negative individuals (Paper II), the highest mean AL and the highest mean AL progression was seen in the individuals positive for the JP2 genotype of *A. actinomycetemcomitans* during the two year follow-up period. Based on the results obtained from this study cohort, the presence of the JP2 genotype provides evidence for its specific virulence characteristics shown in this geographic area. The progression of AL related to the presence of *A. actinomycetemcomitans* in the cohort studied
provide evidence for the importance of the different virulence traits of the LtxA shown in vitro (Johansson 2011).

In vitro data apparently suggest that the Cdt acts as a virulence factor. However, we were not able to show a clear association between the presence of the Cdt activity and an enhanced risk for progression of AL (Paper III). According to the serotypes analyzed, all isolates of the serotype b examined revealed Cdt activity. Perhaps there could be a synergistic effect of the two exotoxins in the pathogenesis of periodontitis.

Recent studies of bacterial virulence factors and toxins have resulted in an increased understanding of the way in which pathogenic bacteria manipulate the cellular processes of the host (Los et al., 2013). In view of the mounting problems related to antibiotic-resistant bacteria, one novel approach would be to disarm bacteria by targeting bacterial virulence. For the study population of the present thesis the LtxA might be an attractive target for the development of tools for LtxA neutralization, relevant to use for a preventive purpose. Hopefully, in the future, new approaches for the management of periodontal infections might be developed. Strategies with anti-virulence agents, which prevent the expression or activity of virulence traits of oral pathogens, would be welcome tools (Scheie and Petersen, 2004).
CONCLUSIONS

- In the Ghanaian population studied, the overall detection frequency of *A. actinomycetemcomitans* was high, and the presence of the bacterium was associated with the presence of AL. The highly leukotoxic JP2 genotype of *A. actinomycetemcomitans* was prevalent in this population.

- The JP2 genotype of *A. actinomycetemcomitans* was shown to be strongly associated with the progression of AL. The presence of the non-JP2 genotypes of *A. actinomycetemcomitans* were also, however less pronounced, but still significantly associated with the progression of AL.

- The distribution pattern in the studied isolates of *A. actinomycetemcomitans* according to serotype, *cdt* genotype and Cdt activity, was in line with results based on other previously studied populations.

- The presence of *A. actinomycetemcomitans* was significantly associated with disease progression, in particular the b serotype, whereas the association with progression of AL was not particularly related to the *cdt* genotype or the Cdt activity.

- The risk for developing progression of AL was most pronounced in individuals that harboured isolates of *A. actinomycetemcomitans* with a high leukotoxicity.

- The main proportion of the serotype b isolates was distributed within the category with a high leukotoxicity. The analyses of the non-JP2 genotypes of serotype b indicated a genetic diversity linked to the leukotoxic activity. A subgroup of the serotype b isolates of the non-JP2 genotypes of *A. actinomycetemcomitans* exhibited equal levels of leukotoxicity as the highly leukotoxic JP2 genotype of *A. actinomycetemcomitans*. 
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