Epidemiological and microbiological aspects of aggressive periodontitis in Sudan

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UNIVERSITETET I BERGEN

To my parents

Scientific environment

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Abbreviations

AgP	Aggressive periodontitis
LAgP	Localized aggressive periodontitis
GAgP	Generalized aggressive periodontitis
CP	Chronic periodontitis
CEJ	Cemento-enamel junction
DNA	Deoxyribonucleic acid
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
VZV	Varicella zoster virus
EBV	Epstein-Barr virus
HCMV	Human cytomegalovirus
HHV-6	Human herpes virus type 6
HHV-7	Human herpes virus type 7
HHV-8	Human herpes virus type 8
PCR	Polymerase chain reaction
LAMP	Loop mediated isothermal amplification of DNA
PSU	Primary sampling units
PPD	Probing pocket depth
BOP	Bleeding on probing
PAL	Periodontal attachment loss
OR	Odds ratio
CI	Confidence interval

List of papers

This thesis is based on the following papers:

Paper I

Elamin AM, Skaug N, Ali RW, Bakken V, Albandar JM.

Ethnic disparities in the prevalence of periodontitis among high school students in Sudan. J Periodontol. 2010, 81:891-6.

Paper II

Elamin A, Albandar JM, Poulsen K, Ali RW, Bakken V.

Prevalence of *Aggregatibacter actinomycetemcomitans* in Sudanese patients with aggressive periodontitis: a case-control study. J Periodontal Res. 2011, 46:285-91.

Paper III

Elamin A, Ali RW, Bakken V

Putative periodontopathic bacteria and herpes viruses in patients with aggressive periodontitis: a case-control study in Sudan. *In manuscript.*

1 INTRODUCTION

1.1 Aggressive periodontitis

Periodontitis is one of the most widespread infectious diseases of mankind. The term is collective for a group of pathological alterations of the periodontium, the support system for the dentition. The disease is associated with loss of the supporting connective tissue and resorption of the alveolar bone surrounding the teeth. Clinical presentations of periodontitis include gingival inflammation (manifested as bleeding on probing), gingival recession, gingival bleeding, halitosis, periodontal attachment loss, pathological tooth mobility and/or migration and alveolar bone loss. A particular type of periodontitis is aggressive periodontitis, a distinct disease entity characterized by rapid progression of attachment loss in otherwise healthy subjects; this condition tends to have familial trends (1). Untreated cases of aggressive periodontitis may eventually suffer tooth loss at early age (2).

Over the years, numerous classifications and definitions have been adopted for aggressive periodontitis. In 1999, the International Workshop for Classification of Periodontal Diseases and Conditions (2) accepted a Consensus Report (1) to discard former terminologies that relied on age as a diagnostic criterion and to emphasize instead the rate of destruction caused by the disease. Accordingly, the term "Early Onset Periodontitis” was replaced by “aggressive periodontitis” and “adult periodontitis” was replaced by “chronic periodontitis”.

1.1.1 Clinical and radiological manifestations

Aggressive periodontitis can be distinguished from other forms of periodontitis by its distinctive features (Figure 1); these include the following:

(i) Apart from aggressive periodontitis, individuals are otherwise clinically healthy;

(ii) The severity of periodontal destruction manifested in aggressive periodontitis is intensive with respect to the low levels of plaque and calculus typically observed in chronic periodontitis;

(iii) The rate of progression of aggressive periodontitis is rapid in comparison with chronic periodontitis;

(iv) Familial aggregation within families is another distinctive feature of the disease.

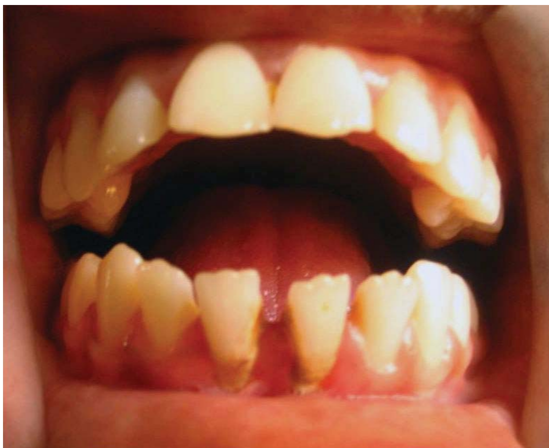


Figure 1. Clinical appearance of medically healthy 14-year-old African female with localized aggressive periodontitis. Note the minimal amounts of supra-gingival plaque. Gingival recession, attachment loss and pathological tooth migration are most obvious in the mandibular first incisors.

Age of onset is considered a key diagnostic feature of localized aggressive periodontitis with a circum-pubertal debut, while the generalized form is suggested mostly to affect subjects under 30 years of age but they may be older. The two forms of aggressive periodontitis, localized and generalized, are associated with two different patterns of destruction. The localized form, as implied by the name, is a localized interproximal attachment loss on at least two permanent teeth, one of which

is the first molar, and involving no more than two teeth other than first molars and incisors. On the other hand, the generalized form exhibits more extensive interproximal attachment loss affecting at least three permanent teeth other than the first molars and the incisors. The two forms of aggressive periodontitis differ with respect to their etiology, pathogenesis and clinical presentation, and in the host response patterns (1). It is clear that localized aggressive periodontitis is not merely a localized form of generalized aggressive periodontitis (3). Whether generalized aggressive periodontitis is the result of untreated or inadequately localized aggressive periodontitis, or is a specific form of aggressive periodontitis, is a matter of controversy.

The radiographic appearance of localized aggressive periodontitis is characteristic, with angular defects around incisors and vertical or arch-shaped bone defects around the affected molars. On the other hand, a generalized bone loss around the teeth is observed in the case of generalized aggressive periodontitis.

1.1.2 Global prevalence diversities

In epidemiology, prevalence of a given disease is defined as the proportion of persons in the population with the disease at a given time. Principally, aggressive periodontitis is a condition with lower prevalences compared with chronic periodontitis and it affects younger individuals. There is strong evidence that certain ethnic groups have a higher risk of developing aggressive periodontitis. The global prevalence estimates in general populations show that Africa has the highest prevalence of aggressive periodontitis (0.5-5%), followed by Asia (0.4-1%), South America (0.3-1%), North America (0.4-0.8%) and Western Europe (0.1-0.5%). The prevalence of aggressive periodontitis in the various ethnic groups are: Africans and

African Americans (1-3%), Hispanic and South Americans (0.5-1%), Asians (0.4-1%) and Caucasians (0.1-0.2%) (4). Nevertheless, comprehensive global comparisons remain biased and unreliable due to methodological inconsistencies, including poor case definition, selection criteria and disease detection methods (5).

There is a lack of studies reporting aggressive periodontitis in the Sudanese population. Those studies that exist (6-8) were conducted decades ago and provided only limited knowledge of the disease. Moreover, none of the studies was designed to assess prevalence of aggressive periodontitis (Table 1).

Table 1. Prevalence estimates of periodontal diseases among adolescents in Sudan in the literature.

Author and year	Region	Study sample (*n*)	Age (years)	Index	Prevalence
Emslie 1966 (6)	Butana, Gezira, Khartoum city and Kordofan river	995	4-60	Russell index (PI)	-High prevalence of periodontal disease associated with poor oral hygiene. - Three cases of periodontosis were found among 645 (age group 15-19 years), yielding a prevalence of 0.47%
Dowty 1982 (7)	South Sudan (Jonglei Province and Juba)	426	4-17	No data	-High prevalence of calculus and intense gingivitis. - Advanced periodontal diseases confined to anterior mandibular teeth 8% (among 15-year-old).
Ali 1994 (8)	Khartoum city and El-Obeid	264	15-64	CPITN	-High prevalence of periodontal diseases among adolescents. - 95.2% having pockets 4-5 mm -4% having pocket depths 6 mm.

1.2 Microbiological characteristics of aggressive periodontitis

The approximately 215 cm² (mucosal and dental) surface area of the oral cavity (9) presents numerous sites for microbial colonization (10). Over 700 bacterial species have been identified in the oral cavity by molecular methods; more than half of these species are uncultivable (11), with the periodontal pocket harbouring more than 400 bacterial species (12). For decades, investigators have studied extensively microbiota associated with periodontal diseases, using both culture and culture-independent techniques. The majority of these investigations focused on the identification of putative pathogens implicated in the etiology of periodontal diseases. However, the task of identifying microbiota associated with periodontal diseases remains hampered because of several factors:

- *(*i) The complex matrix in which oral microbiota exist, known as oral biofilms; these microorganisms live in a state of ecological balance, with constant interactions with the local environment and the host;
- (*ii*) Periodontal diseases are considered to have a polymicrobial etiology, *i.e.* mixed infections with diverse pathogens incriminated as commensal opportunistic pathogens or exogenous pathogens;
- (*iii*) Difficulty in determining the debut of the periodontal infection and ascertaining when the disease is active or inactive;
- (*iv*) Variations with respect to host susceptibility to colonization by microorganisms which is influenced by genetic and environmental risk factors;
- (*v*) Differences in periodontal pathogen virulence, which is supported by the consistent finding that putative periodontal pathogens are often found in

periodontally healthy subjects for long periods without progressing to periodontal destruction;

(*vi*) Inter-study variations in case definitions, sampling techniques and detection methods (13).

1.2.1 Putative periodontal pathogens

In the 1999 International Workshop for Classification of Periodontal Diseases and Conditions, the microbial feature of aggressive periodontitis was considered a secondary feature for distinction between aggressive and chronic periodontitis. The reports suggested "an elevated proportion of *Aggregatibacter actinomycetem-comitans* and in some populations, *Porphyromonas gingivalis"*(1). These two organisms meet Socransky's criteria for periodontal pathogens (14).

Other bacteria that have putatively been associated with aggressive periodontitis and with other forms of periodontitis were proposed by the 1996 World Workshop in Periodontics (15). They include mainly cultivable gram-negative anaerobic bacteria such as *Tannerella forsythia, Prevotella intermedia, Campylobacter rectus* and *Treponema denticola,* as well as some gram-positive anaerobes including *Peptostreptococcus micros* and *Eubacterium* species (16). Current advances in culture-independent molecular techniques have led to the recognition of several diverse putative pathogens (13,17,18).

1.2.2 *Aggregatibacter actinomycetemcomitans*

Aggregatibacter actinomycetemcomitans (previously known as *Actinobacillus actinomycetemcomitans*) (19) is a fastidious, facultative, non-motile, non-spore forming, gram-negative anaerobic rod first described by Klinger in 1912 (20). More than sixty years later, the pathogen was identified in dental plaque (21), linked to

aggressive (juvenile) periodontitis (22,23) and its leukotoxin discovered (24). The organism is categorized into seven serotypes a, b, c, d, e, f and g (25-27). *A. actinomycetemcomitans* may be detected in the dental plaque of healthy individuals where it constitutes a persistent portion of the normal flora (10,28,29). It is known to be part of the HACEK group (includes *Haemophilus* species, *A. actinomycetemcomitans, Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella* species) of pathogens which has the capacity to produce endocardial (30) and other extra-oral infections (31). Moreover, *A. actinomycetemcomitans* possesses various virulence factors that may activate different inflammatory and immune responses in host tissues and may inflict periodontal destruction (32). The virulence traits of *A. actinomycetemcomitans* include numerous molecules and organelles and several virulent mechanisms including:

(*i*) Pore forming leukotoxin that belongs to the RTX (repeat in toxin) family of bacterial cytolysin which destroys polymorphnuclear cells, monocytes and subsets of lymphocytes;

(*ii*) Cytotoxin: CDT (cytolethal distending toxin);

(*iii*) Iron binding proteins and lipopolysaccharide that can bind to haemoglobin;

(*iv*) *A. actinomycetemcomitans* can adhere to host cells and invade primary and transformed human oral epithelial cells (33,34); it has been demonstrated *in vivo* in buccal epithelial cells collected from human subjects (35) and is known to induce apoptosis (36);

(*v*) Activation of T and B cells and other bacterial components that promote bone loss (30,32).

It is clear that *A. actinomycetemcomitans,* with its potent virulence factors, is capable of causing marked alterations in the host and plays a significant etiological role in

aggressive periodontitis as well as some other forms of periodontitis (32,37). Strains of *A. actinomycetemcomitans* vary with regard to their pathogenicity and the expression of their leukotoxin (38). The observation that *A. actinomycetemcomitans* is found in the dental plaque of both diseased and healthy subjects suggests that isolates may differ with respect to their pathogenic potential (32). Moreover, the findings that *A. actinomycetemcomitans* is found to be part of the normal flora of periodontally healthy people suggest categorizing it as an opportunistic pathogen (29). A particular clone of *A. actinomycetemcomitans* known as JP2 (serotype b) is characterized by 530 base pair deletion in the promoter region of the leukotoxin gene operon. This deletion accounts for a 10-20 fold elevated leukotoxic activity compared with non-JP2 types of *A. actinomycetemcomitans* (38). The clone was first isolated from an 8-year-old male with deep periodontal pockets and advanced alveolar bone loss affecting the primary molar teeth (39). It was suggested that the JP2 clone may have a predilection for colonizing certain genetic population lines, particularly persons of African, Afro-Arab or Berber origins (40-43). It is seldom found among other ethnic lines *e.g.* Caucasians and Asians (42,44-46); only a few studies have reported detection of the clone among Caucasians individuals (41,47-49). Longitudinal studies have demonstrated a strong association between harbouring the JP2 clone and an elevated risk of developing aggressive periodontitis (50,51). Nevertheless, both cross-sectional and longitudinal studies have shown that the non-JP2 types of *A. actinomycetemcomitans* remain strongly associated with aggressive periodontitis in other geographically distinct populations (52-55).

1.2.3 Bacteria and virus interactions in periodontal diseases

Members of the herpes virus family are composed of a double stranded DNA genome contained within nucleocaspid surrounded by a lipid envelope. To date, the

family of herpes viruses includes eight viruses, namely, herpes simplex virus (HSV-1 and HSV-2), Varicella Zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), and the human herpes viruses 6-8 (HHV-6, HHV-7 and HHV-8). The viruses can cause a wide range of infections in humans and have the ability to establish latent infections that persist for life (56). In the past decade, lines of evidence implicating periodontal HCMV and EBV-1 in the etiopathogenesis of periodontal diseases by inducing several inflammatory and immune responses leading to periodontal tissue destruction, have been suggested by some researchers (57,58). Herpes viruses may trigger periodontal breakdown by the release of virally mediated proinflammatory cytokines and chemokines from host cells and/or the alteration of periodontal immune responses leading to possible upgrowth of periodontopathic bacteria (59). The hypothesis suggests that an active herpes viral infection leads to initiation of a number of immune and inflammatory events that may facilitate potential increase in the number of, or the virulence of, the resident bacterial pathogens in these lesions (60). Together the periodontal herpes viruses and putative bacteria have an ecological synergy that acts on periodontal tissues, resulting in eventual periodontal destruction. Clearly, the proposed hypothesis goes beyond incriminating bacteria as classical causative agents in periodontal diseases and it suggests the involvement of periodontal herpes viruses. However, the researchers based their hypothesis on a series of findings including:

(*i*) The association between the subgingival presence of herpes viruses and different types of periodontal diseases (57,61-64);

(*ii*) The high HCMV and EBV-1 DNA loads in aggressive periodontitis lesions (65);

(*iii*) The significant associations found between the subgingival presence of HCMV and EBV-1 and co-infection with periodontopathic bacteria and severity of periodontal diseases (66,67);

(*iv*) The findings that periodontitis occurs more frequently and progresses more rapidly at sites infected with HCMV and EBV-1 than non-infected sites (68,69);

(*v*) Periodontal therapy may result in marked reduction of periodontal herpes virus counts (58,70);

(*vi*) The bacterial etiology of the periodontal diseases may seem insufficient to elucidate important clinico-pathological features of periodontal diseases (65,71,72), and

(vii) The generally accepted fact that acute viral infections predispose individuals to bacterial infections (73,74).

1.2.4 Risk factors for aggressive periodontitis

Risk is defined as the probability that an individual will develop a particular disease or condition within a specified period of time (75). Risk assesses the association between potential risk factors (exposures) and a given condition (outcome). Longitudinal studies provide the most powerful evidence of associations. However, cross-sectional and case-control studies are capable of offering equivalent strength when proper study design and representative study samples are employed (76). It is well recognized that, in addition to the microbial etiology, there are several non-microbial risk factors associated with aggressive periodontitis. Several epidemiological studies have been conducted to identify demographic, environmental and genetic risk factors. Only a few risk factors were found to be exclusively associated with aggressive periodontitis. Increasing age is a risk factor found to be associated with higher prevalence and severity of periodontal attachment loss (77).

Moreover, gender was suggested to be a risk indicator, with males showing a higher prevalence of the disease than females (78-80), although the evidence to support gender-dependence of the disease is still controversial (81,82). The socioeconomic level of individuals may play a role in developing aggressive periodontitis; individuals with lower socioeconomic status exhibit higher prevalence of the disease than their counterparts with higher socioeconomic status (78,83,84). Smoking is an environmental risk factor, which has been found to contribute to increased disease extent and severity, especially the generalized form of the disease (85,86). The habit does not distinguish between aggressive and chronic periodontitis (87). Poor oral hygiene is another environmental risk indicator associated with accumulation of plaque biofilms and increased risk of periodontal destruction (87,88). According to the 1999 classification for periodontal diseases, aggressive periodontitis is associated with minimal levels of dental plaque (1). One of the main significant risk factors for aggressive periodontitis is ethnicity, with Africans and Hispanics showing higher prevalence of the disease than Asians and Caucasians (4,88). It is suggested that the ethnic disparities are due to certain genetic predispositions (78). Genetic polymorphisms (single nucleotide polymorphism, SNP) are suggested to be associated with greater risk of periodontal disease progression (89,90), although it might be argued that there is no clear-cut distinction between genetic markers for aggressive and chronic periodontitis. Moreover, investigators have reported that the pathogenesis of aggressive periodontitis may be associated with host immune defects in the neutrophils and monocytes in some patients (91,92). In conclusion, disparities in prevalence of aggressive periodontitis between different populations may be attributed to various risk factors and predisposition indicators. Further investigations are therefore needed to identify potential risk factors associated with

different populations that lead to increased susceptibility of individuals to aggressive periodontitis.

1.2.5 Detection of periodontopathic bacteria

Over the years, a variety of microbiological diagnostic tests has been developed for detection of pathogenic microorganisms. These include microscopy, culture, immunodiagnosis, enzymatic assays, nucleic acid probes and polymerase chain reaction (PCR). Methods based on culture are considered the gold standard, with antibiotic sensitivity having a distinctive advantage that distinguishes it from other microbiological techniques. The method has several limitations, including the difficulty of growing several bacteria, it is relatively slow and has lower sensitivity when compared with other culture-independent approaches (93).

1.2.6 Polymerase chain reaction (PCR)

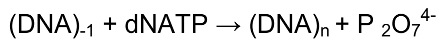
PCR is a powerful molecular technique (re)developed by Kary Mullis and co-workers in the mid-eighties that involves amplification of minute amounts of DNA flanked by primers and employs a heat-stable DNA polymerase (e.g. Taq polymerase) in a cyclic process to produce large amounts of DNA copies that can be analysed (94). It has marked advantages over the gold standard method, including higher sensitivity, viability of the microorganism is not a requirement, rapidity and it overcomes the problem of microorganisms not or only very slowly susceptible to cultivation (93). The 16S rRNA-based PCR is a method that targets phylogenetically informative genes that are conserved across bacteria and archaea species (95). The method uses probes that target 16S rRNA gene and are highly specific to individual species (95). Moreover, the method enables researchers to identify novel pathogens, uncultured

bacteria, rarely isolated, phenotypically abnormal strains and provides information on bacterial species diversity (12,96).

1.2.7 Loop mediated isothermal amplification (LAMP)

The LAMP is a novel autocycling and strand displacement DNA synthesis method that emerged a decade ago (97). Since its debut, it has been employed to detect wide ranges of microorganisms in different biological samples (98-100). The method entails a set of two inner primers (FIP, BIP) and two outer primers (F3, B3) and a set of additional loop primers (LF, LP). The inner and outer primers span a total of six distinct regions within the target DNA, labelled as F3, F2, F1, B1c, B2c and B3c from the 5' end (97). The loop primers, on the other hand, accelerate the amplification (101). The amplification is executed at an isothermal temperature range 63-65°C and large fragment *Bst* DNA polymerase with strand displacement activity is required. The end amplification product is a stem-loop of DNA with inverted repeats of DNA and cauliflower-like structure with multiple loops (101). The target DNA is amplified with up to 10^{9} copies that can be detected by gel electrophoresis, turbidity, fluorescence, or colorimetric detection by adding intercalating dye to the reaction solution (102-104). The agarose gel analysis of the LAMP product reveals a ladder-like pattern due to the stem loop DNA with alternately inverted repeats of the target sequence. This pattern is characteristic of LAMP rather than the typical single band seen with PCR (97). The turbidity that is yielded as a white precipitate is attributed to the formation of magnesium pyrophosphate, which is an amplification bi-product. Furthermore, the turbidity is found to be proportional to the amount of amplification product synthesised. Therefore, real-time monitoring of the reaction is feasible through real-time measurement of the turbidity using a turbidity meter (102).

The white precipitate produced in the LAMP reaction can be represented by the following reaction (102): (See the reactions below)



$(DNA)_{-1} + dNATP \rightarrow (DNA)_n + P_2O_7^{4-}$

$P_2O_7^{4-} + 2MgSO_4 \rightarrow Mg_2P_2O_7$

The principles of the LAMP method are illustrated in Figure 2.

The LAMP method has several advantages over PCR-based methods (105). These advantages include:

1. Rapidity: the isothermal nature of the reaction involves no loss of time caused by temperature changes, which occur with thermal cycling in PCR. Moreover, the reaction time requires a maximum of one hour to detect the amplification products. In addition, employing loop primers accelerates the reaction.
2. High specificity: due to the use of four inner and outer primers.
3. The detection limit may be higher or equal to PCR with shorter detection time.
4. High efficiency: The inhibition reaction, which is a typical problem with PCR that disturbs the amplification at later stages and is caused by blocking necessary reagents and binding to the polymerase, is less likely to take place with the LAMP method.
5. Ease of detection of amplification product through turbidity that can be observed by the naked eye.
6. Cost effectiveness: the isothermal reaction can be performed in a water bath or a heat block instead of thermocycler.
7. Quantification of the initial template DNA is feasible through the use of a real-time turbidimeter designed for the LAMP reaction.

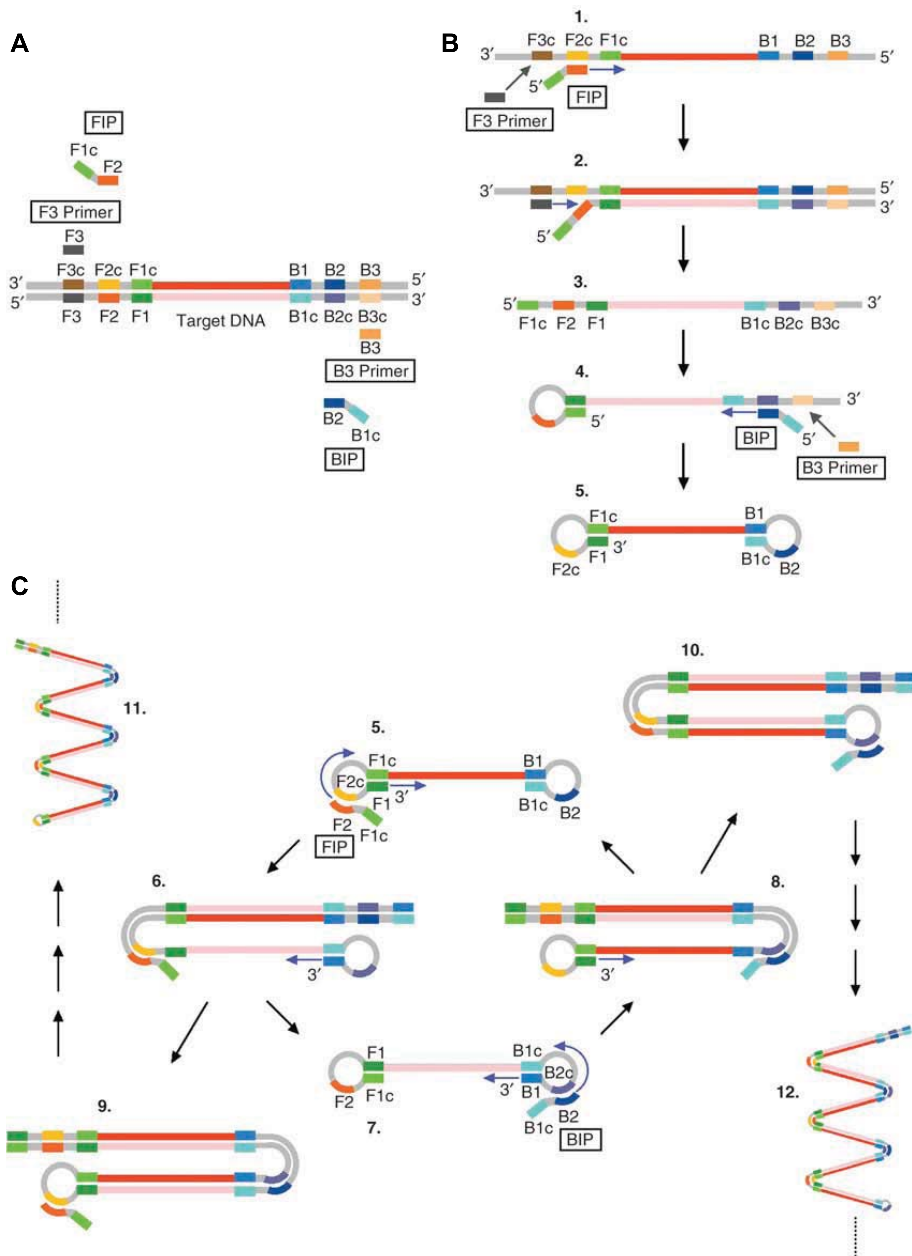


Figure 2. Schematic representation of the principle of Loop Mediated Isothermal Amplification (LAMP) method: **A**) Primer design of the LAMP reaction. **B**) Starting structure producing step. **C**) Elongation and cycling amplification step. FIP, Forward Inner Primer; F3 Primer, Forward Outer Primer; BIP, Backward Inner Primer; B3 Primer, Backward Outer Primer. See main text for further explanation. Adapted by permission from Macmillan Publishers Ltd: Nature Protocols, Tomita et al. (103), copyright 2008.

8. Potentially valuable diagnostic tool: all of the above-mentioned advantages make the LAMP method a valuable tool for application in field work and laboratories with limited resources. Moreover, the method can be used as a chair-side diagnostic method and the amplification end product can be observed by the naked eye.

2 Aims of the thesis

The general aim of this PhD-project was to study aggressive periodontitis among adolescents in Khartoum state, Sudan.

Specific aims

1. To assess the prevalence of aggressive periodontitis in a representative sample of adolescents who attended high schools in the Khartoum metropolitan area, Sudan; to describe the clinical occurrence of the disease and to compare the prevalence of the disease among the different ethnic groups in this population.
2. To determine the presence of putative periodontal pathogens such as *P.gingivalis, T. forsythia* and *T. denticola* in the periodontal lesions of these subjects.
3. To examine the periodontal presence of HCMV and EBV-1 in subjects with aggressive periodontitis and periodontally healthy subjects.
4. To assess the potential colonization by JP2 clone and non- JP2 types of *A. actinomycetemcomitans* in the subgingival biofilms of aggressive periodontitis and periodontally healthy subjects.

3 Working hypotheses

1) Aggressive periodontitis is highly prevalent among high school students in Khartoum state, Sudan.
2) There is a significant association between the prevalence of aggressive periodontitis and the ethnic make-up of the participating individuals.
3) *A. actinomycetemcomitans* is a key pathogen associated with aggressive periodontitis in this population.
4) Sudanese patients with aggressive periodontitis may harbour subgingivally a JP2 clone of *A. actinomycetemcomitans.*
5) There are significant associations between aggressive periodontitis and the following factors:
    a) Certain periodontopathic bacteria e.g. *P.gingivalis, T. forsythia* and *T. denticola*.
    b) Subgingival co-infection with HCMV and EBV-1.

4 Materials and methods

4.1 Study population

The target population was Sudanese subjects attending public and private high schools in Khartoum state, Sudan. Khartoum state is located south of the confluence of the Blue and White Nile rivers. Khartoum city is the national capital of Sudan and the capital of Khartoum state. The state has an area of 22,122 km^2 and a population of approximately 6.2 million inhabitants, of whom 56% were 15-64 years old (data from

2007). Khartoum state includes seven municipalities: Khartoum, Omdurman, Bahri, Sharq Elnil, Karari, Jabal Awliya and Umbaddah (Figure 3).

The seven municipalities represent the 19 highly diverse ethnic groups of Sudan and almost 600 subgroups (Figure 4). Khartoum state is the main location for most of Sudan's top educational institutes and industrial centres. In 2007, the gross enrolment ratio (GER) of both genders in Khartoum state was 68%, which is much higher than the national ratio (106). Geographical disparities existed due to political instability, the internally displaced and nomadic populations. The population in this state works mainly in government service and trades. Arabic is spoken by most of the inhabitants. The educational system in Sudan consists of 11 grades, with high school education lasting three years (grade 9-11), followed by college. In the school year 2006-2007, 151,126 students (28,477 girls and 22,978 boys) distributed in 744 schools (331 public and 413 privates) attended high school (107) (Table 2, Figure 5).

Figure 3. A) Map of Sudan showing Khartoum state (coloured red). **B**) Map of Khartoum state showing the seven municipalities.

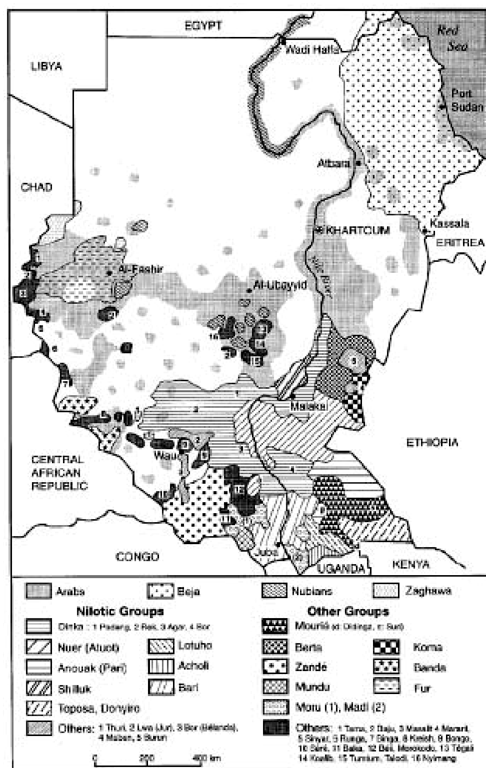


Figure 4. Map of Sudan showing the distribution of the main ethnic groups. Adapted from Mosely, A.L., 1998 (157).

Table 2. Distribution of high school students in the seven municipalities in Khartoum state.

Municipality	Private schools		Public schools	
	girls	boys	girls	Boys
Khartoum	2,882	2,019	6,581	7,678
Omdurman	7,021	6,066	6,077	5,667
Bahri	3,369	2,427	7,031	7,177
Jabal Awliya	3,039	2,326	7,599	5,842
Umbaddah	2,787	2,416	8,442	6,846
Karari	4,515	3,096	7,337	6,570
Sharq Elnil	4,868	4,628	9,433	7,391
Total	28,477	22,978	52,500	47,171
	51,455		99,671	

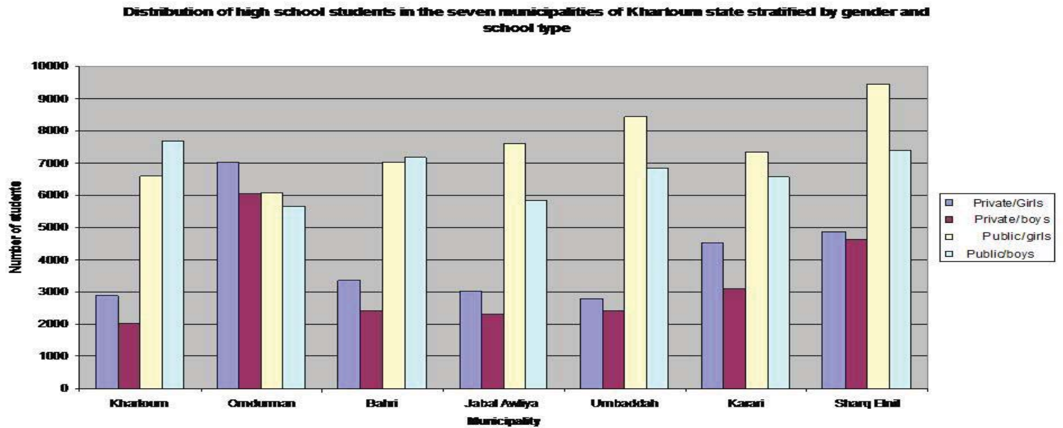


Figure 5. Distribution of high school students in the seven municipalities of Khartoum state stratified by gender and school type.

4.1.1 Study sample, examination and microbial sampling

4.1.1.1 Paper I

This cross-sectional study used a multistage stratified sampling design to assess the prevalence of aggressive periodontitis and periodontal attachment loss among high school students in Khartoum state, Sudan (Figure 6). The clusters sampled in the first stage consisted of high schools stratified by school type and municipality and referred to as "primary sampling units" (PSU). The PSU were selected in proportion to the population within each municipality. The second stage consisted of random selection of three classes from each school. All the students within each class were invited to participate in the study, forming the third stage. Complete details of sampling selection and inclusion criteria are available (108).

Accordingly, a total of 1,247 subjects (age 13-19 years old) selected from 38 schools were eligible, but only 1,200 subjects (604 females and 596 males) consented to participate in the study. All subjects were interviewed using a structured questionnaire and data on personal traits, periodontal, dental, and medical histories were collected. Moreover, enrolled subjects were clinically examined by one examiner (Elamin A.), under field conditions. Measurements were made at six sites per tooth (mesiobuccal, midbuccal, distobuccal, distolingual, midlingual and mesiolingual) for the incisors, first and second molars only. Detailed descriptions of the examination protocol are available (108).

Aggressive periodontitis in this population was defined as subjects with at least 4 teeth with interproximal sites showing ≥4 mm attachment loss, or at least 3 teeth with interproximal sites showing ≥5 mm attachment loss.

4.1.1.2 Paper II

In this case control study, 17 patients with aggressive periodontitis (cases) and 17 periodontally healthy subjects were recruited to serve as controls (14-19 years of age). The 17 cases were a subset of 41 patients with aggressive periodontitis identified during the first study. Controls were randomly selected from the same class of the cases and they were matched in gender and ethnicity (Figure 6). Subgingival plaque samples were collected from the participants using paper points (#35, Zipperer, Munich, Germany) to determine the prevalence of JP2 clone and non-JP2 genotypes of *A. actinomycetemcomitans*. Loop mediated isothermal amplification method (LAMP) and 16S-rRNA PCR were used for assessing the presence of the target pathogens (Figure 7). Detailed narratives of the study sample and methods are available (109).

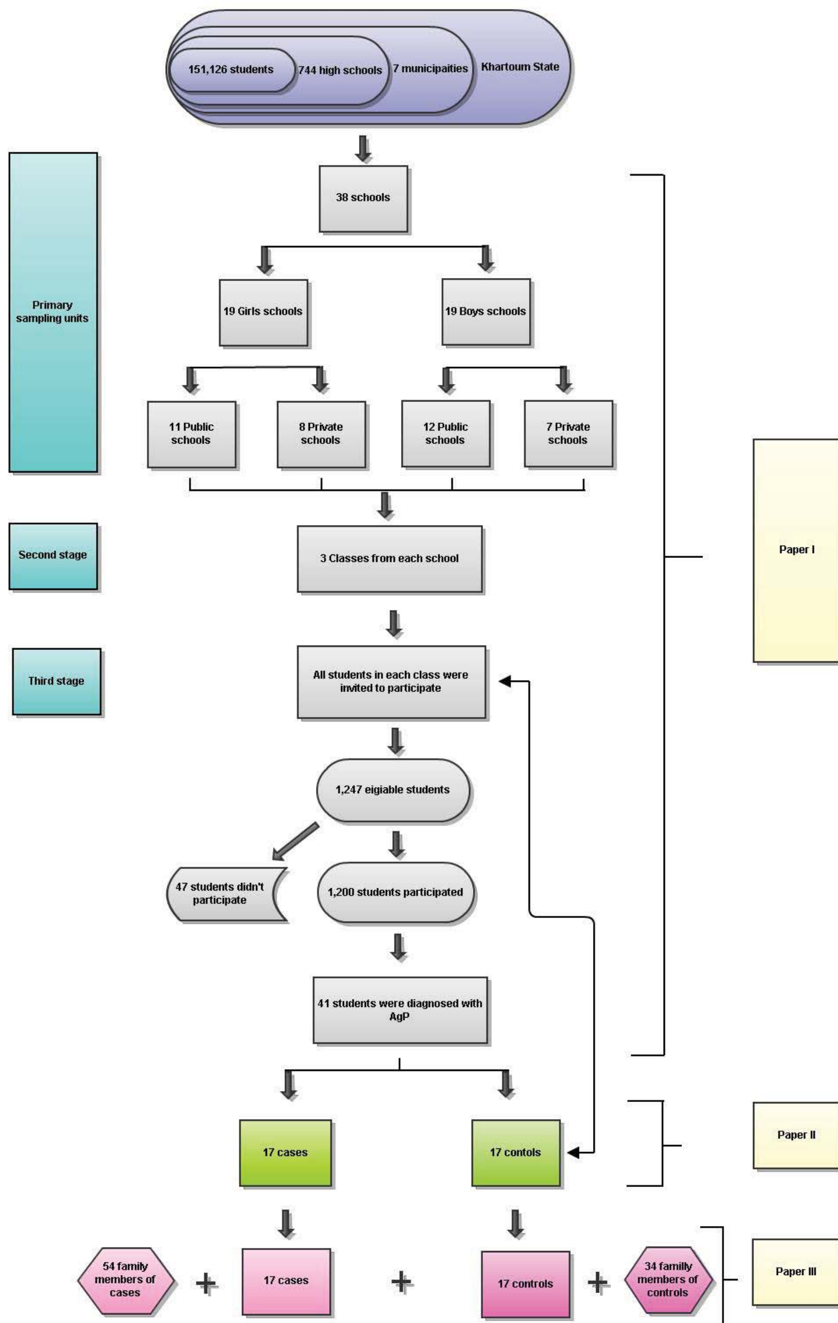


Figure 6. Selection procedure and study design of the PhD project.

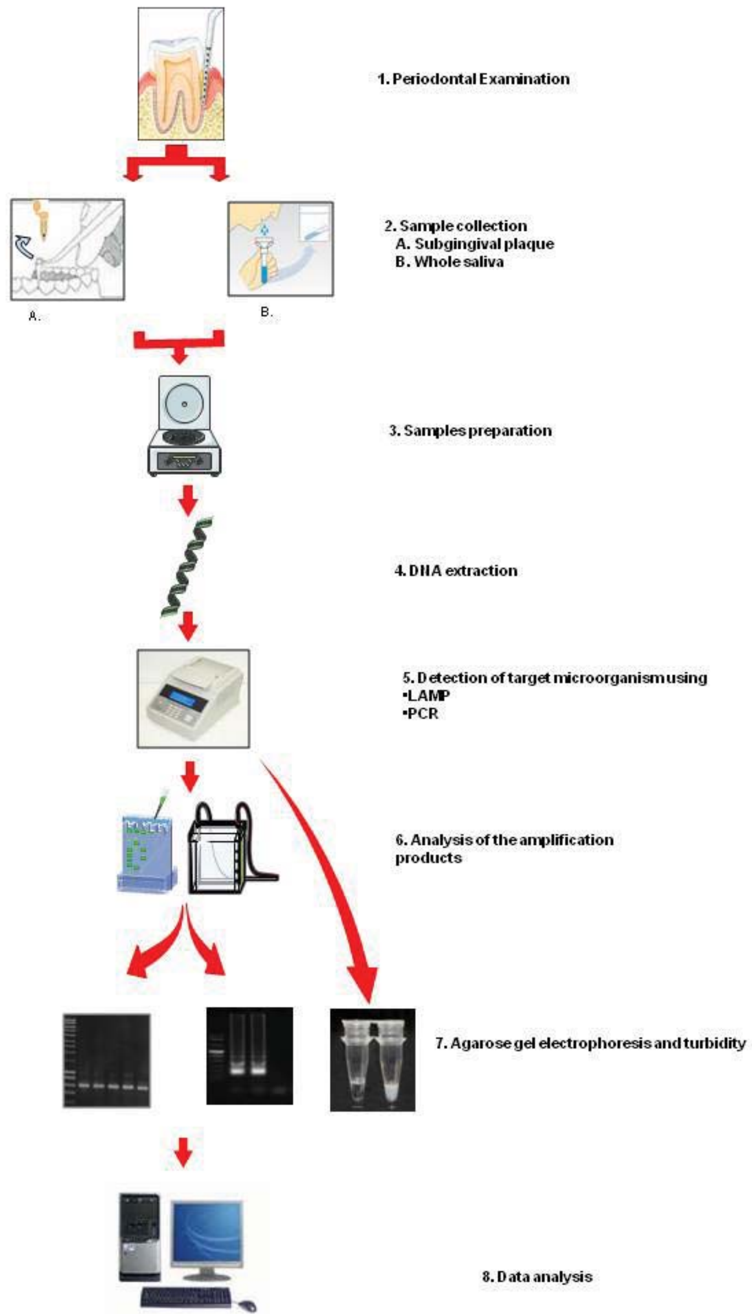


Figure 7. Schematic representation for clinical and microbial examinations.

4.1.1.3 Paper III

The same seventeen patients with aggressive periodontitis (cases) and 17 periodontally healthy subjects (controls) enrolled in study II were recruited in this study (Figure 6). In addition, relatives of cases and controls were invited to participate in this study. Fifty-four relatives of cases (mean age 33.1, ±20.1 years), and 34 relatives of controls (mean age 35.7, ±15.3 years) accepted to participate. Each case or control had at least one biological family member (parent or sibling) recruited in the study. Mothers made up the main group of participants among family members surveyed. Whole stimulated saliva samples were collected from 17 cases, 17 controls and the 88 participating relatives at their homes. Furthermore, 17 cases, 17 controls and a subset of the enrolled relatives (13 relatives of cases, and 8 relatives of controls) agreed to contribute subgingival plaque samples. The LAMP method was used to asses the prevalence of the four periodontopathic bacteria *A. actinomycetemcomitans, P.gingivalis, T. forsythia* and *T. denticola* and two periodontal herpes viruses EBV-1 and HCMV in whole stimulated saliva and the subgingival plaque of the participants. LAMP products were analysed by visual inspection to detect turbidity (Figure 8), fluorescent using fluorescent detection reagent (Figure 9), and 2% agarose gel electrophoresis.

Figure 8. Visual inspection of LAMP products by detecting white turbidity. *A. actinomycetemcomitans* primers were used to amplify positive control and water was the negative control.

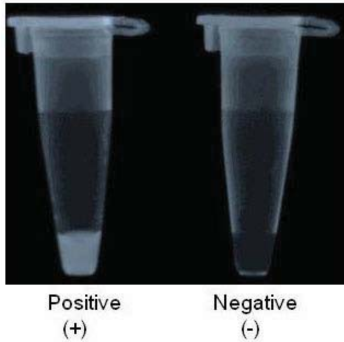


Figure 9. Visual detection negative and positive controls under UV light of LAMP using fluorescent detection reagent (ethidium bromide 1mg/ml). *P.gingivalis* primers were used to amplify positive control and water was the negative control.

4.1.2 Inclusion criteria

Subjects, who attended public or private schools in grade 9-11 in Khartoum state, were invited to participate in these studies. Subjects were informed about the aims of the studies and examination methods, and they were provided with general information about oral diseases and the effects of these diseases on systemic health and wellbeing. Only consenting parents/subjects were admitted to the studies. The three studies were based on the permanent dentition only. Subjects with acute infections and those requiring antibiotic cover were excluded (Paper I). High school students (age 13-19 years) who had used antibiotics within the previous three months, received periodontal treatment before, and those who had acute infections were excluded (Papers II and III). Family members were recruited in the study if they had no recognized systemic disease, if they were not receiving periodontal treatment or had not used antibiotic or antiviral treatment within three months prior to the study. Pregnant and lactating women and fully edentulous individuals were excluded (Paper III).

4.1.3 Ethical issues

Ethical clearance for the studies was granted by ethical committees at the University of Science and Technology (UST), Omdurman and the Ministry of Health and the Ministry of Education in Sudan. Written consent was obtained from the participants and the parents or guardians as well as from school principals. Written consent was obtained from the parents or guardian, when participants were less than 18 years old. Participants were informed of the study objectives and methods, that their participation was voluntary and they were at liberty to withdraw at any time.

5 Results

5.1 Paper I

A total of 41 subjects were identified with aggressive periodontitis, a prevalence of 3.4%. Moreover, 16.3% and 8.2% of the subjects had at least one tooth with ≥4 mm and ≥5 mm attachment loss, respectively. African tribes had significantly higher prevalences of aggressive periodontitis compared with Afro-Arab tribes (6.0% vs. 2.3%, $p<0.01$), and had a higher prevalence of attachment loss of ≥4 mm and ≥5 mm. (19.8 vs. 14.7%, $p<0.02$; and 12.0% vs. 6.4%, $p<0.004$, respectively). African ethnicity was also associated with higher risk of being diagnosed with this aggressive periodontitis (OR 2.7, 95% CI 1.6-4.4) and with a significantly higher number of teeth with attachment loss than Afro-Arab ethnicity ($p< 0.01$). Gender played a role in the prevalence of aggressive periodontitis in this population as well, with a significantly higher proportion of males showing the disease (4.9% vs. 2.0%, $p<0.01$), and a higher risk for this disease (OR 2.5, 95% CI 1.2-4.9) than females. No significant difference

was found in the prevalences of subjects with attachment loss ≥4 mm and ≥5 mm in the two gender groups.

5.2 Paper II

Twelve aggressive periodontitis patients (70.6%) and only one (5.9%) control subject harboured non-JP2 types of *A. actinomycetemcomitans* in their subgingival plaque, showing a significantly higher frequency of detection in cases than in controls ($p<0.0001$). On the other hand, the JP2 clone of *A. actinomycetemcomitans* was not detected in the subgingival plaque of either the cases or the controls. The odds ratio for the detection of non-JP2 types of *A. actinomycetemcomitans* in the subgingival plaque of the aggressive periodontitis cases was 38.4 (95% CI 4.0 - 373.0).
Total agreement between PCR and LAMP methods was found in identifying JP2 clone and non-JP2 types of *A. actinomycetemcomitans*.

5.3 Paper III

The prevalence of *A. actinomycetemcomitans*, *P. gingivalis* and HCMV were significantly higher in the subgingival plaque of aggressive periodontitis patients than in the periodontally healthy controls (70.6% vs. 5.9%, $p< 0.0001$, 82.4% vs. 41.2%, $p<0.01$, 70.6% vs. 11.8%, $p<0.001$, respectively). Higher prevalences of T*. forsythia* and EBV-1 were found in the subgingival plaque of cases compared with controls, in contrast to *T. denticola* the prevalence of which was found to be higher among controls than cases, although not significantly so. *A. actinomycetemcomitans*, on the other hand, was found to have significantly higher prevalence in the saliva of cases than in the controls ($p<0.001$). The odds ratio for the detection of *A. actinomycetemcomitans* in the subgingival plaque of patients with aggressive periodontitis was 38.4 (95% CI 3.9 to 373.1), HCMV (OR 18.0, 95% CI

2.9 -109.7) and *P. gingivalis* (OR 6.7, 95% CI 1.4 - 32,3).

Subgingival co-infection with *A. actinomycetemcomitans*, HCMV and/or EBV-1 was restricted to the cases and their family members (p<0.001). On the other hand, co-infection with *P. gingivalis*, EBV-1and HCMV was significantly higher in the subgingival plaque of cases than controls (p<0.001). Comparison between the presence of the pathogens in the subgingival plaque vs. whole stimulated saliva revealed significantly higher prevalences of *A. actinomycetemcomitans* and *P. gingivalis* in subgingival plaque than in saliva in this population (58% vs. 38%, p<0.0001, 60% vs. 40%l, p<0.04, respectively). Increased risk of aggressive periodontitis was highest when *A. actinomycetemcomitans* was detected together with EBV-1 (OR 49.0, 95% CI 2.5-948.7) and HCMV (OR 39.1, 95% CI 2.0 - 754.6). Moreover, the dual presence of herpes virus with *A. actino-mycetemcomitans* or *P. gingivalis* resulted in an increased risk of aggressive periodontitis (OR 31.3, 95% CI 1.6-604.1).

The characteristics of the family members of cases and controls are presented in Table 3.

Table 3. Demographic characteristics and clinical parameters of family members of cases and controls.

	Family members of cases	family members of controls
Number of subjects ***(n)***	54	34
Sex		
Female	61,11 %	73.53%
Male	38,89 %	26.47%
Ethnicity		
African	62,96 %	58.82%
Afro-Arab	37,04 %	41.18%
Age in years - mean (SD)	33.13(20.14)	35.73(15.34)
PPD (mean ±SD) mm	8.8±1.5	5.9±1.6
BOP(%) of positive sites	64.3	49.7
PAL (mean ±SD) mm	6.5±1.2	4.3±0.4
Number of teeth present (mean ±SD)	23.6±4.6	26±2.3
Subjects with previous periodontal treatment (%)	9.2%	8.8%

6 Discussion

This section discusses important methodological considerations and main findings which make up the present thesis. It is based on cross-sectional and case-controls studies. The data were collected in the period August 2007-March 2008.

6.1 Methodological considerations

6.1.1 The study design

Cross sectional studies investigate the presence or absence of an event and the associated factors at one point in time (78). The finite data on aggressive periodontitis in Sudan required the employment of a cross-sectional study (survey) to asses, firstly, the prevalence and extent of the disease in this African region. In the field of periodontal epidemiology, selection of the study sample and selection of

periodontal recording protocol are important issues that may influence study outcome (5). In Paper I, we used a multistage stratified sampling design, which is a complex form of cluster sampling. The design is employed in most large surveys (110,111). Selection of proper primary sampling units (PSU) is a vital step in this type of sample design. In our study, the PSU (schools) were proportional to the number of students in each municipality. This study design has the advantage of being more cost effective and more accurate than cluster sampling for a given sample size and more efficient, since it relies on multiple randomizations (112). It is well recognized that school-based studies have the advantage of overcoming selection bias when compared with corresponding hospital-based studies (113). The nature of African/Sudanese health settings, characterised by inadequate access to dental care and health facilities due to long distances and costs of treatments, required recruiting subjects from non-hospital settings. Moreover, it is suggested that the burdens of African life eclipse the need to seek periodontal treatment (114). Thus, we based our study on school students. With an estimated drop-out of 3.32% (115), our results are representative only for the population attending high schools in Khartoum state. As mentioned previously, aggressive periodontitis is known to have a circumpubertal onset (1). Therefore, students at the age group of high schools (13-19 years) were considered an ideal target population.

It well recognized that detection of *A. actinomycetemcomitans* in the subgingival plaque of subjects can be used as a marker of risk for initiation and development of aggressive periodontitis (55). Aggressive periodontitis can be considered a disease with relatively low prevalence. The presence of the JP2 clone of *A. actinomycetem-comitans* among some aggressive periodontitis populations - e.g. the Moroccan population - has been found to be endemic and a significant risk factor for initiation of

the disease in that population (51). The Sudanese population is of diverse ethnic backgrounds and, in that way, to some extent, it resembles the Moroccan population in being of both Arabic and African origin. The second paper of this thesis used a case-control design to assess the presence of JP2 clone and non-JP2 types of *A. actinomycetemcomitans* as a risk factor for developing aggressive periodontitis among Sudanese adolescents. The case-control study design is often used to identify associations between diseases or medical conditions (usually of low prevalence or rare) and the risk factors for this disease, by assessing the risk of the condition among cases and controls (112,116).

It could be argued that the number of participants in Studies II and III was relatively small. On the other hand, in both studies, cases and controls were matched by gender and ethnicity and belonged to the same age group (13-19 years). Lists of eligible matched controls were prepared for each case, and then a control was randomly selected, which reinforced the study design.

Several investigators have reported recruitment of families in school-based studies to be challenging (117). Case-control studies may sometimes suffer from substantially different participation rates between cases and controls, cases being keener to participate than controls (118). In our studies, family members of cases showed a considerably higher response rate than families of controls. It has been suggested that "an individual - in order to take a voluntary health action - must have readiness to act" (119). In these studies, every effort was made to explain to the participants about the sterilization method, the sampling procedures, objectives, the study rationale and the importance of the early diagnosis of the disease. To encourage participation, an incentive was offered after examination (toothpaste, toothbrushes and an offer of free treatment at UST dental clinics if subjects were diagnosed with aggressive

periodontitis) (120). A majority of the participants lacked dental awareness and dental knowledge. Several unconventional beliefs, perceptions and ideas about oral health were noted among the recruited subjects in these studies and among their families. The main reasons for non-participation were fear of HIV/AIDS and other infectious disease transmission during sampling and some of the non-participants believed that periodontal problems were minor problems; they felt it natural for people to lose all their teeth in old age, thus eventual loss of teeth caused by aggressive periodontitis was not a hazard. It has been suggested that the difficulties of African life, dealing with life threatening diseases and infections and poverty override other problems relating to dental diseases (114). In this context, the non-participating subjects preferred putting up with the periodontal destruction rather than acquiring more serious diseases. Traditional beliefs such as refusal to donate biological samples due to fear of black magic practices and religious beliefs and fear of the examination and shortage of time also contributed to a reluctance to participate among some people. Another major inconsistency in participation was observed in Study III. A higher participation rate was observed in this study for saliva sampling compared with subgingival plaque sampling. This finding could be interpreted as confirming the advantage of non-invasive saliva sampling compared with subgingival plaque sampling.

6.1.2 Interview

A structured written questionnaire was used to interview students at their schools (Appendix 1, Paper I) and relatives of students at their residences (Paper III) (Figure 10A). The questionnaire gathered demographic and behavioural information, medical data and oral-health related information. The demographic information included ethnicity and tribe. Tribe was reported by naming the tribe. Ethnicity, on the other hand

was self-declared by participants, based on a structured ethnical chart and categorized into Afro-Arabs and Africans (Table 4).

Table 4. Main ethnic and linguistic groups in Sudan. Adapted from Mosely, A.L. , 1998 (157).

Afro-Arabs (40%)	Africans (60%)	
	Southern people (34%)	Non-Arab peoples of Northern Sudan (26%)
• **Ja’aliyin Arab**: Danagla Arabs, Hassaniya, Kawahla, Gima, Husainat • **Juhayna Arab**: Jamala (Kababish, Shukriya), Baqqara (Silaim, Hawazma, Misiriya, Humr, Rizaiqat, Ta’aisha, Bani Rashid, Rashaida, Habaniya) • **Gezira Arab**: Mesellimiya, Halawin, Rufa’a • **Zibaidiya Arab** • **Hawawir Arab** (Berber stock): Hawawit, Jellaba, Hawara, Korobat • **Mixed Arab-Nubian**: Shaiqiya, Manasir, Rubatab, Mirifab • **Christian Arab**:Copt, Syrian Orthodox.	• **Nilotic linguistic groups**: Dinka, Nuer , Shilluk, Anouak, Acholi, Bor Bélanda, Jur, Shilluk Lwo, Pari • **Nilo-Hamitic linguistic groups**: Bari-speaking (Bari, Kuku, Pojulu, Kakwa, Nyangwara, Mundari, Nyepo, Lokoyo, Luluba, Latuko, Logit, Lango, Toposa, Domjiro, Jiye, Mourle Group • **Sudanese linguistic groups:** Azande , Muru, Ndogo, Sere, Mundo, Biri (Balanda/Fertit), Madi, Bongo (Fertit), Baka, Feroge	• **Beja**: Beni Amer, Amarar, Bisharin, Hadendowa • **Dar Fur:** Fur, Daju, Beigo, Zaghawa, Berti, Masalit, Gimr, Tama. • **Nuba :** over 50 groups, including Nyimang, Temein, Katla, Tima, Tegali, Koalib-Moro (Heiban, Shwai, Otoro, Tira, Moro), Daju, Tulishi, Keiga, Miri, Kadugli, Korongo, Talodi-Mesakin, Lafofa, and “Hill Nubians”. • **Nubian** • **West African** (fallata) : Fulani, Hausa, Kanuri, Songhai (Zabarma).

6.1.3 Clinical examination

The subjects received an in-school examination conducted under field conditions (Appendix 2, Figure 10B). Instruments were sterilized prior to the examination day using a portable steam sterilizer (Figure 10C) (121). It might be argued that conducting the clinical examination under field conditions could contribute to higher measurement error. However, every effort was made to ensure the examiner had adequate visibility and accessibility during the examinations. Moreover, estimates of measurement reproducibility showed adequate accuracy of the assessments. It has been customary to use a threshold of ≥3 mm of periodontal attachment loss, although lower and higher thresholds have also been used (5). We used more stringent thresholds of ≥4 mm and ≥5 mm of attachment loss for the diagnostic criteria of aggressive periodontitis to minimize the number of false positives (122). The use of radiographic examination was not feasible in this study so we prioritized clinical examinations over radiographic. It has been suggested that projection geometry with unstandardized radiographs may disguise incipient bone loss and thereby underestimate the prevalence of disease (114). Moreover, results based on radiographic assessment are influenced by the clinical judgment of the examiner, variations in the radiographic techniques and physiological alterations in alveolar bone level (82). On the other hand, longitudinal studies have shown that soft tissue destruction (periodontal attachment loss) precedes hard tissue destruction (alveolar bone loss) (123,124). In the field of periodontal epidemiology, different sampling protocols are used to measure periodontal attachment loss within subjects, including full mouth assessment or partial recording protocols. It has been suggested that examining selected teeth in the mouth may underestimate the prevalence of the disease. Localized aggressive periodontitis typically involves incisors and first molars,

although few studies suggest that this is a site specific association rather than a tooth specific one (125). Moreover, molars and incisors were found to be associated with the highest occurrence of attachment loss (111,126,127). Accordingly, we examined a representative set of teeth including all incisors, first and second molars. This provided a total of 96 sites per subject, i.e. six sites per tooth to be examined, in this study. It is worth noting that despite not performing radiographic and full mouth examinations on this population, the prevalence of aggressive periodontitis was still high.

Figure 10. Examination in the field. **A**) Interviewing a family member of an aggressive periodontitis subject to complete the questionnaire at the home. **B**) Clinical examination at schools, with the examiner wearing a head light and an assistant recording the findings. **C**) Portable steam sterilizer developed by World Health Organization and the United Nations Children's Fund.

6.1.4 Microbial examination

We collected pooled subgingival plaque samples from the deepest periodontal pockets, one in each quadrant from the participants (Study II and Study III). Associations between deep pockets and high recovery rates of putative periodontal pathogens, notably *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia,* have been reported in earlier studies (128,129). We regarded the deepest periodontal pocket in each quadrant as representative sites for recovering putative pathogens (130,131). Obtaining representative subgingival plaque samples is crucial for determining colonization with putative pathogens. Several techniques have been employed, of which paper points and curettes are the most widely used. The sampling efficiency of both techniques has been controversially discussed by several investigators (132-135). Curettes were argued to sample both tightly attached subgingival plaque as well loosely adherent plaque. However, paper points were found to collect plaque from the outer layer of the biofilms, in which most of the pathogenic bacteria reside and higher proportions of putative bacteria were found in these layers (93,135,136). Moreover, sampling with paper points has the advantage that the complete tips can be placed in a transport vial after probing. A study, comparing the sampling of subgingival bacteria using paper points and curettes, reported that curettes harvest significantly higher amounts of subgingival bacteria (134). In addition, paper points are considered less invasive compared with curettes, which made paper points our method of choice for sampling in both apprehensive and co-operative subjects (137). No significant difference was reported in the composition of subgingival plaque with respect to the selected target pathogens, making both paper-points and curettes suitable for microbiological diagnostics. Pooling paper-points is a widely established technique that minimizes the effect of

sampling different sites in the same subjects and it retains the subject as unit of analysis (138). Only non-culture based techniques were applied in these studies, due to the previously mentioned advantages of these techniques over culture based ones. Employing non-culture based techniques helped overcome some field work difficulties we encountered. It is well recognized that members of *Pasteurella* family *e.g. A. actinomycetemcomitans,* survive best at 37 °C (139,140). We considered that conducting the sampling procedures in our study in the hot climatic conditions of Sudan (45-47 °C), had contributed to loss of viability of *A. actinomycetemcomitans*, despite the storage in generous amounts of dry ice. Therefore, the use of non-culture based methods was considered a useful approach to tackling such field work challenges.

6.2 Discussion of the findings

6.2.1 Findings on epidemiology of aggressive periodontitis

Paper I shows that the prevalence of aggressive periodontitis among Sudanese adolescents is high. This is in agreement with previous epidemiological studies investigating the prevalences of aggressive periodontitis in African populations. In a series of longitudinal and cross sectional studies, Haubek *et al.* reported a Moroccan population of 301 adolescents aged 14-19 years to have a high prevalence of 7.6%(51,141). In Nigeria, a study based in Lagos surveyed 1001 students aged 12-19 years; aggressive periodontitis was identified in 0.8% of the subjects (142). On the other hand, a higher prevalence of the disease, 1.6%, was reported among 17-34 years old subjects residing in Ibadan, Nigeria (113). Prevalence estimates in Uganda showed a high prevalence of the disease, where researchers reported a prevalence of 28.8% among school attendees aged 12-25 years (79). Relatively lower

prevalences were reported in other African populations. A study among participants of a national youth training program in Kenya surveyed 350 subjects aged 18-26 years and identified persons with aggressive periodontitis, giving a prevalence of only 0.3% (143). The authors of this study suggested that inclusion criteria used in their study may have influenced the results. Remarkable inconsistencies are observed between earlier and later aggressive periodontitis studies conducted on African populations; there has been a propensity for a few early studies to report lower prevalences of the disease (6,144,145). Therefore, results drawn from inter-study comparisons must be interpreted with caution. These inconsistencies might be due to differences in study design, case definition or the type of the target population (114). In this thesis, we assess the prevalence of aggressive periodontitis using a larger sample size compared with the previously mentioned studies and used a study design that allows multiple randomizations.

Moreover, we evaluated ethnicity as a risk factor for developing aggressive periodontitis. With ethnicity categorized as Afro-Arab or African, subjects of African origin were found to be twice as likely to have attachment loss ≥ 5 mm and three times more likely to have aggressive periodontitis than Afro-Arab subjects. Several studies have emphasized the role of ethnic background as a risk factor for aggressive periodontitis and for harbouring specific pathogens associated with the disease (40,146). Subjects of African, African American and Afro-Caribbean origins (blacks) have a higher prevalence of the disease compared with other ethnic groups (141,146-149). Furthermore, higher responses of neutrophil chemotaxis to n-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) antigens were observed in black patients with aggressive periodontitis, compared with white patients, suggesting that the association between ethnic origin and aggressive periodontitis may be due to

genetic predisposition (150). Thus, we anticipate that a higher prevalence of aggressive periodontitis will be observed in other regions of Sudan where African tribes are the majority population. It would be interesting to assess other risk factors associated with the disease.

Moreover, a significant association between gender and aggressive periodontitis was found, although no significant differences were found between attachment loss ≥ 5 mm and gender. Male participants were found to be 2.5 times more likely to have aggressive periodontitis than female ones in this population, which is in agreement with reports on the role of gender as a risk factor for aggressive periodontitis (88). Although an increased severity of attachment loss and the prevalence of the disease might be expected with increasing age, this association was not observed in our study population. This may be attributed to the fact that the age range of participants in Paper I (13-19 years) is small compared with other studies in which the recruited subjects belonged to wider age ranges (80,83,111). Increased severity of periodontal destruction is suggested to be associated with accumulated tissue destruction, rather than being age-related (151-153). The narrow range of age evaluated in this study did not permit testing the effect of this accumulated damage on the prevalence of the disease.

6.2.2 Findings on microbiology of aggressive periodontitis

It is well established that the microbial profile of aggressive periodontitis subjects has a distinct geographical distribution. Papers II and III deal with the microbial profile of aggressive periodontitis patients and with the association of the putative pathogens with the disease. In Paper II, the case-control study, we evaluated the association between outcome (aggressive periodontitis) and risk factor (*A. actinomycetem-comitans*). The results revealed a strong association between subgingival

colonization and aggressive periodontitis (OR 38.4). On the other hand, the JP2 clone of *A. actinomycetemcomitans* was absent from the studied sample population. We further compared these aggressive periodontitis patients with periodontally healthy subjects. The results obtained from Paper II have clearly indicated *A. actinomycetemcomitans* as a risk factor for developing aggressive periodontitis. It will be of great interest to characterize non-JP2 types identified in this population.

In Paper III, we further determine the prevalence of putative periodontal bacteria and herpes viruses. Collectively, we found that *A. actinomycetemcomitans*, HCMV and *P. gingivalis* are linked to aggressive periodontitis (OR 38.4, 18.0 and 6.7 respectively). These results are in agreement with previous reports on the role of these pathogens on aggressive periodontitis and periodontal destruction (13,154). High prevalences of herpes virus infections were reported in this population, which is in accordance with reports showing increased estimates of these infection in low-income countries compared with high-income countries (155). *A. actinomycetemcomitans* is the strongest indicator of aggressive periodontitis in this population, followed by HCMV. Moreover, we found a strong tendency towards associations between bacterial-herpes virus co-infections and aggressive periodontitis, where we showed that co-infection with EBV- *A. actinomycetemcomitans* (OR 49.0, 95% CI 2.5-948.7) and HCMV- *A. actinomycetemcomitans* (OR 39.1, 95% CI 2.0 - 754.6) are the main risk indicators for developing aggressive periodontitis in these subjects. This finding of a close association between the pathogens supports the evidence that active infection with HCMV facilitates adherence of *A. actinomycetemcomitans* to the epithelium of periodontal pocket (156).

Findings in this study support the evidence on the role of bacterial-viral paradigm on aggressive periodontitis (154). We suggest that the etiology of aggressive

periodontitis in this population is mutlifactorial with several risk factors associated with disease. We propose that those subjects with aggressive periodontitis had an active herpes virus infection which increased the virulence of the commensal bacteria *e.g. P. gingivalis* or enhanced the adherence of opportunistic bacteria such as *A. actinomycetemcomitans* to the periodontal tissues. The aberrant immune responses caused by the active herpes virus infections could predispose individuals with aggressive periodontitis to increased bacterial carriage and impaired antibacterial immune responses, giving rise to increased numbers of bacteria. However, more research is needed to examine the pathogenesis of aggressive periodontitis.

7 Conclusions and future perspectives

The studies constituting this thesis have generated additional knowledge on the prevalence of aggressive periodontitis among Sudanese adolescents and risk factors associated with the disease in this population, by employing epidemiological and molecular approaches. Collectively, the prevalence of aggressive periodontitis was found to be high among high school students in Khartoum state, Sudan. African ethnicity, being male, subgingival colonization with *A. actinomycetemcomitans,* HCMV and *P.gingivalis* were identified as the risk factors for developing aggressive periodontitis in this population. Whole stimulated saliva sampling was an alternative and/or concurrent tool, along with subgingival sampling, for detection of *T. forsythia* and *T. denticola,* EBV-1 and HCMV. Whole stimulated saliva may not be an appropriate sampling tool for detection of *A. actinomycetemcomitans* and *P. gingivalis* in this population.

Our results have uncovered several unanswered issues that require elucidation. Data in the present thesis should encourage the further collection of clinical samples from

this population to examine intra-species phylogenetic differences and investigate the possibility of identifying especially virulent pathogenic strains, clones or subpopulations of *A. actinomycetemcomitans* associated with aggressive periodontitis. Substantial increase in the sample size of the clinical samples would help in formulating more definitive conclusions to questions raised about the etiology of aggressive periodontitis in young Sudanese adults. Longitudinal studies would be ideal for following this cohort of subjects, to determine alterations in attachment loss and the severity of aggressive periodontitis, the status of the disease (active vs. stable) and their associations with present risk factors. Our currently available data on the microbial profile of subjects with aggressive periodontitis needs to be revisited using a quantitative approach (real-time PCR). Findings in Paper III advocate further exploration to relate the severity of aggressive periodontitis in this population with the load of herpes virus and the status of the viral infection (active vs. latent) using molecular approaches. Data generated by Papers II and III strongly indicate the need to raise the dental health awareness and promote oral health programs among the Sudanese population. School-based oral health programs are recommended for early diagnosis of aggressive periodontitis in this population.

8 References

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