Association between CD4+T lymphocyte levels and "red complex" pathogens of Chronic Inflammatory Periodontal disease in HIV-positive patients

By

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KEY WORDS

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ABSTRACT

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Background: Infection with HIV results in gradual loss of immunologic functions, especially those mediated by CD4+T helper cells with consequent impairment of the immune response leading to severe manifestations of periodontal disease. The lower the CD4+T lymphocyte cell count or the higher the level of immunosuppression, the higher the incidence of periodontal disease in those patients will be. Putative periodontopathic bacteria namely *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia,* commonly referred to as "red complex", and many other bacterial species have been implicated in the initiation and progression of periodontal disease. **Objective:** The present study tests the association between different CD4+T lymphocyte levels and "red complex" pathogens using BANA, in HIV-positive patients with chronic inflammatory periodontal disease (CIPD). **Methods:** 120 HIV-positive patients from the infectious disease clinic at Tygerberg hospital participated in the study with a mean age of 33.3 years. The CD4+T lymphocyte counts were obtained from patient's medical records. The six Ramjford teeth were used for evaluating periodontal clinical parameters such as plaque index, gingival index, periodontal probing depth and clinical attachment loss. Subgingival plaque samples were collected and analyzed by the enzymatic BANA test for the detection of the "red complex". **Results:** The CD4+T lymphocyte mean level was 293.43cells/mm^3.Statistically significant associations were found between CD4+T cell counts and probing depth (p= 0.0434) and clinical attachment loss (p= 0.0268). Significant associations were found between BANA with all the clinical indices (p= <0.05). However no association was found between CD4+T cell counts and BANA. **Conclusion:** HIV-positive patients show a high prevalence of "red complex" pathogens subgingivally. Immunosuppression seems to favour the colonization of these species, resulting in periodontal disease manifestations.

DECLARATION

I declare that this work is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references

Cathy Nisha John   **18-09-2012**

Signed: ……………………..

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DEDICATION

I dedicate this thesis to my parents, my husband and my son for their unreserved encouragement, love and sacrifice for my success.
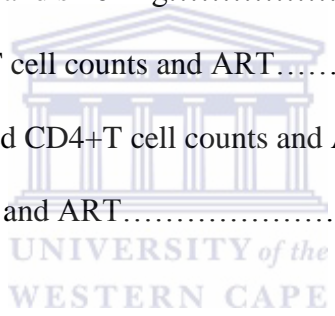
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1: <u>LITERATURE REVIEW</u>

1:1 <u>INTRODUCTION</u>

According to the World Health Report in 1996, a global crisis exists across the world in respect to infectious diseases. Among the infectious diseases, human immunodeficiency virus infection, which eventually results in AIDS, remains as a global health problem. The Sub-Saharan African populations account for about 70% of the World's total bulk of HIV-infected individuals and AIDS patients (WHO, 1996 a). HIV infection is characterized by the decline of CD4+T lymphocytes, thus lowering the immune status of the patients.

Periodontal disease has been associated with HIV (Holmstrup, 1994). The presence and severity of periodontal disease may be considered as one of the first clinical presentations of a previously undiagnosed HIV infection (Howard, 1991). Periodontal disease is an inflammatory disease with a multifactorial etiology. Inadequate oral hygiene and a decrease in CD4+T lymphocyte cell count results in plaque formation and ultimately, periodontal inflammation with destruction of the supporting tooth structures (Kroidl, 2005). Microbial complexes of subgingival plaque in HIV-positive patients with periodontal disease have been studied (Aas *et al*, 2007; Goncalves *et al*, 2007) and more than 500 bacterial species are capable of colonizing the oral cavity (Socransky, 1998; Wilson, 1997; Kolenbrander, 1995).

A literature search identified more than 383 publications regarding the relationship between periodontal disease and HIV infection between 1986 and June 2000, and yet this area of research remains poorly understood (Robinson, 2002). The progression of HIV infection and the severity of periodontal inflammation and subsequent destruction of the periodontium have been clearly demonstrated (Drinkard, 1991) with a recent study reporting an increased risk of dental and periodontal disease in HIV-positive and AIDS patients among American Indian and Alaska Natives when compared with the general population (Goddard *et al*, 2005). Moreover, many studies have also related the degree of immunosuppression and HIV viral load to the progression of periodontal disease (McDonell, 1990; Mellors, 1997) and proposed that the increased number of mast cells, macrophages and neutrophils in chronic periodontitis may release inflammatory mediators causing loss of periodontal tissue and severe destruction of the periodontium in HIV-positive individuals (Myint *et al*, 2002). However; several other studies do not indicate a strong association between the periodontal condition and HIV infection (Martinez-Canut *et al*, 1996; Smith *et al*, 1995; Tomar *et al*, 1995; Doshi *et al* 2008;

Goncalves, 2005 a). The purpose of the current study was to investigate the presence of subgingival "red complex" periodontal pathogens in HIV-positive patients, with clinical indices used in the diagnosis of chronic inflammatory periodontal disease at various CD4+T lymphocyte levels.

1:2 AETIOLOGY AND PATHOGENESIS OF PERIODONTAL DISEASES

The primary aetiology of periodontal diseases is the formation and adhesion of dental plaque on the surfaces of the tooth (Carranza, 2009 a). Dental plaque can be defined as the diverse community of micro-organisms found on the tooth surface as a biofilm, embedded in an extracellular matrix of polymers of host and microbial origin (Marsh, 2004 a). The dental plaque biofilm develops under different conditions and environments. According to recent studies, dental plaque consists of at least 800 bacterial species (Aas *et al,* 2005, 2008; Preza *et al,* 2008; Paster *et al,* 2006). Formation of plaque involves three major phases: (1) pellicle formation on the tooth surface, (2) initial adhesion and bacterial attachment, (3) colonization and plaque maturation. The pellicle consists of glycoproteins, phosphoproteins and enzymes. The mechanism involved in enamel pellicle formation includes electrostatic, van der Waals and hydrostatic forces. Transport of the bacteria to the tooth surface takes place by Brownian motion and initial adhesion is by the electrostatic bonding between the host receptors and bacterial adhesins (fimbriae). The bacteria from the pellicle undergo proliferation to form colonies and co-aggregation of bacteria contributing to the complexity and composition of dental plaque formation (Filoche, 2010).

Dental plaque is classified according to location into supragingival and subgingival plaque (Carranza, 1997 b). Supragingival plaque is a thick, non-calcified mass of plaque found at or above the gingival margin and consists predominantly of Gram-positive saccharolytic bacterial flora, which are cariogenic. Subgingival plaque is located entirely within the gingival sulcus or periodontal pocket, and is mainly composed of proteolytic Gram-negative bacterial microflora responsible for periodontal diseases (Slots, 1992; Genco, 1990). Hence dental plaque can be considered as an aetiologic agent for both dental caries and periodontal diseases (Marsh, 2006 b).

The periodontium serves as a reservoir for microorganisms. The first microscopic examination of the complexity of subgingival microbiota was recognized by Van Leeuwenhoek in 1683 (Tal, 1980). Inadequate oral hygiene, leads to the accumulation of

bacteria in the gingival crevice and with the gradual increase in the proportions of Gram-negative, anaerobic periodontopathogens there is a gradual progression to periodontal infections. Evidence in the literature indicates that the destructive process begins with biofilm accumulations that contain bacterial masses in the range of 1 to 2×10^{11} bacteria per gram at or below the gingival margins (Gibbons, 1980). The destruction proceeds with the release of toxic products such as lipopolysaccharides (LPS) and hydrolytic enzymes, from the pathogenic plaque bacteria (AAP, 1998 b). LPS induce the release of proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) which may enter the blood stream and trigger the induction of systemic inflammatory responses (Yiorgos, 2006).

Dental plaque becomes mineralized from mineral components of saliva and becomes calculus. The rough surface of calculus provides an ideal medium for further plaque growth, threatening the health of gingiva (Lindhe, 2008). The understanding of the aetiology and pathogenesis of periodontal disease has remarkably changed over the last 30 years (Page, 1998). At the 1996 World Workshop, it was concluded that most of the human periodontitis is caused by *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans* (earlier named as *Actinobacillus actinomycetemcomitans*) (Consensus Report, 1996). There are other species in the mouth which in most of the cases are the primary pathogens causing periodontal disease, but they act indirectly in the progression of periodontal disease. A diverse group of Gram-negative species coaggreggate with the initial primary colonizers such as the streptococci, with minor proportions of *Actinomyces*, *Gemella*, *Neisseria*, *Rothia* and *Veillonella* species. The succession of bacterial colonization influences plaque maturation leading to the establishment of periodontal pathogens depending on the increase in bacterial diversity over time (Kolenbrander *et al*, 2006). On day one of plaque formation, *Actinomyces* species show unusually high proportions followed by *Fusobacterium nucleatum* on day four of plaque development, which in turn coaggregates with several late colonizers. In moderately deep pockets, the surface layers of subgingival plaque are inhabited by *Treponema denticola* while *Porphyromonas gingivalis* is predominantly restricted to the inner layers. However, both bacterial species co-exist in deeper pockets (Kigure *et al*, 1995). *Fusobacterium nucleatum* releases fatty acids which are required for the growth and metabolism of *Treponema denticola* (Miyakawa and Nakazawa, 2010) and also provides nutrional requirements for *Tannerella forsythia* (Tanner, 1986). Apparently, *Fusobacterium nucleatum* facilitates nutrional interdependence with other

oral microorganisms and coaggregates with all other oral bacteria (Whittaker, 1996) and should therefore be considered as an important contributor for the oral biofilm formation.

Periodontal pathogens are essential but not sufficient to initiate the destruction of the periodontium (van Winkelhoff, 2002). The susceptibility of the host in the initiation and progression of the disease is also an important factor. The virulence of microorganisms depends on its ability to invade and grow within a susceptible host. The inflammatory host response to the periodontal pathogens leads to clinical manifestation of the disease (Kinane, 2001 a). Experimental evidence indicates that any shift in the T cell immune response leads to periodontal disease progression (Geatch *et al*, 1997; Preshaw *et al*, 1998; Seymour *et al*, 1993). For the progression of periodontitis into an advanced disease, both immune and inflammatory responses are essential and are confounded by various intrinsic (eg: genetics) and induced (eg: pollutants) host-related factors (Page and Kornman, 1997; Offenbacher, 1996; Taubman *et al*, 2007). These environmental responses lead to the bacterial succession favouring the ecological plaque hypothesis. The ecological shifts in the environment are responsible for the pathogenecity and virulence factors of the bacterial species (Marsh, 1991 c). According to the ecological plaque hypothesis, periodontal disease is an opportunistic endogenous infection brought about by an ecological shift in the plaque biofilm from a predominantly Gram-positive facultatively anaerobic microflora to a Gram-negative obligate anaerobic or micro-aerophilic flora, resulting from host-microbial and microbe-microbe interactions, creating an anaerobic environment which favours their growth (Konopka, 2006). With synergy prevailing over antagonism, the oral microbial species in the dental plaque respond to the ecological changes and react as a single unit rather than as individual species (Caldwell *et al*, 1997). A relationship exists between the community members of the oral microbiota favouring the formation of the plaque biofilm (Dawes, 2008; Mineoka *et al*, 2008).

Dental plaque is initially composed of Gram-positive cocci which are gradually replaced by Gram-positive filamentous forms of microorganisms followed by an abundance of Gram-negative colonisation of microorganisms in the gingival sulcus (Kolenbrander *et al*, 1985). Approximately 1,200 predominant species in the oral cavity were revealed in an analysis of more than 36,000 16Sr DNA gene clones (Dewhirst *et al*, 2010). Cooperative and competitive mmunity interactions among the oral bacteria may influence metabolic communication; as for example, the release of vitamin K by the *Veillonella* species supports

the growth of *Prevotella* and *Porphyromonas* species (Hojo *et al*, 2009). The specific interactions among the oral bacteria in the form of co-aggregates have been considered responsible for the development of oral biofilms (Kolenbrander, 2000). Coaggregation can be intrageneric, intergeneric or multigeneric cell-to-cell recognition (Kolenbrander, 1989 a) and is achieved by the colonisation of a synergestic species. Intergeneric coaggregations are found between *Fusobacterium* and other bacteria such as *Aggregatibacter actinomycetemcomitans* (Rosen *et al*, 2003), *Tannerella forsythia* (Sharma *et al*, 2005), and oral *Treponema* species (Kolenbrander *et al*, 1995). Intrageneric coaggregations are found among different strains of oral fusobacteria (Kolenbrander *et al*, 1995), *Porphyromonas gingivalis* (Lamont *et al*, 1992), oral streptococci, and *Actinomyces* species (Kolenbrander and Andersson, 1989 b). Herpes viruses such as *Epstein-Barr virus-1 (EBV-1)* and *Cytomegalovirus (CMV)* may also influence the pathogenesis of chronic periodontitis facilitating synergistic mechanisms with *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* (Chalabi *et al*, 2010; Dawson *et al*, 2009; Slots, 2010 a). Loss of epithelial attachment and adult periodontitis is associated with a consortium of bacteria (Moore, 1994; Darveau *et al*, 1997) or more likey several clonal types of subgingival pathogens rather than one species. *Aggregatibacter actinomycetemcomitans* (Sreenivasan, 1993) and *Porphyromonas gingivalis* (Duncan *et al*, 1993; Lamont *et al*, 1995) invade gingival epithelial cells and mediate extensive tissue destruction.

1:3 CHRONIC INFLAMMATORY PERIODONTAL DISEASES- GINGIVITIS AND PERIODONTITIS

Gingivitis and periodontitis are inflammatory and immunological reactions to the dental plaque or oral biofilm, composed of predominantly Gram-negative anaerobic microorganisms (Grossi, 1994). The initial inflammatory reaction is known as gingivitis and is limited to the gingiva without affecting the deeper connective tissue attachment. In advanced lesions, gingivitis may lead to the destruction of the connective tissue attachment and underlying alveolar bone resulting in periodontitis (Tucker, 2006). By definition, gingivitis is the result of a non-specific inflammatory reaction response to an increase in the mass of bacteria (either Gram-negative or Gram-positive) at or under the gingival crevice (Darveau *et al*, 1997) and periodontitis appears to be an inflammatory disease of specific bacterial origin (predominantly Gram-negative) (AAP, 2001 a). Gram-negative anaerobes predominate as

gingivitis and periodontitis progress clinically (Slots, 1979 b). An advanced and established periodontal disease can cause pain and tooth mobility due to a high degree of alveolar bone loss. Tooth loss may be the ultimate consequence of destructive periodontal disease, leading to impaired function and changes in facial appearance.

Gingivitis is caused by dental plaque which is the most common cause of gingival disease, or it can be of non-plaque origin, including gingival lesions due to bacterial, viral, fungal, genetic or systemic conditions (Lindhe, 2008). Non-plaque induced gingivitis may be induced by microorganisms such as *Treponema pallidum*, *Neisseria gonorrhoeae*, streptococci, herpesviruses and oral *Candida* species (Armitage, 1999). According to the clinical observations and scientific research compiled by Marsh and Martin (2000) as well as Liebana (2002), periodontitis is classified according to the extent of lesions (localized or generalized), rate of progress (slow, rapid and chronic), and the age of onset in affected individuals (aggressive and chronic).

1:4 PREVALENCE OF PERIODONTAL DISEASE

Depending on the diagnostic criteria, periodontal disease is one of the most ubiquitous diseases with a reported prevalence varying between 10% and 60% in adults (Albandar, 2002; Offenbacher, 2001). The prevalence of periodontal disease increases with age (Loesche, 2001) but recently this concept has been challenged and it is considered that with a proper oral hygiene maintenance, periodontal disease can be prevented (Lindhe, 2008). According to the data published in 1996 World Workshop in Periodontics, advanced adult periodontitis does not exceed a prevalence of 10-15% in most populations (Papapanou, 1999 a). About 75-80% of the individuals show varying degrees of susceptibility to periodontal diseases. In addition, about 10% of the population appears to be completely resistant, despite the presence of plaque, and the reason for this is not known (Tucker, 2006)

1:5 MICROBIAL COMPLEXES IN SUBGINGIVAL PLAQUE

As previously mentioned, periodontal disease is initiated by the overgrowth of certain Gram-negative, anaerobic bacteria growing in subgingival areas. The bacterial species associated with periodontal disease are harboured within the dental plaque or oral biofilm both above and below the gum margins. In 1998, Socransky and colleagues examined over 13,000 subgingival plaque samples using cluster analysis, and divided these bacteria into clusters or

microbial complexes, with colour designation assigned for each complex (Table.1). Each cluster was created based on similarities and differences in nutritional and atmospheric environments and was considered to influence other clusters, in addition to being related to a specific periodontal state, indicating that these microbes are very closely associated with one another (Sbordone, 2003; Socransky, 2000). The "blue", "green", "yellow" and "purple" complexes were considered as early plaque colonisers. As the biofilm matures, organisms of the "orange" complex appear which are required for the subsequent colonisation of the more pathogenic "red" complex. The "orange" and "red" complex bacterial species are considered to be the major etiologic agents of periodontal diseases (Socransky, 1998). Furthermore, microbial species found in the "red" cluster were rarely observed without the presence of the "orange" cluster species, showing a great deal of inter-species communication (Socransky, 2000). The "orange" complex microorganisms were associated with pocket depth but less frequently related with the other clinical parameters of periodontal disease, while the "purple", "yellow" and "green" complexes were considered to be more strongly associated with each other than with either the "red" or "orange" complexes, suggesting a possible pattern or sequence of microbial colonization (Feres *et al*, 2004).
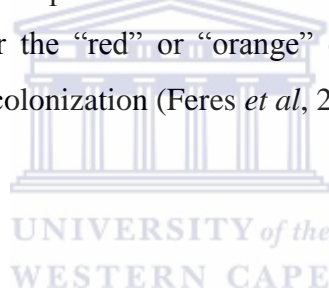
Table 1: Bacterial clusters described by Socransky *et al* (1998)

Complex	Bacterial cluster
Red	*Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia*
Orange	*Fusobacterium nucleatum, Prevotella intermedia, Prevotella nigrescens, Peptostreptococcus micros* (Associated species included, *Eubacterium nodatum, Campylobacter rectus, Campylobacter showae, Streptococcus constellatus, Campylobacter gracilis*)
Green	*Capnocytophaga, Campylobacter concisus, Eikenella corrodens, Aggregatibacter actinomycetemcomitans* (serotype a)
Yellow	*Streptococcus sanguis, Streptococcus oralis, Streptococcus mitis, Streptococcus gordonii, Streptococcus intermedius*
Purple	*Veillonella parvula, Actinomyces odontolyticus, Aggregatibacter actinomycetemcomitans* (serotype b), *Selenomonas noxia, Actinomyces naeslundii* genospecies 2 (*Actinomyces vicosus*)

1:6 <u>RED COMPLEX PREVALENCE</u>

Periodontal disease is a poly-microbial infection. Most forms of periodontal inflammation appear to be associated with elevated proportions of spirochetes of the genus *Treponema* within the subgingival plaque samples. In the progression of periodontal disease, the "red complex" is considered as the most significant of the bacterial clusters since they increase in numbers and prevalence with increasing clinical parameters of the disease (Gmur *et al*, 1989, Haffajee, 1994; Socransky *et al*, 1998; Hosaka, 1994; Simonson, 1992, Holt and Ebersole, 2005), such as in sites with deeper pockets (Kigure *et al*, 1995) and in areas that show bleeding on probing (Kasuga, 2000). The "red complex" microorganisms invade the periodontal pocket and are attacked by the host's white blood cells. The ability of *Tannerella*

forsythia to induce apoptosis eliminates the host's immune or pre-immune cells resulting in bacterial colonization in the periodontal pocket (Holt and Ebersole, 2005). Moreover, they are significantly associated with attachment loss and alveolar bone loss eventually resulting in tooth loss (Machtei *et al*, 1999). Elimination of these pathogenic bacteria by the traditional debridement procedures such as scaling and root planing, have been associated with an improved clinical response (Haffajee *et al*, 1997; Christersson, 1991). The synergestic mechanisms between species of the "red complex" contribute to immune-inflammatory responses (Kesavalu *et al*, 2007) and their interaction with the epithelial cells in the subgingival environment leads to the destruction of host tissues.

Porphyromonas gingivalis interacts with *Actinomyces naeslundii*, which is an early colonizer of oral biofilms via coaggregation properties. *Tannerella forsythia* acts as a precursor for the growth and colonisation of *Porphyromonas gingivalis* and *Treponema denticola* (Dashper *et al*, 2011), and along with *Porphyromonas gingivalis,* coaggregates with *Fusobacterium nucleatum* enhancing colonization of subgingival plaque (Kapatral *et al*, 2002). *Porphyromonas gingivalis* also coaggregates with *Prevotella interme*dia to form a more mature periodontal biofilm (Kamaguchi *et al*, 2001). A study by Kesavalu *et al* (1998) demonstrated that in mice, an infection with *Porphyromonas gingivalis* and *Treponema denticola* together was more pathogenic than infection with either of the microorganisms alone. Individuals with high proportions of *Porphyromonas gingivalis* were previously reported to have few or absence of *Prevotella intermedia* and vice versa (Africa, unpublished data; Loesche *et al*, 1988). However, a mutual tolerance now appears to exist between them either due to their interactions with other emerging species or due to their clonal diversity (Africa, 2012).

1:7 MICROBIOLOGICAL DETECTION METHODS FOR PERIODONTAL PATHOGENS

The presence of periodontal pathogens has been identified by various detection techniques such as microscopy, culture, immunoassays, enzyme tests and DNA based techniques (Dzink, 1988; Grossi, 1995). However, for various reasons they have been replaced by more rapid and sensitive tests.

Darkfield and phase contrast microscopy

Increased motility of the bacterial plaque indicates a highly inflamed periodontium. Microscopic monitoring of bacterial morphotypes, particularly spirochaetes, in freshly sampled plaque is a definite diagnostic aid but the technique requires special equipment and is time consuming. Since most of the putative pathogens such as *Porphyromonas gingivalis, Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* are non-motile, this technique fails to identify these species and gives an end result only for a small portion of the ecological system (Conrads, 2001). Fluorescence microscopy has been used to detect specific antibodies to plaque bacteria but with higher possibilities of obtaining false negative and positive results (Gmur, 1988). Although an accurate assessment of disease, the inability to identify species is a limitation for darkfield microscopy.

Culture techniques

Culture methods are still considered as a golden standard for routine diagnostic procedure in medical microbiology (van Steenbergen, 1996) yet many of the periodontal pathogens are anaerobic and are still uncultivable (Wardle, 1997). However, the limitations of the method are that the procedure is time consuming and the bacteria are killed by the oxygen very rapidly, thus requiring rapid transportation to the laboratory.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is an immunological method designed to detect periodontal pathogens in dental plaque, by using serum antibodies to detect antigens on target bacteria (Di Murro, 1997). But sensitivity of these tests is relatively low, requiring 10^4 to 10^5 bacteria per sample and the disease activity remains unclear due to elevated antibody titres developed by past infection.

DNA based techniques

DNA based microbiologic tests are able to extract bacterial DNA from the plaque sample obtained and amplify the specific DNA sequence of the target periodontal pathogens. DNA probes can detect non-viable species by colorimetric or chemiluminescence techniques (Keller, 1991). The requirement of quantitative assessment of the target bacteria resulted in the development of quantitative Polymerase Chain Reaction (PCR) methods. The technique provides large quantities of DNA in a simplified and automated manner (Dawson, 1996). Both the techniques can detect uncultivable species but requires sophisticated technology and are expensive.

<u>Enzyme assay</u>

According to Walter Loesche and his co-workers at Michigan University, a rapid and simple chair side bacteriological test could be reliably used in everyday practice to identify the presence or prevalence of periodontal microorganisms (Loesche *et al*, 1992). The presence of *Treponema denticola*, *Tannerella forsythia* and *Porphyromonas gingivalis* can be determined or detected by an enzymatic test known as BANA (N-benzoyl-DL-arginine-naphthylamide). These "red complex" pathogens are cultivable plaque species which possess an enzyme capable of hydrolyzing the synthetic peptide of the BANA reagent (Loesche, *et al*, 1990). A positive BANA assay may indicate a high level of "red complex" members in the plaque sample as well as *Capnocytophaga* species (Loesche *et al*, 1987).

In clinical periodontal practice, the BANA test has proved to be efficient in the diagnosis of periodontal disease and also in the bacteriologic monitoring of periodontally involved patients during the different phases of periodontal treatment (Grisi, 1998). BANA has shown to correlate well with the depth of the periodontal pocket and clinical indices which are used to diagnose periodontal disease (Grisi *et al*, 2001). A positive BANA result shows the incidence of 10,000 colony forming units of bacterial species in each plaque sample. The test is a reliable indicator to be used in large epidemiologic studies concerning the severity of periodontal infections (Puscasu, 2006). Above all, the test provides equal accuracy in detecting these "red complex" species comparable to that obtained with DNA probes and immunology. However, Andrade *et al* (2010) reported a sensitivity of 90% and an accuracy of 83% for the BANA test, taking DNA probes as the primary reference. These authors concur that the BANA test is more effective in the initial diagnosis of chronic periodontitis, when the "red complex" pathogen levels are high.

The limitation of the BANA test is that it does not identify which of the three BANA positive species is present in the plaque, though it may not be necessary since these species are all anaerobes and coexist in the same plaques. Moreover other species such as *Capnocytophaga* can also give false positive results. However, BANA helps the clinician to detect the presence of an anaerobic infection (Haffajee, 1998).

1:8   THE   AETIOLOGY   AND   EPIDEMIOLOGY   OF   HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

HIV-AIDS is caused by a retroviral pathogen, the human immunodeficiency virus (HIV) which was identified in 1984 (Relman, 1988), and has evolved from a mysterious illness to a major global pandemic. AIDS was, and still is, one of the pathologies of greatest interest which has become a social problem for the well-being of the individual. Since its discovery in 1981, AIDS killed more than 25 million people worldwide (Mataftsi *et al*, 2010). The number of people living with HIV still continues to grow, as new infections occur each year. WHO has estimated about 38 million individuals infected with one of the 10 known subtypes of HIV (Margiotta, 1999). South Africa has the largest HIV-positive population in the world followed by Nigeria and India (McNeil DG Jr, 2008). AIDS affects individuals of all age groups, with 98% prevalence for adults and among them 19% are women (CDC, 2003 a).

HIV is a RNA virus with a particular affinity for the CD4 receptor found on the T-helper lymphocytes, the cells that get depleted in HIV infection. CD4 cells coordinate the immune response, signalling other cells in the immune system to perform their special functions. Thus progressive HIV infection results in loss of immune response, especially those coordinated by CD4+ T cells (Alves, 2006). Depletion of CD4+T lymphocytes renders the host vulnerable to opportunistic infections of the mouth (Coffin *et al*, 1997).

The CD4+T lymphocyte cell count is widely used for the following purposes:
1. To stage HIV disease
2. For differential diagnosis of present complaints/symptoms
3. To determine prophylaxis for opportunistic infections
4. To decide on the initiation of antiretroviral therapy   (Doshi *et al*, 2008)

HIV-infected patients can be grouped depending on the number of CD4+T-cells/mm^3 (< 200, 200-500, >500) and total lymphocytes (>2000, 1000-2000, <1000) (WHO, 1990 b).

According to the 1993 Revised Classification System for HIV infection developed by Centers for Disease Control and Prevention, the three CD4+T lymphocyte categories are defined as follows:

- Category 1: ≥ 500 CD4+T lymphocyte cells/mm^3
- Category 2: 200-499 CD4+T lymphocyte cells/mm^3
- Category 3: < 200 CD4+T lymphocyte cells/mm^3

The most significant prediction from the classification was that, in HIV-infected individuals, a CD4+T lymphocyte count ≤ 200cells/mm^3 or CD4+T lymphocyte percentage less than 14% is considered as an AIDS-defining condition. As the CD4+T lymphocyte count decreases, the risk of opportunistic illnesses increases. The first appearance of systemic and oral opportunistic infection indicates the initial immunosuppression by CD4 levels below 500cells/mm^3 (Lewis, 2003). Lack of oral hygiene and reduction in CD4+T lymphocyte cell count less than 400 makes the host unable to control infection by the periodontal pathogens resulting in periodontal inflammation (Murray, 1994a). This indicates that the gradual depletion of CD4+T lymphocyte cells and subsequent deterioration of the immune system would compromise the host defence in the dento-gingival region too (Lifson, 1989). Altered antibody production may activate complement, stimulating periodontal inflammation (Tolo, 1991).
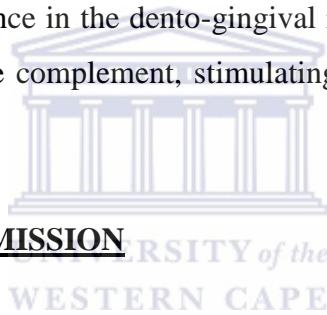
1:9 ROUTES OF HIV TRANSMISSION

Epidemiologic research has shown that, human immunodeficiency virus infection has been transmitted mainly by three routes, namely, sexual contact, parenteral transmission and perinatal transmission (Von Reyn, 1987; Morison, 2001)

1. Sexual contact - Although previously considered to be a disease associated with homosexuals only, the incidence in heterosexual transmission is rapidly increasing (Hansson et al, 2008). In sub-Saharan Africa, young women are the most frequently affected and the main mode of transmission of HIV is heterosexual contact (UNAIDS 2010; Subbiah *et al*, 2010; Schmidt *et al*, 2004).

2. Parenteral transmission - This mode of transmission occurs in three groups of high risk populations:

a) Intravenous drug abusers who share unsterilized needles/syringes and other skin penetrating instruments (Godinho *et al*, 2005; Rhodes *et al*, 1999).

b) Haemophiliacs who have received large amounts of factor VIII concentrates from pooled blood components from multiple donors (Lifson, 1986; Simmonds *et al*, 2007).

c) Recipients of blood and blood products who have received multiple transfusions of whole blood or components like platelets and plasma (Vaz *et al*, 2010).

3. Perinatal transmission- HIV infection is transmitted transplacentally from the infected mother to the foetus during pregnancy or in the immediate post-partum period through contamination with infected maternal blood, amniotic fluid or breast milk (McGowan and Shah, 2000). The overall risk of HIV transmission from an infected mother to an infant ranges from 25%-50% (Mok, 1987).

HIV has been isolated from a number of body fluids and tissues such as: semen, vaginal secretions, cervical secretions, breast milk, CSF, synovial, pleural, peritoneal, pericardial and amniotic fluid (Friedland, 1987; Campo *et al*, 2006; Grivel *et al*, 2010). Very rarely, the oral cavity has been reported as a site of HIV transmission (Cohen *et al*, 2000; Jotwani *et al*, 2004; Cutler and Jotwani, 2006). There are certain factors such as traumas in the mouth, ulcers or erosions of oral and/or genital mucosa, gingivitis or periodontitis, sexually transmitted infections, viral RNA and other oral infections (*Herpes Simplex* virus, lactobacillus, etc) which make the oral cavity a route for HIV transmission thus increasing the risk of HIV acquisition by oral-genital contacts (Scully and Porter, 2000).

1:10 <u>PATHOGENESIS OF HIV INFECTION</u>

The pathogenesis of HIV infection involves a certain sequence of events and is shown in Fig.1 (Mohan, 2005). HIV infection is manifested by both continual virus replication and a vigorous immune response (Andrews, 1996). The pathogenesis of HIV infection is extensively related to depletion or reduction of CD4+T helper cells causing profound immunosuppression. However, monocytes, macrophages, Langerhans cells and some neuronal and glial brain cells are also attacked by the human immunodeficiency virus without any cytopathic effect on these cells. Replication of the virus takes place often in the lymphoreticular tissues of lymph nodes, spleen, gut-associated lymphoid cells and macrophages (Fauci, 1993). Several factors such as viral, host and environmental factors influence the progression of HIV to AIDS within 10 years of HIV infection. However, the rate of progression differs with individuals (Buchbinder, 1994). .

Interaction of gp 120 of HIV to CD4+T cell

Internalisation of virion

Uncoating of virion

Reverse transcriptase

Other virus infection   Proviral DNA   CD4+Tcell lysis

Unintegrated, integrated

Activated CD4+T cell   Inactive CD4+T cell

Latent phase

Budding of virus particle

Syncytia formation

Cytopathic phase

Quantitative   Qualitative

depletion of CD4+T cells   failure of CD4+T cells

to respond to antigens

Fig.1 Diagrammatic representation of sequence of events in the pathogenesis of HIV infection (Mohan, 2005)

1:11 <u>PERIODONTAL DISEASES AND HIV INFECTION</u>

In 1987, Winkler and Murray reported the presence of a distinctive form of periodontal disease in HIV seropositive individuals. Since then, HIV-associated periodontal disease has been considered as one of the earliest clinical features of HIV infection (Robinson, 2002). The periodontal diseases frequently associated with HIV infection are the following: (Armitage 1999; Coogan 2005)

- Linear gingival erythema (LGE)
- Necrotizing ulcerative gingivitis (NUG) and Necrotizing ulcerative periodontitis (NUP)
- Acute necrotising stomatitis (ANS) or Necrotising ulcerative stomatitis (NUS)
- Chronic periodontitis with an increased rate of attachment loss

The primary difference between these oral diseases is their clinical severity. LGE or "red band" gingivitis is characterized by a distinct red band along the gingival margin, usually associated with anterior teeth but commonly extending to the posterior teeth. It may or may not be accompanied by bleeding or discomfort. Occasionally it appears as petechia-like patches on attached or free gingiva (Reznik, 2006). NUG is characterized by an acute painful inflammation of the gingiva with rapid destruction of soft tissues, while NUP is involved by a bleeding and sharp pain with extensive soft tissue necrosis and advanced loss of periodontal attachment, possibly exposing bone (Greenspan and Greenspan, 2008). NUG and NUP, if untreated, can progress to ANS which is manifested by the extension of necrosis past the periodontium into the mucosa and the osseous tissue (Robinson *et al*, 1998 a).

Factors which may predispose an individual to aggressive gingivitis or periodontitis unresponsive to conventional therapy (Winkler, 1992; Schenkein, 1994) may include sex, race, age, smoking, poor oral hygiene, malnutrition, stress, and socioeconomic status of individuals, some systemic diseases such as diabetes , impaired neutrophil function and state of immunosuppression (AIDS, leukaemia) (Fleming, 1999; Umeda, 1998; Tucker, 2006). Studies report that NUG and NUP are significantly associated with severe immunosuppression indicated by a CD4+T cell counts less than 200 cells/mm^3 (Margiotta *et al*, 1999; Kroidl *et al*, 2005). It is considered that the severe immunosuppression in HIV patients may exacerbate pre-existing periodontitis (Kinane, 1999 b). The course of the disease

is further associated with increasingly severe gingival inflammation, progressive destruction of ligament fibers and alveolar bone loss (Hilton, 1995; Salvi, 1997).

HAART or highly active antiretroviral therapy is defined as a treatment regimen that included at least three different antiretroviral drugs from at least two different classes, i.e, nucleoside/nucleotide analogues (NUC), protease inhibitors (PI), non-nucleotide reverse transcriptase inhibitors (NNRTI) and entry or fusion inhibitors (EI) (Vernon *et al*, 2009). A study conducted after the introduction of HAART, suggested that these diseases were not related to HIV serostatus or CD4+T cell counts (Schuman *et al*, 1998). According to a study by Patton (2000 a), LGE has a predictive value of 70% for immunosuppression, whereas, in another study by Glick *et al* (1994), NUP showed a positive predictive value of 95.1% which makes NUP a good marker for immune deterioration. A recent study conducted in South Africa, demonstrated a significant correlation between necrotizing diseases and severe immnnunosuppression, with a positive predictive value of 69.6% (Shangase *et al*, 2004). Moreover, a study conducted by Bolscher *et al* (2002), reported that the presence of oral lesions and periodontal disease results in a continuous shedding of HIV infected blood from mucosal and gingival lesions into the oral cavity, with an increased possibility for the transmission of HIV.

Researchers have suggested a higher prevalence and severity of periodontal diseases in HIV infected individuals ranging from 1-66% for gingivitis and 0-91% for periodontitis (Ranganathan *et al*, 2000; Eyeson *et al*, 2000). However, controversy exists, as investigations demonstrated that the prevalence of the disease in HIV infected patients is highly variable (Laskaris, 1992; McKaig, 1998 b) with some studies reporting differences in clinical indices between HIV-positive and HIV-negative subjects, while others report no differences in clinical indices regardless of CD4+T cell counts and viral loads (Alves, 2006). The reason for the conflicting reports may be due to the different populations studied, lack of consensus criteria for disease, patient's state of immunosuppression, antiretroviral therapy, study location and biased samples (Murray, 1994 b). The implication of HIV as a risk factor for susceptibility to periodontal disease is based on evidence from cross-sectional surveys and not longitudinal studies (Papapanou, 1998 b). A cross-sectional study conducted by McKaig *et al* (1998 b) included 326 HIV-infected individuals in whom the presence of adult periodontitis was demonstrated by measurement of probing depth and clinical attachment loss of ≥5mm in 62% and 66% respectively. In their study, only a few HIV-positive patients

(n=15) showed the presence of HIV-associated periodontitis. As mentioned earlier, periodontal disease is initiated by the overgrowth of certain Gram-negative, anaerobic bacteria growing in the subgingival sites during infection (Xiong, 2006). According to recent research, these bacteria or their toxins can be released into the blood stream, thus activating the immune system (Fowler, 2001; John, 2002). Several studies have reported HIV infection as a risk factor for the progression of chronic periodontitis, while others suggest that the presence of oral opportunistic pathogens and periodontal diseases in HIV-positive patients may influence HIV reactivation and progression (Mataftsi *et al*, 2010; Imai *et al* 2012) Whether necrotising periodontal disease is exacerbated by HIV infection or whether the progression and reactivation of HIV infection occurs as a result of periodontal disease remains unclear (Robinson, 2002; Bascones-Martinez and Escribano-Bermejo, 2005). Defects in the systemic host immune response are known to play a characteristic role in aggressive periodontitis (Schenkein, 1994), with the severity of probing pocket depth, attachment loss and gingival recession reported to correlate well with the degree of immunosuppression (McKaig, *et al*, 2000 a, Pedreira *et al*, 2008). Studies of chronic periodontitis (Barr *et al*, 1992; Yeung *et al*, 1993; Lucht *et al*, 1991; Reichart *et al*, 2003). and necrotising periodontitis (Clearinghouse; Shibosky *et al*, 2009) reported a higher risk of attachment loss in HIV-positive patients with chronic periodontitis, exhibiting CD4+T lymphocyte counts of <200cells/mm^3. An increase in the proportions of Th2 type lymphocytes in HIV-infected patients was reported when comparing chronic gingivitis with NSG (necrotising SG) (Gomez *et al*, 1997).

In a workshop conducted by Robinson *et al* (2002), a need for further research was recommended to determine the effectiveness of interventions for the management of necrotizing periodontal disease and HIV-associated morbidity and mortality (Slots 2003 c). Earlier intervention with antiretroviral therapies and the widespread use of HAART in 1995, could limit the exposure to immunosuppression (CD4+T cell count less than 200cells/ mm^3), hence reducing the incidence of many AIDS-related opportunistic infections (Paul *et al*, 2002). The advantage in the use of HAART is that it increases the CD4+T cell counts and decreases the level of HIV RNA, thus reducing the morbidity in HIV disease for a short-term (Palella *et al*, 1998). Even though the introduction of HAART highly reduces the frequency and severity of oral diseases, the majority of HIV-infected individuals with limited access to anti-HIV therapy continue to develop oral disease (Frezzini *et al*, 2005). The literature reports a reduction of about 47-85% in the overall prevalence of oral lesions prior to the introduction

of HAART, with a gradual decline of 32-46% following the introduction of HAART (Gaitan Cepeda *et al*, 2008), thereby demonstrating a lack of substantiating evidence of the effect of HAART on the progression of periodontal disease (Schmidt-Westerhausen *et al*, 2000). Feller *et al* (2006), during his study on oral diseases among HIV-infected individuals, reported that standard periodontal therapy and adequate oral hygiene maintanence may improve the clinical condition of HIV or AIDS patients. A retrospective cross-sectional study conducted by Engeland *et al* (2008) established that tooth loss due to periodontal disease in HIV-infected individuals was not influenced by their CD4+T cell counts or their viral loads. They observed no significant differences among the HIV-positive patients before dental treatment, after initial periodontal treatment and following a 2-year maintenance period even during antiretroviral therapy.

Drug interactions occur between the drugs used in periodontal therapy and antiretroviral medications and it is therefore important for the dentist to have a proper understanding of the drugs used for the treatment of periodontal disease in HIV-positive patients (Goncalves *et al*, 2010). The progression of chronic periodontitis may lead to severe invasion of opportunistic infections and destructive inflammatory response in HIV-positive patients highlighting the need for the introduction of newer systemic therapies to improve the immunocompetence of the patients (Ryder, 2002).

Oral lesions in HIV infection should therefore always be considered significant (Chapple and Hamburger, 2000) since they may**:**
1. indicate HIV infection in previously undiagnosed cases
2. predict the severity of HIV disease
3. represent early clinical features of clinical AIDS (for example, oral Kaposi sarcoma)
4. act as a traditional entry or exit determinant for antiretroviral therapy
5. determine anti-opportunistic infection therapy
6. be used in disease staging and classification
7. individually correlate with CD4 levels in severely immunosuppressed patients
8. correlate with CD4 levels when grouped together (for CD4 counts $<200 \times 10^6/\mu l$)
9. act as cofactors affecting the rate of HIV

Careful oral screening among the HIV-infected individuals is necessary for an early diagnosis and treatment of periodontal disease and other oral manifestations (Gennaro *et al*, 2008), and the combined effort of the physician and the dentist are necessary to diagnose and manage these conditions effectively (Weinert, *et al*, 1996).

1:12 <u>SUBGINGIVAL MICROBIAL COMPLEXES IN HIV-POSITIVE PATIENTS</u>

HIV-associated periodontitis can be regarded as an important diagnostic and prognostic marker (Coogan *et al*, 2005), since oral manifestations are among the first clinical expressions of HIV infection (Piluso *et al*, 1993). Thus far, no specific single organism has been implicated for periodontal disease in HIV seropositive individuals (Murray *et al*, 1989) although several reports indicate the presence of mixed microflora not usually associated with oral infections, such as *Escherichia coli, Clostridium, Mycoplasma* and *Klebsiella pneumoniae* in HIV seropositive and seronegative individuals (Zambon *et al* 1990; Patel *et al*, 2003). This may be due to the immune status of those patients, since humoral and cellular immunity changes can facilitate the colonization and overgrowth of atypical pathogenic species (Paster *et al*, 2002). High concentrations and diffuse invasion of these bacteria into the gingival tissues may lead to increased secretion of inflammatory mediators resulting in rapid periodontal destruction (Murray *et al*, 1994). The increased prevalence of periodontitis and oral diseases in HIV-infected patients caused by non oral pathogens in the mouth may also result in HIV reactivation (Gonzalez *et al*, 2009). Since periodontitis is a polymicrobial infection, the presence of Gram-positive and Gram-negative pathogens may exhibit synergestic mechanisms eliciting the activation of HIV immune cells (Huang *et al*, 2011), while the virulence mechanisms of the periodontopathogens are responsible for the occurrence of chronic inflammatory periodontal disease in HIV patients contributing to AIDS.

Several studies report that microbial complexes of HIV-associated periodontal diseases are not significantly different from those in HIV-negative individuals with periodontal diseases (Brady *et al*, 1996; Tsang and Samaranayake, 2001; Teanpaisan *et al*, 2001; Alpagot *et al*, 2004) although some increase in Gram-negative anaerobes (Barr, 1995; Patel *et al*, 2003) and *Candida* have been reported (Zambon *et al*, 1990). IgG subclass antibody responses to periodontal pathogens have also demonstrated no difference between HIV-positive and HIV-negative patients (Yeung *et al*, 2002). The "red complex" microorganisms namely the potent periodontopathogenic bacteria *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*, are considered as risk factors in HIV patients (Goncalves *et al*, 2004 b). Cross and smith (1995) compared plaque from HIV-positive and HIV-negative periodontal patients and detected a 75-100% prevalence in HIV-positive patients for *Aggregatibacter actinomycetemcomitans, Campylobacter rectus, Veillonella parvula,*

Capnocytophaga ochracea, Porphyromonas gingivalis, Prevotella intermedia and *Tannerella forsythia* (Table.2). However, other studies reported a lower prevalence of these putative bacteria in HIV-positive compared with HIV-negative individuals (Goncalves, 2007; 2009). The pathogenic potential of *Porphyromonas gingivalis* and *Tannerella forsythia* in adult periodontitis has been reported by van Winkelhoff *et al* (2002), with a recent study demonstrating that *Porphyromonas gingivalis* infection could increase HIV infection transmission through the oral cavity (Giacaman *et al*, 2007). Conversely, a high prevalence of *Tannerella forsythia* (81.8%) was in individuals with a low prevalence and severity of adult periodontitis (Tran *et al*, 2001). Consequently, the microbial associations of HIV associated periodontal diseases are not thoroughly understood (Aas *et al*, 2007).

The immunosuppression in HIV-infected individuals triggers the re-activation of human herpes virus (HHV) infection leading to severe chronic periodontitis and HIV-associated periodontal disease. (Slots and Contreras, 2000; Cappuyns, 2005) with several herpes viruses, especially Cytomegalovirus (HHV 5), Epstein-Barr virus type-I (HHV4) (Contreras *et al*, 1999) and HHV-6, HHV-7 and HHV-8 detected with higher frequency in subgingival plaque samples from HIV-positive individuals with periodontal disease than from healthy or HIV-negative subjects (Mardrossian *et al*, 2000).

Table.2 presents bacteria and viruses associated with periodontal disease in HIV-positive individuals and Table.3 presents some of the oral bacteria found in HIV-positive individuals but not associated with periodontal disease.

Table.2 Bacteria and viruses associated with periodontal diseases in HIV-positive individuals

BACTERIAL SPECIES	REFERENCES
Aggregatibacter actinomycetemcomitans *Fusobacterium nucleatum* *Porphyromonas gingivalis* *Prevotella intermedia* *Tannerella forsythia* *Treponema denticola* *Veillonella parvula* *Capnocytophaga ochracea*	Murray *et al*, 1989 Cross and Smith, 1995 Scully *et al*, 1999 Alpagot *et al*, 2004
Candia albicans and other *Candida* species	Jabra-Rizk *et al*, 2001
Eikenella corrodens *Campylobacter rectus*	Murray *et al*, 1991 Rams *et al*, 1991 Moore *et al*, 1993
Cytomegalovirus (HHV 5) *Epstein-Barr virus type-I (HHV4)*	Contreras *et al*, 1999
HHV-6 *HHV-7* *HHV-8*	Mardrossian *et al*, 2000

Table.3 Oral bacteria found in HIV-positive individuals, but not associated with periodontal disease

BACTERIAL SPECIES	REFERENCES
Enterobacter faecalis	Zambon *et al*, 1990 Nakou *et al*, 1997 Goncalves *et al*, 2004; 2007
Clostridium clostridiiforme	Zambon *et al*, 1990
Clostridium difficile	Zambon *et al*, 1990 Nakou *et al*, 1997 Goncalves *et al*, 2007
Klebsiella pneumonia *Escherichia coli*	Zambon *et al*, 1990 Nakou *et al*, 1997 Botero *et al*, 2007 a Goncalves *et al*, 2007
Mycoplasma salivarium	Zambon *et al*, 1990 Moore *et al*, 1993 Nakou *et al*, 1997 Goncalves *et al*, 2007
Pseudomonas aeruginosa	Nakou *et al*, 1997 Botero *et al*, 2007 a Goncalves *et al*, 2007
Acinetobacter baumanii	Nakou *et al*, 1997 Goncalves *et al*, 2007
Enterobacter cloacae	Nakou *et al*, 1997 Botero *et al*, 2007 a
Entamoeba gingivalis *Enterococcus faecalis* *Enterococcus avium*	Chattin *et al*, 1999 Lucht *et al*, 1998
Campylobacter pylori	Aas *et al*, 2007

2: SUMMARY

Although the oral cavity may be a primary source of infection in any individual, it is a frequently undervalued, yet significant source of diagnostic and prognostic information in patients with HIV disease. HIV infection results in impaired immune response mediated by CD4+T cell lymphocyte counts contributing to severe periodontal manifestations. Many studies referred to above, show the association between periodontal clinical indices and immunosuppression in HIV-infected individuals supporting HIV infection as a risk factor for chronic periodontitis. However, several studies failed to associate any relationship between the immunosuppression of the patients and periodontal disease. Strong evidence suggests that periodontal disease is a polymicrobial infection, associated with potential periodontopathogens including *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, commonly referred to as the "red complex".

The above literature highlights the need to determine whether any association exists between the immunosuppression in HIV-infected individuals and periodontal disease. This study will focus on the presence of "red complex" pathogens using the BANA test and will investigate and compare the degree of immunosupppression of the patients with the presence and severity of periodontal disease by measurement of their clinical indices.

3: AIMS AND OBJECTIVES

3:1 AIMS

To determine the association of periodontal diseases and the presence of "red complex" pathogens in HIV-positive patients with different CD4+T lymphocyte levels.

3:2 OBJECTIVES

1. To determine an association between clinical indices used in the diagnosis of periodontal disease across different CD4+T lymphocyte levels.
2. To seek an association between CD4+T lymphocyte counts and the presence of *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* ("red complex") using the BANA test.
3. To associate the clinical indices of periodontal disease with the BANA test

3:3 NULL HYPOTHESIS

No significant association exists between CD4+T lymphocyte levels and "red complex" pathogens of chronic inflammatory periodontal disease in HIV-positive patients.

4: MATERIALS AND METHODS

4:1 STUDY DESIGN

A cohort descriptive study of HIV-positive patients was conducted.

4:2 ETHICAL CONSIDERATIONS

Informed consent was obtained from the participants in verbal and written form as defined by the "World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects" of 2008 (Appendix 4:2 A).

Ethical clearance was obtained from the UWC research ethics committee (Appendix 4:2 B) and a translator was available in the clinic to ensure that the patients fully understood the nature of the study and the benefits and potential risks of the study. Their right to refuse

participation at any time was accepted (Appendix 4:2 C). A questionnaire was completed by them containing items designed to obtain information regarding medical history, factors predisposing for periodontal disease, and oral hygiene habits (Appendix 4:2 D). Patients requiring treatment for periodontal disease were referred to the Tygerberg Oral Health Centre (UWC) and were treated by well experienced dental professionals.

4:3 <u>SAMPLE SIZE</u>

The study sample included 120 HIV-positive patients attending the infectious disease clinic at Tygerberg Medical Hospital, Western Cape. A randomized selection of the patients was conducted. No oral hygiene instructions were given prior to the sample collection.

4:4 <u>INCLUSION CRITERIA</u>

- All the HIV-positive patients with various CD4+T lymphocyte counts
- Patients with or without ART

4:5 <u>EXCLUSION CRITERIA</u>
- Patients on antibiotic treatment
- Patients with cardiovascular disease, diabetes, TB, autoimmune diseases
- Pregnancy
- Patients who have undergone dental treatment in the past 3 months (since chances of finding infections will be less in those patients)

4:6 <u>MATERIALS</u>

4:6:1 <u>For clinical examination:</u>

- Sterile mouth mirrors
- Sterile Williams periodontal probe
- Chlorhexidine solution

4:6:2 <u>For BANA test:</u>

- Sterile cotton-tipped swabs
- Sterile cotton rolls

- Sterile scaling curette (Gracey curette 11/12, 13/14)
- BANA test strips
- BANA incubator
- Distilled water
- Cotton balls or wetting applicator bottle
- Sterile clear, non-porous tape
- Biohazard waste bags

4:6:3 Technical specifications of BANA incubator:

- Voltage: 120 VAC, 60 Hz
- Amperage: 170 mA
- Watts: 20 W

4:7 METHODS

4:7:1 Clinical assessment

The clinical assessment included patient history, recording of clinical indices and oral lesions (if present). The CD4+T lymphocyte cell counts were obtained from their medical records (Appendix 4:7:1 E).

Periodontal measurements were taken on the mesial aspect of the six Ramfjord teeth. The following teeth were selected:

- Maxillary right first molar (16)
- Maxillary left central incisor (21)
- Maxillary left first bicuspid (24)
- Mandibular left first molar (36)
- Mandibular right central incisor (41)
- Mandibular right first bicuspid (44)

Missing teeth were substituted by the following teeth:

- 16: by the mesial of 15, if not by the distal of 17
- 21: by the distal of 22
- 24: by the distal of 25

- 36: by the mesial of 35, if not by the distal of 37
- 41: by the distal of 42
- 44: by the distal of 45

The amount of plaque present was assessed using the criteria of plaque index (PI) introduced by Silness and Loe (1964) and were scored from 0-3 (Table.4).

Table.4 Plaque Index (Silness and Loe, 1964)

Scores	Criteria
0	No plaque in the gingival area
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be recognized only by running the probe across the tooth surface.
2	Moderate accumulation of soft deposits within the gingival pocket and on the gingival margin and/or adjacent tooth surface that can be seen by the naked eye.
3	Abundance of soft matter within the gingival pocket and or on the adjacent tooth surface and gingival margin.

Gingival inflammation was assessed by the gingival index (GI) introduced by Loe and Silness (1963) and was scored from 0-3 (Table.5).

Table.5 Gingival Index (Loe and Silness, 1963)

Scores	Criteria
0	Absence of inflammation
1	Mild inflammation- slight change in colour and little change in texture of any portion of the marginal or papillary gingival unit
2	Moderate inflammation- glazing, redness, edema and hypertrophy of marginal or papillary gingival unit. Bleeding on probing
3	Severe inflammation, marked redness and hypertrophy of the marginal or papillary unit. Spontaneous bleeding, congestion or ulceration.

Probing pocket depth (PPD) was measured from the gingival margin to the base of the pocket using Williams periodontal probe with markings to the nearest millimetre. Clinical attachment loss (CAL) was measured from the cementoenamel junction to the base of the pocket using the above mentioned probe. The probing depth and/or attachment loss were measured based on the periodontal disease classification system recommended by the 1999 International Workshop for classification of periodontal diseases and conditions, accepted by American Academy of Periodontology (AAP) (Colin, 2000). They were measured as follows;

Slight = 1-2mm

Moderate = 3-4mm

Severe = ≥ 5mm

4:7:2 BANA hydrolysis assay

The BANA reagent strip consisted of a plastic strip to which two separate reagent matrices were attached;

- The lower white reagent matrix was impregnated with N-benzoyl-DL-arginine-B-napthylamide (BANA).
- The upper buff reagent matrix contained a chromogenic diazo reagent, Fast Black K.

A BANA test strip was taken from the bottle just prior to use, as the test reagents were sensitive to light and humidity. The patient's name, date and site information were recorded in the marked space on the test card.

4:7:2:1 Subgingival plaque sampling

Subgingival plaque sample was collected from the site with the maximum pocket depth. The supragingival plaque was removed from the specific tooth prior to sampling and the tooth was isolated using sterile cotton rolls. A gracey curette was inserted deep into the apical third of the pocket and subgingival plaque collected. The specimen taken was dispersed on the lower reagent matrix of the BANA test strip.

4:7:2:2 BANA testing

After the desired plaque sample has been transferred to the lower matrix, the upper matrix was moistened with distilled water on a cotton swab. Care was taken to just moisten and not wet the upper matrix with too much water thus diluting the blue colour, resulting in a false negative interpretation. The BANA test strip was folded at the perforation mark so that the

upper and the lower matrices meet. The BANA strip was then placed into one of the top slots of the incubator. The incubator cycle began when the indicator light was on and was incubated for 15 minutes at 55˚c. The BANA test strip was then removed from the incubator and the lower strip at the perforation was discarded as contaminated waste. The reason for this disposal was that the lower portion of the strip may contain traces of pathogenic bacteria, viruses and beta-napthylamine, a potentially carcinogenic end product of BANA hydrolysis. Care was also taken to avoid handling the lower portion of the test strip which would contain the original plaque sample.

If BANA positive species were present when the test strip was opened, a permanent blue colour was obtained on the upper matrix. The higher the concentration of bacterial species, the darker the blue colour obtained. Results were reported as positive, weakly positive or negative.

BANA test results were interpreted as the following:

- Positive reaction- This appeared as a distinct blue colour in very small zones or over the entire contact area of the plaque sample. A positive reaction indicated the presence of more than 10,000 colony-forming units of the BANA anaerobes in the specimen. These levels may indicate the incidence of clinical periodontal disease and/or oral malador in those patients.
- Weakly positive reaction- This appeared as a faint blue colour in small zones or over the entire contact area of the specimen. A faint intensity indicated the presence of low levels of BANA positive species in a plaque sample and was evaluated by the patient's history of periodontal disease and current clinical status.
  For statistical analyses, both strong and weakly positive results were recorded as positive.
- Negative reaction- Absence of colour indicated the absence of BANA anaerobes in the plaque specimen, i.e, below 10,000 colony forming units at the sample site. A negative BANA test showed periodontal health or no oral malador.

The result thus obtained was recorded in the respective patient's data collection chart and the upper coloured strip was preserved by sealing with clear, non porous tape and attached to the patient's chart for future reference.

4:8 <u>DATA ANALYSIS</u>

Data was analyzed using statistical programmes such as SAS (SAS Institute Inc., Cary, NC, USA). All questionnaires, oral examinations and laboratory data were entered into a computer spread sheet programme, Excel. The Wilcoxon rank sum test was used to determine the association between CD4+T cell counts and each of the clinical periodontal parameters with BANA. Bivariate relationships between continuous variables were determined by Spearmans rank correlation test. Pearsons chi-square test was applied to determine the association between "red complex" and periodontal status. A significance level of <0.01 rather than the usual <0.05 level indicated a highly significant result, while results with a p-value between 0.01 and 0.05 were referred to as marginally significant.

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5: RESULTS

5:1 Health Risk Assessment Questionnaire

Prior to the clinical examination, all the patients were given a set of consent forms and questionnaires. They were asked to sign the consent forms to show their valuable participation in the study and also to complete the questionnaires in order to identify any predisposing or risk factors that might lead to periodontal disease. Table.6 shows the frequency distribution of the patients and their response to the questionnaires.

Of the 120 patients examined, 54.2% were females and 45.9% were males. The majority of patients were blacks (59.2%) followed by coloureds (31.7%), expatriates (8.4%) and whites (0.9%). About 19.2% of them had no formal education followed by 44.2% and 32.5% who attended primary and high school respectively. Only 4.2% had university education. Around fifty percent lived in flats and 45.9% lived in shacks while 3.4% were homeless.

Seventy percent reported brushing once a day and only 30% brushed twice a day. Only 21.7% used interdental aids to clean their teeth whereas 78.4% never used interdental aids. About 19.2% of the individuals admitted to never visiting a dentist while 10.9% claimed to maintain their dental visit every year for five years. Very few of them claimed to visit the dentist three times (4.2%) and four times (2.5%) in five years, whereas, 51.7% and 11.7% maintained their dental checkups only once and twice in five years respectively.

Among the study population, half of them were current smokers (50%) and half of them were (50%) were non-smokers. Most of the patients (60.9%) were on antiretroviral therapy and the rest (39.2%) were not on therapy. Other sexually transmitted diseases were reported by 41.7% whereas 58.4% were free of other sexually transmitted diseases.

Table.6 Characteristics and frequency distribution of the study population

Characteristics	Frequency	Percentage
Gender		
Males	55	45.9%
Females	65	54.2%
Race		
Blacks	71	59.2%
Whites	1	0.9%
Coloureds	38	31.7%
Others	10	8.4%
Level of Education		
No formal education	23	19.2%
Primary school	53	44.2%
High school	39	32.6%
University	5	4.2%
Living Conditions		
Homeless	4	3.4%
Flat	61	50.9%
Shack	55	45.9%

Table.6 (continued)

Dental History		
Dental visits in 5years		
Never	23	19.2%
Once	62	51.7%
Two times	14	11.7%
Three times	5	4.2%
Four times	3	2.6%
Five times	13	10.9%
Brushing Frequency		
Once a day	84	70%
Twice a day	36	30%
Use of Interdental aids		
Yes	26	21.7%
No	94	78.4%
Current Smokers		
Yes	60	50%
No	60	50%
Antiretroviral Therapy		
Yes	73	60.9%
No	47	39.2%
STD		
Positive	50	41.7%
Negative	70	58.4%

5:2 <u>Oral hygiene and clinical indices</u>

5:2:1 <u>Association between clinical indices with the frequency of brushing</u>

Among the clinical indices only plaque index (p= 0.0352) showed a significant association with the brushing habit of the patients. The median for the clinical indices were higher for those who brushed once a day compared to those who brushed twice a day. Table.7 shows significant association found between frequency of brushing and plaque index using Wilcoxon rank sum test. None of the other clinical indices showed any significant associations with brushing.

5:2:2 <u>Associations between clinical indices with the use of interdental aids</u>

Plaque index (p= 0.0110), gingival index (p= 0.0102) and clinical attachment level (p= 0.0336) showed significant associations with interdental aids except for the probing depth Table.8 shows significant results between the use of interdental aids with plaque index, gingival index and clinical attachment level using Wilcoxon rank sum test.

Table.7 Clinical indices relative to frequency of brushing

Frequency of Brushing							
	Once a day (N=84)			Twice a day (N= 36)			
Variables	Mean(SD)	Median	Minimum-Maximum	Mean(SD)	Median	Minimum-Maximum	P value
Plaque index	2.63(0.4)	2.9	1.2-3	2.38(0.58)	2.4	0.8-3	**0.0352**
Gingival index	2.79(0.4)	3	1.4-3	2.67(0.53)	2.9	0.5-3	0.0911
Probing depth	4.86(1)	5	2.9-6.8	4.55(1)	4.15	3-6.8	0.1376
Clinical attachment level	5.39(1.1)	5.6	3-7.3	5.06(1)	5	3.3-7	0.1101

Table.8 Clinical indices relative to interdental aids

	Use of Interdental aids						
	Yes (N= 26)			No (N= 94)			
Variables	Mean(SD)	Median	Minimum-Maximum	Mean(SD)	Median	Minimum-Maximum	p value
Plaque index	2.27(0.6)	2.2	0.8-3	2.63(0.49)	2.9	1.2-3	**0.0110**
Gingival index	2.57(0.59)	2.85	0.5-3	2.8(0.39)	3	1.4-3	**0.0102**
Probing depth	4.45(1.17)	4	3-6.5	4.86(0.99)	5	2.9-6.8	0.0762
Clinical attachment level	4.89(1.12)	4.6	3.3-6.9	5.3(1.07)	5.6	3-7.3	**0.0336**

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5:3 Descriptive statistics of age, CD4+T cell counts and periodontal indices

The mean age of the study population was 33.3years with a median of 32years (Table.9). The mean plaque index score (2.55) was the lowest among the periodontal indices with the highest being the mean clinical attachment level (5.29mm). The mean probing depth was 4.77mm and the mean gingival index score was 2.75 with a median score of 4.9mm for probing depth and 3 for gingival index.

The actual CD4+T lymphocyte mean level was 293.5cells/mm^3. The HIV infected patients were grouped depending on their CD4+T cell counts into those with <200 cells/mm^3, 200-500cells/mm^3 and >500cells/mm^3. Fig.2 shows CD4+T cell counts grouped by intervals of 100 rather than the coarser grouping of <200, 200-500 and >500cells/mm^3. Thirty percent had a CD4+T cell count <200cells/mm^3 indicating severe immunosuppression with a lower mean 135.67cells/mm^3 compared to 59.2% individuals with CD4+T cell counts of 200-500cells/mm^3 with a mean of 321.27cells/mm^3. Only 10.9% of the patients had a CD4+T cell count >500cells/mm^3 with the highest mean (578.24cells/mm^3) compared to other groups.

Table.9 Mean (SD) and Median of age, CD4+T cell counts and periodontal indices

Variables	N	Mean (SD)	Median	Minimum-Maximum
Age (years)	120	33.3(7.42)	32	20-55
CD4+T cell counts	120	293.5(151.06)	294.5	36-859
Plaque index	120	2.55(0.54)	2.8	0.8-3
Gingival index	120	2.75(0.45)	3	0.5-3
Probing depth	120	4.77(1.04)	4.9	2.9-6.8
Clinical attachment level	120	5.29(1.1)	5.35	3-7.3
CD4+T cell groups				
<200 cells/mm^3	36 (30%)	135.67(37.5)	136	36-190
200-500 cells/mm^3	71(59.2%)	321.27(87.9)	312	200-500
>500 cells/mm^3	13(10.9%)	578.24(100.0)	534	510-859
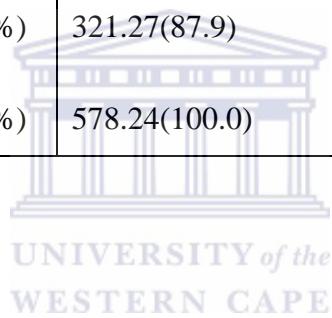
Grouped CD4 counts
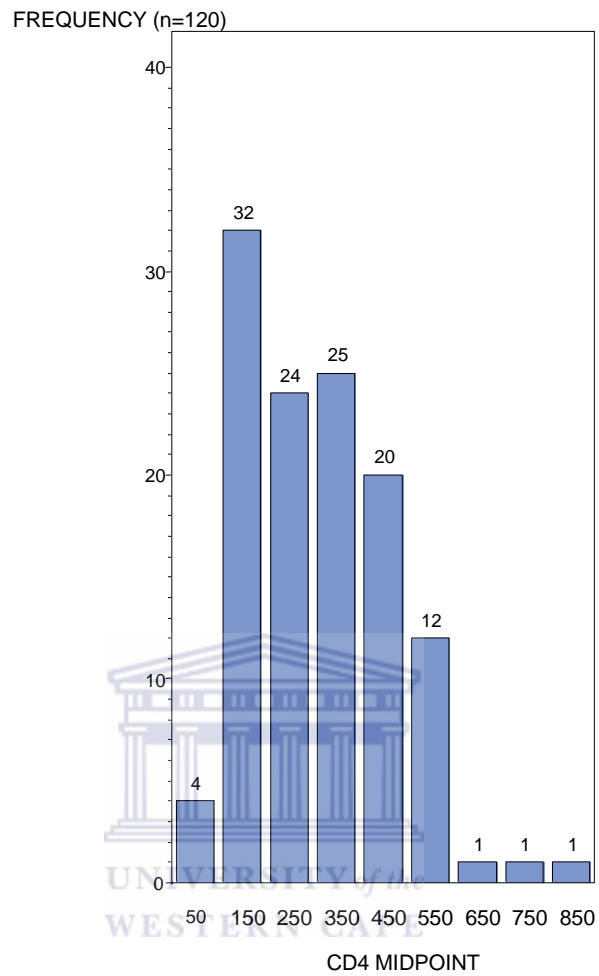
Fig.2 Frequency distribution of CD4+T cell counts grouped into intervals of 100 and plotted against the midpoint

Table.10 Descriptive statistics for grouped CD4+T cell counts relative to clinical indices

Group	N	Variables	N	Mean(SD)	Median	Minimum-Maximum
<200cells/mm^3	36	Plaque index	36	2.59(0.49)	2.9	1.3-3
		Gingival index	36	2.8(0.36)	3	1.9-3
		Probing depth	36	4.6(0.95)	4.16	2.9-6.4
		Clinical attachment level	36	5.09(1.06)	5	3-6.9
200-500cells/mm^3	71	Plaque index	71	2.52(0.55)	2.8	0.8-3
		Gingival index	71	2.74(0.48)	3	0.5-3
		Probing depth	71	4.83(1.07)	4.9	3-6.8
		Clinical attachment level	71	5.37(1.07)	5.5	3.3-7.3
>500cells/mm^3	13	Plaque index	13	2.59(0.56)	2.8	1.2-3
		Gingival index	13	2.7(0.52)	3	1.4-3
		Probing depth	13	4.86(1.17)	5	2.9-6.5
		Clinical attachment level	13	5.44(1.36)	5.9	3-7

5:4 <u>Grouped CD4+T cell counts and clinical indices</u>

The median for plaque index was higher in HIV-positive patients with CD4+T cell counts <200cells/mm^3 compared to those with CD4+T cell counts >500cells/mm^3 and those with 200-500cells/mm^3. Table.10 shows the descriptive statistics for grouped CD4+T lymphocyte levels with clinical indices. While no difference was found between gingival index in HIV-positive patients with CD4+T cell counts <200cells/mm^3, 200-500cells/mm^3 and >500cells/mm^3, the median for probing depth and clinical attachment level were higher in HIV patients with a CD4+T cell count >500cells/mm^3 than those with CD4+T cell counts between 200-500 cells/mm^3 and <200cells/mm^3.

Table.11 Spearman Rank Correlation

	Plaque index	Gingival index	Probing depth	Clinical attachment level
A rho	0.28149	0.18813	0.18842	0.15461
p	0.0018	0.0396	0.0393	0.0918
B rho	-0.02748	-0.03339	0.09761	0.12376
p	0.7657	0.7173	0.2889	0.1781
C rho	0.01514	-0.01133	0.18472	0.20212
p	0.8696	0.9023	0.0434	0.0268

*A = Age of the study population

*B = Grouped CD4+T cell counts

*C = CD4+T cell counts

*rho = coefficient of correlation

*p = p value

5:5 Correlations between clinical indices with demographic and clinical variables

5:5:1 Age of the population and clinical indices

There was a statistically significant positive correlation between age and plaque index (p= 0.0018), and gingival index (p= 0.0396) and probing depth (p= 0.0393). The correlations were 0.28149, 0.18813 and 0.18842 respectively. There was no significant correlation found between clinical attachment level and age at survey (Table.11).

5:5:2 CD4+T cell counts and clinical indices

There were positive correlations found between CD4+T cell counts with probing depth and clinical attachment level showing a significance of 0.0434 and 0.0268 respectively, but when the CD4+T cell counts were grouped, these correlations were no longer evident (Fig.3 and Table.11). Although, plaque and gingival indices respectively showed positive (0.01514) and negative (-0.01133) correlations with CD4+T cell counts (Table.11), no significant associations (as reflected in p values) were observed.
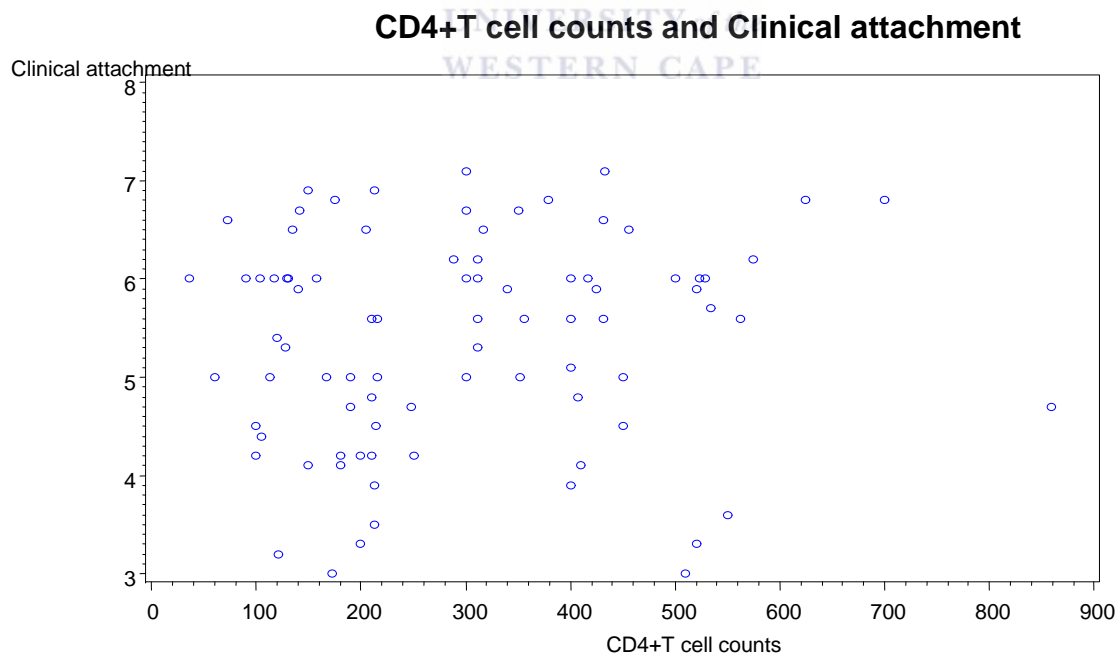
Fig.3 Scatter plot showing correlation between CD4+T cell counts and clinical attachment level

5:6 <u>BANA hydrolysis</u>

The BANA hydrolysis test was performed to detect the presence of periodontal pathogens referred to as "red complex" microorganisms. Of the 120 HIV-positive patients, 83 (69.2%) were reported as BANA-positive and 37 (30.9%) were BANA-negative (Tabe.12).

Table.12 Frequency of BANA results

Characteristics	Frequency	Percentage
BANA positive	83	69.2%
BANA negative	37	30.9%

5:6:1 <u>CD4+T cell counts and BANA</u>

There was no significant association found between BANA with CD4+T cell counts (Wilcoxon test, p= 0.7075). Significant positive correlations were found between BANA with clinical indices. The median for CD4+T cell counts in patients with positive BANA were higher than those who were BANA negative. The median for clinical indices of BANA-positive patients were higher than for the BANA-negative patients, except for the gingival index where no marked difference was found for BANA-positive patients compared to BANA-negative patients. Table.13 shows no statistical significance result observed between CD4+T cell counts and with BANA test using Wilcoxon rank sum test. However, highly significant associations were observed between BANA and probing depth and clinical attachment level, while marginal significances were observed between BANA and plaque and gingival indices using Wilcoxon rank sum test.

5:6:2 <u>Grouped CD4+T cell counts and BANA</u>

No statistical significance observed between BANA with grouped CD4+T cell counts using Pearsons chi-square test (p= 0.9989) (Table.14).

Table.13 CD4+T cell counts and clinical indices relative to BANA

	BANA						
	Positive (N= 83)			Negative (N= 37)			
Variables	Mean(SD)	Median	Minimum-Maximum	Mean(SD)	Median	Minimum-Maximum	p value
CD4+T cell counts	299.07(157.16)	300	61-859	280.79(137.6)	256	36-562	0.7075
Plaque index	2.64(0.46)	2.9	1.3-3	2.35(0.64)	2.5	0.8-3	**0.0248**
Gingival index	2.83(0.35)	3	1.5-3	2.59(0.59)	2.9	0.5-3	**0.0348**
Probing depth	5.06(1.02)	5	2.9-6.8	4.12(0.76)	4	2.9-5.8	**<.0001**
Clinical attachment level	5.6(1.1)	5.9	3-7.3	4.59(0.83)	4.4	3-6.1	**<.0001**

Table.14 Grouped CD4+T cell counts relative to BANA

BANA	Grouped CD4+T cell counts (p= 0.9989)			Total
	<200	200-500	>500	
Negative	11(29.8%)	22(59.5%)	4(10.9%)	37(30.4%)
Positive	25(30.2%)	49(59%)	9(10.9%)	83(69.2%)

5:7 Associations between Oral hygiene and CD4+T cell counts

Statistically significant differences were found between CD4+T cell counts relative to brushing and the use of interdental aids with p values of 0.0190 and 0.0170 respectively. A higher mean of 313.14 (152.63) was found in those who brushed once a day 84 (70%) compared to those who brushed twice a day 36 (30%) with a mean of 247.45 (138.6). Likewise, the majority of the population 94 (78.4%) never used interdental aids and a higher

mean of 310.6 (149.6) and a median of 308 was found between them compared to those who used interdental aids 26 (21.7%). Table.15 shows statistically significant results between CD4+T cell counts with brushing and interdental aids using Wilcoxon rank sum test

Table.15 CD4+T cell counts relative to oral hygiene

Variables	N	Mean (SD)	Median	Minimum-Maximum	p value
Brushing frequency					**0.0190**
Once a day	84(70%)	313.14(152.63)	312	61-859	
Twice a day	36(30%)	247.45(138.7)	213	36-700	
Interdental aids use					**0.0170**
Yes	26(21.7%)	231.35(142.28)	211.5	36-700	
No	94(78.4%)	310.6(149.6)	308	90-859	

5:8 Associations between Oral hygiene and BANA

5:8:1 BANA and the frequency of brushing

Out of 83 (69.2%) BANA-positive patients 62 (74.8%) brushed once a day and about 21 (25.4%) patients brushed twice a day (Table.16). Among 37 (30.9%) BANA-negative patients, 22 (59.5%) admitted brushing once a day and 15 (40.6%) patients admitted brushing twice a day. There were no statistical significant associations found between BANA and frequency of brushing using Pearsons chi-square test (p= 0.0925).

Table.16 BANA relative to frequency of Brushing

BANA	Brushing (p= 0.0925)		Total
	Once a day	Twice a day	
Negative	22(59.5%)	15(40.6%)	37(30.9%)
Positive	62(74.8%)	21(25.4%)	83(69.2%)

5:8:2 BANA and the use of interdental aids

Statistically significant association was found between BANA and the use of interdental aids (p= 0.0168). A majority of 70 (84.4%) of BANA-positive patients never used interdental aids while 13 (15.7%) of them reported using interdental aids. Among the BANA-negative patients, 24 (64.9%) never used interdental aids and 13 (35.2%) used interdental aids. Table.17 shows statistical significance results observed between BANA positivity with interdental aids using Pearsons chi-square test

Table.17 BANA relative to interdental use

BANA	Interdental aids (**p= 0.0168**)		Total
	No	Yes	
Negative	24(64.9%)	13(35.2%)	37(30.9%)
Positive	70(84.4%)	13(15.7%)	83(69.2%)

5:9 Associations between CD4+T cell counts and Smoking

CD4+T cell counts (p=0.0540), plaque index (p= 0.0180), probing depth (p= 0.0191) and clinical attachment level (p= 0.0109) appeared to increase significantly relative to the smoking status of the study population. However there was no significant association found between gingival index and smoking, with the median for gingival index being similar for smokers and non-smokers. Table.18 shows statistically significant results observed towards CD4+T cell counts, plaque index, probing depth and clinical attachment level relative to smoking using Wilcoxon rank sum test (significance 0.01<p<0.05).

Table.18 CD4+T cell counts and clinical indices relative to smoking status

	Smoking						
	Yes (N= 60)			No (N= 60)			
Variables	Mean(SD)	Median	Minimum-Maximum	Mean(SD)	Median	Minimum-Maximum	p value
CD4+T cell counts	322.45(164.46)	312	61-859	264.4(131.4)	214	36-562	**0.0540**
Plaque index	2.66(0.49)	2.9	0.8-3	2.44(0.56)	2.55	1.2-3	**0.0180**
Gingival index	2.8(0.45)	3	0.5-3	2.70(0.45)	3	1.4-3	0.1500
Probing depth	4.99(0.9)	5	3-6.8	4.54(1.04)	4.15	2.9-6.4	**0.0191**
Clinical attachment level	5.55(1)	5.75	3.4-7.3	5.03(1.13)	5	3-7.1	**0.0109**

5:10 Association between BANA and Smoking

60% of the smokers and 40% of the non-smokers were BANA-negative. Among the BANA-positive patients, 78.4% were smokers and 21.7% were non-smokers. A statistical significance was found between BANA positivity and smoking using Pearsons chi-square test (p= 0.0297) (Table.19).

Table.19 BANA relative to smoking

BANA	Smoking (**p= 0.0297**)		Total
	No	Yes	
Negative	24(40%)	36(60%)	60(50%)
Positive	13(21.7%)	47(78.4%)	60(50%)

5:11 Association between CD4+T cell counts and ART

A highly significant positive relationship was found between CD4+T cell counts with ART (p=<.0001) (Table 20). The median for CD4+T cell counts of patients who were on ART were higher than those who were not on ART. Of the 47 (39.2%) patients who were without ART, 68.1% belonged to <200 CD4+T cell counts group, 29.8% belonged to 200-500 CD4+T cell counts group and a few (2.2%) belonged to >500 CD4+T cell counts group. Among the 73 (60.9%) patients with ART, majority of the them (78.1%) belonged to 200-500 CD4+T cell counts group and a few of 5.5% patients belonged to <200 CD4+T cell counts group. Significant positive relationships were found between ART with probing depth (p= 0.0065) and clinical attachment level (p= 0.0029) with a higher median than those who were not on ART. However there were no significant relationships found between ART with plaque and gingival indices and the median appeared similar to that of patients without ART. Table.20 shows highly statistical significance between CD4+T cell counts, probing depth and clinical attachment level with ART using Spearmans rank sum correlation test.

Table.20 CD4+T cell counts and clinical indices relative to ART

	ART						
	Yes (N= 73)			No (N= 47)			
Variables	Mean(SD)	Median	Minimum-Maximum	Mean(SD)	Median	Minimum-Maximum	Spearmans correlation (p value)
CD4+T cell counts	377.74(126.97)	352	140-859	162.47(70.67)	158	36-520	0.77004 (**<.001**)
Plaque Index	2.58(0.53)	2.8	0.8-3	2.51(0.55)	2.8	1.3-3	0.03499 (0.7044)
Gingival index	2.75(0.48)	3	0.5-3	2.76(0.4)	3	1.8-3	-0.01551 (0.8665)
Probing depth	4.97(1)	5	2.9-6.8	4.45(1)	4	2.9-6.4	0.24716 (**0.0065**)
Clinical attachment level	5.53(1.03)	5.6	3-7.3	4.92(1)	4.6	3-6.9	0.26956 (**0.0029**)

5:12 Association between Grouped CD4+T cell counts and ART

CD4+T cell count groups showed a highly significant relationship with ART (p= <.0001). A greater percentage of HIV-positive patients with ART had CD4+T cell counts between 200-500 cells/mm^3 and a major percentage of the patients with CD4+T cell count <200cells/mm^3 indicating severe immunosuppression were without ART. Statistical significance observed between grouped CD4+T cell counts with ART using Pearsons chi-square test (Table.21).

Table.21 Grouped CD4+T cell counts relative to ART

ART	Grouped CD4+T cell counts (p= <.0001)			Total
	<200	200-500	>500	
Negative	32(68.1%)	14(29.8%)	1(2.2%)	47(39.2%)
Positive	4(5.5%)	57(78.1%)	12(16.5%)	73(60.9%)

5:13 Association between BANA and ART

There was no significant association found between BANA and ART (p= 0.8422). No statistical significance observed between BANA with ART using Pearsons chi-square test (Table.22).

Table.22 ART relative to BANA

BANA	ART (p= 0.8422)		Total
	No	Yes	
Negative	14(29.8%)	33(70.3%)	47(39.2%)
Positive	23(31.6%)	50(68.5%)	73(60.9%)

6: **DISCUSSION**

Much controversy continues to exist regarding the association between HIV infection and periodontal disease. Although some studies have demonstrated an increase in the severity and prevalence of periodontal diseases in HIV-positive populations (McKaig *et al* 1998 b; Masouredis *et al*, 1992; Riley, 1992; Drinkard *et al*, 1991), others have reported no differences between HIV-positive and HIV-negative patients in CD4+T cell counts and the clinical presentation of periodontal disease (Smith, 1995; Tomar *et al*, 1995; Scheutz, 1997; Matee, 1999; Mulligan *et al*, 2004). Reports have indicated that low CD4+T cell counts and high viral loads resulted in severe periodontal and oral manifestations in HIV-infected patients and that a better prognosis could be obtained by reducing their viral load and restoring their immune system (Patton, 1999).

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The present study evaluated the association of CD4+T lymphocyte cell counts and the "red complex" subgingival pathogens among HIV-positive patients with chronic inflammatory periodontal disease. The examination of the mesial aspect of the Ramfjord teeth in this study was considered to be useful in predicting the whole mouth periodontal status since they have frequently been used in epidemiology and prevalence studies (Silness and Roynstrand, 1988; Baelum *et al*, 1995; Mumghamba *et al*, 2004) and provided a standardized sample site for all subjects, whether diseased or not. A limitation of sampling diseased sights only, wherever in the mouth they may occur, may often not be a true reflection of what is actually taking place at a particular site at a particular time since different sites on the same tooth may present with different measurements, thus introducing a bias.

The overall CD4+T lymphocyte mean level of the study population was 293.5cells/mm^3 and the mean clinical attachment level was 5.29. Statistically significant positive relationships were found between CD4+T cell counts and probing depth (p= 0.0434) and clinical attachment level (p= 0.0268). This is contrary to other studies where one study reported an association between clinical attachment destruction but not probing depth with progressive HIV infection (Robinson *et al*, 1996 b) and another demonstrated no difference between the level of clinical attachment loss and probing depth in HIV-positive and HIV-negative women irrespective of their CD4+T cell counts and viral load (Alves, 2006).

In the present study, the mean gingival index score was 2.75 showing a high prevalence of gingival inflammation. Plaque and gingival indices showed no significant associations with CD4+T cell counts, although positive (rho= 0.01514, p= 0.8696) and negative (rho= -0.01133, p= 0.9023) relationships were demonstrated. Doshi *et al* (2008) observed an increase in the gingival bleeding, a predictor of severe gingival inflammation, with reduced CD4+T cell counts (<200cells/mm^3), suggesting that CD4+T cells may be an important indicator for clinical aggravation of HIV infection. However, they too could not report a significant positive correlation of bleeding index with CD4+T cell counts (p = 0.14).

In a longitudinal study including 33 HIV-positive patients, Alpagot *et al* (2003) established an association between gingival crevicular fluid levels of interferon gamma (GCF IFN-gamma) and periodontal clinical parameters (p= <0.001), revealing that sites with increased GCF IFN gamma levels carry a higher risk of periodontal disease progression.

In a cross-sectional study, Tomar *et al* (1995) failed to determine an association between periodontal disease and CD4+T cell counts. Their cut-off point for CD4+T cell counts was <300cells/mm^3; nor was a statistical association found between CD4+T lymphocyte levels with higher prevalence of chronic periodontitis in a Brazilian population (Goncalves *et al*, 2005 a). The reasons for this variation in the findings may be due to the fact that the study excluded individuals with severe periodontal conditions and also because all of the 64 HIV-positive Brazilian patients included, were on trimethoprim and sulfamethoxazole prophylaxis to prevent opportunistic infections, thus limiting the severity of periodontal disease. Findings by Howard (1991) considered periodontal disease as a later complication, rather than one of the first signs, of HIV infection.

An interesting finding in the present study was that no significant associations were found between any of the periodontal indices and CD4+T cell counts when grouped into <200cells/mm^3, 200-500cells/mm^3 and >500cells/mm^3. On the contrary, a study by Vastardis *et al* (2003) determined that for individuals with moderate or severe immunosupression (CD4+T cell count <500cells/mm^3), a significant positive correlation existed between CD4+T cell counts with modified gingival index and bleeding index with a marginal significance for clinical attachment level (p= 0.0560), while individuals with extreme immunosupression (CD4+T cell count <200cells/mm^3) showed less periodontal disease compared to those with higher CD4+T cell counts. In their study they could not find any significant association

between periodontal indices and CD4+T cell counts for all the individuals examined. Moreover a relatively small number of 39 patients were used in their study compared to the present study with the sample size of 120.

As generally expected, the lower the immunosuppression of an individual, the higher the severity of periodontal disease detected. The present study revealed that a relationship existed between the immunosuppression of the individuals and their periodontal status. However, the stages of immunosuppression do not favour the severity of periodontal disease. The reason may possibly be due to the differences in the study groups and the method used to collect the periodontal data. The study population selected had moderate to severe forms of periodontal disease and the subgingival specimens were taken from the sites with maximum probing depths. The majority of individuals in the study had probing depths and clinical attachment levels >5mm, indicating a higher prevalence of periodontal disease. Both male and female patients were included in the study with the probable exception of pregnant patients and any patients with recent antibiotic therapy and dental treatment.

BANA test was used to detect the presence of anaerobic infections caused by the subgingival pathogens *Treponema denticola, Porphyromonas gingivalis* and *Tannerella forsythia*. Although the test does not differentiate between the three species of bacteria in the plaque sample, it was considered that if any of the three "red complex" species was present, the BANA test would appear positive. The accuracy of the BANA test was established in a study by Bretz *et al* (1990) with BANA showing an accuracy of 93% for *Treponema denticola*, 76% for *Tannerella forsythia* and 96% for *Porphyromonas gingivalis* compared with indirect immunofluorescent antibody techniques. In this study, BANA test showed significant associations with all the periodontal indices, with clinical attachment and probing depth measurements demonstrating a highly significant (p= <.001) association, while lower levels of significance were found between plaque (p= 0.0248) and gingival (p= 0.038) indices and BANA. This investigation's results clearly indicate the ability of BANA positive species to cause periodontal disease manifestations. Similar results suggesting an association between BANA and clinical signs of periodontal disease have been reported (Grisi *et al*, 2001; Figueiredo *et al*, 2000). Findings by Loesche *et al* (1987); Bretz et *al* (1989); Socransky *et al* (1998) and Schmidt *et al* (1988) showed a strong correlation between BANA and increasing probing pocket depth, while a high frequency of BANA positive results (66.1%) was found even at a minimum pocket depth of 2 to 3mm, with a significant correlation between gingival

index and BANA, based on the presence or absence of bleeding on probing, thus demonstrating that gingival changes even at a small pocket depth may indicate colonization by BANA positive microorganisms (Grisi *et al*, 1998).

There were significant positive relationships found between gingival index (p= 0.0396) and probing depth (p= 0.0393) with the age of the HIV-positive population. Plaque index showed a highly significant (p= 0.0018) positive relationship with age. The study group aged between 17-55 years. This growing age of the study population may support the fact that as the person gets older the chances of developing periodontal disease manifestations among them are high. Likewise, a study conducted by Taani *et al* (2003) also reported a significant association between the increase of age and probing depth. Martinez-Canut *et al* (1995) established a statistical significance between age and periodontitis. Since probing depth was directly related to age and BANA positive tests, this study may correlate with the studies of Rodenburg *et al* (1990), Savitt and Kent (1991) and Yoshihiko *et al* (2000) who reported age as one of the risk factors for *Porphyromonas gingivalis* infection. Yalcin *et al* (2002) reported no association between age and clinical parameters.

A study of 61 adult patients with gingivitis or periodontitis, demonstrated a statistical correlation with the severity of periodontal destruction and BANA but failed to determine a statistical correlation between plaque index and BANA (Puscasu *et al*, 2006). However their study used both supragingival and subgingival plaque deposits for BANA, thus accounting for the lack of correlation with plaque index since less anaerobic bacteria would be detected in supragingival than in subgingival plaque. However, in the present study, only subgingival plaque samples were collected, thus detecting the "red complex", where they colonise abundantly. About 30.83% patients showed BANA negative results. As earlier stated, BANA negative plaques may also influence changes in periodontal disease activity even though they remain below the detection limit of the BANA test.

The oral hygiene behaviours as explained by the frequent brushing habits and the use of interdental aids, as additional cleaning devices to clean the teeth, were significantly related to CD4+T cell counts of the patients. Most of the HIV patients examined, brushed only once a day (70%) and never used interdental aids (78.33%). This indicates less effort on the patient's part to maintain better oral hygiene, ultimately leading to gingival inflammation and periodontal diseases. The frequency of brushing was significantly associated with plaque

index (p= 0.0352) and not with the other periodontal indices. However, the use of interdental aids showed significant associations with all of the periodontal indices except the probing depth. Inadequate oral hygiene can be considered as a risk indicator, risk factor, and/or risk predictor in the development of periodontal manifestations (Bakdash, 1994) which may be exacerbated by HIV infection.

As one would expect, more frequent brushing and interdental use tends to provide better plaque control and improved gingival health. Microbial plaque is reduced in the mouths of individuals with good oral hygiene. A knowledge about the living and educational background of the study population was important in order to understand their interest in maintaining good oral hygiene which was mandatory for their general health. The majority of patients in the study population had a minimum level of education with better living conditions. However, less interest was seen among them in maintaining better oral health.

Overall, the oral hygiene status among the individuals was poor. A large part of the patients brushed only once a day instead of brushing twice a day and never used interdental aids to keep their teeth clean. However, only 10.83% of them managed regular dental visits. Many of the patients were not aware of the fact that their oral health may have a huge impact on their medical health. Lack of proper oral hygiene may lead to the accumulation of periodontal microorganisms in the mouth resulting in a positive response to the BANA test. Although BANA was not significantly related to the frequency of brushing (p= 0.0925), there was a significant relationship between BANA and the use of interdental aids (p= 0.01). The immmunosuppression of HIV patients influences the prevalence of periodontal diseases which in turn is exacerbated by their poor oral hygiene.

The association between CD4+T cell counts with periodontal measures may be influenced by other factors. For instance, it was found that cigarette smoking, showed a significant association with CD4+T cell counts (p = 0.0540). The plaque index (p = 0.0180), probing depth (p = 0.0191) and clinical attachment level (p= 0.0109) scores of the HIV patients examined, were also significantly related to their smoking habits, except for their gingival index scores (p= 0.150). Hence the present study established that smoking is a true risk factor for periodontal disease as was found in previous studies (Bergström and Preber, 1994; Tomar *et al*, 1995; AAP, 1996 c; Barbour *et al*, 1997) with smokers demonstrating a higher susceptibility to advanced and aggressive forms of periodontitis than non-smokers (Calsina *et*

al, 2002). A comparison of seropositive and seronegative patients revealed higher scores of gingival index and probing depth in seropositive individuals (Ndiaye *et al*, 1997) and in individuals using smokeless tobacco compared to the non-tobacco control subjects (Poore *et al*, 1995).

A significant correlation was also established in the present study between smoking and BANA positivity (p= 0.0297) in HIV-positive individuals. Other studies reported that smokers have 10 times more BANA positive plaques than non-smokers (Zambon 1996 a; Kazor e*t al*, 1999) with a statistically significant correlation in smokers with deep pockets (p = 0.02) and shallow pockets (p = 0.0002) when compared with non-smokers (Theagarayan *et al*, 2010). When examining for members of the red complex, dental plaque in smokers showed higher levels of *Tannerella forsythia* (Zambon *et al*, 1996 b; Kazor *et al*, 1999), *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* compared to non-smokers (Kazor *et al*, 1999). On the contrary, a study by Boström *et al* (2001) used checkerboard DNA-DNA hybridization technology, to demonstrate that smoking exerts little, if any, influence on the periodontally associated subgingival pathogens including the "red complex". Hence the present study agrees with these studies which confirm the use of BANA as a rapid chair-side diagnostic test for the detection of the "red complex" microbiota (Andrade *et al*, 2010; Loesche *et al*, 1992). BANA was not found to be correlated with total CD4+T cell counts nor with grouped CD4+T cell counts in the present study.

A highly significant association was found between ART with both total CD4+T cell counts and CD4+T cell counts categorized into groups (p= <.0001). A large percentage of the individuals without ART suffered with severe immunosuppression (<200 cells/mm^3). This clinical finding confirms that antiretroviral therapy in HIV-positive patients has a strong effect on their immune system and unlike patients on ART who demonstrate an increase in their CD4+T cell counts; these patients show evidence of being severely immunosuppressed. As in the study by Smith *et al* (2004), the majority of patients in the present study showed higher CD4+T cell counts as a result of undergoing long-term antiretroviral therapy. Not all the patients who participated in this study were on ART or any other form of antimicrobials. This may have contributed to their higher prevalence of periodontal disease. It can be suggested that an early introduction of HIV patients with CD4+T cell counts <200cells/mm^3 to ART may limit their exposure to immunosuppression and periodontal disease.

In the present study, ART showed significant relationships with probing depth and clinical attachment level but not with the plaque and gingival indices. The patient compliance with ART, besides being positive for the virus, was thought to determine the severity of periodontal destruction. A study by Vernon *et al* (2009) including 112 HIV-positive adults on HAART, reported a significant association between CD4+T cell counts <200 cells/mm^3 and higher levels of clinical attachment loss and periodontal pathogens thus proposing periodontal disease as a deadly challenge to the long-term oral health of HIV-infected individuals. Few studies have reported on the effectiveness of HAART and the reduced incidence of oral damage (Mulligan *et al,* 2004; Greenspan *et al*, 2004). Some studies have claimed that HAART reduces the incidence of HIV-related oral manifestations (Patton *et al*, 2000 b; Hardy *et al*, 2002; Nicolatou-Galitis *et al*, 2004), while yet others demonstrated no correlation between CD4+T cell count and ART duration with probing depth and suggested that these immune factors appeared to have less impact on acute periodontal disease (Beck and Offenbacher, 2002).

No significant association was found between BANA and ART (p= 0.8422). This observation can be supported by the study conducted by Botero *et al* (2007 b) establishing that ART has no effect on the composition of subgingival microbiota in HIV patients with periodontal disease. Moreover there was no association found between BANA and CD4+T cell counts in the HIV-positive patients in the study which could explain the lack of any association between BANA and ART.

As discussed earlier, the subgingival microbiota in periodontal disease consists of a pool of several microorganisms coaggregating with each other in the establishment of a pathogenic community facilitating the progression of periodontal inflammation. Since the current investigation was strictly restricted for the detection of "red complex" subgingival pathogens only, knowledge of the relation with one or a combination of other periodontopathic bacteria causing destruction of the periodontium cannot be ignored. This could be considered as a limitation of the study since other bacterial species that might have been present subgingivally were not detected. The virulence factors of the "red complex" species in synergy with other oral bacteria should be considered pathogenic.

CONCLUSION

To conclude, the above study establishes an association between CD4+T lymphocyte levels and periodontal disease indices in HIV-positive patients. The clinical signs and symptoms of gingival and periodontal disease with reduced CD4+T cell counts remain as a significant complication of HIV infection. This shows the importance of higher periodic clinical control to prevent these periodontal manifestations. Long term ART greatly reduces the exposure to immunosuppression and also to some extent may reduce the incidence of periodontal disease.

Special attention to the presence of Gram-negative bacteria is essential. BANA has proved to be effective in the early diagnosis of periodontal disease. In the present study, the "red complex" subgingival pathogens were closely related to the clinical parameters of inflammation and periodontal destruction and appear meaningful in periodontal diagnosis. However, the presence of the "red complex" pathogens were not associated with the immunosuppression of the HIV-positive patients. A positive BANA test after the regular maintenance program indicates the repopulation of these bacteria subgingivally. Therefore, it further helps the clinician to monitor the periodontal disease progress. The BANA test also helps to determine the diseased sites that require any form of periodontal treatment.

Oral hygiene maintenance and smoking habits appeared to be strongly related to periodontal disease, forming risk factors for gingivitis and periodontitis. A greater emphasis on oral health and prevention of periodontal disease is necessary especially among HIV-infected individuals. Comprehensive dental care should be advised to all the immunosuppressed HIV patients especially those who neglect their oral health.

Therefore, this study determined an association between CD4+T lymphocyte cell counts and periodontal disease progression in HIV-positive patients. Although, "red" complex pathogens were detected by the BANA test in HIV-positive patients with indicators of periodontal disease, no association was dertermined between the "red" complex pathogens and CD4+T lymphocyte cell counts or immunosuppression in these patients. .

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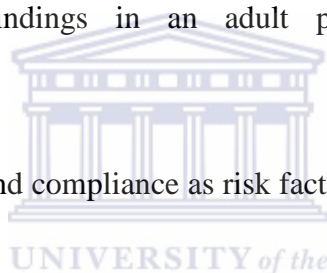
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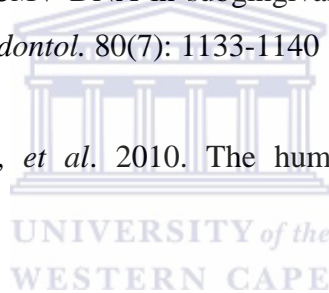
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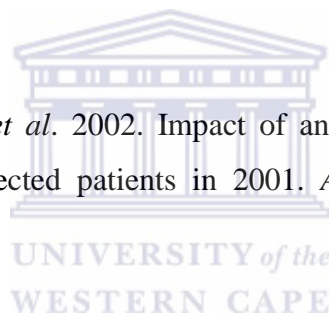
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CONSENT FORM FOR PARTICIPATION IN RESEARCH PROJECT

Title of Project: Association of CD4+T lymphocyte levels and "red" complex pathogens of chronic inflammatory periodontal disease in HIV-positive patients

Names of Researchers: Dr. Cathy Nisha John, Prof. LXG Stephen, Prof CWJ Africa

If you would like to participate in this study please tick the relevant boxes:

1. Have you read the attached information sheet and has the purpose of the research project been explained to you?   Yes or No

2. Do you understand the method of sample collection and any risks involved?
   Yes or No

3. Do you grant permission for information from your medical records to be disclosed to the research team as and when necessary?   Yes or No

4. Do you agree that samples collected for research or diagnostic testing can be stored for possible use in future research projects conducted by the above named researchers and /or other research collaborators? Yes or No

I declare that my participation in this research is voluntary and that I am free to withdraw my approval for use of the sample(s) at any time without my medical treatment or legal rights being affected. I understand that any information contained in my file will remain confidential and that I or my doctor will be informed if any of the results of the medical tests done (as part of the research) have implications for my health. I know how to contact members of the research team should I change my mind about participating in this study.

……………………………   ……………….   …………………
Name of patient   **Date**   **Signature**
(BLOCK CAPITALS)

……………………………   ……………….   …....……………
Name of person taking consent   **Date**   **Signature**

…………………………   ……………….   …………………
Name of researcher   **Date**   **Signature**

THANK YOU FOR AGREEING TO PARTICIPATE IN THIS RESEARCH

Office of the Deputy Dean
Postgraduate Studies and Research

Faculty of Dentistry & WHO Collaborating Centre for Oral Health

UNIVERSITY OF THE WESTERN CAPE

Private Bag X1, Tygerberg 7505

SOUTH AFRICA

Date: 20th July 2011

For Attention: Dr CN John
Department of Oral Medicine and Periodontology

Dear Dr John

STUDY PROJECT: Association of CD4+ T lymphocytes levels and "RED" complex pathogens of chronic periodontal diseases in HIV positive patients

PROJECT REGISTRATION NUMBER: 11/5/19

ETHICS: Approved

At a meeting of the Senate Research Committee held on Friday 10th June 2011 the above project was approved. This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project title and registration number in all further correspondence. Please carefully read the Standards and Guidance for Researchers below before carrying out your study.

Patients participating in a research project at the Tygerberg and Mitchells Plain Oral Health Centres will not be treated free of charge as the Provincial Administration of the Western Cape does not support research financially.

Due to the heavy workload auxiliary staff of the Oral Health Centres cannot offer assistance with research projects.

Yours sincerely

Professor Sudeshni Naidoo

INFORMATION SHEET

Prospective participants are requested to read this information sheet carefully and to ask questions where necessary, before signing the attached consent form. This sheet must be detached and retained by the participant and the consent form filed for record.

Evidence has related the progression of HIV infection and the severity of periodontal inflammation and subsequent destruction of the periodontium. Based on the risk assessment, HIV infection is a risk indicator that increases the susceptibility to periodontal diseases. HIV infection results in gradual loss of immunologic functions, especially those mediated by CD4+T helper cells with consequent impairment of immune response leading to severe periodontal conditions. The bacterial species associated with periodontal disease are located within the dental plaque or oral biofilm both above and below the gum margins. Therefore, the purpose of this study is to investigate the presence of subgingival periodontal pathogens in HIV positive patients, with chronic inflammatory periodontal disease at various CD4+T lymphocyte levels and to shed light on its association with periodontal disease progression.

The clinical procedure will entail the measurement of oral clinical indices for use in making a clinical diagnosis of periodontal disease and the removal of plaque samples from the selected teeth to investigate the presence of periodontal pathogens. The clinical measurements and sample collection are non-invasive and safe and will be carried out with the utmost care to ensure the comfort of the patient.

Patients will be required to sign the attached form granting consent for these procedures to be carried out and for the subsequent use of the samples donated and clinical parameters recorded. The patient will also be required to grant permission for her HIV status and other medical history to be disclosed. Participants will not be recorded by name, but samples and information will be coded to protect the identity of the individual. However, the coding will be used by the clinic to trace the individual if relevant information (as a result of the study) should be passed to her or her doctor. Where necessary, participants with severe periodontal disease will be referred for treatment. Permission will also be sought for the use of additional biological material collected in the clinic, which is usually discarded but which the researchers may find useful for future research.

Participation in this study is voluntary and refusal to participate will not prejudice the treatment of the patient in any way. Consent to participate will be recorded by completing the attached form. Should individuals agree to participate and later change their minds, they may withdraw by calling the following persons:

Prof LXG. Stephen, Department of Periodontics and Oral Medicine, University of the Western Cape, Tel: 021 9373131, or Prof CWJ. Africa, Department of Medical Biosciences, University of the Western Cape, Tel: 021 9592341, or Dr Cathy Nisha John, Tygerberg dental hospital, Tel: 021 9373167.

APPENDIX 4:2 D

Health Risk Assessment Questionnaire

Dear patient,

As part of this study we need to collect information pertaining to your lifestyle so that an assessment of your health risks may be made. Any information contained on this sheet will be held in the strictest confidence and I would urge you to respond to the questions with accuracy. No details of personal identification will be included for your protection.

Age (yrs)………..   Gender (M/F)………

Race (White/ black/ coloured/others)…………..

1. What is your level of formal education? High school/ Primary school/ University/ No formal education	2. Do you live in a house? Flat/ Shack/ House
3. How many people share your home?	4. How long have you been living at your present address? <5years, 5-10years, >10years
5. (i) Have you ever had a sexually transmitted disease? Yes/No (ii)If yes, do you know what it was and was it treated?	6. How long have you been diagnosed with HIV infection? Days…../ Months…..../ Years…….
7. (i) Are you being treated with antiretroviral drugs? Yes/No (ii) If yes, what is the duration of the treatment?	8. (i) How frequently do you visit a dentist? Never/ Once a year/ Twice a year/ Whenever (ii)When was the last time you visited a dentist? (iii)Do your gums bleed when you brush your teeth? Yes/No (iv)How frequently do you brush your teeth? Once a day/ Twice a day/ After every meal (v)Do you use any interdental aids? Yes/No
9. (i) Do you smoke? Yes/No/Sometimes (ii)If yes, how many a day? <5/ 5-10/ >10 (iii)How long have you been smoking? <5years/ 5-10years/ >10years	10. What does your diet mainly consist of ? Bread/ Meat/ Fruit and Veg

Thank you for your participation

APPENDIX 4:7:1 E

CLINICAL MEASUREMENTS

Patient number: …………………… Date of examination: ……………………

Date of Birth: …………………….. CD4+T cell count: …………………….

	16m	21m	24m	36m	41m	44m
PI						
GI						
PD						
CAL						
BANA						

Observations

……
……
……

Examiner…………………………

Assisted by……………………….