PREVALENCE OF INTERLEUKIN-1 POLYMORPHISMS AND ITS ASSOCIATION WITH PERIODONTAL DISEASE STATUS IN THE XHOSA POPULATION OF SOUTH AFRICA

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Keywords

Periodontitis

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ABSTRACT

Background: Periodontitis is a bacterially induced chronic inflammatory disease that destroys the supporting tissues of teeth. Clinical observations and experimental evidence indicates that there is a genetic influence of risk factors for periodontitis. A specific pattern of IL-1 polymorphisms (known as the composite IL-1 genotype) has been found to influence the severity of chronic periodontitis.

Objective: To evaluate the association between the IL-1 gene polymorphisms and the severity of periodontal disease in a selected Xhosa population of South Africa.

Methods: Ninety-nine subjects, 35-60 years of age who were non-smokers and free of systemic disease were enrolled in a case-control study depending on their periodontal status (healthy to mild vs. moderate to severe disease). A buccal smear was obtained from the patient; the DNA was isolated then amplified by polymerase chain reaction (PCR). Allele identification was either by real-time PCR or by the size of fragment after restriction digestion and separation in polyacrylamide gel.

Results: Logistic regression analysis was conducted to estimate the effects of gender, age, plaque index, gingival index, number of teeth lost and the IL-1 allele status on the severity periodontal disease. IL-1A polymorphism had a statistically significant effect on the severity of periodontitis. The prevalence of allele 2 was 46.9 % in cases and 22 % in controls at the IL-1A +4845 locus, and 15.8 % in cases and 14.3 % in controls at the IL-1B +3954 locus.

Conclusions: This study demonstrated that the IL-1 composite polymorphism is not related to the severity of periodontitis in the Xhosa population of South Africa. This supports the recent findings that the composite genotype does not show the same association in patients of different ethnic groups.

SUMMARY

Periodontitis is a bacterially induced chronic inflammatory disease that destroys the bone and connective tissue attachment to the teeth. Host response to periodontal disease is a protective, as well as a destructive factor and the role of pro-inflammatory cytokines has been reported. A specific pattern of IL-1 polymorphisms has been found to be associated with increased risk for severe generalized periodontitis.

Aim: The aim of this study was to evaluate the association between the IL-1 gene polymorphisms and the severity of periodontal disease in the Xhosa population of South Africa

Materials & Methods: Ninety-nine subjects, 35-60 years of age non smokers, free of systemic disease were enrolled in a case-control study, depending on their periodontal status (healthy to mild vs. moderate to severe disease). A buccal smear was obtained from the patient; the DNA was isolated then amplified by PCR. Allele identification was either by real-time PCR or by the size of fragment after restriction digestion.

Results: Interleukin-1A polymorphism had a statistically significant effect on the periodontal condition. The carrier rate of allele 2 at IL-1B failed to distinguish between cases and controls. Thus, 15.8 % of the cases and 14.3 % of the controls carried at least one #2 allele at the IL-1B +3954 locus, and 46.9 % of the cases and 22 % of the controls at the IL-1A +4845 locus. Logistic regression analysis was conducted to estimate the effects of gender, age, plaque index, gingival index, number of lost teeth as well as Interleukin-1 allele status on the periodontal disease condition.

Conclusion: The findings of the study demonstrated that the IL-1 composite polymorphism is not related to the severity of periodontitis in the Xhosa population of South Africa. The findings supported the recent findings that the composite IL-1α-4845 and IL-1β +3953 genotype reported to be associated with severity of adult periodontitis in Northern European non-smokers does not show the same association in patients of other ethnic groups.

DECLARATION

I hereby declare that 'PREVALENCE OF INTERLEUKIN-1 POLYMORPHISMS AND ITS ASSOCIATION WITH PERIODONTAL DISEASE STATUS IN THE XHOSA POPULATION OF SOUTH AFRICA' is my own work, that it has not been submitted before for any degree or examination in any university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Abu Saleh, Tareq

May 2007

Signed:……

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DEDICATION

To my family for their constant sacrifice support and love when they needed me to be with them

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CAL: Clinical attachment loss

CEJ: Cemento enamel junction

GCF: Gingival crevicular fluid

CP: Chronic periodontitis

PI: Plaque index

GI: Gingival index

DNA: Deoxyribonucleic acid

PCR: Polymerase chain reaction

dNTPs: Deoxyribonucleotide triphosphate

$MgCl_2$: Magnesium Chloride

AP: Aggressive periodontitis

IL-1: Interleukin-1

TNF: Tumor necrosis factor

TBE: Tris Borate Ethylenediaminetetraacetic acid (EDTA)

CHAPTER 1

INTRODUCTION

Bacterial pathogens are the primary etiologic factors in periodontal disease.[1] The disease process is modified by several risk factors, including; smoking, systemic diseases and genetic influences.[2]

Host response to periodontal disease is a protective as well as a destructive process and the role of pro-inflammatory cytokines, notably interleukin-1 and tumor necrosis factor in bone resorption and attachment loss has been reported.[3]

Recent studies have demonstrated that a specific polymorphism in the interleukin-1 gene cluster is associated with severity of periodontitis.[4, 5, 6]

Studies designed to estimate the genetic risk for periodontitis indicated that these diseases are etiologically complex and that both smoking habits and ethnicity are confounding factors .[4, 7, 8]

The prevalence of periodontitis appears to vary between populations with different genetic backgrounds, and findings from one ethnic group may not be extrapolated to other ethnic groups. Perusal of the literature indicates that this is the first study to evaluate the prevalence of the interleukin-1 composite genotype and its association with chronic periodontal disease in a population of African ancestry, namely the Xhosa population of South Africa.

CHAPTER 2

LITERATURE REVIEW

2.1- INTRODUCTION AND DEFINITIONS

Periodontitis is a bacterially induced chronic inflammatory disease.[1] Toxins produced by bacteria in the plaque activate the body's inflammatory and immune mechanisms which can lead to the destruction of the bone and periodontal tissues supporting the teeth. [3] Periodontal disease is progressive, and it can be classified by the magnitude of tissue destruction as mild, moderate, or severe.[9] Approximately 10 -15% of the adult population have a severe generalized form of the disease, that might result in tooth loss or require surgical intervention. [10]

Smoking has been strongly associated with an increased prevalence and severity of periodontitis. Other reported risk factors of periodontal disease are age, diabetes, stress and malnutrition .[3]

The clinical observations and experimental evidence indicate that there is a genetic component to risk for periodontitis in adults.[2,4,5,11] Studies of identical twins demonstrated that genetics plays a significant role in the variability of chronic periodontitis.[12] There is also evidence that aggressive periodontitis has a genetic component. However; this type of periodontitis of low prevalence in the population and as it tends to cluster in families, the likelihood of inheriting aggressive priodontitis may be high.[13]

Linkage and association studies [11] showed that genetic polymorphisms associated with risk for various forms of periodontitis in different populations were in genes that code for inflammatory cytokines such as interleukin-1 and tumor necrosis factor, immunoglobulin receptors, and the vitamin D receptor. [6,14,15, 16]

One of the primary components of the inflammatory and immune responses is cytokine production. Cytokines are peptide/protein immunomodulators that are produced by activated immune cells including T lymphocytes, B lymphocytes and monocyte/macrophages. These cytokines include interleukins (IL-1 through to IL-15), tumor necrosis factors (TNFs alpha. & beta.) interferons (IFN alpha. beta. & gamma.) and colony stimulating factors (CSFs) for granulocytes and/or macrophages (CSF-G, CSF-M) [6, 14,15,16]

The IL-1 family consists of three genes: IL-1A (IL-1a), IL-1B (IL-1b) and IL-RN (IL-1 receptor antagonist). Cytokines such as (IL)-1b and (TNF) have an important role in collagen and bone destruction in periodontal disease. Other modulators such as prostaglandins (such as PG E2), and matrix metalloproteinases (MMPs) also play a significant role. [3,16,17]

Some studies have shown that allelic polymorphisms controlling the inflammatory and other immune responses in certain diseases with inflammatory components affects the susceptibility or severity of these diseases.[5,16] Attempts have been made to elucidate the association of single nucleotide polymorphisms (SNPs) between patient populations with regard to specific disease patterns.[14]

In a report by Kornman and co-workers it has been suggested that a specific pattern of IL-1 polymorphisms was associated with a

significantly increased risk for developing severe generalized periodontitis. This genotype includes two polymorphisms of the IL-1 gene cluster on chromosome 2 and has been refered to as the composite genotype. [5,14, 16,18]

According to Kornman the composite interleukin –1 genotype was described as allele 2 at the IL-1A (-889) locus plus allele 2 at the IL-1B (+3953) locus.[5] Another polymorphism at IL-1 (+4845) was found to be 100% concordant with the IL-1 (-889) locus, and technically it is less difficult to be assayed. The IL-1B polymorphism was renumbered to IL-1B (+3954) .[18] It is now accepted that the composite interleukin –1 genotype is described as allele 2 (Thymidine substitutes Guanine) at the IL-1A (+4845) locus plus allele 2 (Thymidine substitutes Cytocine) at the IL-1B (+3954) locus.[18,19]

Kornman et al.[4] reported that the IL-1 genotype is significantly associated with severe generalized periodontitis in non-smoking adults. In smokers, severe disease was not correlated with genotype. [4] Several other studies were conducted in different populations that either reported similar results or challenged Kornman's findings. [5,6, 15, 20-23]

In this review the prevalence and etiological factors of chronic periodontitis are discussed to give a general understanding of the role of IL-1 polymorphisms as a potential risk factor for chronic periodontitis.

No relevant information was found about the periodontal health and the relationship to IL-1 polymorphisms in South African populations of African ancestry, in the last decade. This study investigated the

prevalence of Interleukin-1 genotype and its association with the periodontal disease status in the Xhosa population of South Africa.

Definitions

For more convenient reading the definitions are listed at the end of the thesis, *see appendix A*

2.2- PREVALENCE OF PERIODONTAL DISEASE

Periodontitis, a chronic multifactorial disease in adults, is associated with gram-negative bacteria (see page 9). The most common form of periodontitis is chronic periodontitis, which has been reported to affect more than 30% of the population, and it has been claimed that about 10 % of the adult population have a severe generalized form of the disease.[10] Chronic periodontitis can be explained as a result of an interaction between the host immunoinflammatory response and gram-negative bacteria.

The general understanding about periodontitis was that it is universally prevalent in middle age people as a result of bacterial plaque accumulation on teeth and so preventative measures were directed at controlling the bacterial challenge. However, studies found that even with high plaque challenges, severity of periodonitits was variable.[11]

In 1986, Löe and co-workers [24] conducted a study on the natural history of periodontal disease in man.[24] Over a period of 15 years, they examined a group of male tea workers in Sri Lanka (age range 14 to 31 years). These men did not follow any conventional oral hygiene measures and had no access to dental care; they presented with large amounts of plaque, calculus, and stains on their teeth, and almost all the gingival units were inflamed thus allowing the study of the natural progression of untreated periodontal disease.

On the basis of interproximal loss of attachment, it was found that 81% of the men had moderate progression in attachment loss, 11% did not progress beyond gingivitis and the remaining group; 8% exhibited rapid loss of attachment, losing between 10 and 32 teeth over 15 years.

The group exhibiting rapid progression had a mean loss of attachment of approximately 9 mm at age 35 years, which increased to approximately 13 mm at age 45 years, with an annual rate of destruction of 0.1 to 1.0 mm.

In individuals exhibiting moderate progression, the mean loss of attachment was approximately 4 mm at age 35 years and 7 mm at age 45 years, with an annual rate of destruction between 0.05 and 0.5 mm. The mean loss of attachment in individuals with no progression of disease was 1 mm at age 35 years, with an annual rate of destruction between 0.05 and 0.09.

Conclusions that the authors drew from this study are that periodontitis could be a group of diseases that differ in extent and severity rather than a single disease entity. Severe periodontitis was found in a small percentage of the population and a fraction of persons were immune to periodontitis. Also contrary to popular theory not all cases of gingivitis will proceed into periodontitis.

Several studies indicated that other factors may play a significant role in periodontitis. [25-27]

Clinical differences in the prevalence and severity of chronic periodontitis could be due to genetic host's makeup. A patient's resistance to periodontal disease is influenced by his genetic make-up,

and this has been determined from a variety of sources: studies of twins, studies of aggressive periodontitis, natural history studies, and cytokine genetics studies. [2,4,5,11,12,13,24]

These findings have shifted the focus of periodontal research over the last few decades into the host susceptibility risk factors such as genetic factors to periodontal disease.

2.3- RISK FACTORS OF PERIODONTAL DISEASE

2.3.1- ROLE OF BACTERIA IN PERIODONTAL DISEASE

Introduction

It is generally accepted that the primary etiologic factor for periodontal disease is bacterial plaque.[28] The composition of dental plaque varies considerably between patients and among different sites in the same patient.

It has been demonstrated that specific elements of host susceptibility, such as an individual's systemic disease state and immune response, are important factors affecting the progression of disease.[25-27]

Studies have identified three bacterial species to be consistently prevalent in plaque associated with severe disease. These bacteria are now officially designated as 'periodontal pathogens'. These are *Actinobacillus actinomycetemcomitans(Aa), Porphyromonas (Bacteroides) gingivalis* (*Pg*) and *Tannerella forsythia(Tf).* [28-31]

Other species of bacteria likely to contribute to the etiology of periodontitis both in adults and children disease are *Treponema denticola, Selenomonas* and *Eikenella* species. [29,30]

Recent work suggested that bacterial species exist in complexes within subgingival plaque, and it is these microbial complexes that contribute and exacerbate the disease processes.[28-30] It seems most likely that the organisms benefit from nutritional and metabolic interdependencies and collectively are able to resist the host defenses more efficiently. The most significant complex is that which includes *P. gingivalis, T. forsythia* and *T. denticola* because species in this complex

increase in numbers and prevalence with increasing pocket depth and bleeding on probing.[30]

A larger complex of organisms is implicated with advanced periodontitis include the black-pigmented *Prevotella intermedia* (formerly *Bacteroides intermedius*) and *Prevotella nigrescens*, *Peptostreptococcus micros* and *Fusobacterium nucleatum.*[29,30]

A. actinomycetemcomitans serotype b isolates are uniquely associated with aggressive periodontitis. These isolates are rarely found within a specific bacterial complex, but occasionally they cluster with *A.actinomycetemcomitans* serotype a, which in turn is found in association with Eikenella corrodens and various Capnocytophaga species.

Initiation and progression of disease

Dental plaque is a soft deposit that accumulates on teeth. Plaque can be defined as a complex microbial community, with approximately 10^{10} bacteria per milligram. It has been estimated that as many as 400 distinct bacterial species may be found in plaque. In addition to the bacterial cells, plaque contains a small number of epithelial cells, leukocytes, and macrophages. The cells are contained within an extracellular matrix, which is formed from bacterial products and saliva. The extracellular matrix contains proteins, polysaccharides and lipids.[28, 30]

Bacterial plaque is essential for the initiation and progression of periodontitis. However, it now appears that once the bacteria are present, the amount of periodontitis that a patient develops is due to factors related to the body's response to the bacterial challenge. In

this context the value of plaque sampling in non-responding patients is of growing interest.[30] One principal reason for the differences in how patients respond to plaque, manifest disease, and respond to treatment is that there are different types of plaque.

The fastest growth of the plaque occurs at the gingival margin, where plaque accumulations usually are visible after several days. This plaque may, in some instances, provoke gingivitis where *Spirochetes* and *Actinomyces viscosus* are prominent members of the plaque flora. [28,30] If this plaque remains undisturbed, the flora gradually shifts toward an anaerobic, Gram-negative flora which includes black pigmented bacteroides and several types of spirochetes. [28] The increase in growth potential of these anaerobic organisms can be explained by the low oxidation-reduction potential of the aged plaque and by nutrients derived from the inflammatory exudate at the site.

Disease episodes ensue from an upset in the balance between bacteria and host, such as changes in the relative numbers and pathogenic potential of certain bacteria, or modulation of host factors.

The process of periodontal disease initiation is not fully understood but it seems that the so-called 'primary colonizers', including *Streptococcus*, and *Actinomyces*, which bind strongly to saliva-coated surfaces and to gingival epithelial cells, are recognized by later (secondary) colonizers, which include some periodontal pathogens, with the former acting as a base for the latter to adhere to. [28,30]

The primary colonizing bacteria also create an anaerobic environment and provide nutrients for the late colonizers.

Bacteria in the plaque, touching the tissue, elaborate various compounds, such as hydrogen sulfide, ammonia, amines, endotoxins lipopolysac-charide (LPS), enzymes (such as collagenases) and antigens, all of which penetrate the gingiva and activate host cells (i.e., fibroblasts, macrophages, and polymorphonuclear leukocytes [PMNs]) to secrete proinflammatory cytokines such as interleukin-1 beta (IL-Ib) and tumor necrosis factor alpha (TNF-a). [28,32] This inflammatory response, although protective, appears to be responsible for a net loss of periodontal supporting tissue, and leads to periodontal pocket formation, mobility of the teeth, and eventual tooth loss.

Clinical evidence has demonstrated that not all individuals have the same response to similar amounts of plaque accumulation. There are patients with moderate and advanced disease who have very little plaque while other patients with little disease have large amounts of plaque. Most importantly, large studies that have assessed the relationship of plaque quantity, as well as the presence of specific bacteria, to the severity of periodontitis indicate that a substantial part of the variation in clinical severity of disease may be explained by factors other than the bacterial challenge.

2.3.2- THE RELATIONSHIP BETWEEN AGE AND PERIODONTAL DISEASE

The prevalence of periodontal disease increases with age.[33, 34] This could be due to the presence of the potential risk factors for a longer period or due to the consequences of aging. Although aging is associated with metabolic changes, it has not been directly linked with periodontal attachment loss. While the rate of periodontal destruction increases after the age of 70 years, up to that age, the rate of attachment loss is the same.[35]

Studies of natural history of periodontitis showed that patients might have different rates of periodontal destruction and therefore the amount of periodontal loss may be different at a specfic age in different individuals. [24] An important theory is that some risk factors might be potentiated in certain age groups. [4]

2.3.3- SMOKING AND PERIODONTAL DISEASE

Smoking is strongly associated with periodontitis. It has been reported that in adults aged 19-40 tooth loss is strongly associated with smoking more than 15 cigarettes a day.[36] Grossi and co-workers[37] found that, a light smoker (<10 cigarettes a day) was 2.0 times more likely to have alveolar bone loss as compared to a nonsmoker. In a heavy smoker (>10 cigarettes a day), the odds ratio was 7.3. That report revealed a dose–response relationship between smoking and bone loss.

The biological basis for smoking as a risk factor for periodontal disease is evident and results in inhibition of neutrophil function in saliva and connective tissues, [38] suppression of IgG2 antibody response and

increase of the release of (IL-1ß), which greatly affects osteoblast function.[39] In addition, some ingredients in tobacco constrict the gingival blood vessels, which accounts, in part, for the lack of bleeding on probing observed in most smokers. It has been reported that the response to periodontal therapy, whether surgical or non surgical, is less in smokers than in non-smokers, and a higher recurrence to disease was observed in smokers.[40-41]

2.3.4- EFFECT OF DIABETES ON PERIODONTAL DISEASE

The likelihood of periodontal disease increases when diabetes is poorly controlled.[42] People with diabetes mellitus are more likely to be edentulous than people without the disease (odds ratio 15). On the other hand well-controlled diabetics, with good oral hygiene and on a regular supportive periodontal treatment have the same chance of developing severe periodontitis as healthy subjects.

The biological mechanism is complex. The small blood vessels of people with diabetes have thickened basement membranes, thus leading to a reduction in transport across the vessel walls. There is a reduction in collagen production by gingival and periodontal fibroblasts. In addition, high levels of pro-inflammatory mediators responding to endotoxin from gram-negative bacteria lead to an increase in collagen breakdown. [42]

2.3.5-GENETIC FACTORS AND PERIODONTAL DISEASE

Introduction

Chronic periodontitis appears to be multifactorial, meaning that susceptibility involves multiple genes and environmental factors.

The genetics of the more common chronic forms of periodontitis appear to be complex. There is no clear evidence of a simple Mendelian pattern of genetic transmission that would support an etiologic role for a single gene mutation in chronic periodontitis.

The search for susceptibility genes for complex diseases is becoming more extensive, by non-molecular approaches (segregation analysis by studying twins, family studies, or unrelated individuals in a case-control design), and by molecular approaches (family studies or case-control approaches coupled with analyses of genetic marker polymorphisms). However, molecular approaches offer better analyses to assess whether specific genes or candidate regions are associated with complex diseases such as chronic periodontitis. [43]

Complex diseases may be studied using both molecular and non-molecular approaches to more completely understand their genetic and environmental nature.

It has been reported that specific mutations were able to define the genetic basis of some rare syndromic conditions, for example, the cathepsin C gene in Papillon-Lefèvre syndrome, [44] the CHS gene in Chédiak-Higashi syndrome, [45] and the beta-2 integrin chain gene in leukocyte adhesion deficiency type 1.[46]

While understanding rare forms of disease may expand the understanding of periodontitis, it may not provide specific genetic tests to guide practitioners in managing the majority of patients.[47]

There have been remarkably few studies of chronic periodontitis in families, and although some studies reported a significant sibling effect for clinical parameters of disease in non-twins, [48] others have concluded that the basis of this similarity is the shared environment or culture, but not genes.[49-51]

Studies of aggressive periodontitis

Familial aggregation of severe early-onset forms of non-syndromic aggressive periodontitis has been noted in the literature. [2, 52, 53]

Although heritable differences among individuals appear to play an important role in risk for early-onset periodontitis (EOP), the specific nature of genetic risk is unclear. These conditions are more complex than the simple Mendelian syndromes.[54-56] Some studies suggested familial transmission of juvenile periodontitis as an autosomal trait (both dominant and recessive transmission), consistent with a gene of major effect.[57] Boughman and coworkers reported localization of an EOP gene to chromosome 4q, apparently providing direct evidence for the existence of a gene of major effect for EOP in a genetically unique population isolate.[58] Segregation analysis and linkage analysis indicate that there are multiple different genetic forms of aggressive periodontitis but it is currently unclear how many genes may be involved in aggressive periodontitis.[57-60]

Aggressive periodontitis is rare and it is difficult to collect enough information from families to provide sufficient evidence to identify disease-related genes.[55, 56]

It appears that some genes of major effect are associated with aggressive periodontitis and some rare syndromic forms of periodontitis. There is evidence that some genes may modify the severity of periodontal disease. Environmental and microbial agents also modify the risk and expression of disease.

Some studies suggested that a single gene of major effect may be etiologically responsible for chronic periodontitis, while other studies support a more complex genetic etiology and it is likely that the synergistic effect of multiple genes is pivotal of disease susceptibility in chronic periodontitis.[2, 61] Furthermore, these models suggest that these genes interact with other genes and with environmental factors over a substantial period of time to ultimately influence susceptibility.[62]

The genetic influence in chronic diseases is difficult to study since so many confounding factors are interacting during the progress of the disease. Besides, it is not practical to study families in different generations in chronic diseases. Therefore, in chronic periodontitis with an older age of onset, evidence for a genetic component for periodontitis susceptibility comes primarily from twin studies.[2, 11, 12]

Studies of Twins

The most convincing demonstration of a genetic influence on adult periodontitis came from studies of twins by Michalowics and coworkers.[11, 12] Twin studies are typically used to estimate heritability for a trait or disorder. In general terms, heritability is the proportion of the variance in the population that is due to individual differences in genetic makeup.

Comparison of concordance rates of disease (i.e. if both twins in a pair are affected, the concordance rate is 100%) in monozygotic or dizygotic twins is the classic method for determining whether familial patterns of disease are the result of common genetic or common environmental factors. The higher the concordance rate is in monozygotic twins compared with dizygotic twins, the more influencing are the genetic factors. The concordance rate in monozygotic twins is not expected to be 100%, due to environmental effects.[63]

Studies on periodontal disease in twins suggested that approximately 50% of susceptibility may be due to genetic factors.[11]

Michalowicz and coworkers[12] assessed the effect of environmental and host genetic factors to the clinical parameters of periodontal disease in adult twins.[12] The study group included 77 monozygous twins and 33 dizygous twins. A significant genetic component was identified for plaque, gingivitis, probing depth, and attachment loss based on ratios of within-pair variances or heritability estimates. It was found by heritability estimates that genetic factors resulted in a population

variance between 38 and 82% for the studied clinical parameters of disease.[12]

Another study by Michalowicz and coworkers[12] determined that the population variances for alveolar bone height (between-pair and within-pair) of the monozygous and dizygous twins were similar.[12] Calculated intraclass correlations and heritability estimates were for the reared-together monozygous (0.70) for dizygous twins (0.52), and for the reared-apart monozygous twins (0.55). The study indicated that there is significant genetic variance for alveolar bone height in the studied population. [64]

2.4- IMMUNOLOGY OF PERIODONTAL DISEASE

2.4.1- INTRODUCTION

The host defense response to pathogens depends on the immune system and there are many genetic loci that have been associated with the immune response of the host to microbial infection. The most studied genetic factor is the major histocompatibility complex (MHC) on chromosome 6, which defines the HLA system that is involved in many interactions between the cells of immune response.

Gene polymorphisms for Fc-gamma receptor (CD32 and CD16) can result in reduced phagocytic capacities, which provide a mechanism for heritable susceptibility to microbial infections. Genetic polymorphisms in Fc-gamma receptors have been associated with both early-onset periodontitis and refractory periodontitis.[65]

The role of IL-1 in the immunology of the periodontal disease will be discussed as the link between the genetic polymorphisms (the genotype) and the periodontal disease status (the phenotype).

2.4.3- ROLE OF INTERLEUKIN-1 IN PERIODONTAL DISEASE

Chemicals in the tissues that provide communications between cells are generally referred to as cytokines. The role of inflammatory cytokines has been studied extensively in periodontal diseases.[66]

An important cytokine (IL-1) plays a critical role in many different systems in the body and has been strongly implicated in the risk and severity of adult periodontitis.

The possible roles of interleukin 1 have recently received considerable attention. IL-1 facilitates the flow of inflammatory cells into sites of infection; stimulates eicosanoid release by monocytes and fibroblasts; stimulates matrix metalloproteinases; promotes bone resorption; and contributes to the inflammatory cascade of the microbial immune response. It also activates specific protective mechanisms and is involved in wound healing and bone and connective tissue metabolism. [3, 25, 26,43,67,68]

IL-1 levels have been reported to be elevated in periodontal tissues and gingival crevicular fluid associated with periodontitis. Higher production of these cytokines has also been associated with response to infection, where local induction of IL-I and TNF facilitates the elimination of the microbial invasion. However, classic studies also report that in some infectious conditions very high levels of monocytic cytokines are produced and initiate a cascade of concomitant events, such as tissue catabolism, vascular reactivity, and hypercoagulation, with damaging effects on the host. [15, 69]

The relationship between IL-I and periodontitis has been extensively reviewed by Offenbacher.[3] While the inflammatory process, in general, increases the local tissue levels of IL-I, differences between people in cytokine production rates have been fixed, which indicates that some people are more likely to produce cytokines upon stimulation.[3]

IL-I genes (IL-IA, IL-IB, and IL-IRN) cluster on chromosome 2q. [13] Interleukin- IA and IL- 1 B encode for the proinflammatory cytokines IL- Ia and IL-Ib, and IL-IRN encodes IL-lra, a receptor antagonist.

Several genetic polymorphisms have been identified in the genes of the IL-I cluster. Pociot and coworkers[17] reported that a TaqI polymorphism in the interleukin-1β gene correlated with secretions of interleukin-1β in vitro.[17] Other studies found that it was correlated to endotoxins produced by Gram-negative bacteria.[69]
Kornman and coworkers.[4] reported that the presence of a composite genotype (allele 2 at two interleukin-1 gene polymorphisms) was associated with increased severity of periodontal disease in adult non-smokers of Northern European ancestry.[4] This was based on the premise that patients with the composite IL-I genotype have higher levels of IL-I in the gingiva than those observed in genotype-negative patients.

Engebretson and coworkers[15] have determined the levels of IL-1a and IL-1b in gingival biopsies and the levels of IL-1b in gingival crevicular fluid (GCF).[15] The IL-1 genotype positives had higher levels of IL-1b in GCF, and the greatest differences being recorded between genotype-positive and genotype-negative patients in sites of minimal probing depth. [15]

Gingival tissue levels of IL- 1b were also higher and levels of IL- 1a were higher in genotype-positive patients. These findings were dramatic and indicate that IL-1 genotype-positive patients will have higher levels of IL-1 in periodontal tissues when there is a bacterial challenge.

2.5- GENOTYPE PREVALENCE IN DIFFERENT POPULATIONS

As polymorphisms originally arise from mutations the frequency of genetic polymorphisms often varies among different ethnic groups. Therefore, it is necessary to determine the prevalence of specific alleles in ethnic categories to determine if information derived from one group can be extrapolated to another.

In general terms, pathogenic gene mutations are rare alleles that dramatically affect the expression or function of a gene product, so that they are deterministic of disease. Genetic mutations underlie most simple genetic diseases. In contrast, complex genetic diseases may result from the combined effect of multiple functional genetic polymorphisms interacting with each other and with environmental factors to such an extent that over time, they modulate disease risk. In this complex disease model, a single functional genetic polymorphism associated with disease (at a population level) is not sufficient to cause disease, and therefore itself is not deterministic of disease. Consequently, such functional polymorphisms may be found in individuals with no evidence of disease and who may not be at great risk for disease. A fundamental characteristic of this genetic model is that such genetic polymorphisms are more frequent in the population than mutations, and the correlation between genetic polymorphisms and disease is generally much weaker than the relationship between a functional mutation and disease phenotype.[12]

When studying the genetic basis of a disease, it is important to clarify the genetic model of disease and to apply appropriate statistical tests

to determine the degree of association of a specific gene in individuals with disease and also in individuals without disease.

Several studies have indicated that 28 to 44 percent of Caucasians possess the composite genotype [4, 70-72] Reported rates in the literature on the carriage of allele 2 at the IL-1B +3954 locus are 44.1%, 40.6%, and 33.7%. [4,6,73] In the IL-1A −889 locus, which is essentially 100% concordant with the IL-1A +4845 locus, allele 2 carriage rates were shown to be 50.5% and 40.6%. [4, 6] It becomes increasingly clear, however, that such rates should be expected to vary greatly between ethnically and/or racially distinct populations. In contrast, Armitage and colleagues[8] revealed allele 2 carriage rates of 3.3% (IL-1B +3954) and 17% (IL-1A +4845), and a prevalence of the positive composite genotype of only 2.3%.[8] Furthermore, Walker and colleagues[13] reported that less than 15 percent of African-Americans in their study population were genotype-positive. [13]

The positive composite genotype was found to occur at rates of 23% and 35% in 2 samples of periodontally healthy subjects, one predominantly consisting of Hispanics and the second comprising both Caucasians and African Americans. [74, 75]

It is clear that the prevalence of allele 2 at both the IL1A+4845 and IL1B+3954 loci is different in different populations. Thus, it can be concluded that the prevalence of specific genotypes may vary among different ethnic groups, and information derived from one group can not be extrapolated to another without verification of its accuracy.

2.6- CORRELATION BETWEEN INTERLEUKIN-1 GENOTYPES AND CLINICAL CONDITIONS

2.6.1- IL- I POLYMORPHISMS AND CHRONIC PERIODONTITIS

Since Kornman et al.[4] in 1997 several studies explored the relationship between IL-1 polymorphisms and periodontitis but the results were not unanimous in supporting or refuting a positive relationship between presence of the composite genotype and the severity of periodontal disease. [4,6,7,8,72,73,76]

Several studies have evaluated the correlation between bone loss and genotype status and reported an association between genotype positivity and the development of severe periodontitis.

Kornman and colleagues[4] concluded that nonsmokers with a specific genotype were 6.8 times more likely (6.8 odds ratio) to have severe periodontitis than genotype-negative patients. However, 14 (38 percent) of 37 patients with moderate disease and 10 (23 percent) of 44 healthy patients in their study also were genotype-positive (that is, false positive findings).

The association between severe periodontitis and the genetic polymorphism in the IL- 1 genes was present only when smokers were excluded, which confirmed the importance of smoking as a risk factor for periodontitis. This was the first study that identified a genetic polymorphism that corresponds with a phenotypic immune response variable (IL- 1 production) in chronic periodontitis patients.[4] The IL- 1 genotype identified in this study appears to be a marker of a strong biological change that results in severe periodontitis, without regard to the amount of bacterial challenge as suggested by Offenbacher.[3] The

first findings indicate that the significant association between the IL-1 genotype and severity of periodontitis did not require any adjustments for the amount of bacterial plaque. The combination of having either the specific genotype or smoking accounted for the majority (86%) of severe cases of periodontitis. The IL-1 genotype was a very strong predictor of severe periodontitis in non-smokers aged 40 to 60 years (odds ratio 18.90). Among similar-aged individuals with mild periodontitis, 84% were genotype negative, which indicates the importance of an age factor on periodontal disease. [4]

It is noteworthy that the association between the genetic polymorphism in IL- 1 genes with severe periodontitis was only evident when smokers were excluded. This data supports the importance of other environmental factors, such as smoking, as a risk factor for periodontitis.[4] The association of severe periodontitis with smoking and the IL-1 genotype suggests that both factors play an important role in the pathogenesis and clinical course of adult periodontitis. Smoking, by itself, is a strong risk factor for more severe periodontitis. It is therefore reasonable to expect that some IL-I genotype-negative patients who are heavy smokers will be susceptible to more severe periodontitis. This will certainly confuse the data analysis of research studies unless this factor is taken into account in the analysis.

McDevits and colleagues [7] reported that genotype-positive patients were 3.75 times more likely to have moderate-to advanced periodontitis than were genotype negative patients. On the other hand, Mark and colleagues,[77] found no relationship between genotype status and the presence of mild-to-moderate periodontitis.

Elevated tissue and gingival fluid levels of IL-I b in particular have been repeatedly associated with the severity of periodontitis.

Gore and colleagues [6] reported a significant association between the IL-1 B polymorphism and severity of disease.[6] In their study, the composite genotype did not offer advantages over just the IL-1B markers. Such differences among the studies are not surprising as the undertaken genetic studies had small numbers of subjects.

In most of the populations that have been tested, the composite genotype occurs in approximately 30% of individuals.[4] Although it is expected that the IL-1 genotype will have the same relationship to disease in all populations, the IL-1 genotype may be found less frequently in some populations than in Caucasians.

It is also possible that other genetic factors may play a role in other ethnic groups.

Researchers have noted different genetic polymorphisms associated with chronic periodontitis and aggressive periodontitis.[13,43,78] The finding that the prevalence of allele 1 and not allele 2 of the IL1B+3953 locus was higher among people with aggressive periodontitis has been interpreted by several authors to indicate there may be genetic differences related to different types of periodontal diseases.[13,43,78] Other explanations for these results include the possibility that other genes closely linked to the tested IL-1 polymorphisms may be etiologic for periodontal disease susceptibility.[32]

At present, it seems reasonable to expect that, as with risk factors for cardiovascular disease, multiple factors such as IL-I genotype and smoking carry increased risk of more severe disease. It is likely that larger studies will help to define the magnitude of interactions between smoking and the IL-I genotype as well as other risk factors.

2.6.2- RELATIONSHIP BETWEEN INTERLEUKIN-1 BETA LEVEL AND GENOTYPE STATUS

The biological model that explains the relation between the genetic polymorphisms and the periodontal disease status is that the level of IL-1ß secreted in the crevicular fluid and periodontal tissues is related to genetic polymorphisms, specifically, that allele 2 (homo- or heterozygous) of IL-1B is associated with an increased potential to secrete IL-1ß after bacterial stimulation.

This hypothesis was based on the in vitro work of Pociot, [17] who found that people with allele 2 at the IL1B+3953 produced increased amounts of IL-1ß. This finding was not consistent in several other studies.[27, 28, 30, 38-41] For example, Engebretson and colleagues[15] found that genotype-positive patients had an elevated level of IL-1ß in the gingival crevicular fluid from shallow pockets (less than 4 millimeters), and not in deeper probing depths (deeper than 4 mm). Mark and colleagues[77] also noted that the level of IL-1ß was not related to genotype status. Other investigators who examined the levels of IL-1ß and a single polymorphism at allele 2 of the IL1B+3953 locus also reported no significant relationship with regard to the level of IL-1ß.[39, 40]

It appears that the relationship between genotype status and the level of IL-1ß is not completely understood, and that polymorphisms detected by the genetic susceptibility test cannot be used to reliably predict which patients will manifest elevated levels of IL-1ß.

2.6.3- IL- I POLYMORPHISMS AND BLEEDING ON PROBING

Lang and colleagues[70] assessed the relationship between IL-1 polymorphisms and bleeding on probing in patients receiving maintenance therapy after periodontal surgery, to determine if a correlation existed between the incidence of bleeding on probing and the presence of the positive genotype. Statistically, no relationship was found between bleeding on probing and the composite genotypes when smokers were included. However, in non-smokers, a statistically significant more bleeding was found in genotype-positive patents.

This finding may be due to the fact that smoking is a risk factor for periodontitis, and it is not possible to determine if genetic assessments reveal patients who are predisposed to develop a more pronounced host response to a microbial challenge.[24] Goodson and colleagues[75] also noted an increased amount of bleeding on probing among genotype-positive patients in a gingivitis study. Other researchers found that among untreated[16, 26] and treated patients, [20, 27] there was no correlation between the genetic susceptibility test results and the amount of bleeding on probing.[28] Different levels of oral hygiene, professional maintenance therapy and small study populations could account in part for these conflicting results.

Thus, it can be concluded that the relationship between bleeding on probing and the patient's genotype is also conflicting, and studies have not verified that individuals with the associated genotype are more prone to develop increased bleeding on probing.

2.6.4- IL- I POLYMORPHISMS AND TOOTH LOSS

McGuire and Nunn have reported an increased susceptibility to tooth loss after periodontal therapy in IL-1 genotype-positive patients.[72] Ultimately, the aim of performing dental procedures is to maintain teeth in a state of health and comfort for the life of the patient. Thus, it would be beneficial to be able to predict which people are at increased risk of losing teeth, so that this information could be incorporated into a patient's risk profile. To test this concept, McGuire and Nunn[72] performed a retrospective study, genotyping 42 patients they had followed up over 13 years.[72] They reported that genotype positive patients were associated with a 2.7 times greater chance of losing teeth as a result of periodontal problems. The odds ratio might have been even higher in a group of patients who did not comply with maintenance recall appointments.

On the other hand, the study results were unclear with regard to the number of teeth that were lost among smokers and nonsmokers and the number of teeth were lost per patient. It is interesting that 26 (62 percent) of 42 patients with periodontal disease in this investigation were genotype-negative which is the opposite of what would be expected in a group of patients in a periodontal practice.

In contrast, several other investigators genotyped patients who had been monitored during maintenance therapy for 10 or more years after periodontal treatment and did not find an increased incidence of tooth loss among those who were genotype positive.[74, 76, 80] Thus, conflicting data preclude the concept that genotype assessments could help predict which patients are predisposed to losing teeth as a result of periodontitis.

2.6.5- RELATIONSHIP BETWEEN GENOTYPE AND MAINTENANCE OF CLINICAL ATTACHMENT AFTER PERIODONTAL THERAPY

A study that involved periodontal treatment showed a synergistic risk when a patient is both a smoker and is IL-1 genotype positive.[43]

Another study suggested that genotype-positive patients were more prone to develop additional clinical attachment loss after treatment than were genotype-negative patients.[20]

A prospective study conducted over five years by Cullinan and colleagues[22] evaluated the relationship between periodontitis and the IL-1 genotype. They concluded that IL-1 polymorphisms may be a contributing risk factor for periodontal disease progression, but are not essential for it to occur, and by themselves may have only a small effect.

It seems reasonable to assume that if the IL-I genotype is associated with more severe disease; the genotype may also influence response to therapy.

Data from McGuire and Nunn validate that assumption. [72] This study determined that the significant predictors of tooth loss in periodontal patients who were monitored for 14 years after active therapy were smoking (increased risk for tooth loss where heavy smokers had a 288% increased risk of losing teeth after therapy as compared to non-smokers or light smokers) and the IL-I genotype (increased risk for tooth loss of 2.66*).* Patients who were both heavy smokers and IL-I type positive were 7.7 times more likely to lose teeth after periodontal therapy than all other patients. It is relevant that even in IL-I

genotype-positive patients, conventional periodontal therapy and good maintenance care allowed the successful retention of most teeth. [72]

Studies so far have shown evidence that IL-polymorphism analysis can provide valuable insight into an individual patient's likely response to various interventions. However, several other clinical trials that monitored patients after periodontal therapy did not find a greater predilection for attachment loss among genotype-positive patients treated nonsurgically[34, 35] or surgically.[34] Therefore, it cannot be concluded that genotype-positive patients are more susceptible to develop additional attachment loss after periodontal therapy.

Additional studies are necessary to provide greater insight into the relationships between genetic factors and periodontal and restorative therapy. The composite polymorphism marker is diagnostic; and may be used as a prognostic test to identify individuals who have a much higher susceptibility to adverse reactions to plaque.[67]

The finding of the association between the IL-polymorphism and an increase in IL- I b production and more severe periodontal disease is consistent with the current model of how genetic factors influence common chronic diseases. If this model is applied to periodontitis, it would involve a disease initiating factor, which would probably be specific bacterium and modifiers of disease mechanisms that explain the clinical severity, including certain systemic diseases, smoking, psychosocial factors, and the IL-I genotype.

2.6.6- IL- I POLYMORPHISMS AND IMPLANT LOSS

Genotyping might be able to predict survival for implants. To investigate this possibility, Wilson and Nunn[81] assessed the relationship between IL-1 genotype and implant loss. They found no increased loss of implants among patients who were genotype-positive.[81] Siervo and colleagues[82] confirmed this finding. Rogers and colleagues[83] performed IL-1 genotyping on 119 white subjects and 60 white healthy control subjects. The investigators concluded that the simultaneous presence of allele 2 at the IL1A-889 and IL1B+3953 loci was not found more often among subjects with early onset or adult periodontitis or among subjects with failed implants. Thus, similar to tooth loss, the current data do not support the assumption that dental implants are at greater risk of being lost among genotype-positive people.

Finally, The Xhosas are part of the southern Nguni African migration and they are well established African population in the Eastern Cape and Western Cape provinces in South Africa.[85] This study evaluated the prevalence of interleukin-1 polymorphisms and its association with the periodontal disease status in the Xhosa population of South Africa.

CHAPTER 3

AIMS AND OBJECTIVES

3.1 – AIM OF THE STUDY

The aim of this study was to determine the prevalence of the IL-1a and IL-1b polymorphisms in the Xhosa population of South Africa, as well as to determine whether or not there is an association between these polymorphisms and the severity of periodontal disease.

3.2 – OBJECTIVES OF THE STUDY

The objectives of this study were to:

- Evaluate periodontal health status in the Xhosa population of South Africa
- Determine the prevalence of IL-1A +4845 polymorphisms in this population.
- Evaluate the association between IL-1A polymorphism and severity of chronic periodontitis
- Determine the prevalence of IL-1B +3954 polymorphisms in the Xhosa population of South Africa.
- Evaluate the association between IL-1B polymorphism and severity of chronic periodontitis
- Evaluate the association between the composite genotype and severity of chronic periodontitis in the Xhosa population of South Africa.

3.3 – NULL HYPOTHESIS

There is no significant association between the IL-1 composite genotype and the severity of chronic periodontitis.

CHAPTER 4

MATERIALS & METHODS

4.1- STUDY DESIGN AND PREPARATION

The study design was a 1:1 matched case-control study, and the study population was of patients attending at department of oral medicine and periodontics at Mitchell's Plain Oral Health Center, Gugulethu dental clinic and Tygerberg Hospital.

Although the researcher was aiming at maximum 50 subjects at the beginning of the research (as a pilot study), the size of the sample was doubled to achieve better statistical analysis after power calculation (p-value was set at <0.05 for significance, to have 95% confidence interval and 80% power), and a total of 108 patients were finally enrolled in the study.

Subjects included were 35-60 years of age, free of systemic disease, non smokers, with no history or evident signs of aggressive periodontitis, and both parents and grand parents of the Xhosa population. The Xhosas are part of the southern Nguni African growth and they are well established population in the Eastern Cape and Western Cape provinces in South Africa.[84] Subjects had no first degree siblingship to other subjects.

Patients with moderate to severe chronic periodontitis were regarded as investigation subjects (case group).

Patients with no or mild chronic periodontitis were regarded as controls.

All subjects were screened for the degree of periodontal disease in each of three parameters. These parameters were pocket depth, recession and clinical attachment loss (CAL). In addition, a medical

and dental history of each subject was obtained including specific questions on family history of diabetes, cardiovascular disease as well as whether or not they were smokers. All subjects were of low to middle socioeconomic class. Subjects who agreed to participate in the study signed a consent form (In the Xhosa language).
The protocol was approved by ethical and research committee of the University of the Western Cape, Cape Town, South Africa and participation in the study was on voluntary basis.
All statistical tests were done using commercial software: SPSS 14.0 (SPSS corp.2005).

4.2 CLINICAL EXAMINATION:

In order to determine periodontal disease status, each patient underwent an examination by a calibrated examiner, including a full mouth measurement of probing depth (PD) and recession (R). All clinical assessments were performed by one calibrated examiner who started by examining few patients randomly scoring more than 80% intra-examiner reproducibility. Smears of the patients were obtained in accordance with the instructions of the genetic laboratory as a training phase before proceeding into sample collection.
Probing depth was defined as the distance between the gingival margin and the bottom of the probeable pocket to the nearest whole mm.[21]
Location of the gingival margin was considered as the distance between the cementoenamel junction (CEJ) and the gingival margin recorded to the nearest whole mm. This measure was given a positive sign in case of gingival recession and a negative sign when the gingival margin was located coronal to the CEJ. [21]

The algebraic sum of the above 2 parameters was used to compute the clinical attachment level. Modified Plaque index, and modified Gingival index were used to denote the gingival health and oral hygiene status of the patients.[84]

Plaque was recorded without any use of disclosing agents.

Bleeding on probing was also recorde and was deemed positive if it occurred within 15 seconds after the assessment of probing depth.

The number of lost teeth was recorded by the examiner in cases and controls. The minimum number of remaining teeth for the subject to be included in the study was 14 teeth.

All clinical variables were calculated on six surfaces (distobuccal, buccal, mesiobuccal, mesiolingual, lingual and distolingual) on each tooth (excluding third molars) for up to 168 sites. The Williams® probe was used for clinical examination.

Chronic periodontitis was defined by the history of disease status rather than active signs and symptoms.

Moderate to severe disease was defined as patients presenting with mean loss of attachment of 2.5mm or loss of clinical attachment (CAL) of 3mm or above in 7 sites.[4,7]

Panoramic radiographs were only adjunctive aids in diagnosis. Severe disease was defined as the presentation with 50% bone loss on radiographs, in 7 interproximal sites, with a total mouth mean bone loss greater than 30%.[4, 7]

4.3-DETERMINATION OF INTERLEUKIN-1 POLYMORPHISMS

In order to take a buccal smear, candidate subjects should not have eaten for about one hour before collection of the sample, and for females lipsticks were to be avoided. Commercial polyester tipped applicators (sterile Dacron®) were used to collect the samples. Two buccal smears were obtained from each patient, by rubbing the buccal mucosa on the right and left side, for at least 30 seconds as suggested by the manufacturer. They were coded for identification purposes, stored in special sealed bags to avoid sun and temperature, and then sent to the laboratory for molecular analysis (Figure 4.1).

The laboratory personnel responsible for genotyping did not know the clinical status or identity of the patient.

DNA was extracted by GeneCare extraction kit at GeneCare Molecular Genetics private laboratory and it was stored there for future analysis. The extraction procedure was as follows:

In short, two 2.0 ml microtubes were labeled per sample. The applicator tip of the buccal swab was inserted into a labeled 2.0 ml microtube and the handle was broken off. 400ul PBS was added and mixed on vortex for 20s, and then the applicator tip was shaken in PBS for 20 min (Figure 4.2).

800ul cold reagent C-A was pippetted into a labeled 1.5ml microtube and 300Ul of the PBS-cell suspension was added and inverted several times to mix. The mixture was incubated at room temperature for 2 min. The mixture then was centrifuged at 14000 rpm for 60s. The cells formed a pellet as supernatant (Figure 4.3) which was removed and 50ul of reagent C-B was added and mixed by continuous pippitting. The new mix was incubated in a water bath at 80 C for 15 min. 5ul reagent C-C was added and mixed and then placed on a shaker for 10

min. In this way, the DNA was extracted and stored at -20° for future PCR reactions.

The DNA concentration for each sample was measured using NanoDrop® ND-1000 spectrophotometer (NanoDrop technologies, USA) (Figure 4.4) and the results were used in order to calculate the volume of DNA to be added to the reaction mix of the PCR (Figure 4.5).

Detecting the IL-1A polymorphisms:

The DNA was isolated and target sequences were then amplified using the polymerase chain reaction (PCR). Oligonucleotide DNA primers that target the specific polymorphic DNA region within the genes of interest were determined so that in the PCR reaction amplification of the target sequences was achieved.

For IL-1A the primer sequence was as follows (Inqaba biotech, SA):

Forward: 5' ACTAGACTGTGTATATCATCTGTGTT 3'

Reverse: 5' GCTCGAATTATACTTTGATTGAGGG 3'

Cycling was carried out for 1 cycle at 95°C for 1 minute,
35 cycles each at 94°C for 1 minute,
65°C for 1 minute,
72°C for 2 minutes,
and 1 cycle at 72°C for 5 minutes.

The DNA sequences were amplified and analyzed using Real-time PCR for IL-1a.

The basic theory behind Real-time polymerase chain reaction is that the more abundant a particular DNA is in a sample, the earlier it will be detected during repeated cycles of amplification. Typically,

amplification of a given DNA sequence over time follows a curve, with an initial flat-phase, followed by an exponential phase. Finally, as the experiment reagents are used up, DNA synthesis slows and the exponential curve flattens into a plateau.

In Real-time PCR There is continuous collection of fluorescent signal from polymerase chain reaction throughout cycles.

Rapid-cycle PCR was used (LightCycler®, Roche)(Figure 4.6), which is a powerful technique for nucleic acid amplification and analysis. Samples amplified by rapid-cycle PCR are immediately analyzed by melting curve analysis in the same instrument. In the presence of fluorescent hybridization probes, melting curves provide dynamic dot blots for fine sequence analysis, including SNPs (Figure 4.7). In melting curve (dissociation) analysis: Every piece of DNA has a melting point (Tm) at which temperature 50% of the DNA is single stranded. The temperature depends on the length of the DNA, sequence order and the G:C content. When DNA-binding dyes are used, as the fragment is heated, a sudden decrease in fluorescence is detected when Tm is reached (due to dissociation of DNA strands and release of the dye). This point is determined from the inflection point of the melting curve (Figure 4.8) or the melting peak of the derivative plot (Figure 4.9) (Derivative plot is the negative first-derivative of the melting curve and it is easier to be interpreted). The same analysis can be performed when hybridization probes are used as they keep intact after PCR. Mismatch between a hybridization probe and the target results in a lower Tm. Melting curve analysis can be used in known and unknown (new) mutation analysis as a new mutation will create an additional peak or change the peak area.

Detecting the IL-1B polymorphisms:

The restriction fragment length polymorphisms and gel electrophoresis was used for IL-1b to determine the genetic polymorphisms present in the amplified sequences and thereby provide a genetic polymorphism profile of the patient.

The PCR set up for IL-1B (+3954) was as follows:

Forward primer: 5' - CTC AGG TGT CCT CGA AGA AAT CAA - 3'.

Reverse primer: 5' - GCT TTT TTG CTG TGA GTC CCG - 3'.

After optimization of the reaction the following reaction mixture was used: DNA (100 ng/ul), BSA (0.4 ng/ul): 2ul, MgCl2 (2mM): 2ul, reaction buffer to a final concentration (1X): 5ul, Taq polymerase (Biotaq, Bioline limited, UK)(1.5 units): 0.3ul, dNTPs (100uM): 2.5ul, F' primer (50 pM): 1ul, R' primer (Inqaba Biotech, SA)(50 pM): 1ul, and distilled water to get finally 50ul reaction mix.

Cycling was carried out for 1 cycle at 95°C for 2 minutes, 38 cycles for 1 minute each at 94°C, 53°C and 72°C, and 1 cycle at 72°C for 8 minutes in Eppendorf thermal cycler.

The resulting amplicon of 194 base pairs were resolved on 2% agarose gel alongside a 100bp ladder (Fermentas, SA) on 1X TBE solution. It was visualized via Ethidium bromide staining (1ul/75 ml of agarose gel), then it was viewed under UV-transilluminator (UVITEC).

The resulting PCR products were digested overnight at 370C with each reaction containing 5 µl of the respective PCR products, 10 Units Taq I and 1x reaction buffer. Restriction products were resolved on a 8% polyacrylamide gel and yielded either two cut fragments of 85 + 97 bp (in allele1[C]) or a single uncut intact 182 bp fragment (allele 2[T] when the subject is homozygous) or both cut and uncut fragments when the patient is heterozygous (allele 2 on one copy of the chromosomes).For IL-1a, allele identification was visual at the time of

PCR, while for IL-1b it was by the size of fragments after restriction digestion and separation in polyacrylamide gel.

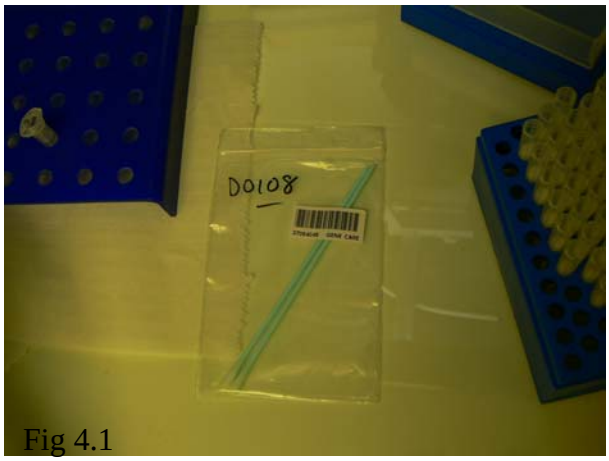

Fig 4.1

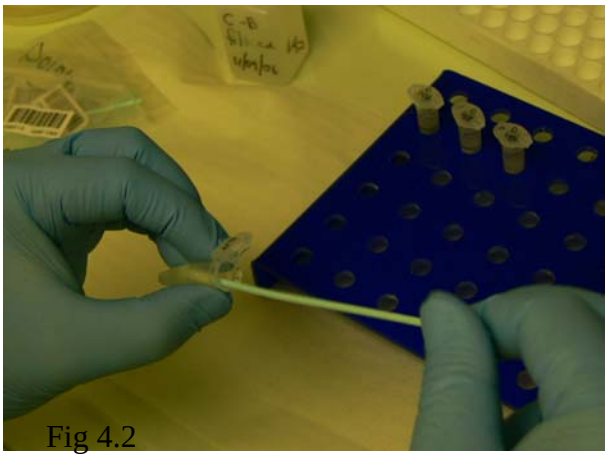
Fig 4.2

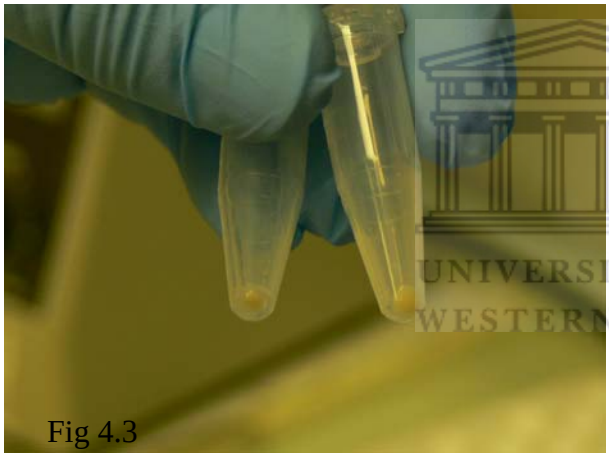
Fig 4.3

Fig 4.4

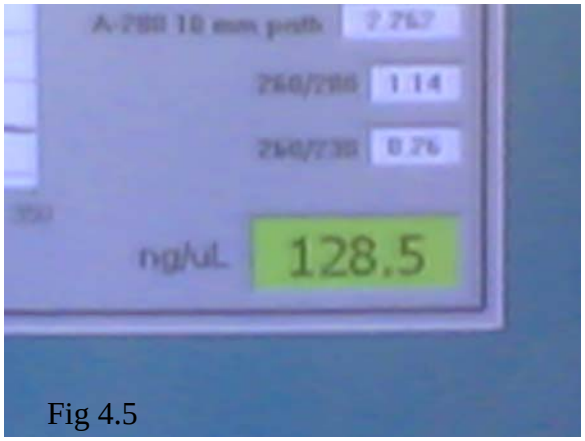

Fig 4.5

Fig 4.6

Figures 4.1: Dacron applicators, 4.2: Collection of cells from smears, 4.3: Pellet formation, 4.4: Nanodrop spectrophotometer, 4.5: DNA concentration and 4.6: Roche Light cycler PCR machine.

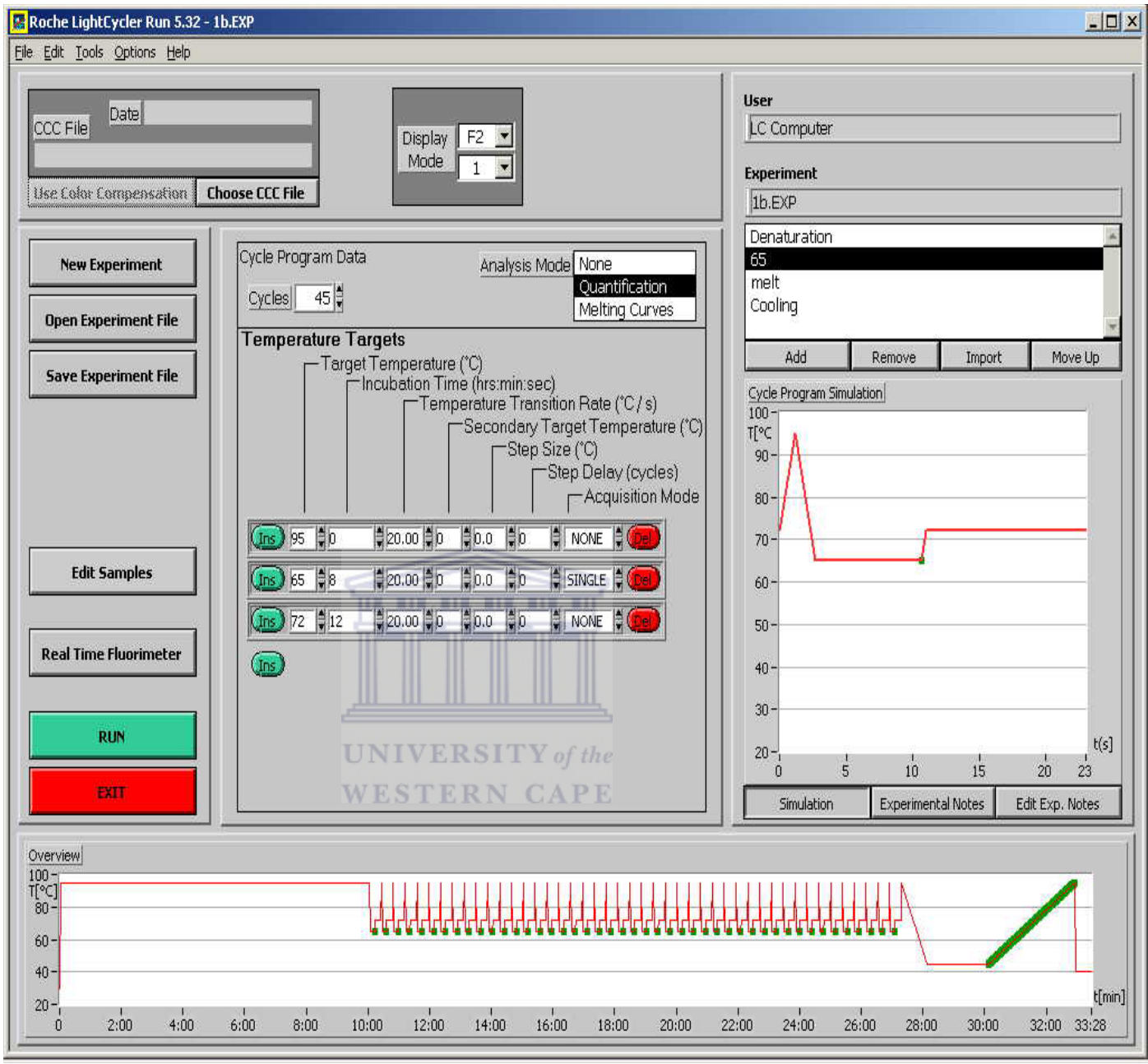

Figure 4.7 Programming of the PCR machine with the required cycles

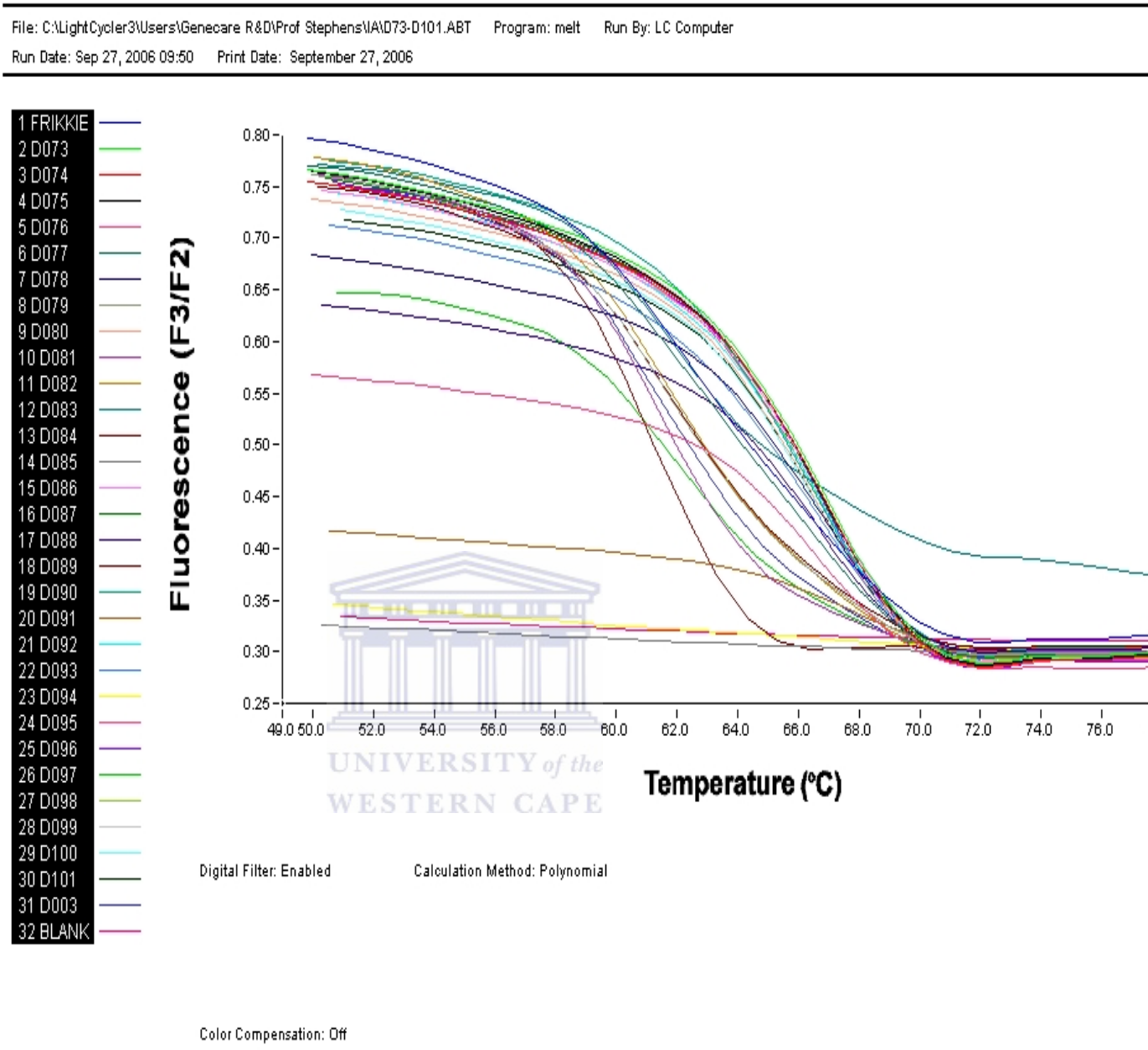

Figure 4.8 Inflection point of the melting curve coincides with the melting temperature (Tm).

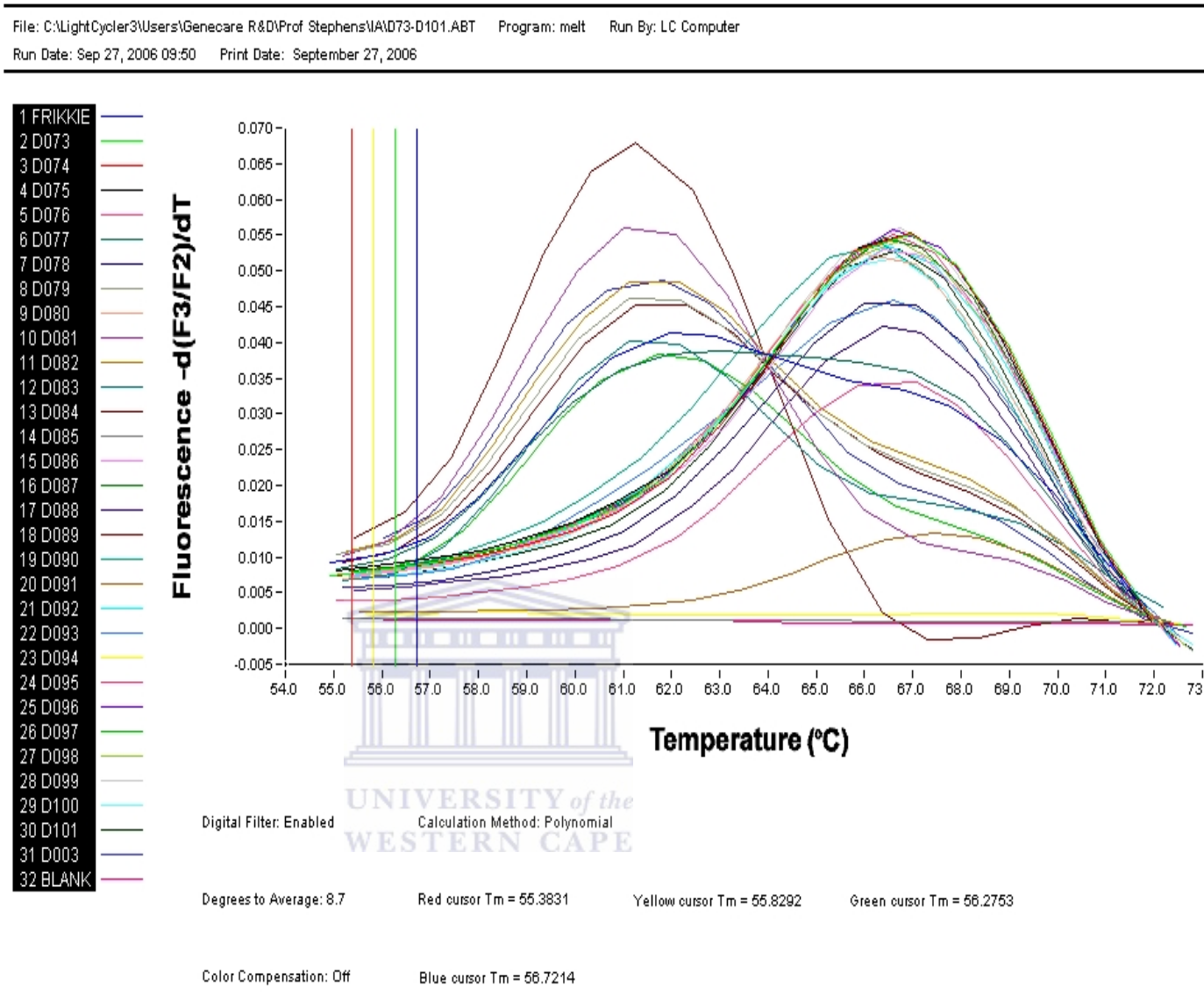

Figure 4.9 Derivative plot is the negative first-derivative of the melting curve; the peak coincides with (Tm).

CHAPTER 5

RESULTS:

5.1- OVERVIEW:

The main findings of this study can be summarized as follows:
No difference in the prevalence of the positive composite IL-1 genotype was observed between a group of periodontitis patients and a control group of age and gender matched periodontally healthy to mild subjects; and the components of this hypothetical genotype were analyzed separately.
When Interleukin-1 B gene polymorphism was studied individually no significant difference was observed between cases and controls. A positive association was observed, however, between the severity of periodontal disease and the presence of the gene polymorphism on IL-1A.

In a separate analysis the potential causative factors were run in a multivariate logistic regression model. Age, gender, PI, GI and number of lost teeth were not significantly associated factors with the severity of periodontal disease. IL-1A polymorphism was significantly associated with severity of periodontitis when the regression model was adjusted for confounding factors.

5.2- DEMOGRAPHIC AND DESCRIPTIVE CLINICAL RESULTS:

A total of 99 samples were successfully analyzed at the end of the study (table 5.1), 49 were for cases and 50 were for controls. Females presented more than males for treatment, and out of the 99 subjects 63 (63.6%) were females and 36 (36.4%) were males (table 5.2). Females presented more often in both cases and controls taken individually as well, but they had the same percentage among cases and controls (table 5.3).

	Frequency	Percent
Healthy to mild (controls)	50	50.5
Moderate to severe (cases)	49	49.5
Total	99	100.0

Table 5.1 The distribution of the sample into cases and controls

	Frequency	Percent
Male	36	36.4
Female	63	63.6
Total	99	100.0

Table 5.2 The distribution of gender in the sample

			Gender		
			Male	Female	Total
Periodontal status	Healthy to mild	Count	18	32	50
		% within Periodontal status	36.0%	64.0%	100.0%
	Moderate to severe	Count	18	31	49
		% within Periodontal status	36.7%	63.3%	100.0%
Total		Count	36	63	99
		% within Periodontal status	36.4%	63.6%	100.0%

Table 5.3 The distribution of gender among cases and controls

The mean age of the study population was 46 ± 9 in cases and 44 ± 8 in controls measured in whole years. Due to the reported higher mean age in cases than in controls a statistical test was necessary to assess if the age is related to periodontal status.

Using distribution charts it was found that data in cases and controls were non-parametric, so the Mann-Whitney Test was used and the P-value was 0.251. That meant that there was no significant difference in the two groups with regard to age.

The mean clinical attachment loss was 2.5 ± 0.5 in cases and 0.5 ± 0.4 in controls measured in millimeters. This difference can be attributed to the fact that the clinical attachment loss defines the periodontal disease status and varies considerably between cases and controls (See 4.2). Data were non-parametric and Mann-Whitney test showed a significant difference between cases and controls (p= 0.00)

With regard to the number of lost teeth, the mean value was 4.4± 3.1 in cases and 4.0± 2.9 in controls. The p-value was 0.522 for Mann-

Whitney test and there was no significant difference in the two groups with regard to the lost teeth.

The PI and GI were tested both in cases and controls, and it was found that the mean PI in cases was 1.6± 0.7 and in controls 1.0 ± 0.7 while for the GI the mean was 1.7± 0.7 in cases and 1± 0.8 in controls.

Data were non-parametric both for PI and GI and Mann-Whitney tests showed that the differences were highly significant between cases and controls with a p-value of 0.00 for both PI and GI.

Table 5.4 summarizes the distribution of mean age, clinical attachment loss, number of lost teeth, plaque index and gingival index among cases and contols.

	Periodontal status			
	Healthy to mild		Moderate to severe	
	Mean	Std Deviation	Mean	Std Deviation
Age (in years)	44	8	46	9
Clinical attachment loss (in mm)	0.5	0.4	2.5	0.5
Tooth loss (Number of lost teeth)	4.0	2.9	4.4	3.1
Plaque index (PI)	1.0	0.7	1.6	0.7
Gingival index (GI)	1.0	0.8	1.7	0.7

Table 5.4 Distribution of some demographic and descriptive clinical findings in cases and contols

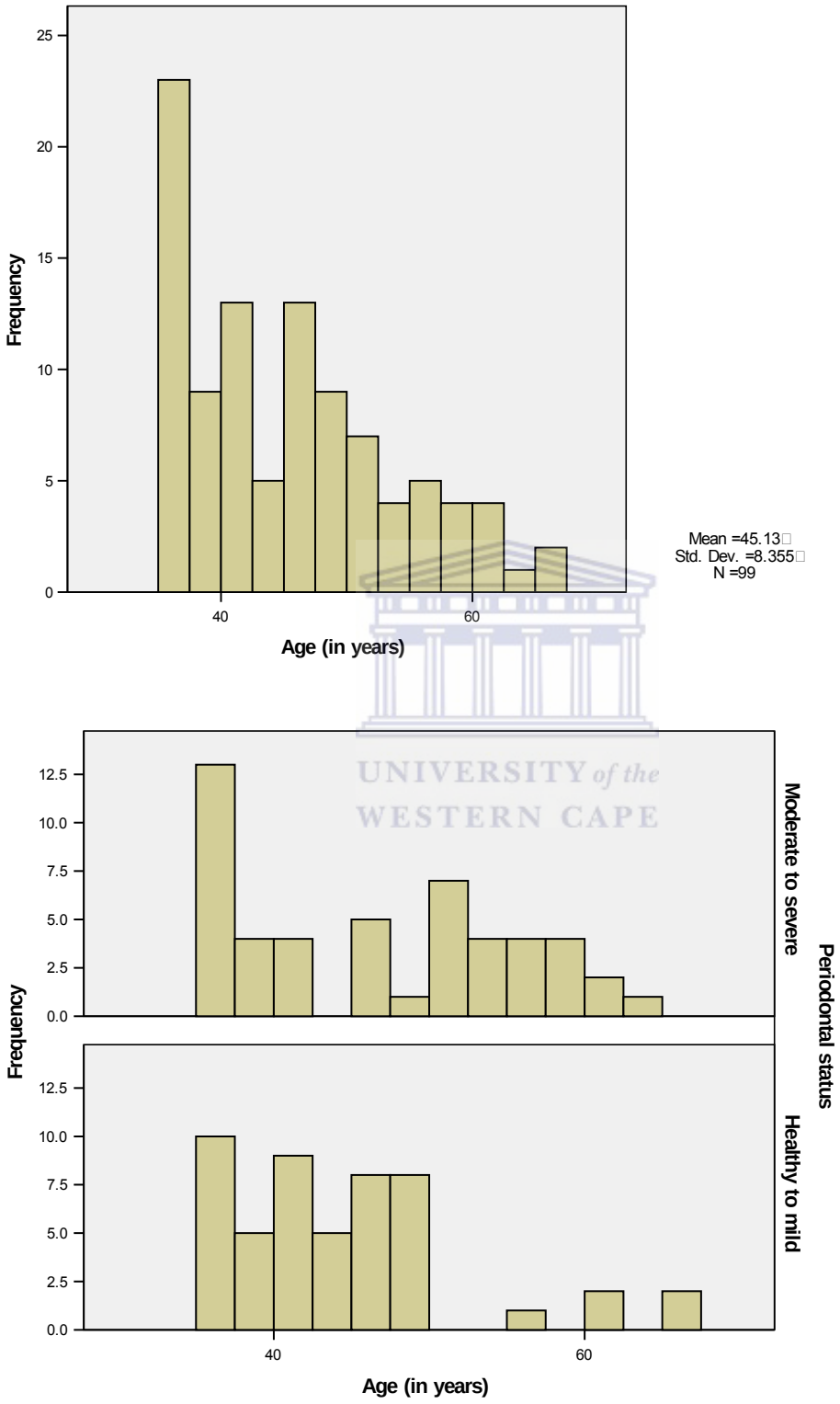

Figure 5.1 Distribution of Age in the study population and in cases and controls

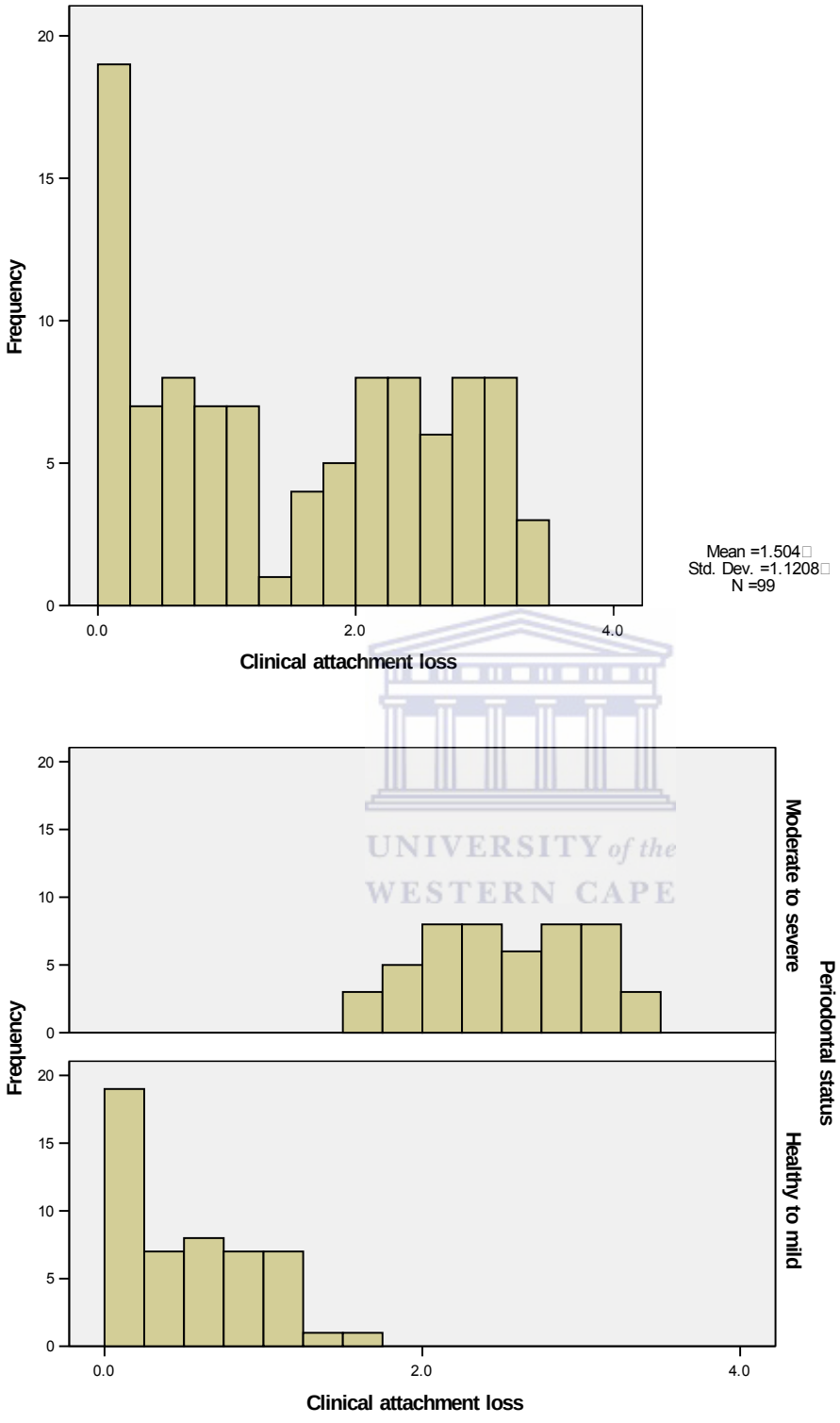

Figure 5.2 Distribution of CAL in the study population and in cases and controls

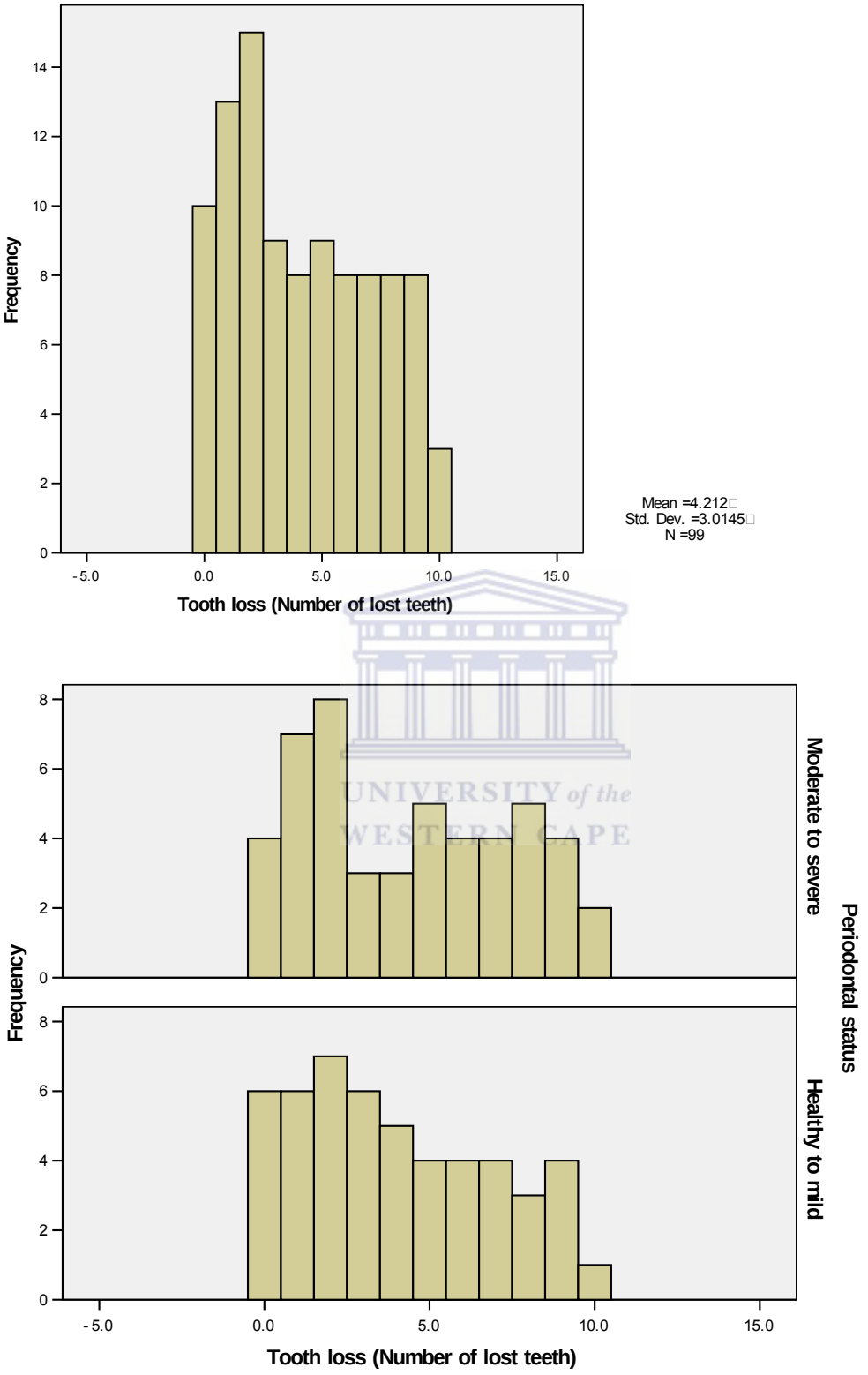

Figure 5.3 Distribution of Tooth loss in the study population and in cases and controls

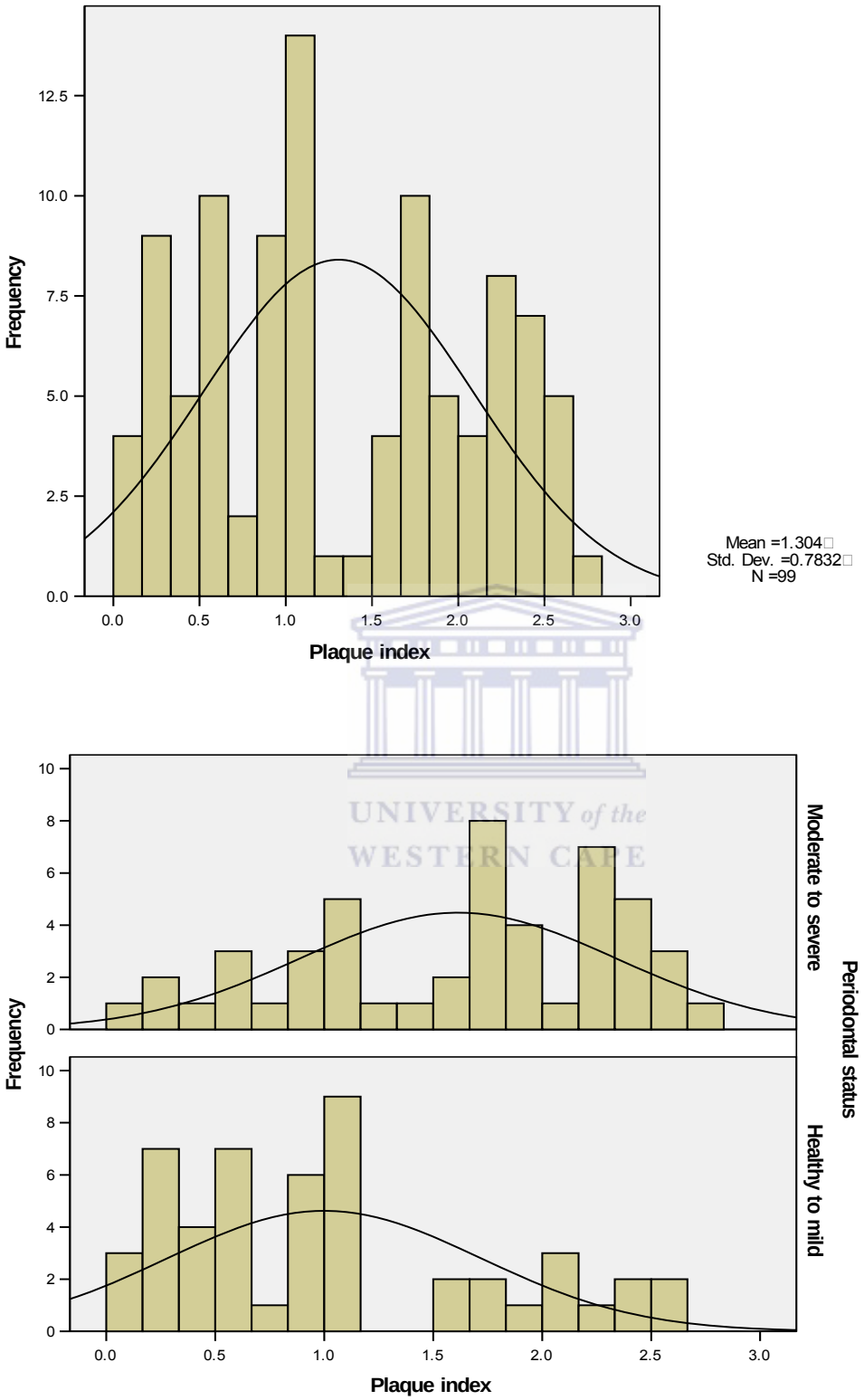

Figure 5.4 Distribution of PI in the study population and in cases and controls

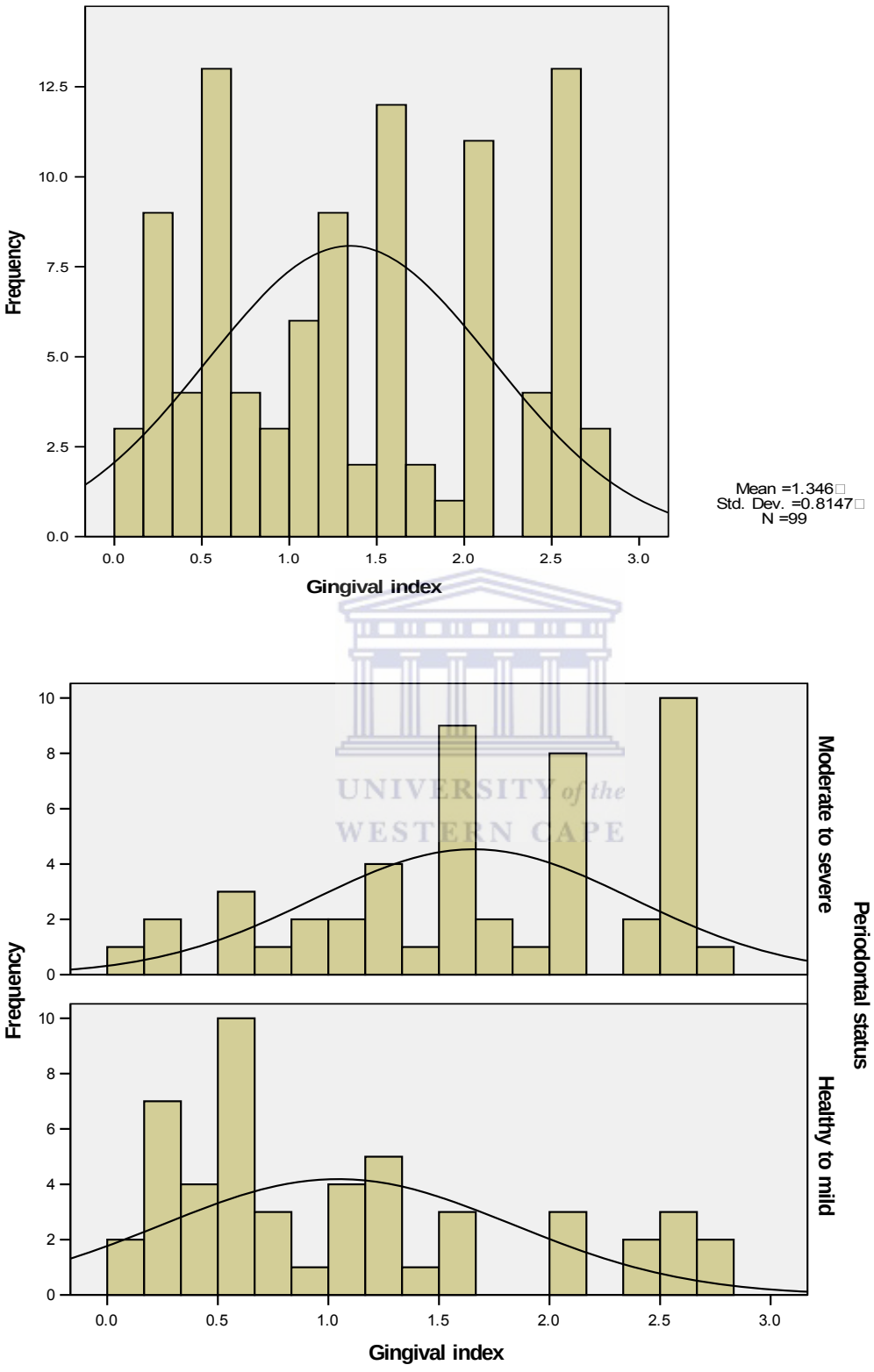

Figure 5.5 Distribution of GI in the study population and in cases and controls

5.3- INTERLEUKIN-1A AND THE PERIODONTAL STATUS:

The prevalence of allele 2 of IL-1A was 34% in the study population, composed of 28% who were heterozygous and 6% who were homozygous for the allele. Looking at the distribution between cases and controls it was found that in cases the allele 2 occurred more frequently than in the controls (prevalence in cases was 53% allele 1, 38.8% allele 2 heterozygous, and 8.2% allele 2 homozygous, while in controls allele 1 prevalence was 78.0% , allele 2 heterozygous was 18.0% and allele 2 homozygous was 4.0%) (Table 5.5).

			IL-1A polymorphisms			Total
			Allele 1	Allele 2 Heterozygous	Allele 2 Homozygous	
Periodontal status	Healthy to mild	Count	39	9	2	50
		% within Periodontal status	78.0%	18.0%	4.0%	100.0%
	Moderate to severe	Count	26	19	4	49
		% within Periodontal status	53.1%	38.8%	8.2%	100.0%
Total		Count	65	28	6	99
		% within Periodontal status	65.7%	28.3%	6.1%	100.0%

Table 5.5 The distribution of IL-1A alleles in cases and controls

	Homozygous Allele 1	Heterozygous	Homozygous Allele 2	Allele 1	Allele 2	Total # Alleles
Cases	26 (0.531)	19 (0.388)	4 (0.082)	71 (0.724)	27 (0.276)	98
Controls	39 (0.78)	9 (0.18)	2 (0.04)	87 (0.87)	13 (0.13)	100

Table 5.6 Genotype and Allele frequencies for the IL-1A polymorphism in cases and controls (Frequencies are given in brackets).

The genotypes in Table 5.6 have been examined for adherence to Hardy Weinberg distribution and both cases (p=0.98) and controls (p=0.352) do not deviate from Hardy Weinberg equilibrium.

It was noted that the cells were low in number in some categories which would compromise the accuracy of the proposed statistical tests for association of the periodontal status with the occurrence of allele 2 polymorphism.

In order to reduce the influence of very low sample numbers the data was examined with the numbers for the homozygous Allele 2 individuals recoded as combined with the heterozygous individuals (thus allowing a comparison of samples carrying allele 2 vs those not carrying allele 2) The recalculated data with the "collapsed" genotype distributions are shown in Table 5.7

Chi-squared test was used with one degree of freedom.

The occurrence of allele 2 was significantly associated with the presence of the severe form of periodontal disease when the cells were collapsed (p-value was 0.009).

			IL-1A polymorphisms		
			Allele 1	Allele 2	Total
Periodontal status	Healthy to mild	Count	39	11	50
		% within Periodontal status	78.0%	22.0%	100.0%
	Moderate to severe	Count	26	23	49
		% within Periodontal status	53.1%	46.9%	100.0%
Total		Count	65	34	99
		% within Periodontal status	65.7%	34.3%	100.0%

Table 5.7 Distribution of IL-1A alleles among cases and controls

(Allele 2 homozygous was collapsed with heterozygous)

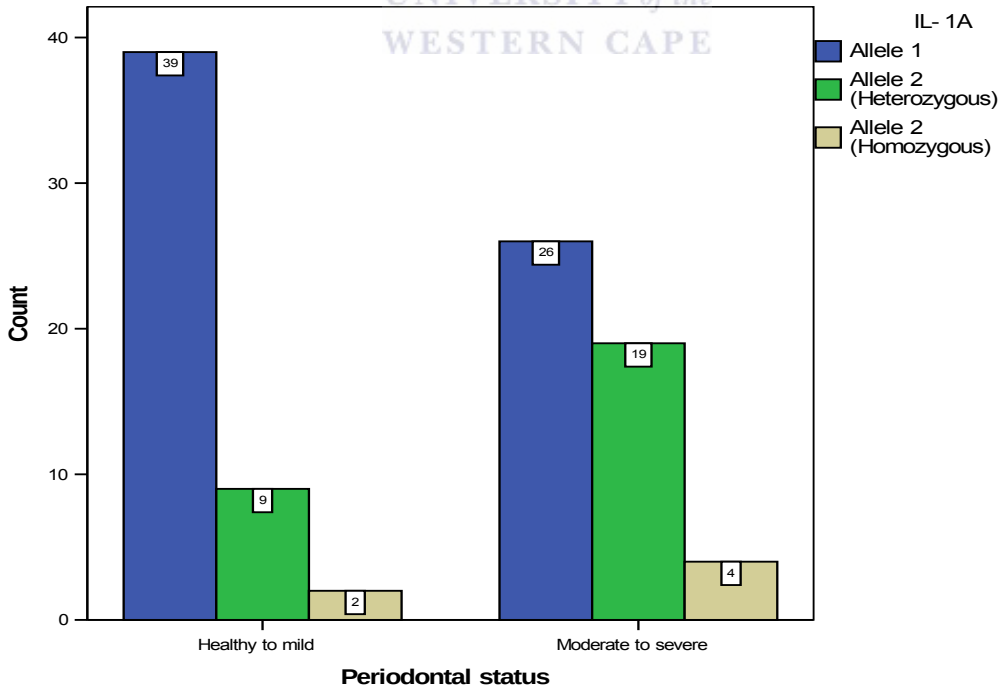

Figure 5.6 The distribution of IL-1A alleles among cases and controls

5.4- INTERLEKIN-1B POLYMORPHISMS AND THE PERIODONTAL STATUS:

In order to calculate the prevalence of IL-1B- allele 2 polymorphism, 40 samples were run as a pilot, consisting of 19 cases and 21 controls. The allele 2 occurrence was very low as it occurred only in 10% as heterozygous and 5% as homozygous while 85% of the sample had allele 1. Also, the difference in occurrence of allele 2 between cases and controls was minor: as in the case group the prevalence of allele 2 heterozygous was 10.5% and the homozygous was 5.3% while in the controls group the prevalence of allele 2 heterozygous was 9.5% and the homozygous was 4.8% (table. 5.8)

			IL-1B polymorphisms			Total
			Allele 1	Allele 2 (Heterozygous)	Allele 2 (Homozygous)	
Periodontal status	Healthy to mild	Count	18	2	1	21
		% within Periodontal status	85.7%	9.5%	4.8%	100.0%
	Moderate to severe	Count	16	2	1	19
		% within Periodontal status	84.2%	10.5%	5.3%	100.0%
Total		Count	34	4	2	40
		% within Periodontal status	85.0%	10.0%	5.0%	100.0%

Table 5.8 The distribution of IL-1B alleles among cases and controls

	Homozygous Allele 1	Heterozygous	Homozygous Allele 2	Allele 1	Allele 2	Total # Alleles
Cases	16 (0.842)	2 (0.105)	1 (0.053)	34 (0.89)	4 (0.11)	38
Controls	18 (0.857)	2 (0.095)	1 (0.048)	38 (0.90)	4 (0.01)	42

Table 5.9 Genotype and Allele frequencies for the IL-1B polymorphism in cases and controls (Frequencies are given in brackets).

The genotypes in Table 5.9 have been examined for adherence to Hardy Weinberg distribution and both cases (p=0.157) and controls (p=0.122) do not deviate from Hardy Weinberg equilibrium.

As was the case in IL-1A, the tables in IL-1B had cells with very low numbers and the tables had to be modified accordingly, in order to test for the association of the periodontal status with the occurrence of allele 2 polymorphisms accurately (Table 5.10).
In order to reduce the influence of very low numbers of samples the data was examined with the numbers for the homozygous Allele 2 individuals recoded as combined with the heterozygous individuals (thus allowing a comparison of samples carrying allele 2 vs those not carrying allele 2). The recalculated data with the "collapsed" genotype distributions are shown in Table 5.10.

It was found that the occurrence of allele 2 was not significantly associated with the presence of the severe form of periodontal disease.
The Fischer exact test was used and the p-value was 0.619 upon merging of cells; that is not significant.

			IL-1B polymorphisms		
			Allele 1	Allele 2	Total
Periodontal status	Healthy to mild	Count	18	3	21
		% within Periodontal status	85.7%	14.3%	100.0%
	Moderate to severe	Count	16	3	19
		% within Periodontal status	84.2%	15.8%	100.0%
Total		Count	34	6	40
		% within Periodontal status	85.0%	15.0%	100.0%

Table 5.10 Distribution of IL-1B alleles among cases and controls

(Allele 2 homozygous was collapsed with heterozygous)

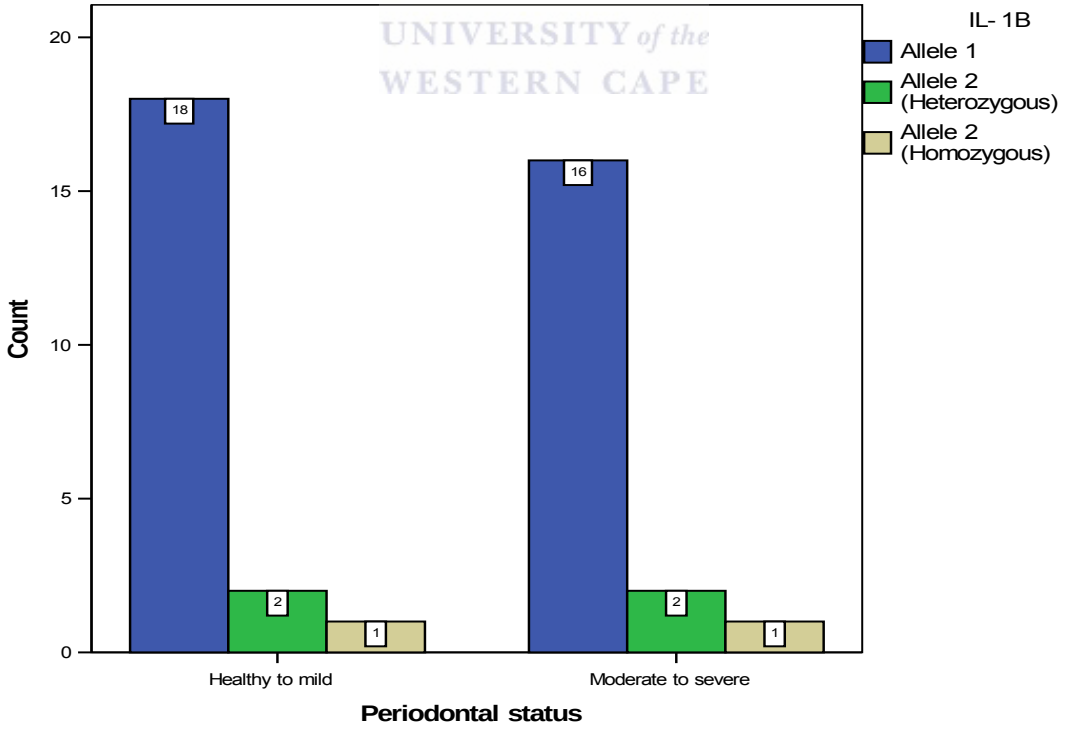

Figure 5.7 The distribution of IL-1B alleles among cases and controls

5.5- LOGISTIC REGRESSION MODEL:

In order to evaluate the effect of all potential contributing factors on the periodontal status, a multiple logistic regression analysis was run. The effect of IL-1A allele2 (collapsed) was tested in the presence of other factors like gender, age, PI and GI, and it was found that the IL-1A polymorphism had a significant effect on the periodontal disease status (p-value= 0.025).

On the other hand, some factors which had a significant effect at the univariate association models (like PI and GI) were not significantly associated with the disease status in the presence of other factors.

	B	S.E.	Wald	df	Sig.	Exp(B)
Gender	.231	.476	.237	1	.627	1.260
Age	.036	.027	1.725	1	.189	1.036
PI	1.348	.992	1.846	1	.174	3.850
GI	-.226	.942	.057	1	.811	.798
IL1A(collapse)	-1.104	.494	4.990	1	.025	.332
Constant	-2.433	1.365	3.178	1	.075	.088

Table 5.11 Variables entered on logistic regression model: Gender, Age, PI, GI and IL1A collapse.

CHAPTER 6

DISCUSSION

6.1 – INTRODUCTION

It is generally agreed that bacteria are the initiators of periodontitis, but there is increasing evidence to suggest that host factors, such as diabetes, smoking, and genetic influences contribute to the severity of destruction and thereby affect the clinical presentation and distribution of lesions.[3] Risk factors can be understood as aspects of personal behavior or lifestyle, environmental exposure, inborn or inherited characteristics, which are epidemiologically known to be associated with a health-related condition.

The multifactorial aetiology of periodontitis is similar to many common diseases involving multiple factors that together determine the clinical presentation of the disease in a specific individual.[3] Environmental and acquired risk factors affect the onset, rate of progression and can control the severity of periodontal disease as well as the response to therapy. Hence widespread more advanced disease occurs primarily in a fraction of the population.[10]

Genetic factors play an important role in determining the actual clinical presentation of periodontitis as evidenced by studies of twins, studies of aggressive periodontitis and epidemiological studies of the natural history of periodontitis.[11,12] It has been estimated by some investigators that less than 20% of the variability in periodontal disease expression can be explained by the quantity of specific bacteria found in disease associated plaque.[4] These findings can explain the clinical observation that widespread advanced disease is not always associated with large amounts of plaque.

Specific bacteria initiate the inflammatory state in periodontitis. As the IL-1 polymorphism is involved in immuno-inflammatory processes, the clinical effects of this genetic factor depend on the presence of bacteria to initiate the inflammation. Although the risk factor may not be a proven cause of a particular disease, its presence implies an increase in the probability of the disease occurring, and not all patients with this genetic factor are therefore prone to severe periodontitis. It can be expected that removing the bacteria would decrease the effect of other risk factors such as the IL-1 genotype. In this study the indicators of bacterial challenge and oral hygiene level like the Plaque Index and Gingival Index were assessed.
All subjects selected were free of systemic diseases to reduce the effect of a well-known confounding factor on periodontal disease.

A history of smoking is considered to be an important risk factor for periodontal disease and this effect is dose dependent.[36,37] Previous investigations[17,20,24] have shown that individuals smoking >10 cigarettes per day (one-half pack) have an increased disease severity and a less favorable response to therapy.[37] Importantly, the IL-1 polymorphism was found to have a significant effect on attachment loss in non-smoking patients.[7]

This study excluded smokers and persons who had been smokers in the previous 5 years in order to reduce the cumulative effect of smoking on periodontal disease.

In finding a relationship between the putative risk factor and periodontitis, it is important that the association should be temporal,

consistent, strong and makes some biologic sense, though those elements are interrelated and can not be studied separately.

As periodontal disease generally progresses slowly, persons studied over a long period may be exposed to multiple potential risk factors, making determination of a cause–effect relationship more difficult.

In the present study patients included were of ages between 35-60 year, in an attempt to reduce the range of the age as possible, as age could be a confounding factor to the disease. They were also chosen to ensure that they presented with the chronic (adult) periodontitis rather than the aggressive (early onset) types of disease.

Consistency is the repeated demonstration, by different investigators, of an association between exposure to a putative cause (e.g., genetic factors) and the outcome of interest (e.g., periodontal attachment loss). Studies concerning the relationship between IL-1 polymorphisms and periodontal diseases were generally inconclusive, and this aspect will be discussed next section.

The strength of the evidence is determined by the study design. Prospective studies are needed to calculate the probability of developing a disease. For example, if 30 of 100 patients who were genotype-positive developed periodontitis, then the risk of developing the disease could be calculated (30 percent chance of developing the disease). This type of calculation can be done only in prospective studies; it cannot be done in retrospective investigations in which patients are selected after they already had the disease.

The best evidence can be drawn from a randomized clinical trial, but it is not ethical to produce periodontal disease in some patients (cases) and not practical to ensure that others (controls) will not develop disease, and so the clinical trial could not be applied in this study.

In prospective cohort studies, a group of people with and without exposure to a suspected cause over time (e.g. a particular genetic polymorphism) are followed in order to determine who develops the disease of interest (e.g. chronic periodontal disease). Unfortunately, cohort studies are expensive and time consuming, and could not be used in this study.

A case-control study examines people with the disease in question (e.g. chronic periodontal disease) and matches them with "controls" who are disease free to determine differences between the 2 groups in the rate of exposure to a potential cause (e.g. comparing the 2 groups for rates of the genetic polymorphism). Although results from this type of study are associated with a greater potential for bias than those from a cohort study or randomized trial, they are often the only evidence available.

Furthermore, clinical examination of patients provided information about the cumulative disease history of the patient and based on the current information the patient's likelihood of future disease and expectation of response to therapy can be assessed, but it is not possible to read the future even with the presence of a risk factor. This study was a case-control study that consisted of 49 cases with moderate to severe periodontitis and 50 age and gender matched controls who were healthy or with mild periodontitis.

The other element of interest is that there should be some reasonable biologic explanation for the putative cause to play a role in the outcome. This can be shown as IL-1 is a powerful regulator of the

inflammatory process and increased levels of IL-1 were associated with the immuno-pathology of the periodontitis.[3, 4, 15,25,26,69] Interleukin-1 (especially IL-1b) is also known to be a critical determinant of bone and collagen destruction. IL-1 levels are higher in GCF from sites with periodontitis, [15] and recent findings indicated that specifically blocking both IL-1 and TNFα significantly reduced periodontal bone destruction in periodontitis, in spite of a heavy bacterial challenge.[38] It is assumed, therefore, that the positive composite genotype does, in fact, translate into a phenotype of enhanced IL-1 production to a given bacterial challenge.[15,17]

6.2 – INTERLEUKIN-1 POLYMORPHISMS AND PERIODONTAL DISEASE STATUS

To the best of knowledge, there has been no information published about the association of IL-1 polymorphisms and chronic periodontitis in any indigineous African population. However, there have been studies that investigated the early-onset type of periodontal diseases in African-American subjects. [13, 43]

In order to determine this association in the present study, the researcher had to assess first the prevalence of the IL-1α +4845 and IL-1β +3954 genetic polymorphisms in a population of African ancestry.The relationship between these polymorphisms to the periodontal disease status was subsequently assessed in these persons.

The IL-1α +4845 polymorphism is reported to be in almost complete linkage disequilibrium (>99%) with the previously tested IL-1α-889 polymorphism, [18] and it is easier to be evaluated technically.

In the first test, (IL-1A) real time PCR genotyping was done for 99 unrelated subjects. In the healthy to mild periodontal disease group the prevalence of allele 2 IL-1α +4845 was 22%, while for allele 1 it was 78%. There was a significant difference in the prevalence of the IL-1α +4845 genotype in the case group where 46.9% had allele 2 and 53.1% carried allele 1. This difference was significant when it was tested by logistic regression model with the presence of other factors such as age, PI and GI.

However, in literature it is established that IL-1B is the more important polymorphism as related to periodontitis.[6]

Diehl and coworkers [43] reported that the IL-1β +3953 polymorphism was more significantly associated with early onset periodontitis than the IL-1α-889 polymorphism. In an African-American control population, the prevalence of the IL-1β +3954 was 73% (1,1), 26% (1,2), and 1% (2,2). This prevalence was similar to that observed in the early onset population examined, with 84% (1,1), 16% (1,2), and 0% (2,2).[13] In our African control population, the prevalence of the IL-1β +3954 was 85.7% (1,1), 9.5% (1,2), and 4.8% (2,2). This prevalence was similar to that observed in the moderate to severe periodontitis, cases group, with 84.2% (1,1), 10% (1,2), and 5.3% (2,2).

A case-control study comprising gingivitis patients, patients with advanced periodontal disease and control populations, [73] reported a significantly higher prevalence of allele 2 at IL-1B +3954 in the advanced periodontitis group when compared to the reference population (55% versus 26.7%). Another case-control study comprising 32 Caucasian adult periodontitis patients age- and gender matched with healthy controls, revealed a statistically significantly increased frequency of allele 2 at the IL-1B +3954 locus among patients with advanced periodontitis and subjects with mild or moderate disease; however, a comparison of the advanced disease group to the controls did not reveal a statistically significant different occurrence rate. [6]

On the contrary, the findings suggested that allele "1" of the IL-1β +3953 polymorphism is the most prevalent allele in the general African population of South Africa. The majority of cases and control population were homozygous for the IL-1β +3953 "1" allele (1/1 genotype).

These findings highlighted the high prevalence of the 'normal' allele in the African population, as well as the resemblance to the results of the African-American population.

The fact that the genotype frequencies for IL-1A and IL-1B in both cases and controls do not deviate from Hardy Weinberg equilibrium shows that there is not selection bias in the groups studies. The sample size in both cohorts is small and larger numbers of samples may change this result.

The occurrence of the positive genotype found in this study is significantly lower than the 29.1% rate found in Northern European subjects of unknown periodontal status or the reported 36.3% prevalence in non-smoking periodontitis patients, or as comparable to the 40.6% rate in Australians of essentially European heritage.[4,22]

The concept of the composite genotype as a susceptibility factor for periodontitis was challenged in this study similar to previous studies that were done in non-Caucasian populations.[8, 13] It was the IL-1B in particular which occurred in a low prevalence that made it difficult to draw any relationship between the proposed composite genotype and the periodontal disease status.

It was concluded that this polymorphism alone does not carry significant risk for severe chronic periodontitis.

6.3 – CLINICAL APPLICATION

It is relevant in the present study that 22% of the healthy or mild periodontitis patients had allele 2 at IL-1A. As is seen with some risk factors in other multifactorial diseases, the IL-1 genotype can be present without always being associated with clinical disease. On the other hand, the genotype positive patients who did not have periodontitis could possibly have controlled other factors that are necessary for disease, such as bacterial load.

In periodontal therapy, there is great reliance on patient participation. Individuals that are at increased risk are advised to perform stringent plaque control methods, and frequent professional care may be an appropriate strategy to manage the increased risk to develop periodontitis.

In general, the findings demonstrated that the IL-1 composite polymorphism is not related to the severity of periodontitis in subjects with disease.

At the same time, it is important that knowledge of the IL-1 genotype of a patient could be useful, if longitudinal data of disease progression or treatment outcomes reveal that genotype positive subjects have a poorer prognosis.

A study where 42 patients were maintained over 14 years, found that IL-1 genotype-positive subjects had a 2.7 increased risk for tooth loss.[72] Contrary to these findings, a study of 48 patients scheduled for regular recall visits and followed-up for two years after non-surgical periodontal therapy, reported no differences in the survival rates of teeth or in the progression of attachment loss between genotype positive and negative subjects. [76]

Additional data is needed to clarify these effects and it is recommended that a larger prospective project shall include persons of different ethnic backgrounds in South Africa. The objective of the study will be to provide longitudinal assessments in both cases and controls, and to determine the relative importance of the IL-1 genotype against other risk factors for disease progression as well as to identify new or different mutations in different ethnicities.

In addition, in the future such information might be utilized to modify the patient's risk and to guide prevention and therapy by identification of theses persons with higher risk to develop the disease in the future.

CHAPTER 7

LIMITATIONS

This study had several limitations, as related to the structure and design, time frames, technical aspects and availability of the resources.

The study was case-control and not prospective in design, and having strict inclusion criteria the sample had to be selected out of more than thousand of patients screened. The primary researcher was foreign student and had to go to different areas in order to meet the specific population, which had a language unfamiliar to the researcher, who is not even an English speaker. Females presented more often than males in the clinics and in order to match cases and controls males were to be selected. The researcher did not have a clinical assistant due to financial constraints, and other than the full mouth clinical examination of the samples he had to occasionally undertake "routine" emergency treatment for patients whether they were included or not in the study. Those factors could have resulted in observer bias or selection bias in the clinical part of the study.

The laboratory part was complicated twice by absence of researchers dedicated for this research and some results were unverified and were disregarded in the statistical part.

The authorundertook did two tuition courses in molecular genetics, including his actual participation in DNA extraction from cells and performance of PCR and running the products

into gels. He also attended beginner and intermediate courses in statistics with SPSS. It is relevant that the author was a dentist and so the literature used was mainly dental, and the scope of the study might be expected on this regard.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

Conclusions drawn from the literature on the role of the positive composite IL-1 genotype, or the ability of specific alleles at the two investigated loci, to discriminate between subjects with and without of periodontal disease were inconclusive. It should be noted, that the previous studies differed considerably in their methodology, therefore, direct comparisons are not readily feasible.

However, with regard to the limitations, and based on the findings of this case-control study, to evaluate the prevalence and association of interleukin-1 polymorphisms among subjects with moderate to severe periodontal disease and age and gender matched controls non-smokers of the Xhosa population of South Africa, the following can be concluded:

Screening of subjects for IL-1 gene polymorphisms in view of making assumptions on their risk for periodontal disease does not appear to be justified. However, the data clearly indicated a significant effect of Interleukin-1A on the severity of chronic periodontal disease. This association was kept both when the genotype was tested alone with the disease status and also when it was analyzed in the presence of other confounding factors.

It is important to note as well, that the cause-effect relationship of chronic diseases, such as periodontal disease, is not easy to prove.

Thus, additional prospective studies that enroll a sufficient number of subjects are needed to investigate valid predictive rates of chronic periodontitis associated with a genotype-positive test result.

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. Appendix A: General definitions of the terms used in this thesis (See: www.geneclinics.org)

Allele – one of several possible alternative forms of a given gene differing in DNA sequence assumed to arise by mutation and often affecting the function of a single product. Humans carry two sets of chromosomes, one from each parent. Single nucleotide polymorphisms may render two sets of equivalent genes different.

Alternative splicing – the generation of multiple protein isoforms from a single gene via the splicing together of nonconsecutive exons during RNA processing of some but not all of RNA transcripts. Believed to be the mechanism involved with the high number of proteins produced from a smaller number of genes in humans.

Autosome – chromosomes other than sex chromosomes.

Autosomal dominant – Describes a trait or disorder in which the phenotype is expressed in those who have inherited only one copy of a particular gene mutation (heterozygotes); specifically refers to a gene on one of the 22 pairs of autosomes (non-sex chromosomes)

Autosomal recessive – Describes a trait or disorder requiring the presence of two copies of a gene mutation at a particular locus in order to express observable phenotype; specifically refers to genes on one of the 22 pairs of autosomes (non-sex chromosomes)

Complementary DNA (cDNA) – the DNA sequence produced by the enzyme called reverse transcriptase from messenger RNA. Very frequently used in cloning experiments.

Chromosome – Physical structure consisting of a large DNA molecule organized into genes and supported by proteins called chromatin

Cloning – An identical copy of a DNA sequence or entire gene; one or more cells derived from and identical to a single ancestor cell; to isolate a gene or specific sequence of DNA

Dizygotic twin – fraternal twins as a result of fertilization of two separate eggs. They are no more similar genetically than are siblings.

Exon – Coding sequence of DNA present in mature messenger RNA

False-positive rate: A test result which indicates that an individual is affected and/or has a certain gene mutation when he or she is actually unaffected and/or does not have the mutation; i.e., a positive test result in a truly unaffected individual

False-negative rate: A test result which indicates that an individual is unaffected and/or does not have a particular gene mutation when he or she is actually affected and/or does have a gene mutation; i.e., a negative test result in an affected individual

Frameshift mutation – A type of mutation as a result of an insertion or deletion of one or more nucleotides into a gene causing the coding regions to be read in the wrong frame.

Gene – The basic unit of heredity, consisting of a segment of DNA arranged in a linear manner along a chromosome. A gene codes for a specific protein or segment of protein leading to a particular characteristic or function.

Gene expression – the process involving use of the information in a gene via transcription and translation leading to production of a

protein affecting the phenotype of the organism determined by that gene.

Genetic code – in RNA and DNA, the consecutive nucleotide triplets (codon) that specify the sequence of amino acids for protein synthesis (translation).

Genetic screening: Large-scale testing of defined populations for a genetic disease or disease-causing gene.

Genome – a term used to refer to all the genes carried by an individual or cell.

Genotype – the genetic makeup of an organism or cell distinct from its expressed features or phenotype.

Haplotype – the collection of one allele of each gene comprising the genotype.

Homozygous – the presence of identical alleles of one or more specific genes (e.g. A/A).

Heterozygous – the presence of differing alleles of one or more specific genes (e.g. A/B).

Intron – the intervening (non-coding) portion of DNA or RNA that is removed during RNA processing.

Isoforms – a protein with equivalent function and similar or identical sequence but derived from a different and usually tissue-specific gene.

Ligand – any particular molecule that binds to another such as a hormone to its receptor.

Linkage: A greater association in inheritance of two or more non-allelic genes than is to be expected from independent assortment. Genes are linked because they reside in close proximity on the same chromosome.

Linkage analysis: A method of determining the chromosomal location of a gene of interest.

Linkage disequilibrium: The tendency of specific combinations of alleles at two or more linked loci to occur together on the same chromosome more frequently than would be expected by chance.

Locus (plural loci) – the physical location a gene occupies within a chromosome or portion of genomic DNA.

LOD (logarithm of the odds) score: Computation performed to evaluate support for/against a linkage hypothesis. Specifically, it is a common logarithm of the ratio of the likelihood of linkage at a specific recombination fraction to the likelihood of no linkage.

Monozygotic twin – identical twins having identical sets of nuclear genes as a result of separation of blastomeres.

Mutation – alteration of the genomic sequence compared to a reference state. Not all mutations have harmful events (silent mutation).

Phenotype – the observable characteristics displayed by an organism as influenced by environmental factors and independent of the genotype of the organism.

Polymorphism – a region on the genome that varies between individual members of a population present in a significant number of individuals.

Sequencing – the linear arrangement of nucleotides (in RNA or DNA) or amino acids (in protein).

Segregation analysis: Method of assessing relative support between various hypothesized inheritance patterns for a trait of interest.

Sensitivity of a genetic test: The proportion of cases that are correctly identified by the genetic test.

Silent mutation – a mutation resulting in no noticeable change in the biological activity of the protein encoded by the affected gene.

Single nucleotide polymorph (SNP) – a polymorphism caused by the change in a single nucleotide believed to be the most common genetic variation between individual humans.

Signal transduction – the cascade of cellular events by which an extracellular signal such as a hormone or growth factor interacting with a receptor on the cell surface triggers an internally-directed response. This stepwise occurrence usually results in changes in gene expression in the nucleus.

Single nucleotide polymorphism (SNP): A small genetic change or variation that can occur within a person's DNA sequence. The genetic code is specified by the four nucleotide "letters" A: adenine; C: cytosine; T: thymine; and G: guanine. SNP variation occurs when a single nucleotide, such as an A, replaces (substitutes for) one of the other three nucleotide letters C, G, or T.

Specificity of a genetic test: The proportion of controls that are correctly identified by the genetic test.

Splicing – the removal of introns from transcribed RNAs. The removal of exons results in the formation of 'splice variants' or 'alternatively spliced' protein isoforms allowing different proteins to be produced from the same initial RNA or gene.

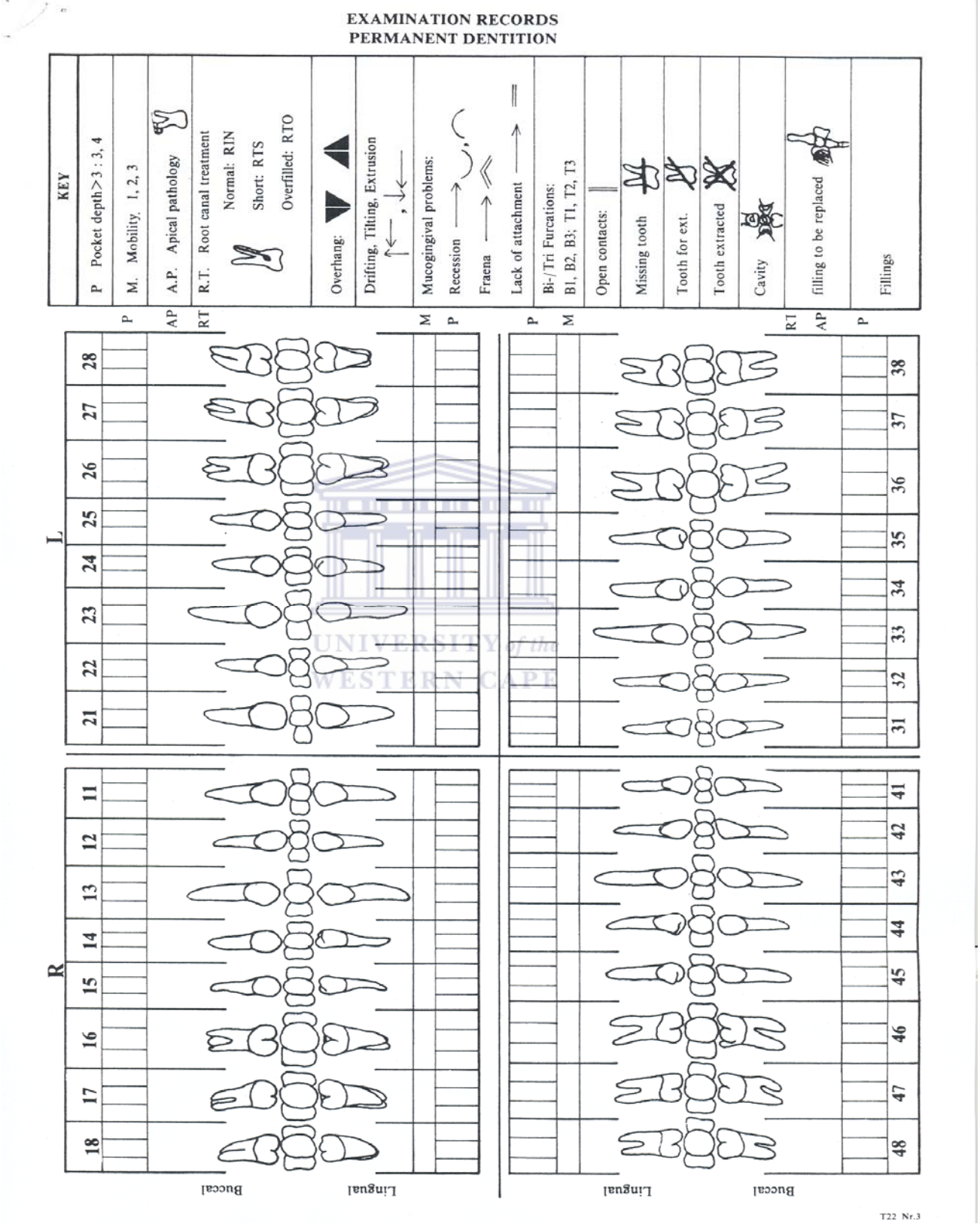
X-linked disease – a disease of genetic origin as a result of a mutation on the X-chromosome.

Vector – common term for a carrier of and organism, DNA, RNA, or protein to be transferred from one organism to another. Examples include plasmids, viruses, and mosquitoes.

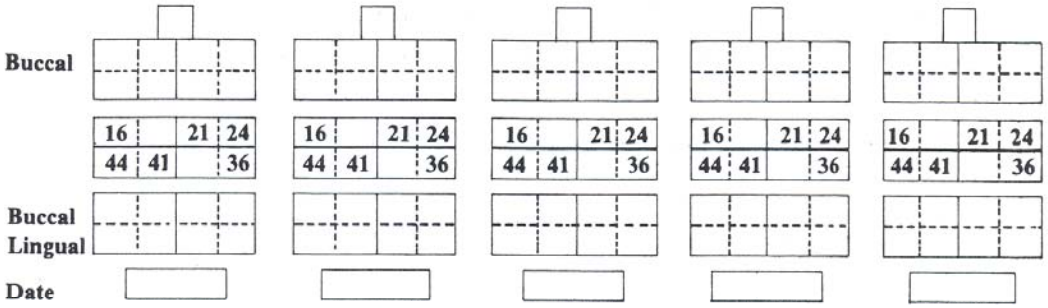
Wild type – the non-mutated, naturally-occurring form of a gene.

Note: Ethidium Bromide (EtBr) is strongly mutagenic. Toxic effects of this chemical may be observed upon swallowing, inhalation or absorption through the skin. Gloves should be worn at all times when handling EtBr.

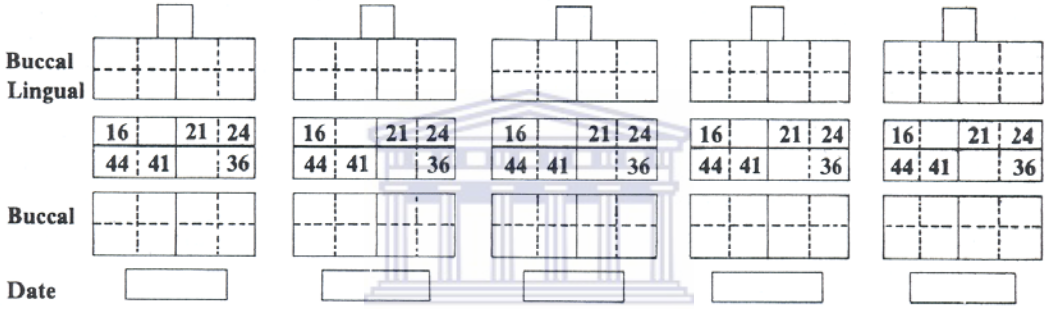
Appendix B: Charting sheets



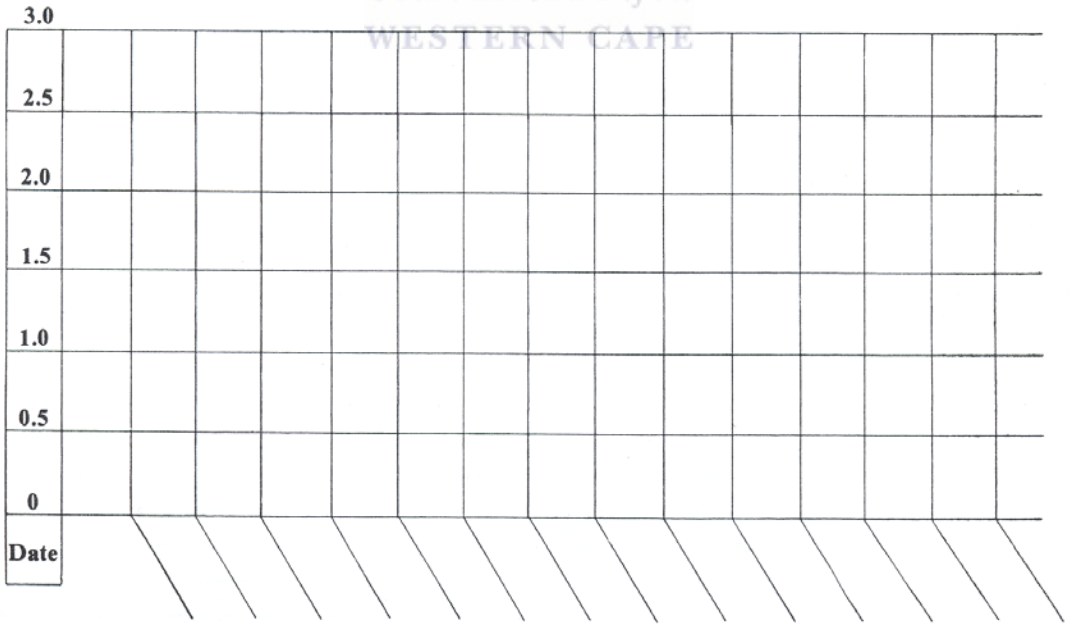
PLAQUE INDEX



GINGIVAL INDEX



P.I., G.I.



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Appendix C: consent form English copy

Consent Form

Patient information sheets

Prevalence of Interleukin –1 gene polymorphism and its association with periodontal status in the Xhosa population of South Africa

Researchers : Dr . Abu Saleh T

Prof. Stephen LXG

Faculty of Dentistry

University of the Western Cape

The purpose of this study is to determine the prevalence of the Interleukin-1 composite genotype in individuals of the Xhosa population of South Africa , as well as to evaluate if there is an association between the composite genotype and the severity of periodontal disease. People who will participate in this study will be examined for periodontal disease, and then a buccal smear will be obtained. Samples will be given codes and the DNA in the sample will be analyzed in the laboratory of molecular genetics for the presence of IL-1 polymorphisms, without knowledge of patient names or records.

Patients will be treated or referred for treatment, and they have the right to withdraw from the study at any stage without affecting their treatment or referral procedure .The results of this study will help determine which people are of higher risk to develop severe periodontal disease.

The information obtained in this study will be treated with utmost confidentiality and the names of the participants will not be divulged for any other purposes.

For any further information please contact:

Dr Abu Saleh ,Tareq

E-mail: 2558431@uwc.ac.za

Consent

I have read the information, asked questions and received answers concerning areas that were unclear and I willingly agree to participate in this study. My participation is completely voluntary. I may withdraw at anytime. I will not have waived any of my legal rights by signing this consent form. Upon signing this form, I will receive a copy of the entire consent.

……	…….	……
Participant name	Signature	Date
……	…….	……
Witness name	Signature	Date

Appendix D: consent form Xhosa copy

I- phepha le Mvume

Ukuvela kwe interleukin-imeko yokuzala isifo ekuthiwa yi polymorphism - nebanga le sisifo sama zinyo neentsini kubantu abangama Xhosa kum Zantsi Africa
Lomsebensi wenziwa ngu gqira Abu Saleh T
no Proffesor Stephen LX
Kwiziko lemfundiso enomsila ibizwa ngokuba yi universityof the Western Cape .
Imbanghe yale mfuniso yeyo kwenza ukukwazi okuba isifo samazinyo neetsi zibonakala kanjani kubantu ababizwa ngokuba ngama Xhosa emzantsi Africa , nokoku kwazi ukuba ikhona indibaniso yokuvela kwezisifo nububanzi kwavo
Abantu abaza kuthathela inxaxeba kweli bando baya kufumana ibuccal smear , lento yenziwa ngokusulwa kwamazinyo neentsi nge cephe elithambilyo lithunyelwe e laboratory . Akukho mfuneko le gama lomtu .
Isigulana siyaku nyangwa , oko unayo imvume yokuba uthi akufuni oku thatha inxaxeba kulomvange .
Olwaziso liyimfihlelo kwabanye abantu. Ndiyabulela .

Dr Abu Saleh
E-mail 2558431@uwc.ac.za

Imvume .
Ndiyifundile ulwaziso, ndibuze imibuzo, kwaze ndafumana iimpendulo, kwaze kwindawo ebendigaziqondi ndizichazelwe .
Ndiya yazi ukuba ndigayeka nokuba kunini na .

Igama lakho	Ubhale	Imini
……	…….	……

Ingqina	Ubhale	Imini
…….	……	……

Appendix E: Statistical Results

Ranks

	Periodontal status	N	Mean Rank	Sum of Ranks
Age (in years)	Healthy to mild	50	46.73	2336.50
	Moderate to severe	49	53.34	2613.50
	Total	99		

Test Statistics(a)

	Age (in years)
Mann-Whitney U	1061.500
Wilcoxon W	2336.500
Z	-1.147
Asymp. Sig. (2-tailed)	.251

a Grouping Variable: Periodontal status

Ranks

	Periodontal status	N	Mean Rank	Sum of Ranks
Clinical attachment loss	Healthy to mild	50	25.50	1275.00
	Moderate to severe	49	75.00	3675.00
	Total	99		

Test Statistics (a)

	Clinical attachment loss
Mann-Whitney U	.000
Wilcoxon W	1275.000
Z	-8.584
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Periodontal status

Ranks

	Periodontal status	N	Mean Rank	Sum of Ranks
Tooth loss (Number of lost teeth)	Healthy to mild	50	48.18	2409.00
	Moderate to severe	49	51.86	2541.00
	Total	99		

Test Statistics(a)

	Tooth loss (Number of lost teeth)
Mann-Whitney U	1134.000
Wilcoxon W	2409.000
Z	-.640
Asymp. Sig. (2-tailed)	.522

a Grouping Variable: Periodontal status

Ranks

	Periodontal status	N	Mean Rank	Sum of Ranks
Plaque index	Healthy to mild	50	38.94	1947.00
	Moderate to severe	49	61.29	3003.00
	Total	99		

Test Statistics(a)

	Plaque index
Mann-Whitney U	672.000
Wilcoxon W	1947.000
Z	-3.877
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Periodontal status

Ranks

	Periodontal status	N	Mean Rank	Sum of Ranks
Gingival index	Healthy to mild	50	39.18	1959.00
	Moderate to severe	49	61.04	2991.00
	Total	99		

Test Statistics(a)

	Gingival index
Mann-Whitney U	684.000
Wilcoxon W	1959.000
Z	-3.795
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: Periodontal status

	Periodontal status			
	Healthy to mild		Moderate to severe	
	Mean	Std Deviation	Mean	Std Deviation
Age (in years)	44	8	46	9
Clinical attachment loss	.5	.4	2.5	.5
Tooth loss (Number of lost teeth)	4.0	2.9	4.4	3.1
Plaque index	1.0	.7	1.6	.7
Gingival index	1.0	.8	1.7	.7

IL-1* periodontal status

Crosstab

			IL-1A			
			Allele 1	Allele 2 (Heterozygous)	Allele 2 (Homozygous)	Total
Periodontal status	Healthy to mild	Count	39	9	2	50
		% within Periodontal status	78.0%	18.0%	4.0%	100.0%
	Moderate to severe	Count	26	19	4	49
		% within Periodontal status	53.1%	38.8%	8.2%	100.0%
Total		Count	65	28	6	99
		% within Periodontal status	65.7%	28.3%	6.1%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.829(a)	2	.033
Likelihood Ratio	6.939	2	.031
Linear-by-Linear Association	5.731	1	.017
N of Valid Cases	99		

a 2 cells (33.3%) have expected count less than 5. The minimum expected count is 2.97.

Periodontal status * IL-1B

Crosstab

			IL-1B			
			Allele 1	Allele 2 (Heterozygous)	Allele 2 (Homozygous)	Total
Periodontal status	Healthy to mild	Count	18	2	1	21
		% within Periodontal status	85.7%	9.5%	4.8%	100.0%
	Moderate to severe	Count	16	2	1	19
		% within Periodontal status	84.2%	10.5%	5.3%	100.0%
Total		Count	34	4	2	40
		% within Periodontal status	85.0%	10.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.018(a)	2	.991
Likelihood Ratio	.018	2	.991
Linear-by-Linear Association	.015	1	.902
N of Valid Cases	40		

a 4 cells (66.7%) have expected count less than 5. The minimum expected count is .95.

Periodontal status * IL-1A Crosstabulation

			IL-1A		
			Allele 1	Allele 2 (Heterozygous)	Total
Periodontal status	Healthy to mild	Count	39	9	48
		% within Periodontal status	81.3%	18.8%	100.0%
	Moderate to severe	Count	26	19	45
		% within Periodontal status	57.8%	42.2%	100.0%
Total		Count	65	28	93
		% within Periodontal status	69.9%	30.1%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.081(b)	1	.014		
Continuity Correction(a)	5.017	1	.025		
Likelihood Ratio	6.172	1	.013		
Fisher's Exact Test				.023	.012
Linear-by-Linear Association	6.016	1	.014		
N of Valid Cases	93				

a Computed only for a 2x2 table

b 0 cells (.0%) have expected count less than 5. The minimum expected count is 13.55.

Periodontal status * IL-1A Crosstabulation

			IL-1A		
			Allele 1	Allele 2	Total
Periodontal status	Healthy to mild	Count	39	11	50
		% within Periodontal status	78.0%	22.0%	100.0%
	Moderate to severe	Count	26	23	49
		% within Periodontal status	53.1%	46.9%	100.0%
Total		Count	65	34	99
		% within Periodontal status	65.7%	34.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.826(b)	1	.009		
Continuity Correction(a)	5.765	1	.016		
Likelihood Ratio	6.935	1	.008		
Fisher's Exact Test				.011	.008
Linear-by-Linear Association	6.757	1	.009		
N of Valid Cases	99				

a Computed only for a 2x2 table

b 0 cells (.0%) have expected count less than 5. The minimum expected count is 16.83.

Periodontal status * IL-1B Crosstabulation

			IL-1B		
			Allele 1	Allele 2 (Heterozygous)	Total
Periodontal status	Healthy to mild	Count	18	2	20
		% within Periodontal status	90.0%	10.0%	100.0%
	Moderate to severe	Count	16	2	18
		% within Periodontal status	88.9%	11.1%	100.0%
Total		Count	34	4	38
		% within Periodontal status	89.5%	10.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.012(b)	1	.911		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.012	1	.911		
Fisher's Exact Test				1.000	.656
Linear-by-Linear Association	.012	1	.912		
N of Valid Cases	38				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.89.

B Crosstabulation1-IL *Periodontal status

			IL-1B		
			Allele 1	Allele 2	Total
Periodontal status	Healthy to mild	Count	18	3	21
		% within Periodontal status	85.7%	14.3%	100.0%
	Moderate to severe	Count	16	3	19
		% within Periodontal status	84.2%	15.8%	100.0%
Total		Count	34	6	40
		% within Periodontal status	85.0%	15.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.018(b)	1	.894		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.018	1	.894		
Fisher's Exact Test				1.000	.619
Linear-by-Linear Association	.017	1	.896		
N of Valid Cases	40				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.85.

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