QUALITATIVE AND QUANTITATIVE ANALYSIS OF SALIVA IN A GROUP OF HIV INFECTED INDIVIDUALS

Thesis submitted in partial fulfilment of the requirements for the degree of Master of Dental Surgery

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This thesis is dedicated to Tony

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DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis being made available for loan and photocopying.

Elizabeth A. Coates December 1997

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LIST OF ABBREVIATIONS

AZT	Zidovudine
Aci	Aciclovir
AIDS	Acquired Immune Deficiency Syndrome
ANUG	Acute necrotising ulcerative gingivitis
aPRPs	Acidic proline rich proteins
bPRPs	Basic proline-rich proteins
Bac	Bactrim
CD4	Cluster differentiated (4) T-cell lymphocytes
CD8	Cluster differentiated (8) T-cell lymphocytes
Clar	Clarithromycin
CMV	Cytomegalovirus
DDC	Hivid/ Zalcitabine
DDI	Didanosine
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EEC	European Economic Community
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
Flu	Fluconazole
Fun	Fungilin
GP160	HIV glycoprotein
HIV	Human Immunodeficiency Virus
HIV-G	HIV-related gingivitis
HIV-SGD	HIV-related salivary gland disease
HSV	Herpes simplex virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
I	Imipramine

Itra	Itraconazole
kD	Kilodaltons
Ket	Ketoconazole
KS	Kaposi's sarcoma
LGE	Linear Gingival Erythema
Lit	Lithium
MAC	Mycobacterium avium complex
Met	Methadone
MG	mucous glycoproteins
MWt	molecular weight
Nap	Naprosan
NUG	Necrotising Ulcerative Gingivitis
NUP	Necrotising Ulcerative Periodontitis
NUS	Necrotising Ulcerative Stomatitis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
PM	Polymedication
PRPs	Proline rich proteins
Pul	Pulmacort
Rif	Rifampicin
Sep	Septrim
SLPI	Secretory leukocyte protease inhibitor
Thal	Thalidomide
Ven	Ventolin
Vit	Vitamins
WHO	The World Health Organisation
ZDV	Zidovudine
Zyl	Zyloprim

CHAPTER 1

INTRODUCTION

Human Immunodeficiency Virus has made a huge impact on the health of infected individuals, either by direct infection of organs or by its ability to deplete the immune system rendering people susceptible to opportunistic infections. Early in the epidemic it became apparent that throughout the course of HIV infection oral health was severely affected. One of the oral problems that has been noted by investigators was the presence of dry mouth, with or without associated swelling of the salivary glands. Few studies on HIV related xerostomia and salivary gland disease had been undertaken and most reports were of an epidemiological nature and generally anecdotal.

The hypothesis for this study was that quality and quantity of saliva may contribute to the prevalence of oral disease in HIV infection and that there maybe significant variance of saliva quality and quantity between early and late stage HIV infection. It was considered important to verify whether saliva could be a major contributing factor to oral problems in HIV infected patients as the value of instituting appropriate preventive therapies at the time of diagnosis of HIV infection could then be justified and both the quality and quantity of life for people infected with HIV improved.

The hypothesis was tested by collecting saliva samples from sixty five individuals with HIV infection and from thirty three control subjects. The saliva samples were tested for pH, volume, calcium and protein content. Results of the salivary analyses were correlated with a range of clinical parameters in subjects with HIV, for example the presence of specified oral diseases, stage of HIV infection and medications taken.

This study demonstrated that it is more likely that subjects with HIV infection will have reduced stimulated salivary flow than subjects without HIV. This reduction of salivary flow in the subjects studied was not related to the stage of HIV infection, nor to medication taken. The presence of the oral manifestations investigated, HIV periodontal disease, candida, caries and erosion, appeared to be unrelated to salivary flow. The pH of the resting phase salivary samples was lower in subjects with HIV and subjects with low salivary pH were more likely to exhibit candidiasis.

The investigations carried out in this research demonstrated the importance of xerostomia as an oral manifestation of HIV and the necessity for ongoing salivary research in subjects with HIV infection.

CHAPTER 2

LITERATURE REVIEW

1 Human Immunodeficiency Virus (HIV)

Introduction

The purpose of this research project was to investigate certain aspects of changes in saliva patterns in HIV-infected people and to ascertain the significance of these changes.

The following review examines Human Immunodeficiency Virus (HIV) infection, particularly in relation to specific oral conditions where salivary changes could be relevant. Reports of the presence of HIV in saliva are also reviewed because of the interest in using salivary assays for assessment of the extent of world-wide epidemics.

1.1 HIV Definition and Pathogenesis

HIV is a virus which causes a dynamic infection in humans, resulting in chronic illness of many years' duration (Stewart *et al.* 1996).

HIV is an enveloped virus of the lentivirus group, a subfamily of retroviruses. It has an enzyme, reverse transcriptase, which copies the genetic viral RNA into deoxyribonucleic acid which is eventually incorporated into the host T-cell genetic material (Cunningham *et al.* 1996).

HIV infects three major cell types that are critical for the immune response. These are monocytes and macrophages, dendritic cells, and CD4 helper-inducer T-cells. HIV also infects a wide range of other host cells with a CD4 receptor, for example Langerhans cells in the skin and microglial cells in the brain.

In addition, Qureshi *et al.* (1997) demonstrated that epithelial cells in the mouth can be productively infected by HIV proviral DNA, probably as a result of infection of the basal layer cells by submucosal HIV-infected lymphocytes. Once infected, the epithelial cells containing the virus move through to the more superficial layers and are finally shed from the surface into the oral cavity.

Acquired Immune Deficiency Syndrome (AIDS) results from the combined effects of HIV destruction of the infected cells causing immunodeficiency, and by direct infection by HIV of host organs such as the brain. The immune cells either suffer depletion or dysfunction, resulting in immunodeficiency of the host (Cunningham *et al.* 1996).

The immune system controls the plasma levels of virus until a significant depletion of immune cells occurs. Damage to the immune system does occur during the asymptomatic phase but there are no clinical signs because the immune system clears the virus and is not fully depleted. Eventually, destruction of the immune system, specifically CD4+ helper-inducer T-cells, leaves the body defenceless against opportunistic pathogens (Ffrench *et al.* 1996).

Clearance of the virus by the body is complex. HIV replication occurs continuously at high rates throughout the course of HIV infection. Perelson *et al.* (1996) used potent protease inhibitors to demonstrate that the average total production of HIV was 10.3×10^9 virions per day and that the HIV-1 has a life cycle of 1.2 days. The time between the release of a new virion and its infection of another cell is estimated at 2.5 days.

Viral load has been defined by Cunningham *et al.* (1996) as the amount of virus present in plasma. It is now believed by many researchers that the amount of virus in the plasma is indicative of HIV activity and potential immune destruction (Cunningham *et al.* 1996; O'Brien *et al.* and the Veterans Affairs Cooperative Study Group on AIDS 1996; Nadler 1996). Consequently, viral load should have a predictive value in determining the prognosis of an individual infected with HIV. According to Ionnidis *et al.* (1996), viral load does have predictive value, even if the time of seroconversion, acquisition of HIV, was unknown. They also stated that serial measurements of viral load would assist in determining therapy. This concept was supported by O'Brien *et al.* (1996) who found that changes in the plasma HIV-1 RNA level combined with the CD4 lymphocyte count were predictors of progression of disease and could be used to demonstrate efficacy of treatments. Nadler (1996) discussed the dynamics of HIV pathogenesis further and defined the new goal of HIV therapy as *"maximal viral suppression and preservation of the immune system".* He stated that as immunologic decline and chronic clinical illness resulted from viral load it would be reasonable to treat all patients in whom HIV replication is detected.

One of the features of HIV infection, from seroconversion to AIDS, is infection by opportunistic pathogens. Advanced HIV infection leads to multiple opportunistic infections that are mostly related to defects in the host's cellular immunity and the microbial environment (Marriott and McMurchie 1996). The usual microorganisms that cause opportunistic infections in HIV infection are ubiquitous in soil and water or reside latently in the host after a previous infection.

Interestingly, some common pathogens such as *Legionella pneumophila* that normally infect patients with immunosuppression as a result of medication, transplants or idiopathic causes, are not common in HIV infection. However other pathogens commonly causing opportunistic infections in HIV infection, for example cytomegalovirus (CMV) and Mycobacterium avium complex (MAC) infections, are rarely encountered in other immunocompromised patients (Ffrench *et al.* 1996).

The presentation of opportunistic infections in HIV infection is also varied and complex. For example, candidial infection of the skin and mucosa is common due to alterations in cell mediated immunity, whereas disseminated candidial infection is uncommon as it is usually associated with severe neutropenia plus a T-cell deficiency. In addition, oral hairy leukoplakia does not normally develop in healthy Epstein-Barr virus (EBV) carriers or immunosuppressed patients who are not infected with HIV (Crowe and Kornbluth 1996). The complexity of opportunistic infection was further supported by Boulter *et al.* (1996) who concluded that oral opportunistic infection with EBV was related to both behavioural and HIV specific immunological changes. These authors provided further evidence to support the work of Crowe and Kornbluth (1996) who concluded that the pathogens involved in HIV-related opportunistic infections differ from those generally infecting humans, either in disease presentation or by their pathogenicity.

The complexity of HIV infection is compounded by the possibility of these opportunistic pathogens impacting on or causing other disease processes, such as neoplasias. Gaidano *et al.* (1996) investigated opportunistic pathogens and

concluded that human Herpes virus - 8 is associated with selected types of AIDS related neoplasias, such as Kaposi's sarcoma and body cavity based lymphomas, but not with other types of AIDS malignancies. Other authors have suggested that the Non-Hodgkin's lymphoma found in HIV infection is related to the Epstein-Barr virus for example Milliken *et al.* (1996) stated that EBV-DNA has been discovered in 100% of all HIV-related cerebral lymphoma and in 30% of other lymphomas.

1.2 Epidemiology

1.2.1 Incidence of HIV infection

Approximately 10,000 people acquire HIV every 24 hours. Almost half of these infections occur in the Asian Pacific region and the infections in women outnumber those in men. An estimate of new infections for South East Asia for the year 1995 is two and a half million. The World Health Organisation predicts an epidemic of tuberculosis will accompany the HIV epidemic (Dwyer, Mahathir and Nath 1996).

Over 19,000 Australians have been infected with HIV since the introduction of HIV antibody testing and over 4,000 have died of AIDS (Communicable disease Intelligence 1996).

In an article entitled *Global Burden of HIV pandemic*, Quinn (1996) reported that estimates of the extent of the pandemic are "generally quite awful" and that the number of infected people world wide will be "phenomenal". Dwyer *et al.* (1996) outlined the essential steps required to control and minimise the potential devastation, including careful monitoring of the course of the epidemic.

Quinn (1996) supported careful monitoring and presented a solid argument for epidemiological research within the clinical setting stating that:

"Clinical assessment alone can provide considerable information on prognosis. Knowledge of the sequence in which opportunistic infection and other complications occur within each country is invaluable. This can be learnt from clinical experience and from clinical research in centres with a high case load..."

Two of the principles outlined by Quinn (1996) and Dwyer (1996) for management in resource-poor settings were that (1) early detection of HIV would optimise prophylaxis and treatment at all stages of HIV and would be cost effective, and (2) management should focus on common opportunistic infections and symptoms that are easy to diagnose, and treatable within the confines of a country's available resources. These authors suggested that the many oral opportunistic infections must also continue to be documented and monitored.

1.2.2 Incidence of oral manifestations

Several authors have studied the incidence of HIV infection and oral manifestations. There appears to be a wide variation in reporting of oral manifestations and the incidence of many oral lesions shows significant discrepancies between different countries. Nevertheless, there appears to be uniformity in the statistical incidence for many oral manifestations. This variation and correlation is epitomised by two studies. Laskaris, Hadjivassiliou, and Stratigos (1992) described a wide spectrum of oral manifestations in Greece, and a clinical diagnosis of HIV for 16 of the patients studied was made on the presentation of an oral manifestation. They found candidiasis in 70% of the group, hairy leukoplakia in 24% of their patients, and 19% had periodontal problems. Coates *et al.* (1996) assessed the incidence of several oral manifestations occurring in a dental clinic dedicated to the oral health management for HIV-infected patients. Hairy leukoplakia was present in 24% of attending patients, which was identical to the results of Laskaris *et al.* (1992). However, a much lower percentage of patients presented with candida (32%) and a higher percentage had periodontal problems (51%).

Variation between studies can be accounted for by many factors. Access to prophylactic antifungal treatment can reduce the prevalence of fungal infections, and both behavioural factors, genetic factors and HIV-specific immunological changes will influence the incidence of periodontal disease. Of interest is a study of a group of HIV patients in Zaire in Africa, where the male/female ratio was 0.8 (46/83), which gave a large prevalence of women (Tukutuku *et al.* 1990) in the group studied. These authors reported an incidence of 94% for oral fungal infections and a relatively high proportion of aggressive periodontal lesions (33%), especially in the younger age bracket. Winkler and Robertson (1992) reviewed information drawn from studies performed in USA, Montreal, Amsterdam and England, and found that the incidence of periodontal conditions associated with HIV infection ranged from 16%-48%.

Several other studies have documented the incidence of oral manifestations of HIV infection in various countries. Gillespie and Mariño (1993) in a group of Pan-American HIV patients, recognised oral candidiasis as the greatest presenting oral problem whereas Firth, Rich, and Reade (1994) reviewed Australian patients with HIV, and considered that viral infections such as hairy leukoplakia, herpes simplex ulceration, cytomegalovirus ulceration and warts caused by human papilloma virus were all frequent manifestations requiring diagnosis and management. Schulten, ten Kate and van der Waal (1990) studied 70 HIV infected patients, of which 35 had AIDS, and found that 50% showed oral candidiasis, 14% oral hairy leukoplakia, 13% periodontal disease, and 4% Kaposi's sarcoma. Overall, 74% of their patients presented with oral mucosal lesions.

Barone *et al.* (1990) produced similar results when they examined a group of 217 Italian patients of whom 65% were intravenous drug users. Porter *et al.* (1989), in a study of 44 patients in Britain, presented figures for oral manifestations which were consistent with the above studies. Whereas a study of 75 Dutch patients with HIV by Schulten *et al.* (1989) presented lower figures for oral hairy leukoplakia (16%) than other studies, an overall 52% of patients had candidiasis, a result in concordance with the findings of Coates *et al.* (1996). Of significance is the fact that all of these studies reported oral manifestations as being significant in HIV infection. Furthermore, they presented in all infected groups: women, men, heterosexual, homosexual, and injecting drug users.

The importance of adequate standardisation of results and documentation of lesions, given the significant number of oral manifestations in HIV infection, led the European Economic Community (EEC) to devise a classification for oral manifestations of HIV infection (Pindborg 1993).

The significance of diagnosis and management of oral manifestations is evident when investigators such as Jones (1993) points out the outcomes associated with a missed diagnosis of HIV. He cited that 95% of patients presenting with AIDS had oral candidiasis, and 37% had oral hairy leukoplakia. The failure to diagnose HIV infection may adversely influence the progression and morbidity of the disease. Schulten *et al.* (1990) noted that ten of twelve suspected HIV-infected patients (83%) whose serostatus had yet to be determined showed oral lesions consistent with HIV infection. These findings highlight the necessity for ongoing research into

oral lesions, which Barr (1994) states may be as prevalent as 70% in HIV infection.

Greenspan *et al.* (1990) described oral manifestations of HIV and their management and proposed that early diagnosis of HIV infection was important for improving survival. Greenspan and Greenspan (1993) verified these proposals when they found that 59% of HIV-infected patients with oral candidiasis went on to develop AIDS. Further support for early diagnosis and monitoring of oral lesions was forthcoming from Glick *et al.* (1994) who concluded that oral manifestations were *'highly predictive markers of severe immune deterioration and disease progression.'*

1.2.3 Progression of disease

Progression of HIV infection and AIDS is dependent on many factors. It will vary from country to country depending on the subtypes and virulence of viruses that are causing the epidemics. Dore *et al.* (1996) and Quinn (1996) described multiple epidemics of HIV infection throughout the world. These epidemics presented with varying proportions of the population infected and with varying degrees of pathogenesis. Dore *et al.* (1996) reported nine known subtypes of HIV-1, and the explosive spread of disease in Thailand has been attributed to HIV-1 subtype E because of its affinity for the Langerhans cells in the vaginal wall. However spread of HIV-1 subtypes A and D in Africa and subtype B in the Caribbean and South America suggest that heterosexual spread may occur irrespective of the subtype.

Perelson *et al.* (1996) demonstrated that by using potent protease inhibitors the viral load can be reduced. This led to advances in the understanding of HIV pathogenesis and has resulted in the use of combination antiretroviral therapy for treatment of HIV. This is likely to change the progression of HIV infection in countries where advanced treatments are readily available, thereby extending the lifespan of those infected and prolonging the chronicity of the disease (Collier *et al.* 1996).

There are currently four protease inhibitors available in Australia, 5 nucleoside reverse transcriptase inhibitors and two nonnucleoside reverse transcriptase inhibitors. These drugs are generally given as a combination of three drugs: two nucleoside reverse transcriptase inhibitors with one protease inhibitor (Nadler 1996; Vella and Floridia 1996; Williams 1996). The purpose of the multiple therapy is to target the virus at multiple replication sites and make it difficult for resistant strains to develop and propagate (3rd International Congress on Drug Therapy in HIV Infection 1996; Stewart *et al.* 1996). Unfortunately, the virus has the potential to develop rapid resistance to antiretroviral medication so the efficacy of the treatment is monitored regularly using viral load tests (Carpenter 1996; Medical Frontiers International Management of HIV Infection: Cornerstones of therapy. A report from the 4th Conference on Retroviruses and Opportunistic Infections).

1.2.4 New Treatments

- **Nucleoside reverse transcriptase inhibitors**
- **HIV protease inhibitors**
- **Non-nucleoside reverse transcriptase inhibitors**

Nucleoside reverse transcriptase inhibitors

Nucleoside reverse transcriptase inhibitors or nucleoside analogues have been used as antiretroviral medication for many years. Zidovudine (ZDV/AZT) was one of the first available antiretroviral drugs and has been shown to prevent infection of uninfected CD4 lymphocytes and limit viral production. Used as a monotherapy, however, it can not eradicate HIV infection. It effectively crosses the blood-brain barrier. Unfortunately resistance has been known to develop after six months of therapy and bone marrow suppression is a serious side effect (Lewin *et al.* 1996). Since the introduction of Zidovudine, other nucleoside analogues have been developed and proven effective as antiretroviral medication (Vella and Floridia 1996). Oral side effects of nucleoside analogues have been few; pigmentation of the oral mucosa has been seen following use of Zidovudine (Lewin *et al.* 1996), and Hivid (DDC) has been implicated in the development of oral ulceration. Importantly, Didanosine (DDI) has been the only antiretroviral drug specifically noted to cause salivary gland dysfunction as one of its side effects.

HIV protease inhibitors

The establishment of a second target for inhibiting HIV replication led to the development of protease inhibitors which target HIV protease, preventing maturation of core proteins and rendering the virion noninfectious. There are four protease inhibitors currently being used in treatment protocols (Williams 1996). Saquinavir is taken three times a day and it is suggested that the drug be taken with grapefruit juice which blocks the action of cytochrome P450, resulting in higher serum drug levels (Lewin *et al.* 1997). The acid of the juice combined with a possible direct salivary gland side effect of protease inhibitors may lead to considerable oral morbidity.

Non nucleoside reverse transcriptase inhibitors

Non nucleoside reverse transcriptase inhibitors result in a significant reduction in plasma viral load, and have a rapid anti-HIV activity, but development of antiviral drug resistance is also rapid (Lazarus 1996).

The effect of combination therapy has resulted in greatly reduced quantities of circulating virus, thereby permitting improved overall health and improvement or recovery of the immune system (Nadler 1996; 3rd International Congress on Drug Therapy in HIV Infection, UK). One of the disadvantages of any polymedication regime are the oral side effects of mucosal ulceration, xerostomia and dental disease (Levine 1989a).

1.3 Opportunistic Infections

There is a wide range of organisms that take advantage of HIV induced deficiencies in the immune system (particularly lowered T-cell numbers and function and altered macrophage function) to cause opportunistic infections. The opportunists include bacteria, especially *Mycobacteria* and *Salmonella* species. Viruses such as cytomegalovirus and herpes viruses predominate, and fungi are common opportunists with *candida*, *cryptococcus*, and *aspergillus* species all prevailing in Australia. Rare opportunists are also found in HIV infection. Protozoa, especially *Pneumocystis Carinii* and *Toxoplasma gondii,* cause significant morbidity and mortality (Crowe and Kornbluth 1996), and *microsporidia* and *cryptosporidia* are organisms commonly causing diarrhoea and weight loss (Sievert *et al.* 1996).

1.3.1 Treatments for opportunistic infections

Many drugs are used as either primary or prophylactic treatment of the opportunistic infections which develop as the immune system becomes depleted in HIV-infected patients. Antibacterial, antiviral and antifungal drugs are all required in the management of infective secondary outcomes. In addition, chemotherapeutic drugs for the management of neoplasia, and drugs used in the management of HIV-related psychiatric disorders are frequently prescribed. The combined effects of polymedication, both antiretroviral and for opportunistic infections, impact on the health of the oral cavity. For example, many of the antidepressant drugs are highly xerostomic, some drugs impact on taste sensation, and others give rise to mouth ulceration (Buhrich and Judd 1996; Milliken *et al.* 1996; Wong and Shumach 1996).

1.4 Oral manifestations

- **Candidiasis**
- **Decay and erosion of teeth**
- **Periodontal disease**

Many of the opportunistic pathogens are capable of causing oral disease in HIV. Schulten *et al.* (1990), Greenspan and Greenspan (1993), Barr (1994), Coates *et al.* (1996), Foltyn and Marroitt (1996) and Quail (1997) all described the extensive range of oral manifestations which includes bacterial, fungal and viral lesions, as well as those of unknown etiology.

1.4.1 Candidiasis

Barr (1994) outlined four different types of candidiasis found in HIV infection, including hyperplastic candidiasis. The World Health Organisation (1992) has subsequently eliminated hyperplastic candidiasis from the list of HIV-related oral manifestations. Candida appears relatively early in HIV disease (Robinovitch *et al.* 1993). Greenspan and Greenspan (1993) described candidiasis as creamy white plaques or erythematous patches on the mucosal surfaces. The white plaques are readily removable by wiping.

The causative agent of candidiasis in HIV infection has been the subject of considerable investigation. Foltyn and Marroitt (1996) stated that candidiasis is most frequently caused by *Candida albicans*. However Heald *et al.* (1996) demonstrated that treatment of the condition with systemic antifungals such as fluconazole resulted in HIV-infected patients suffering from recurrent infections

which often yielded fungal species other than *Candida albicans*, as well as the development of drug resistant strains of *Candida albicans*. HIV-infected people also tend to yield more than one species of candida concurrently, although none of these species necessarily gives rise to symptomatic disease. McCullough, Ross and Reade (1995) revealed that genetically atypical strains of Candida in HIV infection were more virulent. This raises the question of why so many people with HIV carry multiple strains or species of Candida, some of which are more virulent, but do not necessarily manifest as opportunistic infections.

Coates *et al.* (1994) and Glick *et al.* (1994) found a definite correlation between CD4 counts and the presence of symptomatic infection, thereby indicating that the immune system has a definite bearing on the presence of Candidiasis. Nevertheless, this is only part of the explanation, as other factors obviously impact on the ability of the fungal organisms to express symptomatic infection. Barr (1994) stated that oral candidiasis can occur when factors other than immunosuppression exist. Radiation treatment, and the use of oral antibiotics, chemotherapeutic agents and steroids, all predispose people to Candida infections.

1.4.2 Caries and erosion of teeth and xerostomia

Previous studies have not concentrated on problems with teeth or dryness of the mouth, presumably because other complications are believed to have more serious outcomes. Much of the information on decay, erosion and dryness of mouth is either anecdotal or hypothetical. Greenspan *et al.* (1990) proposed that if HIV-related salivary gland disease results in xerostomia, then the development of caries is a possibility. These authors suggested that complaints of dry mouth are not uncommon and this could be due to salivary gland disease or to the antidepressant medication that is commonly prescribed for people with HIV infection. Where salivary flow is shown to be reduced, topical fluorides have been advocated and it has been recommended that strict attention should be given to oral hygiene.

Glick *et al.* (1994) observed increasing sinus problems led to the development of mouth breathing and dryness of the anterior part of the oral cavity and suggested that this might be a contributory factor to the development of xerostomia. Levine (1989) suggested that mouth breathing was a significant cause of decay and erosion of teeth.

1.4.3 Periodontal conditions

Although periodontal conditions are found in both early and late stage HIV infection, only Glick *et al.* (1994) observed HIV-related periodontal lesions as an early oral manifestation of HIV infection. Coates *et al.* (1995) reported a case of necrotising ulcerative stomatitis (NUS) occurring early in HIV infection, less than 24 months after the patient had seroconverted. The patient had a high CD4 count, suggesting that, in this patient, factors other than the state of the immune system may be responsible for the development of periodontal manifestations.

Continuing aggressive periodontal disease as CD4 cells declined and throughout the progression of AIDS was noted by all investigators (Winkler and Robertson 1992; Greenspan and Greenspan 1993; Smith *et al.* 1993; Yeung *et al.* 1993; Yeung and Serb 1994).

Classification of HIV-related periodontal lesions created much confusion until the WHO classification of HIV-related periodontal conditions (1992) was published. Smith *et al.* (1993) and Yeung *et al.* (1993) described the periodontal lesions but inaccurate differentiation between HIV-related gingivitis (HIV-G) and acute necrotising ulcerative gingivitis (ANUG) led to inconsistencies by these and other authors in their documentation of lesions. Yeung and Serb (1994) elaborated on the problems of accurate measurement of disease prevalence because of misinterpretation of the classification of HIV-related periodontal disease. The WHO (1992) has now determined that Necrotising Ulcerative Gingivitis (NUG) relates to the necrotising and painful presentations of gingivitis in HIV infection and Linear Gingival Erythema (LGE) relates to a non-painful erythematous condition of the gingiva.

Characteristics of HIV-related periodontal diseases include lack of plaque, soft tissue destruction and loss of periodontal attachment of the teeth usually associated with severe pain. Yeung and Serb (1994) found no increase in plaque build up in the HIV-infected patients, yet observed an increasing loss of attachment over a period of 18 months. CD4 cell counts declined in the cohort studied over this period and these authors concluded that there was a strong correlation between progression of HIV illness and loss of periodontal attachment. Yeung and Serb (1994) also found that microbiological studies failed to discover any new organisms or new organism relationships, and concluded that the tissue breakdown might indeed be a host response phenomenon. Additional research into HIV-related periodontal diseases has been described by Robinson (1997).

The WHO (1992) has determined that progressive, destructive periodontal destruction is classified as Necrotising Ulcerative Periodontitis (NUP). Where ulceration extends in to the non-attached mucosa of the mouth, the lesion has been termed Necrotising Ulcerative Stomatitis (NUS).

2 HIV and saliva

2.1 HIV-related salivary gland disease (HIV-SGD)

Greenspan *et al.* (1990) described HIV-related salivary gland disease (HIV-SGD) as an enlargement of predominantly the parotid but sometimes the submandibular salivary glands, that was a soft diffuse swelling and most often bilateral. Some, but not all people with this syndrome may show a reduced salivary flow rate. Autoimmune disease is now recognised as a manifestation of HIV infection. Often the disorder results from a direct attack by autoreactive T-cells. In HIV-related salivary gland disease, for example, the glands are infiltrated with CD8 lymphocytes, whereas non-HIV-infected patients with Sjögrens syndrome usually have a CD4 cell infiltrate of the salivary glands (Gala and Fulcher 1996; Challacombe and Sweet 1997).

Ficarra (1992) described HIV-SGD more broadly and suggested a multifactorial etiology, namely autoimmune, viral and bacterial infections, acute and chronic inflammation and tumours. Regarding the CD8 cell lymphocytosis syndrome, a major histocompatibility complex antigen HLADR5 was suggested responsible for pathogenesis.

Schiødt (1992) described the onset of salivary gland swelling as varying from early to late in HIV infection. This author found that patients with HIV-SGD and parotid swelling had significantly reduced saliva flow rate.

Schiødt (1992) also reported that there was increased albumin in saliva but the results were equivocal for other components such as Immunoglobulin A (IgA). The CD4/CD8 ratio was 0.5 (with Sjögrens Syndrome it is 3-8) and the serology markers for Sjögrens were usually absent. The etiology is unknown, and whether HIV, CMV or a herpes 6 type virus are implicated has yet to be determined.

2.2 Xerostomia

Few researchers have documented the presence of xerostomia as a specific problem in HIV infection. This may be due to the frequent use of polymedication in the management of HIV infection and the erroneous assumption that xerostomia occurs as a direct result of medication. Many of the medications prescribed for treatment of HIV and opportunistic infections cite xerostomia as a side effect, so the incidence of this condition resulting directly from viral infection, specifically HIV, tends to have been discounted or overlooked. Furthermore, there has been a tendency to discount xerostomia as a significant health problem in comparison to the other life-threatening or predictive opportunistic infections of HIV.

Ficarra (1992) is one of the few researchers to have placed emphasis on salivary gland disease in HIV infection. He cites xerostomia as an oral lesion of iatrogenic and undefined etiology, and claims it to be a *"fairly common oral symptom in HIV seropositive patients"*. The potential causes listed for HIV-associated xerostomia are Sicca syndrome (diffuse infiltrative CD8 lymphocytosis syndrome), salivary gland infection (CMV, bacteria, others), emotional distress, anaemia (Zidovudine), nutritional deficiencies, anticholinergic medication, radiation treatment (lymphoma, oral KS), HIV-related neuropathies (polyuria), and dehydration.

Barr (1994) mentioned that xerostomia has been reported in HIV infection and proposed a correlation with medications, or treatments such as radiation treatment for Kaposi's sarcoma.

Xerostomia was documented as a condition of unknown etiology by Greenspan *et al.* (1990). These authors reported that in a sample of HIV-infected patients from San Francisco, 10% had xerostomia and, in Copenhagen, xerostomia was observed in 5% of HIV-infected patients. An incidence of 10-13% was reported for patients with AIDS, but no distinctions were drawn between patients who might have had xerostomia from polymedication or as a result of HIV.

Schiødt (1992) found that in children with HIV infection the incidence of salivary gland disease varied from 0%-58%. HIV-SGD was often pronounced in children, with the parotid glands demonstrating marked swelling.

Laskaris *et al.* (1992) found a significant proportion of their subjects presented with xerostomia (42%) but this may have been based upon reported symptoms, *"that people with HIV infection frequently complain of a dry mouth"*, rather than a clinical assessment. On the other hand, frequent complaints of a dry mouth may be the best method of determining the actual prevalence of xerostomia in HIV infection rather than a clinical evaluation. Gillespie and Mariño (1993) reported results from a Peruvian study which indicated that xerostomia was a significant manifestation, being present in 80% of the group studied. In most of the countries

documented in their overview, xerostomia was not recorded at all, suggesting that the significance of xerostomia and its contribution to the development of oral manifestations is not being considered.

This bias in the documentation of xerostomia is again evident in a study of 44 patients in Britain. Porter *et al.* (1989) noted only one subject as having xerostomia and high caries. The attempt to correlate high caries as an indication of xerostomia ignores both the subjective and other objective measurements of xerostomia, such as measurement of salivary flow (Dawes 1987; Levine 1989 b,c; Levine 1993). Foltyn and Marriott (1996) highlighted xerostomia as being the most significant oral manifestation of HIV but, like the British study, these authors correlated the effects of dry mouth with rampant dental destruction. Bobek *et al.* (1993) are the only investigators who considered the potential wider ramifications of a dry mouth, suggesting xerostomia as a cause for an increase in oral infections such as candidiasis.

Symptoms of xerostomia associated with HIV infection also vary. There appear to be two distinct patterns in the presentation of HIV-related salivary gland disease. The salivary glands are either affected by benign lymphoepithelial lesions or cysts, or by a classic Sjögrens-like syndrome. In a review by Schiødt (1992), none of 84 patients with benign lymphoepithelial lesions or cysts presented with xerostomia, whereas 91% of patients with Sjögrens-like syndrome had a dry mouth.

One of the effects of the multiple medications taken for both primary therapy and for treatment of opportunistic infections is to establish multiple parameters that may affect salivary gland function and subsequently the health of oral structures.

Even though the immune system is advantaged by the advances in treatment of HIV infection, often the major side effects of the medication impacts on the mouth. This appears to be especially true of the protease inhibitors where patients complain of increased decay and sensitive teeth (Collier *et al.* 1996; Foltyn and Marroitt 1996).

2.3 Presence of HIV in saliva

2.3.1 Quantity and significance of HIV in saliva

Interest in the presence of HIV in saliva is intense for two reasons. Firstly, investigators wish to know if the disease is likely to be spread via oral transmission and, secondly, investigators are eager for simple diagnostic tests to be developed to allow for easy diagnosis of HIV infection.

Phillips *et al.* (1994) demonstrated that T-cell free HIV-1 RNA was present in a significant proportion of saliva samples collected from infected individuals, reporting isolation rates of 0-21%. However, HIV-1 proviral DNA has been recovered in up to 50% of samples, suggesting that either the infected cells do not produce infectious virus, or that replication is at a very low level.

There may be a correlation between plasma viral load and the presence of HIV in saliva, but the wide variation in results from these studies (as much as 100-fold) suggests that it was difficult to determine whether the virus came from the blood plasma, or whether it was produced by the epithelial and T-cells located in the mucosa (Phillips *et al.* 1994).

Saliva also contains anti-HIV antibody. It is found in a much lower concentration than in plasma but it is still sufficient for a diagnosis of HIV to be made. Hunt *et al.* (1993), Frerichs *et al.* (1994) and Lamey *et al.* (1996) all found that mixed unstimulated saliva (whole saliva) samples gave a reliable indication of infection, especially when a modification of the ELISA antibody assay (GACELISA) was used for analysis.

2.3.2 Inhibitory factors of HIV activity in saliva

Having found that little virus was present in the mouth, and that the virus present tended to be non-infectious, the attention of investigators turned to identification of putative antiviral factors existing in the oral cavity. Subsequently, inhibitory factors were identified in saliva. Robinovitch *et al.* (1993) found no correlation between total protein content and electrophoretic patterns and the presence or absence of anti-HIV activity. However, these authors did not use a filtration step in their study, and reported that their results are a conservative estimate of an anti HIV assay. A filtration step involves the removal of the mucinous components of saliva that are believed to entrap virus.

Moore *et al.* (1993) concluded that salivary factors render the virus non-infectious. Only one sample of unstimulated saliva collected from 21 HIV positive patients revealed the presence of infectious virus, and in this patient there were low or undetectable levels of salivary viral inhibitors. Non-infectious virus was also collected from an additional five patients, in which moderate to high levels of non-specific salivary viral inhibitors were found. These authors concluded that salivary mucin displays broad antiviral activity, including activity against HIV.

Archibald *et al.* (1993) suggested that complexes between whole saliva and the HIV glycoprotein, GP160, block monoclonal antibody binding. These authors believed this demonstrates that the loss of infectivity of HIV-1 on exposure to whole saliva is not due to proteolytic degradation. It was suggested that complexes of negatively charged salivary mucins could interact with the positive charged areas of the HIV-1 envelope protein, which was thought to be critical in inhibiting viral replication. Bergey *et al.* (1993) found that HIV particles were entrapped by precipitated salivary components, salivary proteins and glycoproteins. These not only included the mucins but also lysozyme, peroxidase, IgA, amylase, statherin, and detectable amounts of the proline-rich proteins.

Malamud and Friedman (1993) reviewed the literature on the presence of HIV and inhibitory factors in saliva, and reported a study by Ho (1985) in which HIV was cultured from blood samples from 28/83 seropositive individuals, but could only be cultured from one saliva sample from the same patients. There was inadequate

information to determine if this one sample was blood or cell contaminated. Overall, the viral titre was much lower in the saliva samples. This was supported by Yeung *et al.* (1993) who found that the level of HIV in saliva was low, even in subjects with severe periodontal disease.

It is possible that some antibacterial functions of saliva also act antivirally, especially against enveloped viruses. Malamud and Friedman (1993) found that submandibular saliva possessed the greatest anti HIV activity, and that prefiltration of saliva tends to reduce most of the inhibition against HIV. These authors concluded that:

- the presence of infectious virus in the oral cavity is an uncommon event
- Polymerase chain reaction (PCR) data indicate that HIV sequences may be found in the oral cavity at reasonably high frequency
- saliva appears to contain potent anti-HIV activity that may be responsible for the low oral virus titre
- oral secretions are a reliable source for monitoring anti-HIV antibodies.

Malamud *et al.* (1997) further investigated the antiviral properties of saliva, and found that the potent antiviral efficacy is directed against HIV-1, in particular the laboratory isolates of the virus. In contrast, the antiviral activity is not directed against adenovirus, simian virus or HIV-2. There is a moderate antiviral effect against Herpes simplex virus (HSV). These investigators also concluded that the anti HIV-1 properties of saliva are directed towards the virus itself. There is limited inhibitory activity against infected cells, and human cells incubated with saliva

actually become more readily infected with HIV-1 than non-incubated cells. When the virus itself was incubated with saliva, its potential for infecting cells was dramatically reduced. These authors were unable to determine what factor/s in saliva were responsible for the marked HIV-1 inhibition of infectivity.

Wahl *et al.* (1997) suggested that a particular component of saliva may be responsible for HIV inhibition. They isolated a 12kD endogenous protein which is a secretory leukocyte protease inhibitor (SLPI) that blocks HIV 1 infection *in vitro.* These authors also proposed that the increased risk of oral transmission of HIV in infants and children may be related to the lack of fully developed salivary glands. Shugars, Sauls and Weinberg (1997) demonstrated the ability of SLPI to inhibit infection of both primary monocytes and mononuclear cells by HIV-1.

2.4 Sialochemistry in HIV infection

Rudney (1995) concluded that the recently developed interest in the role of saliva in the health of HIV infected patients appears to have arisen because of the absence or low levels of HIV in saliva. Increased oral infections during HIV infection also indicated a role for saliva in controlling the oral ecology. Fox (1992) also deduced that, because saliva is essential in maintaining oral health, changes in the oral cavity found in HIV infection indicate that salivary performance may be impaired.

Fox (1992) also outlined the possible changes in salivary secretions as a result of HIV infection. Composition was altered, with chloride and lysozyme elevated, and greater levels of total protein, lactoferrin and IgA. The levels of lysozyme and histatins were higher if yeast hyphae were present. These changes suggested that the salivary glands were affected by chronic inflammation and responses to HIV infection. Fox (1992) stated that up to 10% of patients complained of xerostomia.

However, these were subjective results as flow rates had not been measured. This author was also concerned that the numbers and types of medication had not been accounted for in dry mouth assessment in these studies, and believed that many of the medications taken by subjects with HIV infection could impact upon salivary flow or the perception of salivary flow.

A study by Yeh *et al.* (1988) included measurements of salivary flow rates which were significantly reduced in stimulated secretions but not unstimulated secretions, compared to non-infected control subjects. The HIV subjects were not taking medication. Results from the studies reviewed by Fox (1992) indicated that decreases in flow rate were predominantly associated with submandibular and sublingual secretions, but not with parotid secretions.

Schiødt *et al.* (1992) studied parotid saliva in patients with HIV-related salivary gland disease. In this study, there was decreased salivary protein, increased salivary IgA, lysozyme and albumin, and no changes in the electrolytes, sodium,

chlorine and potassium. These results differed from the studies reviewed by Fox (1992), possibly because whole saliva was examined rather than a single gland secretion.

Other investigators have studied saliva changes in HIV-1 infection and measured salivary flow rates. Barr *et al.* (1992) undertook a longitudinal study of 102 HIV-infected individuals and examined oral lesions in relation to T-cell counts, P24 antigen and salivary flow rates. The flow rates for whole saliva in the presence of oral candidiasis and oral hairy leukoplakia were higher than those in subjects without these manifestations. The authors did not indicate if flow rates were different between the 102 HIV-infected subjects and the 33 non-infected control subjects. The presence of candidiasis was confirmed by Sabouraud culture and KOH smear. Barr *et al.* (1992) concluded that the significance of oral lesions and comparisons between studies would be far more accurate if immunological and viral markers, such as P24 antigen and CD4 cell counts, were recorded, and also if saliva flow rates were assessed.

Mandel, Barr and Turgeon (1992) examined the parotid salivary flow rate and saliva chemistry in 78 HIV-infected men. In a subgroup of these subjects, a low salivary flow rate was found, associated with the presence of oral candidiasis. For the subjects overall, the flow rate was within normal parameters and subjects secreted normal or elevated proteins. However, a subgroup of these subjects were monitored over time and showed reduced flow and reduced histatins.

Challacombe and Sweet (1997) examined the mucosal and salivary immune responses to HIV and found that, in early HIV infection, the levels of salivary IgA antibodies against *Candida albicans* are elevated in contrast to those of subjects without HIV infection. These levels only fell below normal values in late stage HIV infection or AIDS. These authors concluded that patients with HIV infection appeared to secrete unimpaired specific antibody responses to infection and that these were only affected in late HIV infection.

Whole saliva contains blood, serum, and cells, as well as the saliva from major and minor salivary glands. Whereas HIV has been recovered from only one pure salivary secretion, antibodies are commonly found in saliva, and testing for seropositivity using saliva HIV antibodies has been proposed as an easy, non-invasive method of screening (Fox 1992; Wisnom *et al.* 1997).

3 SALIVA

Introduction

Saliva has many important functions in maintaining oral and systemic health. It protects the body, in particular the mouth, against numerous pathogenic organisms and chemical toxins (Tenovuo 1989). The presence of disease or altered physiology in the human body can lead to changes in the flow and composition of saliva which may impact upon the ability of saliva to perform its protective functions.

The following review outlines the basic physiology of saliva, collection of saliva samples and methods of saliva analysis.

3.1 Composition of Saliva

Saliva is a term that refers to the combined mucosal fluids found in the mouth that wet the body surface (Schenkels, Veerman and Amerongen 1995). Strictly, it should refer to the combined mucous and serous secretions from the major and minor salivary glands. Water constitutes 99% of saliva and the remainder is composed of organic and inorganic components (Mandel 1987; Tenovuo 1989). Whole saliva is a mixture of salivary gland secretions, both major and minor, and also contains dead cells, blood cells, organisms such as bacteria and fungi, and crevicular fluid from the teeth (Söderling 1989).

Saliva functions as a 'pleasant lubricant' that assists speech, swallowing and mastication and provides physical and chemical protection for the human body.

Shenkels *et al.* (1995) summarized the functions of saliva as follows:

Protective functions	Tissue coating (mucosal and tooth pellicle)
	Lubrication
	Humidification
	Remineralization of the teeth
Host defence functions	Immunological activity
	Anti-bacterial activity
	Anti-viral activity
	Antifungal activity
Digestion	Digestive enzymes
	Bolus formation
	Taste

Levine (1993) explained the importance of the various functions of saliva. These include flushing of the oral cavity by physical and chemical means, tissue coating both for lubrication and as a permeable barrier, modulation of oral flora, antacid and neutralization functions, regulation of calcium/phosphate equilibrium, digestion and extracellular post-translational processing of salivary molecules.

The ability of saliva to perform all of these functions is due to the complex nature of the secretions forming saliva in both physiological and pathological circumstances.

Considerable intra-individual and inter-individual variation exists in the composition of normal saliva, and there is a broad range of physical and chemical values that encompass normal flow and consistency of saliva (Dawes 1987). The variation in saliva composition mainly arises from changes in protein content and/or in changes in electrolyte balance.

The organic component of saliva consists of numerous proteins, enzymes, free amino acids, urea, uric acid, glucose and lipids. The inorganic component of saliva consists of major and minor inorganic ions (Levine 1989b; Slomiany, Slomiany and Mandel 1989; Levine 1993).

3.2 Salivary Proteins

Proteins such as mucins, acidic proline-rich proteins (aPRPs), alpha-amylase, basic proline-rich proteins (bPRPs), basic PRG, secretory immunoglobulin A (IgA), cystatins, and statherin are all found in significant quantities in saliva. Other proteins, such as immunoglobulin G (IgG), EP-GP, VEGh, histatins, lysozyme, kallikrein, lactoferrin, lactoperoxidase, haptocorrin, ß-Microseminoprotein, immunoglobulin M (IgM), albumin and Zn-α2 glycoprotein are found in lesser amounts.

3.2.1 Proline rich proteins (PRPs)

Proline rich proteins (PRPs) make up 70% of the total protein in human saliva. PRPs are major constituents of both parotid and submandibular salivary gland secretions and have been widely investigated. The concentration of PRPs ranges from 19-80 mg% in human parotid saliva (Carlson 1993).

PRPs are a heterogeneous group of proteins which can be classified into three groups: acidic (mw 16kD), basic (6-9kD) and glycosylated (36kD). More than 20 PRPs have been described in human saliva. Acidic as well as some basic PRPs are phosphorylated (Bennick 1987). Acidic PRPs have been found in salivary secretions but not in other mucosal secretions (Shenkels *et al.* 1995).

PRPs show complex electrophoretic patterns that demonstrate polymorphism of PRPs between individuals. For example, acidic PRP phenotypes may show a double electrophoretic band (Azen 1993).

3.2.2 Mucoproteins

Mucins are highly glycosylated proteins, providing much of the viscosity of saliva, and forming the main component of submandibular and sublingual secretions, thus explaining the higher viscosity of these secretions compared with those of the parotid gland. Saliva has two types of mucous glycoproteins (MG), MG2(150-200kD) and MG1 (>1000kD).

As well as these major organic components there are enzymes, such as lysozyme and peroxidases, present in saliva. Again, the variation between the secretions of various glands is pronounced, with parotid saliva containing large amounts of amylase and lesser amounts of lysozyme. Submandibular secretions consist predominantly of cystatins and lysozyme but have less amylase (Cohen and Levine 1989; Shenkel *et al.* 1995).

3.2.3. Histidine-rich proteins

Another major group of salivary proteins are the histidine-rich proteins (histatins). Oppenheim *et al.* (1986, 1988) studied the histidine-rich proteins and found that, like the PRPs, both neutral and basic forms may complex with each other in saliva. These investigators found that histatins 1, 3, and 5 were a family of histidine-rich proteins in human parotid secretions.

3.3 Function of salivary proteins

Proteins form numerous functions in saliva many of which are yet to be defined (Shenkels *et al.* 1995). For example, Tenovuo *et al.* (1987) and Scannapieco (1994) found there was little or no correlation between the antimicrobial properties of individual salivary proteins and oral health. These authors suggested that synergistic activity between classes of proteins as well as other salivary components such as electrolytes may be the mechanism whereby saliva regulates oral health and ensures microflora levels are regulated.

Furthermore, the roles of salivary proteins in pellicle formation (Shenkels *et al.* 1995), calcium regulation, tissue hydration, and protection and control of the oral microflora, have all been investigated (Levine *et al.* 1987; Johnson *et al.* 1995).

The only proteins that are specific for saliva in comparison to other mucosal fluids are the histatins and acidic proline-rich proteins. It is thought that these proteins have predominant functions specific to the oral cavity (Shenkel *et al.* 1995). PRPs must play an important role in regulating the oral ecology, both microbial and chemical, since they constitute such a large portion of the organic content of saliva (Shenkel *et al.* 1995). It is known that they contribute to the formation of the pellicle, enhance mastication, and may contribute to the protective function of lubrication of tissues and teeth in combination with other proteins such as mucins (Mandel 1987). PRPs also help to maintain high calcium concentrations in the saliva, in particular the calcium phosphate supersaturation, thereby protecting and maintaining the mineralised state of the tooth surface (Mandel 1987; Scannapieco 1994; Johnson *et al.* 1995; Shenkels *et al.* 1995).

Acidic PRPs are known to inhibit apatitic crystal growth which assists in the maintenance of tooth surface uniformity yet allows for remineralisation by free calcium ions. Belford *et al.* (1984) and Hay and Moreno (1989) concluded that PRPs had a high reactivity for hydroxyapatite and enamel, inhibited crystal growth of calcium phosphate salts, and bound calcium ions. These authors concluded that the major anionic PRPs were responsible for these phenomena, thereby

providing protection of the tooth substance while inhibiting calcium salt formation within the salivary glands. These properties could also explain how PRPs neutralize toxic substances in the diet.

There has been considerable interest in the antibacterial activity of PRPs. Azen (1993) postulated that it was the polymorphism of PRPs which might account for bacterial agglutination and hydroxyapatite affinity, and that this would explain the mechanism for protection of enamel surface, remineralisation, and antimicrobial activity. Shenkels *et al.* (1995) hypothesized that glycosylated basic PRPs functioned as lubricants and interacted with several different types of microorganisms. Further evidence for the protective function of PRPs is gained from dietary protein studies on rats (Johnson *et al.* 1995). Solid low protein diets resulted in a significant increase in the basic proline rich protein (SP-3) in rats, whereas amylase and acidic PRPs did not vary according to dietary intake. Moderate protein deficiency altered salivary protein and, although whole saliva showed no change in volume of secretion, the protein concentration as well as the bacterial agglutinating activity were reduced.

Interestingly, secretory granules have been shown by immunocytological investigations to consist of, or contain, proline rich proteins, although their exact role has not been determined (Tandler 1987).

Apart from these specific functions, other functions of PRPs are unknown. They are considered to be complex, and may act in co-ordination with other organic components such as enzymes and mucins to augment their function. For example, Levine (1993) discussed the multifunctional nature of salivary molecules and drew attention to the fact that PRPs are responsible not only for mineralisation, but also for tissue coating. PRPs may also be involved in the bitter taste sensation (Mandel 1987; Tenovuo 1989).

The functions of other salivary proteins, histatins, statherin, the mucins and salivary enzymes such as the peroxidases, amylaze and lysozyme have all been investigated. Levine *et al.* (1987) explained that salivary molecules such as the mucins or histatins may have either a protective or detrimental effect against oral microorganisms, and De Jong and van der Hoeven (1987) found that oral streptococci could be grown continuously in human saliva and could degrade the salivary glycoproteins needed for their growth.

Several authors have studied the antimicrobial activities of saliva. Oppenheim *et al.* (1986, 1988) examined the antifungal activity of salivary histatins 1,3, and 5. All three histatins exhibited the ability to kill the pathogenic yeast *Candida albicans*. Saitoh and Isemura (1993) showed that histatin 5 is a salivary antimicrobial protein and also a salivary proteinase inhibitor. Levine (1993) explained that histatins were responsible almost solely for the antifungal activity of saliva.

Other salivary proteins such as IgA, IgM, IgG, cystatin and amylase are also strongly implicated in host defence functions. Immunoglobulins in saliva act in conjunction with the complement system. IgA has been shown to have an antiviral effect, as discovered with the polio vaccine (Levine 1989a). IgG is found in the crevicular fluid of teeth, and seeps into the saliva. It has been difficult to determine exactly what role immunoglobulins play in oral health or to verify their efficacy.

Levine (1993) pointed out that cystatins and mucins both have an antiviral activity as well as an antibacterial activity. They are aided in the latter by amylases, histatins and peroxidases. Some cystatins have been demonstrated to bind to Herpes simplex 1 and to block the replication of that virus. Robinovitch, Iversen and Resnick (1993) postulated that the functional significance of salivary proteinase inhibitors may explain the HIV inhibition found in human saliva.

The viscous nature of saliva depends on the presence of the mucins which function as lubricants and have roles in cytoprotection, protection against dehydration, and maintenance of viscoelasticity in secretions. Scannapieco (1994) found that mucin, specifically MG1, adheres to the tooth surface, thereby limiting attack by acid. Mucins also coat and adhere to mucosal surfaces, and, being resistant to proteolysis, they form an effective barrier against bacterial proteases which have the potential to ulcerate mucosa (Levine *et al.* 1987).

Shenkels *et al.* (1995) stated that "Mucins promote the clearance of some bacteria by masking their surface adhesins". This could explain why Johnson *et al.* (1995) found that the lack of mucins in patients with xerostomia contributed to a shift in oral flora to aciduric organisms such as lactobacilli and yeasts.

The physical protective functions of mucins occur either solely or in combination with other components. Mandel (1987) and Levine (1993) stated that both cystatins and histatins assisted PRPs in mineralisation and cystatins and mucins also assisted PRPs in tissue coating.

3.4 Salivary Lipids

Saliva contains neutral lipids, glycolipids and phospholipids, and there are between 8-10 mg of lipids per 100 ml of saliva. Interestingly, people with heavy concentrations of lipids in their saliva form significant deposits of calculus on their teeth. Lipids facilitate the penetration of oral mucosa by lipophilic substances and may alter calcium/protein interaction. They may also influence caries resistance (Slomiany, Slomiany and Mandel 1989).

3.5 The inorganic components of saliva

There are major and minor inorganic ions in saliva. The important ions are Na^+, K^+, Cl^-, HCO_3^-, Ca^{++}, Mg^{++}, and HPO_4^-, and the minor ions include I^-, SCN^-, and F^- (Levine 1989c; Levine 1993).

Salivary electrolytes function to maintain the pH of the oral cavity and also buffer both the acids formed in plaque and those which are ingested. The minerals are important in maintaining tooth structure (Johnson *et al.* 1995). The concentration of electrolytes is affected by the flow of saliva. Some electrolytes will actually decrease if the flow rate increases, and low flow rates might contribute to low bicarbonate levels (Ferguson 1989).

The carbonic acid/bicarbonate system is the most important buffer system of saliva, as salivary pH is regulated by the ratio between carbonic acid and bicarbonate. The partial pressure of carbon dioxide in saliva is generally constant

and independent of secretion rate. Salivary pH, therefore, varies with different levels of the bicarbonate concentration of saliva (Birkhed and Heintze 1989). The bicarbonate ion (HCO_3^-) provides about 85% of the buffering capacity in stimulated saliva whereas the concentration of HCO_3^- in resting saliva is low where other buffering systems, such as the phosphate system or the protein buffering system, predominate (Levine 1989c).

The importance of the electrolytes in protecting the tooth structure was demonstrated by Vehkalahti, Nikula-Sarakorpi and Paunio (1996) who studied the effect of buffering capacity on the incidence of caries and found a correlation between the presence of caries and poor buffering capacity.

Calcium is an important electrolyte in saliva as, together with phosphate ions, it is responsible for the mineralised state of the tooth (Johnson *et al.* 1995).

Approximately one half of the calcium in saliva occurs in an ionic state that is altered by pH, and the rest is complexed with proteins and other electrolytes (Söderling 1989). Ferguson (1989) explained that the parotid gland has a mean calcium concentration which amounts to two thirds that of the other glands.

Therefore, the concentration of total calcium of resting saliva will be greater than that of stimulated saliva because of the diminishing contribution of the submandibular glands to the total saliva during stimulation.

The following table provides the dynamics of calcium levels, in milli-mols (mM), for all glands and for flow rates (Ferguson 1989).

Calcium	US		STIM		effect on flow
	Mean±S.D	Range	Mean±S.D	Range	
Whole	1.35±0.45	0.5- 2.8	1.7 ± 1.0	0.2- 4.7	decreases
Parotid	1.05±0.35	0.5- 2.1	1.6±0.8	0.2- 2.7	increases
Subm	1.56±0.45	0.5 - 5.0	2.4±0.6	0.7-3.7	decreases
Subl			2.1±0.4	1.7-2.95	increases

Blood calcium levels are almost double that of salivary secretions, ranging from 2 to 2.5 mM (Menaker 1980).

Calcium, sodium and chloride concentrations have been noted to be lower in older subjects. The significance of the role played by salivary calcium and other electrolytes in oral health is not fully understood. For example, no correlation has been noted between variations in calcium levels and caries incidence although Ferguson (1989) indicated that phosphate concentrations were significantly higher and calcium concentrations slightly higher in caries free subjects.

Levine (1989b) described how saliva can provide calcium and phosphate ions to plaque thereby preventing demineralisation, and Scannapieco (1994) outlined the possible role that calcium might play in the formation of the pellicle, and these hypothesised roles for calcium are supported by results from a study by Milesovic and Dawson (1996). In their research into toothwear in bulimics, these authors found that there was more calcium in the enamel in the non-tooth wear group of bulimics (determined from enamel biopsies) providing the pellicle was left in situ. The research by Milesovic and Dawson (1996) emphasised the complex dynamic relationship between saliva, enamel mineralisation and the tooth pellicle.

3.6 SALIVA AND ORAL HEALTH

The mouth is often the presenting site for pathogens and toxic agents in the body and saliva plays an integral role in their removal, processing and nullification. Saliva has immunological and non-immunological roles in maintaining oral and systemic health.

Rudney (1995) stated that specific changes in saliva might produce alterations in oral ecology under circumstances less drastic than major loss of salivary gland function. Thus, although an individual might register on the low side of normal salivary flow for a population, for that individual such a shift from a higher value represents a significant change for their indigenous flora and other host factors. Oral disease may then occur. Bobek, Tsai and Levine (1993) supported this concept, pointing out that saliva components have many protective functions that

are crucial for oral health, and that the antimicrobial activities are significant in the human 'non-immune defence system'.

Levine (1993) further developed the relationship between disease and saliva quality. He stated that the first concept to be established is that the conformation or shape of the salivary molecule is 'critical for its biological function' and that, secondly, most salivary molecules are multifunctional. Furthermore, the functions of the molecules overlap, therefore suggesting a variance in how individuals are affected by changes in salivary flow and consistency.

3.6.1 Saliva and microorganisms

There are four main types of salivary protein-microorganism interaction;

- aggregation
- adherence
- inhibition
- cell killing

In addition, it is likely that nutrition and salivary proteins that inhibit growth of microorganisms *in vitro* perform that function *in vivo* (Scannapieco 1994).

Numerous authors have studied the oral microbial ecology in subjects with either impaired salivary flow or quality, or existing oral disease. Rudney (1995) examined subjects with Sjögren's syndrome or radiation-induced salivary gland change. These subjects showed increased levels of *Streptococcus mutans* and other acidogenic and aciduric species of organisms. Salivary protein levels also

changed in these groups, with sIgA, lactoferrin and salivary peroxidase being increased. Even in fluoride treated patients, caries risk is elevated if there is a salivary flow rate of less than 0.1ml/min (Rudney 1995).

In a study of patients with epilepsy, Henskens *et al.* (1996) found that pH and flow rate of both stimulated and unstimulated saliva were not affected by epileptic drugs, and that the total salivary protein tended to be enhanced, perhaps due to an increase in amylase.

Milosevic and Dawson (1996) examined the impact of saliva on disease processes. These investigators studied the role of salivary factors as aetiological agents for pathological tooth wear in a group of vomiting bulimics. They found the stimulated salivary flow rate to be less in the tooth wear group and the bicarbonate concentration higher. The bulimic subjects had reduced flow in comparison to the healthy subjects.

Oppenheim *et al.* (1988) found that one neutral histidine-rich polypeptide isolated from parotid saliva was a potent inhibitor of *Candida albicans* germination *in vitro.* Consequently, in xerostomia, lack of such inhibitors may precipitate oral infections such as candidiasis.

As one function of saliva is to maintain high calcium concentrations in order to protect and maintain the mineralised state of the tooth surface (Johnson *et al.* 1995), a number of investigators have studied the impact of saliva and oral

ecology in relation to the development of dental caries. Mäkinen *et al.* (1996) studied the effects of saliva and plaque on the incidence of dental caries, and concluded that little correlation could be established, possibly because of the difficulties engendered by clinical trials and saliva collection.

Previously Tenovuo *et al.* (1987) had reported a similar conclusion, stating that correlation between salivary parameters and the development of caries might be unfeasible because caries is generally a "long term disease experience". Mandel (1993), however, felt that saliva does have value as a diagnostic tool in determining caries risk, a conclusion supported by studies by Vehkalahti, Nikula-Sarakorpi and Paunio (1996) who examined saliva in a group of caries susceptible teenagers. The latter group found that low flow rates and low buffering capacity were indicators of caries experience.

As well as changes to the microflora, there are physical changes created when saliva quality and quantity are altered. For example, there are decreases in the mechanical cleansing ability of saliva, and in buffering capacity, when flow is reduced. Wright (1987) cited oral changes ranging from rough scaly cracked lips and angular cheilosis to intraoral redness, candida and traumatized mucosa, when saliva volume is reduced. Wright also reported that tooth surfaces may be normal or decalcified in such circumstances.

Dawes (1987) posed a model for dry mouth symptoms. This author suggested that there was a residual volume of saliva left in the mouth after swallowing, ('*RESID*'),

and that swallowing was not induced until a maximum volume was attained. The mouth should always therefore have a saliva volume somewhere between '*RESID*' and '*Maximum*'. Dawes (1987) suggested that dry mouth occurred when the salivary flow rate was less than the sum of water absorption and evaporation, and that the water lost through air passing through the oral cavity was more significant than the absorption of saliva by the oral mucosa.

Electrolytes may also be affected by changes in saliva caused by disease. Ferguson (1987) examined the diagnostic uses of saliva and found that saliva is increasingly being used in diagnosis and assessment of disease. For example, calcium and protein levels are raised in sialadenitis and radiation damage. Ferguson (1989) also noticed increases in calcium levels in saliva in digoxin stimulated patients and in patients with cystic fibrosis.

3.7 Methods of collection and analysis of saliva

3.7.1 Collection

Standardisation

Whole saliva is a mixture of salivary gland secretions, from both major and minor glands, and it also contains dead cells, blood cells, organisms such as bacteria and fungi, and crevicular fluid from the teeth. The proportion of salivary fluid from different glands depends on the collection method. For example, stimulated saliva will increase the parotid gland contribution. To obtain meaningful results, the collection of samples needs to be standardised (Söderling 1989).

Reference values established by investigators are useful as a guide but their accuracy is limited because of the difficulties encompassed in standardization of samples. The total number of parameters, the amount collected, the type of saliva, the storage of saliva, and any pre-treatment of samples need to be determined for clear results.

Some of the factors influencing the flow rate and composition are the donor, glandular source, plasma condition and circadian rhythm. The nature of the stimulus exercise and physical and chemical collection circumstances also affect the sample. Of paramount importance, acute and chronic diseases and medication also affect the saliva sample.

The choice of saliva (specific glandular or whole saliva) used for a study determines the parameters for standardisation. Rudney (1995) found that whole saliva was relatively easy to collect. There was no direct evidence as to whether salivary protein concentrations in whole or specific glandular salivas were more relevant to the presence of oral disease, but the choice of saliva to be studied will affect the results of any study.

Apart from the glandular type of saliva collected, composition and flow rates of saliva will vary according to whether the samples are stimulated or unstimulated saliva. Ferguson (1987) found the use of unstimulated saliva is preferred by clinicians for investigations and may be less susceptible to physiological variation than specific gland or stimulated samples. Rudney (1995) found that one

individual will tend to secrete similar levels of proteins in either stimulated or unstimulated saliva, but, as unstimulated saliva is present in the oral cavity for most periods, it might be considered to be more relevant to any salivary protein interaction with microbes.

Söderling (1989) outlined the following instructions for standardisation that were supported by other investigators (Mandel 1987; Oppenheim *et al.* 1988; Levine 1989c; Rudney 1995).

- The subject should not eat or drink (except water) for 1-2 h before collection.
- The subject should not undertake heavy physical exercise before collection.
- Saliva should be collected at the same time of the day from each subject.
- The saliva collection should be performed under standardized environmental conditions.
- A prestimulation period (e.g. 30 secs) is recommended.
- A fixed collection time (e.g. 5 min) should be used, or a fixed volume should be collected.
- In repeated collections, the same stimulant should be used.
- All acute or chronic diseases should be considered.
- Samples containing visible blood should be discarded.
- The choice of saliva to be sampled must be kept uniform.

Stimulation

There were two methods of stimulating salivary flow for collection, mechanical stimulation or chemical stimulation. The type of stimulation used depended on the parameters being evaluated and whether whole or glandular saliva was required. A set time of stimulation or a specified volume of stimulated saliva is necessary for collection of stimulated samples in order to achieve consistency in results (Rudney 1995).

Mechanical stimulation

An inert material such as paraffin was generally used for mechanical stimulation of saliva. Chewing the paraffin for a set time, usually five minutes, was one method of stimulation although some investigators preferred a set volume to be sampled (Vehkalahti, Nikula-Sarakorpi and Paunio 1996). Dawes (1987) stated that the subject should chew on a standard weight of material at a constant rate, about 70 chews /min. Paraffin is a solid oil or a carbohydrate (Berg and Tjell 1969) that has been considered biologically neutral and should therefore be a suitable salivary stimulant for most studies. Nevertheless, Berg and Tjell (1969) found that chewing paraffin as a salivary stimulant resulted in the adsorption of mucins. Accordingly, if mucins are the subject of the study, then a different salivary stimulant needs to be considered. Henskens *et al.* (1996) used paraffin for salivary stimulation in their study of a group of epileptic patients, and their results reflected the neutral quality of paraffin as a stimulant.

Other investigators have used alternative stimulants. Barr *et al.* (1992) used an elastic band to stimulate whole flow in their study of HIV-infected individuals, whereas Mäkinen *et al.* (1996) and Milosevic and Dawson (1996) used sugar-free chewing gum as a saliva stimulant. The latter investigators examined the properties of whole saliva when different chewing gums were consumed and their subjects maintained a chewing frequency of between 60 to70 strokes/ minute.

Chemical stimulation

Candies and citric acid can be used to provide both mechanical and gustatory stimulation, the latter being especially useful for the collection of specific glandular saliva. Rudney (1995) and Archibald *et al.* (1993) used lemon candy for the collection of stimulated salivas for testing the effect on HIV proteins, and McCartan, Lamey and Wallace (1996) used 1 ml of 10% citric acid as a stimulant in their study on the evaluation of the levels of salivary cortisol. Barr *et al.* (1992), in a study of HIV-infected individuals, also used citric acid for parotid stimulation. Nedefors and Dahlöf (1996) used citric acid as a stimulant for collecting parotid and submandibular saliva, but at a concentration of 3%.

Other chemicals used for salivary stimulation include pilocarpine, a systemic drug, which is a potent salivary stimulant but also has potential side effects such as sweating, palpitations and shortness of breath (Johnson *et al.* 1995).

Flow rate / Volume

Variation in flow rate is considerable given a constant stimulus and saliva source. According to Rudney (1995), flow rates for different salivary glands and for collections taken using different stimuli are not comparable. Flow rate was determined by collecting salivary samples under constant conditions for a specified period of time. Milosevic and Dawson (1996) collected saliva using chewing gum stimulation for a total of 9 minutes with 3 x 3 minute collections. These authors measured the initial 3 minute flow rate and the final 9 minute flow rate.

Dawes (1987) and Levine *et al.* (1993) summarized four methods for collection.

(1) the subject bent their head forward and allowed saliva to drip off the lower lip into a graduated cylinder and/or a preweighed container.
(2) the subject spat out every 60 secs.
(3) the saliva was sucked from the floor of the mouth with a suction tube.
(4) preweighed absorbent pads were used.

Unstimulated saliva should be taken from subjects at least one hour after they have eaten, otherwise studies of unstimulated saliva were not comparable with each other (Rudney 1995).

When there is a reduction in salivary flow then the flow rate of whole saliva rather than specific glandular saliva should be measured (Dawes 1987).

Variation within studies

Many investigators found that results of salivary analyses showed considerable variation. Rudney (1995) demonstrated that circadian rhythm affected the concentration of proteins in samples. If samples were collected at a set time of the day, higher correlation was achieved but this was not always possible with human subjects.

Age had little bearing on protein concentration, especially proline rich proteins, or on flow rate of healthy nonmedicated subjects (Rudney 1995).

Medication will alter salivary flow and composition, and medicated subjects showed significant alterations in protein concentration and flow rate (Rudney 1995).

Although few studies have examined the impact of disease on saliva, Schiødt *et al.* (1992) examined a group of subjects with HIV infection, and found that salivary gland function may be altered in systemic disease.

All of the above factors (except for age) have been shown to cause a variability in results which was likely to be extensive, even in well-controlled studies. To balance the effects of variation, Rudney (1995) suggested that the sample of subjects being tested needed to be of suitable size.

Calcium

Calcium analysis of salivary samples can be determined from stored frozen samples. For measurement of ionic calcium, the samples are taken and stored anaerobically. If the samples are stored away from environmental contact then no treatment of the samples is required. Special collection conditions are required for determining ionic calcium, although atomic absorption spectrophotometry is accurate for the determination of total calcium in whole saliva. Large volumes of saliva are required for conventional atomic absorption spectrophotometry.

Whereas the concentration of calcium in samples can be affected by pH, salivary proteins and the presence of magnesium or hydrogen ions do not appear to cause significant variation in calcium analyses (Söderling 1989; Jun, Lima, and Montenegro 1994).

Samples of saliva are usually stored as frozen aliquots at - 20°C. Precipitation of mucins was considered to be a problem with storage of saliva samples, but other components were generally unaffected (Henskens *et al.* 1996; McCartan *et al.* 1996).

3.7.2 Saliva Analysis

Proteins

The use of ELISA is becoming the preferred method for assay of salivary proteins (Tenovuo *et al.* 1987; Tenovuo 1989; Rudney 1995). These investigators cited problems with accurate quantitation of submandibular/sublingual proteins, and with whole saliva because of protein complexes.

Several studies have used gel electrophoresis for salivary protein analysis. O'Connell, Pearson and Bowen (1994) examined saliva collected from rats, using sodium-dodecyl-sulfate polyacrylamide gel electrophoresis to separate the salivary proteins (12% gels for parotid saliva samples and 10% gels for submandibular samples). The gels had ten lanes and each lane was loaded with 25µg protein. Gels were stained with Coomassie Brilliant blue R250 and the major protein components were identified by comparison with previously published studies.

Oppenheim *et al.* (1986) used sodium dodecylsulfate polyacrylamide gel electrophoresis. The authors employed 10 or 15% gels in identifying PRPs. The gels were stained with Coomassie Brilliant blue R250, destained and treated with ENHANCE, dried, and placed on Kodak XAR film at -70°C.

pH and Buffering capacity

A stimulated salivary pH of <5 was considered low and >7 as normal or high for whole saliva and resting pH varied between 6.7 and 7.4 for whole saliva (Levine 1989c). Vehkalahti *et al.* (1996) tested saliva for buffering capacity using a commercial colorimetric strip test, Dentobuff (Orion Diagnostica, Espoo, Finland).

Calcium

The use of the atomic absorption spectrophotometer for calcium analyses originated in the 1950's when urine, blood and plasma calcium were evaluated in a flame spectrometer. Until 1952, atomic spectra had been evaluated by emission spectrometry. The physicist, Alan Walsh, subsequently developed the original atomic absorption spectrophotometer. This involved the principle of light passing through atomic vapour that was absorbed by the elements and occurred independently of temperature range. The system was significantly more sensitive than emission spectrometry (Willis 1993).

The samples needed to be pretreated with solutions such as EDTA to remove protein in order to obtain accurate measurements of calcium levels. The spectrophotometers used for these analyses were double beam systems.

The system was further developed by Jun *et al.* (1994) using a sequential flow injection manifold involving tubular electrodes. These investigators measured both ionic and total calcium on the same equipment without any treatment of the saliva samples. The ionic calcium was recorded with a digital potentiometer and the total calcium was measured with an atomic absorption spectrophotometer.

Calcium levels fall in whole saliva as flow rate increases. However there may be variations in results, and Coroneo *et al.* (1981) found that parotid total calcium will increase with increasing flow rate, whereas in submandibular saliva the total calcium will remain relatively constant. Therefore, it is possible that a stimulated sample will provide a greater calcium reading than a non-stimulated sample.

4 CONCLUSIONS AND RATIONALE FOR THE PRESENT STUDY

From the above review, it is apparent that there are real and significant oral problems associated with the epidemics caused by the Human Immunodeficiency Virus. Oral opportunistic infections found in HIV infection are complex with a multi-factorial aetiology. The combination of a deficient immune syndrome and some rare opportunistic pathogens leads to the conclusion that social, sexual and environmental factors appear to be contributory in symptomatic infections. To blame the immune system alone for oral manifestations could be misguided, and factors such as absence of saliva, smoking and medications must be taken into account. This was emphasized by the conflicting disease pattern noted in periodontal infections. The question needs to be addressed is why these bacterial infections occur when the immune system (as indicated by immune markers) is relatively intact.

Although many investigators have suggested a role for xerostomia in the aetiology of oral manifestations of HIV infection, little research has been done on sialochemistry in HIV infection. From previous studies, it has become apparent that quality of life is affected by oral problems for those people with HIV infection, who have generally found dry mouth a problem of significance. Poly-pharmacy will result in oral morbidity and the importance of analyzing the changes in saliva in HIV and in managing the effects of those changes is becoming increasingly urgent.

There are many areas in the field of sialochemistry that remain to be studied. The roles and functions of salivary proteins and electrolytes need to be elucidated. Numerous investigators allude to the role that saliva plays in maintaining health, yet few studies have examined saliva in various disease states. In reviewing the literature, it is apparent that there are many difficulties to be overcome in saliva research. The collection and handling of samples needed to be consistent. Mandel *et al.* (1992), Schiodt *et al.* (1992), Scannapieco (1994), Dawson (1996), and Milesovic (1996) confirmed the difficulties in determining the individual roles that both electrolytes and the organic components of saliva play in oral health and ecology.

The many oral opportunistic infections in HIV could be related to changes in saliva. Therefore research into simple assays for detection of the virus in saliva should be encouraged. Changes in saliva composition can be evaluated, and recognition of oral signs and symptoms related to these changes should continue to be documented and monitored. Accordingly clinical therapies can then be applied on a scientific basis and patient management improved.

Rationale for the present study

Impact of oral health problems on quality of life

Many authors have discussed the wide range of oral manifestations of HIV and the complexity of treatment required for their management (Greenspan *et al.* 1990; Schulten *et al.* 1990; Barr 1994; Glick *et al.* 1994). Coates *et al.* (1996) concluded that significantly greater problems were experienced with oral health in a cohort of HIV-infected individuals compared with eligible subjects, age and sex matched, attending for dental treatment at South Australian Dental Service clinics. Those authors found that much of the oral pain, cited by the subjects with HIV-infection, could possibly be attributed to HIV infection. Fox (1992) in his review implicated altered salivary performance as a result of HIV infection as a major contributing factor in deteriorating oral health, and Mandel *et al.* (1992) also proposed that the most likely pathway for changes in the oral environment would be from changes in the salivary gland function. They specified not only altered immune factors in saliva, but also impact on saliva flow rate and composition, as integral components in the development of opportunistic oral infections.

The aims of the present study were:

- to evaluate the quality and quantity of saliva in a group of HIV infected patients
- to identify possible causes for any salivary changes discovered
- to determine whether a correlation exists between any salivary changes and oral health of the HIV infected patients.

CHAPTER 3

MATERIALS AND METHODS

3.1 SUBJECTS

3.1.1 Subjects with HIV infection

Sixty five HIV infected individuals were selected for this study. All subjects were assessed for changes in saliva flow, pH and calcium and protein levels. Fifty two were tested for protein content of saliva. Sixty three subjects were male and two were female. Subjects had been infected with HIV for periods ranging from 2-12 years. Twenty subjects were defined as having AIDS according to the WHO Classification for HIV/AIDS (EC-Clearinghouse on Oral Problems Related to HIV Infection and WHO Collaboration Centre on Oral Manifestations of the Immunodeficiency Virus; London 1992. An Update of the Classification and Diagnostic Criteria of oral lesions in HIV Infection).

3.1.2 Subjects without HIV infection

Thirty three control subjects who did not have HIV infection were selected. Thirty two of these were on no medication and had no apparent disease. Thirty were male and three were female. One subject had a history of radiation treatment for a brain tumour and exhibited a rampant caries pattern. Volume, weight and pH of saliva were recorded for all thirty three control subjects. Calcium and protein analyses were carried out for 21 of the non-HIV infected saliva samples.

3.1.3 Ethics and informed consent

All subjects were entered into the study with the approval of The University of Adelaide Ethics Committee for Human Experimentation. Subjects were advised of the reasons for the project and fully informed consent for participation in the project was obtained before proceeding (Appendix 1).

3.1.4 Recording of clinical information

All subjects with HIV were examined by the same operator in a dedicated dental clinic at the Adelaide Dental Hospital. All available relevant clinical data was recorded when the saliva samples were taken. Data recorded included CD4 cell counts and CD4 percentage; β2 microglobulin levels; medications being taken, (both antiretroviral and for opportunistic infections), or medications for HIV-unrelated purposes; time since seroconversion with HIV infection if known and any other relevant medical history.

On examination, the presence or absence of HIV-related oral manifestations including candida infection, periodontal disease, erosion and decay were described and recorded for the HIV subjects. The classification and diagnostic criteria used for oral lesions were those outlined by the EC-Clearinghouse on Oral Problems Related to HIV Infection and WHO Collaboration Centre on Oral Manifestations of the Immunodeficiency Virus; (1992). All clinical lesions were photographed using a Pentax SFXN camera with a ring flash and Kodachrome 64 ASA 35mm transparency film.

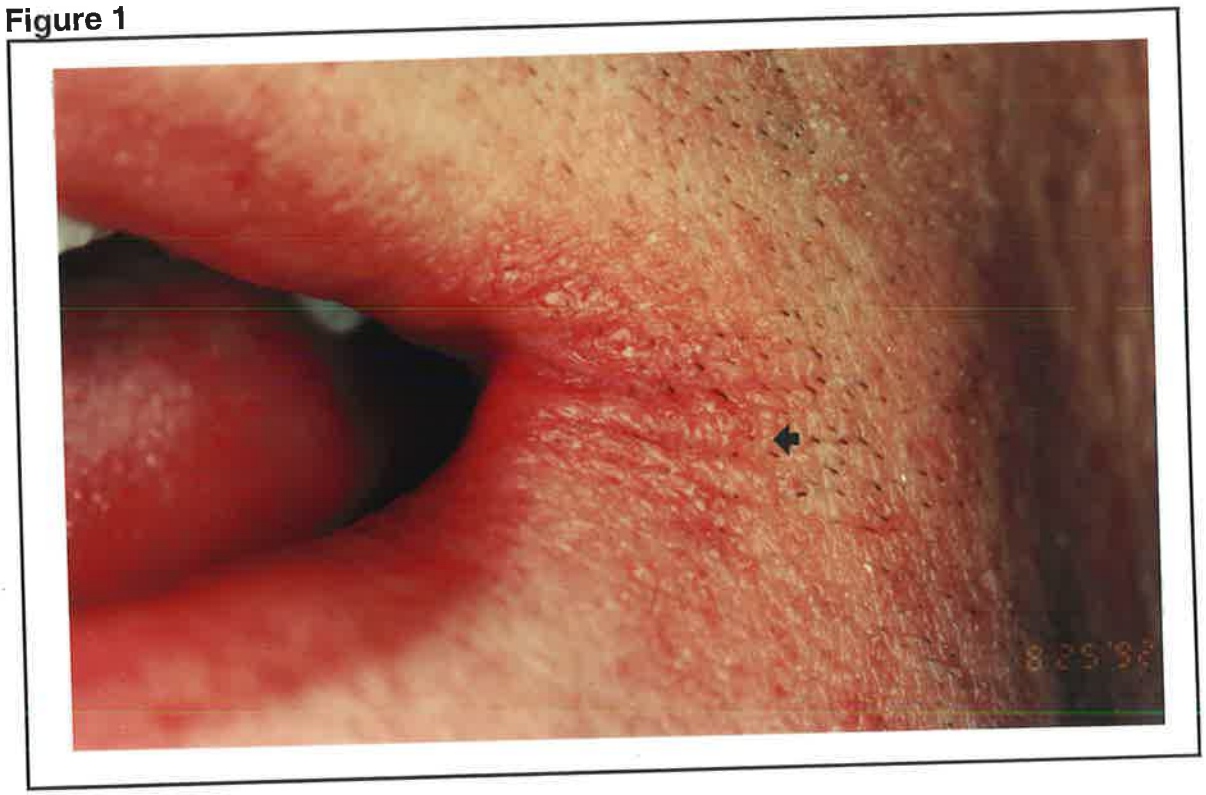
HIV-related candidiasis was recorded as: angular cheilitis, erythematous candidiasis, and pseudomembranous candidiasis (Figures 1-4). The presence of candidiasis was confirmed by Sabouraud culture and KOH smear (Figure 5).

HIV-related periodontal manifestations recorded were: Linear Gingival Erythema (LGE), Necrotising Ulcerative Gingivitis (NUG), Necrotising Ulcerative Periodontitis (NUP), and Necrotising Ulcerative Stomatitis (NUS) (Figures 6-11).

Decay was noted if the carious lesions were active. Previous dental work was not recorded as being indicative of caries activity. Carious lesions were diagnosed by clinical examination, orthopantomogram and bitewing radiographs (Figure 12).

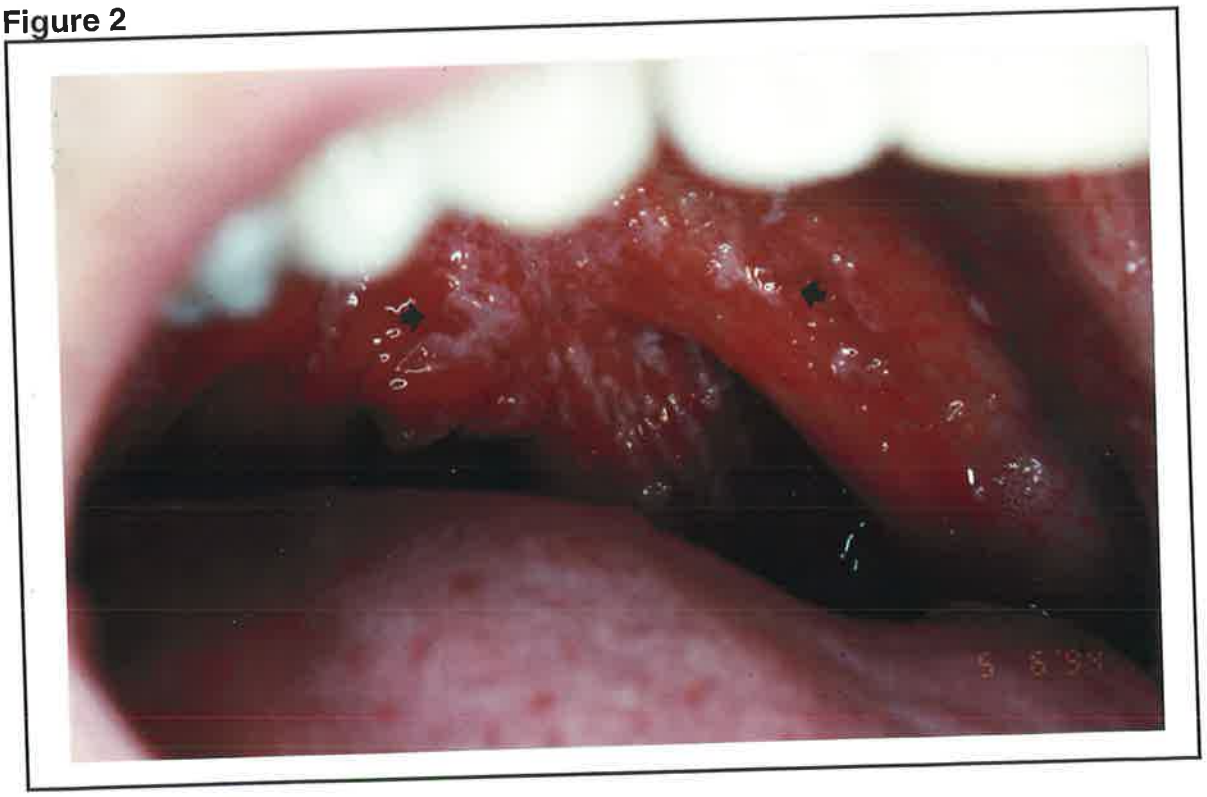
Erosive lesions of the teeth (Figures 13-15) were recorded if the enamel surface was etched or eroded. Erosive lesions were not recorded if causes other than HIV infection or management of HIV infection were elicited from the history, for example excessive use of citric juices, soft drinks, or recreational drugs and mechanical trauma of teeth by brushing.

Figure 1



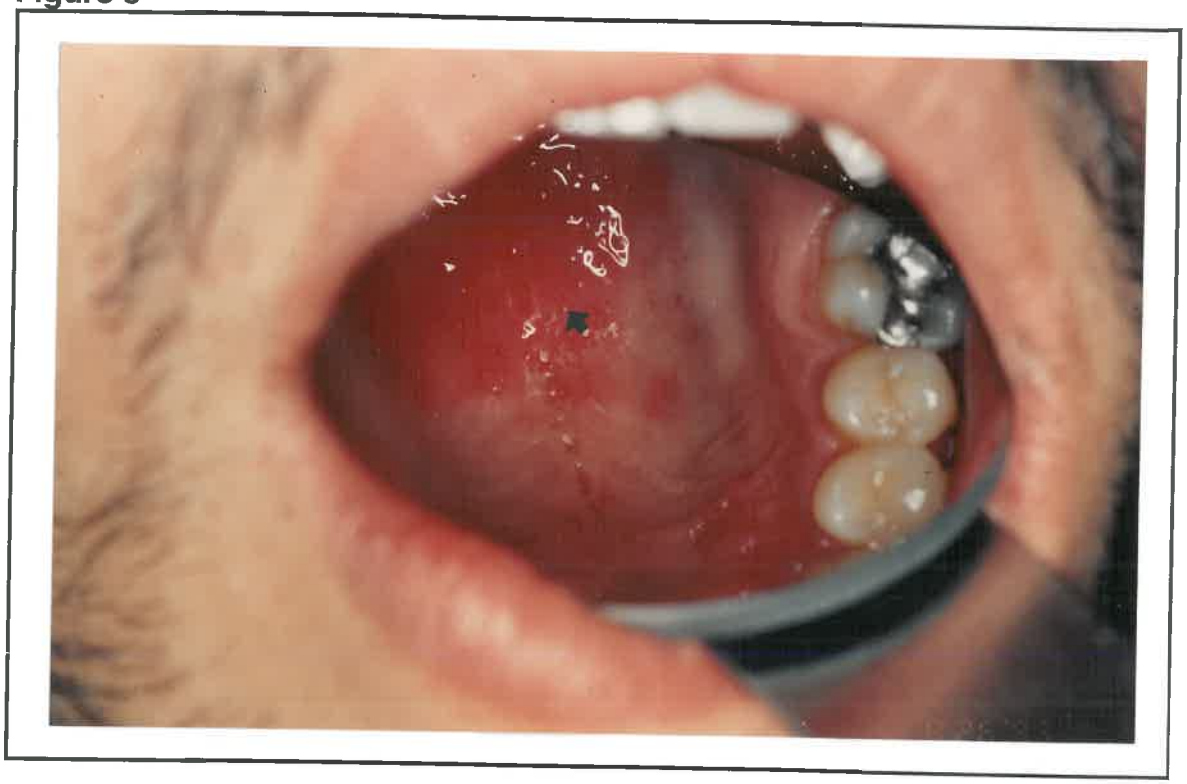
Angular cheilitis (arrow)

Figure 2



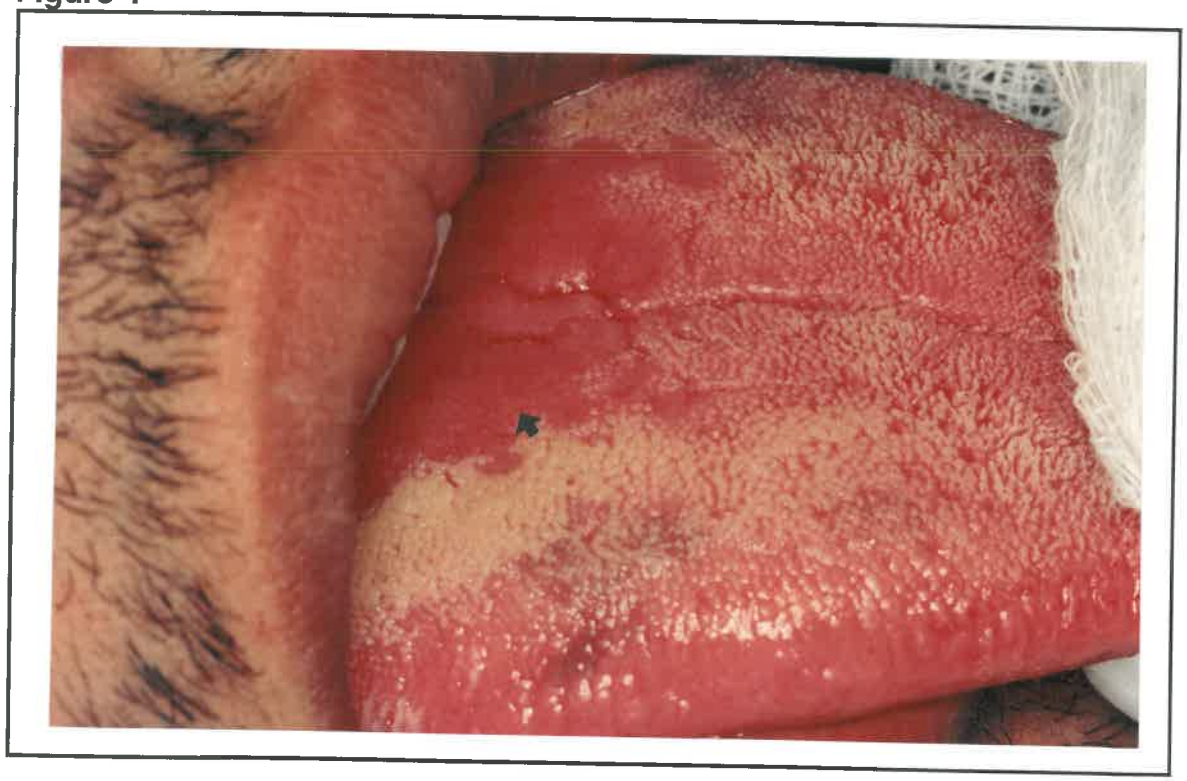
Pseudomembranous candidiasis of the oropharynx (arrows)

Figure 3



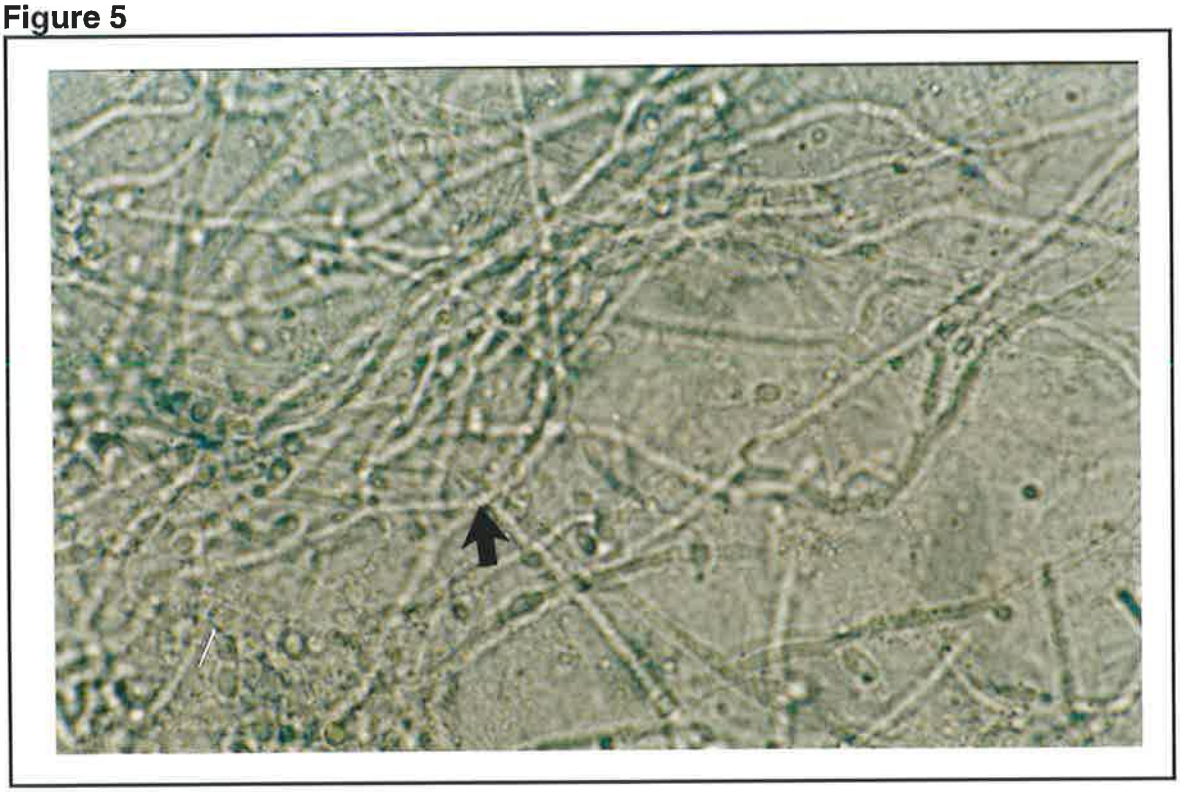
Erythematous candidiasis of the palate (arrow)

Figure 4



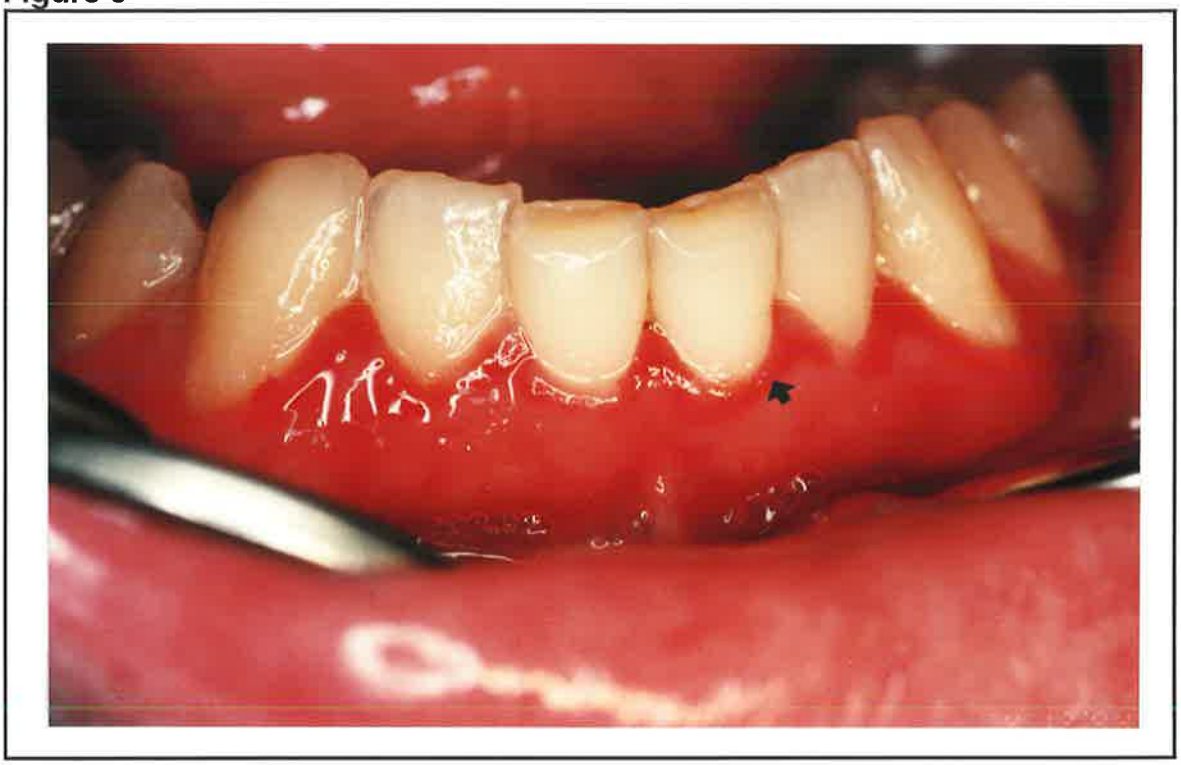
Erythematous candidiasis of the tongue in the same patient (arrow)

Figure 5



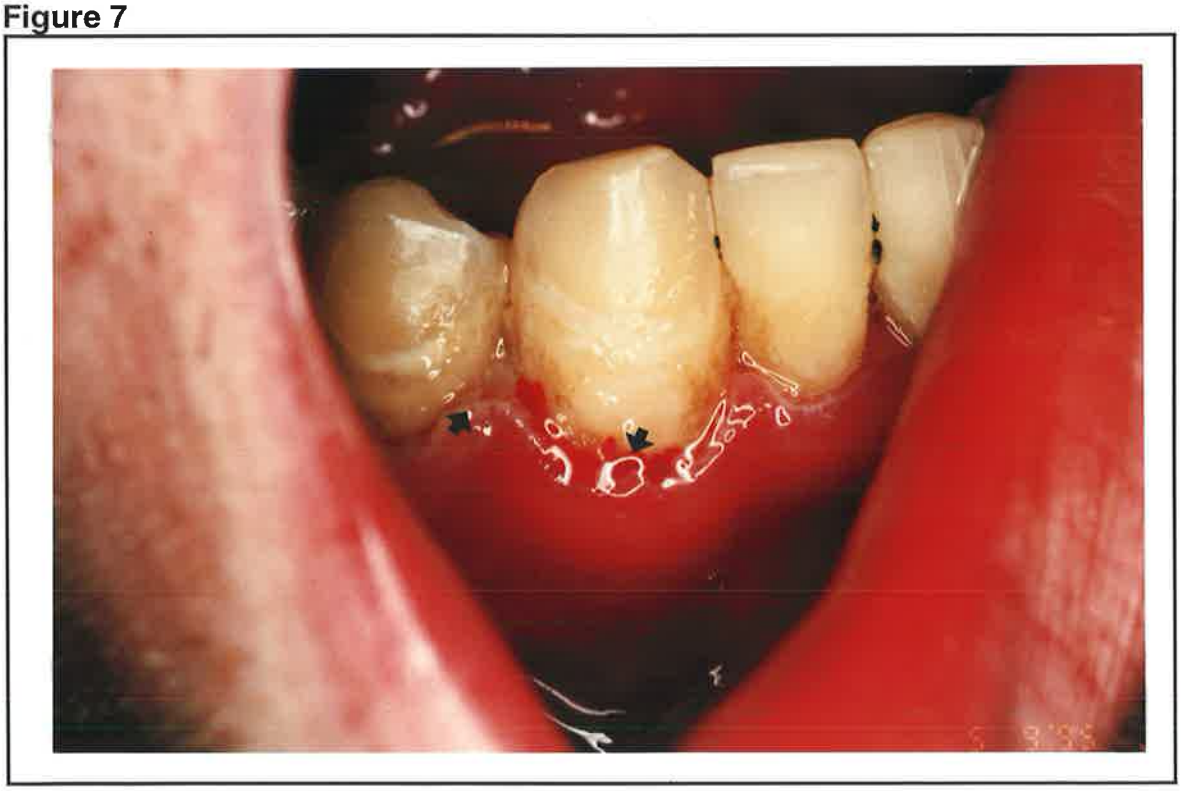
The presence of candida was confirmed by smear microscopy using 10% potassium hydroxide. The presence of pseudohyphae indicating active replication are evident (arrow).

Figure 6



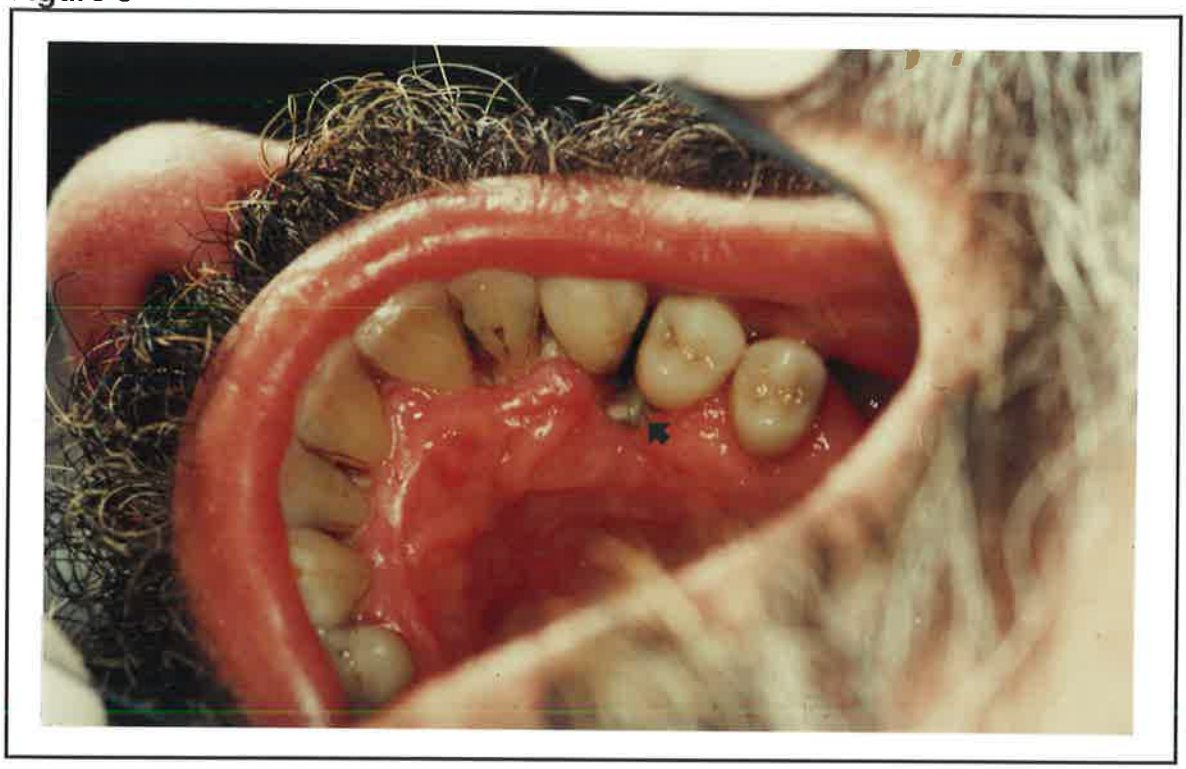
Linear Gingival Erythema (LGE); characterised by a fiery red band at the gingival margin with minimal plaque formation (arrow)

Figure 7



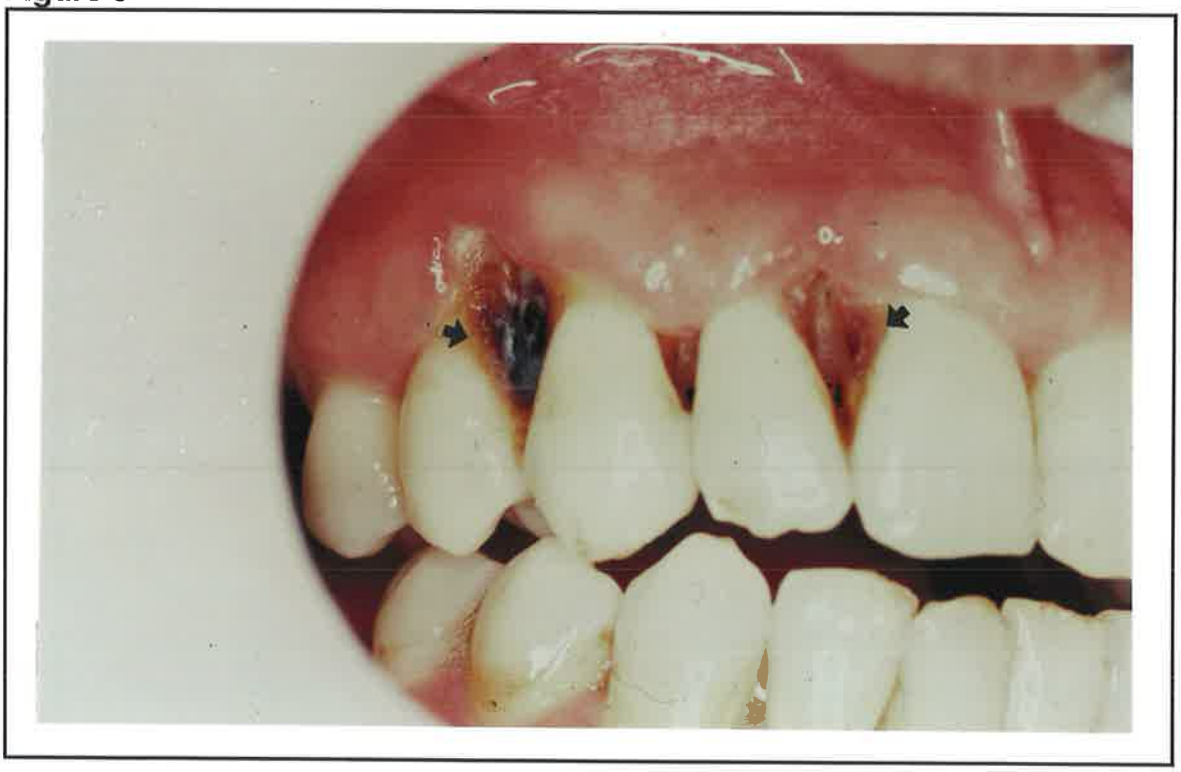
Necrotising Ulcerative Gingivitis (NUG) showing spontaneous bleeding (arrow) and necrosis of the gingival tissues (arrow)

Figure 8



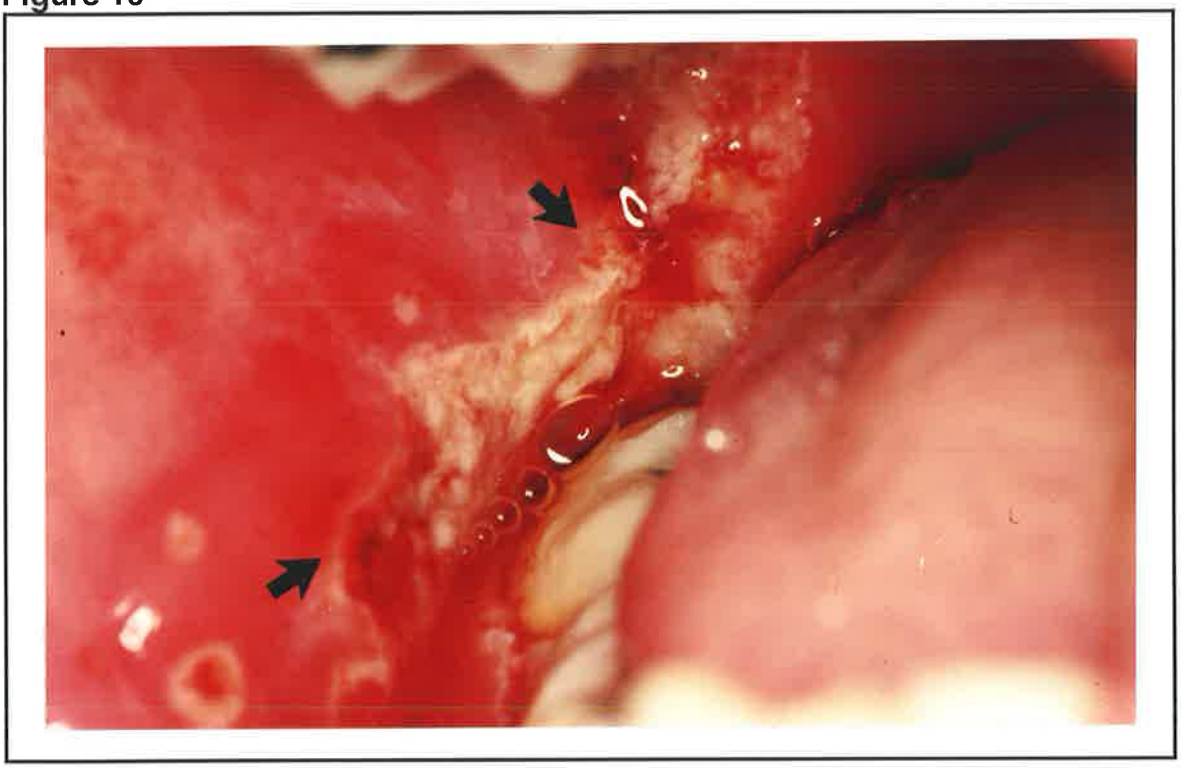
Necrotising Ulcerative Periodontitis (NUP). A spontaneous bony sequestrum is evident on the palate distal to the left canine (arrow).

Figure 9



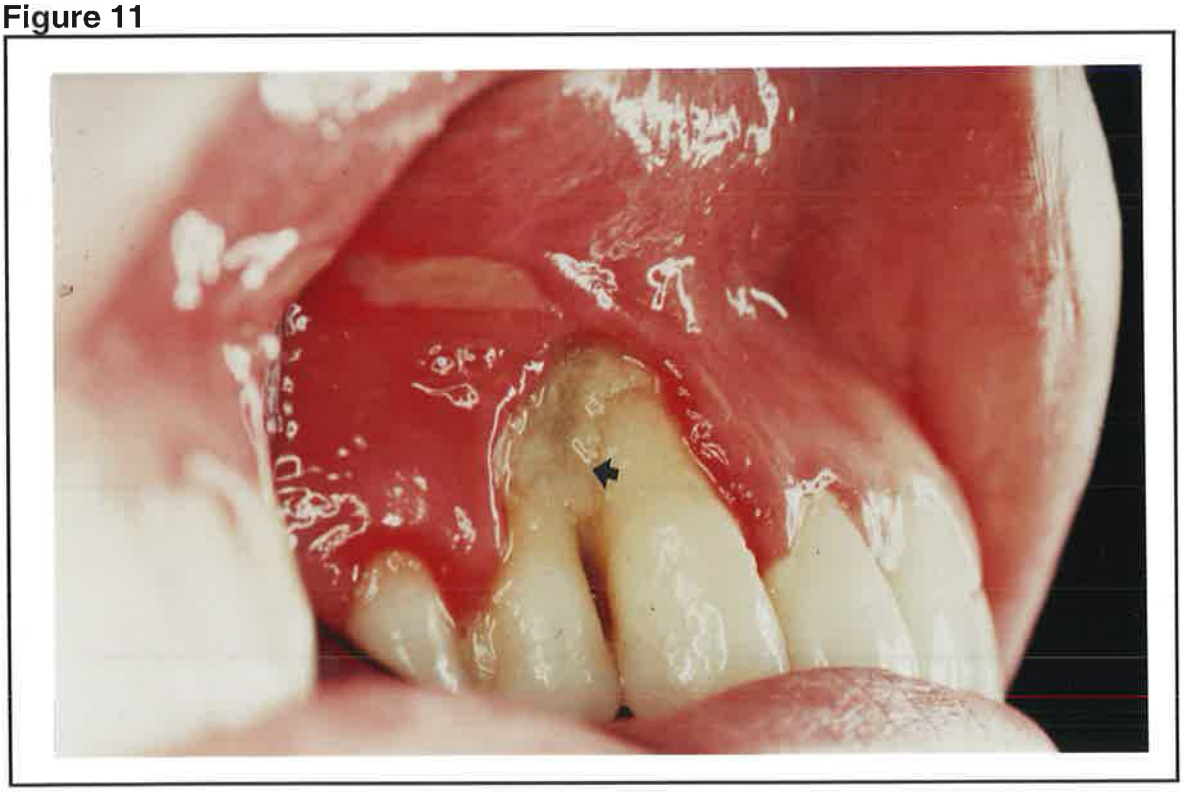
Extensive bone and soft tissue loss are a feature of NUP and in this patient obvious root caries are also present (arrows).

Figure 10



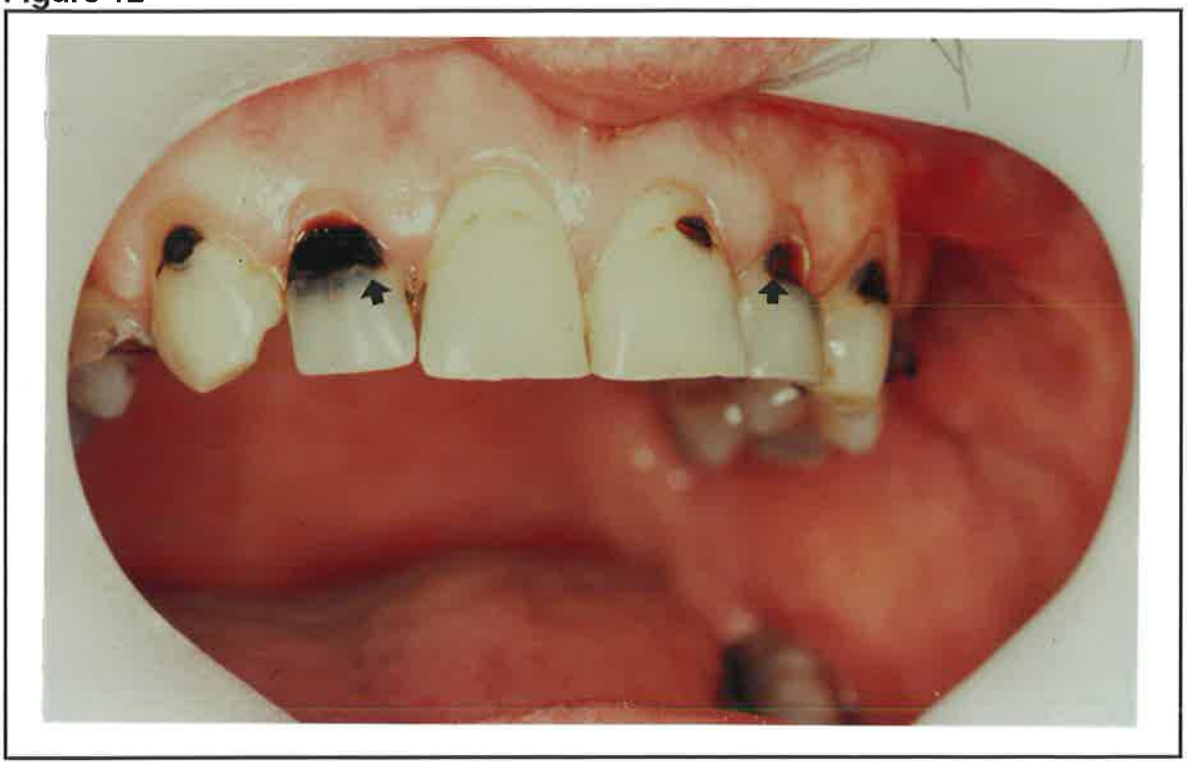
Necrotising Ulcerative Stomatitis(NUS); spontaneous bleeding, and necrosis of the buccal mucosa are evident (arrows).

Figure 11



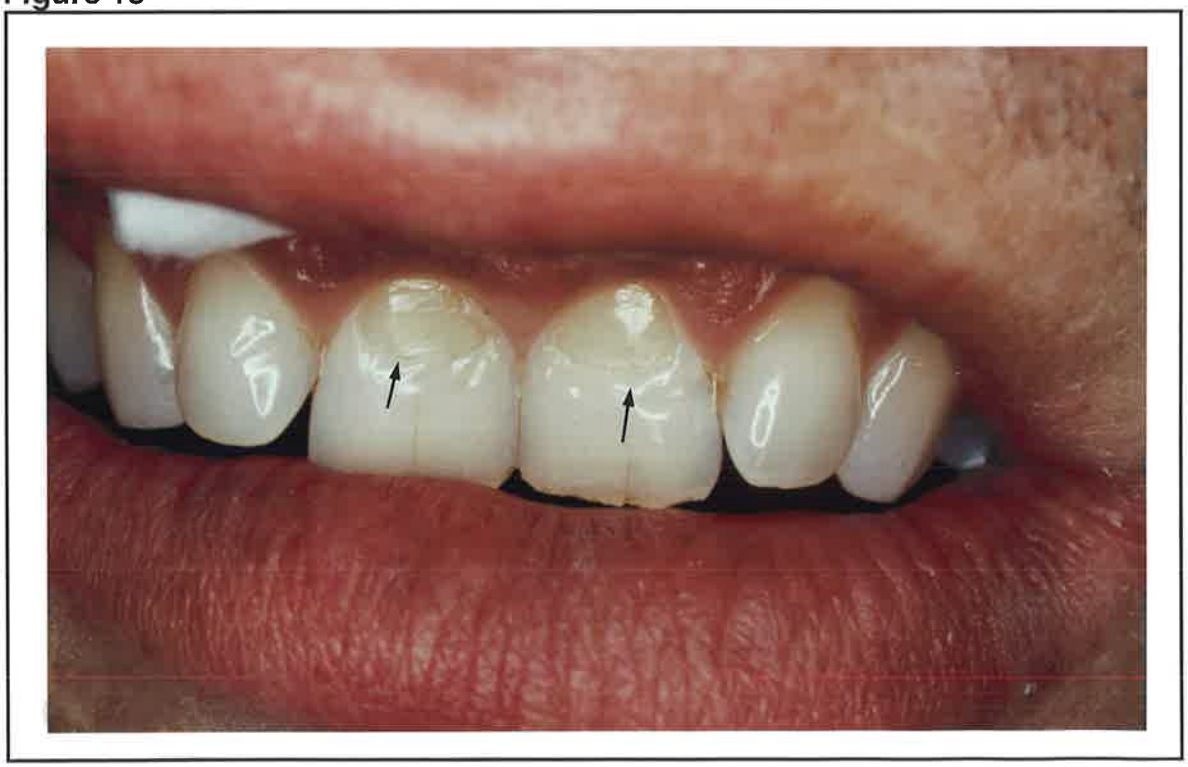
A spontaneous bony sequestrum (arrow) in the same patient as shown in Figure 10

Figure 12



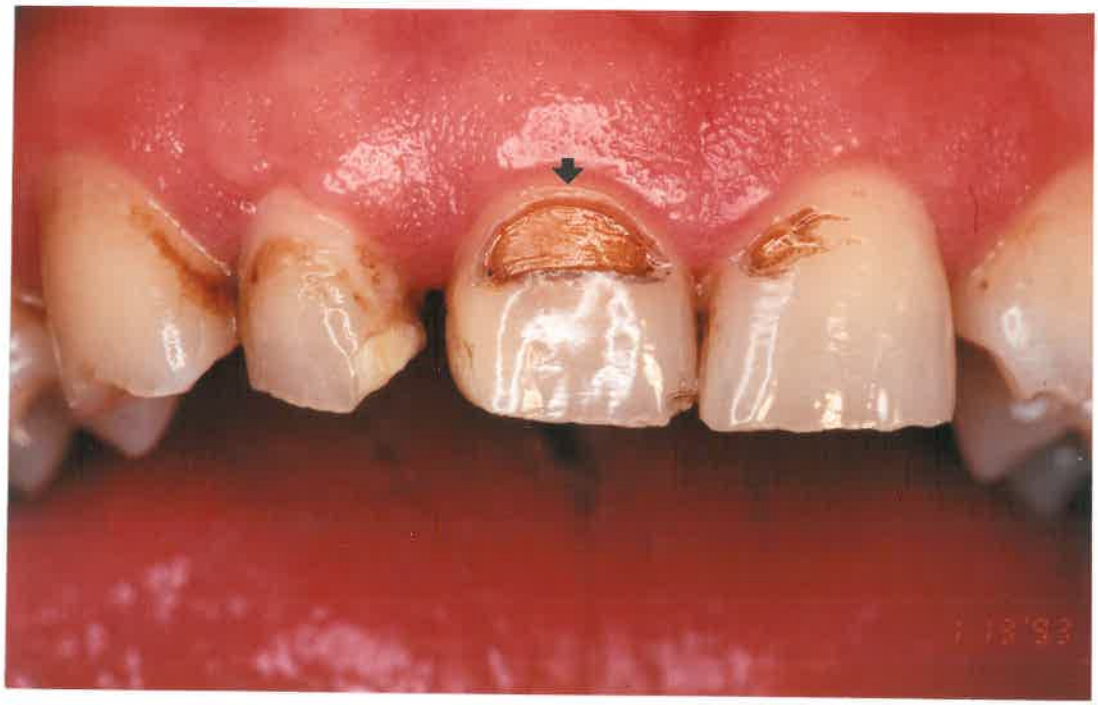
Rampant caries in a patient with low stimulated salivary flow (arrows)

Figure 13



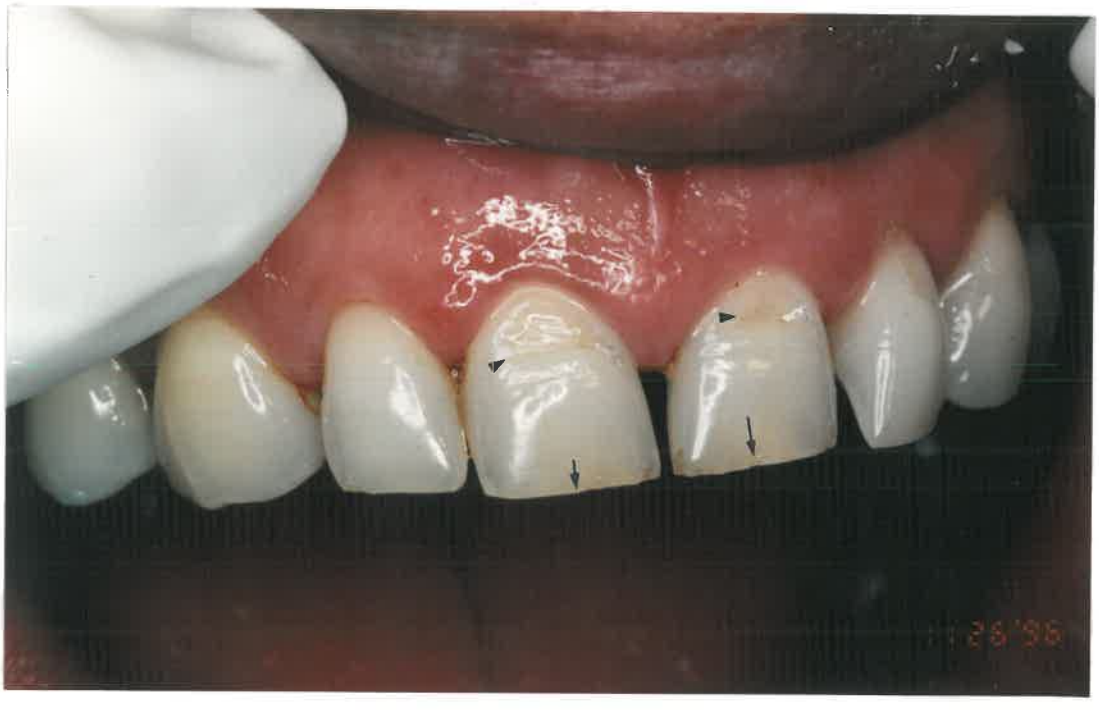
Erosion of enamel: a distinct pattern on the labial surfaces of the upper teeth (arrows)

Figure 14



Erosion of labial surfaces of teeth resembling tooth cavity preparation (arrow). This damage was noted by the patient in the 3 months prior to his dental appointment.

Figure 15



Erosion of teeth is associated with wear of incisal surfaces (arrow).

3.2 SALIVA COLLECTION

3.2.1 Recording of pH

Immediately prior to the collection of the resting phase sample, the subjects' salivary pH was recorded (unstimulated salivary pH) using Duotest (Machery-Nagel GmbH & Co KG) pH 5.0 - 8.0 indicating paper (Figure 16). The pH paper was placed on the floor of the mouth. The colour change of the saliva dampened paper was compared to a Standard Duotest colour chart and results recorded. Immediately following the completion of the stimulated phase saliva collection, a pH recording was taken again from each subject (stimulated salivary pH).

3.2.2 Method and time of collection of samples

Time and place of collection

The saliva samples were taken late morning or early afternoon. The subjects had not eaten or drunk immediately prior to the collection of saliva nor undertaken any physical exercise.

The saliva samples were all taken in a clinical setting, with uniform temperature and lighting. A fixed collection time of five minutes was set for both unstimulated and stimulated samples. All samples collected were of whole saliva.

Figure 16

Duotest pH paper showing a stimulated salivary sample reading

Stimulation

Parafilm "M" (American National Can, Greenwich, CT 06836) was the stimulant used to obtain all stimulated saliva samples. A two centimetre square of parafilm was chewed by each subject for five minutes during stimulated saliva collection.

Method for collection of unstimulated and stimulated saliva

The subjects were asked to tilt their head and allow any saliva that collected to drip off their lips into an autoclavable (heat resistant) graduated tube (Figure 17). The tubes were preweighed and labelled with a code number.

Subjects were asked to drip any saliva into the tube over a period of five minutes whilst sitting and relaxing. This constituted the resting phase saliva sample. Tubes were handled using gloves and when collection was completed the tubes were placed in a sealable screw lid container which had not been contaminated with any bodily fluid (Figure 18).

Following collection of resting phase saliva, subjects were given parafilm to chew for five minutes. All saliva that accumulated in the mouth whilst chewing on parafilm was collected in a second labelled and weighed tube. This constituted the stimulated phase saliva sample. The second tube was sealed and placed with the first tube in the sealed container.

Figure 17

Saliva was collected in a graduated tube. Two millilitres was removed by pipette for protein analysis.

Figure 18

Saliva was weighed on a Sauter K1200 electronic balance, inside a sealed container.

3.2.3 Recording of weight and volume

Volume of saliva

The tubes were removed from the sealed carrying container and the amount of saliva collected in each tube was recorded by reading the level corresponding to the graduations on the tubes (Figure 17). Recordings were taken for resting phase and stimulated phase.

Weight of saliva

Samples were weighed using a Sauter K1200 electronic balance (Figure 18). For each sample, the sealed carrying container was placed on the electronic balance which was recalibrated to zero. The graduated tube with the saliva sample was then placed in the carrying container and the weight recorded. The original pre-recorded weight of the graduated tube was deducted from this figure. Weights for the resting and stimulated phase samples were tabulated. Universal precautions applied at all stages.

3.2.4 Samples for protein analysis

Two millilitres of stimulated saliva was removed from each graduated collection tube with an individual disposable transfer pipette and placed in a Nalgene ® (New York, USA) cryogenic vial suitable for freezing at -80°C. Individual vials containing saliva samples from each subject were labelled and frozen at -18°C (Figure 17).

3.3 PROTEIN ANALYSIS

3.3.1 Materials

Electrophoresis was carried out using standard sodium dodecylsulfate AGE techniques for proteins in the range 6-200 kD (Appendix 2).

The following materials were used in this phase of the study:

- Novex™ 4-20% poly acrylamide tris-glycine gel, with an acrylamide/bis-acrylamide matrix of 1.00mm thickness (Appendix 3).
- Deep reservoir Hoeffer Electrophoresis equipment powered by a ECPS 3000/150 powerpack (Pharmacea).
- Running voltage: 50 volts.
- Twenty microlitres of sample buffer (Appendix 4) was added to the saliva samples together with the tracking dye, bromophenol blue, to provide a leading edge marker.
- Broad range molecular markers were prepared in the conventional manner according to the suppliers (Biorad).
- Reservoir buffer (Appendix 5) at a pH of 8 was added to the reservoir well of the electrophoresis equipment. All reservoir and sample buffer solutions were freshly prepared.
- The gels were stained with Coomassie Brilliant blue R250.
- The gels were destained after the electrophoresis was completed (Appendix 6).

3.3.2 Methods

Preparation of saliva samples

Twenty microlitres of thawed saliva was placed in an Eppendorf tube and diluted with an equal amount of sample buffer (pH 6.8) that contained mercapto-ethanol and to which five microlitres of leading edge marker, bromophenol blue to 0.001%, had been added. The Eppendorf tube was then sealed, placed in a rack and boiled for ninety seconds to disrupt the disulphide bonds.

Electrophoresis preparation

The protein samples were electrophoresed on prepacked Novex™ 4-20% poly acrylamide tris-glycine gels.

Ten microlitres of denatured saliva in sample buffer were placed into nine of the reservoir wells; the remaining well was loaded with molecular markers.

Electrophoresis was run at a constant voltage of 50Volts until the leading edge marker Bromophenol blue had reached the bottom of the gel.

Destaining and Recording of results

At the completion of electrophoresis the gels were placed in Destainer for 2-3 hours (Appendix 7).

The destained gels were then photocopied for immediate record (and for relative investigation) and photographed for intensity. The gels were photographed using Ilford Pan F film.

3.4 CALCIUM ANALYSIS

3.4.1 Collection and Preparation of samples

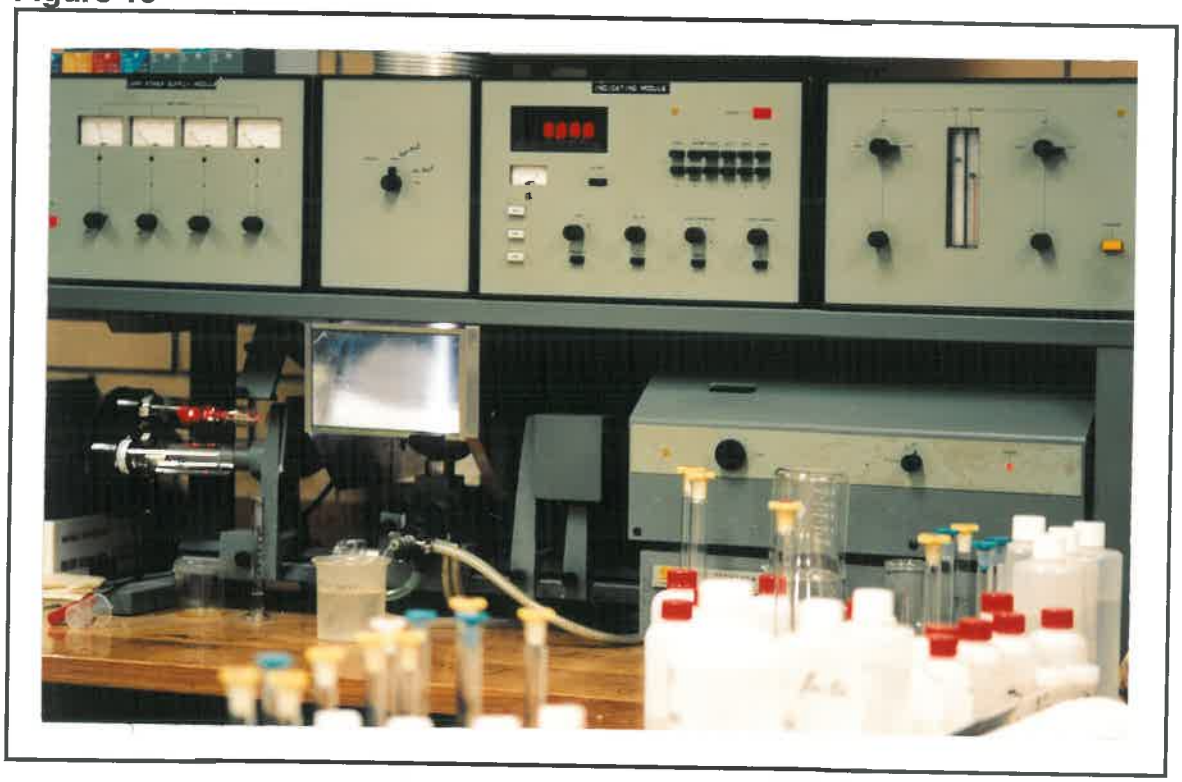
After the volume and weight of saliva samples was recorded for all samples and 2ml was removed by transfer pipette for protein analysis from the stimulated saliva samples, the saliva remaining in the large graduated collection tubes was then taken to the central sterilising department in the Adelaide Dental Hospital and autoclaved in saturated steam, 206 kPa, at 134 degrees. The resulting autoclaved samples were then tested for calcium content using Calcium Spectrophotometry Analysis.

3.4.2 Atomic Absorption Spectrophotometry Calcium Analysis

A single beam Atomic Absorption Spectrophotometer (Figure 19) was used for calculating total calcium levels. An angled burner was used, with an acetylene flame and nitrous oxide as an oxidant. The Atomic Absorption Spectrophotometer was set at zero and calibrated by testing solutions of known calcium standards (Appendix 7).

Saliva from the autoclaved samples was drawn up the catheter and burnt in nitrous oxide flame. The level of calcium was determined from the electronic readout and recorded to two decimal places.

Figure 19



The Atomic Absorption Spectrophotometer

3.5 ANALYSIS OF DATA

The statistical analysis of results was undertaken by Mr P. Leppard of the Department of Statistics, The University of Adelaide, using BMDP Statistical software, UCLA, United States of America (Program 4F). Chi squared analysis was performed on the data.

CHAPTER 4

RESULTS

4.1 AGE AND SEX DISTRIBUTION OF SUBJECTS

Saliva samples from sixty-five HIV infected individuals were analysed for changes in salivary flow rate, pH and calcium content. Sixty-three subjects were male and two subjects were female. The mean age was 36 years. Protein analysis was done on samples from fifty-two of these subjects. The mean age of these individuals was 37 years. The mean age of the thirteen HIV infected subjects who did not have protein analysed was 35 years (Table 1).

Thirty-three HIV negative subjects volunteered as sources of control saliva. Salivary flow rate, weight and pH of saliva were recorded for all thirty-three. The mean age of these subjects was 32 years. Thirty subjects were male and three were female. Calcium and protein analyses were done for 21 of the non-HIV infected saliva samples. The mean age of these subjects was 30 years and 18 were male and three were female (Table 1).

TABLE 1

Subjects	mean age years	Female	Male	Total
HIV all patients	36	2	63	65
HIV prot (52)	37	1	51	52
HIV no prot(13)	35	1	12	13
Control all	32	3	30	33
Control calc + prot	30	3	18	21

Age and sex distribution, relative to investigations carried out, of all subjects in the study.

4.2 pH OF SALIVA SAMPLES

4.2.1 pH of Resting Phase Saliva

Sixty five subjects with HIV infection had the pH of their unstimulated saliva recorded. Thirty three subjects without HIV also had their salivary pH recorded. The two groups were compared using a salivary pH of 6.5 and above as normal for unstimulated saliva.

Thirty of the sixty five (46%) HIV infected subjects recorded saliva pH values less than 6.5. Thirty five (54%) recorded values of 6.5 or greater. Of the thirty three subjects without HIV infection, 7 (21.2%) recorded values less than 6.5 and 26 had values equal to or greater than pH 6.5 (Appendix 8).

Chi-square analysis indicated a significant association between presence of HIV infection and a low resting phase salivary pH (Table 2, Figure 20).

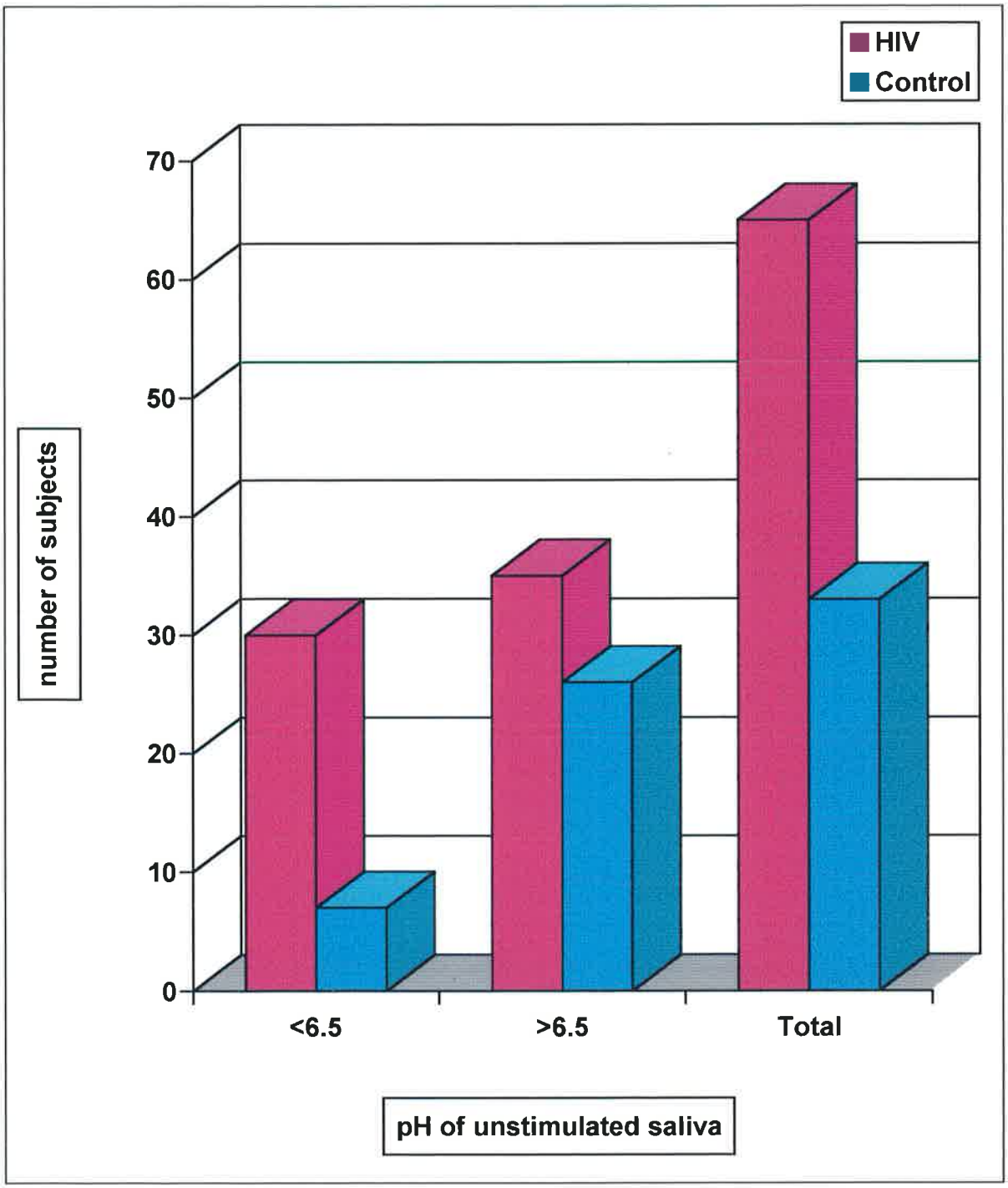
TABLE 2

Group	US salivary pH < 6.5	US salivary pH > 6.5	Total
HIV (n=65)	46.2% (n= 30)	53.8% (n= 35)	100% (n= 65)
Control (n=33)	21.2% (n= 7)	78.8% (n= 26)	100% (n= 33)
Total (n=98)	37.8% (n= 37)	62.2% (n= 61)	100% (n= 98)

Variation in pH of unstimulated saliva between HIV subjects and control subjects $p<0.03$ ($X^2= 4.781$, 1 degree of freedom).

Figure 20

Comparison of unstimulated salivary pH between control and HIV infected subjects p<0.03 D.F.1



4.2.2 pH of Stimulated Phase Saliva

There was no significant difference in the pH of stimulated saliva between the control group and those with HIV infection. Seven of the sixty five (11%) HIV infected subjects recorded saliva pH values less than 6.8. Fifty eight (89%) recorded values of 6.8 or greater.

Of the thirty three subjects without HIV infection, 3 (9%) recorded values less than 6.8 and 30 (91%) had values equal to or greater than pH 6.8.

4.2.3 pH and CD4 cell counts

There appeared to be no correlation between unstimulated salivary pH values and CD4 cell counts in subjects with HIV infection (Table 3, Figure 21).

Chi-square analysis indicated no significant association between CD4 cell counts and a low resting phase salivary pH. $p<0.2$ ($X^2 = 3.386$, 2 degrees of freedom).

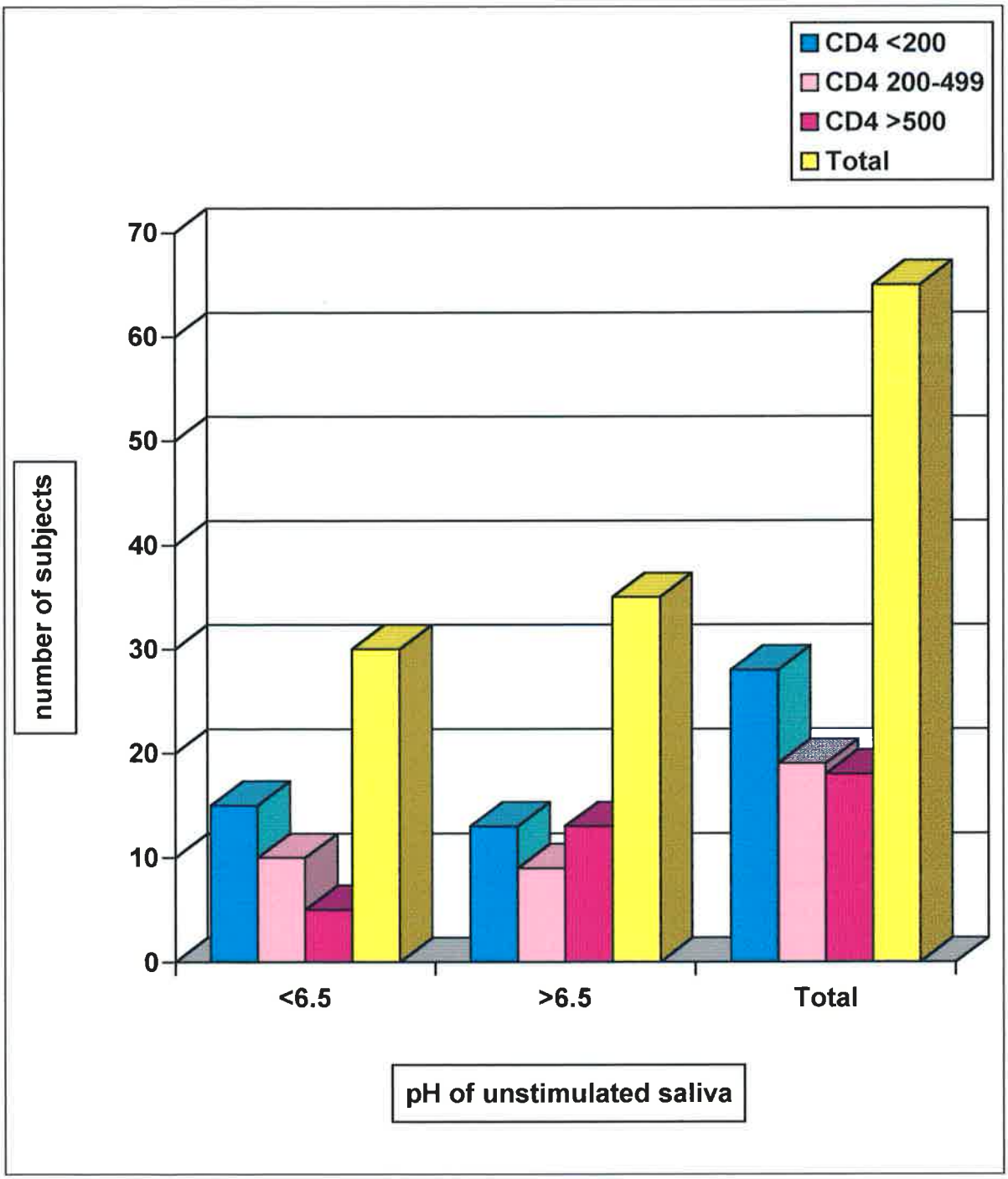
TABLE 3

CD4 cell count	US salivary pH<6.5	US salivary pH>6.5	Total
<200	15	13	28
200-499	10	9	19
>500	5	13	18
Total	30	35	65

HIV subjects with unstimulated salivary pH values relative to CD4 cell count

Figure 21

CD4 cell counts relative to unstimulated salivary pH demonstrating no statistical correlation between low pH values and immune status, as measured by CD4 markers p<0.2 D.F.2



4.2.4 Presence of oral manifestations related to pH

Four clinical conditions (candida, periodontal disease, caries, erosion) were evaluated with respect to pH for the sixty five HIV infected subjects.

Candida was more prevalent in HIV subjects with an unstimulated saliva pH value of less than 6.5 compared with HIV subjects having a pH >6.5. This was statistically significant at the 5% level (Table 4, Figure 22).

TABLE 4

Candida	pH <6.5	pH>6.5	Total
yes	50%	20%	33.8%
no	50%	80%	66.2%
Total	100%	100%	100%

HIV subjects: comparison of candida prevalence relative to unstimulated salivary pH

$p<0.03$ ($X^2=5.222$, 1 degree of freedom)

There was no correlation between unstimulated salivary pH values and the presence of periodontal infection, caries or erosion (Tables 5-7). The uniform spread of the these oral manifestations in low and normal resting phase salivary pH is shown in Figures 23- 25.

Figure 22

HIV subjects: comparison of candida prevalence relative to unstimulated salivary pH $p<0.03$ D.F.1

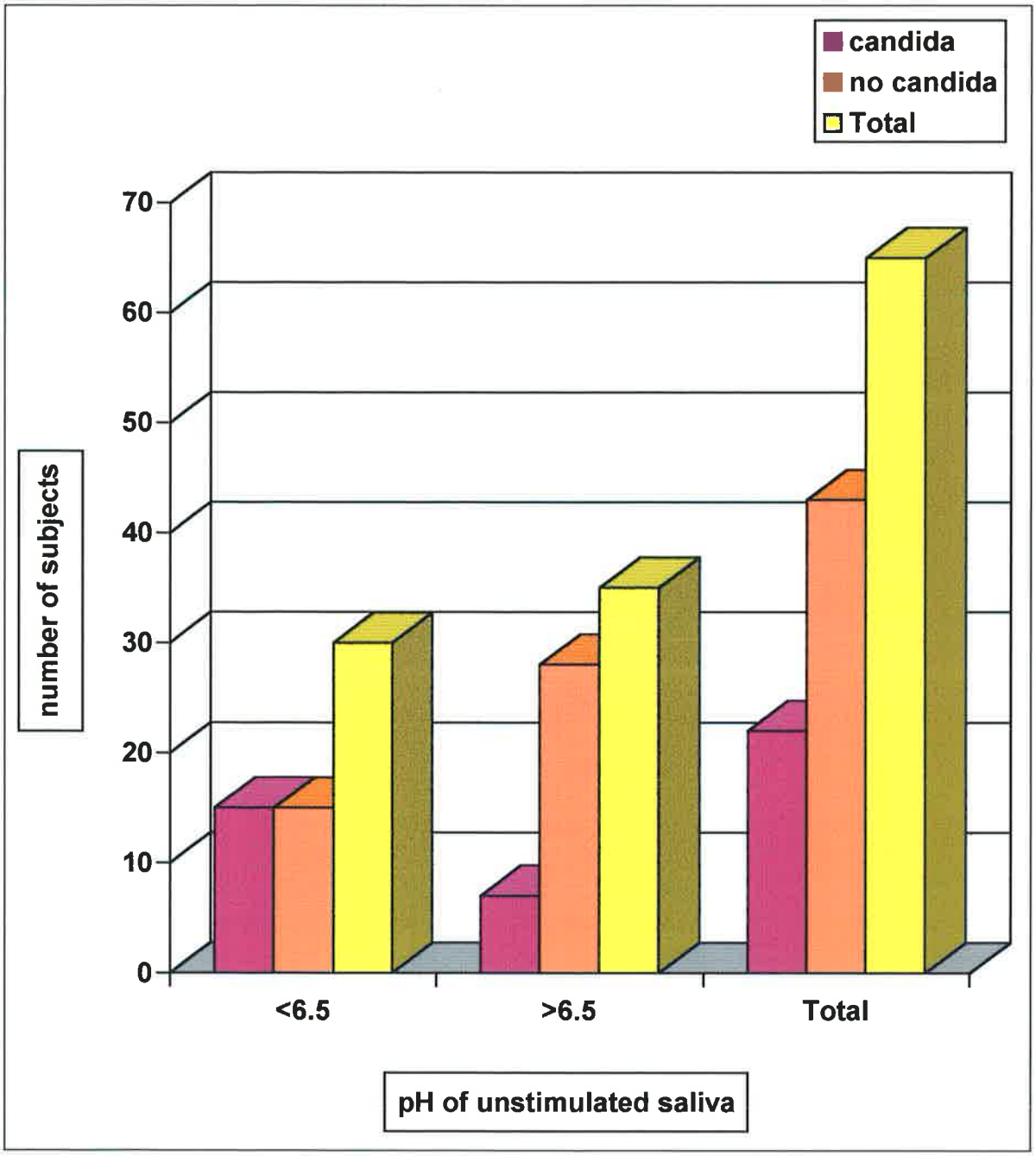


Table 5

Caries	**pH unstimulated saliva**		
	<6.5	**>6.5**	Total
yes	40% (n=12)	22.9% (n=8)	30.8% (n=20)
no	60% (n=18)	77.1% (n=27)	69.2% (n=45)
Total	100% (n=30)	100% (n=35)	100% (n=65)

The presence of caries is unrelated to pH values

$p<0.3$ ($X^2=2.229$, 1 degree of freedom)

Figure 23

The relationship between caries and unstimulated salivary pH

$p<0.3$ D.F.1

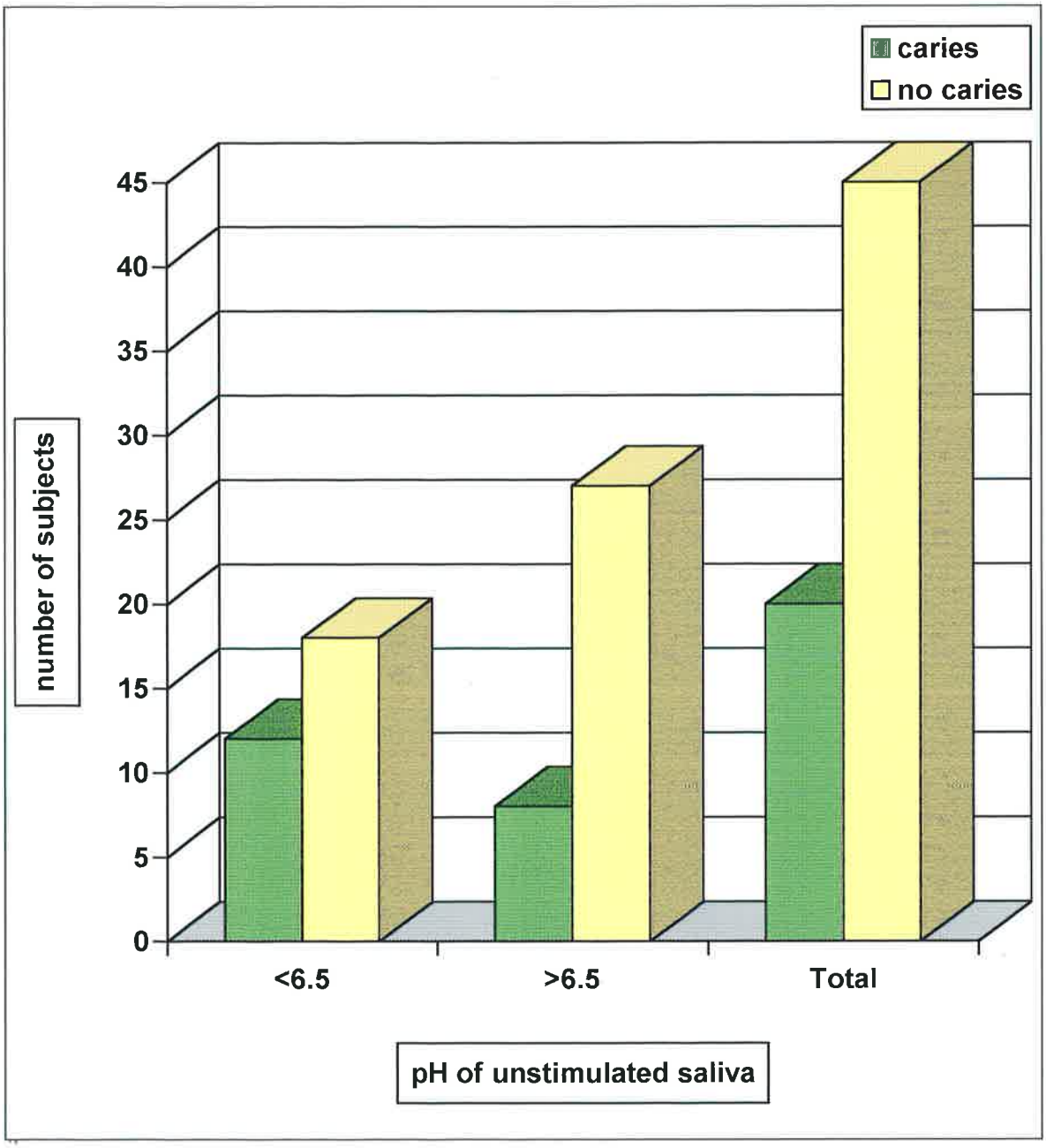


Table 6

erosion	**pH unstimulated saliva**		
	<6.5	**>6.5**	Total
yes	33.3% (n=10)	34.3% (n=12)	33.8% (n=22)
no	66.7% (n=20)	65.7% (n=23)	66.2% (n=43)
Total	100% (n=30)	100% (n=35)	100% (n=65)

The presence of erosion is unrelated to pH values

$p<1$ (X^2=.007, 1 degree of freedom)

Figure 24

Erosion in subjects with HIV relative to pH of unstimulated saliva p<1, D.F.1

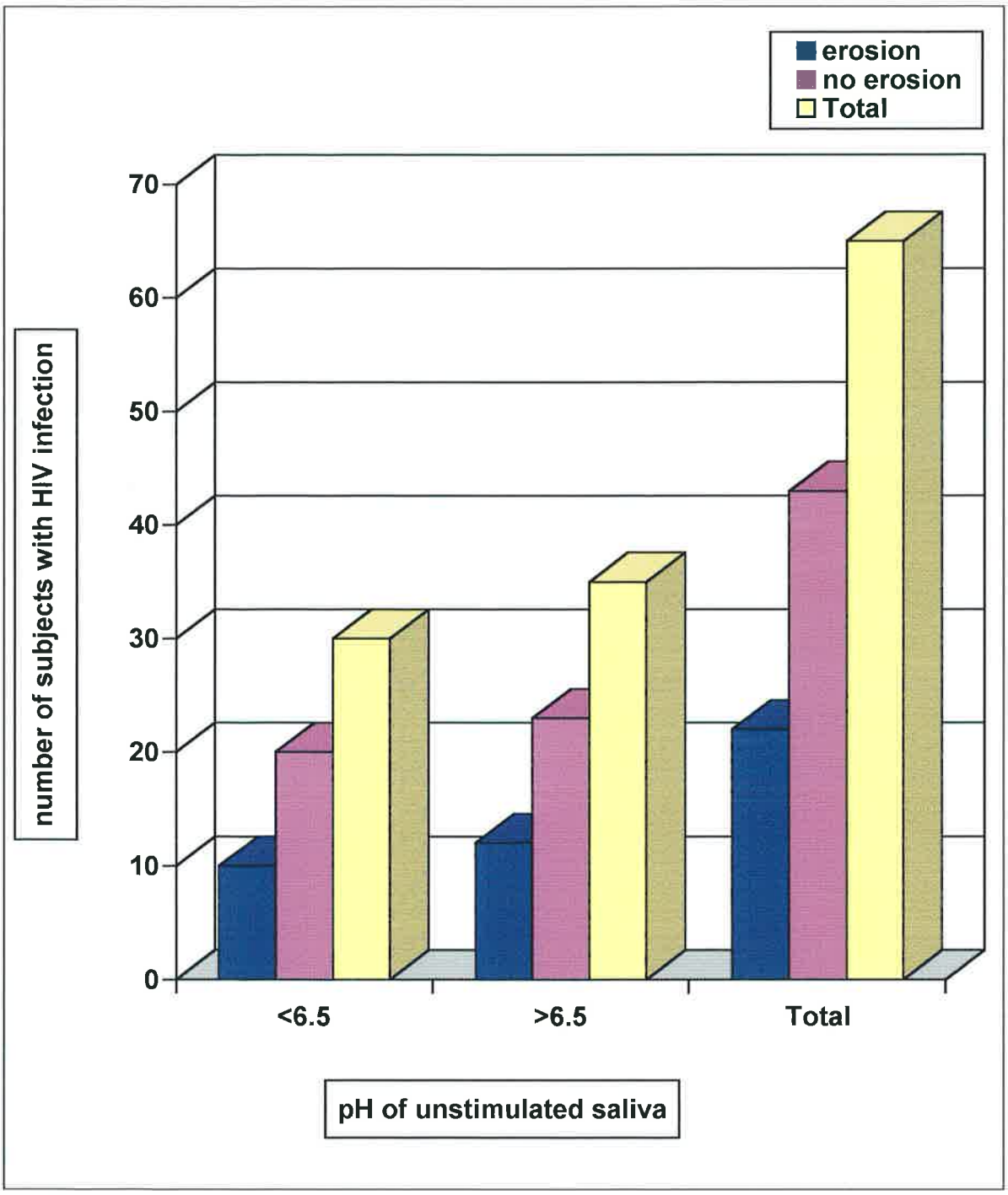


Table 7

Perio	**pH unstimulated saliva**		
	<6.5	**>6.5**	Total
yes	60% (n=18)	37.1% (n=13)	47.7% (n=31)
no	40% (n=12)	62.9% (n=22)	52.3% (n=34)
Total	100% (n=30)	100% (n=35)	100% (n=65)

The presence of periodontal disease is unrelated to pH values

$p<0.2$ ($X^2=3.38$, 1 degree of freedom)

Figure 25

Periodontal manifestations in relation to unstimulated salivary pH p<0.2 D.F.1

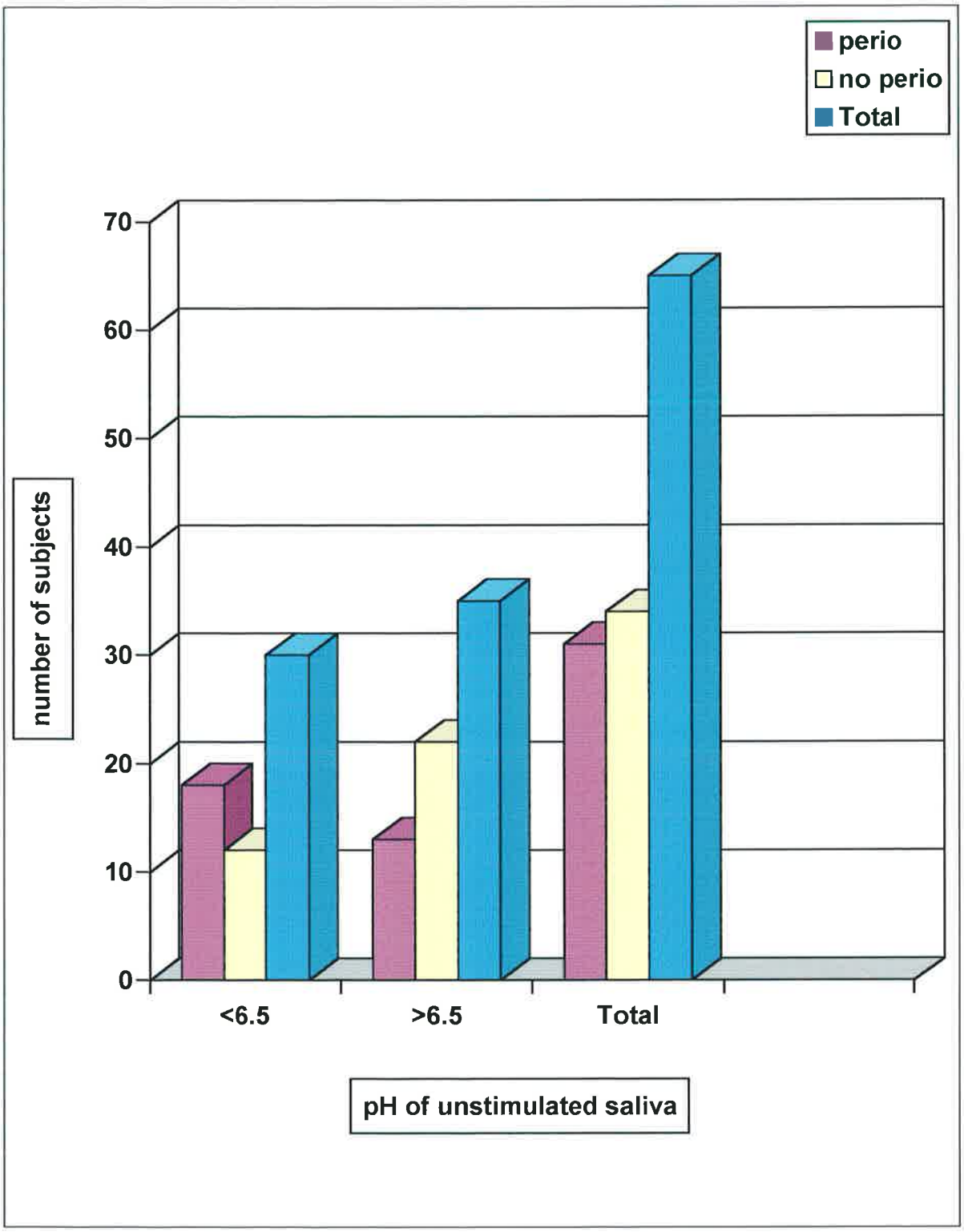
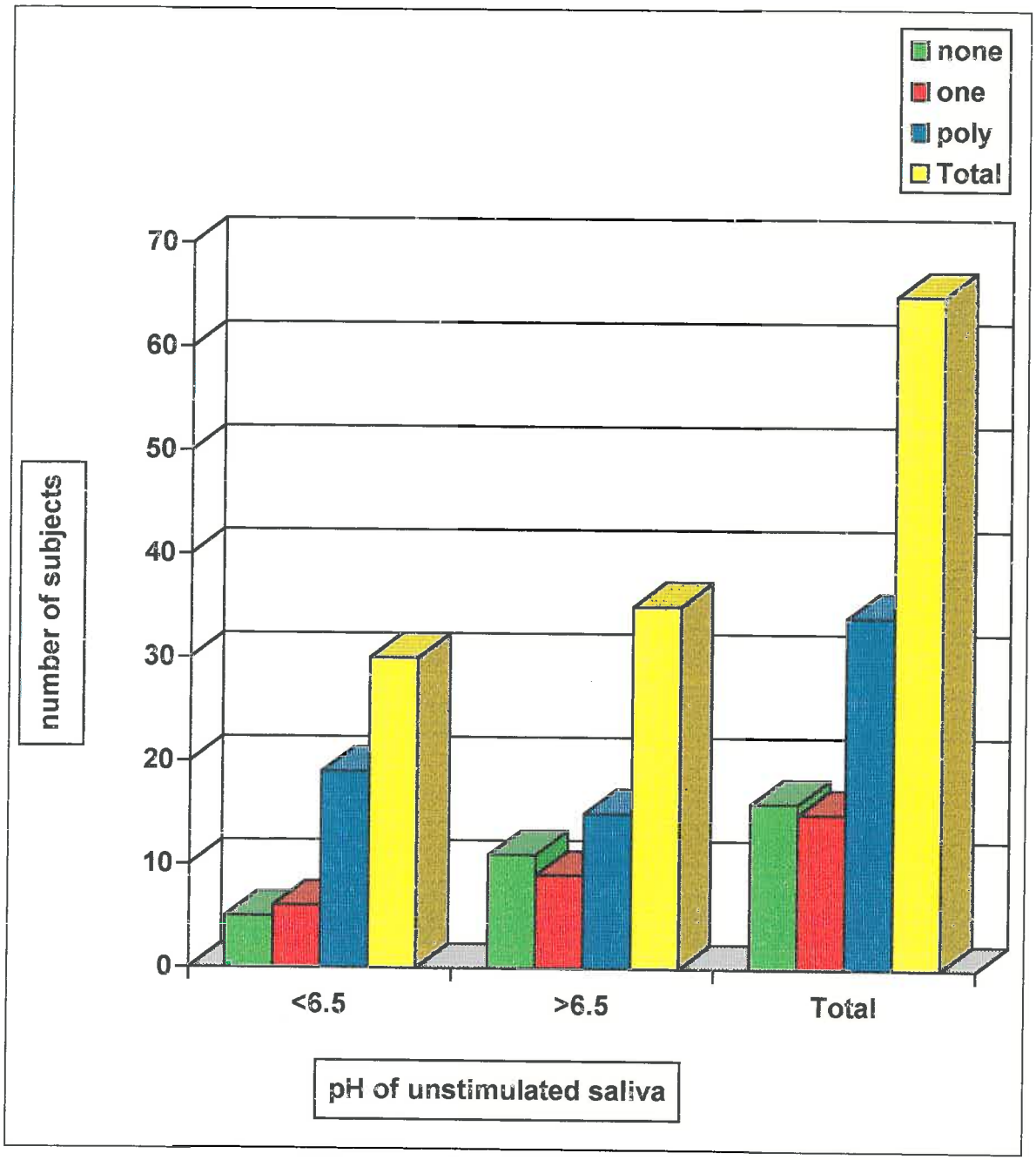


Figure 26

Relationship between medication and pH of unstimulated saliva

p<0.2 D.F.2



Chi-square analysis indicated no significant association between a low resting phase salivary pH and the taking of medication. p<0.3 (X^2= 2.953, 2 degrees of freedom)

4.3 STIMULATED SALIVARY FLOW RATE

Analysis of salivary flow in this study was carried out by comparing results with previously published studies and reference tables (Appendices 8-10). Reference values used for comparison were selected from studies where the age of the subjects corresponded to the age of subjects in this study.

4.3.1 Comparison between HIV subjects and the control group

There was a significant difference between the stimulated salivary flow rate in subjects with HIV infection and the control group. The values for stimulated salivary flow were grouped into three categories as follows: very low flow < 3.5ml; low flow 3.5-5 ml; and normal flow > 5ml.

Thirty-five subjects (54%) with HIV registered low flow rates for stimulated saliva and 30 subjects (46%) with HIV registered normal salivary flow. In the control group, 5 (15%) subjects registered low stimulated salivary flow and 28 (85%) exhibited normal salivary flow values. Of the 35 HIV infected subjects with low salivary flow, 20 had very low flow readings whilst in the control group only one subject registered a very low salivary flow. The observation that subjects with HIV infection had reduced stimulated salivary flow was statistically significant at $p<0.001$ (Table 8, Figures 27, 28).

TABLE 8

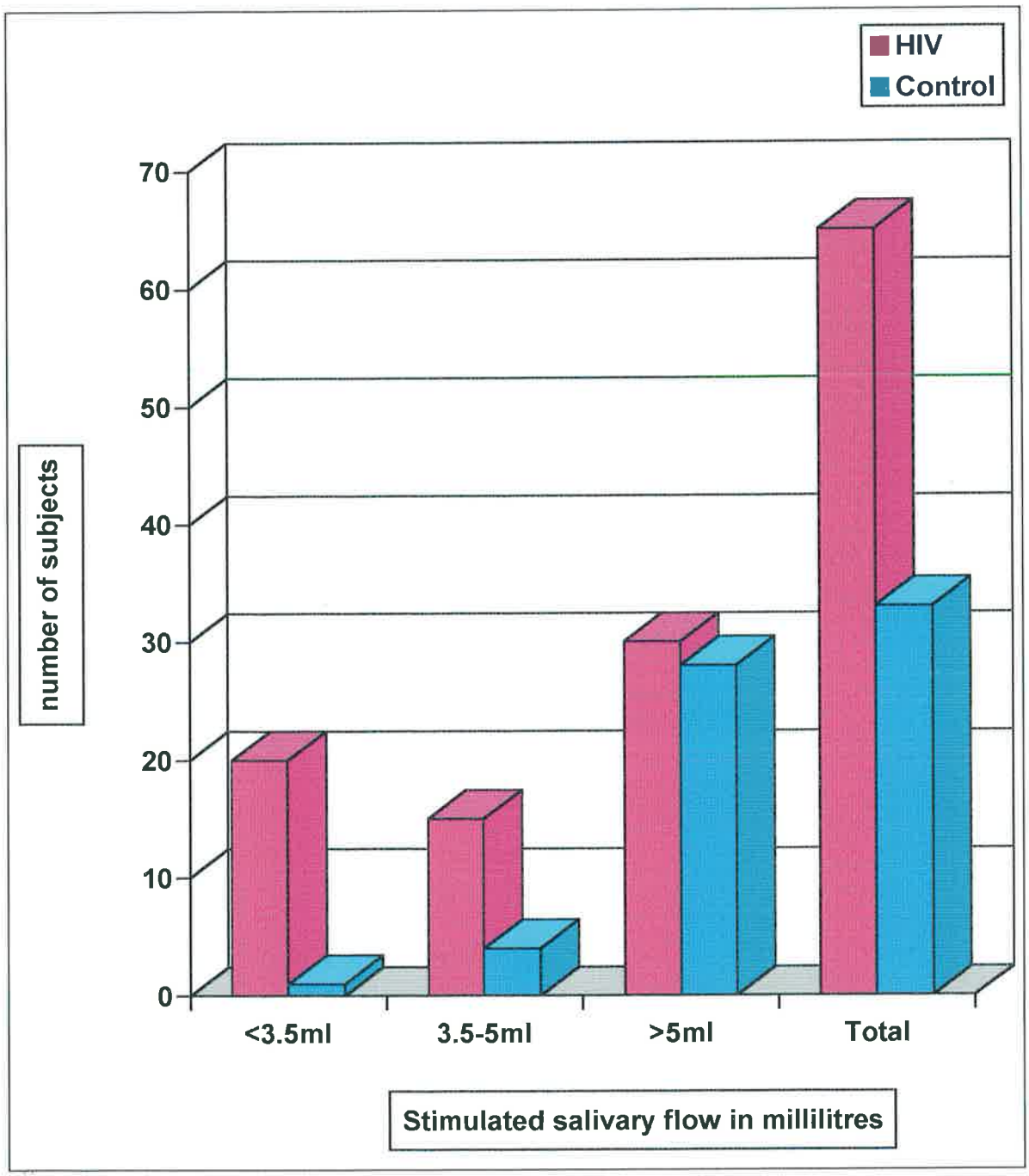
Group	Very Low Flow <3.5 ml	Low Flow 3.5-5 ml	Normal Flow >5 ml	Total no. of patients
HIV	30.8% (n=20)	23.1% (n=15)	46.2% (n=30)	100% (n=65)
Control	3.0% (n=1)	12.1% (n=4)	84.8% (n=28)	100% (n=33)
Total	21.4% (n=21)	19.4% (n=19)	59.2% (n=58)	100% (n=98)

Stimulated salivary flow rates for HIV infected subjects and the control group $p<0.001$ ($X^2=14.3$, 2 degrees of freedom).

Chi-square analysis indicated a significant association between presence of HIV infection and a low salivary flow rate.

Figure 27

Comparison of stimulated salivary flow between control and HIV infected subjects $p<0.001$ D.F.2



4.3.2 Medication related to stimulated salivary flow in HIV subjects

Available data from medical histories for the subjects with HIV infection was analysed to determine if there were other factors that contributed to the observed reduction in stimulated salivary flow rates. The results of analyses revealed no statistical differences between HIV infected subjects taking medication and those not on medication. The type of medication was not related to flow rate reduction (Table 9, Figure 29).

TABLE 9

Medication	Very Low Flow <3.5 ml	Low Flow 3.5-5 ml	Normal Flow >5 ml	Total HIV subjects
none	25% (n=5)	20% (n=3)	26.7% (n=8)	24.6% (n=16)
one	15% (n=3)	26.7% (n=4)	26.7% (n=8)	23.1% (n=15)
poly	60% (n=12)	53.3% (n=8)	46.7% (n=14)	52.3% (n=34)
Total	100% (n=20)	100% (n=15)	100% (n=30)	100% (n=65)

Comparison of stimulated salivary flow rates with medication history in subjects with HIV $p<0.9$ ($X^2=1.411$, 4 degrees of freedom)

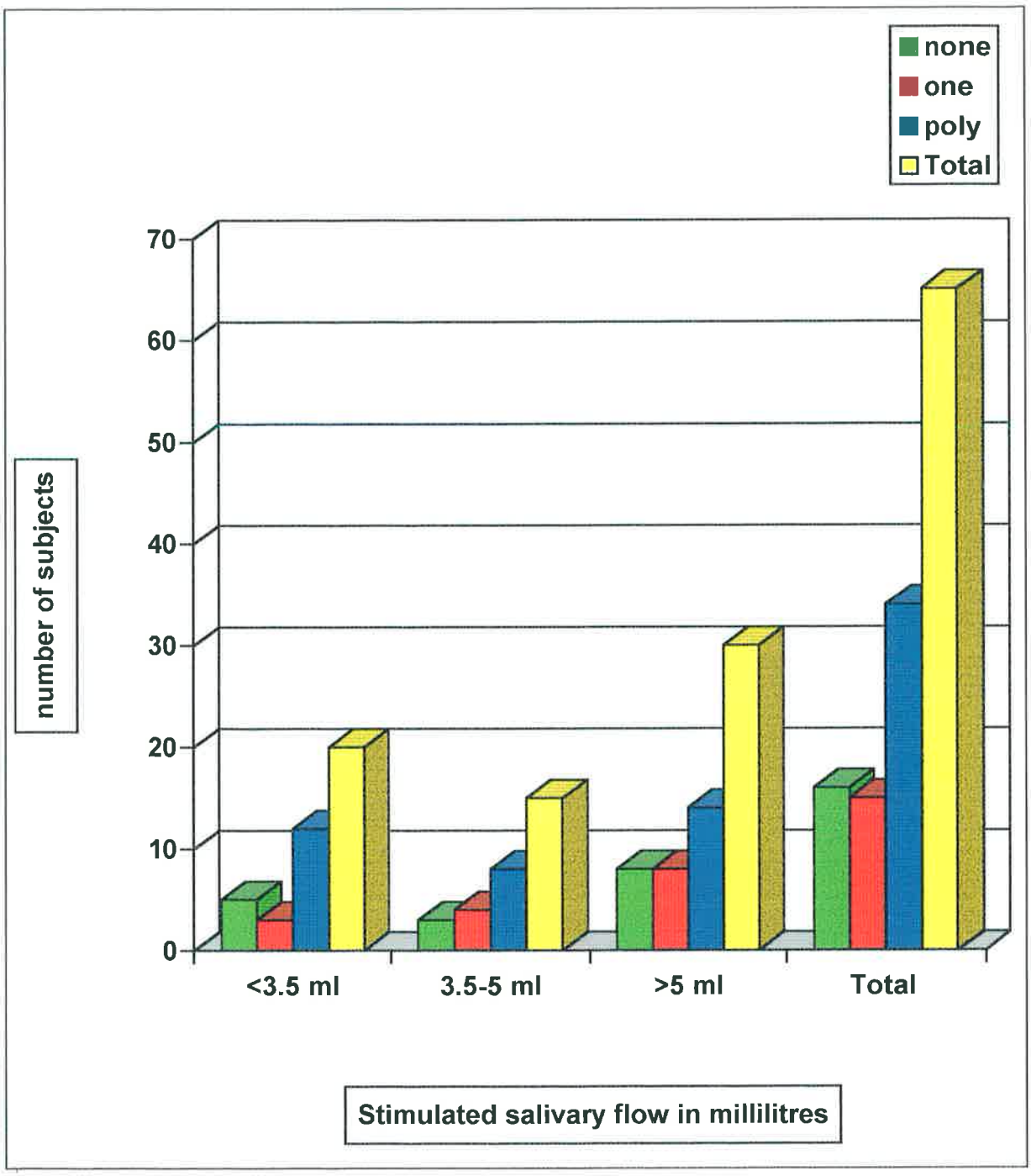
none = no medication

one = one medication

poly = more than one medication

Figure 29

Comparison of stimulated salivary flow rates with medication history in subjects with HIV p< 0.9 D.F.4



4.3.3 Relationship between CD4 cell count and stimulated salivary flow in HIV subjects

The stimulated salivary volume was also evaluated with respect to CD4 cell counts for HIV infected subjects. There was no statistical significant difference in stimulated saliva flow rates for patients with high or low CD4 values (Table 10, Figure 30).

TABLE 10

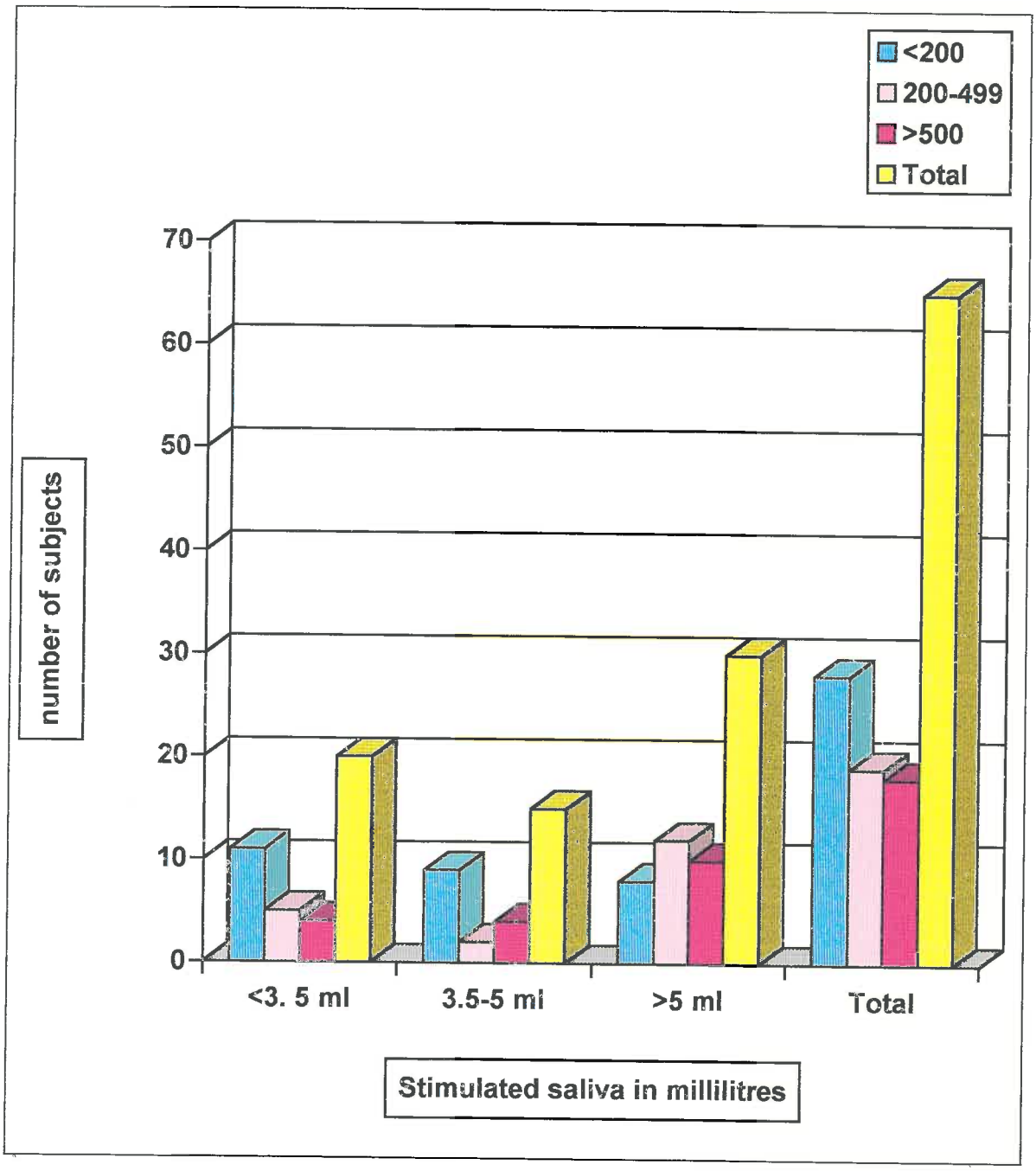
CD4 cell count	Very Low Flow <3.5 ml	Low Flow 3.5-5 ml	Normal Flow >5 ml	Total
< 200 CD4	55% (n=11)	60% (n=9)	26.7% (n=8)	43.1% (n=28)
200 - 499 CD4	25% (n=5)	13.3% (n=2)	40% (n=12)	29.2% (n=19)
> 500 CD4	20% (n=4)	26.7% (n=4)	33.3% (n=10)	27.7% (n=18)
Total	100% (n=20)	100% (n=15)	100% (n=30)	100% (n=65)

Correlation between CD4 cell counts and stimulated salivary flow in HIV infected subjects $p<0.2$ ($X^2=6.920$, 4 degrees of freedom)

Figure 30

The range of CD4 cell counts related to stimulated salivary flow

p<0.2 D.F.4



Tables 11 and 12 detail individual subject CD4 cell counts, medication stimulated salivary flow values, and a postulated cause for the reduction in stimulated salivary flow rates for each of the 35 HIV subjects with reduced stimulated salivary flow.

Abbreviations

Aci	Aciclovir	**Lit**	Lithium
AZT	Zidovudine	**Met**	Methadone
Bac	Bactrim	**Nap**	Naprosan
Clar	Clarithromycin	**PM**	Polymedication
Delta	Delta trial	**Pul**	Pulmacort
DDC	Zalcitabine	**Rif**	Rifampicin
DDI	Didanosine	**Sep**	Septrim
Flu	Fluconazole	**Thal**	Thalidomide
Fun	Fungilin	**Ven**	Ventolin
Hae	Haemophilia	**Vit**	Vitamins
I	Imipramine	**Zyl**	Zyloprim
Itra	Itraconazole	*	AIDS
Ket	Ketoconazole		

TABLE 11

Subject	Volume of saliva	Weight of saliva	CD4 Count	Anti-viral medication	Other medication	Possible Cause
1	0.6	1.49	20*	AZT/DDI	Flu/bac	AIDS/PM
2	1.8	1.99	400	NIL	NIL	Hep B
3	1	0.46	396	AZT	Sep/Achy	PM
4	1.9	1.7	300	AZT	NIL	High VL
5	2	2.15	70*	DDI	Itraconazole	AIDS/PM
6	3	3.19	460	AZT	Ket/Aci	PM
7	2	2.4	70*	AZT	NIL	AIDS/Haem
8	2.9	3.06	80*	AZT/DDI	Bac	AIDS/PM
9	2	2.11	90*	NIL	Flu/Fun	AIDS
10	1.5	1.39	1100	NIL	met	Med/PM
11	2.7	2.87	250*	DDI	NIL	AIDS
12	3	3.03	100*	AZT	Aci/Flu/Bac	AIDS/PM
13	1.7	1.72	170*	DDC/AZT	Aci	AIDS/PM
14	3.2	3.7	260	DDC	Flu	PM
15	2.3	3.03	290	NIL	Nil	?
16	3	2.93	90	DDI	Bac.Lit.I	AIDS/PM
17	0.7	0.5	220	AZT	Zyl.Nap.Ven.Pul	PM.VL
18	3	3.1	689	NIL	NIL	?
19	2.6	3.48	550	NIL	NIL	?
20	1.8	1.4	600	NIL	Met	Medic

Saliva characteristics of 20 HIV infected subjects with very low stimulated salivary flow rates relative to CD4 count and medication

TABLE 12

Subject Low flow	Volume of saliva	Weight of saliva	CD4 count	Anti-viral medication	Other Medication	Possible cause
1	4.4	4.93	540	DDC/AZT	Bac, Achy	Hep C
2	3.5	4.3	362	NIL	NIL	**TIME**
3	4.1	4.03	100	AZT	Thal, Cla	AIDS, PM
4	4.1	4.37	800	NIL	NIL	Cleft palate
5	3.5	3.31	44	AZT, Delta	Nil, Vit	AIDS
6	4.9	4.93	0	NIL	Bac	AIDS
7	3.5	3.63	10	NIL	NIL	AIDS
8	3.5	3.76	28	AZT	Bac, Achy	AIDS, PM
9	4.6	4.96	600	DDI	Achy, Bac, Rif	AIDS, PM
10	4.7	5	420	AZT	NIL	**TIME**
11	4.7	4.52	177	AZT	Flu, Bac	AIDS, PM
12	3.5	3.9	700	AZT	NIL	Sero C
13	3.8	4.36	0	nil	Clar,Rif,Flu	AIDS/PM
14	4.1	4.59	600	nil	Flu	?
15	3.5	3.67	10	nil	Sep,Met,Fun	AIDS/PM

Saliva characteristics of 15 HIV infected subjects with low stimulated salivary flow rate relative to CD4 count and medication

Reference values for low stimulated salivary flow are :

0.7 - 1 ml / min (3.5 - 5 ml / 5 min)

4.3.4 Presence of oral manifestations related to stimulated salivary flow levels

Four oral manifestations (candida, periodontal disease, caries, erosion) were evaluated with respect to stimulated salivary flow rates for the sixty five HIV subjects. There was a marked tendency for increased caries in the HIV group with low stimulated salivary flow rate, but this tendency was not evident in the very low stimulated salivary flow group. Overall, there was a greater chance of carious teeth when the stimulated flow rates were less than accepted normal values (Table 13, Figure 31).

TABLE 13

Caries	Very Low Flow <3.5 ml	Low Flow 3.5-5 ml	Normal Flow >5 ml	Total
Yes	30% (n=6)	60% (n=9)	16.7% (n=5)	30.8% (n=20)
No	70% (n=14)	40% (n=6)	83.3% (n=25)	69.2% (n=45)
Total	100% (n=20)	100% (n=15)	100% (n=30)	100% (n=65)

Caries of teeth related to stimulated salivary flow in subjects with HIV

$p<0.02$ ($X^2=8.823$, 2 degrees of freedom)

There were no statistically significant correlation found between periodontal disease, candida or erosion and stimulated salivary flow rates (Table 14, Figures 32-34). For subjects with a low flow rate, there were as many who did not develop candida as those who did. Furthermore, a low stimulated salivary flow rate did not necessarily correlate with lower pH levels.

Table 14 shows the distribution of manifestations for the 65 subjects with HIV infection in relation to stimulated salivary flow.

TABLE 14

Manifestation	No. of Subjects very low flow (20)	No. of Subjects low flow (15)	No. of Subjects normal flow (30)
Candida	6	7	8
Periodontal	9	8	13
Caries	7	8	5
Erosion	6	7	9

Oral Manifestations recorded for 65 subjects with HIV

Figure 31

Caries of teeth related to stimulated salivary flow in subjects with HIV $p<0.02$ ($X^2=8.823$, 2 degrees of freedom)

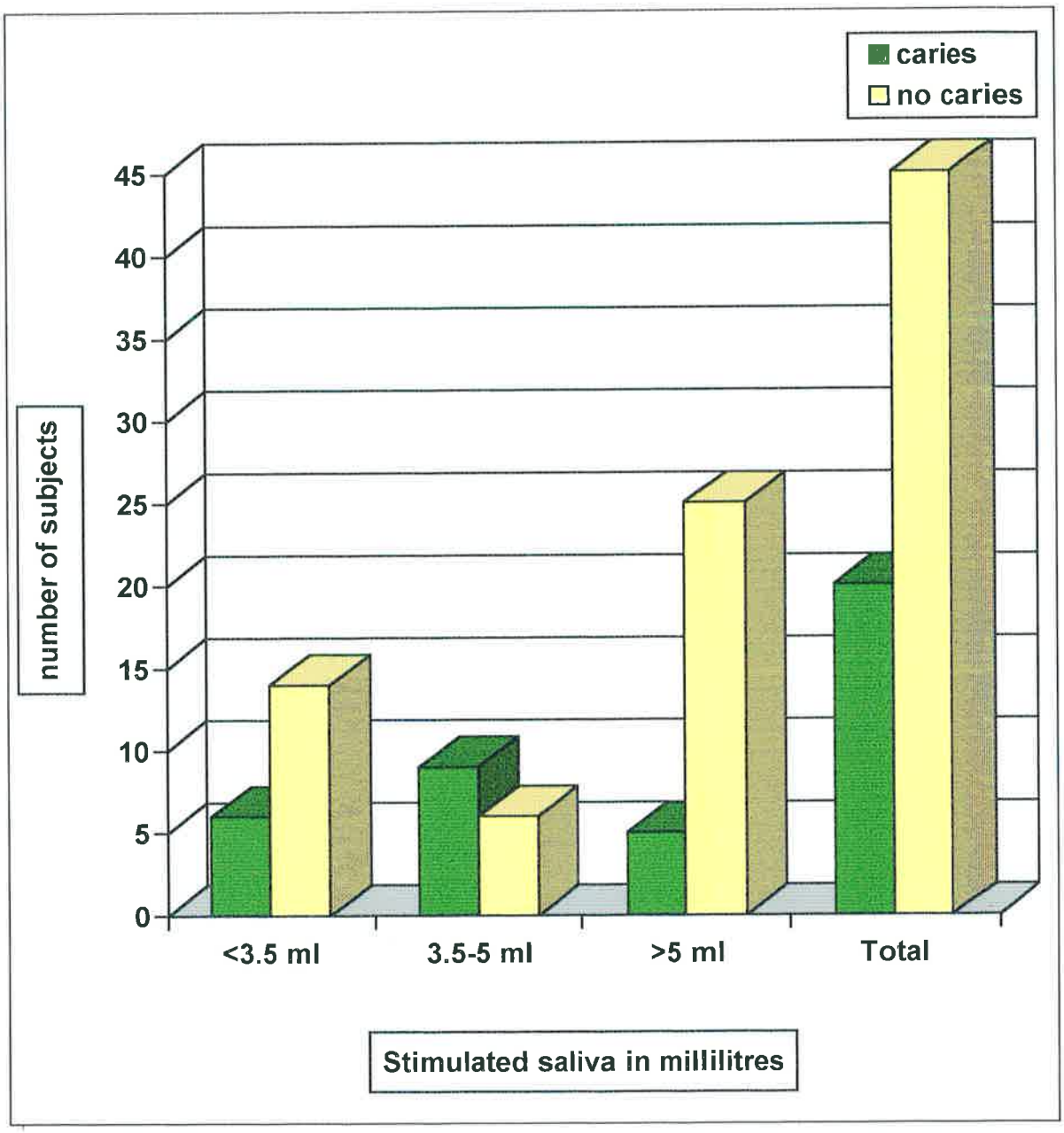


Figure 32

Periodontal manifestations related to stimulated salivary flow

p<0.6 D.F.2

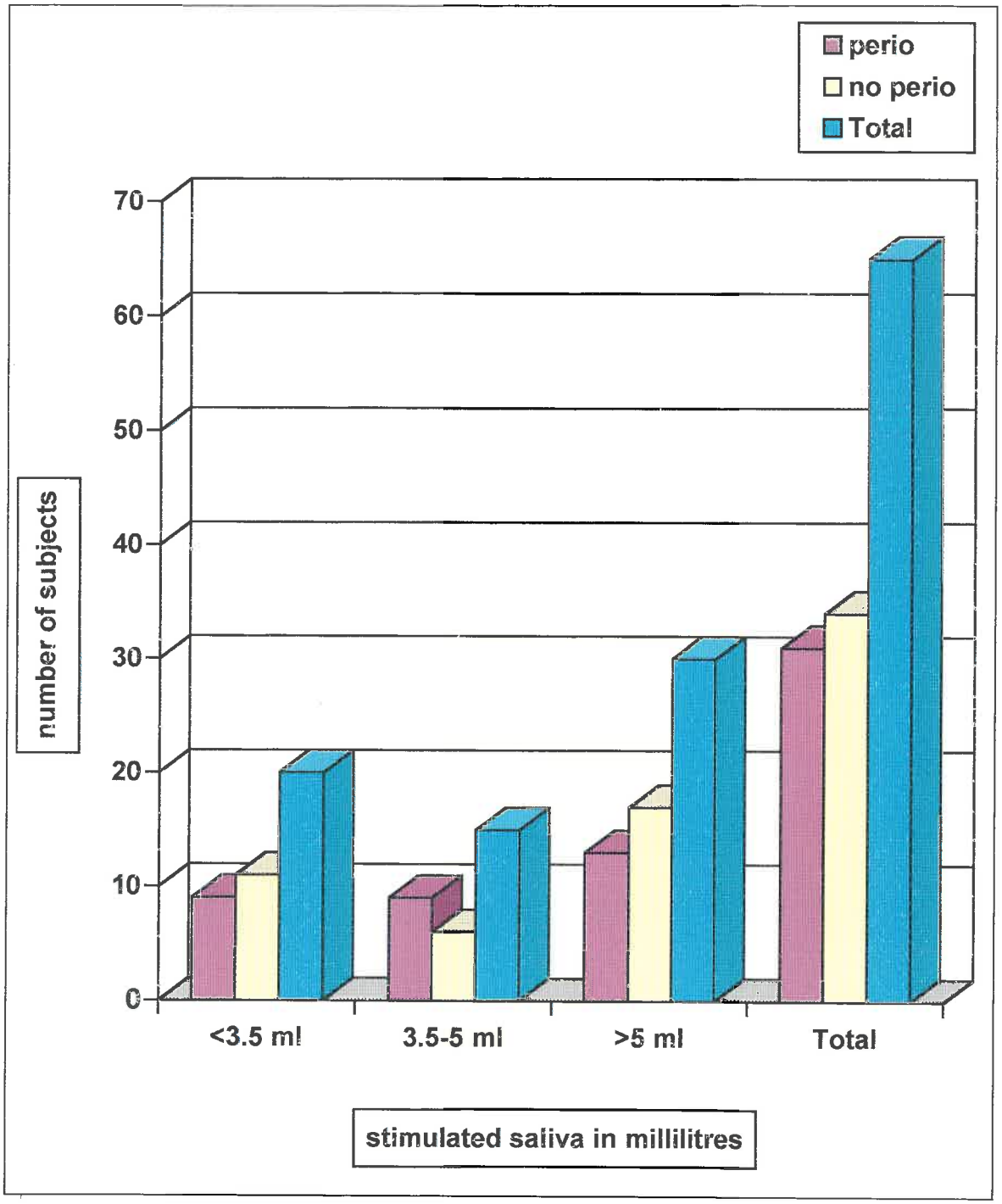


Figure 33

Candida in HIV subjects related to stimulated salivary flow

p<0.2 D.F.2

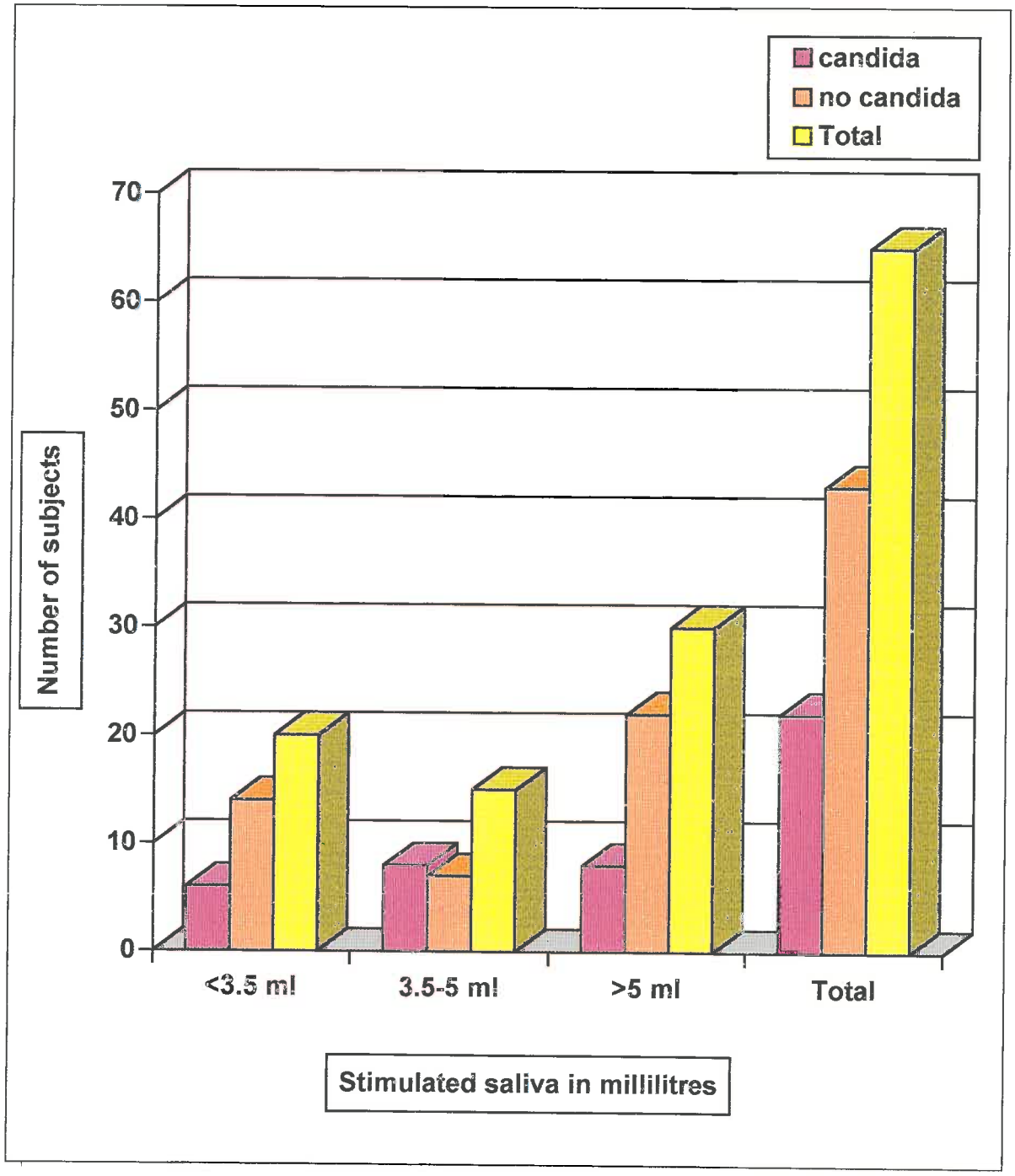
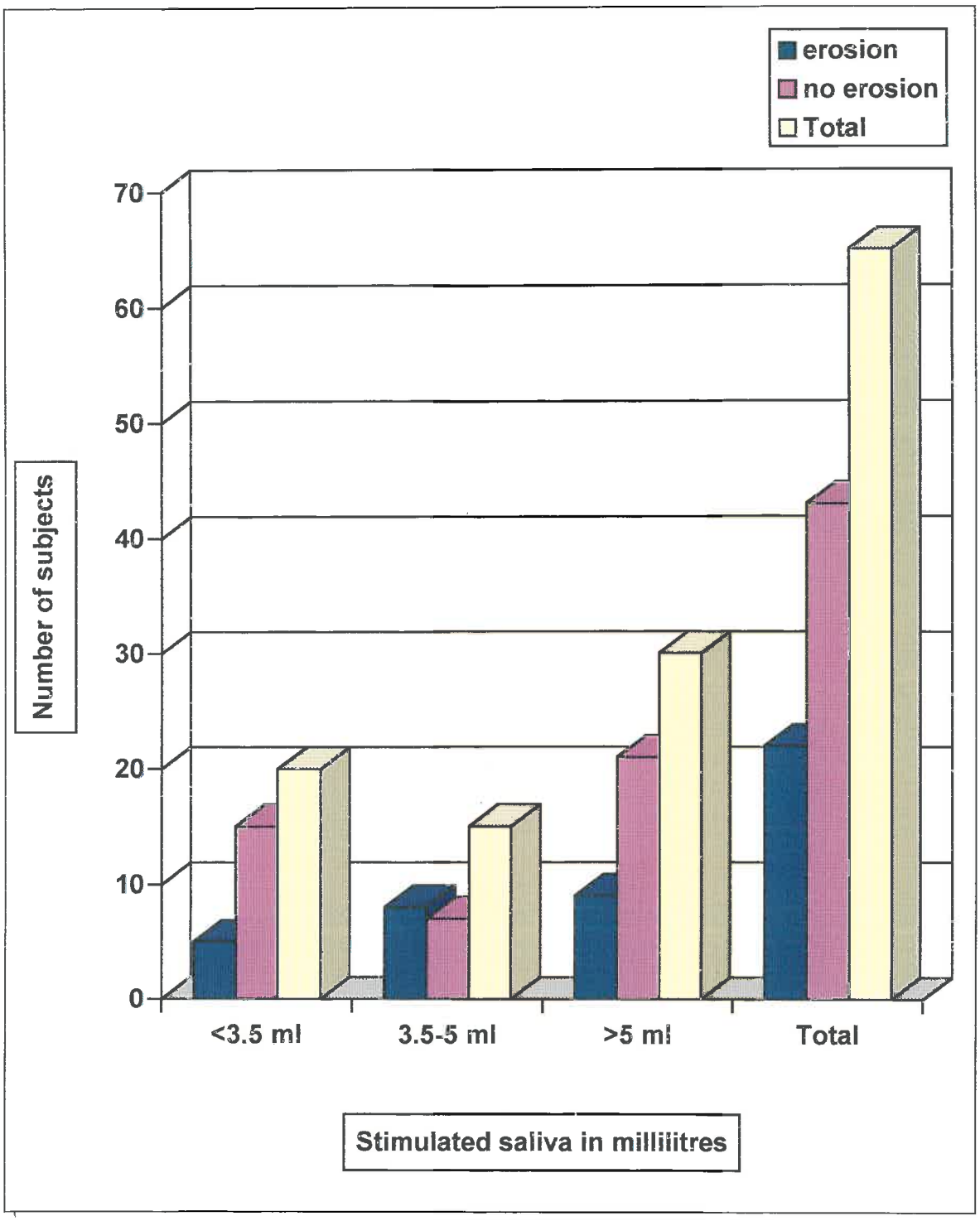


Figure 34

Erosion relative to stimulated salivary flow. p<0.2 D.F.2



4.4 RESTING SALIVARY FLOW RATE

Thirteen (20%) of 65 subjects with HIV recorded very low or low resting salivary flow rates (Table 15). The expression of the four selected oral manifestations in subjects with low and very low resting phase salivary flow subjects is shown in Table 16.

Fifty two (80%) of the 65 subjects with HIV infection recorded normal resting salivary flow rates. Candida infection was present in 15 of these subjects, 15 had dental caries, 18 had some degree of erosion, and 23 showed periodontal disease. Twenty six of the subjects were polymedicated and 15 subjects had more than one oral manifestation of HIV (Table 16).

The resting salivary flow rate was recorded for 21 of the 33 control subjects who did not have HIV infection. All 21 subjects recorded normal resting flow rates, including one subject who had undergone intensive medical treatment for a metastatic brain tumour three years earlier.

TABLE 15

Subject	Volume of saliva	Weight of saliva	CD4 count	Anti-viral Medication	Other medication	Possible cause
1	0.1	0.63	20	AZT/DDI	Flu,Bac	AIDS/PM
2	0.25	0.35	1100	NIL	Meth,Amil	PM
3	1	.81	396	AZT	Sept,Aci	PM
4	0.75	0.77	300	AZT	NIL	Viral L
5	1.2	2.98	44	AZT/Delta	Nil, Vit	AIDS
6	0.7	0.82	600	DDI	Acy,Bac,Rif	AIDS
7	0.5	2.03	98	DDC	Aci, Flu	AIDS/PM
8	0.5	0.72	170	DDC,AZT	Achy	AIDS/PM
9	1.2	0.99	0	NIL	Clar,Flu,Rif	AIDS/PM
10	1	2	90	DDI	Bac,Lit,Imi	AIDS/PM
11	1.2	1.3	220	AZT	Zyl,Nap,Pul,V	PM
12	1	1.39	689	NIL	NIL	?
13	0.7	1.27	10	NIL	Sep,Meth,Fu	AIDS

Subjects with HIV (13/65) with low and very low Resting Salivary Flow rates

Abbreviations

Aci	Aciclovir	**Hae**	Haemophilia	**Rif**	Rifampicin
AZT	Zidovudine	**I**	Imipramine	**Sep**	Septrim
Bac	Bactrim	**Itra**	Itraconazole	**Thal**	Thalidomide
Clar	Clarithromycin	**Ket**	Ketoconazole	**Ven**	Ventolin
Delta	Delta trial	**Lit**	Lithium	**Vit**	Vitamins
DDC	Zalcitabine	**Met**	Methadone	**Zyl**	Zyloprim
DDI	Didanosine	**Nap**	Naprosan	*	AIDS
Flu	Fluconazole	**PM**	Polymedication		
Fun	Fungilin	**Pul**	Pulmacort		

TABLE 16

Manifestation	No. of Subjects Very Low Flow (2)	No. of Subjects Low flow (11)	No. of Subjects Normal flow(52)
Candida	1	4	16
Perio	0	7	14
Caries	1	4	16
Erosion	0	5	22

Expression of candida, periodontal disease, caries and erosion in 65 subjects with HIV correlated to Resting Phase Salivary Flow Rate

4.5 RESTING SALIVARY WEIGHT

The reference value for normal resting mean weight is given as 0.47g / min or 2.35g / 5min (Tenovuo 1989).

Twenty one (32%) of the 65 HIV infected subjects recorded salivary weights of less than 2.35g / 5min (Table 17).

Three (14%) of the 21 non HIV infected group recorded weights less than 2.35g / 5min, including the one subject with a medical condition (Table17).

TABLE 17

Subjects	<2.35gm / 5min	>2.35gm / 5min	Total
HIV	21	44	65
Control	3	18	21

Resting salivary weight for control and HIV-infected subjects

4.6 SALIVA CALCIUM LEVELS

No statistical difference was found for saliva calcium levels between the control group and the HIV-infected patients. Furthermore, there were no significant differences between the calcium values for stimulated or unstimulated saliva samples in either group.

There were some low values for calcium in both groups. There were 11 subjects with HIV (out of sixty-five individuals) who had salivary calcium values less than 0.2millimoles. In the control group, there were four subjects with similar readings. Five of the eleven HIV infected subjects with low calcium values had AIDS. Of the other six HIV subjects with low salivary calcium values, three had high CD4 counts well within normal healthy values.

4.7 PROTEIN ANALYSIS

Salivary proteins were identified by electrophoretic migration in sodium dodecylsulfate on a polyacrylamide gel (4-20%) and comparing the resultant electrophoretic bands with protein standards (Bio-Rad).

Results from this study demonstrated differences both in numbers and density of electrophoretic bands between patients with HIV infection and control subjects.

The saliva samples from the patients with HIV infection expressed more proteins than the saliva samples from the control subjects. This was demonstrated by the presence of electrophoretic bands over a broad range of molecular weights, from 200kD to 6.5kD in the HIV positive samples and absence of those same bands from the control group samples.

One of the proteins, more likely to be found from HIV positive salivary samples, was represented on gel electrophoresis by molecules with a nominal molecular weight of about 14kD when compared with the electrophoretic standard. Ninety percent of patients with HIV infection demonstrated this protein in contrast to only 30% of control subjects.

An even smaller protein (MWt 6.3kD) was also prevalent in saliva from patients with HIV infection (69%) with the co-migrating band found rarely in saliva from control subjects (9.5%).

Similarly, proteins co-migrating with a 21kD standard were strongly represented in the saliva from patients with HIV infection. Ninety percent of these patients demonstrated the protein with this notional molecular weight whereas only 33% of saliva samples from control subjects showed a band at this level. In addition, the intensity of the electrophoretic bands in the control saliva was reduced.

Three bands consistently co-migrated together in the region of the 31kD marker. The proportion of patients with HIV secreting saliva with these proteins was similar to that of control subjects, but greater than 20% of the control subjects secreted more of the faster of the three bands.

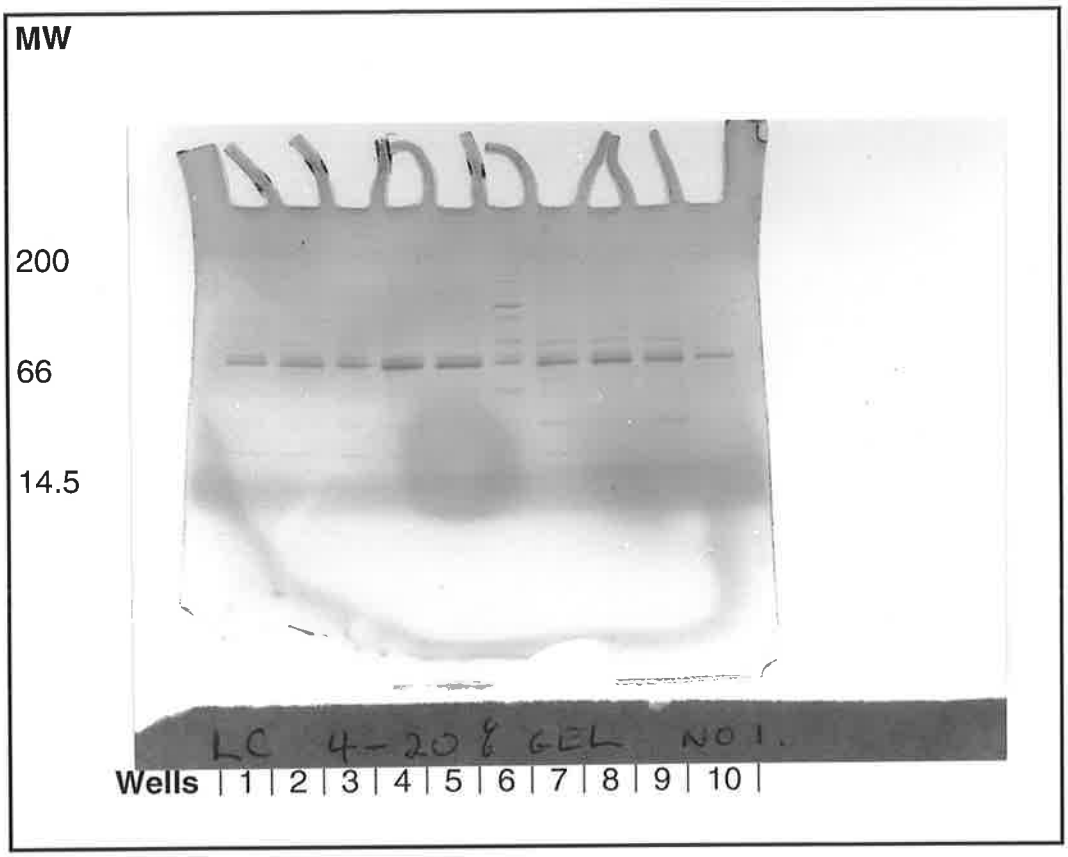
Thirty percent of saliva samples from patients with HIV infection contained proteins co-migrating with 94 and 116 kD standards, while 15% of saliva samples from control subjects contained these proteins.

Ten percent of patients with HIV infection secreted proteins of greater than 200kD. None of those proteins was detected in saliva from control subjects.

Proteins between 45, 66, and 80kD appeared ubiquitous in saliva from both control subjects and patients with HIV infection.

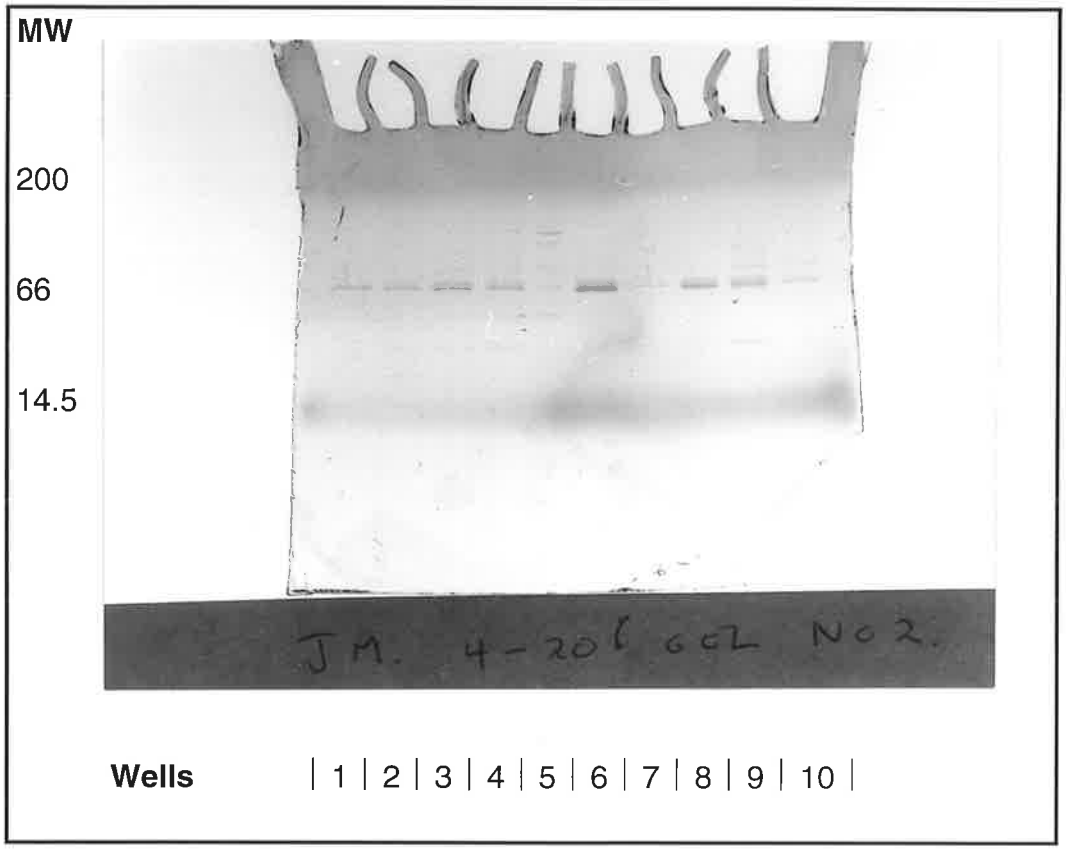
Overall the intensity of electrophoretic bands, at all molecular weight standards, was more marked in saliva from HIV patients than that from control subjects.

Figure 35



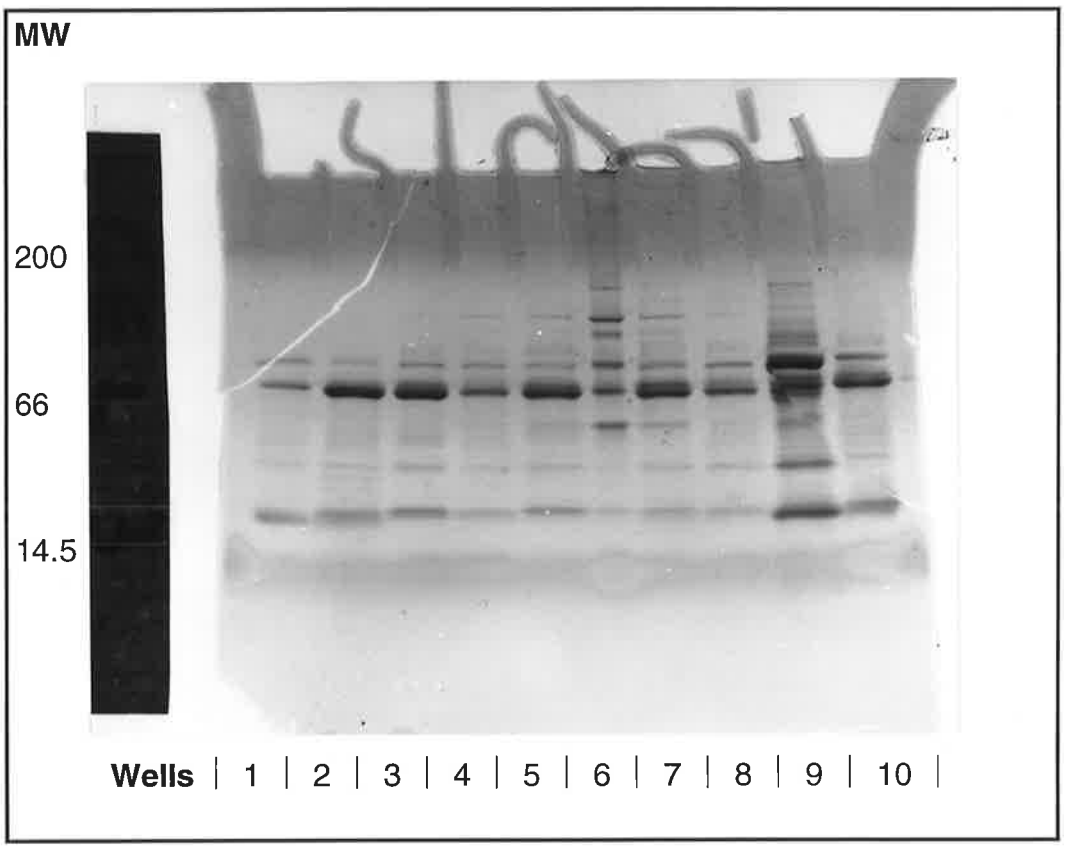
Gel 1; Electrophoresis gel of saliva samples from one subject with HIV; well number 9, and nine control subjects in wells; 1-5, 7-8, and 10. The Bio-Rad standard marker is in well 6. The one subject with HIV, represented in well 9, was polymedicated and had a history of candidiasis in the past. However he had an extremely good salivary flow rate 8.5ml/5min, normal unstimulated salivary pH of 6.5 and a high CD4 count. There is no significant difference in the protein migration of this subject with that of the control subjects.

Figure 36



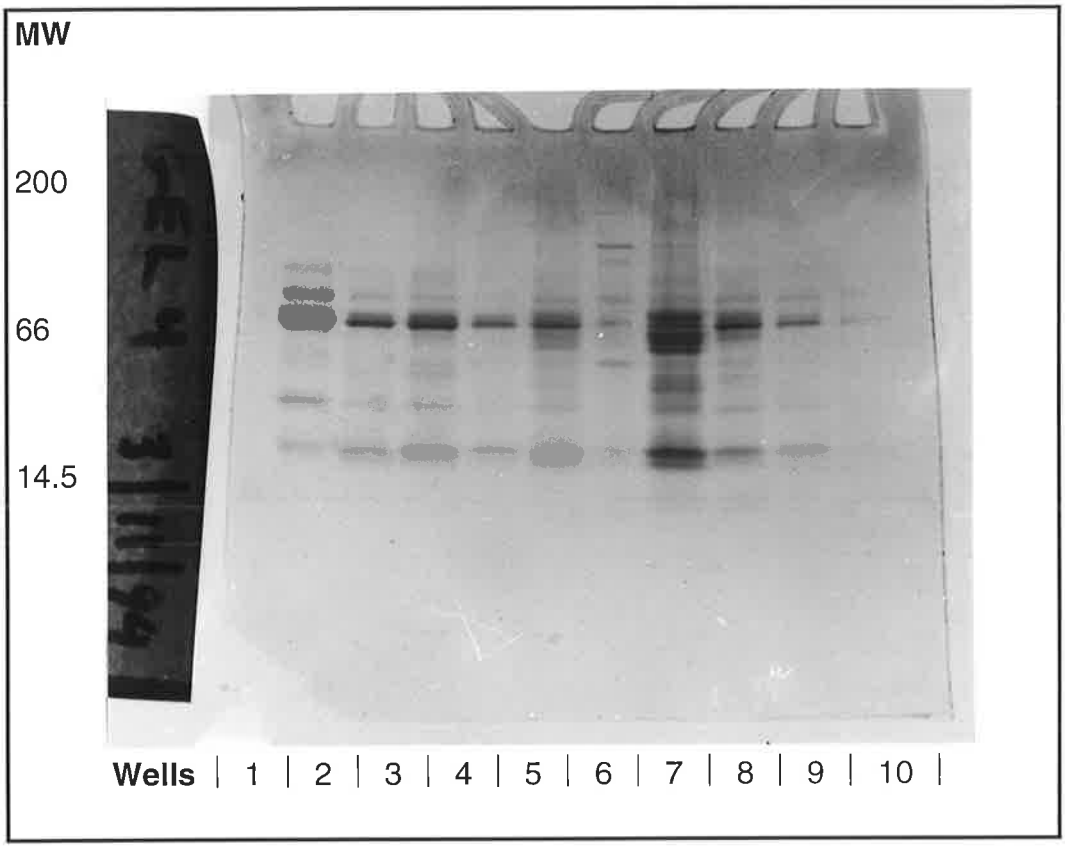
Gel 2; Electrophoresis gel of saliva samples from nine subjects with HIV; well numbers1-4, 6-10. The Bio-Rad standard marker is in well number 5 Although the bands are lightly stained in this gel, the density of the bands at the 21kD area compared to the protein standard is evident. This gel was electrophoresed and then stained concurrently with gel one, and both numbers of bands and density of bands are more pronounced in this gel containing saliva samples from HIV infected patients compared with gel one which contained saliva samples predominantly from control subjects.

Figure 37



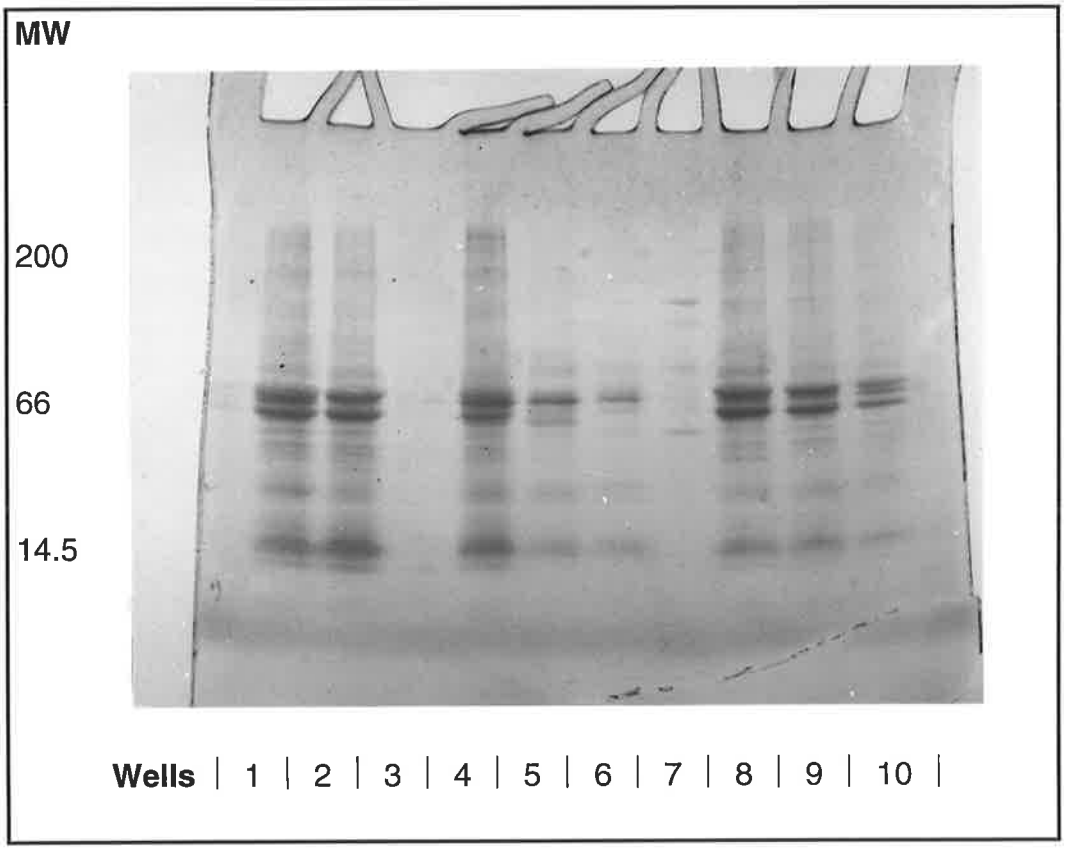
Gel 3; Electrophoresis gel of saliva samples from eight subjects with HIV; well numbers 1-5, 7-9, and one control subject in well number 10. The standard marker is in well 6. There is a heavy band approximating the nominal molecular weight of about 14kD which is particularly intense in well 9. This saliva sample came from a patient with AIDS who had reduced salivary flow. Samples in wells 1, 2, 5 and 7 also came from patients with AIDS and demonstrated a broad spectrum of bands, many of marked intensity.

Figure 38



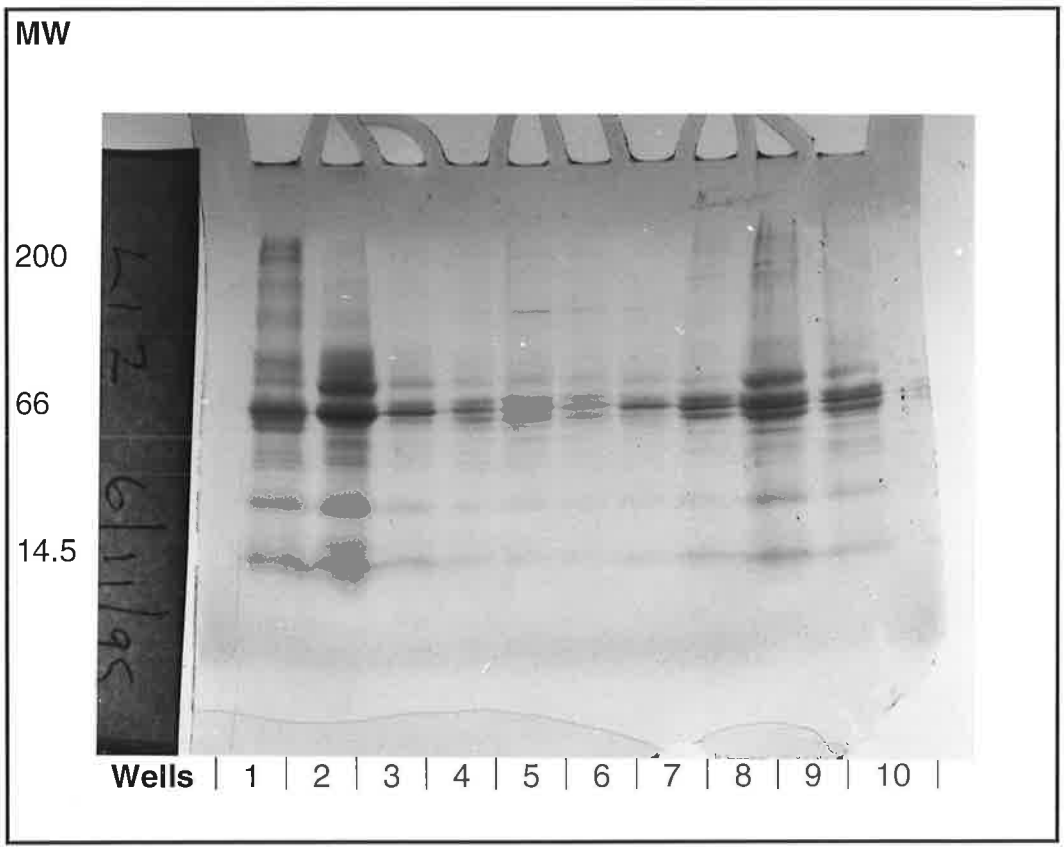
Gel 4; Electrophoresis gel of saliva samples from eight subjects with HIV; well numbers 1, 3-5, 7-10, and one control subject in well number 2. The standard marker is in well 6. The protein band of nominal molecular weight of about 14kD is again well represented on this gel and the subjects with HIV demonstrate a wider diversity of bands, and of greater intensity, than the control subject in well 2. The subjects represented by wells 3 and 5 both had AIDS and show strong band density at both the 14kD and 31kD region. The subject represented by well number 7, demonstrating extremely dense banding, had very low salivary flow, 1ml/5min, had erosion of teeth, was polymedicated, had normal resting phase pH and had a CD4 count of 396.

Figure 39



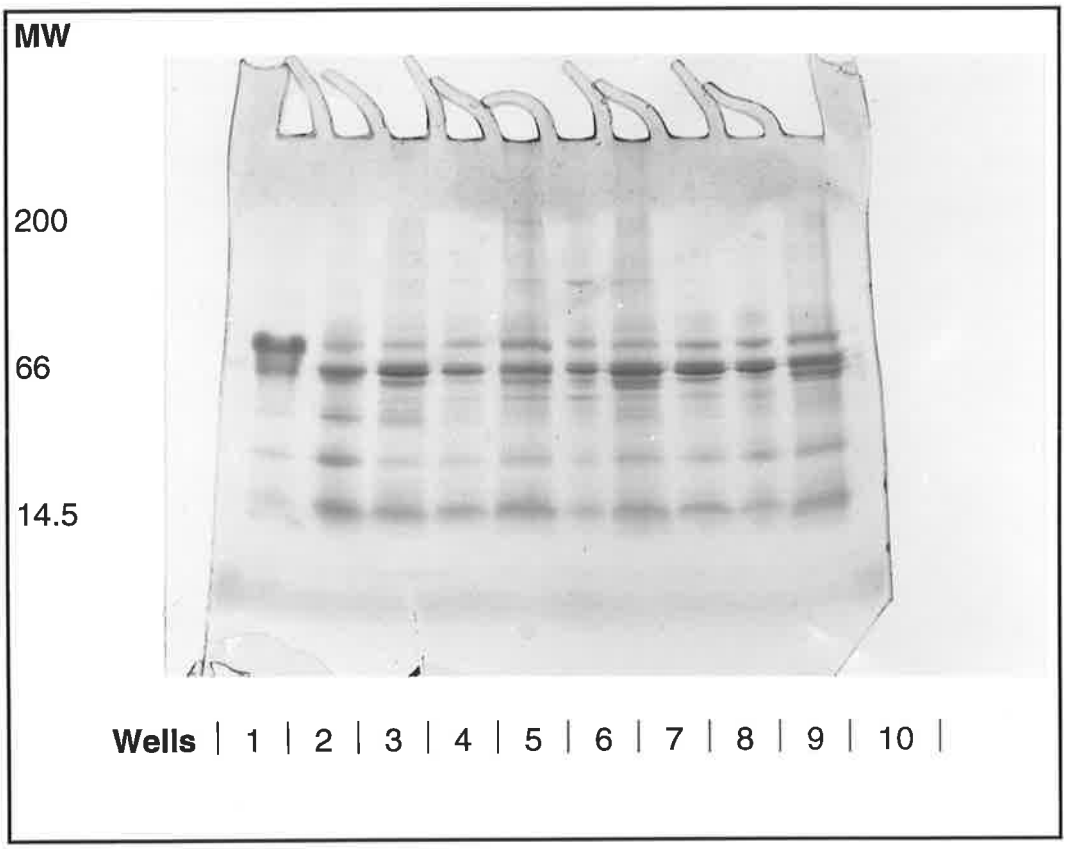
Gel 5: Electrophoresis gel of saliva samples from eight subjects with HIV (well numbers 1-4, 6, 8-10) and one control subject (well number 5). The standard marker is in well 7. The saliva sample in well one was from a female patient with AIDS and the sample in well two was from a patient recently diagnosed with HIV, who had a very low stimulated salivary flow, 1.9ml/5min, a low resting phase salivary pH (5.9) and a CD4 count of 300. Both these samples demonstrate dense banding at the low molecular weight range. The saliva sample represented in well 3 appears to have been inadequate with no evidence of any protein banding. The sample in well 4 was from a patient who had seroconverted with HIV as early as 1980, and has proven to be a non-progressor with a CD4 count of 1100. He takes multiple medication, including methadone and amyltryptilline and he registered a very low stimulated salivary flow.

Figure 40



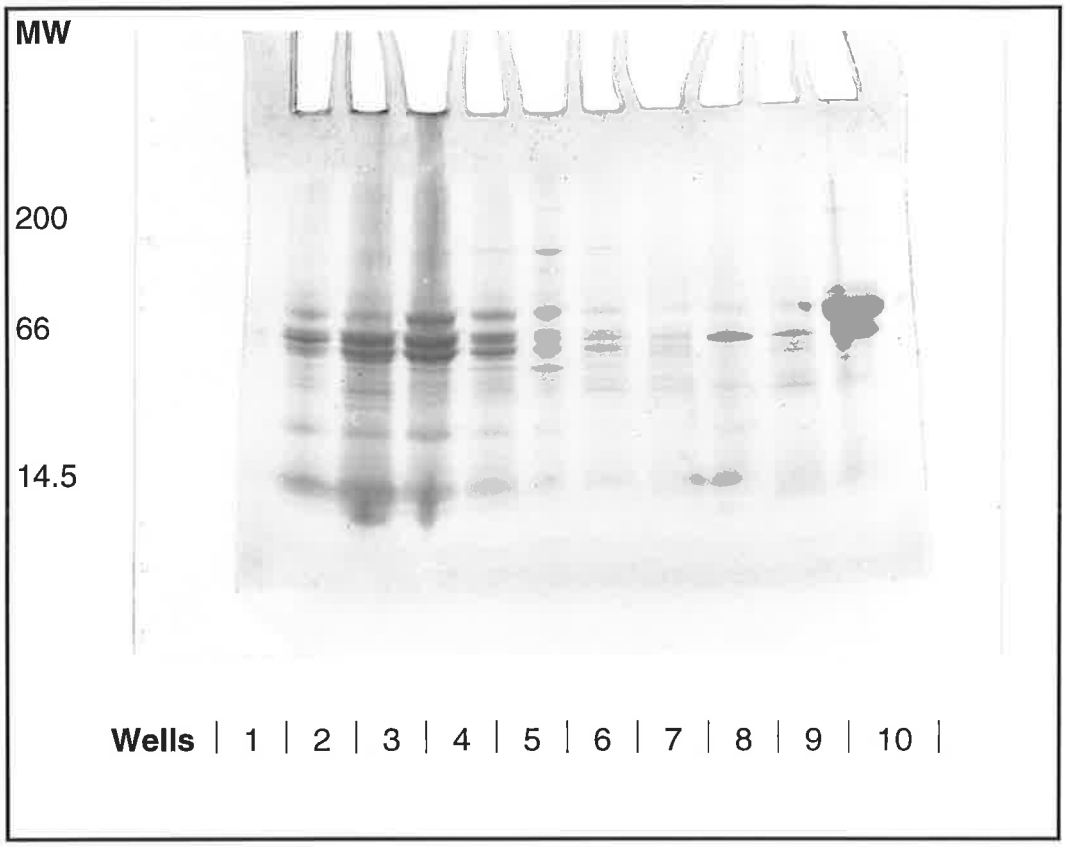
Gel 6; Electrophoresis gel of saliva samples from eight subjects with HIV; well numbers 1-4, 6, 8-10, and one control subject in well number 7. The standard marker is in well 5. The protein bands are particularly intense in wells numbers 1, 2, 9 and 10. The saliva samples in all these wells were taken from patients with AIDS.

Figure 41



Gel 7 Electrophoresis gel of saliva samples from eight subjects with HIV; well numbers 2-5, 7-10. The protein bands in well number one are derived from a urine sample. The standard marker is in well number 6. Protein bands of nominal molecular weight of about 14kD are well represented.

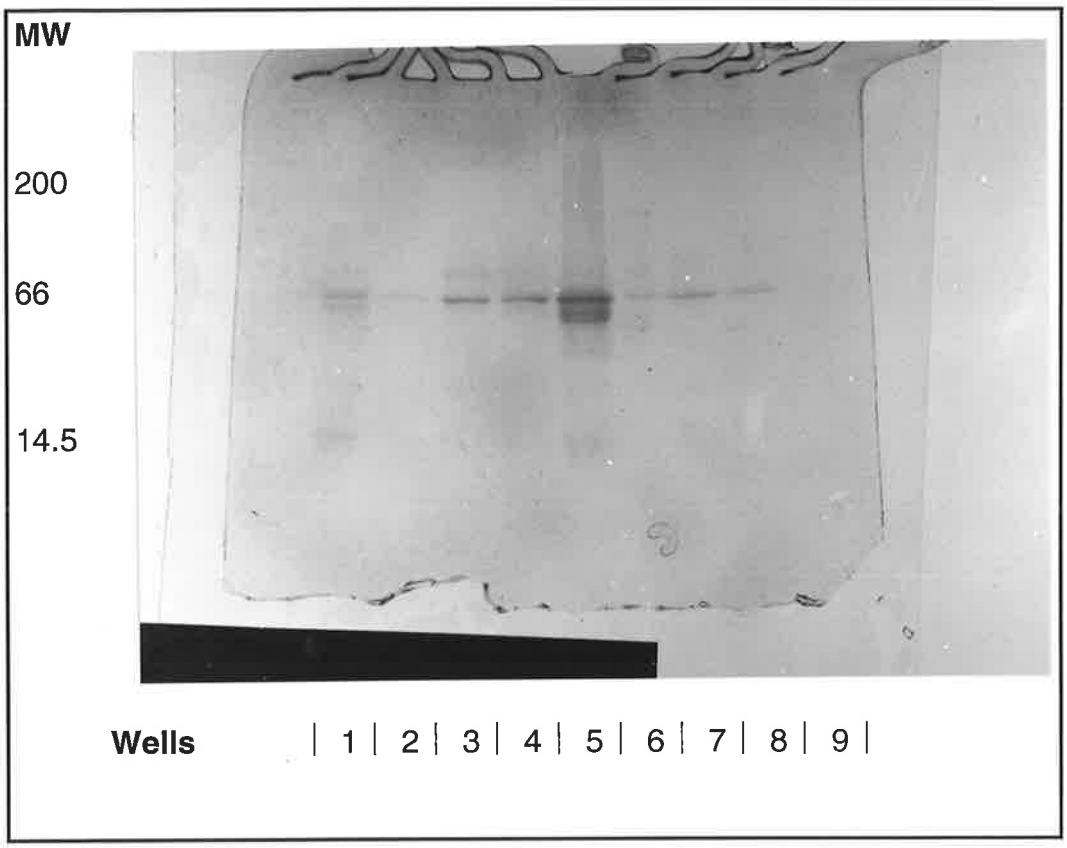
Figure 42



Gel 8; Electrophoresis gel of saliva samples from three subjects with HIV; well numbers 1-3, and from five control subjects in wells 4 and 6-9. The protein bands in well number ten are derived from a urine sample. The standard marker is in well 5.

The differences between salivary samples from the HIV patients and control subjects are highlighted in this gel. The intensity of bands is marked in wells 1-3 and there appears to be stronger representation of protein bands at all ranges of molecular weights.

Figure 43



Gel 9; Electrophoresis gel of saliva samples from eight control subjects in wells; 1-5, and 7-9. The standard marker is in well 6. The proteins have stained poorly on this gel. The saliva sample in well nine was derived from a subject who had received radiation treatment for a brain tumour. No bands are evident in this well. This patient had low salivary flow. The saliva in well 5 demonstrates intense protein banding compared to that of other wells. The subject from whom this saliva was collected registered a low resting phase salivary pH.

CHAPTER 5

DISCUSSION

Introduction

The results of this study highlight the difficulties in establishing and then proving hypotheses when the subject matter, saliva, is associated with so many inherent variables. For instance, although collection of saliva samples was standardised, the results of this study for many of the investigations show little conclusive evidence of change in saliva for many of the HIV-infected patients. However, that no statistical correlations between HIV infection and salivary changes were proven in these patients does not necessarily indicate that they have normal saliva production, merely that the samples collected at that time failed to demonstrate any change. Conversely, for those patients where significant changes were discovered, repeat sampling could have resulted in a sample approximating one of normal values. However, even allowing for *inter* and *intra* individual variation in saliva (Ferguson 1989; Söderling 1989; Rudney 1995), the results of the project have identified definite trends indicating an impact by HIV infection on saliva quality and quantity. Other investigators (Tenovuo *et al.* 1987; Ferguson 1989; Schiødt *et al.* 1992; Scannapieco 1994) have shown that chronic and acute systemic conditions impact on oral health, and on saliva production in particular, and in general this study has supported those findings.

The hypothesis that there might be a relationship between specific oral manifestations of HIV and saliva quantity and quality has been demonstrated in this study for some of the specified manifestations such as candida and the parameter of salivary pH. Interestingly, however, the lack of conclusive evidence for the presence of certain other oral diseases relative to salivary changes is of

possibly equal significance. Where definite correlation was anticipated, for example the presence of candida or caries associated with poor salivary flow, the results of this study show little or no evidence to support any relationship.

Other investigators (Ficarra 1992; Schiødt 1992; Gala and Fulcher 1996; Challacombe and Sweet 1997) have determined that salivary gland disease and resultant xerostomia is a problem with HIV infection although the reported incidence of these complications varies widely (Greenspan *et al.* 1990; Ficarra 1992; Laskaris *et al.* 1992; Schiødt 1992; Barr 1994). This study attempted to verify if there was any significant variation in saliva quality and quantity between early and late stage HIV infection. Challacombe and Sweet (1997) in their studies found distinct discrepancies in salivary secretory immune response in late stage HIV infection indicating that saliva production is definitely altered as immune function deteriorates. Furthermore, there is evidence of a definite correlation between CD4 counts and the presence of symptomatic opportunistic infection in HIV disease (Glick *et al* 1994) suggesting that the findings of the present study should support the concept of salivary changes occurring more frequently as people progress towards AIDS. However, the results of this study indicate that changes in saliva, for example low pH of resting saliva (Table 3), low stimulated salivary flow rate (Table 8), and the presence of HIV-related oral manifestations (Tables 14, 16) often appear to occur when immune function is adequate and therefore they are unrelated to the stage of HIV infection.

Subject age and gender

The mean age of HIV-infected subjects and that of the control group varied by 4 years. No significant changes in salivary flow or pH, with respect to age or sex, were noted when comparing the control group and those infected with HIV. In addition no differences in salivary flow or pH were found on the basis of age and sex when individuals were compared from within the same group, either the control subjects or those with HIV. This finding is consistent with those of other investigators (Söderling 1989; Rudney 1995).

A predominance of male subjects was selected for the control group as the majority of HIV-infected subjects were male. Only six female subjects took part in this investigation, three with HIV infection and three in the control group.

The number of females in the study was too low to provide information of any statistical significance with respect to parameters such as variation in saliva quantity or quality resulting from gender difference. The three females with HIV infection showed similar variation in saliva flow and pH as did the HIV-infected male subjects. The three female control subjects had similar salivary flow and pH values as the male subjects.

Salivary pH

The salivary pH data in this project were assessed by comparing them with "standard values" from previous studies. On this basis and for the purpose of the present study a stimulated saliva pH of less than 5 was considered low, greater

than 5 was normal and greater than 7 was considered normal or high (Ericsson 1976; Birkhed and Heintze 1989; Appendix 8). A resting salivary pH of 6.5 and above was taken as normal for unstimulated saliva (Levine 1989c).

The salivary pH readings in this study were performed using litmus paper with a sensitivity between pH 5 and pH 8. This provided reasonably accurate measurements within this range. However it did not allow for evaluation of subjects with very low unstimulated salivary pH values. Subjects recorded as having a pH of 5 could well have had much lower values. Subsequent testing of resting phase salivary pH has been carried out on patients with HIV infection, as part of their ongoing clinical assessment. These pH recordings have been done with both the Duotest 5-8 pH range of testing paper and repeated with paper recording pH values in the range of 1-9. In these patients, the resting phase salivary pH was as low as 2 or 3.

In the present study it was apparent that stimulated saliva needs to be tested with the Duotest 5-8 range of pH values as no subject in the study registered a stimulated salivary pH value of less than 5.5. Furthermore, there was no difference in the stimulated salivary pH results between the HIV positive and the control groups of subjects. In addition, HIV-infected patients registered predominantly normal to high stimulated salivary pH values. These observations would suggest that there is little or no effect on stimulated salivary pH as a result of HIV infection and that consequently stimulated salivary pH values should have little clinical significance in the possible development of dental or oral disease.

In this study a statistical difference ($p<0.03$) between resting salivary pH in HIV-infected individuals and the control group of subjects was observed, with HIV-infected patients having a greater probability of having a lower resting phase salivary pH. The implications of this result are interesting. A continual resting phase salivary pH that is low should result in an acidic oral environment. Mandel (1987) and Levine (1989) cite the importance of saliva in not only maintaining the pH in the oral cavity but also the pH of bacterial plaque and the pH of the oesophagus. These investigators consider that salivary pH at rest is controlled less by bicarbonate activity and more by the buffering activities of histidine rich peptides, amino acids and phosphates. The results of the present study would suggest that there may be diminished activity by the histidine rich peptide, amino acid and phosphate buffering systems in subjects with HIV infection.

The consequences of a low resting phase salivary pH for HIV-infected subjects may be important in several regards. For example, if the oesophageal pH is affected, it is possible that this might be a predisposing factor in the development of oesophageal candidiasis, an AIDS defining condition (Marriott and McMurchie 1996). Further, teeth should be prone to erosion or caries as a result of decreased bacterial plaque pH resulting in demineralisation of enamel. Levine (1989) demonstrated the critical role resting salivary pH plays in tooth demineralisation and remineralization. However, in the present study, a correlation was not

established between low resting pH and increased prevalence of tooth caries or erosion. There are possible explanations for this;

- Firstly, the subject sample size in the study may have been too small to establish evidence of a trend for tooth damage as a result of increased mouth acidity.
- Secondly, only caries or evident erosion was recorded in the study, not the incidence or amount of previous dental treatment. Therefore other factors, such as good oral hygiene, fluoride prevention treatments and regular dental care may have contributed to the lack of any correlation between low resting pH and decay or erosion of teeth. Preventive and treatment protocols have been advocated by numerous authors in dental management for patients with HIV infection (Greenspan et al. 1990; Greenspan and Greenspan 1993; Barr 1994; Coates et al 1996; Foltyn and Marroitt 1996) and many of the patients before entering this study had been regularly attending the clinic for oral care.

Fox (1992) considered that salivary secretions become increasingly affected over time and that factors such as concurrent viral infections, oral health, general health status, medications, and in particular, the stage of HIV infection should be carefully evaluated in the change in salivary function. In the present study the development of a low resting phase salivary pH occurred independently from the CD4 count suggesting that salivary changes are not affected by the stage of HIV infection (Table 4). However, the period of time from seroconversion in HIV-infected individuals may impact on salivary change as many of the subjects in the present study who had reasonable immune systems (as indicated by CD4 counts)

had been infected for many years. Several of the other factors described by Fox (1992) were investigated in the present study. Neither oral health, as judged by caries and periodontal disease, nor the taking of medications appeared to be responsible for alteration in resting phase salivary pH values. However, in this investigation oral candidiasis was more likely to be associated with low resting phase pH.

Salivary pH and Candidiasis

In this study, patients infected with HIV who had a low resting phase salivary pH (value less than 6.5), had a greater likelihood of having candidiasis. This was statistically significant at the 5% level. This finding may suggest that salivary pH may play a role in the aetiology of symptomatic oral candidiasis. Alternatively, the presence of oral candidiasis may be a contributing factor in the lowering of salivary pH, although an argument against this premise is that 50% of HIV-infected patients with low resting phase salivary pH did not have candidiasis.

The reasons why HIV-infected individuals with low resting phase pH values have a higher likelihood of having oral candidiasis may be several;

- It is possible that resting phase oral acidity promotes the development of drug resistant strains of candida or the colonisation by different fungal species, both of which are commonly found in HIV related candidiasis (McCullough *et al* 1995).
- Alternatively, the effect of increased acidity on the mucosal tissues could create dryness and inflammation thus providing an environment favourable for yeast colonisation (Mandel 1986; Levine 1989; Shenkels *et al.* 1995).

- Finally, the interaction of a local factor such as low resting phase salivary pH with a depleted immune system may explain why candidiasis rarely causes systemic infection (Crowe and Kornbluth 1996) in HIV individuals.

Barone *et al* (1990) found 74% of subjects with HIV infection presented with mucosal lesions such as candida, ulceration or hairy leukoplakia. In the present study only candida was examined with respect to pH of saliva but it is conceivable that the low resting salivary pH could also be a contributing factor in relation to the clinical expression of herpes and aphthous ulceration.

In the present study no correlation was found between salivary pH and salivary flow, indicating that the changes in resting phase salivary pH values are not being caused by dryness.

Stimulated salivary flow

In this study a statistical difference ($p<0.01$) between stimulated salivary flow rate in HIV-infected individuals and a control group of subjects was observed. The control subjects (15%) who registered low flow recorded values just below those of normal flow, (5ml/min), whereas subjects with HIV (54%) who registered low flow had values significantly below normal (Fig 28).

The results of reduced stimulated salivary flow values recorded for the subjects with HIV in this investigation lead to several observations;

- Parotid glands are responsible for much of the stimulated salivary flow (Söderling 1989) and these are also the salivary glands predominantly implicated in HIV-related salivary gland disease (HIV-SGD) (Greenspan *et al.* 1990). In addition, Schiødt (1992) observed that xerostomia was associated with parotid swelling in HIV-SGD (for example see Figure 45). If the reduced stimulated salivary flow is representative of decreased glandular function (Dawes 1987), the results of the present study may suggest that HIV-SGD could be much more prevalent in individuals with HIV infection than previously thought (Schiødt 1992). Salivary gland enlargement (SGE) (for example see Figures 44-46) has rarely been seen in adult HIV infection but, as noted by Greenspan and Greenspan (1993) the syndrome is increasing, possibly as the life expectancy of HIV-infected individuals increases.
- Schiødt (1992) considered that xerostomia manifested only in the Sjögrens type of HIV-SGD, not in the lymphoepithelial cyst type of parotid swelling sometimes seen in HIV-infected individuals (for example see Figure 47). The results of the present study would indicate that measuring salivary flow would be a simple

clinical test for differentiating between the two types of HIV-SGD. If salivary gland enlargement was present in an HIV-infected individual, then reduced salivary flow would suggest the Sjögrens type of HIV-SGD was the underlying pathology.

- Stimulated saliva provides the main buffering system of saliva (Levine 1989). It is conceivable then, that HIV subjects with low stimulated salivary flow rates might have reduced buffering capacity, especially at meal times or when taking in salivary stimulants such as acidic drinks. The concept of reduced buffering capacity and increased potential for oral damage is supported by Milosevic and Dawson (1996) who demonstrated in their study that reduced stimulated salivary flow was evident in two groups of Bulimics with or without pathological tooth wear.
- The composition of saliva is intimately related to flow rate, with protein and most electrolytes increasing with increasing flow rate (Levine 1989). The apparently reduced capacity for subjects with HIV to produce normal quantities of stimulated saliva could mean that the delicate balance of protein and electrolytes is altered. This could possibly result in disruption to the formation of the tooth pellicle as well as affecting the remineralisation of teeth.

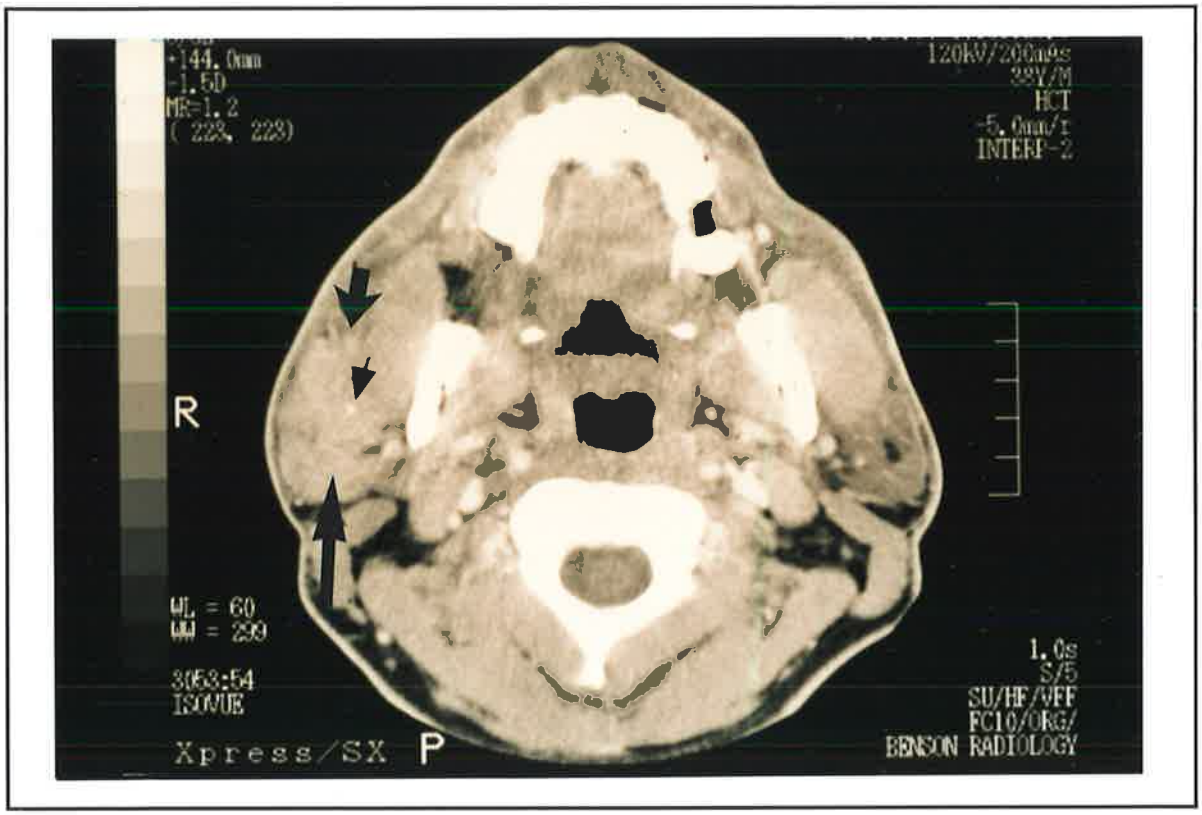


Figure 44: CT scan demonstrating Sjögrens type of parotid swelling in HIV-SGD (arrows) in a South Australian HIV-infected subject. A salivary calculus (small arrow) is present.

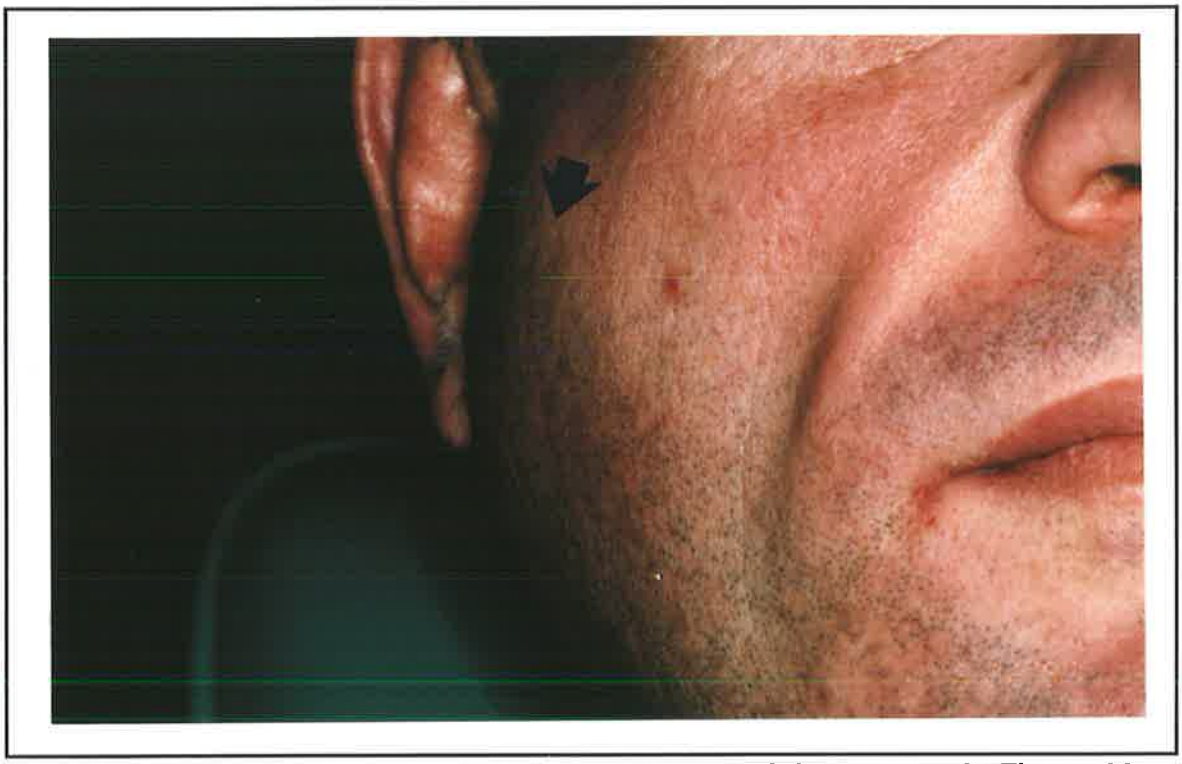


Figure 45 HIV-SGE (arrow) in the patient who had the CT scan seen in Figure 44

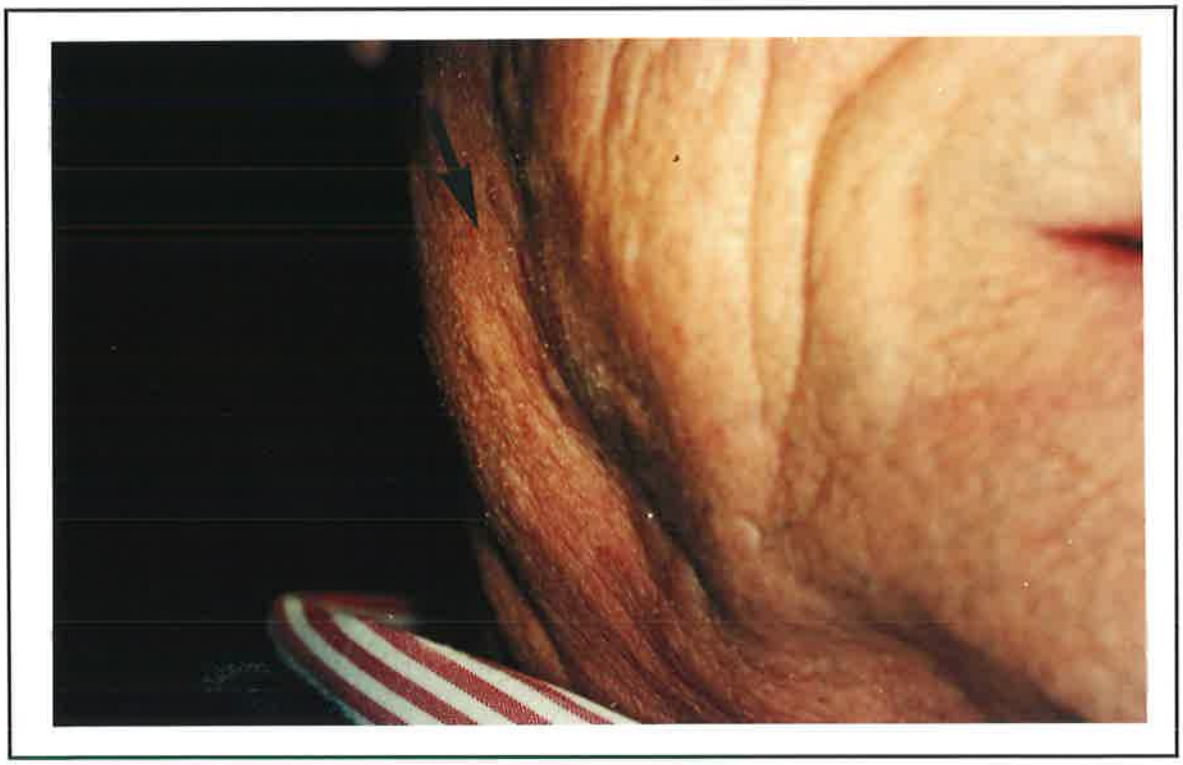


Figure 46 HIV-SGE (arrow) in a HIV-infected patient whose serum amylase was elevated (salivary amylase). HIV-SGE was also confirmed by CT scan.

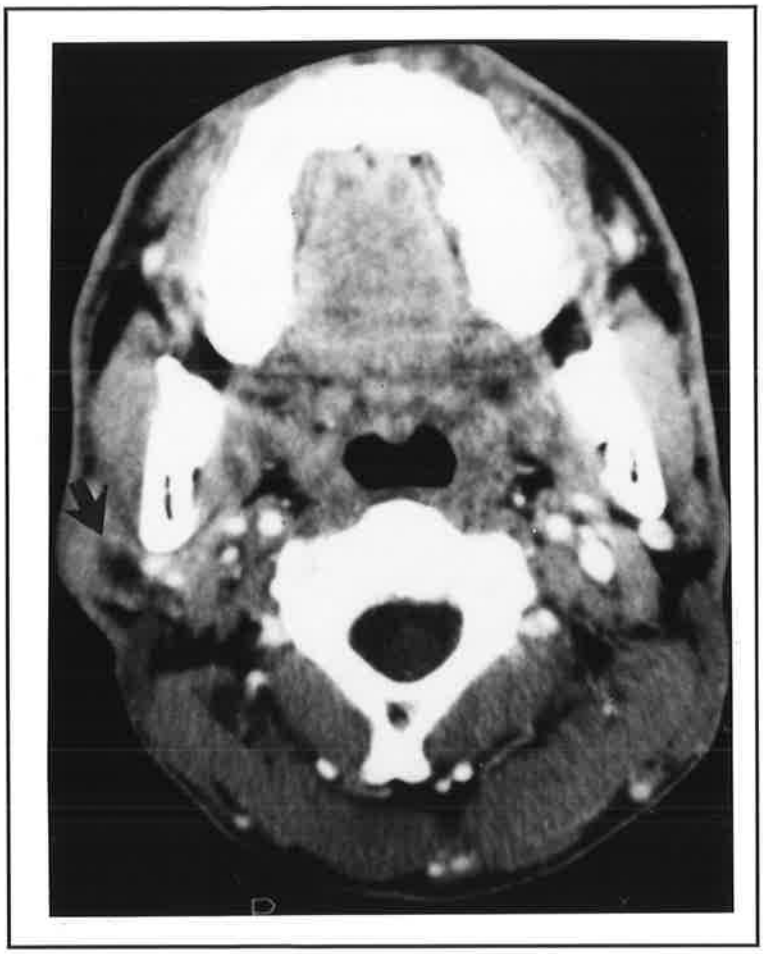


Figure 47: CT scan demonstrating the lymphoepithelial cyst type of parotid swelling (arrow) associated with HIV-SGD

Stimulated salivary flow relative to CD4 cell counts, medication, and pH

- Statistical analysis of the results failed to find a correlation between low stimulated salivary flow in subjects with HIV infection and a range of factors including medication, pH and immune status. There are several possible reasons for this:
- Firstly, the number of subjects with HIV that fitted into each category (low or high CD4 cell counts; multiple or single medication) may have been too small to be a reliable sample size for analysis. However, in this investigation, several subjects not on medication and with adequate immune function registered reduced stimulated salivary flow rates. Yeh *et al.* (1988) also found in their study of HIV-infected subjects that medication was not implicated in the reduction of salivary flow.
- Secondly, duration of HIV infection may be a factor contributing to reduced stimulated salivary flow. After a prolonged period of infection by some other viral infections, such as hepatitis or diffuse infiltrative CD8 lymphocytosis syndrome, salivary glands have shown diminished production (Ficarra 1992, Wright 1987). Duration of disease is also important in Sjögrens Syndrome as progressive diminution of salivary flow varies in relation to the rate of destruction of parotid gland salivary acini (Levine 1989). In the present study there were 12 subjects who had been infected with HIV for several years yet had immune function greater than a CD4 cell count of 250 and who had developed few or no opportunistic infections. Two of these individuals recorded

low stimulated salivary flow, had no obvious possible cause for xerostomia and the duration of HIV infection was postulated as a possible cause for the reduced flow (Table 12).

- Thirdly, the amount of HIV in the blood (viral load) may contribute to reduced salivary flow. Viral load was not assessed in this research project as viral load tests were unavailable at the start of the project. One study subject had a very low stimulated salivary flow, was not polymedicated and had recently been diagnosed with HIV infection. This individual had rapidly progressive disease and suffered nightsweats and a declining CD4 cell count consistent with an active viral infection. His plasma viral load, taken shortly after the present investigation was completed, ranged from 120,000 copies to 150,000 copies.
- Fourthly, medical problems unrelated to HIV infection may impact on low stimulated salivary flow rate in HIV-infected individuals. For example, one subject in this study was on no medication, had a relatively high CD4 cell count (400) and showed no apparent reason for having a very low stimulated salivary flow (1.8ml/5min). The viral load tests performed on this patient, since completion of this project, remain low but he has since been confirmed as a chronic carrier of Hepatitis B virus and his liver function tests remain abnormal indicating active viral hepatitis replication. Ficarra (1992) and Wright (1987) have both proposed infections, particularly viral infections, as a cause for reduced salivary gland secretion.
- Finally, the reduced salivary flow possibly might be a result of the Human Immunodeficiency Virus infecting the lymphatic tissue of the salivary glands.

Alternatively, autoimmune changes caused by HIV infection could impact directly on salivary gland function. For example, salivary gland enlargement is considered to be due to a CD8 cell infiltrate (Greenspan *et al* 1990; Ficarra 1992; Schiødt 1992; Schiødt *et al* 1992; Barr 1994).

Medication as a cause for reduced stimulated salivary flow

Greenspan *et al* (1990), Ficcarra (1992) and Barr (1994) all proposed that the taking of multiple medications was a possible cause for reduced salivary flow in HIV-infected individuals. Six of the thirty-five subjects in the present study who recorded low and very low flow were polymedicated or taking medication that causes a dry mouth and a further twelve subjects were also polymedicated and also had an AIDS defining condition. However, when the results were further analysed, fifteen of thirty HIV-infected subjects who recorded normal stimulated salivary flow were also taking multiple medications.

No statistical significance was found between reduced stimulated salivary flow and the taking of medication, whether it was antiretroviral medication, medication for opportunistic infections or medication unrelated to HIV infection such as asthmatic medication.

Medications with a known xerostomic side effect such as asthmatic treatments and the recreational drug substitute, methadone, should impact on salivary flow (Levine 1989; Greenspan *et al* 1990). In this study the number of subjects on these medications was too low for specific analyses to be performed in relation to these medications.

Presence or absence of oral manifestations in relation to stimulated salivary flow

There was a uniform spread of oral manifestations of HIV infection found in all stimulated salivary flow groups in subjects with HIV infection (Table 14). The number of subjects in each group was too small to draw any statistically relevant conclusions. However, it is interesting that there were more HIV-infected subjects with periodontal disease who registered normal stimulated salivary flow. Of further interest was the presence of candida in 8/30 HIV-infected subjects with normal stimulated salivary flow. A "dry" environment is supposed to enhance the risk of developing candida infection (Barr 1994, Ficarra 1992). The results from this project indicate that a "dry" environment is probably of lesser importance in the manifestation of oral candidiasis in HIV infection than has hitherto been thought. However, in the present study the resting phase salivary pH was implicated in the presence of candidiasis. Interestingly there was no correlation between low salivary flow or pH values.

Caries and stimulated salivary flow

It is difficult to determine why the presence of caries was more evident, (p 0.02), in the low, yet not in the very low flow, stimulated saliva samples of HIV-infected subjects (see Table 13). The presence of caries in this study showed no correlation with low resting phase salivary pH and it was hypothesised that factors such as dental treatment, oral hygiene and prevention programs could have impacted on these results. These same factors may have determined why caries was not recorded in the HIV subjects with very low flow stimulated saliva values. The difficulties in interpretation of any caries propensity /saliva flow correlation are

epitomised by the conclusions of Rudney(1995) and Bobek *et al.* (1993). They found it difficult to determine what roles electrolytes and organic components of saliva played in determining oral health. It is important to note that the presence of caries was more likely to occur in HIV-infected subjects with low stimulated salivary flow as this indicates the need for preventive oral health strategies.

Resting phase salivary results and salivary weight

All resting saliva values were recorded on the basis of weight as well as flow. There is wide variation in normal resting phase salivary weight and flow. One reference value for normal resting mean weight is given as 0.47gram/min or 2.35 G/ 5 minutes (Tenovuo 1989). If 2.35G/5minutes is taken as a normal value for salivary resting phase weight then 32% of subjects with HIV in this study recorded values lower than this. In contrast, only 14% of control subjects recorded less than 2.35G in 5 minutes. The number of subjects is too low to get statistical corroboration of what appears to be a trend to lower resting phase salivary secretion. No control subjects recorded low flow. However, the variation in accepted normal values is so extensive that any trend indicated by these results needs to be verified by larger sample groups.

Calcium.

No statistical difference was found for calcium levels when the control group and the HIV subjects were compared. There also was no significant difference between the calcium values for stimulated or unstimulated saliva samples in either group. Fox (1992) found that chloride levels were elevated in HIV infection

whereas Schiødt *et al* (1992) in their study of parotid saliva in patients with HIV-related salivary gland disease found no changes in chloride, potassium or sodium levels. The results from our study would tend to support the latter investigators in their conclusions that electrolytes remained unchanged in saliva from subjects infected with HIV.

There were some very low values for calcium in subjects from both groups. When the subjects with very low calcium values were examined, there were 11/65 subjects with HIV who had values less than 0.2. In the control group there were 4/21 who had similar readings. Of the eleven HIV subjects with low values, five had AIDS, but of the other six, three had extremely high CD4 counts well within normal CD4 range. It is therefore possible to suggest that AIDS does not impact on calcium values.

Coroneo *et al.* (1981) and Ferguson (1989).both observed differences in calcium values between stimulated and resting phase salivary secretions. In the present study, the lack of any statistical significance in calcium levels between stimulated and resting phase saliva samples may have occurred as a result of the number of subjects in the study. Alternatively the processing of samples could have affected the results. HIV is known to be present in saliva and for the present study it was deemed necessary to render the samples non-infectious for transfer between laboratories. There is no mention in the literature of the effect of autoclaving on the calcium in saliva samples. Theoretically the calcium concentration should be unaffected. However, as protein is denatured by autoclaving, it is possible that the

reading of protein bound calcium may have been influenced. Nevertheless, the samples in this study appeared to give readings that were comparable to those of previous studies (Ferguson 1989; Söderling 1989) so it was assumed that autoclaving of saliva samples for calcium analysis did not severely impact upon the results.

Scannapieco (1994) outlined the possible role that calcium might play in the formation of the pellicle that is critical to tooth protection. In addition, Milesovic and Dawson (1996) have found interesting results in their research into toothwear in Bulimics. In our study no difference was noted in the calcium values when subjects with HIV and control subjects were compared. Neither was there any difference in calcium levels between stimulated or resting phase saliva samples. However, the results have shown saliva flow is definitely reduced in subjects with HIV infection and therefore it is possible that there would be correspondingly less calcium present in the mouth at any given time. Therefore, although the calcium per ml of saliva is unaffected, the overall lack of saliva could still result in diminished electrolyte concentration. Whether this is significant in relation to tooth mineralisation can only be hypothesised, although decay was a problem in HIV-infected subjects in this study who had a low salivary flow rate.

Protein analysis

Previous studies of salivary proteins in HIV infection have concentrated on specific proteins such as IgA, peroxidases, lysozymes and lactoferrin (Challacombe and Sweet 1997; Shugars, Sauls and Weinberg 1997; Wahl *et al* 1997). The results

from these studies appear to be equivocal with some investigators finding raised protein levels and others finding decreased levels of proteins. A possible cause for the disparities between these studies could have been the design of the saliva studies (Fox 1992; Mandel, Barr and Turgeon 1992; Schiødt *et al* 1992).

Most studies have concentrated on the immune response of saliva to HIV or opportunistic infections, the presence of HIV in saliva, or why saliva should have such potent inhibition of HIV infectivity (Fox 1992; Malamud and Friedman 1993; Challacombe and Sweet 1997; Malamud *et al* 1997; Qureshi *et al* 1997; Shugars *et al* 1997; Wahl *et al* 1997; Wisnom *et al* 1997). In our study the total protein of stimulated whole saliva of individuals with HIV was compared to total protein of stimulated whole saliva from individuals with no known disease by comparing bands on gel electrophoresis. The salivary proteins were not deglycosylated and therefore interpretation of all electrophoretic patterns needed to be performed on a comparative basis (that is noting discrepancies between the control and HIV-infected subjects).

In this investigation, several observations were made on apparent differences in protein content of saliva between HIV-infected individuals and the control group of subjects;

- Firstly, there were more proteins expressed in salivary samples from the HIV-infected patients than in control subjects. This is in contrast to the findings of the study by Schiødt *et al.* (1992) on patients with HIV-related salivary gland

disease. These investigators found that there was decreased salivary protein in HIV-infected subjects. However, these investigators studied only parotid salivary secretions.

- Secondly, the proteins that were present in the salivary samples from HIV-infected patients tended to stain with a greater intensity than salivary proteins of the control subjects. This would indicate that not only was there a wider distribution of proteins according to molecular weight but also that the proteins were more concentrated in HIV subjects.
- Thirdly, proteins from salivary samples of the HIV-infected subjects expressed a different migration pattern throughout the gel with numerous samples demonstrating a marked concentration of proteins in the high molecular weight range. In addition they also showed an aggregation of proteins in the very low molecular weight range. The presence of proteins in the high molecular weight range suggests that either individuals with HIV infection secrete more of these proteins or that the proteins themselves are defective and tend to conjugate causing strong representation at the top of the electrophoresis gels. The accumulation of proteins at the lower molecular weight range suggests that these also are secreted more actively by subjects with HIV infection. Interestingly, some support for the concept that individuals with HIV infection may secrete more lower molecular weight protein comes from investigations by Wahl *et al.* (1997). These investigators isolated a 12kD endogenous protein which is a secretory leukocyte protease inhibitor (SLPI) that blocks HIV 1 infection *in vitro.* It is accepted that little infectious virus can be isolated from

the mouth (Malamud and Friedman 1993), suggesting that inhibitory factors such as SLPI may be prevalent in people with HIV infection. This might possibly account for some of the heavy banding at 14kD noticed on the electrophoresis gels in the present study. However, as deglycolysation of the salivary proteins was not performed prior to electrophoresis, it is not possible to definitively confirm that the protein represented by the 14kD banding is the protein identified by these investigators, merely that there is increased banding at the approximate relevant level. Wahl *et al.* (1997). also proposed that the increased risk of oral transmission of HIV in infants and children may be related to the lack of fully developed salivary glands and the presence of increased expression of proteins in HIV-infected subjects in this study tends to offer circumstantial support for this theory.

The assumption prior to starting this project was that there would be less rather than more salivary protein in HIV-infected individuals. Electrophoresis of the salivary proteins tended to indicate that subjects with HIV infection had both a wider range and more concentrated salivary proteins per millilitre of saliva. However, observations of the stimulated salivary flow rate indicate that there is often less saliva in HIV- infected subjects. Therefore, the total amount of salivary protein present in the mouth will or could still be diminished.

The delicate balance of the oral environment (Bobek, Tsai and Levine 1993; Rudney 1995) is dependent on adequate salivary protein secretion. Therefore,

although the range and concentration of salivary proteins per millilitre appeared to be higher in HIV-infected subjects, because this group tended to have a low stimulated salivary flow rate it is possible their total salivary protein secretion was reduced compared to the control subjects. Consequently, it is theoretically possible that the balance of the oral environment in HIV-infected subjects could be disrupted. However, the fact that there is a concentration of low molecular weight and also very high molecular weight salivary proteins in HIV-infected individuals suggests that either compensatory mechanisms occur to raise the amount of total salivary protein, or that the presence of oral disease, such as candidiasis, stimulates the production of specific proteins. The lower molecular weight salivary proteins supposedly inhibit oral fungal infections (Oppenheim *et al.* 1988) and the concentrated banding noted on the gels in the range of 14kD, 21kD, and 31kD might suggest that this represents a response to the presence of infection.

The presence of very high molecular weight proteins could also suggest a deficiency in normal protein metabolism and clearance of organic material from the mouth. Alternatively, a raised serum proteinaemia resulting from HIV infection might elevate secretion of proteins from the salivary glands.

Since the project was commenced, improved methods have been developed to assess viral activity (Viral Load) and immune system damage (Collier *et al* 1996, Ffrench *et al* 1996, Ionnidis *et al* 1996, O'Brien *et al* 1996, Perelson *et al* 1996).

This project did not correlate changes in saliva quality or quantity with the amount of circulating virus in a person with HIV (Viral Load). However, information about viral load, available retrospectively, for some subjects who presented with significant salivary changes, such as lowered pH and reduced flow, suggests that this may be another extremely important factor in salivary alteration along with duration of HIV infection. It is possible that it could be the level of circulating virus or localised tissue reservoir virus that determines alterations in saliva production. CD4 cell count, used as an immune marker, does not indicate either the level of viral load or more importantly, localised tissue reservoir virus.

CHAPTER 6

CONCLUSIONS

- Resting phase salivary pH is likely to be low in a significant number of subjects with HIV infection. This may not be related to medication being taken, or to the immune status or stage of HIV infection in affected subjects.
- Subjects with HIV infection appear more likely to have symptomatic candidiasis if they have a low resting phase salivary pH value.
- Low resting phase salivary pH values does not appear to correlate with the presence of HIV-related periodontal disease in subjects with HIV infection. Neither does a low resting phase salivary pH contribute to the likelihood of increased caries or erosion of teeth.
- Stimulated salivary flow is reduced by the presence of HIV infection in an individual. This appears to be unrelated to medication being taken, or to the stage of HIV infection. The reduction in stimulated salivary flow in subjects with HIV infection may occur without noticeable swelling of the salivary glands (HIV-SGD). The recording of salivary flow is non invasive and there is no reason for this not to be performed in the routine clinical setting.
- In this study no evidence was found to indicate that reduced stimulated salivary flow in subjects with HIV infection contributed to the presence of HIV-related oral candidiasis, HIV-related periodontal infections or erosion of teeth. Caries was more likely to occur in subjects with HIV infection who had low stimulated salivary flow.
- The calcium content of saliva appears to be unaffected by the presence of HIV infection in individuals.
- There are changes in the salivary proteins of individuals with HIV infection. These changes require further investigation.

APPENDICES

APPENDIX I

THE UNIVERSITY OF ADELAIDE

CONSENT FORM

See also Information Sheet attached.

1. I .....(please print) hereby consent to take part in the research project entitled:

   QUALITATIVE AND QUANTITATIVE ANALYSIS OF SALIVA IN A GROUP OF HIVE INFECTED INDIVIDUALS.

2. I acknowledge that I have read the Information Sheet entitled:

   THE STUDY OF SALIVA IN HIV INFECTED PATIENTS

3. I have had the project, so far as it affects me, fully explained to my satisfaction by the research worker. My consent is given freely.

4. Although I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

5. I have been given the opportunity to have a member of my family or a friend present while the project was explained to me.

6. I have been informed that, while information gained during the study may be published, I will not be identified and my personal results will not be divulged.

7. I understand that I am free to withdraw from the project at any time and that this will not affect medical advice in the management of my health, now or in the future.

8. I am aware that I should retain a copy of this Consent Form, when completed, and the relevant Information Sheet.

SIGNED ..... DATE .....

NAME OF WITNESS ..... SIGNED .....
(Please print)

DATE .....

I ..... have described to .....
(Please print)

the nature of the procedures to be carried out. In my opinion she/he understood the explanation.

SIGNED ..... DATE .....

STATUS IN PROJECT .....

INFORMATION SHEET

THE STUDY OF SALIVA IN HIV INFECTED PATIENTS

People infected with HIV often have problems with their mouths both at the start of and during their HIV infection. Loss of appetite, self-esteem and weight loss can all occur as a result of infection in the mouth. The Adelaide Dental Hospital has recognised that people with HIV need better and more frequent access to dental care and have established a clinic to serve this purpose. One of the observations made during the operation of the clinic is that many people with HIV who are experiencing troubles with their mouth also have what appears to be a poor amount of saliva or else poor quality saliva.

One supposition that can be drawn but which is not necessarily correct is that inadequacies of saliva are contributing to, if not directly causing the mouth problems such as gum disease, tooth decay and "thrush". In order to test this theory it is necessary to collect saliva from people with HIV and perform tests to find out the acidity, protein content and mineral content of the saliva.

You will be asked to spit into a plastic tube for about five minutes. The saliva will be stoppered and taken away for tests. A second spit will be required, only this time you will be asked to chew gum for a two to five minute period. This sample will also be taken away for testing.

The samples will be numbered but not named. All data will be analysed in an anonymous way. Your name or any other information which could identify you will not be recorded. Your participation in the study involves your agreement to have your saliva collected and analysed. Your participation in the project is voluntary. Your eligibility for dental treatment now and in the future will not be affected by your decision to either participate or not.

The results of the study will help to identify or eliminate saliva as a cause of poor oral health in people infected with HIV. This in turn will focus clinical efforts aimed at treatment prevention of oral problems associated with HIV infection.

Investigator;

Dr E. Coates Ph. 2239236

Administrator

Dr P. Telfer Ph.2239272

Supervisor

Dr D Wilson

APPENDIX II

Electrophoresis and Blotting Standards

Protein Standards

Table 7. Standard Protein Migration on SDS-PAGE Gels

The three ranges of Bio-Rad standards were run on SDS-PAGE gels: H=high range: 45,000 to 200,000; L = low range: 14,400 to 97,400; B = broad range: 6,500 to 200,000. The molecular weight of each band is given in kD. Migrations are based on the dye front being run to the bottom of a 2.67% crosslinked gel. The various gel percentages (%T) in the chart are available as precast Ready Gels (see pages 199–201). For a poster size copy of the protein migration chart, request bulletin 1787.

Ordering Information

Catalog Number	Product Description
161-0304	**SDS-PAGE Standards,** low range, 200 µl
161-0303	**SDS-PAGE Standards,** high range, 200 µl
161-0317	**SDS-PAGE Standards,** broad range, 200 µl
161-0314	**Silver Stain SDS-PAGE Standards,** low range, 200 µl
161-0315	**Silver Stain SDS-PAGE Standards,** high range, 200 µl
161-0320	**2-D SDS-PAGE Standards,** 500 µl
161-0306	**Biotinylated SDS-PAGE Standards,** low range, 250 µl
161-0311	**Biotinylated SDS-PAGE Standards,** high range, 250 µl
161-0319	**Biotinylated SDS-PAGE Standards,** broad range, 250 µl

Catalog Number	Product Description
161-0307	**Biotinylated SDS-PAGE Standards Kit,*** low range, Avidin-HRP
161-0308	**Biotinylated SDS-PAGE Standards Kit,*** low range, Avidin-AP
161-0312	**Biotinylated SDS-PAGE Standards Kit,*** high range, Avidin-HRP
161-0313	**Biotinylated SDS-PAGE Standards Kit,*** high range, Avidin-AP
161-0321	**Biotinylated SDS-PAGE Standards Kit,*** broad range, Avidin-HRP
161-0322	**Biotinylated SDS-PAGE Standards Kit,*** broad range, Avidin-AP
170-6528	**Avidin-HRP,** 2 ml
170-6533	**Avidin-AP,** 1 ml

* Each kit contains 250 µl biotinylated standards, 2 ml Avidin-HRP or 1 ml Avidin-AP, and complete instructions.

BIO-RAD

APPENDIX III

Instructions for the use of Novex™ precast gel

I. GENERAL GEL INFORMATION

Gel Specifications:

Gel Matrix	Acrylamide/Bis-Acrylamide
Gel Thickness	1.0mm or 1.5mm
Gel Size	8cm x 8cm (H x W)
Cassette Size	10cm x 10cm (H x W)
Cassette Material	Styrene Copolymer (recycle code 7)
Sample Well Configuration	10, 15, 1, 2, 5, 12 or 2D/Preparative Wells

Sample Well Volume:

1.0mm	700µl	210µl	60µl	25µl	20µl	15µl	600µl
1.5mm				37µl		25µl	

Recommended Amount of Sample per Band:

Tris-Glycine Gel	0.10 to 0.50 µg per band detected with Coomassie™ Blue
TBE Gel	10 to 100 ng of nucleic acid per band with Ethidium Bromide (values stated are for 10 well, 1.0mm thick gels).

Shipping and Handling:

Shipping:	Gels are shipped overnight delivery on wet ice.
Handling:	**Packaging buffer contain 0.02% sodium azide and low levels of residual acrylamide monomer. Gloves should be worn at all times when handling gels.**
Packaging:	10 gels/box. Individually packaged in clear pouches with 10 mls of packaging buffer.
Storage:	Store at 4°C. DO NOT FREEZE. Gels should be used immediately from the refrigerator. Extended exposure to room temperature seriously impairs the performance of the gel. For *in vitro* research use only.

II. GEL COMPOSITION AND PERFORMANCE RANGE

Gel	% Acrylamide	%Bis-Acrylamide	pH	Performance Range
Tris-Glycine	4%	1.3%	8.6	100-400kDa
	6%	2.6%	8.6	60-300kDa
	8%	2.6%	8.6	40-250kDa
	10%	2.6%	8.6	30-200kDa
	12%	2.6%	8.6	10-120kDa
	14%	2.6%	8.6	6-80kDa
	16%	2.6%	8.6	5-60kDa
	18%	2.6%	8.6	5-60kDa
	4-12%	2.6%	8.6	30-300kDa
	8-16%	2.6%	8.6	14-200kDa
	4-20%	2.6%	8.6	6-200kDa
	10-27%	2.6-5.0%	8.6	5-100kDa
Tricine	10%	2.6%	8.3	8-100kDa
	16%	2.6%	8.3	2-40kDa
	10-20%	2.6%	8.3	2-100kDa
TBE	6%	2.6%	8.3	60-2500 bp
	10%	2.6%	8.3	80-1500 bp
	20%	2.6%	8.3	10-900 bp
	4-20%	2.6%	8.3	10-3000 bp
DNA Retardation	6%	2.6%	8.3	60-2500 bp
Zymogram	10%	2.6%	8.6	30-200kDa
IEF, 3.0-7.0	5%	2.6%	5.0	3.0 - 7.0 pI
IEF, 3.0-10.0	5%	2.6%	6.0	3.5 - 8.5 pI

III. INSTRUCTIONS FOR RUNNING GELS AND OPENING CASSETTES

For safety reasons, wear protective gloves and safety glasses when working in a laboratory environment. Please note that the Xcell™ Mini-Cell requires volumes of 150ml for the upper buffer chamber and 300ml for the lower buffer chamber. The Xcell II Mini-Cell requires volumes of 200ml for the upper buffer chamber and 600ml for the lower buffer chamber.

1. Cut open the gel pouch with scissors and remove the gel cassette.

2. Drain away the packaging buffer and rinse gel cassette with D.I. water.

3. Peel the tape off of the bottom of the cassette.

4. In one quick motion, pull the comb out of the cassette.

5. Orient two gels in the Mini-Cell so that the notched "well" side of the cassette faces the buffer core. Please refer to the Mini-Cell instructions for detailed instructions on how to assemble the Mini-Cell. Note: If you are running only one gel, the square plastic buffer dam replaces the second gel cassette.

6. Use a pipet to wash the sample wells with 1x running buffer. Leave the wells full of running buffer. Make sure that air bubbles are displaced from the wells.

7. Load samples into the wells. For best results, load sample buffer in all of the wells, whether or not they contain samples. This ensures uniform band width in all lanes.

8. Fill the buffer chambers with the appropriate amount of running buffer. (Please refer to Mini-Cell instructions for details).

9. With the power OFF, place the lid on the buffer core and connect the electrode wires to the power supply. Turn on the power supply and run gel(s).

10. When the run is complete, shut off the power, disconnect electrodes, and remove gel(s) from the Mini-Cell.

11. Separate each of the three bonded sides of the cassette by inserting the gel knife's beveled edge into the gap between the cassette's two plates. The notched ("well") side of the cassette should face up. Push down gently on the knife handle to separate the plates. Repeat on each side of the cassette until the plates are completely separated.

12. Carefully remove and discard the top plate, allowing the gel to remain on the bottom (slotted) plate. Note: Trying to remove the gel at this point will result in the gel tearing. Proceed to step 13 before removing the gel.

13. Remove the gel from the cassette plate by one of the following two methods:

    A. Use the sharp edge of the gel knife to remove the bottom lip of the gel. The gel knife should be at a 90° angle, perpendicular to the gel and the slotted half of the cassette. Push down on the knife, and then repeat the motion across the gel to cut off the entire lip. Hold the cassette plate and gel over a container with the gel facing downward and use the knife to carefully loosen one lower corner of the gel and allow the gel to peel away from the plate.

    B. Hold the cassette plate and gel over a container with the gel facing downward. Gently push the gel knife through the slot in the cassette, until the gel peels away from the plate. Cut the lip off of the gel after fixing, staining, or transferring, but before drying.

14. Fix, stain or transfer as desired.

IV. GEL TYPE AND PROTOCOLS

TRIS-GLYCINE GELS

NOVEX offers a variety of single percentage and gradient Tris-Glycine "Laemmli" Page gels available in 7 different well formats and 2 thicknesses. Since NOVEX pre-cast gels **do not contain SDS**, they are suitable for standard SDS and native applications. Please refer to the migration table in our catalog to select the proper gel for your application.

Recommended Buffers

For SDS Gels: (Denaturing, non-reducing)
Tris-Glycine SDS Running Buffer, (10x) 500ml, Cat. No. LC2675
Tris-Glycine SDS Sample Buffer, (2x) 20ml, Cat. No. LC2676

For Native Gels:
Tris-Glycine Native Running Buffer, (10X) 500ml, Cat. No. LC2672
Tris-Glycine Native Sample Buffer, (2x) 20ml, Cat. No. LC2673

For Reducing Conditions:
NOVEX sample buffer does not contain a reducing agent. Typically, 2.5% β-mercaptoethanol or 1% dithiothreitol final concentration is sufficient to reduce the sample. Add 0.5ml BME to 10ml of 2x sample buffer or 0.2g of DTT to 10ml of 2x sample buffer and proceed to the protocol for sample preparation.
NOTE: It is best to prepare fresh reducing sample buffer since the reducing agent will oxidize over time.

Buffer recipes are located on pages 21-23.

Protocol:

1. Prepare your sample by adding one part of the appropriate NOVEX Tris-Glycine Sample Buffer (2x), (Laemmli or Native), to one part sample and mix well. For denaturing conditions, heat the sample at 85° for 2 minutes. For native conditions, do not heat.

2. Dilute the NOVEX Tris-Glycine Running Buffer (10x), (Laemmli or Native), 1:9 before use. Fill the upper and lower buffer chambers of the Mini-Cell with the appropriate amounts of running buffer.

3. Run the gel according to either of the following running conditions.

Laemmli SDS:

Voltage:	125V constant
Current:	Start: 30-40mA/gel
	End: 8-12mA/gel
Run Time:	Approximately 90 minutes. The run is complete when the bromophenol blue tracking dye reaches the bottom of the gel.

Native Tris-Glycine:

Voltage:	125V constant
Current:	Start: 6-12mA/gel
	End: 3.6mA/gel
Run Time:	1-12 hours

4. Fix, stain or transfer as desired. (For more information on staining, please request Bulletin EP009).

V. BUFFER RECIPES

GEL TYPE	SAMPLE BUFFER		RUNNING BUFFER	
Laemmli	Catalog # LC2676 (2x)		Catalog # LC2675 (10x)	
Tris-Glycine, SDS-PAGE	0.5M Tris-HCl,pH 6.8	2.5ml	Tris Base	29g
denaturing, non-reducing	Glycerol	2.0ml	Glycine	144g
	10% (w/v) SDS	4.0ml	SDS	10g
	0.1% bromophenol blue	0.5ml	Distilled water	to 1 liter
	Distilled water	to 10.0ml		

* 1x running buffer should be ~pH 8.3. Do not use acid or base to adjust pH.

APPENDIX IV

Formula for Sample Buffer 2x concentrated

pH 6.8

0.0625M	TRIS	
	HCl	pH 6.8
2%	SDS	
10%	Glycerol	
5%	mercapto-ethanol	

Leading edge marker bromophenol blue to 0.001% 5 microlitre of bromophenol blue into sample buffer

APPENDIX V

Formula for Reservoir Buffer (Running Buffer)

pH 8

0.025M TRIS (organic buffer tris hydroxymethylanine methane)

0.192M Glycine

0.1% SDS (Sodium Dodecyl Sulphate= Sodium Lauryl Sulphate= detergent)

To reach the required concentration of solutions the following amounts were mixed together;

5.3 gram Tris in 1000 ml distilled water =0.025M

14.4gram Glycine in 1000 ml distilled water = 0.192M

1.0gram SDS in 1000ml distilled water =0.1%

APPENDIX VI

Formula for Destainer

30% MeOH methanol

10% AcH acetic acid

APPENDIX VII

The range of calcium standards used and the corresponding readings from the atomic absorption photospectometer

N_2O flame with angled burners

Calcium	Value
5ppm	.051
10ppm	.086
20ppm	.172
50ppm	.385
100ppm	.633

CaH_2	8.25
N_2O	6.75

APPENDIX VIII

Table 12
BUFFER CAPACITY AND pH OF WHOLE SALIVA FROM SUBJECTS AGED 15 TO 55 YEARS, NORMAL AND VERY LOW VALUES ACCORDING TO ERICSSON AND HARDWICK[9]

	Resting saliva	Stimulated saliva
Buffer capacity		
Normal final pH	4.25—4.75	5.75—6.50
Very low final pH	<3.5	<4.0
pH		
Normal	6.5—6.9	7.0—7.5
Very low	<6.3	<6.8

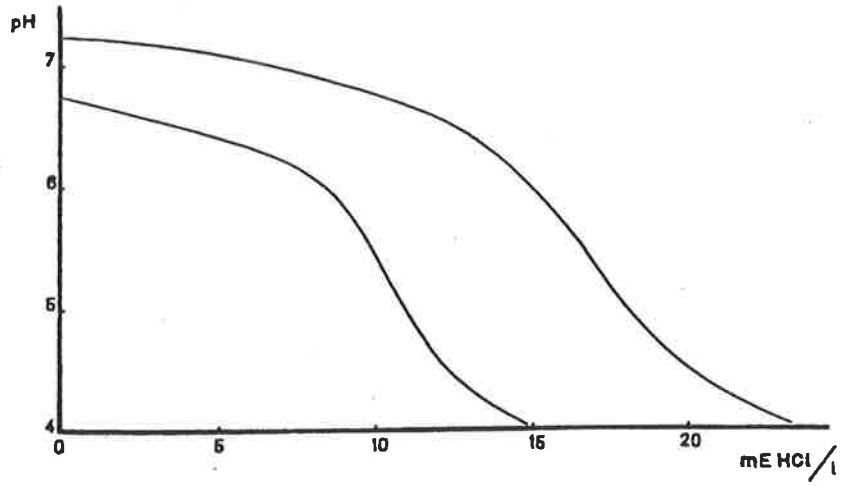


FIGURE 14. Buffer titration curves for salivary samples taken from the same patient. The lower curve represents resting saliva, and the higher curve stimulated saliva. Note the low buffer capacity between pH 6 and pH 4. (After Ericsson, Y., *Nordisk lärobok i cariologi*, Sveriges Tandläkarförbunds förlagsförening u p a, Stockholm, 1976. With permission.)

Tables taken from Tenovuo JO (Ed). Human Saliva: Clinical Chemistry and Microbiology. CRC Press Boca Raton, Florida 1989

Table 1
CLASSIFICATION OF SALIVARY SECRETION RATE ACCORDING TO THE REFERENCE INTERVALS OF ERICSSON AND HARDWICK[9] FOR RESTING AND PARAFFIN-STIMULATED WHOLE SALIVA

Secretion rate (ml/min)	Very low	Low	Normal
Resting saliva	<0.1	0.1—0.25	0.25—0.35 (mean: 0.30)
Stimulated	<0.7	0.7—1	1—3 (mean: 1.5)

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