Prevalence of oral and oropharyngeal human papillomavirus (HPV) in a sample of selected South African males: a pilot study

by

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Submitted in fulfilment of the requirements for the degree of Master of Science (Odontology) in the School of Dentistry, Faculty of Health Sciences, University of Pretoria

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DECLARATION OF OWN WORK

I declare that the dissertation, which I hereby submit for the degree of Master of Science (Odonotology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Christy Davidson

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SUMMARY OF DISSERTATION

Prevalence of oral and oropharyngeal human papillomavirus (HPV) in a sample of selected South African males: a pilot study

Oral human papillomavirus (HPV) infection and its association with head and neck cancers (HNCs) have been established by many studies. The characteristics of HPV-associated HNCs are distinguishable from those of non HPV-associated HNCs. HPV-associated HNCs are related to sexual behaviour, particularly the lifetime number of oral sex partners. The oral and oropharyngeal HPV epidemiology in South African men has not yet been researched.

The objective of this study was to determine the oral and oropharyngeal HPV strain prevalence and associated factors in a selected male population in Pretoria, South Africa. Male factory workers were recruited on a voluntary basis to be part of this study. Oral rinse and gargle samples were tested for 37 HPV types using the HPV linear array genotyping kit (Roche Molecular System). A questionnaire was utilised to obtain information regarding age, medical conditions, substance and alcohol use and sexual behaviour. HIV testing was optional.

The HPV prevalence was 5.6% among the men (n=125) aged 17-64 years. High risk HPV (hrHPV) types 16 and 68 were found in two men. Amongst the majority of the participants oral sex seemed to be an uncommon practice however, those participants with hrHPV did practice oral sex. A statistically significant association between HPV infection and an increased number of sexual partners (p=0.027) was seen but not between substance use, HIV-status or clinical mucosal pathology.

Considering the oral and oropharyngeal HPV prevalence found in this study compared to those reported in other countries. It is therefore proposed that a larger nationwide study be conducted to give a more representative view of the burden of oral and oropharyngeal HPV infection in South Africa.

Key terms: Human papillomavirus (HPV), men, South Africa, oropharyngeal squamous cell carcinoma, sexual partners, tobacco use, alcohol use, oral sex, HIV, oral mucosal lesions

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
HPV	Human papillomavirus
HIV	Human immunodeficiency virus
DNA	Deoxyribonucleic acid
LCR	Long control region (same as Upstream regulatory region, URR)
E1BS	E1 binding site
kDa	Kilodalton
p53	Tumour suppressor protein
Rb	Retinoblastoma
Brd4	Bromodomain containing 4
EDGFR	Epithelial derived growth factor receptor
PDGFβR	Platelet derived growth factor receptor beta
MHC	Major histocompatibility class I
Bak	Pro- apoptotic factor of the Bcl 2 family
E6-AP	E6 associated protein
hrHPV	High risk HPV type
lrHPV	Low risk HPV type
PBM	PDZ binding motif
PDZ	PSD-95, DLG/Zo
hTERT	Catalytic subunit of the telomerase complex
OCSCC	Oral cavity squamous cell carcinoma
OSCC	Oropharyngeal squamous cell carcinoma
PBS	Phosphate buffered saline
OHP	Occupational health practitioner

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cigarettes and drank alcohol. He disclosed that he suffered from high blood pressure. (pg.36)

Fig.13: Hyperkeratosis can be seen on the edentulous alveolar ridge. The brownish-black pigmentation could be either a form of racial pigmentation or could be representative of post-traumatic pigmentation in this edentulous area. Subgingival calculus and gingivitis is seen in association with tooth 43. (pg.37)

Fig. 14: Clinically this white hyperkeratotic lesion on the left buccal mucosa looked like leukoplakia or frictional keratosis. The participant was referred to the Oral Medicine Clinic of the Oral and Dental Hospital, University of Pretoria for biopsy. The participant was HPV negative and the HIV-status of this participant was unknown as he refused HIV testing. He smokes cigarettes and drinks alcohol. No medical conditions were disclosed in the questionnaire. (pg.38)

Fig. 15: Another case with leukoedema of the buccal mucosa and retromolar area. A small papillomatous lesion was also seen posterior to the tooth 38 on the left palato-glossal arch. On testing, this patient was not positive for HPV and although the possibility of a small lymphoepithelial cyst was considered on clinical grounds, the patient did not opt to come for follow up. The participants HIV status remained unknown as he declined HIV testing. The participant did smoke cigarettes and drink alcohol. No medical conditions were disclosed in the questionnaire. (pg.39)

Fig. 16: This erythematous discoid lesion was found on the on the right posterior ventral aspect of the tongue. No similar lesions were present anywhere else in the oral mucosa. There was no clinical explanation for it and the participant was referred to the Oral Medicine Clinic, Oral and Dental Hospital, University of Pretoria for biopsy. This participant was HPV negative and declined HIV testing. He did not smoke any substances but did drink alcohol. This participant did not show up for his appointment for follow up at the Oral Medicine Clinic. (pg.40)

Fig. 17: This participant presented with lingual swellings suggestive of mandibular tori. He was referred to a local dental clinic for further investigation. He was HPV and HIV negative, smoked cigarettes and drank alcohol. He suffered from high blood pressure. (pg.41)

Fig. 18: This participant presented with these small collections of white material that could be wiped off and the clinical impression was that of *Candidiasis.* The participant was referred to his oral health practitioner for management. This participant was HPV

negative and HIV negative. The participant did not smoke or drink alcohol and disclosed no medical conditions in the questionnaire. (pg.42)

Fig. 19: This participant presented with a possible bony cyst in the 12/13 region. The participant was referred to the Oral Medicine Clinic of the Oral and Dental Hospital, University of Pretoria for further investigation. The participant was HPV negative and HIV negative. The participant did not smoke any substances but drank alcohol. He did not declare any medical conditions in the questionnaire. (pg.43)

Fig. 20: Multiple small white papillomatous lesions were seen on the mucoperiostial mucosa of the anterior maxillary alveolar ridge of this participant. He was not positive for HPV on testing and tested negative for HIV. The nature of these lesions remains uncertain. The participant however did smoke and drink alcohol and suffers from high blood pressure. (pg.44)

Fig. 21: This participant presented with racial pigmentation on large areas of the oral mucosa. He also had this lesion that looked like frictional keratosis on the lateral ventral tongue secondary to trauma of the adjacent teeth. The lesions could not be wiped off. The participant did not come for his follow up appointment for biopsy of the lesion but the implications of leukoplakia with dysplasia were explained to him. He was HPV negative and decline HIV testing. He did not smoke any substances but did drink alcohol. No medical conditions were declared in his questionnaire. (pg.45)

Fig. 22: The lower lip of this HIV-negative and HPV-negative participant had these hypopigmented areas intermingled with areas of racial pigmentation. The pathogenesis of these lesions was uncertain. The participant did smoke cigarettes and drink alcohol and declared no medical conditions in his questionnaire. (pg.46)

Fig. 23: This participant presented with median rhomboid glossitis, a type of Candidiasis on the central aspect of the tongue. He did not have the typical “kissing lesion” on the opposing palatal area. Black hairy tongue can be seen on the postero-lateral aspects of the tongue. This is secondary to lengthening of the filliform papillae of the tongue with staining due to his cigarette smoking and/ or coffee or tea. This participant was HPV negative and HIV positive. He also drank alcohol. No medical conditions were declared in the questionnaire and he was referred to the OHP for treatment. (pg.47)

Fig. 24: This HIV-positive participant on antiretroviral therapy presented with these corrugated white lesions on the lateral tongue with the clinical appearance of oral hairy leukoplakia, an EBV-related lesion commonly encountered in HIV-infected

individuals. This can however only be confirmed with surgical biopsy and special investigative techniques such as *in situ* hybridisation for demonstration of the EBV. The pigment in this patient could be racial pigmentation but pigment secondary to the use of anti-retroviral therapy is also a well-known possibility. The participant did smoke cigarettes and drink alcohol and did not disclose any medical conditions in his questionnaire. (pg.48)

Fig. 25: This participant presented with linea alba buccalis, a traumatic lesion on the occlusal surface of the left buccal mucosa. The participant was HPV and HIV negative, smoked cigarettes and drank alcohol. This participant disclosed that he suffered from Diabetes. (pg.49)

Fig. 26: Multiple white lesions on the facial gingiva of the anterior maxilla in the region of the mucogingival junction. Gives the idea of HPV papillomas but participant was HPV negative. He was also HIV negative and did not smoke or drink any substances. He does however suffer from high blood pressure. (pg.50)

Fig. 27: Participant no 88 presented with this solitary fibrotic lesion on the facial gingiva of the anterior maxilla. A dental problem such as a healed parulis was considered clinically and the participant was referred to a dental clinic for further investigation. A list of dental clinics nearby his work was provided and no feedback was received. This participant was HPV and HIV negative, did not smoke but drank alcohol. He disclosed no medical conditions in his questionnaire. (pg.51)

Fig. 28: Participant no 116 presented with a severely painfull, non-healing lip ulcer on the lower left lip. A possible autoimmune disease or chronic ulcerative stomatitis of HIV was considered clinically. He was referred to the oral medicine clinic for a biopsy and treatment but he did not come for this appointment. The participant was HPV negative and his HIV status was unknown as he declined HIV testing. He was a smoker and did drink alcohol. He disclosed in his questionnaire that he suffers from high blood pressure. (pg.52)

Fig. 29: This participant presented with these white lesions that could be wiped off. The clinical impression was that of oral *Candidiasis* accompanied by underlying inflammatory erythema of the mucosa (see Fig. 30). (pg.53)

Fig. 30: This participant presented with these white lesions that could be wiped off. The clinical impression was that of oral *Candidiasis* accompanied by underlying inflammatory erythema of the mucosa. A smear biopsy was performed and sent to the

Department of Oral Pathology and Oral Biology, University of Pretoria for evaluation. Histological features confirmed the presence of fungal hyphae and a diagnosis of Candidiasis was confirmed in the report. He was referred to the Company Health Practitioner for further management. He was HPV negative and HIV positive. He did smoke and drink alcohol and he disclosed in his questionnaire that he was HIV positive and that he was suffering from TB (pg.54)

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CHAPTER 1

Introduction

The human papillomavirus (HPV) has recently been shown to play an important role in a subset of oropharyngeal squamous cell carcinoma. The natural history of this virus in the oropharynx and oral cavity environment has not been clarified and it was decided to investigate the prevalence of oral and oropharyngeal HPV in South African men.

1.1 Aims of this study:

The aims of this pilot study were:

1. to determine the oral and oropharyngeal HPV strain prevalence in a selected male population in Pretoria, South Africa
2. to determine if an association exists between the presence of HPV and sexual practices, substance use and/ or HIV status

1.2 Study Approvals:

Approval for the study was obtained from the Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Ethics number 101/2012) (Annexure A) and from the Research Committee (RESCOM) (Annexure B) of the School of Dentistry, University of Pretoria (DENT 2012/06). Consent (Annexure C) from the factory management to conduct the study was also obtained prior to study commencement.

CHAPTER 2

Literature Review

2.1 Human Papillomavirus

2.1.1 Structure

The human papillomaviruses (HPV) is a group of small DNA viruses that contain an icosahedral outer protein capsule shell. This shell encloses a circular double strand of DNA that consists of 8000 base-pairs. These base pairs can be divided into three regions: the E region that codes for the early proteins (E1-E7); the L region coding for the major and minor capsid proteins (L1, L2) and a non-coding region/ long control region (LCR) which contains the cis-elements that are required for replication and transcription of the viral genome (1) (Fig.1).

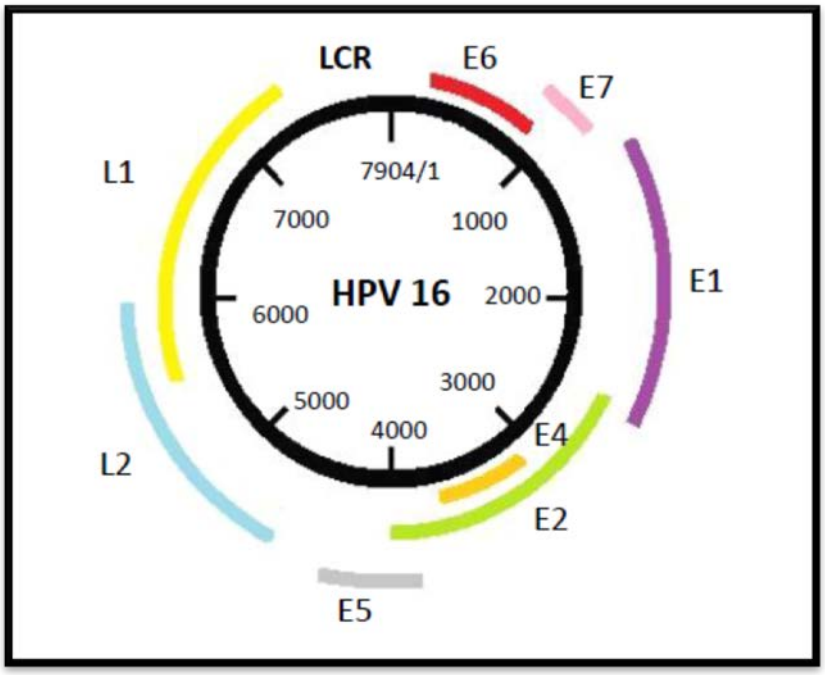


Fig.1: The genomic organisation of the high risk HPV 16 (2).

Adapted from Papillomavirus Research: from natural history to vaccines and beyond (pg.1). Edited by M. Saveria Campo, 2006. Norfolk, England: Caister Academic Press. Copyright ©2006.

2.1.2 The functions of the viral proteins are as follows:

The Early (E1-E7) coding proteins:

E1 protein:

E1 codes for a 70-80 kDa protein which is the primary replication protein essential in initiating viral DNA replication. It does so by binding to a specific DNA sequence on the viral origin of replication in the LCR region called the E1 binding site (E1BS). The E2 protein loads E1 onto this site. The E2 binding site is located adjacent to this E1BS. With this support from E2, E1 forms a hexameric complex, which promotes DNA unwinding (3-5) and provides the DNA template for future progeny DNA (6). The E2 protein is then displaced. E1 proteins are ATPases and helicases making them proteins with enzymatic activity. This ATPase activity is the source of energy for E1's helicase unwinding function (7-9). E1, by interacting with cellular DNA polymerase α is also involved in destabilisation of the chromatin structure (10).

E2 protein:

The E2 gene of the papillomaviruses encodes for a 40-45 kDa protein which is expressed in the early and intermediate stages of the viral life cycle. All of the E2 proteins are sequence specific proteins that bind to a 12 base pair DNA sequence of the viral genome, mainly in the LCR. The E2 proteins are multifunctional but are mainly associated with the transcription and replication of the viral genome (2, 3, 11, 12).

The E2 protein has the following functions:

- **Initiation of viral replication and regulates transcription**

  There are three stages of replication in the papillomavirus lifecycle:

  - Genome amplification when the virus first enters the host cell
  - Maintenance of the viral genome at a constant copy number in the proliferating basal cells. This step requires both viral genome replication and segregation.

- Production of progeny virions in differentiated cells (2, 11).

In order for the virus to continue through its life cycle after initial infection, it must replicate its own genome. This is accomplished by the viral proteins E1 and E2 as explained earlier (4, 11, 13, 14). Thus in summary, E2 is the viral origin recognition complex and E1 is the primary replication protein responsible for the initiation of DNA replication. The process of viral DNA replication occurs in nuclear foci which are dependent on E2 for their formation. Within these foci, a DNA damage response is induced through viral DNA replication and the viral proteins. This process is thought to be important in recruiting cellular repair proteins to synthesise viral DNA (15, 16).

- **Regulation of transcription**

E2 is the main transcriptional regulator of the papillomaviruses. It performs this function mainly by recruiting cellular factors which can either activate or repress the transcriptional processes depending on the binding sites, the nature of the associated cellular factors and the level of the E2 protein. The repression/activation of transcription by E2 is said to occur dose dependently and hinders the binding of cellular factors to the promoter elements (11, 17-19). Repression of viral DNA replication occurs through the E8^E2 protein. This is a spliced form of the full length E2 protein (20, 21). E8^E2 is responsible for regulating the levels of viral replication in the maintenance phase of replication in the high risk (hr) HPV types (HPV 16, HPV 18 and HPV 31) (11, 20, 22).

There are four E2 target sequences. Two of these can be found 3-4 base pairs upstream from the TATA box, themselves separated by only 3-4 base pairs. The third target sequence is found 80-100 base pairs upstream. An A/T rich region that constitutes the viral origin of DNA replication is found between these two E2 target sequence locations (23, 24). The fourth E2 target sequence is situated 400-500 hundred base pairs upstream from the third E2 sequence. Between this target sequence and the two target sequences surrounding the origin of

replication is an epithelial specific enhancer responsible for mediating cell type specific transcriptional activation from the adjacent promoter. This organisation of E2 target DNA sequences is retained in all HPV's, both cutaneous and mucosal (Fig.2).

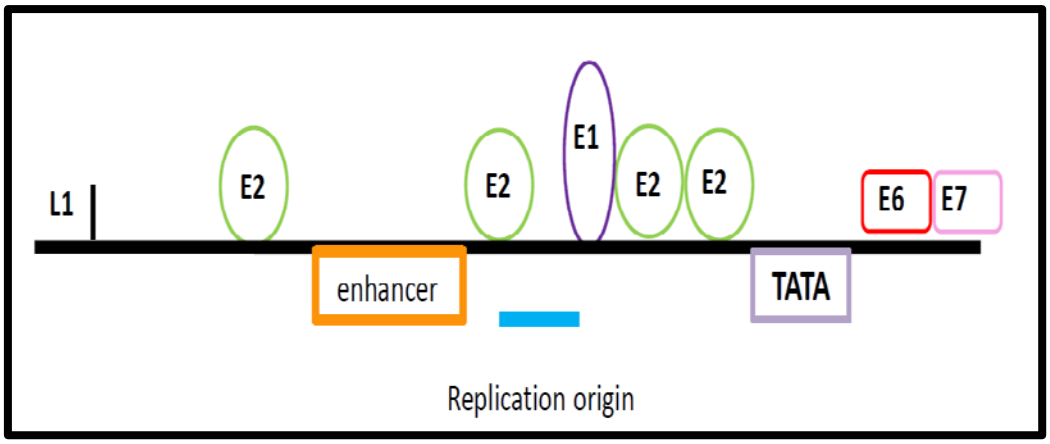


Fig. 2: A simple diagram illustrating the binding sites of the E2 viral protein (2).

Adapted from Papillomavirus Research: from natural history to vaccines and beyond (pg.43). Edited by M. Saveria Campo, 2006. Norfolk, England: Caister Academic Press

- **Cell cycle arrest and apoptosis**

Transient over expression of the E2 protein suppresses expression of the viral proteins E6 and E7 (25, 26). E6 and E7 are required for continued proliferation of the HPV-infected cell lines (see later). Suppression of E6 and E7 promotes reactivation of the cellular p53 and Rb pathways resulting in cell cycle arrest. Normal expression of E2 in the hrHPV types causes cell death through apoptosis and by arresting the cell cycle. The exact mechanisms of how this occurs is said to be different for the different HPV types. For HPV 16 this cell cycle arrest occurs through a p53 dependent pathway while as for HPV 18 this occurs independently of the p53 pathway (27).

- **Genome segregation**

  After initial infection, it is essential that the viral DNA is segregated into the daughter cells. E2 ensures a comparable distribution of DNA in these cells. Brd4, a chromatin adapter protein, interacts with E2 and prevents the association of E2 and the mitotic chromatin. The exact mechanism of how Brd4 interacts with HPV E2 functions still requires further investigation. Recently it was shown that Brd4 localises to the replication foci that is formed by E1 and E2 proteins (16, 28), although in HPV 31, a mutated E2 protein unable to bind with Brd4 is still capable of maintaining extrachromosomal viral genomes and amplification can still occur in differentiated keratinocytes (29, 30).

E4 protein:

In the final stage of the viral life cycle, the E4 protein is expressed as a fusion protein with E1 (E1^E4). In this document it will be referred to as E4. In the late stages of the viral life cycle this protein is found in the middle and upper layers of epithelium and differs from one HPV type to another (31-33).

The E4 protein has the following functions:

- **Genome amplification**

  E4 has been shown to optimise genome amplification, as well as the expression of capsid proteins for HPV 16 (34), HPV 18 (35) and for HPV 31 (36). The exact mechanism of how this occurs is not yet clearly understood, although it was seen that HPV 16 and HPV 18 E4 causes arrest of the cell cycle in dividing epithelial cells (37, 38).

- **Viral assembly , viral release and transmission**

  Expression of E4 occurs before the expression of the minor capsid protein L2, and the expression of L2 precedes the expression of L1, the major capsid protein. This sequence of expression ensures that capsid proteins are only produced in E4 positive epithelial cells (39-41). HPV 16

E4 has the ability to reorganise the cytokeratin network in keratinocytes (42-45). Studies are yet to explain the exact role that E4 plays in the viral life cycle but its late expression and its ability to reorganise the cytokeratin network suggests that it functions in viral release.

E5 protein:

Many, but not all, of the papillomaviruses encode for an E5 protein. It is small, hydrophobic protein that modulates a variety of cellular proteins. The carcinogenic potential of the virus correlates with the presence of an E5 gene in the viral genome (46-48).

The functions of E5 are as follows:

- **Oncogenic activity**

E5 causes an increase in cellular proliferation by slowing down the degradation of the epidermal growth factor receptor (EDGRF), platelet dervived growth factor β receptor (PDGFβR), colony stimulating factor 1 receptor and p185 neu via direct interaction (49, 50). E5 itself has weak transforming capability but by enhancing E6 and E7 ability it immortalises primary human keratinocytes (51), thus causing increased motility and invasiveness of the keratinocyte cell line(52, 53).

- **Effects on the cell cycle**

E5 protein helps maintain keratinocyte proliferation in cells which are undergoing maturation and favours expression of the late HPV proteins (54). This is accomplished by different pathways which increase the transcription factors c-fos and c-jun (55-57), thereby forcing the cells through the cell cycle and stimulating the transcription of the E6 and E7 proteins (58, 59).

- **Immune surveillance escape**

E5 is thought to play a role in the escape from the host immune surveillance by means of retaining the major histocompatibility complex (MHC) class I in the Golgi apparatus of the host cell. This impedes transport to the cell surface and therefore immune recognition (50, 60, 61).

E6 protein:

E6 and E7 are the two viral proteins responsible for the oncogenic potential of HPV through a process of cellular transformation (62). E7 induces an S-phase state within the infected host cell. This re-programs the host cells to enter the cell cycle once again and they can express the proteins that are required for the replication of the viral genome. E6 assists in E7 functions by counteracting the various apoptotic programs that are activated in response to unscheduled DNA replication caused by E7. This ensures the continued survival of the virus infected cells.

The functions of E6 are as follows:

- **Preventing apoptosis**

E6 prevents cell apoptosis through two mechanisms, one p53-dependent and the other p53-independent. p53, a tumour suppressor protein, through its functions as a transcription factor can prevent neoplastic cellular transformation through temporary/permanent cell cycle arrest or by triggering apoptosis in these defective cells.

- Both high risk(hr) and low risk (lr) HPV E6 binds to p53 by means of the recruitment of a protein ligase E6-associated protein (E6-AP) (63, 64). This leads to ubiquitin mediated degradation of p53 (65) resulting in epithelial immortalisation. The hrHPV E6 protein effectively degrades p53 whereas the lrHPV E6, although still binds to p53, exerts little

degradatory activity. Importantly this distinguishes the difference in oncogenic potential between the low risk and the high risk HPV types.

- The second mechanism whereby E6 prevents apoptosis is through a p53-independent pathway. E6 interacts with *Bak* (66), a pro-apoptotic factor of the Bcl-2 family, that is abundantly expressed in the upper layers of epithelium. Both the hrHPVs and lrHPVs E6 binds to *Bak* and degrades it (66, 67).

- **E6 interactions with other cellular proteins**

A defining characteristic of the hrHPV E6 is the ability to bind to a variety of other cellular proteins that are involved in many regulatory processes, allowing their degradation e.g. Drosophilia disc large tumour suppressor protein; Scribble; MAGI-1. These cellular proteins contain PDZ domains that serve as receptors for binding to the PDZ (PSD-95, DLG/Zo-1) binding motif (PBM) on the hrHPV E6 carboxy terminus. This binding motif is not present on the lrHPV types. (68, 69). This specific domain sequence is only found on the hrHPVs E6 and is absent from the lrHPV types. These domains serve as receptors/docking stations for many different cellular proteins (68-71). The oncogenic potential of hrHPVs could be attributed to this difference in the targeting of cellular proteins by mechanisms other than just through that of its association with E6AP.

- **E6 can enhance telomerase activity**

The E6 proteins of the hrHPVs enhance telomerase activity (72, 73). Telomerase is inactive in normal cells and for this reason the telomeric DNA at the ends of chromosomes in proliferating cells is continuously shortened with each cell division (74). This loss leads to chromosomal instability and eventual senescence of the cell (72, 75). However, in immortalised cancers cells, telomerase activity is reactivated and the telomere length will be maintained thus promoting cell survival (76, 77). The activation of telomerase by hrHPV E6 involves the upregulation of hTERT (the catalytic subunit of the telomerase complex) by Myc (73, 78, 79).

- **E6 affects gene transcription**

E6 can either repress or activate gene transcription. It causes repression of transcription by binding to and inhibiting transcriptional co-activators like CREB-binding protein (CBP) and p300 by a yet unknown mechanism (80-82). CBP/p300 normally activates transcription. When E6 binds to CBP/p300 the acetylation of p53 is prevented (82). p53 is then unable to carry out its normal functions. Both the hrHPVs and lrHPVs have this function; the only distinguishing factor is that the hrHPVs E6 binds strongly to CBP/p300 whereas lrHPVs E6 binds weakly to CBP/p300. E6 also up-regulates vascular endothelial growth factor (VEGF) (83) which is responsible for initiating angiogenesis necessary for the recruitment of new blood vessels for expansive tumours (84).

E7 protein:

The E7 proteins of high and low risk HPV types are about 98 amino acids in length. They are responsible for deregulating the cell cycle causing cell proliferation, immortalisation of the host cells and finally transformation of these cells. E7 works together with E6 to carry out these functions but E7 seems to be the dominant protein in this relationship.

The functions of E7 are as follows:

- **Effects on the cell cycle and transcription**

E7 carries out its function mainly through its interaction with the retinoblastoma (Rb) family of proteins. These tumour suppressor proteins prevent DNA replication and progression through the cell cycle (85, 86). The normal function of Rb protein is dependent on its phosphorylation state. Active, hypophosphorylated Rb binds to and prevents E2F-mediated transcription (87).

HPV E7 can affect E2F-mediated transcription through its interactions with Rb or independently of Rb in different ways:

- E7 binds to and inactivates hypophosphorylated Rb thereby releasing the E2F transcription factor and promoting cell cycle progression. E7 can also bind directly to E2F without the involvement of Rb.

- An important mechanism of regulating gene transcription is through chromatin remodelling by histone acetylation. E7 and its association with histone deacetylase (HDAC) complexes (88) aids in the evasion of tumour suppression. E7 interacts with HDAC-1 causing HDAC to be displaced from Rb causing de-repression of gene transcription (87, 89-91). HDACs also inactivate E2F factors by deacetylation.

- E7 binds to the cyclins A and E and to the cyclin-dependent kinase (cdk) inhibitors (p21 and p27) affecting the phosphorylation state of Rb and leads to its inactivation (85, 92, 93). The result is the release of the E2F transcription factor allowing the production of proteins involved in DNA synthesis and the productive replication of the virus in the suprabasal differentiating keratinocytes (94-96). This interaction also leads to the degradation of Rb through the unbiquitin-proteasome pathway (97).

- **Effects on cellular metabolism**
  - Proliferating cells express M2 type iso-enzyme of pyruvate kinase (M2-PK). This enzyme is involved in the exit of cells from the glycolytic pathway. E7 expressing cells have been shown to have a significant increase in the total glycolytic rate (98). This suggests that E7 ensures glucose to the synthetic processes and concomitantly reduces the cell's requirement for oxygen. These are important characteristics of tumour cells.

  - When cells switch to malignant transformation, changes in the cellular carbohydrate metabolism are seen. Slowly growing cells are converted to highly proliferating cells which is accompanied by a

depletion of the intracellular glycogen stores. This occurs through an unknown mechanism under the control of E7 on alpha-glucosidase (99).

- E7 can bind to members of the basal transcription machinery (eg. TBP and TBP-associated factor-110) (100, 101). The effects that this causes are still unclear but it suggests that E7 could have other functions that are not yet known.

The Late coding proteins (L1, L2):

L1 protein:

It is a 55 kDa major structural protein with the ability to self-assemble into virus-like particles (VLPs). These particles are potent immunogens (102, 103). This finding laid down the foundation for the manufacturing of the current prophylactic vaccines. L1 plays a role in infectious entry of the virus which is accomplished by L1-heparin sulphate proteoglycan (HSPG) interaction. This interaction occurs on the host extracellular basement membrane (104). The current thought is that infection of all papillomaviruses requires initial binding to HSPG (105, 106). This attachment results in minor conformational changes allowing exposure of a specific portion of the L2 capsid. This exposure allows the cleavage of L2 by cellular furin protease (107, 108). This induces sequential conformational changes that promotes the binding of the virion to the keratinocyte cell surface through a secondary receptor (109). These secondary receptors are still unknown.

L2 protein:

L2 is a 55 kDa minor structural protein. Both L1 and L2 have DNA-binding ability suggesting that they may aid in viral encapsidation (110, 111). L2 interacts with various host cell proteins such as chaperon protein cyclophillin B (CyPB) (112), cytosolic adaptor protein Sortin Nexin 17 (SNX17) (113, 114)

and tSNARE syntaxin 18 protein (115, 116) enabling entry of the virus and trafficking of the virus to the nucleus of the host cell. Following viral dissociation, the viral DNA must escape the vesicular compartment to be able to travel to the host cell nucleus. Various studies have shown regions of L2 to be involved in this process (117-119). These processes deliver the virus to replication centres and aid in the packaging of the viral DNA into capsids.

Upstream Regulatory Region/Long Control Region:

This region consists of 400-850 base pairs which are found between the L1 and E6 genes. This LCR contains most of the elements that are necessary for replication and transcription of the viral genome. Transcription of the early and late coding genes occurs in one direction from promoters located in the LCR or in the E6 or E7 genes (Fig 1).

2.1.3 Human papillomavirus types

Human papillomaviruses belong to the *Papillomaviridae* family. They are a heterogeneous group of viruses that can be categorized into five different genera based on their genomic sequence: alpha, beta, gamma, mu and nu (120, 121). Papillomaviruses can affect different species but for the purpose of this study only those affecting humans (HPVs) will be discussed. More than 150 types of HPVs have been identified, with only 10% difference in the L1 nucleotide sequence distinguishing the different types (3, 122, 123).

HPVs can also be classified according to the epithelium that they infect (either cutaneous or mucosal), or according to their association with malignant or benign lesions (low or high risk). In this regard cutaneous HPV types are typically “low risk” as they are generally associated with benign/non-malignant lesions. Most of the mucosal types are also low risk (e.g. HPV 6 and HPV 11), but a few of the mucosal types are termed high risk as they are carcinogenic and typically associated with malignant lesions (eg. HPV 16, HPV 18 and HPV 31). This does not however mean that there are no

exceptions to the statements above, as studies have shown the presence of low risk HPV in high-grade lesions and high risk types in low-grade lesions (124-127).

The low risk HPV types are associated with common warts, squamous papillomas, condyloma acuminatum and focal epithelial hyperplasia (125, 128, 129). The most common high risk HPV types are HPV 16 and HPV 18 which are responsible for on average 70% of all cervical cancers worldwide (130) and HPV 16 is said to account for at least 90% of oropharyngeal squamous cell carcinomas (OSCCs) (123, 131-134).

2.1.4 Transmission

HPV affects the basal epithelial cells of the skin and mucosa. Both conventional and oral sexual contacts are means of transferring the virus. Oral HPV infection can therefore be acquired through oral-genital contact, by oral to oral contact or possibly by autoinoculation (135, 136). The transmission of HPV from environmental surfaces is at present questionable and although HPV DNA may be found on a surface it is not necessarily infectious (137). Studies on nonsexual methods of transmission have shown that HPV DNA has been found in blood, reproductive and placental cells and orally in children that had not been involved with any form of sexual activity (138-142). HPV can be transmitted from mother to child through different potential routes (141, 143, 144) and *Medeiros et al* in 2005 showed that this vertical transmission of HPV only developed in a third of neonates whose mothers were infected with the virus (145). The clearance rate of HPV by the infants was roughly six months, although it was shown that some of these HPV infections do persist (141, 146). Persistence of oral HPV in infants is associated with the mother having a hrHPV type at 36 months follow-up, if her age of sexual debut was between 14-16 years, early age of oral contraception (14-16 years) and if she had warts on her hands. Approximately 90% of all HPV infections resolve spontaneously within 1-2 years

(147). Further investigations into the mechanisms of transmission of HPV are required (148).

2.1.5 HPV life cycle

HPV infects the basal cells of the squamous epithelium and the life cycle of the virus is intimately linked to the differentiation of these epithelial cells. The specific reason why HPV s infects these basal cells is thought to be due to their associated cell surface receptors (149-151). These receptors allow binding of the virus to the epithelial cells promoting internalisation of the viral particles. It is thought that heparan sulphate proteoglycans found in the basement membrane may play an initial role in the primary binding of the virus (149, 150, 152). Infection with HPV also requires a secondary receptor to physically gain entry into the epithelial cell. The first secondary HPV receptor described was α6β4 integrin (153, 154) and recently others have been suggested (CD151, HPV-HSPG-growth factor complexes and annexin A2) (155-158). Host epithelial cell entrance via calveolar or clathrin-mediated endocytosis has also been described (149, 159-161). Once the virus has gained entry into the epithelial cell, uncoating of the virus occurs and the circular viral genome is transported to the nucleus of the host cell (161, 162).

In order for the virus to continue through its life cycle after entry and transport to the nucleus it must replicate its own genome as a stable episome in the basal layer of cells (6). The viral genome is maintained at a low copy number of around 10-200 copies per cell in the basal layer of epithelium (163) and infection in the basal layer cells can persist as a latent infection (164).

In normal epithelium, uninfected with HPV, the basal epithelial cells would now undergo mitosis. One daughter cell would remain as a basal cell and the other would migrate into the suprabasal cell layer. This cell will have exited the cell cycle and undergo terminal differentiation (165, 166).

However, HPV infected basal cells will undergo mitosis with one daughter cell remaining as a basal cell i.e. latency of the virus in that cell and the other

daughter cell would migrate into the suprabasal layer. This cell would progress through the cell cycle and cellular differentiation would be blocked and mitosis continues.

Viral synthesis occurs in the granular epithelial layer once viral genome amplification is complete. The down regulation of E6 and E7 transcription by E2 causes a release of p53 and Rb proteins as well as activates the L1 and L2 genes. Before the late viral proteins are expressed we see the expression of E4 (39). This viral protein is involved in the release of the viral particles from the host cells and has the ability to reorganise the cytokeratin network in keratinocytes (42-45). The capsid proteins, L1 and L2 are expressed and encapsidate the newly synthesised viral genomes (41). Virus-like particles have the capability to assemble in the absence of L2 but L1 is thought to enhance viral packaging and infectivity (167, 168). After completion of the reproductive process the host cell dies and the newly produced virions are released when the cornified layers of epithelium are shed. The newly formed viral particles are fully infective and now have the ability to infect other cells.

2.2 Oral and Oropharyngeal HPV associated cancers

Head and neck squamous cell carcinoma (HNC) is the sixth most common epithelial malignancy in the world (169). Oral cavity squamous cell carcinoma (OCSCC) is categorised as malignant epithelial tumour of the oral cavity comprised of the buccal mucosa, floor of the mouth, gingiva, hard palate and the tongue (3). Oropharyngeal cancers (OPC) include cancers of the mucosa of the base of the tongue (lingual tonsils), palatine tonsils, soft palate and the posterior pharyngeal wall (170, 171).

Tobacco and alcohol consumption are known risk factors for OSCC and with the worldwide decrease in tobacco usage there has been a decline in the incidence of this malignancy in recent years (172, 173). Despite this downward trend of OCSCC, squamous cell carcinoma (OSCC) of the oropharynx on the other hand has been on the rise even with the decreasing

trend in tobacco use (174-179). The presence of HPV in a subset of OSCCs suggests a pathogenic role for HPV in this subset of malignancies.

2.2.1 HPV

The prevalence of oral HPV infection in normal epithelium in the literature varies greatly (<1% - >50%) (180, 181) and can be ascribed to the variances in the methods used to collect, to process and to test for the virus (182-185). It could also be attributed to the difference in study populations. The association of hrHPV types with this subset of OSCC has now been established by many studies (123, 132-134, 186). It has been shown that oral HPV infection, and specifically HPV 16 infection, causes up to a 50 fold increase in HPV positive OSCC (131). In South Africa there has been a proven increase in OPC since 1995 and ninety per-cent of these were associated with HPV 16. Men being affected more frequently than females (187). Gillison et al. in 2012 published the first population-based study that concurrently examined the epidemiology of oral HPV infection among men and women and showed an almost 3-fold higher HPV prevalence in men (188). In their study the HPV 16 prevalence was 5-fold higher in the men than the women. The overall oral HPV prevalence in their study was 6.9% and 10.1% in men specifically (188).

Prevalent oral HPV infections among men have been linked with a variety of risk factors which include: race (189), age (190), current smoking (191), circumcision (192), sexual orientation (190), oral sex (193, 194) and number of vaginal sexual partners (195-197).

2.2.2 Smoking and Alcohol

The literature on cigarette smoking as a risk factor for oral HPV infection is conflicting. Much of what we know and understand regarding oral HPV infection is based on HPV's role in cervical cancer. In cervical cancer studies a synergistic effect with smoking and the invasiveness of cervical cancer has

been seen. Smoking can modify the acquisition and clearance of HPV by compromising the immune system (198-203). Cigarette smoking has been shown to increase cellular proliferation and metaplasia in various cell types and tissues which could increase replication and production of HPV (199, 204, 205). There are three different schools of thought regarding HPV associated Head and Neck Squamous Cell Carcinomas (HNSCC) and smoking of cigarettes. They are: those that believe that there is an elevated risk of HPV associated HNSCC in non-smokers (131, 133, 206-208), those that believe that there is no difference in HPV associated HNSCC in smokers or non-smokers (209-211)and those that believe that smoking cigarettes does in fact increase the risk of HPV associated HNSCC (197, 212). Marijuana use, especially increased intensity and duration, has been shown to cause biological changes and is considered a strong risk factor for HPV positive HNSCC (131).

Alcohol has the ability to modify mucosa possibly allowing easier entry of the virus. It is also known to modify the host immune response, thereby increasing the susceptibility to HPV infection (213, 214). A study by *Urashima et al.* in 2013 showed that heavy alcohol consumption causes distinct genetic alterations in HNSCCs (215). Alcohol however, has not been shown to increase oral HPV infection (197, 216) but could potentially alter behaviour which could increase the likelihood of acquiring HPV.

2.2.3 Sexual History

HPV is a virus that can be sexually transmitted and therefore, HPV associated cancers and oral HPV infection is intimately linked with sexual behaviour. These types of behaviours include: young age at first sexual encounter, a high number of lifetime sexual and oral sex partners as well as open-mouthed kissing of an infected person (131, 197).

2.2.4 HIV

HIV infected individuals have been reported to have an up to a six fold higher risk for HPV-related OSCC (217, 218). Recent literature indicates that the overall oral and oropharyngeal HPV prevalence in individuals infected with HIV ranges between 20% and 40% and the prevalence with oncogenic types is between 12% and 26% (219-223). HIV infected individuals with normal mucosa have also been found to be at a greater risk of acquiring more than one type of HPV orally (194). It is thought that HIV and HPV function as a combined risk factor, as HIV-positive individuals have more frequent HPV infections but the natural history of these infections might be altered due to altered behaviour and immunity(182).

CHAPTER 3

Materials and Methods

3.1 Participant selection

Male participants were voluntarily recruited from an industrial factory in Pretoria in 2012. All male employees working at the factory were invited to be part of the study. Flyers (Annexure D) were printed and distributed to the workers beforehand explaining what the envisioned HPV study entailed and HPVs association with OSCC in men specifically. Any questions regarding the study, HPV and OSCC were handled by the occupational health practitioner (OHP) of the company and the research team on the day of sampling and throughout the study.

On the day that sampling commenced participants were invited to the clinic on the work premises. They were allowed to enter free of choice and ask any questions they had regarding the study and what would be expected of them if they choose to participate. The study included a compulsory oral rinse sample (HPV), a self-questionnaire (Annexure E) and an optional HIV test which was offered to each participant but was not a criterion for participation in the study. The HIV testing was optional as it was uncertain whether or not the participants would be willing to test. The HPV prevalence was the main aim of the study and it was decided not to enforce HIV testing. Even so, the association between HIV and HPV is well-known and where possible, the HIV status of the participants willing to test was correlated with the HPV status.

Informed consent (Annexure E) to participate in the HPV study was signed by all participants before sampling commenced. Separate consent was obtained for the optional HIV testing and pre-HIV test counselling was provided by the OHP or the Matron of the Oral and Dental Hospital. The HIV testing was done after the oral rinse sample was obtained and the

questionnaire completed. Participants were permitted to leave the study at any time during the proceedings.

3.2 Study Logistics:

The study had four components to it:

1. Clinical oral examination (compulsory)
2. Saliva sample collection (compulsory)
3. Self-Questionnaire (compulsory)
4. HIV testing (optional)

3.2.1 Clinical examination

Before the saliva sample was obtained, every participant underwent a thorough oral clinical screen by a senior dentist and/or oral pathologist. All dental and other soft tissue abnormalities were noted and photographed. Any pathology detected and requiring further management was referred to either the OHP or to a local dental clinic. A list of oral health clinics in and around Pretoria was provided to the participants. All soft tissue lesions requiring biopsy for definite diagnosis were referred to the Oral Medicine Clinic of the University of Pretoria situated in the Oral and Dental Hospital (Pretoria, South Africa).

3.2.2 Saliva sample collection:

Oral rinse samples were collected by means of a 20 second oral rinse and gargle with 5 ml phosphate-buffered saline (PBS, p4417 Sigma Life Sciences, USA). The 40 ml sterile collection cup (Lasec, South Africa) filled with the PBS was given to the participant that had to alternately swish and gargled every 5 s and then expectorate the saliva back into the sterile collection cup. Some of the participants commented on the taste for the PBS saying it was very

salty. For this reason, the participant was offered a drink (Coke a Cola) once the saliva sample collection was completed.

The saliva collection cups were numbered to correspond with the questionnaire number and HIV test number (should the participant have opted to test). This ensured patient anonymity as no names were used. All the samples were stored on ice and then transported within twenty four hours to the molecular laboratory at the University of Pretoria for sample preparation.

Fig. 3: Participants waiting in line for their oral examination and saliva sample collection (Permission was granted by all participants for the taking of all the photographs).

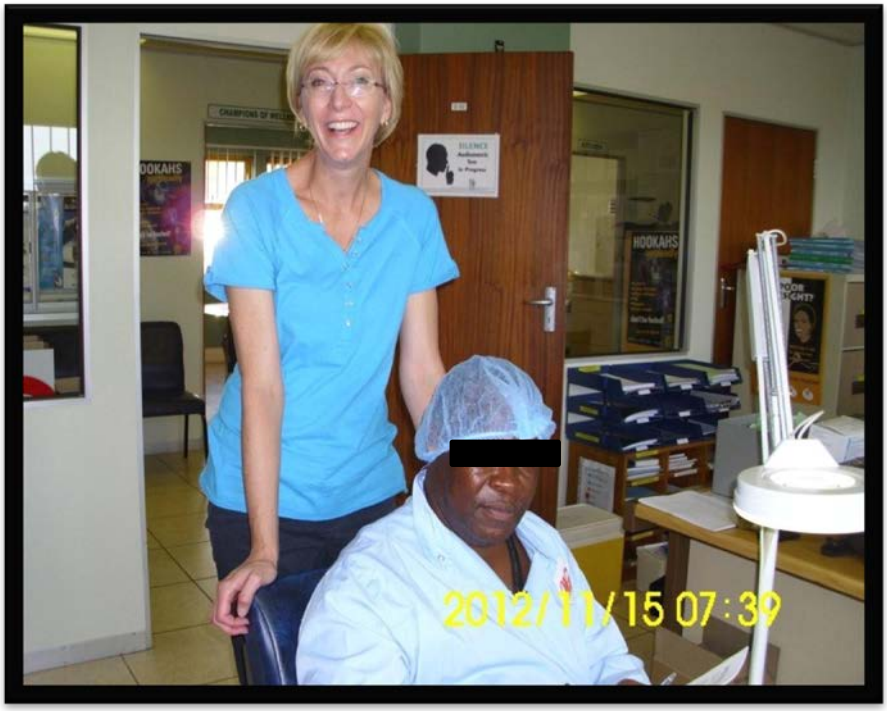


Fig.4: Prof Sonja Boy (oral pathologist) performing an oral examination and saliva sample collection**.**

Fig. 5: Dr Christy Davidson (senior dentist) performing an oral examination and saliva sample collection.

3.2.3 Questionnaire

Each participant was required to complete a questionnaire. The questionnaire content (Annexure E) comprised of specifically formulated questions pertaining to substance and alcohol use, any medical conditions and previous and present sexual histories. The questions for the questionnaire were based on the literature available at that time and included possible highlighted risk factors for oropharyngeal SCC (pg. 27) and oral HPV infection in men. A basic medical history and age was also included. The questionnaire was designed by the statisticians to ensure as much data be collected as possible. This included the quantity and frequency of use of the substances.

A male field worker trained to deal with all aspects of the questionnaire was present throughout the proceedings to further explain or translate the content of the questionnaire to any participant. Once the questionnaire was completed and a participant chose not to test for HIV he placed his own questionnaire in a locked box (Fig.7) which was only opened at the end of the study. All information was confidential and only the OHP of the company had the participant names that corresponded to the participant numbers in order to provide feedback if and when necessary.

Fig. 6: Mr Solly Mafiri, the male field worker involved in the study was available to explain or translate any questions the participants had.

Fig. 7: All questionnaires were kept in a locked wooden box which remained locked ensuring participant anonymity.

3.2.4 HIV counselling and testing

If a participant agreed to test for HIV, the completed questionnaire accompanied the individual participant to the OHP/ matron for HIV pre-test counselling and testing in a private room. This ensured that the OHP/matron had the correct participant number to match the HIV test result.

A rapid point of care HIV screening test (U test HIV/AIDS, Humor Diagnostica, South Africa) which detects HIV-1 and -2 antibodies was then performed by the sisters according to the manufacturer's instructions as follows:

The participants' finger was cleaned with a sterile swab and pricked with a lancet slightly off centre. The finger was massaged towards the puncture site to obtain the required volume of blood. The correct end of the test pipette was held against the finger to capture the drop of blood. The blood was pipetted into the round sample well and seven drops of diluent were added. A positive result could be interpreted after 5 min; 20 min was the maximum time to confirm a negative result. As stipulated by the manufacturer's instructions, no readings were interpreted after 30 min from the time of assay.

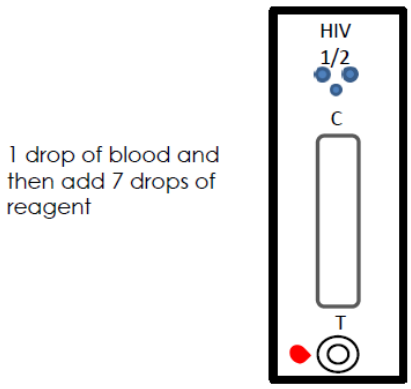


Fig. 8: The diagram represents the rapid HIV U test used in this study to test the HIV status of the participants who consented for HIV testing.

Feedback on the HIV-result with the applicable post-test counselling was provided immediately by the OHP. Confirmatory follow up testing by means of an Elisa test was recommended and conducted by the OHP of the company in all positive cases. Once HIV counselling and testing was completed, the participant again personally placed the questionnaire in the secure wooden box.

Fig. 9: Matron Ella Khumalo doing HIV counselling and testing in a private room.

3.3 Molecular Laboratory Techniques

3.3.1 Specimen preparation

Saliva sample preparation for HPV genotyping commenced within twenty four hours of sampling. The steps for each sample preparation were as follows:

- Each sample was vortexed for 5 s and then transferred to a 15 ml sterile centrifuge tube (Sterilin). Centrifuge tubes were numbered with the corresponding participant number.
- Sterile PBS was added to the sample to obtain a total volume of 6 ml (first wash).
- The samples were then centrifuged in an ALC PK121 centrifuge (ALC International, Italy) at 3000 rpm for 10 min.
- Thereafter the supernatant was decanted into a container of 1:10 ml NaClO solution.
- Again sterile PBS was added to the cell pellet up to a final volume of 8 ml (second wash).
- The sample was resuspended by vortexing for 20 s.
- This resuspended sample was pelleted by centrifugation at 3000 rpm for 5 min in the same ALC PK121 centrifuge.
- The supernatant was once again decanted into a container of 1:10 ml NaClO solution.
- Sterile PBS was again added to the cell pellet up to a final volume of 3 ml (third wash).
- After vortexing by pulse action for 20 s each resuspended cell sample was divided into two equal samples of 1.5 ml each.
- Each was placed in two 1.5 ml microfuge tube (Lasec, South Africa) and both numbered with the participant number.
- One sample was sent for genetic testing and the other was stored as a back-up.

The original sample was therefore washed three times will sterile PBS. All samples were stored at -70°C until subsequent DNA extraction. The supernatant in the NACIO solution was left to denature for three days and then disposed as medical waste. Infection control procedures were followed throughout the entire sample preparation procedure.

3.3.2 DNA extraction

Specimens for extraction were centrifuged at 3000g for 10 min. The cell pellets were resuspended in 200 µl of sterile PBS. DNA extraction on these cell pellets was performed in batches using the DNA Isolation Kit (Roche Molecular Systems®, Branchburg, NJ) on the MagNA Pure automated extraction system. Strict quality control procedures were followed to prevent contamination. DNA extraction was performed by Lancet Laboratories.

3.3.3 HPV DNA amplification and genotyping

HPV DNA amplification and genotyping was done using the Linear Array HPV Genotyping Test (Roche Molecular Systems®, Branchburg, NJ). The pool of primers in the kit are designed to amplify HPV DNA from 15 high -risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), three probable high-risk genotypes (26, 53 and 66) and 19 low/undetermined risk types (6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39 and CP6108) (224). The β-globin gene was amplified concurrently for each sample and served as an internal control for cellular adequacy, extraction efficiency and amplification.

CHAPTER 4

Results

4.1 Statistical Analysis

The relationship between the prevalence of HPV and the prevalence of variables included in the study, namely substance use, alcohol consumption, sexual history, HIV and oral mucosal lesions were investigated by making use of the Chi-square test for independence or where appropriate the Fisher Exact test. The Fisher's Exact test was used when sample size was too small or when information in specific cells of the two-way tables analysed was too sparse. The Kruskal-Wallis test was used to analyse whether or not any of the variables investigated had any significant influence on the HPV-status of the participants.

4.2 Study results

4.2.1 Clinical oral examination

- **Clinical mucosal lesions**

Eighteen of 125 participants (14.4%) presented with oral mucosal pathology on clinical examination. The lesions encountered included white lesions, red lesions, ulcerative lesions and *Candida* infection. The latter were confirmed by microscopic examination of smear biopsies by the Department of Oral Pathology and Oral Biology, School of Dentistry, University of Pretoria.

None of the seven HPV positive participants had any clinically visible mucosal pathology. The oral mucosal pathology of the participants that was seen is summarised below:

Participant 5

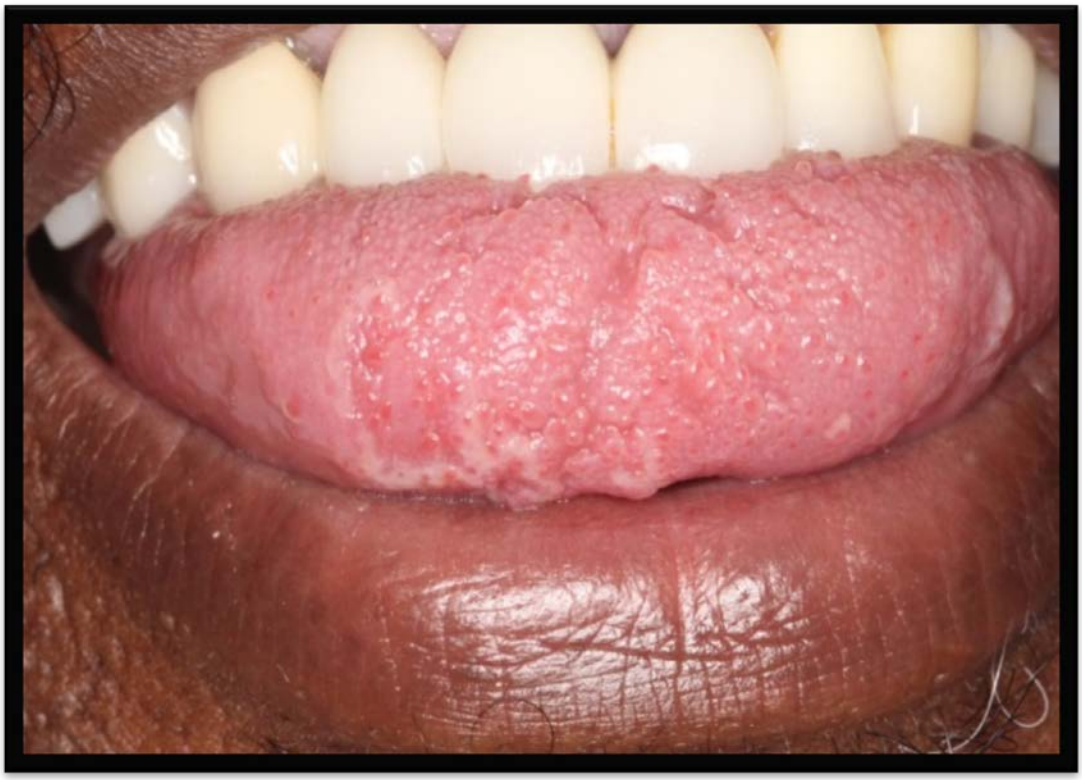


Fig. 10: Migratory glossitis of the dorsal tongue. This participant was HPV negative and HIV negative. He did not smoke any substances but did drink alcohol. He disclosed in the questionnaire that he has both high blood pressure and Diabetes.

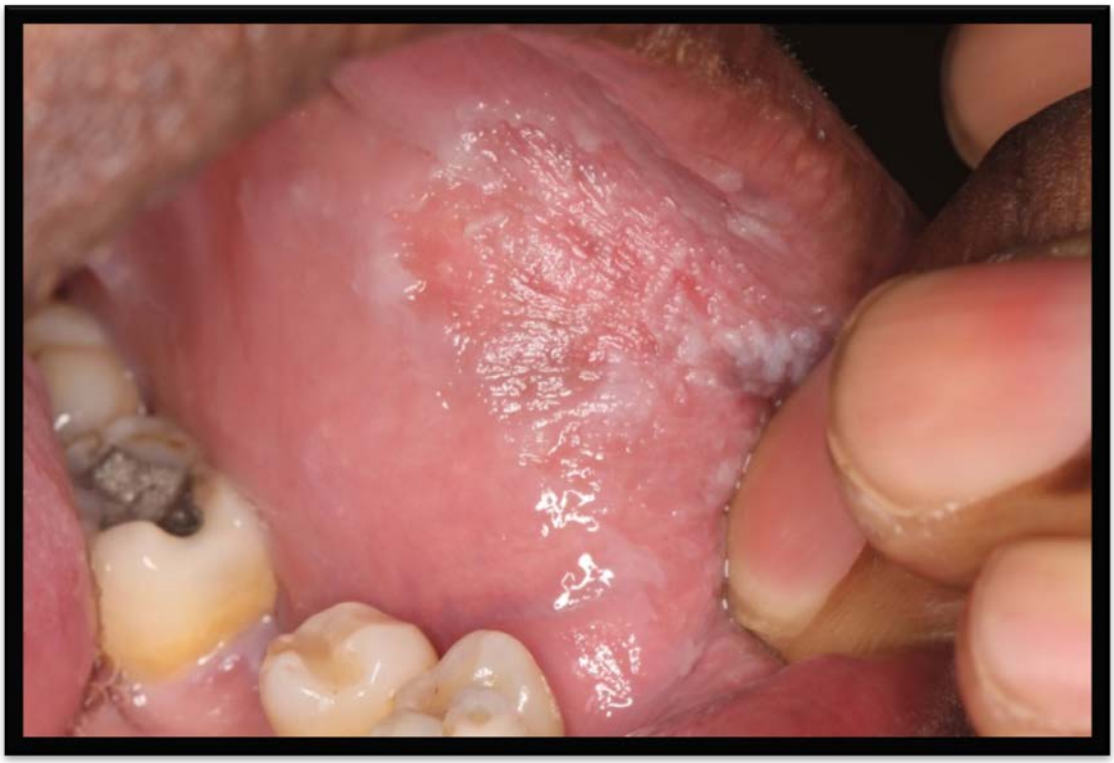


Fig. 11: The lateral aspect of the ventral tongue mucosa shows features of traumatic erosion and frictional keratosis. This is most probably a traumatic lesion secondary to trauma associated with absent tooth 46 and sharp restoration and tooth edges on tooth 47.

Participant 16

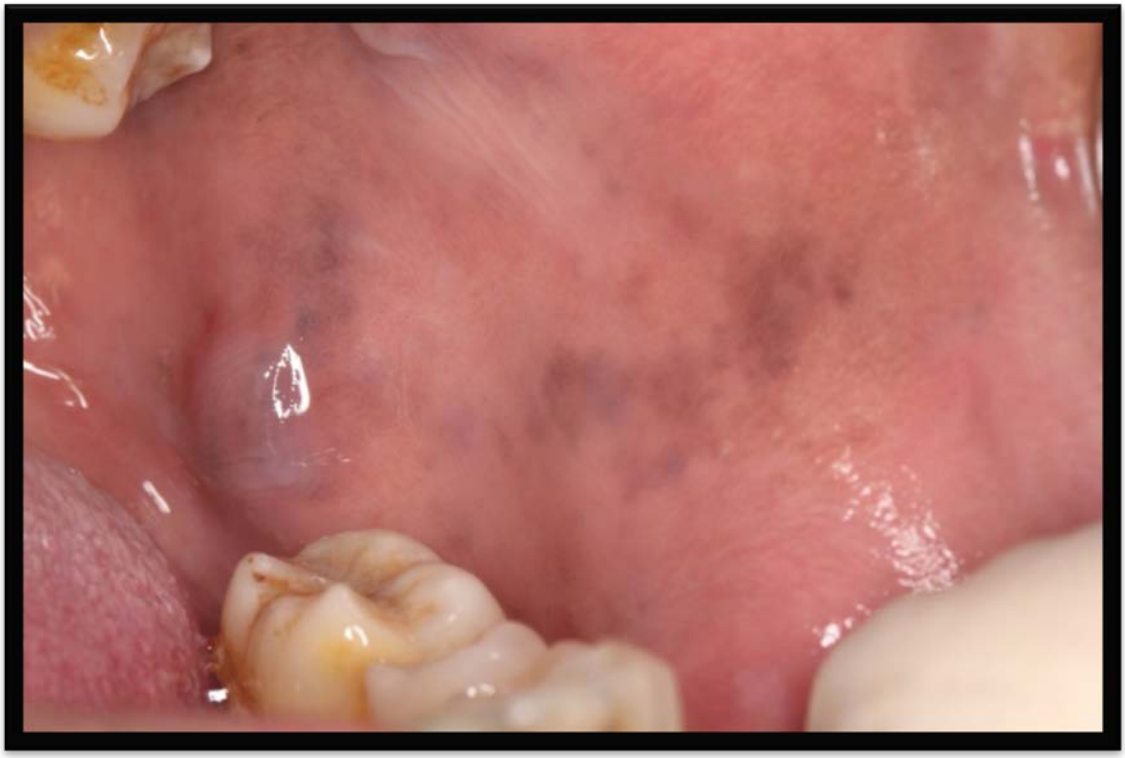


Fig.12: Leukoedema was a common finding in many of the participants, and especially the smokers. This smokey-white "film" caused by intracellular oedema of the spinous layer epithelium could be seen on the buccal mucosae, soft palate and retromolar areas. This participant was HPV negative and HIV negative. He was a current smoker of cigarettes and drank alcohol. He disclosed that he suffered from high blood pressure.

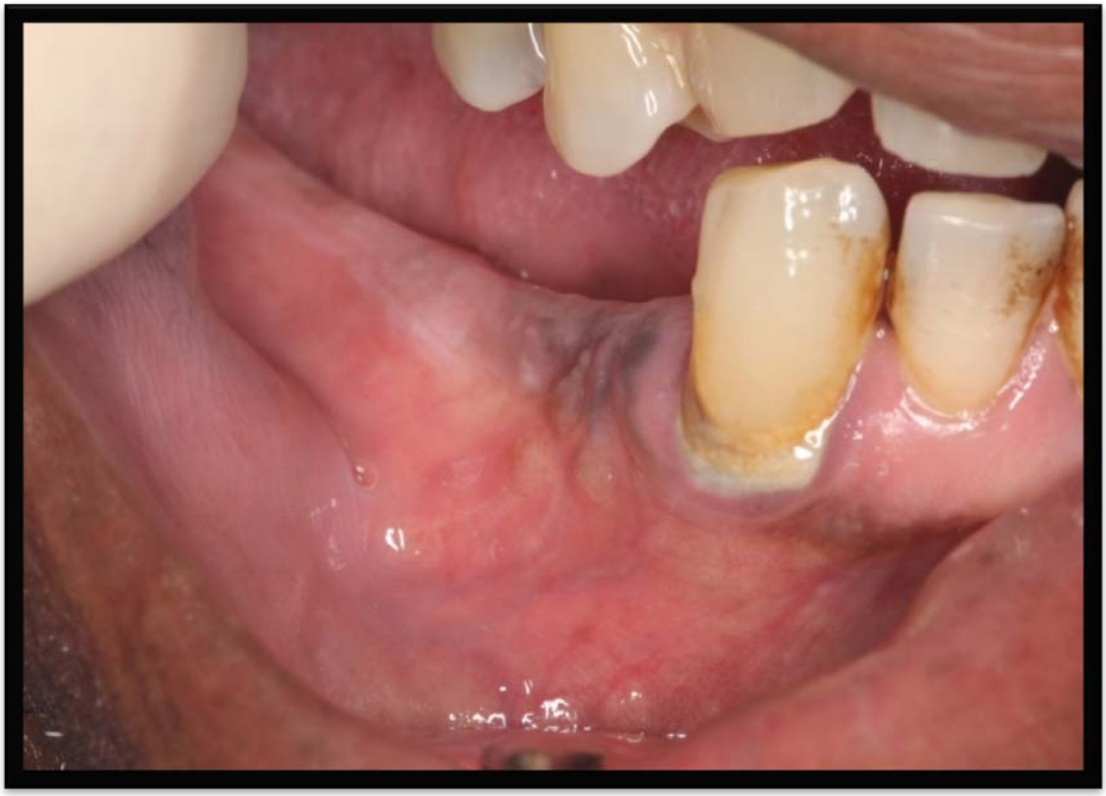


Fig.13: Hyperkeratosis can be seen on the edentulous alveolar ridge. The brownish-black pigmentation could be either a form of racial pigmentation or could be representative of post-traumatic pigmentation in this edentulous area. Subgingival calculus and gingivitis is seen in association with tooth 43.

Participant 17

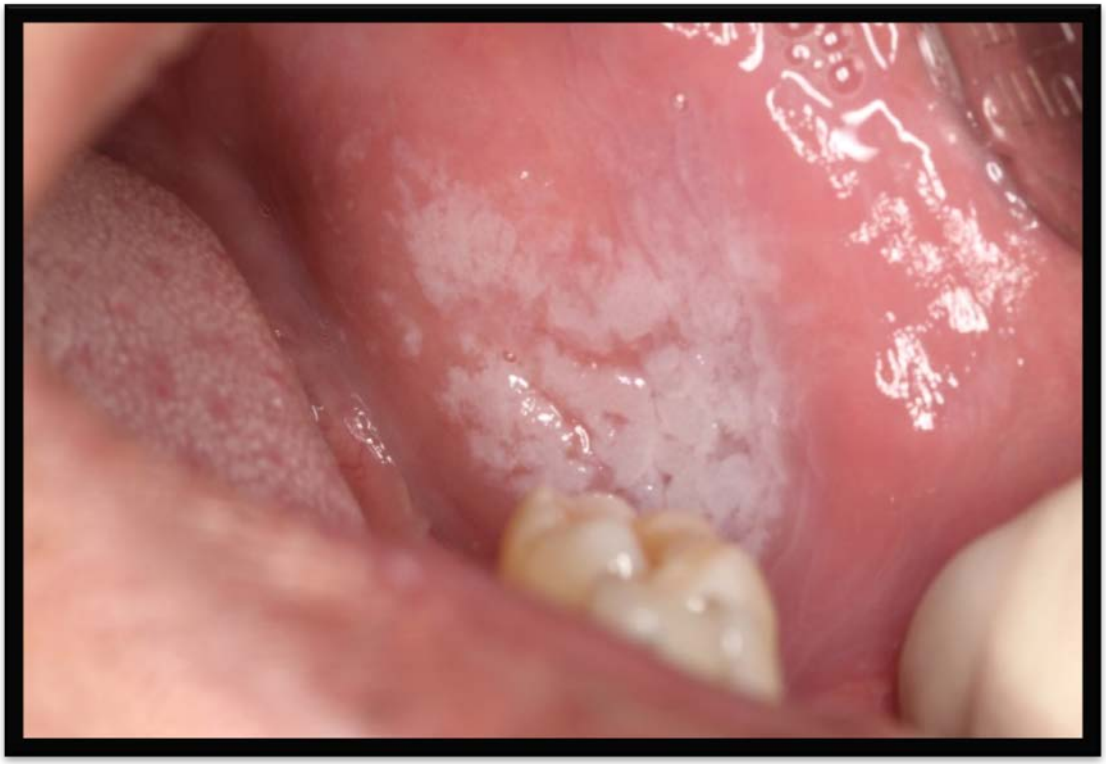


Fig. 14: Clinically this white hyperkeratotic lesion on the left buccal mucosa looked like leukoplakia or frictional keratosis. The participant was referred to the Oral Medicine Clinic of the Oral and Dental Hospital, University of Pretoria for biopsy. The participant was HPV negative and the HIV-status of this participant was unknown as he refused HIV testing. He smokes cigarettes and drinks alcohol. No medical conditions were disclosed in the questionnaire.

Participant 19

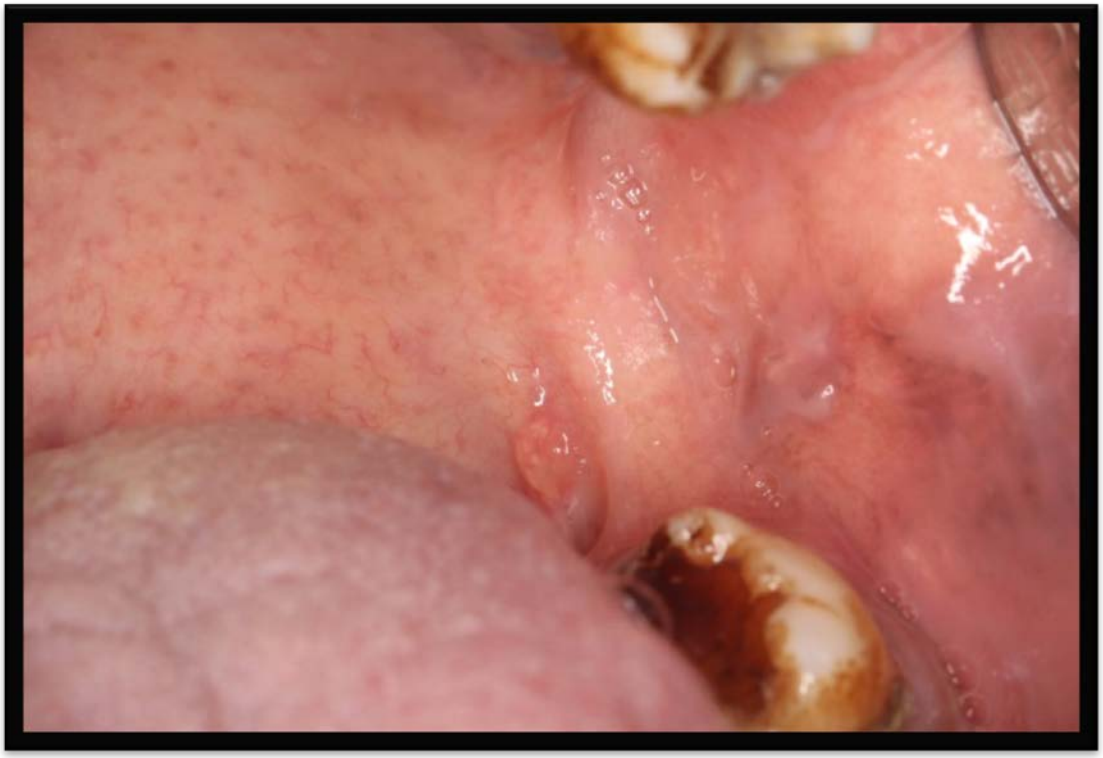


Fig. 15: Another case with leukoedema of the buccal mucosa and retromolar area. A small papillomatous lesion was also seen posterior to the tooth 38 on the left palato-glossal arch. On testing, this patient was not positive for HPV and although the possibility of a small lymphoepithelial cyst was considered on clinical grounds, the patient did not opt to come for follow up. The participants HIV status remained unknown as he declined HIV testing. The participant did smoke cigarettes and drink alcohol. No medical conditions were disclosed in the questionnaire.

Participant 21

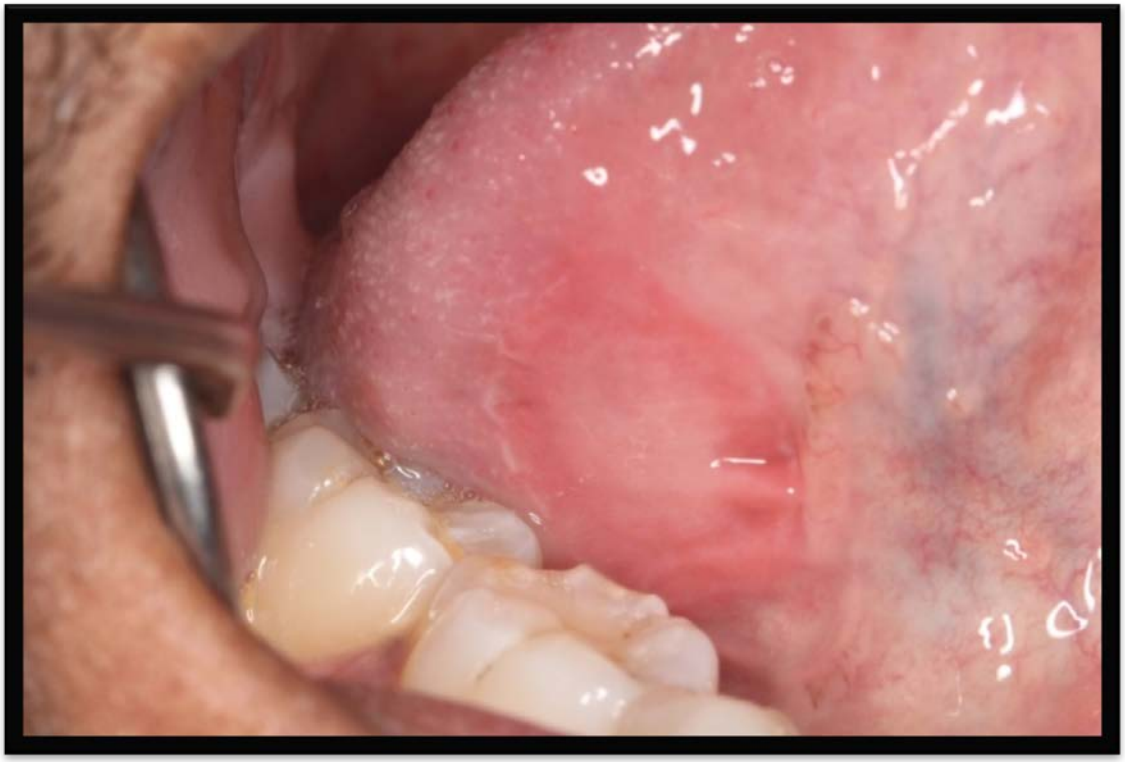


Fig. 16: This erythematous discoid lesion was found on the on the right posterior ventral aspect of the tongue. No similar lesions were present anywhere else in the oral mucosa. There was no clinical explanation for it and the participant was referred to the Oral Medicine Clinic, Oral and Dental Hospital, University of Pretoria for biopsy. This participant was HPV negative and declined HIV testing. He did not smoke any substances but did drink alcohol. This participant did not show up for his appointment for follow up at the Oral Medicine Clinic.

Participant 23:

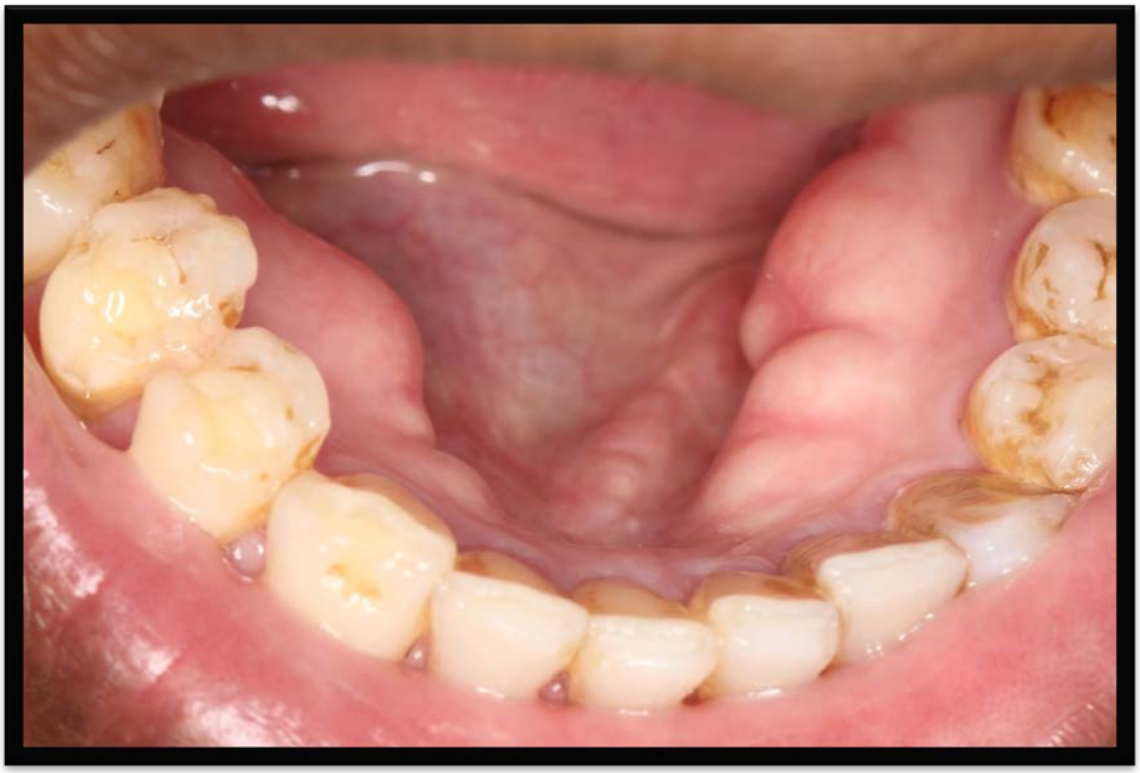


Fig. 17: This participant presented with lingual swellings suggestive of mandibular tori. He was referred to a local dental clinic for further investigation. He was HPV and HIV negative, smoked cigarettes and drank alcohol. He suffered from high blood pressure.

Participant 34

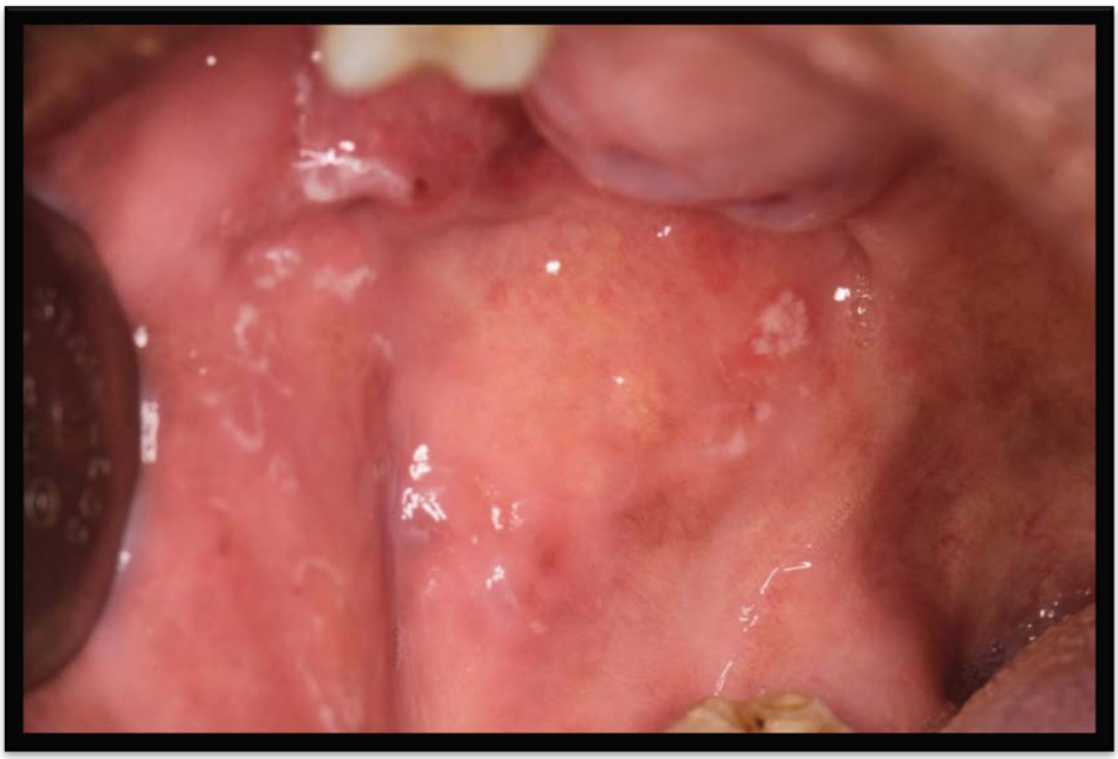


Fig. 18: This participant presented with these small collections of white material that could be wiped off and the clinical impression was that of *Candidiasis.* The participant was referred to his oral health practitioner for management. This participant was HPV negative and HIV negative. The participant did not smoke or drink alcohol and disclosed no medical conditions in the questionnaire.

Participant 49

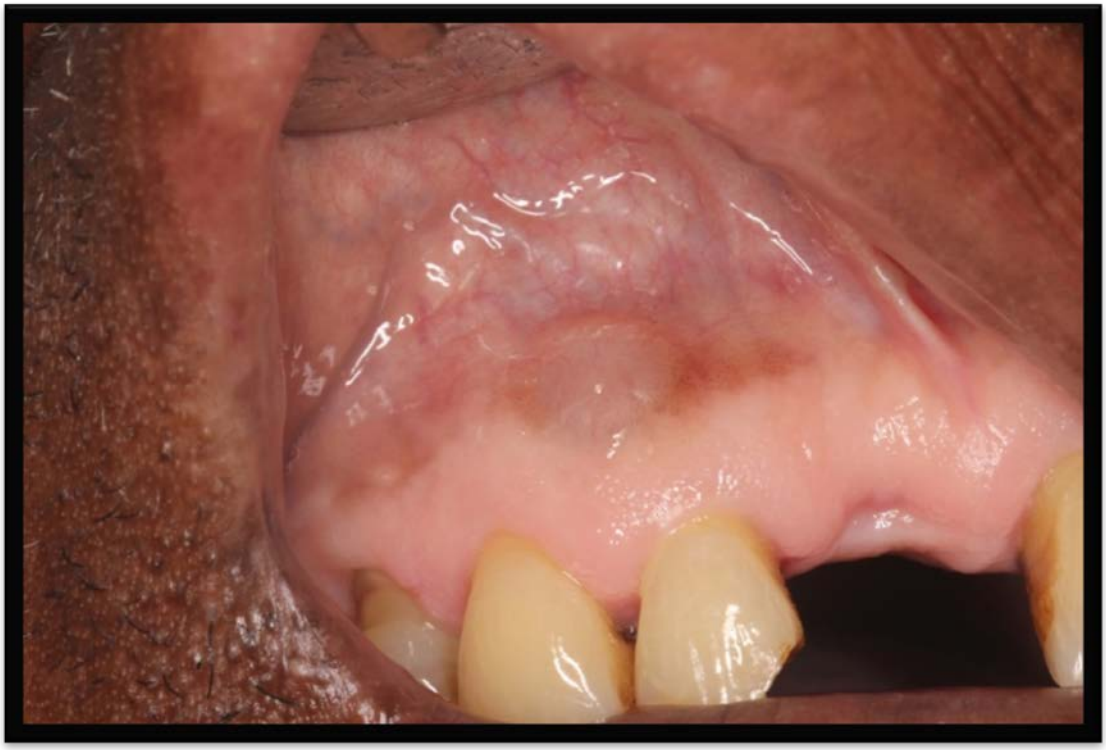


Fig. 19: This participant presented with a possible bony cyst in the 12/13 region. The participant was referred to the Oral Medicine Clinic of the Oral and Dental Hospital, University of Pretoria for further investigation. The participant was HPV negative and HIV negative. The participant did not smoke any substances but drank alcohol. He did not declare any medical conditions in the questionnaire.

Participant 50

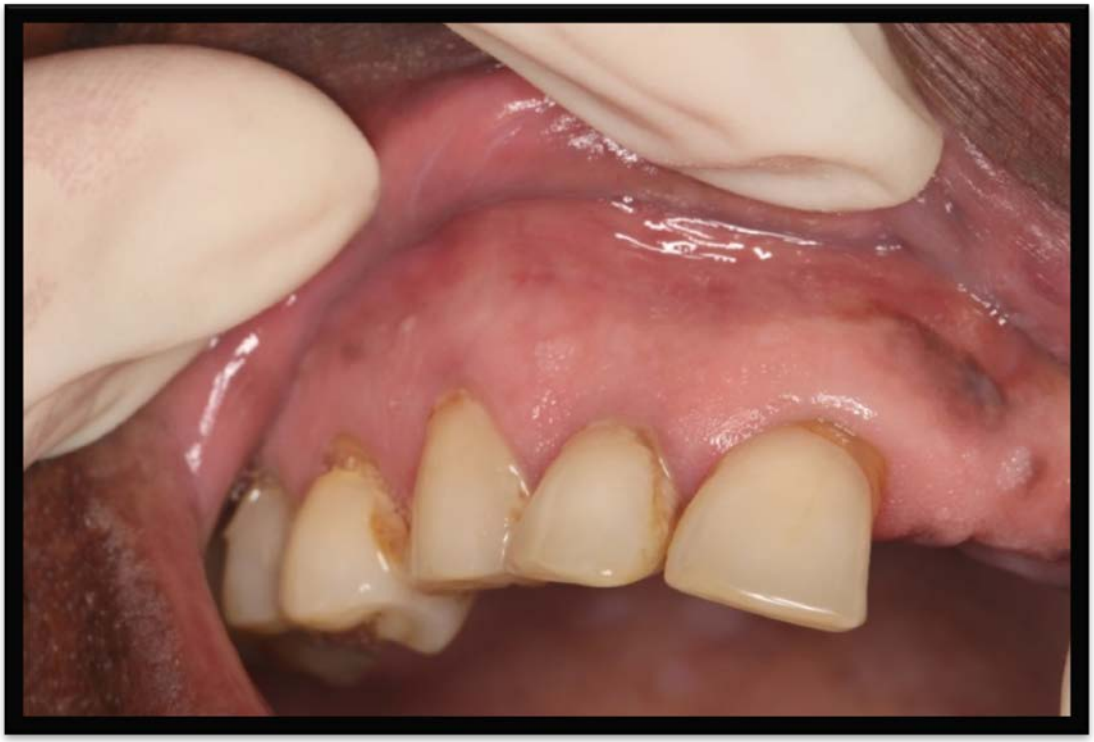


Fig. 20: Multiple small white papillomatous lesions were seen on the mucoperiostial mucosa of the anterior maxillary alveolar ridge of this participant. He was not positive for HPV on testing and tested negative for HIV. The nature of these lesions remains uncertain. The participant however did smoke and drink alcohol and suffers from high blood pressure.

Participant 51

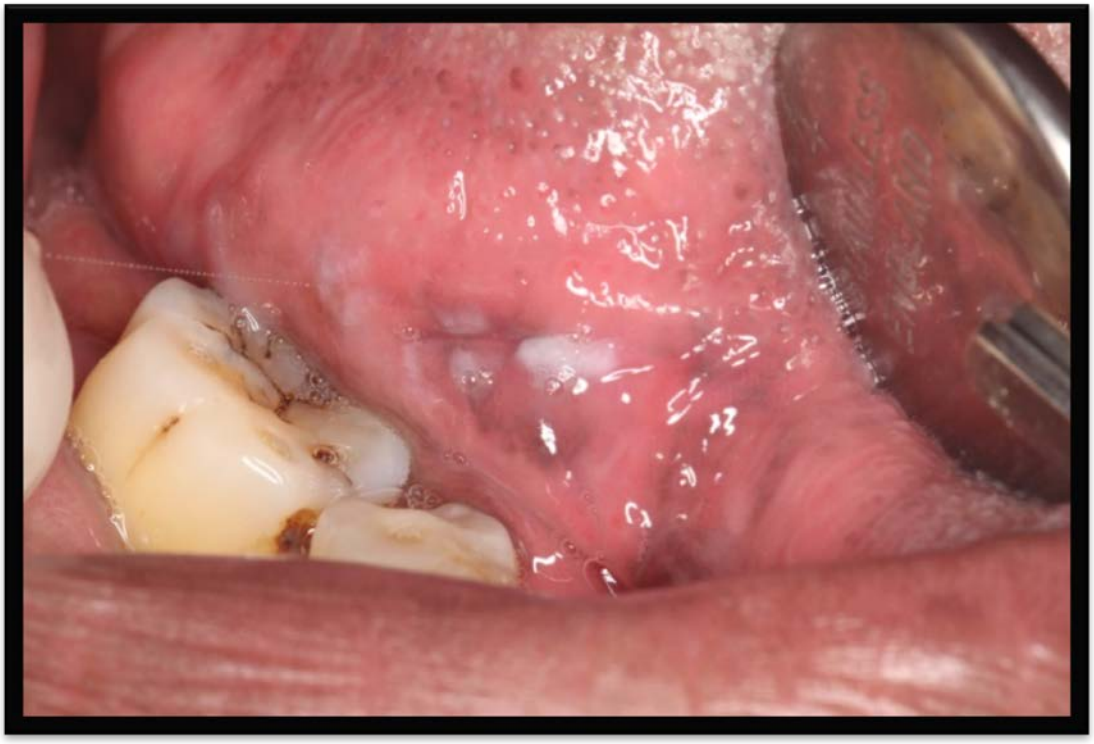


Fig. 21: This participant presented with racial pigmentation on large areas of the oral mucosa. He also had this lesion that looked like frictional keratosis on the lateral ventral tongue secondary to trauma of the adjacent teeth. The lesions could not be wiped off. The participant did not come for his follow up appointment for biopsy of the lesion but the implications of leukoplakia with dysplasia were explained to him. He was HPV negative and decline HIV testing. He did not smoke any substances but did drink alcohol. No medical conditions were declared in his questionnaire.

Participant 54

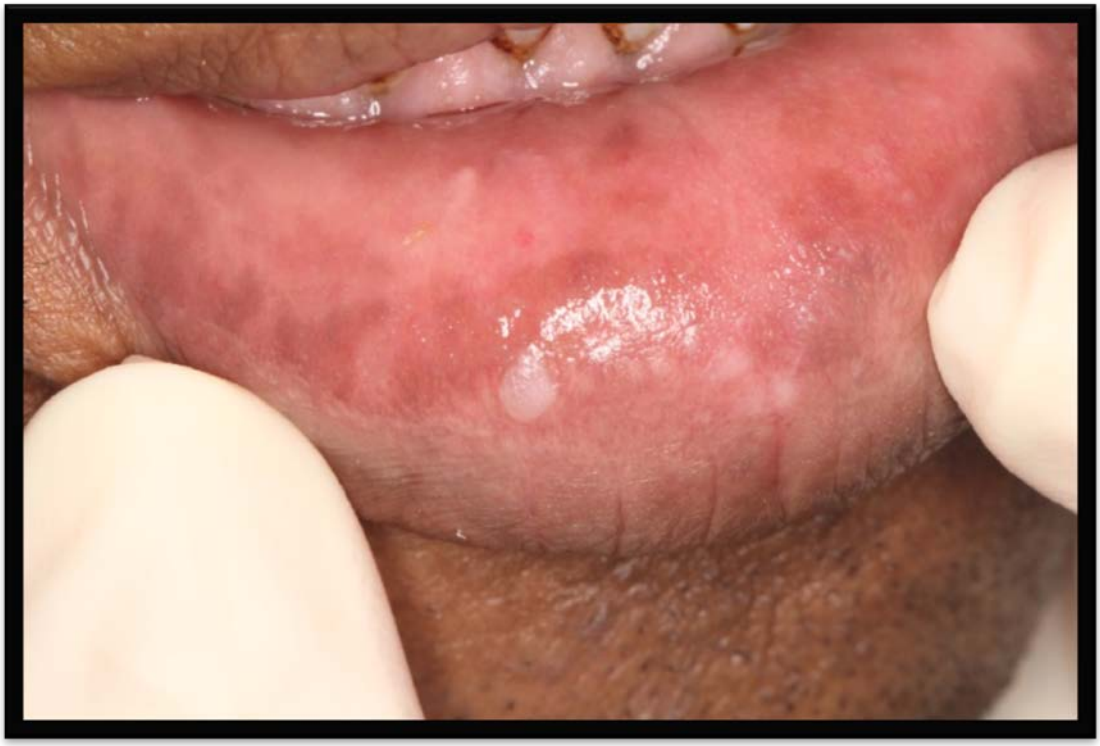


Fig. 22: The lower lip of this HIV-negative and HPV-negative participant had these hypopigmented areas intermingled with areas of racial pigmentation. The pathogenesis of these lesions was uncertain. The participant did smoke cigarettes and drink alcohol and declared no medical conditions in his questionnaire.

Participant 59

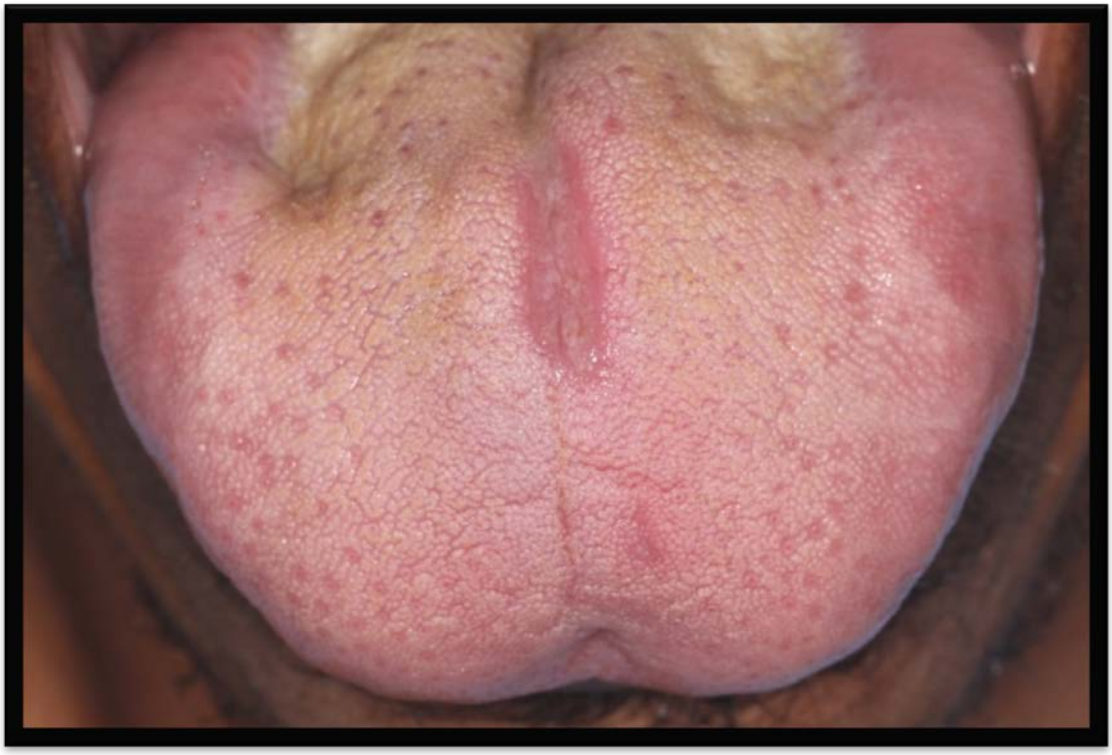


Fig. 23: This participant presented with median rhomboid glossitis, a type of Candidiasis on the central aspect of the tongue. He did not have the typical "kissing lesion" on the opposing palatal area. Black hairy tongue can be seen on the postero-lateral aspects of the tongue. This is secondary to lengthening of the filliform papillae of the tongue with staining due to his cigarette smoking and/ or coffee or tea. This participant was HPV negative and HIV positive. He also drank alcohol. No medical conditions were declared in the questionnaire and he was referred to the OHP for treatment.

Participant 61

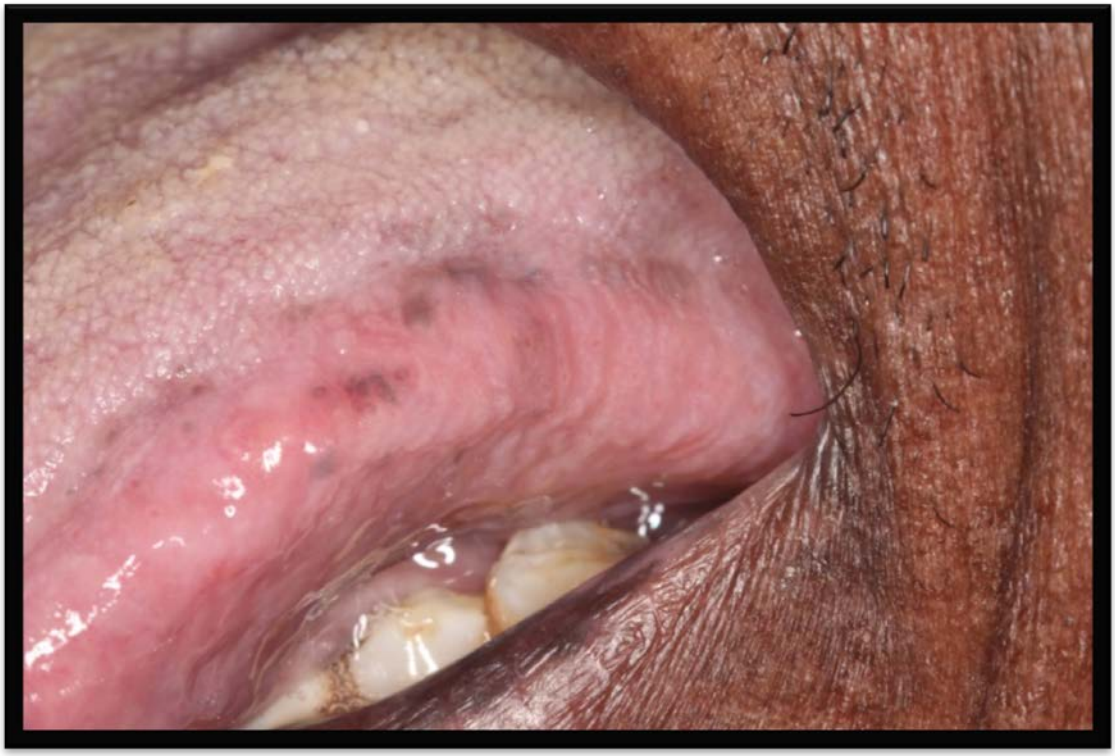


Fig. 24: This HIV-positive participant on antiretroviral therapy presented with these corrugated white lesions on the lateral tongue with the clinical appearance of oral hairy leukoplakia, an EBV-related lesion commonly encountered in HIV-infected individuals. This can however only be confirmed with surgical biopsy and special investigative techniques such as *in situ* hybridisation for demonstration of the EBV. The pigment in this patient could be racial pigmentation but pigment secondary to the use of anti-retroviral therapy is also a well-known possibility. The participant did smoke cigarettes and drink alcohol and did not disclose any medical conditions in his questionnaire.

Participant 81

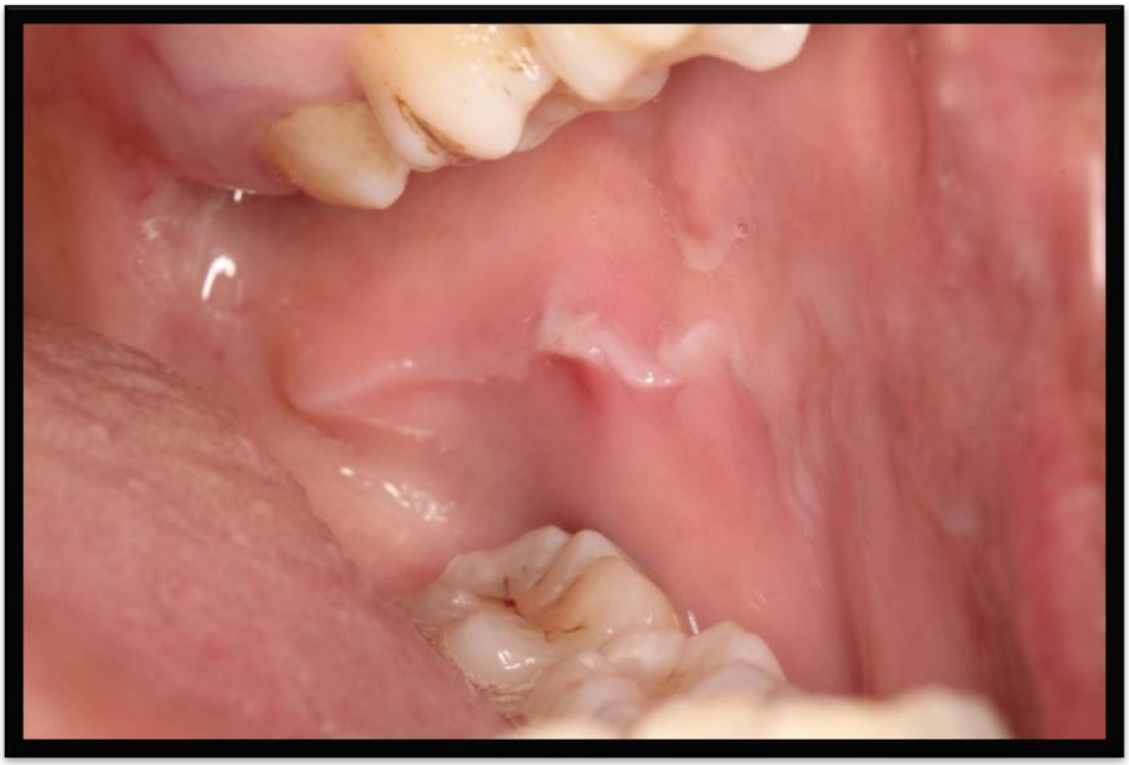


Fig. 25: This participant presented with linea alba buccalis, a traumatic lesion on the occlusal surface of the left buccal mucosa. The participant was HPV and HIV negative, smoked cigarettes and drank alcohol. This participant disclosed that he suffered from Diabetes.

Participant 86

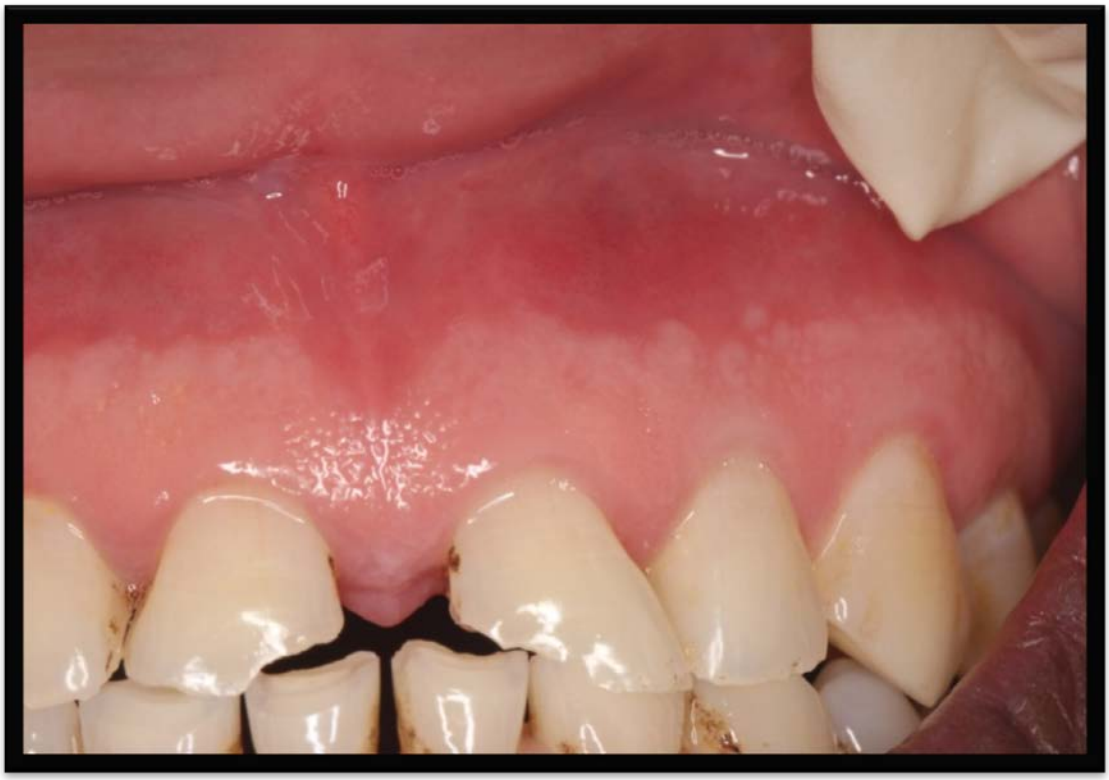


Fig. 26: Multiple white lesions on the facial gingiva of the anterior maxilla in the region of the mucogingival junction. Gives the idea of HPV papillomas but participant was HPV negative. He was also HIV negative and did not smoke or drink any substances. He does however suffer from high blood pressure.

Participant 88

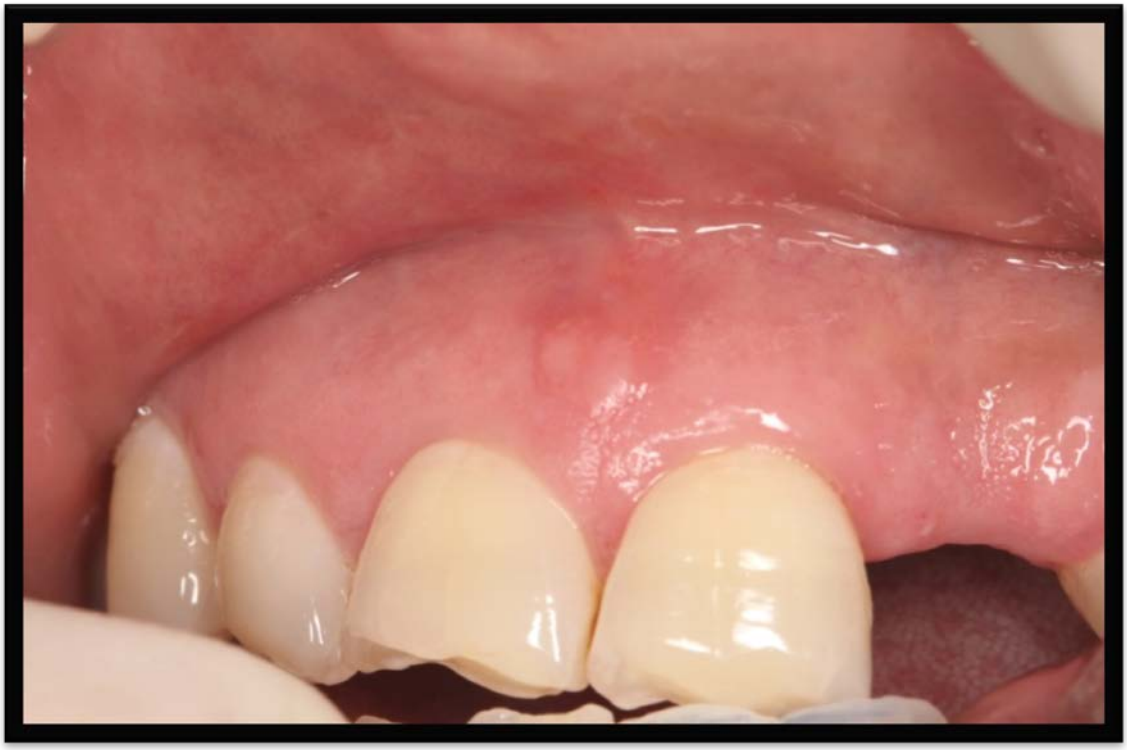


Fig. 27: Participant no 88 presented with this solitary fibrotic lesion on the facial gingiva of the anterior maxilla. A dental problem such as a healed parulis was considered clinically and the participant was referred to a dental clinic for further investigation. A list of dental clinics nearby his work was provided and no feedback was received. This participant was HPV and HIV negative, did not smoke but drank alcohol. He disclosed no medical conditions in his questionnaire.

Participant 116

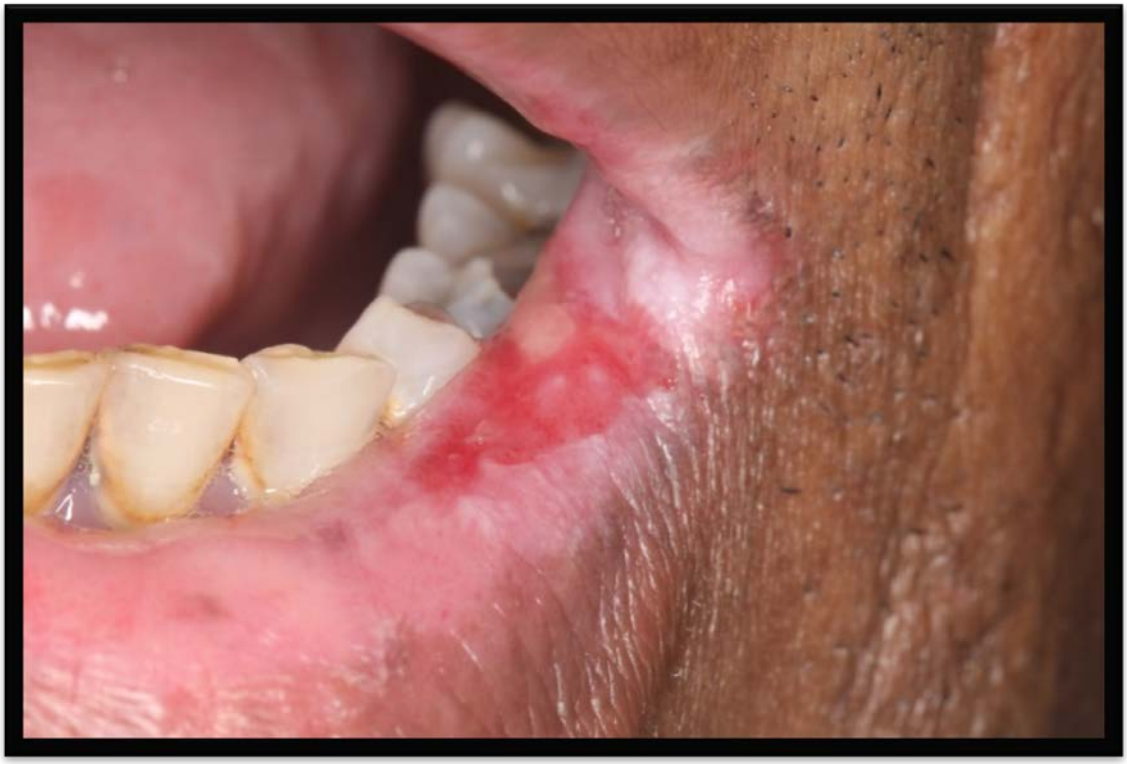


Fig. 28: Participant no 116 presented with a severely painfull, non-healing lip ulcer on the lower left lip. A possible autoimmune disease or chronic ulcerative stomatitis of HIV was considered clinically. He was referred to the oral medicine clinic for a biopsy and treatment but he did not come for this appointment. The participant was HPV negative and his HIV status was unknown as he declined HIV testing. He was a smoker and did drink alcohol. He disclosed in his questionnaire that he suffers from high blood pressure.

Participant 127

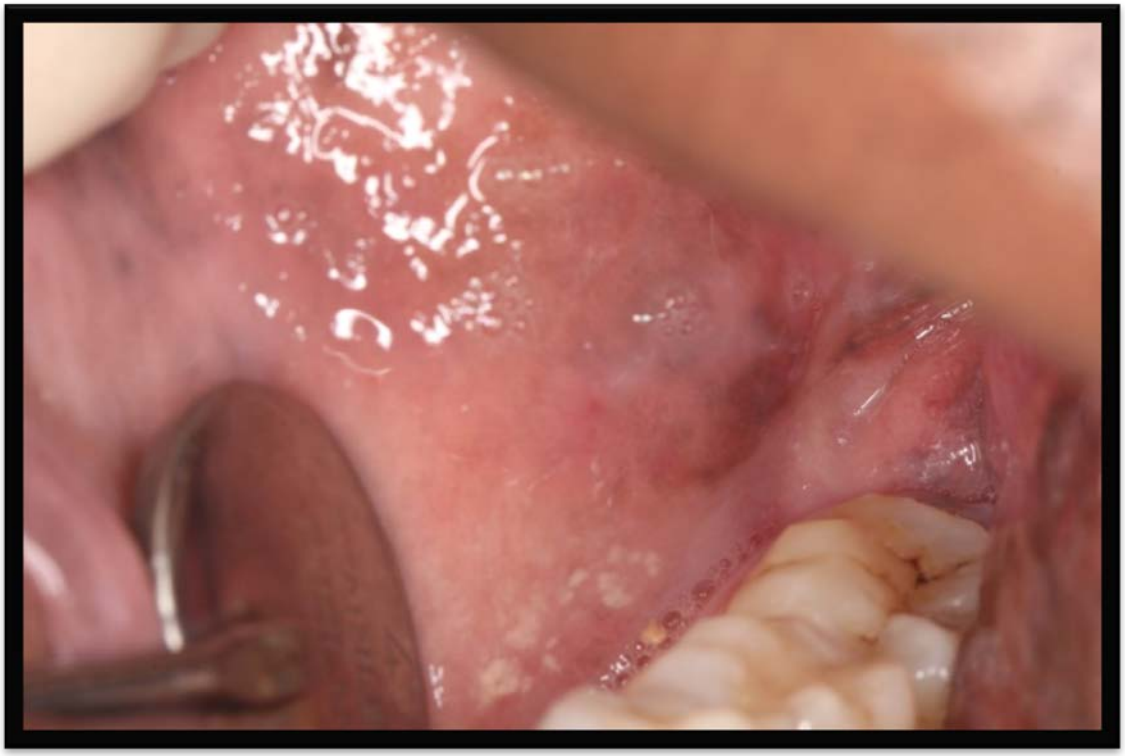


Fig. 29: This participant presented with these white lesions that could be wiped off. The clinical impression was that of oral *Candidiasis* accompanied by underlying inflammatory erythema of the mucosa (see Fig. 30)

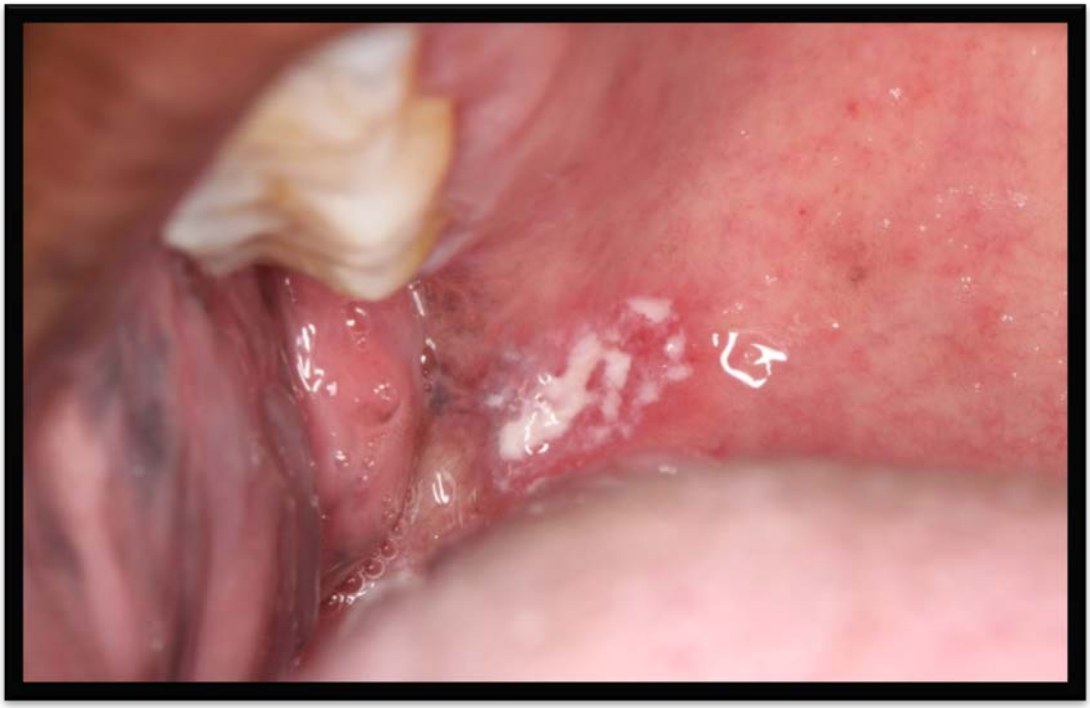


Fig. 30: This participant presented with these white lesions that could be wiped off. The clinical impression was that of oral *Candidiasis* accompanied by underlying inflammatory erythema of the mucosa. A smear biopsy was performed and sent to the Department of Oral Pathology and Oral Biology, University of Pretoria for evaluation. Histological features confirmed the presence of fungal hyphae and a diagnosis of Candidiasis was confirmed in the report. He was referred to the Company Health Practitioner for further management. He was HPV negative and HIV positive. He did smoke and drink alcohol and he disclosed in his questionnaire that he was HIV positive and that he was suffering from TB.

4.2.2 Saliva sample collection

Oral rinse specimens were collected from 128 males between the ages of 17-64 years with a median age of 50 years. The participants originated from all over South Africa and were not only from this specific geographical location. Three participants who did not complete questionnaires were excluded from the study leaving a total of 125 participants. The results for each of the variables examined during the study will be discussed separately.

- **HPV**

Seven of the125 participants (5.6%) tested positive for HPV. One participant was co-infected with HPV types 71 and 72. Two participants tested positive for hrHPV types namely HPV 16 and HPV 68 respectively. Table 1 summarises all the HPV types found by genotyping.

Table 1: This table represents a summary of all the HPV types found in the HPV-positive participants.

Participant Number	HPV Type found
2	HPV 68 (**hr**)
22	HPV 71,72
25	HPV 55
28	HPV 62
118	HPV 16 (**hr**)
119	HPV 72
125	HPV 70

hr = high risk type

4.2.3 Questionnaire

- **Age**

The majority, 87/125 (69.5%) of the total participants in the study were older than 40 years of age. Five of the seven (71%) HPV positive cases were older than 40 years and both of the participants that tested positive for hrHPV types were over 50 years of age.

Table 2: This table represents a summary of the ages of all the participants in relation to their HPV status.

HPV	**Age**		
	>= 40 years	**> 40 years**	***Total***
HPV negative	**36**	**82**	**118**
Row %	30.51%	69.49%	
Column %	94.74%	94.25%	
HPV positive	**2**	**5**	**7**
Row %	28.57%	71.43%	
Column %	5.26%	5.75%	
Total	**38**	**87**	**125**

- **Medical conditions**

A list of all the medical conditions that the participants declared in the questionnaire can be seen in Table 3. This information was as provided by the participants themselves and not confirmed by a medical practitioner. The most common aliments declared were high blood pressure (27/125) and diabetes (10/125). The overall clinical health of the participants was good.

Table 3: This table represents a summary of the medical conditions declared by the participants in their questionnaires.

Code	Medical Condition	Number of participants suffering from this condition
01	High blood pressure	27
02	Diabetes	10
03	Sinusitis	2
04	Stomach Ulcer	2
05	Flu (as defined by patient)	2
06	Low blood pressure (as defined by patient)	1
07	Skin problems	1
08	Prostate cancer	1
09	Skin cancer	1
10	Cholesterol levels abnormal *	1
11	Gout	1
12	Depression	1
13	Hearing loss	1
14	Tuberculosis	1
15	HIV(declared by participant)	1
16	Asthma	1

*The participant did not stipulate high or low cholesterol

- **Substances:**

The substances smoked and alcohols consumed that were included in the questionnaire are listed in Table 4 below. Due to the small numbers, the quantity and frequency of the different substances/alcohol used were not investigated. Only an overall score which reflected whether a respondent used or didn't use any of the substances/alcohol in the questionnaire was calculated.

Table 4: This table represents a summary of the types of substances smoked and alcohol consumed as asked in the questionnaire.

Substances smoked	Alcohol consumed
Cigarettes	Beer
Cigars	Spirits
Pipe	Wine
Hubbly bubbly	
Marijuana	
Chewable tobacco	
Snuff	

- **Smoking of substances**

Fifty eight of the 125 participants (46%) smoked one/more substances as included in the questionnaire, whilst 4/7 HPV positive participants (57%) reported to smoke one/ more substances (See Table 5). The association between HPV and smoking was not statistically significant (p-value=0.7034). (Table 5)

Table 5: This table represents the smoking habits of the participants in the study in relation to their HPV status.

HPV	**Smoking one/more substances**		
	Don't Smoke	**Do Smoke**	***Total***
HPV negative Row % Column %	**64**	**54**	**118**
	54.24%	45.76%	
	95.52%	93.10%	
HPV positive	**3**	**4**	**7**
Row % Column %	42.86%	57.14%	
	4.48%	6.90%	
Total	**67**	**58**	**125**

- **Alcohol consumption**

The overall alcohol consumption prevalence was 80% with 100 of the 125 participants drinking one/more types of alcohol (Table 6). All seven of the participants that were positive for HPV consumed alcohol. No association was seen regarding the use of alcohol and having HPV (p=0.3430).

Table 6: This table represents a summary of the alcohol consumption of the participants in the study in relation to the HPV status.

HPV	**Alcohol**		
	Don't drink	**Do drink**	***Total***
HPV negative	**25**	**93**	**118**
Row %	21.19%	78.81%	
Column %	100%	93%	
HPV positive	**0**	**7**	**7**
Row %	0%	100%	
Column %	0%	7%	
Total	**25**	**100**	**125**

- **Sexual practices**

All the participants that took part in this study were sexually active with 90/125 (72%) of them reporting to have had their first sexual encounter between 15-20 years of age (Table 8). Six of the seven HPV positive participants had 5/more sexual partners with four of them reporting to have had more than 20 (Table 9). The association between HPV and the number of sexual partners was statistically significant (p=0.0270). Eighty nine per cent (111/124) of all the participants claimed to have between one and three sexual partners in the last six months (Table 10).

Table 7: This table indicates the ages at which the participants had their sexual debut.

HPV	**Age of Sexual Debut**			
	8-14 years	**15-20 years**	**>20 years**	***Total***
HPV negative	**3**	**85**	**30**	**118**
Row %	2.54%	72.03%	25.42%	
Column %	100%	94.44%	93.75%	
HPV positive	**0**	**5**	**2**	**7**
Row %	0%	71.43%	28.57%	
Column %	0%	5.56%	6.25%	
Total	**3**	**90**	**32**	**125**

Table 8: This table indicates the total number of lifetime sexual partners the participants had.

HPV	Total number of sexual partners			
	0-4	**5-20**	***>20***	***Total***
HPV negative Row % Column %	**19**	**79**	**19**	**117***
	16.24%	72.03%	25.42%	
	95%	94.44%	93.75%	
HPV positive	**1**	**2**	**4**	**7**
Row % Column %	14.29%	28.57%	57.14%	
	5%	2.47%	17.39%	
Total	**20**	**81**	**23**	**124**

* 1 answer was missing

Table 9: This table indicates the total number of sexual partners the participants had in the last six months.

HPV	Total number of sexual partners in last 6 months			
	0	**1-3**	***>3***	***Total***
HPV negative Row % Column %	**3**	**104**	**10**	117*
	2.56%	88.89%	8.55%	
	100%	93.69%	100%	
HPV positive	**0**	**7**	**0**	7
Row % Column %	0%	100%	0%	
	0%	6.31%	0%	
Total	3	111	10	124

* 1 answer was missing

The prevalence of oral sex was found to be 40%, with only 51/125 participants ever engaging in this activity. Three of the seven (42.8%) of the HPV positive participants, including both positive for hrHPV, practiced oral sex (Table 11).

Table 10: This table indicates the total number of lifetime oral sex partners the participants had as well as the age of oral sexual debut.*

HPV	Total number of oral sex partners and age of oral sex debut *				
	Don't do	**10-19 years**	**20-30 years**	**> 30 years**	***Total***
HPV negative	**70**	**9**	**29**	**10**	**118**
Row %	59.32%	7.63%	24.58%	8.47%	
Column %	94.59%	90%	96.67%	90.91%	
HPV positive	**4**	**1**	**1**	**1**	**7**
Row %	57.14%	14.29%	14.29%	14.29%	
Column %	5.41%	10%	3.33%	9.09%	
Total	**74**	**10**	**30**	**11**	**125**

*Taking into account that the "oral sex" was not specifically defined and was interpreted in several different ways by the participants.

Table 11: This table indicates the total number of oral sex partners that the participants had in the last six months.

HPV	Total number of oral sex partners in the last 6 months				
	None	**1**	**2**	**> 2**	***Total***
HPV negative	**66**	**19**	**16**	**17**	**118**
Row %	55.93%	16.10%	13.56%	14.41%	
Column %	94.29%	100.00%	94.12%	89.47%	
HPV positive	**4**	**0**	**1**	**2**	**7**
Row %	57.14%	0.00%	14.29%	28.57%	
Column %	5.71%	0.00%	5.88%	10.53%	
Total	**70**	**19**	**17**	**19**	**125**

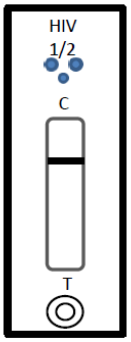
4.2.4 HIV counselling and testing

The interpretation of the HIV results was recorded as follows: (225)

HIV Negative-

When only one colour line was visible in the control region of the HIV U test (123, 226):

This result indicated that at the time of testing no HIV-1 and HIV-2 antibodies were detected. This could either be because there are no antibodies present or that the concentration of HIV antibodies at that stage was below the detection limit of the test. A negative result does therefore not preclude the possibility of HIV infection.

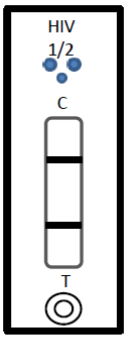


Negative

HIV Positive-

Two colour lines were visible, one in the control region and one in the test region of the HIV U test.

Any positive results on the HIV U test were to be followed up with a confirmatory laboratory based blood test.

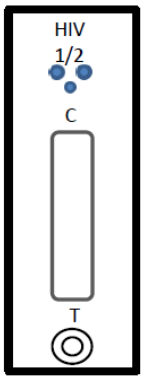


Positive

Invalid HIV Result-

When no colour lines were present, the test was interpreted as invalid and repeated.

Either proper procedures may not have been followed in performing the assay or the test may have deteriorated.



Invalid

In the subgroup of 90 men (72%) that volunteered for HIV testing, only 5/90 (5.5%) tested positive. Four of the seven (57.1%) HPV positive participants opted for HIV testing. All of the HPV-positive participants who tested for HIV were negative (Table 13). Although not statistically significant, 4/5 (80%) HIV positive participants were older than 40 years of age.

Table 12: This table represents a summary of those participants that declined to test for HIV and the HIV test results of those that agreed to test.

HPV	**HIV**			
	Negative	**Positive**	**Declined testing**	***Total***
HPV negative	**81**	**5**	**32**	**118**
Row %	68.64%	4.24%	27.12%	
Column %	95.29%	100%	91.43%	
HPV positive	**4**	**0**	**3**	**7**
Row %	57.14%	0%	42.86%	
Column %	4.71%	0%	8.57%	
Total	**85**	**5**	**35**	**125**

Table 13: This table represents a summary of all the variables investigated in the study in association with the HPV status of the participants.

	HPV + (%) [n=7]	HPV – (%) [n=118]	Total number [n=125]	p-value (p<0.05)
Age:				
≤ 40 years	2 (28.6%)	36 (30.5%)	38 (30.4%)	
> 40 years	5 (71.4%)	82 (69.5%)	87 (69.6%)	p=1.000
Smoking:				
Do	4 (57.1%)	54 (45.8%)	58 (46.4%)	
Don't	3 (42.9%)	64 (54.2%)	67 (53.6%)	p=0.703
Alcohol:				
Drink	7 (100.0%)	93 (78.8%)	100 (80.0%)	
Don't	0	25 (21.2%)	25 (20.0%)	p= 0.343
Lifetime sexual partners:				
0-5	1 (14.3%)	19/117*(16.2%)	20/124 (16.1%)	
5-20	2 (28.6%)	79/117*(67.5%)	81/124 (65.3%)	
> 20	4 (57.1%)	19/117*(16.2%)	23/124 (18.5%)	p=0.027
Sexual partners in the last 6 months:				
None	0	3/117 (2.6%)	3/124 (2.4%)	
1-3	7	104/117 (88.9%)	111/124(89.5%)	
> 3	0	10/117 (8.5%)	10/124 (8.1%)	p=1.000
Practice oral sex:				
Do	3 (42.9%)	48 (40.7%)	51 (41.0%)	
Don't	4 (57.1%)	70 (59.3%)	74 (59.0%)	p=0.525
Number of lifetime oral sex partners:				
1	0	19/48 (39.6%)	19/51 (37.3%)	
2	1/3 (33.3%)	16/48 (33.3%)	17/51 (33.3%)	
> 2	2/3 (66.7%)	17/48 (35.4%)	19/51 (37.3%)	p=0.592
Oral sex partners in the last 6 months:				
None	4 (57.1%)	83 (70.3%)	87 (69.6%)	
1	2 (28.6%)	26 (22.0%)	28 (22.4%)	
> 1	1 (14.3%)	9 (7.6%)	10 (8.0%)	p= 0.518
HIV:				
Positive	0	5 (4.2%)	5 (4.0%)	
Negative	4 (57.1)	81 (68.6%)	85 (68.0%)	
Declined testing	3 (42.9%)	32 (27.1%)	35 (28.0%)	p=0.563
Clinical Lesions:				
With	0	18 (15.3%)	18 (14.4%)	
Without	7 (100.0%)	100 (84.7%)	107 (85.6%)	p= 0.592

*One questionnaire was not completed

Table 14: This table indicates what types of HPV were found in the HPV-positive participants as well as a summary of the associated variables investigated as part of the study.

	HPV Type/s	Age (years)	Smoking	Alcohol	Sex	Oral sex	HIV status	Lesions
1	16	51	Yes	Yes	Yes (m)	Yes	Declined	No
2	55	59	Yes	Yes	Yes(m)	No	Declined	No
3	62	46	No	Yes	Yes(m)	No	Negative	No
4	68	58	No	Yes	Yes	Yes	Negative	No
5	70	29	Yes	Yes	Yes(m)	No	Declined	No
6	71,72	64	No	Yes	Yes(m)	No	Negative	No
7	72	32	Yes	Yes	Yes(m)	Yes	Negative	No

m = multiple sexual partners

CHAPTER 5

Discussion

The natural history of oral and oropharyngeal mucosal HPV infection in men is unknown. Currently, known risk factors for oral and oropharyngeal HPV infection include: life time number of sexual partners (131, 188, 227), the number of oral sex partners (133, 197), substance use (188, 197, 212, 213), and infection with HIV(136, 182, 219, 228). A recent study found the oral HPV prevalence in men with normal mucosa in the United States of America to be around 10% (188). This pilot study among a group of South African men had an unexpected lower HPV prevalence of only 5.5%. This could be attributed to the smaller sample size or to the observation that oral sex did not seem to be a common practice by the group of men included in this study.

Sexual contact, either genital or oral, remains the primary mode of HPV transmission and it is well documented that multiple sexual partners increases the prevalence of oral and genital HPV (131, 188, 229, 230). A study conducted in Kwa-Zulu Natal, South Africa reported male undergraduate students to have had a median of four sexual partners in the previous year (227). In many African cultures it is acceptable for men to have multiple partners and engage in sex outside of the relationship (231, 232). A statistically significant association between the number of sexual partners and presence of oral and oropharyngeal HPV was seen in this study (p=0.027) with 4/7 HPV positive participants having had more than 20 sexual partners and 2/7 having had between 5-20 lifetime sexual partners. The numbers of sexual partners of the HPV positive participants ranged from one to three in the last six months.

Oral HPV infection is said to be bimodal in age distribution with a high prevalence in younger men (30-34 years of age) and again in later years (60-64 years) (188). The participants that were HPV positive in this study were between 29-64 years of age and those with hrHPV were between 50-60 years of age. It was previously speculated that higher HPV prevalence at older ages could be

due to increased duration of infections at older ages, rather than an increased acquisition of new HPV infections (233). In this study however, all the participants' positive for HPV had between one and three sexual partners in the last six months, suggesting that this could be newly acquired HPV.

The HIV pandemic and the prevention thereof have increasingly highlighted sexual practices other than penetrative sex. Oral sex, especially amongst teenagers, has increased notably due to the false perception that sexually transmitted diseases such as HIV-infection could not transmit in this manner(197, 234). Studies on oral sex practices in the USA have shown that between 19.6%-78% of young adults had engaged in oral sex in their lifetime (235). Gillison, in line with other studies found oral sex performed on women as one possible explanation for the higher prevalence of oropharyngeal HPV in men who, in their study on average had more partners than the females(188). Sexual behaviour studies in South Africa are few and those that do exist are not specific in its data regarding genital versus oral sexual practices and the gender differences that do exist.

After the first day of this study we realised that there was a small problem regarding the questions on oral sex in the questionnaire. What we intended to evaluate with the question on oral sexual practice was if the participant himself performed oral sex (his oral mucosa to have contact with the genital mucosa of the other individual) on a woman/man as direct mucosal contact is how HPV is transferred. However, this was not stipulated as such in the questionnaire. Through the field worker it was realised that many participants interpreted "oral sex" to mean that the participant "received" the oral mucosal contact on his genital mucosa and in fact not the other way around. This would obviously influence the outcome of the interpretations of this question. For the sake of continuity in the study we decided not to influence the participant group on the second day and did not provide further explanations. This is however extremely important and the setting of questions in any further studies of this nature should be clear in the definition and direction of oral sexual practice. More research regarding the practice of oral sex in South African men is required. If oral sex is uncommon, then why has there been a proven increase

in OPC in South Africa since 1995 with men being more affected than women? Are the oral sexual practices different amongst the subpopulations of South African men? Are certain subpopulations therefore more susceptible to acquiring this subset of HPV-associated oropharyngeal SCC?

The understanding of the definition of oral sex could explain the lower HPV prevalence in our study. Only 51 of all participants (40%) and 3/7 (43%) HPV positive participants answered 'yes' to the question if they have practiced oral sex, again keeping in mind how they interpret the definition thereof. Of note was that both the individuals with hrHPV types answered 'yes' to oral sex with more than one partner.

The medical conditions, as declared by the participants in their questionnaires, suggests that the overall health of the participants was good. The most common illnesses that the participants declared were high blood pressure (27/125) and diabetes (10/125).

Tobacco use is an accepted aetiological factor of head and neck cancers but is not considered a strong risk factor of HPV associated OSCC (131, 133). We found no statistically significant association between smoking and the presence of HPV (p=0.7034), although the only participant with HPV 16 in our study was a current cigarette smoker. An increased oral HPV prevalence in men with normal mucosa has been shown to be associated with current and previous smoking, but the exact pathogenic mechanisms are unknown (197, 212).

Alcohol has the ability to modify mucosal tissue allowing easier entry of the virus and is also known to modify the host immune response thereby increasing the susceptibility to HPV infection for both cancers associated with HPV and in normal healthy mucosa (213, 214). In our study we found no statistical association between alcohol use and the presence of HPV (p=0.3430) although all the HPV-positive participants are current alcohol users.

In South Africa, the total estimated number of adults 15-49 years living with HIV in 2013 was 5.26 million with a HIV prevalence rate of 10% (236). The HIV prevalence in this study was only 5.5%. Only four of the HPV positive participants (n=7) volunteered to test for HIV and were all negative whilst the two

participants with hrHPV declined HIV testing and their HIV status remained unknown. The factory where the study was conducted has an HIV clinic where HIV counselling and testing is routinely offered. The workers have a good relationship with the OHP running this clinic and are well cared for. Of the participants that chose to test as part of this study, no new HIV positive results were found. A much larger nationwide study with HIV-prevalence more representative of that reported in SA will be needed in order to determine the role of HIV-infection in the natural history of oral and oropharyngeal HPV infection in this country. Interestingly, other associations with HIV were seen in this study. Clinically 3/5 HIV positive participants (p-value of 0.0295) presented with oral lesions commonly associated with HIV infection. The HIV status of the participants was also shown to be associated with the use of alcohol (p=0.0527) (Fishers exact test) as well as with the number of sexual partners the participant had in the last six months (p=0.0251) (Fishers exact test). 4/5 of the HIV positive participants had between 1-3 sexual partners in the last six months.

The eighteen participants that presented with oral mucosal lesions were all negative for HPV. Two of the participants that presented with clinical lesions had systemic conditions that could explain the presence of these lesions (diabetes, HIV and TB). A close association of those participants that presented with oral mucosal lesions was seen with the smoking of substances (p value=0.076) and all these participants were older than 40 years of age (p-value=0.0585). Both smoking and older age could result in reduced local immunity and may have some role to play in the presence of the oral mucosa lesions.

This pilot study once again confirmed that the lifetime number of sexual partners is a risk factor for acquiring oral and oropharynheal HPV. Although not statistically significant, those participants with hrHPV types had one or more sexual partners in the last six months and engaged in oral sex. This highlights the possible role that sexual and oral sex practices can play in the likelihood of acquiring HPV associated diseases. A much larger nation-wide analysis with inter-institutional collaboration will be necessary to confirm if the prevalence of

5.5% of oral and oropharyngeal HPV is in fact truly representative of oral and oropharyngeal HPV in the normal mucosa of South African men.

CHAPTER 6

Short comings and study limitations

The greatest short coming of the study was the sample size; of which funding was the major determinant.

If going forward with a larger nationwide study of this nature the questionnaire requires several amendments. A particularly important aspect to address regarding the questions on the sexual history was highlighted: the oral sex questions need to be very specific. The participants must be very clear on what is expected from them such as in which direction the oral sex was performed. In other words, did the participant perform oral sex with his oral mucosa touching the genital mucosa of his partner(s) or did he merely receive oral sex, in other words, that his genital mucosa was involved only but not his oral mucosa. More sexual practice studies are needed to investigate whether oral sex is common practice amongst South African men as this can influence acquiring the virus. There is the issue of over/under inflation of the numbers of sexual partners. It is very important that the participants be quiet and on their own when they complete this questionnaire as being in the same room with one another might result in them inflating the numbers, especially the younger men. Once again, the questionnaire has to be user friendly enough not to require a field worker but still to be able to attain all the correct information with the questionnaire.

If someone should wish to include a larger number of participants in this type of study, I would recommend calibration in the method of oral sample collection.

CHAPTER 7

Conclusion

Up to date the oral HPV prevalence in the South African population was unknown. The oral HPV prevalence found in this pilot study is lower than reported in other countries. There may be several reasons for this such as a small sample size and the cohort tested.

An association of oral HPV and having multiple sexual partners was found and oral sex, although an uncommon practice amongst the majority of participants, was common in those with hrHPV types. This pilot study gives us an insight into what the oral HPV prevalence could be in men living in South Africa. Although the sample size was small many issues were highlighted that could assist future studies of this nature. The development of the questionnaire is an especially important aspect of a study such as this and needs to be carefully attended to. It should be easy to read and interpret and the questions have to be concise but very clear.

The diversity of the South African population has to be considered in any study of this nature going forward. A larger nationwide study will give a more representative view of oral HPV in South Africa but one would have to include individuals from all over South Africa and from all different cultures and backgrounds.

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ANNEXURE A

Approval from the Ethics Committee of the Faculty of Health Sciences, University of Pretoria

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* **FWA** 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
* **IRB** 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 28/06/2012

NUMBER	**101/2012**
TITLE OF THE PROTOCOL	Prevalence of oral and oropharyngeal Human Papillomavirus (HPV) in a sample of selected South African males: a pilot study.
PRINCIPAL INVESTIGATOR	**Student Name & Surname:** Dr Christy Davidson **Dept:** Oral Pathology and Oral Biology, School of Dentistry, Faculty of Health Sciences;University of Pretoria. **Cell:** 0832984610 **E-Mail:** christy.davidson@up.ac.za
SUB INVESTIGATOR	Prof Sonja Boy and Dr Karin Richter
STUDY COORDINATOR	Dr Christy Davidson
SUPERVISOR (ONLY STUDENTS)	Prof Sonja Boy **E-Mail:** sonja.boy@up.ac.za
STUDY DEGREE	MSc (Odont)
MEETING DATE	27/06/2012

The Protocol and Informed Consent Document were approved on 27/06/2012 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid for 3 years period [till the end of December 2014], and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Dr NK Likibi	MBB HM – Representing Gauteng Department of Health) MPH
Dr MP Mathebula	(female)Deputy CEO: Steve Biko Academic Hospital; MBCHB, PDM, HM
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM; LLD(UP); PhD; Dipl.Datametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MbChB (Natal) FCS (SA)
Snr Sr J Phatoli	(female) BCur(Eet.A); BTec(Oncology Nursing Science) – Nursing representative
Dr R Reynders	MBChB (Prêt), FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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ANNEXURE B

Approval from the Research Committee (RESCOM) of the Faculty of Dentistry, University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences
School of Dentistry

2012/06/05

Dr CL Davidson

PROTOCOL APPROVAL: DENT 2012/06

We would like to inform you that your Protocol and Research project, as recommended by the Research Committee, has been approved by the Dean.

Title: **"Prevalence of oral and oropyharyngeal Human Papillomavirus (HPV) in a sample of selected South African males: a pilot study"**

Good luck with your studies!

PROF PJ VAN WYK
CHAIRMAN: RESEARCH COMMITTEE

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ANNEXURE C

Letter of permission to conduct the study at Willards, Snackworks Rosslyn

Snackworks
That's Good Times!

Reference: Sister Marina Jordaan
Tel: (012) 529 5300/5315
Email: MarinaJ@snackworks.co.za

19 April 2012

Dr Christy Davidson
Department of Oral Pathology and Oral Biology
University of Pretoria

Dear Dr Davidson

PERMISSION TO CONDUCT ORAL CANCER AWARENESS SURVEY

On behalf of The Company, Snackworks Rosslyn, I would like to officially grant you permission to conduct the Oral Cancer Awareness Survey at the Rosslyn site.

I trust that the survey will be conducted without any major disruption to our core business.

For further detail pertaining to this event, please contact our Occupational Health Practitioner: Sister Marina Jordaan, at the above contact details.

Kind regards

Shrine Nolan

OPERATIONS EXECUTIVE – SNACKWORKS
A DIVISION OF NATIONAL BRANDS LTD

ANNEXURE D

HPV flyer that was distributed to the workers of the factory prior to commencement of the study

HPV AND ORAL CANCER

15 NOV & 22 NOV 2012

Human Papillomavirus (HPV)

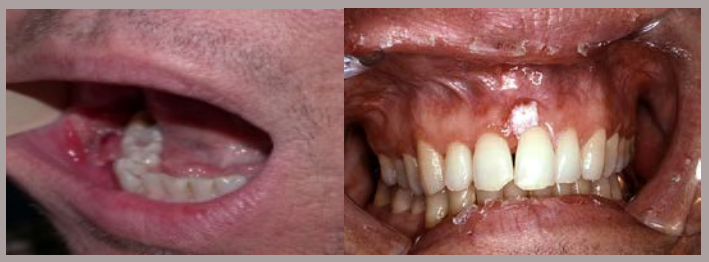
Human Papilloma Virus (HPV) is a common virus that is responsible for giving you warts on your hands, genitals and mouth, but even more importantly it has also been found to cause deadly cancer of the throat and mouth in **men**.

There are two dangerous types of this virus (HPV-16 and HPV-18) causing this cancer but we don't know how many men have this virus in their mouths as the virus can be there without anyone knowing it is there.

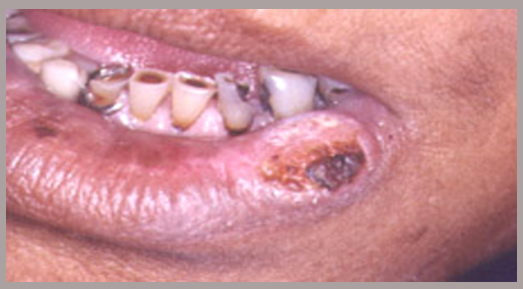
What will we do for you?

If you are between 20 – 40 years:

- Examine your mouth for any signs of the virus
- Spit into a small cup
- Complete some short questions
- We will offer a free HIV test for anyone that wishes to be tested for HIV but this is **NOT compulsory**



What we can see in the mouth



Oral Cancer on the Lip

Caption goes here

Please come and help us see if this virus is found in the mouths of men

DELETE BOX, OR PLACE LOGO HERE

- You will not be identified in any way but you will be helping us get important information that can prevent this type of cancer in men!!!

You will also be told if you have this virus in your mouth and what to do if you do have it.

ANNEXURE E

Patient information leaflet provided to the participant before commencement of the study, consent forms and questionnaire.

Patient Information Leaflet for:

Prevalence of oral and oropharyngeal Human Papillomavirus (HPV) in a sample of selected South African males: a pilot study

Dear Participant,

My name is Dr Christy Davidson from the University of Pretoria and I would like to invite you to participate in my study that will look if the Human Papillomavirus (HPV) is present in the mouths and throats of a sample of South African males between 20-40 years. We would also like to know what types of this common virus are present and if your sexual practices, smoking and drinking habits and/or HIV-status have any role in it being there.

This information leaflet will help you to decide if you want to participate. Before you agree to take part, you should fully understand what is involved. If you have any questions that this leaflet does not fully explain, please do not hesitate to ask the interviewer, any of the doctors present or your occupational health sister.

If you have any questions or need further explanation please contact me on:

Tel: (012) 319 2530

Reasons why we are doing this study:

The Human papillomavirus (HPV) is a common virus that is responsible for a wide range of small lesions (such as common warts on the skin of the hands and genital warts) but is also the main cause of cancer in the female genital tract (cervix cancer), the main killer amongst females in South Africa. This is the reason why an injection was developed to immunise young girls against the virus even before they have sex for the first time.

Not too long ago though, scientists also discovered it also plays an important role in cancer of the back of the mouth and throat (oropharynx). What is very important about this is that more and more men are getting this type of cancer and dying from it in South Africa and other parts of the world. The virus is mainly passed on by sexual contact with someone that already has this virus. Unfortunately, this virus does not always give us signs that it is there and this means that someone could have the virus without them knowing it. HIV infection can possibly be a risk factor for infection with HPV and for this reason we have included the option to volunteer for HIV testing in this study.

The **purpose** of this study is to see how many men between 20 and 40 years in sample of South Africa males carry this virus in their mouths. We would also like to know what types of this virus are present and if your sexual practices, smoking and drinking habits and/or HIV-status have any role in it being there.

The findings from this study will hopefully help us to do larger studies which could make the health authorities aware that the immunisation of boys may help us prevent, not only cancers of the female cervix, but also this form of throat cancer so common in men.

Please note that this is a voluntary study which means that you can choose if you want to participate in it or not and if you choose not to take part in the study you will not be disadvantaged in any way.

What will you have to do?

You will have to fill out a **questionnaire** answering personal questions about your sex life and alcohol/smoking habits. There will be someone to help you should you struggle with any of the questions. Some of the questions asked are of a very sensitive nature so you do not have to answer them should you not want to. We are not here to judge you in any way but it is very important to answer these questions as honestly as possible so that we can have truthful information that will be of help to the study. This questionnaire will **only have a number** on it and no-one from the research team will know that you filled it out. **Your name will never be used in this study.**

You will **rinse and gargle** with a teaspoon full (5ml) of watery, slightly salty liquid in your mouth for 30 seconds and then **spit it into a sterile container** that we will give you.

If you decide to do the HIV-test you will have to sign consent. The Occupational Health Sister at your company will then tell you about HIV. The HIV test for this study will be done by gently pricking your finger for a single drop of blood. The same number as on the questionnaire will be given to the blood sample and **your name will not be used on any of the paper work of this research project.** You can also **decide if you want your HIV-test results or not**. The result **will not disadvantage** you in any way and will only be given to you by your Occupation Health Sister,

Sr_____.

Overview of the process on day that the research team are at your company:

Step 1: Go to your Occupational Health Sister and **state whether you want to participate in this study** and **whether you want only the tests for the papillomavirus or both the tests for the papillomavirus and the HIV test**. . You will have to **sign consent forms** for both. If you choose to test for HIV then the sister will talk to you about HIV (counselling). The sister will prick your finger to get a small drop of blood. You then have to **decide if you want the results of your tests as soon as it is ready or not.**

Step 2: In the next room **fill out the questionnaire honestly** and once completed, **seal it in the envelope yourself and place it in the box** provided. If at any point you do not understand anything within the questionnaire, Mr MJ Mokgere (Bobo) and the occupational health Sister of your company will be there to help you or even help you to fill out the questionnaire from start to end.

Step 3: A **dentists/specialist in a private room will now look in your mouth** and see if you have any lesions or even rotten teeth. They will tell you if there are any problems and what you should do about them. You will also be given names of clinics in and around Pretoria to go to should you have teeth that need to be fixed. The doctor will give you **a teaspoon full of fluid to gargle and rinse your mouth** with for 30 seconds after which you will **spit it into a container**. The spit sample will be sent to a laboratory to test for the presence of the papillomavirus and you will receive the result at a later date through your Occupational Health Sister.

Your spit sample, the HIV result and your self-questionnaire will only be used for this study and nothing else. It will be kept for the duration of the study and then be destroyed. Under no circumstances will it be used for any other study or purpose. All test results (the HPV and HIV-results) will be kept **strictly confidential** and at no point will we or anyone but the Occupational Health Sister be able to connect your name to your test results. The sister will not have access to your questionnaire and will only be able to provide you with the test results should you decide that you want to know the results.

Side Effects, Risks and Discomforts:

The only discomfort you might experience in this study would be the prick to your finger should you decide to do the HIV test. There are no side effects with any of the tests.

What are the benefits in being part of this study?

You will get information on papillomaviruses (HPV) and its role in Cancer of the mouth and throat.

You will receive a free examination by a Dentist/ Dental Specialist with a list of names where you may be treated should you need any.

You will receive a free HPV test with information on what to do if you have this virus in your mouth or throat. A list of contact numbers has been given at the end this leaflet for you to contact should we find that you have a high risk virus in your mouth/throat. In order to do this, the researchers will return after the study has been completed to do a feedback session to all of those men who are interested.

You will receive free HIV counselling and an HIV test if you decide to do the HIV-test.
Your participation in this study might help the authorities to give permission for both boys and girls to be vaccinated against the virus that is responsible for two common cancers in South Africa namely cervix cancer and throat cancer.

Confidentiality:

The oral rinse sample, questionnaire and HIV test (voluntary) will be marked with the same **random number and your name will not appear on any of these.**

You will seal the questionnaire in an envelope as soon as you have filled it out and drop it into a sealed box. The box will only be opened for collection once all sampling for the day has been completed. There will be no way for the researchers to link your name to the numbers on the envelopes in the box.

If you choose to have the HIV test then only the sister involved will have your contact information given by you for feedback of the results should you want them. **NO** other employee of the company will have access to this information. This information will only be known by her and not the rest of the research team. She, will be the one to give you your virus results but she will have no access to the questionnaire you filled out.

The results of this study will be published in a scientific journal but all participants involved will remain anonymous and no names will ever be used at any time during this research project.

Thank you for your willingness to be part of this study

Consent to participate in the study: (HPV testing and questionnaire)

I have been invited to participate in this study on the Human Papillomavirus and I know that I will be required to give an oral rinse and gargle sample (spit) and fill out a questionnaire honestly.

I have read the information regarding the study and the steps that will be required, or it has been read and explained to me. I was able to ask questions about any aspect of this study and any questions that I have asked have been answered to my satisfaction. I voluntarily consent to participate in this research and understand that should I wish not to participate or withdraw at any time; it will have no effect on my working environment or my medical care.

Print Name of Participant: _____

Signature of Participant: _____ Date: _____
Day/month/year

If illiterate:

A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team).

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness: _____

Signature of witness: _____ Date: _____
Day/month/year

Consent for HIV Testing **follows on the next page ...**

Consent for HIV Testing

I have been invited to participate in the study on the Human Papillomavirus (HPV) and as part of this study HIV testing has been offered to me voluntarily.

If I choose to be tested for HIV, I will receive counselling before the testing from my Occupational Health Sister. I was able to ask questions about HIV and any questions that I have asked have been answered to my satisfaction. I understand that this HIV testing is voluntary. I have the right to refuse this test. I am aware that I will not be allowed to have the HIV test without consenting to be part of the rest of the study but that I can do the HPV testing and questionnaire without doing the HIV test. If I choose not to go ahead with the HIV testing I will not be disadvantaged in any way. My test results will remain confidential.

Please choose OPTION A (Agree to do HIV test) or OPTION B (Decline to do HIV test)

OPTION A: I hereby AGREE to the HIV testing:

Print Name of Participant: _____

Signature of Participant: _____ Date: _____
Day/month/year

If illiterate:

A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team).

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness: _____ AND Thumb print of participant

Signature of witness: _____ Date: _____
Day/month/year

Option B: DECLINE HIV testing

I hereby **decline** the HIV testing but still wish to take part in the rest of the study knowing that my Occupational Health Sister will be the one to give me my HPV results.

Print Name of Participant: _____

Signature of Participant: _____

Date: _____
(Day/month/year)

Signature of Occupational Health Sister: _____

Date: _____
(Day/month/year)

The Questionnaire follows on the next page ...

For Office Use

HPV Study ***Participant Questionnaire***

The answers you provide in this questionnaire will remain anonymous and will not be linked to you in any way. Your honest answers to each question will be highly appreciated.

The aim of this questionnaire is to see to what degree a person's sexual behaviour puts him at risk of HPV infection of which certain types are known to be involved in certain forms of cancer.

Participant number V1 | | | | 1

Please circle the appropriate number in a shaded box to indicate your answer and answer all the questions. Where you do not have an answer please do not indicate anything.

SECTION A ***GENERAL HEALTH***

1. What is your **age**?

	V2	5

2. What **diseases** do you have?

None that I am aware of	**0**	V3	8
Condition 1 (specify)		V4	10
Condition 2 (specify)		V5	13
Condition 3 (specify)		V6	16
Condition 4 (specify)		V7	19
Condition 5 (specify)		V8	22

Question 3 ***follows on the next page ...***

For Office Use

3. For each of the following **substances** please indicate the **number** you **use** and **how often** you use this substance and for **how long** you have been using this substance?
(*If one or more substances are not used please leave blank!)*

Number of cigarettes smoked		How often smoked		For how long smoked			
	cigarettes 1		per day 1		days 1	V9	25
	packets 2		per week 2		weeks 2	V11	30
			per month 3		months 3	V13	35
					years 4		

Number of cigars smoked		How often smoked		For how long smoked			
	cigars 1		per day 1		days 1	V15	40
			per week 2		weeks 2	V17	45
			per month 3		months 3	V19	50
					years 4		

Number of pipes smoked		How often smoked		For how long smoked			
	pipes 1		per day 1		days 1	V21	55
			per week 2		weeks 2	V23	60
			per month 3		months 3	V25	65
					years 4		

Number of Hubbly Bubbly smoked		How often smoked		For how long smoked			
	Hubbly Bubbly 1		per day 1		days 1	V27	70
			per week 2		weeks 2	V29	75
			per month 3		months 3	V31	80
					years 4		

Number of gunja (marijuana) smoked		How often smoked		For how long smoked			
	zols 1		per day 1		days 1	V33	85
			per week 2		weeks 2	V35	90
			per month 3		months 3	V37	95
					years 4		

Number of plugs of chewable tobacco used		How often used		For how long used			
	plugs 1		per day 1		days 1	V39	100
			per week 2		weeks 2	V41	105
			per month 3		months 3	V43	110
					years 4		

Number of pinches of snuff used		How often used		For how long used			
	pinches snuff 1		per day 1		days 1	V45	115
			per week 2		weeks 2	V47	120
			per month 3		months 3	V49	125
					years 4		

I do not use any substances	0	V51	130

Question 4 follows on the next page

For Office Use

4. For each of the following **types of alcohol** please indicate the **number** of units you **drink** and **how often** you drink this type of alcohol and for **how long** you have been drinking this type of alcohol
(*If one or more are not appropriate please leave blank!)*

Number of beers (e.g. castle)			**How often consumed**			**For how long consumed**				
	cans	**1**		per day	**1**		days	**1**	V52	132
	dumpies	**2**		per week	**2**		weeks	**2**	V54	137
	quarts	**3**		per month	**3**		months	**3**	V56	142
							years	**4**		

Spirits consumed (e.g. vodka, cane)			**How often consumed**			**For how long consumed**				
	tots	**1**		per day	**1**		days	**1**	V58	147
	glasses	**2**		per week	**2**		weeks	**2**	V60	152
	bottles	**3**		per month	**3**		months	**3**	V62	157
							years	**4**		

Wine consumed			**How often consumed**			**For how long consumed**				
	glasses	**1**		per day	**1**		days	**1**	V64	162
	bottles	**2**		per week	**2**		weeks	**2**	V66	167
				per month	**3**		months	**3**	V68	172
							years	**4**		

I do not consume alcohol	**0**	V70	177

SECTION B: ***SEXUAL HISTORY***

5. At what **age** did you have penetrative sex for the first time?

Age:		V71	179
I have never had penetrative sex before	**0**		

6. **Who** have you had penetrative **sex** with?
(Mark all that are applicable)

Virgins	**1**	V72	182
Sexually active women	**2**	V73	184
Sexually active men	**3**	V74	186
I have never had penetrative sex with anyone	**4**	V75	188

7. Up to the present time, how **many** people have you had **penetrative sex** with?

	V76	190

Question 8 ***follows on the next page ...***

For Office Use

8. How **many** people have you had **penetrative sex** with **in the last 6 months**?

V77 [ ][ ][ ] 194

9. Do you use **sexual protection** during **penetrative sex** in the form of male condoms?

Never	**1**	V78 [ ] 198
Occasionally	**2**	
Always	**3**	

10. Which **aspects** best **describe** the forms of **unprotected sexual activity** you have been involved in?
(Mark all that are applicable)

Open mouth kissing (not oral sex)	**1**	V79 [ ] 200
Mouth-genital contact	**2**	V80 [ ] 202
Mouth-anal contact	**3**	V81 [ ] 204
Genital-genital contact	**4**	V82 [ ] 206
I am not involved in unprotected penetrative sexual activity	**5**	V83 [ ] 208

11. At what **age** did you have **oral sex** for the **first** time?

Age:		V84 [ ][ ] 210
I have never had oral sex before	**0**	

12. Up to the present time, how **many** people have you had **oral sex** with?

V85 [ ][ ][ ] 213

13. How **many** people have you had **oral sex** with in the **last 6 months**?

V86 [ ][ ][ ] 217

Thank you for your time and co-operation in this study

ANNEXURE F

Article published in South African Medical Journal (SAMJ), May 2014

Prevalence of oral and oropharyngeal human papillomavirus in a sample of South African men: A pilot study

C L Davidson,[1] BChD; **K L Richter**,[2] MMed (Path) (Virology); **M van der Linde**,[3] PhD; **J Coetsee**,[3] MCom (Statistics); **S C Boy**,[1] PhD

[1] *Department of Oral Pathology and Oral Biology, School of Dentistry, Faculty of Health Sciences, University of Pretoria, South Africa*
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[3] *Department of Statistics, Faculty of Natural and Agricultural Sciences, University of Pretoria, South Africa*

Corresponding author: *C Davidson (christy.davidson@up.ac.za)*

Background. Human papillomavirus (HPV) infection is well known to be associated with head and neck cancers (HNCs). HPV-associated HNCs are related to sexual behaviour, particularly the lifetime number of oral sex partners, but the epidemiology of oral and oropharyngeal HPV in South African men has not yet been studied.
Objectives. To determine the oral and oropharyngeal HPV strain prevalence and associated factors in a selected male population in Pretoria, South Africa (SA).
Methods. Male factory workers were recruited. Oral rinse and gargle samples were tested for 37 HPV types using the Linear Array HPV Genotyping Test (Roche Molecular Systems). A questionnaire was used to obtain information regarding age, medical conditions, substance and alcohol use and sexual behaviour. HIV testing was optional.
Results. The HPV prevalence was 5.6% among men (*N*=125) aged 17 - 64 years. High-risk HPV (hrHPV) types 16 and 68 were found in two men. Oral sex seemed to be an uncommon practice in the majority of respondents, but the two respondents with hrHPV did practise oral sex. There was a statistically significant association between HPV infection and an increased number of sexual partners (p=0.027), but not between HPV and substance use, HIV status or clinical mucosal pathology.
Conclusion. The prevalence of oral and oropharyngeal HPV was lower than reported in other countries. An association between oral HPV and having multiple sexual partners was found. A larger nationwide study would give a more representative view of the burden of oral and oropharyngeal HPV infection in SA.

S Afr Med J 2014;104(5):358-361. DOI:10.7196/SAMJ.7542

Oropharyngeal squamous cell carcinoma (OSCC) may originate in the soft palate, tongue base, pharyngeal walls or tonsils. The association of high-risk human papillomavirus (hrHPV) types with increased risk for a subset of OSCC has been established by many studies.[1-5] It has been shown that oral human papillomavirus (HPV) infection, and specifically HPV type 16 infection, causes an up to 50-fold increase in HPV-positive OSCC.[6] The pathogenic association of OSCC with HPV was therefore recently accepted by the International Agency for Research on Cancer.[7] Head and neck cancer (HNC), of which OSCC is a subset, is the sixth most common cancer in the world. In South Africa (SA) there has been a proven increase in OSCC since 1995, and 90% of these cancers were associated with HPV 16.[8] Males were affected more frequently than females.[8] Gillison *et al.*[9] recently published the first population-based study that concurrently examined the epidemiology of oral HPV infection among males and females and showed an almost three-fold higher HPV prevalence in males; the HPV 16 prevalence was five-fold higher in males than in females.

The aims of this pilot study were to determine the oral and oropharyngeal HPV strain prevalence in a selected male population in Pretoria, SA, as well as the association of HPV with sexual practices and substance use.

Methods

Participant selection

Male participants were voluntarily recruited from an industrial factory in Pretoria in 2012. Written informed consent was obtained from each participant for HPV testing and answering the questionnaire. Separate consent was obtained for voluntary HIV testing. Refusal of HIV testing did not serve as a criterion for exclusion from the study, but refusal of HPV testing did. Approval for the study was obtained from the Ethics Committee of the Faculty of Health Sciences, University of Pretoria (no. 101/2012).

Clinical examination and saliva sample collection

Participants underwent a thorough oral and oropharyngeal clinical examination by a senior dentist/oral pathologist. All dental and other soft-tissue abnormalities were noted and photographed.

Oral rinse samples were collected by means of a 20-second oral rinse and gargle with 5 ml phosphate-buffered saline (PBS) (Sigma Life Sciences, USA) given to the patient in a 40 ml sterile collection cup (Lasec, SA). Participants alternately swished and gargled and then expectorated the saliva into the cup. All samples were numbered consecutively and stored on ice until sample preparation.

Questionnaire

The questionnaire comprised specifically formulated questions pertaining to past and present sexual histories, substance and alcohol use and any medical conditions. The numbers of the questionnaires corresponded with the oral rinse samples, and their content was treated as confidential.

Voluntary HIV counselling and testing

Participants who opted for HIV testing received pre-test counselling from the occupational health practitioner (OHP) of the company. A rapid point-of-care HIV screening test (U test HIV/AIDS, Humor Diagnostica, SA) that detects HIV-1 and HIV-2 antibodies was then performed according to the manufacturer's instructions. Confirmatory follow-up testing and continued care were conducted by the same OHP.

Molecular laboratory techniques

Specimen preparation

Saliva samples were prepared for HPV genotyping within 24 hours of collection. The samples were washed three times with sterile PBS and the cell pellets were stored at -70°C until subsequent DNA extraction.

DNA extraction

DNA extraction from the cell pellets was performed using the DNA Isolation Kit (Roche Molecular Systems, USA) on the MagNA Pure automated extraction system.

HPV DNA amplification and genotyping

HPV DNA amplification and genotyping was done using the Linear Array HPV Genotyping Test (Roche Molecular Systems). The pool of primers in the kit are designed to amplify HPV DNA from 15 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), 3 probable high-risk genotypes (26, 53 and 66) and 19 low/undetermined risk types (6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39 and CP6108).[10] The β-globin gene was amplified concurrently for each sample and served as internal control for cellular adequacy, extraction efficiency and amplification.

Statistical analysis

The relationship between the prevalence of HPV and the prevalence of the variables substance use, alcohol use, sexual history, HIV and oral lesions was investigated using the chi-square test for independence or, where appropriate, Fisher's exact test. The Kruskal-Wallis test was used to analyse whether or not any of the variables investigated had any significant influence on the HPV status of the participants.

Table 1. Summary of human papillomavirus status and results for the variables investigated in all the participants

	HPV+ (*N*=7) *n* (%)	HPV- (*N*=118) *n* (%)	Total (*N*=125) *n* (%)	*p*-value†
Age (years)				1.000
≤40	2 (28.6)	36 (30.5)	38 (30.4)	
>40	5 (71.4)	82 (69.5)	87 (69.6)	
Smoking				0.703
Yes	4 (57.1)	54 (45.8)	58 (46.4)	
No	3 (42.9)	64 (54.2)	67 (53.6)	
Alcohol				0.343
Yes	7 (100.0)	93 (78.8)	100 (80.0)	
No	0	25 (21.2)	25 (20.0)	
Lifetime sexual partners				0.027
0 - 5	1 (14.3)	19/117* (16.2)	20/124 (16.1)	
5 - 20	2 (28.6)	79/117* (67.5)	81/124 (65.3)	
>20	4 (57.1)	19/117* (16.2)	23/124 (18.5)	
Sexual partners in the past 6 months				1.000
None	0 (0)	3/117 (2.6)	3/124 (2.4)	
1 - 3	7 (100)	104/117 (88.9)	111/124 (89.5)	
>3	0 (0)	10/117 (8.5)	10/124 (8.1)	
Practise oral sex				0.525
Yes	3 (42.9)	48 (40.7)	51 (40.8)	
No	4 (57.1)	70 (59.3)	74 (59.2)	
Lifetime oral sex partners				0.592
1	0	19/48 (39.6)	19/51 (37.3)	
2	1/3 (33.3)	16/48 (33.3)	17/51 (33.3)	
>2	2/3 (66.7)	17/48 (35.4)	19/51 (37.3)	
Oral sex partners in the past 6 months				0.518
None	4 (57.1)	83 (70.3)	87 (69.6)	
1	2 (28.6)	26 (22.0)	28 (22.4)	
>1	1 (14.3)	9 (7.6)	10 (8.0)	
HIV				0.563
Positive	0	5 (4.2)	5 (4.0)	
Negative	4 (57.1)	81 (68.6)	85 (68.0)	
Declined testing	3 (42.9)	32 (27.1)	35 (28.0)	
Clinical lesions				0.592
With	0	18 (15.3)	18 (14.4)	
Without	7 (100.0)	100 (84.7)	107 (85.6)	

HPV+ = HPV-positive; HPV- = HPV-negative.
*One questionnaire was not completed.
†Fisher's exact test.

Results

Oral rinse specimens were collected from 128 males between the ages of 17 and 64 years (median 50). The participants originated from all over SA. Three participants who did not complete questionnaires were excluded from the study. Only 7/125 participants (5.6%) tested positive for HPV. One participant was co-infected with HPV types 71 and 72. Two

Table 2. Summary of human papillomavirus-positive results for all the variables investigated

	HPV type/s	Age (years)	Smoking	Alcohol	Sex	Oral sex	HIV status	Oral lesions
1	16 (hr)	51	Yes	Yes	Yes (m)	Yes	Declined testing	No
2	55	59	Yes	Yes	Yes (m)	No	Declined testing	No
3	62	46	No	Yes	Yes (m)	No	Negative	No
4	68 (hr)	58	No	Yes	Yes	Yes	Negative	No
5	70	29	Yes	Yes	Yes (m)	No	Declined testing	No
6	71, 72	64	No	Yes	Yes (m)	No	Negative	No
7	72	32	Yes	Yes	Yes (m)	Yes	Negative	No

HPV = human papillomavirus; hr = high-risk HPV type; m = multiple sexual partners.

participants tested positive for hrHPV types, namely HPV 16 and HPV 68, respectively. Table 1 summarises the findings for all the variables investigated. The quantity and frequency of the different substances used were not investigated. An overall score was calculated, which only reflected whether a respondent used or did not use any of the substances listed in the questionnaire.

All participants were sexually active, 90/125 (72.0%) of them reporting having had their first sexual encounter between 15 and 20 years of age. The association between HPV and the number of sexual partners was statistically significant (p=0.027).

In the subgroup of 90 men (72.0%) who volunteered for HIV testing, only five (5.5%) tested positive. Unfortunately only 4/7 HPV-positive participants (57.1%) opted for HIV testing; one of these was found to have an hrHPV type. All the HPV-positive participants who tested for HIV were negative.

Only 18 of the 125 participants (14.4%) were found to have oral mucosal lesions on clinical examination. The lesions encountered included white lesions, red lesions, ulcerative lesions and *Candida albicans* infection. None of the HPV-positive participants had clinically visible mucosal pathology.

Table 2 summarises the findings for the variables investigated in the HPV-positive participants.

Discussion

The natural history of oral and oropharyngeal HPV infection in men is unknown. A recent study found the prevalence of oral HPV in men in the USA to be around 10%.[9] This pilot study among a group of SA men had an unexpectedly low HPV prevalence of only 5.6%. This could be attributed to the small sample size, or to the observation that oral sex did not seem to be a common practice among the group of men studied. A much larger nation-wide analysis with inter-institutional collaboration will be necessary to confirm whether this figure is in fact truly representative of oral and oropharyngeal HPV in SA men. Known risk factors for oral and oropharyngeal HPV infection currently include lifetime number of sexual partners,[6,9,11] number of oral sex partners,[1,12] substance use[9,12-14] and infection with HIV.[15-18]

Sexual contact, either genital or oral, remains the primary mode of HPV transmission, and it is well documented that having multiple partners increases the prevalence of oral and genital HPV.[6,9,19,20] A study in KwaZulu-Natal, SA, reported male undergraduate students to have had a median of four sexual partners in the previous year.[11] In many African cultures it is acceptable for men to have multiple partners and engage in sex outside the primary relationship.[21,22] A statistically significant association between the number of sexual partners and the presence of oral HPV was seen in this study (p=0.027), with 4/7 HPV-positive participants having had >20 sexual partners and 2/7 having had between five and 20 partners. The numbers of sexual partners of the HPV-positive participants in the past 6 months ranged from one to three.

Tobacco use is an accepted cause of HNCs, but it is not considered a strong risk factor for HPV-associated oropharyngeal cancers.[1,6] We found no statistically significant association between smoking and the presence of HPV (p=0.703), although the only participant with HPV 16 in our study was a current cigarette smoker. An increased oral HPV prevalence has been shown to be associated with current and previous smoking, but the exact pathogenic mechanisms are unknown.[12,13] Alcohol has the ability to modify mucosal tissue, allowing easier entry of the virus, and is also known to modify the host immune response, thereby increasing susceptibility to HPV infection.[14,23] In our study we found no statistical association between alcohol use and HPV (p=0.343), although all the HPV-positive participants were current alcohol users.

The HIV pandemic and attempts to prevent spread of the virus have increasingly highlighted sexual practices other than penetrative sex. Oral sex has increased to a marked extent, especially among teenagers, as a result of the false perception that sexually transmitted diseases such as HIV infection cannot be passed on in this manner.[12,24] Studies on oral sex practices in the USA have shown that 19.6 - 78% of young adults had engaged in oral sex in their lifetime.[25] Gillison *et al.*,[9] in line with other studies, found oral sex performed on women to be one possible explanation for the increased prevalence of oropharyngeal HPV in males, who in their study on average had more partners than the females. Sexual behaviour studies in SA are few, and those that do exist are not specific in their data regarding genital versus oral sexual practices and the gender differences that exist. However, it was later discovered through the male fieldworker that most men in our study interpreted oral sex to mean contact of the female's oral mucosa with the man's genital mucosa rather than the other way around. This could explain the low prevalence of oral HPV. Only 51 of all participants (40.8%) and 3/7 HPV-positive participants (42.9%) answered 'yes' to the question whether they had practised oral sex. Considering the participants' understanding of the definition of oral sex, one should be cautious in interpreting these figures. Of note was that both the individuals with hrHPV types said that they had had oral sex with more than one partner.

Oral HPV infection is considered to be bimodal in age distribution, with a high prevalence in younger men (30 - 34 years of age) and in later years (60 - 64).[9] The participants who were HPV-positive in this study were between 29 and 64 years of age, and those with hrHPV were between 50 and 60 years of age. It has been speculated that the higher HPV prevalence at older ages could be due to increased duration of infections at older ages, rather than an increased

acquisition of new HPV infections.[26] In our study, however, all the participants who were positive for HPV had had between one and three sexual partners in the past 6 months, suggesting that their HPV infections could have been newly acquired.

HIV-infected individuals have been reported to have an up to six-fold increased risk for HPV-related OSCC.[27] It is thought that HIV and HPV function as a combined risk factor, as HIV-positive individuals have more frequent HPV infections, but the natural history of these infections may be altered when they co-exist.[15] In SA, the total estimated number of adults aged 15 - 49 years living with HIV in 2013 was 5.26 million, giving an HIV prevalence of 10%.[28] The HIV prevalence in this study was only 5.5%. Only four of the HPV-positive participants (n=7) volunteered to test for HIV, and all were negative. One of the participants who was found to have hrHPV 16, which is associated with OSCC, declined HIV testing. A much larger nationwide study with an HIV prevalence more representative of that for the country as a whole will be needed to determine the role of HIV infection in the natural history of oral and oropharyngeal HPV infection.

The 18 participants who presented with oral mucosal lesions were all negative for HPV. All patients with dental or mucosal pathology were referred to peripheral dental clinics or the Oral Medicine Clinic of the Oral and Dental Hospital, Pretoria, for surgical biopsies and clinical follow-up.

Study strength

Ours is the first study of this nature to investigate the oral and oropharyngeal HPV prevalence in SA men. Although the sample size was small, we did find a statistical association between HPV and the number of lifetime sexual partners.

Study limitations

The greatest limitation of this study is the small sample size, which was restricted owing to funding. Another limitation is the fact that not all the participants opted to test for HIV, which may also have an influence on the conclusions. Because this was a pilot study some of the conclusions made, including the role of HIV, will need to be verified by a larger, nationwide study.

Conclusion

In contrast to the prevalence of genital HPV in SA females and the role of the virus in the pathogenesis of cervical carcinoma, little is known about the prevalence of HPV in the oropharyngeal mucosa of SA men, who are known to acquire HPV-related OSCC more frequently than women. The prevalence of oral and oropharyngeal HPV in this study is lower than reported in other countries. Even though the number of participants was low, a statistically significant association was shown between oral and oropharyngeal HPV and having multiple sexual partners. Oral sex, although not practised by the majority of participants, was practised by those identified with hrHPV types. The high number of deaths from HPV-related cervical carcinoma and the increase in HPV-related OSCC, especially in males, makes it urgently necessary to investigate the prevalence of oral and oropharyngeal HPV in the larger SA population. Specific reference to the roles of sexual practices and HIV in the natural history of HPV is essential. Deeper investigations into the nature of sexual practices among South Africans are needed, and the diversity of the SA population will have to be considered in any future study of this nature.

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GLOSSARY

1. **Cis-responsive elements**: region of DNA/RNA that regulates the expression of genes located on that same molecule of DNA. It may be located upstream of the coding sequence of the gene it controls or downstream of the genes coding sequence (on the same molecule of DNA as the gene they regulate). They are often binding sites for one/more trans-acting factors.
2. **Deacetylation**: the removal of an acetyl group from a molecule.
3. **De-repression**: to induce the operation of a gene by deactivating the repressor.
4. **Encapsidation**: process whereby a virus' nucleic acid is enclosed in a capsid.
5. **Enhancer**: is the DNA sequence which increases transcription of a related gene.
6. **Episome**: an extrachromosaomal replicating unit that exists autonomously or functions with a chromosome.
7. **Exon**: an expressed sequence/ region of a gene which is not excised during transcription forming part of the mature mRNA and therefore specifying part of the primary structure of the gene product.
8. **Homeotic genes**: genes which are involved in controlling the development of a region/compartment of an organism producing proteins/factors which regulate gene expression by the binding particular DNA sequences.
9. **Homobox**: a stretch of approximately 180 base pairs conserved in different homeotic genes.
10. **Homodimers**: a protein composed of two identical subunits/proteins.
11. **Homodimerisation**: the process of joining two identical subunits to form a single compound.
12. **Intron**: is an intervening sequence/region of DNA which generates that part of precursor RNA which is spliced out during transcription and does not form mature mRNA and therefore does not specify the primary structure of the gene product.
13. **Open Reading Frames (ORFs):** a portion of a genes sequence that contains a sequence of bases, uninterrupted by stop sequences that could potentially encode a protein.
14. **Oncogene**: a gene that causes the transformation of normal cells into cancerous tumour cells, especially a viral gene that transforms a host cell into a tumour cell.
15. **Promoter**: is the initiation site and associated 'upstream' region. It attracts RNA polymerase and is the recognition sequence for the binding of RNA polymerase.

16. **Recombination:** is the cross-over between two linked loci.
17. **Silencer**: a negative 'enhancer', the normal action of which is to repress gene expression.
18. **Splicing**: removal of the introns and joining of the exons in RNA transcription with introns being spliced out and exons being spliced together.
19. **TATA box**: a DNA sequence occurring in the promoter region 25 to 35 base pairs upstream from the transcriptional start site.
20. **Transcription**: is the process whereby genetic information is transmitted from DNA to RNA (mRNA).
21. **Transcription factors**: these are genes which include the Hox, Pax and zinc finger containing genes which control RNA transcription by binding to specific DNA regulatory sequences forming complexes which initiate transcription by RNA polymerase.
22. **Translation**: the process where cellular ribosomes create proteins (mRNA produced by transcription is decoded by a ribosome complex to produce a specific amino acid chain/polypeptide that will later fold into an active protein).
23. **Virion**: virus particles which are inert carriers of the genome. It consists of protein shell and an inner core of nucleic acid. They are infective.

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* **FWA** 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
* **IRB** 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

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Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 28/06/2012

NUMBER	**101/2012**
TITLE OF THE PROTOCOL	Prevalence of oral and oropharyngeal Human Papillomavirus (HPV) in a sample of selected South African males: a pilot study.
PRINCIPAL INVESTIGATOR	**Student Name & Surname:** Dr Christy Davidson **Dept:** Oral Pathology and Oral Biology, School of Dentistry, Faculty of Health Sciences;University of Pretoria. **Cell:** 0832984610 **E-Mail:** christy.davidson@up.ac.za
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STUDY COORDINATOR	Dr Christy Davidson
SUPERVISOR (ONLY STUDENTS)	Prof Sonja Boy **E-Mail:** sonja.boy@up.ac.za
STUDY DEGREE	MSc (Odont)
MEETING DATE	27/06/2012

The Protocol and Informed Consent Document were approved on 27/06/2012 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid for 3 years period [till the end of December 2014], and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Dr NK Likibi	MBB HM – Representing Gauteng Department of Health) MPH
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Faculty of Health Sciences
School of Dentistry

2012/06/05

Dr CL Davidson

PROTOCOL APPROVAL: DENT 2012/06

We would like to inform you that your Protocol and Research project, as recommended by the Research Committee, has been approved by the Dean.

Title: **"Prevalence of oral and oropyharyngeal Human Papillomavirus (HPV) in a sample of selected South African males: a pilot study"**

Good luck with your studies!

PROF PJ VAN WYK
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That's Good Times!

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19 April 2012

Dr Christy Davidson
Department of Oral Pathology and Oral Biology
University of Pretoria

Dear Dr Davidson

PERMISSION TO CONDUCT ORAL CANCER AWARENESS SURVEY

On behalf of The Company, Snackworks Rosslyn, I would like to officially grant you permission to conduct the Oral Cancer Awareness Survey at the Rosslyn site.

I trust that the survey will be conducted without any major disruption to our core business.

For further detail pertaining to this event, please contact our Occupational Health Practitioner: Sister Marina Jordaan, at the above contact details.

Kind regards

Shrine Nolan

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