CHAPTER ONE

INTRODUCTION

1.1 Background

Lifestyle factors accounts for up to 80% of all tumour cases (Giri *et al.,* 2009), with tobacco and alcohol consumption being the most common lifestyle factors seen amongst commercial bus drivers. In Nigeria, tobacco is consumed mainly in two forms, smoked and smokeless tobacco products. Tobacco usage in any form constitutes a major risk factor of oral cavity neoplasms (Farhadi *et al*., 2016a; Khlifi *et al*., 2013). According to Singh *et al*., (2016), oral cancer is a tobacco-related disease. Farhadi *et al*., (2016a) reported that the carcinogenic effect of tobacco use may be related to its ability to induce genotoxicity in the oral mucosal cells of users. Carcinogenesis may be initiated by the destructive effect on the cells of the oral cavity by the heat generated during smoking (Farhadi *et al*., 2016a), or by the interaction of chemical components of tobacco with the cells. The tar in tobacco has for long been associated with development of cancers (Johnson *et al*., 2000). According to Pautassi *et al*., (2010) cigarette smoke contains about 4700 chemical compounds, with about 70% of them already known as carcinogens. These include polycyclic aromatic hydrocarbons, aromatics amines, nitrosamines, heavy metals, poisonous gases and pesticide residues to mention but a few.

Haveric *et al*., (2010) established that a dose and time dependent relationship exist between tobacco use and development of cancers. The form in which the tobacco is used also determines the degree of risk of developing oral cavity lesions. The use of tobacco has been

associated with various lesions in the oral cavity which include tooth stains, abrasions, smoker's melanosis, acute necrotizing ulcerative gingivitis and other periodontal conditions, burns and keratotic patches, black hairy tongue, nicotinic stomatitis, palatal erosions, leukoplakia, epithelial dysplasia and squamous-cell carcinoma (Mirbod and Ahing, 2000). A study carried out by Biswas *et al*., (2014) revealed that tobacco users have significantly higher oral cellular abnormalities when compared to healthy control subjects, with chewers of tobacco having worse cellular abnormalities than smokers. Prominent cellular abnormalities observed by these authors include condensed chromatin, pyknosis, karyolysis, and bi nucleation, with the degree of atypia being age-dependent.

Alcohol consumption has long been associated with oral lesions (American Cancer Society, (ACS) 2014). Alcohol is the term used for ethanol or ethyl alcohol found in alcoholic beverages such as beer, wine, liquor, as well as in some medicines, mouthwashes, household products, and essential oils (scented liquids taken from plants). It is a product of fermentation of sugar and starch by yeast. Reports by the International Agency for Research on Cancer (IARC) working group on the evaluation of carcinogenic risks to humans in 2010 and 2012, confirmed a strong association of alcohol and cancers (IARC, 2010; IARC, 2012). These reports further revealed that increased consumption of alcohol increases the risk of developing cancers. According to ACS (2014), alcohol use accounts for 3-4% of all cancer deaths annually. Alcohol drinkers who consume up to ≥50 g of alcohol per day have 2-3 times greater risk of developing oral cancer than non drinkers (Baan *et al*., 2007).

Increased risk of development of oral cancer had been reported in alcohol drinkers who also use tobacco (Ogden, 2005; Hashibe *et al*., 2009; ACS, 2014). The mechanism by which alcohol initiates tumourigenesis is not completely understood.

ASC (2014) reported that tumourogensis may be initiated by the following mechanism; damage to body tissues, effects on other harmful chemicals, lower level of folate and other nutrients, effects on estrogen and other hormones, effects on body weight and generation of reactive oxygen species. Furthermore, alcohol affects cellular structure and function. It may influence the proliferative cells by both intracellular and intercellular pathways with the carcinogenic exposure of the proliferating stem cells in the basal layer being regulated through these pathways (Ogden, 2005). The etiology of premalignant lesions is generally accepted to be multifactorial, with tobacco and alcohol being reported as important cofactors in transition from pre-malignancy to malignancy (Humayun and Prasad, 2011).

The expression pattern of k-i67, p53 and p16 in malignant and pre-malignat lesions generally and in oral lesions in particular has been reported, both as predictive and prognostic biomarkers (Scholzen and Gerdes, 2000; de Vicente *et al*., 2002; Jamaroon *et al*., 2004; Humayun and Prasad, 2011; Christos *et al*., 2012). Though, the genes expression pattern have been amply reported for tumours such as lymphomas, breast and prostate lesions, there still remain paucity of report of their expression in oral lesions. Ki-67 is a nuclear protein present in peri-chromosomal region, which in human is encoded by the MKI16 gene (Bullwinkel *et al*., 2006). It is strictly associated with cell proliferation and therefore, widely used in pathology as a proliferative marker (Humayun and Prasad, 2011). The expression pattern is directly proportional to the rate of cell division. This makes it a

useful biomarker for predicting the outcome of premalignant conditions of the oral cavity epithelial cells. Similarly, p53, known as tumour suppressor gene, is a protein encoded by the TP53 gene (Humayun and Prasad, 2011). According to the authors, this protein is not expressed in normal tissues because of its very short half life, but however, remains active for a longer time when mutated, due to defect in degradation pathway or when bound to other proteins.

The function of p53 protein is that of preventing accumulation of genetic damage in cells either by allowing for repair of the damage before cell division or by causing death of the cell. This function is impaired in the mutant variant leading to the loss of the tumour suppressor function of the protein (Nylander *et al*., 2000; Reibel, 2003). Sequel to this, p53 has become a valuable predictive biomarker, especially for screening population at risk of developing malignancy. It is used to predict the behavior of dysplastic and non dysplastic cells in high risk individuals. Gissi *et al*., (2015) reported the role of p53 both as a single predictive marker and in association with Ki-67, thereby agreeing with earlier report by Jamaroon *et al*., (2004) who noted and reported the co-expression of p53 and Ki-67 in oral squamous cell carcinoma. Christos *et al*., (2012) in an independent study observed the over expression of p53 and Ki-67 in oropharyngeal squamous cell carcinoma. Moreover, p16, also known as cyclin-dependent kinase inhibitor 2A (CDKN2A) is a tumour suppressor protein which in humans is encoded by the *CDKN2A* gene. It is involved in the p16/cyclin dependent kinase/retinoblastoma gene pathway of cell cycle control by decelerating cells from progressing from G1 to S phase transition of the cell cycle (Lin *et al*., 2003). The p16 gene when mutated or hypermethylated loses its cell cycle regulation role, leading to

uncontrolled cell division and cancer (Shaw *et al*., 2006; Romagosa *et al*., 2011). Over expression of p16 in oral cancer has been reported by some authors (Shaw *et al*., 2006; Riechelmann, 2010; Romagosa *et al*., 2011). The association of p16 and high risk *Human papilloma* virus (HPV) infection with head and neck cancers has also been reported (Ai *et al*., 2003; Wittekindt *et al*., 2005; Kuo *et al*., 2008; Riechelmann, 2010; Wittekindt *et al*., 2012). According to the authors, HPV infection is major risk factors of oral squamous cell carcinoma and p16 expression is used to detect the involvement of the virus in carcinogenesis.

Commercial bus drivers are well known for tobacco use and alcohol consumption, which constitute the major risk factors for development of cellular changes of the oral cavity (Mohan, 2010). Oral cavity carcinogenesis usually begins with cellular changes such as bi-nucleation, prominent nucleoli, karyolysis, increased nuclear/cytoplasmic ratio, micronuclei, to mention but a few (Farhadi *et al.,* 2016a). Repair index (RI) is the ratio of the sum of karyorrhexis and karyolysis and broken egg nuclei and micronuclei [*KR+KL/BE+MN*] (Farhadi *et al.,* 2016b). It is a scientific measure of the severity of nuclear damage and predictor of the likelihood of progression of genotoxic cellular damage to pre-malignant lesions. Farhadi *et al.,* (2016b) were the first to apply RI to determine and compare the degree of cellular changes between smokers and non smoker control groups. A direct proportional relationship exists between increasing of value of RI and favourable outcome. This implies that the higher the value of RI the more the likelihood of dysplastic cells reversing to normal cells and vice versa.

Ki-67, p53 and p16 genes are known biomarkers of oral carcinomas. Their expression patterns, especially in high risk individuals may help to predict the progression of cellular dysplasia to malignancy. It could also serve as a useful tool for screening apparently healthy individuals and individuals exposed to mutagenic effects of tobacco and/or alcohol consumption and in monitoring of progress during treatment of oral cancer.

1.2 Statement of the Problem

Lifestyle factors such as of tobacco and alcohol use, which are commonly found amongst commercial bus drivers, account for 80% risk factors of all cancers (Giri *et al*., 2009). Tobacco and alcohol use, and *Human Papilloma* Virus (HPV) infection have been attributed the major risk factors of oral squamous cell carcinoma (Mohan, 2010). Opubo *et al* (2009) in a review of the current evidence on the burden of head and neck cancers in Nigeria noted a report by Otoh *et al* (2004) who observed in their study that 20-24 new cases were reported in the Northern part of Nigeria, while in the Southern part of Nigeria, head and neck cancers constitute 6.2% of all cancers. Similarly, a yearly incidence of 33-38 new cases had been reported in the South Western part of the country (Amusa *et al*., 2004). In a more recent study, Kodiya *et al* (2016) noted and reported that head and neck cancers is one of the common health problems in our environment, affecting mostly the youths. The authors further attributed paucity of literature on community based studies in Nigeria to determine the incidence of the disease as the factor which obscures its burden, pattern and magnitude. The expression pattern of k-i67, p53 and p16 in malignant and pre-malignat lesions generally and in oral lesions in particular has been reported, both as predictive and prognostic biomarkers (Scholzen and Gerdes, 2000; de Vicente *et al*., 2002; Jamaroon *et al*., 2004; Humayun and Prasad, 2011; Christos *et al*., 2012). Though, the gene expression

patterns have been amply reported for tumours such as lymphomas, breast and prostate lesions, there still remain paucity of report of their expression in oral lesions.

In view of these, the need to determine the prevalence of nuclear changes and gene expression pattern of ki-67, p53 and p16 in the oral cavity of commercial bus drivers (a high risk population) using tobacco and alcohol in Nnewi becomes necessary.

1.3 Justification of the study

The findings of this study will among other things evaluate the prevalence of nuclear changes in the oral mucosa of a high risk population, relevance or otherwise of ki-67, p53 and p16 as predictive, diagnostic and prognostic biomarkers of oral lesions. The study will emphasize on the significance of oral mucosa cytology as an efficient non invasive screening tool for oral squamous cell carcinoma, especially for screening in high risk populations. This may prove to be an indispensible preventive strategy in the wake of increasing lifestyle risk factors vis-à-vis increasing incidence of oral cancers. Furthermore, the findings of this study hope to open a new interest in Environmental/Public Health Pathology which will encourage development of healthcare policies and programmes to deal with lifestyle factors and risk factors of not only oral lesion but other neoplasms.

1.4 Aim of the Study

This study was aimed to evaluate the prevalence of nuclear changes and gene expression patterns of ki-67, p53 and p16 in the oral cavity of commercial bus drivers using tobacco and alcohol in Nnewi.

1.5 Objectives of the Study

The following study objectives were used to achieve the aim:

1. To evaluate and compare the presence and degrees of various forms of nuclear changes in the cells of the oral cavity amongst commercial bus drivers actively using tobacco and alcohol and control subjects.
2. To determine and compare the expression patterns of ki-67, p53 and p16 in the cells of oral the cavity, amongst commercial bus drivers using tobacco and alcohol and control group.
3. To associate the expression pattern of ki-67, p53 and p16 with presence and degrees of various forms of changes in nuclei of cells of the oral cavity amongst tobacco and alcohol users.
4. To Assess and compare the presence and degrees of various forms of oral nuclear changes and gene expression patterns amongst different age groups.
5. To determine and compare time dependence of the presence and degrees of various forms of oral nuclear changes in cells of the oral cavity and gene expression patterns.

1. 6 Significance of the study

Oral cancer is the most common (90%) malignancy of the oral and maxillofacial region (Johnson *et al*., 2011). It is the 4th most common cancer in males and 6th in females (Farhadi *et al*., 2016a), accounting for 2% of all cancers diagnosed in the United States of America (USA). Tobacco is considered the greatest disease producing chemicals to humans (Biswas *et al*., 2104). Tobacco use of any sort is associated with 75% cases of oral carcinoma

(Farhadi *et al*., 2016a). The association of tobacco and alcohol use with oral cancer has been established by many authors (Proia *et al*., 2006; Mohan, 2010; Biswas *et al*., 2104; Farhadi *et al*., 2016a). Besides tobacco and alcohol use, HPV infection has also been established as a major risk factor of oral cancer. Lifestyle factors such as of tobacco and alcohol uses and sexual promiscuity, which are common amongst commercial bus drivers, account for 80% risk factors of all cancers. The significance of bio-monitoring of nuclear abnormalities in smokers using exfoliated cytology of oral mucosa cells has been reported by Farhadi *et al*., (2016b). The role of exfoliated cytology of oral mucosa cells in screening for micro nuclei, genotoxic nuclear damage and other cellular abnormalities has been established by many authors (Kausar *et al*., 2009; Biswas *et al*., 2104; Farhadi *et al*., 2016a). This study therefore, becomes imperative to unravel the pattern and extent of nuclear changes amongst this high risk population and to determine the expression pattern of some biomarkers of oral pre malignant lesions.

1.7 Research Questions

1. What is the prevalence and degree of oral epithelial nuclear changes in commercial drivers who actively use tobacco and alcohol compared to control subjects?

2. What is the expression pattern of Ki-67, p53 and p16 in the oral cavity cells of commercial drivers who use tobacco and alcohol compared to control subjects?

3. Is there co-expression of these genes among commercial bus drivers who use tobacco and alcohol and also control subjects?

4. What is the link between ki-67, p53 and p16 expression and tobacco and alcohol consumption?

1.8 Research Hypotheses

1. There will be a significant difference in the presence and degrees of various forms of oral epithelial nuclear changes amongst the study population when compared with normal control subjects.

   There will be a no significant difference in the presence and degrees of various forms of oral epithelial nuclear changes amongst the study population when compared with normal control subjects.

2. There will be a significant difference in the presence and degrees of various forms of oral epithelial nuclear changes between cigarette smokers/alcohol drinkers and control group.

   There will be a no significant difference in the presence and degrees of various forms of oral epithelial nuclear changes between cigarette smokers/alcohol drinkers and control group.

3. There will be a significant difference in the presence and degrees of various forms of oral epithelial nuclear changes amongst cigarette smokers/alcohol drinkers, snuff users/alcohol drinkers and control group.

   There will be a no significant difference in the presence and degrees of various forms of oral epithelial nuclear changes amongst cigarette smokers/alcohol drinkers, snuff users/alcohol drinkers and control group.

4. There will be a positive association between oral epithelial nuclear changes and ki-67, p53 and p16 expression patterns.

There will be no association between oral epithelial nuclear changes and ki-67, p53 and p16 expression patterns.

CHAPTER TWO

LITERATURE REVIEW

2.1 Histology of the Oral cavity

The oral cavity is the point of entry for digestive and respiratory tracts (Mohan, 2010). The mucous membrane of the mouth consists of squamous epithelium covering vascularised connective tissue. The epithelium is keratinized over the hard palate, lips and gingiva, while elsewhere it is non-keratinised. Mucous glands (minor salivary glands) are scattered throughout the oral mucosa. The lip is lined by thin skin covered by stratified squamous keratinized epithelium. Blood vessels are close to the surface which impart red colour of the lip. Sebaceous glands, sweat glands, and mucus secreting labial glands are present in the region of the lips, with the core containing skeletal muscle called orbicularis oris. The tongue arises from the ventral ends of the branchial arches. An ectoderm-covered oral part from the mandibular arches forms the body of the tongue: an endodermal pharyngeal part from the second branchial arch gives rise to the rest of the tongue. The junction between ectoderm and endoderm is just anterior to the sulcus terminalis. The tongue is made up of interlacing skeleton muscle fibers with the surface covered by surface elevations, known filiform, fungiform, and circumvallate papillae. Filiform papillae constitute the most numerous and smallest covering of the tongue but lack taste buds. The mushroom shaped fungiform papillae on the other hand are less numerous but larger and contains taste buds. Circumvallate papillae are the largest. They occupy the back of tongue, and have furrows, underlying serous glands, and taste buds.

Foliate papillae are rudimentary in humans. Posterior lingual glands in the connective tissue open ontodorsal surface of tongue (Eroschenko, 2008).

The taste buds are located within the foliate, fungiform, circumvallate papillae; pharynx, palate, and epiglottis.They contain taste pores and occupy the thickness of the epithelium with neuroepithelial cells associated with afferent axons being the receptors for taste. Substances that are tasted are first dissolved in saliva and then enter taste pore. Serous glands wash peripheral taste buds in the furrows of circumvallate papillae. The tip of tongue is sensitive to sweet and sour; posterior tongue is sensitive to bitter, and lateral to sour taste. Lymphoid tissue is present in the form of tonsils and adenoids. Palatine and lingual tonsils are covered by stratified squamous epithelium and show crypts whereas pharyngeal tonsil is single and covered by pseudostratified ciliated epithelium. Some lymph nodules contain germinal centers. The major and minor salivary glands are considered to arise from ectoderm. All begin from the epithelial surface as buds that grow and branch, treelike, to form a system of solid ducts. Terminal twigs, which eventually form intercalated ducts, round out at their ends to form secretory tubules and acini. This is followed by canalization of the ducts. The sublingual gland differs slightly in that several buds form from the oral epithelium and each remains a discrete gland. The parotid appears first, followed by the submandibular, sublingual, and, later still, minor salivary glands. Mesenchyme surrounding the epithelial primordia provides the capsule and septae of the salivary glands (Eroschenko, 2008).

The teeth have a dual origin; enamel being ectodermal while dentin, pulp, and cementum are mesodermal. Tooth development begins with the appearance of the dental lamina, a plate of epithelium on which knoblike swellings (enamel organs) appear at intervals.

These give rise to enamel and also act as molds for tooth development. The enamel organs grow into the mesenchyme to form inverted, cuplike structures invaginated at the bases by dentalpapillae. The latter are condensations of mesenchyme that give rise to the dentin and pulp of teeth (Eroschenko, 2008).

2.2 Pathology of Oral cavity

The oral cavity is the site of numerous congenital and acquired diseases. Besides, many systemic diseases have oral manifestations (Mohan, 2010). The mouth is a mirror of health or disease and sentinel or early warning system (Gupta and Jawanda, 2015). According to the authors, the oral cavity might well be thought as a window to the body because oral manifestations accompany many systemic diseases, oral involvement preceding the appearance of other symptoms or lesions at other locations in many instances (Mehrotra *et al*., 2010). The pathology of oral cavity will be discussed under the following headings: developmental defects, mucocutaneous lesions, inflammatory diseases, pigmentary lesions and tumours and tumour-like lesions.

2.2.1 Developmental defects

These are group of congenital disorders arising from under development of certain organs during embryogenesis. Mohan, (2010) classified them as follows:

i. Facial cleft: Facial clefts (FCs) are common congenital malformations of the lip, palate, or both caused by complex genetic and environmental factors (Agbenorku, 2013). It may involve the lip, the roof of the mouth (hard palate), or the soft tissue in the back of the mouth (soft palate) as well as structures around the oral cavity which can extend onto the facial structures resulting in oral, facial, and craniofacial deformity (Mossey and Little, 2009).

The prevalence of FCs varies from 1/1500 to 1/2500 births depending on the geographic origin, racial and ethnic backgrounds, and socioeconomic status (Agbenorku, 2013). This condition may result from the inability of the facial processes to fuse. The first branchial arch fails to fuse with the frontal process giving rise to cleft of upper lip and/or cleft palate. It is the most common facial abnormalities (Mohan, 2010). The etiology of FCs is obscure and complex, involving multiple genetic and environmental factors (Agbenorku, 2013; Kesande *et al*., 2014). The authors in separate studies reported that oral clefts frequently occur in combination with a wide range of chromosomal abnormalities and syndromes (trisomy 13, amniotic band anomalad, Fryns syndrome, Meckel syndrome, Stickler syndrome, Treacher Collins syndrome, van der Woude syndrome and Velocardiofacial syndrome. The impact of environmental factors such as medication during pregnancy, maternal alcohol consumption and smoking, dietary and vitamin deficiencies, diabetes, environmental toxins, altitude, birth order, socioeconomic

status, and parental age have also been reported (Vieira *et al*., 2002; Little *et al*., 2004; Yang *et al*., 2008; Agbenorku, 2013).

ii. Fordyce's granules: These are symmetric, small, light yellow macular spots on the lips and buccal mucosa, representing collections of sebaceous glands which remain undeveloped until puberty but occur quite commonly in adults (Mohan, 2010). Scully, (2013) defined it as visible sebaceous glands.

iii. Leukoedema: This is an asymptomatic condition occurring in children and is characterised by symmetric, grey white areas on the buccal mucosa which partially disappears when the mucosa is stretched. It occurs in 90% of Blacks and 10–90% in Whites. This variation may be due to the difficulty in observation of leukoedema in non-pigmented mucosa. Leukoedema is accentuated in smokers.

iv. Histologically**,** there is pronounced intracellular oedema with no increased malignant potential (Mohan, 2010). Intracellular edema of the superficial epithelial cells coupled with retention of superficial parakeratin is thought to account for the white appearance.

v. Developmental defects of the tongue: Mohan, (2010) described the following classes of tongue defects:

a) Macroglossia is the enlargement of the tongue, usually due to lymphangioma or haemangioma, and sometimes due to amyloid tumour.

b) Microglossia and aglossia are rare congenital anomalies representing small-sized and absence of tongue respectively.

c) Fissured tongue (scrotal, furrowed or grooved tongue) is a genetically-determined condition characterized by numerous small furrows or grooves on the dorsum of the tongue. It is often associated with mild glossitis.

d) Bifid tongue is a rare condition occurring due to failure of the two lateral halves of the tongue to fuse in the midline.

e) Tongue tie occurs when the lingual fraenum is quite short, or when the fraenum is attached near the tongue tip (Chaubal and Dixit, 2011).

f) Hairy tongue is not a true developmental defect, but is mentioned here because of its similarity with other conditions discussed. The filiform papillae are hypertrophied and elongated. These 'hairs' (papillae) are stained black, brown or yellowish-white by food, tobacco, oxidising agents or by oral flora.

2.3 Mucocutaneous lesions

Most of the oral mucosa is derived embryologically from an invagination of the ectoderm and perhaps not surprisingly, this, like other similar orifices, may become involved in disorders that are primarily associated with the skin (Gupta and Jawanda, 2015). Common mucocutaneous lesions include lichen planus and vesicular lesions. .

Lichen planus (LP) is a chronic mucocutaneous disorder of the stratified squamous epithelium that affects oral and genital mucous membranes, skin, nails, and scalp (Gupta and Jawanda, 2015). The oral mucosal presentation of cutaneous LP is designated as oral lichen planus (OLP) (Lavanya *et al*., 2011). OLP may appear in several forms, ranging from reticular to erosive and some forms appearing as plaques. The reticular form is characterized

by lacy, white lines, while the erosive form, besides these classical features has areas of erosion and ulceration (Lavanya *et al*., 2011). The authors further reported that the plaque pattern, the lacy pattern, is lost with lesion appearing as white macules. The etiology of OLP is unknown; however, it is largely believed to be immune mediated (Mayank *et al*., 2015). A number of vesicular diseases of the skin have oral lesions (Gonsalves *et al*., 2007; Mohan, 2010). The commonly identified lesions, according to the authors include; Pemphigus vulgaris, Pemphigoid, Erythema multiforme, Stevens-Johnson syndrome and Epidermolysis bullosa.

2.4 Inflammatory Diseases

These are group of diseases, occurring in the course of several different diseases, involving inflammation of the mucus membrane of the buccal cavity. Stomatitis as they are generally designated may be seen in the following different diseases;

2.4.1 Aphthous ulcers (Canker sores)

This is one of the most common oral diseases (Field and Allan, 2003; Mohan 2010). According to the authors, the exact incidence is unknown, but estimates range from 20% to 60% of the population. Lesions appear as single or multiple painful ulcers, ranging in size from less than 1 mm to 2 centimeters. Aphthous ulcers are classified into minor and major aphthous ulcers (Field and Allan, 2003). Small lesions (<0.5 cm) are referred to as minor aphthous ulcers while large lesions (>0.5 cm) are called major aphthous ulcers. An uncommon presentation of this disease, termed herpetiform pattern, appears as multiple, pinpoint areas of ulceration that seldom exceed 1 mm (Porter and Leao, 2005).

Each lesion begins as a red macule, less often a papule but not as a blister. It soon ulcerates and the ulcer becomes covered by a pyogenic membrane producing the characteristic yellow-white center with surrounding erythematous flare. The shape is usually round to oval but may be elongated in natural folds such as the vestibule. Aphthous stomatitis occurs on freely movable mucosa that does not overlie bone. The lips, cheeks, soft palate, floor of mouth, ventral and lateral tongue are often involved but attached gingival, hard palate and dorsal tongue are seldom affected. Aphthous lesions affect all age groups from young to old but young adults and females are more affected (Porter and Leao, 2005). Elapsed time between recurrences is extremely variable; some unfortunate patients have almost continuous disease whereas others go from months to years between episodes.

The etiology of aphthous ulcers is unknown (Mohan, 2010). The concept that canker sores are caused by a microbiologic agent has been superseded by theories revolving around an immunopathogenesis. The deposition of antibodies and complement within epithelium and basement membrane during the early stages of the disease suggests a humoral immune response, and the influx of lymphocytes rather than neutrophils in early lesions points to a cellular immune reaction as well (Mayank *et al*., 2015). However, it is yet to be learned if the immune response is directed against self (autoimmunity) or against an extrinsic antigen such as bacteria or viruses. To further cloud the issue, a variety of other factors have been implicated. Withdrawal of certain foods such as cheese, tomato products and gluten, as well as sodium lauryl sulfate-containing toothpastes, has been claimed to help some patients whereas in others, correction of iron, B12 and folate deficiencies have brought about a cure. Improvement of aphthous lesions during the last stages of pregnancy with exacerbation after

delivery suggests that gonadal hormones may play a role. The occurrence of canker sores during menstruation also suggests a hormonal basis. To add a final element of mystery, aphthous stomatitis has been reported to worsen when cigarette smoking is discontinued. There are too many theories for them all to be correct. Aphthous stomatitis may not be a single disease with a single cause but instead a variety of diseases all manifested by painful mouth sores. The role of emotional factors such as stress, allergy, hormonal imbalance, nutritional deficiencies, gastrointestinal disturbances, and trauma in etiology has also been reported by (Mohan, 2010).

2.4.2 Herpetic stomatitis

This is an acute disease mostly seen in infants and young children, with a most common manifestation of primary infection with herpes simplex virus (Mohan, 2010). Oral herpetic stomatitis occurs in three clinical forms.

The most common type consists of recurrent small blisters on the lips commonly referred to as fever blisters or secondary herpes labialis. The second type is a generalized oral infection called primary herpetic stomatitis while the third and least common form of oral herpes infection consist of small ulcers usually localized on palatal mucosa (Field and Allan, 2003). The lesions are in form of vesicles around the lips. Similar lesions may be seen on the genital skin. The etiologic agent of herpetic stomatitis is herpes simplex virus mostly type I.

2.4.3 Necrotising stomatitis (Noma or Cancrum oris)

This condition occurs more commonly in poorly-nourished children like in kwashiorkor; infectious diseases such as measles, immunodeficiencies and emotional stress. The lesions are characterised by necrosis of the marginal gingiva and may extend on to oral mucosa,

causing cellulitis of the tissue of the cheek. The condition may progress to gangrene of the cheek (Mohan, 2010).

2.5 Mycotic infections

Actinomycosis and candidiasis are two most common mycotic infections of the oral cavity (Mohan, 2010). Infection with *Candida albicans* accounts for about 80% of all cases of oral candidiasis (Raju and Rajappa, 2011). These are commensals which are found in almost all human organs and tissues with the skin, mouth and genitalia, being the most common sites.

When there is disturbance in the flora/host balance and under debilitating conditions of the host, they become pathogenic (Raju and Rajappa, 2011). Oral candidiasis, also known as oral moniliasis or thrush varies in severity ranging from localized to generalized form of stomatitis. It is characterized by ulceration of mucous membrane of the mouth with a characteristic white slough consisting necrotic mucosa and organisms (Field and Allan, 2003). There is the presence of speckled white patches over the red lesions, due to uneven distribution of the lesions. Candida may also present as red lesions having been referred to as erythematous candidiasis. This is especially common under dentures. *Cervicofacial actinomycosis* is the commonest form of the disease developing at the angle of the mandible (Mohan, 2010). Other forms of mycotic infections with oral manifestation include; glossitis, tuberculous lesions, syphilitic lesions and infection with human immunodeficiency virus (HIV).

2.6 Pigmentary lesions

Oral and labial melanotic pigmentation may be observed in certain systemic and metabolic disorders such as Addison's disease, Albright syndrome, Peutz-Jeghers syndrome and

haemochromatosis (Mohan, 2010). The author further reported that all types of pigmented naevi as well as malignant melanoma can occur in oral cavity. Exogenous pigmentation such as due to deposition of lead sulfide can also occur. Oral malignant melanoma has been reported by Ali *et al*., (2016).

2.7 Tumours and Tumour-like lesions

A good number of benign, premalignant, and malignant as well some tumour-like lesions are associated with the oral soft tissues.

2.7.1 Benign Tumours

The different parts of the mouth have mesodermal tissues with keratinizing and non keratinizing epithelia cells (Mohan, 2010). In view of this fact, the oral tissues, like other body tissues are prone to a variety of benign neoplasm. The common benign oral tumours include:

i. Sqaumous Papilloma

The squamous papillomas are benign exophytic masses of the oral cavity usually appearing as pedunculated or sessile, white or normal colored cauliflower-like projections that arise from the mucosal surface (Naveena *et al*., 2012). They involve the proliferation of the stratified epithelium andoccur most frequently on the hard and soft palate, uvula, tonsil, or epiglottis, but any surface of the oral cavity can be affected (Hilal *et al*., 2015). The soft plate is the most common site, accounting for 20% of all lesions (Naveena *et al*., 2012). Oral squamous papillomas are usually asymptomatic and progress slowly but could mimic

condyloma acuminatum, verrucous carcinoma, or exophytic carcinoma because their clinical appearance is similar (Carneiro *et al*., 2009; Feller and Lemmer; 2012; Hilal *et al*., 2015). The authors, however, reported that the tumour is amenable to surgical removal with laser ablation, electrocautery, intralesional injections of interferon, cold-steel excision, and cryosurgery.

According to Prashant *et al*., (2014) squamous papillomas are traditionally divided into two types: Isolated-solitary, usually found in an adult's oral cavity and multiple-recurring.

The former is usually found in an adult's oral cavity, while the latter is mostly found in a child's laryngotracheobronchial complex. The pathogenesis of squamous papillomas is related to infection with HPV types 6 and 11, the occurrence being influenced by tobacco smoking, co-existent infections, dietary deficiencies and hormonal changes (Prashant *et al*., 2014; Hilal *et al*., 2015). The authors further reported that the route of transmission of HPV is yet to be determined. These lesions commonly occur between the ages of 30 and 50 years, and sometimes can occur before the age of 10 years (Prashant *et al*., 2014). Frigerio *et al*., (2015), however, reported a slight male predilection of the tumour which according to them, occurred mostly in the sixth decade of life.

ii. Haemangioma

Haemangioma is an abnormal proliferation of blood vessels that may occur in any vascularized tissue (Gangavati *et al*., 2015). It is a term that encompasses a heterogeneous group of clinical benignmvascular lesions that have similar histologic features (Alparslan *et al*., 2009).They may be identified by rapid endothelial cell proliferation in early infancy, followed by involution over time. Kamala et *al*.,(2014) on the other hand defined

haemangioma as a developmental vascular abnormalities characterized by a proliferative growth phase and by very slow inevitable regression (involutive phase). Considerable debate existed as to whether these lesions are neoplasms, hamartomas or vascularmalformations (Gangavati *et al*., 2015). Recent studies, however, classified them as neoplasms.Hemangiomas are considered as benign tumors, being characterized by 3 stages: Endothelial cell proliferation, rapid growth and at last spontaneous involution (Kamala et *al*., 2014). Though hemangioma is considered one of the most common soft tissue tumors of the head and neck (Alparslan *et al*., 2009) it is rarely reported in the oral cavity. They may be cutaneous, involving skin, lips and deeper structures; mucosal, involving the lining of the oral cavity; intramuscular, involving masticator and perioral muscles; or intra-osseous, involving mandible and/or maxilla (Alparslan *et al*., 2009). The lip is the most common site. According to the authors haemangioma may be classified either as capillary or cavernous based on histological appearance.

Richter and Friedman (2012) further classified haemagioma as either infantile or congenital. Other sub-classifications were also reported by the authors. Infantile hemangiomas are the most common tumor in infancy and occur in approximately 10% of the population (Richter and Friedman 2012). They also reported that female sex, prematurity, low birth weight, and fair skin as common identifiable risk factors. The pathophysiology of hemangiomas is attributed to genetic and cellular factors, mainly to monocytes, which are considered the potential ancestors of hemangioma endothelial cells (Kamala et *al*., 2014). According to these authors, imbalance in the angiogenesis, results in an uncontrolled proliferation of vascular elements. This in association with substances such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (BFGF) and indole-amine 2,3-dioxygenase

(IDO), which are over expressed during proliferative stages, are believed to be the cause of haemangiomas.

iii. Lymphangioma

Lymphangioma is a benign hamartomatous tumor of lymphatic vessels with a marked predilection for the head and the neck region (Stănescu *et al*., 2006). It was first described by Redenbacher in 1828 (Pammer *et al*., 2015) and is currently classified as malformations and not as neoplasms (Stănescu *et al*., 2006). The authors further reported that lymphangiomas represent about 6% of the total number of benign tumours of the smooth tissue in patients aged less than 20 years. They arise from sequestrations of lymphatic tissue and have marked predilection for head and neck region (Pammer *et al*., 2015). This tumour is relatively uncommon in the oral cavity (Usha *et al*., 2014) but common on the tongue with a predilection for anterior two-third of the tongue (Pammer *et al*., 2015) where they appear as solitary or circumscribed lesions. The occurrence of lesion in other sites, such as palate, buccal mucosa, gingiva, and lips has been reported (Usha *et al*., 2014; Pammer *et al*., 2015). They are usually found at birth with about 90% of cases developing within the 2nd year of life (Singh and Yadav, 2010). Occasionally, they are associated with cystic hygroma (Stănescu *et al*., 2006), a special variety of lymphangioma that occur on the lateral side of neck of children (Mohan, 2010).

iv. Fibroma

Fibroma is a benign neoplasm of fibroblastic origin, rare in the oral cavity (Shankargouda *et al*., 2014). It is the most common oral mucous membrane mass, appearing as discrete superficial pedunculated mass (Mohan, 2010). Shankargouda *et al*., (2014) reported that the majority of the fibromas occurring in the oral cavity are reactive in nature and represent a

reactive hyperplasia of fibrous connective tissue in response to local irritation or trauma rather than being a true neoplasm.

v. Fibromatosis gingiva

Fibromatosis gingiva on the other hand is a fibrous over growth of unknown etiology involving the entire gingival, the fibrous overgrowth sometimes being so much that the teeth are covered by fibrous tissue (Mohan, 2010). Gingival fibromatosis (GF) is a rare, benign, slowly-growing fibrous overgrowth of the gingiva, with great genetic and clinical heterogeneity which can be inherited as an isolated trait (hereditary gingival fibromatosis) and/or as a component of a syndrome, or can be drug induced (Poulopoulos *et al*., 2011). Further reported by the authors is that the clinical manifestation of this disorder is usually associated with generalized hypertrichosis, mental retardation, or epilepsy and its related syndromes are mainly inherited in an autosomal-dominant manner, but autosomal-recessive inheritance has also been reported. In severe cases functional, periodontal, esthetic and psychological problems may occur (Gawron *et al*., 2016).

The authors further reported that it may also develop in susceptible individuals as a side effect of systemic medications, including the anti-seizure, immunosuppressant, or calcium channel blockers; however, in some cases the etiology of the enlargement remains unknown.

vi. Tumours of minor salivary gland

Minor salivary gland tumors are frequently encountered on the palate, followed by the lip, cheek, tongue and floor of the mouth (Jain *et al*., 2015). The most common example of this group of disorders is pleomorphic adenomas (PA) (Mohan, 2010). This is an asymptomatic, slow progressing, parotid gland swelling tumour which usually does not involve facial nerve

involution (Jain *et al*., 2015). Reddy *et al* (2015) described PA as a solitary slow growing painless rubbery mass. It is the most common salivary gland tumours accounting for two third (56-65%) of all salivary gland neoplasms (Jain *et al*., 2015), and 80% of all salivary gland benign neoplasms (Reddy *et al*., 2015). The authors further reported that the incidence of PA in intraoral minor salivary glands is 40-50%, parotid glands (85%), minor salivary glands (10%), and the submandibular glands (5%).

vii. Granular cell myoblastoma

Granular cell lesions of the oral mucosa, jaws, and salivary glands constitute a heterogeneous group of lesions which may be either odontogenic, salivary gland, or metastatic in origin (Yogesh and Sowmya, 2011). Granular cell tumour (GCT) was first reported in 1926 by Abrikossoff and for a long timeconsidered to be of myoblastic origin hence the term ‘granular cell myoblastoma’ (Loo *et al*., 2015).

According to the authors, the lesion is believed to arise from Schwann cells, does rarely metastasize and the majoritybeing primarily localized on the lower extremities. In an earlier report by Alka *et al*., (2013) GCT is characterized by the accumulation of plump cells with abundant granular cytoplasm. According to the authors, a wide variety of cell types have been proposed as cell of origin involved. These include; histiocytes, fibroblasts, myoblasts, neural sheath cells, neuroendocrine cells, and undifferentiated mesenchymal cells.GCT has been reported in a variety of organs, but the tongue is the most commonly affected (Yogesh and Sowmya, 2011; Alka *et al*., 2013; Gopinath *et al*., 2016; Loo *et al*., 2015). The head and neck accounts for 45-65% of all GCT cases while 70% of lesions are located intraorally (Alka *et al*., 2013). The authors in their independent studies also reported a slight female

predilection, usually between the 4th and 6th decade of life. It occurs very rarely in children; however, similar lesion seen in infants is termed as congenital epulis (Mohan, 2010).Both benign and malignant lesions have been reported; although malignancy occurrence is rare, comprising of 2% of all GCT cases (Alka *et al*., 2013). The tumour is composed of large polyhedral cells with granular, acidophilic cytoplasm; the covering epithelium usually shows pronounced pseudoepitheliomatous hyperplasia (Mohan, 2010).

Neurilemmoma, neurofibroma, lipoma, giant cell granuloma, rhabdomyoma, leiomyoma, solitary plasmacytoma, osteoma,chondroma, naevi and vascular oral lesions seen in hereditary haemorrhagic telangiectasia (Osler-Rendu-Weber syndrome) and encephalofacial angiomatosis (Sturge-Weber syndrome) are examples of oral soft tissue benign tumours which are classified as rare benign tumours of the oral cavity (Mohan, 2010).

2.8 Pre-malignant tumours

World Health Organization (WHO) defines premalignant tumours as lesions with a predisposition to malignant transformation (Yardimci *et al*., 2014). Commonly encountered oral precancerous lesions are oral leukoplakia, oral submucous fibrosis (OSMF), and oral erythroplakia. Actinic cheilitis, some miscellaneous inherited diseases such as xeroderma pigmentosum and Fanconi's anemia, and immunodeficiency are less commonly encountered potentially malignant disorders for oral carcinoma (Van derWaal, 2009). Erythroplakia, even though, less frequent its risk of malignant progression is the highest among the oral potentially malignant disorders (Villa *et al*., 2011). The need for early detection of oral

premalignant lesions vis-à-vis prevention and management of oral cancers has been advocated (Epstein *et al*., 2008; Villa *et al*., 2011; Yardimci *et al*., 2014).

2.8.1 Oral leukoplakia (white lesions)

Leukoplakia (white plaque) may be clinically defined as a white patch or plaque on the oral mucosa, exceeding 5 mm in diameter, which cannot be rubbed off nor can be classified into any other diagnosable disease (Mohan, 2010). According to Yardimci *et al*., (2014), Leukoplakia is defined as a white plaque of questionable risk having excluded known diseases or disorders that carry no increased risk for cancer. From the pathologists' point of view, leukoplakia' is reserved for epithelial thickening which may range from completely benign to atypical and to premalignant cellular changes (Mohan, 2010).These potentially premalignant lesions vary in size, shape, and consistency, and macroscopically said to be homogenous and nodular (Carnelio *et al*., 2011). It can affect any part of the oral and oropharyngeal cavity, however, the sites of predilection, in descending order of frequency, are: cheek mucosa, angles of mouth, alveolar mucosa, tongue, lip, hard and soft palate, and floor of the mouth (Mohan, 2010). Oral leukoplakia is more common in individuals below the 4^{th} decade of life, occurring more in males than in females (Mohan, 2010; Yardimci *et al*., (2014). An earlier study by Bánóczy, (2001) reported an incidence rate of 1.1-11.7%, however, a recent report revealed that about 4-6% cases of leukoplakia, carcinomatous change (Mohan, 2010; Carnelio *et al*., 2011). The authors noted the difficulty in telling which lesion undergoes malignant transformation and in view of this advocated for investigation of all cases of white lesions. The risk factors of oral leukoplakia are similar to those of oral squamous cell carcinoma with lesion having strong association with tobacco use, alcohol consumption and HPV infection. Yardimci *et al*., (2014), in a study observed

that leukoplakia is seen 6 times more in smokers than in non smokers. According to Van der Waal *et al*., (2014), the etiologic role of alcohol may be synergistic.

2.8.2 Oral erythroplakia (Red lesions)

Erythroplakia is defined as "A fiery red patch that cannotbe characterized clinically or pathologically as any other definable disease (Yardimci *et al*., 2014). The term describes a red plaque or macular lesion in the mouth for which a specific clinical diagnosis cannot be established (Hosni *et al*., 2009). Erythroplakia was further defined as erythematous patch of the oral mucosa and is associated with significantly higher rates of dysplasia (Carnelio *et al*., 2011). The original term, erythroplasia, was used to describe a precancerous red colour that develops on the penis (Villa *et al*., 2011). Yardimci *et al*., (2014) described the clinical appearance as flat or even depressed erythematous change of the mucosawithout a patch lesion. Both red and white changesin the same lesion have been reported (Villa *et al*., 2011; Carnelio *et al*., 2011; Yardimci *et al*., 2014). It was referred to as erythroleukoplakia.

However, WHO currently recommends the term, speckled leukoplakia for such lesions (Hosni *et al*., 2009). Prevalence of erythroplakia varies between 0.02% and 0.83%, occurring mainly at the middle age with a predilection for male gender (Villa *et al*., 2011; Yardimci *et al*., 2014). Mostly, commonly affected areas as reported by most authors are the soft palate, the floor of the mouth, and the buccal mucosa. Erythroplakia, though the least common oral pre malignant tumour, has the highest rate of transformation to cancer (Villa *et al*., 2011. In a study carried out between 1971and 2007, Villa *et al*., (2011) reported that out of 258 oral potentially malignant lesions were identified, 62 lesions developed oral cancer with a malignant transformation rate of44.9%. The malignant transformation rate according

to Yardimci *et al*., (2014) varies between 14 to 50%. Etiopathogenesis is of erythroplakia still remains obscure; however], chewing tobacco and alcohol use are the possible strong etiologic factors (Yardimci *et al*., 2014). Morphologically, typical lesion of oral erythroplakia is lessthan 1.5 cm in diameter, but lesios less than 1 cm and greater than 4 cm have been reported (Reichart and Philipsen, 2005). Microscopically, moderate or severe dysplasia was usually seen in lesion with erythroplakia.

2.9 Malignant Tumours

Mmalignant tumours of the oral cavity are oral squamous cell carcinoma (OSCC), malignant melanoma, lymphoepithelial carcinoma, malignant lymphoma, malignant tumours of minor salivary glands, and various sarcomas like rhabdomyosarcoma, liposarcoma, alveolar soft part sarcoma, Kaposi's sarcoma and fibrosarcoma. Metastatic tumours can also occur in the soft tissues of the mouth (Mohan, 2010). Oral squamous cell carcinoma is the most prevalent malignant tumour of the head and neck and is discussed in detail here.

2.9.1 Oral Sqamous cell Carcinoma (OSCC)

This is the malignant proliferation of the squamous cells of the buccal cavity. OSCC has very poor prognosis, due to inability to detect and treat lesions when small. It is 6^{th} most common cancers in the world, accounting for 90% of oral cancers (Massano *et al*., 2006; Feller and Lemmer, 2012; Feller.*et al*., 2013; Pires *et al*., 2013) and 2-4% of all cancers (Markopoulos, 2012). Pires *et al*., (2013) in a study of the clinicopathologicalfeatures from 346 cases from a single Oral Pathology service during an 8-year period in Brazil reported that oral cavity ranges from the 6th to the 9th most common anatomical location for cancers.

This, however, varies for different countries, different regions within a country and even between gender. The prevalence is as high as 10% in Pakistan, and around 45% in India (Markopoulos, 2012) According to the author, over 300,000 new cases of oral and oropharyngeal cancers were diagnosed worldwide between 2004 and 2009; during which period, over 7,000 affected individuals died of these cancers.

The peak incidence of oral squamous cell carcinoma varies for different countries and races. This may be due to life style habits, exposure to risk factors and screening and preventive measures put in place. The peak incidence in the UK and the USA is from 55 to 75 years of age, 40 to 45 years in India, where as in Nigeria it is 21 to 30 year of age (Mohan, 2010; Kodiya *et al*., 2015). The very low peak incidence age in Nigeria corresponds to a world Health Organization report that the incidence of oral SCC in persons under the age of 45 is increasing (Warnakulasuriya, 2009). Oral cancer is a very frequent malignancy in India, Sri Lanka and some Eastern countries, probably related to habits of betel-nut chewing and reversed smoking (Mohan, 2010; Markopoulos, 2012; Kodiya *et al*., 2105).

Oral SCC can occur anywhere within the buccal cavity. Common sites in decreasing order of incidence are the lips (more commonly lower), tongue, anterior floor of mouth, buccal mucosa in the region of alveolar lingual sulcus, and palate (Mohan, 2010). In a similar report by Feller and Lemmer, (2012) oral SCC affects the tongue in 20% - 40% of cases and the floor of the mouth in 15% - 20% of the cases in Western countries with these sites accounting for about 50% of all cases of oral SCC. The authors further reported that the gingivae, palate, retromolar area and the buccal and labial mucosa are oral sites less frequently affected while the ventral surface of the tongue and the floor of the mouth are the

sites most commonly affected. This according to the author may be due to thin non-keratinized epithelium which lines these sites. Carcinogens not readily penetrate this thin epithelium to reach the progenitor cell compartment, but-carcinogens, particularly tobacco products and alcohol in solution, constantly accumulate in the floor of the mouth and bathe the tissues of the floor of the mouth and the ventrum of tongue (Naville and Day, 2002; Feller and Lemmer, 2012).

a. Etiology of Oral SCC

The etiology of oral squamous cell carcinoma, like most malignant tumours is unknown, however, certain risk factors are associated with carcinogenesis. Tobacco use, alcohol consumption and HPV infection are considered the major risk factors (Mohan, 2010; Markopoulos, 2012; Pires *et al*., 2013; Chi *et al*., 2015). Chronic irritation from ill-fitting denture or jagged teeth, sub mucosal fibrosis as seen in Indians consuming excess of chillies, poor orodental hygiene, nutritional deficiencies, exposure to sunlight, exposure to radiation, plummer-Vinson syndrome (characterized by atrophy of the upper alimentary tract), Epstein-Barr Virus and Hepatitis C Virus infections are among the risk factors, though with weak association with oral SCC (Mohan, 2010; Markopoulos, 2012).

b. Major Risk Factors

i. Tobacco

Tobacco consumption continues to be a major risk factor for Oral-SCC and Pharyngeal – SCC (Laawal *et al*., 2013; Khilif *et al*., 2013; Saranya and Sudha, 2014; Chi *et al*., 2015). According to Chi *et al*., (2015), the International Agency for Research on Cancer classifies tobacco smoking group 1 carcinogen for both the oral cavity and the pharynx and classifies

smokeless tobacco as a group 1 carcinogen for the oral cavity. This was based on sufficient evidence of carcinogenicity tobacco in humans. WHO (2013) in a report on global tobacco epidemic revealed a decline in the use of tobacco in many high-income countries and an increase of its use in many low-income and middle-income countries, where nearly 80% of the world's one billion smokers currently reside. A meta-analysis by Gandini *et al* noted a relative risk of 6.76% for Pharyngeal -SSC and 3.43% for Oral-SCCamong current tobacco smokers compared with nonsmokers (Chi *et al*., 2015).

A dose and time dependent association of smoking-associated risk factor has been reported by several authors ((Lawal *et al*., 2013; Khlifi *et al*., 2013; Chi *et al*., 2015; Farhadi *et al*., 2016a). The effects on cells are reversed when smoking is quitted.

Although cigarettes represent the predominant form of tobacco used worldwide, tobacco types abound and vary in popularity by region ((Lawal *et al*., 2013; Khlifi *et al*., 2013; Chi *et al*., 2015) Lawal *et al* (2013) reported that tobacco is commonly consumed in betel quid or pan consisting of tobacco mixed with chopped areca nut, slaked lime and catechu, wrapped in a leaf of piper betelvine. Besides these, Indians add spices such as cardamom, cloves and aniseed. The authors further reported that a mixture of tobacco, ash and lime in water or oil called nass or nasswar, commonly held in the mouth is commonly seen in North Africa and the Middle East. Also in their report Lawal *et al*., (2013) noted that many different forms of snuff are placed in contact with oral mucosa in northern Europe, France, the USA and parts of Africa including Sudan, Southern Egypt and Saudi Arabia. The information was based on a study by Johnson, (2011). All forms of tobacco use have been strongly linked to the development of oral cancer with cigar and pipe smokings are

associated with a greater risk of the development of oral cancer when compared with cigarette smoking (Lawal, 2013). The very high risk associated with pipe and cigar may be due to lack of filters which allows full of carcinogen that will come in contact with the oral mucosa.

The Centers for Disease Control and Prevention reported progressive exponential increase in the total annual number of these products consumed from 2008 to 2011 (Chi *et al*., 2015). It could be said from these reports that all forms of tobacco use are unsafe and could initiate carcinogenesis. However, the riskfor OC-SCC appears to be greater with dry snuff (relative risk, 4-13) compared with moist snuff and chewing tobacco (relative risk, 0.6-1.7) (Chi *et al*., 2015). The risk and severity may also be increased in subjects who combine tobacco and alcohol use. Contrary to popular opinion that the risk of developing Oral SCC is associated with tobacco use, Oji and Chukwuemeka (2007) reported no association of oral SCC with tobacco and alcohol uses in a study carried out in Enugu, south East Nigeria. The authors on the hand postulated that poverty, malnutrition, lack of education, poor oral hygiene and chronic malaria may be more important in the aetiology and severity of oral cancer than tobacco and alcohol use.

Farhadi *et al*., (2016b) reported that the carcinogenic effect of tobacco use may be related to its ability to induce genotoxicity in the oral mucosal cell of users. Carcinogenesis may be initiated by the destructive effect on the cells of the oral cavity, the heat generated during smoking (Farhadi *et al*., 2016b), or by the interaction of chemical components of tobacco with the cells. The tar in tobacco has for long been associated with development of cancers

(Johnson *et al*., 2000). According to Pautassi *et al*., (2010) cigarette smoke contains about 4700 chemical compounds, with about 70% 0f them already known as carcinogens.

These include polycyclic aromatics hydrocarbons, aromatics amines, nitrosamines, heavy metals, poisonous gases and pesticide residues to mention but a few. Carcinogens in tobacco (mainly polycyclic aromatic hydrocarbons), can cause an accumulation of genetic mutations in oral epithelial cells including p53 mutation, mutation and loss of heterozygosity (H-RAS) and amplification (K-RAS and N-RAS) of the RAS oncogenes leading to abnormal and uncontrollable cell division and growth (Lawal *et al*., 2013).

ii. Alcohol

The role of alcohol in the etiology of most cancers has been well documented (Ogden, 2005; Lawal *et al*., 2013, ACS, 2014).Alcohol is the term for ethanol or ethyl alcohol found in alcoholic beverages such as beer, wine, and liquor, as well as in some medicines, mouthwashes, household products, and essential oils (scented liquids taken from plants). It is a product of fermentation of sugar and starch by yeast. Reports by IARC working group on the evaluation of carcinogenic risks to humans in 2010 and 2012, confirm a strong association of alcohol and cancers (IARC, 2010; IARC, 2012). These reports further revealed that the more alcohol one drinks of time the more the risk of developing cancers.

According to ACS (2014), alcohol use accounts for 3-4% of all cancer deaths annually. Alcohol drinkers who consume up to ≥50 grams of alcohol per day have 2-3 times greater risk of developing oral cancer than non drinkers (Baan *et al*., 2007). Increased risk of development of oral cancer had been reported in alcohol drinkers who also use tobacco (Ogden, 2005; Hashibe *et al*., 2009; ACS, 2014). The mechanism by which alcohol initiates

carcinogenesis is not completely understood. ASC (2014) reported that this may be initiated by the following mechanism; damage to body tissues, effects on other harmful chemicals, lower level of folate and other nutrients, effects on estrogen and other hormones, effects on body weight and generation of reactive oxygen species. Furthermore, alcohol affects cellular structure and function. It may influence the proliferative cells by both intracellular and intercellular pathways with the carcinogenic exposure of the proliferating stem cells in the basal layer being regulated through these pathways (Ogden, 2005).

Alcohol as a risk factor in development of cancer was previously thought to act indirectly and through its synergistic effect with tobacco. According to Lawal *et al*., (2013), the effect of alcohol has been thought to occur through its ability to irritate the oral mucosa and to act as a solvent for carcinogens (especially in tobacco) Furthermore, contaminants and additives with carcinogenic potentials that are found in alcoholic drinks have also been thought to have a role in oral cancer development. Acetaldehyde, a metabolite of alcohol is known to be a direct carcinogen by causing alteration of the p53 gene and Ras oncogenes (Lawal *et al*., 2013). This compound is also known to be cytotoxic and causes production of free radicals. Chronic alcohol consumption is known to interfere with repair of alkylated DNA (Ogden, 2005).

Oji and Chukwuemeka, (2007) reported low association alcohol use and risk of oral SCC.The etiology of premalignant lesions is generally accepted to be multifactorial, with tobacco and alcohol being reported as important cofactors in transition from premalignancy to malignancy (Humayun and Prasad, 2011).

iii. Human Papilloma Virus (HPV) infection

The association of HPV with head and neck tumours have been reported (Ai *et al*., 2003; Wittekindt *et al*., 2005; Kuo *et al*., 2008; Riechelmann, 2010; Wittekindt *et al*., 2012).The increasing incidence of oral squamous cell carcinoma in young people and especially in those who do not smoke or use alcohol has indicated a possible aetiologic role for infections such as HPV (Lawal *et al*., 2013). HPV16, 18 and 33 which are considered high risk types are well-established initiators of cervical and anogenital carcinogenesis (Mohan, 2010; Riechelmann, 2010; Wittekindt *et al*., 2012; Monsjou *et al*., 2012; Lawal *et al*., 2013, Melchers *et al*., 2015). The oncogenic potential of HPV is attributable to its ability to insert specific viral DNA fragments (early genes E5, E6 and E7) into the host cellular genome (Lawal *et al*., 2013), thereby abrogating the function of tumour suppressor factors. The function of p16, p21, p53 and pRb pathways are mostly affected (Monsjou *et al*., 2013; Lawal *et al*., 2013, Melchers *et al*., 2015).As a result of this integration, some key functions of tumour suppressor factors are abrogated (p21, p53 and pRb pathways, respectively), This leads to defects in apoptosis, DNA repair mechanisms, cell cycle regulation and, finally, to cellular immortalization (Ragin *et al*., 2007; Lawal *et al*., 2013, Melchers *et al*., 2015). According to Lawal *et al* (2013), the risk factors for HPV positive head and neck squamous cell carcinoma are mainly related to sexual habits rather than tobacco and alcohol use in HPV negative cancer. Hennessey *et al* (2009) in an earlier report noted that the risk increases with increasing numbers of both oral and vaginal sexual partners, a history of genital warts, and a younger age at first intercourse. Conversely, Castro and Filho (2006) noted that the route of transmission of HPV to establish oral cavity carcinogenesis is indeterminate. Previous studies have consistently shown that HPV infection conferred a

higher risk of oropharyngeal cancer when compared with oral cavity cancer (Lawal*et al*., 2013). A meta-analysis of 17 studies, as reported by Lawal.*et al*., (2013) found that HPV is most strongly associated with tonsillar cancer, intermediate for oropharyngeal cancer and weakest for oral cancer. Additionally, many studies have shown that persons with HPV-positive oropharyngeal cancers are more responsive to treatment and have better rates of disease-specific survival than those with HPV-negative oropharyngeal cancers (Castro and Filho 2006).

Preliminary reports from a study in our centre using PCR to detect HPV showed that all the oral cancer cases studied were HPV negative and HPV may not be important in the aetiogenesis of oral cancer in Nigerians (Lawal *et al*., 2013).

2.10 Carcinogenesis

Carcinogenesis is the process by which normal cells or tissues become transformed into cancer through the action of carcinogens (Rivera, 2012). Carcinogen therefore is a substance which will transform normal cells to malignant cells. Carcinogens can be essentially divided into DNA-reactive genotoxic and nongenotoxic types on the basis of their mechanisms of action (Rivera, 2012).They can cause initiated cells to develop into pre-neoplastic and neoplastic lesions by stimulating clonal proliferation (Miranda *et al*., 2011; Rivera, 2012). According to the authors, a large number of agents, which includes chemical and physical carcinogens, oncogenic viruses and other microorganisms, are capable of producing genetic damage, thereby inducing malignant transformation. Cellular alterations at molecular level occur first during this process. Rivera, (2012) in his report noted that molecular changes are not detectable by conventional histopathology. The accumulation of these molecular

changes over time, lead to massive individual cell alteration which can be detected histologically or cytologically, as well as macroscopically with significant number of altered cells. Theses cellular changes are seen as the various types of dysplasia in the oral cells.

Oral carcinogenesis is a highly complex multifocal process that takes place when squamous epithelium is affected by several genetic alterations (Tanaka and Rikako 2011). Oral carcinogenesis is a multistep process in which genetic events lead to the disruption of the normal regulatory pathways that control basic cellular functions including cell division, differentiation, and cell death (Rivera, 2012).The use of several molecular biology techniques to diagnose oral precancerous lesions and cancer may markedly improve the early detection of alterations that are invisible under the microscope, thereby identifying individuals at a high risk of developing oralcancer. Microarray technology, methylation microarrays, gene expression microarrays, array comparative genomic hybridization, proteomics, mitochondrial arrays, and micro-RNA arrays are some of the approaches to understanding the molecular basis of carcinogenesis that have been reported (Viet and Schmidt 2010;Tanaka and Rikako 2011). The authors further noted that high thorough- put techniques aimed at detecting biomarkers in body fluids such as saliva and blood are being used these days.

Oral cancer, like carcinomas in other tissues, develops over many years, during which period, multiple sites of neoplastic transformation occur throughout the oral cavity (Tanaka and Rikako 2011).The author also reported the existence of mutations of certain genes in

various sites of premalignant lesions and carcinoma in the same oral cavity . Reductions in tumor suppressor activity by the gene and the development of mutations in p53 have been associated with smoking and an increased risk for oral carcinoma development (Tanaka and Rikako 2011). Therefore, multifocal presentations and mutational expressions of tumour suppressor genes may be the consequence of long-term exposure to various environmental and exogenous factors. The continual presence of mutations may also signify changes in DNA repair and apoptosis, thereby increasing the susceptibility for future transformation (Tanaka1and Rikako 2011; Polz-Gruszka *et al*., 2014). The role of oncogenes in oral carcinogenesis has been reported by Polz-Gruszka *et al*., (2015). Oncogenes are mutated proto-oncogenes, which are known to control cell division and differentiation processes and also regulate apoptosis. According to Polz-Gruszka *et al* (2014), mutated proto-oncogenes are over-expressed, thereby stimulating cell proliferation which further leads to tumour formation and cancer development. The role of infectious agents such as Human papilloma virus (HPV), Epstein-Barr virus (EBV), Herpes Simplex virus type 1 (HSV-1 and Helicobacter pylori was also reported by the authors. Several genes such as p53, p16 and ki-67 to mention but a few have also been associated with oral carcinogenesis (Ragin *et al*., 2007; Lawal *et al*., 2013; Polz-Gruszka *et al*., 2014; Melchers *et al*., 2015).

The presence of cellular/nuclear abnormalities in the oral cells has become an indispensible biomarker of genetic damage since its introduction in 1983 (Farhadi *et al*., 2016b). According to the authors, it is believed that the number of nuclear changes is related to the increasing effects of carcinogens. Also noted was the fact that this event precedes the clinical symptoms of cancer, therefore, early detection of them may be useful in more

effective treatment and reduction of its complications. Farhadi *et al*., (2016b) also noted that despite the availability of best medical options in the treatment of oral cancers, the survival rate is markedly low. This is due to lack of early diagnostic markers, with diagnosis possible only at an advanced stage. However, the use of micronucleus assay provides a simple, yet reliable indicator of genotoxic damage. This was according report of Farhadi *et al*., (2016b) , who also emphasized that micronucleus assay is a well validated method for testing genotoxic effects of various agents; the changes in buccal mucosa of tobacco chewers and cigarette smokers had been demonstrated by micronucleus assay. A micronucleus (MN) is a small extra nucleus separated from the main one, generated during cellular division by late chromosomes or by chromosome fragments, microscopically visible round to oval cytoplasmic chromatin mass in the extra nuclear vicinity, induced in cells by numerous genotoxic agents that damage the chromosomes (Farhadi *et al*., 2016b). MN mainly originate from acentric chromosome fragments, acentric chromatid fragments or whole chromosomes that fail to be included in the daughter nuclei at the completion of telophase during mitosis because they did not attach properly with the spindle during the segregation process in anaphase (Fenech *et al*., 2011).Kausar *et al*., (2009) in an earlier study noted that the buccal mucosa is a suitable site for monitoring of genotoxins amongst subjects exposed to occupational and environmental risk factors. The authors reported significant increase in genotoxic indices amongst tobacco users of different forms. The nuclear anomalies present as nuclear buds (indicative of gene 3 amplification), bi-nucleation (caused by cytokinesis-failure or arrest), broken egg nuclei and micronucleus (referred to chromosomal alterations) and various forms of cell death measured as condensed chromatin, karyorrhexis, karyolysis,

pyknosis as well as the frequency of basal and fully differentiated cells chromosomes (Farhadi *et al*., 2016b).

Pyknosis (condensation of nuclear chromatin) is an irreversible condensation of chromatin of cell undergoing either necrosis or apoptosis. It is seen as the first indicator of irreversible cell injury which usually progresses to karyorrhexis. Karyorrhexis on the other hand is a destructive fragmentation of nuclei of dying cells whereby its chromatin is distributed irregularly throughout the cytoplasm. This usually results from apoptosis, senescence or necrosis and mostly progresses to karyolysis. Karyolysis is the complete dissolution of nuclear chromatin of lethally injured or necrotic cells due to enzymatic degradation. It is usually preceded by pyknosis and karyorrhexis. Moreover, karyomegaly is the abnormal enlargement of cell nuclei due largely to accumulation of chromation, thereby resulting to increased nuclear/cytoplasmic ratio. Karyomegaly is a hallmark of malignancy while pyknosis, karyorrhexis and karyolysis are known hallmarks of cellular injury. They are therefore; known as essential biomarkers of cellular injury and have since become indispensible tools in the evaluation of cellular genotoxicity (Farhadi *et al*., 2016a; Biswas *et al*., 2014). Suffice it to state here that even though, karyomegaly is a hallmark of malignancy, it must be reported alongside other hallmarks. Bi-nucleate cell is a cell with two nuclei, most commonly seen and associated with neoplasia. It could result from variety of causes, which include; cleavage furrow regression, failed cytokinesis, multipolar spindle and merging of newly formed cells. The value of bi nucleated cells as biomarkers of cellular damage has been documented (Farhadi *et al*., 2016a; Biswas *et al*., 2014). Broken egg nucleus is a nucleus appearing very much like the micro nucleus but connected to the main nucleus by means of stalk. It is most often confused with micronucleus (Armen, 2005).

Broken egg nucleus is of great value in calculating the repair index and also a marker of genotoxic cellular damage.

Micronucleus (MN) is a small nucleus that forms whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division. It is a small extra nucleus separated from the main one, generated during cellular division by late chromosomes or by chromosome fragments, microscopically visible as round to oval cytoplasmic chromatin mass in the extra nuclear vicinity. Micronuclei are induced in cells by numerous genotoxic agents that damage the chromosome and originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division (Farhadi *et al*., 2016b). These authors further reported that Micronucleus assay is a well validated method for testing genotoxic effects of various agents being the only biomarker that allows the simultaneous evaluation of both clastogenic and aneugenic effects in a wide range of cells and are therefore, widely used for evaluation of exposure to genotoxins or carcinogens. The significantly high prevalence of micronuclei in the test subjects when compared to control subjects, undoubtedly infers the genotoxic and/or carcinogenic effects of both tobacco and alcohol on the oral mucosal cells, thereby agreeing with Mohan (2010) who reported tobacco and alcohol use as the major risk factor of oral squamous cell carcinoma. Perinuclear halo, also known as koilocytosis is a cytopathic effect which present as a clear area around the nucleus, usually resulting from HPV infection. It could also be induced by mutagenic agents.

2.11 The Ki-67 Gene

Ki-67 is a cell cycle associated with human nuclear protein present in peri-chromosomal region, the expression of which strictly associated with cell proliferation and which is widely used in pathology as a proliferation marker to measure the growth fraction of cells in human tumours (Humayun and Prasad, 2011). It is the monoclonal antibody which recognizes MKI67, the human homologue of the product, which is used as a tumor proliferation marker (Ntzeros *et al*., 2015).Antigen KI-67 is also defined as a nuclear protein that is associated with and may be necessary for cellular proliferation (Bullwinkel *et al*., 2006). It is associated with ribosomal RNA transcription, thus, inactivation of antigen KI-67 leads to inhibition of ribosomal RNA synthesis (Rahmanzadeh *et al*., 2007).The expression of the human Ki-67 protein is strictly associated with cell proliferation (Scholzen and Gerdes, 2000). The authors further noted that during interphase, the antigen is exclusively detected within the nucleus but relocate to the surface of the chromosomes in mitosis.

The fact that the Ki-67 protein is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells G(0)), makes it an excellent marker for determining the so-called growth fraction of a given cell population (Scholzen and Gerdes, 2000).This proliferation biomarker has an estimated half life of 60-90 minutes, with expression starting in the S phase, progressively increasing through S and G2 phases and reaching a plateau at mitosis (Humayun and Prasad, 2011).

The authors equally reported that the cells return to G1 with a stock of ki-67 antigen after cell division, whose level decreases rapidly during this phase. It has also been reported that the half life increases when mutated.

MKI67 is located on chromosome 10 at locus 10q26.2, starting at 128096659 and ending at 128126204 bp (Ntzeros *et al*., 2015). The gene size is 29,545 bp consisting of 15 exons and 14 introns and has a minus strand orientation. The promoter of MKI67 is located upstream of the transcription start site (Ntzeros *et al*., 2015). Several studies have shown that the 5' flanking region of the gene has promoter activity via reporter gene expression assays and deletion analysis (Zambon, 2010; Pei *et al*., 2012; Ntzeros *et al*., 2015). The authors in independent studies showed that the promoter region was shown to contain a TATA-less, GC rich region with several putative Sp1 binding sites and two evolutionary conserved E2F transcription factor binding sites. MKI67 has two mRNA splice variants which are shown to be processed through translation (Ntzeros *et al*., 2014). These two mRNA variants are named as "long type", which has 15 exon and total annotated spliced exon length of 12497 bp and "short type", which has 14 exon and total annotated spliced exon length of 11417 bp. The two differ only by the presence of 7^{th} exon in "long type" and its absence in the "short type". The two variants correspond to the isoforms of MKI67 protein; the heavy isoform produced from the "long type" mRNA and the light isoform translated from the "short type" mRNA, lacking the 7th exon. The heavy isoform is referred to as Antigen Ki-67 isoform 1 while the light one is known as Antigen Ki-67 isoform 2. Ntzeros *et al*., (2015) moreover reported the presence of MKI67 Pseudogene present in chromosome X and located at the Xp11 chromosomal locus.

2.11.1 Ki-67 and cancer

Ki-67 is widely used as a proliferation biomarker, being associated with dividing cells only. However its contribution to carcinogenesis and tumour development is not very clear

(Ntzeros *et al*., 2015). Buban *et al*., (2004) gave a clue to its role in carcinogenesis when they discovered eight different point mutation of this gene in four tumour cell lines during their study. These mutations which include a deletion in position 1496 resulting in a truncated product, a base exchange silent mutation in position 433 (A433T) and six other exchange mutations resulting in residue changes are enough evidence that MKI67 might provide a genetic background in tumor development (Ntzeros *et al*., 2015). MKI67 gene though not associated with any specific type of human cancer as a causative factor, it is implicated in many of them as a prognostic factor based on the expression profile of this gene at tumor cells (Humayun and Prasad, 2011; Ntzeros *et al*., 2015).Besides this, the role of MKI67 expression in diagnosis of certain diseases such as lymphoma has been reported by these authors.

The expression of Ki-67 in breast lesions has been extensively reported (Brown and Gatter, 2002; Kontzoglou *et al*., 2013; Inwald *et al*., 2013; Ntzeros *et al*., 2015).

Common histopathological parameters of breast cancer have positive association with ki-67 expression which also correlates very strongly with tumour grades (Inwald *et al*., 2013). The prognostic value of this gene in determining the overall and event free survival rate was equally reported by the authors. Pathmanathan *et al*., (2014) in a recent study showed that ki-67 expression correlates with survival in node negative breast cancer and therefore, can be used to monitor treatment success. Keam *et al*., (2011) employed the expression pattern of Ki-67 in classifying triple negative breast cancers, while Ishihara *et al*., (2013) reported an association of its expression with early central nervous system metastases. Grade 3 breast cancer which expresses MS110, Lys27H3, vimentin and Ki-67 are at high risk of carrying

BRCA1 mutations (Hassanein *et al*., 2013). Ki-67 and p53 expression pattern in phyllodes tumour is associated with tumour grade and is useful tool in differentiating benign from malignant lesion (Kucuk *et al*., 2013). The role of Estrogen positive breast cancer and ki-67 expression in distinguishing between Toker cells and cells of Paget's disease was reported by Park and Suh, (2009). Furthermore, Konofaos *et al*., (2013) has established the prognostic value of Ki-67 in samples collected via fine needle aspiration is comparable to histology samples.

Despite the fact that Ki-67 has been of value as a proliferation biomarker, there are paucity of reports validating its prognostic or diagnostic role in endometrial lesions as with breast lesions (Ntzeros *et al*., 2015). However, Markova *et al*., (2010) associated over expression of both Ki-67 and p53 with endometrial cancer and further reported direct relationship between expression and malignant phenotype with poor differentiation of tumour cells. Mourtzikou *et al*., (2012) in a similar study reported association expression of this gene in high grade endometrial carcinoma and its negative expression in atrophic endometrium. In this vein, high ki-67 expression correlates with poorly differentiated carcinomas, invasion of the myometrium, stage III tumor (Stoian *et al*., 2011) and morphologic features of aggressiveness and high grade of endometrial cancer (Konstantinos *et al*., 2013). On the other hand, the expression of Ki-67 has a role in determining the proliferation index of malignant cells, associated with specific characteristics of cervical tumor but not as a prognostic factor of survival or disease relapse (Ntzeros *et al*., 2015). Ancuta *et al* (2009), however, reported no significant association between Ki-67 expression and classic prognostic markers. Koo *et al* (2013) in their study noted a direct relationship between p16

and ki-67 expression with histologic severity of cervical tumours. Sequel to this, the authors reported that the expression of these genes could be more accurate in predicting high grade lesions than HPV testing. Additionally, they could be used for low-grade squamous intraepithelial lesions triage better than HPV DNA testing and reduce referral colposcopy to almost the half by detecting the more severe cases of CIN3 (Wentzensen *et al*., 2012; Ntzeros *et al*., 2015). Ki-67 expression has been associated with histopathological characteristics in ovarian cancer and overall survival in some subtypes (Ntzeros *et al*., 2015). However more studies are needed to establish its specific role.

The role of Ki-67 in prognosis, diagnosis, grading and management of vulvar cancer (Gincheva *et al*., 2009), bladder cancer (Wang *et al*., 2013), gliomas (Liu *et al*., 2013), meningiomas (Pavelic *et al*., 2014), pituitary adenomas (Paek *et al*., 2005), gastric cancers (Giaginis *et al*., 2011), gastrointestinal stromal tumours (Liu *et al*., 2013), colorectal cancer (Ma *et al*., 2010), lung cancer (Jakobsen and Sorensen, 2013), lymphoma (Bryant, 2006; Kim *et al*., 2007), neuroendocrine tumours (Panzuto *et al*., 2012), thyroid cancer (Saltman *et al*., 2006; Mian *et al*., 2011) and a lot of other malignant and non malignant lesions has been reported (Ntzeros *etal*., 2015).

2.11.2 Ki-67 and Oral lesions

The prognostic relevance of Ki-67 expression in oral cancers was reported by Schliephake, (2003). The author in a review study on the prognostic relevance of molecular markers of oral cancers noted that 52.2% of all oral cancer samples showed over expression of the gene and therefore, affirmed its relevance in predicting the outcome of oral cancers. de Vicente *et*

al., (2002) in an independent study noted that the Ki-67 labelling index was significantly higher in tumors with poor histologic grade of differentiation and therefore, suggested that it could be a marker to help determine the appropriate treatment for patients with oral squamous cell carcinoma. The expression of p53 and Ki-67 was found to be significantly higher in oral squamous carcinoma than in oral pre-malignant dysplasia, oral hyperkeratosis, and normal oral mucosa (Jamaroon *et al*., 2004). In view of this finding the authors suggested that the co-expression of p53 and Ki67 may play roles in carcinogenesis of OSCC.

Similarly, Adegboyega *et al* (2005) reported that the pattern of Ki67 and p53 expression in verrucous carcinoma is readily reproducible and distinctly different from that observed in epithelial hyperplasia and that seen in invasive squamous cell carcinoma, thus could be reliable adjuncts in resolving diagnostic problems associated with verrucous carcinoma. The role of Ki-67 antigen as markers of malignant transformation and carcinogenesis in oral premalignant lesions was validated by Humayun and Prasad, (2011). According to the authors, Ki-67 expression pattern in future may serve as prognostic tools in the early detection of malignant transformation in oral premalignant lesions and conditions. Zargaran *et al* (2013) in a more recent study on the suitability/unsuitability of cell proliferation as an indicator of malignant potential in oral lichen planus (OLP) noted that the expression of Ki67 was significantly higher in OLP than that of epithelial hyperplasia with no significant difference from that of mild epithelial dysplasia. It was based on this that the authors concluded that even though that it may not be possible to definitely consider malignant transformation potential for OLP, Ki-67 expression pattern may offer guide in follow up of

OLP lesions to detect potential subtle changes at an early stage. In an earlier study Zargaran *et al* (2012) reported thatKi-67 expression is not a good immunohistochemical marker to assess invasion status and differentiate oral verrucous carcinoma OVC from well-differentiated oral SCC and also cannot be used as a diagnostic tool to distinguish between variants of OSCC with similar grade. Perisanidis *et al* (2012) also noted that Ki-67 expression has no impact on treatment response and survival in patients with oral and oropharyngeal cancer treated with preoperative chemoradiation.

2.12 The p53 gene

p53, also known as TP53, tumour protein, cellular tumour antigen p53, phosphoprotein p53, tumor suppressor p53, antigen NY-CO-13, or transformation-related protein 53 (TRP53) is any isoform of a protein encoded by TP53 in humans and Trp53 in mice (George, 2011). It is a gene that codes for a protein that regulates the cell cycle and hence functions as a tumour suppression. The role of p53 in conserving the stability of cells by preventing genome mutation has made it to be widely referred to as "the guardian of the genome" (George, 2011). Arnold Levine, David Lane and William Old working at Princeton University, Dundee University (UK) and Sloan-Kettering Memorial Hospital, respectively discovered the p53 gene in 1979.

The gene was first thought to be an oncogene, but 10 years later was shown to be a tumor suppressor gene by a research team lead by Bert Vogelstein and Ray White, then studying colon cancer (George, 2011). P53 gene is located at the short arm of chromosome 17, precisely at 17p13.1 (George, 2011; Suzuki and Matsubara, 2011). The gene according to the authors encompasses 20kb of DNA with 11 exons which on transcription gives a 3.0 kb

mRNA having 1179bp open reading frame and on translation, this mRNA produces a 53kDa protein, hence the name p53. The p53 protein is a phosphoprotein made of 393 amino acids and consists of four domains (Bell *et al*., 2002).

2.12.1 Functions of p53

The p53 protein has broad range of functions, including regulation of the cell cycle, apoptosis, senescence, DNA metabolism, angiogenesis, cellular differentiation, and the immune response (Suzuki and Matsubara, 2011). Transcriptional, posttranscriptional, and posttranslational roles of p53 were also reported by the authors. The role of p53 protein is categorized into three major functions viz: growth arrest, DNA repair and apoptosis. DNA instability has been known as a prominent feature of tumomourogenesis and p53 responds to DNA damage by eliciting cell cycle arrest or apoptosis (George, 2011; Suzuki and Matsubara, 2011).The author in their reports further noted that p53, under normal steady state, is latent and does not interfere with cell cycle progression or cell survival or essential for the normal cellular function in the body. However, a variety of conditions such as DNA damage, resulting from any cause, can stimulate p53 activity (George, 2011; Suzuki and Matsubara, 2011; Ghanghoria *et al*., 2015; Yang *et al*., 2015). The authors in independent studies reported that accumulation of genomic aberrations is a key carcinogenic mechanism; the rapid induction of p53 activity in response to genomic damage thus serves to ensure that cells carrying such damage are effectively taken care of. They further note the role p53 in direct or indirect, DNA repair processes. Besides, p53 activity is triggered by a variety of oncogenicproteins, including Myc, Ras, adenovirus E1A, and β-catenin **(**George, 2011). The author also reported that p53 activation may involve a change in subcellular localization; whereas latent p53 may often be cytoplasmic, at least during part of the cell cycle, exposure

to stress results in its accumulation in the nucleus, where it is expected to exert its biochemical activities.

i. p53 and cell cycle arrest

The p53 protein suppresses progression through the cell cycle in response to DNA damage, thereby allowing DNA repair to occur before replicating the genome; hence, preventing the transmission of damaged genetic information from one cell generation to the next (George, 2011). It suppresses tumor formation by causing cell cycle arrest. This however, depending on the type of cellular stress can be achieved by inducing G1 arrest through activationof transcription of the cyclin-dependent kinase inhibitorp21 (Suzuki and Matsubara, 2011).This process is well known and has been extensively studied and reported (George, 2011; Suzuki and Matsubara, 2011; Rivilin *et al*., 2011; Naga *et al*., 2011; Muller and Vousden, 2014; Ghanghoria *et al*., 2015; Yang *et al*., 2015). According to the authors, p53 regulates cell cycle progression to s phase by activating p21 which inhibits cdk2 enzyme, required for the progression.p53 also regulates the G2/M transition by the activation of p21, GADD45 or 14-3-3 inhibits cdc2 enzyme required in the process(George, 2011; Suzuki and Matsubara, 2011; Ghanghoria *et al*., 2015). According to George, (2011) the cell cycle suppression is achieved by p53 binding to E2F transcription factor, which prevents it from binding to the promoters of protooncogenes such as *cmyc* and *cfos*. It should be noted that transcription of of *cmyc*and*cfos*is needed for mitosis therefore, blocking the transcription factor needed to turn on these genes prevents cell division.Suzuki and Matsubara, (2011) in an earlier study however reported that these transient cell cycle arrests may not lead to tumour eradication because a cell with oncogenic potential that cannot be repaired may resume proliferation.

Sequel to this, they noted that cellular senescence may play a crucial role in p53-mediate tumour suppression.

ii. p53 and apoptosis

p53 initiates apoptosis if the damage to the cell is severe and works as an emergency brake on cancer development by killing cells that attempt to proliferate in oxygen-deficient regions of tumors (George, 2011). The mechanism by which p53 induces apoptosis have been reported by several authors reported (George, 2011; Suzuki and Matsubara, 2011; Rivilin *et al*., 2011; Naga *et al*., 2011; Muller and Vousden, 2014; Ghanghoria *et al*., 2015; Yang *et al*., 2015). There is agreement by the authors that wild-type p53 gene can bind the *bax* gene promoter region and regulate *bax* gene transcription. Bax, a member of the Bcl-2 family forms heterodimers with Bcl-2 thereby inhibiting its activity (Ghanghoria *et al*., 2015). The Bcl-2 protein family is known to plays an important role in apoptosis and cancer (George, 2011). Bcl-2 is known to control the release of cytochrome c from the mitochondria,which activates the apoptotic pathway by activating caspase9which in turn activates executioner caspase 3. The role of both caspases in the apoptotic pathway has been reported (Yip and Reed, 2008; Cotter, 2009).

2.11.2 p53 and cancer

The evolution of a normal cell toward a cancerous one is a complex process, accompanied by multiple steps of genetic and epigenetic alterations that confer selective advantages upon the altered cells with the alterations underlying tumorigenesis considered to endow the evolving tumour with self-sufficiency of growth signals, insensitivity to antigrowth signals, evasion from programmed cell death, unlimited replicative potential, sustained angiogenesis,

and finally, the ability to invade and metastasize (Rivlin *et al*., 2011).According to the authors, a two stage carcinogenesis process of initiation and promotion was discovered by Isaac Berenblum and Philippe Shubik in 1947 whereas Knudson in 1971, leveraging on the on the earlier work proposed the 'Knudson two hit hypothesis of tumour development'. Theses earlier studies paved way deeper understanding of the role of tumour suppressor genes in development of tumours with a huge diversity in the genes implicated in tumourigenesis (Rivlin *et al*., 2011). The p53 gene stands out as a key tumour suppressor and a master regulator of various signaling pathways involved in this process. Indeed, *TP53* mutations were reported to occur in almost every type of cancer at rates varying between 10% in hematopoietic malignancies and close to 100% in high-grade serous carcinoma of the ovary (Rivlin *et al*., 2011; George, 2011). Suzuki and Matsubara, (2011) in a related report showed that he frequency of TP53 mutation varies from approximately 10% in hematopoietic malignancies to 50–70% in ovarian, colorectal, and head and neck malignancies with more than 26,000 somatic mutation data of p53 appearing in the international agency for research on cancer (IARC) TP53 database version R14.

Rivlin *et al*., (2011) and George, (2011) in independent reports noted the contrary to deletion or truncating mutations which inactivate the majority of tumour suppressor genes, such as *RB* or *BRCA1* during cancer progression, the *TP53* gene in human tumours is often found to undergo missense mutations, in which a single nucleotide is substituted by another.

This results in the production of a full length protein containing only a single amino acid substitution. Sequel to this, cancer associated *TP53* mutations are very diverse in their locations within the p53 coding sequence and their effects on the thermodynamic stability of the p53 protein (Rivlin *et al*., 2011; George, 2011). According to Suzuki and Matsubara,

(2011) Most TP53 mutations in human cancers result in mutations within the DNA binding domain, thus preventing p53 from transcribing its target genes. The authors also noted that mutant p53 has not only led to a loss of normal function of the wild-type protein but also led to new abilities to promote cancer.

Germinal Mutation of p53 gene is associated with the rare familial Li-Fraumeni syndrome (Rivlin *et al*., 2011; George, 2011; Suzuki and Matsubara, 2011; Naga *et al*., 2011). According to the authors Li-Fraumeni syndrome is a dominantly inherited disease with affected individuals predisposed to developing sarcomas, osteosarcomas, leukemia and breast cancer, at unusually early ages. Somatic mutation of p53 gene has been reported in 50% of all human cancers with the non mutated alleles generally lost (George, 2011). According to the author, the frequency and the type of mutation may vary from one tumor type to another; and the mutations tend to cluster in central DNA-binding domain. Breast, colon, lung, liver, prostate, bladder and skin cancers are the most frequently reported cancers involved. The p53 gene can also be inactivated by certain viruses, thereby interfering with its tumour suppressing function.

The role of Simian Virus 40 (SV40), Hepatitis and Human Papillomavirus in the gene inactivation has been reported (George, 2011).

2.11.3 p53 and oral lesions

The expression of p53 protein in various forms of oral lesions has been well documented (Klieb and Raphael, 2007; Denaro *et al*., 2011; Yang *et al*., 2015; Ghanghoria *et al*., 2015; Gissi *et al*., 2015; Singh *et al*., 2016). Klieb and Raphael (2007) in comparative study of the expression of p53, Ki67, E-cadherin and MMP-1 in Verrucous hyperplasia and Verrucous

carcinoma of the oral cavity noted significant staining trends of Ki67, p53, and MMP-1 in formalin fixed oral tissues. According to the authors, p53 immunohistochemistry panel may serve as a useful diagnostic adjunct in difficult cases, although a properly oriented hematoxylin–eosin-stained section including normal marginal tissue is considered to be the gold standard for differentiation of OVH and OVC.

In a similar study Denaro *et al*., (2011) in a review of the role of p53 and MDM2 in head and neck cancer confirmed P53 as a useful biomarker of risk, prognosis, and predictors of response in head and neck cancers. The authors reported a correlation between p53 and increased risk and development of head and neck cancer at an early age. They further noted that though MDM2 and p53 together could be used as predictive markers of response, neither p53 alone nor MDM2 correlates with patients overall survival. Yang *et al*., (2015) in an earlier report showed that the expression of expression of p53 significantly correlated with tumour stages and pathological grade of oral SCC. They also noted that the expression pattern of the gene correlated with tumourigenesis and prognosis of oral SCC and may serve as a prognostic bio marker.

Ghanghoria *et al*., (2015) similarly reported over-expression of p53 in oral squamous cell carcinomas which according to them could serve as a significant biomarker of carcinogenesis as well as for clinical evaluation, diagnosis and prognosis of disease. Gissi *et al*., (2015) in a longitudinal study to evaluate the predictive role of p53 and Ki-67 proteins alone or in combination in a group oral leukoplakia (OL) without dysplasia reported that the presence of either high p53 expression or low/normal p53 expression associated with high Ki67 expression the risk of transformation of OL to oral SCC is considered high. The

authors concluded that the combined immunohistochemical expression of p53 and Ki67 proteins could be a useful and simple molecular marker for early detection of non-dysplastic OL at risk of developing oral cancer. Singh *et al*., (2014) studied p53 mutation spectrum and its role in prognosis of oral cancer patients and reported report a very high frequency and a diverse pattern of p53 mutations in cases oral cancer. Interestingly, the authors discovered three distinct novel mutations in exons 4 and 9 and therefore concluded that analyzing p53 mutation status in tumour tissues at an early stage could serve as an important prognostic factor. In a much earlier study, Brennan *et al*., (1995) observed that a history of tobacco and alcohol use was associated with a high frequency of p53 mutations in patients with squamous-cell carcinoma of the head and neck. Based on this finding, the authors suggest an association of tobacco in the molecular progression of squamous-cell carcinoma of the head and neck and supported the epidemiologic evidence that abstinence from smoking is important to prevent head and neck cancer. Gibbons *et al*., (2014) corroborated this report in a study of the role of p53 mutation in lung cancers.

2.13 The p16 Gene

The Gene p16, also known as cyclin-dependent kinase inhibitor 2A (CDKN2A), multiple tumour suppressor 1(MTS1) and a host of other synonyms is a tumour suppressor protein which in humans is encoded by the *CDKN2A* gene. It is involved in the p16/cyclin dependent kinase/retinoblastoma gene pathway of cell cycle control by decelerating cells from progressing from G1 to S phase transition of the cell cycle (Ai *et al*., 2003). The p16Ink4a, a principal member of the Ink4 family of CDK inhibitors is codified by a gene localized on the short arm of chromosome 9 (9p21.3) within the INK4a/ARF locus, which

encodes for two different proteins with different promoters: p16Ink4a and p19ARF (Romagosa *et al*., 2011). The gene encompasses 6.6 kb of DNA; 3 exons, 471 nucleotides mRNA, 156 amino acids and 16.5 kDa protein. The p16 gene generates several transcript variants that differ in their first exons and at least three alternatively spliced variants encoding distinct proteins have been reported. Two encode structurally related isoforms known to function as inhibitors of CDK4, while the one contains an alternate open reading frame (ARF) that specifies a protein that is structurally unrelated to the products of the other variants (Roussel, 1999; Ai *et al*., 2003). The ARF product can interact with MDM2 and therefore, functions as a stabilizer of the p53 protein. The gene is moderately expressed in many organs as thymus, liver, pancreas, prostate, lung, or kidney.

2.13.1 Functions of p16

The *CDKN2A* gene provides instructions for making several proteins with p16(INK4a) and the p14(ARF being the most well-studied (Cánepa *et al*., 2007). The gene, p16, is a cyclin-dependent kinase (CDK) inhibitor which slows down cell cycle by prohibiting progression from G1 phase to S phase (Rayess *et al., 2012*). According to the authors, CDK4/6 binds cyclin D and forms an active protein complex that phosphorylates retinoblastoma (pRB) which once phosphorylated, disassociates from the transcription factor E2F1, liberating it from its cytoplasm bound state and allowing it to enter the nucleus. E2F1 when present in the nucleus promotes the transcription of target genes essential for transition from G1 to S phase of cell division. As a tumour suppressor gene, p16 binds to CDK4/6 thereby preventing inhibiting cyclin D–CDK4/6 complex formation and CDK4/6-mediated phosphorylation of Rb family members and ultimately inhibits the downstream activities of

transcription factors, such as E2F1, and arrests cell proliferation (Ai *et al*., 2003; Cánepa *et al*., 2007; Romagosa *et al*., 2011; Rayess *et al.,* 2012). The role of p16 in senescence, a growth arrest mechanism that protects the cell from hyperproliferative signals and from various forms of stress, was also reported by Romagosa *et al*., (2011). According to the authors, senescence and cell cycle arrest in non-senescent cells seem to share, at least in part, the same molecular mechanisms, involving the p16Ink4a/Rb and p14ARF/p53 pathways. This is in line with an earlier report by Zindy *et al*., (1997) and Ressler *et al*., (2006) who observed marked increased expression of p16 with increasing age of mouse tissues and human skin and kidney tissues. Besides the cell regulation function of p16, the role of the gene in apoptosis, cell invasion and angiogenesis which are related to it's over expression in cancer has also been reported (Romagosa *et al*., 2011).

The p16 gene regulation is complex involving the interaction of several transcription factors and several proteins involved in epigenetic modification through methylation and repression of the promoter region *(*Rayess *et al.,* 2012). According to the authors, PRC1 and PRC2 are the two protein complexes which modify the expression of p16. This is made possible through the interaction of various transcription factors that execute methylation patterns that can repress transcription of the gene.

2.13.2 p16 and cancer

p16 as a negative regulator of cell proliferation is one of the main factors which avert tumourigenesiswith about 50% of all human cancers showing p16 inactivation ranging from 25 to 70% (Romagosa *et al*., 2011). In the authors report, head and neck, esophagus, biliary tract, liver, lung, bladder, colon cancers as well as breast carcinomas, leukemia, lymphomas

and glioblastomas are mostly involved in p16 inactivation. Mutations resulting in deletion or reduction of function of the CDKN2A gene are associated with increased risk of a wide range of cancers and alterations of the gene are frequently seen in cancer cell lines. Homozygotic deletions, loss of heterozygosity, point mutations and promoter methylation are some of the several mechanisms by which p16 gene is inactivated (Romagosa *et al*., 2011). According to Ai *et al*., (2003) a review by Rocco and Sidransky in 2001 revealed that p16 inactivation was seen in 68% of primary human tumours and was caused by homozygous deletions in 27%, by inactivating mutations in 11%, and by promoter hypermethylation in 30% of the cases respectively. The authors further reported the occurrence of loss of p16 function early in cancer progression and pointed out that this gene may be the earliest detectable genetic abnormality in head and neck squamous cell carcinoma.

p16 promoter hypermethylation turns off the production of p16, leading to reduced concentration of the gene in the cells Cánepa et *al*., (2007). The consequence of this is proliferation of cells which invariably leads to cancer. The association of p16 promoter hypermethylation with human can has been amply reported (Huang *et al*., 2002; Nakahara *et al*., 2006; Shaw, 2006; Romagosa *et al*., 2011). Mutations affecting the *CDKN2A* gene are associated with skin cancer (melanoma) breast cancer, colorectal cancer, cervical cancer, lung cancer, and pancreatic cancer (Volgareva *et al*., 2004; Russo *et al*., 2005; Cánepa *et al*., 2007). p16 overexpression has been observed at the invasive front of endometrial, colorectal and basal cell carcinoma (Romagosa *et al*., 2011). According to these authors these mutations are germ line mutations and are found in all body cells. It was further reported that germ line p16 mutations are associated with development of only one type of cancer in

some families where as in some other families; they may lead to a cancer predisposition syndrome, which increases the risk of developing multiple types of cancer.

There are ample studies establishing the down regulation of p16 in cancers as a tumour suppressor protein (Romagosa *et al*., 2011). The authors however reported that the over expression of the tumour suppressor gene in tumours has been elucidated in some studies. (Mulvany *et al*., 2008) in an earlier study established that p16 over expression in cervical cancer, head and neck cancer and perianal lesions which are directly associated with infection by high-risk genotypes of HPV can be used as a diagnostic tool. The finding of Lewis *et al*., (2012), who reported positive correlation between p16 expression and HPV infection in oral squamous cell carcinoma, agreed with the earlier report. In a review study, Romagosa *et al*., (2011) reported that during immortalization of cancer cells, the p16–Rb pathway is often targeted by viral oncoproteins because of its critical activity to prevent inappropriate cell proliferation.

The authors further explained that the presence of the viral oncorotein E6 and E7 is the molecular mechanism that explains the over expression of p16 in HPV related lesions. Rb protein is inactivated by interaction with the high-risk HPV oncoprotein E7, resulting in the release p16 from its negative feedback control, causing an increase in the levels of the protein, which attempts to inhibit uncontrolled cellular replication. The over expression of p16 gene in the cytoplasm of certain neoplasms, which corresponds to tumour progression and disease prognosis has been reported (Romagosa *et al*., 2011). It is noteworthy however, that cell cycle regulation, a function confined in the cell nuclei, is classically the only function of p16. In breast cancer, for example nuclear staining of p16 was observed in fibro

adenoma but nuclear/cytoplasmic or exclusively cytoplasmic staining was found in carcinoma (Di Vinci *et al*., 2005). In a related study, the authors reported that in colorectal cancer, adenomas and primary or metastatic adenocarcinomas express strong nuclear/ cytoplasmic positivity (80%) while negativity or low nuclear expression is observed in normal mucosa and in benign conditions.

Besides these, O'Neill *et al*., (2007) showed p16 over expression can be detected in both the nucleus and the cytoplasm of non-epithelial tumors such as astrocytomas or uterine leiomyosarcomas and that such staining pattern is associated with high-grade malignant phenotypes. In a more recent study, Haller *et al*., (2010) reported that cytoplasmic p16 over expression and nuclear p16 down regulation correlates with poor prognosis in gastrointestinal stromal tumour. Romagosa *et al*., (2011) reported that nuclear p16 down regulation was associated with an over expression of E2F, conversely, cytoplasm p16 over expression has no relation with E2F expression.

These results, according to the authors, support the hypothesis that p16 has different roles in different sub-cellular locations, and that the control of cell cycle is mainly regulated by nuclear p16. The authors concluded that p16 localization in the cytoplasm may represent an alternative mechanism for modulating different pathways, instead of simply a way to inactivate its cell cycle control function. A progressive increase of p16 expression has been described in the transformation from normal tissue to pre-neoplastic lesions, and from pre-neoplastic lesions to carcinoma in several types of cancer was also reported by the authors.

The expression pattern of this gene could also be used in differentiating the diagnosis from benign to malignant lesions in tumors showing a progressive increase of its expression.

2.13.3 p16 and oral lesions

The expression of p16 gene in oral malignant and pre malignant lesions has been reported (Sartor *et al*., 1999; Huang *et al*., 2002; Ai *et al*., 2003; Russo *et al*., 2006; Buajeeb *et al*., 2008; Kuo *et al*., 2008; Westra, 2009; Riechelmann, 2010; Lewis *et al*., 2012). In a western blot analysis of oral tissues, Sartor *et al*., (1999) observed down regulation of p16 in both oral squamous carcinoma and pre malignant lesions. In a similar report, Huang *et al*., (2002) showed an inverse relationship between p16 promoter and the gene expression, in their conclusion, the authors reported that the methylation of p16 in oral SCC occurs in both pre-cancerous and cancerous stages, thereby resulting in decreased p16 expression, which is heterogeneous in the cancer cells. Furthermore, Ai e*t al.*, (2003) observed p16 promoter hypermethylation and apparent loss of p16 protein expression were detected in 27% and 74% of head and neck squamous cell carcinoma, respectively with history of alcohol or tobacco being significantly correlated with the loss of the protein expression.

Russo *et al*., (2006) in an independent study agreed with the finding of Ai e*t al.*, (2003). In another study Buajeeb *et al*., (2008) detected p16 expression in 3 out of 16 cases of oral SCC and 4 0ut of 15 cases of oral leukoplakia and therefore, concluded that p16 is not reliable marker for oral mucosal dysplasia and malignant transformation. The association between HPV infection and p16 expression in oral SCC, which also correlates with favourable prognosis, has been reported (Kuo *et al*., 2008; Riechelmann, 2010). Similarly,

Lewis *et al*., (2012) reported p16 expression amongst HPV related oral lesion and negative staining in non HPV related tumours.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study was carried out in Nnewi Metropolis, Nnewi North Local Government Area Anambra State Nigeria. Nnewi, which is made up of four (4) quarters: Otolo, Uruagu, Umu-dim and Nnewi-Ichi. Nnewi North Local Government Area is located in Anambra South senatorial district. It is a metropolitan city and the second largest commercial city in Anambra State, with a population of about 391,227 people as of 2006 National Population Census (National Population commission, 2010). The population is made up of vast number of professionals, transporters, businessmen, industrialists, artisans' public servants and students.

3.2 Ethical Clearance

Ethical clearance to carry out this study was obtained from the Ethics Committee of the Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Nnewi Campus (Appendix I).

3.3 Study population

The study population comprised of commercial bus drivers. A study sample of commercial bus drivers who actively use tobacco and alcohol were recruited.

3.4 Determination of sample size

The sample size was determined using Yaro Yamane formula as stated by Osemeke (2012).

$n = N/1+N\,(e)^2$ where,

n= sample size

N= the finite population

e= level of significance (0.05)

1= unity (constant)

Calculation of sample size:

n =N/1+N (e) ^{2} N= 134, e =0.05. n= 134/1+134 $(0.05)^2$, n= 134/1+134(0.0025)

n = 164/1+0.335, n= 164/1.335 =122.85 ~123

Therefore (sample size) n= 123. A total 145 subjects were recruited for the study. This was meant to enhance categorization.

3.5 Study Design

The study was a cross sectional survey and the study groups were made up of the following:

i. Cigarette / Alcohol users : n =42

ii. Snuff / Alcohol users: n= 41

iii. Alcohol users: n=22

iv. Control: (non cigarette, snuff and alcohol users) n=40

Cigarette / Alcohol users and Snuff / Alcohol users were further categorized as follows:

i. Short term: Subjects who habitually smoke cigarette and/or inhale snuff for ≤ 5years.
ii. Middle term: Subjects who habitually smoke cigarette and/or inhale snuff for ≤ 10 years but not ≤ 5years.
iii. Long term: Subjects who habitually smoke cigarette and/or inhale snuff for > 10 years.

3.6 Recruitment of subjects

The study subjects were recruited from three popular motor parks in Nnewi. These are Eastern Mass Transit Limited Nnewi, Nnobi/Ekwulobia Park Nnewi and Onitsha Park Nnewi. Drivers and Motor Park workers who do not actively use tobacco in any form and do not drink alcohol were recruited from the various Motor Parks. These served as control group.

3.7 Inclusion criteria

Commercial drivers within the study area and who actively use tobacco and/or drink any alcoholic beverage and who signed a written informed consent form, were included in the study.

3.8 Exclusion criteria

The exclusion criteria are as follws:

1. Those that neither actively use tobacco products nor drink alcoholic beverage;
2. Those already diagnosed of any malignant or benign lesion;
3. That with disease conditions such HIV, diabetes, high blood pressure, tuberculosis and other chronic disease conditions.

These were determined through the instrument of self constructed questionnaire.

3.9 Sample collection

Each participant read and signed a written informed consent form (Appendix II) while bio data, demographic data and risk factor data were obtained from each participant through the instrument of questionnaire. Buccal smear was collected from the oral mucosa and tongue of each participant with the aid of wooded spatula. This was preceded by rinsing of the buccal cavity with table water to avoid contamination with debris. The smear was immediately emulsified in 10 ml of 70% ethyl alcohol in a plastic universal container, labbled with identification numbers, and allowed to stand at room temperature for 48 hours, to complete fixation of cells. A modification of Farhadi *et al*., (2016b) was used.

3.9.2 Sample processing

The cell suspension in each universal container was mixed by shaking the container after which the content was emptied into a centrifuge tube. The samples were spun in a centrifuge for 5 mins at 1200rpm, the supernatant was discarded, the deposit re-suspended in the remaining fixative. A drop each was placed in six different pre-coated and pre-labeled

slides, and monolayer smears made with aid of wire loop. The smears were allowed briefly to air dry and the slides carefully transferred to slide box pending staining.

3.9.3 Staining

i. Papanicolaou Staining Method (Farhadi *et al*., 2016b**)**

Principle

Haematoxylin, a basic dye with affinity for acidic substances stains the DNA filled cell nuclei dark blue, Orange-G 6 (O-G6) an acid dye on the other hand reacts with mature superficial cells, giving them orange colouration, while Eosin Azure 50 (EA50) another acid dye reacts with less mature cells, staining the light blue to green. Papanicolaou staining method thus, gives a sharp differential staining of nuclear and cytoplasmic morphology of cells. The haematoxylin nuclear stain demonstrates chromatinic patterns of normal and abnormal cells while the counter stains, O-G and EA have a high alcoholic concentration which provides cytoplasmic transparency, thus enabling clear visualization through areas of overlapping cells, mucus and debris.

Procedure

Smears were transferred to 50% ethanol and then to distilled water for 15 Seconds each. They were thereafter immersed in a jar of Harris haematoxylin for 5 minutes, rinsed by dipping for 10 times in distilled water and differentiated in 0.5% hydrochloric acid by 2 quick dips in the solution. The differentiated smears were washed in distilled water for 15 seconds, blued for a few seconds in 0.1% ammoniated water and passed through 50%, 70%, and 95% ethanol for 15 seconds each, before being stained in O-G 6 for 2 minutes. The smears thereafter were dipped 10 times each in three changes of 95% ethanol, stained in EA 50 for 3

minutes, rinsed by dipping in 95% ethanol for 10 times and dehydration completed in absolute ethanol.

The stained dehydrated smears were cleared in xylene (10 dips) and finally mounted in DPX using a 22x40 mm cover slip.

ii Feulgen reaction

Principle of Feulgen reaction (Shin *et al*., 2005)

Hydrolysis of DNA by hydrochloric acid librates two purine bases namely adenine and guanine from the nucleic acid, which in the presence of an acidic environment becomes converted to aldehyde groups, the free adldehyde present re-colours Schiff's reagent to a magenta coloured dye (Shin *et al*., 2005). Feulgen reaction is a highly specific test for DNA and has been widely employed in the study of nuclear details (Fenech *et al*., 2011). It was also reported the specificity is attributed to the removal of the purine bases which allows precise localization of DNA molecule. RNA does not take part in the reaction because presence of hydroxyl group on carbon 2 of the ribose sugar prevents acid hydrolysis of RNA.

Procedure

Smears were taken to 50% ethanol and then to distilled water for 15 Seconds each. They were treated for 4 minutes in N HCL at 60^{o} C in order to hydrolyse DNA and there after rinsed in distilled water for 30 seconds. The rinsed smears were transferred to Schiff's reagent for 60 minutes and there after wash in running tap water for 10 minutes. Stained smears were

dehydrated by passing through 70%, 90% and two changes of absolute ethanol (10 dips in each bathe) and finally cleared in xylene and mounted in DPX using 20 x 40 mm cover slip.

iii **Immunocytochemical Staining (ICC) and Scoring (**Humayun and Prasad, 2011)

Immunocytochemical staining of oral mcossal smears were carried according to a method described by Humayun and Prasad, (2011). Monoclonal antibodies Ki-67, p53 and p16 were employed. Exposed Mouse and Rabbit Specific HRP/DAB detection IHC kit were employed for immunostaining while detection of immunoreactivity was performed according to manufacturer's instruction. Appendix tissue was used as positive control. Both antibodies and detection kits were procured from Bridge Biotech limited Cambridge Uk, through Bridgestone Scientifik Enterprises Ilorin. The immunohistochemical staining was semi-quantitatively scored according to Zlobec *et al*., (2006). This was based on the percentage of cells that stained positive and the intensity of the staining (strong, moderate, weak). A score of 5+ was assigned to 80% or more of epithelial cells that stained positive with strong intensity, 4+ was assigned to 50% or more (but less than 80%) of epithelial cells with strong intensity or 80% of cells or more with moderate to weak intensity; 3+ was assigned to 30% or more of epithelial cells with strong intensity or 50% or more (but less than 80%) of positive cells with moderate to weak intensity; 2+ was assigned to 10% or more of cells that stained positive with strong intensity or 30% or more (but less than 50%) that stained moderate to weak and 1+ was assigned to 10% or more of cells (less than 30% of positive cells) that stained positive with moderate to weak intensity; 0 was assigned to less than 10% of positivity irrespective of the intensity of staining.

Principle of Immunocytochemistry

When an antibody is introduced into a tissue section or smear, it reacts with specific antigen if present, forming antigen-antibody reaction complex *in situ* which is then localized and visualized through an appropriate enzymatic or fluorescent detection system.

Procedure

Smears were taken to water by passing through 50% ethanol for 15 seconds and then to distilled water for 15 seconds. Slides were arranged in slide racks and treated in protein block and biotin block solutions for 25 minutes in each solution. Thereafter, they were arranged on a staining rack and flooded with phosphate buffer saline (PBS) solution to prevent drying. The smears were afterwards drained, the exact portions of smears on slides carefully ringed with a hydrophobic pen and diluted antibodies (1: 100) (anti ki-67, p53 and p16) applied onto smears with the aid of Pasteur pipette and allowed to incubate at room temperature for 1 hour. After incubation in the primary antibodies, the smears were washed w2ith PBS, flooded with a secondary antibody for 25 minutes, washed with PBS, drained and diaminobenzidine (DAB) applied for 5 minutes. Finally, the smears were washed with PBS, counter stained in Harris Haematoxylin for 5 minutes, washed in water and differentiated by dipping 10 times in 1 % acid alcohol. They were later washed and blued in tap water, dehydrated by passing through 70%, 90% and two changes of absolute ethyl alcohol for 15 seconds each, cleared in xylene and mounted in DPX. Ki-67, p53and p16 positive immune positive control sections were also stained along with test and control smears.

3.10 Repair Index (Farhadi *et al*., 2016a)

The repair index for each sample was calculated by the formular described by Farhadi *et al* (2016a). It is the ratio of sum of Karyorrhexis and Karyolysis and Broken nuclei and Micronuclei [*KR+KL/BE+MN*] and measures the severity of nuclear damage and the fate of dysplastic cell.

3.11 Microscopy and Photomicrography

All stained slides were examined under optical microscope using x10 and x40 objective lenses. Two hundred (200) cells were critically evaluated for cytolasmic and nuclear details for PAP and Feulgen stained slides while stained cells and staining intensity, as afore described, were examined for ICC stained slides. Photomicrographs of each slide were taken for reference and presentation purposes. Nuclear abnormalities such as pyknosis (P), karyorrhexis (KH), karyolysis (KL), karyomegaly (KM), binucleate (BN), broken egg nuclei (BE), micro nuclei (MN) and perinuclear halo (NH) were sought for.

3.12 Data Analyses

All numerical data were summarized using midian, mean rank and chi square, whereas categorical data were presented using frequency and percentages. Significance differences in the prevalence of various forms of nuclear changes between cigarette smokers/alcohol drinkers and control group and snuff users/alcohol drinkers and control group were determined using Kruskal walis H-test, P values of <0.05 were considered significant. Comparison of the prevalence of the various forms of nuclear changes amongst cigarette smokers/alcohol drinkers, snuff users/alcohol drinkers and control group was carried out using Kruskal walis H-test (ANOVA) with P values of <0.05 also considered significant.

Moreover, Kruskal walis H-test was employed to compare the presence and degrees of various forms of nuclear changes amongst short term, middle term and long term cigarette smoking/alcohol drinking and snuff using/alcohol drinking. The associations between the various forms of nuclear changes and Ki-67, p53 and p16 expression patterns were determined with Spearman's correlation test. These statistical analyses were carried out with the aid of Statistical Package for Social Scientist (SPSS) version 22.0

CHAPTER FOUR

RESULTS

The present study evaluated the prevalence of nuclear changes and ki-67, p53 and p16 expression pattern in the oral cavity smears of commercial bus drivers who actively use tobacco and drink alcohol in Nnewi.

Fig 4.1 Age distribution of Participants

The age range of test subjects was from 35- >64years (mean age: 42.5 years), while that of control subjects ranged from 22- >62years (mean age: 35 years). Greater number (66) of study subjects were >50 while the least number (14) were within the age range of 35 to 40 years. The rest were as follows; 41-45 years, 20, and 46-50 years, 15 in number (Fig 4.1).

Fig 4.2 Distribution of Nuclear changes amongst study subjects and control group

The following nuclear changes were detected in both test and control groups; Pyknosis (P), Karyorrhexis (KH), Karyolysis (KL), Karyomegaly (KM), binucleate (BN), Broken egg nucleus (BE), Micronucleus (MN) and Nuclear halo (NH). Micronuclei were the most frequently occurred, making the greatest percentage of all nuclear changes across all study groups. Broken egg nuclei and peri nuclear haloes were the least frequently ecountered. Broken egg nuclei were not ecountered in alcohol user and control groups, while peri nucear halo was not ecountered in the control group. All nuclear changes were ecountered at varying degrees in the cigarette /alcohol user and snuff/alcohol user groups (Fig 4.2).

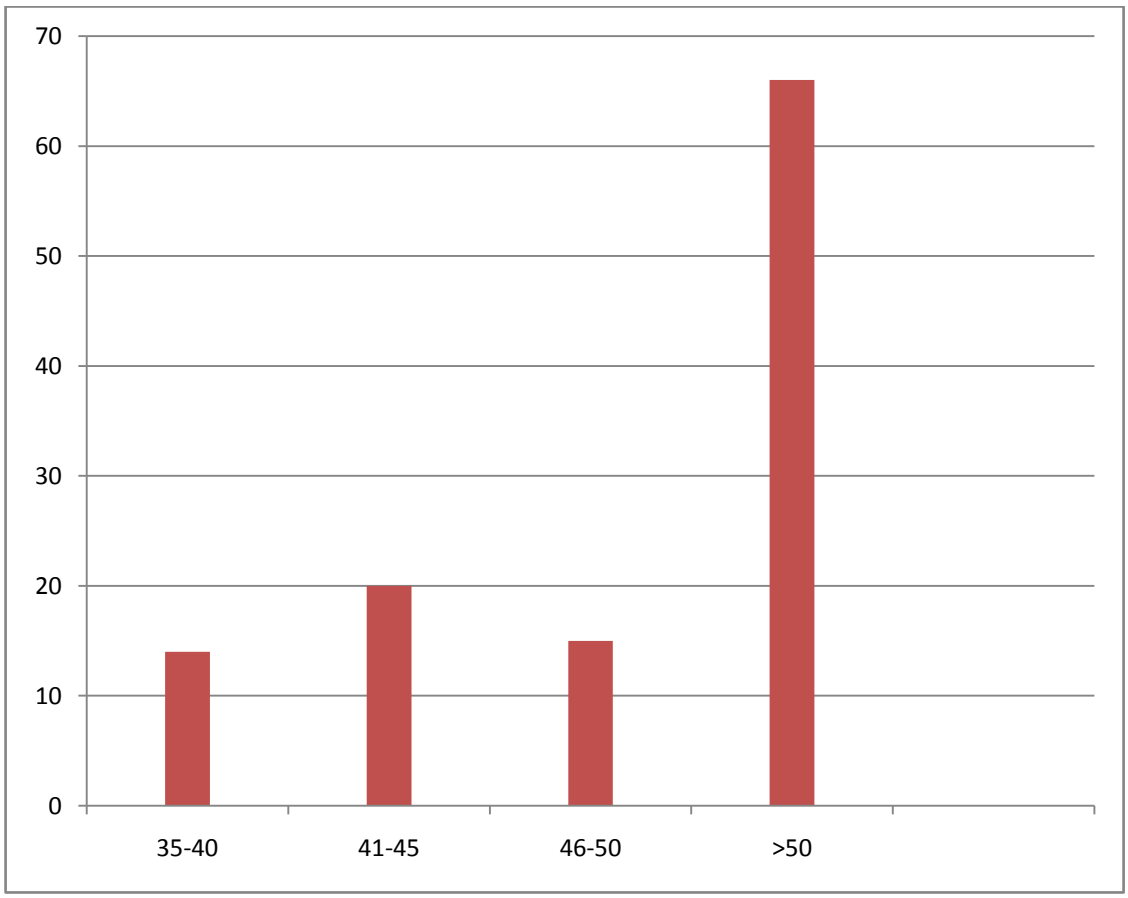


Fig 4.1: Age distribution of study subjects (years) (Mean age: 42.5 years)

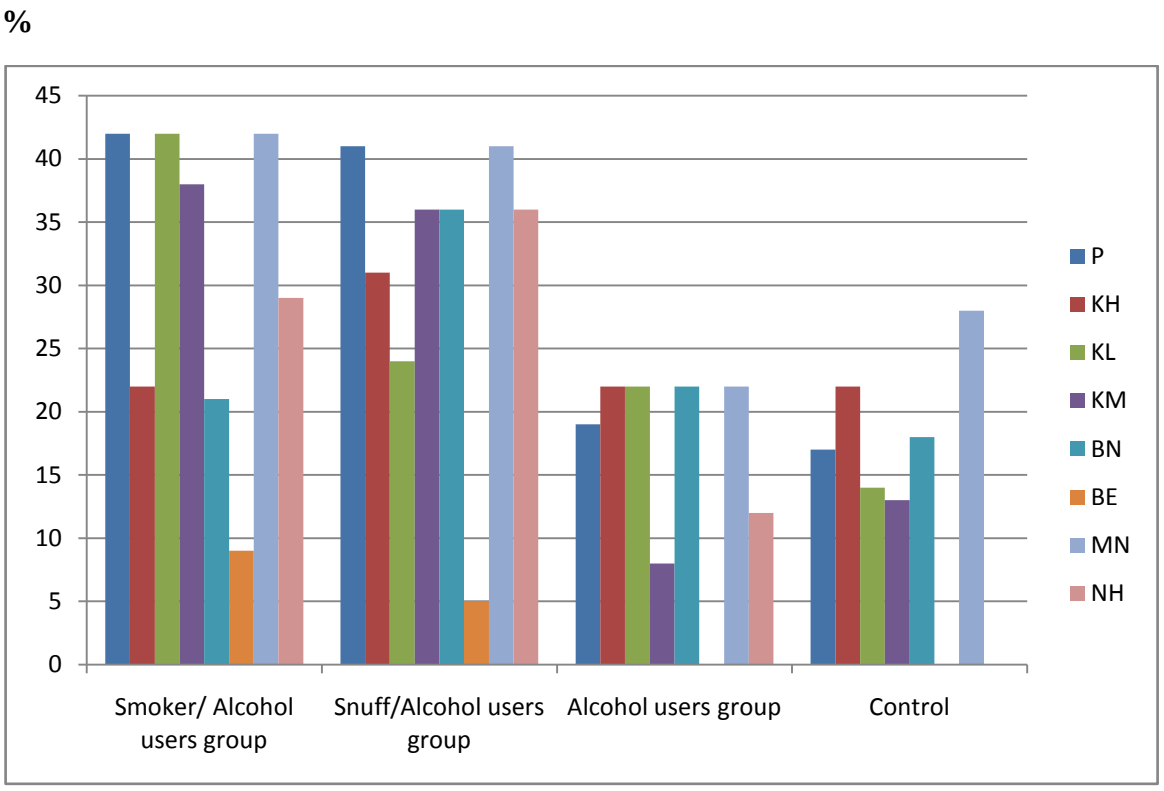


Fig 4.2: Distribution of nuclear changes amongst Smokers/Alcohol users, Snuff/Alcohol users, Alcohol users and Control group.

Fig 4.3: Prevalence of nuclear changes amongst Cigarette/Alcohol users, Snuff/Alcohol users, Alcohol users and Control groups

The prevalence of nuclear changes was highest amongst snuff/alcohol user group, (33.97%), followed by cigarette/alcohol user group (33.29%), alcohol user group (17.53%) and Control (15.21%) (Fig 4.3).

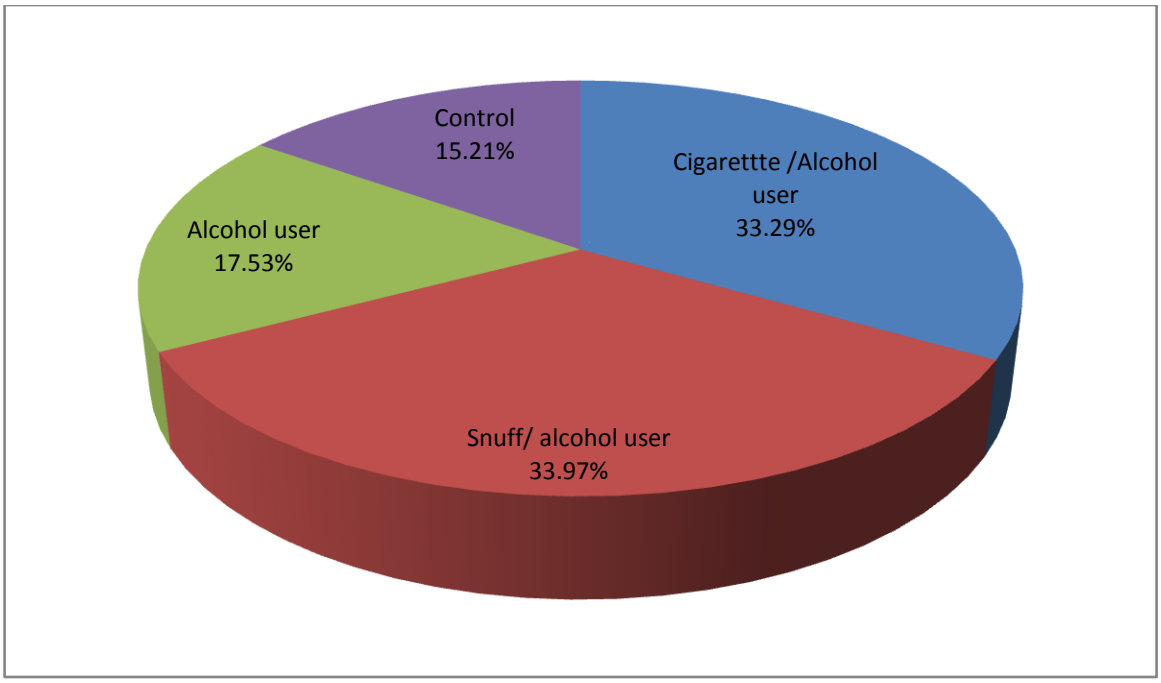


Fig 4.3: Prevalence of nuclear changes amongst Cigarette/Alcohol users, Snuff/Alcohol users, Alcohol users and Control groups

Table 4.1: Distribution of specific of nuclear changes amongst Smokers/Alcohol users, Snuff/Alcohol users, Alcohol users and Control groups

Micronuclei were the most prevalent nuclear changes both in test subjects (100%) and control groups (70%), while broken egg nuclei were the least prevalent (15.2%). The rest were as follows; Pyknosis, 97.1%, Karyorrhexis, 61.9%, Karyolysis, 74.3%, Karyomegaly, 78.1%, Bi-nucleate, 75.2% and Nuclear halo, 73.3% (Table 4.1).

Table 4.1: Distribution of specific nuclear changes amongst Cigarette/Alcohol users, Snuff/Alcohol users and Alcohol users groups

	Number/%				
Nuclear changes	**Cigarette / Alcohol users**	**Snuffer/ Alcohol users**	**Alcohol users**	**Control**	**Prevalence in test groups**
Pyknosis	42 (100%)	41 (100%)	19(86.4%)	17 (42.5%)	(97.1%)
Karyorrhexis	22 (52.4%	31 (75.6%)	12 (54.5%)	22 (55%)	61.9%
Karyolysis	42 (100%)	24 (58.3%	12 (54.5%)	14 (35%)	74.3%
Karomegaly	38 (90.5%)	36 (87.8%)	8 (36.4%)	13 (32.5%)	78.1%
Binucleate	21 (50%)	36 (87.8%)	22 (100%)	18 (45%)	75.2%
Broken egg nuclei	9 (21.4%)	5 (12.2%)	2 (9.1%)	0 (0%)	15.2%
Micronuclei	42 (100%)	41 (100%)	22 (100%)	28 (70%)	100%
Nuclear halo	29 (69%)	36 (87.8%)	12 (54.5%)	0 (0%)	73.3%

Table 4.2: Immuno-staining pattern of Ki-67, p53 and p16 amongst Cigarette, Snuff, Alcohol users and Control groups

The immuno-staining pattern showed that 50% of subjects in the cigarette /alcohol user groups variously expressed ki-67 and p53, while 71% expressed p16. Similarly, 17% of the snuff/alcohol user group expressed ki-67, 34% expressed p53 while 58.5% expressed p16. Moreover, 45.5% of subjects in the alcohol user group expressed ki-67, 54.5% expressed p53 while 68% expressed p16. As for the control group, 15% expressed ki-67 and p16 while 27.5% of the subjects expressed p53. P16 was the most frequently expressed gene (42.4%), followed by p53 (32.7%) and ki-67 (24.9%). Cigarette /Alcohol users showed more gene expression than all other test groups. The immuno-staining scoring showed a consistent pattern of weak to mild positivity (1+ and 2+), with the exception of p53, where 4 subjects in the Cigarette /Alcohol user group showed high intensity staining (4+) (Table 4.2).

Table 4.3: Co-expression pattern of ki-67, p53 and p16 amongst Cigarette, Snuff, Alcohol users and Control groups

Ki-67, p53 and p16 genes were co-expressed in 17(40.5%) subjects in the cigarette/alcohol user group while 3(7%) subjects co-expressed ki-67 and p53. Conversely, there was no co-expression of the three genes in the snuff/alcohol user group. However, 7(17%) subjects co-expressed ki-67 and p16 while 5(12%) co-expressed p53 and p16. The three genes were also co-expressed in 12(54.5%) subjects in the alcohol user group whereas 4 (10%) control subjects co-expressed ki-67 and p16 genes (Table 4.3).

Table 4.2: Immuno-staining pattern of Ki-67, p53 and p16 amongst Cigarette, Snuff, Alcohol users and Control groups

	Cigarette /Alcohol users n=42			Snuff/Alcohol users n=41			Alcohol users n=22			Control n=40		
Score (+)	**Ki-67**	**p53**	**p16**	**Ki-67**	**p53**	**p16**	**Ki-67**	**p53**	**p16**	**Ki-67**	**p53**	**p16**
0	21	20	12	34	27	17	12	10	7	34	29	36
	50%	47.6%	28.6%	82.9%	65.9%		54.5%	45.5%)	31.8%	85%	72.5%	90%
+1	9	4	17	7	7	17	10	12	13	6	11	4
	21.4%	9.5%	40.5%	(17.1%	17.1%		45.5%	54.5%	59.1%	15%	27.5%	10%
+2	12	13	13	0	7	7	0	0	2	0	0	0
	28.6%	30.9%	30.9%		17.1%				9.1%			
+3	0	0	0	0	0	0	0	0	0	0	0	0
+4	0	4	0	0	0	0	0	0	0	0	0	0
		9.5%										

Table 4.3: Co-expression pattern of ki-67, p53 and p16 amongst Cigarette, Snuff, Alcohol users and Control groups

Genes	Cigarette/Alcohol users n=42 Frequency	Snuff/Alcohol users n=41 Frequency	Alcohol users n=22 Frequency	Control n=40 Frequency
Ki-67/p53/p16	17	0	12	0
Ki-67/p53	3	0	0	0
Ki-67/p16	0	7	0	4
P53/p16	0	5	0	0

Table 4.4a and b: Comparison of nuclear changes and repair index across cigarette, snuff, alcohol users and control groups

A Kruskal Walis test, which compared the median values of occurrence nuclear changes and repair index amongst cigarette, snuff, alcohol users and control groups revealed a statistically significant difference in the median of all detected nuclear changes ($p< 0.05$). The mean rank values of pyknosis revealed a consistent decrease from cigarette/alcohol user, snuff/alcohol user, Alcohol user to Control groups. The repair index conversely, showed a progressive increase from cigarette/alcohol user, snuff/alcohol user, alcohol user to Control groups (Table 4.4).

Table 4.5: Pair wise comparison of nuclear changes and repair index across Control Cigarette, Snuff and Alcohol user groups

A pair wise comparison of median values of nuclear changes and repair index between control and cigarette, snuff, alcohol users groups showed an increased statistically significant difference in pyknosis, karyolysis, micronuclei and nuclear halo in all the groups. Karyomegaly and broken egg nuclei showed increased statistically significant differences when compared between control and cigarette/alcohol user groups and control and snuff/alcohol user groups. Karyorrhexis and binucleate, similarly, showed increased statistically significant differences when compared between control and snuff/alcohol user groups and control and alcohol user groups. Repair index decreased significantly between control and cigarette/alcohol user groups and control and snuff/alcohol user groups (Table 4.5).

Table 4.4a: Comparison of nuclear changes and repair index across Cigarette, Snuff, Alcohol users and Control groups

Nuclear changes	Study group	Mean Rank	Median	P-value
Pyknosis	Cigarette/Alcohol users	103.06	3.00	0.000
	Snuff/Alcohol users	94.16	2.00	
	Alcohol users	56.23	1.00	
	Control	28.98	0.00	
Karyorrhexis	Cigarette/Alcohol users	56.02	1.00	.
	Snuff/Alcohol users	77.78	1.00	0.000
	Alcohol users	112.68	2.00	
	Control	64.10	1.00	
Karyolysis	Cigarette/Alcohol users	95.98	1.00	
	Snuff/Alcohol users	70.93	1.00	0.000
	Alcohol users	92.64	1.00	
	Control	40.20	0.00	
Karyomegaly	Cigarette/Alcohol users	89.30	2.00	
	Snuff/Alcohol users	100.62	2.00	0.000
	Alcohol users	49.59	0.00	
	Control	40.45	0.00	

Significant level: $P<0.05$

Table 4.4b: Comparison of nuclear changes and repair index across Cigarette, Snuff, Alcohol users and Control groups

Nuclear changes	Study group	Mean Rank	Median	P-value
Bi-nucleate	Cigarette/Alcohol users	57.00	0.50	0.000
	Snuff/Alcohol users	103.73	2.00	
	Alcohol users	88.09	1.00	
	Control	50.00	0.00	
Broken egg nuclei	Cigarette/Alcohol users	80.54	0.00	0.021
	Snuff/Alcohol users	73.84	0.00	
	Alcohol users	71.59	0.00	
	Control	65.00	0.00	
Micro-nuclei	Cigarette/Alcohol users	92.67	3.00	0.000
	Snuff/Alcohol users	84.18	3.00	
	Alcohol users	101.32	4.00	
	Control	25.31	1.00	
Nuclear halo	Cigarette/Alcohol users	87.07	1.00	0.000
	Snuff/Alcohol users	101.88	2.00	
	Alcohol users	62.32	1.00	
	Control	34.50	0.00	
Repair index	Cigarette/Alcohol users	58.36	0.50	0.000
	Snuff/Alcohol users	65.41	0.67	
	Alcohol users	82.64	0.75	
	Control	90.85	1.00	

Significant level: $P<0.05$

Table 4.5: Pair wise comparison of nuclear changes and repair index across Control Cigarette, Snuff and Alcohol users groups

Nuclear changes	Control vs. Cigarette/ Alcohol users		Control vs. Snuff/ Alcohol users		Control vs. Alcohol users	
	X^2	P value	X^2	P value	X^2	P value
Pyknosis	52.595*	0.000	63.220*	0.000	9.888*	0.002
Karyorrhexis	8.076	1.000	5.199*	0.023	27.055*	0.000
Karyolysis	23.553*	0.000	4.504*	0.034	21.678*	0.000
Karyomegaly	30.444*	0.000	37.357*	0.000	0.095	0.758
Bi-nucleate	0.463	0.496	29.975*	0.000	6.875*	0.009
Broken egg nuclei	9.628*	0.002	5.199*	0.023	3.758	0.053
Micronuclei	59.911*	0.000	49.795*	0.000	38.803*	0.000
Nuclear Halo	42.731*	0.000	63.220*	0.000	27.055*	0.000
Repair index	8.443*	0.004	13.743*	0.000	8.214	1.000

* The Chi square is significant if $p < 0.05$.

Table 4.6: Pair wise comparison of nuclear changes and repair index across Cigarette/Alcohol users, Snuff/Alcohol users and Alcohol users

There were decreased statistically significant differences when the median values of karyorrhexis, karyomegaly and binucleate were compared between cigarette/alcohol users and snuff/alcohol users and cigarette/alcohol users and alcohol users. Conversely, an increase was observed when karyomegaly and binucleate were compared between snuff/alcohol users and alcohol users. Pyknosis significantly increased when compared between cigarette/alcohol users and snuff/alcohol users and cigarette/alcohol users and alcohol users. Similarly, nuclear halo significantly increased when compared between cigarette/alcohol users and alcohol users and snuff/alcohol users and alcohol users. There were no statistically significant differences in the median of karyolysis and repair index across all groups (Table 4.6).

Table 4.6: Pair wise comparison of nuclear changes and repair index across Cigarette/Alcohol users, Snuff/Alcohol users and Alcohol users

Nuclear changes	Cigarette/Alcohol users vs. snuff users		Cigarette/Alcohol users vs. Alcohol users		Snuff/Alcohol users vs. Alcohol users	
	X^2	P value	X^2	P value	X^2	P value
Pyknosis	7.689*	0.006	12.353*	0.000	1.929	0.165
Karyorrhexis	5.450*	0.020	28.196*	0.000	13.033*	0.000
Karyolysis	2.261	0.133	0.000	0.987	1.651	0.199
Karyomegaly	17.251*	0.000	7.921*	0.005	11.744*	0.001
Bi-nucleate	22.271*	0.000	3.148*	0.076	7.954*	0.005
Broken egg nuclei	1.261	0.261	1.544	0.214	0.104	0.709
Micronuclei	3.165	0.075	0.792	0.373	5.729*	0.017
Nuclear Halo	1.261	0.261	12.126*	0.000	23.755*	0.000
Repair index	1.517	0.218	0.408	0.523	0.333	0.564

* The Chi square is significant $p < 0.05$.

Table 4.7: Comparison of the immuno-staining pattern of ki-67, p53 and p16 amongst Cigarette/Alcohol users, Snuff/Alcohol users, Alcohol users and Control

There were statistically significant differences in the mean rank values of immuno-staining pattern of ki-67, p53 and p16 genes, ($p<0.05$) when compared across cigarette/alcohol users, snuff/alcohol users, alcohol users control (Table 4.7).

Table 4.8: Pair wise comparison of the immuno-staining pattern of ki-67, p53 and p16 between Control and Cigarette/Alcohol users, Snuff/Alcohol users and Alcohol users

In pair wise comparisons between control and cigarette users, snuff and alcohol users, p16 immuno-staining pattern increased significantly across all the groups. Mean rank ki-67 expression pattern increased significantly when control group was compared with cigarette and alcohol users, but not statistically significant in Control versus snuffer comparison. On the other hand p53 immuno-staining pattern increased significantly in control versus cigarette users comparison but were not significant in control versus snuff and alcohol users comparisons ($p>0.05$) (Table 4.8).

Table 4.7: Comparison of the immuno-staining pattern of ki-67, p53 and p16 amongst Cigarette/Alcohol users, Snuff/Alcohol users, Alcohol users and Control

Genes	Study group	N0 of subjects(n)	Mean Rank	P-value
	Cigarette/Alcohol users	42	89.82	
Ki-67	Snuff/Alcohol users	41	61.35	
	Alcohol users	22	86.27	0.000
	Control	40	59.98	
	Cigarette/Alcohol users	42	86.32	
P53	Snuff/Alcohol users	41	69.27	
	Alcohol users	22	77.00	0.014
	Control	40	60.64	
	Cigarette/Alcohol users	42	85.05	
P16	Snuff/Alcohol users	41	72.07	
	Alcohol users	22	107.14	0.000
	Control	40	42.53	

Significant level: $P<0.05$

Table 4.8: Pair wise comparison of the immuno-staining pattern of ki-67, p53 and p16 between Control and Cigarette/Alcohol users, Snuff/Alcohol users and Alcohol users

Study group	Cigarette/Alcohol users		Control vs. Snuff/Alcohol users		Control vs. Alcohol users	
Genes	Mean Rank	P value	Mean Rank	P value	Mean Rank	P value
Ki-67	29.846*	0.001	1.379	1.000	26.298*	0.023
P53	25.648*	0.010	8.631	0.100	16.362	0.565
P16	42.523*	0.000	29.548*	0.004	64.611*	0.000

* Significant level: $p < 0.05$.

Table 4.9: Pair wise comparison of the immuno-staining pattern of ki-67, p53 and p16 between Cigarette/Alcohol users, Snuff/Alcohol users and Alcohol users

There was increased statistically significant difference in the mean rank of ki-67 expression when compared between cigarette and snuff user groups and a significant decrease when snuff verses alcohol user groups were compared. In this same vein p16 expressions significantly decreased when mean rank of cigarette versus alcohol users and snuff versus alcohol user groups were compared. Conversely, mean rank expression of p53 immuno-staining pattern showed increased statistically significant difference only in the comparison of snuff versus alcohol user groups (Table 4.9).

Table 4.9: Pair wise comparison of the immuno-staining pattern of ki-67, p53 and p16 between Cigarette/Alcohol users, Snuff/Alcohol users and Alcohol users

Study Group	Cigarette vs. Snuff users		Cigarette vs. Alcohol users		Snuff vs. Alcohol users	
Genes	**Mean Rank**	**P value**	**Mean Rank**	**P value**	**Mean Rank**	**P value**
Ki-67	28.468*	0.000	3.549	0.695	-24.919*	0.006
P53	17.053*	0.035	9.321	0.336	-7.732	0.427
P16	12.974	0.131	-22.038*	0.032	-35.063*	0.001

* Significant level: $p < 0.05$.

Table 4.10a and b: Comparison of nuclear changes and repair index across duration of Cigarette /Alcohol use amongst the Cigarette users group

There were statistically significant differences ($p<0.05$) in the median values of Pyknosis, karyolysis micronuclei and repair index when compared across short term, middle term and long term duration (in years) of smoking amongst the cigarette user group. Conversely, the median values of karyorrhexis, karyomegaly, binucleate, broken egg nuclei and perinuclear halo had no statistically significant differences ($p>0.05$) (Table 4.9a and 4.9b).

Table 4.11: Comparison of nuclear changes and repair index across short term, middle term and long term Smoking/Alcohol use amongst the Cigarette /Alcohol users group

There were increased statistical significant differences ($p<0.05$) in the median values of Pyknosis and micronulei, when compared between short term and middle term and a decrease in middle term versus long term comparison. Similarly, karyolysis and repair index increased significantly in a comparison between short term and middle term and middle term and long term duration of smoking respectively (Table 4.11).

Table 4.12: Comparison of immuno-staining pattern of ki-67, p53 and p16 in duration of Cigarette /Alcohol use amongst Cigarette users group

There were no statistically significant difference in the mean rank values of ki-67, p53 and p16 genes expression, when compared amongst short term, middle term and long term duration of tobacco use (Table 4.12).

Table 4.10a: Comparison of nuclear abnormalities and repair index across duration of Smoking/Alcohol use amongst the Cigarette users group

Nuclear changes	Duration	Mean Rank	Median	P-value
Pyknosis	Short Term	11.17	1.00	
	Middle Term	32.00	4.00	0.000
	Long Term	20.83	3.00	
Karyorrhexis	Short Term	16.17	0.50	
	Middle Term	19.64	1.00	0.409
	Long Term	25.06	1.50	
Karyolysis	Short Term	17.00	1.00	
	Middle Term	31.45	2.00	0.000
	Long Term	17.28	1.00	
Karyomegaly	Short Term	22.88	1.00	
	Middle Term	16.86	1.00	0.340
	Long Term	22.28	1.00	

Significant level: $P<0.05$

Table 4.10 b: Comparison of nuclear changes and repair index across duration of Smoking/Alcohol use amongst the Cigarette users group

Nuclear changes	Duration	Mean Rank	Median	P-value
Bi-nucleate	Short Term	17.50	0.00	
	Middle Term	21.18	0.00	0.175
	Long Term	23.22	0.00	
Broken egg nuclei	Short Term	24.33	0.00	
	Middle Term	18.27	0.00	0.289
	Long Term	20.44	0.00	
Micronuclei	Short Term	26.50	4.00	
	Middle Term	9.27	3.00	0.005
	Long Term	24.50	4.00	
Nuclear halo	Short Term	13.83	1.00	
	Middle Term	18.82	1.00	0.330
	Long Term	27.11	1.00	
Repair index	Short Term	14.67	0.25	
	Middle Term	28.91	0.67	0.002
	Long Term	20.39	0.50	

Significant level: $P<0.05$

Table 4.11: Comparison of nuclear changes and repair index across short term, middle term and long term Cigarette/Alcohol use amongst the Cigarette/Alcohol users group

Nuclear changes	Short term vs. Middle term		Short term vs. Long term		Middle term vs. Long term	
	X^2	P value	X^2	P value	X^2	P value
Pyknosis	19.301*	0.000	1.733	0.188	12.900*	0.000
Karyolysis	5.282*	0.022	0.455	0.500	0.684	0.408
Micronuclei	11.244*	0.001	2.056	1.000	9.327*	0.002
Repair index	0.023	0.879	0.455	0.500	12.900*	0.000

* The chi sequare is significant p< 0.05.

Table 4.12: Comparison of immuno-staining pattern of ki-67, p53 and p16 in duration of Cigarette /Alcohol use amongst Cigarette users group

Genes	Study group	N0 of subjects(n)	Mean Rank	X^2	P-value
Ki-67	Short Term	12	25.08	2.728	0.255
	Middle Term	11	17.23		
	Long Term	19	21.71		
P53	Short Term	12	26.38	3.057	0.217
	Middle Term	11	19.18		
	Long Term	19	19.76		
P16	Short Term	12	16.25	5.670	0.059
	Middle Term	11	19.50		
	Long Term	19	25.97		

Significant level: P<0.05

Table 4.13a and b: Comparison of nuclear changes and repair index across duration of Snuff/Alcohol use amongst the Snuff users group

Medians values of pyknosis, karyolysis, karyomegaly, bi nucleate, micronuclei and repair showed statistically significant differences ($p<0.05$) when compared across short term, middile term and long term duration (in years) of inhalation of snuff amongst the snuff user group. Conversely, karyorrhexis, broken egg nuclei and nuclear halo were not statistically significant different ($p>0.05$) (Table 4.13a and 4.13b).

Table 4.14 Comparison of nuclear changes and repair index across short term, middle term and long term Snuff/Alcohol use amongst the Snuff/Alcohol users group

There were increased statistical significant differences ($p<0.05$) in the median values of Pyknosis, when compared between short term and middle term and a decrease in middle term versus long term inhalation of tobacco comparison. Karyomegaly on the other hand increased significantly when compared between short term and middle term, short term and long term but decreased in middle term versus long term tobacco inhalation comparison. Median values of karyolysis, binucleate and micronuclei similarly increased significantly when compared across short term and middle term, short term and long term and middle term and long term tobacco inhalation. The repair index values conversely decreased significantly when compared between short term and middle term but increased in short term versus long term and middle term versus long term tobacco inhalation comparison. There was no statistical significant difference in karyorrhexis, broken egg nuclei and nuclear halo when compared across duration of tobacco inhalation (Table 4.14).

Table 4.13a: Comparison of nuclear changes and repair index across duration of Snuff/Alcohol use amongst the Snuff users group

Nuclear changes	Duration	Mean Rank	Median	P-value
Pyknosis	Short Term	16.50	2.00	0.000
	Middle Term	37.00	4.00	
	Long Term	16.50	2.00	
Karyorrhexis	Short Term	7.50	1.00	0.108
	Middle Term	25.50	1.00	
	Long Term	24.52	1.00	
Karyolysis	Short Term	37.00	4.00	0.000
	Middle Term	28.00	3.00	
	Long Term	12.00	0.00	
Karyomegaly	Short Term	12.50	1.00	0.020
	Middle Term	26.50	2.00	
	Long Term	22.17	2.00	

Significant level: P<0.05

Table 4.13b: Comparison of nuclear changes and repair index across duration of Snuff/Alcohol use amongst the Snuff users group

Nuclear changes	Duration	Mean Rank	Median	P-value
Bi-nucleate	Short Term	5.00	0.00	
	Middle Term	23.50	2.00	0.011
	Long Term	26.28	2.00	
Broken egg nuclei	Short Term	18.50	0.00	
	Middle Term	18.50	0.00	0.108
	Long Term	22.96	0.00	
Micro-nuclei	Short Term	20.50	3.00	
	Middle Term	5.00	2.00	0.006
	Long Term	27.46	3.00	
Nuclei halo	Short Term	12.50	2.00	
	Middle Term	28.00	2.00	0.108
	Long Term	21.59	2.00	
Repair index	Short Term	28.00	1.33	
	Middle Term	37.00	2.00	0.000
	Long Term	12.00	0.33	

Significant level: $P<0.05$

Table 4.14 Comparison of nuclear changes and repair index across short term, middle term and long term Snuff/Alcohol use amongst the Snuff/Alcohol users group

Nuclear changes	Short term vs. Middle term		Short term vs. Long term		Middle term vs. Long term	
	X^2	P value	X^2		X^2	P value
Pyknosis	18.000*	0.000	2.319	0.128	16.099*	0.000
Karyolysis	18.000*	0.000	32.000*	0.000	32.000*	0.000
Karyomegaly	18.000*	0.000	8.568*	0.003	4.174*	0.041
Bi-nucleate	18.000*	0.000	4.900*	0.027	4.900*	0.027
Micronuclei	18.000*	0.000	5.692*	0.017	5.692*	0.017
Repair index	18.000*	0.000	16.099*	0.000	16.099*	0.000

* Significant level: $p < 0.05$.

Table 4.15: Comparison of immuno-staining pattern of ki-67, p53 and p16 in duration of Snuff/Alcohol use amongst Snuff/Alcohol users group

The mean rank values of p53 and p16 expression were statistically significantly different when compared amongst short term, middle term and log term duration of snuff use, while pki-67 expression pattern showed no statistically significant difference in the mean rank values when compared across duration of snuff use (Table 4.15).

Table 4.16: Pair wise comparison of immuno-staining pattern of p53 and p16 between Short term, Middle term and Long term Snuff/Alcohol users

Mean rank values of p53 expression increased significantly when compared between short term and middle term but dereased in a middle term versus long term duration of snuff use comparison. P16 gene expression on the other hand increased significantly when compared between short term versus long term and middle term versus long term duration of snuff use. There was no statistical significant difference in the mean rank values of ki-67when compared across duration of snuffing (Table 4.16).

Table 4.15: Comparison of immuno-staining pattern of ki-67, p53 and p16 in duration of Snuff/Alcohol use amongst Snuff/Alcohol users group

Genes	Duration	N0 of subjects(n)	Mean Rank	Chi-Square	P-value
Ki-67	Short Term	9	19.00	3.384	0.184
	Middle Term	9	19.00		
	Long Term	23	22.57		
P53	Short Term	9	14.50	31.207	0.000
	Middle Term	9	37.00*		
	Long Term	23	17.28		
P16	Short Term	9	12.00	23.126	0.000
	Middle Term	9	12.00*		
	Long Term	23	28.04		

*Significant level: P<0.05

Table 4.16: Pair wise comparison of immuno-staining pattern of p53 and p16 between Short term, Middle term and Long term Snuff/Alcohol users

Genes	Short term vs. Middle term		Short term vs. Long term		Middle term vs. Long term	
	Mean Rank	P value	Mean Rank	P value	Mean Rank	P value
P53	-22.500*	0.000	-2.783	0.471	19.717*	0.000
P16	0.00	1.000	-16.043*	0.000	-16.043*	0.000

*Significant level: P<0.05

Table 4.17a and b: Comparison of nuclear changesand repair index across the various age groups of the study subjects

Median values of pyknosis, karyolysis, karyomegaly, binucleate, broken egg nuclei, micronuclei and repair index were all statistically significanty different ($p<0.05$) when compared across various age groups of the study subjects. Karyorrhexis and nuclear halo conversely were not statistically significanty different ($p>0.05$) (Table 4.17a and 4.17b).

Table 4.18a and b: Pair wise comparison of the median of nuclear changes and repair index across the various age groups of the study subjects

There were decreased statistically significant differences in the median values of pyknosis when compared between 35-40 years and>50 years and 41-46 years and >50 years. Karyolysis on the other hand increased significantly, when compared between 35-40 years and 41-45 years, 35-40 years and>50 years, 41-46 years and>50 and 46 years and>50 years of age. Karyomegaly decreased significantly, when compared between 35-40 years and 41-45 years, 35-40 years and>50 years and 41-46 years and >50 years and 46 years and>50 years of age. Binucleate, micronuclei and repair index conversely increased significantly, when compared between 35-40 years and 41-45 years, 35-40 years and>50 years and 41-46 years and >50 years of age. Median value of micronuclei however, decreased significantly in a pair wise comparison between 41-46 years and >50 years of age (Table 4.18a and b).

Table 4.17a: Comparison of nuclear changes and repair index across the various age groups of the study subjects

Nuclear changes	Age group (years)	Mean Rank	Median	P-value
Pyknosis	35-40	61.71	4.00	0.003
	41-45	51.70	3.00	
	46-50	36.00	2.00	
	>50	30.82	2.00	
Karyorrhexis	35-40	41.64	1.00	0.105
	41-45	42.50	1.00	
	46-50	27.17	1.00	
	>50	48.40	1.00	
Karyolysis	35-40	58.00	2.00	0.000
	41-45	44.80	1.00	
	46-50	30.50	1.00	
	>50	38.84	1.00	
Karyomegaly	35-40	61.18	2.00	0.006
	41-45	23.90	1.00	
	46-50	44.17	2.00	
	>50	43.79	2.00	

Significant level: P<0.05

Table 4.17b: Comparison of nuclear changes and repair index across the various age groups of the study subjects

Nuclear changes	Age group (years)	Mean Rank	Median	P-value
Bi-nucleate	35-40	23.29	0.00	
	41-45	45.40	1.00	0.000
	46-50	24.50	0.00	
	>50	55.43	2.00	
Broken egg nuclei	35-40	33.50	0.00	
	41-45	41.80	0.00	0.000
	46-50	61.17	1.00	
	>50	37.16	0.00	
Micro-nuclei	35-40	32.50	3.00	
	41-45	41.30	3.00	0.001
	46-50	56.50	4.00	
	>50	39.93	3.00	
Nuclear halo	35-40	51.86	2.00	
	41-45	42.60	1.00	0.175
	46-50	34.83	1.00	
	>50	40.75	1.00	
Repair index	35-40	56.43	1.00	
	41-45	44.40	0.67	0.001
	46-50	17.50	0.25	
	>50	45.46	0.71	

Significant level: P<0.05

Table 4.18a: Pair wise comparison of the median values of nuclear changes and repair index across the various age groups of the study subjects

Nuclear changes	35-40 vs. 41-45		35-40 vs. 46-50		35-40 vs. >50	
	X^2	P value	X^2	P value	X^2	P value
Pyknosis	1.943	0.163	2.778	0.096	25.412*	0.000
Karyolysis	3.173	0.075	29.000*	0.000	5.876*	0.015
Karyomegaly	8.374*	0.004	6.473*	0.011	0.746	0.388
Bi-nucleate	7.323*	0.007	0.077	0.782	19.765*	0.000
Broken egg nuclei	3.173	0.075	14.246*	0.000	1.318	0.251
Micronuclei	7.323*	0.007	14.246*	0.001	4.561*	0.033
Repair index	8.993*	0.003	29.000*	0.000	3.630	0.340

* The Chi square is significant $p < 0.05$.

Table 4.18b: Pair wise comparison of the median values of nuclear changes and repair index across the various age groups of the study subjects

Nuclear changes	41-45 vs. 46-50		41-45 vs. >50		46-50 vs. >50	
	X^2	P value	X^2	P value	X^2	P value
Pyknosis	0.163	0.686	18.179*	0.000	0.914	0.339
Karyolysis	7.778*	0.005	0.007	0.932	8.647*	0.003
Karyomegaly	7.778*	0.005	6.631*	0.010	4.218*	0.040
Bi-nucleate	7.778*	0.005	4.731*	0.030	20.753*	0.000
Broken egg nuclei	7.778*	0.005	1.394	0.238	17.866*	0.000
Micronuclei	15.200	0.221	1.069	0.301	7.048*	0.008
Repair index	13.686*	0.000	16.213*	0.000	0.506	0.447

* The Chi square is significant p< 0.05.

Table 4.19: Comparison of immune-staining pattern of ki-67, p53 and p16 amongst the various age groups of the study subjects

There were statistically significant differences in the mean rank gene expression of ki-67 and p53 genes when compared amongst the different age groups while the mean rank gene expression of p16 was not statistically significantly different in the comparison across age groups (Table 4.19).

Table 4.20a and b: Pair wise comparison of immuno-staining pattern of ki-67, and p53 and amongst the various age groups of the study subjects

The mean rank expression pattern of ki-67and p53 decreased significantly in a pair wise comparison between 35-40 verses 46-50 and 35-40 verses > 50 years of age (Table 4.20a and b)

Table 4.19: Comparison of immune-staining pattern of ki-67, p53 and p16 amongst the various age groups of the study subjects

Genes	Study group	N0 of subjects(n)	Mean Rank	P-value
Ki-67	35-40	14	70.68	
	41-45	20	62.00	
	46-50	15	45.50*	0.008
	>50	56	47.38	
P53	35-40	14	72.14	
	41-45	20	64.10	
	46-50	15	49.67	0.002
	>50	56	45.14	
P16	35-40	14	52.50	
	41-45	20	52.60	
	46-50	15	40.50	0.288
	>50	56	56.62	

*Significant level: P<0.05

Table 4.20a: Pair wise comparison of immuno-staining pattern of ki-67, and p53 and amongst the various age groups of the study subjects

Age	35-40 vs. 41-45		35-40 vs. 46-50		35-40 vs. >50	
Genes	**Mean Rank**	**P value**	**Mean Rank**	**P value**	**Mean Rank**	**P value**
Ki-67	8.679	0.357	25.179*	0.012	23.304*	0.004
P53	8.043	0.398	22.476*	0.027	27.000*	0.001

*Significant level: P<0.05

Table 4.20b: Pair wise comparison of immuno-staining pattern of ki-67, and p53 and amongst the various age groups of the study subjects

Age	41-45 vs 46-50		41-45 vs >50		46-50vs >50	
Genes	Mean Rank	P value	Mean Rank	P value	Mean Rank	P value
Ki-67	16.500	0.074	14.625	0.038	-1.875	0.811
P53	14.433	0.122	18.957	0.008	4.524	0.569

*Significant level: P<0.05

Table 4.21: Association of nuclear changes with ki-67, p53 and p16 expression

There were significant positive associations between pyknosis, karyomegaly and nuclear halo and ki-67, p53 and p16 expression. Karyorrhexis and karyolysis had positive association with p16 and p53 gene expression respectively. Similarly binucleate and broken egg nuclei had significant positive association only with p16 gene expression. Besides, there was significant association between repair index and p16 expression, while ki-67and p53 were non significant (Table 4.21).

Table 4.21: Association of nuclear changes and repair index with ki-67, p53 and p16 expression

Nuclear changes	Ki-67		P53		P16	
	R	P value	R	P value	R	P value
Pyknosis	0.263*	0.001	0.444*	0.000	0.274*	0.001
Karyorrhexis	0.139	0.094	-0.051	0.542	0.235*	0.004
Karyolysis	0.103	0.214	0.297*	0.000	0.018	0.832
Karyomegaly	0.282*	0.001	0.331*	0.000	0.327*	0.000
Bi-nucleate	-0.116	0.164	-0.127	0.126	0.318*	0.000
Broken egg nuclei	-0.081	0.329	-0.045	0.593	0.165*	0.46
Micronuclei	0.338*	0.000	0.116	0.162	0.421*	0.000
Nuclear Halo	0.377*	0.000	0.507*	0.000	0.238*	0.004
Repair index	-0.055	0.577	0.188	0.055	-0.240*	0.014

*. Correlation is significant at $p < 0.05$

Photomicrographs of Papanicolaou stained buccal smears of the study subjects illustrating pyknosis, karyorrhexis, karyolysis, karyomegaly, bi-nucleate, broken egg nuclei, micronuclei and nuclear halo were shown bellow (Plate 4.1-4.7). Similarly, ki-67, p53 and p16 immno-positive test smears and ki-67, p53 and p16 immno-positive control slides were as shown (Plate 4.8- 4.10).

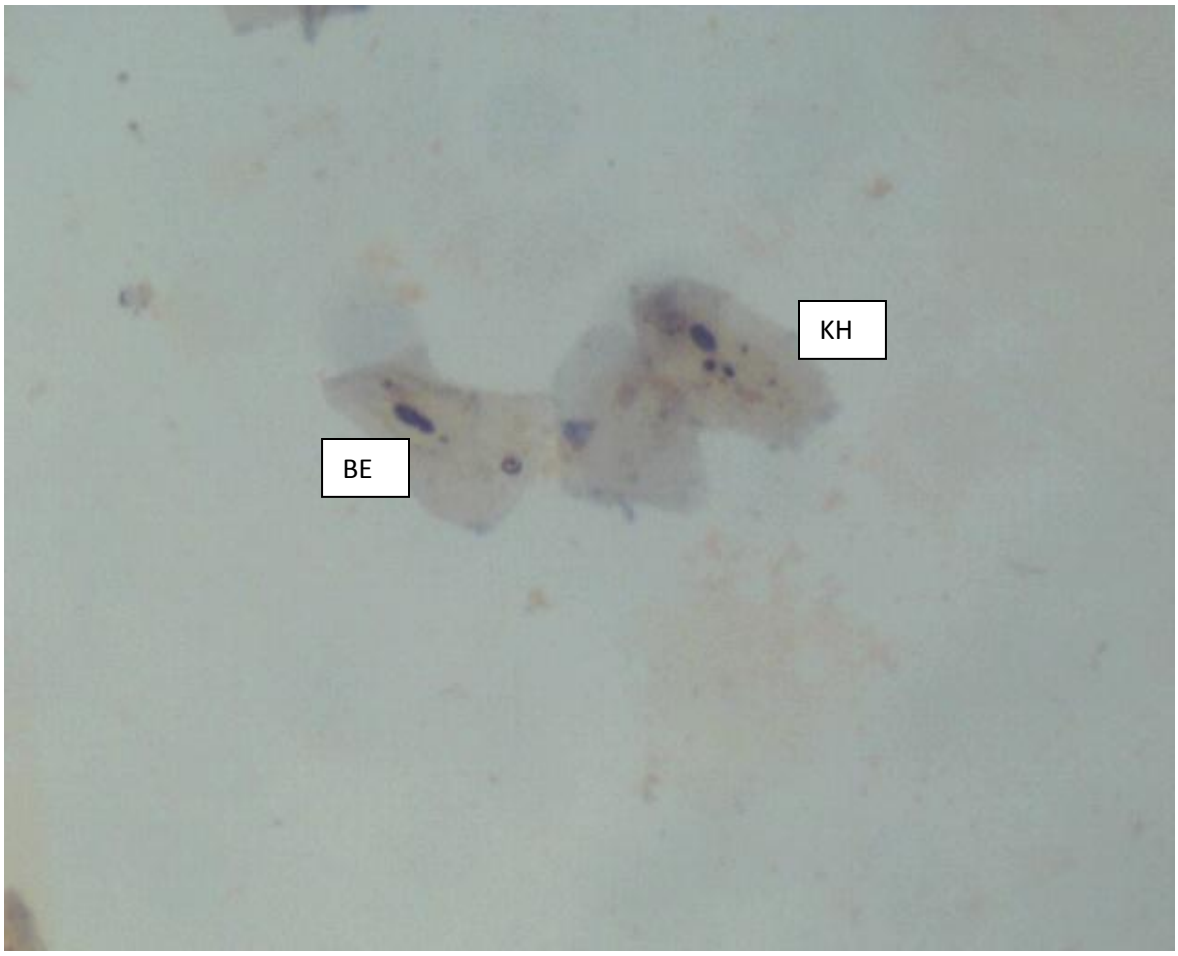


Plate 4.1: Photomicrograph of buccal smear showing broken egg nucleus (BE) and karyorrhexis (KH) (X 100, PAP Method)

Oral mucosal smears show cell with broken egg nucleus (BE) and progressive nuclear fragmentation (KH).

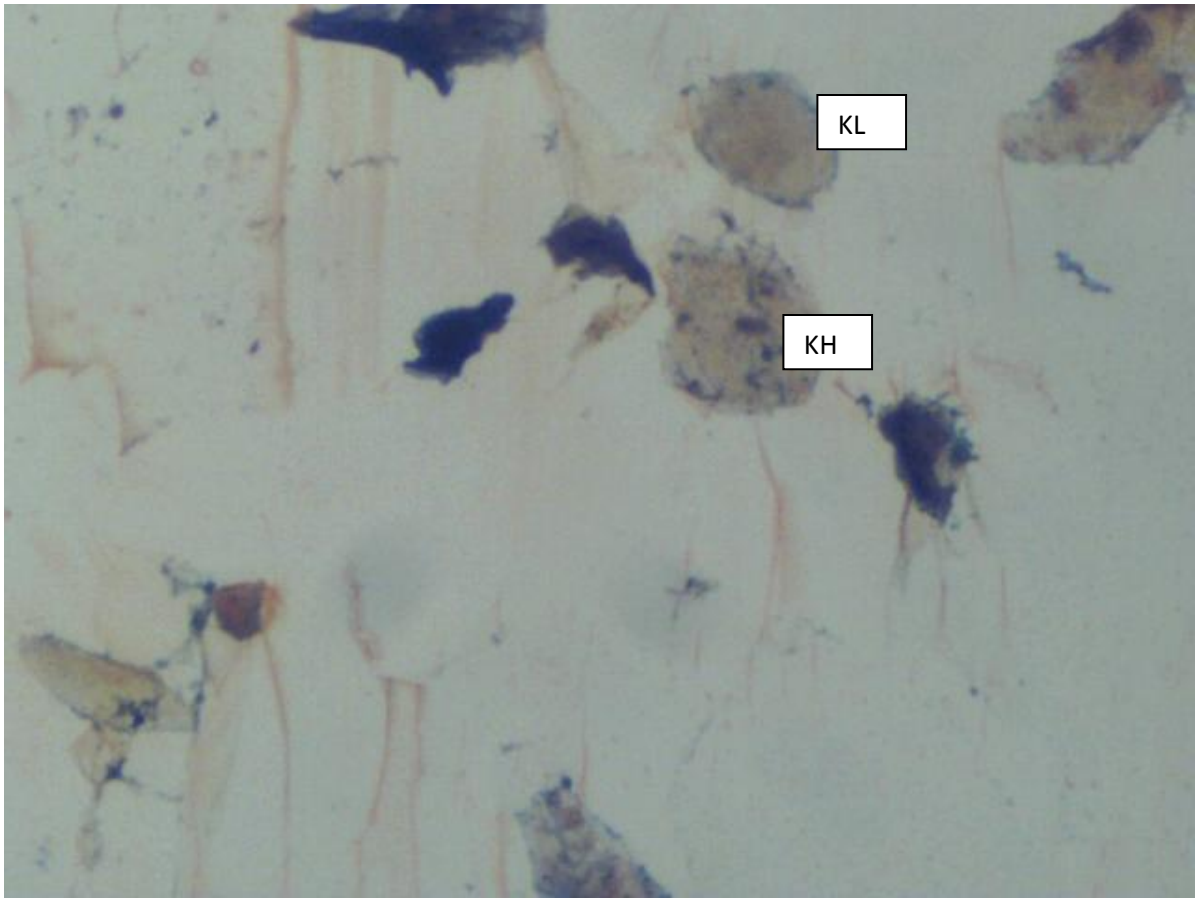


Plate 4.2: Photomicrograph of buccal smear showing karyorrhexis (KH) and karyolysis (KL) (X 100, PAP Method)

Oral mucosal smear shows cells with with progressive nuclear fragmentation (KH) and dissolusion (KL).

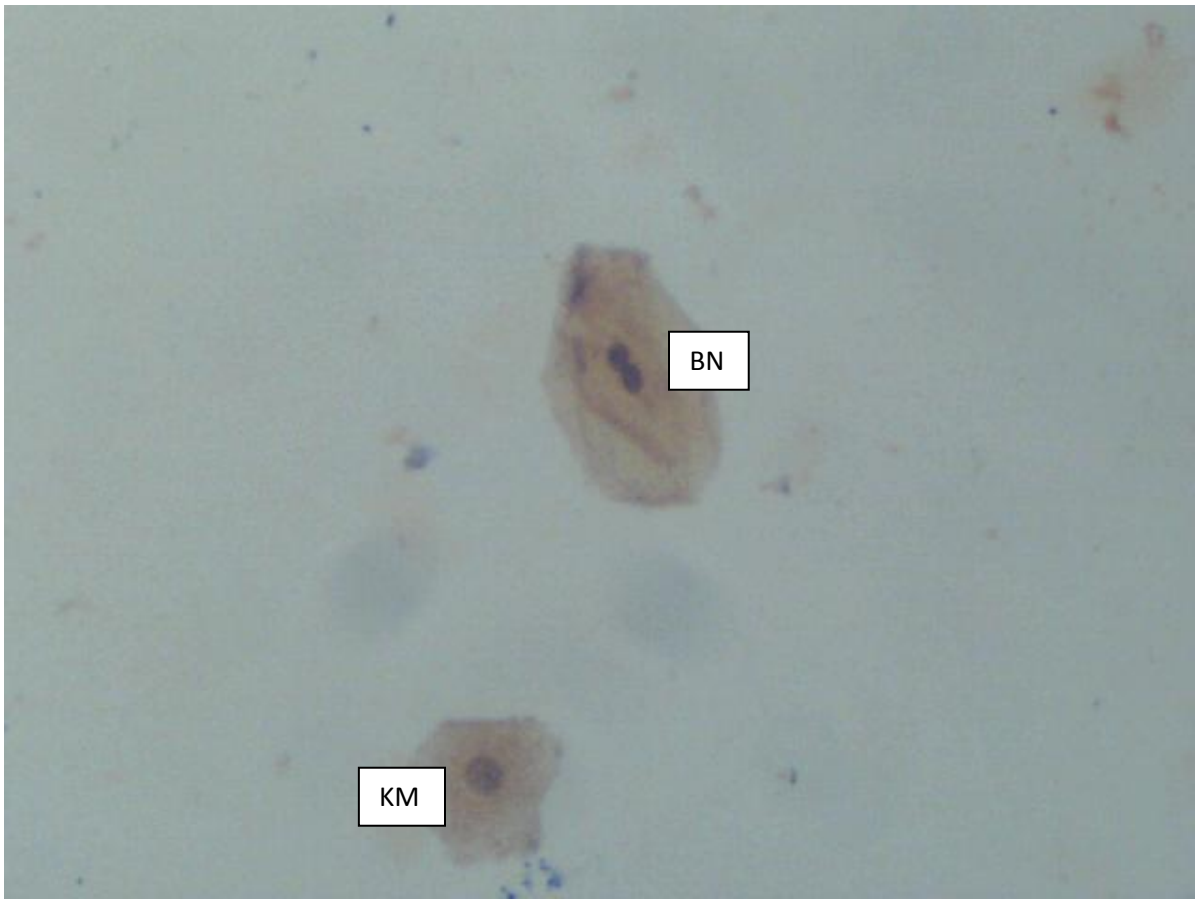


Plate 4.3: Photomicrograph of buccal smear showing bi-nucleate (BN) karyomegally (KM) (X 100, PAP Method)

Oral mucosal smears show cells with double nuclei (BN) and enlarged nucleus (KM).

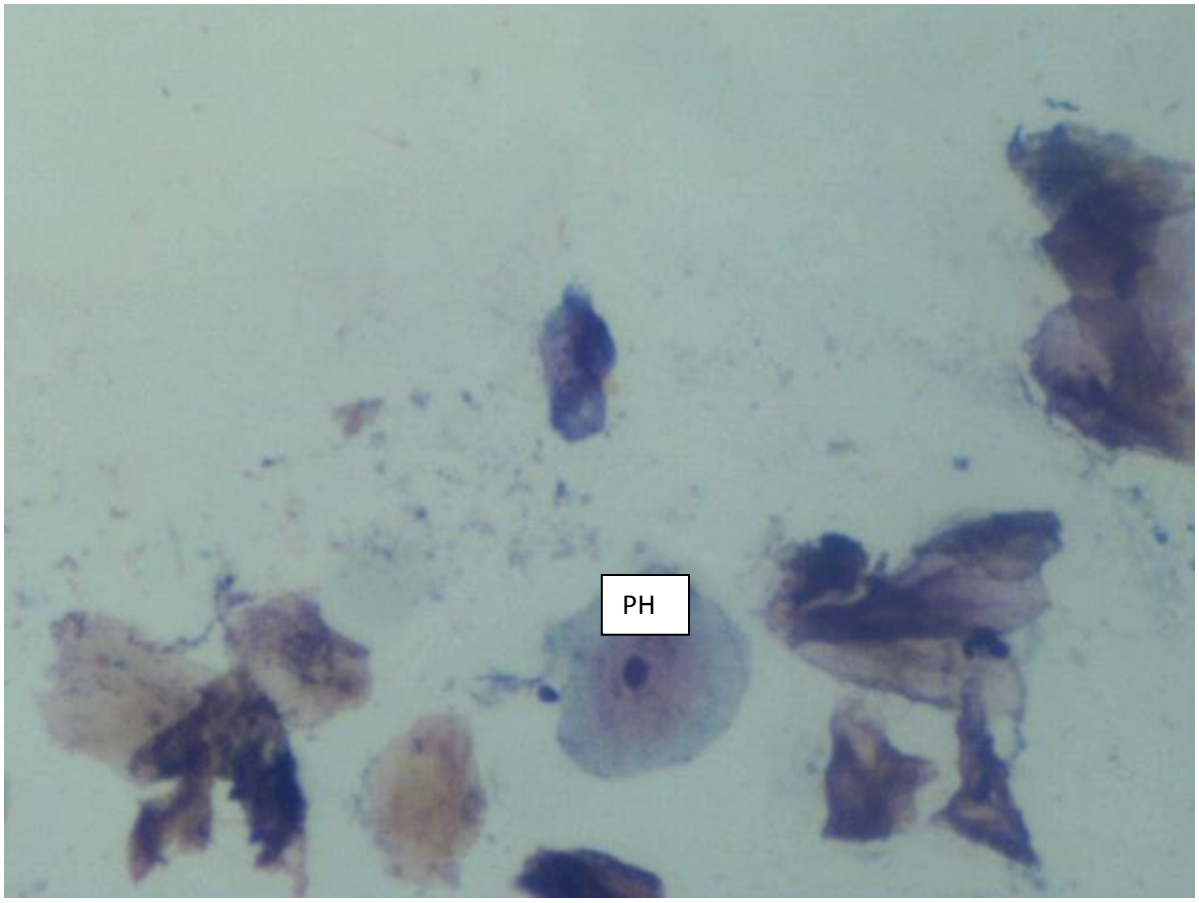


Plate 4.4: Photomicrograph of buccal smear showing peri nuclear clearing (PH) (X 100, PAP Method)

Oral mucosal smear shows stained cells one of which has a nucleus with clearing around the nuclear membrane (PH).

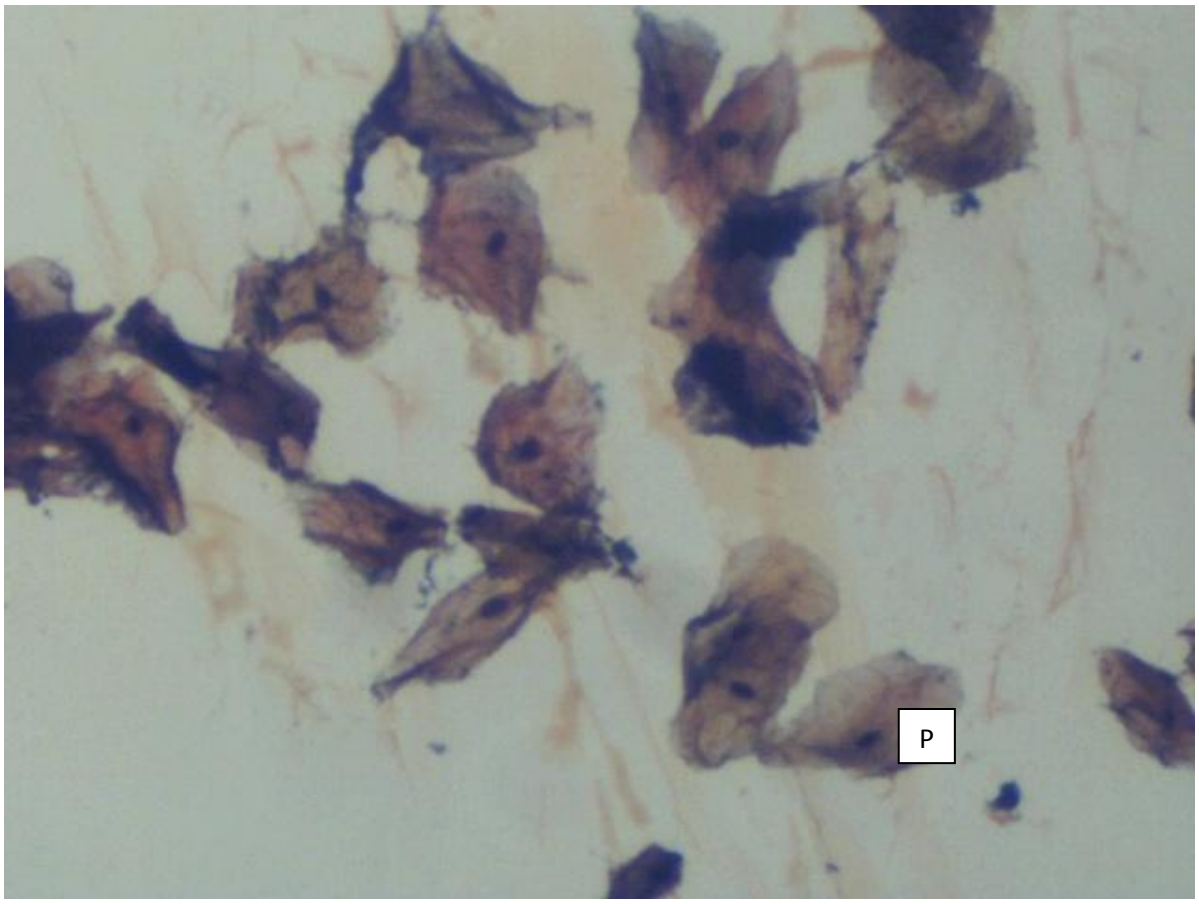
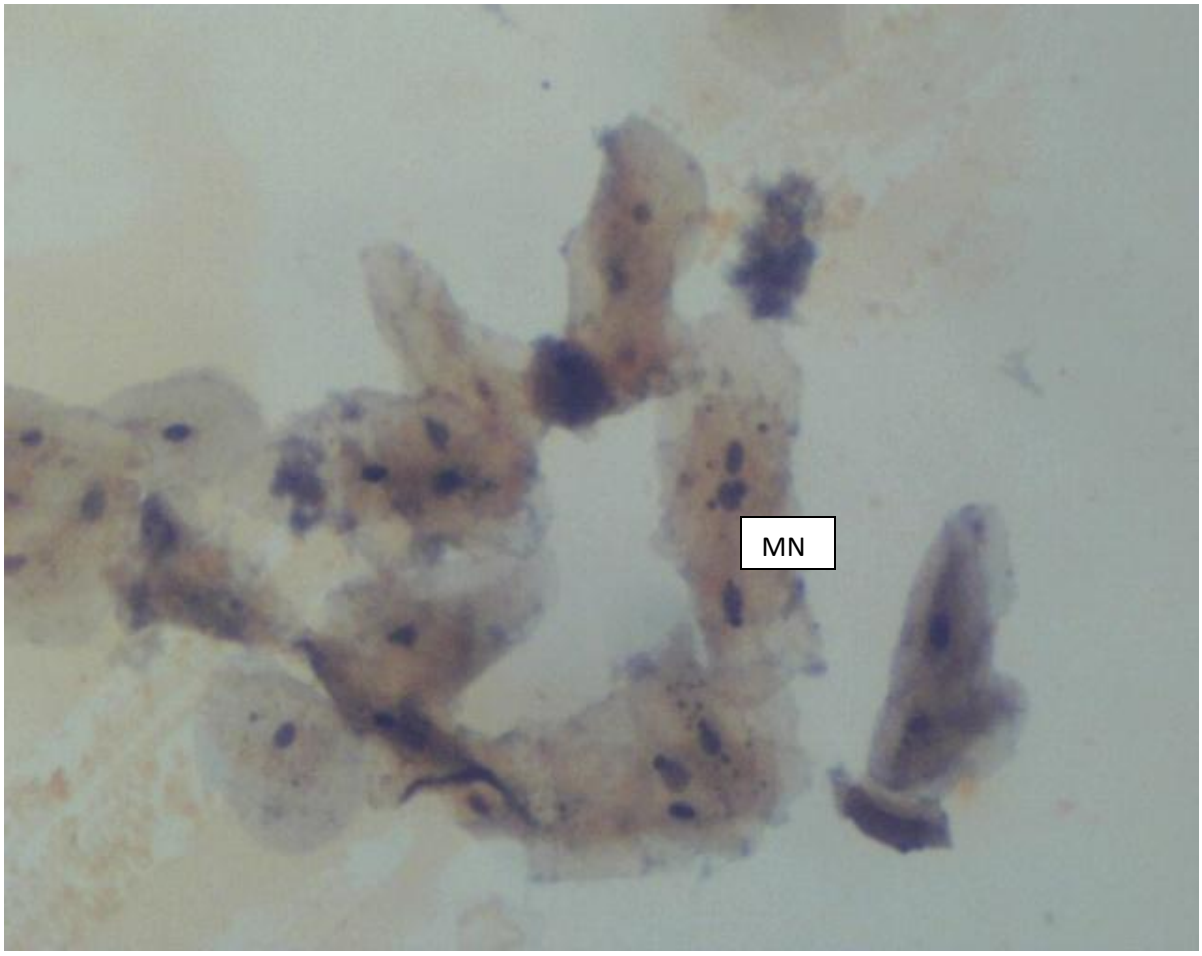


Plate 4.5: Photomicrograph of buccal smear showing pyknotic nucleus (P) (X 100, PAP Method)

Oral mucosal smear show stained cells with some of them undergoing progressive nuclear condensation (P). This is evident when compared with the nuclear size of the other cells.



Pate 4.6: Photomicrograph of buccal smear showing micronucleus (MN) (X 100, PAP Method)

Oral mucosal smears show stained cells with prominent micronuclei (MN).

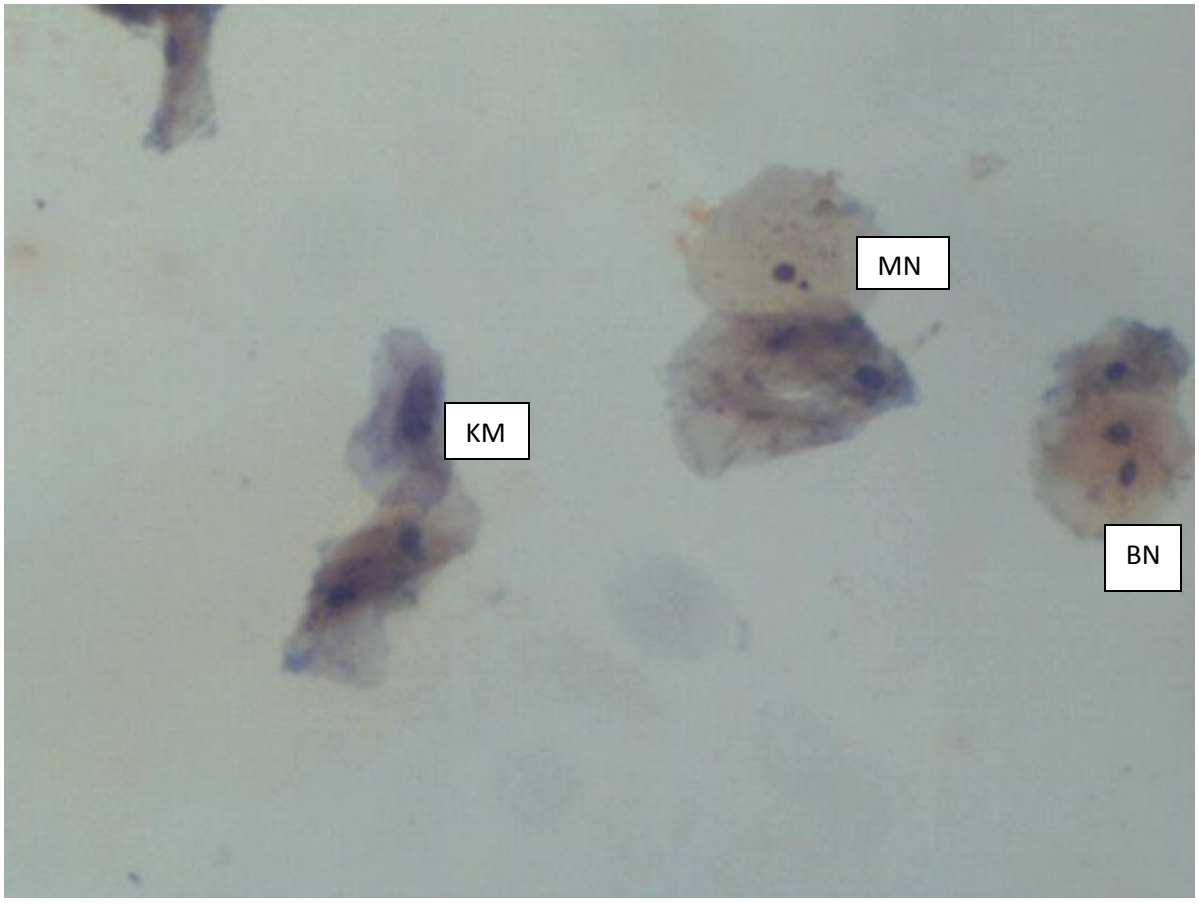


Plate 4.7: Photomicrograph of buccal smear showing Karyomegaly (KM) Binucleate (BN) and Micronucleus (X 100, PAP Method)

Oral mucosal smear shows stained cells with highly enlarged nucleus (KM), micronucleus (MN) and duplication of nuclei within a cell (BN).

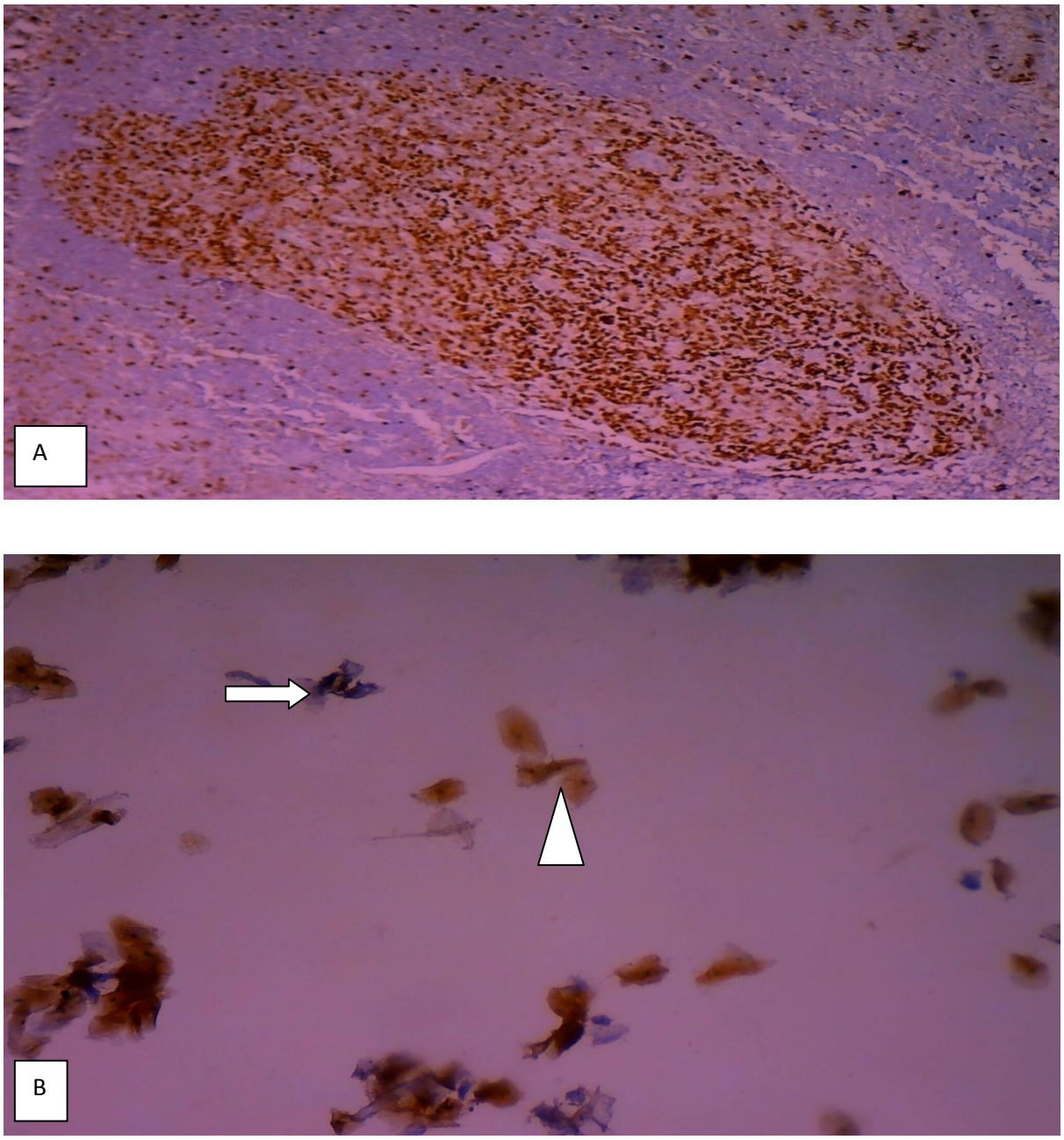


Plate 4.8: Photomicrograph of Ki-67 immuno reactivity (A) Positive control slide (B) Test smear (smoker group) (x 100).

Plate 4.8 shows a ki-67 positive control slide (A) with the positive cells showing brown colour and test smear (B) showing ki-67 immuno reactive cells (brown: arrow head) and non reactive cells (blue: arrow).

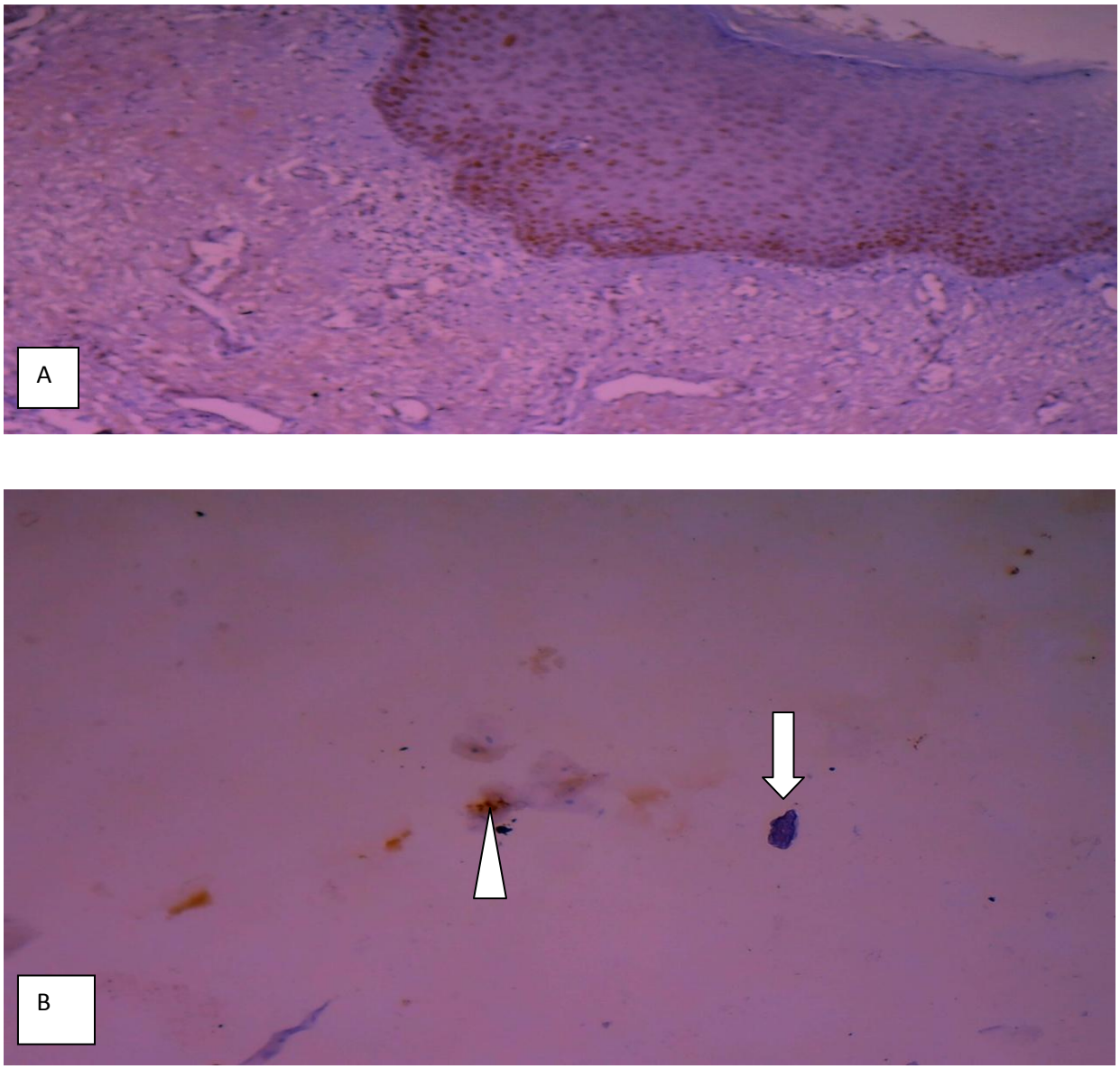


Plate 4.9: Photomicrograph of p53 immuno reactivity (A) positive control slide (B) Test smear (snuffer group) (x 100).

Plate 4.9 shows a p53 positive control slide (A) with the positive cells showing brown colour and test smear (B) showing p53 immuno reactive cells (brown: arrow head) and non reactive cells (blue: arrow).

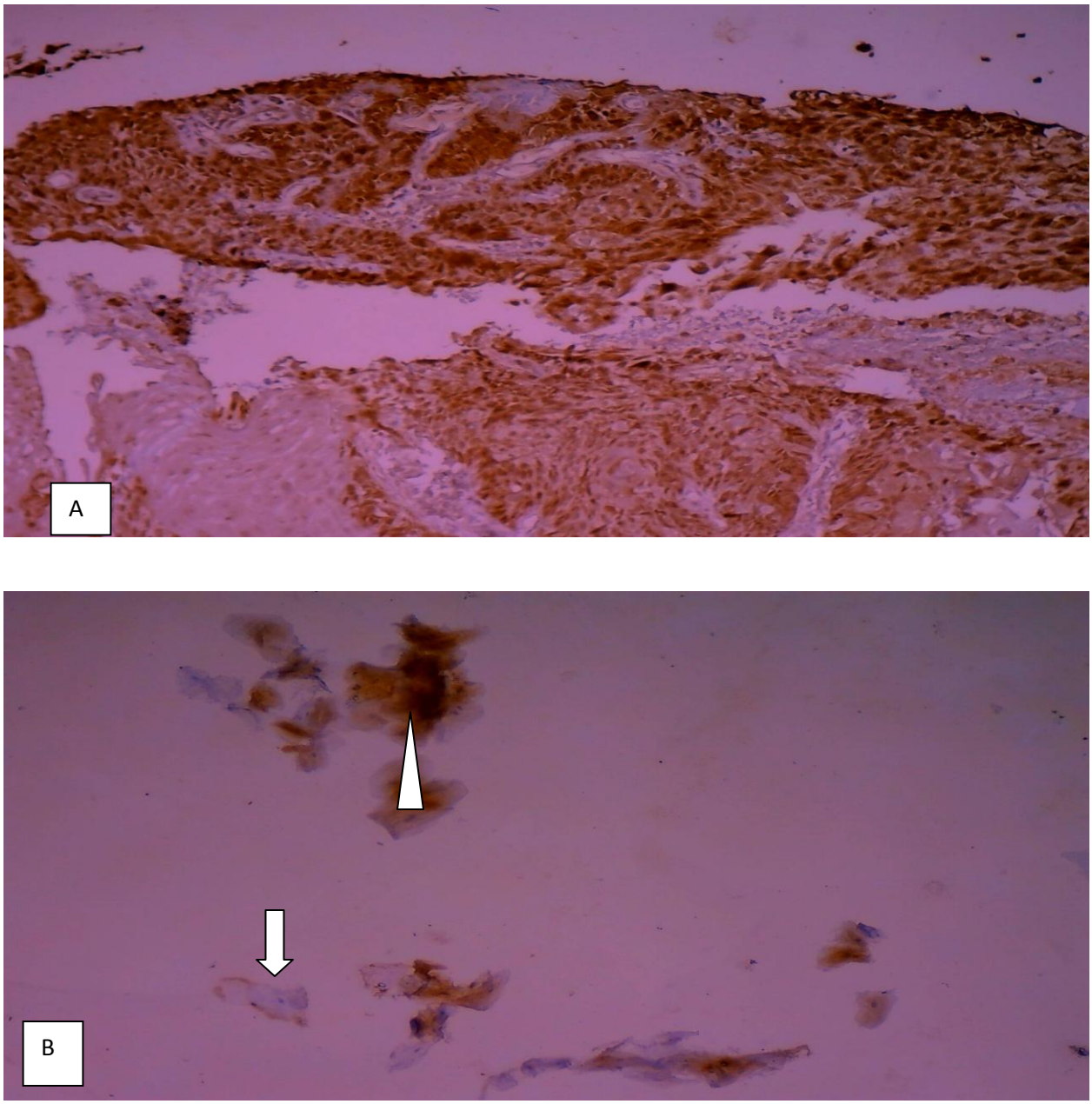


Plate 4.10: Photomicrograph of p16 immuno reactivity (A) positive control slide (B) Test smear (Alcohol drinker group) (x 100).

Plate 4.10 shows a p16 positive control slide (A) with the positive cells showing brown colour and test smear (B) showing p16 immuno reactive cells (brown: arrow head) and non reactive cells (blue: arrow).

CHAPTER FIVE

DISCUSSION

Buccal mucosal nuclear changes in subjects habitually and occupationally exposed to risk factors of oral lesions have been studied by a good number of researchers (Farhadi *et al*., 2016b; Singh *et al*., 2014; Biswas *et al*., 2014; Pautassi *et al*., 2010; Johnson *et al*., 2000; Khlifi *et al*., 2013). However, none seemed to have targeted the study population and the genes, which form the focus of the present study .The habitual use of tobacco and alcohol amongst commercial bus drivers in Nigeria, though yet to be properly documented, is a common knowledge. Mohan (2010) earlier associated tobacco use; alcohol consumption and HPV infection with oral squamous cell carcinoma and according to him constitute the major risk factors of oral neoplasia.

The present study reported highest percentage of participants within the age group of 50 years and above and majority of participants being smokers. Though most of the earlier studies paid little attention to age distribution, it could be noted here that majority of participant being within the age group of 50 years and above. This is likely related to the nature of the study population, who were mainly intra State transports. Sequel to advancing age, a good number of them preferred intra State routes to the youth dominated inter State routes. The report more so corroborated that of Khlifi *et al* (2013) who in a study of the cytogenetic abnormality in exfoliated buccal epithelial cells of head and neck cancer (HNC) patients in a Tunisian population, reported that 60% of study subjects were ≥ 50 years of age. It should also be noted that out of the 105 study subjects recruited, only 22 of them

neither smoke nor inhale snuff, but drink alcohol (drinkers only). Those who smoke cigarette and inhale snuff also drink alcohol. The two vices are usually practiced together.

Pyknosis (P), karyorrhexis (KH), karyolysis (KL), karyomegaly (KM), binucleate (BN), broken egg nuclei (BE), micronuclei (MN) and nuclear halo (NH) were the nuclear changes reported in the oral mucosal cells of the subjects, with micronuclei being the most prevalent while snuff using subjects were the most affected. This report corroborates partly the report of Farhadi *et al* (2016b) who reported higher occurrence of micronuclei in a study that involved smokers and non smokers. Besides Farhadi *et al* (2016b), Khlifi *et al* (2013) reported significantly elevated frequency of micronuclei and binucleated cells in the buccal mucosa of in head and neck cancer patients when compared with healthy controls. Khlifi *et al* (2013) further reported a significant association of MN with tobacco smoking and chewing, and alcohol consumption. Saranya and Sudha (2014) in a similar study on the cytomorphological changes in buccal epithelial cells of khaini chewers in different age groups also observed various types of nuclear changes hence, the report agreeing with findings of the present study. These authors report of karyomegaly in the buccal cells of khaini (a product of tobacco) chewers also agreed with the finding of the present study. The nuclear changes herein reported also corroborates with a much earlier study by Proia *et al* (2006) who reported that diverse buccal cellular changes are associated with smoking and smokeless tobacco. Furthermore, Biswas *et al* (2014) reported that condensed chromatin (CC), karyolysis (KL), bi-nucleation and pyknosis were the nuclear anomalies most frequently associated with tobacco smoking and chewing, thus strongly agreeing with the report of the present study. In view of this, it could deduced that tobacco and alcohol consumption induce varying nuclear changes in the oral mucosal cells of their consumers.

The high prevalence of nuclear changes amongst tobacco consumers (cigarette smokers and snuffers) though not directly reported in earlier studies by other authors, agree partly with the finding of Farhadi *et al* (2016b). The authors, who did not report the prevalence of these changes amongst tobacco smokers reported very high occurrence of them amongst smokers when compared to non smokers. The prevalence of nuclear changes, though highest amongst snuffers, was only very slightly higher than that of smokers.

This finding disagrees with the report of Khlifi *et al* (2013) who observed higher occurrence of nuclear changes amongst tobacco chewers than cigarette smokers. It could be explained by the fact that the cigarette filters, which filter out some toxic agents of tobacco thereby preventing direct contact with oral mucosa, could be akin to the nasal hairs and mucous secretion of epithelial cells, which trap some toxic agents of tobacco, preventing also direct contact with the oral mucosa. This is not the case with tobacco chewers, whose oral mucosal cells are directly exposed to all the toxic substances of tobacco. Sequel to this, it could be inferred that both cigarette smoking and nasal inhalation of tobacco (snuffing) impact similar genotoxic effects on the oral mucosal cells. The relatively lower prevalence of nuclear changes amongst alcohol drinkers when compared to smokers and snuffers could be partly as a result of combined effect of tobacco and alcohol and partly due to reduced number of drinkers when compared to smokers and snuffers. This study also reported 100% prevalence of micronuclei, 97% of pyknosis and high prevalence of all other detected abnormalities. This agrees with most earlier studies (Farhadi *et al*., 2016b; Singh *et al*., 2014; Biswas *et al*., 2014; Pautassi *et al*., 2010; Johnson *et al*., 2000; Khlifi *et al*., 2013). The relatively high prevalence of micronuclei in the control group (70%) did not wholly agree with the earlier report of Farhadi *et al* (2016b) and Khlifi *et al* (2013).This finding though surprising is not unexplainable. This may be attributed to passive exposure to other agents of genotoxicity such as passive smoking, petrochemical products to mention but a few.

The present study nonetheless, reported significantly high prevalence of oral mucosal cell nuclear changes in the test subjects when compared with control subjects. This undoubtedly infers the genotoxic and/or carcinogenic effects of both tobacco and alcohol on the oral mucosal cells, thereby agreeing with Mohan (2010) who reported tobacco and alcohol use as the major risk factor of oral squamous cell carcinoma. It could also be inferred from the finding that, though Micronucleus assay is a well validated method for testing genotoxic effects of various agents being the only biomarker that allows the simultaneous evaluation of both clastogenic and aneugenic effects in a wide range of cells, it should be used in conjunction with other biomarkers.

Furthermore, there were statistically significant differences when the median values of nuclear changes and repair index were compared between control and smoker, snuffer and alcohol drinker groups and this agrees with earlier reports (Khilif *et al*., 2013; Farhadi *et al*., 2016b). These authors reported statistically significant differences in the mean distribution of pyknosis, karyolysis, karyorrhexis, bi-nucleate, broken egg nuclei, micronuclei and repair index, when compared in control and tobacco user group, which were also seen in this study. As afore noted, these forms of nuclear changes are validated biomarkers of cellular injury and genotoxicity and their significant increase in test subjects when compared to control is a clear indication that tobacco and alcohol are agents of genotoxicity. The higher value of repair index in control and more so, the increased statistically significant difference in the median, when compared with the test groups corroborates with the above assertion. This implies that, though mild forms of nuclear changes (resulting maybe from inflammatory reactions) may be present in the buccal mucosa of non users of tobacco and alcohol, the tendency of repair and reversal to normal cells is higher than in the dysplastic cells seen amongst tobacco and alcohol users which more likely will progress from atypical to neoplastic cells, especially with continued cell damage. This could be better explained if one notes that repair index,

which is the ratio of the sum of karyorrhexis and karyolysis and broken egg nuclei and micronuclei (*RI=KR+KL/BE+MN*) (Farhadi *et al.,* 2016) predicts the likelihood of neoplastic cellular transformation and invariably measures the degree of cellular damage. An inverse relationship therefore, exists between repair index and the likelihood of neoplastic cellular transformation. In the light of this, therefore, it could be substantiated that tobacco and alcohol induced genotoxic effects on oral cells of the users. It is noteworthy, however, that a threshold level for repair index is yet to be determined and will be the focus of further study.

The study reported significantly increased pyknosis, karyolysis, broken egg nuclei and micronuclei amongst cigarette smokers when compared with snuffers. This may not conclusively infer more genotoxic and/or mutagenic effect of smoked tobacco compared with smokeless tobacco. This assertion could be held true owing to the fact that karyorrhexis, karyomegaly, bi-nucleate and peri nuclear halo (other biomarkers of genotoxicity) were more prevalent amongst snuffers, who also had highest overall number of nuclei anomalies. However, the increased statistically significant difference of the medians of pyknosis, karyomegaly and bi-nucleate when compared between smokers and snuffers tend to support the fact that tobacco smoking slightly predispose more risk to oral lesions than snuffing. This point could further be buttressed with the lower mean rank value of repair index of smokers, though there was a non significant difference in the distribution of their medians in a pair wise comparison within the groups. This holds true also for alcohol drinkers, who though with the least number of subjects had comparable prevalence of nuclear changes.

Ki-67, p53 and p16 genes were reported expressed in different patterns in all the study groups with p16 being the most commonly expressed gene. This finding agrees partly with the reports of (Singh *et al*., 2016; Ghanghoria *et al*., 2015; Gissi *et al*., 2015; Yang *et al*., 2015; Zargaran *et al*., 2013; Lewis *et al*., 2012; Humayun and Prasad, 2011; Riechelmann, 2010; Westra, 2009; Klieb and Raphael 2007; Adegboyega *et al*., 2005; Jamaroon *et al*., 2003; Schliephake, 2003).

It should be recalled that the earlier report of Gissi *et al* (2015) in a longitudinal study to evaluate the predictive role of p53 and Ki-67 proteins alone or in combination, in a group of subjects with oral leukoplakia (OL) without dysplasia noted that the presence of either high p53 expression or low/normal p53 expression associated with high Ki67 expression is considered high and correlated with increased risk of transformation of OL to oral SCC. The expression of these proteins, which are known biomarkers of genotoxicity, in the oral epithelial cells of tobacco and alcohol users, more unequivocally affirmed gentoxic and/or mutagenic effect of tobacco and alcohol, thereby establishing the role they play in the etiopathogenesis of oral lesions. Also reported in the present study is co-expression of ki-67, p53 and p16 in both smoker and alcohol drinker groups, ki-67/p53 and ki-67/p16 in smoker and snuffer groups respectively whereas p53 and p16 were co-expressed in snuffer group only. This agrees wholly with the report of Humayun and Prasad (2011) and Jamaroon *et al* (2003). These authors in separate studies observed and reported co-expression of ki-67/p53 genes in dysplastic, premalignant and malignant oral cavity lesions. Ghanghoria *et al* (2015) in an earlier study reported expression of p53 gene in oral cancers, thus also agreeing to an extent with the present report. George (2011) reported that p53 gene is the most commonly mutated genes in human cancers and that alone may explain

expression of this gene in users of tobacco and alcohol which have been known for their mutagenic ability.

Furthermore, the more prevalent expression of p16 gene in the study groups may be seen to support the report of Adeyemi *et al* (2011). The authors in a study of the relationship between socioeconomic status, tobacco and alcohol use to oral squamous carcinoma noted that most patients with the lesion do not use tobacco or alcohol and are of low socioeconomic status and therefore, attributed etiology to HPV infection. It should be emphasized here that Lingboa e*t al* (2003) observed p16 promoter hypermethylation and apparent loss of p16 protein expression in 27% and 74% of head and neck squamous cell carcinoma, respectively with history of alcohol or tobacco being significantly correlated with the loss of the protein expression. The report of this present study does not agree with that of Lingboa *et al* (2003) since ki-67, p53 and p16 were all expressed amongst tobacco users and alcohol drinkers, with p16 gene expression being found in 72% of the participants. It could be said that though, there is higher expression of p16, compared with ki-67 and p53 it may not be absolutely connected to oral nuclear abnormalities, since p16 protein is expression is always connected to HPV infection. However, HPV infection on the other hand causes oral cellular dysplasia which in turn may induce mutation and over amplification of p53 and ki-67**.** The expression of the trio genes amongst tobacco and alcohol users may be said to synergistic. It is noteworthy, however, that the association of HPV infection and p16 expression in oral SCC, which also correlates with favorable prognosis, has been reported (Kuo *et al*., 2008; Riechelmann, 2010).

It could therefore, be deduced from the present study, that combined immunohistochemical expression of p53 and Ki-67 proteins or single expression of either gene could be a useful and simple molecular biomarker for early detection of non-dysplastic oral lesions at risk of developing oral cancer.

Expression patterns of ki-67, p53 and p16 genes revealed almost the same trend across smoker, snuffer and alcohol drinker groups with much more cellular immuno positivity when compared to control group. Statistical significant difference in the mean rank of gene expression patterns were reported when study groups were compared with control. Ki-67 and p53 genes were more expressed in smoker/alcohol drinker, followed by alcohol drinkers and snuffer/alcohol drinker, while p16 was the most expressed amongst alcohol drinker group. Surprisingly but interestingly, snuffer/alcohol drinker group, though, with the highest prevalence of nuclear abnormalities had the least gene expression patterns. This could be explained by the fact that though, all nuclear abnormalities are indicators of cell injury, some, such as karyorrhexis, karyolysis, micronucleus and broken egg nucleus, more unequivocally determines genotoxicity and therefore, amplification and over expression of these nuclear proteins. Sequel to this, it could be inferred that nuclear genotoxic effects of tobacco and alcohol were responsible for their amplification and hence, expression. All the genes were least expressed in the control group. There were statistically significant differences in the mean rank expression patterns when control was compared with study groups. This therefore, confirms the role of tobacco and alcohol as agents in buccal cell tumourigenesis. Moreover, with the more expression pattern of ki-67, p53 and p16 genes, which were adjudged biomarkers of neoplastic transformation, amongst smokers than

snuffers, one could easily infer that tobacco smokers are more at risk of oral lesions than snuffers. This agrees with the findings of Khilif *et al* (2013) and Farhadi *et al* (2016b). The association of nuclear changes with ki-67, p53 and p16 genes expression as evidenced in the significant positive correlation of a good number of the nuclear changes and the genes expression patterns, further validates the inference. It is noteworthy also, that though statistically significant associations were not established between ki-67 and p53 and repair index, a negative association exist between p16 and repair index Suffice it to state that the observed nuclear changes vis-à-vis gene expression pattern may not be specifically associated to smoking or snuffing only, as both groups were also drinkers of alcohol. Genotoxicity of buccal mucosal cells have been reported more with exposure to multiple mutagenic agents (Khlifi *et al*., 2013). This among others things may explain the reason for increased percentage occurrence of nuclear changes and gene expression pattern amongst the smoker/alcohol drinker and snuffer/alcohol drinker groups relative to alcohol drinker group.

A comparison of the median values of nuclear changes between short term, middle term and long term tobacco use, showed statistically significant differences in all nuclear changes except karyorrhexis, broken egg nuclear and nuclear halo. It was noted that there was progressive increase in the number of nuclear changes from short term to middle term and a slight decrease in long term use. This report did not wholly agree with that of Khilif *et al* (2013) who found and reported a progressive increase in cellular abnormalities from short term to long term exposure to genotoxic agents. These authors however, did not consider middle term. The progressive increasing pattern of nuclear changes from short term to

middle term and followed by a slight decrease in long term tobacco use, may be explained by the usual behavior of injured cells. When cells at normal steady state are subjected to noxious stimuli and are injured, they initially try to adapt by any of the cellular adaptation mechanisms. However, with continued stimuli and increased concentration of the agents, more severe effects are impacted to the cells which tend to involve more cells. These cells expectedly progress from reversibly injured cells to irreversibly injured cells, with more cellular involvement at the middle term, with a slight decline at long term, which may eventually progress to cell death, with continued exposure. The statistically significant difference in median values of virtually all the detected nuclear changes when compared amongst duration of usage in both groups and in multiple comparisons between groups validates the assertion. It should however be noted the non significant difference in the median of the repair index when compared amongst different duration exposure to both substances may suggest that short term, middle term and long term exposure have seemly, equal likelihood of cellular transformation or otherwise. This may further be explained by the non consistent expression patterns of ki-67, p53 and p16 genes across duration of exposure to tobacco.

The present study further reported a near consistent pattern of direct proportional relationship between nuclear changes and age. This agrees with the finding of Suhda *et al* (2014) who reported a direct proportional relationship between increased age and increased cytoplasmic diameter in a study cytomorphological changes in buccal epithelial cells of khaini chewers in different age groups. Statistically significant differences exist when the median values of the nuclear changes were compared amongst age groups. Comparisons

between 35-40 years verse >50 years and 41-46years verses > 50 years revealed statistically significant differences in nearly all detected nuclear changes and repair index. In view of this finding one could deduce that advanced age coupled with exposure to tobacco and/or alcohol increases buccal cellular changes vis-à-vis increased risk of oral lesions. This also corroborates the already known epidemiology of oral squamous carcinoma which according to Mohan (2010) is most prevalent amongst older adults. The expression pattern of the genes though not clearly supporting this also could be said to lend explanation to the assertion.

5.1 Conclusion

It could be concluded from the findings of this present study that tobacco and/or alcohol use induced nuclear changes on the buccal mucosal cells of their users. Tobacco smoking impacted more severe effect than snuff inhalation, while combined use of tobacco and alcohol aggravated cellular damage. Ki-67, p53 and p16 genes were all expressed amongst smokers, snuffers and alcohol drinkers with the expression pattern very high when compared to non users of tobacco and alcohol. Tobacco smokers had more gene expression whereas p16 expression pattern was the most prevalent amongst the three genes. A significant association existed between increased occurrence of nuclear changes and expression of ki-67, p53 and p16 genes with ki-67, p53 and p16 co-expressing on smokers. There was a consistent increasing pattern of percentage occurrence of nuclear changes with increased number of years of use of tobacco and/or alcohol. The direct proportional relationship between age, coupled with exposure to mutagens, and the rate of cellular damage was also observed. Repair index decreased with increase in the occurrence of nuclear changes.

5.2: Recommendations

In view the findings of the present study, it is recommended that individuals who consume tobacco and/or alcohol should routinely undergo buccal smear screening test, especially older adults. Further studies should be carried out to determine the nature and level of mutations, and associate them with gene expression, repair index and specific nuclear changes. The use of repair index as a predictive indicator of neoplastic cellular transformation should be standardized and harmonized. Ki-67, p53 and p16 should be included in screening of oral lesions haven been found useful as predictive and prognostic biomarkers.

5.3: Contributions to Knowledge

The present study made the following contributions to existing knowledge:

1. That the prevalence of oral nuclear changes is high on tobacco and alcohol consumers in our environment.
2. That ki-67, p53 and p16 genes are all expressed in dysplastic buccal cells and therefore, could be used either singly or in combination to monitor cellular progression from atypia to malignancy. They can be used as predictive and prognostic biomarkers of oral lesions.
3. That there is an association between ki-67, p53 and p16 genes expression with nuclear changes.
4. Repair index decreased with increase in the occurrence of nuclear abnormalities and therefore, could scientifically predict the fate of damaged cells.

5. That immunoctochemical staining can be performed on liquid based cytology smears and should be used routinely as an ancillary for diagnostic cytology.

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Appendix I

Ethical Approval

FACULTY OF HEALTH SCIENCES AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
NNAMDI AZIKIWE UNIVERSITY, NNEWI CAMPUS
P.M.B 5001, NNEWI, ANAMBRA STATE-NIGERIA

OFFICE OF THE DEAN

*Our Ref:*_____ *Your Ref:*_____ *Date:* 9th February, 2017

SAMUEL IFEDIORANMA OGENYI
Reg. No: 2012617012P
Department of Medical Laboratory Science
Faculty of Health Sciences & Technology
Nnamdi Azikiwe University
Nnewi Campus.

Dear Samuel,

RE: PREVALENCE OF CELLULAR ABNORMALITIES AND SOME GENE EXPRESSION PATTERN IN THE ORAL CAVITY OF COMMERCIAL BUS DRIVERS USING TOBACCO AND ALCOHOL IN NNEWI

We write to inform you that after due consideration of your research proposal, approval is hereby conveyed for you to commence the study.

Best wishes in your research endeavours.

Thank you.

Yours Sincerely,

Dr. J.O. Umunnah
(Chairman)
For FHST Ethical Committee.

Appendix II

Questionnaire

A QUESTIONNAIRE ON THE PREVALENCE OF NUCLEAR ABNORMALITIES AND GENE EXPRESSION PATTERN OF KI-67, P53 AND P16 IN THE ORAL CAVITY OF COMMERCIAL BUS DRIVERS USING TOBACCO AND ALCOHOL IN NNEWI

Dear Respondent,

I am a PhD student of Medical Laboratory Science Department, Nnamdi Azikiwe University, Nnewi Campus, currently carrying out a project on **"PREVALENCE OF NUCLEAR ABNORMALITIES AND GENE EXPRESSION PATTERN OF KI-67, P53 AND P16 IN THE ORAL CAVITY OF COMMERCIAL BUS DRIVERS USING TOBACCO AND ALCOHOL IN NNEWI"**

It is a research intended for award of academic degree. Moreover, the result of this study will be of immense benefit both to you in particular and to humanity in general. Your participation and honest responses will be appreciated and treated with utmost confidence. You may wish not to write your name or address on the questionnaire. How these may be needed for follow up study. Once again, be assured that information supplied will be treated with utmost confidentiality.

Thanks for your kind attention and anticipated co-operation and participation.

INSTRUCTION:- Write or Tick ( ) in the box provided below where appropriate.

Biodata

1. Name (Optional)

2. ID No

3. Sex: (a) male ( ) (b) female ( )

4. Age: (a) 25-30( ) (b) 30-35( ) (c) 35-40 ( ) (d) 40-45 ( ) (e) 45-50 ( ) (f) >50 ( )

5. Marital Status: (a) Single ( ) (b) Married ( )

6. No of children …….

7. Highest School attended; Primary ( ) Secondary ( ) Tertiary ( ) others (specify)

8. State of origin----------

9. LGA ----------

Risk factor data

10. Do you smoke cigarette? Yes ( ) No ( )

11. If yes how long have you smoked: less than 1 year ( ), 2-5 years ( ), 5-10 years ( ), greater than 10years ( ).

12. Please indicate no of sticks per day ( ).

13. Do you smoke any other thing apart from cigarette? Yes ( ) No ( )

14. If yes, indicate type of substance----------

14. Do you use snuff? Yes ( ) No ( )

15. If yes, indicate method of application. Inhalation ( ), Oral application ( ), Chewing of leaf ( )

16. How long have you used snuff: less than 1 year ( ), 2-5 years ( ), 5-10 years ( ), greater than 10years ( ).

17. Do you drink alcoholic beverage? Yes ( ) No ( )

18. If yes, indicate type of drink. Beer ( ), Spirit/Gin/Hot ( ), others, Specify ( )

19. How long have you been drinking: less than 1 year ( ), 2-5 years ( ), 5-10 years ( ), greater than 10years ( ).

20. Please indicate no of bottles/shuts/ glasses per day ( ).

21. Please indicate below which chronic condition(s) you have:

Diabetes ( ) Asthma ( )

Emphysema or COPD ( )

Other lung disease Type of lung disease:----------

Heart disease Type of heart disease:----------

Arthritis or other rheumatic disease Specify type: ----------

Cancer Type of cancer: ------ ----------

Other chronic condition Specify: ----------

22. Please indicate below which symptom(s) you have

a. weakness ( )

b. Dizziness ( )

c. Pain in the mouth ( )

d. Bleeding gum ( )

e. White or red patches in the mouth ( )

f. constant headache ( )

g. Others specify ( )

Phone Number (optional) ----------

Appendix III

INFORMED CONSENT

Dear Sir,

My name is Samuel Ifedioranma Ogenyi, a PhD student of Medical Laboratory Science Department, Nnamdi Azikiwe University, Nnewi Campus. I am currently carrying out a study on **"PREVALENCE OF CELLULAR ABNORMALITIES AND GENE EXPRESSION PATTERN OF KI-67, P53 AND P16 IN THE ORAL CAVITY OF COMMERCIAL BUS DRIVERS USING TOBACCO AND ALCOHOL IN NNEWI"**

PURPOSE OF THE STUDY

To determine cellular abnormalities, ki67, p53 and p16 gene expression patterns amongst commercial bus drivers in Nnewi Anambra State.

PROCEDURE

The procedure involves gently scraping the oral (mouth) cavity using a wooden spatula, staining the smear with Papanicoloau staining technique and immuncytochemical methods.

INCLUSION CRITERIA

All commercial bus drivers who use tobacco and/or drink any form of alcoholic beverage will be included in this study.

EXCLUSION CRITERIA

All commercial bus drivers who do not use tobacco and/or drink any form of alcoholic beverage or has been previously diagnosed of any cancer/ and or chronic diseases will be excluded in the study.

VOLUNTARISM

One has right to agree or decline from participating in the study, and also has the right to withdraw from participating in the study at any time. However, I will appreciate your decision to take part in this study.

CONFIDENTIALITY

Be assured that any information provided during the course of this research will be treated with utmost confidentiality, and will be used only for the research purpose. Your name and address is optional. It will be needed if you need to get the result of the investigation.

RISK/ BENEFIT

No risk involved.

The participant will be able to know his oral cytology. This will aid in informed decision risks of oral cancer amongst the study group. The result of this research may identify biomarkers for oral cancer screening amongst high risk population.

Thank you for accepting to participate in the study.

Name/Id no: ----------

Signature---------- **Date** ----------

Appendix IV

Frequency of nuclear changes amongst Smoker group

N0 of cells	P	KH	KL	KM	BN	BE	MN	NH	TOTAL 315
0	0	20	0	4	21	33	0	13	70
	(0%)	(47.6%)	(0%)	(9.5%)	(50.0%)	(78.6%)	(0%)	(31.0%)	(22.2%)
1	9	22	23	15	11	9	0	12	101
	(24.1%)	(52.4%)	(54.8%)	(35.7%)	(26.2%)	(21.4%)	(0%)	(28.6%)	(32.1%)
2	8	0	11	23	10	0	4	8	64
	(19.0%)	(0%)	(26.2%)	(54.8%)	(23.8%)	(0%)	(9.5%)	(19.0%)	(20.3%)
3	9	0	8	0	0	0	20	9	46
	(21.4)	(0%)	(19.0%)	(0%)	(0%)	(0%)	(47.6%)	(21.4%)	(14.6%)
4	12	0	0	0	0	0	18	0	30
	(28.6)	(0%)	(0%)	(0%)	(0%)	(0%)	(42.9%)	(0%)	(9.5%)
5	4	0	0	0	0	0	0	0	4
	(9.5%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(1.3%)
Total	42	22	42	38	21	9	42	29	245
	(100%)	(52.4%)	(100%)	(90.5%)	(50%)	(21.4%)	(100%)	(69%)	(77.8%)

Appendix V

Frequency of nuclear changes amongst Snuffer group

N0 of cells	P	KH	KL	KM	BN	BE	MN	NH	TOTAL (328)
0	0 (0%)	10 (24.4%)	17 (41.5%)	5 (12.2%)	5 (12.2%)	36 (87.8%)	0 (0%)	5 (12.2%)	78 (23.8%)
1	5 (12.2%)	26 (53.4%)	12 (29.3%)	10 (24.4%)	5 (12.2%)	5 (12.2%)	0 (0%)	10 (24.4%)	73 (22.3%)
2	24 (58.5%)	0 (0%)	(0 (0%)	12 (29.3%)	19 (46.3%)	0 (0%)	7 (17.1%)	21 (51.2%)	83 (25.3%)
3	5 (12.2%)	5 (12.2%)	7 (17.1%)	7 (17.1%)	12 (29.3%)	0 (0%)	24 (58.5%)	5 (12.2)	65 (19.8%)
4	7 (17.1%)	0 (0%)	5 (12.2%)	7 (17.1%)	0 (0%)	0 (0%)	5 (12.2%)	0 (0%)	24 (7.3%)
5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (12.2%)	0 (0%)	5 (1.5%)
Total	41 (100%)	31 (75.6%)	24 (59.5%)	36 (87.8%)	36 (87.8%)	5 (12.2%)	41 (100%)	36 (87.8%)	250 (76.2%)

Appendix VI

Frequency of nuclear changes amongst Drinker group

N0 of cells	P	KH	KL	KM	BN	BE	MN	NH	TOTAL (156)
0	3 (13.6%)	0 (0%)	0 (0%)	14 (63.6%)	0 (0%)	20 (90.9%)	0 (0%)	10 (45.5%)	27 (17.3%)
1	14 (63.6%)	10 (45.5%)	10 (45.5%)	4 (18.2%)	12 (54.5%)	2 (9.1%)	0 (0%)	12 (54.5%)	64 (41.1%)
2	2 (9.1%)	12 (54.5%)	12 (54.5%)	4 (18.2%)	10 (45.5%)	0 (0%)	0 (0%)	0 (0%)	40 (25.6%)
3	3 (13.6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	10 (45.5%)	0 (0%)	13 (8.3%)
4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	12 (54.5%)	0 (0%)	12 (7.7%)
5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	(0.0%)
Total	19 (86.4%)	12 (54.5%))	12 (54.5%))	8 (36.4%)	22 (100%)	2 (9.1%)	22 (100%)	12 (54.5%)	129 (82.7%)

Appendix VII

Frequency of nuclear changes amongst Control group

N0 of cells	P	KH	KL	KM	BN	BE	MN	NH	TOTAL (295)
0	23 (57.5%)	18 (45%)	26 (65%)	27 (67.5%)	22 (55%)	40 (100%)	12 (30%)	40 (100%)	183 (62.03%)
1	17 (42.5%)	22 (55%)	14 (35%)	13 (32.5%)	12 (30%)	0 (0%)	22 (55%)	0 (0%)	112 (37.95%)
2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	6 (15%)	0 (0%)	4 (10%)	0 (0%)	10 (3.4%)
3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0.0%)
4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (5%)	0 (0%)	2 (0.7%)
5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	(0.0%)
Total	17 (42.5%)	22 (55%)	14 (35%)	13 (32.5%)	18 (45%)	0 (0%)	28 (70%)	0 (0%)	112 (37.95%)