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Human papillomavirus infection and oral cancer: a case-control study

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Statement of originality

When I became involved with this project in 1996, only one case-control study had been published on human papillomavirus infection (HPV) and oral cancer, and this study had several weaknesses. The original protocol for the present project was prepared by Drs. Rolando Herrero, Nubia Muñoz, and Silvia Franceschi at the International Agency for Research on Cancer (IARC) after discussions with several of the principal investigators responsible for the individual centres, including Montreal. I participated in the conception of the study for Montreal, together with Drs. Eduardo Franco and Jocelyne Feine, in the discussion of the protocol, as well as in modifications to the questionnaire, many of which were in fact incorporated into the design of the IARC study. I participated in the preparation of the grant application that was funded by the National Cancer Institute of Canada (NCIC) to carry out this investigation.

I was responsible for the research in Montreal. I was the study coordinator, contacting the main clinics in Montreal which treat and follow up patients with oral cancer. I coordinated the process of obtaining ethical approval for the project in the various hospitals involved in the project. I coordinated with the participating clinicians the best ways to identify eligible patients and to contact them, as well as the best circumstances to interview participating patients and to obtain all the clinical samples. I also coordinated with other clinics in participating hospitals the best way to recruit control subjects. I was responsible for the training of the research nurses who participated in the project on all aspects of questionnaire administration and collection of clinical samples. Finally, I prepared and maintained the database and conducted all data analyses.

My unique contribution to the design of this study was to propose the use of the PGMY09/11 set of PCR primers for detection of HPV DNAas an exclusive technical addendum to the Montreal investigation. This decision was taken because of my belief that this protocol is the most sensitive and reliable for HPV testing. Of the laboratory results produced by the IARC this study only used the HPV testing in biopsies, whereas examination of serological response and HPV DNA in oral exfoliated cells was done specifically for this project.

Several studies have analyzed oropharyngeal cancers independently of buccal cancers, following an anatomical criterion. To my knowledge, there is no published case-control study that grouped cancers of the palatine and lingual tonsils, following a histopathological criterion, as I did in this project. Regarding the analysis of HPV serology and oropharyngeal cancer, only one study has been published, and it appeared last year.

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Abstract

Introduction

Human papillomavirus (HPV) has been detected with varying frequency in oral cancers and in normal oral tissues. The main objective of the present study was to examine the association between HPV infection and risk of developing oral cancer.

Methodology

This investigation, as a component of an international multi-centre study coordinated by the IARC, followed a hospital-based case-control design. Cases consisted of newly diagnosed patients with primary squamous cell carcinoma of the oral cavity, including mouth and oropharynx. Controls were frequency matched to cases by sex, age, and hospital. All subjects were interviewed to elicit detail information on known and putative risk factors.

Oral exfoliated cells were collected from all subjects for detection of HPV DNA using the PGMY09/11 PCR protocol. Antibodies against HPV 16, 18, and 31 capsids were detected in patients' plasma using an immunoassay technique. Logistic regression was used for estimation of odds ratios (ORs) and 95% confidence intervals (CI) of oral cancer for HPV and other candidate risk factors.

Results

A total of 72 cases and 129 controls were recruited. HPV DNA was detected in 19% of cases (14 out of 72), and in 5% of controls (6 out of 129). Analysis for cancers related to Waldeyer's ring (palatine tonsil and base of tongue) showed that the OR of disease for detection of high

risk HPV types was 19.32 (95%CI:2.3-159.5), after adjustment for socio-demographic characteristics, tobacco and alcohol consumption. The adjusted OR of disease for HPV 16 seropositivity was 31.51 (95%CI:4.5-219.7). Analysis for non tonsillar oral cancers showed that the OR for detection of high risk HPV DNA in oral cells and for seropositivity were 2.14 (95%CI:0.4-13.0) and 3.16 (95%CI:0.8-13.0), respectively.

Discussion

The results from this study provide evidence supporting a strong association between HPV infection and cancers of the oropharynx, especially those arising from Waldeyer's ring. On the other hand, the association with non tonsillar oral cancers was of much lower magnitude. The biological evidence establishing a firm etiologic link remains to be established for the latter subsites, whereas the association between HPV and Waldeyer's ring carcinomas is consistent with a causal link.

Résumé

Introduction

La prévalence de détection du virus du papillome humain (VPH) chez des individus atteints de cancer oral est variable et les études cas-témoins portant sur le VPH et le cancer oral n'ont pas fourni de résultats cohérents. L'objectif principal de cette étude était d'examiner l'association entre l'infection par le VPH et le risque de développement du cancer oral.

Méthodologie

Ce projet, une composante d'une étude internationale multicentrique coordonnée par le Centre international de recherche sur le cancer (CIRC), était basé sur un devis d'étude cas-témoins menée dans un hôpital. Les cas étaient des patients venant d'obtenir un diagnostic de carcinome primaire des cellules épidermoïdes de la cavité orale, incluant la bouche et l'oropharynx. Les sujets témoins, recrutés dans les mêmes hôpitaux, étaient assortis par fréquence aux cas, selon le sexe et l'âge. Tous les sujets ont été interviewés afin de recueillir des informations détaillées sur les facteurs de risque connus et potentiels.

Des cellules orales exfoliées ont été recueillies chez tous les sujets pour la détection de l'ADN du VPH par le protocole PGMY09/11 basé sur la réaction de polymérisation en chaîne (PCR). Les anticorps dirigés contre les capsides des VPH 16, 18 et 31 ont été détectés dans le sérum des sujets. La méthode de régression logistique non conditionnelle a été utilisée pour l'estimation des ratios de cotes (ORs) du cancer oral pour le VPH et les autres facteurs de risque potentiels.

Résultats

Un total de 72 cas et 129 sujets témoins ont été recrutés. L'ADN du VPH a été détecté chez 19% des cas (14 sur 72) et 5% des témoins (6 sur 129). L'analyse pour les cancers reliés à l'anneau de Waldeyer (amygdales et base de la langue) a démontré que le OR de cancer pour la détection de VPH à risque élevé était de 19,32 (IC95% : 2,3-159,5), après contrôle pour les caractéristiques sociodémographiques et l'usage de tabac et d'alcool. Le OR ajusté pour la séropositivité pour le VPH 16 était de 31,51 (IC95% : 4,5-219,7). L'analyse pour les cancers oraux non amygdaliens a suggéré des ORs respectifs de 2,14 (IC95% : 0,4-13,0) pour la détection d'ADN du VPH à haut risque et de 3,16 (IC95% : 0,8-13,0) pour la séropositivité.

Discussion

Les résultats de ce projet, combinés à ceux d'autres études, suggèrent une forte association entre l'infection au VPH et les cancers de l'oropharynx, plus spécifiquement ceux de l'anneau de Waldeyer. Par contre, l'association pour les cancers oraux non amygdaliens est plus faible et les données biologiques et expérimentales n'ont pas permis d'établir un lien étiologique solide.

Acknowledgments

I am extremely thankful to my supervisor, Dr. Eduardo Franco, my tutor and my friend, who always supported me to carry out this project. He teaches and guides with passion and honesty; with him I learnt a lot more than epidemiology. Not only he provided me with all the resources needed to complete this work, but also motivated and encouraged me all these long years. He guided my work in a friendly and generous way, and gave me the freedom to work and to learn in my own way. He was always available when I needed his assistance, even when I rushed him, and he assisted me during my periods of despair. My entire gratitude to him, forever.

I wish to express my sincere gratitude to Drs. Jim Hanley and Jocelyne Feine, members of my supervisory committee, who gave me advice and support to complete this work. My only regret is not having profited more their knowledge and experience.

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This project would not have been possible without the collaboration of Dr. François Coutlée at Notre Dame Hospital, who tested the samples for HPV DNA, and Dr. Raphael Viscidi at The Johns Hopkins Hospital, who performed the serological analysis. I thank them for having delivered so well, even when I rushed them.

I am very thankful to all my colleagues at the Department of Cancer Epidemiology: Marie-Claude Rosseau, Anita Koushik, Susan Brogly, and Nicolas Schlecht. They all gave me support and encouragement, each one in her or his own way. Additional thanks to Marie-Claude, who translated my abstract to French. I am also very thankful to Dr. Eliane Duarte-Franco, my office mate, who had to bear my unpleasant presence all these years, and who gave me support and assistance whenever I needed it.

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My sincere gratitude to all the patients who participated in this study.

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1. INTRODUCTION

The International Agency for Research on Cancer (IARC) has concluded that there is compelling evidence, both from the biologic and epidemiologic standpoints, to consider human papillomavirus (HPV) infection as the main determinant of cervical cancer (IARC, 1995).

The mucosal epithelia of the uterine cervix and of the oral cavity are histologically very similar; they have the same embryological origin, both are exposed to the action of environmental carcinogens, and most of the resulting malignancies are squamous in type. In fact, similar environmental carcinogens (such as tobacco and HPV) have been implicated in the etiology of both oral and cervical cancers.

HPV involvement in carcinomas of the upper digestive and respiratory tracts has initially been suggested on the basis of histologic and immunohistochemical studies. With the advent of highly sensitive techniques used in molecular biology for viral detection, there is evidence suggesting that HPV infection may play a role in the etiology of oral malignant tumours, in addition to the one played in anogenital neoplasms.

In 1996, when I became involved in this project, the existing epidemiological evidence on this topic was scarce. Several case-series (with or without a comparison group) had dealt with the association between HPV and cancer of the oral cavity, but most of them were very small in size and not epidemiologically sound. Only one case-control study had been published, and it had several weaknesses (Maden et al., 1992). Since then better design epidemiological studies have been published assessing the association of

i) detection of viral DNA with buccal and oropharyngeal cancers, ii) serological response to HPV with buccal and oropharyngeal cancers, iii) detection of HPV DNA and HPV serological response with buccal cancer. No published study have assessed the association of both markers of HPV infection –detection of oral HPV DNA and serological response- with buccal and oropharyngeal cancers.

This project is part of a multinational multi-centre case-control study, coordinated by the IARC. In the process of designing this study, IARC researchers contacted several of the principal investigators responsible for the individual centres, including Montreal.

The primary objective of the present project was to examine the association between HPV infection and risk of developing oral cancer, and with two subset of oral cancers: i) tonsillar related carcinomas (palatine tonsil and base of tongue), and ii) oral cancers not related to Waldeyer's ring. Secondary objectives were to assess the role of other known (i.e., smoking, alcohol drinking, fruit and vegetable intake) or putative (i.e., oral health, sexual behaviour) risk factors for cancer of the oral cavity in the study population, to assess the effect modification between HPV infection and tobacco smoking or alcohol drinking on oral cancer risk, and to examine sexual behaviour as a route of transmission for oral HPV infection.

2. REVIEW OF THE LITERATURE

2.1 CANCER OF THE ORAL CAVITY

In the medical literature, oral cancer sometimes includes cancers of the mouth alone, cancers of the mouth and the pharynx, or cancers of the mouth and the oropharynx. In this thesis, oral cancer will be defined to include malignant neoplasms of the lip [International Classification of Diseases, 9th Revision(ICD-9) 140], tongue (ICD-9 141), gum (ICD-9 143), floor of the mouth (ICD-9 144), other parts of the mouth –including cheek mucosa, palate, and uvula- (ICD-9 145), and oro-pharynx (ICD-146). These cancers are usually studied together because they share similar etiological and biological features. Cancers of the salivary glands (ICD-9 142), nasopharynx (ICD-9 147), and hypopharynx (ICD-9 149) will be excluded from this group.

Histologically, over 90% of tumours of the oral cavity are squamous cell carcinomas (Jacobs, 1990; Chen et al., 1990; Muir and Weiland, 1995; Ostman et al., 1995). The incidence of these malignant lesions peaks in the 6th and 7th decades in most populations (Jensen et al., 1990; Chen et al., 1991). These cancers are more common in males than females: the M:F sex ratio is 2.0 for cancers of the mouth, and 4.4 for pharyngeal cancer (Parkin et al., 1999).

2.1.1 Descriptive Epidemiology

2.1.1.1 Incidence

Cancers of the oral cavity are a major health problem in many parts of the world. These cancers account for over 300,000 incident cases around the world annually, and they

represent 6% of new cancer cases worldwide among males, and 3% of all cancers among females (Parkin et al., 1999). In less developed countries oral cancers are ranked as the sixth most frequent cancer in men, and the eighth malignancy in women (Parkin et al., 1999).

Table 1 shows the age-standardized incidence rates for the different regions of the world. The highest incidence of oral cancer is found in Melanesia (Papua New Guinea and Solomon Islands). In 1990 it was estimated in 38.8 per 100,000/per year for males, and 23.6 for females. In Europe the highest incidence is seen in the Western part of the continent. The main contributors to this high incidence are France and Luxembourg, with annual rates of 34.9 and 26.8/100,000 for males, and 3.5 and 3.0 for females, respectively. The lowest incidence rates around the world are seen in Eastern Asia, China being the country with the lowest estimated annual incidence: 1.3 for males, and 0.8/100,000 for females.

In Canada, the estimated number of oral cancer cases in 1998 was 3150, 2200 among males and 950 among females. They represent 3.4% of all new cancer cases, and 2.2% of all cancer deaths (NCIC, 1998). The annual incidence rate is 15/100,000 for men and 5/100,000 for women. In Quebec, the estimated total number of cases for 1998 was 770, 580 among men and 190 among women.

Not only the incidence rate of oral cancer varies between different geographical regions, but also its distribution among anatomical subsites. In men, both buccal and pharyngeal cancer are common in Western and Southern Europe, and South Asia, whereas mouth cancers (but not pharynx) are particularly frequent in Melanesia, Southern Africa, and

Table 1. Age-standardized[1] incidence rates of oral cancer for the different regions of the world (per 100,000), around 1990[2]

		Males	Females
Africa			
	Eastern Africa	9.7	5.6
	Middle Africa	6.8	3.3
	Northern Africa	5.7	2.2
	Southern Africa	17.3	3.7
	Western Africa	6.8	2.9
America			
	Carribean	12.2	6.2
	Central America	6.3	2.7
	South America	11.9	3.2
	North America	11.1	4.7
Asia			
	Eastern Asia	1.9	1.0
	South-Eastern Asia	6.3	3.4
	South Central Asia	19.9	8.8
	Western Asia	4.7	2.8
Europe			
	Eastern Europe	13.2	2.1
	Northern Europe	6.9	2.6
	Southern Europe	15.5	2.0
	Western Europe	21.1	3.0
Oceania			
	Australia/NewZealand	18.5	5.4
	Melanesia	38.8	23.6
	Micronesia	4.9	8.6
	Polynesia	14.2	3.6
Worldwide		**10.3**	**3.6**

1. Direct age standardization based on the world population of 1960.
2. Source: Parkin et al., 1999

Australia/New Zealand. While tongue and other mouth cancers predominate among oral cancers in South India, oropharyngeal cancer represents a high proportion of oral malignancies in France, Central and Eastern Europe. Lip cancer predominates in several regions of Australia and Canada; it accounts for more than half of oral cancers in Newfoundland, Saskatchewan, and South Australia (Parkin et al., 1997).

The variation in incidence and distribution by subsite is due to differences in the relative distribution of specific risk factors: smoking and alcohol consumption are high in Western and Southern Europe and Southern Africa, while the chewing of betel quid is highly prevalent in South-central Asia and Melanesia. The high rate of oral cancer in Australia is mainly due to lip cancer, a disease related to UV light exposure due to solar irradiation. Also, to a certain extent, misclassification by subsite may explain some of the differences in the distribution of anatomical subsites, especially in the cases of advanced cancers (Sankaranarayanan et al., 1998).

2.1.1.2 Mortality and Survival

Worldwide, an estimated 197,000 deaths from oral cancer occur per year. Mortality from oral cancer is notably high among males in Melanesia (23.9/100,000), followed by South Central Asia (13.1/100,000). Worldwide, the estimated mortality rate for oral cancer per 100,000 is 6.6 for males, and 2.3 for women (Pisani et al., 1999).

Survival of patients with oral cancer is generally lower than that for other cancers, such as breast, prostate, or bladder. The 5-year survival rate in the province of Quebec is 49% for males, and 59% for females (NCIC, 1995). For cancers of the lip, the 5-year survival rate is higher than 80%. The prognosis for patients with regional spread of the

disease (lymph node involvement) and/or distant metastasis is very poor, with survival rates after 5 years of less than 40%, and less than 20%, respectively (Wingo et al., 1995). A substantial proportion of surviving patients develop second primary cancers related to tobacco and alcohol consumption (Franco et al., 1991).

Not only is the length of survival short in comparison with many other neoplasms, but the quality of life of patients is considerably deteriorated due to the disfigurement and dysfunction that result from treatment. Coping with the permanent threat of disease recurrence and death is further complicated by the loss of function in communicating.

2.1.1.3 Trends

Incidence rates for oral cancer have been slowly declining in India, Hong Kong, Brazil, and US whites. On the other hand, the rates are rising in most regions of the world (Blot et al., 1994). In Europe, a slow but steady increase in incidence is evident among males in most populations; however, the increase is more pronounced in Central and Eastern Europe, where some populations have experienced more than 100% increase in the rates during the last decades (Sankaranarayanan et al., 1998). Two-to-three-fold mortality increases have been recorded in these regions in the last three decades (La Vecchia et al., 1992), especially for younger males (La Vecchia et al., 1997).

In the Americas, rates have been stable or declining slowly in most populations. A declining trend is observed in US whites, but rates are stable among US blacks. In Canada, though there is a declining trend in the incidence of oral cancer, there has been a steady increase in tongue and pharyngeal cancer in several regions (Sankaranarayanan et al., 1998; Parkin et al., 1997).

2.1.2 Risk factors for oral cancer

Although oral cancer is a disease with multifactorial etiology, tobacco use and alcohol consumption are the major risk factors. The distribution of risk factors varies considerably across populations. Tobacco smoking, alcohol drinking, dietary practices, occupational and environmental exposures, and genetic susceptibility vary geographically as a function of economic, cultural, ethnic, and demographic characteristics.

2.1.2.1 Tobacco and Alcohol

Tobacco and alcohol have long been implicated as the most important risk factors for oral cancers. Tobacco, whether smoked, chewed, or snuffed, is a major carcinogen causing both initiation and promotion of cancer of the oral cavity. There is extensive evidence of the carcinogenic role of tobacco in humans (IARC, 1986).

The incidence and mortality rates of oral cancer among smokers are substantially greater than those observed for never smokers. Although estimates vary, most studies have reported risk ratios for smokers versus never smokers ranging from 3 to 15, or even higher. Blot et al. (1988) analyzed data from a case-control study, comprising more than one thousand cases of oral cancer and population-based controls in U.S. metropolitan areas. Risk of disease for smokers was three to five times higher than that among non smokers. They showed a clear dose response relationship between intensity and duration of smoking with risk of oral cancer.

Kabat et al. (1989) investigated risk factors for oral cancer among females in New York. Current smoking was implicated with a risk of oral cancer three times higher than that of never smokers. Ex-smokers had a similar risk to that of never smokers. Franco et al. (1989) found odd ratios (ORs) of more than ten for heavy smokers, compared to never smokers in Southern Brazil.

In Italy, Franceschi et al. (1990) conducted a hospital-based case-control study on head and neck cancers. The ORs for current smokers, after controlling for alcohol and other confounders, were 11.1 for cancer of the mouth and 12.9 for pharyngeal cancer. The risk increased with number of cigarettes smoked daily, and with duration of smoking. Among ex-smokers, those who had quit smoking for more than ten years showed ORs close to unit for cancer of the mouth, and 3.7 for cancer of the pharynx. For smokers of only pipe or cigars, the risk for oral cancer was greater than for those who smoked only cigarettes (Blot et al., 1988; Franceschi et al., 1990; Schlecht et al., 1999).

Smokeless tobacco (such as snuff and chewing tobacco), which is common in some parts of North America, has also been shown to be carcinogenic for the oral cavity. Winn et al. (1981) studied women living in rural North Carolina. They found a four-fold increased risk of oral cancer among nonsmokers who dipped snuff. There is also evidence that betel quid chewing, a common habit in the Eastern hemisphere, when consumed with tobacco, is carcinogenic (IARC, 1985). Sankaranarayanan et al. (1989) conducted a case-control study of gingival cancer in Southern India. They found strong associations between cancer risk and pan(betel)-tobacco chewing, as well as with bidi and cigarette smoking.

Most patients who develop oral cancer drink alcohol. Although alcohol consumption is highly correlated with tobacco smoking the effect of alcohol drinking seems to be not only an effect modifier of smoking, but also it has an independent effect in increasing oral cancer risk. Several studies found an increased risk of oral cancer among smokers who did not drink, and among drinkers who did not smoke. (Blot et al., 1988; Franceschi et al., 1990; Schlecht et al., 1999)

All three forms of alcohol –wine, beer, and hard liquor- have been associated with oral cancer, although hard liquor seems to be the type of alcohol associated with the highest risk (Mashberg et al., 1981; Spitz et al., 1988; Merletti et al., 1989; Franceschi et al., 1990; Ng et al., 1993; Schlecht et al., 2001). Most studies found a dose-response trend between intensity of alcohol drinking and increased risk of oral cancer. Several mechanisms have been suggested to implicate alcohol as an oral carcinogen (Fraumeni Jr., 1979; Doll and Peto, 1981; IARC, 1988). Firstly, alcohol may act as a solvent, facilitating the passage of carcinogens through cellular membranes. Another mechanism may be the alteration of the cellular metabolism of the epithelial cells at the target site by ethanol, which may also be aggravated by nutritional deficiencies (Rossing et al., 1989). Also ethanol enhances liver metabolism, therefore it may activate some carcinogenic substances.

Despite the tendency for alcohol consumption to be related to tobacco smoking, Hindle et al. (2000), in their study of the association between oral cancer and surrogate markers of smoking and alcohol consumption, supplied evidence that for males at least, alcohol may be more important than cigarette smoking in the etiology of oral cancer.

Most authors who analyzed the effect of both alcohol and tobacco have concluded that the combined effect of both exposures is at least greater than the additive effects, and, in most cases, greater than their multiplicative effects (Elwood et al., 1984; Cann et al., 1985; Blot et al., 1988; Franco et al., 1989; Franceschi et al., 1990; Schlecht et al., 1999).

It has been calculated that tobacco smoking and alcohol drinking play a causal role in about 75% of all oral cancer in North America (Blot et al., 1988), and in Italy (Merletti et al., 1989; Negri et al., 1993), and in 80% of the cases in temperate South America (Franco et al., 1989).

2.1.2.2 Dietary factors

Despite the strong association between tobacco and alcohol with oral cancers, other factors, such as diet, have also been implicated in the etiology of oral cancer.

Several epidemiological studies have shown that intake of fruits and vegetables rich in vitamin A and carotenes is inversely related to subsequent development of cancer (Graham et al., 1977; Winn, 1995). Low intake of fruits and/or vegetables, which are the primary source of beta-carotene, has been linked to increased oral cancer risk and mortality (McLaughlin et al., 1988; Franco et al., 1989; Singh and Gaby, 1991; Franceschi et al., 1991). Garewal (1994) summarized the findings of 54 studies that evaluated fruit and vegetable intake in the development of cancers of the upper aerodigestive tract: 52 of the 54 studies reviewed had shown a protective effect.

Numerous animal studies have demonstrated the inhibitory action of vitamin A in the development of epithelial tumours. A major physiologic role of vitamin A is to control cell differentiation (De Luca et al., 1972). Deficiency of vitamin A causes cellular alterations similar to those induced by chemical carcinogens. Beta carotene is metabolized into retinol, that controls expression of genes involved in cell differentiation and proliferation (Sporn and Roberts, 1983). Carotene itself may protect against oxidative reactions within the cell, thus limiting damage to DNA (Willett and MacMahon, 1984).

Charcoal grilling, which introduces polycyclic aromatic hydrocarbons in food, was shown to be associated with increased risk of oral cancer in Brazil (Franco et al., 1991) but not in the U.S. (McLaughlin et al., 1988). *Maté* drinking, a tea-like beverage typical of temperate South America, which is drunk very hot, has been shown to be associated with increased risk (De Stefani et al., 1988; Pintos et al., 1994).

2.1.2.3 Genetic and Family factors

Until recently, little attention has been paid to possible hereditary factors in oral cancer. There is now increasing epidemiological evidence from case-control studies that a family history of head and neck cancer may be a risk factor for the development of oral cancers. Studies by Copper et al. (1995) in Holland, by Foulkes et al. (1995) in Brazil and in Montreal (Foulkes et al., 1996), all found an increased risk for developing head and neck cancer if first degree family members had had the same disease. The three studies found ORs in the vicinity of 3.5. However, Goldstein et al. (1994), who studied oral cancer, failed to find an association (OR=1.2; 95% CI: 0.7-2.3).

Several criticisms can be aimed at these studies. For example, cancer among relatives was not confirmed in any of the studies by checking their medical records. First degree relatives tend to share life style behaviours. In some studies (Goldstein et al., 1994; Copper et al., 1995) no attempt was made to collect information on strong confounders - such as smoking and alcohol consumption- from relatives who had developed cancer, or the measurement was poor.

Nevertheless, during the last decade, several authors have studied whether increased host susceptibility may play a role in the etiology of oral cancer. Individuals may be at increased cancer susceptibility due to less efficient detoxification of carcinogens, or more efficient activation of co-carcinogens, or a failure to maintain adequate DNA repair after carcinogen exposure (Jefferies and Foulkes, 2001).

Cytochromes P450 (CYPs) is a superfamily of enzymes metabolizing various drugs and foreign chemicals. Some of them are important for carcinogen metabolism and activation: particularly those belonging to families 1-3. Gluthathione transferases (GSTs) and N-acetiltransferases (NATs), on the other hand, play an important role in the inactivation of carcinogens. If a polymorphism of one or more genes encoding for these enzymes leads to increased activation of carcinogens or decreased capacity to inactivate them (or both), it is possible that such an individual faces an increased risk of cancer when exposed to carcinogens. Scully et al.(2000) reviewed the association of oral cancer susceptibility with various genotypic polymorphisms such as cytochrome P-450 (CYP1A1) and glutathione-S-transferase (GSTM1). Several studies (Katoh et al., 1999; Sato et al., 1999; Tanimoto et al., 1999) have shown that individuals with the

GSTM1 and/or CYP1A1 genotype have a higher susceptibility for oral cancer, particularly with a low dose of cigarette smoking.

Certain polymorphisms in the p53 tumour suppressor gene have been studied in relation to cancer. It has been reported that patients with homozygous arginine alleles at codon 72 of p53 were at increased risk of HPV-related cervical cancer (Storey et al., 1998; Makni et al., 2000). No excess of this polymorphism was seen in a study of 163 cases of head and neck cancers, compared with 163 matched controls (Hamel et al., 2000), although HPV expression status was not determined

2.1.2.4 Other factors

There is some evidence suggesting that poor oral hygiene, or improperly fitting dental prostheses, may be associated with oral cancer. However, there is contradictory evidence in the estimation of the risk after controlling for tobacco and alcohol consumption. Graham et al. (1977) and Velly et al. (1998) found that poor dentition, tooth brushing frequency, and ill-fitted dentures, were associated with cancer, whereas Gorsky and Silverman (1984) did not find an association between use of dentures and oral cancer.

A possible role for environmental exposures has not been demonstrated. Elwood et al. (1984) did not find any association between occupational exposures and oral cancer. On the other hand, some studies have suggested that indoor air pollution may be linked with increased risk of oral cancer (Dietz et al., 1995, Pintos et al., 1998).

2.1.2.5 Human Papillomavirus

With the advent of highly sensitive techniques used in molecular biology for viral detection, there is evidence suggesting that human papillomavirus (HPV) infection may play a role in the etiology of oral malignant tumours, in addition to the one played in anogenital neoplasms. Before reviewing the evidence on the association between HPV and oral cancer, is necessary to briefly discuss some features of the virus and its interaction with the host cells, to better understand its carcinogenic role in humans.

2.2 Biology of the Human Papillomavirus

Human papillomavirus is a non-enveloped double-stranded small DNA virus with an icosahedral capsid (Almeida et al., 1962). HPV usually exists as non-integrated episomal plasmids in benign and premalignant lesions, but it frequently integrates in the host cell genome in malignant lesions (zur Hausen, 1989). The number of identified and characterized HPV types has increased considerably over the last 10 years DNA genomes of 82 HPV types have been cloned and characterized to date, numbered according to the chronological order in which they were isolated. An additional 60 to 70 putative new HPV types have been partially identified through amplification of DNA fragments by PCR (de Villiers et al., 1999).

HPVs are strictly epitheliotropic and they can be divided into HPV types that infect mainly the skin (the so-called cutaneous types) and HPV types that infect the mucosa of the anogenital and upper aerodigestive tract (mucosotropic types). Alternatively, based on their association with either benign or premalignant and malignant lesions, HPVs can be grouped into "low risk" and "high risk" types, respectively (zur Hausen and Schneider,

1987). HPV 6 and 11 –low risk types- are the most common types found in benign lesions such as genital condylomas and laryngeal papillomas, while HPV 16 and 18 – high risk types-, are the most prevalent types associated with cervical high grade intraepithelial lesions, cervical squamous cell carcinomas, and cancers of the upper aerodigestive tract.

2.2.1 Molecular mechanisms of HPV-induced carcinogenesis

HPV genomes code for at least eight proteins: six early proteins, and two late proteins. The E6 and E7 proteins coded by high risk HPV types have transforming properties. Both proteins are consistently expressed in HPV related anogenital tumours (zur Hausen, 2000).

Matlashewski et al. (1986) observed that the levels of p53, a protein involved in maintaining cellular integrity after DNA damage, were very low in cells infected with HPV. This finding led to the suggestion that HPV proteins may inactivate p53 through degradation. This hypothesis was supported by the findings by Werness et al. (1990), who observed that the HPV E6 protein binds with p53, causing the functional inactivation of p53, a protein which plays a key role in tumour suppression. Other interactions with human cellular proteins have been described, that result in a large number of modifications in the respective host cells. It appears, at present, that the two most prominent functions of E6 can be summarized as follows: mutagenic and antiapoptotic effect (reviewed by zur Hausen, 2000).

The properties of the E7 protein have been reviewed by Münger and Phelps (1993), and zur Hausen (2000). Similar to the functions of E6, the HPV E7 protein has several

oncogenic functions. A key observation of the oncogenic properties of E7 was its binding with the human retinoblastoma (pRB) tumour suppressor protein (Dyson et al, 1989).

Integration of HPV DNA is regularly observed in malignant tumours. This contrasts remarkably with premalignant lesions which regularly contain episomal (non integrated) HPV DNA (zur Hausen, 1994). Integration in the host cell genome usually occurs with disruption of the viral E2 gene. Since the E2 gene encodes a repressor for transcription of the E6 and E7 genes, integration leads to an overexpression of these two oncogenes (reviewed by zur Hausen, 1994).

2.2.2 Methods for detection of HPV infections

Classical virus detection methods, such as virus cultivation, cannot be used for HPV detection since the virus cannot be propagated in tissue culture. Diagnosis of HPV infection is done by detection of viral genome sequences in infected tissues. HPV infection may also be inferred from cytological, histological, and clinical findings.

The link between koilocytosis and HPV infection was established by cytological and histological studies (Meisels and Fortin, 1976). The presence of koilocytes is a highly specific marker of productive viral infection. Colposcopic visualization of the cervix after application of acetic acid is also highly specific for diagnosis of HPV infection. However, the advent of techniques for HPV DNA detection has shown that cytological, histological and clinical diagnosis were not very sensitive to detect HPV infection. Most HPV positive specimens do not show cytological changes (Bauer et al., 1991).

Before the advent of polymerase chain reaction (PCR), several techniques had been used for detection of HPV DNA, such as filter *in situ* hybridization (FISH), Southern blot (SB) hybridization, dot blot (DB), and *in situ* hybridization (IARC, 1995). Since the arrival of PCR, several detections protocols have been developed using DNA amplification. Of the technologies currently available for detection of HPV genomic sequences, the three techniques most widely used are: Hybrid Capture II (HCII) HPV test, and the PCR based GP5+/6+ and PGMY09/11 systems.

2.2.2.1 Hybrid Capture II (HCII)

The HCII HPV assay is currently the only commercially available product for HPV DNA testing (Digene Inc., Gaithersburg, USA), although it is likely that new technologies for HPV DNA testing will be introduced in the market in the near future. One the advantages of HCII is that it does not require a laboratory with expertise in molecular biology to be used. This assay, not based on amplification of DNA segments, has a slightly lower sensitivity than PCR based techniques (Peyton et al., 1998). One of the disadvantages of HCII is that it does not allow for identification of the specific HPV types. However, it can distinguish between low risk and high risk types (Lorincz, 1996).

2.2.2.2 PCR based techniques

PCR based assays allow the in vitro amplification of specific HPV DNA target segments in order to generate sufficient copies for subsequent detection and analysis. The first step in this process requires the separation of the double-stranded DNA (denaturation) by heating the sample at 95°C. The next step (annealing) involves cooling the reaction to 40-60°C to allow the hybridization of short synthetic single-stranded DNA (oligonucleotides) with their complementay sequence of the target DNA. The hybridized

oligonucleotides then act as primers for the last step in the reaction (extension), in which DNA polymerase enzyme –at 72°C- catalyses the formation of two new double-stranded DNA molecules (amplicons), using each of the original target DNA single strands as templates. By repeating this cycle of denaturation, annealing, and extension, each newly synthesized double-stranded DNA molecule can serve as a template for the next cycle. Theoretically, PCR can produce 10^6 identical copies for a single double-stranded DNA molecule after 30 cycles of amplification, therefore achieving its exceptionally high sensitivity.

The most commonly PCR based assays for detection of HPV DNA target segments of the viral L1 gene, a highly conserved region among different HPV types. The MY09/11 system amplifies a region of approximately 450 bp (Manos et al., 1989). The GP5+/6+ system was developed by de Roda Husman et al. (1995), and targets a segment of the HPV genome of approximately 140-150 bp. Some studies have compared the performance of the GP5+/6+ and the MY09/11 systems in clinical samples, and the sensitivity of both systems are totally comparable, and their correlation in detection of positive and negative samples is extremely good. Gravitt et al. (2000) redesigned the MY09/11 primers to improve the sensitivity for HPV DNA detection, creating the PGMY09/11 set of primers. To my knowledge, there is no published study comparing the performance of the GP5+/6+ and the PGMY09/11 systems, which has a higher sensitivity than the original MY09/11 protocol (Gravitt et al., 2000). The performance of this two detection systems - GP5+/6+ and PGMY09/11 – will be presented in further detail in the discussion section.

2.3 HUMAN PAPILLOMAVIRUS AND CANCER

2.3.1 HPV and cervical cancer

The International Agency for Research on Cancer (IARC) has concluded that there is compelling evidence, both from the biologic and epidemiologic standpoints, to consider HPV infection as the main determinant of cervical cancer (IARC, 1995). The biological evidence to involve HPV as an oncogenic agent was briefly discussed in section 2.2.1.

There is also strong epidemiological evidence to implicate HPV in the etiology of other anogenital tumours. Some landmark studies have shown a very strong link between HPV and premalignant and malignant lesions of the cervix, one of the strongest associations known between a virus and human cancer. Koustky et al. (1992) have shown that HPV infection of the cervix precedes the development of cervical premalignant lesions. A case-control study carried out in Spain and Colombia showed that women with HPV infection had a risk of developing invasive cervical cancer 29 times higher than women without HPV infection (Muñoz et al., 1992). Another epidemiological study estimated that women with cervical infection due to HPV types 16 or 18 had 50 times greater risk of developing a cervical intraepithelial lesion than HPV negative women (Schiffman et al., 1993).

Franco (1996) reviewed several case-control and cohort studies that have demonstrated the link between HPV infection and risk of cervical neoplasia, either pre-invasive (CIN) or invasive, showing the pooled estimates of these studies. The combined OR for studies which used PCR based methods was 19.8 (95%CI: 15.2-25.8). For invasive cervical cancer, the pooled OR for PCR studies was 34.5 (95%CI: 21.5-55.4). The ORs

for most of the studies reviewed (Franco, 1996) are in the 20-70 range, which places HPV infection as the strongest risk factor for cervical cancer, with a magnitude of association that is greater than the one for the association between smoking and lung cancer.

The IARC carried out a study to determine the prevalence of HPV in cervical cancer. Over 1000 frozen biopsy specimens were collected from around the world, and HPV DNA was detected in 93% of the samples (Bosh et al., 1995). Specimens originally classified as HPV negative (7%) were retested, excluding specimens considered inadequate for testing (Walboomers et al., 1999). Combining the results from both studies, the worldwide HPV prevalence in cervical carcinomas is 99.7%. The presence of HPV in virtually all cervical cancers implies the highest worldwide attributable fraction so far reported for a specific cause of any major human cancer, suggesting that HPV is a necessary cause of cervical cancer.

2.3.2 HPV and oral cancer

The mucosal epithelia of the uterine cervix and the oral cavity are histologically identical; they have the same embryological origin, both are exposed to the action of environmental carcinogens, and most of the resulting malignancies are squamous in type. Histological similarities have been noted between experimentally induced oral dysplasias and squamous carcinoma, and corresponding lesions of cervical intraepithelial neoplasia (CIN) and invasive cervical squamous carcinoma (Howell et al., 1986). Consistent with the multistage model for carcinogenesis, a synergistic effect of cofactors (e.g., chemical, physical or viral) has been cited as necessary for malignant transformation (zur Hausen, 1982). In fact, similar environmental carcinogens (such as

tobacco and HPV) have been implicated in the etiology of both oral and cervical cancers. An American study, using incidence data from the National Cancer Institute, has shown that women with an initial cervical cancer were at a significantly increased risk both for subsequent buccal cavity and laryngeal cancer (Spitz et al., 1992). The same study also showed that the risk for cervical cancer subsequent to an initial oral or laryngeal cancer was also significantly elevated.

HPV involvement in carcinomas of the upper digestive and respiratory tracts has initially been suggested on the basis of histologic and immunohistochemical studies. Histologic examination of laryngeal squamous cell carcinomas has revealed the presence of condylomatous changes, suggestive of HPV infection in a substantial proportion of cases (Syrjanen and Syrjanen, 1981). Pathological examination of specimens of oral squamous cell carcinomas revealed that 16 of the 40 biopsies examined showed histologic changes suggesting an HPV infection, and eight of them showed positive staining with antiserum obtained by immunization with papillomavirus structural antigens (Syrjanen et al., 1983).

The present review of the literature on HPV and oral cancer summarizes all articles published in English reporting case series that included at least twenty cases of cancers of the head and neck, and that included cases of oral cancer. All studies that included a comparison group, regardless of the number of cases studied, are also summarized.

Initial investigations on the role of HPV in oral lesions relied on light microscopy (LM), electron microscopy (EM), and immunohistochemical staining. Under LM, identification of koilocytosis ('ballooning' of keratinocytes within the intermediate layers of the

epithelium) is indicative of HPV infection (Meisels and Fortin, 1976). The HPV capsids may be visualized with EM. However, HPV genetic material may be present in epithelial cells in the absence of the EM appearance of HPV capsids. Immunohistochemical staining has revealed the presence of HPV capside antigens in HPV infected cells. However, capsid antigens have rarely been detected in high grade neoplasias or invasive cancer, probably because such tissue contain limited numbers of highly differentiated squamous epithelial cells (McKaig et al., 1998). Table 2 summarizes studies on HPV and oral cancer using light microscopy for identification of koilocytosis or immunohistochemical staining. There was a great variability in the detection rate of HPV infection using these techniques, and their reproducibility is very low, at least partially explained by the fact that interpretation of results is highly subjective.

Since at present it is not possible to culture the virus *in vitro*, the introduction of newly developed molecular biologic methods (especially PCR) has opened novel ways to examine the role of HPV in the development of oral carcinomas.

2.3.2.1 Detection of HPV DNA in oral squamous cell cancers using hybridization techniques

Methods for detecting HPV in oral tissues have been reviewed by Miller and White (1996), and Chang et al. (1991). Hybridization techniques –Southern blot, dot blot, and in situ hybridization- have been used to identify specific viral genetic sequences in cells and tissues. Southern blot and dot blot methodologies require the isolation and purification of cellular DNA from clinical specimens. In Southern blots, DNA is digested with restriction enzymes, separated by size, transferred to filters, and probed with radiolabeled or chemi-illuminescent probes specific for a given HPV type. In situ and in

Table 2. Case series for detection of HPV in oral lesions using light microscopy for detection of koylocytes or indirect immunoperoxidase staining

Author, year, study area	Site	Type of lesion[1]	Type of material	Method[2]	HPV detection		
					Positive	Tested	Percentage
Syrjanen et al., 1983 Finland	Mouth	SCC	Fixed biopsies	IP-PAP	0	6	0%
Syrjanen et al., 1983 Finland	Mouth	SCC	Fixed biopsies				
				LM	14	40	35%
				IP-PAP	8	16	50%
Loning et al., 1985 Germany	Mouth	SCC	Fixed biopsies	IP-PAP	3	6	50%
Lind et al., 1986 Norway	Mouth	Hyperplasias	Fixed biopsies	IP-PAP	13	20	65%
Loning et al., 1987 Germany	Mouth		Fixed biopsies				
		SCC		LM	6	13	46%
		Dysplasia		LM	3	4	75%
Ahmed and Jafarey, 1995 Pakistan	Mouth			IP-PAP			
		SCC	Fixed biopsies		0	56	0%
			Total		**47**	**161**	**29%**

1. SCC: squamous cell carcioma
2. LM: light microscopy; IP-PAP: indirect immunoperoxidase staining

filter in situ hybridization do not require DNA isolation and purification from tissues, but rather probe directly for the presence of viral sequences in tissues and smears. The major advantage of in situ hybridization compared to other methods is the preservation of the tissue morphology, and allows localizing the HPV genomic sequences within the cells.

Researchers have used hybridization techniques to investigate the presence of HPV not only in malignant lesions, but also in benign and premalignant lesions, such as papillomas, leukoplakia, condyloma, focal epithelial hyperplasia, verruca, lichen planus, and dysplasia. Tables 3 and 4 summarize case series reports that utilized in situ hybridization (ISH), and Southern blot (SBH) and dot blot hybridization (DBH), respectively, for identification of HPV DNA in oral squamous cell carcinomas as well as in benign and premalignant oral lesions. It was impossible to differentiate benign from premalignant lesions, since most articles do not provide enough information to discriminate detection rates between lesions.

There is great variability in the detection rate of viral DNA using either technique. The detection rate of HPV DNA in oral squamous cell carcinomas for studies that used ISH (table 3) ranged form 0 to 70%, and in benign and premalignant oral lesions ranged from 0 to 50%. Studies that used SBH and DBH (table 4) also showed great variability: the detection rate in oral cancers ranged from 0 to 100%. The average detection rate in oral cancers was very similar for both techniques: 22% (102 out of 472) 181 for ISH, and 25% (42 of 171) for studies that used SBH or DBH.

Table 3. Case series for detection of HPV in oral lesions using in situ hybridization (ISH)

Author, year, study area	Site	Type of material	Squamous cell carcinomas			Benign and Premalignant Lesions		
			HPV +	Tested	Percentage	HPV +	Tested	Percentage
Loning et al., 1987 Germany	Mouth	Fixed biopsies	4	7	57%			
Syrjanen et al., 1986 Finland	Mouth	Fixed biopsies				10	32	31%
Gassenmaier and Hornstein, 1988 Germany	Mouth	Fixed biopsies	16	68	24%	19	103	18%
Syrjanen et al., 1988 Finland	Mouth	Fixed biopsies	6	51	12%	6	21	29%
Niedobitek et al., 1990 Germany	Tonsils	Fixed biopsies	6	28	21%			
Greer et al., 1990 U.S.A.	Mouth	Fixed biopsies	3	50	6%	2	60	3%
Chang et al., 1990 Finland	Mouth	Fixed biopsies	1	40	3%			
Zeuss et al., 1991 U.S.A. (Kentucky)	Mouth	Fixed biopsies	0	20	0%	0	15	0%

(cont'd)

Table 3 (cont'd). Case series for detection of HPV in oral lesions using in situ hybridization (ISH)

Author, year, study area	Site	Type of material	Squamous cell carcinomas			Benign and Premalignant Lesions		
			HPV +	Tested	Percentage	HPV +	Tested	Percentage
Young and Min, 1991 U.S.A. (Oklahoma)	Mouth	Fixed biopsies	0	17	0%	0	3	0%
Shroyer and Greer, 1991 U.S.A. (Colorado)	Mouth	Fixed biopsies	1	10	10%	4	24	17%
Frazer et al., 1993 Australia	Mouth and Pharynx	Frozen biopsies	2	25	8%			
Miller et al., 1994 Venezuela	Mouth	Fixed biopsies	0	29	0%			
Donofrio et al., 1995 Italy	Mouth	Fixed biopsies	14	26	54%	3	6	50%
Cerovac et al., 1996 Croatia	Mouth Pharynx		9 5	26 25	35% 20%			
Premoli-De-Percoco et al., 1998 Venezuela	Mouth	Fixed biopsies	35	50	70%			
		Total	**102**	**472**	**22%**	**44**	**264**	**17%**

Table 4. Case series for detection of HPV in oral lesions using in Southern blot (SBH) and dot blot hybridization (DBH)

Author, year, study area	Site	Type of material	Method	Squamous cell carcinomas			Benign and Premalignant Lesions		
				HPV +	Tested	Percentage	HPV +	Tested	Percentage
De Villiers et al., 1985 Germany	Mouth	Fixed biopsies	SBH	3	7	43%			
Loning et al., 1987 Germany	Mouth	Fixed biopsies	DBH	5	13	38%	3	4	75%
Brandsma & Abramson, 1989 U.S.A.	Mouth	Frozen biopsies	SBH	2	21	10%			
	Tonsils		SBH	2	7	29%			
Chang et al., 1989 China	Mouth	Frozen biopsies	SBH	13	17	76%			
Yeudall and Campo, 1991 U.K.	Mouth	Frozen biopsies	SBH	3	39	8%			
Watts et al., 1991 U.S.A.	Mouth	Fixed biopsies	SBH	8	8	100%			
	Tonsils		SBH	2	3	67%			
Tsuchiya et al., 1991 Japan	Mouth	Frozen biopsies	SBH	3	23	13%			
Kellokoski et al., 1992 Finland	Mouth	Frozen biopsies	SBH				9	60	15%
Howell and Gallant, 1992 Canada	Mouth	Frozen biopsies	SBH	1	8	13%			
Frazer et al., 1993 Australia	Mouth and Pharynx	Frozen biopsies	SBH	0	25	0%			
			DBH	0	25	0%			
		Total		**42**	**171**	**25%**	**12**	**64**	**19%**

2.3.2.2 Detection of HPV DNA in oral squamous cell cancers using PCR techniques

Most of the studies carried out during the last decade used PCR based methods for HPV DNA detection, and the presence of HPV was confirmed in a variable proportion of oral, carcinomas. Table 5 summarizes studies that used PCR based techniques for detection of viral DNA in oral cancers, and in oral benign and premalignant tissues. It was impossible to discriminate between benign and premalignant lesions, given that most studies did not provide enough information to determine detection rates specific for the different lesions. As with other techniques, there was great variability in the detection of HPV DNA. Detection rates ranged from 0 to 100%. The great variability of detection rate among different studies could be due in part to the different types of clinical specimens used, to the different PCR protocols utilized in the detection of the virus, to the different populations studied, and to the variable level of expertise in HPV detection assays between different research groups.

The overall HPV detection rate in benign and premalignant lesions was 40% (95%CI:34-46%). The average detection rate in oral cancers was 30% (95% CI: 28-32%), being higher than for studies that used techniques other than PCR. On average, HPV DNA was detected in a higher proportion of tonsillar carcinomas (45%, 83 out of 185) than in cancers of the mouth (28%, 659 out of 2334). Studies that examined oropharyngeal cancers without reporting subsites -tonsils or other oropharyngeal cancers- showed an average detection rate of 40% (85 out of 214). The study of Balaram et al., (1995) that showed one of the highest prevalence rates (74%), is of special interest, given that PCR products of positive samples were confirmed as containing HPV DNA by direct sequencing.

Table 5. Case series for detection of HPV in oral lesions using PCR based techniques

Author, year, study area	Site	Type of material	Squamous cell carcinomas			Benign and Premalignant lesions		
			HPV +	Tested	Percentage	HPV +	Tested	Percentage
Kiyabu et al., 1989 U.S.A.	Mouth	Fixed biopsies	5	15	33%			
Chang et al., 1990 Finland	Mouth	Fixed biopsies	11	40	28%			
Watts et al., 1991 U.S.A.	Mouth	Fixed biopsies	9	13	69%			
	Tonsils		1	5	20%			
Shroyer et al., 1991 U.S.A.	Mouth	Fixed biopsies	1	10	10%	4	24	17%
Shindoh et al., 1992 Japan	Mouth	Fixed biopsies	8	24	33%			
Kellokoski et al., 1992 Finland	Mouth	Fixed biopsies				7	7	100%
Holladay and Gerald, 1993 U.S.A.	Mouth	Fixed biopsies	10	46	22%	7	27	26%
Ogura et al., 1991 Japan	Mouth	Fixed biopsies	0	15	0%			
	Tonsils		1	4	25%			
Miller et al., 1994 Venezuela	Mouth	Fixed biopsies	19	29	66%			
Balaram et al., 1995 India	Mouth	Frozen&Fixed	67	91	74%			
Fouret et al., 1997 France	Mouth	Fixed biopsies	2	21	10%			
	Oropharynx		7	26	27%			

(cont'd)

Table 5 (cont'd). Case series for detection of HPV in oral lesions using PCR based techniques

Author, year, study area	Site	Type of material	Squamous cell carcinomas			Benign and Premalignant lesions		
			HPV +	Tested	Percentage	HPV +	Tested	Percentage
Shindoh et al., 1995 Japan	Mouth	Fixed biopsies	24	77	31%			
Van Rensburg et al., 1996 South Africa (Black Pop'n)	Mouth	Fixed biopsies	3	146	2%			
Haraf et al., 1996 U.S.A.	Mouth	Fixed biopsies	0	14	0%			
	Tonsil		9	18	50%			
	Other Oropharynx		1	8	13%			
Nielsen et al., 1996 Denmark	Mouth	Fixed biopsies	20	49	41%			
Mao et al., 1996 U.S.A.	Mouth	Fixed biopies	15	61	25%	5	13	38%
Wen et al., 1997 China	Mouth	Fixed biopies	14	45	31%			
Portugal et al., 1997 U.S.A.	Mouth	Fixed biopies	4	58	7%			
	Tonsils		7	42	17%			
Wilczynski et al., 1998 U.S.A.	Tonsil	Frozen & Fixed	14	22	64%			
Elamin et al., 1998 U.K.	Mouth	Fixed biopsies	14	28	50%	4	12	33%
Ibrahim et al., 1998 Sudan	Mouth	Fixed biopsies	0	28	0%			

(cont'd)

Table 5 (cont'd). Case series for detection of HPV in oral lesions using PCR based techniques

Author, year, study area	Site	Type of material	Squamous cell carcinomas			Benign and Premalignant lesions		
			HPV +	Tested	Percentage	HPV +	Tested	Percentage
Adams et al., 1999 Switzerland	Mouth Tonsil	Fixed biopsies	2 1	15 5	13% 20%			
Pintos et al., 1999 Canada	Mouth Oropharynx	Fixed biopsies	3 6	29 20	10% 30%			
Aggelopoulou et al., 1999 Greece	Oral	Fixed biopsies	40	81	49%	10	21	48%
Shima et al., 2000 Japan	Mouth	Fixed biopsies	25	46	54%			
Bouda et al., 2000 Greece	Mouth	Fixed biopsies	18	19	95%	30	34	88%
Sand et al., 2000 Sweden	Mouth	Fixed biopsies	3	24	13%	8	29	28%
Niv et al., 2000 Israel	Mouth Tonsils	Fixed biopsies	1 3	19 4	5% 75%			
Tsuhako et al., 2000 Japan	Mouth Oropharynx	Fixed biopsies	40 7	46 12	87% 58%			
Yeudall and Campo, 1991 U.K.	Mouth	Frozen biopsies	18	39	46%			
Snidjers et al., 1992 Netherlands	Tonsils	Frozen biopsies	10	10	100%			
Frazer et al., 1993 Australia	Mouth & Pharynx (Subsite NS)	Frozen biopsies	4	25	16%			

(cont'd)

Table 5 (cont'd). Case series for detection of HPV in oral lesions using PCR based techniques

Author, year, study area	Site	Type of material	Squamous cell carcinomas			Benign and Premalignant lesions		
			HPV +	Tested	Percentage	HPV +	Tested	Percentage
Ogura et al., 1993 Japan	Mouth	Frozen biopsies	0	15	0%			
	Tonsils		1	4	25%			
Tyan et al., 1993 China	Mouth	Frozen biopsies	1	9	11%			
Lee et al., 1993 U.S.A.	Mouth and Pharynx	Frozen biopsies	5	45	11%			
Ostwald et al., 1994 Germany	Mouth	Frozen biopsies	16	26	62%			
Lewensohn-Fuchs et al., Sweden, 1994	Mouth	Frozen biopsies	4	9	44%			
	Tonsils		1	4	25%			
Anderson et al., 1994 North America	Mouth	Frozen biopsies	6	27	22%			
Brandwein et al., 1994 U.S.A.	Mouth	Frozen biopsies	8	43	19%			
	Tonsils		6	16	38%			
Barten et al., 1995 Germany	Mouth	Frozen biopsies	26	37	70%			
Cruz et al., 1996 Netherlands	Mouth	Frozen biopsies	19	35	54%			
Snijders et al., 1996 U.K.	Mouth	Frozen biopsies	5	25	20%			
	Oropharynx		2	7	29%			
Chiba et al., 1996 Japan	Mouth	Fresh biopsies	8	38	21%			

(cont'd)

Table 5 (cont'd). Case series for detection of HPV in oral lesions using PCR based techniques

Author, year, study area	Site	Type of material	Squamous cell carcinomas			Benign and Premalignant lesions		
			HPV +	Tested	Percentage	HPV +	Tested	Percentage
Paz et al., 1997 U.S.A.	Mouth Tonsil	Frozen biopsies	9 9	71 15	13% 60%			
Fouret et al., 1997 France	Oropharynx	Frozen biopsies	13	20	65%			
Riethdorf et al., 1997 Germany	Mouth Tonsils	Frozen biopsies	33 2	78 3	42% 67%			
Alvarez et al., 1997 Spain	Mouth Oropharynx	Frozen biopsies	1 4	2 19	50% 21%			
Andl et al., 1998 Germany	Tonsil	Frozen biopsies	11	21	52%			
Atula et al., 1997 Finland	Mouth Oropharynx	Frozen biopsies	8 0	39 6	21% 0%			
Penhallow et al., 1998 U.K.	Mouth	Frozen biopsies	14	28	50%	4	12	33%
Hoffmann et al., 1998 Germany	Oropharynx	Frozen biopsies	6	23	26%			
Matzow et al., 1998 Sweden	Mouth	Frozen biopsies	1	33	3%	0	3	0%
D'Costa et al., 1998 India	Mouth	Frozen biopsies	15	100	15%	27	80	34%
Miguel et al., 1998 Brazil	Mouth Tonsil	Frozen biopsies	1 3	18 6	6% 50%			

(cont'd)

Table 5 (cont'd). Case series for detection of HPV in oral lesions using PCR based techniques

Author, year, study area	Site	Type of material	Squamous cell carcinomas			Benign and Premalignant lesions		
			HPV +	Tested	Percentage	HPV +	Tested	Percentage
Mineta et al., 1998 Japan	Mouth	Frozen biopsies	3	14	21%			
	Oropharynx		5	13	38%			
Badaracco et al., 2000a Italy	Mouth	Frozen biopsies	8	25	32%			
	Tonsil		2	2	100%			
Saranath et al., 1999 India	Mouth	Frozen biopsies	12	83	14%			
Koch et al., 1999 U.S.A	Mouth	Frozen biopsies	37	211	18%			
Gillison et al., 2000 U.S.A.	Mouth	Frozen biopsies	10	84	12%			
	Oropharynx		34	60	57%			
Sisk et al., 2000 U.S.A.	Oral	Frozen biopsies	11	22	50%			
Badaracco et al., 2000b Italy	Mouth	Frozen biopsies	10	38	26%			
	Tonsil		2	4	50%			
Mao, 1995 U.K.	Mouth	Exfoliated cells	8	26	31%			
	Subtotal Mouth carcinomas		**659**	**2334**	**28%**			
	Subtotal Tonsillar carcinomas		**83**	**185**	**45%**			
	Subtotal Orophryngeal carcinomas		**85**	**214**	**40%**			
	TOTAL		**827**	**2733**	**30%**	**106**	**262**	**40%**

So far I have summarized prevalence studies on HPV DNA detection in oral squamous cell carcinomas and other oral lesions. The interpretation of the significance of HPV detection in these cancers should be made with caution. The virus could colonize malignant tissues without playing any role in the natural history of the disease, and there is a variety of normal epithelial tissues in the head and neck that can harbour HPVs. Fortunately, there are several comparison studies that included normal tissues, and can help us to better assess the role of HPV in oral cancers.

2.3.2.3 Case series with a comparison group

At least 18 studies have examined the presence of HPV DNA in oral squamous cell carcinomas and in comparison series. Most studies included non cancer patients as controls, while two investigations (Chang et al., 1990; Howell and Gallant, 1992) used normal tissues of the index cases as a comparison group. One study (Ostwald et al., 1994) used both non cancer controls and non cancer tissues from cases as comparison series. Most investigations provide little or no information on sex, gender, or smoking status of the participating subjects. Four studies utilized hybridization techniques without amplification (SBH or ISH) for detection of HPV DNA; thirteen studies used PCR based techniques, whereas one investigation (Yeudall and Campo, 1991) used both methods for viral detection.

Table 6 summarizes studies with comparison groups that used for viral detection hybridization techniques withouth DNA amplification. Brandsma and Abramson (1989), studied 101 cases of head and neck cancers and 116 tissues from matched anatomic sites (control group) for the presence of HPV. Control patients were, on average, 7.5 years younger than cancer patients. The control group of tissues included epithelial mucosa from benign lesions, congenital and structural abnormalities, and clinically normal sites adjacent to benign lesions. Among all the studied subjects, 28 had oral cancer, and 38 were the controls. Presence of HPV DNA was examined with SBH, using DNA probes specific for HPV types 6, 11, and 16. HPV 16 was detected in two of 21 mouth cancers (10%), and in two of seven tonsillar cancers (29%). None of the control tissues was positive.

Chang et al. (1989), in Taiwan compared HPV detection rates in 17 mouth cancer tissues, and 17 normal oral tissues from patients who underwent dental extractions. Presence of HPV DNA was examined using SBH, with HPV 6, 11, 16, and 18 probes, and 13 of the 17 (76%) carcinomas were shown to contain episomal HPV 16. Of the normal tissues, only one of the 17 were positive for HPV 16. None of the specimens examined were positive for HPV 6, 11, or 18. The authors did not provide information on age or sex.

Niedobitek et al. (1990) examined 28 tonsillar carcinomas, and as a control group they used 30 tonsils removed because of chronic inflamation. Control patients were comparable to cases on the basis of age and sex. Presence of DNA from HPV 6, 11, and 16 was assessed using ISH. Six of the tonsillar carcinomas (21%) turned out

Table 6. Studies of HPV and oral carcinomas with a comparison group using hybridization techniques (SBH and ISH)

Author, year, study area	Site	Type of material	Method	Squamous cell carcinomas			Controls tissues		
				HPV +	Tested	Percent.	HPV +	Tested	Percent
Brandsma and Abramson U.S.A., 1989	Mouth Tonsils	Frozen biopsies	SBH	2 2	21 7	10% 29%	0 0	18 20	0% 0%
Chang et al., 1989 Taiwan	Mouth	Fixed biopsies	SBH	13	17	76%	1	17	6%
Niedobitek et al., 1990 Germany	Tonsils	Fixed biopsies	ISH	6	28	21%	0	30	0%
Howell and Gallant, 1992 Canada	Mouth	Frozen biopsies	SBH	1	8	13%	0	7	0%
Yeudall and Campo, 1991 U.K.	Mouth	Frozen biopsies	SBH	3	39	8%	0	25	0%
		Total		**27**	**120**	**23%**	**1**	**117**	**1%**

positive for HPV 16, while none of the 30 control tissues were positive. Hybridization to the HPV 6 and 11 probes gave negative results for both cases and control tissues.

Howell and Gallant (1992) examined oral tumors from eight patients, and normal mucosa from seven of those patients. HPV DNA was detected by SBH in one of the eight tumours, and in none of the seven normal tissues.

Yeudall and Campo (1991), using both SBH and PCR, analyzed biopsies from 39 primary oral carcinomas, and from 25 control samples of normal buccal mucosa from cancer-free individuals. By SBH, three of the 39 tumours and none of the 25 controls were positive for HPV.

Combining the previous five studies (table 6), a total of 120 tumour specimens and 117 non cancer tissues have been examined. HPV DNA was detected in 23% of the carcinomas (27 out of 120), and in 1% of the tumour free specimens (1 out of 117). HPV positivity rate ranged from 8% to 76% in tumours, and from 0 to 6% in control tissues. All five studies showed a higher positivity rate in tumours than in cancer free specimens.

Table 7 summarizes investigations that used PCR based techniques for detection of viral DNA. Yeudall and Campo (1991) examined samples using HPV 16 and 18 type specific PCR. Of 39 cases, 10 (26%) were positive for HPV 16, and eight further cases (21%) were positive for HPV 18. In addition, two of the 25 (8%) tumour free samples were positive for HPV18. The authors did not provide any information on sex, gender, or any other characteristics of participating subjects.

Table 7. Studies of HPV and oral carcinomas with a comparison group. PCR based techniques

Author, year, study area	Site	Type of material	Squamous cell carcinomas			Controls tissues		
			HPV +	Tested	Percent.	HPV +	Tested	Percent.
Yeudall and Campo, 1991 U.K.	Mouth	Frozen biopsies	18	39	46%	2	25	8%
Chang et al., 1990 Finland	Mouth	Fixed biopsies	11	40	28%	0	40	0%
Snijders et al., 1992 Netherlands	Tonsils	Frozen biopsies	10	10	100%	0	7	0%
Tyan et al., 1993 China	Mouth	Frozen biopsies	1	9	11%	1	11	9%
Watanabe et al., 1993 Japan	Tonsils and other oropharynx	Frozen biopsies	3	12	25%	4	28	14%
Holladay and Gerald, 1993 U.S.A.		Fixed biopsies	10	46	22%	1	6	17%
Ostwald et al., 1994 Germany	Mouth	Frozen biopsies	16	26	62%	1	97	1%
Mao, 1995 U.K.	Oral	Exfoliated cells	8	26	31%	4	26	15%
Cruz et al., 1996 Netherlands	Mouth	Frozen biopsies	19	35	54%	0	12	0%
Mao et al., 1996 U.S.A.	Mouth	Fixed biopies	15	61	25%	0	6	0%
Wang et al., 1998 China	Oral	Frozen biopsies	11	30	37%	4	30	13%
Sand et al., 2000 Sweden	Mouth	Fixed biopsies	3	24	13%	0	12	0%
Mellin et al., 2000 Sweden	Tonsils	Fixed biopsies	26	60	43%	0	10	0%
Bouda et al., 2000 Greece	Mouth	Fixed biopsies	18	19	95%	0	16	0%
		Total	**169**	**437**	**39%**	**17**	**326**	**5%**

Chang et al. (1990) examined 40 surgically removed oral squamous cell carcinomas, and the tumour-free resection margins of the same tumours. Presence of HPV DNA was examined using type specific PCR for HPV types 6, 11, 16, and 18. HPV DNA was detected in 11 of the 40 tumours (28%), while no viral DNA could be detected in the biopsies derived from the tumour free margins. HPV 16 was the most common type detected (9 of 11).

Snijders et al. (1992) assessed prevalence of HPV infection using a consensus PCR technique. HPV DNA was detected in all of the 10 biopsies of tonsillar carcinomas tested and in none of the seven biopsies of tonsillitis used as controls. The authors did not provide information on control subjects.

Tyan et al. (1993) studied nine oral tumours and eleven normal tissues by type specific PCR for HPV 6, 11, 16, 18, and 33. The authors did not state whether the control tissues came from patients with oral cancer or from cancer free patients, nor did they give any information regarding characteristics of patients. HPV DNA 16 was detected in one of the nine oral carcinomas, and in one of the eleven oral normal tissues. No other HPV type was detected.

Watanabe et al. (1993) examined presence of HPV DNA in tonsillar and other oropharyngeal tumours using type specific PCR type 16 and 18. As control group they used chronic tonsillitis specimens. On average, controls were younger than cases. Three of the 12 cases (25%) and four of the 28 controls (14%) were HPV positive.

Holladay and Gerald (1993) examined oral tissues using a consensus PCR based method, that allows for detection of several HPV types. Of the 46 oral carcinomas examined, 10 were HPV positive (22%). Control tissues consisted of six specimens of normal oral mucosa: one turned out to be HPV positive. No information was given on characteristics of patients.

Ostwald et al. (1994) studied 26 patients with oral cancers, using consensus PCR, and typing for HPVs 6, 11, 16, and 18. They examined the presence of HPV DNA in samples from three different sites: biopsy and scrapings of the surface of the tumour, scrapings of the tumour free mucosa adjacent to the tumour, and scrapings from oral mucosa distant from the tumour. HPV DNA was detected in 50% of the tumour samples, although the authors did not specify whether these results correspond to the examination of the biopsies or surface scrapings. HPV 16 was the type most frequently detected. Examination of scrapings obtained from peritumoural mucosa were HPV positive in seven of the 26 cases (27%). Only 1 sample (4%) of distant oral mucosa was HPV positive. In addition, they examined exfoliated oral cells from 97 healthy volunteers. Only one of these samples turned out to be HPV positive. Control subjects were younger than case patients.

Mao (1995) assessed HPV infection in oral exfoliated cells from 26 patients with oral cancer and volunteers with the use of type specific PCR for detection of HPV 16. Healthy volunteers were matched to cases on the basis of sex and age. HPV 16 DNA was detected in 8 of the 26 cases (31%) and in 4 of the 26 controls (15%).

Cruz et al (1996) compared HPV detection rates in 35 oral carcinomas and in 12 biopsies of normal gingival mucosa collected from volunteers. Using consensus PCR, they detected HPV DNA in 19 of the 35 oral tumours (54%), and in none of the 12 normal biopsies. Most HPV positive samples were positive for HPV 16. Healthy volunteers were younger than cases.

Mao et al. (1996) examined 61 oral carcinomas, and six biopsies from non cancer controls. Using consensus PCR, they detected HPV DNA in 15 of 61 tumours (25%) and none of the six control tissues. Control tissues came from patients with periodontal disease.

Wang et al. (1998) assessed prevalence of HPV 16 infection in 30 patients with primary oral squamous cell carcinoma, and 30 healthy controls. They examined biopsies using a type specific PCR for detection of HPV 16. HPV DNA was detected in 11 of 30 tumours and in 4 of 30 control tissues.

Sand et al. (2000) examined 24 oral carcinomas, and 12 control tissues from healthy volunteers, using a consensus PCR technique. HPV DNA was detected in three of 24 cancers, and none of the 12 normal biopsies.

Mellin et al. (2000) investigated the frequency of HPV DNA detection in 60 biopsies of tonsillar carcinomas and in 10 non malignant tissues from patients with chronic tonsillitis. By consensus PCR, HPV was detected in 26 of the 60 cancers (43%), and in none of the cancer free tissues. HPV 16 was present in all HPV positive tumours.

Bouda et al. (2000) analyzed 19 tumour tissues from cases of oral carcinomas, as well as 16 oral scrapings form healthy individuals. Using a nested consensus PCR, they detected HPV DNA in 18 of the 19 tumours (95%), and in none of the 16 samples from non cancer controls. While the age distribution of cases was similar to the age distribution of controls, the type of specimens used (biopsies *versus* scrapings) was not comparable.

A majority of these comparison studies utilized the MY09/11 or the GP5+/6+ PCR protocols (consensus PCR techniques). Detection of HPV DNA in carcinomas varied widely from one study to another, but in all studies the detection rate was substantially higher in tumours than in cancer free samples.

Combining all studies, viral DNA was detected in 169 of 437 cancers (39%), and in 17 of 326 non cancer samples (5%). HPV 16 was the most prevalent type detected, being present in 80% to 100% of all HPV positive carcinomas. All but three studies (Mao, 1995; Ostwald et al., 1994; Bouda et al., 2000) used biopsies from both cases and controls: Mao (1995) used oral exfoliated cells from both cases and controls, whereas Ostwald et al. (1994) and Bouda et al. (2000) compared detection rates using biopsies from cases and exfoliated cells from controls. Excluding these two studies, where the type of specimen of cases and controls were not comparable, the average detection rate of HPV DNA for studies which used PCR based techniques was 34% in carcinomas (135 out of 392) and 8% in cancer free samples (16 out of 213).

Some reviews have been published summarizing the prevalence of HPV detection in the head and neck mucosa (Franceschi et al., 1996; Miller and White, 1996, McKaig et al.,

Table 8. Reviews on detection of HPV in oral tissues

Author, year	Site	Type of tissue	Detection technique[1]	HPV+/ tested	Rate
Franceschi et al., 1996	Head&Neck	Non tumoral tissues	PCR	27/235	11%
			No amplification	9/199	5%
		Squamous cell carcinomas	PCR	112/254	44%
			No amplification	39/173	23%
Miller and White, 1996	Mouth	Normal Mucosa	PCR	91/358	25%
			No amplification	48/668	7%
		Benign leukoplakia	No amplification	78/526	15%
		Intraepithelial Neoplasia	PCR	20/48	42%
			No amplification	34/252	13%
		Squamous cell carcinomas	PCR	130/355	37%
			No amplification	145/696	21%
McKaig et al., 1998	Head&Neck	Benign and premalignant lesions	SB	33/92	36%
			DB	8/34	24%
			ISH	180/972	19%
		Squamous cell carcinomas	SB	66/269	25%
			DB	20/319	6%
			ISH	52/286	18%
			PCR	416/1205	35%

1. Abbreviations: PCR, Polymerase Chain Reaction, SB, Southern Blot, DB, Dot blot, ISH, *In situ* hybridization.

1998). These reviews compiled the existing evidence on HPV detection rates according to detection techniques and type of tissue (table 8). In average, all reviews show that PCR based techniques detected the virus in a higher proportion of samples than non amplification techniques. Also HPV was detected more often in cancers than in non tumoural tissues. The detection rate in benign and premalignant lesions is rather similar to the detection in malignant tissues.

2.3.2.4 Case-control studies

Six studies followed a design that could be defined as a case-control study. In these studies there is a description of eligibility criteria for cases and controls, as well as an attempt to define the study base, either primary or secondary. These studies are summarized in table 9.

Maden et al. (1992) carried out a population-based case-control study in the Washington state area to examine the relationship between HPV and risk of oral cancer in men. Cases were identified form a cancer registry covering the area, and controls were selected by random digit dialing. The study comprised a total 131 cases of oral carcinoma and 136 controls matched to the cases by age. The presence of HPV was investigated in exfoliated cells from the oral cavity, collected with a soft toothbrush from both cases and controls, using type specific PCR for HPV 6 and HPV 16. HPV 6 was detected in 22 of 118 tested cases (19%), and 10 of 112 tested controls (9%), for a crude OR of 2.9 (95%CI:1.1-7.3). Adjustment for age, smoking, and alcohol consumption, did not substantially change the point estimate. HPV 16 was detected in 6 of 108 the cases (6%), and in 1 of 106 controls (1%). The crude OR of disease for HPV 16 was 6.2 (95%CI:0.7-52.2).

Table 9. Case-controls studies of HPV detection and risk of oral carcinomas

Author, year, study area	Site	Type of material	HPV type	Cases			Controls			OR	95%CI	Study Population
				HPV+	N	Percent.	HPV+	N	Percent.			
Maden et al., 1992	Mouth	Exfoliated	HPV 6	22	118	19%	10	112	9%	2.3	(1.1-5.2)	Prevalent cases
U.S.A (Seattle)		cells	HPV 16	6	108	6%	1	116	1%	6.8	(0.8-57.1)	Population-based
Schwartz et al., 1998	Mouth	Exfoliated	All types	22	237	9%	40	435	9%	0.9	(0.5-1.6)	Prevalent cases
U.S.A (Seattle)		cells	HPV 6,11	6	237	3%	19	435	4%	0.5	(0.2-1.4)	Population-based
			HPV16,18	14	237	6%	18	435	4%	1.3	(0.6-2.9)	
Smith et al., 1998	Mouth	Exfoliated	All types	14	93	15%	10	205	5%	3.7	(1.5-9.3)	Incident cases
U.S.A (Iowa)		cells										Clinic controls
Summersgill et al.,	Mouth	Exfoliated	All types	58	202	29%	62	333	19%	1.8	(1.1-2.7)	Incident cases
U.S.A (Iowa), 2000		cells	High Risk	46	202	23%	37	333	11%	2.4	(1.4-3.9)	Clinic controls
Nishioka et al., 1999	Mouth	Biopsies	HPV16,18	3	14	21%	0	14	0%	ND[1]	ND	Controls with
Japan	Pharynx			0	15	0%	0	17	0%			benign disease
Herrero et al., 2000	Oral	Exfoliated	All types	86	1625	5%	81	1532	5%	1.0	(0.7-1.4)	Incident cases
International		cells										Hospital controls

1. ND: Not determined

The authors identified from the cancer registry 241 cases of oral cancer diagnosed between 1985 and 1989, and 58 of them (24%) were deceased before recruitment. One of the major concerns with this study is that exfoliated cells from cancer patients were collected after treatment of the disease. Surgical removal of the tumour and surrounding areas, and radiation treatment, will affect the detection of tumoural HPV infection. Another investigation by the same research group (Schwartz et al., 1998), which will be summarized below, assessed the difference in HPV detection rates before and after treatment.

Schwartz et al. (1998) conducted a case-control study following a methodology very similar to the study by Maden et al. (1992). They recruited cases diagnosed between 1990 and 1995 in Washington State. Of a total of 449 eligible subjects, they recruited 284. Of 729 potential control subjects selected by random digit dialing, they recruited 477. Oral exfoliated cells from both cases and controls were collected at the time of the interview, that took place after treatment of the disease (median time following diagnosis: 8 months). Presence of HPV DNA was examined using a consensus PCR protocol (MY09/11), as well as type specific PCR for HPV 6, 11, 16, 18, and 31/33/15. The detection rate for any HPV type was 9% for both cases an controls, for an OR of 0.9 (96%CI:0.5-1.6). In addition to exfoliated cells, 248 archival tumour specimens from participating cases were examined using the same detection technique. HPV DNA was found in 64 of the 248 tumours (26%), a higher detection rate than the one seen in exfoliated cells (9%). HPV 16, the most common type detected, was present in 11% of carcinomas of the mouth (22 out of 193), in 15 of 44 tonsillar tumours (34%), and in four

of 11 tumours of other oropharyngeal tumours (36%). These two studies did not find any association between sexual practices (including oral sex) and risk of oral cancer.

In another case-control study carried out in Iowa, U.S.A. (Smith et al., 1998), a total of 93 newly diagnosed patients of oral cancer and 205 control patients were recruited. Controls, frequency matched to cases on the basis of age and gender, were selected from nondiseased patients who attended family practice and dentistry clinics. HPV was evaluated from a mouth rinse collection of cells in the oral cavity, and tested with consensus PCR (MY09/11). HPV DNA was identified in 14 of the 93 oral cancer (15%) and in 10 of 205 controls (5%). The OR of cancer associated with HPV infection was 3.7 (95%CI:1.5-9.3), adjusted for tobacco and alcohol use. When the authors compared the prevalence of HPV infection according to oncogenicity, high risk types were detected in 71% of HPV positive cases, and in 30% of HPV positive controls. The analysis of markers of sexual behaviour did not show significant difference between cases and controls. The authors did not provide information on subsite analysis, comparing detection rates on mouth, tonsils, and other oropharyngeal cancers.

The same research group published a second case-control study (Summersgill et al., 2000) using the same elegibility criteria for recruitment of cases and controls, and the same methodology for sample collection and HPV DNA detection as the previous study. Oral exfoliated cells from 202 patients with oral cancer and 333 controls, frequency matched on age and gender, were evaluated by consensus PCR (MY09/11) for the presence of the virus. HPV DNA was detected in 29% of cases, and in 19% of controls, for a crude OR of 1.8 (95%CI:1.1-2.7). The detection rate of high risk HPV types in

cases and controls was 23% and 11%, respectively (OR=2.4; 95%CI:1.4-3.9). Again, the authors did not provide any information on analysis by subsite.

Nishioka et al. (1999), in Japan, examined patients with head and neck carcinomas and controls with head and neck benign lesions. A total of 15 patients with cancer of the mouth and 14 with pharyngeal cancer were recruited. Controls were matched to cases in terms of anatomical site of the lesion, age, gender, and smoking status (smoker or non smoker). Presence of HPV DNA was examined using type specific PCR in surgically removed tumours for cases, and in surgically removed benign lesions for controls. The detection rate was very low: 3 of the 14 cancers of the mouth (21%) were HPV positive, while none of the pharyngeal cancers or mouth and pharyngeal controls were positive.

Herrero et al. (2000) presented results from the multinational case control study in HPV and oral cancer. Cases and controls were recruited from 14 centers around the world. Information on risk factors was obtained through an interview, and oral exfoliated cells were collected before any treatment took place. HPV DNA was examined using the GP5+/6+ PCR method. Laboratory results were available for 1711 cases and 1613 controls. In exfoliated cells, HPV was detected in approximately 5% of both cases and controls, with higher detection in cancer of the tonsils (10%). Biopsy specimens from case tumours were also examined with the same method. Preliminary results showed a low overall prevalence of HPV detection in biopsies (8%), being higher in cancers of the tonsils (15%) compared to other sites (6%).

Of the six case-control studies (table 9), only two found a possitive association between detection of HPV and oral cancer (Smith et al., 1998; Summersgill et al., 2000). The study by Nishioka et al (1999) may be considered the less rigorous. Although there is a definion of eligible cases and controls, the authors did not attempt to explore any potential confouding due to other risk factors. The prevalence of HPV DNA detection was very low in cases and nil in controls.This study is the lest informative among those in table 9 since the number of participating subjects was the lowest.

Four studies were conducted in the U.S.A. by two research groups. The studies conducted in the Seattle area (Maden et al., 1992; Schwartz et al.; 1998) did not find a positive association between HPV and cancer of the mouth. The interpretation of these results should take into account the fact that both studies assessed the main exposure in cases that had already been treated for their disease. Most likely, cancer treatment affected the detection of tumoural HPV infection.

The two other studies carried out in the U.S.A. (Smith et al., 1998; Summersgill et al., 2000) showed a positive association between presence of the virus and oral cancer. The two investigations enrolled cases before they received treatment. The main purpose of the study by Summersgill et al. (2000) was to evaluate the association between p53 polymorphism at codon 72, and HPV infection in the oral cavity and oral cancer. Nevertheless, the authors did not discuss the difference in HPV detection rates reported in the two publications by the same reseach group (Smith et al., 1998, and Summersgill et al., 2000). Having used the same methodology, the HPV infection rate in the former study was 15% in cases and 5% in controls, while in the latter study was 29% and 19%,

respectively. It is not clear whether the patients recruited for the first study were also included in the second publication.

The study with the largest number of patients (Herrero et al., 2000), failed to find any association. The main similarities betweeen this study and the previous two are that the three investigations recruited newly diagnosed cases –before any treatment was received-, and that presence of HPV was examined in oral exfoliated cells. The main differences are the study populations and the detection techniques: Herrero et al. (2000) utilized the GP5+/GP6+ PCR method, while the other two investigations used the MY09/MY11 protocol. The difference in results are unexpected and very difficult to explain, since both detection techniques have very similar sensitivity, and it is very unlikely that the difference in study populations may explain alone such a difference. These studies will be further discussed in the discussion section.

2.3.2.5 Oral cancer and HPV serology

Studies of humoral immunity to HPV have been hampered by the lack of suitable antigenic targets for serological assays, since neither clinical lesions nor *in vitro* culture systems are practical source of viruses (Stanley, 2001). These limitations have been overcome by the demonstration that the expression of the L1 capsid protein via recombinant vectors results in the assembly of the protein into a conformationally correct virus like particle (VLP), also known as capsids. Kirnbauer et al. (1994) developed a standard enzyme linked immunosorbent assay (ELISA) based on HPV 16 VLPs used as antigen bound to the solid phase. This immunological assay showed a good concordance between serological response and detection of viral DNA in the cervix. In this study, serum IgG antibodies against HPV 16 VLPs were found in 59% of

women testing positive for cervical HPV 16 DNA, whereas only 6% of women negative for cervical HPV or positive for the benign HPV 6 or 11 had these antibodies (Kirnbauer et al. 1994). The lack of perfect concordance could be due to several reasons: i) there are at least 130 HPV types described, and serological cross-reactions between different types cannot be ruled out; ii) most HPV infections are transient, and they are cleared spontaneously. Many people testing negative for HPV DNA may have had a previous infection; iii) seropositivity may have resulted from antigenic exposure in the oral cavity and not in the genital tract; and iv) not all seroconversions against HPV VLPs are seen immediately after acquisition of HPV infection; at least in some individuals, seroconvertions may take several months (Wikström et al., 1995; Dillner, 1999).

Validation of serological assays has been studied using follow up studies. Assessment of sensitivity and specificity of humoral response has been done using detection of HPV DNA as a reference despite the above caveats. In general, well designed studies have found a sensitivity of at least 50%. All studies addressing type specific serology for HPV capsids have found a high specificity (reviewed by Dillner, 1999). Formal studies on testing-retesting variability and variability between different laboratories have found good agreement (af Geijersstam et al., 1998; Strickler et al., 1997).

Studies of HPV serology and cervical HPV infection or cervical lesions showed that HPV seropositivity was more strongly associated with markers of sexual activity than with detection of cervical HPV DNA, specially in populations with low prevalence of HPV infection. These findings suggest that serological response is more a marker of past cumulative HPV exposure rather than current HPV infection, and that most

seroconversions are persistent (Nonnenmacher et al., 1996; Dillner et al., 1995a; reviewed by Dillner, 1999).

Only a few investigations have been carried out on HPV serology and oral cancer. A few more have been done on esophageal carcinomas and on head and neck cancers, including a small number of oral malignancies. These investigations are summarized in table 10. Dillner et al. (1995b) conducted a nested case-control study using a serum bank comprising samples collected between 1968 to 1972 from 39,268 healthy individuals in Finland. Registry linkage with the Finish cancer registry identified 39 cases of esophageal cancer, and 89 cases of mouth cancer that had occurred in the cohort up to 1991. For each cancer patient, two controls (free of cancer at baseline) were selected, matched for sex, age, and municipality. Detection of IgG against HPV 16 capsids was performed by ELISA. Eight of 39 esophageal cancers (21%) were positive for HPV 16 capsids at the preassigned cutoff levels, for a smoking adjusted ORs of 13.1 (95%CI: 1.6-108). For cancers of the mouth, 5 of 89 cancers were seropositive (6%); smoking adjusted OR of 0.5 (95%CI: 0.1-4.5).

Han et al. (1996) also studied esophageal cancers. They carried out a hospital-based case control study in China that included 90 cases of esophageal cancer and 121 cancer-free control subjects, matched to cases on the basis of age and sex. Blood samples were drawn at the moment of recruitment, and presence of HPV 16 antibodies was determined by ELISA using HPV 16 VLPs. The mean seroreactivity was significantly higher among cases compared to controls. Using a preassigned cutoff point for HPV 16 seropositivity, 24% of cancer patients were seropositive compared with 7% of the control subjects, yielding an OR of 4.5 (95%CI:1.8-11.9).

Table 10. Case-controls studies of HPV serology and oral carcinomas

Author, year, study area	Site	HPV type	Cases			Controls			OR	95%CI	Characteristics of the study
			+	N	Perc.	+	N	Perc.			
Dillner et al., Finland, 1995b	Esophagus	HPV 16	8	36	22%	2	78	3%	13.1	(1.6-108.0)	Serum Bank
	Mouth	HPV 16	5	89	6%	ND[1]	ND	ND	0.5	(0.1-4.5)	Nested case-control
Han et al., China, 1996	Esophagus	HPV 16	24	90	27%	7	121	6%	4.5	(1.8-11.9)	Hospital-based
Bjorge et al., Norway, 1997	Esophagus	HPV 16	9	57	16%	2	171	1%	6.2	(1.0-6.7)	Serum Bank
		HPV 18	11	57	19%	5	171	3%	2.3	(0.6-7.6)	Nested case-control
		HPV 33	12	57	21%	3	171	2%	4.5	(1.1-21.0)	Adjusted for cotinine levels
Schwartz et al., U.S.A., 1998											
HPV - tumors	Oral	HPV 16	73	139	53%	156	446	35%	2.5	(1.6-3.8)	Prevalent cases
HPV + tumors	Oral	HPV 16	28	37	76%	156	446	35%	6.8	(3.0-15.2)	
Lagergren et al., Sweden, 1999	Esophagus	HPV 16	6	113	5%	19	288	7%	1.0	(0.5-2.0)	Population based
		HPV 18	4	119	3%	9	281	3%	0.5	(0.2-1.1)	Adj. for smoking and alcohol
Zumbach et al., Germany, 2000	Head and Neck	HPV 16/18	11	92	12%	10	288	3%	3.8	(1.4-10.0)	E6 & E7 proteins as antigens
Mork et al., Scandinavia, 2001	Head and Neck	HPV 16	35	292	12%	102	1568	7%	2.2	(1.4-3.4)	Serum Bank
		HPV 18	17	292	6%	101	1568	6%	1.0	(0.6-1.8)	Nested case-control
		HPV 33	22	292	8%	154	1568	10%	0.8	(0.5-1.3)	Adjusted for cotinine levels
		HPV 73	15	292	5%	111	1568	7%	0.6	(0.4-1.2)	
	Tongue	HPV 16	9	57	16%	22	302	7%	2.7	(1.2-6.6)	
	Oropharynx	HPV 16	10	26	38%	14	137	10%	14.4	(3.6-58.1)	

1. ND: Not determined.

Bjorge et al. (1997) used a very similar methodology to the one used by Dillner et al. (1995b). They took advantage of the Janus serum bank, in Norway, that contains samples from approximately 300,000 individuals. Data from the serum bank were linked to the cancer registry of Norway to identify cases of esophageal cancer diagnosed after donation of the serum. A total of 57 cases were identified. Three controls per case were selected from the cohort, individually matched on sex, age at serum sampling, storage time, and county of residence. They examined antibodies anti VLPs for HPV 16, 18, and 33 using an ELISA assay. There was an increased risk of esophageal cancer among patients seropositive for HPV 16 (OR=6.2;95%CI:1.0-6.7). For HPV 33, the OR was 4.5 (1.1-2.1). Results for HPV 18 were not significant (OR=2.3;95%CI:0.6-7.6). All ORs were adjusted for serum cotinine levels, a biological marker of tobacco smoking.

Schwartz et al. (1998) determined the seropositivity for HPV 16 capsids among 259 cases of oral cancer, recruited after treatment, and 446 control subjects. Serological response to HPV 16 was determined using an ELISA technique. Mean values of seroreactivity were significantly higher among cases. Using an empirical cutoff point, 51% of cases and 35% of controls were seropositive for HPV 16, with a resulting OR of 2.3 (95%CI:1.6-3.3). The authors stratified the analysis according to detection of HPV DNA in tumours. The OR of disease for patients with tumours positive for HPV DNA was 6.8 (95%CI:3.0-15.2), whereas for patients with HPV negative tumours the OR was 2.5 (95%CI:1.6-3.8). The authors did not present subsite specific analysis, such as for patients with cancer of the mouth, tonsillar cancer, or other oropharyngeal cancers.

Lagergren et al. (1999) conducted a case-control study in Sweden. They assessed increased risk of esophageal cancer according to serological response to HPV 16 and

HPV 18 capsids. They recruited 121 cases of esophageal squamous cell carcinoma, 173 cases of adenocarcinoma of the esophagus, and 302 population based controls. To determine IgG antibodies against HPV 16 and HPV 18 capsids they used an ELISA assay identical to one used in previous studies (Dillner et al., 1995b; Bjorge et al., 1997). Participating subjects were interviewed in person to collect detailed information about several possible risk factors. Age and sex adjusted ORs of squamous cell carcinoma for HPV 16 seropositivity was 1.0 (95%CI:0.5-2.0), and for HPV18 seropositivity was0.5 (95%CI:0.2-1.1). The corresponding ORs for adenocarcinoma were 1.2 (95%CI:0.7-2.2), and 0.2 (95%CI:0.1-0.7). These results differ substantially with those found by in previous studies on esophageal cancer (Dillner et al., 1995b; Han et al., 1996; Bjorge et al., 1997). The main reason proposed by Lagergren et al. to explain these differences is the lack of extensive control of confounding in previous studies. However, in their study, the crude and adjusted ORs did not differ much (Lagergren et al. 1999).

Zumbach et al. (2000), in Germany, studied HPV serology in 92 cases of head and neck cancers, and 288 healthy individuals who served as controls. Among the recruited cases were patients with cancer of the mouth, oropharynx, hypopharynx, larynx, and with unknown primaries. HPV serology was determined in samples taken at the moment of diagnosis using four ELISA assays against HPV type 16 and 18 E6 and E7 proteins, respectively. Antibodies against these proteins were found in 11 of 92 cases (12%) and in 10 of 288 controls (3%). Antibodies against HPV 16 oncoproteins were found in 10 of the 11 seropositive cases. The highest prevalence of seropositivity was seen in patients with laryngeal carcinomas (6 out 31, 19%). The seropositivity rate in patients with oropharyngeal cancer was 9% (5 out of 54), and none of patients with cancers of other sites were seropositive.

Mork et al. (2001) used a very similar methodology to previous studies that used serum banks (Dillner et al., 1995b; Bjorge et al., 1997). Data files from four serum banks in Norway, Finland, and Sweden, comprising 900,000 subjects, were linked to the national cancer registries. Persons who developed head and neck cancer (mouth, pharynx, larynx, and nose and paranasal sinuses) and donated blood at least one month before diagnosis were identified. A total of 292 cases were identified, and five to seven controls per case were selected from the cohort. Controls were alive and free of head and neck cancer at the time of diagnosis of the index case. Controls were individually matched based on sex, age at serum sampling, and storage time. Presence of antibodies against VLPs for HPV 16, 18, 33, and 73 was determined using an ELISA assay. After adjustment for cotinine levels, the OR for squamous cell carcinoma of the head and neck in subjects seropositive for HPV 16 was 2.2 (95%CI:1.4-3.4). No increased risk was observed for other HPV types. The analysis by subsite showed that the highest risk was seen for oropharyngeal cancer (OR=14.4;95%CI:3.6-58.1), and carcinoma of the tongue (OR=2.7;95%CI:1.2-6.6).

Of the seven studies on HPV serology and head and neck cancers, summarized in table 10, four examined esophageal cancers, two examined several head and neck sites, and one (Schwartz et al., 1998) was limited to oral cancers. All studies but one utilized a serological assay to detect IgG against viral capsids, known also as VLPs; the study by Zumbach et al. (2000) used an ELISA for detection of antibodies against oncoproteins E6 and E7 of both HPV 16 and HPV 18. Three studies used serum banks, three collected serum samples at the moment of diagnosis, and one (Schwartz et al., 1998) collected samples from patients after they had received treatment.

Of the four studies on esophageal cancer, all but one (Lagergren et al., 1999) found an increased risk for seropositive individuals; for the positive studies, ORs for HPV 16 capsids ranged from 4.5 to 13.1. Three studies included cases of oral cancer, alone (Schwartz et al., 1998) or together with cases of other head and neck tumors (Zumbach et al., 2000; Mork et al., 2001). The three of them found a positive association between HPV 16 seropositivity and increased risk of cancer. Zumbach et al. (2000) did not report results for cancers of the mouth or oropharynx. Schwartz et al. (1998) found a higher OR for patients with HPV DNA positive tumours, whereas Mork et al. (2001) found the highest OR for patients with oropharyngeal cancers. The only study that controlled for markers of sexual activity was the one by Schwartz et al. (1998); this adjustment did not change the point estimates.

2.3.3 Summary of the evidence

HPV DNA is detected in a substantial proportion of oral squamous cell carcinomas. Studies which compared cancers of the oropharynx with cancers of the oral cavity have found that the detection rate of viral DNA is higher among the former than among the latter. The majority of studies that compared detection rates in oral cancers and in non malignant tissues have shown that HPV DNA was detected more frequently in cancer tissues. Most case-control studies that recruited cases before being treated for the disease found a positive association between detection of viral DNA and oral cancer. The majority of case-control studies that assessed HPV infection using serological assays also found a positive association between the virus and oral cancer, especially cancers of the oropharynx.

3. RATIONALE AND OBJECTIVES

3.1 RATIONALE

Epidemiological studies have assessed the association of i) detection of viral DNA with buccal and oropharyngeal cancers, ii) serological response to HPV with buccal and oropharyngeal cancers, iii) detection of HPV DNA and HPV serological response with buccal cancer. No published study have assessed the association of both markers of HPV infection –detection of oral HPV DNA and serological response- with risk of developing buccal and oropharyngeal cancers, and whether the risk differs between the two subsites.

3.2 OBJECTIVES

The primary objectives of the present project were as follows:

- to examine the association between HPV infection –assessed by detection of HPV DNA in the oral cavity and serological response- and risk of developing oral cancer, and risk of two subset of oral cancers: i) tonsil related carcinomas (palatine tonsil and base of tongue), and ii) oral cancers not related to Waldeyer's ring.
- To assess the potential effect modification between HPV and tobacco smoking or alcohol drinking on oral cancer risk.
- To examine sexual behaviour as a route of transmission for oral HPV infection.
- To assess the role of other known (i.e., smoking, alcohol drinking, fruit and vegetable intake) or putative (i.e., oral health, sexual behaviour) risk factors for cancer of the oral cavity in the study population.

4. METHODOLOGY

This project, as a part of a multi centre study, followed a hospital-based case control design. Cases consisted of patients with cancer the oral cavity, and controls were selected form the same hospital as cases

4.1 STUDY SUBJECTS

Cases and controls were identified at the Ear, Nose, and Throat (ENT) departments at the Jewish General Hospital (JGH) and Royal Victoria Hospital (RVH), hospitals affiliated with McGill University, as well as Notre-Dame Hospital (HND), affiliated with Université de Montréal. Recruitment took place from October 1997 until May 2001.

4.1.1 Selection of Cases

All patients diagnosed with a primary squamous cell carcinoma of the oral cavity were considered for recruitment. The following topographic sites, according to the International Classification for Diseases in Oncology (ICD-O, Percy et al., 1990) were included: inner lip (C00), base of tongue (C01), other and unspecified parts of tongue (C02), gum (C03), floor of mouth (C04), palate (C05), other and unspecified parts of mouth (C06), tonsils (C09), and oropharynx (C10). Patients with cancer of the salivary glands (C07, C08) were not eligible.

Cases consisted of newly diagnosed patients in one of the participating hospitals. Cases might have been diagnosed for the first time outside the participating study hospitals as long as the referral to the participating hospital is for primary therapy, not previously

treated. Cases were enrolled as soon as possible after histological confirmation of squamous cell carcinomas and before receiving any treatment, as any local or systemic therapy may interfere with detection of HPV. A clinical diagnosis was sufficient to have the patient interviewed and specimens collected, pending histological confirmation.

Eligible cases were first introduced to the study by the treating physician, who gave the patient an English and French version of the letter of introduction to the study (English version in appendix 1). This letter, based on the informed consent, explained the nature of the investigation and the collaboration requested to participating patients. A research nurse or the study coordinator attended weekly tumour boards at participating hospitals to identify eligible patients. Once these patients were identified they were contacted by the research nurse at the ENT clinic, admission clinic, or at the hospital room the day before surgery. Patients were further explained about the nature of the study, as well as their right to refuse participation. Patients agreeing to participate were interviewed, and oral exfoliated cells and a blood sample were collected.

4.1.2 Selection of Controls

Controls were selected from the inpatient and outpatient clinics at the same hospitals as the cases. Initially, one control per case was chosen. Afterwards, due to the low number of cases recruited, the proportion of controls was increased, to complete almost two controls per case. Control subjects were frequency matched to cases by sex, age (five year group), and hospital.

Controls were selected among individuals without a personal history of cancer. Patients with admitting diseases related to tobacco and/or alcohol consumption were not eligible,

e.g. chronic lung disease, coronary artery disease, cirrhosis. Severely debilitated patients, or those who were in physical or mental conditions too poor to give reliable answers to the questionnaire or to undergo oral examination and exfoliated cell collection were not included, either as cases or controls. Efforts were made to ensure a good balance in the distribution of diseases among controls, with no single diagnostic group contributing more than 20% of all controls.

4.2 INTERVIEWS OF STUDY SUBJECTS

All subjects, cases and controls, were interviewed by bilingual nurses specially trained for this study. Research nurses, one per hospital, were trained by the study coordinator to understand the purpose of all questions in the questionnaire and to learn how to collect the oral exfoliated cells. Each patient was interviewed by a research nurse by means of a precoded questionnaire. All interviews took place at the hospital in a quit room, to assure patients' confidentiality.

The questionnaire was designed by researchers at IARC, with modifications suggested from investigators responsible for the different centres. It included demographic characteristics, life time history of smoking, and alcohol drinking, detailed cancer family history, history of selected infectious diseases, and recent intake of fruit, vegetables, and a few selected dietary pattern indicators. Information on lifetime sexual practices was also included. Questions about oral health were complemented by a visual examination of the oral cavity by the interviewer. Appendix 2 includes the English version of the questionnaire. After the interview, the study subjects were asked to

provide a blood sample and the oral cells sample. Overall, the interview and collection of samples took on average 30 to 40 minutes.

4.3 CLINICAL SPECIMENS

For all participating subjects, oral exfoliated cells and a blood sample were collected. For cases, in addition to the previos samples, a biopsy ot the tumour was obtained.

4.3.1 Oral exfoliated cells

Collection of oral exfoliated cells was done by the research nurse afer the interview using a soft toothbrush, followed by a mouthwash. This method has been shown to yield good quantities of DNA (Lawton et al., 1992). This oral sample was used for the detection of HPV, therefore it was extremely important to use disposable equipment and to take all measures possible to prevent contamination of one sample with another. This is particularly important because the detection method utilized was PCR, which is very susceptible to contamination.

Cases and controls were instructed to remove dentures if they worn, and then to perform a mouthwash with water. The research nurse examined the oral cavity to assess the general oral hygiene and to register the nature of any visible lesion. Afterwards, she performed the brushing of the oral cavity with a soft toothbrush as follows:

- In control subjects, several (5-10) gentle strokes with the toothbrush were made on each of the following areas: right buccal mucosa (from high to low position),left

buccal mucosa (from high to low position), right side of the tongue, dorsal side of the tongue, left side of the tongue, inside of upper and lower lip.

- In cancer cases, in addition to performing a brushing in a similar way as in controls, any visible lesion was brushed with several (5-10) gentle strokes trying to avoid necrotic areas anc causing any pain.

Immediately after the scraping of the oral mucosa, the toothbrush was introduced in a conic plastic tube of 50 ml containing about 20 ml of Phosphate Buffered Saline (PBS), and was shaken to detach exfoliated cells. Patients were asked to perform energetic washing of the oral cavity, including the throat by performing gargarisms, with 10 ml of salien solutions PBS which will then be poured in the same conic tube.

The conic tube containg the oral cells was kept a at 4°C (normal refrigerator) until processing of the sample, that took place no later than 24 hours after the sample was drawn.

The processing of the oral cells was done as follows: i) the conic tube was centrifuged at 3000 G for 10 minutes; ii) the overnatant solution, two thirds of PBS and one third of saline, was discarded by gently pouring off, leaving the cells pellet in small quantity of solution (2-3 ml); iii) the pellet was diluted in the same volume (2-3 ml); iv) the diluted pellet was aliquoted into three microtubes using a Pasteur pipette; microtubes were labelled with the initials of the subject and the identification number; v) the cell suspensions were frozen at -80°C until they were sent for analysis in batches.

4.3.2 Blood Sample

A sample of 10 ml of blood was collected from each case and control, using the usual sterile technique and heparinized tubes. The sample was kept at 4°C and processed as soon as possible. Most blood samples were processed within a few hours after they were drawn. A few samples were processed the day after.

The heparinized blood was centrifuged at 1500 G for 20 minutes. Three aliquots of plasma were place in microtubes, as well as two aliquots of the buffy coat. Microtubes were frozen at -80°C until they were sent for analysis in batches.

4.3.3 Biopsies from cases

Whenever possible, biopsies were obtained from cases for HPV testing. Some biopsies were obtained at the clinic, using a standard forceps. Some others were obtained by the ENT surgeon at the operating room, or by the pathologist when the fresh surgical specimen was taken to the laboratory. All speciments collected were obtained before initiation of radiotherapy or chemotherapy. The tumour specimens were kept at -70°C until further analysis.

4.4 CLINICAL INFORMATION ON CASES

Clinical information on cases was obtained form the medical charts. The following information was collected: site of the tumour, TNM staging (tumour classification, lymph node involvemnet, and presence of mathastasis), and morphological differention of the tumour.

4.5 ANALYSIS OF BIOLOGICAL SPECIMENS

4.5.1 Detection of HPV DNA in oral exfoliated cells

Detection of HPV DNA in oral exfoliated cells was performed at the laboratory of Dr. François Coutlée, at the Départements de Microbiologie-Infectiologíe, Pavillon Notre Dame, CHUM, in Montreal, using the PGMY09/11 PCR protocol (Gravitt et al., 2000).

Frozen oral cell suspensions were thawed, lysed by addition of Tween 20 at a final concentration of 0.8% (v/v) and digested with 250 µg per ml of proteinase K for 2 hours at 45°C (Coutlée et al., 1997a). After heat inactivation at 95°C for 5 minutes, cell lysates were stored at -70°C until tested.

Five µl of each lysate were tested with PC04 and GH20 primers for the presence of ß-globin to identify samples that contained inhibitors, degraded or inadequate quantities of cellular DNA (Bauer et al., 1991; Coutlée et al., 1997a). Samples testing positive for ß-globin were tested for HPV with PGMY primers (see below). DNA was purified from ß-globin-negative samples by phenol-chloroform extractions and ethanol precipitation at –70°C (Coutlée et al., 1997b) and resuspended in 50 µl of 10 mM Tris-HCl [pH 8.2]. One µg of extracted DNA was then tested for ß-globin. Samples remaining negative for ß-globin were considered inadequate.

ß-globin-positive samples were amplified for HPV with consensus primers PGMY09 and PGMY11 (Gravitt et al., 2000; Coutlée et al., 2002). Amplification of HPV DNA was accomplished using the ultrasensitive amplification profile in a TC 9600 thermal cycler which consisted in the activation of AmpliTaq Gold at 95°C for 9 minutes, denaturation

at 95°C for 1 minute, primer annealing at 55°C for 1 minunte, DNA synthesis at 72°C for 1 minunte for 40 cycles, followed by a five-minute terminal extension step at 72°C.

HPV amplicons were detected and typed with the line blot assay (Roche Molecular systems) as described previously (Coutlée et al., 1999; Gravitt et al., 2000). Twenty seven genital HPV genotypes were detected with the latter assay, including types 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 68, 73, 82, 83, 84 (Gravitt et al., 2000). This PCR assay reliably detects 10 HPV DNA copies and is very specific. Negative, weak (10 HPV18 DNA copies), and strong positive HPV controls, were included in each amplification run. Precautions to avoid contamination were taken at all steps.

4.5.3 HPV serology

Serological response to HPV was performed at the laboratory of Dr. Raphael Viscidi, the The Johns Hopkins Hospital, Baltimore, MD, U.S.A.

4.5.3.1. Production of virus-like-particles (VLPs)

For large-scale production of VLPs, approximately 2 x 10^9 *Trichoplusia ni* (High Five) cells (Invitrogen, Carlsbad CA) were infected with 20 ml of a high titer recombinant baculovirus expressing L1 proteins. After 96 hours of incubation at 27°C, the cells were harvested and lysed by sonication. VLPs were purified and the total protein was measured (Kimbauer et al., 1993, Cook et al., 1999).

4.5.3.2. VLP ELISA

Plasma samples were tested using a HPV VLP based ELISA as described by Viscidi et al. (1997). Wells of 96-well polystyrene flat bottom PolySorp plates (Nunc, Naperville, IL) were coated with 50 ng, 40 ng, or 40 ng of HPV 16, HPV 18, or HPV 31 VLP protein, respectively, in 100-ul of phosphate buffered saline, pH 7.2 (PBS). Following overnight incubation at 40°C, plates were tapped dry on a paper towel and 300 µl per well of blocking solution [0.5% polyvinyl alcohol in PBS (wt/vol)(catalogue number P-8136, Sigma, St Louis MO)] was added. Plates were incubated at room temperature for 3 hours, and then the blocking solution was removed by inversion of the plates and 300 µl per well of PBS was added. The plates were covered with a plastic sealer and stored at –20°C. Before use, plates were thawed at room temperature and washed 3 times with wash solution (PBS-0.05% Tween 20) in an automatic plate washer (Skanwasher 300, Skatron, Lier, Norway). The wash buffer was left in the wells until all plates had been washed in order to prevent drying of VLPs absorbed to the plate. Plates were tapped dry on a paper towel and 100 µl per well of sample dilution buffer (0.5% polyvinyl alcohol in PBS) was added. Pipetting of serum samples was done using a MultiPROBE II robotic liquid handling system (Packard Instruments, Meriden CT). A 1:10 dilution of the sample was made into sample dilution buffer in the well of an uncoated 96 well microtiter plate, and then 10 µl of diluted serum was added to the well of an antigen coated plate containing 100 µl of sample dilution buffer, for a final sample dilution of 1:100. Samples were tested in duplicate on separate antigen coated plates. Plates were incubated at 37°C for 1 hour on a microplate shaker and then washed twice, rotated 1800 and washed 2 more times. Goat anti-human IgG, gamma chain specific, conjugated with horseradish peroxidase (Zymed, San Francisco, CA), was diluted 1:4000 in conjugate buffer [0.8% polyvinyl pyrrolidone (wt/vol) (catalogue number PVP-360, Sigma), 0.5%

polyvinyl alcohol and 0.025% Tween 20 in PBS] and 100 µl were added per well after the wash buffer had been removed by tapping the plates dry on a paper towel. Plates were incubated at 37°C for 30 min on a microplate shaker and then washed as described above. Freshly prepared 2,2'-azinobis(3-ethylbenzthiazolinrsulfonic acid) and hydrogen peroxide solution (Kierkegaard and Perry, Gaithersburg, MD), pre-warmed to 50°C, was added to each well in 100 µl volumes. Plates were incubated at room temperature in the dark for approximately 20 minutes. The first plate in a series was monitored until the weak positive control reached a predetermined optical density (OD) value. The positive controls were human serum samples previously shown to be reactive in the assay. Three controls were included on each plate, a weak positive, a moderate to strong positive and a negative control. The enzyme reaction was stopped by the addition of 100 µl of 1% dodecyl sulfate per well to all the plates. The absorbance was measured at 405 nm, with a reference wavelength of 490 nm, in an automated microtiter plate reader (Molecular Devices, Menlo Park CA). The cutpoint for positive results was determined from the reactivity of plasma samples from self-reported virgins from Costa Rica or from previously established negative control samples. The mean and standard deviation (SD) of OD values for the control samples was calculated and values greater than the mean plus 3 SD were excluded. The analysis was repeated on the remaining samples until no further OD values could be excluded by this criterion. After excluding outliers, the cut point was set as an OD value greater than the mean OD of the negative controls plus either 3 SD or 0.030 OD units, whichever value was higher. The cut points for the HPV 16, 18 and 31 assays were 0.050, 0.040, and 0.045 OD units, respectively.

4.5.4 Detection of HPV DNA in tumour biopsies

Detection of HPV DNA in tumour biopsies was performed at the laboratory of Dr. Peter Snijders, Department of Pathology (Molecular Pathology Section) of the Free University Hospital, Amsterdam.

Frozen biopsies were were thawed, and were digested in a similar way to the already described. Samples were tested with PC04 and GH20 primers for the presence of ß-globin to identify samples with adequate ampunt of cellular DNA.

HPV DNA testing was done using the GP5+/GP6+ PCR protocol (De Roda Husman et al., 1995), that allows the detection of a broad spectrum of mucosotropic HPV genotypes. The main difference between this PCR protocol and to the one described above is the use of a different ser of primers – the GP5+/GP6+ instead of the PGMY09/PGMY11-. Each cycle of amplification included a 1 minute denaturation step at 94°C, an annealing step at 40°C for 2 minutes, and a chain elongation step at 72°C for 1.5 minutes. The last cycle was extended by a 10 min elongation step, and then the tubes were left to soak at 4°C.

4.6 STATISTICAL ANALYSIS

Standard epidmiological methods of data analysis for case-control studies were used (Breslow and Day, 1980). The OR was the measure of association to calculate the rate ratio of disease for each study factor. Multivariate logistic regression was used to assess the effect of candidate risk factors with mutual adjustment for confounders. Adjustment for tobacco and alcohol consumption was based on the lifetime cumulative exposure using the pack-years equivalent of cigarette smoking and the sum over all alcoholic

beverage types in kilograms of ethanol consumption. A pack-year was defined as the cumulative exposure equivalent to smoking one pack of cigarettes daily during one year. Doses were calculated as follows: 20 commercial-brand cigarettes = 4 hand-rolled, black tobacco cigarettes = 4 cigars = 5 pipefuls with pipe tobacco = 1 pack; ethanol concentration in beer = 5%, wine = 10%, hard liquor = 40%.

4.7 ETHICAL CONSIDERATIONS

This project was approved by the Institutional Review Board of McGill University (appendix 3), and those of participating hospitals. Patients were introduced to the study by treating physicians, and cases were contacted after having full knowledge of the nature of their disease. Patients were clearly explained that there were no direct personal benefits for them for participating in the study. Only patients who agreed to participate after reading and signing the informed consent were recruited. English version of informed consent for cases (yellow paper) and controls (green paper) are included in appendix 4.

Results from laboratory analyses and information from questionnaries are kept confidential. Names or other information that could identify patients cannot be linked to data files.

5. RESULTS

5.1 CHARACTERISTICS OF STUDY SUBJECTS

A total of 72 cases were recruited in the study. At the JGH and RVH, 86 patients with newly diagnosed oral cancer were identified during the study period. Of these, 68 agreed to participate in the study: 44 at the JGH, and 28 at the RVH. Of the remaining 18 (20.9%), three patients were not contacted before beginning of treatment, one did not return for treatment, one had extreme weakness, three patients were unable to give consent due to mental conditions, and 10 patients refused to participate. At NDH, I could not establish a good coordination with the clinicians and the research nurses, since they were involved in a clinical trial which included the same eligible patients, and the latter study received priority over mine. Nevertheless, four patients were invited to participate at NDH and they all agreed. It was not possible to gather information on age and sex for the eligible patients who were not contacted at NDH.

A total of 129 controls were recruited for this study: four at NDH, 37 at RVH, and 88 at the JGH. Ten patients refused to participate as controls. Table 11 shows the distribution of participating and non participating cases and controls according to age and sex. Non participating cases were on average older that the recruited cases: mean age 61.6 and 66.8, respectively. Patients aged 70 and older accounted for 25.0% of recruited cases and for 44.4% for non participants. The proportion of females was higher among non participating cases, being a minority in participating cases (29.2%), and a majority among those who did not participate (61.1%). Similar differences in terms of age and sex were seen in the comparison between participating and non participating controls: mean age 60.8, and 69.4, respectively. Females accounted for 28.7% of participating

Table 11. Distribution of participating and non participating cases (top), and controls (bottom) according to age and sex

Variable	Categories	Participating Cases		Non participating Cases	
		N	%	N	%
Age	<50	12	16.7%	1	5.6%
	50-59	19	26.4%	4	22.2%
	60-69	23	31.9%	5	27.8%
	70+	18	25.0%	8	44.4%
	Mean	61.6		66.8	
Sex	Male	51	70.8%	7	38.9%
	Female	21	29.2%	11	61.1%
	Total	**72**	**100.0%**	**18**	**100.0%**

		Participating Controls		Non participating Controls	
		N	%	N	%
Age	<50	24	18.6%	0	0.0%
	50-59	33	25.6%	2	20.0%
	60-69	41	31.8%	3	30.0%
	70+	31	24.0%	5	50.0%
	Mean	60.8		69.4	
Sex	Male	92	71.3%	6	60.0%
	Female	37	28.7%	4	40.0%
Total	**Total**	**129**	**100.0%**	**10**	**100.0%**

controls and 40.0% of non participating potential controls. The potential selection bias introduced by these differences will be presented in the discussion section.

5.1.1 Distribution of cases according to clinical and histopathological variables

The distribution of cases according to main clinical and histopathological characteristics is shown in tables 12 and 13. Table 12 shows the distribution of cancer patients according to site of the primary tumour. The most common site was tongue (excepting base of tongue), with 21 cases (29.2%), followed by floor of the mouth, and tonsillar cancer, with 12 cases each (16.7%). The least common sites were inner lip (one case), gum, and oropharynx (two cases each).

Table 13 shows the distribution of cases according to staging of the disease and histopathological grade. Disease staging of invasive tumors –does not include carcinomas in situ (CIS)- is based on the TNM classification of malignant tumours published by the Union Internationale Contre le Cancer (UICC, 1980). The extent of the disease is assessed by the T classification (local extent), the N classification (regional lymph node involvement), and the presence or absence of distant metastasis (M classification). Three cases (4.2%) were diagnosed with CIS (non invasive), 12.5% of the patients were diagnosed with invasive tumours of 2 cm or less (T1), 45.8% had tumours of 2 to 4 cm (T2), 18.1% had a T3 primary tumour (more than 4 cm), whereas 19.4% of the patients had tumours with extension to neighbouring structures such as bone, muscle, skin, or cartilage (T4).

The diagnosis of regional spread of the disease is done through the assessment of involvement of cervical lymph nodes. Most patients (58.3%) did not present evidence of lymph node involvement at the moment of the diagnosis of the disease, whereas 13.9%

Table 12. Distribution of cases according to topographic site

ICD-O[1]	Topogographic site	N	%
C00	Lip	1	1.4%
C01	Base of tongue	9	12.5%
C02	Other and unespecified parts of tongue	21	29.2%
C03	Gum	2	2.8%
C04	Floor of mouth	12	16.7%
C05	Palate	4	5.6%
C06	Other and unespecified parts of mouth	9	12.5%
C09	Tonsil	12	16.7%
C10	Oropharynx	2	2.8%
	Total	**72**	**100.0%**

1. International Classification for Diseases in Oncology

Table 13. Distribution of cases according to clinical and pathological variables

Variable	Categories	N	%
T Classification	CIS[1]	3	4.2%
	T1	9	12.5%
	T2	33	45.8%
	T3	13	18.1%
	T4	14	19.4%
N Classification	N0	42	58.3%
	N1	10	13.9%
	N2	18	25.0%
	N3	2	2.8%
TNM Stage	CIS	3	4.2%
	Stage I	8	11.2%
	Stage II	23	31.9%
	Stage III	13	18.1%
	Stage IV	25	34.7%
Histopathological grade	CIS	3	4.2%
	Well	14	19.4%
	Moderate	32	44.4%
	Poor	13	18.1%
	Not reported	10	13.9%
	Total	**72**	**100.0%**

1. CIS: carcinoma in situ

of the cases presented movable homolateral metastatic lymph nodes (N1), 25.0% presented movable contralateral or bilateral lymph node involvement (N2), and 2.8% of the patients were diagnosed with fixed (non-movable) metastatic lymph nodes (N3). None of the recruited cases had clinical evidence of distant metastasis at the moment of diagnosis.

The TNM stage is a classification of the extension of the disease which summarizes the local (T classification), regional (N classification), and distant (M classification) spread of the tumour. The TNM stage is one of the most important predictors of survival among individuals with oral cancer. The most common disease stage at diagnosis were stage IV (34.7%), and stage II (31.9%).

Several reports have indicated that histopathologic grading of tumours may have prognostic value for oral cancers (reviewed by Bryne, 1991). The grading is based on differentiation of the tumour according to morphologic characteristics such as degree of keratinization, nuclear polymorphism, number of mitoses, pattern of invasion, and lympho-plasmocytic infiltration (Bryne, 1991). The distribution of patients according to histopathological differentiation is shown in table 13: 14 patients (19.4%) had well differentiated tumours, 32 (44.4%) had moderately differentiated tumours, 13 (18.1%) had poorly differentiated tumours, whereas three patients had CIS (4.2%). Degree of differentiation for ten patients (13.9%) was not reported in their medical charts.

5.1.2 Distribution of admission conditions among hospital controls

The underlying causes of hospital admission among control patients were grouped into 11 diagnostic categories of the ICD-9 (table 14). The most common groups were: diseases of the nervous system and sense organs (mainly patients with diseases of the

Table 14. Distribution of control patients according to diagnostic group

ICD-9 rubric	Main diagnostic group	N	%
001-139	Infectious and parasitic diseases	1	0.8
210-229	Benign Neoplasms	7	5.4
240-279	Endocrine, nutritional and metabolic diseases, and immunity disorders	4	3.1
320-389	Diseases of the nervous system and sense organs	25	19.4
390-459	Diseases of the circulatory system	6	4.7
520-579	Diseases of the digestive system	25	19.4
580-629	Diseases of the genitourinary system	9	7.0
680-709	Diseases of the skin and subcutaneous tissue	4	3.1
710-739	Diseases of the musculoskeletal system and connective tissue	16	12.4
780-799	Symptoms, signs, and ill-defined conditions	24	18.6
800-999	Injury and poisoning	8	6.2
	Total	**129**	**100.0**

eye), diseases of the digestive system diseases (such as cholelithiasis, inguinal hernia, and diverticulitis of the colon), and ill-defined diagnostic conditions (such as fever, abdominal pain, and urinary incontinence).

5.2 Distribution of Cases and Controls According to Study Variables

5.2.1 Distribution of subjects according to socio-demographic characteristics

Table 15 summarizes the distribution of cases and controls according to selected socio-demographic characteristics. By design, the distribution of cases and controls by sex and age was very similar. The age groups most commonly represented were the 55 to 64, and the 65 to 74, with over 25% of subjects within each group. Women accounted for 29.2% of the cases (21 out of 72), and 28.7% of the controls (37 out of 129).

Among other variables included in table 15, cases differed from controls with respect to indicators of social and/or cultural background. On average, cases had fewer years of schooling than controls: 18.6% of controls, and 38.9% of cases, respectively, had fewer that 10 years of formal education, whereas the proportion of controls and cases that had 15 or more years were 45%, and 20.8%, respectively. Regarding ethnic/racial background, the proportion of white subjects was slightly higher among cases than controls (94.4% and 92.2%, respectively). The distribution of participating subjects by religion showed that the proportion of catholics was higher among cases, whereas the proportion of jewish individuals was higher among controls. Despite the efforts by the research nurses to find a good balance in spoken language between cases and controls, the proportion of English speaking subjects was higher among controls. Due to the imbalance in these variables -schooling, race, religion, and language-, they were selected as potential confounders of the relationship of other factors and oral cancer for the purpose of covariate adjustment.

Table 15. Distribution of study subjects according to selected socio-demographic characteristics

Variable	Categories	Controls N	Controls %	Cases N	Cases %
Age in years	25-34	4	3.1%	2	2.8%
	35-44	7	5.4%	4	5.6%
	45-54	27	20.9%	14	19.4%
	55-64	37	28.7%	19	26.4%
	65-74	33	25.6%	20	27.8%
	75-84	21	16.3%	13	18.1%
Sex	Male	92	71.3%	51	70.8%
	Female	37	28.7%	21	29.2%
Schooling	<10 years	24	18.6%	28	38.9%
	10-14 years	47	36.4%	29	40.3%
	15+ years	58	45.0%	15	20.8%
Race	White	118	92.2%	68	94.4%
	Non-white	10	7.8%	4	5.6%
Religion	Catholic	75	58.1%	51	70.8%
	Protestant	19	14.7%	9	12.5%
	Jewish	18	14.0%	4	5.6%
	Other	17	13.2%	8	11.1%
Language	English	54	41.9%	26	36.1%
	French	56	43.4%	40	55.6%
	Other	19	14.7%	6	8.3%
Total		129	100.0%	72	100.0%

5.2.2 Distribution of subjects according to tobacco and alcohol consumption

Before presenting the results from the logistic regression analysis, I will present the distribution of participating subjects according to the main risk factors. The distribution of cases and controls according to tobacco consumption is shown in table 16. As expected, the proportion of smokers and the intensity of the habit was higher among cases. Subjects were classified depending on their smoking status (never, former, or current smokers), their lifetime cumulative exposure, as well as smoking duration (in years), and intensity (average consumption of cigarettes per day). Lifetime cumulative exposure was measured in pack-years as explained in the methodology section.

The prevalence of current smoking was substantially higher among cases than controls: 48.6% versus 14.0%. Former and never smokers were more common among controls. The lifetime cumulative exposure of cigarette smoking showed a high proportion of heavy smokers; 38.9% of cases and 16.3% of controls were in the category of 45 pack-years and more. In the category of 19 to 45 pack-years, the proportions were 30.6% and 21.7%, respectively. The cumulative exposure to cigar and pipe smoking did not differ substantially between cases and controls. Combining all three smoking habits (cigarette, cigar, and pipe), the distribution of total tobacco consumption was similar to that of cigarette smoking alone.

Regarding duration of smoking, 41.7% of cases smoked for 39 or more years, and 25.0% smoked between 26 and 38 years. For controls, the proportions were 16.3%, and 22.5%, respectively. The distribution of average intensity of smoking, measured in cigarettes per day (or its equivalent), showed that 34.7% of cases and 22.5% of controls

Table 16. Distribution of study subjects according to tobacco smoking

Variable	Categories	Controls		Cases	
		N	%	N	%
Smoking status	Never	41	31.8%	11	15.3%
	Former	70	54.3%	26	36.1%
	Current	18	14.0%	35	48.6%
Cigarettes (in pack-years)	No smoker	44	34.1%	12	16.7%
	≤ 18	36	27.9%	10	13.9%
	19-45	28	21.7%	22	30.6%
	45+	21	16.3%	28	38.9%
Cigars (in pack-years)	No smoker	119	92.2%	65	90.3%
	≤ 9	6	4.7%	3	4.2%
	10+	4	3.1%	4	5.6%
Pipe (in pack-years)	No smoker	117	90.7%	64	88.9%
	≤ 2.5	5	3.9%	5	6.9%
	2.5+	7	5.4%	3	4.2%
Total tobacco (in pack-years)	Never	41	31.8%	11	15.3%
	≤ 19	40	31.0%	12	16.7%
	20-48	27	20.9%	21	29.2%
	49+	21	16.3%	28	38.9%
Duration (in years)	< 1	44	34.1%	12	16.7%
	1-25	35	27.1%	12	16.7%
	26-38	29	22.5%	18	25.0%
	39+	21	16.3%	30	41.7%
Intensity (cigarettes/day)	< 1	44	34.1%	12	16.7%
	1-14	32	24.8%	13	18.1%
	15-24	24	18.6%	22	30.6%
	25+	29	22.5%	25	34.7%
Total		129	100.0%	72	100.0%

smoked on average 25 or more cigarettes per day during their life as smokers. The proportions of cases and controls who smoked an average of 15 to 24 cigarettes per day were 30.6%, and 18.6%, respectively.

Table 17 shows the distribution of subjects according to alcohol drinking. On average cases drank alcohol for a longer time than controls, but the difference was not of great magnitude. On the other hand, the difference in intensity of drinking -measured in number of drinks per day or week- was more remarkable. One drink was defined as one small bottle of beer (330 ml), medium glass of wine (125 ml), or a small glass of hard liquor (50 ml). Approximately one fifth of controls (20.9%) and more than half of cases (55.6%) had on average at least one drink per day. The proportion of subjects having one or fewer drinks per week (including non drinkers) was 40.3% for controls, and 20.8% for cases. The cumulative lifetime consumption of alcohol, measured in kilograms of alcohol as explained in the methodology section, was higher among cases than controls. The proportion of non drinkers, defined as lifetime consumption of less than one kilogram of alcohol, was similar between cases and controls: 9.7% and 11.6%, respectively. In contrast, the proportion of heavy drinkers was considerably higher among cases. The proportion of subjects with a total alcohol consumption of more than 400 kilograms was 52.8% among cases, and 17.1% among controls.

The type of alcoholic beverage consumed also differed between cases and controls. The proportion of mainly wine drinkers (more than 50% of the total alcohol consumption) was 31.0% for controls, and 16.7% for cases. In contrast, the proportion of mainly beer drinkers was 25.6%, and 48.6%, respectively. The distribution of subjects according to the percentage of hard liquor (such as whisky, cognac, vodka, and gin) of the total alcohol drinking did not differ substantially between cases and controls.

Table 17. Distribution of study subjects according to alcohol drinking

Variable	Categories	Controls N	Controls %	Cases N	Cases %
Duration (in years)	< 5	16	12.4%	7	9.7%
	6 -10	39	30.2%	16	22.2%
	31-44	39	30.2%	24	33.3%
	≥ 45	35	27.1%	25	34.7%
Intensity (in number of drinks)	≤ 1/week	52	40.3%	15	20.8%
	2-6/week	50	38.8%	17	23.6%
	≥ 1/day	27	20.9%	40	55.6%
Cumulative consumption (in kgs)	Non drinker[1]	15	11.6%	7	9.7%
	1-80	47	36.4%	10	13.9%
	81-400	45	34.9%	17	23.6%
	≥ 401	22	17.1%	38	52.8%
Wine (% total alcohol)	Non drinker	15	11.6%	7	9.7%
	< 20%	28	21.7%	19	26.4%
	20-49%	28	21.7%	13	18.1%
	≥ 50%	40	31.0%	12	16.7%
	Other alcohol	18	14.0%	21	29.2%
Beer (% total alcohol)	Non drinker	15	11.6%	7	9.7%
	< 20%	13	10.1%	7	9.7%
	20-49%	25	19.4%	8	11.1%
	≥ 50%	33	25.6%	35	48.6%
	Other alcohol	43	33.3%	15	20.8%
Hard liquor (% total alcohol)	Non drinker	15	11.6%	7	9.7%
	< 20%	13	10.1%	16	22.2%
	20-49%	20	15.5%	9	12.5%
	≥ 50%	30	23.3%	14	19.4%
	Other alcohol	51	39.5%	26	36.1%
Total		129	100.0%	72	100.0%

1. Non drinker: lifetime consumption of alcohol of less than 1 kg.

5.2.3 Distribution of subjects according other risk factors

Table 18 shows the distribution of study subjects according to consumption of dietary items during the last year before disease symptoms developed. These variables were categorized in approximate tertiles, to allow a balanced distribution of cases and controls in each category. Cases had a higher consumption of ham, salami, and sausages than controls: 38.9% of cases consumed these items at least twice a week, compared to 24.8% of controls. The consumption of read meat was slightly higher among cases than controls.On the other hand, intake of fish, fresh vegetables (including tomatoes, cruciferae, and carrots), as well as fresh fruits was higher among controls. The proportion of controls who declared a consumption of fish of at least twice a week was 44.2%, compared with 22.3% of cases. The intake of fresh tomatoes was slightly higher among controls than cases, whereas the intake of cruciferae vegetables (such broccoli, cabbage, and Brussels sprout) and carrots was substantially higher. The proportion of controls who declared to had consumed cruciferus vegetables at least three times a week was 42.6%, and carrots was 55.0%, compared to 19.4% and 31.9% for cases, respectively. The consumption of fresh fruits and fruit juices did not differ much between cases and controls.

Table 19 shows the history of cancer among first degree relatives –excluding children- of study subjects. The rate of malignancies in the head and neck region, lung, and all sites did not differ substantially between first degree relatives of cases and controls. Unfortunately, the rates of cervical cancer shown in the table were not valid, since many patients who declared having mothers or sisters who developed cancer of the uterus

Table 18. Distribution of study subjects according to consumption of dietary items

Variable	Categories	Controls		Cases	
		N	%	N	%
Ham, salami, and sausages	< 1/week	68	52.7%	23	31.9%
	1/week	29	22.5%	21	29.2%
	2+/week	32	24.8%	28	38.9%
Read meat	≤2/week	43	33.3%	18	25.0%
	3-5/week	50	38.8%	30	41.7%
	6+/week	36	27.9%	24	33.3%
Fish	< 1/week	25	19.4%	20	27.8%
	1/week	47	36.4%	36	50.0%
	2+/week	57	44.2%	16	22.2%
Tomatoes	≤2/week	28	21.7%	22	30.6%
	3-5/week	41	31.8%	21	29.2%
	6+/week	60	46.5%	29	40.3%
Cruciferus vegetables	< 1/week	26	20.2%	23	31.9%
	1-2/week	48	37.2%	35	48.6%
	3+/week	55	42.6%	14	19.4%
Carrots	< 1/week	12	9.3%	19	26.4%
	1-2/week	46	35.7%	30	41.7%
	3+/week	71	55.0%	23	31.9%
Fresh vegetables	< 1/day	22	17.1%	30	41.7%
	1/day	66	51.2%	17	23.6%
	2+/day	41	31.8%	25	34.7%
Fresh fruit	< 1/day	41	31.8%	27	37.5%
	1/day	57	44.2%	21	29.2%
	2+/day	31	24.0%	24	29.2%
Total		129	100.0%	72	100.0%

Table 19. History of cancer among first degree relatives of cases and controls

Relative	Site of cancer of relative	Controls			Cases		
		N /	Total[1]	Perc.	N /	Total[1]	Perc.
Father	Any site	35 /	118	29.7%	18 /	71	25.4%
	Head and neck	4 /	118	3.4%	2 /	71	2.8%
	Lung	3 /	118	2.5%	4 /	71	5.6%
	Unkown		11			2	
Mother	Any site	37 /	127	29.1%	12 /	67	17.9%
	Head and neck	0 /	127	0.0%	0 /	67	0.0%
	Lung	4 /	127	3.1%	2 /	67	3.0%
	Cervix	1 /	127	0.8%	1 /	67	1.5%
	Uterus[2]	3 /	127	2.4%	2 /	67	3.0%
	Unkown		2			4	
Sibling	Any site	34 /	403	8.4%	16 /	317	5.0%
	Head and neck	2 /	403	0.5%	1 /	317	0.3%
	Lung	7 /	403	1.7%	4 /	317	1.3%
	Cervix	0 /	188	0.0%	0 /	154	0.0%
	Uterus	5 /	188	2.7%	1 /	154	0.6%
First degree relative	Any site	106 /	648	16.4%	46 /	455	10.1%
	Head and neck	6 /	648	0.9%	3 /	455	0.7%
	Lung	14 /	648	2.2%	10 /	455	2.2%
	Uterus[1]	8 /	315	2.5%	3 /	221	1.4%

1. Number of relatives for all subjects.
2. Includes cancer of the cervix and the uterine body, when patients did not know specific site.

could not specify whether the primary site of the malignancy was the cervix or the uterine body.

The distribution of subjects depending on oral health variables is shown in table 20. The main disparity between cases and controls was seen in the use of denture. Only one third of cases (33.3%) did not wear a denture, compared with 60.5% of controls. The proportion of individuals wearing a complete denture (either upper, lower, or both) was more than half for cases (52.8%), and one fifth for controls (20.2%). Regarding the frequency of tooth brushing, gum bleeding after brushing, and use of mouthwash solutions, the distribution of subjects did not differ appreciably between cases and controls.

Table 21 shows the distribution of male subjects according to markers of sexual activity. The categories of the traditional markers -number of female sexual partners and age at first intercourse- did not differ considerably between cases and controls. Approximately one fifth of both male controls (22.8%) and male cases(18.4%) reported having had at most one lifetime sexual partner, whereas the proportion reporting more than 20 female sexual partners was 20.7% for controls and 24.5% for cases. Male cases had a slightly higher age at first intercourse, with 29.2% of controls and 34.7% of cases reporting age at first intercourse of older than 20. Markers of oral sex (cunnilingus), such as frequency and age at first time of oral sex, as well as past or present history of homosexuality, did not differ substantially between cases and controls. The main difference was seen in number of prostitute female partners and in personal antecedents of sexual transmitted diseases (STDs) other than HIV/AIDS -syphilis, gonorrhea, or genital warts-. The proportion of male cases and male controls who reported having had intercourse with more than five female prostitutes was 17.6%, and 9.8%, respectively. The rate of male

Table 20. Distribution of study subjects according to oral health characteristics

Variable	Categories	Controls		Cases	
		N	%	N	%
Denture	No Denture	78	60.5%	24	33.3%
	Incomplete	25	19.4%	10	13.9%
	Complete	26	20.2%	38	52.8%
Frequency of tooth brushing	<1/day	11	8.5%	11	15.7%
	1/day	47	36.4%	21	30.0%
	2/day	46	35.7%	27	38.6%
	3+/day	25	19.4%	11	15.7%
Gum bleeding when brushing	Never	76	59.8%	40	61.5%
	Sometimes	51	40.2%	25	38.5%
Use of mouthwash	Never	67	52.3%	40	57.1%
	1-4/week	27	21.1%	9	12.9%
	1+/day	34	26.6%	21	30.0%
Total		129	100.0%	72	100.0%

Table 21. Distribution of **male** study subejcts according to markers of sexual activity

Variable	Categories	Controls N	Controls %	Cases N	Cases %
Number of sexual partners	0-1	21	22.8%	9	18.4%
	2-5	26	28.3%	16	32.7%
	6-20	26	28.3%	12	24.5%
	21+	19	20.7%	12	24.5%
Age at first intercourse	<18	36	40.4%	19	38.8%
	18-20	27	30.3%	13	26.5%
	21+	26	29.2%	17	34.7%
Age first time oral sex (cunnilingus)	Never	33	37.1%	16	34.0%
	<=18	18	20.2%	12	25.5%
	19-24	21	23.6%	8	17.0%
	25+	17	19.1%	11	23.4%
Frequency of oral sex (cunnilingus)	Never	33	37.1%	16	33.3%
	Seldom	36	40.4%	19	39.6%
	Often	20	22.5%	13	27.1%
Number of female prostitute partners	Never	70	76.1%	34	66.7%
	1-5	13	14.1%	8	15.7%
	6+	9	9.8%	9	17.6%
Homosexuality	No	87	94.6%	46	93.9%
	Yes	5	5.4%	3	6.1%
STDs	No	81	88.0%	38	76.0%
	Yes	8	8.7%	9	18.0%
	Unknown	3	3.3%	3	6.0%
Partners w/STDs	No	76	83.5%	37	78.7%
	Yes	15	16.5%	10	21.3%
Total		92	100.0%	51	100.0%

cases who reported STDs was 18.0%, compared with 8.7% of controls. Finally, the proportion of male cases who reported having had sex with women with known STDs was slightly higher than the proportion among male controls: 21.6%, and 16.5%, respectively.

The distribution of female subjects according to markers of sexual activity is shown in table 22. The distribution by number of male sexual partners, age at first intercourse, age at first time and frequency of oral sex (fellatio), as well as personal history of STDs was not considerably different between cases and controls. The main difference was seen in age at first sexual intercourse, where on average cases were older than controls: the rate of individuals with age at first intercourse older than 20 was 31.4% for controls, and 57.1% for cases.

5.3 Odds Ratios (ORs) of Oral Cancer According to Study Variables

5.3.1 Tobacco smoking and Alcohol drinking

As expected, the risk of oral cancer was increased due to tobacco smoking. Table 23 shows the OR of disease due to lifetime cumulative smoking, measured in pack-years. There was a trend in increased risk with increasing pack-years. Heavy smokers (> 48 pack-years) were almost five times more likely to develop oral cancer than never smokers: adjusted OR = 4.71, 95%CI:1.7-12.8.

Table 24 shows the analysis for tobacco smoking discriminating between former and current smokers. The increased risk of oral cancer for former smokers was relatively small, whereas the increased risk for current smokers at the moment of diagnosis was substantially high. The crude OR point estimates for heavy current smokers, compared

Table 22. Distribution of **female** study subejcts according to markers of sexual activity

Variable	Categories	Controls		Cases	
		N	%	N	%
Number of sexual partners	0-1	22	59.5%	11	52.4%
	2-5	11	29.7%	8	38.1%
	6-20	4	10.8%	2	9.5%
Age at first intercourse	<18	8	22.9%	3	14.3%
	18-20	16	45.7%	6	28.6%
	21+	11	31.4%	12	57.1%
Age first time oral sex (fellatio)	Never	15	42.9%	10	47.6%
	<=24	13	37.1%	6	28.6%
	25+	7	20.0%	5	23.8%
Frequency of oral sex (fellatio)	Never	15	42.9%	10	47.6%
	Seldom	16	45.7%	10	47.6%
	Often	4	11.4%	1	4.8%
STDs	No	36	97.3%	20	95.2%
	Yes	1	2.7%	1	4.8%
Total		37	100.0%	21	100.0%

Table 23. Odds Ratios (ORs) of oral cancer associated with cumulative tobacco smoking

Smoking (in pack-years)	Cases/ controls	Crude		Adjusted[1]	
		OR	95%CI	OR	95%CI
Never smokers	11 / 41	1.00		1.00	
<=19	12 / 40	1.12	0.4 - 2.8	0.87	0.3 - 2.5
20-48	21 / 27	2.90	1.2 - 7.0	2.68	1.0 - 7.0
49+	28 / 21	4.97	2.1 - 11.9	4.71	1.7 - 12.8

1. Adjusted for age, sex, schooling, race, religion, and language.

Table 24. Odds Ratios (ORs) of oral cancer associated with cumulative tobacco smoking and current smoking status

Smoking (in pack-years)	Cases/ controls	Crude		Adjusted[1]	
		OR	95%CI	OR	95%CI
Never smokers	11 / 41	1.00		1.00	
Former smokers					
<=19	9 / 31	1.08	0.4 - 2.9	0.92	0.3 - 2.8
20-48	7 / 22	1.19	0.4 - 3.5	1.00	0.3 - 3.3
49+	7 / 16	1.63	0.5 - 4.9	1.58	0.5 - 5.5
Current smokers					
<=19	3 / 9	1.24	0.3 - 5.4	0.93	0.2 - 5.0
20-48	14 / 5	10.44	3.1 - 35.3	9.15	2.5 - 33.1
49+	21 / 5	15.65	4.8 - 51.0	11.55	3.2 - 41.8

1. Adjusted for age, sex, schooling, race, religion, and language.

Table 25. Odds Ratios (ORs) of oral cancer associated with tobacco smoking and time since quitting

Smoking (time since quitting)	Cases/ controls	Crude		Adjusted[1]	
		OR	95%CI	OR	95%CI
Never smokers	11 / 41	1.00		1.00	
Stopped (> 10 yrs. ago)	18 / 53	1.27	0.5 - 3.0	1.16	0.4 - 3.0
Stopped (< 10 yrs. ago)	5 / 16	1.17	0.3 - 3.9	0.93	0.3 - 3.4
Current smokers	38 / 19	7.45	3.1 - 17.7	6.43	2.5 - 16.8

1. Adjusted for age, sex, schooling, race, religion, and language.

to never smokers, was 15.65. Adjustment for socio-demographic variables reduced the OR of disease to 11.55 (95%CI:3.2-41.8).

Patients who quit smoking had this risk reduced substantially. Table 25 shows the ORs of disease according to time since quitting. Patients who stopped more than 10 years ago had a risk almost similar to never smokers, as well as those who stopped less than 10 years before recruitment. It should be noted that the estimate is not very precise due to the low number of patients, and most patients in this category quitted the habit more than 5 years before enrollment.

Alcohol drinking also showed to be an important determinant of the disease. Table 26 shows the OR of oral cancer due to lifetime cumulative alcohol drinking, measured in kilograms. The crude OR for heavy drinkers (> 400 kgs. of alcohol) was 4.92 (95%CI: 1.3-10.5). Adjustment for socio-demographic variables and tobacco smoking reduced the OR to 3.01 (95%CI: 0.8-11.8). Table 27 shows the increased risk of oral cancer due to intensity and frequency of alcohol drinking. As expected, increasing the frequency and intensity of alcohol consumption increased the risk of disease. However, the trend was not very clear, probably due to the lack of precision in the OR estimates.

Table 28 shows the analysis by alcohol type. Mainly beer drinkers (patients with more than 50% of their total alcohol consumption corresponding to beer) had a greater risk of disease than mainly wine drinkers, or mainly hard liquor drinkers. Heavy drinkers of beer (more than 400 kgs.) showed an adjusted OR of oral cancer of 11.14 (95%CI: 2.0-61.6), whereas heavy drinkers of wine showed an adjusted OR of 7.00 (95%CI: 0.5-97.4). Mainly drinkers of hard liquors showed lower increased risk of disease.

Table 26. Odds Ratios (ORs) of oral cancer associated with cumulative alcohol drinking

Alcohol drinking (in kgs)	Cases/ controls	Crude		Adjusted[1]		Adjusted[2]	
		OR	95%CI	OR	95%CI	OR	95%CI
Non drinkers	7 / 15	1.00		1.00		1.00	
1-80	10 / 47	0.46	0.1 - 1.4	0.45	0.1 - 1.5	0.40	0.1 - 1.4
81-400	17 / 45	0.81	0.3 - 2.3	1.24	0.4 - 4.1	1.15	0.3 - 4.0
401+	38 / 22	3.70	1.3 - 10.5	4.92	1.4 - 17.4	3.01	0.8 - 11.8

1. Adjusted for age, sex, schooling, race, religion, and language.
2. Adjusted for age, sex, schooling, race, religion, language, and tobacco smoking.

Table 27. Odds Ratios (OR) of oral cancer associated with intensity and frequency of alcohol drinking

Duration (in years)	Cases/ controls	Frequency of drinks					
		≤ 1/week		2-6/week		1+/day	
		OR	95%CI	OR	95%CI	OR	95%CI
Non drinkers	7 / 15	1.00		1.00		1.00	
< 30	16 / 40	0.54	0.1 - 3.0	0.00	ND - ND	1.54	0.3 - 8.2
31-44	24 / 39	0.54	0.1 - 2.6	0.71	0.2 - 3.3	2.52	0.5 - 13.3
45+	25 / 35	1.70	0.3 - 8.5	5.35	1.1 - 26.5	2.86	0.6 - 12.9

1. Adjusted for age, sex, schooling, race, religion, language, and tobacco smoking.

Table 28. Odds Ratios (ORs) of oral cancer associated with type of alcoholic beverage

Alcohol drinking (in kgs)	Cases/ controls	Crude		Adjusted[1]	
		OR	95%CI	OR	95%CI
Non drinkers	7 / 15	1.00		1.00	
Mainly[2] Wine drinkers					
1-80	4 / 19	0.45	0.1 - 1.8	0.46	0.1 - 2.2
81-400	4 / 17	0.50	0.1 - 2.1	0.78	0.2 - 4.0
401+	3 / 1	6.43	0.6 - 73.4	7.00	0.5 - 97.4
Mainly[2] Beer drinkers					
1-80	3 / 13	0.50	0.1 - 2.3	0.28	0.0 - 1.6
81-400	7 / 15	1.00	0.3 - 3.6	1.35	0.3 - 6.6
401+	25 / 5	10.71	2.9 - 39.9	11.14	2.0 - 61.6
Mainly[2] Hard liquor drinkers					
1-80	2 / 10	0.43	0.1 - 2.5	0.26	0.0 - 1.9
81-400	2 / 8	0.54	0.1 - 3.2	0.66	0.1 - 5.3
401+	10 / 12	1.79	0.5 - 6.1	1.42	0.3 - 7.3

1. Adjusted for age, sex, schooling, race, religion, language, and tobacco smoking.
2. Mainly drinker of a specific alcohol type refers to patients where more than 50% of their total alcohol consumption corresponds to that specific acohol type.

It was impractical to assess the potential effect modification between tobacco smoking and alcohol drinking. Due to the lack of moderate and heavy drinkers among never smokers, and lack of smokers among non drinkers, it was impossible run any logistic models including the original variables plus the interaction terms. Table 29 shows the results for the fitted model. The OR for those subjects who were heavy smokers and heavy drinkers was 8.04 (95%CI: 2.3-28.2), compared to individuals non smokers and non drinkers.

5.3.2 Other risk factors

The analysis of dietary items as potential risk factors is shown in table 30. Frequent consumption of ham, salami, and sausages was shown to be positively associated with the disease. People who consumed these items at least twice a week had an increased risk of oral cancer compared to subjects who consumed them less than weekly: adjusted OR=2.36, 95%CI:1.0-5.6. Frequent consumption of fish, cruciferus vegetables, carrots, and fresh vegetables in general, showed a decreased risk of disease. The highest reduction was seen for consumption of carrots: the OR of oral cancer for subjects who consumed this vegetable three times or more per week was 0.29 (95%CI:0.1-0.8), compared to individuals who consumed it less than once a week.

Table 31 shows the association of oral cancer with family history of cancer. Due to the low number of cancer cases among relatives the estimates for familial history of head and neck cancer and cervical cancer were extremely imprecise, and no conclusion can be drawn from them. However, it seems that family history of cancer at any site was not associated with development of oral cancer.

Table 29. Odds Ratios (ORs) of oral cancer associated with cumulative tobacco smoking and alcohol drinking

Cumulative Smoking (in pack-years)	Cases/ controls	Cumulative Alcohol drinking (in kgs)							
		Non drinkers		1-80		81-400		401+	
		OR	95%CI	OR	95%CI	OR	95%CI	OR	95%CI
Never smokers	11 / 41	1.0[1]		0.40	0.1 - 1.4	1.15	0.3 - 4.0	3.01	0.8 - 11.8
<=19	12 / 40	0.72	0.2 - 2.2	0.29	0.1 - 1.3	0.83	0.2 - 3.3	2.17	0.5 - 9.7
20-48	21 / 27	1.77	0.6 - 5.1	0.71	0.2 - 3.0	2.03	0.5 - 7.6	5.32	1.5 - 18.9
49+	28 / 21	2.67	0.9 - 8.3	1.08	0.2 - 4.8	3.07	0.7 - 13.2	8.04	2.3 - 28.2

1. Referent

Table 30. Odds Ratios (ORs) of oral cancer according to dietary items

Variable Categories	Cases/ controls	Crude		Adjusted[1]		Adjusted[2]	
		OR	95%CI	OR	95%CI	OR	95%CI
Read meat							
≤ 2/week	18 / 43	1.00		1.00		1.00	
3-5/week	30 / 50	1.43	0.7 - 2.9	1.54	0.7 - 3.4	1.23	0.5 - 2.9
6+/week	24 / 36	1.59	0.7 - 3.4	1.75	0.8 - 4.0	1.70	0.7 - 4.4
Tomatoes							
≤ 2/week	22 / 28	1.00		1.00		1.00	
3-5/week	21 / 41	0.65	0.3 - 1.4	0.79	0.3 - 1.8	1.00	0.4 - 2.6
6+/week	29 / 60	0.62	0.3 - 1.3	0.66	0.3 - 1.4	0.74	0.3 - 1.8
Fish							
< 1/week	20 / 25	1.00		1.00		1.00	
1/week	36 / 47	0.96	0.5 - 2.0	1.03	0.5 - 2.3	1.54	0.6 - 3.8
2+/week	16 / 57	0.35	0.2 - 0.8	0.42	0.2 - 1.0	0.51	0.2 - 1.4
Ham, salami, and sausages							
< 1/week	23 / 68	1.00		1.00		1.00	
1/week	21 / 29	2.14	1.0 - 4.5	2.11	0.9 - 5.0	1.34	0.5 - 3.5
2+/week	28 / 32	2.59	1.3 - 5.2	2.82	1.3 - 6.2	2.36	1.0 - 5.6
Cruciferae							
< 1/week	23 / 26	1.00		1.00		1.00	
1-2/week	35 / 48	0.82	0.4 - 1.7	0.93	0.4 - 2.0	1.45	0.6 - 3.6
3+/week	14 / 55	0.29	0.1 - 0.6	0.28	0.1 - 0.7	0.48	0.2 - 1.3
Carrots							
< 1/week	19 / 12	1.00		1.00		1.00	
1-2/week	30 / 46	0.41	0.2 - 1.0	0.50	0.2 - 1.3	0.67	0.2 - 1.9
3+/week	23 / 71	0.21	0.1 - 0.5	0.22	0.1 - 0.6	0.29	0.1 - 0.8
Vegetables							
< 1/day	30 / 22	1.00		1.00		1.00	
1/day	17 / 66	0.19	0.1 - 0.4	0.22	0.1 - 0.5	0.25	0.1 - 0.6
2+/day	25 / 41	0.45	0.2 - 0.9	0.55	0.2 - 1.2	0.59	0.2 - 1.4
Fresh fruit							
< 1/day	27 / 41	1.00		1.00		1.00	
1/day	21 / 57	0.56	0.3 - 1.1	0.53	0.2 - 1.2	0.68	0.3 - 1.6
2+/day	24 / 31	1.18	0.6 - 2.4	1.09	0.5 - 2.5	1.57	0.6 - 3.9

1. Adjusted for age, sex, schooling, race, religion, and language.
2. Adjusted for age, sex, schooling, race, religion, language, tobacco, and alcohol drinking.

Table 31. Odds Ratios (ORs) of oral cancer according to family history of cancer

Relative Cancer site	Cases/ controls	Crude		Adjusted[1]		Adjusted[2]	
		OR	95%CI	OR	95%CI	OR	95%CI
Father							
Head&Neck	2 / 4	0.89	0.2 - 5.0	0.80	0.1 - 5.3	1.03	0.1 - 8.7
Lung	4 / 3	2.47	0.5 - 11.4	3.39	0.6 - 18.8	2.27	0.4 - 14.6
All sites	18 / 35	0.90	0.5 - 1.7	0.98	0.5 - 2.0	1.01	0.5 - 2.2
Mother							
Lung	2 / 4	0.89	0.2 - 5.0	1.53	0.2 - 10.0	1.97	0.3 - 14.2
Cervix	1 / 1	1.80	0.1 - 29.3	1.25	0.1 - 23.4	0.57	0.0 - 11.6
Uterus[3]	3 / 2	1.20	0.2 - 7.4	2.12	0.3 - 16.2	2.00	0.2 - 20.9
All sites	12 / 37	0.50	0.2 - 1.0	0.44	0.2 - 1.0	0.47	0.2 - 1.2
Sibling							
Head&Neck	1 / 2	0.89	0.1 - 10.0	1.58	0.1 - 38.6	2.47	0.1 - 46.5
Lung	4 / 7	1.03	0.3 - 3.6	0.68	0.2 - 2.7	0.62	0.1 - 2.8
Uterus	1 / 5	0.35	0.0 - 3.0	0.35	0.0 - 3.3	0.44	0.0 - 4.7
All sites	16 / 34	0.80	0.4 - 1.6	0.66	0.3 - 1.4	0.63	0.3 - 1.5
First degree relative							
Head&Neck	3 / 6	0.89	0.2 - 3.7	0.95	0.2 - 4.8	1.38	0.2 - 7.6
Lung	8 / 13	1.12	0.4 - 2.8	1.11	0.4 - 3.1	0.89	0.3 - 2.8
Uterus	3 / 8	0.66	0.2 - 2.6	0.82	0.2 - 3.6	0.88	0.2 - 4.6
All sites	34 / 77	0.60	0.3 - 1.1	0.58	0.3 - 1.1	0.62	0.3 - 1.2

1. Adjusted for age, sex, schooling, race, religion, and language.
2. Adjusted for age, sex, schooling, race, religion, language, tobacco, and alcohol drinking.
3. Includes cancer of the cervix and the uterine body, when patients did not know specific site.

Table 32 presents the association between oral health characteristics and risk of oral cancer. No association was found between frequency of tooth brushing, use of mouthwash, and gum bleeding after brushing. There was a strong association between use of a complete denture and oral cancer in the crude analysis, and after adjustment for socio-demographic variables. This association decreased susbstantially after further adjustment for tobacco smoking and alcohol drinking (OR=2.05, 95%CI:0.8-5.4).

The association between markers of sexual activity and oral cancer among male subjects is shown in table 33. No clear significant associations were found, probably due to the low numbers. However, there were some estimates that may indicate a possible relation between certain markers of sexual activity and the disease among males, such as the analysis for past history of sexual intercourse with female prostitutes, antecedents of STDs, and having had sex with partners with STD. Markers of oral sex (cunnilingus), such as frequency and age at first oral sexual experience, were not associated with the disease.

All estimates for markers of sexual activity among female subjects (table 34) were very imprecise due to the low number of women recruited in the study.

Table 32. Odds Ratios (ORs) of oral cancer according to oral health characteristics

Variable Categories	Cases/ controls	Crude		Adjusted[1]		Adjusted[2]	
		OR	95%CI	OR	95%CI	OR	95%CI
Denture							
No Denture	24 / 78						
Incomplete	10 / 25	1.30	0.5 - 3.1	1.31	0.5 - 3.5	0.76	0.2 - 2.3
Complete	38 / 26	4.75	2.4 - 9.3	4.26	1.9 - 9.6	2.05	0.8 - 5.4
Tooth brushing							
<1/day	11 / 11						
1/day	21 / 47	0.45	0.2 - 1.2	0.45	0.1 - 1.4	0.61	0.2 - 2.1
2/day	27 / 46	0.59	0.2 - 1.5	0.74	0.2 - 2.3	1.28	0.4 - 4.6
3+/day	11 / 25	0.44	0.1 - 1.3	0.49	0.1 - 1.7	0.57	0.1 - 2.4
Gum bleeding							
Never	40 / 76						
Sometimes	25 / 51	0.93	0.5 - 1.7	0.90	0.5 - 1.8	1.02	0.5 - 2.1
Mouthwash							
Never	40 / 67						
1-4/week	9 / 27	0.56	0.2 - 1.3	0.38	0.1 - 1.0	0.27	0.1 - 0.8
1+/day	21 / 34	1.04	0.5 - 2.0	0.78	0.4 - 1.7	0.64	0.3 - 1.5

1. Adjusted for age, sex, schooling, race, religion, and language.
2. Adjusted for age, sex, schooling, race, religion, language, tobacco, and alcohol drinking.

Table 33. Odds Ratios (ORs) of oral cancer among **males** according to sexual activity

Variable Categories	Cases/ controls	Crude		Adjusted[1]		Adjusted[2]	
		OR	95%CI	OR	95%CI	OR	95%CI
Number of sexual partners							
0-1	9 / 21						
2-5	16 / 26	1.44	0.5 - 3.9	2.31	0.7 - 7.4	1.53	0.4 - 5.7
6-20	12 / 26	1.08	0.4 - 3.0	1.61	0.5 - 5.3	1.10	0.3 - 4.4
21+	12 / 19	1.47	0.5 - 4.3	2.59	0.7 - 9.1	1.35	0.3 - 5.8
Age at first intercourse							
<18	19 / 36						
18-20	13 / 27	0.91	0.4 - 2.2	0.99	0.4 - 2.7	1.10	0.3 - 3.9
21+	17 / 26	1.24	0.5 - 2.8	1.05	0.4 - 2.9	1.70	0.5 - 6.0
Frequency of oral sex (cunnilingus)							
No oral	16 / 33						
Seldom	19 / 36	1.09	0.5 - 2.5	1.41	0.5 - 4.0	1.77	0.5 - 6.3
Often	13 / 20	1.34	0.5 - 3.4	1.55	0.5 - 4.9	1.11	0.3 - 4.6
Age first time oral sex (cunnilingus)							
No oral	16 / 33						
<=18	12 / 18	1.38	0.5 - 3.5	1.60	0.5 - 5.7	0.99	0.2 - 4.6
19-24	8 / 21	0.79	0.3 - 2.2	1.08	0.3 - 3.8	1.43	0.3 - 7.2
25+	11 / 17	1.34	0.5 - 3.5	1.59	0.5 - 5.1	1.81	0.4 - 7.4
Female prostitute partners							
No prost	34 / 70						
1-5	8 / 13	1.27	0.5 - 3.3	1.61	0.5 - 5.1	1.72	0.4 - 6.6
6+	9 / 9	2.06	0.7 - 5.7	2.55	0.8 - 8.2	1.80	0.5 - 6.7
Homosexuality							
No	46 / 87						
Yes	3 / 5	1.14	0.3 - 5.0	1.22	0.2 - 7.2	0.98	0.1 - 7.2
STDs							
No	38 / 81						
Yes	9 / 8	2.40	0.9 - 6.7	2.33	0.7 - 7.4	3.55	0.8 - 15.9
Partners w/STDs							
No	37 / 76						
Yes	10 / 15	1.37	0.6 - 3.3	1.52	0.5 - 4.5	1.87	0.5 - 7.4

1. Adjusted for age, schooling, race, religion, and language.
2. Adjusted for age, schooling, race, religion, language, tobacco, and alcohol drinking.

Table 34. Odds Ratios (ORs) of oral cancer among **females** according to sexual activity

Variable Categories	Cases/ controls	Crude		Adjusted[1]		Adjusted[2]	
		OR	95%CI	OR	95%CI	OR	95%CI
Number of sexual partners							
0-1	11 / 22						
2-5	8 / 11	1.45	0.5 - 4.7	2.25	0.5 - 9.7	3.50	0.5 - 25.1
6-20	2 / 4	1.00	0.2 - 6.3	1.62	0.2 - 16.4	1.11	0.1 - 22.4
Age at first intercourse							
<18	3 / 8						
18-20	6 / 16	1.00	0.2 - 5.1	1.37	0.1 - 13.5	0.70	0.1 - 9.8
21+	12 / 11	2.91	0.6 - 13.8	4.42	0.5 - 39.2	3.71	0.3 - 41.7
Frequency of oral sex (fellatio)							
Never	10 / 15						
Seldom	10 / 16	0.94	0.3 - 2.9	1.06	0.2 - 5.0	1.05	0.2 - 6.9
Often	1 / 4	0.38	0.0 - 3.9	0.38	0.0 - 4.8	0.21	0.0 - 6.0
Age first time oral sex (fellatio)							
Never	10 / 15						
<=24	6 / 13	0.69	0.2 - 2.4	0.69	0.1 - 3.6	0.62	0.1 - 4.0
25+	5 / 7	1.07	0.3 - 4.3	1.25	0.2 - 7.7	2.48	0.2 - 32.0
STDs							
No	20 / 36						
Yes	1 / 1	1.80	0.1 - 30.4	2.16	0.1 - 53.3	1.17	0.0 - 152.5

1. Adjusted for age, schooling, race, religion, and language.
2. Adjusted for age, schooling, race, religion, language, tobacco, and alcohol drinking.

5.4 Human Papillomavirus (HPV) and Oral Cancer

Of the 201 oral cell samples, a total of 20 (10.0%) were HPV positive for DNA. The distribution of cases and controls according to HPV positivity is shown in table 35. HPV DNA was detected in oral exfoliated cells of six out of 129 controls (4.7%) and 14 out of 72 cases (19.4%). The distribution of HPV DNA positivity among controls according to socio demographic characteristics (sex, language, religion, income, and schooling) did not differ in an appreciable way, except for age, where young controls (less than 60 years of age) were more likely to be HPV positive (8.5%) than older controls (1.5%).

The distribution of subjects according to HPV oncogenicity showed that most viral infections among cases harboured high risk HPV types. In case of multiple infections, samples with the presence of at least one high risk HPV type were classified as high risk. Among cases, 13 out of 14 samples harboured high risk types, compared to four of the six HPV positive controls. HPV 16 was not detected in samples from controls, whereas 13 of the 14 positive samples from cases harboured HPV 16.

The HPV types detected in oral cells are summarized in table 36. Five of the six HPV positive samples among controls, and nine of the 14 among cases, were single infections. HPV 16 was the most common type found, and among cases it was detected in most single infection samples and in all multiple infection samples. Infections with multiple HPV types were seen in samples from one control, and in samples from 5 cases.

Table 35. Distribution of cases and controls according to HPV DNA detection

Definition of positivity	Categories	Controls		Cases	
		N	%	N	%
Overall	Negative	123	95.3%	58	80.6%
	Positive	6	4.7%	14	19.4%
Oncogenic risk grouping	Negative	123	95.3%	58	80.6%
	Low risk	2	1.6%	1	1.4%
	High risk	4	3.1%	13	18.1%
Oncogenic risk grouping with HPV 16	Negative	123	95.3%	58	80.6%
	Low risk	2	1.6%	1	1.4%
	High risk	4	3.1%	0	0.0%
	HPV 16	0	0.0%	13	18.1%
Single *vs.* multiple infections	Negative	123	95.3%	58	80.6%
	Single type	5	3.9%	9	12.5%
	Two types	1	0.8%	2	2.8%
	Three types	0	0.0%	2	2.8%
	Four types	0	0.0%	1	1.4%
		129	100.0%	72	100.0%

Table 36. Distribution of HPV positive samples according to risk group and single *versus* multiple infections

HPV types	Controls N	Controls %	Cases N	Cases %	Total N	Total %
Low Risk types						
11	1	0.8%			1	0.5%
66	1	0.8%			1	0.5%
84			1	1.4%	1	0.5%
High Risk types						
16			8	11.1%	8	4.0%
55	1	0.8%			1	0.5%
58	2	1.6%			2	1.0%
Subtotal for single infections	**5**	**3.9%**	**9**	**12.5%**	**14**	**7.0%**
16,31			1	1.4%	1	0.5%
16,35			1	1.4%	1	0.5%
56,58	1	0.8%			1	0.5%
16,39,53			1	1.4%	1	0.5%
16,51,55			1	1.4%	1	0.5%
6,16,39,53			1	1.4%	1	0.5%
Subtotal for multiple infections	**1**	**0.8%**	**5**	**6.9%**	**6**	**3.0%**
Total	**6**	**4.7%**	**14**	**19.4%**	**20**	**10.0%**

5.4.1 Detection of HPV DNA according to clinical and pathological characteristics of the disease

Table 37 shows the distribution of HPV DNA positivity according to topographic site of the tumour. The sites with the highest positivity rate were tonsil (50.0%) and base of tongue (33.3%). Other sites of the oral cavity had a considerably lower rate. For example, only one of 21 tumours (4.8%) of other parts of the tongue and two of 12 tumours of the floor of the mouth (16.7%) turned out to be HPV positive. Grouping together tonsils and base of the tongue, the rate of HPV infection was 42.9% (12 out of 21). I grouped together these two sites because both are part of the Waldeyer's ring, a region rich in lymphatic tissue especially organized as a first line of defense against microbial antigens from the external environment.

Table 38 summarizes detection of HPV DNA according to histopathological characteristics of the tumour. The detection rate was slightly higher in larger tumours (T3,T4) than less advanced tumors (CIS, T1, T2). Oral cell samples from tumours with lymph node involvement were more likely to be HPV positive (33.3%) than tumours without regional spread (9.5%). In consequence, the HPV positivity rate was higher among advanced disease samples (TNM stage III, IV) than in those from cases with less advanced disease (CIC, stage I, II): 28.9%, and 8.8%, respectively. The rate of HPV detection was higher for poorly differentiated tumours (38.5%), compared with moderately (15.6%), and well differentiated tumours (11.8%). These differences in detection of HPV DNA are mainly explained by the fact that most tonsillar cancer cases were characterized by advanced disease: large tumours, with lymph node involvement, stage III-IV, and poorly differentiated.

Table 37. HPV DNA positivity by topographic site among cases of oral cancer

Site of primary tumour	HPV negative N	%	HPV positive N	%	Total N	%
Lip	1	100.0%	0	0.0%	1	100.0%
Base of tongue	6	66.7%	3	33.3%	9	100.0%
Other and unspecified parts of tongue	20	95.2%	1	4.8%	21	100.0%
Gum	2	100.0%	0	0.0%	2	100.0%
Floor of mouth	10	83.3%	2	16.7%	12	100.0%
Palate	3	75.0%	1	25.0%	4	100.0%
Other and unspecified parts of mouth	8	88.9%	1	11.1%	9	100.0%
Tonsil	6	50.0%	6	50.0%	12	100.0%
Oropharynx	2	100.0%	0	0.0%	2	100.0%
Total	**58**	**80.6%**	**14**	**19.4%**	**72**	**100.0%**
Tonsil & Base of tongue	12	57.1%	9	42.9%	21	100.0%
Other sites	46	90.2%	5	9.8%	51	100.0%
Total	**58**	**80.6%**	**14**	**19.4%**	**72**	**100.0%**

Table 38. HPV DNA positivity according to clinical and histopathological characteristics of oral cancers

Variable	Categories	HPV negative N	HPV negative %	HPV positive N	HPV positive %
Tumour classification					
	CIS[1]-T1-T2	37	82.2%	8	17.8%
	T3-T4	21	77.8%	6	22.2%
Lymph node involvement					
	No	38	90.5%	4	9.5%
	Yes	20	66.7%	10	33.3%
TNM Clinical Stage					
	CIS,I-II	31	91.2%	3	8.8%
	III-IV	27	71.1%	11	28.9%
Differentiation					
	Well	15	88.2%	2	11.8%
	Moderate	27	84.4%	5	15.6%
	Poor	8	61.5%	5	38.5%
	Unknown	8	80.0%	2	20.0%
Total		**58**	**80.6%**	**14**	**19.4%**

1. CIS: carcinoma in situ

5.4.2 Odds Ratios (ORs) of oral cancer according to detection of HPV DNA

The analysis of detection of HPV DNA as a risk factor for the disease was done using two classifications: i) dichotomous (positive *versus* negative), and ii) by oncogenicity (high risk types, low risk types, and negative). Table 39 shows the ORs of disease from the crude and adjusted analyses. The crude estimate for overal HPV DNA postivity was 4.95 (95%CI:1.8-13.5). Adjustment for socio-demographic variables reduced the OR to 3.04 (95%CI:1.0-9.3). Further adjustment for tobacco smoking and alcohol drinking did not modify the estimate but imposed a loss of precision (OR=3.14; 95%CI:0.9-10.9).

The analysis of detection of HPV DNA according to oncogenic types showed that the ORs of oral cancer for high risk types were substantially higher than for low risk types. The estimates for low risk types were very close to unity, whereas the crude OR for high risk types was 6.89 (95%CI:2.2-22.1). After adjustment for socio-demographic variables plus tobacco smoking and alcohol drinking the magnitude of association between high risk types and oral cancer persisted at the same level: OR=4.81; 95%CI:1.2-19.4.

In addition to the analysis of all oral cancers, a subsite analysis was done to assess the association between detection of HPV DNA with cancers of the tonsil and base of the tongue as a combined set (table 40). All controls and only cases of these cancers were included in the analysis. The crude OR of disease for high risk types was 23.06 (95%CI:6.2-86.2). Full adjustment for socio-demographic variables plus tobacco and alcohol slightly decreased the estimate (OR=19.32; 95%CI:2.3-159.5), but the association persisted of high magnitude.

Table 39. Odds Ratios (ORs) of oral cancer associated with detection of HPV DNA

HPV DNA	Cases/ controls	Crude		Adjusted[1]		Adjusted[2]	
		OR	95%CI	OR	95%CI	OR	95%CI
Negative	58 / 123	1.00		1.00		1.00	
Positive	14 / 6	4.95	1.8 - 13.5	3.04	1.0 - 9.3	3.14	0.9 - 10.9
Negative	58 / 123	1.00		1.00		1.00	
Low risk	1 / 2	1.06	0.1 - 11.9	0.41	0.0 - 5.3	0.27	0.0 - 4.4
High risk	13 / 4	6.89	2.2 - 22.1	4.41	1.3 - 15.5	4.81	1.2 - 19.4

1. Adjusted for age, sex, schooling, race, religion, and language.
2. Adjusted for age, sex, schooling, race, religion, language, tobacco smoking, and alcohol drinking.

Table 40. Odds Ratios (ORs) of cancer of the tonsils and base of tongue associated with detection of HPV DNA

HPV DNA	Cases/ controls	Crude		Adjusted[1]		Adjusted[2]	
		OR	95%CI	OR	95%CI	OR	95%CI
Negative	12 / 123	1.00		1.00		1.00	
Positive	9 / 6	15.38	4.7 - 50.6	12.70	2.4 - 66.8	18.43	2.2 - 154.5
Negative	12 / 123	1.00		1.00		1.00	
Low risk	0 / 2	0.01	0.0 - ND	0.00	0.0 - ND	0.00	0.0 - ND
High risk	9 / 4	23.06	6.2 - 86.2	18.45	3.3 - 104.5	19.32	2.3 - 159.5

1. Adjusted for age, sex, schooling, race, religion, and language.
2. Adjusted for age, sex, schooling, race, religion, language, tobacco smoking, and alcohol drinking.

Table 41. Odds Ratios (ORs) of oral cancer other than tonsils and base of tongue associated with detection of HPV DNA

HPV DNA	Cases/ controls	Crude		Adjusted[1]		Adjusted[2]	
		OR	95%CI	OR	95%CI	OR	95%CI
Negative	46 / 123	1.00		1.00		1.00	
Positive	5 / 6	2.23	0.6 - 7.7	1.42	0.4 - 5.7	1.29	0.3 - 6.3
Negative	46 / 123	1.00		1.00		1.00	
Low risk	1 / 2	1.34	0.1 - 15.1	0.55	0.0 - 7.3	0.33	0.0 - 5.5
High risk	4 / 4	2.67	0.6 - 11.1	2.01	0.4 - 9.7	2.14	0.4 - 13.0

1. Adjusted for age, sex, schooling, race, religion, and language.
2. Adjusted for age, sex, schooling, race, religion, language, tobacco smoking, and alcohol drinking.

The results for all oral cancers except tonsil and base of tongue are shown in table 41. The ORs of disease for high risk HPV types was 2.67 (95%CI:0.6-11.1) for the crude model, and 2.14 (95%CI: 0.4-13.0) for the fully adjusted model. These OR estimates were considerably lower than the ones for tonsil and base of tongue.

Effect modification between detection of HPV DNA and certain established determinants of the disease was assessed running models including and excluding interaction terms (table 42). None of the interaction terms added to the fitted model reached statistical significance. However, despite the relatively small sample size of the present study, some estimates could point to a potential effect modification. Regarding the analysis of effect modification between age and HPV DNA in oral samples, the OR of oral cancer for older patients (more than 55 years) with HPV positive samples was 7.21 (95%CI: 1.7-31.3) in the fitted model, whereas in the model with the interaction term the OR was 16.31 (95%CI: 1.8-144.6). In both models, the reference category were younger patients with HPV negative oral samples. The analysis for tobacco smoking showed that heavy smokers (more than 30 pack-years) with HPV positive samples had higher risk estimates in the model including interaction terms than the fitted model. The same situation occurred with alcohol drinking. Regarding interaction of detection of HPV DNA and sex, the estimates for both models were rather similar.

5.4.3 Detection of HPV DNA in exfoliated oral cells and tumour biopsies

Of the 72 cases recruited in the study, a tumour biopsy was collected for 41 of them and sent for HPV DNA testing. Biopsy samples were tested for the amplification of the β-globin gene to assess the quality of the sample for further testing for HPV DNA. Of the 41 biopsies, 35 were positive for the β-globin gene. These biopsies were further tested

Table 42. Assessment of effect modification between detection of HPV DNA and other factors on the risk of oral cancers

Variables	Categories	Cases/ controls	Fitted Model				Assuming interaction				p value[1]
			HPV negative		HPV positive		HPV negative		HPV positive		
			OR	95%CI	OR	95%CI	OR	95%CI	OR	95%CI	
Age	<=55	22 / 40	1.00		5.45	1.9 - 15.4	1.00		3.27	0.9 - 12.0	0.2104
	>55	50 / 89	1.32	0.7 - 2.6	7.21	1.7 - 31.3	1.14	0.6 - 2.3	16.31	1.8 - 144.6	
Tobacco	Never	11 / 41	1.00		5.18	1.8 - 15.0	1.00		8.89	0.7 - 109.0	0.7352
(in pkyrs)	1-30	19 / 55	1.17	0.5 - 2.8	6.07	1.6 - 22.8	1.31	0.5 - 3.3	4.45	0.9 - 21.2	
	>30	42 / 33	4.61	2.0 - 10.5	23.85	6.1 - 93.4	4.72	2.0 - 11.3	35.45	3.9 - 321.2	
Alcohol	<1	7 / 15	1.00		4.46	1.5 - 12.9	1.00		0.67	0.1 - 7.9	0.1438
(in kgs)	1-200	16 / 69	0.62	0.2 - 1.8	2.74	0.5 - 14.0	0.42	0.1 - 1.3	2.00	0.2 - 17.9	
	>200	49 / 45	2.70	1.0 - 7.6	12.02	2.5 - 58.5	1.73	0.6 - 5.0	22.02	2.3 - 212.9	
Sex	Females	21 / 37	1.00		5.16	1.9 - 14.3	1.00		1.80	0.1 - 30.4	0.4467
	Males	51 / 92	0.83	0.4 - 1.6	4.28	1.4 - 13.2	0.79	0.4 - 1.5	4.68	1.5 - 15.0	

1. p value (likelihood ratio test) for the improvement in goodness of fit for the model adding the interaction term(s).

for the presence of HPV DNA using the GP5+/GP6+ technique. A total of seven of the 35 biopsies (20%) were positive for HPV DNA, all of them for HPV 16. Tumours of the tonsil and base of tongue had a higher positivity rate (45.4%; 5 out of 11) than other oral cancer tumours (4.2%; 2 out of 24).

Detection of viral DNA in oral exfoliated cells and in tumour biopsies were highly correlated. Table 43 presents the agreement between detection of HPV DNA in both types of samples. Despite the use of different PCR protocols and the different nature of biological materials there was agreement in measurement for 31 of the 35 samples. All the oral cell samples included in the table were positive for HPV 16 DNA.

Table 43. Agreement between detection of HPV DNA in oral exfoliated cells and tumour biopsies

	Biopsies		Total	Kappa statistic	*p* value[1]
Oral cells	Negative	Positive			
Negative	26	2	28	0.643	<0.0001
Positive	2	5	7		
Total	28	7	35		

1. Association between HPV positivity in oral cells and biopsies.

5.4.5 HPV serology and Oral Cancer

Plasma samples for serology testing were obtained from 66 of the 72 cases, and 128 of the 129 controls. Table 44 presents the mean optical density (OD) values for the ELISA assays carried out for three anti-capsid antibodies: HPV 16, HPV 18, and HPV 31. The

Table 44. HPV capsid serum antibodies (in absorbance values) according to patient status (cases and controls)

Capsid antibody	Status	N	Mean OD	Difference in means	95% CI	*p* value[1]
HPV16	Controls	128	0.014	0.067	0.021 - 0.113	0.005
	Cases	66	0.082			
HPV18	Controls	128	0.011	0.004	0.000 - 0.007	0.048
	Cases	66	0.015			
HPV31	Controls	128	0.026	0.036	-0.009 - 0.081	0.115
	Cases	66	0.062			

1. *p* value for independent T-test.

Table 45. HPV capsid serum antibodies (in absorbance values) among **cases** according to detection of HPV DNA in oral exfoliated cells

Capsid antibody	Detection of HPV DNA	N	Mean OD	Difference in means	95% CI	*p* value[1]
HPV16	Negative	52	0.030	0.243	0.059 - 0.427	0.014
	Positive	14	0.273			
HPV18	Negative	52	0.011	0.016	0.002 - 0.030	0.031
	Positive	14	0.027			
HPV31	Negative	52	0.023	0.183	-0.024 - 0.391	0.079
	Positive	14	0.206			

1. *p* value for independent T-test.

mean OD values were higher among cases than controls for the three antibodies tested. The highest difference was seen for HPV 16: 0.067 (95%CI: 0.021-0.113).

Cases with oral exfoliated cell samples positive for detection of HPV DNA were compared with cases with oral samples that were HPV negative (table 45). The mean OD values were higher for all three HPV types among HPV DNA positive cases compared to HPV DNA negative cases. Again, the highest difference was seen for HPV 16: 0.243 (95%CI:0.059-0.427).

5.4.6 Odds Ratios (ORs) of oral cancer according to HPV seropositivity

The association between HPV serology and risk of oral cancer is summarized in table 46. The highest association between seropositivity and oral cancer was seen for the HPV 16 capsid antigen. The OR of disease for this antigen, after adjustment for socio-demographic variables as well as tobacco smoking and alcohol drinking (model 3), was 7.48 (95%CI:2.1-27.2). The ORs for the other two HPV types were substantially lower: the adjusted OR (model 3) for HPV 18 was 2.31 (95%CI: 0.4-13.4), and for HPV 31 was 2.25 (95%CI:0.8-6.3). As expected, given their correlation, further adjustment for detection of HPV DNA in the oral cavity decreased the estimates for HPV seropositivity (model 4): e.g. the OR estimate for HPV 16 decreased form 7.48 (model 3) to 6.45 (95%CI:1.6-25.3).

The definition of seropositivity used in the analysis presented in table 46 corresponded to the predefined cutpoint for OD values as porposed by Dr. Viscidi's laboratory. Table 47 presents the ORs of oral cancer for different cutpoints to define seropositivity for the HPV 16 ELISA assay. Different cutpoints were defined according to the distribution of results among control subjects: 70th percentile, 80th percentile, 90th percentile, the

Table 46. Odds ratios (ORs) of oral carcinoma according to HPV seropositivity

Seropositivity	Cases/ Controls	Model 1		Model 2		Model 3		Model 4	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
HPV 16									
Negative	58/123	1.00		1.00		1.00		1.00	
Positive	14/5	**6.62**	2.3 - 19.3	**5.79**	1.7 – 19.3	**7.48**	2.1 – 27.2	**6.45**	1.6 – 25.3
HPV 18									
Negative	57/120	1.00		1.00		1.00		1.00	
Positive	4/3	**2.69**	0.6 – 12.4	**2.31**	0.4 – 12.5	**2.31**	0.4 – 13.4	**1.53**	0.2 – 10.2
HPV 31									
Negative	49/110	1.00		1.00		1.00		1.00	
Positive	13/11	**2.61**	1.1 – 6.2	**2.13**	0.8 – 5.5	**2.25**	0.8 – 6.3	**1.84**	0.6 – 5.5

1. Crude
2. Adjusted for socio demographic variables
3. Adjusted for the above plus tobacco and alcohol
4. Adjusted for the above plus detection of HPV DNA

predefined cutpoint –that corresponded to the 96th percentile-, and the value that provided the maximum positive likelihood ratio –corresponding to the 99th percentile-. As expected, the highest association between HPV 16 seropositivity and oral cancer was found for the latter value (crude OR=28.2: 95%CI:3.6-222.4). The crude OR for the preassigned cutpoint was 6.62 (95%CI:2.3-19.3). I preferred to use this cutpoint for the analysis because I will be able to compare the results form this investigation with results from other studies, given that most studies on HPV serology defined seropositivy the same way.

Table 47. Crude odds ratios (ORs) of oral cancer according to different cutpoint for HPV 16 seropositivity

Cutpoint	OD value	Controls		Cases		OR	95%CI	
		Neg	Pos	Neg	Pos			
70th percentile	0.0115	91	37	26	40	3.78	2.0	7.1
80th percentile	0.0131	101	27	34	32	3.52	1.9	6.7
90th percentile	0.0165	115	13	43	23	4.73	2.2	10.2
Preassigned (96th perc)	0.0500	123	5	52	14	6.62	2.3	19.3
Maximun +LR (99th perc)	0.0862	127	1	54	12	28.22	3.6	222.4

The subsite analysis for tonsil plus base of tongue, and for all other cancers, was carried out only for HPV 16 serology (table 48). The magnitude of association between HPV 16 seropositivity and cancers of the tonsil and base of tongue was extremely high. The fully adjusted model, including adjustment for detection of HPV DNA in oral cells, yielded an OR of 99.34 (95%CI:3.2–3089.9). As expected, the OR estimates were very imprecise, due to the low number of cases in the subsite analysis and the low number of

Table 48. Odds ratios (ORs) of oral carcinoma according to **HPV 16** seropositivity

Seropositivity by subsite	Cases/ Controls	Model 1		Model 2		Model 3		Model 4	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Tonsils & Base of Tongue									
Negative	8/123	1.00		1.00		1.00		1.00	
Positive	9/5	**27.68**	7.5 – 102.2	**31.51**	4.5 - 219.7	**182.27**	7.0 - 4753.0	**99.34**	3.2 - 3089.9
Oral cancer other than Tonsil & Base of tongue									
Negative	44/123	1.00		1.00		1.00		1.00	
Positive	5/5	**2.80**	0.8 - 10.1	**3.16**	0.8 - 13.0	**3.87**	0.9 - 17.5	**3.93**	0.9 - 18.0

Model 1. Crude
Model 2. Adjusted for socio-demographic variables
Model 3. Adjusted for socio-demographic variables plus tobacco and alcohol
Model 4. Adjusted for socio-demographic variables, tobacco, alcohol plus detection of HPV DNA

seropositive controls. The risk estimates for other cancers were much smaller. The OR for the fully adjusted model was 3.93 (95%CI:0.9-18.0).

5.4.7 HPV serology and detection of HPV DNA

Detection of viral DNA in oral cells and the results form HPV serology were highly correlated. Table 49 shows the agreement between serology for HPV 16 and HPV DNA. As expected, the agreement between HPV 16 seropositivity and HPV 16 DNA was higher than the correlation with any HPV DNA.

Table 49. Agreement between HPV DNA and serology measurements

Detection of viral DNA in oral cells		HPV 16 serology		Total	Kappa statistic	*p* value
		Negative	Positive			
Any HPV type						
	Negative	166	8	174	0.515	<0.0001
	Positive	9	11	20		
HPV 16						
	Negative	173	8	181	0.660	<0.0001
	Positive	2	11	13		
Total		175	19	194		

Table 50 shows the ORs of oral cancer according to detection of HPV DNA and HPV 16 seropositivity, each one alone and together. The analysis was done for all oral cancers, tonsil and base of tongue together, and other oral cancers. When detection of HPV DNA and serology were each one alone in the model, the OR estimates for HPV serology were higher than the estimates for HPV DNA for all three outcomes considered. The models that included both variables mutually adjusted showed that for all oral cancers

Table 50. Odds Ratios (ORs) of oral cancer for HPV serology and detection of viral DNA, alone and mutually adjusted

		Cases/ controls	Crude		Mutually adjusted		Further[1] adjusment	
			OR	95%CI	OR	95%CI	OR	95%CI
All Oral Cancer Substies								
HPV DNA	Negative	58 / 123	1.00		1.00		1.00	
	Positive	14 / 6	4.95	1.8 - 13.5	3.02	1.0 - 9.4	3.56	1.1 –12.5
HPV 16 serology	Negative	52 / 123	1.00		1.00		1.00	
	Positive	14 / 5	6.62	2.3 - 19.3	4.00	1.2 - 13.1	4.29	1.2 – 15.0
Tonsil & Base of Tongue								
HPV DNA	Negative	12 / 123	1.00		1.00		1.00	
	Positive	9 / 6	15.38	4.7 - 50.6	9.49	2.2 – 41.2	13.01	1.9 - 87.9
HPV 16 serology	Negative	8 / 123	1.00		1.00		1.00	
	Positive	9 / 5	27.68	7.5 - 102.2	12.00	2.7 – 53.2	23.38	3.5 – 156.0
Other Oral Cancers[2]								
HPV DNA	Negative	46 / 123	1.00		1.00		1.00	
	Positive	5 / 6	2.23	0.6 - 7.7	1.90	0.5 – 6.9	2.09	0.5 – 8.4
HPV 16 serology	Negative	44 / 123	1.00		1.00		1.00	
	Positive	5 / 5	2.80	0.8 - 10.1	2.40	0.6 – 9.1	2.56	0.6 – 10.6

1. Adjustment for markers of sexual activity. See text for details.
2. Other than tonsil and base of tongue.

both estimates decreased compared to the crude models. For detection of HPV DNA, the OR estimate decreased from 4.95 (95 %CI: 1.8-13.5) to 3.02 (95 %CI: 1.0-9.4). The OR for HPV 16 seropositivity decreased form 6.62 (95 %CI: 2.3-19.3) to 4.00 (95 %CI: 1.2-13.1). The analysis for cancer of tonsil and base of tongue also showed a decrease in both estimates: for HPV DNA from 15.38 (95 %CI 4.7-50.6) in the crude model, to 9.49 (95 %CI:2.2-41.2) in the mutually adjusted model, and the for HPV 16 seropositivity from 27.68 (95 %CI: 7.5-102.2) to 12.00 (95 %CI:2.7-53.2). For cancers other than tonsil and base of tongue, there was a slight decrease for both HPV infection markers. An important caveat to consider in these analyses is the relatively low precision of the estimates.

The last column of table 50 presents the above analyses plus further adjustment for some markers of sexual activity. The variables included in the model -in addition to HPV DNA detection and HPV 16 seropositivity- were antecedents of sexual intercourse with prostitutes and with sexual partners with STDs for males, and antecedents of personal STDs for both males and females. These variables were chosen because they are the ones most likely to be associated with the disease, as suggested by the results presented in tables 33 and 34. Adjustment for these markers of sexual activity did not decrease the magnitude of the estimates for HPV DNA or serological response, suggesting that the seropositivity is not explained by a genital infection.

Table 51 presents the distribution of subjects according to HPV posivity combining the results of viral DNA detection and serological response. The proportion of cases positive for both measurements was substantially higher for cancers related to Waldeyer's ring (palatine and lingual tonsil) than for other oral cancers.

Table 51. Distribution of subjects according to HPV DNA detection and HPV 16 seropositivity

HPV DNA	HPV serology	Controls		All oral cancers		Other than tonsils and base of tongue		Tonsils and Base of Tongue	
		N	rate	N	rate	N	rate	N	rate
Negative	Negative	117	91.4%	49	74.2%	42	85.7%	7	41.2%
Positive	Negative	6	4.7%	3	4.5%	2	4.1%	1	5.9%
Negative	Positive	5	3.9%	3	4.5%	2	4.1%	1	5.9%
Positive	Positive	0	0.0%	11	16.7%	3	6.1%	8	47.1%
	Total	**128**	**100.0%**	**66**	**100.0%**	**49**	**100.0%**	**17**	**100.0%**

It was not possible to estimate ORs of oral cancer due to positivity with both measurements since none of the control subjects was in this category. To circumvent this problem, a case-only analysis was performed to assess the magnitude of association of markers of HPV infection with tonsillar related cancers, compared to oral cancers not related to palatine and lingual tonsils. These results are shown in table 52.

Table 52. Case-only analysis: odds ratios (ORs) of tonsillar[1] cancers (compared to non tonsillar cancers) for markers of HPV infection

HPV marker	Categories	Non tonsillar[1] cases/ Tonsillar[1] cases	OR	95%CI
HPV DNA	Negative	46/12	1.00	ref.[2]
	Positive	5/9	6.90	1.9 - 24.4
HPV 16 serology	Negative	44/8	1.00	ref.
	Positive	5/9	9.90	2.6 - 37.3
Combined tests	Both Negative	42/7	1.00	ref.
	Only HPV DNA positive	2/1	3.00	0.2 - 37.7
	Only serology positive	2/1	3.00	0.2 - 37.7
	Either test positive	7/10	8.57	2.4 - 30.0
	Both tests positive	3/8	16.00	3.4 - 75.3

1. Tonsillar cases: include palatine and lingual tonsils,
   Non tonsillar cases: other than palatine and lingual tonsils.
2. Reference category.

The magnitude of association of both markers of HPV infection (HPV DNA and serology) with tonsillar related cancers was substantially higher than for either marker alone. These results suggest that not only the association of HPV infection with cancers of the

palatine and lingual tonsils is much stronger than for other oral cancers, but also that assessment of HPV infection with immunological assays supplements the detection of viral DNA in the oral cavity.

6. DISCUSSION

The results from the present project show that there is a clear association between HPV infection and oral cancer, or at least with a subset of oral malignancies. Two markers of viral infection were used: detection of HPV DNA in the oral cavity, and serological response to HPV capsids. Both markers were strongly correlated with the disease.

HPV DNA was detected in approximately 20 % of subjects with oral cancer, and in 5% of controls. For HPV 16 DNA, the rates were 18% and 0%, respectively. The rates for any HPV type are in the middle range of detection compared to other case-control studies that used oral exfoliated cell samples and newly diagnosed cases. Our rates are comparable to those in the study by Smith et al. (1998) (15% among cases and 5% among controls); they were lower compared with the study by Summersgill et al. (2000), (29% and 19%, respectively), and higher than the rates shown in the research by Herrero et al. (2000) (5% for both cases and controls).

6.1 ORAL CANCERS NOT RELATED TO LINGUAL AND PALATINE TONSILS

The analysis for oral cancers excepting palatine tonsil and base of tongue indicated that detection of high risk HPV DNA in the oral cells was associated with a two-fold increase in the risk of disease (OR=2.14, 95%CI:0.4-13.0), after adjustment for age, sex, socio-demographic factors, and tobacco and alcohol consumption. Although lacking precision, this estimate was comparable to the results from the study by Summersgill et al. (2000): OR=2.4 (95%CI:1.4-3.9), and the results from Smith et al. (1998): OR for all HPV types of 3.7 (95%CI:1.5-9.3). These two studies included cases with cancer of the mouth only. Herrero et al. (2000), who included patients with cancers of the mouth and of the oropharynx, failed to find an association between the disease and detection of viral DNA

in oral cells. Two case-control studies that included a considerable proportion of patients already treated for oral cancer found a relatively low detection rate of high risk HPV DNA in both cases and controls: less than 10% (Maden et al., 1992; Schwartz et al., 1998).

A recent meta-analysis by Miller and Johnstone (2001) comprised studies on HPV and cancer of the mouth published from 1982 to 1997. The authors reported a pooled OR of 5.37 (95%CI: 2.5-11.6) for studies comparing prevalence of HPV in normal mucosa and squamous cell carcinomas of the mouth. This estimate took into consideration sample size, age, sex, method of tissue preservation, and type of assay. Tobacco and alcohol consumption were also considered cofactors, but availability of detailed information on these potential confounders was scanty.

This study's positive association between detection of HPV DNA and oral cancers not related to Waldeyer's ring does not necessarily mean that the virus plays an etiologic role. HPV appears to be a common comensal in the oral mucosa (Scully, 2002), and there is the possibility that the virus infects preferentially already premalignant or malignant tissues. Conversely, a virus may be an etiologically important factor in the development of a tumour and still not be present in tumour cells ("hit and run" model). Studies on serological response to HPV may better represent the temporal relationship between the virus and the tumour. Serological response is more a marker of past cumulative HPV exposure rather than current HPV infection, and most seroconversions are persistent (Dillner et al., 1995; Dillner, 1999).

In the present project a positive association was found between serological response to the HPV 16 capsid and cancers of the oral cavity other than tonsil and base of tongue (OR=3.87, 95%CI:0.9-17.5, adjusted for socio-demographic variables and tobacco and

alcohol consumption). These findings are in agreement with the results by Schwartz et al. (1998), who found an association between seropositivity for HPV 16 capsids and oral cancers (OR=2.3; 95%CI:1.6-3.3). The authors did not present subsite specific analysis, such as for patients with cancer of the mouth, tonsillar cancer, or other oropharyngeal cancers. Similar results were also found by Mork et al. (2001). Using a matched case-control study design and serum banks, the authors showed an OR for squamous cell carcinoma of the head and neck in subjects seropositive for HPV 16 of 2.2 (95%CI:1.4-3.4). Plasma samples were obtained several years before the diagnosis of the disease. The analysis of carcinomas of the tongue showed and OR of 2.7 (95%CI:1.2-6.6). It is not reported what proportion of these tumours had base of tongue as the primary site.

In summary, there is a positive epidemiological association between HPV infection and cancers of the oral cavity not related to the lingual and palatine tonsils, which was detected in this study with somewhat reduced precision. However, the biological evidence for a firm etiologic link is not well established. PCR's extreme sensitivity could detect latent infections not related to the tumour. A study by Gillison et al. (2000) showed that non tonsillar oral tumours positive for HPV by PCR were rarely positive by Southern blot *or in situ* hybridization, suggesting a lack of clonal association between the virus and cancer cells. Nevertheless, some reports have shown that the HPV 16 genome was integrated into cancer cells of the mouth, and that the viral genome was transcriptionally active (Steenbergen et al., 1995). This latter evidence, plus the association between HPV seropositivity and cancers of the mouth found in this and other studies, gives support to the idea that at least some squamous cell carcinomas not related to the tonsils are etiologically linked to HPV infection. However, given that the biological evidence is scarce, the proportion of cancers of the mouth linked to the virus is likely to be small.

6.2 CANCERS OF THE PALATINE TONSIL AND BASE OF TONGUE

The evidence linking HPV infection and cancers related to Waldeyers's ring is very strong in the present study. This ring comprises lymphatic tissue especially organized for a direct contact with antigens from external environment, hence its role in regional and general immune response. One of the first observations suggesting that tonsillar cancers may be etiologically linked to HPV was provided by Snijders et al. (1992) . The authors detected HPV DNA in all of the 10 biopsies of tonsillar carcinomas tested and in none of the seven biopsies of tonsillitis used as controls. Since then, several reports found that tonsillar cancers, or cancers of the oropharynx, presented a higher prevalence rate of HPV infection than other oral cancers (studies were summarized in table 5). Gillison et al. (2000) found that oropharyngeal tumours were six times more likely to harbour HPV DNA than other oral cancers (OR=6.2; 95%CI:3.1-12.1).

In the present investigation detection of HPV DNA was strongly associated with cancers of the palatine tonsil and base of tongue. After controlling for the potential confounding effect of socio-demographic factors, tobacco smoking, and alcohol drinking, the OR of disease for high risk HPV types was 19.32 (95%CI:2.3-159.5). Serological response to HPV 18 and HPV 31 were not strongly correlated with cancer. On the other hand, presence of antibodies against HPV 16 capsids was associated with a more than thirty-fold increase in risk compared to seronegatives, after adjusting for socio-demographic variables (OR=31.51; 95%CI:4.5-219.7). Further adjustment for tobacco and alcohol consumption did not decrease this estimate. When both detection of HPV DNA and seropositivity to HPV 16 are included in the model, both estimates decreased. This reduction is not surprising given the high correlation of both measurements. The decrease in the estimate for serology indicates that the serological response is mainly due to oral HPV infection and not to a genital infection. Moreover, this association did

not decrease after adjustment for markers of sexual activity, indicating that genital infections are not likely to explain the serological response to HPV 16. The fact that there is still a strong association between seropositivity and cancer after including detection of HPV DNA in the model may reflect that HPV was not detected in some samples, or that the viral genome was lost after having been involved in the development of the tumour ("hit and run" model). In any case, the results for both markers of HPV infection combined showed that serology supplemented detection of HPV DNA in the assessment of oral HPV infection.

To my knowledge, the only published study on HPV serology that focused specifically on the association with cancer of the oropharynx is the research by Mork et al. (2001). The analysis for this subsite showed an OR of 14.4 (95%CI:3.6-58.1). This magnitude of association is comparable to the one measured in the present project. The epidemiological evidence from this and other studies shows that the magnitude of association between HPV infection and squamous cell carcinomas related to the Waldeyer's ring is very strong. In addition to the epidemiological evidence, there is consistent biological evidence suggesting that HPV positive cancers arising from the palatine and lingual tonsils are a distinct entity etiologically linked to infection by high risk HPV types, especially HPV 16. Gillison et al. (2000) showed that HPV positive oropharyngeal cancers had predominantly a basaloid morphology, fewer p53 mutations, improved survival, and were less likely to be associated with alcohol drinking and perhaps tobacco smoking than HPV negative oropharyngeal cancers. Most HPV positive oropharyngeal cancers harboured HPV 16, which was localized by *in situ* hybridization within the nuclei of cancer cells, and Southern blot hybridization patterns were consistent with viral integration (Gillison et al., 2000). Furthermore, Van Houten et al. (2001) found that HPV positive oral tumors that were also positive for expression of the viral E6 gene

(E6 mRNA) lacked a p53 mutation. On the other hand, p53 mutations were very common among HPV negative tumours and HPV positive tumours that did not express the E6 gene. In agreement with this study, a recent report by Wiest et al. (2002) showed that among HPV positive tumours of the head and neck regions, most tumours of the oropharynx expressed the viral oncogenes E6 and E7 and presented the wild type p53, whereas most tumours arising form other oral regions presented a mutated p53 and did not express the viral oncogenes. Finally, on the clinical side, Gillison et al. (2000), Lindel et al. (2001), and Schwartz et al. (2001) have reported that patients with HPV positive oropharyngeal tumours have a better survival than patients with HPV negative cancers.

In summary, the results from this project added to other studies provide epidemiological evidence supporting a strong association between HPV infection and cancers of the oropharynx, especially those arising form the Waldeyer's ring. In recent years several studies have provided biological evidence supporting an etiological link between the virus and these malignancies.

6.3 Route of transmission for HPV Oral Infection

HPV appears to be a common comensal of the oral mucosa, although the source of viral infection remains uncertain. Oral HPV infections in newborns of infected mothers are rare (Watts et al., 1998), and infections through fomites cannot be discarded.

Despite the association between cervical cancer and subsequent head and neck cancers (Spitz et al., 1992; Frisch et al., 1999; Hemminki et al., 2000), the evidence for a sexual route of transmission is not consistent. Kellokoski et al. (1992) examined the presence of HPV DNA in biopsies of clinically normal buccal mucosa of women with genital HPV infections. Using Southern blot and PCR techniques they detected HPV in

15% and 29% of normal samples, respectively. The HPV types detected in the genital and oral mucosa of index patients differed in most cases. On the other hand Premoli-De-Percoco et al. (1998) found a strong correlation between HPV detection in oral squamous cell carcinomas and that in cervical smears. Using in situ hybridization for detection of the virus, they detected HPV DNA in 70% (35 of 50) of oral cancers and in 56% (28 of 50) of cervical smears. In 23 of the 28 patients who were positive for HPV sequences in the cervicovaginal tract, the same HPV type was found in the oral cavity.

Given the strong association between markers of sexual activity and cervical cancer, some studies have examined the relationship between sexual activity and oral carcinomas. Schwartz et al. (1998) found an increased risk of oral cancer among males with younger age at first intercourse, higher lifetime number of opposite sex partners, and prior diagnosis of genital warts. However, they did not find a relationship ever performing oral sex and lifetime number of oral sex partners. A second case-control study (Smith et al., 1998) failed to find any association between indicators of sexual behaviour and oral cancer: HPV related genital lesions, sexual oral behaviour, and lifetime number of sexual partners differed little between cases and controls. A third case-control study (Maden et al., 1992) found paradoxical results: while lifetime number of sexual partners was positively associated with oral cancer, ever practicing oral sex was shown to be protective of the disease (OR=0.4; 95%CI:0.2-0.8). The results from the present study concerning markers of sexual activity were not consistent with a clearcut role. Some markers seemed to be associated with oral cancer among males, such as past history of sexual intercourse with female prostitutes, personal antecedents of STDs, and having had sex with partners with STD. However, none of these associations were significant. On the other hand, indicators of oral sexual activity, such

as frequency and age at first oral sexual experience, were not associated with the oral malignancies.

6.4. Interaction between HPV and Other Factors

Another aspect of the role of HPV in oral cancers that needs further investigation is the potential synergism or independence of effects between the virus and tobacco and alcohol consumption, the main risk factors for oral cancer. Most of the evidence indicates that HPV linked tumours are more likely to occur in non smokers. Koch et al. (1999) found a marginally higher rate of HPV infection in head and neck cancers among non smokers compared to tobacco users. Fouret et al. (1997) analyzed the presence of HPV in head and neck tumours. The rate of viral detection among smokers was 8.5% (15 of 171), whereas among non smokers was 50% (5 of 10). Gillison et al. (2000) found that HPV positive oropharyngeal cancers were less likely to occur among moderate to heavy drinkers (OR of exposure = 0.17; 95%CI:0.05-0.61) and smokers (OR of exposure = 0.16; 95%CI:0.02-1.4) compared to HPV negative oropharyngeal cancers. Results from the present investigation do not show a clear interaction between HPV infection and tobacco or alcohol: despite the imprecise estimates, the ORs point more to a positive than to a negative effect modification.

6.5 Advantages and Limitations of the Present Study

One of the limitations of the present study is the relatively small number of patients with oral cancer who were recruited. Despite intense efforts to involve all major clinics in Montreal, the centre that treats the highest number of patients did not participate. The small sample size prevented the estimation of more precise parameters.

Another potential limitation, intrinsic to case-control studies in which exposure and outcome are assessed at the same point in time, is the uncertainty to confirm whether the exposure precedes the outcome. It could be argued that a reverse causality bias could partially explain the association between HPV detection and non tonsillar oral cancers. However, the positive association found between serology and the cancers of these subsites make this explanation less likely, since seropositivity is more a marker of past than recent infection. It is extremely unlikely that reverse causality bias could explain the association between HPV infection and cancers of the palatine and lingual tonsils. Not only is the magnitude of the association very high, but also the biological evidence supporting the carcinogenic effect of viral infection in these tumours is compelling.

The comparison between participating and non participating subjects showed that the latter were on average older than participants, and the proportion of females was higher. These differences are unlikely to affect the validity of the study because they would not have affected the distribution of HPV results between cases and controls to any appreciable extent. Furthermore, a selection bias is also unlikely, because participants and non participants were totally unaware of their HPV status.

The distribution of subjects according to socio-demographic characteristics, already presented in table 15, shows that cases and controls differed in some aspects: controls were more likely than cases to have more years of formal education, to speak English, to be Jewish, and less likely to be Catholic. These factors are associated with life style factors potentially linked to oral cancer, therefore there is the possibility of a selection bias. To prevent it, these variables were treated as potential confounders of the relationship between putative factors and oral cancer. Nevertheless, even if some bias

was introduced by the imbalance of socio-demographic variables between cases and controls, it is highly unlikely that these differences could explain the magnitude of association found between HPV and cancers, particularly the ones of palatine tonsil and base of tongue.

Ideally, population controls would reflect better than hospital controls the exposure experience in the study base. However, the use of population controls in this study would have had an important limitation concerning response rate given the need for clinical specimens. In addition, the quality of information given by hospital controls is more comparable to cases because they share the same environment and they have similar motivations to recall exposures. A typical concern with the use of hospital controls is the possibility that they may not represent the study base, therefore introducing the possibility of selection bias. The most important requisite for a control group is that it should reflect the population from which cases would arise: a subject is a member of the base only if he or she would be enrolled as a case if diagnosed with the disease during the recruitment time. It is reasonable to assume that in this study if a control had developed oral cancer he or she would have been recruited as a case, thus satisfying this requisite.

Selection bias is introduced when the sampling fractions unknowingly depend on an exposure variable. There is no bias (or confounding) when the selection probability depends on a factor that is unrelated to the exposure (Breslow and Day, 1980). In theory, if there is complete evidence that a single disease is unrelated to the exposure of interest, the whole control series may be selected from patients with that disease. However, inclusion of patients with several diseases minimizes potential bias if any of the control diseases is associated with the exposure (Breslow and Day, 1980).

6.5.1 Assessment of HPV infection

HPV infection was assessed using two markers: HPV DNA and serology. Individually, both markers showed similar association with the disease, whereas combined they supplemented each other.

The most commonly PCR based assays for detection of HPV DNA found in the literature are the GP5+/6+ system (de Roda Husman et al., 1995) and the MY09/11 system (Manos et al., 1989). The sensitivity and specificity of both systems are very similar. (Zehbe and Wilander, 1996; Zehbe and Wilander, 1997; Qu et al., 1997). The MY09/11 primers were redesigned into the PGMY09/11 primers (Gravitt et al., 2000) to increase the sensitivity of amplification across a broader spectrum of HPV types, and is considered an improvement of the previously described MY09/11 protocol. The performance of the PGMY09/11 primer system relative to that of the standard MY09/11 system was evaluated with a set of 262 cervicovaginal lavage specimens (Gravitt et al., 2000). There was a 91.5% overall agreement between the two systems. The PGMY09/11 system appeared to be significantly more sensitive than the MY09/11 system, detecting an additional 20 HPV-positive specimens, for a detection rate of 62.8% and 55.1%, respectively. The PGMY09/11 protocol has become the HPV detection method of choice in many ongoing international epidemiologic studies of HPV and cancer, not only because of its high sensitivity and specificity, but also due to the availability of a simple line blot system to type samples (Gravitt et al., 1998, Gravitt et al., 2000; Coutlee et al., 2002). The use of less sensitive techniques may miss focal HPV infections, especially in anatomic sites related to Waldeyer's ring, which would have introduced substantial misclassification of exposure status.

There is no gold standard to determine HPV infection, and there is not a perfect agreement between different detection techniques or within the same technique repeated over time. Therefore, it is likely that determination of HPV status by PCR based techniques include false positive and false negative results. Determination of HPV status in this study suffered from the same limitation. However, some of our results suggest that misclassification was not a major concern. Despite the use of different PCR protocols in different laboratories, and the different nature of biological materials, there was a very good agreement in the detection of HPV DNA between oral exfoliated cells and tumour biopsies. Furthermore, there was also a very good agreement between detection of HPV 16 DNA and serological response to HPV 16 capsids, despite the fact that both techniques were measuring different dimensions of the same infection (i.e., past *versus* present exposure) at least in most cases, and the combined analysis showed that both markers supplemented each other. Any HPV measurement error that could have existed in this study is of minor extent, and is more likely to be non differential than differential, therefore decreasing and not increasing the magnitude of association between HPV infection and oral cancer.

In summary, the association found in this investigation between HPV and oral cancer seems to be genuine. Selection bias and measurement error are an unlikely explanation for the association found between the viral infection and oral cancers, particularly tonsillar squamous cell carcinomas. The association seems to be largely independent form the influence of smoking and alcohol, the two established causal factors for oral cancers. In addition, the association was consistently detected using both PCR and serological techniques, which further supports its validity.

7. HIGHLIGHTS AND CONCLUSIONS

The evidence produced by this study, in addition to epidemiological and biological evidence from other studies, strongly support an etiological role for HPV infection in the development of a substantial proportion of oropharyngeal squamous cell carcinomas, especially those tumours related to Waldeyer's ring. The magnitude of association between the viral infection and tonsillar tumours is comparable to that of smoking and head and neck cancers.

The route of transmission for oral HPV infection remains controversial. Some evidence points to sexual behaviour as the main route, though the association with oral cancer is rather weak, and much lower than the association between markers of sexual activity and cervical cancer.

Several other gaps in knowledge remain, such as the potential interaction between HPV and certain genetic polymorphisms, as well as interaction with other factors such as tobacco smoking and alcohol drinking. Also remains to be investigated the proportion of tonsillar cancers etiologically linked to the virus across different populations.

Epidemiological and biological future research should focus in the natural history of oral HPV infection, to examine which infections are likely to be transient and clinically irrelevant, and which infections are more likely to be involved in the development of premalignant and malignant lesions. Biological research should help us to better understand the molecular mechanisms involved in the carcinogenic process.

The evidence linking HPV infection as a determinant of oral cancers not related to Waldeyer's ring is far from conclusive. Several studies, including this project, have found a relatively weak positive association between the virus and these tumours. Most of these studies are cross-sectional (including case-control studies), in which HPV exposure is assessed concomitantly with the outcome, a circumstance that cannot rule out that the viral infection is more a consequence than a determinant of the disease. In addition, the biological evidence is limited, compared to that for oropharyngeal tumours. Nevertheless, it is possible that a proportion of non tonsillar oral cancers is etiologically linked to HPV, although this proportion is much lower than for carcinomas of the palatine and lingual tonsils.

Future research should focus in the natural history of HPV infection and the development of premalignant and malignant lesions of the mouth, to assess which are the infections that precede the disease and which ones are associated with its progression.

The prospect of therapeutic and/or preventive HPV vaccines which are currently being developed for cervical cancer eventually may be used for prevention of an unknown proportion of oral tumours. However "HPV is clearly neither *necessary* nor *sufficient* for all tumor production and it must be remembered that much oral squamous cell carcinoma is induced by the known risk habits involving tobacco and alcohol" (Scully, 2002).

8. REFERENCES

Adams V, Schmid S, Zariwala M, Schmid M, Kleihues P, Briner J, Schafer R. Prevalence of human papilloma virus DNA in head and neck cancers carrying wild-type or mutant p53 tumor suppressor genes. Anticancer Res 19:1-6, 1999.

af Geijersstam V, Wang Z, Lewensohn-Fuchs I, Schiller JT, Forsgren M, Dillner J. Trends in seroprevalence of Human Papillomavirus type 16 among pregnant women in Stockholm, Sweden, during 1969-1989. Int J Cancer 76:341-344, 1998.

Aggelopoulou EP, Skarlos D, Papadimitriou C, Kittas C, Troungos C. Human papilloma virus DNA detection in oral lesions in the Greek population. Anticancer Res 19:1391-1395, 1999.

Ahmed S, Jafarey NA. Association of herpes simplex virus type-I and human papilloma virus with carcinoma of the oral cavity and oropharynx. J Environ Pathol Toxicol Oncol 14:193-196, 1995.

Alvarez Alvarez I, Sanchez Lazo P, Ramos Gonzalez S, Rodrigo Tapia JP, Nunez Batalla F, Suarez Nieto C. Using polymerase chain reaction to human papillomavirus in oral and pharyngolaryngeal carcinomas. Am J Otolaryngol 18:375-381, 1997.

Almeida JD, Howatson AF, Williams MG. Electron microscope study of human warts: Sites of virus production and nature of inclusion bodies. J Invest Dermatol 38:337-345, 1962.

Anderson JA, Irish JC, McLachlin CM, Ngan BY. H-ras oncogene mutation and human papillomavirus infection in oral carcinomas. Arch Otolaryngol Head Neck Surg 120:755-760, 1994.

Andl T, Kahn T, Pfuhl A, Nicola T, Erber R, Conradt C, Klein W, Helbig M, Dietz A, Weidauer H, Bosch FX. Etiological involvement of oncogenic human papillomavirus in tonsillar squamous cell carcinomas lacking retinoblastoma cell cycle control. Cancer Res 58:5-13, 1998.

Atula S, Auvinen E, Grenman R, Syrjanen S. Human papillomavirus and Epstein-Barr virus in epithelial carcinomas of the head and neck region. Anticancer Res 17:4427-4433, 1997.

Badaracco G, Venuti A, Bartolazzi A, Morello R, Marzetti F, Marcante ML. Overexpression of p53 and bcl-2 proteins and the presence of HPV infection are independent events in head and neck cancer. J Oral Pathol 29:173-179, 2000a.

Badaracco G, Venuti A, Morello R, Muller A, Marcante ML. Human papillomavirus in head and neck carcinomas: prevalence, physical status and relationship with clinical/pathological parameters. Anticancer Res 20:1301-1305, 2000b.

Balaram P, Nalinakumari KR, Abraham E, Balan A, Hareendran NK, Bernard HU, Chan SY. Human papillomaviruses in 91 oral cancers from Indian betel quid chewers—high prevalence and multiplicity of infections. Int J Cancer 61:450-454, 1995.

Barten M, Ostwald C, Milde-Langosch K, Muller P, Wukasch Y, Loning T. HPV DNA and p53 alterations in oropharyngeal carcinomas. Virchows Arch 427:153-157, 1995.

Bauer HM, Ting Y, Greer CE, Chambers JC, Tashiro CJ, Chimera J, Reingold A, Manos MM. Genital human papillomavirus infection in female university students as determined by a PCR-based method. J Am Med Assoc 265:472-477, 1991.

Bjorge T, Hakulinen T, Engeland A, Jellum E, Koskela P, Lehtinen M, Luostarinen T, Paavonen J, Sapp M, Schiller J, Thoresen S, Wang Z, Youngman L, Dillner J. A prospective, seroepidemiological study of the role of human papillomavirus in esophageal cancer in Norway. Cancer Res 57:3989-3992, 1997.

Blot WJ, McLaughlin JK, Winn DM, Austin DF, Greenberg RS, Preston-Martin S, Bernstein L, Schoenberg JB, Stemhagen A, Fraumeni JF Jr. Smoking and drinking in relation to oral and pharyngeal cancer. Cancer Res 48:3282-3287, 1988.

Blot WJ, Devesa SS, McLaughlin JK, Fraumeni JF, Jr. Oral and pharyngeal cancers. Cancer Surveys 19-20: 23-42, 1994.

Bosch FX, Manos MM, Muñoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 87:796-802, 1995.

Bouda M, Gorgoulis VG, Kastrinakis NG, Giannoudis A, Tsoli E, Danassi-Afentaki D, Foukas P, Kyroudi A, Laskaris G, Herrington CS, Kittas C. "High risk" HPV types are frequently detected in potentially malignant and malignant oral lesions, but not in normal oral mucosa. Mod Pathol 13:644-653, 2000.

Brandsma JL, Abramson AL. Association of papillomavirus with cancers of the head and neck. Arch Otolaryngol Head Neck Surg 115:621-625, 1989.

Brandwein M, Zeitlin J, Nuovo GJ, MacConnell P, Bodian C, Urken M, Biller H. HPV detection using "hot start" polymerase chain reaction in patients with oral cancer: a clinicopathological study of 64 patients. Mod Pathol 7:720-727, 1994.

Breslow NE, Day NE. Statistical methods in cancer research. Vol. I, The analysis of case-control studies. IARC Scientific Publications No 32, International Agency for Research on Cancer, Lyon, 1980.

Cann CI, Fried MP, Rothman KJ. Epidemiology of squamous cell cancer of head and neck. Otolaryngol Clin North Am 18:367-388, 1985.

Cerovac Z, Sarcevic B, Kralj Z, Ban J. Detection of human papillomavirus (HPV) type 6, 16 and 18 in head and neck squamous cell carcinomas by in situ hybridization. Neoplasma 43:185-194, 1996.

Chang KW, Chang CS, Lai KS, Chou MJ, Choo KB. High prevalence of human papillomavirus infection and possible association with betel quid chewing and smoking in oral epidermoid carcinomas in Taiwan. J Med Virol 28:57-61, 1989.

Chang F, Syrjanen S, Nuutinen J, Karja J, Syrjanen K. Detection of human papillomavirus (HPV) DNA in oral squamous cell carcinomas by in situ hybridization and polymerase chain reaction. Arch Dermatol Res 282:493-497, 1990.

Chang F, Syrjanen S, Kellokoski J, Syrjanen K. Human papillomavirus (HPV) infections and their associations with oral disease. J Oral Pathol Med 20:305-317, 1991.

Chen JK, Katz RV, Krutchkoff DJ. Intraoral squamous cell carcinoma. Epidemiologic patterns in Connecticut from 1935 to 1985. Cancer 1990; 66:1288-1296, 1990.

Chen JK, Eisenberg E, Krutchkoff DJ, Katz RV. Changing trends in oral cancer in the United States, 1935 to 1985: a Connecticut study. J Oral Maxillofac Surg 49:1152-1158, 1991.

Chiba I, Shindoh M, Yasuda M, Yamazaki Y, Amemiya A, Sato Y, Fujinaga K, Notani K, Fukuda H. Mutations in the p53 gene and human papillomavirus infection as significant prognostic factors in squamous cell carcinomas of the oral cavity. Oncogene 12:1663-1668, 1996.

Cook JC, Joyce JG, George HA, Schultz LD, Hurni WM, Jansen KU, Hepler RW, Ip C, Lowe RS, Keller PM, Lehman ED. Purification of virus-like particles of recombinant human papillomavirus type 11 major capsid protein L1 from Saccharomyces cerevisiae. Protein Expr Purif 17:477-484, 1999.

Copper MP, Jovanovic A, Nauta JJ, Braakhuis BJ, de Vries N, van der Waal I, Snow GB. Role of genetic factors in the etiology of squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 121:157-160, 1995.

Coutlee F, Hankins C, Lapointe N. Comparison between vaginal tampon and cervicovaginal lavage specimen collection for detection of human papillomavirus DNA by the polymerase chain reaction. The Canadian Women's HIV Study Group. J Med Virol 51:42-47, 1997a.

Coutlee F, Trottier AM, Ghattas G, Leduc R, Toma E, Sanche G, Rodrigues I, Turmel B, Allaire G, Ghadirian P. Risk factors for oral human papillomavirus in adults infected and not infected with human immunodeficiency virus. Sex Transm Dis 24:23-31, 1997b.

Coutlee F, Gravitt P, Richardson H, Hankins C, Franco E, Lapointe N, Voyer H. Nonisotopic detection and typing of human papillomavirus DNA in genital samples by the line blot assay. The Canadian Women's HIV study group. J Clin Microbiol 1999; 37:1852-1857, 1999.

Coutlee F, Gravitt P, Kornegay J, Hankins C, Richardson H, Lapointe N, Voyer H, Franco E. Use of PGMY primers in L1 consensus PCR improves detection of human papillomavirus DNA in genital samples. J Clin Microbiol 40:902-907, 2002.

Cruz IB, Snijders PJ, Steenbergen RD, Meijer CJ, Snow GB, Walboomers JM, van der Waal I. Age-dependence of human papillomavirus DNA presence in oral squamous cell carcinomas. Eur J Cancer B Oral Oncol 32B:55-62, 1996.

D'Costa J, Saranath D, Dedhia P, Sanghvi V, Mehta AR. Detection of HPV-16 genome in human oral cancers and potentially malignant lesions from India. Oral Oncol 34:413-420, 1998.

De Luca L, Maestri N, Bonnani F, Nelson D. Maintenance of epithelial cell differentiation: the mode of action of Vitamin A. Cancer 30:1326-1331, 1972.

de Roda Husman AM, Walboomers JMM, van den Brule AJC, Meijer CJLM, Snijders PJF. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 76:1057-1062, 1995.

De Stefani E, Correa P, Oreggia F, Deneo-Pellegrini H, Fernandez G, Zavala D, Carzoglio J, Leiva J, Fontham E, Rivero S. Black tobacco, wine and mate in oropharyngeal cancer. Rev Epidemiol Sante Publique 36:389-394, 1988.

de Villiers EM, Weidauer H, Otto H, zur Hausen H. Papillomavirus DNA in human tongue carcinomas. Int J Cancer 36:575-578, 1985.

de Villiers EM, Lavergne D, Chang F, Syrjanen K, Tosi P, Cintorino M, Santopietro R, Syrjanen S. An interlaboratory study to determine the presence of human papillomavirus DNA in esophageal carcinoma from China. Int J Cancer 81:225-228, 1999.

Dietz A, Senneweld E, Maier H. Indoor air pollution by emisions of fossil fuels single stoves: Possibly a hitherto underrated risk factor in the development of carcinomas in the head and neck. Otolaryngol Head Neck Surg 112:308-315, 1995.

Dillner J. The serological response to papillomaviruses. Semin Cancer Biol:9:423-430, 1999

Dillner J, Wiklund F, Lenner P, Eklund C, Frederiksson-Shanazarian V, Schiller JT, Hibma M, Hallmans G, Stendahl U. Antibodies against linear and conformational epitopes of human papillomavirus type 16 that independently associate with incident cervical cancer. Int J Cancer 60:377-382, 1995a.

Dillner J, Knekt P, Schiller JT, Hakulinen T. Prospective seroepidemiological evidence that human papillomavirus type 16 infection is a risk factor for oesophageal squamous cell carcinoma. BMJ 311:1346, 1995b.

Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. J Natl Cancer Inst 66:1191-1308, 1981.

Donofrio V, Lo Muzio L, Mignogna MD, Troncone G, Staibano S, Boscaino A, De Rosa G. Prognostic evaluation of HPV-associated precancerous and microinvasive carcinoma of the oral cavity: combined use of nucleolar organiser regions (AgNOR) and proliferating cell nuclear antigen (PCNA). Eur J Cancer B Oral Oncol 31B:174-180, 1995.

Dyson N, Howley PM, Münger K, Harlow E. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243: 934-937, 1989.

Elamin F, Steingrimsdottir H, Wanakulasuriya S, Johnson N, Tavassoli M. Prevalence of human papillomavirus infection in premalignant and malignant lesions of the oral cavity in U.K. subjects: a novel method of detection. Oral Oncol 34:191-197, 1998.

Elwood JM, Pearson JC, Skippen DH, Jackson SM. Alcohol, smoking, social and occupational factors in the aetiology of cancer of the oral cavity, pharynx and larynx. Int J Cancer 34:603-612, 1984.

Foulkes W, Brunet JS, Kowalski LP, Narod SA, Franco EL. Family history of cancer is a risk factor for squamous cell carcinoma of the head and neck in Brazil: a case-control study. Int J Cancer 63:769-773, 1995.

Foulkes W, Brunet JS, Sieh W, Black MJ, Shenouda G, Narod SA. Familial risks of squamous cell carcinoma of the head and neck: retrospective case-control study. BMJ 313:716-721, 1996.

Fouret P, Monceaux G, Temam S, Lacourreye L, St Guily JL. Human papillomavirus in head and neck squamous cell carcinomas in nonsmokers. Arch Otolaryngol Head Neck Surg 123:513-516, 1997.

Franceschi S, Talamini R, Barra S, Baron AE, Negri E, Bidoli E, Serraino D, La Vecchia C. Smoking and drinking in relation to cancers of the oral cavity, pharynx, larynx, and esophagus in northern Italy. Cancer Res 50:6502-6507, 1990.

Franceschi S, Bidoli E, Barón AE, Barra S, Talamini R, Serraino D, La Vecchia C. Nutrition and cancer of the oral cavity and pharynx in north-east Italy. Int J Cancer 47:20-25, 1991.

Franceschi S, Munoz N, Bosch XF, Snijders PJ, Walboomers JM. Human papillomavirus and cancers of the upper aerodigestive tract: a review of epidemiological and experimental evidence. Cancer Epidemiol Biomarkers Prev 5:567-575, 1996.

Franco EL, Kowalski LP, Oliveira BV, Curado MP, Pereira RN, Silva ME, Fava AS, Torloni H. Risk factors for oral cancer in Brazil: a case-control study. Int J Cancer 43:992-1000, 1989.

Franco EL, Kowalski LP, Kanda JL. Risk factors for second cancers of the upper respiratory and digestive systems: a case-control study. J Clin Epidemiol 44:615-625, 1991.

Franco EL. Epidemiology of anogenital warts and Cancer. Obstet Gynecol Clin North Am 23:597-623, 1996.

Fraumeni JF Jr. Epidemiological opportunities in alcohol-related cancer. Cancer Res 39:2851-2852, 1979.

Frazer IH, Leonard JH, Schonrock J, Wright RG, Kearsley JH. HPV DNA in oropharyngeal squamous cell cancers: comparison of results from four DNA detection methods. Pathology 25:138-143, 1993.

Frisch M, Biggar RJ. Aetiological parallel between tonsillar and anogenital squamous-cell carcinomas. Lancet 354:1442-1433, 1999.

Garewal H. Chemoprevention of oral cancer: beta-carotene and vitamin E in leukoplakia. Eur J Cancer Prev 3:101-107, 1994.

Gassenmaier A, Hornstein OP. Presence of human papillomavirus DNA in benign and precancerous oral leukoplakias and squamous cell carcinomas. Dermatologica 176:224-233, 1988.

Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 92:709-720, 2000.

Goldstein AM, Blot WJ, Greenberg RS, Schoenberg JB, Austin DF, Preston-Martin S, Winn DM, Bernstein L, McLaughlin JK, Fraumeni JF Jr. Familial risk in oral and pharyngeal cancer. Eur J Cancer B Oral Oncol 30B:319-322, 1994.

Gorsky M, Silverman S Jr. Denture wearing and oral cancer. J Prosthet Dent 52:164-166, 1984.

Graham S, Dayal H, Rohrer J, Swanson M, Sultz H, Shedd D, Fischman S. Dentition, diet, tobacco and alcohol in the epidemiology of oral cancer. J Natl Cancer Inst 59:1611-1618, 1977.

Gravitt PE, Peyton CL, Apple RJ, Wheeler CM. Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. J Clin Microbiol 36:3020-3027, 1998.

Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlee F, Hildesheim A, Schiffman MH, Scott DR, Apple RJ. Improved amplification of genital human papillomaviruses. J Clin Microbiol 38:357-361, 2000.

Greer RO, Eversole LR, Crosby LK. Detection of human papillomavirus-genomic DNA in oral epithelial dysplasias, oral smokeless tobacco-associated leukoplakias, and epithelial malignancies. J Oral Maxillofac Surg 48:1201-1205, 1990.

Hamel N, Black MJ, Ghadirian P, Foulkes WD. No association between P53 codon 72 polymorphism and risk of squamous cell carcinoma of the head and neck. Br J Cancer 82:757-759, 2000.

Han C, Qiao G, Hubbert NL, Li L, Sun C, Wang Y, Yan M, Xu D, Li Y, Lowy DR, Schiller JT. Serologic association between human papillomavirus type 16 infection and esophageal cancer in Shaanxi Province, China. J Natl Cancer Inst 88:1467-1471, 1996.

Haraf DJ, Nodzenski E, Brachman D, Mick R, Montag A, Graves D, Vokes EE, Weichselbaum RR. Human papilloma virus and p53 in head and neck cancer: clinical correlates and survival. Clin Cancer Res 2:755-762, 1996.

Hemminki K, Dong C, Frisch M. Tonsillar and other upper aerodigestive tract cancers among cervical cancer patients and their husbands. Eur J Cancer Prev 9:433-437, 2000.

Herrero R, Muñoz N, Franceschi S, Castellsagué X, Meijers CJL, Walboomers JMM, Snijders P, IARC International Oral Cancer Study Group. International case-control study of HPV and cancer of the oral cavity and pharynx. 18th International Papillomavirus Conference; July 23-28, p.184. Barcelona, Spain, 2000.

Hindle I, Downer MC, Speight PM. The association between intra-oral cancer and surrogate markers of smoking and alcohol consumption. Community Dent Health 17:107-113, 2000.

Holladay EB, Gerald WL. Viral gene detection in oral neoplasms using the polymerase chain reaction. 517: Am J Clin Pathol 100:36-40, 1993.

Hoffmann M, Kahn T, Mahnke CG, Goeroegh T, Lippert BM, Werner JA. Prevalence of human papillomavirus in squamous cell carcinoma of the head and neck determined by polymerase chain reaction and Southern blot hybridization: proposal for optimized diagnostic requirements. Acta Otolaryngol 118:138-144, 1998.

Howell RE, Danforth FA, Bray BA, Mathews FR. Human cervical and hamster oral epithelial neoplasia: colposcopic similarities. Gynecol Oncol 24:17-22, 1986.

Howell RE, Gallant L. Human papillomavirus type 16 in an oral squamous carcinoma and its metastasis. Oral Surg Oral Med Oral Pathol 74:620-626, 1992.

IARC - International Agency for Research on Cancer. IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. Tobacco habits other than smoking: betel-quid and areca-nut chewing; and some related nitrosamines. Vol 37. International Agency for Research on Cancer, Lyon, France, 1985.

IARC - International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks of chemicals to humans. Tobacco Smoking. Vol 38. International Agency for Research on Cancer, Lyon, France, 1986.

IARC - International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks of chemicals to humans. Alcohol Drinking. Vol 44. International Agency for Research on Cancer, Lyon, France, 1988.

IARC - International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks of chemicals to humans. Human papillomaviruses. vol 64, International Agency for Research on Cancer, Lyon, France, 1995.

Ibrahim SO, Bertelsen B, Kalvenes MB, Idris AM, Vasstrand EN, Nilsen R, Johannessen AC. Expression of keratin 13, 14 and 19 in oral squamous cell carcinomas from Sudanese snuff dippers: lack of association with human papillomavirus infection. 156: APMIS 106:959-969, 1998.

Jacobs CD. Etiologic considerations for head and neck squamous cancers. In: Jacobs C (ed.). Carcinomas of the Head and Neck: Evaluation and Management. Kluwer Academic Publishers, Boston, pp 265-282, 1990.

Jefferies S, Foulkes WD. Genetic mechanisms in squamous cell carcinoma of the head and neck. Oral Oncol 37:115-126, 2001.

Jensen OM, Esteve J, Moller H, Renard H. Cancer in the European Community and its member states. Eur J Cancer 26:1167-1256, 1990.

Kabat GC, Hebert JR, Wynder EL. Risk factors for oral cancer in women. Cancer Res 49:2803-2806, 1989.

Katoh T, Kaneko S, Kohshi K, Munaka M, Kitagawa K, Kunugita N, Ikemura K, Kawamoto T. Genetic polymorphisms of tobacco- and alcohol-related metabolizing enzymes and oral cavity cancer. Int J Cancer 83:606-609, 1999.

Kellokoski JK, Syrjanen SM, Chang F, Yliskoski M, Syrjanen KJ. Southern blot hybridization and PCR in detection of oral human papillomavirus (HPV) infections in women with genital HPV infections. J Oral Pathol Med 21:459-464, 1992.

Kirnbauer R, Taub J, Greenstone H, Roden R, Durst M, Gissmann L, Lowy DR, Schiller JT. Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. J Virol 67:6929-6936, 1993.

Kirnbauer R, Hubbert NL, Wheeler CM, Becker TM, Lowy DR, Schiller JT. A virus-like particle ELISA detects serum antibodies in a majority of women infected with human papillomavirus type 16. J Natl Cancer Inst 86:494-499, 1994.

Kiyabu MT, Shibata D, Arnheim N, Martin WJ, Fitzgibbons PL. Detection of human papillomavirus in formalin-fixed, invasive squamous carcinomas using the polymerase chain reaction. Am J Surg Pathol 13:221-224, 1989.

Koch WM, Lango M, Sewell D, Zahurak M, Sidransky D. Head and neck cancer in nonsmokers: a distinct clinical and molecular entity. Laryngoscope 109:1544-1551, 1999.

Koutsky LA, Holmes KK, Critchlow CW, Stevens CE, Paavonen J, Beckman AM, DeRouen TA, Galloway DA, Vernon D, Kiviat NB. A cohort study of the risk of cervical intraepithelial neoplasia grade 2 or 3 in relation ro papillomavirus infection. N Eng J Med 327:1272-1278, 1992.

Lagergren J, Wang Z, Bergstrom R, Dillner J, Nyren O. Human papillomavirus infection and esophageal cancer: a nationwide seroepidemiologic case-control study in Sweden. J Natl Cancer Inst 91:156-162, 1999.

La Vecchia C, Lucchini F, Negri E, Boyle P, Maisonneuve P, Levi F. Trends of cancer mortality in Europe, 1955-1989: I. Digestive sites. Eur J Cancer 28:132-235, 1992.

La Vecchia C, Tavani A, Franceschi S, Levi F, Corrao G, Negri E. Epidemiology and prevention of oral cancer. Oral Oncol 33:302-312, 1997.

Lawton GM, Thomas SJ, Schonrock J, Monsour FN, Frazer IH. Prevalence of genital human papillomaviruses in normal oral mucosa: a comparison of methods for sample collection. J Oral Pathol Med 21:265-269, 1992.

Lee NK, Ye YW, Chen J, Li X, Waber PG, Nisen PD. p53, retinoblastoma, and human papillomavirus in squamous cell carcinoma and adjacent normal mucosa of the upper aerodigestive tract. Arch Otolaryngol Head Neck Surg 119:1125-1131, 1993.

Lewensohn-Fuchs I, Munck-Wikland E, Berke Z, Magnusson KP, Pallesen G, Auer G, Lindholm J, Linde A, Aberg B, Rubio C. Involvement of aberrant p53 expression and human papillomavirus in carcinoma of the head, neck and esophagus. Anticancer Res 1994 14:1281-1285, 1994.

Lind PO, Syrjanen SM, Syrjanen KJ, Koppang HS, Aas E. Local immunoreactivity and human papillomavirus (HPV) in oral precancer and cancer lesions. Scand J Dent Res 1986 94:419-426, 1986.

Lindel K, Beer KT, Laissue J, Greiner RH, Aebersold DM. Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. Cancer 92:805-813, 2001.

Loning T, Ikenberg H, Becker J, Gissmann L, Hoepfer I, zur Hausen H. Analysis of oral papillomas, leukoplakias, and invasive carcinomas for human papillomavirus type related DNA. J Invest Dermatol 84:417-420, 1985.

Loning T, Meichsner M, Milde-Langosch K, Hinze H, Orlt I, Hormann K, Sesterhenn K, Becker J, Reichart P. HPV DNA detection in tumours of the head and neck: a comparative light microscopy and DNA hybridization study. ORL J Otorhinolaryngol Relat Spec 49:259-269, 1987.

Lorincz AT. Hybrid Capture method for detection of human papillomavirus DNA in clinical specimens: a tool for clinical management of equivocal Pap smears and for population screening. J Obstet Gynaecol Res 22:629-636, 1996.

Maden C, Beckmann AM, Thomas DB, McKnight B, Sherman KJ, Ashley RL, Corey L, Daling JR. Human papillomaviruses, herpes simplex viruses, and the risk of oral cancer in men. Am J Epidemiol 135:1093-1102, 1992.

Makni H, Franco EL, Kaiano J, Villa LL, Labrecque S, Dudley R, Storey A, Matlashewski G. P53 polymorphism in codon 72 and risk of human papillomavirus-induced cervical cancer: effect of inter-laboratory variation. Int J Cancer 87:528-533, 2000.

Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. Use of polymerase chain reaction amplificationfor the detection of genital human papillomaviruses. Cancer cells 7:209-214, 1989.

Mao EJ. Prevalence of human papillomavirus 16 and nucleolar organizer region counts in oral exfoliated cells from normal and malignant epithelia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 80:320-329, 1995.

Mao EJ, Schwartz SM, Daling JR, Oda D, Tickman L, Beckmann AM. Human papilloma viruses and p53 mutations in normal pre-malignant and malignant oral epithelia. Int J Cancer 69:152-158, 1996.

Mashberg A, Garfinkel L, Harris S. Alcohol as a primary risk factor in oral squamous carcinoma. CA Cancer J Clin 31:146-155, 1981.

Matlashewski G, Banks L, Pim D, Crawford L. Analysis of human p53 proteins and mRNA levels in normal and transformed cells. Eur J Biochem 154:665-672, 1986.

Matzow T, Boysen M, Kalantari M, Johansson B, Hagmar B. Low detection rate of HPV in oral and laryngeal carcinomas. Acta Oncol 37:73-76, 1998.

McKaig RG, Baric RS, Olshan AF. Human papillomavirus and head and neck cancer: epidemiology and molecular biology. Head Neck 20:250-65, 1998.

McLaughlin JK, Gridley G, Block G, Winn DM, Preston-Martin S, Schoenberg JB, Greenberg RS, Stemhagen A, Austin DF, Ershow AG, Blot WJ, Fraumeni JF Jr. Dietary factors in oral and pharyngeal cancer. J Nat Cancer Inst 80:1237-1243, 1988.

Meisels A, Fortin R. Condylomatous lesions of the cervix and vagina. I. Cytologic Paterns. Acta Cytol 20:505-509, 1976.

Mellin H, Friesland S, Lewensohn R, Dalianis T, Munck-Wikland E. Human papillomavirus (HPV) DNA in tonsillar cancer: clinical correlates, risk of relapse, and survival. Int J Cancer 89:300-304, 2000.

Merletti F, Boffetta P, Ciccone G, Mashberg A, Terracini B. Role of tobacco and alcoholic beverages in the etiology of cancer of the oral cavity/oropharynx in Torino, Italy. Cancer Res 49:4919-4924, 1989.

Miguel RE, Villa LL, Cordeiro AC, Prado JC, Sobrinho JS, Kowalski LP. Low prevalence of human papillomavirus in a geographic region with a high incidence of head and neck cancer. Am J Surg 176:428-429, 1998.

Miller CS, Zeuss MS, White DK. Detection of HPV DNA in oral carcinoma using polymerase chain reaction together with in situ hybridization. Oral Surg Oral Med Oral Pathol 77:480-486, 1994.

Miller CS, White DK. Human papillomavirus expression in oral mucosa, premalignant conditions, and squamous cell carcinoma: a retrospective review of the literature. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 82:57-68, 1996.

Miller CS, Johnstone BM. Human papillomavirus as a risk factor for oral squamous cell carcinoma: a meta-analysis, 1982-1997. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 91:622-635, 2001.

Mineta H, Ogino T, Amano HM, Ohkawa Y, Araki K, Takebayashi S, Miura K. Human papilloma virus (HPV) type 16 and 18 detected in head and neck squamous cell carcinoma. Anticancer Res 18:4765-4768, 1998.

Mork J, Lie AK, Glattre E, Hallmans G, Jellum E, Koskela P, Moller B, Pukkala E, Schiller JT, Youngman L, Lehtinen M, Dillner J. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. N Engl J Med 344:1125-1131, 2001.

Muir C, Weiland L. Upper aerodigestive tract cancers. Cancer 75:147S-153S, 1995.

Münger K, Phelps WC. The human papillomavirus E7 protein as a transforming and transactivating factor. Biochim Biophys Acta 1155:111-123, 1993

Muñoz N, Bosch FX, De Sanjose S, Tafur L, Izarzugaza I, Gili M, Viladiu P, Navarro C, Martos C, Ascunce N, Gonzalez LC, Kaldor JM, Guerrero E, Lorincz A, Santamaria M, Deruiz PA, Ariztizabal N, Shah K. The causal link between human papillomavirus and invasive cervical cancer: A population-base case-control study in Colombia and Spain. Int J Cancer 52:743-749, 1992.

NCIC - National Cancer Institute of Canada. Canadian Cancer Statistics 1995. Toronto, Canada, 1995.

NCIC - National Cancer Institute of Canada. Canadian Cancer Statistics 1998. Toronto, Canada, 1998.

Negri E, La Vecchia C, Franceschi S, Tavani A. Attributable risk for oral cancer in northern Italy. Cancer Epidemiol Biomarkers Prev 2:189-193, 1993.

Ng SK, Kabat GC, Wynder EL. Oral cavity cancer in non-users of tobacco. J Natl Cancer Inst 85:743-745, 1993.

Niedobitek G, Pitteroff S, Herbst H, Shepherd P, Finn T, Anagnostopoulos I, Stein H. Detection of human papillomavirus type 16 DNA in carcinomas of the palatine tonsil. J Clin Pathol 43:918-921, 1990.

Nielsen H, Norrild B, Vedtofte P, Praetorius F, Reibel J, Holmstrup P. Human papillomavirus in oral premalignant lesions. Eur J Cancer B Oral Oncol 32B:264-270, 1996.

Nishioka S, Fukushima K, Nishizaki K, Gunduz M, Tominaga S, Fukazawa M, Monden N, Watanabe S, Masuda Y, Ogura H. Human papillomavirus as a risk factor for head and neck cancers--a case-control study. Acta Otolaryngol Suppl 1999;540:77-80.

Niv A, Sion-Vardi N, Gatot A, Nash M, Fliss DM. Identification and typing of human papillomavirus (HPV) in squamous cell carcinoma of the oral cavity and oropharynx. J Laryngol Otol 114:41-46, 2000.

Nonnenmacher B, Kruger Kjaer S, Svare EI, Scott JD, Hubbert NL, van den Brule AJ, Kirnbauer R, Walboomers JM, Lowy DR, Schiller JT. Seroreactivity to HPV16 virus-like

particles as a marker for cervical cancer risk in high-risk populations. Int J Cancer 68:704-709, 1996.

Ogura H, Watanabe S, Fukushima K, Masuda Y, Fujiwara T, Yabe Y. Presence of human papillomavirus type 18 DNA in a pharyngeal and a laryngeal carcinoma. Jpn J Cancer Res 82:1184-1186, 1991.

Ogura H, Watanabe S, Fukushima K, Masuda Y, Fujiwara T, Yabe Y. Human papillomavirus DNA in squamous cell carcinomas of the respiratory and upper digestive tracts. Jpn J Clin Oncol 23:221-225, 1993.

Ostman J, Anneroth G, Gustafsson H, Tavelin B. Malignant oral tumours in Sweden 1960-1989--an epidemiological study. Eur J Cancer B Oral Oncol 31B:106-112, 1995.

Ostwald C, Muller P, Barten M, Rutsatz K, Sonnenburg M, Milde-Langosch K, Loning T. Human papillomavirus DNA in oral squamous cell carcinomas and normal mucosa. J Oral Pathol Med 23:220-225, 1994.

Parkin DM, Whelan SL, Ferlay J, Raymond L, Young J. Cancer incidence in five continents Vol. VII. IARC Scientific Publications No.143, International Agency for Research on Cancer, Lyon, 1997.

Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. Int J Cancer 80:827-841, 1999.

Paz IB, Cook N, Odom-Maryon T, Xie Y, Wilczynski SP. Human papillomavirus (HPV) in head and neck cancer. An association of HPV 16 with squamous cell carcinoma of Waldeyer's tonsillar ring. Cancer 1997 79:595-604, 1997.

Penhallow J, Steingrimsdottir H, Elamin F, Warnakulasuriya S, Farzaneh F, Johnson N, Tavassoli M. p53 alterations and HPV infections are common in oral SCC: p53 gene mutations correlate with the absence of HPV 16-E6 DNA. Int J Oncol 12:59-68, 1998.

Percy C, Van Holten V, Muir C. ICD-O: International Classification of Diseases for Oncology, Second Edition. World Health Organization, Geneva, 1990.

Peyton CL, Schiffman M, Lorincz AT, Hunt WC, Mielzynska I, Bratti C, Eaton S, Hildesheim A, Morera LA, Rodriguez AC, Herrero R, Sherman ME, Wheeler CM. Comparison of PCR- and hybrid capture-based human papillomavirus detection systems using multiple cervical specimen collection strategies. J Clin Microbiol 36:3248-3254, 1998.

Pintos J, Franco EL, Oliveira B, Kowalski LP, Curado MP, Dewar R. Mate, coffee, and tea consumption and risk of cancers of the upper aero-digestive tract in Southern Brazil. Epidemiology 5:583-590, 1994.

Pintos J, Franco EL, Kowalski LP, Oliveira BV, Curado MP. Use of wood stoves and risk of cancers of the upper aero-digestive tract: a case-control study. Int J Epidemiol 27:936-940, 1998.

Pintos J, Franco EL, Black MJ, Bergeron J, Arella M. Human papillomavirus and prognoses of patients with cancers of the upper aerodigestive tract. Cancer 85:1903-1909, 1999.

Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 major cancers in 1990. Int J Cancer 80:18-29, 1999.

Portugal LG, Goldenberg JD, Wenig BL, Ferrer KT, Nodzenski E, Sabnani JB, Javier C, Weichselbaum RR, Vokes EE. Human papillomavirus expression and p53 gene mutations in squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 123:1230-1234, 1997.

Qu W, Jiang G, Cruz Y, Chang CJ, Ho GY, Klein RS, Burk RD. PCR detection of human papillomavirus: comparison between MY09/MY11 and GP5+/GP6+ primer systems. J Clin Microbiol 35:1304-1310, 1997.

Premoli-De-Percoco G, Ramirez JL, Galindo I. Correlation between HPV types associated with oral squamous cell carcinoma and cervicovaginal cytology: An in situ hybridization study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 86:77-81, 1998.

Riethdorf S, Friedrich RE, Ostwald C, Barten M, Gogacz P, Gundlach KK, Schlechte H, Becker J, Bregenzer T, Riethdorf L, Loning T. p53 gene mutations and HPV infection in primary head and neck squamous cell carcinomas do not correlate with overall survival: a long-term follow-up study. J Oral Pathol Med 26:315-321, 1997.

Rossing MA, Vaughan TL, McKnight B. Diet and pharyngeal cancer. Int J Cancer 15:593-597, 1989.

Sand L, Jalouli J, Larsson PA, Hirsch JM. Human papilloma viruses in oral lesions. Anticancer Res 20:1183-1188, 2000.

Sankaranarayanan R, Duffy SW, Padmakumary G, Day NE, Padmanabhan TK. Tobacco chewing, alcohol and nasal snuff in cancer of the gingiva in Kerala, India. Br J Cancer 60:638-643, 1989.

Sankaranarayanan R, Masuyer E, Swaminathan R, Ferlay J, Whelan S. Head and neck cancer: a global perspective on epidemiology and prognosis. Anticancer Res 8:4779-4786, 1998.

Saranath D, Tandle AT, Teni TR, Dedhia PM, Borges AM, Parikh D, Sanghavi V, Mehta AR. p53 inactivation in chewing tobacco-induced oral cancers and leukoplakias from India. Oral Oncol ;35:242-250, 1999.

Sato M, Sato T, Izumo T, Amagasa T. Genetic polymorphism of drug-metabolising enzymes and susceptibility to oral cancer. Carcinogenesis 20:1927-1931, 1999.

Schiffman MH, Bauer HM, Hoover RN, Glass AG, Cadell DM, Rush BB, Scott DR, Sherman ME, Kurman RJ, Wacholder S, Stanton CK, Manos MM. Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. J Natl Cancer Inst 85:958-964, 1993.

Schlecht NF, Franco EL, Pintos J, Negassa A, Kowalski LP, Oliveira BV, Curado MP. Interaction between tobacco and alcohol consumption and the risk of cancers of the upper aero-digestive tract in Brazil. Am J Epidemiol 150:1129-1137, 1999.

Schlecht NF, Pintos J, Kowalski LP, Franco EL. Effect of type of alcoholic beverage on the risks of upper aerodigestive tract cancers in Brazil. Cancer Causes Control 12:579-587, 2001.

Schwartz SM, Daling JR, Doody DR, Wipf GC, Carter JJ, Madeleine MM, Mao EJ, Fitzgibbons ED, Huang S, Beckmann AM, McDougall JK, Galloway DA. Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection. J Natl Cancer Inst 1998;90:1626-36.

Schwartz SR, Yueh B, McDougall JK, Daling JR, Schwartz SM. Human papillomavirus infection and survival in oral squamous cell cancer: a population-based study. Otolaryngol Head Neck Surg 125:1-9, 2001.

Scully C. Oral squamous cell carcinoma; from an hypothesis about a virus, to concern about possible sexual transmission. Oral Oncol 38:227-234, 2002.

Scully C, Field JK, Tanzawa H. Genetic aberrations in oral or head and neck squamous cell carcinoma (SCCHN): 1. Carcinogen metabolism, DNA repair and cell cycle control. Oral Oncol 36:256-263, 2000.

Shima K, Kobayashi I, Saito I, Kiyoshima T, Matsuo K, Ozeki S, Ohishi M, Sakai H. Incidence of human papillomavirus 16 and 18 infection and p53 mutation in patients with oral squamous cell carcinoma in Japan. Br J Oral Maxillofac Surg 38:445-450, 2000.

Shindoh M, Sawada Y, Kohgo T, Amemiya A, Fujinaga K. Detection of human papillomavirus DNA sequences in tongue squamous-cell carcinoma utilizing the polymerase chain reaction method. Int J Cancer 50:167-171, 1992.

Shindoh M, Chiba I, Yasuda M, Saito T, Funaoka K, Kohgo T, Amemiya A, Sawada Y, Fujinaga K. Detection of human papillomavirus DNA sequences in oral squamous cell carcinomas and their relation to p53 and proliferating cell nuclear antigen expression. Cancer 1995 76:1513-1521, 1995.

Shroyer KR, Greer RO. Detection of human papillomavirus DNA by in situ DNA hybridization and polymerase chain reaction in premalignant and malignant oral lesions. Oral Surg Oral Med Oral Pathol 71:708-713, 1991.

Singh VN, Gaby SK. Premalignant lesions: role of antioxidant vitamins and beta-carotene in risk reduction and prevention of malignant transformation. Am J Clin Nutr 53:386S-390S, 1991.

Sisk EA, Bradford CR, Jacob A, Yian CH, Staton KM, Tang G, Harris MO, Carey TE, Lancaster WD, Gregoire L. Human papillomavirus infection in "young" versus "old" patients with squamous cell carcinoma of the head and neck. Head Neck 22:649-657, 2000.

Smith EM, Hoffman HT, Summersgill KS, Kirchner HL, Turek LP, Haugen TH. Human papillomavirus and risk of oral cancer. Laryngoscope 1998;108:1098-103.

Snijders PJ, Cromme FV, van den Brule AJ, Schrijnemakers HF, Snow GB, Meijer CJ, Walboomers JM. Prevalence and expression of human papillomavirus in tonsillar carcinomas, indicating a possible viral etiology. Int J Cancer 51:845-850, 1992.

Snijders PJ, Scholes AG, Hart CA, Jones AS, Vaughan ED, Woolgar JA, Meijer CJ, Walboomers JM, Field JK. Prevalence of mucosotropic human papillomaviruses in squamous-cell carcinoma of the head and neck. Int J Cancer 66:464-469, 1996.

Spitz MR, Fueger JJ, Goepfert H, Hong WK, Newell GR. Squamous cell carcinoma of the upper aerodigestive tract. A case comparison analysis. Cancer 61:203-208, 1988.

Spitz MR, Sider JG, Schantz SP, Newell GR. Association between malignancies of the upper aerodigestive tract and uterine cervix. Head & Neck 14:347-351, 1992.

Sporn MB, Roberts AB. Role of retinoids in differentiation and carcinogenesis. Cancer Res 43:3034-3040, 1983.

Stanley MA. Immunobiology of papillomavirus infections. J Reprod Immunol 52:45-59, 2001.

Steenbergen RD, Hermsen MA, Walboomers JM, Joenje H, Arwert F, Meijer CJ, Snijders PJ. Integrated human papillomavirus type 16 and loss of heterozygosity at 11q22 and 18q21 in an oral carcinoma and its derivative cell line. Cancer Res 55:5465–5571, 1995.

Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, Leigh IM, Matlashewski G, Banks L. Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. Nature 393:229-234, 1998.

Strickler HD, Hildesheim A, Viscidi RP, Shah KV, Goebel B, Drummond J, Waters D, Sun Y, Hubbert NL, Wacholder S, Brinton LA, Han CL, Nasca PC, McClimens R, Turk K, Devairakkam V, Leitman S, Martin C, Schiller JT. Interlaboratory agreement among results of human papillomavirus type 16 enzyme-linked immunosorbent assays. J Clin Microbiol 35:1751-1756, 1997.

Summersgill KF, Smith EM, Kirchner HL, Haugen TH, Turek LP. p53 polymorphism, human papillomavirus infection in the oral cavity, and oral cancer. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 90:334-39, 2000.

Syrjanen KJ, Syrjanen SM. Histological evidence for the presence of condylomatous epithelial lesions in association with laryngeal squamous cell carcinoma. ORL J Otorhinolaryngol Relat Spec 43:181-194, 1981.

Syrjanen K, Syrjanen S, Lamberg M, Pyrkonen S, Nuutinen J. Morphological and immunohistochemical evidence suggesting human papilloma virus (HPV) involvement in oral squamous cell carcinogenesis. Int J Oral Surg 12:418-424, 1983.

Syrjanen SM, Syrjanen KJ, Lamberg MA. Detection of human papillomavirus DNA in oral mucosal lesions using in situ DNA-hybridization applied on paraffin sections. Oral Surg Oral Med Oral Pathol 62:660-667, 1986.

Syrjanen SM, Syrjanen KJ, Happonen RP. Human papillomavirus (HPV) DNA sequences in oral precancerous lesions and squamous cell carcinoma demonstrated by in situ hybridization. J Oral Pathol 17:273-278, 1988.

Tanimoto K, Hayashi S, Yoshiga K, Ichikawa T. Polymorphisms of CYP1A1 and GSTM1 gene involved in oral squamous cell carcinoma in association with cigarette dosage. Oral Oncol 35B:191-196, 1999.

Tsuchiya H, Tomita Y, Shirasawa H, Tanzawa H, Sato K, Simizu B. Detection of human papillomavirus in head and neck tumors with DNA hybridization and immunohistochemical analysis. Oral Surg Oral Med Oral Pathol 71:721-725, 1991.

Tsuhako K, Nakazato I, Miyagi J, Iwamasa T, Arasaki A, Hiratsuka H, Sunakawa H, Kohama G, Abo T. Comparative study of oral squamous cell carcinoma in Okinawa, Southern Japan and Sapporo in Hokkaido, Northern Japan; with special reference to human papillomavirus and Epstein-Barr virus infection. J Oral Pathol Med 29:70-79, 2000.

Tyan YS, Liu ST, Ong WR, Chen ML, Shu CH, Chang YS. Detection of Epstein-Barr virus and human papillomavirus in head and neck tumors. J Clin Microbiol 31:53-56, 1993.

van Houten VM, Snijders PJ, van den Brekel MW, Kummer JA, Meijer CJ, van Leeuwen B, Denkers F, Smeele LE, Snow GB, Brakenhoff RH. Biological evidence that human papillomaviruses are etiologically involved in a subgroup of head and neck squamous cell carcinomas. Int J Cancer 93:232-235, 2001.

van Rensburg EJ, Engelbrecht S, van Heerden WF, Raubennheimer EJ, Schoub BD. Human papillomavirus DNA in oral squamous cell carcinomas from an African population sample. Anticancer Res 16:969-973, 1996.

Velly AM, Franco EL, Schlecht N, Pintos J, Kowalski LP, Oliveira BV, Curado MP. Relationship between dental factors and risk of upper aerodigestive tract cancer. Oral Oncol 34:284-291, 1998.

Viscidi RP, Kotloff KL, Clayman B, Russ K, Shapiro S, Shah KV. Prevalence of antibodies to human papillomavirus (HPV) type 16 virus-like particles in relation to cervical HPV infection among college women. Clin Diagn Lab Immunol 4:122-126, 1997.

Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189:12-19, 1999.

Wang J, Li J, Huang H, Fu Y. Detection of the E7 transform gene of human papilloma virus type 16 in human oral squamous cell carcinoma. Chin J Dent Res 1:35-37, 1998.

Watanabe S, Ogura H, Fukushima K, Yabe Y. Comparison of Virapap filter hybridization with polymerase chain reaction and Southern blot hybridization methods for detection of human papillomavirus in tonsillar and pharyngeal cancers. Eur Arch Otorhinolaryngol 250:115-119, 1993.

Watts SL, Brewer EE, Fry TL. Human papillomavirus DNA types in squamous cell carcinomas of the head and neck. Oral Surg Oral Med Oral Pathol 71:701-707, 1991.

Watts DH, Koutsky LA, Holmes KK, Goldman D, Kuypers J, Kiviat NB, Galloway DA. Low risk of perinatal transmission of human papillomavirus: results from a prospective cohort study. Am J Obstet Gynecol 178:365–373, 1998.

Wen S, Tsuji T, Li X, Mizugaki Y, Hayatsu Y, Shinozaki F. Detection and analysis of human papillomavirus 16 and 18 homologous DNA sequences in oral lesions. Anticancer Res 1997 17:307-311, 1997.

Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 248:76-79, 1990.

Wiest T, Schwarz E, Enders C, Flechtenmacher C, Bosch FX. Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control. Oncogene 21:1510-1517, 2002.

Wikström A, Van Doornum GJJ, Quint WGV, Schiller JT, Dillner J. Identification of human papillomavirus seroconversions. J Gen Virol 76:529-539, 1995.

Willett WC, MacMahon B. Diet and cancer. An overview. N Engl J Med 310:633-638, 697-701, 1984.

Wilczynski SP, Lin BT, Xie Y, Paz IB. Detection of human papillomavirus DNA and oncoprotein overexpression are associated with distinct morphological patterns of tonsillar squamous cell carcinoma. Am J Pathol 152:145-156, 1998.

Wingo PA, Tong T, Bolden S. Cancer statistics, 1995. CA Cancer J Clin 45:8-30, 1995.

Winn DM. Diet and nutrition in the etiology of oral cancer. Am J Clin Nutr 61:437S-445S, 1995.

Winn DM, Blot WJ, McLaughklin JK, Austin DF, Greenberg RS, Preston-Martin S, Shoenberg JB, Fraumeni JF Jr. Mouthwash use and oral conditions in the risk of oral and pharyngeal cancer. Cancer Res 51:3044-3047, 1991.

Yeudall WA, Campo MS. Human papillomavirus DNA in biopsies of oral tissues. J Gen Virol 72:173-176, 1991.

Young SK, Min KW. In situ DNA hybridization analysis of oral papillomas, leukoplakias, and carcinomas for human papillomavirus. Oral Surg Oral Med Oral Pathol 71:726-729, 1991.

Zehbe I, Wilander E. Two consensus primer systems and nested polymerase chain reaction for human papillomavirus detection in cervical biopsies: A study of sensitivity. Hum Pathol 27:812-815, 1996.

Zehbe I, Wilander E. Nonisotopic ELISA-based detection of human papillomavirus-amplified DNA. Mod Pathol 10:188-191, 1997.

Zeuss MS, Miller CS, White DK. In situ hybridization analysis of human papillomavirus DNA in oral mucosal lesions. Oral Surg Oral Med Oral Pathol 71:714-720, 1991.

Zumbach K, Hoffmann M, Kahn T, Bosch F, Gottschlich S, Gorogh T, Rudert H, Pawlita M. Antibodies against oncoproteins E6 and E7 of human papillomavirus types 16 and 18 in patients with head-and-neck squamous-cell carcinoma. Int J Cancer 85:815-818, 2000.

zur Hausen H. Human genital cancer: synergism between two virus infections of synergism between a virus infection and initiating events? Lancet 2:1370-1372, 1982.

zur Hausen H. Papillomaviruses as carcinomaviruses. In: Klein G (ed.). Advances in viral oncology, Vol. 8, New York, Raven Press, pp. 1-26, 1989.

zur Hausen H. Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. Curr Top Microbiol Immunol 186:131-146, 1994.

zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 92:690-698, 2000.

zur Hausen H, Schneider A. The role of papillomaviruses in human anogenital cancers. In: Salzman N and Howley PM (eds). The papovaviridae vol 2, New York, Plenum Press, pp. 245-263, 1987.

Appendix 1

English version of introductory letter for patients

McGill

Department of Oncology

Letter of Introduction: McGill University Study of Oral Health

Dear Patient:

We would like to describe to you a new study by a team of physicians and scientists affiliated with this hospital and with McGill University. Our team is studying whether infection of the mouth by a group of viruses called papillomaviruses may cause certain lesions that could later in life become cancerous tumours of the mouth and throat. Parallel studies are being conducted simultaneously in many hospitals in Europe, Asia, and South America, all of which are coordinated by the World Health Organization. It is through the cooperation of these many centres that we will be able to better understand the causes of oral cancers, which would allow us to propose methods for preventing them.

In order to investigate this we need the participation of patients with oral tumours as well as of patients being treated at this hospital for any other diseases except cancer. You are being approached by your doctor because you belong to one or the other group of patients. Your doctor will briefly describe the study to you but later our nurse will contact you again to give further details and to answer any questions you may have. After explaining the study to you, our nurse will invite you to participate by asking you to sign an informed consent form that contains the same information that is written in this letter of introduction. There are no costs to you, direct or indirect. All the tests will be paid out of research funds that our scientific team received to investigate the causes of oral cancer.

If you consent, our nurse will collect a sample of cells from your mouth using a soft toothbrush and you will be asked to perform a mouthwash with saline solution. The fluid that is collected will be taken to the laboratory to be tested for abnormal cells and for your own defences against carcinogens. It will also be necessary to collect a small blood sample (10 ml or, equivalently, about 2 teaspoons) that will be tested for antibodies against papillomaviruses. As you may already know, you may feel a mild, temporary discomfort during the blood sample collection. Our nurse will also interview you to check if you have one or more of the risk factors for cancer of the mouth and throat that we are studying. This interview will have questions about family life, lifestyle, and sexual activity, some of which may be of a sensitive nature. During the interview you may refuse to answer any questions with which you do not feel comfortable. Altogether, you may have to spend about 30-40 min with our nurse for the interview and for the brief oral examination.

There are no direct personal benefits for you due to your participation in the study. By participating, however, you will be contributing to our understanding of what causes oral cancers. Our results could help us to develop new laboratory tests to diagnose the oral tumours before they become malignant, allowing us to cure these lesions. This will help future patients at risk of developing oral cancers.

The results from the laboratory analyses and the responses you give during the interview will be treated very confidentially. No names or other information that could identify you as a patient will be released. All the data from this study will be analyzed in statistical form only. You may refuse to participate in the study now or later, without any negative consequences. Nothing will change in terms of the quality of health care that you are receiving in this hospital.

Our nurse will be contacting you soon. Thank you for your cooperation.

Name of clinicain
Title
Head and Neck Oncology, McGill

Eduardo Franco, PhD
Professor and Director
Division of Cancer Epidemiolgy, McGill

Appendix 2

English version of the questionnaire

dd-mm-yyyy

MULTINATIONAL STUDY OF THE ORAL CAVITY

1. Identification number |__|__| Country |__| Centre |__|__|__| Person number

The identification number is composed of the values for country, centre and person number.
Person numbers are consecutive numbers within each centre.
Initials of the patient should be written with the identification number on each biological sample.

2. General guidelines

- The columns should be filled in justified to the right (example: valid |__| 1| 2|; not valid | 1| 2| |)
- Leave blank if question is not asked or not applicable.
- Avoid missing or unknown codes. Insist to get an answer even if it is only an estimation.
- If you do not succeed in getting an answer or estimation, the columns should be filled in with 9.
- When "specify" is written, note your answer on the uninterrupted line.
- For some variables (occupation, ICD code, etc...) a coding method is not provided. They will be coded by the coordinator after the interview or centrally. Boxes are then in bold italics.

3. Local codes

Local code [LC]. They change from one place to another. Please refer to the local study coordinator (i.e. interviewer, hospital, town and district, tar level and type of tobacco for cigarettes, cigars ...).

Patient's name .....
Contact address .....
.....
.....
Phone .....

Table of contents

MULTINATIONAL STUDY OF THE ORAL CAVITY

Identification number..... |__|__|-|__|-|__|__|__| 1-6
(to be used on biological specimens) Country C Person n.

Country: |_0|_1| 7-8

(01) Italy
(02) Spain
(03) Switzerland
(04) Northern Ireland
(05) Poland
(06) India
(07) Pakistan
(08) Brazil
(09) Cuba
(10) Canada
(11) Australia
(12) France
(14) Sudan
(15) Argentina
(16) Uruguay

Centre (C) (for Italy, Spain, India & Argentina):

(1) Aviano; (2) Udine; (3) Milan
(1) Barcelona; (2) Granada; (3) Sevilla
(1) Bangalore; (2) Madras; (3) Trivandrum
(1) Porto Alegre; (2) Rio de Janeiro; (3) São Paulo

Person n. = consecutive number, by centre

A1 Status: (1) Case; (2) Control ..... |__| 9

Medical record n. _____

A2 Initials (surname - name) ..... |__|__| 10-11

A3 Hospital [LC] ..... |__|__| 12-13

A4 Department: (1) Medicine
(2) Surgery
(3) Ob/Gyn
(4) Orthopaedic
(5) Ear, nose, throat
(6) Dermatology
(7) Ophthalmology
(8) Dentistry
(9) Radiotherapy
(10) Oncology
(11) Out-patient
(12) Other _____ (specify) |__|__| 14-15

A5 Main diagnosis for being in the hospital ..... /__/__/__/-/__/ **16-19**
(I.C.D.9)

_____
(for outpatient with only cancer suspicion = 8888)

A6 Date of hospital admission (or visit) ..... |__|__|-|__|__|-|__|__| 20-25
Day Month Year

A7 Interviewer [LC] ..... |__| 26

Good morning.

My name is ..... and first of all I would like to thank you for having accepted to participate in this study. We are conducting a study in ...[THE CORRESPONDING COUNTRY] and in other countries in order to clarify if certain characteristics and habits of men and women are related to certain diseases. For this purpose, we will interview many patients attending this and other hospitals.

If you agree, I will ask you several questions and the answers will be recorded on this form.

I would like to reassure you that all that is said during the interview will be strictly confidential and that the information collected from several hundreds of people will only be used in scientific reports without any personal name or identifiers being mentioned.

Any likely benefits of the study for the well-being of the population rely on the accuracy of your answers. Therefore, if you do not understand the meaning of any of the questions, please don't be afraid to ask.

At any time you may refuse to continue or to answer specific questions. In addition to the interview, the study includes an examination of your mouth, a collection of some cells and a blood sample.

Can we start now?

GENERAL INFORMATION

B1 Date of interview ..... |__|__|-|__|__|-|__|__| 27-32
Day Month Year

B2 Beginning of interview ..... |__|__| |__|__| 33-36
Hour Min.

B3 Sex: (1) Male; (2) Female ..... |__| 37

B4 Ethnic group: (established by interviewer)
(01) White
(02) Black
(03) Mestizo
(04) Mulatto
(05) Indian
(06) Other _____ (specify) |__|__| 38-39

B5 *How old are you?* ..... |__|__| 40-41

B6 *What is your date of birth?* ..... |__|__|-|__|__|-|__|__| 42-47
Day Month Year

B7 *In what town or district do you live?* [LC] ..... /__/__/__/__/__/__/ **48-52**

_____

B8 *For how many years have you been living there?* ..... |__|__| 53-54
(If less than a year code 00)

B9 *In what town or district were you born?* [LC] ..... /__/__/__/__/__/__/ **55-59**

_____

B10 *What is your religion?*
(01) Catholic
(02) Muslim
(03) Protestant
(04) Jewish
(05) Buddhist
(06) Animist/woodooist
(07) Hindu
(08) None
(09) Other _____ (specify) |__|__| 60-61

B11 (for Canada, Pakistan & Switzerland only):
Which language is spoken in your family?
(01) English
(02) French
(03) German
(04) Italian
(05) Urdu
(06) Punjabi
(07) Sindhi
(08) Baluchi
(09) Pashtu
(10) Other _____ (specify) |__|__| 62-63

EDUCATION AND OCCUPATION

C1 *Did you ever attend school?* (1) Yes; (2) No ..... |__| 64
[if 'no' go to C4]

C2 *For how many years did you go to school?* ..... |__|__| 65-66

C3 *At which age did you stop going to school?* ..... |__|__| 67-68

C4 *Which is or was your longest occupation?* ..... /__/__/ **69-70**

_____ (specify)

Identification number ..... |__|__|-|__|-|__|__|__| 1-6
Country C Person n.

|_0|_2| 7-8

SMOKING AND CHEWING HABITS

D1 *Do you or did you smoke daily for at least one year?* ..... |__| 9
(1) Yes, still; (2) Never; (3) Only in the past [if 'never' go to D6]

Please describe the periods in your life during which you smoked cigarettes, cigars, pipe or bidi, the amounts smoked and other details about the products smoked. Please try to summarize the most important changes in your life regarding the amount and type of each product. Ignore any changes occurring for short periods (less than a year).

Interviewers: Avoid overlapping years for the same product or type of cigarette (b,c), i.e. record 30-40, 41-45 rather than 30-40, 40-45.

D2 *Do you or did you smoke cigarettes?* [if 'no' go to D3]

Cigarette (a)	*From age?*	*To age?*	*Tobacco type* (b)	*Filter* (c)	*Brand*	*Number per day*	
|__|	|__|__|	|__|__|	|__|	_____/__/	|__|__|	10-19
	|__|__|	|__|__|	|__|	|__|	_____/__/	|__|__|	20-28
	|__|__|	|__|__|	|__|	|__|	_____/__/	|__|__|	29-37
	|__|__|	|__|__|	|__|	|__|	_____/__/	|__|__|	38-46
	|__|__|	|__|__|	|__|	|__|	_____/__/	|__|__|	47-55

D3 *Do you or did you smoke cigars?* [if 'no' go to D4]

Cigar (a)	*From age?*	*To age?*	*Brand*	*Number per day*	
|__|	|__|__|	|__|__|	_____/__/	|__|__|	56-63
	|__|__|	|__|__|	_____/__/	|__|__|	64-70
	|__|__|	|__|__|	_____/__/	|__|__|	71-77

D4 *Do you or did you smoke pipe?* [if 'no' go to D5]

Pipe (a)	*From age?*	*To age?*	*Brand*	*Number per day*	
|__|	|__|__|	|__|__|	_____/__/	|__|__|	78-85
	|__|__|	|__|__|	_____/__/	|__|__|	86-92
	|__|__|	|__|__|	_____/__/	|__|__|	93-99

(a) (1) Yes; (2) No
(b) (1) Blond; (2) Black; (9) Don't know
(c) (1) Filter; (2) Non filter; (9) Don't know

D5 *Do you or did you smoke bidi?* [if 'no' go to D6]

Bidi (a)	*From age?*	*To age?*	*Brand*	*Number per day*	
|__|	|__|__|	|__|__|	_____/__/	|__|__|	100-107
	|__|__|	|__|__|	_____/__/	|__|__|	108-114
	|__|__|	|__|__|	_____/__/	|__|__|	115-121

(a) (1) Yes; (2) No

D6 *Have you ever chewed daily tobacco, betel quid, areca nut, pan massala for at least one year?* ..... \|__\| 122

(1) Yes, still; (2) Never; (3) Only in the past [if 'never' go to D8]

Please describe the periods in your life in which you chewed and the amounts. Please try to sumarize the most important changes in your life regarding the amount and type of product.

Product:
(1) Tobacco
(2) Betel quid with tobacco
(3) betel quid without tobacco
(4) Areca nut with tobacco
(5) Areca nut without tobacco
(6) Pan massala
(7) Other (specify) _____

D7 *Product*	*From age*	*To age?*	*How may times a day?*	
|__|	|__|__|	|__|__|	|__|__|	123-129
|__|	|__|__|	|__|__|	|__|__|	130-136
|__|	|__|__|	|__|__|	|__|__|	137-143
|__|	|__|__|	|__|__|	|__|__|	144-150
|__|	|__|__|	|__|__|	|__|__|	151-157
|__|	|__|__|	|__|__|	|__|__|	158-164
|__|	|__|__|	|__|__|	|__|__|	165-171
|__|	|__|__|	|__|__|	|__|__|	172-178

D8 *Have you ever snuffed tobacco daily for at least one year?* ..... \|__\| 179

(1) Yes, still; (2) Never; (3) Only in the past [if 'never' go to next page]

Please describe the periods in your life in which you snuffed tobacco and the amounts. Please try to summarize the most important changes in your life regarding the amount.

D9 *From age?*	*To age?*	*How many times a day?*	
|__|__|	|__|__|	|__|__|	180-185
|__|__|	|__|__|	|__|__|	186-191

Identification number ..... |__|__|-|__|-|__|__|__| 1-6
Country C Person N.

| 0| 3| 7-8

DIETARY HABITS

Before you got sick, which was your frequency of consumption of the following foods and beverages? Only in season, when specified.

	Unit	**Food item**	*How many times/week?* (if less than once a week, code 98, if not consumed at all, code 0)	
E1	1 glass	Milk	|__|__|	9-10
E2	1 pot	Yoghurt	|__|__|	11-12
E3	1 serving	Bread	|__|__|	13-14
E4	1 serving	Pasta or rice	|__|__|	15-16
E5	1 serving	Maize dishes	|__|__|	17-18
E6	1 serving	Meat	|__|__|	19-20
E7	1 serving	Fish	|__|__|	21-22
E8	1 serving	Ham, salami, sausages	|__|__|	23-24
E9	1	Egg	|__|__|	25-26
E10	1 serving	Cheese	|__|__|	27-28
E11	1 medium	Potatoes	|__|__|	29-30
E12	1 serving	Raw green vegetables and salads	|__|__|	31-32
E13	1 serving	Cruciferae (broccoli, cabbage, Brussel sprout)	|__|__|	33-34
E14	1 medium	Carrots	|__|__|	35-36
E15	1 medium	Fresh tomatoes (in season)	|__|__|	37-38
E16	1 serving	Pulses (peas, beans, etc.)	|__|__|	39-40
E17	1 serving	*As a summary, how often would you say that you eat any kind of vegetables (potatoes excluded)?*	|__|__|	41-42
E18	1 glass	Fresh fruit juices	|__|__|	43-44
E19	1 medium	Apples or pears	|__|__|	45-46
E20	1 medium	Citrus fruit (oranges, grapefruit, lemons) (in season)	|__|__|	47-48
E21	1 medium	Bananas	|__|__|	49-50
E22	1 medium	*As a summary, how often would you say that you eat any kind of fresh fruit (including fruit salads)?*	|__|__|	51-52
E23	1 slice or cup	Cakes and desserts	|__|__|	53-54

Which type of fat do you use predominantly:

(1) olive oil	(4) other seed oils	(7) no fat at all
(2) palm oil	(5) butter	(8) other animal fat
(3) coconut oil	(6) margarine	(9) don't know

E24 *to season vegetables etc.?* ..... |__| 55

E25 *for cooking?* ..... |__| 56

E26 *In the last two years, have you been taking vitamin supplements?* ..... |__| 57
(1) Yes; (2) No; (9) don't know [if 'no' go to E29]

E27 *How often have you been taking vitamin supplements?* ..... |__| 58
(1) Every day
(2) At least once a week
(3) At least once a month
(4) Occasionally
(5) Never

E28 *At which age did you start taking vitamins, as an adult?* ..... |__|__| 59-60

E29 *If you remember, can you tell me what was your weight two years ago?* ..... |__|__|__| 61-63
(kg)

E30 *Can you tell me what was your weight at age 30?* ..... |__|__|__| 64-66
(kg)

E31 *What is your height?* ..... |__|__|__| 67-69
(cm)

Identification number ..... |__|__|-|__|-|__|__|__| 1-6
Country C Person N.

| 0| 4| 7-8

DRINKING HABITS

F1 *Have you ever drunk alcoholic beverages at least once a month?* ..... |__| 9
(1) Yes, still; (2) Never; (3) Only in the past [if 'never' go to next page]

F2 *When do you drink?* ..... |__| 10
(1) with meals; (2) between meals; (3) Both

Describe the periods in your life during which you consumed alcoholic beverages. Please try to summarize the most important changes in your life regarding the amount and type of beverage. Ignore any changes occurring for short periods (less than a year) or occasional drinking of one specific beverage.
Interviewers: Avoid overlapping years for the same beverage,
i.e. record 30-40, 41-45 rather than 30-40, 40-45.
Ask about each beverage separately.

F3 *Beverage* (a)	*From age?*	*To age?*	*Unit* (b)	*Consumption How many?*	*Per* (c)	
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	11-19
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	20-28
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	29-37
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	38-46
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	47-55
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	56-64
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	65-73
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	74-82
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	83-91
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	92-100
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	101-109
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	110-118
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	119-127
|__|	|__|__|	|__|__|	|__|	|__|__|	|__|	128-136

(a) (1) Wine
(2) Beer & cider
(3) Hard liquors (≥ 35°)
(whisky, cognac,vodka, brandy, grappa, marc, gin, cachaca, pinga)
(4) Aperitif (<35°)
(Martini, porto, sherry, vermouth)
(5) Others (specify) _____

(b) (1) Small glass (50 ml)
(2) Medium glass (100 ml)
(3) Big glass (250 ml)
(4) 1/2 or small bottle (330 ml)
(5) Bottle (700-750 ml)

c) (1) Day
(2) Week
(3) Month

Identification number ..... |__|__|-|__|-|__|__|__| 1-6
Country C Person N.

|_0|_5| 7-8

MARITAL AND SEXUAL HISTORY

G1 *Have you ever been married or living as married?* ..... |__| 9
(1) Yes; (2) No [if 'no' go to G7]

G2 *Are you still married or living as married?* ..... |__| 10
(1) Yes; (2) Separated/divorced; (3) Widowed

G3 *How many times have you been married or living as married?* ..... |__|__| 11-12

G4 *How old were you at your first marriage or when you first lived as married?* ..... |__|__| 13-14

G5 *For how many years did your last spouse go to school?* ..... |__|__| 15-16

G6 *Which is or was the longest occupation of your last spouse?* ..... /__/__/ **17-18**

_____ (specify)

G7 *How many children have you had, in total?* ..... |__|__| 19-20

IN CASE THE DETAILED QUESTIONNAIRE ON SEXUAL BEHAVIOUR IS NOT FEASIBLE:

G8 *How many sexual partners have you had in total?* ..... |__|__|__| 21-23
(regular and casual)

G9 *If difficult to answer* ..... |__| 24
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

ASK G10 AND G11 TO MEN ONLY:

G10 *Out of them, how many were prostitutes?* ..... |__|__|__| 25-27

G11 *If difficult to answer* ..... |__| 28
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

G12 *Have you ever had oral sex?* ..... |__| 29
(your mouth and your partner's genitals) (1) Yes; (2) No

G13 *How often?* ..... |__| 30
(1) Occasionally
(2) Often
(3) Most of the time

HISTORY OF VARIOUS DISEASES

H1 *Have you ever had skin warts?* ..... |__| 25
(1) Yes; (2) No; (9) Don't know [if 'no' go to H6]

If yes, *where?* (1) Yes; (2) No

H2 Hands ..... |__| 26
H3 Feet ..... |__| 27
H4 Head & Neck..... |__| 28
H5 Other (specify) _____ ..... |__| 29

H6 *During your adult life, have you ever had Candida Albicans/thrush?* ..... |__| 30
(1) Yes; (2) No; (9) Don't know [if 'no' go to H10]

If yes, *where?* (1) Yes; (2) No

H7 Genitals ..... |__| 31
H8 Mouth ..... |__| 32
H9 Other (specify) _____ ..... |__| 33

H10 *Have you ever had herpetic lesions (cold sore)?* ..... |__| 34
(1) Yes; (2) No; (9) Don't know [if 'no' go to H14]

If yes, *where?* (1) Yes; (2) No

H11 Lip ..... |__| 35
H12 Genitals ..... |__| 36
H13 Other (specify) _____ ..... |__| 37

H14 *Have you ever had sexually transmitted diseases?* ..... |__| 38
(1) Yes; (2) No; (9) Don't know [if 'no' go to next page]

If yes, *which ones?* (1) Yes; (2) No; (9) Don't know

H15 Syphilis/ulcer ..... |__| 39
H16 Gonorrhoea/discharge ..... |__| 40
H17 Condyloma/warts ..... |__| 41
H18 HIV/AIDS ... . |__| 42

CANCER FAMILY HISTORY

I am now going to ask some questions about your first degree relatives and spouses.

I1 *How many brothers have you had?* ..... |__|__| 43-44

I2 *How many sisters have you had?* ..... |__|__| 45-46

I3 *How many daughters have you had?* ..... |__|__| 47-48

I4 *How many sons have you had?* ..... |__|__| 49-50

I5 *How many spouses did you tell me you had?* ..... |__|__| 51-52

Identification number ..... |__|__|-|__|-|__|__|__| 1-6
Country C Person N.

|_0|_6| 7-8

Let us speak about your mother/father/sister/brother/daughter/son/spouse

I6 *Is he (she) still alive?*
If yes, *how old is he (she)?*
If no, *how old was he (she) when he (she) died?*

I7 *Did he (she) develop a malignant tumour?*
If yes, *which one?*
how old was he (she)?

Type of relative: [one relative each line]
(1) mother (3) sister (5) daughter (7) spouse
(2) father (4) brother (6) son

Type of Relative	Alive=1 Dead=2	If dead, give age at death; if alive, give present age	Malignant tumour (1) Yes; (2) No (9) Unk.	Tumour type	Age at diagnosis
(9-10)	(11)	(12) (13-14)	(15)	**(16-19)**	(20-21)
|0|1|	|__|	|__|__|	|__|	ICD/I__/__/__/-/__/	|__|__|
|0|2|	|__|	|__|__|	|__|	ICD/I__/__/__/-/__/	|__|__|
|0|3|	|__|	|__|__|	|__|	ICD/I__/__/__/-/__/	|__|__|
|0|4|	|__|	|__|__|	|__|	ICD/I__/__/__/-/__/	|__|__|
|0|5|	|__|	|__|__|	|__|	ICD/I__/__/__/-/__/	|__|__|
|0|6|	|__|	|__|__|	|__|	ICD/I__/__/__/-/__/	|__|__|
|0|7|	|__|	|__|__|	|__|	ICD/I__/__/__/-/__/	|__|__|
|0|8|	|__|	|__|__|	|__|	ICD/I__/__/__/-/__/	|__|__|
|0|9|	|__|	|__|__|	|__|	ICD/I__/__/__/-/__/	|__|__|

Identification number ..... |__|__|-|__|-|__|__|__| 1-6
Country C Person N.

|_0|_6| 7-8

Let us speak about your mother/father/sister/brother/daughter/son/spouse

I6 *Is he (she) still alive?*
If yes, *how old is he (she)?*
If no, *how old was he (she) when he (she) died?*

I7 *Did he (she) develop a malignant tumour?*
If yes, *which one?*
how old was he (she)?

Type of relative: [one relative each line]
(1) mother (3) sister (5) daughter (7) spouse
(2) father (4) brother (6) son

Type of Relative	Alive=1 Dead=2	If dead, give age at death; if alive, give present age	Malignant tumour (1) Yes; (2) No (9) Unk.	Tumour type	Age at diagnosis
(9-10)	(11)	(12) (13-14)	(15)	**(16-19)**	(20-21)
|1|0|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|1|1|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|1|2|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|1|3|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|1|4|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|1|5|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|1|6|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|1|7|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|1|8|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|1|9|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|2|0|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|2|1|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|2|2|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|2|3|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|2|4|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|2|5|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|2|6|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|2|7|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|
|2|8|	|__|	|__| |__|__| _____	|__|	ICD/I__/__/__/-/__/	|__|__|

Identification number ..... |__|__|-|__|-|__|__|__| 1-6
Country C Person N.

|_0|_7| 7-8

ORAL CAVITY HEALTH

J1 *How often do you brush your teeth?* ..... |__| 9
(0) Never
(1) < once a week
(2) 1-2 times a week
(3) Every other day
(4) Once a day
(5) 2 times a day
(6) 3 times a day
(7) > 3 times a day
(8) Not applicable [go to J5]

J2 *What instrument do you use to clean your teeth?* ..... |__| 10
(1) Tooth brush
(2) Finger
(3) Sticks
(4) Other _____ (specify)

J3 *What material do you use with the tooth brush?* ..... |__| 11
(1) Nothing
(2) Toothpaste
(3) Other _____ (specify)

J4 *Do your gums bleed when you wash your teeth?* ..... |__| 12
(1) No; (2) Sometimes; (3) Always or almost always

J5 *How often do you use mouthwashes?* ..... |__| 13
(0) Never
(1) < once a week
(2) 1-2 times a week
(3) Every other day
(4) Once a day
(5) 2 times a day
(6) 3 times a day
(7) > 3 times a day
(8) N/A

J6 *Do you wear denture?* ..... |__| 14
(1) Yes; (2) No [if 'no' go to J9]

J7 *Is it a complete denture?* ..... |__| 15
(1) Yes; (2) No

J8 *At which age did you start wearing dentures?* ..... |__|__| 16-17

J9 *During the last 20 years, how often did you go to see a dentist?* ..... |__| 18
(1) Every year
(2) Every 2-5 years
(3) < every 5 years
(4) Never

J10 *Before any procedure related to your present disease, have you ever had an oral biopsy?* ..... |__| 19
(1) Yes; (2) No [if 'no' go to J13]

J11 *At what age?* ..... |__|__| 20-21

J12 *What did it show?* _____ /__/ **22**
(specify)

J13 *Thank you for having agreed to answer this questionnaire.*

J14 End of interview ..... |__|__||__|__| 23-26
hour min.

J15 Quality of interview (to be established by interviewer) ..... |__| 27
(1) Unsatisfactory
(2) Questionnable
(3) Reliable
(4) High quality

J16 Comments

.....

.....

.....

.....

.....

INSTRUCTIONS FOR COLLECTION OF EXFOLIATED CELLS FROM THE MOUTH

1. Instruct the patient to perform a mouthwash with water.

2. Instruct the patient to remove dentures if worn

3. Perform the brushing of the oral cavity with a soft toothbrush.

In **control** subjects, several (5-10) gentle strokes with the toothbrush will be made on each of the following areas:

Right buccal mucosa (from high to low position)
Left buccal mucosa (from high to low position)
Right side of the tongue
Dorsal side of the tongue
Left side of the tongue
Inside of upper and lower lip

In **cancer cases**, in addition to performing a brushing in a similar way as in controls, the visible lesion will be brushed with **several (5-10) gentle strokes** trying to avoid necrotic areas. In the case of cancer of the salivary gland, gentle squeezing of the tumour should be performed in order to obtain exfoliated cells from the glandular duct.

4. Immediately after the scraping of the oral mucosa, prepare a smear on a slide labelled with the patient's name and study number. The slide must be immediately fixed in 90% alcohol and later stained with the Papanicolaou stain and coverslipped.

5. After performing the smear, introduce the toothbrush in a conic plastic tube of 50 ml containing about 20 ml of Phosphate Buffered Saline (PBS) and shake to detach exfoliated cells.

6. Ask the patients to perform energic washing of the oral cavity, including the throat by performing gargarisms, with 10 ml of PBS which will then be poured in the same conic tube.

7. Process the sample according to protocol.

EXAMINATION BY INTERVIEWER

ANTHROPOMETRIC MEASURES (in some centres)

K1 Weight (kg) ..... |__|__|__| 28-30

K2 Height (cm) ..... |__|__|__| 31-33

ORAL EXAMINATION (to be performed before cell collection)

K3 Examination: (1) accepted; (2) refused ..... |__| 34

K4 Who has made the examination? ..... |__| 35
(1) interviewer; (2) other, specify _____

K5 Date of oral examination (if different from interview) ..... |__|__|-|__|__|-|__|__| 36-41
Day Month Year

K6 General oral hygiene (e.g. tartar, gingival bleeding, etc.) ..... |__| 42
(1) Good; (2) Average; (3) Poor

K7 Missing teeth ..... |__| 43
(1) Less than 5; (2) 6-15; (3) 16 or more

K8 Is there any visible lesion ..... |__| 44
(1) No; (2) Yes; (3) Not sure
If yes, describe .....
.....
.....

If tumoral lesion suspected, please refer to the Principal Investigator

SAMPLE COLLECTION

- Exfoliated cells from the mouth are to be obtained following the instructions on the previous page
- 10 ml blood will be provided in a heparinized tube for later processing according to the protocol.

K9 Exfoliated cells obtained ..... |__| 45
(1) Yes; (2) Only mouth wash; (3) No

K10 Blood sample obtained: (1) Yes; (2) No ..... |__| 46

K11 Date of blood and cell sample collection ..... |__|__|-|__|__|-|__|__| 47-52
[if different from interview] Day Month Year

PRECANCEROUS LESIONS

[ONLY IF A PHYSICIAN CAN PERFORM THE EXAMINATION]

L1	Examination: (1) Done; (2) Not done	|__|	53
		Number of lesions	
L2	Lichen planus	|__|	54
	Leukoplakia (i.e. white patch > 5 mm)		
L3	Homogeneous	|__|	55
L4	Non-homogeneous: ulcerated	|__|	56
L5	nodular	|__|	57
L6	Erythroplakia (i.e., red patches)	|__|	58
L7	Submucous fibrosis	|__|	59
L8	Cancer	|__|	60
L9	Other _____ (specify)	|__|	61

L10 Name of the physician _____

Please annotate location of lesion described above

_____

_____

_____

_____

Identification number ..... |__|__|-|__|-|__|__|__| 1-6
Country C Person n.

|_0|_8| 7-8

FOR CASES ONLY

[FILL ADDITIONAL SHEETS FOR MULTIPLE SYNCHRONOUS TUMOURS]

M1 Describe the macroscopic aspect of the tumour(s) ..... |__| 9
(1) Exophytic (3) Verrucose
(2) Ulcerative (4) Other

M2 Topography, according to ICD-O, 1990 ..... |__|__| 10-11
[fill in more than one, if cancer overlaps two or more regions] |__|__| 12-13
|__|__| 14-15
(01) Base of tongue
(02) Tongue, other and unspecified
(03) Gum
(04) Floor of mouth
(05) Palate
(06) Mouth, other and unspecified
(07) Parotid gland
(08) Salivary gland, other and unspecified
(09) Tonsil
(10) Oropharynx

M3 Morphology, according to ICD-O, 1990 _____/__/__/__/__/__/ **16-20**
_____
_____ (specify)

M4 Number of diagnostic histological specimen(s) (not to code)
— — — — — — — —, — — — — — — — —

M5 Date of surgical operation, if performed ..... |__|__|-|__|__|-|__|__| 21-26
Day Month Year

Using the following table, classify the tumour by TNM stage

UICC	Extension of the tumour		
(1) TIS	Carcinoma in situ		
(2) T1	Tumour ≤ 2 cm		
(3) T2	Tumour > 2 ≤ 4 cm		
(4) T3	Tumour > 4 cm		
(5) T4	Tumour invades adjacent structures	|__|	27
(1) No	No lymph node metastases		
(2) N1	Metastases in single ipsilateral lymph node ≤ 3 cm		
(3) N2	Greater or bilateral lymph node metastases	|__|	28
(1) Mo	No distal metastases		
(2) M1	Distant metastases	|__|	29

M6 If formal staging is not available, what is your estimate of the tumour extension? ..... |__| 30
(1) Local; (2) Regional; (3) Disseminated

M7 Are biopsies/tissue available for this study? ..... |__| 31
(1) Yes, frozen biopsies
(2) Yes, other _____ (specify)
(3) No

M8 Are histological slides available for this study? ..... |__| 32
(1) Yes, punch biopsy
(2) Yes, surgical biopsy
(3) No

PLEASE ENCLOSE A PHOTOCOPY OF HISTOLOGICAL OR CYTOLOGICAL DIAGNOSIS, IF AVAILABLE

M9 Photocopy of histological or cytological diagnosis ..... |__| 33
(1) Enclosed
(2) Not enclosed
(3) Not available

QUESTIONNAIRE ON SEXUAL HABITS - MALES

To be administered at the end of the interview or given in a separate envelope for completion.

The next section of the questionnaire deals with issues regarding sexual behaviour and lifestyle. As you know, our study is being conducted in many different countries and human behaviour in this regard can vary markedly from country to country. However, we have learned how important sexual behaviour is in the origin of many diseases. That is why it is considered an important topic to study.

We would be grateful if you would respond to/complete this part of the questionnaire or as much of it as you feel you can and remember that results of the study will be anonymised and your responses will be dealt with in strictest confidence.

Thanks again for your help.

Identification number ..... |__|__|-|__|-|__|__|__| 1-6

|1|0| 7-8

O1 Have you ever had sexual intercourse with a woman? ..... |__| 9
(1) Yes; (2) No [if 'no', go to O14]

O2 How old were you when you had sexual intercourse with a woman for the first time? ..... |__|__| 10-11

O3 How many female sexual partners have you had in your life? ..... |__|__|__| 12-14
(regular and casual)
998 = difficult to answer

O4 If difficult to answer: ..... |__| 15
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

O5 Have you ever had oral sex with a woman? ..... |__| 16
(your mouth and a woman's genitals)
(1) Yes; (2) No [if 'no', go to O8]

O6 How often? ..... |__| 17
(1) Occasionally
(2) Often
(3) Most of the time

O7 How old were you when you had oral sex with a woman for the first time? |__|__| 18-19

O8 Have you ever had sex with a female prostitute? ..... |__| 20
(1) Yes; (2) No [if 'no', go to O14]

O9 How many female prostitutes have you had sexual contact with? ..... |__|__| 21-22

O10 If difficult to answer: ..... |__| 23
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

O11 Have you ever had oral sex with a female prostitute? ..... |__| 24
(your mouth and a woman's genitals)
(1) Yes; (2) No [if 'no', go to O14]

O12 How often? ..... |__| 25
(1) Occasionally
(2) Often
(3) Most of the time

O13 How old were you when you had oral sex with a female prostitute for the first time? ..... |__|__| 26-27

O14 Have you ever had sexual contact with another man? ..... |__| 28
(1) Yes; (2) No [if 'no', go to O28] [if 'no' in O1 & O14, end]

O15 How often? ..... |__| 29
(1) Rarely
(2) Frequently
(3) Very frequently

O16 How old were you when you had sexual intercourse with a man for the first time? ..... |__|__| 30-31

O17 How many male sexual partners have you had in your life? ..... |__|__|__| 32-34
(regular and casual)
998 = difficult to answer

O18 If difficult to answer: ..... |__| 35
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

O19 Have you ever had oral sex with a man? ..... |__| 36
(your mouth and a man's genitals)
(1) Yes; (2) No [if 'no', go to O22]

O20 How often? ..... |__| 37
(1) Occasionally
(2) Often
(3) Most of the time

O21 How old were you when you had oral sex with a man for the first time? ..... |__|__| 38-39

O22 Have you ever had sex with a male homosexual or bisexual prostitute or transvestite? ..... |__| 40
(1) Yes; (2) No [if 'no', go to O28]

O23 How many male homosexual or bisexual prostitutes or transvestites have you had sexual contact with? ..... |__|__| 41-42

O24 If difficult to answer: ..... |__| 43
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

O25 Have you ever had oral sex with a male homosexual or bisexual prostitute or transvestite? ..... |__| 44
(your mouth and a man's genitals)
(1) Yes; (2) No [if 'no', go to O28]

O26 How often? ..... |__| 45
(1) Occasionally
(2) Often
(3) Most of the time

O27 How old were you when you had oral sex with a male homosexual or bisexual prostitute or transvestite for the first time? ..... |__|__| 46-47

O28 As far as you remember, has any of your sexual partners had:

genital warts ..... (1) Yes; (2) No ..... |__| 48

cancer of the cervix [if 'yes' in O1] ..... (1) Yes; (2) No ..... |__| 49

cancer of the uterus [if 'yes in O1] ..... (1) Yes; (2) No ..... |__| 50

cancer of the anus ..... (1) Yes; (2) No ..... |__| 51

cancer of the penis [if 'yes' in O14] .... (1) Yes; (2) No ..... |__| 52

AIDS ..... (1) Yes; (2) No ..... |__| 53

sexually transmitted diseases..... (1) Yes; (2) No ..... |__| 54
(for example gonorrhea, syphilis, herpes, chlamydia)
Specify _____ ..... |__| 55

QUESTIONNAIRE ON SEXUAL HABITS - FEMALES

To be administered at the end of the interview or given in a separate envelope for completion.

The next section of the questionnaire deals with issues regarding sexual behaviour and lifestyle. As you know, our study is being conducted in many different countries and human behaviour in this regard can vary markedly from country to country. However, we have learned how important sexual behaviour is in the origin of many diseases. That is why it is considered an important topic to study.

We would be grateful if you would respond to/complete this part of the questionnaire or as much of it as you feel you can and remember that results of the study will be anonymised and your responses will be dealt with in strictest confidence.

Thanks again for your help.

Identification number ..... |__|__|-|__|-|__|__|__| 1-6

|0|9| 7-8

N1 Have you ever had sexual intercourse with a man? ..... |__| 9
(1) Yes; (2) No [if 'no' go to N14]

N2 How old were you when you had sexual intercourse with a man for the first time? ..... |__|__| 10-11

N3 How many male sexual partners have you had in your life? ..... |__|__|__| 12-14
(regular and casual)
998 = difficult to answer

N4 If difficult to answer: ..... |__| 15
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

N5 Have you ever had oral sex with a man? ..... |__| 16
(your mouth and a man's genitals)
(1) Yes; (2) No [if 'no', go to N8]

N6 How often? ..... |__| 17
(1) Occasionally
(2) Often
(3) Most of the time

N7 How old were you when you had oral sex with a man for the first time? .... |__|__| 18-19

N8 Have you ever had sex with a bisexual male? ..... |__| 20
(1) Yes; (2) No [if 'no', go to N14]

N9 How many bisexual males have you had sexual contact with? ..... |__|__| 21-22

N10 If difficult to answer: ..... |__| 23
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

N11 Have you ever had oral sex with a bisexual male? ..... |__| 24
(your mouth and a man's genitals)
(1) Yes; (2) No [if 'no', go to N14]

N12 How often? ..... |__| 25
(1) Occasionally
(2) Often
(3) Most of the time

N13 How old were you when you had oral sex with a bisexual male
for the first time? ..... |__|__| 26-27

N14 Have you ever had sexual contact with another woman? ..... |__| 28
(1) Yes; (2) No [if 'no', go to N28] [if 'no' in N1 & N14, end]

N15 How often? ..... |__| 29
(1) Rarely
(2) Frequently
(3) Very frequently

N16 How old were you when you had sexual intercourse with a woman
for the first time? ..... |__|__| 30-31

N17 How many female sexual partners have you had in your life? ..... |__|__|__| 32-34
(regular and casual)
998 = difficult to answer

N18 If difficult to answer: ..... |__| 35
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

N19 Have you ever had oral sex with a woman? ..... |__| 36
(your mouth and a woman's genitals)
(1) Yes; (2) No [if 'no', go to N22]

N20 How often? ..... |__| 37
(1) Occasionally
(2) Often
(3) Most of the time

N21 How old were you when you had oral sex with a woman for the first time? |__|__| 38-39

N22 Have you ever had sex with a female prostitute? ..... |__| 40
(1) Yes; (2) No [if 'no', go to N28]

N23 How many female prostitutes have you had sexual contact with? ..... |__|__| 41-42

N24 If difficult to answer: ..... |__| 43
(1) 2-5
(2) 6-10
(3) 11-20
(4) 21-50
(5) 51-100
(6) more than 100

N25 Have you ever had oral sex with a female prostitute? ..... |__| 44
(your mouth and a woman's genitals)
(1) Yes; (2) No [if 'no', go to N28]

N26 How often? ..... |__| 45
(1) Occasionally
(2) Often
(3) Most of the time

N27 How old were you when you had oral sex with a female prostitute
for the first time? ..... |__|__| 46-47

N28 As far as you remember, has any of your sexual partners had:

genital warts ..... (1) Yes; (2) No ..... |__| 48

cancer of the cervix [if 'yes' in N14] .... (1) Yes; (2) No ..... |__| 49

cancer of the uterus [if 'yes' in N14] .... (1) Yes; (2) No ..... |__| 50

cancer of the anus ..... (1) Yes; (2) No ..... |__| 51

cancer of the penis [if 'yes' in N1] ..... (1) Yes; (2) No ..... |__| 52

AIDS ..... (1) Yes; (2) No ..... |__| 53

sexually transmitted diseases..... (1) Yes; (2) No ..... |__| 54
(for example gonorrhea, syphilis,
herpes, chlamydia)
Specify _____ ..... |__| 55

Appendix 3

Ethical approval for this study from the

Institutional Review Board of McGill University

McGill

Faculty of Medicine Faculté de médecine

CERTIFICATION OF ETHICAL ACCEPTABILITY FOR RESEARCH INVOLVING HUMAN SUBJECTS

The Faculty of Medicine Institutional Review Board consisting of:

NEIL MACDONALD, MD (CHAIR)	CELESTE JOHNSTION, DED
SHARON BATT, MA	NANCY MAYO, PHD
ARTHUR CANDIB, MED	RONALD MELZACK, PHD
HAROLD FRANK, MD	WILSON MILLER, PHD, MD
BENJAMIN FREEDMAN, PHD	GILLES THÉRIAULT, MD
LAWRENCE HUTCHISON, MD	

has examined the research project A03-M56-96 entitled: "Case-Control Study of Human Papillomavirus and Oral Cancer"

as proposed by: EDUARDO FRANCO, PHD (Applicant) to _____ (Granting Agency, if any)

and consider the experimental procedures to be acceptable on ethical grounds for research involving human subjects.

MARCH 13, 1997
Date

Chair, IRB

Dean of Faculty

Institutional Review Board Assurance Number: M-1458

Appendix 4

English version of the informed consent form

for cases and controls.

McGill University Study of Oral Health

Informed Consent Form

Purpose:

We are studying whether infection of the oral cavity by papillomaviruses may influence risk of tumours of the mouth and throat. To investigate this we need the participation of patients with oral tumours. You have been contacted by our nurse because you have been diagnosed with one of these lesions in the mouth or throat that we need to investigate further.

This investigation is being done by clinical and basic scientists at this hospital and at McGill University. Similar studies are being conducted simultaneously in many hospitals in Europe, Asia, and South America, all of which are coordinated by the World Health Organization.

What is necessary?

If you consent, our nurse will collect a sample of cells from your mouth using a soft toothbrush and you will be asked to perform a mouthwash with saline solution. The fluid that is collected will be taken to the laboratory for a variety of tests to detect abnormal cells and to analyze some of your own defences against carcinogens.

We will also ask you to donate a small blood sample (10 ml or, equivalently, about 2 teaspoons) that will be tested subsequently for antibodies against papillomaviruses. Your doctor will perform a biopsy of your lesion that will be checked for tumour cells. We ask your permission to allow us to test the biopsy tissue for signs of infection by human papillomaviruses and for mutations in certain genes.

You will also be interviewed by our nurse who will check if you have one or more of the risk factors for cancer of the mouth and throat that we are studying. This interview should take 20-30 minutes.

Benefits:

There are no direct personal benefits for you consequent to your participation in the study. By participating, however, you will be contributing to our understanding of what causes oral cancers. Our results could help us to design new laboratory tests to diagnose the oral tumours before they become malignant, allowing us to cure these lesions. This will help future patients at risk of developing oral cancers.

Risks:

There are no potential risks from participation in the study. The collection of the blood sample may cause some discomfort, however, and you may have to spend about 30-40 min altogether with our nurse for the interview and for the brief oral examination. During the interview you will

be asked a number of questions about family life, lifestyle, and sexual activity, some of which of a sensitive nature.

Confidentiality:

The results from the analyses of your blood sample, oral cells, and of the biopsy, as well as the responses you gave during the interview will be treated very confidentially. No names or other information that could identify you as a patient will be released. All the data from this study will be analyzed in statistical form only.

Your rights:

You may refuse to participate in the study now or later, without any negative consequences. Nothing will change in terms of the quality of health care that you are receiving in this hospital. During the interview you may also refuse to answer any questions with which you do not feel comfortable.

There are no costs to you, direct or indirect. All the tests will be paid out of research funds that our scientific team received to investigate the causes of oral cancer.

Additional information:

If you would like to obtain additional information about this study you may call Dr. J. Pintos or Dr. E. Duarte at

Your consent:

I understand the general purpose of the study, what will be required of me, and my rights as a participant. I consent to participate in the study. My participation is voluntary and if I agree to participate I may withdraw my consent and discontinue my participation from the study at any time without prejudice or loss of benefits to which I am otherwise entitled. I understand that my participation may be terminated with or without my consent.

Patient's name:	Signature:	Date:
Nurse:	Signature:	Date:

McGill University Study of Oral Health

Informed Consent Form

Purpose:

We are studying whether infection of the oral cavity by papillomaviruses may influence risk of tumours of the mouth and throat. To investigate this we need the participation of patients with oral tumours as well as of patients with any other diseases except cancer, the latter to serve as a control group. You have been contacted by our nurse because you could be one of these control patients.

This investigation is being done by clinical and basic scientists at this hospital and at McGill University. Similar studies are being conducted simultaneously in many hospitals in Europe, Asia, and South America, all of which are coordinated by the World Health Organization.

What is necessary?

If you consent, our nurse will collect a sample of cells from your mouth using a soft toothbrush and you will be asked to perform a mouthwash with saline solution. The fluid that is collected will be taken to the laboratory for a variety of tests to detect abnormal cells and to analyze some of your own defences against carcinogens.

We will also ask you to donate a small blood sample (10 ml or, equivalently, about 2 teaspoons) that will be tested subsequently for antibodies against papillomaviruses.

You will also be interviewed by our nurse who will check if you have one or more of the risk factors for cancer of the mouth and throat that we are studying. This interview should take 20-30 minutes.

Benefits:

There are no direct personal benefits for you consequent to your participation in the study. By participating, however, you will be contributing to our understanding of what causes oral cancers. Our results could help us to design new laboratory tests to diagnose oral tumours before they become malignant, allowing us to cure these lesions. This will help future patients at risk of developing oral cancers.

Risks:

There are no potential risks from participation in the study. The collection of the blood sample may cause some discomfort, however, and you may have to spend about 30-40 min altogether with our nurse for the interview and for the brief oral examination. During the interview you will be asked a number of questions about family life, lifestyle, and sexual activity, some of which of a sensitive nature.

Confidentiality:

The results from the analyses of your blood sample and oral cells, as well as the responses you gave during the interview will be treated very confidentially. No names or other information that could identify you as a patient will be released. All the data from this study will be analyzed in statistical form only.

Your rights:

You may refuse to participate in the study now or later, without any negative consequences. Nothing will change in terms of the quality of health care that you are receiving in this hospital. During the interview you may also refuse to answer any questions with which you do not feel comfortable.

There are no costs to you, direct or indirect. All the tests will be paid out of research funds that our scientific team received to investigate the causes of oral cancer.

Additional information:

If you would like to obtain additional information about this study you may call Dr. J. Pintos or Dr. E. Duarte at

Your consent:

I understand the general purpose of the study, what will be required of me, and my rights as a participant. I consent to participate in the study. My participation is voluntary and if I agree to participate I may withdraw my consent and discontinue my participation from the study at any time without prejudice or loss of benefits to which I am otherwise entitled. I understand that my participation may be terminated with or without my consent.

Patient's name:	Signature:	Date:
Nurse:	Signature:	Date: