ARTICLES

Oral Cancer Risk in Relation to Sexual History and Evidence of Human Papillomavirus Infection

Stephen M. Schwartz, Janet R. Daling, David R. Doody, Gregory C. Wipf, Joseph J. Carter, Margaret M. Madeleine, Er-Jia Mao, E. Dawn Fitzgibbons, Shixuan Huang, Anna Marie Beckmann, James K. McDougall, Denise A. Galloway

Background: **Experimental models and analyses of human tumors suggest that oncogenic, sexually transmittable human papillomaviruses (HPVs) are etiologic factors in the development of oral squamous cell carcinoma (SCC). We conducted a population-based, case–control study to determine whether the risk of this cancer is related to HPV infection and sexual history factors.** ***Methods:*** **Case subjects (n = 284) were 18–65-year-old residents of three counties in western Washington State who were newly diagnosed with oral SCC from 1990 through 1995. Control subjects (n = 477) similar in age and sex were selected from the general population. Serum samples were tested for HPV type 16 capsid antibodies. Exfoliated oral tissue collected from case and control subjects and tumor tissue from case subjects were tested for HPV DNA. Odds ratios (ORs) were calculated after adjusting for age, sex, cigarette smoking, and alcohol consumption.** ***Results:*** **Among males only, oral SCC risk increased with self-reported decreasing age at first intercourse, increasing number of sex partners, and a history of genital warts. Approximately 26% of the tumors in case subjects contained HPV DNA; 16.5% of the tumors contained HPV type 16 DNA. The prevalence of oncogenic HPV types in exfoliated oral tissue was similar in case and control subjects. The ORs for HPV type 16 capsid seropositivity were 2.3 (95% confidence interval [CI] = 1.6–3.3) for all oral SCCs and 6.8 (95% CI = 3.0–15.2) for oral SCCs containing HPV type 16 DNA. The joint association of cigarette smoking and HPV type 16 capsid seropositivity with oral SCC (OR = 8.5; 95% CI = 5.1–14.4) was stronger than predicted from the sum of individual associations with current smoking (OR = 3.2; 95% CI = 2.0–5.2) and seropositivity (OR = 1.7; 95% CI = 1.1–2.6).** ***Conclusions:*** **HPV type 16 infection may contribute to the development of a small proportion of oral SCCs in this population, most likely in combination with cigarette smoking. [J Natl Cancer Inst 1998;90:1626–36]**

Human papillomaviruses (HPVs) are small DNA viruses of which more than 70 different types have been identified based on DNA sequence variation *(1).* Genital infection with high-risk HPV types appears to be a cause of virtually all cases of cervical neoplasia and probably underlies the development of a substantial proportion of vulvar, vaginal, anal, and penile carcinomas *(1).* Mucosatropic HPV strains can infect and cause benign lesions in the human upper aerodigestive tract *(2),* and the possibility that HPVs known to cause genital carcinomas may also contribute to the pathogenesis of upper aerodigestive tract cancers was raised more than a decade ago with initial reports of cytologic and molecular evidence of HPV infection in oral carcinomas *(3–5).* Accumulating molecular and pathologic evidence has strengthened the hypothesis that HPV infection could play a role in the etiology of some oral squamous cell carcinomas (SCCs) *(6–13).*

In contrast to cervical and other genital cancers, there are limited epidemiologic data addressing the relationship between HPV infection and the development of oral cancer. Several studies *(14–16)* have observed a higher prevalence of HPV DNA in tumors of patients with oral cancer compared with normal tissue from the same patient or to normal tissue in patients with other oral conditions, but these investigations lacked a formal epidemiologic design and analysis. For example, such studies have not considered either confounding by, or interactions with, tobacco and alcohol use, which contribute to the development of more than 75% of oral cancers in the United States *(17).* Results from rigorously designed epidemiologic studies have yielded conflicting results. In a prior case–control study conducted by members of our group, the detection of HPV type 6 DNA and the detection of HPV type 16 DNA in exfoliated oral cavity cells were both strongly associated with oral cancer risk *(18).* In contrast, a nested case–control study found that serologic antibody response to the HPV type 16 capsids was not associated with an

Affiliations of authors: S. M. Schwartz, J. R. Daling, M. M. Madeleine, Program in Epidemiology, Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, and Department of Epidemiology, School of Public Health and Community Medicine, University of Washington, Seattle; D. R. Doody, E. D. Fitzgibbons (Program in Epidemiology), G. C. Wipf, J. J. Carter, E.-J. Mao, S. Huang, A. M. Beckmann (Program in Cancer Biology), Division of Public Health Sciences, Fred Hutchinson Cancer Research Center; J. K. McDougall, Program in Cancer Biology, Division of Public Health Sciences, Fred Hutchinson Cancer Research Center and Department of Pathology, School of Medicine, University of Washington; D. A. Galloway, Program in Cancer Biology, Division of Public Health Sciences, Fred Hutchinson Cancer Research Center and Department of Microbiology, School of Medicine, University of Washington.

Correspondence to: Stephen M. Schwartz, Ph.D., Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave., N. (MP-381), P.O. Box 19024, Seattle, WA 98109-1024.

See ''Notes'' following ''References.''

increased risk of oral cancer *(19)*. We report the results of a second population-based, case–control study testing the hypothesis that HPV infection is a risk factor for oral squamous cell cancer.

PATIENTS AND METHODS

Subject Eligibility, Identification, and Recruitment

Eligible case subjects were 18–65-year-old male and female residents of King, Pierce, and Snohomish counties, Washington State, who were diagnosed with incident histologically confirmed SCC of the oral cavity between 1990 and 1995. We identified case subjects through the files of the Cancer Surveillance System (CSS), a participant in the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER)[1] Program. Oral tumors, whether *in situ* or invasive, were included if they arose in one of the following sites: tongue, gum, floor of mouth, other and unspecified part of the mouth, tonsils, or oropharynx. Information on age, stage, site, and histologic type was obtained from the files of the CSS.

Eligible control subjects were 18–65-year-old male and female residents of King, Pierce, and Snohomish counties, Washington State, between 1990 and 1995 who had no history of oral cancer. We sought three control subjects for every two case subjects, similar to the sex and age distribution (18–19, 20–24, 25–29, 30–34, 35–39, 40–44, 45–49, 50–54, 55–59, and 60–65 years) of the case subjects. Potential control subjects were ascertained using random-digit telephone dialing (RDD) *(20,21)*. Briefly, telephone numbers were generated using equal probability random sampling from among all working telephone prefixes in the study areas. Each number was called to determine whether or not it was a residence; numbers that were not answered initially were called again during the day, evenings, and weekends. When a residence was reached, a household census was conducted to determine if an individual who was eligible on the basis of age and county of residence lived in the household. A household census was conducted for 93.1% of the residences.

To recruit a case subject, we first wrote to his or her follow-up physician. For case subjects diagnosed prior to 1992, written or verbal approval from the physician was required to contact each case subject. For case subjects diagnosed from 1992 onward, written or verbal permission was not required but we did not initiate patient recruitment if the follow-up physician notified us that the patient should not be approached. We sent case subjects and control subjects identified through RDD an introductory letter followed by a telephone call from an interviewer. Of 449 case subjects ascertained from the CSS files, we did not attempt to contact 28 (6.2%) at the request of the physician, 69 (15.4%) died before we could recruit them, 45 (10.0%) refused to participate, and 23 (5.1%) were too ill or could not located. Thus, 284 (63.3%) case subjects participated in the study. Of the 284 case subjects that participated, 261 (91.9%) were diagnosed with invasive cancer and 23 (8.1%) were diagnosed with carcinoma *in situ*. Of 729 potential control subjects ascertained through RDD, 234 (32.1%) refused to participate either by requesting that we not send an introductory letter (n = 51) or by refusing after receiving the introductory letter (n = 183). An additional 18 (2.5%) potential control subjects could not be located. Thus, 477 (65.4%) of the potential 729 control subjects participated in this study. The overall response rate for control subjects, calculated as the product of the RDD household screening rate and the interview participation rate, was 60.9%.

The protocol for recruitment of case and control subjects was approved by the institutional review board of the Fred Hutchinson Cancer Research Center.

Data Collection

All data collection activities were conducted following written informed consent from each case or control subject.

Interviews. Case and control subjects participated in structured in-person interviews through which we elicited information on demographic characteristics, lifetime histories of tobacco and alcohol use, clinical history of sexually transmitted diseases, and histories of sexual activity and sexual practices. All questions were directed toward the time period prior to each participant's reference date. The reference date for a case subject was the month and year he or she was diagnosed. Since we did not pair individual control subjects with individual case subjects, the reference date for a particular control subject was assigned at random from among the possible case subject diagnosis dates that had occurred prior to the selection of the control through RDD. Demographic characteristics ascertained included marital status as of reference date, race, highest level of school attended, and combined family income in the reference year. Cigarette smoking data included whether a person had ever smoked more than 100 cigarettes, and if so, details regarding continuous periods (episodes) during which smoking habits (e.g., packs per day) were relatively unchanged. Episodes were delineated according to ages at which each case or control subject reported major changes in smoking habits. Case and control subjects were also asked whether or not they had used smokeless tobacco (chewing tobacco, snuff, or mini-pouches), cigars, or pipes. We ascertained each participant's history of alcohol consumption using an approach similar to that for ascertaining cigarette smoking history. Thus, among participants who reported having had at least four alcoholic beverages in any 1 year, we elicited separate age-defined periods in which alcohol consumption (e.g., frequency of drinking, number of drinks) was relatively unchanged. We asked each participant his or her age at first regular intercourse (where ''regular'' was defined as three or more times per month). Participants who had had any heterosexual relationships were asked about the lifetime number of opposite sex sexual partners, whether oral sex had been performed on any opposite sex partner, and the total number of opposite sex oral sex partners. Each male was asked about his sexual orientation since puberty (exclusively heterosexual, primarily heterosexual, heterosexual and homosexual, primarily homosexual, or exclusively homosexual). We also asked case and control subjects about histories of genital warts and oral warts.

Biologic specimens. Each participating case and control subject was asked to provide a venous blood sample. Aliquots of serum were stored at −70 °C until thawed for assays of antibody response to HPV type 16 capsids (*see below*). In addition, we collected a sample of exfoliated oral tissue according to the following protocol: with the use of a soft-bristled toothbrush, the interviewer performed five complete backward and forward brushes of each of the inside of the upper and lower lips, the left and right sides of the hard palate, buccal mucosa, top and bottom of the tongue, and the surface of the gingiva. After each of these areas was brushed, the toothbrush was squeezed on the side of a tube containing a buffer of 0.5% sodium dodecyl sulfate, 1 m*M* EDTA, and 10 m*M* Tris. The sides of the tube were then rinsed with buffer. The participant proceeded to rinse his or her mouth with 0.5 oz tap water and spat into the tube. Tubes containing the collected material were then frozen at −70 °C prior to DNA extraction and viral genotyping (*see below*). Among those men and women who participated in the in-person interview, we obtained blood specimens from 260 (91.5%) of 284 case subjects and 448 (93.9%) of 477 control subjects and exfoliated oral tissue for 265 (93.3%) of 284 case subjects and 468 (98.1%) of 477 control subjects. Blood and exfoliated oral tissue were collected at the time of the in-person interviews (median time following diagnosis, 8 months). We attempted to obtain paraffin-embedded specimens of primary oral tumors for each case subject who participated in the in-person interview. Since some, but not all, studies have suggested that the presence of HPV DNA in oral carcinoma is associated with a favorable prognosis *(22–25)*, we also attempted to obtain primary oral tumors for each case subject who could not be interviewed because of death prior to recruitment. We selected tumor blocks containing malignant tissue to be tested for HPV DNA sequences (*see below*) based on review of the accompanying pathology report. We obtained one or more tumor specimens for 300 (87.2%) of 344 case subjects; the proportion obtained was higher for interviewed case subjects (89.1% of 284) than for case subjects not interviewed because of death prior to recruitment (78.3% of 60).

Laboratory Methods

We extracted DNA from the exfoliated oral tissue as follows: Tubes containing the specimens were thawed and centrifuged at 1500*g* for 30 minutes at 4 °C and the supernatant was discarded. The pellet was then washed by adding 5 mL 1× phosphate-buffered saline (PBS) followed by vortexing. This step was repeated, 3 μL proteinase K (10 mg/mL) was added to the tube containing the 300-μL sample, and the tube was incubated 4–6 hours at 55 °C or at 37 °C overnight. An equal volume of phenol (300 μL) was added followed by vortexing. The tubes were briefly centrifuged again and the phenol (lower layer) was discarded. We then added 10 μL of 3 *M* sodium acetate, pH 8.0, 600 μL ethanol, and 1.2 μL of 10 mg/mL transfer RNA to the sample tube. The tubes were incubated at 70 °C for 2 hours or at 20 °C overnight. The tubes were then centrifuged at 12 000*g* for 30 minutes at 4 °C after which the liquid was discarded. The pellet was dried in a vacuum centrifuge for 20 minutes and then redissolved in 100 μL H_2O. Finally, the tube was incubated at 95 °C for 8 minutes to inactivate the proteinase K. DNA was extracted from tumor tissue using standard methods for paraffin-embedded specimens *(26)*.

We used oligonucleotide primers (MY09/MY11) complementary to highly conserved sequences in the L1 region of HPV *(27)*, as well as primers complementary to the respective E6 regions of HPV DNA types 6, 11, 16, and 18, to amplify HPV DNA from exfoliated oral tissue and tumor tissue using the polymerase chain reaction (PCR) *(28)*. Amplification reactions were performed using standard conditions previously described *(29)*. Positive controls included both 0.1 and 1.0 pg of HPV plasmid DNA for each HPV type tested. Negative controls were 1.0 μg of human placental DNA. Reaction controls consisting of only PCR buffer (i.e., no sample DNA) were also included in each PCR series to ensure that no contamination occurred during the setup of the reactions. The quality of the DNA for allowing PCR amplification of HPV DNA was determined by a PCR test for a fragment of the human β-globin gene *(30)*. If a DNA specimen tested negative for the β-globin fragment following repeated purifications, that specimen was excluded from further PCR analyses. To ensure the sensitivity and specificity of the PCR, 10 μL of the amplification reaction products were electrophoresed through agarose gels, transferred to nylon membranes, and hybridized with oligonucleotide probes. These probes were derived from sequences internal to the consensus primer pairs *(31)*. Specimens positive for the HPV L1 consensus probes were then typed by sequential hybridizations with probes for HPV types 6, 11, 16, 18, 31, 33, and 35 *(31)*. In all analyses, probes for HPV type 31 DNA, HPV type 33 DNA, and HPV type 35 DNA were combined in a single ''cocktail'' due to their expected infrequent occurrence; in this article we refer to this HPV type as HPV 31/33/35. DNA samples from case and control subjects were included in random sequence within each genotyping batch and blinded as to participant characteristics (e.g., case or control subject status, interview data). For genotyping of DNA from tumor tissue, the laboratory technicians were blinded to the characteristics of the case subjects.

We used an antibody capture enzyme-linked immunosorbent assay (ELISA) to test sera from case and control subjects for antibody response to capsids (also known as virus-like particles) made from conformationally correct HPV type 16 L1 protein. Briefly, we used a vaccinia virus expression system to produce HPV type 16 L1 proteins that self-assemble into capsids. Ninety-six-well ELISA plates (Dynatech Immulon 2 HB; Dynex Technologies, Chantilly, VA) were coated with monoclonal antibody H16V5 raised against the HPV type 16 capsids. The monoclonal antibody was provided by N. Christensen, Milton S. Hershey Medical Center, Hershey, PA. The coated test wells contained HPV type 16 capsids diluted in blocking solution (5% goat serum, 0.05% Tween 20 in PBS), while coated blank wells contained blocking solution without capsids. For each case and control subject, serum was diluted 1/100 in blocking solution and 50 μL was added to three test wells and three blank wells. Plates were incubated at 37 °C for 1 hour and washed with PBS. Goat anti-human immunoglobulin G with conjugated alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, IN) was diluted in blocker and added to the wells, and plates were incubated at 37 °C for 1 hour. After washing with PBS, the plates were developed in the dark at room temperature for 30 minutes by adding phosphatase substrate in buffer. The optical density (OD) was read at 405 nm on an automated plate reader.

Previously unthawed serum aliquots from case and control subjects were provided to the laboratory in random order, blinded as to participant characteristics (e.g., case or control subject, interview data, HPV genotyping results), and analyzed using a single batch of HPV capsids, monoclonal antibodies, and plates. Each plate also contained quality-control aliquots consisting of pooled sera previously observed to produce high values for antibody response to HPV 16 capsids. Plates also contained sera from a series of 25 college-aged women from the University of Washington, Seattle, who reported no history of sexual activity and had no evidence of genital HPV infection as determined by PCR. The ELISA results from this population were used to define seropositivity (*see below*).

We tested DNA extracted from exfoliated oral cells for 257 of 265 case subjects and 461 of 468 control subjects who provided these specimens and tumor blocks from 262 (87.3%) of the 300 case subjects for whom blocks were obtained. We were able to amplify the β-globin gene fragment from DNA extracted from exfoliated oral cells of 237 (92.2%) of 257 case subjects and 435 (94.4%) of 461 control subjects tested and from at least one block of the primary tumors of 253 (96.6%) of 262 case subjects tested. HPV genotyping results for tumors of five of the 253 case subjects were excluded because only tissue samples from metastases were available for testing, leaving 248 tumors for statistical analyses. HPV type 16 capsid antibody response results were available for 259 case subjects and 446 control subjects.

Data Analysis

We created analytic variables to describe each case and control subject's cigarette smoking status as follows: status as of reference date (current smoker, former smoker, or never smoker), recency of cigarette smoking (among former cigarette smokers), total years smoked cigarettes, and total pack-years of cigarette smoking. Similarly, we classified case and control subjects according to their consumption of alcoholic beverages: status as of reference date (current, former, or never), lifetime average number of alcoholic beverages consumed per week, and lifetime total number of alcoholic beverages consumed. For analyses of sexual history other than sexual orientation among males, we excluded case subjects (three males and seven females) and control subjects (four females) who reported never having sexual intercourse and eight male case subjects and eight male control subjects who reported not having exclusively heterosexual relationships. (We did not elicit histories of homosexual activity of female participants.)

We categorized the HPV genotyping results according to whether or not the tissue contained any HPV DNA and, among those with HPV DNA, whether a high-risk type (types 16, 18, and 31/33/35) was detected or whether only low-risk types (types 6 and 11) were detected. Thus, a person categorized as having a high-risk HPV DNA type detected in his or her tissue may also have had low-risk HPV DNA type detected, whereas a person categorized as having low-risk HPV DNA detected in his or her tissue only had low-risk HPV DNA detected. Since multiple tumor specimens were available for many cases, we considered a case subject's tumor specimen to be positive for a particular HPV DNA type if any of the specimens were positive for that type. In some instances, the HPV DNA type was unknown because we were able to amplify the L1 region with consensus primers but did not observe hybridization with L1 region type-specific primers.

For each participant's serum sample, we calculated an ELISA value as the difference between the mean of the log-transformed OD readings of the three test wells and the mean of the log-transformed OD readings of the three blank wells. Log transformation was used to reduce the influence of extreme OD readings on the computation of ELISA values. The cutoff point for the ELISA values was calculated from the sera of 25 college-aged women with no history of sexual activity and without evidence of genital HPV infection by PCR. The ELISA values were scored as positive if they exceeded the ELISA value plus two standard deviations [−0.0604 + (2 × 0.1347)] calculated from the sera of the 25 women. This method of cutoff-point determination was based on the assumption that the respective distributions of ELISA values for infected and uninfected persons overlap and are normally distributed, and that overlapping distributions will misclassify about 5% of uninfected women as infected.

We used standard methods for the statistical analysis of case–control studies *(32)*. The odds ratio (OR) for the association between oral cancer risk and factors under study was estimated using unconditional logistic regression. Ninety-five percent confidence intervals (CIs) were calculated using the standard errors from the corresponding logistic regression models and the normal approximation. All reported *P* values were two-sided. Unless otherwise stated, ORs presented are adjusted for age, cigarette smoking (continuous pack-years), alcohol consumption (continuous average number of alcoholic beverages consumed/week during lifetime), and sex (in analyses combining males and females). Analyses in which we adjusted for cigarette smoking and alcohol consumption using other indices of these behaviors (e.g., current, past, or never cigarette smoking) yielded very similar results. Additional adjustment for race or measures of socioeconomic status (income and education) did not affect the results. In examining associations with antibody response to HPV type 16 capsids, we began by comparing the ELISA values between case and control subjects using nonparametric methods because the distributions were not normal. We then estimated the OR associated with seropositivity for all case subjects combined as well as separately for case subjects according to HPV DNA type found in tumor tissue (any HPV type 16 DNA, only HPV type 6 or 11 DNA, or no HPV DNA) and tumor site (tongue, tonsils, or floor of mouth).

To determine whether the association between antibody response to HPV type 16 capsids and oral cancer was modified by either cigarette smoking or alcohol use, we used the approach described by Rothman *(33)* to create indicator variables representing the combination of these factors. From logistic regression models containing these indicator variables as well as covariates, we calculated the Synergy Index (S) *(33)* to quantify the joint association of antibody response to HPV type 16 capsids and either smoking or alcohol consumption with oral cancer risk. Values of S that are greater than 1 are consistent with statistical interaction on an additive scale. That is, the combination of two causal factors in

some persons in the population results in the development of more cases of disease than predicted based on the sum of the added risks associated with exposure to the individual factors. We computed 95% CIs on S by estimating the variances and covariances of the fitted coefficients of the indicator variables and applying formulas developed by Hosmer and Lemeshow *(34)*.

RESULTS

Case and control subjects were similar with respect to race (Table 1). Low income, but not education, was strongly associated with oral cancer after adjustment for cigarette smoking and alcohol use. Risk was increased to a greater extent among current cigarette smokers than among past cigarette smokers; within these groups, the risk was highest among those persons who had smoked greater than or equal to 20 pack-years. The risk of oral cancer increased with increasing levels of alcohol consumption. The risk of oral cancer associated with the combination of heavy smoking (≥20 pack-years) and heavy drinking (≥15 drinks/week) was approximately 11-fold, exceeding that predicted based on the sum of these factors alone (Table 1). Among men, prior smokeless tobacco use was similar between case and control subjects (6.7% and 5.6%, respectively) (OR = 1.0; 95% CI = 0.4–2.3). Only one female (a control subject) reported smokeless tobacco use.

Table 1. Demographic characteristics, cigarette smoking, and alcohol consumption among oral cancer case subjects and control subjects, total and stratified by sex

	Males		Females		Total		
Characteristic	Case subjects, % (n = 165)	Control subjects, % (n = 302)	Case subjects, % (n = 119)	Control subjects, % (n = 175)	Case subjects, % (n = 284)	Control subjects, % (n = 477)	OR* (95% CI)
Age, y							
18–39	8.5	9.6	5.0	10.3	7.0	9.9	—
40–49	25.5	27.2	11.8	15.4	19.7	22.9	—
50–59	38.2	33.8	39.5	36.0	38.7	34.6	—
60–65	27.9	29.5	43.7	38.2	34.5	32.7	—
Race							
White†	91.5	92.7	95.8	94.9	93.3	93.5	1.0 —
African-American	4.9	2.3	1.7	3.4	3.5	2.7	1.2 (0.5–3.0)
Other	3.6	5.0	2.5	1.7	2.9	3.8	1.0 (0.4–2.5)
Highest level of school attended							
Graduate school	10.3	17.6	7.6	13.1	9.2	15.9	1.0 (0.6–1.7)
College†	38.8	54.6	42.9	42.9	40.5	50.3	1.0 —
Technical school	8.5	6.3	6.7	4.6	7.8	5.7	1.4 (0.7–2.6)
High school or less	42.4	21.5	42.8	39.4	42.6	28.1	1.3 (0.9–1.9)
Annual household income							
<$15 000	18.8	5.0	23.5	5.1	20.8	5.0	4.2 (2.2–8.1)
$15 000–$29 999	21.2	14.6	27.7	30.3	23.9	20.3	1.5 (0.9–2.5)
$30 000–$44 999	26.1	22.9	25.2	26.3	25.7	24.1	1.5 (0.9–2.5)
$45 000–$59 999	14.6	26.8	14.2	17.7	14.5	23.5	1.0 (0.6–1.8)
≥$60 000†	17.6	29.1	6.7	17.7	13.0	25.0	1.0 —
Refused/unknown	1.8	1.7	2.5	2.8	2.1	2.1	—
Cigarette smoking‡							
Never†	15.2	31.3	18.8	50.3	16.7	38.3	1.0 —
Past: 1–19 pack-years	10.9	24.7	7.7	24.6	9.6	24.6	0.9 (0.5–1.6)
Past: ≥20 pack-years	17.0	19.0	13.7	5.1	15.6	13.9	2.5 (1.5–4.3)
Current: 1–19 pack-years	3.6	3.3	7.7	6.9	5.3	4.6	2.2 (1.0–4.5)
Current: ≥20 pack-years	53.3	21.7	52.1	13.1	52.8	18.5	5.5 (3.5–8.6)
Alcoholic beverage consumption§							
<1 drinks/wk†	10.3	15.2	24.4	49.7	16.2	27.9	1.0 —
1–7 drinks/wk	21.8	45.0	42.0	40.6	30.3	43.4	1.1 (0.7–1.8)
8–14 drinks/wk	16.4	17.2	16.8	5.7	16.6	13.0	1.7 (1.0–3.1)
15–29 drinks/wk	17.6	13.9	12.4	1.7	15.5	9.4	2.1 (1.2–4.0)
≥30 drinks/wk	33.9	8.6	4.2	2.3	21.5	6.3	4.0 (2.1–7.7)
Cigarette smoking‡ and alcohol beverage consumption§ combined							
<20 pack-years, <15 drinks/wk†	23.6	49.7	31.6	80.0	27.0	60.8	1.0 —
<20 pack-years, ≥15 drinks/wk	6.1	9.7	2.6	1.7	4.6	6.7	1.9 (0.9–4.0)
≥20 pack-years, <15 drinks/wk	24.9	27.7	51.3	16.0	35.8	23.4	4.0 (2.7–5.9)
≥20 pack-years, ≥15 drinks/wk	45.5	13.0	14.5	2.3	32.6	9.1	11.2 (6.9–18.1)

*Odds ratios (ORs) are computed for males and females combined. ORs for education and income are adjusted for age (continuous), sex, pack-years of cigarette smoking (continuous), and average number of alcoholic beverages per week (continuous); ORs for pack-years of cigarette smoking are adjusted for age (continuous), sex, and average number of alcoholic beverages per week (continuous); ORs for average number of alcoholic beverages per week are adjusted for age (continuous), sex, and pack-years of cigarette smoking (continuous); ORs for cigarette and alcohol consumption combined are adjusted for age (continuous) and sex.

†Reference group for OR calculation. For cigarette smoking and alcohol consumption, the reference groups are those with the lowest level of exposure. For demographic characteristics, the category with the highest frequency among control subjects was chosen as the reference group.

‡Excludes two case subjects and two control subjects for whom pack-years of cigarette smoking could not be determined.

§Average alcohol consumption over lifetime.

HPV DNA was detected in 25.8% (64 of 248) of the tumors of the case subjects for whom we obtained and successfully tested specimens. The subset of tumors from interviewed case subjects had a similar prevalence of HPV DNA in tumors (58 [27.4%] of 212). Forty-three (67.2%) of 64 of the HPV DNA-positive tumors contained a high-risk HPV type; 41 (95.3%) of the 43 tumors with high-risk HPV DNA types contained HPV type 16 DNA either alone or with another HPV DNA type, whereas two (4.7%) contained only HPV type 18 DNA. Thus, the overall prevalence of HPV type 16 DNA was 16.5% (41 of 248 tested). The prevalence of HPV type 16 DNA was similar in males and females (Fig. 1, A). HPV 16 DNA, but not HPV type 6 or type 11 DNA, was more common in case subjects less than 50 years of age at diagnosis than case subjects greater than or equal to 50 years of age at diagnosis (Fig. 1, B). HPV type 16 DNA was detected more frequently in tonsillar carcinomas (34.1%) and oropharyngeal carcinomas (36.4%) compared with other sites with sufficient numbers for separate analysis (<15%); this difference was not observed for HPV type 6 or type 11 DNA (Fig. 1, C). Although the mean age of patients with tonsillar or oropharyngeal carcinomas (53.6 years) was similar to that for other sites (54.6 years), a greater proportion of patients with tonsillar/oropharyngeal carcinoma were less than 50 years of age (33.9% and 22.4%, respectively).

Among males, the age-, smoking-, and alcohol consumption-adjusted risk of oral cancer increased with decreasing age at first regular intercourse and increased with increasing number of opposite sex partners (Table 2). These patterns were not seen among women (Table 2). ORs were not increased among men or women who reported having ever performed oral sex on an opposite sex partner. Among both men and women with greater than or equal to five oral sex partners, however, the ORs were weakly elevated (although not statistically significant) (Table 2). Among males, a higher proportion of case subjects compared with control subjects reported a history of one or more homosexual relationships (4.2% versus 2.7%), but there was no association following adjustment for age, cigarette smoking, and alcohol consumption (OR = 1.0; 95% CI = 0.3–3.4). A history of genital warts was more often reported by male case subjects than male control subjects, leading to an approximately twofold OR following adjustment for cigarette smoking and alcohol use. There was no association with genital warts among females. Four case subjects (1.4%) and three control subjects (0.6%) reported a history of oral warts (OR = 1.6; 95% CI = 0.3–8.6). Particularly among men, the ORs in Table 2 were substantially lower than the ORs adjusted only for age, indicating that these known oral cancer risk factors were important confounders. For example, the age-adjusted OR for the association with a history of greater than or equal to 15 lifetime opposite sex partners was 3.4 (95% CI = 2.1–5.8) compared with 2.3 (95% CI = 1.1–5.0) following adjustment for age, cigarette smoking, and alcohol consumption.

For both sexes combined, the associations with sexual history were strongest for tumors containing HPV type 16 DNA. For example, for greater than or equal to 15 sex partners (compared with <15 partners), the OR for case subjects whose tumors contained HPV type 16 DNA was 2.5 (95% CI = 1.1–5.6), whereas the OR was 0.9 (95% CI = 0.2–4.3) for case subjects with tumors containing HPV type 6 or type 11 DNA and 1.2 (95% CI = 0.7–2.2) for case subjects whose tumors did not contain HPV DNA. Similarly, for a history of greater than or equal to five oral sex partners, the ORs for case subjects with HPV type 16 DNA, HPV type 6 or type 11 DNA, and no HPV DNA in tumors were 2.1 (95% CI = 0.9–4.9), 0.3 (95% CI = 0.03–3.5), and 0.9 (95% CI = 0.5–1.7), respectively.

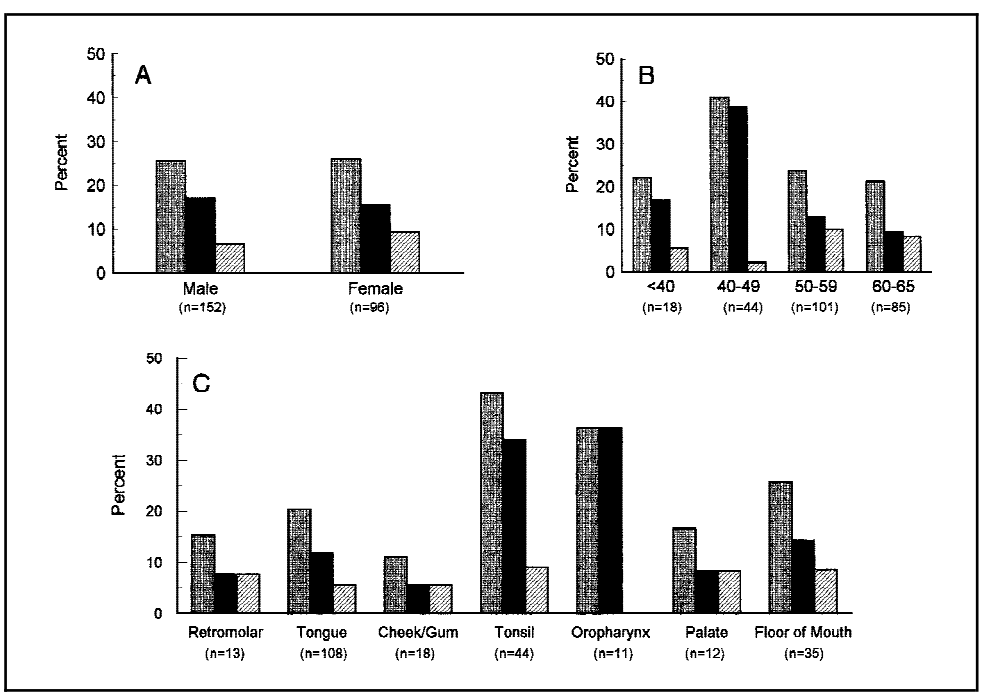

Fig. 1. Prevalence of human papillomavirus (HPV) DNA in tumor tissue of oral cancer case subjects. **A**) By sex; **B**) by age at diagnosis; and **C**) by tumor site. Gray bars = total HPV DNA; black bars = HPV type 16 DNA; and hatched bars = HPV type 6 or type 11 DNA. Includes interviewed and noninterviewed case subjects. HPV type 16 DNA tumors (n = 41) included 31 tumors with only HPV type 16 DNA and 10 tumors with HPV type 16 DNA and one or more other HPV types (seven with HPV type 6 or type 11 DNA, one with HPV type 11 and HPV type 31/33/35 DNA, one with HPV type 6 DNA and an unknown HPV DNA type, and one with unknown HPV DNA type). Two tumors contained only HPV DNA that could not be classified as to a specific type. Not included in C are two tumors arising in the uvula and two tumors for which the site was unknown.

The prevalence of HPV DNA in exfoliated oral tissue was similar in case and control subjects (9.3% versus 9.2%, respectively) (Table 3). Compared with control subjects, HPV DNA detected in exfoliated oral tissue from case subjects was slightly more likely to be of type 16, 18, or 31/33/35. However, there was no association between oral cancer risk and detection of high-risk HPV types in exfoliated oral tissue (Table 3).

Case subjects were more likely than control subjects to have high ELISA values in the HPV type 16 capsid assay (Fig. 2) (Wilcoxon rank sum test: $Z = 4.64$; $P<.0001$). Approximately one half of the case sub-

Table 2. Sexual history among oral cancer case subjects and control subjects stratified by sex

	Males			Females		
Characteristic	Case subjects, % (n = 154)	Control subjects, % (n = 294)	OR* (95% CI)	Case subjects, % (n = 112)	Control subjects, % (n = 171)	OR* (95% CI)
Age at first regular sexual intercourse, y						
⩾25†	7.8	20.1	1.0	10.7	11.2	1.0 —
20–24	38.3	44.9	1.7 (0.8–3.5)	46.4	41.2	1.3 (0.5–3.3)
18–19	20.1	20.4	1.6 (0.7–3.7)	28.6	31.2	0.6 (0.2–1.7)
<18	33.8	14.3	3.4 (1.5–7.5)	14.3	16.5	0.7 (0.2–2.1)
Refused/unknown	0.0	0.3	—	0.0	0.0	—
Lifetime number of opposite sex partners						
1†	7.8	20.8	1.0 —	33.9	38.6	1.0 —
2–4	14.9	21.8	1.3 (0.6–2.0)	28.6	32.2	0.7 (0.4–1.5)
5–14	29.9	31.3	1.5 (0.7–3.1)	31.3	24.0	0.9 (0.4–2.0)
⩾15	47.4	26.2	2.3 (1.1–5.0)	5.4	5.3	1.0 (0.3–3.8)
Refused/unknown	0.0	0.0	—	0.0	0.0	—
Ever performed oral sex on opposite sex partner						
No†	26.6	30.6	1.0 —	49.1	39.8	1.0 —
Yes	73.4	68.7	1.2 (0.7–2.8)	49.1	60.2	0.7 (0.4–1.2)
Refused/unknown	0.0	0.7	—	1.8	0.0	—
Lifetime number of opposite sex oral sex partners						
None†	26.6	30.8	1.0 —	49.1	39.8	1.0 —
1	21.4	26.7	0.8 (0.6–2.1)	22.3	33.9	0.6 (0.3–1.3)
2–4	22.7	19.9	0.9 (0.6–2.2)	18.8	21.6	0.6 (0.3–1.4)
⩾5	29.2	22.3	1.4 (0.8–2.6)	7.1	4.7	1.4 (0.4–5.2)
Refused/unknown	0.0	0.3	—	2.7	0.0	—
Prior diagnosis of genital warts						
No†	87.7	95.6	1.0 —	90.8	93.0	1.0 —
Yes	12.3	4.4	2.2 (1.0–4.9)	9.2	6.4	0.7 (0.3–2.2)
Refused/unknown	0.0	0.0	—	0.0	0.6	—

*Odds ratios (ORs) are adjusted for age (continuous), pack-years cigarette smoking (continuous), and average number of alcoholic beverages per week (continuous). *See* ''Patients and Methods'' for details on numbers of case and control subjects excluded from these analyses.

†Reference group for OR calculation.

Table 3. Oral cancer risk in relation to detection of human papillomavirus (HPV) DNA in exfoliated oral tissue

	Case subjects, %	Control subjects, %	
HPV DNA	(n = 237)	(n = 435)	OR* (95% CI)
None†	90.7	90.8	1.0 —
Any‡	9.3	9.2	0.9 (0.5–1.6)
HPV types 6 or 11	2.5	4.4	0.5 (0.2–1.4)
HPV type 16, type 18, type 31/33/35	5.9	4.1	1.3 (0.6–2.9)

*Odds ratios (ORs) are based on interviewed case and control subjects from whom exfoliated oral tissue was obtained and tested. ORs are adjusted for age (continuous), sex, pack-years of cigarette smoking (continuous), and average number of alcoholic beverages per week (continuous).

†Reference group for OR calculation.

‡HPV DNA was detected in exfoliated oral tissue from 22 case subjects and 40 control subjects. The specific HPV type could not be determined for two case subjects and three control subjects. Thus, the proportion of subjects with HPV types 6, 11, 16, 18, or 31/33/35 (8.4% of case subjects, 8.5% of control subjects) is lower than the proportion of subjects with any HPV DNA detected.

jects compared with one third of the control subjects were seropositive for antibody response to HPV type 16 capsids (OR = 2.3; 95% CI = 1.6–3.3) (Table 4). There was little evidence of confounding by cigarette smoking and alcohol consumption; the OR adjusted for age and sex alone was 2.1 (95% CI = 1.5–2.8). The association was similar among males (OR = 2.2; 95% CI = 1.4–3.4) and females (OR = 2.5; 95% CI = 1.4–4.5) and among persons less than 50 years of age (OR = 2.6; 95% CI = 1.4–4.9) and persons greater than or equal to 50 years of age (OR = 2.2; 95% CI = 1.4–3.3). The OR did not vary according to whether a case subject had blood drawn within 8 months of diagnosis or beyond that time (data not shown). Twenty-six (70.3%) of the 37 interviewed case subjects whose tumors contained HPV type 16 DNA exhibited antibody response to HPV type 16 capsids; the OR for this subset of case subjects was 6.8 (95% CI = 3.0–15.2) (Table 4). The association with HPV type 16 DNA-containing tumors was similar for persons less than 50 years of age (OR = 5.6; 95% CI = 1.7–18.9) and persons greater than or equal to 50 years of age (OR = 7.6; 95% CI = 2.4–24.0). In contrast, there was no association between antibody response to HPV type 16 capsids and oral cancer among the 15 interviewed case subjects whose tumors contained only

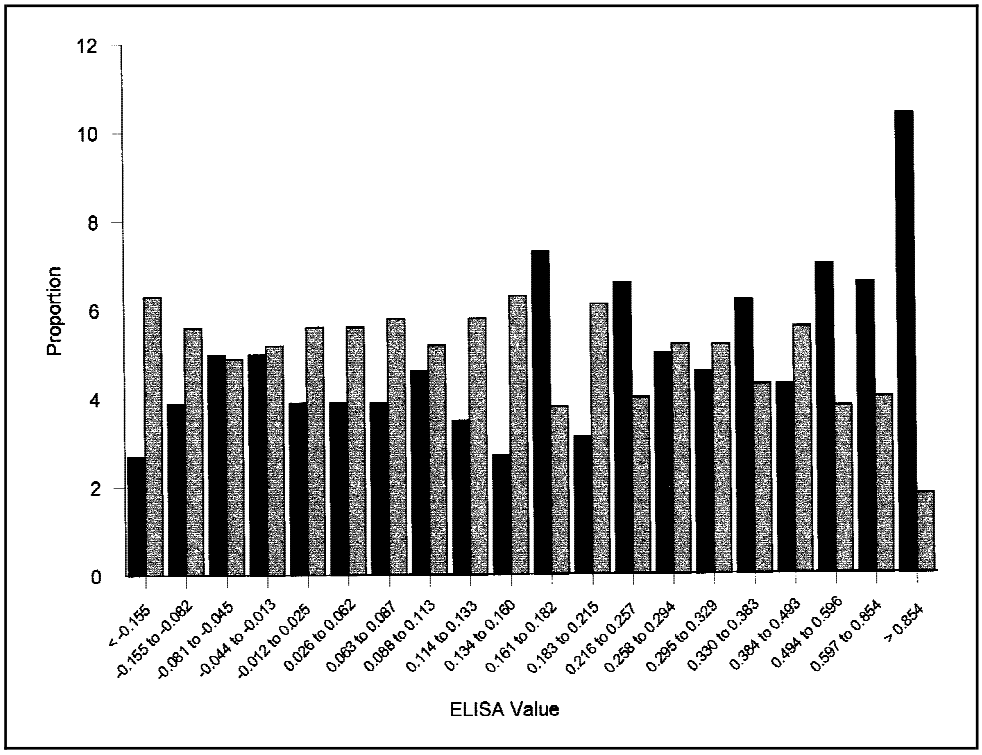

Fig. 2. Distribution of oral cancer case and control subjects by enzyme-linked immunosorbent assay (ELISA) value from the HPV type 16 capsid ELISA. Black bars = case subjects; gray bars = control subjects. ELISA value categories are based on quantiles among case and control subjects combined. The proportion within each category is computed separately for case and control subjects. The ELISA value that defined the cutoff point for seropositivity was 0.209. *See* ''Patients and Methods'' for the definition of ELISA value and determination of cutoff for seropositivity.

HPV type 6 or 11 DNA. The OR for the 139 interviewed case subjects whose tumors did not contain HPV DNA was 2.5 (95% CI = 1.6–3.8). Seropositivity to HPV type 16 capsids was not associated with carcinomas of the floor of the mouth (n = 38; OR = 1.1; 95% CI = 0.5–2.5) but was associated with carcinomas of the tongue (n = 107; OR = 2.4; 95% CI = 1.5–3.8) and tonsils (n = 49; OR = 3.9; 95% CI = 2.0–7.8).

To assess whether the associations observed with antibody response to HPV type 16 capsids were due to sexual transmission of HPV infection, we repeated the analyses above by including terms for age at first intercourse or number of sexual partners in the logistic regression models in addition to sex, age, cigarette smoking, and alcohol use. The ORs for HPV type 16 capsid antibody response and oral cancer were essentially unchanged (data not shown).

Table 5 shows the results of analyses of the joint relationship between antibody response to HPV type 16 capsids and cigarette smoking and alcohol consumption. Relative to persons who were not current smokers and did not have evidence of antibody response to HPV type 16 capsids, the risk of oral cancer was approximately eightfold among persons who were both current smokers and were seropositive. The association with the combined exposure was more than expected (S = 2.6) based on the sum of the associations between current smoking (OR = 3.2) and antibody response alone (OR = 1.7). The effect modification between HPV antibody response and either heavy smoking (⩾20 pack-years) or heavy alcohol use was less strong (S = 1.5). When heavy alcohol consumers and current cigarette smokers were considered together, the strength of the effect modification with antibody response was similar to that between current smoking and antibody response.

HPV genotyping results from both tumor tissue and exfoliated oral tissue were available for 179 case subjects. We found HPV DNA in both types of tissue for four (2.2%) case subjects and did not detect HPV DNA in either type of tissue for 115 (64.2%) case subjects. Among the 127 (70.9%) case subjects for whom no HPV DNA was detected in tumor tissue, we detected HPV DNA in the exfoliated oral tissue samples of 12 (9.4%) case subjects. We detected HPV DNA in the tumor tissue of 48 (29.4%) of the 163 case subjects for whom no HPV DNA was detected in the exfoliated oral tissue. Among the four cases for

Table 4. Risk of oral cancer associated with human papillomavirus (HPV) type 16 capsid antibody response, total and stratified by tumor HPV DNA status

		Case subjects, %*			
	Control subjects, %*	Total†	HPV16 DNA	HPV types 6 or 11 DNA	No HPV DNA
Antibody response	(n = 446)	(n = 259)	(n = 37)	(n = 15)	(n = 139)
Negative‡	65.0	48.7	24.3	66.7	47.5
Positive	35.0	51.4	75.7	33.3	52.5
OR§ (95% CI)	1.0 —	2.3 (1.6–3.3)	6.8 (3.0—15.2)	1.2 (0.4–3.8)	2.5 (1.6–3.8)

*Percentages are based on column totals.

†Sixty-eight case patients are included in total results but not included in results by HPV DNA status due to: no tumor specimens obtained (n = 28), specimens obtained but only tissue from metastases available (n = 2), specimens obtained but not analyzed by conclusion of study (n = 33), specimens analyzed but none positive for β-globin gene fragment (n = 2), and specimen obtained and tested but positive for HPV DNA other than types 6, 11, or 16 (n = 3).

‡Reference group for OR calculation.

§Odds ratios (ORs) associated with positive antibody response to HPV type 16 capsids, adjusted for age (continuous), sex, pack-years of cigarette smoking (continuous), and average number of alcoholic beverages per week (continuous).

Table 5. Risk of oral cancer associated with human papillomavirus (HPV) type 16 capsid antibody response according to cigarette smoking and alcohol consumption

Antibody response	Risk factor	Case subjects, % (n = 259)	Control subjects, % (n = 446)	OR* (95% CI)	S† (95% CI)
	Current cigarette smoking				
Negative	Not current‡	22.0	49.8	1.0 —	2.6 (1.3–5.0)
	Current	26.6	15.2	3.2 (2.0–5.2)	
Positive	Not current	20.1	28.0	1.7 (1.1–2.6)	
	Current	31.3	7.0	8.5 (5.1–14.4)	
	Pack-years cigarette smoking§				
Negative	<20 pack-years‡	13.2	43.9	1.0 —	1.5 (0.9–2.6)
	⩾20 pack-years	35.5	21.2	5.6 (3.4–9.3)	
Positive	<20 pack-years	18.5	24.5	2.8 (1.7–4.6)	
	⩾20 pack-years	32.8	10.4	10.8 (6.2–19.0)	
	Alcohol consumption‖				
Negative	<15 drinks/wk‡	33.2	57.0	1.0 —	1.5 (0.6–3.4)
	⩾15 drinks/wk	15.4	8.1	2.0 (1.1–3.6)	
Positive	<15 drinks/wk	30.1	27.1	2.3 (1.5–3.4)	
	⩾15 drinks/wk	21.2	7.9	4.4 (2.5–7.6)	
	Cigarette smoking and alcohol consumption‖				
Negative	Not current smoking or <15 drinks/wk‡	37.1	61.0	1.0 —	2.4 (0.9–6.3)
	Current smoking and ⩾15 drinks/wk	11.6	4.0	5.8 (3.0–11.1)	
Positive	Not current smoking or <15 drinks/wk	35.5	32.7	2.0 (1.4–2.8)	
	Current smoking and ⩾15 drinks/wk	15.8	2.2	15.0 (7.1–31.8)	

*All odds ratios (ORs) are based on interviewed case and control subjects for whom blood specimens were obtained and tested and are adjusted for age (continuous) and sex. ORs for joint association of HPV type 16 capsid antibody response and current smoking or pack-years of smoking are also adjusted for average number of alcoholic beverages per week (continuous). ORs for joint association of HPV type 16 capsid antibody response and alcohol consumption are also adjusted for pack-years of cigarette smoking (continuous)

†Synergy Index (S) (*33*) defined as $(OR_3 - 1)/(OR_1 + OR_2 - 2)$. OR_3 measures the association between oral cancer risk and the combination of two exposures (e.g., current cigarette smoking and antibody response to HPV capsids), while OR_2 and OR_1 measure the association between oral cancer risk and each of the individual exposures in the absence of the other (e.g., current smoking but no antibody response to HPV capsids, and antibody response to HPV capsids but not current smoking). OR_1, OR_2, and OR_3 are calculated relative to persons without either exposure (e.g., no antibody response to HPV capsids and not current smoking).

‡Reference group for OR calculation.

§Excludes two control subjects for whom pack-years of cigarette smoking could not be determined.

‖Average alcohol consumption over lifetime.

whom results from the exfoliated oral cancer tissue and tumor tissue both indicated the presence of HPV DNA, three subjects had HPV type 16 DNA in the tumor tissue and two of these had HPV type 16 DNA in the exfoliated oral tissue samples, whereas the third had HPV type 6 DNA. A fourth case had HPV type 11 DNA in the tumor tissue, but the DNA in the exfoliated oral tissue was not one of the specific types for which we probed.

Discussion

In this population-based study, we found that 16.5% (41 of 248) of oral SCCs contained HPV type 16 DNA. Case subjects were more likely than control subjects to have serologic antibody response to the HPV type 16 capsids. This association was not confounded by cigarette smoking and alcohol consumption and was similar for males and females and for younger and older persons. The approximately sevenfold increased risk of HPV type 16 DNA-containing tumors associated with antibody response to HPV type 16 capsids and the absence of an association of antibody response with HPV type 6 or type 11 DNA-containing tumors (although based on small numbers) support the hypothesis that oral SCCs containing HPV type 16 DNA may arise as a result of past HPV16 infection. In contrast to our serologic results, case subjects were not more likely than control subjects to have HPV DNA in normal exfoliated oral tissue.

These findings must be interpreted in the context of the limitations of our study. A large proportion of eligible case and control subjects did not participate in the study. Among the case subjects, death prior to recruitment was a major reason for nonparticipation, but the prevalence of HPV DNA in oral tumors of interviewed case subjects was very similar to the overall prevalence that included eligible case subjects who died prior to recruitment. Our results may also have been affected to the extent that antibody response to HPV type 16 capsids was differentially associated with participation among case and control subjects. That associations with HPV type 16 capsid antibody response were not confounded by cigarette and alcohol consumption raises the possibility that underreporting of these behaviors may have impeded our ability to adjust for differences between case subjects and control subjects in these established oral cancer risk factors. However, the strong associations we observed between oral cancer risk and both cigarette smoking and alcohol use as well as the interaction between these factors suggest that any residual confounding is likely to be small and unlikely to account for our findings.

Although molecular detection of viral DNA sequences is the gold standard for determining HPV infection, it has several limitations when comparing case subjects and control subjects in the

present study. First, because oral HPV infection tends to be focal, excision of a malignant lesion is likely to remove the oral tissue harboring HPV DNA. This likely explains the poor correlation we observed between the detection of HPV DNA in tumor tissue and the exfoliated oral tissue among case subjects. Second, studies have suggested that as yet unidentified components of saliva can inhibit PCR assays *(35)*. Third, the detection of viral DNA in normal tissue is likely to be a poor measure of the cumulative effects of a past infection. Finally, we did not obtain exfoliated oral tissue from several sites, such as the tonsils, at which tumors with HPV DNA tended to occur. We therefore incorporated a serologic approach to classify case and control subjects with respect to past HPV infection status. Among young women, serologic antibody response to HPV type 16 capsids is associated with increased sexual activity *(36)* and can be detected in 93% of individuals at 12 months following the first detection of a prevalent genital HPV type 16 infection and 67% of individuals at 18 months following an incident HPV type 16 infection *(37,38)*. In contrast, HPV type 16 capsid antibody response is absent or minimal among young women without genital HPV type 16 infection *(37)*. Additionally, prospective and cross-sectional studies have observed increased risks of cervical neoplasia and other anogenital carcinomas among persons exhibiting serologic response to HPV type 16 capsids *(39–42)*. Taken together, these data strongly suggest that serologic antibody response to HPV type 16 capsids is a marker of persistent genital HPV type 16 infection and can be useful for epidemiologic studies of cancer risk in settings where viral genotyping is not possible or is not likely to capture the relevant aspects of exposure to HPV *(39)*. Nonetheless, we do not know the extent to which an antibody response to HPV type 16 capsids results from oral HPV infection nor can we exclude the possibility that the antibody response we detected resulted from acquisition or reactivation of the virus following the development of cancer and/or treatment. Long-term prospective studies on the natural history of antibody response to HPV type 16 capsids in relation to molecular evidence of both genital and oral HPV infection are needed to refine the interpretation of these results.

Cigarette smoking and alcohol consumption are the major causes of oral SCC in the United States *(17)*. If HPV type 16 contributes to the development of oral cancer, it likely does so primarily through mechanisms that involve one or both of these factors. Our data are consistent with this prediction in that the combination of current cigarette smoking and HPV type 16 seropositivity was associated with additional cases of oral cancer beyond what would be expected by summing the risks associated with smoking and HPV type 16 seropositivity alone. Although our study was not designed to elucidate specific molecular mechanisms underlying the apparent interaction between smoking and HPV, it seems unlikely that a shared pathway would contribute to the development of oral carcinomas with tobacco carcinogen-induced inactivating mutations in the p53 tumor suppressor gene. The pathogenesis of the substantial proportion of oral carcinomas that lack p53 mutations *(43)*, however, could, in some instances, involve cell cycle abrogation by HPV E6 and E7 oncoproteins enhanced through other tobacco-induced mechanisms, such as inhibition of apoptosis by nicotine *(44–46)* or mutations in other tumor suppressor genes. Molecular epidemiologic and *in vitro* studies examining cell cycle kinetics, exposure to HPV, tobacco, alcohol, and betel quid, and expression and mutation of p53 and other candidate tumor suppressor genes (e.g., p16, pRb) are needed to clarify the joint contributions of known oral carcinogens and HPV infection to oral cancer risk in various populations.

The reported detection of HPV DNA in oral carcinoma by PCR varies widely *(23,25,47–51)*. Our results are similar to several U.S. studies reporting prevalences between 20% and 30% *(23,25,50)* but differ substantially from other investigations that found HPV DNA in more than three quarters of the tumors *(47–49,51)*. Variation among these series could be due to heterogeneity in tumor sites studied or the extent to which patients were exposed to potent oral carcinogens *(47,51)*. In addition, studies such as ours based on paraffin-embedded tissue may underestimate the proportion of tumors containing HPV DNA due to degradation of specimens *(52)*. The twofold association we observed between antibody response to HPV type 16 capsids and the risk of oral cancers lacking HPV DNA could be due, in whole or in part, to the inclusion of some tumors in that group that were truly HPV positive. On the other hand, since we did not sequence PCR products, we also cannot exclude the possibility of false-positive results *(51)*. With the use of the same molecular methods on similar archival specimens, however, we found HPV DNA in more than 70% of invasive cervical carcinomas and vulvar carcinomas *in situ* from women living in the same geographic area *(41,53)*. Taken together, our data suggest that if HPV is an oral carcinogen it contributes to a much smaller proportion of oral carcinomas than anogenital carcinomas in our population, primarily or perhaps entirely in combination with cigarette smoking.

Our population-based results bolster the impression derived from hospital-based series that tonsillar carcinomas are particularly likely to harbor HPV type 16 DNA *(25,54)*. Consistent with the high prevalence of HPV type 16 DNA, we observed that tonsillar carcinomas had the strongest association with serologic evidence of HPV type 16 infection in our study. A limitation of this finding is that up to 50% of young to middle-aged persons in our geographic area have had their tonsils removed *(55)* and we did not ascertain which of our control subjects had intact tonsils. Thus, if having intact tonsils is directly related to HPV type 16 capsid antibody response, we may have overestimated the association between tonsillar carcinoma and seropositivity. As in previous studies of HPV in tonsillar carcinomas *(25,54)*, we also did not determine whether viral gene sequences were found in the epithelial or lymphatic tissue. If the HPV DNA is largely or entirely localized in lymphatic tissue, a causal role in tonsillar carcinoma would be less likely. Arguing against this limitation, however, is that the excess HPV DNA prevalence we observed was specific for HPV type 16 DNA. High-risk HPV oncoproteins are expressed and are associated with disrupted cell cycle control in tonsillar carcinomas *(56,57)*. Thus, seroepidemiologic and molecular evidence is accumulating in support of an etiologic role for HPV type 16 in some tonsillar carcinomas.

Two previous epidemiologic studies *(18,19)* examined the association between HPV and oral cancer risk. In a study among males Maden et al. *(18)* found sixfold and threefold associations between oral cancer and detection of HPV type 16 DNA and HPV type 6 DNA, respectively, in exfoliated oral tissue. The

present study used the same protocol for oral tissue collection. However, in the prior study, only primers directed to sequences in the E6 open-reading frame were used, whereas, in the present study, primers directed to sequences in the L1 open-reading frame were also used. Thus, the methods we used in this study should have been more sensitive than in the previous study, yet among males, the prevalence of HPV type 6 DNA was lower than in the prior study (2.0% versus 18.6% for case subjects, 3.6% versus 8.9% among control subjects, respectively). The prevalence of HPV type 16 DNA was about 6% among male case subjects in both studies, but higher among control subjects in the present study than the prior study (4% versus 1%). More recently, a prospective serologic study in Finland that found a 13-fold association between HPV type 16 capsid antibody response and esophageal cancer risk did not observe any association with lip, tongue, or salivary cancer (OR = 0.6; 95% CI = 0.2–2.1) or other oral cancers (OR = 0.4; 95% CI = 0.0–7.1) *(19).* Those results may differ from ours due to inclusion of lip and salivary gland cancers (which we did not study) or if oral carcinomas in general are less likely to be related to HPV infection in Finland. Since the twofold association we observed is compatible with the CIs reported by the Finnish study, sample size differences may also account for the contrasting findings.

Oral HPV infection can result from both sexual and nonsexual viral transmission *(58).* Our analyses of sexual history addressed the question of whether sexual transmission of HPV types commonly found in the genital tract is important in the development of oral cancer. Early age at first intercourse, multiple sexual partners, and a history of genital warts were associated with oral cancer risk, but only among men. The relatively small number of women in the study and the fact that female subjects in this study tended to be older than male subjects may have reduced the statistical power for identifying associations with sexual activity in this subgroup. Nondifferential misclassification in self-reported behaviors might particularly explain the lack of associations between oral sexual practices and oral cancer risk in this and previous studies *(18).* Although we did observe that histories of multiple sexual partners and multiple oral sex partners were related to oral cancers containing HPV type 16 DNA, the strong association between HPV type 16 capsid antibody response and HPV type 16 DNA-positive oral cancer was not diminished by inclusion of these sexual history measures in our statistical models. Taken together, our findings do not provide strong evidence that a sexually transmitted route underlies oral HPV infections associated with oral cancer risk. Given the small numbers of HPV type 16 DNA-positive tumors and the likely misclassification in our measures of both prior HPV infection and sexual history, however, such a mechanism cannot reliably be excluded by our data.

Although additional molecular epidemiologic research on the contribution of HPV infection to the etiology of oral SCC is needed to confirm and extend our findings, clarifying areas of uncertainty will be challenging. Little is known regarding the frequency, natural history, and biology of oral HPV infection *in vivo.* Unlike cervical neoplasia, oral carcinomas are rare in most populations, and the identity, frequency, and rate of progression of precursor lesions are less well established. Thus, prospective epidemiologic studies, which could address the temporal relationship between oral HPV infection and the development of the earliest stages of oral malignancy, will be feasible only in a few, highly selected populations. Documenting a decline in oral cancer rates in the setting of HPV vaccine trials would provide the strongest possible causal evidence. In the interim, reducing population exposure to tobacco, betel quid chewing, and excessive alcohol consumption remain the keys to preventing the vast majority of oral carcinomas throughout the world.

REFERENCES

(1) zur Hausen H. Papillomavirus infections—a major cause of human cancers. Biochim Biophys Acta 1996;1288:F55–78.

(2) Chang F, Syrjanen S, Kellokoski J, Syrjanen K. Human papillomavirus (HPV) infections and their associations with oral disease. J Oral Pathol Med 1991;20:305–17.

(3) Syrjanen K, Syrjanen S, Lamberg M, Pyrhonen S, Nuutinen J. Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. Int J Oral Surg 1983;12:418–24.

(4) de Villiers EM, Weidauer H, Otto H, zur Hausen H. Papillomavirus DNA in human tongue carcinomas. Int J Cancer 1985;36:575–8.

(5) 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 1985;84:417–20.

(6) Snijders P, van den Brule A, Meijer C, Walboomers J. HPV and cancer of the aerodigestive tract. Papillomavirus Report 1995;6:157–62.

(7) Park NH, Min BM, Li SL, Huang MZ, Cherick HM, Doniger J. Immortalization of normal human oral keratinocytes with type 16 human papillomavirus. Carcinogenesis 1991;12:1627–31.

(8) Liu X, Han S, Baluda MA, Park NH. HPV-16 oncogenes E6 and E7 are mutagenic in normal human oral keratinocytes. Oncogene 1997;14:2347–53.

(9) Li SL, Kim MS, Cherrick HM, Doniger J, Park NH. Sequential combined tumorigenic effect of HPV-16 and chemical carcinogens. Carcinogenesis 1992;13:1981–7.

(10) Kim MS, Shin KH, Baek JH, Cherrick HM, Park NH. HPV-16, tobacco-specific *N*-nitrosamine, and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine in oral carcinogenesis. Cancer Res 1993;53:4811–6.

(11) Shin KH, Min BM, Cherrick HM, Park NH. Combined effects of human papillomavirus-18 and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine on the transformation of normal human oral keratinocytes. Mol Carcinog 1994;9:76–86.

(12) Park NH, Gujuluva CN, Baek JH, Cherrick HM, Shin KH, Min BM. Combined oral carcinogenicity of HPV-16 and benzo(*a*)pyrene: an *in vitro* multistep carcinogenesis model. Oncogene 1995;10:2145–53.

(13) Oda D, Bigler L, Lee P, Blanton R. HPV immortalization of human oral epithelial cells: a model for carcinogenesis. Exp Cell Res 1996;226:164–9.

(14) 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 1996;5:567–75.

(15) Cruz IB, Snijders PJ, Steenbergen RD, Meijer CJ, Snow GB, Walboomers JM, et al. Age-dependence of human papillomavirus DNA presence in oral squamous cell carcinomas. Eur J Cancer B Oral Oncol 1996;32B:55–62.

(16) 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 1996;16:969–73.

(17) Blot WJ, McLaughlin JK, Devesa SS, Fraumeni JF Jr. Cancers of the oral cavity and pharynx. In: Schottenfeld D, Fraumeni JF Jr, editors. Cancer epidemiology and prevention. 2nd edition. New York: Oxford University Press; 1996. p. 666–80.

(18) Maden C, Beckmann AM, Thomas DB, McKnight B, Sherman KJ, Ashley RL, et al. Human papillomaviruses, herpes simplex viruses, and the risk of oral cancer in men. Am J Epidemiol 1992;135:1093–102.

(19) 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 1995;311:1346.

(20) Hartge P, Brinton LA, Rosenthal JF, Cahill JI, Hoover RN, Waksberg J.

Random digit dialing in selecting a population-based control group. Am J Epidemiol 1984;120:825–33.
(21) Harlow BL, Davis S. Two one-step methods for household screening and interviewing using random digit dialing. Am J Epidemiol 1988;127:857–63.
(22) Chiba I, Shindoh M, Yasuda M, Yamazaki Y, Amemiya A, Sato Y, et al. Mutations in the p53 gene and human papillomavirus infection as significant prognostic factors in squamous cell carcinomas of the oral cavity. Oncogene 1996;12:1663–8.
(23) Brandwein M, Zeitlin J, Nuovo GJ, MacConnell P, Bodian C, Urken M, et al. HPV detection using ''hot start'' polymerase chain reaction in patients with oral cancer: a clinicopathological study of 64 patients. Mod Pathol 1994;7:720–7.
(24) Riethdorf S, Friedrich RE, Ostwald C, Barten M, Gogacz P, Gundlach KK, et al. 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 1997;26:315–21.
(25) 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.
(26) Wright DK, Manos MM. Sample preparation from paraffin-embedded tissues. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. San Diego: Academic Press, 1990; p. 153–8.
(27) Gravitt PE, Manos MM. Polymerase chain reaction-based methods for the detection of human papillomavirus DNA. IARC Sci Publ 1992;121–33.
(28) Jenison SA, Yu XP, Valentine JM, Koutsky LA, Christiansen AE, Beckmann AM, et al. Evidence of prevalent genital-type human papillomavirus infections in adults and children. J Infect Dis 1990;162:60–9.
(29) Beckmann AM, Acker R, Christiansen AE, Sherman KJ. Human papillomavirus infection in women with multicentric squamous cell neoplasia. Am J Obstet Gynecol 1991;165:1431–7.
(30) Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487–91.
(31) Bauer HM, Ting Y, Greer CE, Chambers JC, Tashiro CJ, Chimera J, et al. Genital human papillomavirus infection in female university students as determined by a PCR-based method. JAMA 1991;265:472–7.
(32) Thompson WD. Statistical analysis of case–control studies. Epidemiol Rev 1994;16:33–50.
(33) Rothman KJ. Modern epidemiology. Boston: Little, Brown; 1986.
(34) Hosmer DW, Lemeshow S. Confidence interval estimation of interaction. Epidemiology 1992;3:452–6.
(35) Ochert AS, Boulter AW, Birnbaum W, Johnson NW, Teo CG. Inhibitory effect of salivary fluids on PCR: potency and removal. PCR Methods Appl 1994;3:365–8.
(36) Dillner J, Kallings I, Brihmer C, Sikstrom B, Koskela P, Lehtinen M, et al. Seropositivities to human papillomavirus types 16, 18, or 33 capsids and to *Chlamydia trachomatis* are markers of sexual behavior. J Infect Dis 1996;173:1394–8.
(37) Carter JJ, Koutsky LA, Wipf GC, Christensen DN, Lee SK, Kuypers J, et al. The natural history of human papillomavirus 16 capsid antibodies among a cohort of university women. J Infect Dis 1996;174:927–36.
(38) Andersson-Ellstrom A, Dillner J, Hagmar B, Schiller J, Sapp M, Forssman L, et al. Comparison of development of serum antibodies to HPV16 and HPV33 and acquisition of cervical HPV DNA among sexually experienced and virginal young girls. A longitudinal cohort study. Sex Transm Dis 1996;23:234–8.
(39) Carter JJ, Galloway DA. Humoral immune response to human papillomavirus infection. Clin Dermatol 1997;15:249–59.
(40) Shah KV, Viscidi RP, Alberg AJ, Helzlsouer KJ, Comstock GW. Antibodies to human papillomavirus 16 and subsequent *in situ* or invasive cancer of the cervix. Cancer Epidemiol Biomarkers Prev 1997;6:233–7.
(41) Madeleine MM, Daling JR, Carter JJ, Wipf GC, Schwartz SM, McKnight B, et al. Cofactors with human papillomavirus in a population-based study of vulvar cancer [published erratum appears in J Natl Cancer Inst 1997;89:1896]. J Natl Cancer Inst 1997;89:1516–23.
(42) Bjorge T, Dillner J, Anttila T, Engeland A, Hakulinen T, Jellum E, et al. Prospective seroepidemiological study of role of human papillomavirus in non-cervical anogenital cancers. Br Med J 1997;315:646–9.
(43) Wong DT, Todd R, Tsuji T, Donoff RB. Molecular biology of human oral cancer. Crit Rev Oral Biol Med 1996;7:319–28.
(44) Wright SC, Zhong J, Zheng H, Larrick JW. Nicotine inhibition of apoptosis suggests a role in tumor promotion. FASEB J 1993;7:1045–51.
(45) Maneckjee R, Minna JD. Opioids induce while nicotine suppresses apoptosis in human lung cancer cells. Cell Growth Differ 1994;5:1033–40.
(46) Aoshiba K, Nagai A, Yasui S, Konno K. Nicotine prolongs neutrophil survival by suppressing apoptosis. J Lab Clin Med 1996;127:186–94.
(47) 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 1989;28:57–61.
(48) 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 1991;71:701–7.
(49) Woods KV, Shillitoe EJ, Spitz MR, Schantz SP, Adler-Storthz K. Analysis of human papillomavirus DNA in oral squamous cell carcinomas. J Oral Pathol Med 1993;22:101–8.
(50) Holladay EB, Gerald WL. Viral gene detection in oral neoplasms using the polymerase chain reaction. Am J Clin Pathol 1993;100:36–40.
(51) Balaram P, Nalinakumari KR, Abraham E, Balan A, Hareendran NK, Bernard HU, et al. Human papillomaviruses in 91 oral cancers from Indian betel quid chewers—high prevalence and multiplicity of infections. Int J Cancer 1995;61:450–4.
(52) 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 1996;82:57–68.
(53) Daling JR, Madeleine MM, McKnight B, Carter JJ, Wipf GC, Ashley R, et al. The relationship of human papillomavirus-related cervical tumors to cigarette smoking, oral contraceptive use, and prior herpes simplex virus type 2 infection. Cancer Epidemiol Biomarkers Prev 1996;5:541–8.
(54) Niedobitek G, Pitteroff S, Herbst H, Shepherd P, Finn T, Anagnostopoulos I, et al. Detection of human papillomavirus type 16 DNA in carcinomas of the palatine tonsil. J Clin Pathol 1990;43:918–21.
(55) Farrow DC, Davis S. Risk of pancreatic cancer in relation to medical history and the use of tobacco, alcohol and coffee. Int J Cancer 1990;45:816–20.
(56) Snijders PJ, Cromme FV, van den Brule AJ, Schrijnemakers HF, Snow GB, Meijer CJ, et al. Prevalence and expression of human papillomavirus in tonsillar carcinomas, indicating a possible viral etiology. Int J Cancer 1992;51:845–50.
(57) Andl T, Kahn T, Pfuhl A, Erber R, Conradt C, Klein W, et al. Etiological involvement of oncogenic human papillomavirus in tonsillar squamous cell carcinomas lacking retinoblastoma cell cycle control. Cancer Res 1998;58:5–13.
(58) Cason J, Kaye JN, Best JM. Non-sexual acquisition of human genital papillomaviruses. Papillomavirus Report 1995;6:1–7.

NOTES

[1]*Editor's note:* SEER is a set of geographically defined, population-based central tumor registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Each registry annually submits its cases to the NCI on a computer tape. These computer tapes are then edited by the NCI and made available for analysis.

Supported by Public Health Service grant CA48996 and contract CN05230 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, with additional support from the Fred Hutchinson Cancer Research Center.

We thank Elizabeth Tickman for managing data and specimen collection activities; the staff of the Cancer Surveillance System for case subject ascertainment; Dan Edelson, Marion Knudson, Scot McIntosh, Barbara Hansen, Laura Callahan, and Aimee Lowe for recruiting and interviewing case and control subjects; Kay Byron, Dick Jacke, Raymond Miller, Kenneth Scholes, and Judith Kuskin for computer support; Jean Jue for data entry; and Heather Jurado for technical assistance. We also thank Dr. Karen J. Sherman for her important contributions.

Manuscript received December 12, 1997; revised August 26, 1998; accepted September 2, 1998.