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Human papillomavirus type 16 modifies the association between fruit consumption and head and neck squamous cell carcinoma

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Abstract

Human papillomavirus type-16 (HPV16) is a risk factor for head and neck squamous cell carcinoma (HNSCC). HPV-positive cancers have distinct disease cofactors and improved survival following treatment. There is conflicting evidence of a protective association of fruit consumption with HNSCC. As HPV-related disease is clinically distinct, we investigated whether the association between fruit consumption and HNSCC risk was modified by exposure to HPV16. We studied 270 cases and 493 controls with fruit intake information and known HPV16 antibody status. Cases were identified at nine Boston-area medical facilities between 1999 and 2003. Controls were randomly selected from the greater population and frequency-matched to cases by age, gender, and town of residence. Controlling for age, gender, race, smoking, alcohol, total energy intake, body mass index and education, the seronegative individuals had a significantly lower risk of HNSCC with increasing total fruit consumption (OR_{tertile 2}: 0.60, (95% CI 0.38, 0.95); OR_{tertile 3}: 0.57, (0.35, 0.95)) and, specifically, increasing citrus fruit consumption (OR_{tertile 2}: 0.61, (0.39, 0.97); OR_{tertile 3}: 0.59, (0.37, 0.96)). However, among the seropositive, risk increased with greater fruit consumption (OR_{tertile 2}: 2.27, (0.92, 5.58); OR_{tertile 3}: 1.40, (0.55, 3.59) and citrus fruit consumption (OR_{tertile 2}: 3.35, (1.36, 8.24); OR_{tertile 3}: 3.15, (1.23, 8.08)). This interaction was statistically significant (p-value <0.05), showing that fruit consumption was associated with a reduced HNSCC risk among HPV16-seronegative individuals but an increased HNSCC risk among the HPV16-seropositive. These findings suggest that dietary factors dramatically alter the pattern of occurrence of HPV-associated HNSCC and show that viral-related disease is clinically and etiologically distinct.

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Keywords

Human papillomavirus (HPV); head and neck squamous cell carcinoma (HNSCC); citrus fruit; vitamin C; effect modification

Introduction

In the United States, an estimated 47,000 incident cases of head and neck squamous cell carcinoma (HNSCC - cancer of the pharynx, larynx, and oral cavity), are expected to occur in 2008, resulting in 11,000 deaths.(1) The major risk factors for HNSCC are tobacco and alcohol use, which are thought to account for about 75% of all cases.(2) More recently, Human papillomavirus (HPV) has been recognized as an important risk factor for HNSCC. (3, 4)

HPV type-16 (HPV16) is the most commonly identified HPV type in HNSCC tumors.(5) In case-control studies of HNSCC, presence of antibodies against HPV16 has been associated with an approximately four-fold increased HNSCC risk.(6–8) Consistent with the belief that HPV16 is transmitted sexually, prevalence of antibodies against HPV16 has been associated with the number of sexual partners and with the number of oral sex partners in studies of HNSCC.(7, 9–11) Moreover, it has been suggested that HPV16-related HNSCC has a distinct etiology, as evidenced by recent analyses showing that alcohol consumption and tobacco use are not associated with HNSCC risk among HPV16 positive individuals.(12–14) Additionally, it has been shown that the clinical outcomes of patients with HPV16-related HNSCC are significantly better than those with non-HPV-related disease.(4, 7) In particular, the strongest evidence for a relationship with HPV16 has been observed for pharyngeal cancer.(14)

Diet is also thought to play a role in HNSCC risk; in particular, fruit consumption has been linked to decreased risk. Several case-control studies have reported that total fruit intake is inversely related to disease risk after adjustment for known risk factors.(15–20) A meta-analysis of case-control studies found that high fruit intake significantly decreased the risk of oral and pharyngeal cancer (Odds Ratio (OR): 0.53, 95% confidence interval (CI): 0.37, 0.76) and laryngeal cancer (OR: 0.73, 95% CI: 0.64, 0.84), after adjusting for smoking.(21) A more recent meta-analysis of 16 studies also reported an overall protective effect of fruit consumption on oral cancer risk, although not all comparisons were statistically significant. (22) Importantly, results have been somewhat inconsistent with several studies reporting no association between total fruit intake and risk of HNSCC(23), or oral and pharyngeal cancer. (24, 25)

There are fewer prospective studies investigating the impact of diet on HNSCC. The European Investigation into Cancer and Nutrition (EPIC) study reported the highest quintile of fruit intake was associated with lower risk of upper aero-digestive squamous cell cancers (Relative Risk (RR): 0.63, 95% CI: 0.42, 0.96).(26) A recent prospective analysis of the National Institutes of Health (NIH)-AARP Diet and Health Study detected a modest inverse association between head and neck cancers and total fruit and vegetable intake (Hazard Ratio (HR): 0.94, 95% confidence interval (CI): 0.89, 0.99), although the relationships with fruit intake alone (HR: 0.87, 95% CI: 0.86, 1.11) and citrus fruit (HR: 0.90, 95% CI: 0.75, 1.08) were not statistically significant.(27) In an analysis of the Health Professional's Follow-Up Study, Maserejian et al. did not find a significant reduction in risk of oral premalignant lesions with total fruit intake in multivariate models (highest quintile RR: 0.77, 95% CI: 0.47, 1.27).(28)

Consumption of citrus fruit, specifically, has been shown to have a strong inverse relationship with HNSCC and oral premalignant lesions.(24, 28) In multivariate analyses, De Stefani et al. reported an odds ratio of 0.3 (95% CI: 0.2, 0.7) for oral and pharyngeal cancer comparing the highest quartile of citrus fruit consumption to the lowest.(20) Levi et al. found similar results, with an OR of 0.38 (95% CI: 0.20, 0.73) for the highest tertile of citrus fruit intake.(16) A case-control study from the early 1990s also reported an inverse association with fruits, and oranges and tangerines in particular.(29) However, this study did not find an association with dietary vitamin C intake. Not all studies show significant reductions with citrus fruit consumption, as Fernandez Garrote et al. reported an OR of 0.78 (95% CI: 0.40, 1.51) for the highest tertile of intake.(30) It has been suggested that the higher vitamin C concentration of such foods may be responsible for the apparent protective association, while other components such as flavonoids and polyphenols may also play a anti-carcinogenic role.(29, 31)

In addition, a review of epidemiologic studies regarding the influence of diet on cervical carcinogenesis reported a protective association of fruits and vegetables against persistent HPV infection.(32) However, there is little data assessing the role of fruit consumption in negating risk of cervical neoplasia.(32) A separate study of cervical cancer suggested a weak inverse relationship with plasma Vitamin C levels after careful adjustment for HPV status.(33) Goodman et al. also observed that high plasma levels of antioxidants may be protective against cervical squamous intraepithelial lesions, regardless of HPV status.(33) While this research suggests that dietary modification of cancer risk may differ by HPV status, the extent to which the effect of diet on HNSCC is modified by the presence of HPV16 remains unclear.

Accordingly, here we have examined whether the association between fruit consumption and HNSCC differs in the presence of HPV16. To our knowledge there is little data that address whether diet and its association with HNSCC risk varies by HPV status, but as HPV status so profoundly influences the etiology of the disease and its outcome, we hypothesized that it may also modify the effect of fruit consumption on risk.

Method

Study Population

The study design has been described in detail previously.(34) Briefly, incident cases of HNSCC were identified at nine Boston-area medical facilities between December 1999 and December 2003. Eligible cases were at least 18 years old, resided in the Greater Boston Metropolitan Area, were alive at time of initial contact, and were diagnosed within the previous 6 months. The Greater Boston Metropolitan Area is a region consisting of 249 cities and towns within an hour drive from Boston. For this study, HNSCC was defined as carcinoma of the tongue, gum, floor of mouth, other location in mouth, oropharynx, hypopharynx, ill-defined site within lip oral cavity and pharynx, and larynx corresponding to *International Classification of Disease, Ninth Revision* (ICD-9) codes 141, 143, 144, 145, 146, 148, 149, and 161, respectively. Patients with carcinoma *in situ*, lip, salivary gland, or nasopharyngeal cancers were excluded. Prevalent and recurring cancers were also excluded. Cases were histologically confirmed by a study pathologist. The Massachusetts town book methodology was used to identify potential controls.(35) Controls were frequency-matched (1:1) to cases by age (± 3 years), gender, and town of residence. The institutional review boards at the nine participating medical facilities and the Harvard School of Public Health approved all study protocol and materials. All study participants provided informed consent.

Questionnaire

Baseline characteristics, including information on socio-demographics, alcohol consumption, and smoking habits were collected from a comprehensive, self-administered questionnaire. Decade-specific data on alcohol consumption and tobacco use were collected. These data were used to estimate pack-years (packs smoked per day multiplied by years smoked) and average alcoholic drinks consumed per week.(14) Diet information was collected using a semi-quantitative food frequency questionnaire (FFQ) assessing food consumption five years prior to diagnosis for cases and date of enrollment for controls. The FFQ has been validated previously.(36, 37) Fruit intake was calculated as the amount consumed in one week. Total fruit intake included fresh apples/pears, apple juice, bananas, blueberries, cantaloupe, grapefruit, grapefruit juice, oranges, orange juice, other fruit juice, prunes, raisins, strawberries, and peaches/apricots/plums. Citrus fruit intake consisted of grapefruit, grapefruit juice, oranges and orange juice. Vegetable intake was also calculated as the amount consumed in one week. Total vegetable intake included tomatoes, tomato juice, tomato sauce, string beans, broccoli, cabbage, cauliflower, Brussels sprouts, raw and cooked carrots, corn, peas, beans, winter squash, yams/sweet potatoes, zucchini/summer squash, eggplant, raw and cooked spinach, kale, iceberg and romaine lettuce, celery, green peppers, and onions. Green vegetable intake consisted of raw and cooked spinach, kale, and romaine lettuce. Subjects who completed less than half of the FFQ were eliminated from the analysis.

HPV16 Serology

Beginning with subjects enrolled in 2001 or later, venous blood samples were collected. Serum was separated within 24 hours of blood drawing and all samples were frozen at -80°C . Frozen samples were shipped to Merck & Co., Inc. laboratory in West Point, PA for testing. HPV16 serological titer was determined by HPV competitive Luminex immunoassay (cLIA).(38) A cut-off point of 12 milli-Merck units per milliliter was used to classify a positive result. The methods have been described elsewhere.(7)

HPV16 Viral DNA in Tumor Samples

The methods for the collection of HNSCC tumor samples and extraction of DNA have been described previously.(7, 39) The assays were conducted by investigators blinded to patients' HPV16 serology, sexual history, and other risk factors. The short fragment polymerase chain reaction (SPF) assay was used to detect HPV16 in tumor DNA.(7) The beta-actin locus was amplified for use as a control, modifying the previously published methods of Kleter, et al. (40) In addition, DNA from Siha and Ca33 cells was used for positive and negative controls, respectively.

Statistical Analysis

Subjects were excluded if their non-alcohol caloric intake was outside the plausible range of 800 to 4200 kcal/day for males, and 500 to 3500 kcal/day for females.(23) Subjects missing body mass index (BMI) were also excluded. We used unconditional logistic regression to calculate odds ratios (ORs) and 95% confidence intervals (CIs) using SAS Version 9.1 (SAS Institute, Cary, NC). Total fruit and citrus fruit consumption were analyzed using tertiles of intake as determined by the distribution in the controls. We used the nutrient residual method to assess the impact of dietary Vitamin C intake (excluding nutritional supplements). (41) Total and green vegetable intake was also assessed in a similar manner, following reports of a possible association with HNSCC.(16, 18)

All estimates were adjusted for age, gender, race (Caucasian vs. other), pack-years (linear), average alcoholic drinks per week, total energy intake, BMI, and education ($\leq$ high school

diploma vs. higher education). Total energy intake excluded calories from alcohol, and was divided into quartiles based on the distribution in controls. Alcohol was divided into quartiles determined by the joint distribution in cases and controls. BMI was calculated using self-reported height and weight five years prior to enrollment. BMI categories were assigned based on the current guidelines (Underweight: <18.5 ; Normal: $18.5\text{--}24.9$; Overweight: $25.0\text{--}29.9$; Obese: ≥ 30.0) as determined by the Centers for Disease Control and Prevention.(42) Due to the low number of underweight participants ($n = 7$), the underweight and normal BMI categories were combined as the referent group. Spearman correlation coefficients were calculated to assess possible collinearity between risk factors.

We used joint effects models and models stratified by HPV16 serological status to analyze the relationship between fruit consumption and risk of HNSCC, using those who are in the lowest quartile of fruit consumption in each stratum as the referent group. Statistical interaction was evaluated using a likelihood ratio test comparing a model containing the fruit categories and HPV16 serology, and their cross products, to a model without the cross products. This was a two-sided test with two degrees of freedom. P-values less than 0.05 were considered statistically significant. In the joint effects models, those who were HPV16 seronegative and were in the lowest fruit consumption tertile served as the referent group. These analyses were also carried out restricting to cases of pharyngeal cancer, since the literature points to a particularly strong relationship between the virus and this subtype of HNSCC. In addition, a case-only analysis of 151 HNSCC cases with tumor DNA data was conducted to determine if presence of HPV16 DNA in tumors differed by fruit consumption.

Results

Of the 823 eligible cases identified for this study, 57 refused to participate, and 44 did not complete the questionnaire. The final enrollment of 722 cases resulted in an 88% case participation rate. For the controls, 1,643 eligible individuals were contacted to participate in the study, although 828 refused. Of the 815 subjects who consented, 771 were finally enrolled, although six controls were later withdrawn when matched to an ineligible case. The completed enrollment of 765 control subjects represents an overall control participation rate of 47%. Following the initiation of blood collection in 2001, blood was successfully obtained from 81% of cases and 80% of controls enrolled after that time. There were 298 cases and 498 controls who completed the FFQ and whose HPV16 serologic status was determined. After excluding 55 subjects not meeting the caloric intake and 36 missing BMI, there were 270 cases and 493 controls in the analysis, including 122 cases of pharyngeal cancer, 101 cancers of the oral cavity, and 46 of the larynx. DNA was obtained from paraffin-embedded tumors from 151 eligible case patients with dietary data.

Baseline characteristics for cases and controls are shown in Table 1. There was no difference between cases and controls by race, energy intake, or BMI while controlling for age and gender. However, cases were significantly less likely to have achieved a higher education and more likely to smoke and consume more alcohol compared with controls. Cases also consumed less citrus fruit, and less fruit overall than did controls. Furthermore, while only 10.8% of controls tested positive for HPV16 antibodies, 30.4% of cases had serologically detectable antibodies against the virus. The Spearman correlation coefficients revealed no significant correlation between smoking and fruit intake among controls ($r = -0.07$) or the total study population ($r = -0.15$). Similar results were found for alcohol and fruit intake among controls ($r = -0.04$) and the total study population ($r = -0.12$).

Baseline characteristics were also compared by HPV16 serological status among controls only (data not shown). There were no differences between the HPV16 seropositive compared to the seronegative with respect to BMI, alcohol intake, total fruit, or citrus fruit

intake. However, HPV16 seropositive individuals smoked less compared with the HPV16 seronegative individuals.

Results from an analysis of total fruit consumption and HNSCC stratified by HPV16 serology are presented in Table 2. Among the HPV16 seronegative, those with greater fruit consumption had a significantly lower frequency of HNSCC (OR_{tertile 2}: 0.60, 95% CI: 0.38, 0.95; OR_{tertile 3}: 0.57, 95% CI: 0.35, 0.95). However, among the HPV16 seropositive, risk of HNSCC was elevated with increasing fruit consumption (OR_{tertile 2}: 2.27, 95% CI: 0.92, 5.58; OR_{tertile 3}: 1.40, 95% CI: 0.55, 3.59). The interaction between HPV16 serology and fruit consumption indicated that these differences were statistically significant ($p < 0.05$). Looking within the lowest tertile of fruit consumption in the joint effects model, HNSCC risk was greater for the HPV16 seropositive relative to the HPV16 seronegative (OR: 2.31, 95% CI: 1.21, 4.40). The upper tertiles of fruit consumption were similarly related to an increased risk of HNSCC in the HPV16 seropositive, compared to the lowest tertile of fruit consumption in the seronegative (OR_{tertile 2}: 5.24, 95% CI: 2.44, 11.27; OR_{tertile 3}: 3.23, 95% CI: 1.45, 7.21).

The same differences in HNSCC risk were observed by looking at citrus fruit consumption alone (Table 2). Greater citrus fruit consumption was associated with a reduced risk of HNSCC among the HPV16 seronegative (OR_{tertile 2}: 0.61, 95% CI: 0.39, 0.97; OR_{tertile 3}: 0.59, 95% CI: 0.37, 0.96), although among the HPV16 seropositive, citrus fruit consumption increased HNSCC risk (OR_{tertile 2}: 3.35, 95% CI: 1.36, 8.24; OR_{tertile 3}: 3.15, 95% CI: 1.23, 8.08). The interaction between HPV16 serology and citrus fruit was statistically significant ($p < 0.05$). An increased risk of HNSCC with citrus fruit consumption was also observed in the joint effects analysis, with each tertile of citrus fruit intake in the HPV16 seropositive compared to the lowest tertile of citrus fruit intake in the seronegative (OR_{tertile 1}: 1.51, 95% CI: 0.79, 2.87; OR_{tertile 2}: 6.61, 95% CI: 3.01, 14.55; OR_{tertile 3}: 5.19, 95% CI: 2.34, 11.54).

We further examined this relationship by focusing on the dietary component of Vitamin C (Table 3). Increasing Vitamin C intake was associated with a reduced risk of HNSCC among the HPV16 seronegative, but among the HPV16 seropositive, greater Vitamin C consumption was associated with an increasing HNSCC risk. However, these differences were not statistically significant. (p for interaction = 0.21). Vitamin C intake including that from nutritional supplements was also assessed, but the conclusions were the same (data not shown).

When restricted to cases of pharyngeal cancer, the relationship between HNSCC and total fruit intake was similar to that reported for total HNSCC cases (Table 4). The protective effect observed among HPV16 seronegative participants was slightly stronger in cases of pharyngeal cancer (OR_{tertile 2}: 0.47, 95% CI: 0.24, 0.93; OR_{tertile 3}: 0.39, 95% CI: 0.17, 0.87), while the association in the HPV16 seropositive was of similar magnitude (OR_{tertile 2}: 2.32, 95% CI: 0.87, 6.19; OR_{tertile 3}: 1.69, 95% CI: 0.59, 4.82). An increased risk of pharyngeal cancer for the HPV16 seropositive was also observed in the joint effects analysis, when compared with the lowest tertile of fruit intake in the seronegative (OR_{tertile 1}: 3.85, 95% CI: 1.81, 8.19; OR_{tertile 2}: 8.92, 95% CI: 3.80, 20.95; OR_{tertile 3}: 6.49, 95% CI: 2.62, 16.05). Results from the stratified analysis for citrus fruit revealed a strong inverse relationship among the HPV16 seronegative (OR_{tertile 2}: 0.31, 95% CI: 0.15, 0.65; OR_{tertile 3}: 0.36, 95% CI: 0.17, 0.76), and a statistically significant increased risk of pharyngeal cancer in the HPV16 seropositive (OR_{tertile 2}: 3.55, 95% CI: 1.28, 9.86; OR_{tertile 3}: 4.20, 95% CI: 1.53, 11.51). Results of the joint effects analysis can be found in Table 4.

To further explore the interaction of HPV16 and diet in HNSCC risk, we conducted a case-only analysis using data on detectable HPV16 DNA in the tumors (Table 5). Of the 151 cases in the analysis, 48 (32%) had detectable HPV16 DNA in their tumors and 103 (68%) did not. Overall, cases that had detectable HPV16 DNA in tumors consumed less fruit, citrus fruit, and Vitamin C. However, there were no statistical differences between fruit intake and having HPV16 DNA in the tumors.

Analyses looking at total vegetable intake ($p = 0.46$) and green vegetable intake ($p=0.48$) on HNSCC risk did not reveal a significant interaction with HPV16 status (data not shown).

Discussion

HPV16 seronegative individuals with greater fruit or greater citrus consumption had a significantly lower frequency of HNSCC than those with low fruit or low citrus intake; however fruit intake appeared to increase risk of HNSCC among the HPV16-seropositive. These findings remained significant when we analyzed the interactions among pharyngeal cancer cases, the HNSCC site most strongly associated with HPV16. The divergence by HPV16 serologic status was evident for Vitamin C intake as well, but the interaction was not statistically significant, suggesting that Vitamin C alone may not be responsible for this relationship.

In analyzing how dietary factors influenced HNSCC risk in our study population, we previously found no significant association between fruit consumption and HNSCC after adjustment for potential confounders.(23) The results of the present analysis suggest that this observation was due, in part, to effect modification by HPV16 serologic status (which might bias the effect of fruit towards the null). Given the existing literature on the relationship between fruit consumption and HNSCC, the observed interaction with HPV16 was unexpected. However, it raises the issue that the inconsistent findings between overall fruit or citrus fruit and HNSCC risk in the literature may be partially explained by differences in the prevalence of HPV16 exposure across populations. That is, populations with a lower prevalence of HPV16 exposure may be more likely to observe a protective association between fruit and HNSCC, whereas populations with a higher prevalence of HPV16 would be less likely to observe a reduced risk of HNSCC with fruit consumption.

Although we are unaware of similar studies conducted in HNSCC populations, the relationship between HPV16 and fruit consumption has been investigated in subjects with HPV-related cervical cancer. One study reported a possible protective effect of increased fruit intake against persistent HPV infection.(32) Fruits were also considered protective against invasive cervical cancer by an expert committee in 1998.(43) Interestingly, a recent case-control study reported an insignificant trend of increasing risk of *in situ* cervical cancer ($p = 0.06$) and invasive cancer ($p = 0.89$) with increasing intake of foods high in Vitamin C for cases compared with controls.(44) However, these investigators found no significant association between intake of foods high in Vitamin C and incidence of *in situ* or invasive cervical cancer after adjustment for HPV. Overall, evidence relating dietary factors to development of cervical cancer has been inconsistent, and few studies have considered the relationship within strata of HPV status, since almost all cervical cancers are HPV-positive. (32)

The relationship of nutrient intake with viral infections and immune system status has been well studied. The association of nutrition and vitamin intake with disease status in patients with Human Immunodeficiency Virus (HIV) suggests that the virus' behavior may be mediated at least in part by the nutritional status of its host.(45, 46) One cross-sectional study reported that HIV-positive subjects on highly active antiretroviral therapy (HAART)

with high serum zinc levels had lower log viral load levels than subjects in the lowest quartile of serum zinc.(47) Micronutrient deficiency has been linked to faster progression of the disease, particularly in HIV-positive women. A randomized, double-blind, placebo-controlled trial assessed the impact of giving vitamin supplements to pregnant women in Tanzania on the risk of vertical transmission and disease progression.(48) The study found benefits from multivitamins on adverse pregnancy outcomes, lowered viral loads, and increased CD cell counts.(46, 48)

Our data show a significant interaction between HPV16 and fruit intake and the risk of HNSCC, although the mechanism is unclear. However, it appears that the observed association is not explained by correlation between fruit intake and other HNSCC risk factors. It is possible that the ingestion of acidic foods including citrus fruit may erode the mucosal lining of the gastrointestinal tract, enhancing the likelihood of infection upon exposure to HPV16. It is known that a higher proportion of inflammatory cells are observed in the buccal mucosa of smokers as compared to non-smokers(49) and that inflammation caused by tobacco and alcohol use could increase permeability of mucosal surfaces to virus particles. High fruit intake and acidic fruit in particular may combine with these established risk factors to cause mucosal wounding and allow the virus to better access the bloodstream.

It is also possible that nutrient intake modifies the immune response. The development of persistent infection with an oncogenic strain of HPV is associated with higher risk of cancer.(50) Consequently, consumption of fruits might enhance the efficacy of the immune response to DNA damage-associated carcinogenic clonal expansion while paradoxically promoting a persistent serologically detectable response to HPV. Overall, it is unlikely that fruit consumption patterns are related to the risk of acquiring HPV, as fruit intake in our study did not differ by HPV status.

In addition, clinical research has shown that the consumption of grapefruit, grapefruit juice, or Seville orange juice may alter disease-associated metabolism.(51–53) Consumption of these products impairs the metabolism of many prescription drugs, including components of HAART (54); this is thought to be due primarily to interactions between the citrus fruits and drug-metabolizing enzyme cytochrome P4503A (CYP3A).(53) The grapefruit flavonoid naringenin has also been reported to reduce secretion of hepatitis C virus (HCV) in infected cells.(55) These findings suggest that citrus fruit consumption may affect the body's response to viral infections and their treatment. However, a direct link between these modulations and cancer risk has not been established.

There are several methodological issues that were taken into consideration for this population-based case-control analysis. Low control participation in this study (47% overall) could result in a control population that does not accurately represent the study base that gave rise to the cases, resulting in selection bias. Furthermore, 378 cases (35.1%) from the initial study population did not complete the FFQ, compared to only 50 controls (6.3%). It is possible that participating controls were more health-conscious than non-participants, and may adhere to a healthier diet and lifestyle. Low control participation would not explain the observed associations unless participation was differential by HPV16 status. Although we are unable to determine the extent of such differences, we do not believe that participation rates differed based on HPV16 antibody status. The prevalence of HPV16 among controls included in the final analysis is 10.8%. A similar estimate for the seroprevalence of HPV16 in the general US population aged 50–59 years (10.6%, 95% CI: 8.1–13.8%) was reported in a study using data from the third National Health and Nutrition Examination Surveys (NHANES III), suggesting that control participation rates were not determined differentially by HPV16 status.(56)

To further investigate the possible impact of selection bias, we compared the distribution of risk factors (age, gender, race, education, smoking status, and alcohol intake) between those cases included in the analysis and those who were not included. We found no significant differences in age, pack-years of smoking, alcoholic drinks per week, race, or education between the two groups, indicating that the potential for selection bias to account for these findings is small. The same analysis comparing the distribution of these risk factors between controls included in the analysis and those not included yielded the same conclusions.

There is always the possibility that unmeasured confounders or residual confounding could explain the observations reported in this paper. We further adjusted for potential confounding by socio-economic status by including household income in the multivariate models. However this addition did not change the effect estimates, and the variable was not retained in final models. Smoking status and alcohol consumption were assessed through self-reported questionnaire data, and as such, are vulnerable to bias despite detailed measurement. In order for such bias to impact our findings, our study population would have had to report smoking and alcohol consumption differentially by HPV16 serological status. Since participants did not initially know that HPV16 serology would be determined, it is unlikely to have affected their reporting accuracy. Future analyses on this topic could explore the relationship between fruit consumption, HPV16, and HNSCC in never-smokers to further reduce residual confounding by smoking, and elucidate the etiological importance of HPV16. We were unable to restrict our analysis to non-smokers and non-drinkers due to small sample sizes in our population.

In this analysis, dietary data were self-reported using a validated FFQ. Cases and controls were asked to report their diet five years prior to date of diagnosis or date of enrollment, respectively. This measure reduced the influence of recent diet changes due to progression of HNSCC. It is highly unlikely that HPV16 status influenced a participant's ability to accurately recall fruit consumption, and thus we do not believe that recall bias could wholly account for our findings.

The results of our analysis underscore the importance of measuring HPV16 status in studies of HNSCC. Our findings of an increased risk of HNSCC among HPV-positive individuals with increasing total fruit and citrus intake require confirmation in different populations. In particular, using non-smokers or low drinkers as the referent group for future analyses would further reduce the influence of residual confounding, and help elucidate the relationship with HPV16-related HNSCC. Our data highlight the fact that it is important to account for HPV16 status when studying HNSCC, as HPV16-related HNSCC represents a distinct disease, and failure to do so may affect study results.

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Table 1

Selected characteristics for head and neck squamous cell carcinoma cases and controls

	Cases (n=270) (n,%)	Controls (n=493) (n,%)	p-value *
Age			
Mean (SD)	59.9 ($\pm$ 11.7)	61.0 ($\pm$ 11.5)	
Sex			
Male	190 (70.4)	359 (72.8)	
Female	80 (29.6)	134 (27.2)	
Race			
White	250 (92.6)	457 (92.7)	0.98
Other	20 (7.4)	36 (7.3)	
Highest Education Level			
High school or less	107 (39.6)	150 (30.4)	0.01
More than high school	163 (60.4)	343 (69.6)	
BMI			
<25	103 (38.2)	162 (32.9)	0.08
25 to <30	115 (42.6)	210 (42.6)	
$\geq$ 30	52 (19.3)	121 (24.5)	
Tobacco, pack-years			
Never	53 (19.6)	164 (33.3)	<0.01
>0 to < 16	50 (18.5)	114 (23.1)	
16 to 41	67 (24.8)	125 (25.4)	
>41	100 (37.0)	90 (18.3)	
Alcohol Consumption, drinks/week			
$\leq$ 3.1	48 (17.8)	138 (28.0)	<0.01
>3.1 – 7.3	48 (17.8)	139 (28.2)	
>7.3 – 22.1	63 (23.3)	135 (27.4)	
>22.1	111 (41.1)	81 (16.4)	
HPV16 Antibody Titer			
Positive	82 (30.4)	53 (10.8)	<0.01
Negative	188 (69.6)	440 (89.3)	
Total Calories			
$\leq$ 1532.4	69 (25.6)	123 (25.0)	0.18
1532.5–1920.5	57 (21.1)	123 (25.0)	
1920.6–2404.9	58 (21.5)	124 (25.0)	
>2404.9	86 (31.1)	123 (25.0)	
Total Fruit Consumption, servings/week			
$\leq$ 9.4	123 (45.6)	165 (33.5)	<0.01
>9.4 – 16.7	81 (30.0)	164 (33.3)	
>16.7	66 (24.4)	164 (33.3)	
Citrus Fruit Consumption, servings/week			
$\leq$ 2.2	116 (43.0)	159 (32.3)	0.02

	Cases (n=270) (n,%)	Controls (n=493) (n,%)	p-value*
>2.2 – 7.2	82 (30.4)	167 (33.9)	
>7.2	72 (26.7)	167 (33.9)	
Vitamin C Intake			
Tertile 1	108 (40.0)	146 (29.6)	0.02
Tertile 2	79 (29.3)	175 (35.5)	
Tertile 3	83 (30.7)	172 (34.9)	

* Tests controlled for age and gender.

Table 2
Association between fruit consumption and risk of head and neck squamous cell carcinoma by HPV16 serology

Fruit consumption	Servings/ week	HPV16 serology	Cases		Controls		Stratified Model		Joint Effects Model	
			n	%	n	%	OR*	95% CI	OR*	95% CI
Total	≤9.4	Negative	91	33.7	140	28.4	Referent		Referent	
Fruit	>9.4 to 16.7		52	19.3	151	30.6	0.60	0.38, 0.95	0.60	0.38, 0.95
	>16.7		45	16.7	149	30.2	0.57	0.35, 0.95	0.57	0.35, 0.95
	≤9.4	Positive	32	11.9	25	5.1	Referent		2.31	1.21, 4.40
	>9.4 to 16.7		29	10.7	13	2.6	2.27	0.92, 5.58	5.24	2.44, 11.27
	>16.7		21	7.8	15	3.0	1.40	0.55, 3.59	3.23	1.45, 7.21
$P_{\text{interaction}}=0.03$										
Citrus	≤2.2	Negative	89	33.0	133	27.0	Referent		Referent	
Fruit	>2.2 to 7.2		51	18.9	153	31.0	0.61	0.39, 0.97	0.61	0.39, 0.97
	>7.2		48	17.8	154	31.2	0.59	0.37, 0.96	0.59	0.37, 0.96
	≤2.2	Positive	27	10.0	26	5.3	Referent		1.51	0.79, 2.87
	>2.2 to 7.2		31	11.5	14	2.8	3.35	1.36, 8.24	6.61	3.01, 14.55
	>7.2		24	8.9	13	2.6	3.15	1.23, 8.08	5.19	2.34, 11.54
$P_{\text{interaction}}<0.001$										

* Odds ratios adjusted for age, gender, race, pack-years of smoking, alcohol, total energy intake, BMI and education

Association between Vitamin C intake and risk of head and neck squamous cell carcinoma stratified by HPV16 serology

Table 3

HPV16 serology	Vitamin C	Cases		Controls		OR [*]	95% CI
		n	%	n	%		
Negative	Tertile 1	83	30.7	128	26.0	Referent	
	Tertile 2	45	16.7	154	31.2	0.62	0.38, 0.99
	Tertile 3	60	22.2	158	32.1	0.81	0.51, 1.28
Positive	Tertile 1	25	9.3	18	3.7	Referent	
	Tertile 2	34	12.6	21	4.3	1.35	0.56, 3.25
	Tertile 3	23	8.5	14	2.8	1.83	0.69, 4.84
							<i>P</i> _{interaction} = 0.21

* Odds ratios adjusted for age, gender, race, pack-years of smoking, alcohol, total energy intake, BMI and education

Table 4

Association between fruit consumption and risk of pharyngeal cancer by HPV16 serology.

Fruit consumption	Servings/week	HPV16 serology	Cases		Controls		Stratified Model		Joint Effects Model	
			n	%	n	%	OR*	95% CI	OR*	95% CI
Total	≤9.4	Negative	36	29.5	140	28.4	Referent		Referent	
Fruit	>9.4 to 16.7	Positive	16	13.1	151	30.6	0.47	0.24, 0.93	0.47	0.24, 0.93
	>16.7		11	9.0	149	30.2	0.39	0.17, 0.87	0.39	0.17, 0.87
	≤9.4		22	18.0	25	5.1	Referent		3.85	1.81, 8.19
	>9.4 to 16.7		21	17.2	13	2.6	2.32	0.87, 6.19	8.92	3.80, 20.95
	>16.7		16	13.1	15	3.0	1.69	0.59, 4.82	6.49	2.62, 16.05
Citrus	≤2.2	Negative	39	32.0	139	28.2	Referent		$P_{interaction}=0.01$	
Fruit	>2.2 to 7.2	Positive	11	9.0	147	29.8	0.31	0.15, 0.65	0.31	0.15, 0.65
	>7.2		13	10.7	154	31.2	0.36	0.17, 0.76	0.36	0.17, 0.76
	≤2.2		19	15.6	28	5.7	Referent		2.37	1.13, 5.0
	>2.2 to 7.2		19	15.6	12	2.4	3.55	1.28, 9.86	8.41	3.43, 20.62
	>7.2		21	17.2	13	2.6	4.20	1.53, 11.51	9.96	4.16, 23.83
$P_{interaction}<0.001$										

* Odds ratios adjusted for age, gender, race, pack-years of smoking, alcohol, total energy intake, BMI and education.

Table 5

Association between presence of HPV DNA in tumors and fruit consumption

	HPV16 DNA+ Tumors (n=48) (n, %)		HPV16 DNA- Tumors (n=103) (n, %)		OR (95% CI)*
Total Fruit, servings/week					
≤9.4	18	37.5	46	44.7	Referent
>9.4 to 16.7	17	35.4	28	27.2	1.01 (0.41, 2.53)
>16.7	13	27.1	29	28.2	0.74 (0.26, 2.05)
Citrus Fruit, servings/week					
≤2.2	19	39.6	43	41.8	Referent
>2.2 to 7.2	12	25.0	35	34.0	0.47 (0.18, 1.25)
>7.2	17	35.4	25	24.3	0.97 (0.38, 2.48)
Vitamin C					
Tertile 1	18	37.5	32	31.1	Referent
Tertile 2	14	29.2	37	35.9	0.43 (0.16, 1.14)
Tertile 3	16	33.3	34	33.0	0.50 (0.19, 1.31)

* Odds ratios adjusted for age, gender, race, pack-years of smoking, alcohol, total energy intake, BMI and education.