HUMAN PAPILLOMAVIRUS (HPV) AND LIFESTYLE IN THE TRANSLATIONAL EPIDEMIOLOGY OF HEAD AND NECK CANCER

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Head and neck squamous cell carcinomas (HNSCC) afflict 600,000 persons and cause 300,000 deaths annually worldwide. Recent changes in HNSCC epidemiology demonstrate the importance of disease heterogeneity in prevention and treatment. This research investigated heterogeneity in HNSCC pathobiology, etiology, and survival in three separate studies. In the first study, N=67 formalin-fixed, paraffin-embedded HNSCC (27 human papillomavirus (HPV)-positive, 40 HPV-negative) were retrieved from storage and expression of three tumor angiogenesis markers--epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and NOTCH receptor 1 (NOTCH1)--were compared according to HPV status using immunohistochemistry. HPV-positive tumors under-expressed EGFR relative to HPV-negative ($P<0.01$) but VEGF ($P=0.82$) and NOTCH1 ($P=0.68$) were unrelated to HPV status. EGFR-VEGF, and NOTCH1-VEGF associations were observed in HPV-negative tumors only; and the NOTCH1-EGFR association was observed in HPV-positive tumors only. HPV-positive HNSCC may be less angiogenic than HPV-negative HNSCC.

The second study assessed the association between childhood passive smoke exposure (CPSE) and HNSCC using a case-control design (N=862 cases, N=806 frequency-matched controls). CPSE was associated with HNSCC (odds ratio (OR)=1.28, 95% confidence interval (CI): 1.01-1.63) after controlling for adult smoking. Among never-adult-smokers (N=184 cases, N=415 controls) CPSE was associated with oropharyngeal cancer (which is typically HPV-

related) more strongly than other HNSCC (OR=2.04, 95% CI: 1.02-4.08 vs. OR=1.08, 95% CI: 0.71-1.66; P-for-heterogeneity=0.08). Assuming a causal association, 16.9% (95% CI: 0.8%-29.4%) of HNSCC would not occur without CPSE. Limiting CPSE may reduce HNSCC risk.

The third study assessed overall and disease-specific survival associated with metabolic enzyme genotype in N=159 HNSCC cases. After adjustment for tumor site and stage, N-acteyltransferase-2 (*NAT2*) fast acetylators had improved survival (vs. slow acetylators) when treated with surgery alone (hazard ratio (HR)=0.26; 95% CI: 0.10-0.66) but not chemoradiotherapy (HR=1.21; 95% CI: 0.54-2.73) or radiotherapy (HR=0.67; 95% CI: 0.31-1.59) (P-for-interaction=0.04). Reduced activity glutathione S-transferase pi-1 (*GSTP1*) was associated with improved disease-specific survival in men only (HR=0.12; 95% CI: 0.02-0.91; women: HR=2.29; 95% CI=0.41-12.69; P-for-interaction=0.02). Metabolic enzyme genotype modifies HNSCC survival.

This research contributes to public health by demonstrating biological differences in HNSCC exploitable for therapy; encouraging public policy to reduce HNSCC incidence; and supporting individualized therapy.

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PREFACE

I would like to take this opportunity to thank several organizations and people who were instrumental in making this research possible. First, I would like to thank the National Cancer Institute (NCI) for offering the R25 training grant that enabled my doctoral experience. I would also like to thank the University of Pittsburgh Cancer Institute, and especially the Head and Neck Specialized Program of Research Excellence (SPORE), for making available the resources necessary to conduct this research. I would specifically like to thank the Head and Neck SPORE data managers, Jennifer Ridge Hetrick and Kerry Trent, for assisting me with numerous data extraction requests. I would also like to thank the many Head and Neck SPORE investigators for attending my lectures and asking thought-provoking questions that were helpful in moving this research forward.

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for me the importance of checklists--even for experts--and I believe this has served me well in the design and execution of my research, as well as in conducting self-appraisals of my work as an epidemiologist. But most of all, I would like to thank Dr. Weissfeld for introducing me to the importance of storytelling in epidemiology and I hope that my dissertation follows in this tradition.

Finally, I would like to thank my friends and family who have supported me in my pursuit of a PhD. My dream to become an epidemiologist began eight years before this dissertation was completed and represented a major life-change, as it involved a departure from my original professional career as a software engineer. I would particularly like to thank my parents, Sol and Roberta Troy, for their encouragement and confidence in my success. Finally, I would like to dedicate this work to my numerous friends and family who have had personal struggles with cancer. I firmly believe the glory of science belongs to those whose lives are positively impacted by its work, and I sincerely hope that someday my research will contribute to the reduction of suffering and death from cancer.

"Non nobis solum nati sumus. (Not for ourselves alone are we born.)"

-- *Marcus Tullius Cicero*

1.0 INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) are epithelial malignancies occurring in the oral cavity, pharynx, and larynx, and contribute substantially to the worldwide burden of cancer, accounting for over 630,000 new cancer cases and 360,000 deaths worldwide in 2008.[1] The strongest risk factors for HNSCC are tobacco and alcohol use, and recent declines in incidence of HNSCC in the United States, Canada, and Europe have been attributed to declines in smoking.[2-4] However, despite the decline in incidence of HNSCC overall, the incidence of oropharyngeal cancer has risen among younger persons with little or no smoking history.[2,3,5,6] It is now known that these tumors are caused by the sexually transmitted human papillomavirus (HPV).[7] Thus, the United States and other developed nations are now experiencing an epidemic of HPV-positive HNSCC believed to have emerged as a result of changes in sexual behavior over time.[7] HPV-positive HNSCC are recognized as a distinct disease entity, as they are associated with better prognosis, have unique histopathology, and exhibit molecular abnormalities that HPV-negative HNSCC do not.[7] The emergence of HPV-positive HNSCC as a public health problem has stimulated the need to explore heterogeneity in HNSCC that might reveal unique therapeutic approaches for patient subgroups, improved understanding of disease etiology necessary for prevention of HNSCC, and identification of factors influencing HNSCC survival.[7]

One possibly important source of heterogeneity in HNSCC is angiogenesis: the development of tumor-infiltrating blood vessels in response to growth factors released from the

tumor.[8] Angiogenesis is required for tumor growth and can facilitate metastasis by providing a path to other anatomical sites via the vasculature.[8] Immunohistochemical (IHC) studies showing lower expression of proteins upstream of proximal angiogenesis markers in HPV-positive compared with HPV-negative HNSCC,[9-13] combined with clinical observations linking HPV-positive tumors with smaller size[14-17] are suggestive of reduced growth potential in HPV-positive HNSCC. However, it is unclear whether this specifically reflects differences in angiogenesis in HPV-positive and HPV-negative HNSCC. In addition, preliminary evidence has identified a potentially unrecognized actor in HNSCC angiogenesis--the NOTCH1 receptor.[18,19] NOTCH1 is involved in cell-to-cell signal transduction events associated with cellular differentiation, yet its function in cancer as an oncogene or tumor suppressor appears to vary across tumor types.[20] The function of NOTCH1 in HNSCC is yet to be defined,[21,22] although some studies suggest it may be associated with angiogenesis in oral and oropharyngeal cancer.[18,19] However, no studies have compared NOTCH1 with well known markers of angiogenesis in HNSCC stratified by tumor HPV status. Anti-angiogenesis therapies are currently being tested in clinical trials for HNSCC,[23] although it is not yet understood how the heterogeneity of HNSCC might effect response to these therapies. Exploration of differences in angiogenesis comparing HPV-positive and HPV-negative HNSCC might reveal patient subgroups likely to respond to anti-angiogenesis therapy, and identify potential biomarkers of treatment response or new therapeutic targets.

The increasing importance of HPV in HNSCC has also stimulated the need for improved understanding of etiologic cofactors in HPV-positive HNSCC.[7] Although patients diagnosed with HPV-positive HNSCC are less likely to have a smoking history themselves,[7] these patients were born during a time when smoking was more common and had substantial opportunity for exposure to passive cigarette smoke during childhood.[6,24] The International Agency for Research

on Cancer identifies passive smoke as a human carcinogen[25] and the United States Surgeon General concluded that adult exposure to passive smoke causes lung cancer in lifetime never-smokers.[26] The health of children is particularly threatened by passive smoke as children are more likely to live with a smoker than non-smoking adults.[26] Childhood passive smoke exposure is associated with reduced lung function, increased risk of respiratory and ear infections, development of asthma, and suppression of humoral and cellular immune responses.[26] While studies have shown increased risk of adult nasopharyngeal carcinoma associated with childhood passive smoke exposure,[27,28] this head and neck tumor is pathologically distinct from HNSCC.[29,30] The relationship between childhood passive smoke exposure and HNSCC is not extensively explored,[31] and no studies have examined this exposure separately in HPV-positive and HPV-negative HNSCC.

Exploration of heterogeneity in HNSCC also has the potential to yield badly needed improvements in survival for HNSCC patients, who continue to experience five-year relative survival of only 60% whereas patients suffering from more common tumors such as breast and prostate cancers experience five-year relative survival in excess of 90%.[32] Although several determinants of survival have been identified in HNSCC, studies have focused mainly on characteristics of the tumor rather than germline genetics.[7] Germline variation in tobacco metabolizing enzymes modifies the risk of HNSCC in the presence of smoking.[33-35] However, it is unclear whether polymorphisms in these genes also effect survival. This is a concern given 20-40% of HNSCC patients continue smoking after cancer diagnosis,[36-39] and given the additional role of these enzymes in metabolism of chemotherapy used in HNSCC treatment,[40] and carcinogens present in burned fossil fuels[41] and cooked meat.[42]

The pursuit of discoveries leading to novel therapies in HNSCC, a reduction in population burden of HNSCC, and improved survival for HNSCC patients are unique efforts with a common theme embodied in the translational research approach: to quickly bring results of scientific research to bear on reducing cancer morbidity and mortality at the population level.[43] The objective of the research described here is to apply epidemiology to make discoveries that ultimately impact the lives of HNSCC patients and those at risk for developing the disease. Specifically, this research seeks to: 1) explore the role of the NOTCH pathway in tumor angiogenesis in HPV-positive and HPV-negative HNSCC, 2) evaluate the association between childhood passive smoke exposure and adult HNSCC, and 3) evaluate the association between polymorphisms in tobacco and alcohol metabolizing enzymes and survival in HNSCC.

2.0 LITERATURE REVIEW

2.1 BACKGROUND

The World Health Organization (WHO) classifies solid tumors occurring at a variety of anatomic sites in the head and neck as "head and neck tumors," with the notable exceptions of the thyroid, esophagus and brain.[44] Approximately 85-95% of these tumors are squamous cell carcinomas (SCC) occurring in the oral cavity, pharynx, and larynx.[44,45] HNSCC are caused by tobacco smoking, alcohol abuse, and HPV infection.[46] The following literature review discusses the epidemiology of HNSCC with a focus on data from the United States. SCC of the lip are not discussed here (unless otherwise noted) as the primary etiology for these tumors is prolonged sun exposure.[47]

2.2 THE EPIDEMIOLOGY OF HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) IN THE UNITED STATES

HNSCC accounted for 3.2% of incident cancers and 2.0% of cancer deaths in the United States during 2008[48] and are the ninth most common cancer in American men and the fourteenth most common cancer in American women.[49] The epidemiological picture of HNSCC is complex, with changing incidence patterns over time according to sex, race, and tumor site; persistent racial

disparities; and an emerging viral etiology.[46] The following sections describe these trends and provide a brief discussion of the epidemiological evidence for the emergence of HPV-related HNSCC. A more detailed discussion of HNSCC etiology is provided later.

2.2.1 Overview of Incidence Trends in HNSCC in the United States

Systematic collection of cancer incidence data in the United States dates back to 1935 when the Connecticut Tumor Registry was established.[50] This registry is now part of the National Cancer Institute (NCI) Surveillance, Epidemiology, and End Results (SEER) program, which provides statistics on cancer cases occurring in the United States since 1973 among a representative* sample of United States residents.[51] Data from the Connecticut Tumor Registry and SEER show notable changes in HNSCC incidence over time in the United States. First, HNSCC incidence increased substantially from 1935-1984, with distinct patterns according to age, birth cohort, and sex.[52] Second, incidence of HNSCC overall began to decline after 1984.[2,53] However, as the overall incidence of HNSCC declined, the incidence of oropharyngeal cancer began to increase among younger white males.[54] These incidence patterns separate the occurrence of HNSCC into two etiologic eras: the tobacco/alcohol era spanning approximately the first 75% of the 20th century, and the HPV-related era, which began in the early 1970s.[6,54]

2.2.2 Incidence of HNSCC and Tobacco Use in the United States.

Using data from the Connecticut tumor registry, Chen et al.[52] examined incidence of oral cavity cancers from 1935 to 1984. Incidence of oral cavity cancer increased substantially during this

* In terms of income, education, urban/rural residence, and foreign/native birth status.

period, although more drastically among women (6.6-fold increase) compared with men (1.7-fold).[52] Because of this drastic increase among women, the male:female ratio of oral cavity cancers decreased rapidly over time from 8.4:1 in 1935-1939, to 4.8:1 in 1955-1959, and finally to 2.0:1 in 1980-1985.[52] The peak age at diagnosis in both sexes was 60-69 and the majority of tumors diagnosed during this time period were tongue (which included base of tongue and lingual tonsil; 41.2%) and floor of mouth (FOM; 24.2%) cancers.[52] Tumors at these sites were also the top two incident cancers for men (tongue: 41.8%, FOM: 25.8%) and women (tongue: 39.5%, FOM: 19.3%).[52] Increases in incidence among men were primarily due to increases among men aged 50-69, whereas incidence increased over the study period in women aged 40 and over.[52] Birth cohort analysis showed incidence rates began to level off or decrease among men at age 75 in successive birth cohorts after 1880.[52] An opposite pattern was observed in women: the incidence rate among women aged 55 and older increased with successive birth cohorts starting in 1910.[52]

Morse, et al.[53] analyzed data on pharyngeal cancer incidence in Connecticut during 1935-1994 and found a similar pattern, with men experiencing a 1.7-fold increase in incidence and women experiencing a 3.2-fold increase. When examining oral cavity and pharynx cancers combined, incidence among men increased starting with cohorts born in 1890 and 1900, peaked with the 1920 birth cohort, and leveled off or declined in subsequent birth cohorts.[53] In women, incidence increased among women age 35-74 starting with the 1910 birth cohort and then began to drop in women of all ages starting with cohorts born after 1930.[53]

These patterns in the occurrence of oral and pharyngeal cancer in Connecticut have been attributed to patterns in cigarette smoking during this time, which mirror the incidence trends almost exactly.[52,55] For example, by 1920 approximately 1/2 of adult men were smokers, but it

wasn't until 1950 that 1/3 of all women were smokers.[55] The number of ever-smoking men dropped precipitously with each birth cohort after 1920, but a similar decline was not observed in women until post-1940 birth cohorts.[55] In addition, smoking cessation rates have been consistently higher among American men than women across all birth cohorts.[55] These data are consistent with the observation that smoking became socially acceptable among young adult women around 1920-1940, well after it had become common among men.[52]

2.2.3 Recent Incidence Trends in HNSCC and Evidence Suggesting An Emerging Viral Etiology

Approximately 75% of HNSCC are caused by smoking and heavy alcohol consumption.[56] Therefore, as smoking rates have declined in the United States there has been a concomitant decline in incidence of HNSCC.[2] However, the incidence of oropharyngeal cancer in the United States has been rising--at first gradually and then at a much steeper rate in recent years--despite continuingly unprecedented low rates of smoking.[54] During this period, there have been no dramatic changes in alcohol consumption or in detection methods for oropharyngeal tumors. Therefore, it is suspected that an alternate etiology exists for at least a subset of oropharyngeal tumors.

2.2.3.1 Incidence Trends in HNSCC vs. Oropharyngeal Cancer

During 1965, 43% of American adults reported being current smokers.[2] This number declined by approximately half to 21% in 2005. During roughly the same period (1970-2005) the per-capita consumption of cigarettes in the United States decreased by approximately 50%.[2] Approximately 10 years after the start of the decline in cigarette consumption there were noticeable declines in

incidence of HNSCC in the United States.[2] When examining tumor site in more detail however, subtle trends are evident in oropharyngeal cancer incidence rates. Using SEER data from 1975-2004, Chaturvedi, et al.[54] observed an increasing trend in oropharyngeal cancer incidence during 1975-2004 (annual percent change (APC) 0.80, $P < .001$). This upward trend in incidence was attributable to increases in base of tongue (APC=1.27, $P < .001$) and tonsil (APC=0.60, $P < .001$) cancer incidence as incidence of other oropharyngeal tumors did not change during this period (APC=-0.35, $P = .196$).[54] Increasing incidence of oropharyngeal cancer was particularly notable among persons aged 40-49 (1.93 APC, 1975-2004, $P < .05$) and 50-59 (4.85 APC, mid 1990s to 2004, $P < .05$).[54] Finally, while oropharyngeal cancer incidence was increasing during 1975-2004, the incidence of oral cavity cancers was declining.[54] The pattern of increasing incidence of oropharyngeal cancers was noted only in white and other-race males, with incidence of both oropharyngeal and oral cavity cancers declining over time in black males and females, white females, and males and females of other races.[54]

Although the decrease in incidence of HNSCC in the United States from the mid 1980s through the mid 1990s may have been due to decreasing prevalence of smoking,[2] the moderate increase in incidence of oropharyngeal tumors during the same period, followed by a drastic increase beginning in the mid 1990s, combined with the continued low prevalence of smoking is suggestive of an alternate etiology for a subset of oropharyngeal cancers. The specificity of the incidence trend--occurring in a younger white male demographic, and primarily affecting incidence of tonsil and base of tongue cancers--is further suggestive of a unique etiology for these tumors. In fact, evidence suggests a sexually transmitted viral infection, HPV, may be responsible for the rising incidence of tonsil and base of tongue cancers. HPV is known to be oncogenic in epithelial tissue--particularly in the uterine cervix--through a well-described

mechanism, and the virus is also known to infect oral and oropharyngeal mucosa.[7] First recognized as having a potential role in oral cancer in the 1980s, etiological studies suggest associations between HPV infection and risk of oropharyngeal cancer; e.g., patients who have HPV-related anal or genital cancers are at higher risk of tonsil SCC, husbands of cervical cancer patients have an elevated risk of tonsil cancer compared to husbands of women without cervical cancer, number of lifetime oral sexual partners is positively associated with diagnosis of HPV-positive oral or oropharyngeal cancer, and patients with HPV-positive tumors typically have little or no history of smoking or heavy alcohol use.[57] Furthermore, HPV DNA has been detected in approximately half of all tonsil cancers, with the predominant viral subtype being the oncogenic HPV-16.[57] It is currently believed that oral HPV infection may be associated with as many as 60% of tumors occurring in the oropharynx, where it appears to have a particular affinity for the tonsils.[7]

2.2.3.2 Epidemiological Data Supporting an HPV-related Etiology

The HPV-related etiological hypothesis for the increasing incidence of tonsil and base of tongue cancer is supported by comparing the clinical and molecular profile of HPV-positive tumors with epidemiological trends. Specifically, patients with HPV-positive tumors tend to be younger, have little or no history of alcohol and tobacco use, have better response to radiation and chemotherapy and experience better survival--despite presentation with later stage, higher grade tumors (possibly due to the more frequent presence of wild type p53 in HPV-positive tumors)--compared with patients who have HPV-negative tumors.[7] These clinical observations are borne out in the epidemiological literature, which shows a declining age at diagnosis over time for putatively HPV-related tumors,[54] an increase in incidence of moderate and poorly differentiated tongue and tonsil tumors over time (with concomitant decrease in well-differentiated tumors), a

shift in grade (e.g., the ratio of grade 3 to grade 1 tonsil tumors in 1975 was 1.4:1, whereas in 2006 it had become 10.6:1), and a stunning 105% increase in survival for poorly differentiated tonsil cancer between 1975-1979 and 2000-2004 that is not attributable to advances in screening or treatment.[6] Furthermore, at least one study in the United States has demonstrated an increase over time in the ratio of HPV-positive to HPV-negative tumors present in a clinical tumor archive (from 8:11 in during 1990-1995 to 42:11 during 1995-2001) using polymerase chain reaction (PCR) techniques to detect HPV DNA in paraffin-embedded tumor blocks.[58]

The aforementioned patterns are not unique to the United States and have been observed in other countries where smoking rates are also declining. Investigators in Canada have noted nearly identical patterns in incidence and survival of oropharyngeal and oral cavity cancers as have occurred in the United States.[3] In England, investigators observed increasing incidence during 1985-2006 of tonsil (men: 5.7% annually, women: 4.3% annually) and base of tongue cancers (men: 6.7% annually, women: 6.5% annually).[59] In Sweden, the incidence of tonsil cancer increased 1.1% annually in women and 2.6% annually in men between 1960-1964 and 2000-2003, mostly among people between the ages of 40-44 and 64-69.[5] A study of N=203 paraffin-embedded tonsil cancers diagnosed in Stockholm, Sweden between 1975 and 2002 showed an increasing prevalence of PCR-detected HPV DNA in the tumor blocks over time, with an HPV DNA prevalence of 23% in the 1970s, 29% during the 1980s, 57% in the 1990s, and 68% during 2000-2002.[60] These findings were observed during the same time period when tonsil cancer incidence increased 2.8-fold in Stockholm.[60] In addition, the mean age of patients in this study with HPV-positive tumors was 55 years vs. a mean age of 65 years among patients with HPV-negative tumors.[60]

The pattern of increasing oropharyngeal cancer incidence at younger ages is not globally universal, however. For example, although incidence of base of tongue and tonsil cancers increased in both men and women in the Netherlands during 1989-2006, incidence of these cancers increased in both the 45-59 and 60-74 age groups.[61] While smoking has also become less common in the Netherlands over the last thirty years, the authors of this study suggest the reason they observed increases in oropharynx cancer at all ages may be due to shifting patterns in other, non-HPV (or non-sexually related) risk factors; e.g., an increasing prevalence of heavy drinking.[61] Therefore, although this study cannot rule out the influence of HPV in oropharynx tumors in the Netherlands, it does serve to demonstrate the complexity of HNSCC etiology as it relates to risk factor patterns in different geographic areas.

2.2.3.3 Root Causes

Because tonsil and base of tongue cancer incidence has increased in younger persons in recent years, and because sexual behavior may be associated with the development of HPV-positive tumors, it has been proposed that an increasing prevalence of HPV-infection and/or changing sexual practices among younger generations--especially regarding oral sexual acts--may explain the rising incidence of tonsil and base of tongue cancers.[7] However, there is little direct evidence to support these hypotheses as the epidemiology of oral HPV infection is not well described, including trends in prevalence over time, natural history of infection, and risk factors related specifically to oral (as opposed to uterine cervical) HPV infection; there is no direct evidence of changing sexual behavior over time; and it is unclear why males--and white males in particular--would be most susceptible to HPV-related oropharyngeal cancer.[7]

Furthermore, it is not immediately clear whether the rising incidence of oropharyngeal cancer represents a true increase in the occurrence of these tumors, or whether secular trends in

other factors associated with HPV-related oropharyngeal cancer are responsible for the apparent increase. For example, one alternate etiological possibility is a decrease in incidence of tonsillectomy, which may have given rise to a higher prevalence of in-tact adult tonsils over time. Given the predilection of HPV for infection of the tonsils, this could plausibly explain at least a portion of the observed increase in tonsil cancer incidence. Indeed, the incidence of adenotonsillectomy (tonsillectomy with adenectomy) performed in an inpatient setting at short-stay hospitals in the United States declined by nearly 50% from 1970-1977.[62] However, tonsil cancer is primarily localized to the palatine and lingual tonsils,[57] and the incidence of tonsillectomy without adnoidectomy remained constant from 1970-1977, although there was a 43% decline in incidence among men aged 20-29 (a demographic that would be at risk for tonsil cancer during the time period of increasing incidence starting in the middle 1990s).[62] A further decline in adenotonsillectomy rates performed in an inpatient setting was observed over the period 1977 to 1987 in children under the age of 15, and the tonsillectomy rate was higher in females than in males over this period (again, corresponding to a pattern observed in tonsil cancer incidence: a primarily male phenomenon).[63] However, more recent data that accounted for adenotonsillectomy and tonsillectomy performed in both the inpatient and outpatient setting among persons under 18 years old during 1996-2006 actually observed an increase in adenotonsillectomy over this period and no change in tonsillectomy rates.[64] At least one "back of the envelope" analysis determined that changes in tonsillectomy rates, if they impacted tonsil cancer incidence at all, were insufficient to account for the entire increase in incidence rates of tonsil cancer in the United States.[65] Furthermore, regardless of whether tonsillectomy (or adenotonsillectomy) rates affected tonsil cancer rates, it is unclear whether or how tonsillectomy could affect base of tongue cancer incidence.

2.2.3.4 Current Opinion Regarding HPV and Oropharyngeal Cancer

Several lines of evidence have combined to form the current prevailing opinion that the increase in oropharyngeal cancer incidence is real and is related to HPV infection. Specifically, the increasing incidence of tonsil and base of tongue cancer, combined with the unchanging incidence of tumors at other oropharyngeal sites, the increasing frequency of clinicopathological factors known to be associated with HPV-positive tumors (e.g., declining age at diagnosis, increasing ratio of poorly differentiated to well differentiated tumors, and improvements in survival despite a lack of dramatic improvements in detection and treatment), the association between sexual behavior (especially oral sexual behavior) and risk of tonsil cancer, a substantially reduced prevalence of smoking and the concomitant decline of oral cavity and larynx cancers, and an increasing prevalence of HPV in tumor archives over time, are all offered as evidence in support of HPV infection as a newly emerging etiological factor in HNSCC.[7]

2.2.4 Disparities in HNSCC

Several studies have reported on national trends in HNSCC rates according to sex, race, and socioeconomic status (SES). However, most of these studies, which have been conducted using SEER data, have used the de-facto SEER tumor site classification that includes lip cancer (not strongly associated with tobacco, alcohol, or HPV), salivary gland tumors (rarely SCC), and group base of tongue and lingual tonsil cancers (often HPV-related) in the same category with typically tobacco-related sites of the oral cavity.[66-70] Therefore, the trends in rates among population subgroups reported in these studies are difficult to interpret in light of what is known regarding the etiology of these tumors. Nevertheless, some of these studies do report disparities by specific tumor site and therefore offer some valuable insight. However, deeper insight into

disparities--especially racial disparities in survival--are available from cohort studies that examine end points for etiologically relevant groupings of tumor sites. The following discussion centers on trends in HNSCC incidence and mortality, stage at diagnosis, and survival according to race and sex--all in the context of tumor sites putatively associated with HPV vs. tobacco and alcohol.

2.2.4.1 Incidence and Mortality

Only 1 study reports trends in incidence during 1975-2004 using the SEER database according to HPV-related and HPV-unrelated tumor sites in the oral cavity and oropharynx.[54] The incidence of all tumors (regardless of HPV-relatedness) was generally higher in men than women, and in blacks than whites.[54] However, different trends were observed in the HPV-related vs. HPV-unrelated tumors according to sex and race.[54] The incidence of HPV-related tumors increased in white and other-race men during the entire period 1975-2004, while the incidence in black men first increased during 1973-1987 (APC=4, P=.009) and then decreased through 2004 (APC=-2.31, P=0.006) when the rate approximately matched that in white men.[54] The pattern of occurrence of HPV-related tumors in women was quite different, with the trend in incidence being consistently downward among all women over the entire period.[54] However, black women consistently had the highest incidence of HPV-related tumors, followed by white women, and women of other races.[54]

The incidence of HPV-unrelated tumors showed an entirely different pattern.[54] The incidence of these tumors consistently decreased over time among men and women of all races.[54] Declines in men were especially sharp for whites during 1984-2004 (APC=-2.11, $P < .001$) and for blacks during 1992-2004 (APC=-6.76, $P < .001$). [54] Although rates were generally higher in black men compared with white men during 1975-2004, a precipitous decline among black men

starting in the early 1990s and resulted in an equalization of incidence with white men by 2004.[54] Among women, the incidence of HPV-unrelated cancers declined for all races and rates were approximately equal for white, black, and other-race women in 2004.[54]

Morse, et al.[69] reported on mortality rates by HNSCC sub-sites. Despite white and black men having similar incidence of HNSCC during recent years--both for HPV-related and HPV-unrelated tumor sites--racial disparities exist in male mortality rates.[69] The age-adjusted mortality rate for tonsil cancer (typically HPV-related) was 2.3-fold higher among black men than white men during 1998-2002, and the mortality rate for tumors of the floor of mouth as well as gum and other oral cavity cancers (typically HPV-unrelated) was approximately 2-fold higher in black men compared to white men during this period.[69] In contrast to male mortality rates, there was little difference in mortality rates for these tumors between black and white females.[69]

Goodwin, et al.[68] used SEER data to report on the incidence and mortality of laryngeal cancers in the United States during 1975-2003. The incidence of laryngeal cancer was consistently higher among blacks than whites during this period, although racial differences in incidence were larger in men than women.[68] While the incidence trend was downward for both black and white men over time, it remained relatively flat for black and white women.[68] Mortality rates from larynx cancer show essentially the same pattern as incidence rates for the same period. Black men continue to experience higher mortality compared with white men, while mortality rates are nearly the same in black and white women.[68]

2.2.4.2 Survival

Five-year relative survival for HNSCC is substantially lower among blacks compared with whites in the United States (Table 1).[45] For example, 5-year relative survival for tumors of the oral cavity and pharynx in whites during 1998-2002 was 56.7%, but among blacks the figure was

only 35.6%.[45] Differences in socioeconomic status and access to health care have been proposed to explain some of these racial disparities in survival. However, emerging evidence suggests this racial disparity may be due to underlying patterns in the occurrence of HPV-positive oropharyngeal tumors between blacks and whites.

Table 1. Five Year Relative Survival for Cases Diagnosed 1998-2002 (N=15,450), SEER 17 Areas

		White		Black		Other/Unknown	
		N	5-Year RS (%)	N	5-Year RS (%)	N	5-Year RS (%)
Oral cavity and pharynx	Male and female	12,582	56.7	1,704	35.6	1,164	58.1
	Male	8,756	57.5	1,281	33.6	773	57.5
	Female	3,826	54.9	423	41.7	391	59.3
Larynx	Male and female	6,900	64.3	1,266	52.7	376	69.3
	Male	5,532	65.2	1,003	54.7	321	69.1
	Female	1,368	60.7	263	45.6	55	70.0

RS=relative survival.
Survival is calculated with SEER*Stat using the actuarial method.
Included cases were malignant tumors only, actively followed, known age, and in the Limited Use Database.
Exclusions were death certificate and autopsy only cases, cases with multiple primaries, and persons who are known to be alive but have no survival time recorded.
Oral cavity and pharynx cancers: ICDO-3 codes 019-024,028-052,058-069,090-091,098-119,130-139.
Larynx cancers: ICDO-3 codes 320-329.
All cases are squamous cell histology as defined by the following ICDO-3 morphology codes: 8050-8076,8078,8083-8084,8094.

Arbes, et al.[71] investigated sources of racial disparity in HNSCC survival using cases of oropharyngeal and oral cavity cancer diagnosed in the SEER 9 areas during 1973-1993. In this study, blacks were 50% more likely to die from HNSCC than whites in an unadjusted analysis (HR=1.5, 95% CI: 1.3-1.7). However, blacks were more likely than whites to present with distant stage disease (17.7% of blacks vs. 9.6% of whites), have tumor sizes >= 4.1 cm (18.2% of blacks vs. 10.6% of whites), present with lymph node involvement (53.2% of blacks vs. 39.0% of whites), and to live in a census tract where the median level of education was less than high school (21.1% of blacks vs. 2.6% of whites) and the per capita income was under $10,000 per year (69.0% of blacks vs. 9.6% of whites).[71] All of these variables were significantly associated

with poor survival. After adjustment for these and other factors, 5-year disease-specific survival (DSS) in blacks was not statistically different from whites (HR=1.1, 95% CI: 0.9-1.4).[71] In this study, the factors most strongly associated with lower survival in blacks were measures of SES at the census tract level, such as median education level and per capita income. Therefore, the authors propose that racial differences in SES could reflect racial differences in access to health care that might in part determine why black patients have worse survival, e.g., through presentation with more advanced disease or receiving different (or no) treatment.[71]

In fact, in a large single-institution study of N=1,128 patients at the Medical College of Georgia, Gourin, et al.[72] demonstrated that after controlling for race, tumor site, comorbidities, and treatment received, the only significant predictor of survival was insurance status. The relationship between insurance status and survival was complex however, with results differing by race. Specifically, insured blacks (HR=0.66, 95% CI: 0.77-1.34) and insured whites (HR=0.55, 95% CI: 0.39-0.78) had a lower risk of death relative to uninsured blacks.[72] However, uninsured whites (HR=0.53, 95% CI: 0.38-0.76) still fared better than uninsured blacks, suggesting that insurance does not fully explain the racial difference in survival.[72]

Other single institution studies have also demonstrated residual black/white survival disparities among patients with similar characteristics, particularly in survival from oropharyngeal cancer. For example, Chen, et al.[73] studied N=362 HNSCC patients who were diagnosed and treated at MD Anderson Cancer Center during 1995-2008 using a paired case-case study in which black HNSCC patients were matched 1-to-1 with non-Hispanic white HNSCC patients (81 blacks/81 whites), and Hispanics were matched 1-to-1 with whites (100 Hispanics/100 whites). The matched factors were age, sex, smoking status, stage, tumor site, nodal status, and treatment received.[73] No difference was observed in recurrence-free, DSS, or

overall survival (OS) between blacks and whites or between Hispanics and whites for all cancer sites combined.[73] However, when analyzing oropharyngeal cancer alone, blacks and Hispanics combined had higher risk of recurrence (OR=3.2, 95% CI: 1.12-11.7), death from disease (OR=3.25, 95% CI: 1.0-13.68) and death from any cause (OR=5.67, 95% CI: 1.64-30.18) than whites.[73] The observed survival disparities for oropharyngeal cancer were stronger in blacks than Hispanics; e.g., recurrence in blacks compared with whites: OR=5.0, 95% CI: 1.07-46.93, and recurrence in Hispanics compared with whites: OR=2.00, 95% CI: 0.43-12.36.[73]

In a study of N=202 patients (47% black, 53% white) receiving combination chemotherapy and radiation therapy at the University of Maryland during 1995-2006 for stage III or IV HNSCC, Settle, et al.[74] observed that race was a significant prognostic factor. In this study, median disease-free survival (DFS) was 33 months in whites but only 12 months in blacks (P=.028).[74] This disparity appeared to be due specifically to racial disparity in survival from oropharyngeal cancer.[74] In a subgroup analysis that included N=106 former smoking patients who were similar with respect to prognostic factors (with the exception of higher former alcohol abuse among blacks) 49% of blacks died from their cancer whereas only 34% of whites died from their cancer (P=.084).[74] A racial survival disparity remained after controlling for the difference in prior alcohol abuse between whites and blacks, with 3-year DFS of 20% in blacks and 53% in whites (P = .003).[74] In a future update of this cohort, Settle et al.[75] reported OS to be vastly different in whites (median: 52.1 months) compared with blacks (median: 23.7 months; P=.009) for all tumor sites. This disparity was due to worse OS from oropharyngeal cancer among blacks (median: 25.2 months) than whites (median: 69.4 months; P=.0006) as there was no difference in OS between blacks and whites for other tumor sites (P=.58).[75]

Approximately 60% of oropharyngeal tumors are HPV-positive and these tumors are associated with better prognosis.[7] Therefore, one possible reason that blacks appear to have worse survival than whites from oropharyngeal cancer is differing prevalence of HPV-related oropharyngeal tumors. This question has been addressed by at least two studies.[75,76] In a prospective study of patients recruited for a clinical trial, Settle, et al.[75] examined the prevalence of HPV in oropharyngeal tumors from blacks vs. whites and estimated OS. In this study of N=224 patients with previously untreated tumors and no prior history of cancer, 29% of HNSCC were HPV-positive and the majority of these were in the oropharynx, which had an HPV-positivity rate of 50%.[75] A total of 34% of whites had HPV-positive tumors whereas only 4% of blacks (1 patient) had an HPV-positive tumor.[75] As expected, OS was higher for HPV-positive tumors than HPV-negative tumors ($P < .0001$).[75] HPV-positive tumors in whites had better survival than HPV-negative tumors in whites and all tumors in blacks combined ($P < .0001$).[75] No difference in median OS was observed between HPV-negative tumors in whites (30.1 months) and tumors in blacks (20.9 months; $P = 0.78$).[75] Furthermore, HPV-positive oropharyngeal cancers (98% of which were in white persons) had better OS than HPV-negative oropharyngeal cancers ($P < .0001$).[75]

In a retrospective analysis of N=140 patients treated at the Medical College of Georgia, HPV status of tumors was classified as HPV-active (HPV DNA positive by PCR and high expression of p16 by IHC), HPV-inactive (HPV DNA positive and low p16 expression), and HPV-negative (HPV DNA negative, low expression of p16).[76] The 5-year OS was higher among patients with HPV-active tumors (59.7%) compared with HPV-inactive and HPV-negative (21%; $P=.003$).[76] No black patients (0%) had HPV-active tumors whereas 21% of white patients had HPV-active tumors ($P=.017$).[76]

The reason for the apparent lower prevalence of HPV in oropharyngeal tumors among blacks is unknown but several hypotheses have been proffered, including bias due to selection of patients (possible in retrospective studies[73,74,76] but unlikely in the prospective analysis by Settle, et al.[75]), a lower likelihood of blacks to engage in oral sexual behavior compared with whites, and earlier exposure to genital HPV in blacks that may reduce the likelihood of future oral HPV infection through antibody response.[77] Therefore, while it appears that the survival disparity between blacks and whites in HNSCC may be due primarily to differences in survival from oropharyngeal tumors, perhaps due to a lower prevalence of HPV in these tumors among blacks, the root cause of these disparities is for the moment unclear.

2.3 ETIOLOGY

Numerous exposures have been investigated as putative causal factors in the development of HNSCC, including body mass index (BMI), diet, marijuana smoking, smokeless tobacco, oral hygiene practices, and family history.[78] By far, the most important etiologic factors identified for HNSCC are cigarette smoking, alcohol consumption, and HPV infection.[46] Together, these risk factors account for approximately 90% of HNSCC, with 75% of HNSCC attributable to smoking and drinking and 15% of HNSCC attributable to HPV infection.[7] The following discussion focuses on evidence that establishes these three exposures as the predominant risk factors in HNSCC. In addition, a brief discussion of body size and prior history of cancer in relation to HNSCC risk is provided.

2.3.1 Cigarette Smoking and Alcohol Consumption

Tobacco and alcohol have been linked to HNSCC since at least the 1950s.[79] Since this time, many etiologic case-control studies have been performed around the world, providing a large body of evidence showing substantially elevated risk for HNSCC associated with these lifestyle factors.[80,81] These studies consistently show strong associations, tumor site-specific effects, and dose-responses.[80,81] However, because smoking and alcohol drinking are highly correlated behaviors, it is difficult in any single study to assess the individual contribution of each factor and to study effects of these factors in subgroup analyses; e.g., blond vs. black tobacco smokers, filter vs. non-filter smokers, and consumers of single vs. multiple alcoholic beverage types. The previous conduct of a large number of studies has allowed the formation of consortia such as the International Head and Neck Cancer Epidemiology Consortium (INHANCE), which brings together data on over 26,000 cases and 34,000 controls.[82] The development of this consortium provided the opportunity to conduct studies with sufficient power to examine the independent effects of alcohol and smoking on the risk of all HNSCC overall, as well as perform analyses by tumor site and within population subgroups. Therefore, important insights have been gained from analysis of this consortium data that were otherwise difficult to obtain from single studies. Specifically, studies in the INHANCE consortium have confirmed earlier evidence that: cigarette smoking is a stronger risk factor for HNSCC than alcohol consumption and smoking-related risk is particularly strong for cancer of the larynx;[56] alcohol consumption is primarily related to cancers of the oral cavity, oropharynx, and hypopharynx;[56] there is a synergistic effect of alcohol and tobacco on HNSCC risk that exceeds what would be expected under a multiplicative model;[83] important differences exist in tobacco and alcohol-related risk of HNSCC by sex and age group;[83] alcohol itself, rather than other constituents of alcoholic beverages, is likely

associated with HNSCC risk;[84] usage patterns (e.g., heavy smoking for a short duration vs. light smoking for a long duration) affect HNSCC risk differently;[85] and quitting smoking and drinking is associated with a reduction in risk of HNSCC.[86]

2.3.1.1 Independent Association of Smoking and Alcohol With HNSCC

Insight into the individual contribution of cigarette smoking and alcohol consumption to HNSCC risk comes from a study of N=10,244 HNSCC cases and N=15,227 controls from Europe, North, Central, and South America, India, and the African nation of Sudan.[56] In this study, the association between cigarette smoking and HNSCC was studied in never-drinkers (N=1,598 cases and N=4,051 controls), and the association between alcohol and HNSCC was studied in never-smokers (N=1,072 cases and N=5,775 controls).[56] Among never-drinkers, ever-smoking was associated with 2-times the odds of HNSCC compared with never smoking (OR=2.13, 95% CI: 1.52-2.98).[56] Dose responses were observed for frequency (cigarettes/day), duration (years), and cumulative lifetime exposure to smoking (pack-years; $P < .001$ for all).[56] Odds ratios at the highest levels of cumulative lifetime exposure were 3.46 (95% CI: 1.97-6.09) for 41-50 pack-years and 5.40 (95% CI: 3.06-9.03) for >50 pack-years.[56] Ever-smoking (among non-drinkers) was associated most strongly with cancers of the larynx (OR=6.84, 95% CI: 4.25-11.01) compared to the oropharynx/hypopharynx (OR=2.02, 95% CI: 1.34-3.05) and oral cavity (OR=1.35, 95% CI: 0.90-2.01).[56] This study estimated that 24% (95% CI: 16%-31%) of HNSCC in the population would be prevented if never-drinkers had not smoked.[56]

Alcohol was observed to be much more weakly associated with HNSCC in this study, with ever-drinking (among never-smokers) being associated with a non-significant 18% increase in odds of HNSCC compared with never drinking (OR=1.18, 95% CI: 0.93-1.50).[56] While a trend was observed with frequency (drinks/day) of consumption and cumulative exposure (drink-

years; P < .001 for both), no trend was observed for duration of drinking (years; P= .319).[56] The association between alcohol use and HNSCC was significant only when comparing consumers of >=3 drinks/day with non-drinkers (OR=2.04, 95% CI: 1.29-3.21).[56] The tumor site most strongly associated with ever-drinking was the oropharynx/hypopharynx (OR=5.5, 95% CI: 2.26-13.36) while other sites showed weaker associations with ever-alcohol use (larynx: OR=2.98, 95% CI: 1.72-5.17; oral cavity: OR=1.17, 95% CI: 0.92-1.48).[56] The population attributable risk (PAR) estimated in this study showed a non-significant 7% (95% CI: -4%-16%) of HNSCC attributable to alcohol use.[56]

2.3.1.2 Synergy Between Alcohol and Tobacco

While the independent contribution of cigarette smoking appears to be greater than alcohol use in HNSCC, it is well known that these two behaviors are highly correlated. It is therefore natural to ask whether there are synergistic effects of these factors on HNSCC risk. Previous case-control studies of American and European populations had estimated that alcohol and tobacco combined account for approximately 75% of HNSCC.[87,88] A study in the INHANCE consortium verifies these earlier reports and examines in more detail the interaction between these factors, providing updated estimates of the PAR associated with each factor alone and in combination.[83] This study, including N=11,211 cases and N=16,152 controls, demonstrated the combined effect of alcohol and smoking on HNSCC risk exceeds that which would be expected if their joint effect was multiplicative.[83] Estimation of PARs showed 72% (95% CI: 61.2%-79.1%) of HNSCC is attributable to drinking or smoking, with only 4% (95% CI: 1.5%-5.3%) of cases attributable to alcohol alone and 33% (95% CI: 42.6%-25.9%) attributable to tobacco alone.[83] The simultaneous use of alcohol and cigarettes was estimated to account for 34.9% (95% CI: 17.2%-48%) of HNSCC.[83] The tumor site most strongly related to alcohol alone was the

oropharynx/hypopharynx, with 5.6% (95% CI: 1.9%-7.3%) of these tumors in the population attributable to alcohol.[83] The use of cigarettes alone was most strongly associated with larynx cancers, with 52.2% (95% CI: 36.0%-77.8%) of these cancers attributable to smoking.[83] The use of both tobacco and alcohol was most deleterious in the oropharynx/hypopharynx, where 41.6% (95% CI: 25%-53%) of the tumors in the population are attributable to combined use of alcohol and cigarettes.[83] Subgroup analyses showed 74% (95% CI: 59.9%-82.8%) of HNSCC among men and 57.4% (95% CI: 45.6%-65.3%) of HNSCC among women would be prevented if tobacco and alcohol use were eliminated from the population.[83] It was also estimated that tobacco and alcohol account for a significant proportion of HNSCC only among older persons (age 45-60: PAR=76.8%, 95% CI: 63.1%-84.8%; age >=60: PAR=72.7%, 95% CI: 62.8%-79.5%) and is not a significant contributor to HNSCC among young persons (age <45: PAR=33.5%, 95% CI: -6.7%-56.8%).[83]

2.3.1.3 Alcoholic Beverage Type and HNSCC

Although metabolites of alcohol, notably acetaldehyde, are carcinogenic it is also known that alcoholic beverages contain other carcinogenic ingredients such as N-nitrosamines and polycyclic aromatic hydrocarbons.[89] The type and concentration of these ingredients has been found to vary in different alcoholic beverages such as hard liquor, wine, and beer.[89] In addition, some beverages (notably red wine) are known to contain certain levels of antioxidants, which may be protective against cancer.[89] Therefore, it is reasonable to expect that the carcinogenic effect of alcoholic beverages may be due to ingredients other than (or in addition to) alcohol; and the carcinogenic effect of alcoholic beverage types may vary. Because of small sample sizes in individual studies, however, it is often difficult to analyze subgroups of beverage drinkers. Again, the INHANCE consortium has provided insight into the relationship between alcoholic

beverage type and HNSCC using a pooled analysis of case-control studies that included N=9,107 cases and N=14,219 controls.[84] This analysis included N=1,844 beer-only drinkers (858 cases; 986 controls); N=1,026 liquor-only drinkers (499 cases; 527 controls); N=3,481 wine-only drinkers (1,021 cases; 2,460 controls); N=12,364 drinkers of multiple beverage types (5,605 cases; 6,759 controls); and N=4,611 never drinkers (1,124 cases; 3,487 controls). The following odds ratios were observed for ever-drinking vs. never-drinking:[84]

- Beer only: OR=2.1, 95% CI: 1.6-2.7
- Liquor only: OR=2.2, 95% CI: 1.4-3.4
- Wine only: OR=1.6, 95% CI: 1.0-2.6

A positive dose-response with frequency (drinks/week) was observed for all beverage types ($P < .0001$ for all), although odds ratio estimates for wine consumption were only significant at high levels (>30 drinks/week: OR=6.3, 95% CI: 2.2-18.6).[84] The authors attributed this pattern to potential residual confounding; e.g., by healthier diet associated with wine-only drinking and a potential for "alcohol washing" (whereas wine is typically consumed with food, the chewing and swallowing of food may impair the carcinogenic effect of wine on the oral mucosa).[84]

Analyses by tumor site showed each beverage type to have stronger associations with oral cavity and oropharynx/hypopharynx cancers rather than larynx cancers.[84] Beer was significantly associated with cancer at these sites for <15 drinks/week (oral cavity: OR=2.0, 95% CI: 1.4-2.8; pharynx: OR=2.3, 95% CI: 1.7-3.1) and >= 15 drinks/week (oral cavity: OR=6.4, 95%CI: 3.9-10.3; pharynx: OR=4.3, 95% CI: 2.7-6.8).[84] Liquor was significantly associated with these tumor sites only for >= 15 drinks/week (oral cavity: OR=3.2, 95% CI: 1.6-6.4; pharynx:

OR=3.6, 95% CI: 2.0-6.3) and the same was observed for wine (oral cavity: OR=5.9, 95% CI: 2.3-15.4; pharynx: OR=4.4, 95% CI: 2.0-9.6).[84]

Overall, the results of this large pooled analysis of case-control studies, which do not show appreciable differences in risk of HNSCC associated with different beverage types, is suggestive of an ethanol-specific carcinogenic effect rather than a carcinogenic effect of particular beverage ingredients.

2.3.1.4 Patterns of Drinking and Smoking Associated With HNSCC

Cumulative lifetime exposure to smoking and alcohol is typically measured in pack-years (for smoking) and drink-years (for alcohol). These measures are calculated simply by multiplying the usual frequency of consumption (e.g., in cigarettes/day or drinks/day) times the duration of the behavior in years. Because of the nature of these calculations, it is possible to obtain a given magnitude of cumulative exposure by measuring either a low frequency and long duration, or a high frequency and short duration. Thus, it is possible that examination of cumulative exposure alone in relation to disease risk can obscure a relationship in which the pattern of use is more important than the cumulative exposure. A study by Lubin, et al.[85] addressed this concern in HNSCC using INHANCE data and observed that while cigarette smoking patterns were important in HNSCC, the effect of alcohol was due mainly to cumulative exposure. Two opposite patterns of HNSCC risk were evident for smoking: one pattern for smoking up to 15 cigarettes/day, and another for smoking over 15 cigarettes per day.[85] For a fixed pack-year history, up to 15 cigarettes per day, smoking more cigarettes/day for a short duration was associated with higher risk of disease than smoking a less cigarettes/day for a long duration.[85] However, for a fixed pack-year history, above 15 cigarettes/day, smoking less cigarettes/day for

a long time was with higher risk of disease compared with smoking more cigarettes/day for a short time.[85] These patterns were similar for all tumor sites but strongest for laryngeal cancer.[85]

While different disease risk was noted according to cigarette smoking habits, a more consistent pattern was observed for alcohol consumption.[85] Specifically, drinking a large number of drinks/day for a short time period was worse than drinking a small number of drinks/day for a long duration.[85] This pattern was evident regardless of the number of drinks per day consumed.[85]

2.3.1.5 Cessation of Smoking and Drinking

While the effects of smoking and drinking are clearly deleterious to the health of the oral cavity, pharynx, and larynx, data from INHANCE also show that cessation of these behaviors can have a beneficial effect on disease risk. In an analysis of N=9,167 cases and N=12,593 controls, quitting smoking was associated with a significant 30% reduction in risk of HNSCC compared with current smoking (OR=0.70, 95% CI: 0.61-0.81).[86] However, risk of HNSCC became equivalent to that of never-smokers (never vs. current: OR=0.25, 95% CI: 0.17-0.36) only at >=20 years after quitting (>=20 years quit vs. current: OR=0.23, 95% CI: 0.18-0.31).[86] Significant reductions in risk for most tumor sites were observed after only 1-4 years since quitting.[86] Risk of laryngeal cancer was reduced 30% after 1-4 years since quitting (compared with current smoking: OR=0.70, 95% CI: 0.56-0.87) and by 89% with >=20 years since quitting (compared with current smoking: OR=0.11, 95% CI: 0.08-0.16).[86] Cessation of drinking was associated with much weaker decreases in risk, with significant reductions observed only after >= 20 years (compared with current drinking: OR=0.60, 95% CI: 0.40-0.89).[86]

2.3.1.6 Prospective Studies of Smoking and Alcohol in HNSCC

Several large prospective studies have been conducted that examine the risk of "upper aerodigestive tract" cancers associated with cigarette smoking and alcohol consumption.[90-94] While these studies typically include some of the same tumor sites covered under the classification of HNSCC, not all HNSCC sites are included and the grouping of tumor sites is different. For example, two studies included lip cancers, base of tongue, and lingual tonsil tumors as "oral cavity" cancer.[90,91] Lip cancers are not typically included in studies of HNSCC because the primary etiology for these tumors is sun exposure,[47] while base of tongue and lingual tonsil tumors are often included as oropharynx (rather than oral cavity) tumors in studies of HNSCC. Thus, it is nearly impossible to relate results of the aforementioned prospective studies conducted under the heading of "upper aerodigestive tract cancers" with prior case-control studies of HNSCC.

Only one prospective study classified tumor site according to the typical HNSCC classification, and this study included only oral cavity cancers.[92] This study used Cox proportional hazards regression to estimate risk of oral cavity cancer associated with alcohol consumption in a cohort of N=32,347 Indian men (mean duration of follow-up: 8.7 years).[92] This study found that, compared with never drinking, current drinking was associated with an 49% increased risk of oral cavity cancer (HR=1.49, 95% CI: 1.01-2.21).[92] Former drinking in this cohort was associated with an even higher risk (HR=1.90, 95% CI: 1.13-3.18) although this may reflect disease-related cessation of drinking.[92] Significant dose-responses were evident for frequency (days/week drinking; P=.006) and duration (years; P = .005).[92]

2.3.2 Passive Smoking and HNSCC

Although the association between active smoking and risk of HNSCC is well established, the role of passive smoke exposure in HNSCC etiology is less clear. The body of evidence examining this relationship consists largely of case-control studies (Table 2). In aggregate, these studies imply a positive relationship between passive smoke exposure and HNSCC.[31,95-97] In addition, three case-control studies[27,28,98] investigated nasopharyngeal carcinoma, with two of these studies reporting a positive association with passive smoke exposure.[27,28] Nasopharyngeal carcinoma is not typically considered under the umbrella of HNSCC because, despite arising in epithelial tissue, this tumor is characterized by a unique histology involving infiltration of the tumor by inflammatory cells.[30] In addition, nasopharyngeal carcinoma is strongly associated with Epstein-Barr virus (EBV) and certain dietary constituents common in Asian populations.[29] However, because the literature on passive smoke exposure and upper airway cancer is limited, these studies are included here for illustration of the carcinogenic effect of passive smoke exposure in epithelium at sites in the head and neck, and for comparison with tumor sites typically considered under the classification of HNSCC.

2.3.2.1 Case-Control Studies of Passive Smoke Exposure and Cancer in the Head and Neck

Three of the seven case-control studies (Table 2) were hospital-based[27,96,97] while four were population-based.[28,31,95,98] The majority of studies were of nasopharyngeal carcinoma (one in Serbia,[27] one in Taiwan,[98] and one in mainland China[28]); one study examined maxillary sinus cancer in a Japanese population;[95] one study from Germany analyzed larynx cancer;[31] and only two studies from the United States looked more broadly at HNSCC: one examining oral cavity, pharynx, larynx, and sinus cancers;[96] the other looking at these sites in addition to lip, salivary

gland, and esophagus cancers.[97] Only two studies enrolled previously untreated patients.[97,98] In three studies, the case series had no prior history of the cancer under study[27,97,98] and in only one study were the cases verified to have no prior history of any cancer.[97] None of the studies verified the controls had no prior history of cancer.[27,28,31,95-98] All seven studies used matching and some used logistic regression modeling in the analysis.[27,28,31,95-98] All except for one study[27] analyzed never-smokers separately from ever-smokers.

Among the three studies of nasopharyngeal carcinoma,[27,28,98] one found an association with adult passive smoke exposure[28] and two found an association with childhood[27,28] exposure. However, two studies observed no association with adult exposure[27,98] and one study observed no association with childhood exposure.[98] Three of the four[31,95-97] studies examining HNSCC observed associations between adult passive smoke exposure at home[28,31,95-97] or at work.[28,96,97] Only one study examined childhood passive smoke exposure and HNSCC and did not observe an association.[31]

Passive Smoke Exposure as an Adult

In a study of Japanese men and women, Fukuda, et al.[95] noted a significant association between passive smoke exposure and maxillary sinus cancer in women only. Although passive smoking was identified as an independent risk factor for maxillary sinus cancer after controlling for sinusitis, woodworking, and active smoking in a logistic regression model, the authors do not report odds ratios from this model.[95] Rather, the reported odds ratios and tests for trend are adjusted only for the matching factors of age, sex, and residence.[95] Among all women, odds ratios (relative to 0 smokers in the household) increased with increasing numbers of household smokers (1 smoker: OR=1.66; >1 smoker: 4.47).[95] A similar pattern was noted among never smoking women (1 smoker: OR=1.40; >1 smoker: OR=5.73).[95] These increases represented a

significant ($P < 0.05$) trend among all and never smoking women.[95] Similar data are not provided for men, and the formal test of interaction between sex and passive smoke exposure is not reported.[95]

Using N=59 never-smoking HNSCC cases from the Cleveland Clinic and N=177 never-smoking internal medicine patients as controls (matched on age, sex, race, and drinking status) Tan, et al.[96] observed a significant association between exposure to passive smoke either in the workplace or home and HNSCC (either setting vs. neither: OR=5.32, P<.001).[96] Exposure in the workplace (ever vs. never: OR=10.16, $P < .001$) was more strongly related to HNSCC than exposure at home (OR=2.8, P=.006).[96] It should be noted, however, that the case series included in this study was atypical. Notably, these 59 cases exhibited a male:female ratio (0.84:1) that differed significantly from the entire set of N=853 HNSCC cases seen at the Cleveland Clinic during the study period (2.27:1).[96] The distribution of tumor sites also varied between the 59-person case series and the entire group of N=853 cases.[96] Whereas the most common tumor among all patients seen during the study period was laryngeal cancer, the most common tumor in the selected N=59 cases was tongue cancer (it is not clear whether base of tongue was included as "tongue" cancer).[96] The gender imbalance in the case series may have arisen due to the way cases were selected: by looking for evidence of lifelong non-smokers in the medical record.[96] Such information is typically not reliably recorded in the medical record and may have been recorded differently for men and women. In addition, the case series included a 14-year old boy as well as ten people with second primary HNSCC.[96] The exposure status of these patients was not reported and it is uncertain whether the heterogeneity in the case series introduced by inclusion of these cases would have biased odds ratio estimates.[96] Finally, the control group used in this study may not have been representative of non-diseased persons in the source population

that gave rise to the cases. Specifically, the controls were selected from the internal medicine department during an unspecified 2-week time period, whereas the cases were diagnosed between 1986 and 1993 (the study was published in 1997).[96] Some opportunity for information bias is also present in this study. Specifically, while controls themselves were interviewed to verify lifelong never-smoking and to collect data on passive smoke exposure, interviews for cases may have been conducted with spouses.[96]

Zhang, et al.[97] studied N=173 first primary HNSCC cases (26 never smokers) recruited from Memorial Sloan-Kettering Cancer Center and N=173 controls (59 never smokers) recruited from the Sloan-Kettering blood bank. This study demonstrated an increasing risk of HNSCC with increasing intensity of passive smoke exposure (never exposed, either home or work exposure [moderate], or both home and work exposure [heavy]; P=0.025) after controlling for pack-years of smoking, age, sex, race, education, alcohol, and marijuana use.[97] Marijuana use, alcohol consumption, and mutagen sensitivity[†] modified the association between passive smoke exposure and HNSCC in this study after controlling for pack-years of smoking, age, sex, race, and education.[97] Compared to persons never exposed to passive smoke and who consumed <100 drinks/month, the addition of passive smoke exposure alone (OR=2.5, 95% CI: 0.8-7.6) or heavy drinking alone (>=100 drinks/month; OR=4.9, 95% CI: 0.3–75.8) was not associated with HNSCC, while passive smoke exposure and heavy drinking together were significantly associated with HNSCC (OR=10.2, 95% CI: 2.7-37.8).[97] Relative to persons never exposed to passive smoke and who never used marijuana, the addition of marijuana use (OR=3.5, 95% CI: 0.4–28.4) or passive smoking alone (OR=2.6, 95% CI: 0.7-9.0) was not associated with HNSCC, although the presence of both risk factors was associated with a 7-fold increase in odds of

[†]Mutagen sensitivity was defined as the number of chromosome breaks/cell induced by in vitro exposure of peripheral blood lymphocytes to Bleomycin.

HNSCC (OR=7.1, 95% CI: 1.5-34.5).[97] Compared with persons never exposed to passive smoke, and who had <1 break/cell, the addition of passive smoking (OR=2.0, 95% CI: 0.2-17.7) or having >=1 break/cell (OR=2.6, 95% CI: 0.1–71.1) not associated with HNSCC whereas the exposure to passive smoking and having >=1 break/cell was associated with a nearly 18-fold increase in odds of HNSCC (OR=17.5, 95% CI: 1.9-162.0).[97]

Yuan, et al.[28] studied nasopharyngeal carcinoma in Chinese people from Shanghai using the largest sample of never smokers of any of the studies reviewed here (N=429 cases and N=546 controls). Among never smokers, results were suggestive of an association between adult exposure and NPC among women only (ever vs. never exposed in the home among men: OR=1.29, 95% CI: 0.62-2.68; among women: OR=1.95, 95% CI: 1.18-3.21; P-for-interaction: p=0.08).[28] A significant positive trend was observed among women with number of years living with a smoking spouse (p=0.004), number of cigarettes/day smoked by the spouse (p=0.02), and pack-years of smoking by the spouse (p < .001).[28] Results were similar for adult exposure to passive smoke in the workplace (coworker ever smoked vs. never among women: OR=2.84, 95% CI: 1.34-6.00; P-for-interaction of sex and passive smoking=0.02).[28]

Ramroth, et al.[31] studied laryngeal cancer in a German population. In analyses controlling for smoking there was no association with exposure to passive smoke through a partner/spouse (OR=1.1, 95% CI: 0.78-1.7), at work (OR=1.2, 95% CI: 0.82-1.7), or combined exposure from work and a spouse/partner (OR=1.2, 95% CI: 0.77-1.8).[31] However, a dose-response was detected for number of hours exposed to smoke from a spouse/partner during the lifetime.[31] The reported odds ratio for 20,000 hours of exposure (vs 0 hours) is 1.2 (95% CI: 1.0-1.4) and the authors indicated that 20,000 hours is equivalent to 2.5 hours of exposure per day for twenty-two years.[31] No such dose-response was detected for exposure to passive smoke in the workplace.[31]

Passive smoke exposure among never smokers was unrelated to laryngeal cancer in this study (ever exposed vs. never: OR=2.0, 0.39-10.7) although this study included only N=9 never-smoking cases.[31]

Finally, Cheng, et al.[98] studied passive smoke exposure in the home among Chinese people from Taipei and Taiwan and found no association with nasopharyngeal carcinoma (ever vs. never: OR=0.7, 95% CI: 0.5-1.2). No trend was observed with increasing number of smokers in household (P=0.9), duration of exposure in person-years (P=0.7), or cumulative lifetime exposure (pack-person-years; P=0.5) after controlling for age, sex, race, educational level, family history of NPC, and drinking status.[98]

Passive Smoke Exposure During Childhood

Data on the association between childhood passive smoke exposure and cancer in the head and neck are more limited in comparison to adult exposure to passive smoke. A total of four case-control studies[27,28,31,98] examined this relationship and two of the four observed a positive association[27,28] between childhood passive smoke exposure and nasopharyngeal carcinoma. Yuan, et al.[28] studied N=429 never-smoking cases (242 women) and N=546 never-smoking controls (306 women) and found childhood passive smoke exposure to be related to nasopharyngeal carcinoma in women (ever vs. never exposed: OR=1.95, 95% CI: 1.18-3.21) but not in men (ever vs. never exposed: OR=1.29, 95% CI: 0.62-2.68; P-for-interaction=0.06). Among women, a significant positive trend was observed for the number of cigarettes/day smoked by the mother (p=0.003) and father (p=0.001) and for all household members (p=0.01).[28] No such dose-responses were observed for men.[28]

Nesic, et al.[27] also observed a positive association between childhood passive smoke exposure and nasopharyngeal carcinoma in Serbian men and women (ever vs. never exposed:

OR=4.04, 95% CI: 1.10-14.85) in a matched (sex, age, and residence) case-control study. This study did not attempt to control for active smoking, nor did it analyze never smokers separately; however, the authors indicate that the proportion of ever smokers is similar in the case and control groups (specific numbers are not provided in the report).[27]

One other study of nasopharyngeal carcinoma found no association with childhood passive smoke exposure.[98] Cheng, et al.[98] studied N=178 never-smoking cases and N=173 never-smoking controls and observed a borderline inverse association between childhood passive smoke exposure and nasopharyngeal carcinoma (ever vs. never exposed: OR=0.6, 95% CI: 0.4-1.0). However, no trend was observed with the number of smokers in the household (P=0.1), duration of exposure in person-years (P=0.0), or cumulative lifetime exposure in pack-person-years (P=0.0).[98]

Finally, Ramroth, et al.[31] studied laryngeal cancer in a German population and observed no relationship with childhood passive smoke exposure after controlling for smoking (OR=0.96, 95% CI: 0.67-1.4).

2.3.2.2 Prospective Studies of Passive Smoke Exposure and HNSCC

Adult exposure to passive smoke at home and in the workplace in relation to pharyngeal and laryngeal cancer was studied in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.[99] Among N=102,923 never smokers a total of thirteen pharyngeal and laryngeal cancers were diagnosed during a median follow-up of seven years per person.[99] Adult passive smoke exposure was not a risk factor for development of pharyngeal and laryngeal cancers among never-smokers (HR=1.02, 95% CI: 0.63-1.66) after controlling for sex, age, country of residence, years of schooling, total energy intake, consumption of fruit and vegetables, and physical activity.[99] However, adult passive smoke exposure was associated with pharyngeal and

laryngeal cancer among former smokers after controlling for the aforementioned factors (HR=2.32, 95% CI: 1.07-5.01).[99]

Table 2. Summary of Case-Control Studies of Passive Smoke Exposure and HNSCC

Study	Population	Never Smokers	Tumor Site	Time Period of Exposure	Results
Fukuda[95]	• Hokkaido, Japan 1982-1986 • 169 cases • 338 controls matched on age, sex, residence	• 35 cases • 74 controls†	• Maxillary sinus	• Adult (home)	• Dose response with increasing number of smokers in the household in women only; among all and never smoking women‡
Tan[96]	• Cleveland Clinic 1986-1993 • 59 never-smoking cases • 177 never-smoking Internal Medicine outpatients matched on age, sex, race, alcohol use	• 59 cases • 177 controls	• Oral cavity, pharynx, larynx, sinus	• Adult (home,work)	• Home (ever vs. never): OR=2.8, P=.006‡ • Work (ever vs. never): OR=10.16, P<.001+
Cheng[98]	• Taipei and Taiwan 1991-1994 • 375 cases • 327 community controls matched on sex, age, residence	• 178 cases • 173 controls	• Nasopharynx	• Childhood • Adult	• No association between passive smoking and nasopharyngeal carcinoma among ever-smokers or never-smokers#

Table 2 continued

Study	Population	Never Smokers	Tumor Site	Time Period of Exposure	Results
Zhang[97]	• Sloan Kettering 1992-1994 • 173 first primary cases • 176 controls from Sloan Kettering blood bank; frequency matched on age/sex; no history of cancer	• 26 cases • 59 controls	• Oral cavity, pharynx, larynx, sinus, lip, salivary glands, esophagus	• Adult (home,work)	• Trend with increasing intensity (P=0.025) controlling for smoking[$] • Never smokers only: OR=1.5 (0.3-6.5)[$] • Marijuana use and mutagen sensitivity may modify risk
Yuan[28]	• Shanghai, China • 935 cases from Shanghai tumor registry • 1,032 controls randomly chosen from Shanghai; frequency-matched on sex/age	• 429 cases • 546 controls	• Nasopharynx	• Childhood • Adult (home,work)	• Among never smokers, passive smoke associated with nasopharyngeal cancer in women only • P-for-interaction: 0.06 (childhood), 0.08 (home), 0.02 (work)[€]
Ramroth[31]	• Germany 1998-2000 • 257 cases • 769 controls randomly selected from population registries where cases lived; frequency matched on age, sex	• 9 cases • 203 controls	• Larynx	• Childhood • Adult (home, work)	• No association with childhood exposure% • Dose response with number of hours exposed at home as an adult, controlling for smoking%

Table 2 continued

Nesic[27]	• Belgrade, Serbia 2001-2003 • 45 cases at a single clinic • 90 controls treated by orthopedists at 2 other clinics in Belgrade; matched on sex, age, and residence	• Not reported	• Nasopharynx	• Childhood • Adult (work)	• Ever vs. never exposed during childhood: OR=4.04 (1.10-14.85)‡ • No association with adult exposure

*All studies enrolled incident cases. OR=odds ratio. Intervals cited are 95% confidence intervals.

†Non-smoking cases and controls shown here are all women. The number of non-smoking male cases and controls is not specified in the manuscript.

‡Adjusted only for the matching factors.

#Adjusted for matching factors as well as education, family history of NPC, and alcohol drinking

$Adjusted for matching factors as well as pack-years of smoking, education, alcohol drinking, and marijuana use

€Adjusted for matching factors as well as education, frequency of eating preserved foods, tangerines and oranges, exposure to rapeseed oil smoke, burning coal while cooking, exposure to chemical fumes at the workplace, history of chronic ear and nose conditions, and family history of NPC

%Adjusted for matching factors as well as smoking (log-pack-years+1), ex-smoking (yes/no), alcohol drinking, and education

2.3.3 HPV Infection

HPV is a sexually transmitted DNA virus that has been established as a necessary cause of uterine cervical cancer.[100] Although there are over one hundred different types of HPV, only a few are considered to be associated with cancer; particularly HPV-16 and HPV-18.[100] The virus is capable of transforming infected cells forcing cells to enter into the cell cycle, and through abrogation of p53-mediated apoptosis.[100] Specifically, the viral oncogene E7 binds to the retinoblastoma (Rb) protein causing Rb to release the transcription factor E2F, which is required for advancing the cell cycle from G1 to S phase; and the viral oncogene E6 binds to p53, causing is ubiquitination.[100] Molecular evidence of oncogenic capability alone cannot be sufficient to establish a viral agent as a causative factor in cancer, however.[101] In 1990 Evans and Mueller[101] published a set of guidelines for establishing a virus as a causative agent in cancer, including a description of the epidemiological evidence required to draw such conclusions, which are paraphrased as follows:

1. The geographic distribution of the virus should match the geographic distribution of the tumor after adjustment for age of infection and the presence of cofactors required for tumorigenesis; and
2. The virus (or a marker for it) should be found more often in cancer cases than in matched controls within the same geographic area; and
3. Infection with the virus should occur prior to development of the tumor and a higher incidence of the tumor should follow in infected persons compared with uninfected persons; and
4. Preventing infection (e.g., through vaccination) should reduce the incidence of the tumor.

The following is a brief overview of studies that address guidelines 1-3 in relation to HNSCC. To our knowledge, no studies have yet shown vaccination against HPV to reduce the incidence of HNSCC.

2.3.3.1 Distribution of HPV Infection in the United States

Markowitz, et al.[102] estimated the prevalence of HPV in the United States by establishing the presence of antibodies to HPV among N=4,303 persons included in the National Health and Nutrition Examination Survey (NHANES) during 2003-2004. A total of 10.3% of persons surveyed were seropositive for the oncogenic HPV-16.[102] Seropositivity was more common in women (15.6% were seropositive) than men (5.1% were seropositive).[102] Seropositivity was observed in women of all ages beginning with 14-19 year olds (4%), 20-24 year olds (13.4%), and 30-39 year olds (21.9%).[102] Seropositivity was also high among older women; e.g., those aged 50-59 (13.9%).[102] The age-specific pattern of seropositivity was similar in men, although the magnitude at each age was substantially smaller (e.g., 0.3% of 20-24 year old men were seropositive).[102] Non-Hispanic Black men (7%) and women (18.5%) had higher seropositivity rates than their white counterparts (5.6% and 16.2% respectively).[102] Older age and lifetime number of sexual partners were positively associated with seroprevalence in men and women.[102]

Data from the NHANES study[102] match the pattern of viral exposure that might be expected given the occurrence of putatively HPV-related HNSCC in the United States. For example, evidence of infection is present at younger ages (in agreement with the occurrence of putatively HPV-related cancers at younger ages[7]) and more blacks show evidence of infection that whites (agreeing with the higher incidence of HPV-related tumors among blacks in the United States during and prior to the survey period[54]). However, seroprevalence is not necessarily evidence of *oral* infection with HPV and could instead represent genital exposure,

and the association between seroprevalence and number of sexual partners is not necessarily associated with oral sex (a possible route of transmission for oral HPV).

The prevalence of oral HPV infection was addressed by D'Souza, et al.[103] in a study of N=332 healthy persons age 25-87 (mean age of 57) using PCR to detect HPV DNA in exfoliated cells from the oral cavity. A total of 4.8% of this cohort had a prevalent oral HPV infection.[103] Ten persons were positive for high risk HPV types but only one person was infected with HPV-16.[103] Factors associated with oral HPV infection (using multivariable logistic regression) were lifetime number of sexual partners (P=0.007), lifetime number of oral sex partners (P=0.003), and current tobacco smoking (compared to never smokers: OR=3.86, 95% CI: 1.17-12.7).[103] In the same study, lifetime number of vaginal sex partners was not associated with oral HPV infection in a separate cohort of college-aged men (P=0.91), whereas lifetime number of oral sexual partners (P=0.31) and number of people open-mouth kissed in the past year (P=0.023) were associated with oral HPV infection.[103] Thus, these data provide some evidence that oral HPV infection is found in young people and that transmission of the infection may be through oral sexual acts and open-mouth kissing, and risk of infection may be modified by cigarette smoking.

To cause cancer, HPV must integrate into the host cell genome and become transcriptionally active.[100] Therefore, a crucial factor for the development of HPV-related HNSCC is persistence of the virus.[100] Unfortunately, the natural history of oral HPV infection is not as well defined as it is in the uterine cervix. However, some preliminary data suggest persistence in the oral cavity is possible and risk factors for viral persistence in the oral cavity may differ from factors associated with persistence in the uterine cervix. D'Souza, et al.[104] evaluated the 6-month natural history of HPV infection in a convenience sample of N=199

women (63 human immunodeficiency virus (HIV)-negative) from the Women's Interagency HIV Study and compared this with the natural history of cervical HPV infection during the same period. The presence of HPV in the oral cavity was ascertained using PCR to detect HPV DNA in oral rinse specimens collected at baseline and at a 6-month follow-up visit.[104] Persistent oral HPV infection was associated with age > 44 (compared to age <= 30: OR=20, 95% CI: 4.1-83.0) and current smoking (compared to not currently smoking: OR=8.0, 95% CI: 1.3-53.0) after controlling for age and CD4-count, while these factors were unrelated to HPV persistence in the uterine cervix (using PCR with cervical vaginal lavage samples).[104] HPV persistence rates were higher in the cervix than the oral cavity for HIV-negative women (32% vs. 7% respectively) and HIV-positive women (65% vs. 15%).[104] While these data demonstrate oral HPV infections do persist, and suggest the existence of unique risk factors for persistence in the oral cavity, generalizability is limited as this study included only women at high risk for, or already infected with, HIV.[104]

The studies discussed above show HPV is present in the United States where the incidence of putatively HPV-related tumors is in the rise, and that the age and race-specific patterns of HPV exposure match what might be expected if HPV caused HNSCC. Furthermore, the above studies--although preliminary--provide evidence that prevalent oral HPV infection is associated with oral sexual behavior, and that oral HPV infections are capable of persisting. What is not known from these studies however is whether any persons developed HNSCC, whether the rate of HNSCC was higher in persons exposed to HPV vs. persons not exposed to HPV, and whether the prevalence of oral HPV infection (or associated sexual behaviors) has changed over time.

2.3.3.2 Case-Control Studies of HNSCC Associated With HPV Exposure

At least thirty case-control studies have examined the association between HPV exposure and HNSCC. Exposure to HPV is generally measured in three different ways: sexual behavior, and especially oral sexual behavior (presumed to be a surrogate for HPV infection), serology (usually antibodies to the capsid antigens L1 or L2, or the early genes E6 and E7), or through the presence of HPV DNA in oral exfoliated cells. The following discussion provides background on a representative set of such studies.

Sexual Behavior and HNSCC

The association between sexual behavior and HNSCC was studied in the INHANCE consortium via a pooled case-control analysis including N=5,642 cases and N=6,069 controls from eleven different countries.[105] Ever having oral sex was inversely associated with oral cavity cancers (OR=0.80, 95% CI: 0.67-0.95) and was not associated with oropharyngeal cancer (OR=1.05, 95% CI: 0.87-1.26).[105] However, ever having oral sex was associated with tonsil cancer in men only (OR=1.59, 95% CI: 1.09-2.33) as was having 4 or more lifetime sexual partners (OR=3.36, 95% CI: 1.32-8.53).[105] Base of tongue cancer was also associated with ever having oral sex but in women only (OR=2.02, 95% CI: 1.19-3.46).[105] No dose responses were observed with number of lifetime sexual or oral sexual partners for either men or women.[105]

HPV Serology

A hospital case-control study conducted during 2000-2005 at Johns Hopkins University enrolled N=100 oropharyngeal cancer cases and N=200 individually matched (on sex and age) controls from the same clinics where the cases were identified.[106] This study measured HPV exposure by testing for the presence of antibodies to HPV-16 L1, E6, and E7 in serum collected from cases

and controls.[106] Seropositivity to L1 was associated with 32-times the risk of oropharyngeal cancer (OR=32.2, 95% CI: 14.6-71.3) after controlling for age, sex, smoking, drinking, tooth brushing, and family history of HNSCC.[106] Seropositivity to E6 and E7 was associated with even greater risk of oropharyngeal cancer (OR=58.4, 95% CI: 24.2-138.3).[106]

A similar study enrolled N=485 cases of HNSCC from nine clinics in the Boston metropolitan area along with N=594 population controls (frequency matched to cases on age, sex, and town of residence) during 1999-2003.[107] The presence of antibodies to HPV-16 L1 in this study was associated with 4.5 times the risk of HNSCC relative to seronegative status (OR=4.5, 95% CI: 3.1-6.5) after controlling for sex, age, race, education, alcohol, and tobacco use.[107] The association between L1 antibodies and HNSCC was strongest for pharyngeal cancers (OR=10.0, 95% CI: 6.6-15.3), weaker for laryngeal cancers (OR=1.7, 95 %CI: 1.5-5.1) and oral cavity cancers (OR=1.7, 95% CI: 1.0-2.8).[107]

The association between HPV-positive serostatus and HNSCC is not limited to the United States. Similar results to the aforementioned United States studies were observed in an international hospital-based case-control study that enrolled N=1,670 cases of oral cavity and oropharyngeal cancer and N=1,732 controls from eleven different countries (not including the United States).[108] In this study, the presence of HPV-16 L1 antibodies was associated with oropharyngeal (OR=3.5, 95% CI: 2.1-5.9) and oral cavity cancers (OR=1.5, 95% CI: 1.1-2.1) after controlling for sex, country, age, smoking, drinking, and paan chewing.[108] The presence of antibodies to either or both E6 or E7 was also associated with oropharyngeal (OR=9.2, 95% CI: 4.8-17.7) and oral cavity cancers (OR=2.9, 95% CI: 1.7-4.8).[108]

HPV DNA in Oral Exfoliated Cells

Because HPV serology is not necessarily indicative of oral infection with HPV, case-control studies that demonstrate an association between seropositivity to HPV antigen and HNSCC are not definitive. Other studies have attempted to address this issue by establishing the presence of HPV DNA in cells exfoliated from the oral cavity. In a study that enrolled N=201 HNSCC cases from the University of Iowa Hospitals and the Iowa City VA Medical Center along with N=333 controls from the hospitals' family and internal medicine clinics, 28% of cases and 18% of controls had an oral HPV infection.[109] More cases (23%) than controls (11%) were positive for high risk HPV.[109] High risk oral HPV infection was associated with HNSCC (OR=2.5, 95% CI: 1.5-4.2) after controlling for age, pack-years of smoking, and number of drinks per week, and oral HPV infection was associated with the presence of HPV DNA in the tumor as determined by PCR.[109] Similar methods were used to detect HPV DNA in oral exfoliated cells in the aforementioned case-control study of oropharyngeal cancer conducted at Johns Hopkins during 2000-2005 (oral HPV infection vs. no infection: OR=12.3, 95% CI: 5.4-26.4).[106] However, no association was observed between oral HPV infection and oropharyngeal (OR=1.0, 95% CI: 0.4-2.5) and oral cavity cancers (OR=0.6, 95% CI: 0.3-1.1) in the IARC international case-control study, which detected HPV DNA in oral exfoliated cells from only 10% of patients who had HPV-positive tumors.[108] The results of the IARC study call to question whether the presence of HPV DNA in oral exfoliated cells is a good indicator of HPV DNA in the tumor.[108] In addition, while the Iowa and Johns Hopkins studies found an association between oral HPV infection and HNSCC,[106,109] the case-control design cannot establish whether oral HPV infection preceded HNSCC.

2.3.3.3 Infection With HPV Occurs Prior to HNSCC

Mork, et al.[110] conducted a case-control study nested within a N=900,000-person Norwegian and Finnish cohort who had donated blood at baseline. A total of N=292 cases who had donated blood >= 1 month prior to diagnosis with HNSCC were selected.[110] Matched controls (on age, sex, and length of serum storage) were selected for each case from the living, cancer-free members of the cohort at the time the cases were diagnosed, yielding a total of N=1,568 controls.[110] Serum was assayed for antibodies to HPV-16 and HPV-18 L1 and L2 proteins, and HPV-73 L1 protein.[110] Risk of HNSCC was associated with seropositivity to HPV-16 (OR=2.1, 95% CI: 1.4-3.2) after controlling for cotinine levels (a biomarker for smoking).[110] Risk was especially high for oropharyngeal (OR=14.4, 95% CI: 3.6-58.1) and tongue cancers (OR=2.8, 95% CI: 1.2-6.6).[110] Although this study does not offer conclusive evidence of *oral* HPV infection preceding development of HNSCC, it does demonstrate a clearly higher risk of HNSCC *after* exposure to HPV; a critical criterion in establishing a causal link between a virus and a cancer.[101]

2.3.4 Body Mass Index (BMI) and HNSCC

Higher BMI is typically associated with increased risk of cancer; e.g., colon and rectum, endometrial, and non-Hodgkin lymphoma.[111] However, there are a small number of cancers--notably lung and pre-menopausal breast cancer--for which higher BMI is associated with lower risk.[111] Lower risk has also been observed with increasing BMI in HNSCC.[78] The INHANCE data confirm the previously detected inverse dose-response ($P < .00001$).[112] Compared with being normal weight (BMI 18.5-24.9 kg/m^2: OR=1.0), odds ratios decreased from underweight (BMI < 18.5 kg/m^2: OR=2.13, 95% CI: 1.75-2.58) to overweight (BMI 25.0-29.9 kg/m^2:

OR=0.52, 95% CI: 0.44-0.60) and obese (BMI >= 30 kg/m^2: OR=0.43, 95% CI: 0.33-0.57).[112] When stratified by drinking and smoking status, there was no association between BMI and HNSCC among never drinkers/never smokers (P=0.49) but there was an inverse dose-response among ever drinkers/ever smokers (P < .00001).[112]

Because these results are obtained from pooled case-control data, they are subject to recall bias and the possibility of reverse-causality (i.e., HNSCC cases have lower BMI because their illness causes them to lose weight prior to diagnosis). However, the possibility of reverse-causality is somewhat refuted in this study because the aforementioned inverse dose-response was detected for BMI 2-5 years prior to diagnosis (or interview for controls; P=0.17 for never smokers/never drinkers, P=0.00042 for ever drinkers/ever smokers).[112] However, this does not eliminate the possibility of recall bias. Only a prospective study can adequately address these issues and to our knowledge none are currently available.

2.3.5 Prior History of Cancer

The SEER program provides population level data on risk of cancer after diagnosis with primary HNSCC in its monograph titled *New Malignancies Among Cancer Survivors: SEER Cancer Registries, 1973-2000*.[113] Risk of buccal cavity and pharynx cancers after primary tumors at other sites is significantly elevated (P < 0.05) over the expected risk in the general population for esophageal cancer (SIR=9.39), anal cancer (SIR=2.55), lung and bronchus (SIR=2.39), uterine cervix (SIR=1.91), vulva cancer (SIR=2.52), penis cancer (SIR=2.33), non-melanoma and non-retinoblastoma ocular cancer (SIR=5.07), Hodgkin (SIR=3.21) and non-Hodgkin lymphoma (SIR=1.40), acute lymphocytic leukemia (SIR=4.61), chronic myeloid leukemia (SIR=1.99), and any childhood (age 0-17) cancer (SIR=16.06).[113] Risk of HNSCC is also elevated after initial

HNSCC. For example, risk of buccal cavity and pharynx cancer is significantly elevated after larynx cancer (SIR=5.69) and nose, nasal cavity, and middle ear cancer (SIR=3.88).[113] In addition, risk of second buccal cavity or pharynx cancer after a first tumor of this type is extremely high <1 year after diagnosis (SIR=16.19), 1-4 years after diagnosis (SIR=21.46), 5-9 years after diagnosis (SIR=24.89), and at any point after diagnosis (SIR=21.83; $P < 0.05$ for all).[113] Increased risk of HNSCC after cancer at other sites, or after initial HNSCC, may be related to genetic predisposition, tobacco and alcohol use, and oncogenic infections like HPV that may be responsible for cancers at multiple sites.[113] It should be noted, however, that cancer at certain sites is associated with significantly ($P < .05$) decreased risk of second primary HNSCC: uterine corpus (SIR=0.71), prostate (OR=0.80), kidney parenchyma (OR=0.71), and multiple myeloma (OR=0.62), and that there exist cancer sites for which there is no association with increased risk of second primary HNSCC.[113]

2.4 ANGIOGENESIS, THE NOTCH PATHWAY, AND HPV IN HNSCC

It has long been recognized that tumors require a blood supply to grow beyond 1-2 cm^3 in size, as blood provides the tumor with nutrients and growth factors necessary for expansion.[114] It is now known that tumors gain access to nutrient-rich blood supply through angiogenesis: the formation of new, tumor-infiltrating blood vessels from existing vasculature.[115] Furthermore, angiogenesis is believed to facilitate metastasis of cancer by providing tumor cells a pathway to other anatomical sites.[116] Because metastasis is the primary cause of death in cancer patients,[116] angiogenesis has long been recognized as an important factor in malignancy and is now viewed as a therapeutic target in cancer, including HNSCC.[117]

The NOTCH pathway is the key regulator of cellular differentiation and is activated through direct cell-to-cell contact.[118] While NOTCH has a variety of roles relevant to carcinogenesis in general, its effects appear to vary across tumor types and the exact role of NOTCH in HNSCC is currently unclear.[21,118] The NOTCH pathway is also active within blood vessels where signaling between adjacent endothelial cells regulates angiogenesis.[119] In addition, NOTCH signaling takes place between tumor and other cell types in the tumor environment, e.g., tumor-cell-to-endothelial-cell signaling.[18] Tumors therefore may influence angiogenesis through transduction of NOTCH signals in adjacent blood vessel endothelium.[18]

Evidence from IHC studies (Table 3) suggests angiogenesis may differ in HPV-positive and HPV-negative HNSCC, and this may hold important consequences for anti-angiogenesis therapy, e.g., in terms of subgroups most likely to respond or selection of therapeutic targets and biomarkers of response. Furthermore, a small number of studies suggest the NOTCH pathway may be associated with angiogenesis in HNSCC (Table 4) but it is unclear whether this association differs in HPV-positive and HPV-negative HNSCC. The following discussion provides background on these topics. First, angiogenesis is described and its importance in HNSCC is discussed. Then, commonly used markers of angiogenesis are discussed along with findings related to these markers in HPV-positive and HPV-negative HNSCC. Finally, the NOTCH pathway is introduced and its potential role in HNSCC angiogenesis is discussed.

2.4.1 Description of Angiogenesis and its Importance in HNSCC

Tumor angiogenesis begins with secretion of growth factors by the tumor that interact with receptors on existing blood vessels to increase permeability of those vessels.[117] This allows endothelial cells to break free from the vessel wall, enter the interstitial space, proliferate, and

form new vessels.[117] While angiogenesis is a normal physiological process, occurring for example during embryo development and in response to injury, key differences exist between angiogenesis in normal tissues and tumors.[117] For example, endothelial cell division is normally a rare event that takes place approximately every seven years.[117] However, endothelial cells involved in tumor angiogenesis divide on the order of every seven to ten days.[117] In addition, whereas normal angiogenesis results in the formation orderly networks of mature vessels, tumor angiogenesis results immature, poorly organized microvessel networks.[117]

Tumor angiogenesis is mediated through expression of several key proteins. Most notable among these are the epidermal growth factor receptor (EGFR)[9] and the vascular endothelial growth factor (VEGF).[8] EGFR is expressed in nearly all HNSCC and plays a key role in tumor growth and metastasis by initiating intracellular signaling through several critical pathways in response to extracellular ligand binding.[9] *In vitro* studies show that EGFR is associated with angiogenesis through its ability to activate the signal transducer and activator of transcription 3 (STAT3).[9] STAT3 in turn induces transcription of the vascular endothelial growth factor (VEGF),[9] which is secreted by tumors to stimulate angiogenesis.[8] The VEGF pathway is the primary mediator of tumor angiogenesis.[8] This pathway consists of a family of growth factors (released by tumors) and receptors (on existing blood vessels nearby the tumor) with specific functions.[8] The VEGF-A growth factor, referred to simply as VEGF, is the most important growth factor in tumor angiogenesis.[8] Release of VEGF by tumors is associated with increased blood vessel permeability as well as growth, division, migration, and survival of endothelial cells.[8]

2.4.2 Studies Comparing Expression of Angiogenesis Markers in HPV-Positive and HPV-Negative HNSCC Using Immunohistochemistry (IHC)

Expression of EGFR and VEGF, both markers of tumor angiogenesis, has been detected in HNSCC using IHC with paraffin-embedded tumor specimens. Several studies show lower expression of EGFR in HPV-positive compared with HPV-negative HNSCC, suggesting possible differences in angiogenesis in these two HNSCC subgroups. However, few studies have examined expression of the primary angiogenesis mediator, VEGF, according to HPV status in HNSCC, and no studies have examined the EGFR-VEGF association separately in HPV-positive and HPV-negative HNSCC.

2.4.2.1 EGFR Expression in HNSCC

As shown in Table 3, several studies have demonstrated lower EGFR expression in HPV-positive compared with HPV-negative OOSCC,[10-13] although results reported in some studies were not statistically significant[120,121] and one study reported no association between EGFR and tumor HPV status.[122] Studies showing non-significant or null associations tended to include small sample sizes and/or low HPV prevalence,[120-122] whereas studies showing statistically significant differences in EGFR expression comparing HPV-positive and HPV-negative HNSCC had larger samples sizes and/or HPV prevalence.[10-13]

Table 3. Immunohistochemical Studies of EGFR Expression in HPV-Positive and HPV-Negative HNSCC

Author	Tumor Site	HPV Positivity Rate	EGFR-HPV Association
Fei[10]	• Tonsil	• 42/85=49%	• HPV-positive have low EGFR • HPV-positive: 67% EGFR+ • HPV-negative: 90% EGFR+ • P=.008
Al-Swiahb[11]	• Oropharynx	• 45/274=16.4%	• HPV-positive have low EGFR • HPV-positive: 30% EGFR+ • HPV- negative: 99% EGFR+ • P=0.01
Hong[12]	• Oropharynx	• 94/249=38%	• HPV-positive have low EGFR • HPV-positive: 78% EGFR+ • HPV- negative: 93% EGFR+ • P=.0005
Kong[13]	• Oral cavity, pharynx, larynx	• 36/82=44%	• HPV-positive have low EGFR • HPV-strong: 6.1% EGFR-strong • HPV-weak: 29.3% EGFR-strong • P=0.0006
Kumar[120]	• Oropharynx	• 25/39=64%	• HPV-positive have low EGFR • HPV-positive: 60% low EGFR • HPV- negative: 29% low EGFR • P=0.10
Reimers[121]	• Oropharynx	• 30/96=31%	• EGFR-positive tumors are less likely to be p16-positive • EGFR-positive: 34.5% p16+ • EGFR-: 65.5% p16+ • P=0.08
Lindquist[122]	• Tonsil, base of tongue	• 20/56=36%	• Not associated (data not tabulated)

2.4.2.2 VEGF Expression in HNSCC

Expression of VEGF in HNSCC was studied in a large meta-analysis that included N=1,002 cases from twelve studies that used IHC.[123] In this study, positive expression of VEGF was associated with higher tumor stage and lymph node metastasis.[123] In addition, VEGF-positive tumors were associated with an 88% increased risk of death relative to VEGF-negative tumors (RR=1.88, 95% CI: 1.43-2.45).[123] However, this estimate was not adjusted for tumor stage or nodal status.[123] Therefore, while it appears that VEGF expression is important in HNSCC survival, it is unclear whether it exerts this importance through a direct influence on survival or associations with other prognostic indicators.

Expression of VEGF with respect to tumor HPV status is reported in two studies.[10,124] One study used PCR to detect VEGF mRNA in N=13 fresh-frozen oropharyngeal biopsy specimens and found elevated levels of VEGF mRNA in HPV-positive compared with HPV-negative tumors ($P<0.01$).[124] However, another study of N=85 tonsil cancers did not observe any relationship between HPV status and VEGF expression measured by immunohistochemistry ($P=0.9$).[10]

2.4.2.3 Association Between EGFR and VEGF Expression in HNSCC

Only a small number of studies examined the association between expression of EGFR and VEGF in HNSCC using IHC. One study reported a positive association in a heterogeneous group of HNSCC,[125] and two studies showed a null association in tonsil[10] and oral cavity cancer.[126] No studies examined the EGFR-VEGF association stratified by tumor HPV status.

2.4.3 The NOTCH Pathway in HNSCC and Angiogenesis

The NOTCH signal transduction pathway was first identified as a critical pathway in embryonic organogenesis where it effects cellular differentiation, apoptosis, and proliferation in a wide variety of cell types.[118] The functions that NOTCH controls in normal cells are also important in carcinogenesis and therefore NOTCH is recognized as an important pathway in cancer.[20] The NOTCH pathway is activated through direct cell-to-cell contact.[20] Signal transduction is accomplished through interaction between NOTCH ligands (JAGGED1, JAGGED2, Delta-like ligand [DLL] 1, DLL3, and DLL4) and receptors (NOTCH1-NOTCH4) on the surface of neighboring cells.[20] Although there are several structural differences among the ligands and among the receptors, the overall signaling scheme appears to be similar for all receptor/ligand interactions.[20] Upon ligand/receptor binding, the intracellular domain of the NOTCH receptor (NOTCH-IC) undergoes two successive cleavages (first by tumor-necrosis-factor-alpha-converting enzyme, and then by the gamma-secretase enzyme), after which NOTCH-IC translocates to the nucleus and binds to the CSL transcription factor.[20] The CSL transcription factor normally represses transcription when NOTCH-IC is not present.[20] However, upon binding with NOTCH-IC, CSL becomes a transcriptional activator.[20] The complete list of genes that are transcribed as a result of the NOTCH-IC/CSL interaction is still under investigation.[20] Among the known targets is p21, which helps bring the cell cycle to a halt and promotes cellular differentiation.[20] However, the physiological consequences of NOTCH signaling are not entirely predictable.[20] The effects of NOTCH ligands may vary (e.g., NOTCH pathway signals induced by DLL1 result in differentiation of hematopoietic precursor cells *in vitro* whereas signals induced by JAGGED1 block differentiation in these cells).[20] In addition, the cell type and concurrent activity in intersecting pathways might determine effects of NOTCH signaling.[20] This

makes NOTCH particularly enigmatic in cancer as it appears to function either as an oncogene or a tumor suppressor depending on the tumor type.[20] For example, NOTCH1 acts as an oncogene in adult T-cell leukemia where a chromosomal translocation leaves precursor T-cells in a permanently undifferentiated and proliferating state.[20] However, NOTCH1 expression appears to be suppressed in basal cell skin cancer, suggesting it functions as a tumor suppressor in this cancer.[20]

The role of NOTCH in HNSCC is still being explored.[21,22] Mutations in NOTCH1-4 have been observed in 22% of HNSCC.[21] NOTCH1 in particular was mutated in 15% of HNSCC, making it the second most commonly mutated gene in HNSCC next to p53.[22] The mutations observed in NOTCH1 are consistent with inactivating mutations, suggesting NOTCH1 may act as a tumor suppressor in HNSCC.[22] Inactivation of the tumor suppressing function of NOTCH1 in HNSCC may result in a blockage normal differentiation in squamous epithelial cells, thus promoting malignant growth.[22]

NOTCH signaling also plays a critical role in normal and pathological angiogenesis through signaling between adjacent endothelial cells.[119] For example, angiogenesis begins with sprout formation, a process in which VEGF-stimulated endothelial cells break free from the blood vessel wall and adopt the tip cell phenotype, characterized by the presence of filopodia that facilitate migration of the blood vessel sprout towards the source of secreted VEGF (e.g., a tumor).[119] Following behind the tip cell are endothelial stalk cells, which eventually form the vessel lumen when the sprout connects with other sprouts.[119] The adoption of endothelial sprout or stalk phenotype is governed by NOTCH signaling between adjacent endothelial cells.[119] In addition, signals transmitted between adjacent endothelial cells initiated by the JAGGED1 ligand

are associated with increased sprout formation, whereas signals induced by DLL4 are associated with reduced sprouting.[119]

In addition to interactions between NOTCH receptors and ligands expressed on cells of the same type (e.g., endothelial cell-to-cell NOTCH signaling, or tumor cell-to-cell NOTCH signaling), the NOTCH ligand/receptor interaction has been observed between cells of different types.[119] For example, a recent study of HNSCC demonstrated formation of microvessel networks *in vitro* as a result of interaction between NOTCH ligands expressed on tumor cells and NOTCH receptors expressed on endothelial cells.[18] In this study, endothelial cells were cultured with two different populations of oropharyngeal tumor cells, one expressing JAGGED1 and one not expressing JAGGED1.[18] More sprouts were formed in the culture containing JAGGED1-expressing tumor cells.[18] In addition, a visually apparent network of microvessels appeared in the culture containing JAGGED1-expressing tumor cells whereas no such phenotypic change was observed in the culture lacking JAGGED1-expressing tumor cells.[18] The appearance of this microvessel network was also shown to be concomitant with activation of NOTCH1 in endothelial cells.[18] Therefore, while VEGF may be the primary driver of angiogenesis, the NOTCH pathway is an important mediator of blood vessel differentiation.[119] Furthermore, while maturation of blood vessels can occur via interactions between neighboring endothelial cells, as is seen in normal human development, it may also occur due to interaction between NOTCH ligands and receptors on tumors and adjacent endothelial cells.[119]

2.4.4 IHC Studies of the NOTCH Pathway and Angiogenesis in HNSCC

Immunohistochemical studies of HNSCC show expression of JAGGED1,[127-129] JAGGED2,[129] NOTCH1,[19,127-131] and NOTCH3[19,129] in tumors and endothelial cells[129] in normal tissue adjacent

to the tumor (Table 4). Furthermore, expression of these proteins in HNSCC has been associated with advanced stage,[129] lymph node metastasis,[19,129] depth of invasion,[19] non-response to platinum chemotherapy,[131] poor survival,[128] expression of VEGF,[19] and tumor microvessel density (MVD) measured using the CD34 immunostain.[19] The majority of studies included oral cancer only,[19,127,129,130] with two studies including pharyngeal tumor sites,[128,131] and only one study including laryngeal cancer.[131] One study assessed NOTCH1 expression in conjunction with EGFR expression.[130] Finally, none of these studies examined expression of NOTCH proteins by tumor HPV status.[19,127-131]

Table 4. Summary of Immunohistochemical Studies of NOTCH in HNSCC

Author, National Origin, and Sample	Findings*			
	Protein Expression	**Disease Progression**	**Outcome**	**Angiogenesis**
• Joo[19] • Korea • N=51 T1 or T2 oral tongue cancer • N=5 normal tongue samples†	• NOTCH1, NOTCH3 not expressed in normal tongue • NOTCH1 expressed in 35 of 51 (69%) tumors • NOTCH3 expressed in 23 of 51 (45%) of tumors	• NOTCH1 expression associated with LN+ and greater depth of invasion • NOTCH3 unrelated to LN status or depth of invasion	• NOTCH1 and NOTCH3 are *not* associated with disease-specific survival	• VEGF expression was associated with LN+ tumors • MVD was higher in VEGF-positive compared to VEGF-negative tumors • MVD was higher in NOTCH1-positive compared to NOTCH1-negative tumors • Higher MVD was associated with greater depth of invasion, but not LN status
• Hijioka[127] • Japan • N=4 oral cavity tumors	• Nuclear expression of the NOTCH1 intracellular domain was observed • JAGGED1 expressed only in the cytoplasm of tumors	• NA	NA	• NA

Table 4 continued

Author, National Origin, and Sample	Findings*			
	Protein Expression	**Disease Progression**	**Outcome**	**Angiogenesis**
• Lin[128] • Taiwan • N=59 T1-T4 tumors (21 oropharynx, 38 oral cavity)	• JAGGED1 expressed in 37 of 59 tumors (62.7%) • NOTCH1 expressed in 25 of 59 tumors (42.4%)	• NA	• High expression of NOTCH1 or JAGGED1 alone is associated with worse overall survival than low expression • Tumors with high expression of both NOTCH1 and JAGGED1 have the worst survival	• NA
• Zhang TH[129] • China • N=74 Tis-T3 oral tongue tumors • N=74 adjacent normal tissues adjacent to the tumor	• No significant difference in expression of JAGGED1/2 comparing tumor vs. normal • NOTCH1 and NOTCH3 are expressed at higher levels in tumor vs. normal tissue • NOTCH1, NOTCH3, and JAGGED1 are expressed in endothelial cells in normal tissue	• JAGGED1 and NOTCH1 are associated with LN+ tumors • Higher stage tumors are more likely to express NOTCH1/3 than lower stage tumors	• NA	• NA

Table 4 Continued

Author, National Origin, and Sample	Findings*			
	Protein Expression	**Disease Progression**	**Outcome**	**Angiogenesis**
• Huang[130] • China • N=41 oral tongue tumors • N=7 normal tongue specimens from tumor-free margins	• Expression of NOTCH1 increased with increasing differentiation (poor, moderate, well) • EGFR expression was inversely related to differentiation‡	• NA	• NA	• NA
• Zhang ZP[131] • China • N=25 (10 oral, 7 pharyngeal, 6 laryngeal, 1 maxillary sinus, 1 esophagus) • N=25 normal squamous epithelium†#	• All tumors expressed NOTCH1 whereas only 35% of normal squamous epithelium expressed NOTCH1	• NA	• Expression of NOTCH1 showed strong negative correlation with response to Cisplatin; i.e., low NOTCH1 expression=sensitive, high NOTCH1 expression=insensitive	• NA

MVD = microvessel density, Tis = T-stage *in situ,* LN+ = lymph node positive, LN- = lymph node negative, NA=not assessed
*All findings are statistically significant ($P < 0.05$) unless otherwise noted
†It is unclear from this report whether the normal specimens are taken from the same patients who donated tumor tissue
‡No direct correlation between NOTCH1 and EGFR expression is reported in this paper. However, the results suggest tumors expressing high levels of NOTCH1 also express low or no EGFR.
#The anatomic site for normal squamous epithelium is not stated

Expression of NOTCH Ligands in HNSCC

A total of three studies[127-129] examined JAGGED1 or JAGGED2 expression in HNSCC (Table 4). Hijioka, et al.[127] observed expression of JAGGED1 in a small sample (N=4) of oral cavity tumors from Japan. JAGGED1 was also expressed in 62.7% of oral and oropharyngeal tumors in a larger study (N=59) from Taiwan by Lin, et al.[128] Using the Kaplan-Meier method, this study showed high expression (>50% of cells staining) of JAGGED1 was associated with worse overall survival (median=15.6 months) than low expression (<=50% of cells staining) of JAGGED1 (median=65.1 months) (P=0.0001).[128] In the same study, high expression of JAGGED1 was also associated with increased risk of death from any cause in a multivariable Cox proportional hazards regression model (HR=3.28, 95% CI: 1.67-6.44).[128] Expression of JAGGED1 and JAGGED2 was observed in another study of a Chinese oral tongue cancer case series reported by Zhang TH, et al.[129] although no significant differences were observed in the expression of either ligand comparing tumor with normal tissue. However, the expression of JAGGED1 was associated with LN status, with 42.9% of LN+ tumors expressing JAGGED1 (>30% of cells staining) compared with only 15.2% of LN- tumors (P=0.03).[129] Expression of JAGGED2 was not related to LN status (LN+ tumors: 82.6% positive for JAGGED1, LN- tumors: 85.7% positive for JAGGED2, P=0.73).[129]

Expression of NOTCH Receptors in HNSCC

A total of six studies[19,127-131] examined expression of NOTCH receptors in HNSCC (Table 4). In a Korean oral tongue cancer case series reported by Joo, et al.,[19] NOTCH1 and NOTCH3 were expressed in 69% and 45% of tumors respectively, with no expression of either protein detected in normal tongue. Zhang TH, et al.[129] also reported higher expression of NOTCH1 and NOTCH3

in oral tongue tumors compared with normal tissue (NOTCH1: tumor=56.8% stained, normal=36.5% stained, P=0.01; NOTCH3: tumor=63.5% stained, normal=47.3% stained, P=0.05). Similar results were observed in a Chinese HNSCC case series reported by Zhang ZP, et al.[131] in which all tumors (including oral, pharyngeal, laryngeal, maxillary sinus, and esophagus) expressed NOTCH1 whereas only 35% of normal squamous epithelial specimens expressed NOTCH1. In addition, three studies observed NOTCH expression in tumors without comparison to normal tissue: 1) Lin, et. al[128] observed NOTCH1 expression in 42.4% of oral and oropharyngeal tumors in a Taiwanese case series, 2) Huang, et al.[130] observed NOTCH1 expression in 82.9% of oral tongue tumors in a Chinese case series, with stronger NOTCH1 expression in well differentiated tumors compared with poorly differentiated tumors, and 3) Hijioka, et al.[127] observed expression of the intracellular domain of NOTCH1 in a small sample (N=4) of oral cancers from Japan.

Two studies examined NOTCH receptor expression in relation to indicators of disease progression in oral tongue cancer, each reporting similar results.[19,129] Joo, et al.[19] observed an association between NOTCH1 expression and LN status, with 88.9% of LN-positive tumors expressing NOTCH1 and 57.6% of LN-negative tumors expressing NOTCH1 (P=0.02). The mean depth of tumor invasion (+/- SE) was also higher in NOTCH1-expressing tumors (11.51mm +/- 5.54) compared with tumors not expressing NOTCH1 (5.69mm +/- 4.33) (P=0.001).[19] Expression of NOTCH3 was unrelated to LN status (P=0.25) or depth of invasion (P-value not reported) in this study.[19] Zhang TH, et al.[129] also reported an association between NOTCH1 expression and LN status. In this study, 75.0% of LN-positive cases expressed NOTCH1 whereas only 45.7% of LN-negative cases expressed NOTCH1 (P=0.01).[129] NOTCH3 was unrelated to LN status in this study (P=0.11).[129] This study also examined tumor stage and

found that stage III and IV tumors more often expressed NOTCH1 (P=0.002) and NOTCH3 (P=0.003) than stage I and II tumors.[129]

Three studies examined NOTCH receptor expression in HNSCC and response to treatment or survival.[19,128,131] Zhang ZP, et al.[131] observed a strong inverse correlation between response to platinum chemotherapy and NOTCH1 expression (R-Spearman= -0.71, P < 0.01) in a HNSCC case series that also included esophageal and maxillary sinus cancers. Lin, et al.[128] used the Kaplan-Meier method to analyze overall survival associated with NOTCH1 in oral and oropharyngeal cancer. Tumors expressing high levels of NOTCH1 (>30% of cells staining) were associated with reduced survival (median=11.5 months) compared with tumors expressing low levels of NOTCH1 (<=30% of cells staining) (median=47.6 months) (P=0.004).[128] This study also observed statistically significant differences in survival (P < 0.001) associated with joint expression of NOTCH1 and JAGGED1.[128] Survival was best for cases with low expression of both NOTCH1 and JAGGED1 (median=65.1 months).[128] Cases with high expression of NOTCH1 (and low JAGGED1) had reduced survival (median=40.0 months), as did cases with high expression of JAGGED1 (and low NOTCH1) (median=9.41 months).[128] High expression of both NOTCH1 and JAGGED1 was associated with the worst survival (median=5.0 months).[128] In contrast to these results, Joo, et al.[19] did not observe any association between expression of NOTCH1 (P=0.34) or NOTCH3 (P=0.48) and disease-specific survival in oral tongue cancer.

Only one study examined NOTCH1 expression in relation to expression of EGFR.[130] Huang, et al.[130] reported that NOTCH1 expression was strongest in well and moderately differentiated oral tongue cancer and not present in poorly differentiated tumors. In contrast, EGFR was expressed in all tumors regardless of grade, but expression was strongest in poorly differentiated tumors.[130]

Finally, only one study examined NOTCH receptor expression in relation to proximal markers of angiogenesis in HNSCC.[19] Joo, et al.[19] reported that VEGF was expressed in 46.9% of oral tongue tumors. A total of 72.2% of LN-positive tumors expressed VEGF (>30% of cells staining) compared with only 32.3% of LN-negative tumors (P=0.009).[19] In addition, MVD (+/- SE) was greater in VEGF-positive tumors (22.5 vessels/mm^2 +/- 6.53) than VEGF-negative tumors (13.42 vessels/mm^2 +/- 5.80) ($P < 0.001$).[19] MVD was also higher in NOTCH1-positive (any staining) tumors (19.91 vessels/mm^2 +/- 7.05) compared with NOTCH1-negative tumors (12.40 vessels/mm^2 +/- 5.99) (P=0.001).[19] In addition, MVD was correlated with the depth of tumor invasion (R=0.36, P=0.01).[19] However, MVD was not significantly correlated with LN status (correlation coefficient is not specified; P=0.08).[19] Finally, NOTCH3 expression was unrelated to MVD in this study.[19]

2.5 HNSCC SURVIVAL AND METABOLIC ENZYME GENOTYPE

Metabolism of endogenous and xenobiotic compounds is accomplished by a 2-phase enzyme system.[132] The Phase I (functionalization) enzymes are responsible for detoxication whereas Phase II (conjugation) enzymes form hydrophilic compounds (by conjugating Phase I products with other molecules) to facilitate excretion from the body. In addition to their detoxicating role, Phase I enzymes are capable of metabolically activating some xenobiotic compounds. This results in formation of reactive oxygen intermediates (ROMs) that are mutagenic through their ability to readily form covalent bonds with specific sites on DNA molecules. Thus, some xenobiotic compounds are termed 'procarcinogens'-- compounds that become carcinogenic only after their enzymatic activation.[132] Genetic variation in Phase I and Phase II enzymes can result

in different metabolic phenotypes, classically defined as combinations of either "high" or "low" Phase I and Phase II enzyme activity, each with different relationships to cancer risk in the context of procarcinogenic xenobiotic exposure. For example, the high Phase I/low Phase II phenotype (denoting the presence of more mutagenic ROMs due to high expression of Phase I enzymes, and the slower excretion of those ROMs due to deficient activity of Phase II enzymes) is generally associated with higher risk of cancer after high levels of procarcinogenic xenobiotic exposure compared with the low Phase I/high Phase II phenotype.[132] This enzyme system is assumed to have evolved for beneficial endogenous purposes, and the activation of xenobiotics is viewed as an accidental consequence of the wide-ranging substrate specificity of many of the metabolic enzymes.[132] This consequence is of particular relevance in HNSCC, as several procarcinogens are found in tobacco smoke.[132] In addition, alcohol is metabolized to the genotoxic acetaldehyde.[133] Thus, genetic variation in some of these metabolic enzymes is associated with increased risk of HNSCC.[33,134] While the etiologic role of such polymorphisms has been explored in many studies of HNSCC, the relationship between these polymorphisms and survival from HNSCC has received less attention in the literature. The association between genotype and survival is potentially relevant for patients who continue to drink or smoke during therapy, and given the roles of some of these enzymes in metabolization of drugs.[132]

2.5.1 Cytochrome P450 Enzymes

The human cytochrome P-450s (CYP450s) are a set of fifty-seven genes organized into eighteen different families that comprise approximately 80% of the human Phase I enzymes.[132] These enzymes have special relevance to HNSCC as alcohol and procarinogenic xenobiotics in tobacco are metabolically activated by CYP450s.[132,133] For example, CYP2E1 accounts for

approximately 10% of ethanol metabolism[133] and activates benzene (found in cigarette smoke);[132] and CYP1A1, CYP1B1, and CYP2E1 have activity against polycyclic aromatic hydrocarbons (PAHs), which are found in cigarette smoke.[132] Although the CYP450s are primarily localized in the liver,[132] PCR showed CYP2E1 was expressed in normal oral epithelial cells as well as those infected with HPV-16 after exposure to PAHs;[135] microarray-based gene expression profiling showed CYP1A1 and CYP1B1 expression in tonsil SCC cells increased after exposure to cigarette smoke condensate;[135] and PCR and Western blot showed increased expression of CYP1A1 and CYP1B1 after exposure to benzo[a]pyrene in cell lines from oral cavity, hypopharyngeal, and laryngeal SCC, as well as gingival tissues specimens from dental patients surgically treated for non-malignant conditions.[136]

Data on the association between germline polymorphisms in CYP450s and clinical behavior of HNSCC is limited. In a 250-person case series consisting entirely of North Indian males treated with combination chemotherapy (Cisplatin + 5-FU) and radiation, the presence of variant alleles (i.e., *1A/non-*1A + non-*1A/non-*1A) of the *CYP2A6* gene was associated with lack of response to chemotherapy.[137] In this study, 57% of variant genotypes were non-responders whereas 43% of patients with wild type *1A/*1A had partial or complete response ($P < 0.0001$).[137] No estimate of survival associated with *CYP2A6* variants was given in this study, however.[137] Another study of N=385 German men and women diagnosed with first primary larynx, pharynx, or oral cavity cancer showed *CYP2E1*5B* (allele frequency of only 5.8%) to be unrelated to stage at diagnosis or nodal status (both of which are associated with poor survival in HNSCC) after adjusting for age, sex, tobacco, and alcohol use.[138] However, a study of N=153 HNSCC cases from Brazil identified *CYP2E1*5B* in ~13% of cases and showed this polymorphism was associated with advance stage at diagnosis ($P=0.022$), although the presence

of this polymorphism did not predict survival in a Kaplan Meier analysis (survival estimates and P-value not reported).[139] However, the presence of *CYP1A2*1C* (homozygous + heterozygous) in the same Brazilian case series was associated with significantly worse disease-free survival (~35% at 60 months) compared to the homozygous wild type (~80% at 60 months; $P_{log\text{-}rank}$=0.0161).[139]

2.5.2 Glutathione S-Transferases

The glutathione S transferases (GST) are Phase II enzymes that catalyze the conjugation of ROMs to glutathione, which detoxifies the ROMs.[140] Two of the GST genes--*GSTT1* and *GSTM1*--are frequently deleted in humans. Homozygous deletion of one or both genes (denoted by *GSTT1*0* or *GSTM*0*) results in complete absence of protein expression and therefore lack of enzyme function. Deletion of these genes is particularly relevant in HNSCC as these enzymes detoxify carcinogens found in tobacco smoke, as well as procarcinogens activated by Phase I enzymes like CYP450s.[140] There is wide variability in deletion of these enzymes across populations; e.g., with *GSTT1*0* found in 62% of Chinese and Koreans vs. 10% in Mexican Americans.[140] Overall, *GSTM1* is deleted in approximately 50%, and *GSTT1* is deleted in approximately 15% of Caucasians.[40] Another GST important in detoxifying chemical compounds in cigarette smoke is *GSTP1*. Polymorphisms in this gene create four different forms of this protein (Table 5) with different levels of enzymatic activity.[40,141] In addition to their activity against carcinogens found in tobacco smoke, these enzymes also have activity against anti-cancer chemotherapies.[40] Therefore, while deletions or polymorphisms in these genes may increase risk of head and neck cancer[33] through the absence of their detoxifying activity, they may actually improve response to therapy (due to the lack of detoxification of the drug).[40] On the

other hand, such genetic variation could plausibly have negative effects on survival in patients who continue to smoke during therapy.

Table 5. GSTP1 Polymorphisms

GSTP1 Variant	Amino Acid @ 105	Amino Acid @ 114	Nucleotides	Enzyme Activity
GSTP1*A (wild type)	Ile	Ala	AC	Normal
GSTP1*B	Val	Ala	GC	Reduced
GSTP1*C	Val	Val	GT	Reduced
GSTP1*D	Ile	Val	AT	Normal
*A total of 10 genotypes are possible: A/A, A/B, A/C, A/D, B/B, B/C, B/D, C/C, C/D, or D/D				

Limited information is available on the relationship between GST polymorphisms and survival in HNSCC patients. In the aforementioned study of North Indian men, Ruwali, et al.[137] identified polymorphisms in *GSTP1* at position 105 and noted non-response to therapy among 75% of those with the wild type allele (Ile/Ile) and 62% of heterozygotes (Ile/Val).[137] Patients homozygous for the polymorphism at position 105 (Val/Val)--a change associated with reduced enzyme activity--had the lowest rate of non-response (25% were non-responders).[137] Since all patients were treated with chemotherapy in this study, these findings agree with previous findings of improved survival associated with the reduced activity *GSTP1* phenotype in lung, colorectal, and ovarian cancers treated with chemotherapy.[40]

Minard, et al.[142] studied the risk of second primary tumors associated with *GSTM1* and *GSTT1* deletion in survivors of stage I or II SCC of the oral cavity, pharynx, and larynx enrolled in placebo-controlled trial of 13-cis-retinoic acid (30 mg/day for 3 years) for reduction in risk of second primaries. Subjects (N=1,081) enrolled in the trial were recruited during 1991-1999 at several centers in the United States and were 18 years or older, had been free of HNSCC for at least sixteen months, and had no prior history of other cancers within five years of enrollment.

Randomization to placebo or active treatment was stratified on disease stage, tumor site, and smoking status. The trial revealed no difference in risk of second primary tumors between treatment groups after 7 years of follow-up (3 years on treatment, plus 4 additional years). Minard, et al.[142] selected 303 Caucasians (from both the active and placebo groups) from among the 1,081 enrolled subjects who had blood available and studied the association between *GSTM1* and *GSTT1* null genotypes and risk of second primary tumors. The *GSTM1* non-null genotype was associated with increased risk of any second primary tumor (compared to null: HR=1.99, 95% CI: 1.11-3.56) and risk of tobacco related second primary cancer (head and neck, lung, kidney, bladder, and pancreas; HR=2.16, 95% CI: 1.01-4.62) after controlling for age, smoking status, alcohol use, tumor site, stage, and treatment group (placebo or 13-cis-retinoic acid).[142] The GSTT1 non-null genotype was unrelated to risk of any second primary tumor (compared to null: HR=0.59, 95% CI: 0.25-1.41) or tobacco related second primary tumors (HR=0.66, 95% CI: 0.23-1.92).[142]

Geisler, et al.[143] investigated overall and disease-specific survival associated with *GSTM1*, *GSTT1*, and *GSTP1* genotypes in a consecutive series of 190 incident cases of laryngeal, pharyngeal, and oral cavity cancer enrolled in a case-control study of genetic predisposition to HNSCC at the UNC Chapel Hill Memorial Hospital during 1994-1997. A total of 47% of patients were treated with surgery and radiation, 26% received surgery only, 8% received radiation only, and 19% received both chemotherapy and radiation.[143] A total of 123 patients (65%) experienced an event in this study: 79 (42%) died during follow-up (65 of them from HNSCC) and 44 (23%) had a recurrence.[143] In an unadjusted analysis the *GSTT1* non-null genotype was associated with greater risk of death overall (HR=1.95, 95% CI: 0.97-3.92) and death from HNSCC (HR=2.97, 95% CI: 1.19-7.42) compared with the null genotype.[143] This

association strengthened after adjusting for stage, age, and treatment differences (death from any cause: HR=2.37, 95% CI: 1.13-4.97; death from HNSCC: HR=3.35, 95% CI: 1.33-8.41).[143] Polymorphisms in GSTP1 (all reduced activity genotypes compared with Ile105/Ile105) were not associated with overall mortality (HR=1.05, 95% CI: 0.64-1.72) or disease specific mortality (HR=1.03, 95% CI: 0.60-1.76) in adjusted analyses.[143] Likewise, deletions in *GSTM1* were not associated with overall mortality (non-null compared to null: HR=0.80, 95% CI: 0.50-1.29) or disease-specific mortality (HR=1.25, 95% CI: 0.75-2.09) in adjusted analyses.[143] Finally, deletions in *GSTT1* and *GSTM1*, and polymorphisms in *GSTP1* were not associated with disease recurrence either in the crude or adjusted analyses.[143] The association between functional *GSTT1* and poor survival in this study was observed irrespective of treatment. Although the reason for this is unclear, residual confounding by smoking history is unlikely as an earlier report that included a subset (N=170) of these cases showed 94% of patients with non-null *GSTT1* were ever smokers whereas 91% of patients in whom *GSTT1* was deleted were ever-smokers.[144] Furthermore, patients with each genotype had similar pack-year histories of smoking.[144] It remains possible however, that selection factors produced this result. Approximately 12% of the 215 cases invited to participate in the study refused, and DNA was unavailable for 5 (2.6%) of the 190 enrolled participants.[143] Differences between the 215 invited and 190 enrolled are not discussed, nor are differences between those with and without available DNA.[143] If participation in the study or availability of DNA were related to survival this may have produced biased results.

3.0 SUMMARY

HNSCC is a worldwide public health problem,[46] and recent changes in the epidemiology of this disease have revealed previously unrecognized heterogeneity that will complicate prevention and treatment.[7] In particular, the molecular heterogeneity, disparate risk factor profiles, and persistently poor survival across subgroups of HNSCC require a renewed focus on development of impactful interventions for HNSCC patients and those at risk for developing the disease.[7] All of this work is the domain of a newly emerged paradigm called translational research.[145] The earliest concept of the translational approach was that of "bench to bedside" research, in which experimental evidence from the basic sciences is joined with clinical research to develop and test new therapies, and integrate these therapies into clinical practice expeditously.[145] The paradigm of translational research has now been expanded to public health, where the outcome of interest is health improvement at the population level; e.g., reduction in population prevalence of high-risk behaviors or changes in morbidity and mortality rates.[146]

Khoury, et al.[147] describe a framework within which epidemiology--the core science of public health--can contribute to translational research at various phases: T0) description and discovery of new knowledge concerning the occurrence, natural history, or biological pathogenesis of disease, T1) applying T0 discoveries to identify candidate applications, e.g., new tests, biomarkers, or therapies, T2) testing of candidate applications in observational studies and clinical trials, T3) development of evidence-based guidelines to incorporate proven candidate

applications as standard clinical practice, and T4) evaluation of the effectiveness of candidate applications by examining trends in population health. Much progress has already been made in translational cancer research and epidemiology has clearly contributed to successes in HNSCC, e.g., through implication of cigarette smoking in HNSCC etiology and the resulting influence of tobacco control efforts on reduction in HNSCC incidence,[2] the identification of shifting patterns in disease incidence that assisted with identification of the HPV-related HNSCC epidemic,[54] and in the development of the targeted therapy cetuximab.[23] However, this represents what can only be the beginning of the impact of epidemiology in HNSCC, as the need for population sciences is rapidly becoming apparent in addressing the complex heterogeneity of HNSCC.[7]

The research described here applies epidemiology, in the translational context, to HNSCC to make basic discoveries relevant to the T0 phase of translational research,[147] with the goal of informing such pursuits as the development of new therapies for HNSCC, providing a scientific basis for public policy aimed at reducing the burden of HNSCC in the population, and by informing selection of therapies based on individual patient characteristics. Therefore, the specific aims of this research are to: 1) explore the role of the NOTCH pathway in tumor angiogenesis in HPV-positive and HPV-negative HNSCC, 2) evaluate the association between childhood passive smoke exposure and adult HNSCC, and 3) evaluate the association between polymorphisms in tobacco and alcohol metabolizing enzymes and survival in HNSCC.

4.0 ARTICLE 1: EXPRESSION OF NOTCH1, EGFR, AND VEGF SUGGEST IMPORTANT DIFFERENCES IN TUMOR ANGIOGENESIS IN HPV-POSITIVE AND HPV-NEGATIVE ORAL AND OROPHARYNGEAL CANCER

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4.1 ABSTRACT

Background. Angiogenesis may differ in human papilloma virus (HPV)-positive and HPV-negative oral and oropharyngeal squamous cell carcinomas (OOSCC). The NOTCH pathway is implicated in OOSCC angiogenesis but expression of NOTCH proteins and markers of OOSCC angiogenesis are not reported by HPV status. **Methods.** Expression of the epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and NOTCH1 were measured using immunohistochemistry in N=67 cases (27 HPV-positive, 40 HPV-negative, by *in situ* hybridization) who completed an interviewer-administered lifestyle questionnaire. A pathologist scored the slides (intensity x percent of cells staining) blinded to HPV status. Box plots and the Wilcoxon rank sum or Kruskall-Wallis tests were used to compare the score by HPV status and lifestyle factors. Associations between EGFR, VEGF, and NOTCH1 were assessed using box plots and Spearman correlation (Rho) in all cases, and stratified by HPV status. **Results.** EGFR and VEGF were unrelated to T- or N-stage ($P > 0.20$ for all). NOTCH1 was over-expressed in T1/2 (median score [range]: 50 [0-240]) compared with T3/4 tumors (20 [0-160]) (P=0.01). HPV-positive OOSCC under-expressed EGFR (7.5 [0-200]) relative to HPV-negative OOSCC (30 [0-300]) (P=0.006). VEGF (P=0.82) and NOTCH1 (P=0.68) were unrelated to HPV status. EGFR was associated with VEGF in HPV-negative (Rho=0.40, P=0.01) but not HPV-positive tumors (Rho=0.25, P=0.20). NOTCH1 and VEGF were associated in HPV-negative (Rho=0.40, P=0.01) but not HPV-positive tumors (Rho= -0.12, P=0.57). NOTCH1 and EGFR exhibited a non-linear association in HPV-positive (P=0.01) but not HPV-negative cases (P=0.57). Alcohol drinking was associated with EGFR over-expression (P=0.03) and obesity was associated with VEGF under-expression (P=0.03). **Conclusions.** HPV-positive OOSCC may be less angiogenic than HPV-negative OOSCC. The NOTCH pathway is

associated with angiogenesis in HPV-negative OOSCC. Further study may identify subgroups likely to respond to anti-angiogenesis therapies, and identify novel anti-angiogenesis targets and biomarkers of treatment response.

4.2 INTRODUCTION

Oral and oropharyngeal squamous cell carcinomas (OOSCC) are typically associated with tobacco and alcohol use and represent a significant worldwide burden of cancer, totaling 400,000 new cases and causing 200,000 deaths in 2008.[1,56] However, despite declining smoking rates in the United States and other developed nations, the incidence of oropharyngeal tumors has increased among persons with little or no smoking history.[2,6,54,60] It is now known that these tumors are caused by the sexually transmitted human papilloma virus (HPV).[7] HPV-positive OOSCC are recognized as a distinct disease entity due to their unique histopathology and improved survival compared with stage-matched HPV-negative OOSCC.[7,23] Certain molecular characteristics unique to HPV-positive OOSCC have been identified. For example, HPV proteins E6 and E7 interact with host cell p53 and Rb to maintain the malignant phenotype.[148] However, these interactions are not sufficient to drive carcinogenesis and thus, the full extent of heterogeneity between HPV-positive and HPV-negative OOSCC is yet to be elucidated.[148] One potentially important difference in HPV-positive and HPV-negative OOSCC is angiogenesis: the process by which new, tumor-infiltrating blood vessels are formed from existing vasculature in response to the release of growth factors from the tumor.[114,115] These blood vessels supply the tumor with nutrients and growth factors necessary for expansion beyond 1-2 cm^3 in size, and provide a path for metastasis to other sites in the body.[114,115] Because of the role of angiogenesis

in tumor growth and metastasis,[114,115] clinical observations linking HPV-positive tumors with smaller size[14-17] are highly suggestive of differences in angiogenic potential comparing HPV-positive and HPV-negative OOSCC.

The strongest biological evidence for differences in angiogenesis comparing HPV-positive and HPV-negative OOSCC comes from immunohistochemistry (IHC) studies of the epidermal growth factor receptor (EGFR), which is expressed at lower levels in HPV-positive compared with HPV-negative OOSCC.[10-13] Results from *in vitro* studies show EGFR is associated with angiogenesis through its ability to activate the signal transducer and activator of transcription 3 (STAT3).[9] STAT3 induces transcription of the vascular endothelial growth factor (VEGF),[9] which is secreted by tumors.[8] VEGF stimulates angiogenesis by binding to receptors expressed on endothelial cells in existing vasculature nearby the tumor.[8] IHC studies show VEGF is over-expressed in OOSCC and is associated with higher tumor stage, lymph node metastasis, and increased risk of death.[123] However, we are aware of only two studies reporting on VEGF expression with respect to tumor HPV status and results are equivocal.[10,124] One study of oropharyngeal tumors observed higher levels of VEGF in HPV-positive compared with HPV-negative tumors.[124] However, another study did not observe any association between VEGF expression and HPV status in tonsil cancer.[10] In addition, few studies have examined the association between expression of EGFR and VEGF in OOSCC using IHC, with one study reporting a positive association in a heterogeneous group of head and neck tumors,[125] and two studies showing a null association in tonsil[10] and oral cavity cancer.[126] We are unaware of any studies that examined the EGFR-VEGF association separately in HPV-positive and HPV-negative OOSCC.

Recent studies suggest the NOTCH pathway may also be associated with OOSCC angiogenesis.[18,19] The NOTCH pathway, consisting of four cell surface receptors (NOTCH1-NOTCH4) and five membrane-bound ligands (JAGGED1, JAGGED2, Delta-like ligand [DLL] 1, DLL3, and DLL4) is associated with cellular differentiation, apoptosis, and proliferation in a wide variety of cell types and was first recognized as important in embryonic organogenesis.[118] Because many functions of NOTCH are also important in tumor growth, this pathway is also considered important in cancer, although its function in promoting or suppressing growth appears to differ across tumor types, and its role in OOSCC is yet to be determined.[20-22] Of particular interest is NOTCH1, which is the second most commonly mutated gene in head and neck tumors after p53.[21,22] NOTCH1 is expressed in OOSCC[19,127-129,131] and has been associated with microvessel density in oral tongue cancer.[19] In addition, at least one study of oropharyngeal cancer demonstrated development of a microvessel network *in vitro* as a result of NOTCH signaling between tumor and endothelial cells.[18] Although these studies are suggestive of a potential role of NOTCH1 in OOSCC angiogenesis, replication of these findings is required. Furthermore, we are unaware of any studies comparing NOTCH1 with canonical mediators of angiogenesis--VEGF and EGFR--in OOSCC.

OOSCC angiogenesis may also be influenced by lifestyle factors associated with OOSCC etiology.[149] For example, head and neck tumors are more often EGFR-positive in smokers than non-smokers.[150] In addition, one study reported increasing intensity of EGFR expression across oropharyngeal tumors from non-smokers, to past smokers, and current smokers.[120] However, studies of EGFR expression in OOSCC and lifestyle are not conclusive, as one study showed smoking to be associated with lower tumor EGFR expression compared to non-smoking,[151] and another study showed no association between smoking history alone, or in combination with

alcohol use, and expression of EGFR.[152] It is difficult to interpret these results in aggregate, however, as most studies were based on small sample sizes,[120,150,152] relied on medical records for assessment of lifestyle factors, and did not apply a uniform definition of smoking or alcohol use.[120,150,151] We are aware of only one study reporting on VEGF expression in relation to smoking in OOSCC.[153] This study showed no association between VEGF and smoking in a small and heterogeneous group of head and neck tumors, and also relied on medical record data for assessment of smoking habit.[153] Finally, we are unaware of any prior reports examining expression of NOTCH pathway proteins in OOSCC in relation to lifestyle factors.

To explore differences in angiogenesis comparing HPV-positive and HPV-negative OOSCC, we performed an IHC study of EGFR, VEGF, and NOTCH1 expression in an OOSCC case series derived from a case-control study of head and neck cancer etiology. All cases in our study completed an interviewer-administered risk factor questionnaire that included data on tobacco/alcohol use and anthropometry, allowing us the unique opportunity to systematically explore associations between lifestyle factors and expression of angiogenic factors in OOSCC. Based on existing literature, we hypothesized the following: 1) EGFR expression is positively associated with VEGF expression; 2) EGFR is expressed at lower levels in HPV-positive tumors compared with HPV-negative tumors; and 3) VEGF expression is lower in HPV-positive compared with HPV-negative tumors. In addition, we engaged in a hypothesis-generating study of NOTCH1 expression in relation to EGFR and VEGF in HPV-positive and HPV-negative OOSCC. Finally, we hypothesized that cigarette smoking and alcohol consumption are associated with increased EGFR expression, and we conducted exploratory investigations of cigarette smoking, alcohol drinking, and body mass index (BMI) in relation to expression of VEGF and NOTCH1 in OOSCC.

4.3 METHODS

4.3.1 Study Population

Between 2000-2010, N=1,170 cases of squamous cell carcinoma (SCC) of the head and neck were recruited at University of Pittsburgh Medical Center otolaryngology clinics for participation in a case-control study of head and neck cancer etiology. Cases were age 18-79 at diagnosis with biopsy-verified primary lip, oral cavity (mouth or anterior tongue) or oropharyngeal (base of tongue, tonsil fossa, or soft palate) SCC within one year of interview, and completed an interviewer-administered questionnaire soliciting tobacco/alcohol use, anthropometry, and personal cancer history. This study provided the basis for the case series included in our report. Because our primary interest was the expression of NOTCH1 and markers of angiogenesis in OOSCC according to tumor HPV status, we began by restricting our search for cases diagnosed during the time period when HPV testing became common (starting in 2007), and later routine practice (pending availability of tissue and starting in 2009), at our institution. Therefore, we first identified from this case series all OOSCC diagnosed during 2007-2010, and who self-reported no prior history of cancer (to remove the effect prior disease or treatment on our results) (N=322). Furthermore, we specifically sought cases with tumor HPV status recorded in the pathology report, as determined by *in situ* hybridization (ISH) (performed on the index tumor in cases later presenting with second or higher order primary tumors) (N=103). Formalin-fixed, paraffin-embedded (FFPE) tumors were requested from storage for these N=103 cases, and tumor blocks were retrieved for N=71 cases. Our analytic sample included cases with more recent diagnoses, consisted of more oropharyngeal tumors, and more often represented node-positive disease than excluded cases ($P < 0.01$ for all) (Appendix A).

4.3.2 Immunohistochemistry

Paraffin tumor blocks were retrieved from off-site storage (N=50) and project archives (N=21) and cut into 5 micron thick sections. Slide preparation and immunostaining were performed by the Tissue and Research Pathology Services laboratory at the University of Pittsburgh Medical Center (Rajiv Dhir, MD, Director). Three slides were prepared per tumor block to be stained with commercially available antibodies to VEGF (Santa Cruz Biotechnology #SC-152), NOTCH1 (Cell Signaling #3608), and EGFR (Sigma Chemical #E3138 (in this order). A single tumor block was available for N=62 cases and two blocks for N=9 cases. Slides were prepared as follows. Heat-induced antigen retrieval was performed in the Dako Biocare Decloaking Chamber using Biocare Medical Borg buffer (catalog # BD1000G1) (EGFR) or Dako PH6 citrate buffer (VEGF and NOTCH1). Engodenous peroxidase was blocked by quenching with 3% hydrogen peroxide (Fisher Scientific) for 10 minutes, after which the reagent was tapped off (not rinsed). Specimens were then incubated with primary antibody as indicated in Table 6, followed by incubation with Biocare Mach 4 Universal HRP (EGFR), Dako Dual Envision+ (VEGF), and Dako Rabbit Envision+ (NOTCH1) secondary antibodies for 30 minutes. All specimens were then washed for five minutes in tris-buffered saline. This was followed by incubation with Dako Substrate Chromagen (catalog #K3468) for five (NOTCH1) or ten (VEGF and EGFR) minutes. All specimens where then washed with deionized water, counterstained with Harris hematoxylin for ten seconds, washed in tap water, blued in ammonia and water, dehydrated, cleared and cover-slipped. Staining was performed on the Dako Autostainer Plus. All incubations were performed at room temperature. Paraffin-embedded tissues were used as positive controls in all experiments (EGFR: squamous cell head and neck cancer, VEGF: normal kidney, NOTCH1: lung cancer). Stains were interpreted as both intensity (0=no stain, 1=weak

stain, 2=moderate stain, 3=strong stain) and the percentage of cells staining. Interpretation was done by a single pathologist (Lin Wang, MD, PhD) who was blinded to tumor HPV status. Representative weak and strong intensity stains for each marker are shown in Figure 1, Figure 2, and Figure 3. A total of N=67 cases (of 71, or 94%) had enough tumor for staining of one or more markers. Cases with insufficient tumor were more often HPV-positive than other cases (Appendix B). Protein expression and tumor HPV status were unrelated to surrogate markers of variation in specimen handling (Appendix C).

4.3.3 Dependent Variable

The primary dependent variable in our analysis was the staining score for EGFR, VEGF, and NOTCH1. This continuous measure was created by multiplying the staining intensity times the percentage of cells staining. For the N=9 cases with two tumor blocks, the average staining score was used. We conducted sensitivity analyses using the maximum score instead and observed no difference in results compared with using the average (data not shown).

4.3.4 Independent Variables

The primary independent variable of interest in this study was tumor HPV status (positive or negative), as determined by ISH. In addition, we defined the following variables to explore confounding and interaction: age at diagnosis (< 50, 50-59, 60-69, and >=70 years), sex (male or female), race (white or other/unknown), year of diagnosis (nominal; 2007-2010), tumor site (oral cavity or oropharynx), clinical T-stage (nominal; 1/2, 3/4, or X [not evaluable]), clinical N-stage (nominal; negative, positive, or X [not evaluable]), clinical M-stage (nominal; positive, negative,

or X [not evaluable]), smoking status (ever/never, where ever-smoking was defined as smoking at least one cigarette per day for 6 months or longer), drinking status (ever/never, where ever-drinking was defined as drinking at least one drink per month for one year or longer), childhood passive smoke exposure (ever/never; defined as ever being exposed to passive cigarette smoke in the home up to age eighteen), and body mass index (BMI) one year prior to diagnosis (<30 kg/m^2 [not obese] or >=30 kg/m^2 [obese]).

4.3.5 Statistical Analysis

We began our analysis by exploring differences in subgroups of the case series defined by demographic, pathological, and lifestyle factors using Fisher's exact test. We then used quantitative and graphical methods to analyze expression of EGFR, VEGF, and NOTCH1 as measured by the protein staining score. First, the median and range of each protein's staining score were compared between HPV-positive and HPV-negative tumors. In addition, we prepared box plots for each protein stratified by clinical T- and N-stage and tumor HPV status. We also used box plots to examine all possible two-way associations between the markers under study in all cases combined, and stratified by tumor HPV status. Associations between demographic/lifestyle factors and protein expression were examined by comparing the median and range of the staining score across subgroups defined by these factors. Statistical significance in these analyses was assessed using the Wilcoxon rank sum and Kruskal-Wallis tests. Finally, we examined the Spearman correlation coefficient (Rho) to assess correlations between protein staining scores for all markers under study. All statistical tests were two-sided and results were considered statistically significant at alpha=0.05. Analyses were performed in SAS 9.2 (SAS Institute, Cary, NC).

4.4 RESULTS

Table 7 shows characteristics of the N=67 cases included in this study. Median age at diagnosis was 55.6 years (range: 20.0-77.1). Cases were predominantly male (74.6%) and white race (94.0%). The majority represented oropharyngeal tumors (58.2%), were diagnosed during 2009 and 2010 (70.1%), and the most common procedure type was resection/excision (68.7%). Most tumors were early clinical T-stage (59.7% stage 1/2) and node-positive (73.1%). Only one case showed clinical evidence of distant metastases. The majority of cases (74.6%) reported ever-smoking or ever-drinking (82.1%). Childhood passive smoke exposure was also common in the case series (79.1%), and a majority (67.2%) were not obese one year prior to cancer diagnosis. A total of N=27 tumors (40.3%) were HPV-positive (2 oral, 25 oropharyngeal) and N=40 tumors (59.7%) were HPV-negative (26 oral, 14 oropharyngeal).

4.4.1 Factors Associated With Tumor HPV Status

Table 7 shows factors associated with tumor HPV status. HPV-positive cases tended to be younger (77.7% <= 59 years old) than HPV-negative cases (62.5% <= 59 years old), but this difference was not significant (P=0.31). We also noted non-significant differences among HPV-positive (85.2% male) and HPV-negative (67.5% male) cases with regard to sex (P=0.15). The majority of HPV-positive cases (92.6%) were oropharyngeal tumors whereas only 35.0% of HPV-negative tumors were oropharyngeal (P < 0.001). The HPV-positivity rate among oropharyngeal tumors was 64.1% (25 HPV-positive out of 39 total). In general, HPV-positive cases were more often diagnosed prior to 2010 (92.6%) than HPV-negative cases (52.5%) (P<0.01). We noted substantial but non-significant differences in tumor size and nodal status

comparing HPV-positive and HPV-negative tumors. A total of 66.7% of HPV-positive tumors were staged clinically as T1/2, whereas only 55.0% of HPV-negative tumors were staged as T1/2 (P=0.09). HPV-positive tumors were also more often node-positive (81.5%) than HPV-negative tumors (67.5%) (P=0.27). Sensitivity analyses treating indeterminate T-stage tumors as T1/2 or T3/4 did not alter these results (data not shown). Cigarette smoking, alcohol drinking, childhood passive smoke exposure, and BMI one year prior to diagnosis were unrelated to tumor HPV status (P > 0.05 for all).

4.4.2 Protein Expression According to Clinical Stage and Tumor Site

Expression of three proteins--EGFR, VEGF, and NOTCH1 --was measured in N=67 cases (27 HPV-positive [2 oral, 25 oropharyngeal] and 40 HPV-negative [26 oral, 14 oropharyngeal]) (Table 7). Expression of EGFR (Figure 4) and VEGF (Figure 5) were unrelated to clinical T- or N-stage (P > 0.05 for all). However, as shown in Figure 6, we noted a statistically significant difference in expression of NOTCH1 according to clinical T-stage, in which NOTCH1 was over-expressed in T1/2 (median score [range]: 50 [0-240]) compared with T3/4 tumors (median score [range]: 20 [0-160]) (P=0.01). The association between NOTCH1 and T-stage was similar in analyses stratified by tumor site as well as tumor HPV status (data not shown). We did not observe any association between NOTCH1 and clinical N-stage (P=0.57) (Figure 6). In addition, there was no difference in expression of EGFR (Figure 7), VEGF (Figure 8), or NOTCH1 (Figure 9) comparing oral cavity and oropharyngeal cancer (P > 0.05 for all).

4.4.3 Protein Expression According to Tumor HPV Status

Table 8 shows results of our analysis comparing expression of EGFR, VEGF, and NOTCH1 in HPV-positive and HPV-negative cases. EGFR was evaluated in N=67 cases and was expressed (i.e., stained > 0% of cells) in fifty-six cases (83.6%). Evaluation of the staining score showed substantial under-expression of EGFR in HPV-positive tumors (median score [range]: 7.5 [0-200]) compared with HPV-negative tumors (median score [range]: 30 [0-300]) (P=0.006). HPV-positive tumors displayed both a lower percentage of cells staining (P=0.004) and a reduced intensity of stain (P=0.03) for EGFR compared with HPV-negative tumors. Expression of EGFR remained lower in HPV-positive tumors even after restricting our analysis to oropharyngeal cases only (Figure 10). VEGF was positive in 64/67 (95.5%) cases but showed no difference in expression comparing HPV-positive and HPV-negative tumors on staining score, percent of cells staining, and intensity (P > 0.05 for all). NOTCH1 was expressed in 58/66 cases (87.9%). There was no difference in expression of NOTCH1 comparing HPV-positive and HPV-negative tumors according to the staining score, percent of cells staining, or intensity (P > 0.05 for all).

4.4.4 Associations Between Expression of EGFR, VEGF, and NOTCH1 in All Cases and Stratified By Tumor HPV Status

Figures Figure 11, Figure 12, and Figure 13 show associations between EGFR, VEGF, and NOTCH1 in all cases and stratified by tumor HPV status. EGFR was positively associated with VEGF in all cases combined (P < 0.01). However, when stratified by HPV status, the EGFR-VEGF association was evident in HPV-negative (P=0.03) but not HPV-positive (P=0.16) tumors ((Figure 11)). Results of our Spearman correlation analysis (Table 9) also echoed this finding,

with a moderate positive correlation between EGFR and VEGF in HPV-negative tumors (Rho=0.40, P=0.01) and no evidence of a correlation in HPV-positive tumors (Rho=0.25, P=0.20). NOTCH1 was not associated with VEGF in all cases combined (P=0.11) or in HPV-positive cases (P=0.77). However, we noted a significant positive association between NOTCH1 and VEGF in HPV-negative cases (P=0.02) (Figure 12). Again, Spearman correlation (Table 9) also showed a stronger association between NOTCH1 and VEGF expression in HPV-negative (Rho=0.40, P=0.01) than HPV-positive (Rho= -0.12, P=0.57) tumors. Finally, we also observed a statistically significant, and apparently non-linear, relationship between NOTCH1 and EGFR expression in (P=0.02) in all cases combined, although this association was statistically significant only in HPV-positive tumors (P=0.01) and not HPV-negative tumors (P=0.57) (Figure 13). Results of our Spearman correlation analysis (Table 9) were also suggestive of a relationship between NOTCH1 and EGFR in HPV-positive tumors (Rho=0.32, P=0.11) and not in HPV-negative tumors (Rho= -0.04, P=0.79), although these results were not statistically significant.

4.4.5 Demographic and Lifestyle Factors Associated With Protein Expression

Table 10 shows results of an analysis of protein expression according to demographic and lifestyle factors collected using our interviewer-administered questionnaire. Overall, demographic and lifestyle factors were not strongly related to protein expression. However, we did observe significantly higher expression of EGFR among ever-drinkers (median score [range]: 25.0 [0-300]) compared with never-drinkers (median score [range]: 7.5 [0-60]) (P=0.03). However, we did not observe any evidence of increasing EGFR expression associated with increasing drinks/day or years drinking among ever-drinkers (data not shown). Obesity (BMI >=

30 kg/m^2) was associated with reduced expression of VEGF (median score [range]: 32.5 [0-150]) compared with not being obese (BMI < 30 kg/m^2) (median score [range]: 80.0 [0-200]) (P=0.03). All other tested associations were not statistically significant.

4.5 DISCUSSION

In this single-institution study of OOSCC, we used IHC to identify statistically significant differences in expression of tumor angiogenesis markers comparing HPV-positive and HPV-negative tumors that we believe have not been previously reported. Specifically, we observed a positive association between EGFR and VEGF that was restricted to HPV-negative tumors. We also observed a positive association between NOTCH1 and VEGF that was evident in HPV-negative tumors only. In addition, we observed associations between NOTCH1 and EGFR, and between NOTCH1 and tumor size. Our study also confirms previous reports that HPV-positive tumors express lower levels of EGFR than HPV negative tumors. Finally, to our knowledge we present the first report of the association between lifestyle factors and tumor angiogenesis in OOSCC based on data collected using a standardized, interviewer-administered questionnaire. Our results showed higher EGFR expression in alcohol drinkers compared with non-drinkers, and an inverse association between VEGF expression and body size. In total, these results suggest: 1) there are biological differences in angiogenesis in HPV-positive and HPV-negative OOSCC, with HPV-positive OOSCC being possibly less angiogenic; 2) the NOTCH pathway may be involved in OOSCC angiogenesis; and 3) lifestyle risk factors for OOSCC are associated with tumor angiogenesis.

4.5.1 Angiogenesis in HPV-Positive and HPV-Negative OOSCC

Our observation that EGFR is associated with VEGF is consistent with biological evidence showing a relationship between EGFR, STAT3, and VEGF in head and neck cancer cell lines.[9] In addition, EGFR and VEGF were positively associated (using a polymerase chain reaction (PCR) assay) in a prior study of oral, pharyngeal, and laryngeal tumors.[125] Although we observed a statistically significant association between EGFR and VEGF in all cases combined, our data indicate that this result was driven largely by an association between EGFR and VEGF in HPV-negative tumors. However, a previous study of oral cancer, which is typically HPV-negative,[7] reported a null association between EGFR and VEGF.[126] While this study appears to be at odds with our findings, the sample size (N=40) was considerably smaller than our study.[126] A larger study (N=85) of an Australian tonsil cancer case series also reported a null association between EGFR and VEGF in all cases combined.[12] Our analytic sample included a higher proportion (60%) of HPV-negative tumors (compared with 51% in the Australian study[12]), possibly allowing us to detect an association in the entire case series that was driven by HPV-negative tumors whereas the Australian study did not.[12] Unfortunately, the Australian study did not report results for EGFR-VEGF stratified by HPV status, so our results are not directly comparable.[12] However, we cannot ignore an important weakness of our result. Specifically, HPV-negative cases in our study included a mixture of oral cavity (N=26) and oropharyngeal (N=14) tumors (Table 7) whereas the HPV-negative cases in the Australian study included tonsil (oropharyngeal) cancer only.[12] Due to small subgroup sizes, we were unable to compare the EGFR-VEGF association between HPV-positive and HPV-negative cancers separately for oropharyngeal and oral cancers. Therefore, we were unable to determine whether the association we observed between EGFR and VEGF was truly a phenomenon restricted to HPV-negative

tumors, or whether it was attributable to tumor site. Thus, replication of our results is necessary in a larger sample.

The association between HPV-positive tumors and low EGFR expression we observed in our case series has been reported previously,[10-13,120,121] and may be suggestive of differences in angiogenesis comparing HPV-positive and HPV-negative OOSCC. A possible explanation for the under-expression of EGFR in HPV-positive tumors may be a reduction in EGFR copy number in HPV-positive compared with HPV-negative tumors, which has been reported in studies of oropharyngeal cancer.[154-157] Reduced expression of EGFR in HPV-positive tumors might suggest a reduction in EGFR/STAT3-mediated transcription of VEGF,[9] and therefore reduced angiogenesis in HPV-positive tumors. However, we observed no difference in VEGF expression comparing HPV-positive and HPV-negative tumors in our case series. We are aware of one similar study that also found no difference in VEGF expression according to tumor HPV status.[10] However, it is possible that differences in VEGF expression are slight when comparing HPV-positive and HPV-negative tumors, and that our study and others[10] were underpowered to detect this difference. Nonetheless, our observation of reduced EGFR expression in HPV-positive OOSCC, combined with no difference in VEGF expression according to HPV status, is suggestive of an EGFR-independent mechanism of angiogenesis in HPV-positive tumors. Indeed, the fact that EGFR was not significantly associated with VEGF in HPV-positive tumors in our study supports this hypothesis. Replication of our results in a larger sample is required.

Finally, in agreement with prior reports,[10-13,120,121] we observed a subset of HPV-positive tumors in our study that expressed high levels of EGFR. This phenomenon is clinically relevant as tumors with this profile have worse disease-specific survival compared with HPV-positive tumors that express low levels of EGFR.[120] In our study, the six HPV-positive cases in the top

tertile of EGFR expression were less likely to be ever-smokers (4/6, or 66.7%) than HPV-positive cases with lower EGFR expression (17/21, or 81.0%), suggesting smoking history may not be related to poor survival in high-EGFR HPV-positive OOSCC.[120] However, HPV-positive cases with high EGFR expression all had a history of drinking, all had a history of cancer in a blood relative, all were exposed to passive smoke during childhood, and were more often female (2/6, or 33.3%) compared with HPV-positive cases expressing lower levels of EGFR (19/21, or 90.5% drinkers; 14/20, or 70.0% having a blood relative with cancer; 16/21, or 76.2% with childhood passive smoke exposure; and 2/21, or 9.5% female). Over-expression of EGFR in HPV-positive OOSCC may result from the viral protein E5 acting as a ligand for EGFR.[158] It is conceivable that other genetic or environmental factors may influence the degree to which EGFR expression occurs as a result of this interaction between viral and host cell proteins. However, because high-EGFR tumors make up the minority of HPV-positive OOSCC,[10-13,120,121] and because HPV-positive tumors account for a minority of OOSC in general,[7] studying the etiology and clinical features of these tumors is difficult. Pooled analyses of case series with available tumor specimens and questionnaire data on lifestyle and family cancer history will be required.

4.5.2 The NOTCH Pathway and Angiogenesis in HPV-Positive and HPV-Negative OOSCC

We observed an association between NOTCH1 and VEGF in our case series in which NOTCH1 expression increased across tertiles of VEGF expression, but in HPV-negative tumors only. To our knowledge, this is the first report of an association between NOTCH1 and VEGF according to HPV status in OOSCC. However, we are not the first to implicate NOTCH1 in angiogenesis in OOSCC.[19] At least one prior showed study NOTCH1 expression was associated with

microvessel density in early stage (T1/2) oral tongue cancer.[19] This study did not report on the NOTCH1-VEGF association, but it did demonstrate an association between microvessel density and VEGF.[19] While tumor HPV status was not assessed in this study,[19] oral tongue cancer is frequently HPV-negative[7] and therefore we believe our results for HPV-negative OOSCC to be in agreement with these findings. Our observation that NOTCH1 was associated with VEGF, but not EGFR, in HPV-negative tumors suggests NOTCH1 may influence angiogenesis independently of EGFR in HPV-negative tumors. In fact, NOTCH1 expression has been positively correlated with STAT3 expression in oral tongue cancer,[159] and STAT3 activates transcription of VEGF.[9]

While our data suggest NOTCH1 is associated with angiogenesis independently of EGFR in HPV-negative tumors, our data also suggest NOTCH1 plays a different role in HPV-positive OOSCC, and this role may be unrelated to angiogenesis. Specifically, our data suggest a relationship between NOTCH1 and EGFR expression that is more evident in HPV-positive tumors. In addition, both NOTCH1 and EGFR were unrelated to VEGF in HPV-positive tumors in our study. We reviewed the literature on this topic and were unable to identify any studies that examined all three markers--EGFR, VEGF, and NOTCH1--simultaneously in HPV-positive and HPV-negative OOSCC. However, we did identify one study that examined expression of NOTCH1 and EGFR in OOSCC.[130] Huang, et. al.[130] used IHC to measure expression of both proteins in N=41 oral tongue cancers. Although NOTCH1 and EGFR are not directly compared in this study, the authors reported increasing expression of NOTCH1 with increasing differentiation (poorly differentiated: 0% of cells stained; moderately differentiated: mean=12.5% of cells stained; well differentiated: mean=23.1% of cells stained; $P < 0.05$).[130] However, expression of EGFR decreased with increasing differentiation (poorly differentiated:

mean=68.1% of cells stained; moderately differentiated: mean=45.7% of cells stained; well differentiated: 24.3% of cells stained; $P < 0.05$).[130] These results suggest an inverse association between NOTCH1 and EGFR in oral cancer. However, we did not detect this pattern when we restricted our analysis of NOTCH1 and VEGF to oral cancers only (data not shown). Furthermore, our results contradict those of Huang, et al.[130] as our data indicate the NOTCH1-EGFR association may be stronger in HPV-positive tumors, which are rare in the oral cavity.[7] Furthermore, we must point out that Huang, et al.[130] observed NOTCH1 expression in the metaplastic tissue surrounding the tumor but not within the tumor itself. Again, this is at odds with our findings in which we observed NOTCH1 expression directly in the tumor, in agreement with several other IHC studies of OOSCC.[19,127-129,131]

We also observed higher NOTCH1 expression in T1/2 tumors compared with T3/4 tumors. This association was independent of tumor site and HPV status in our case series, suggesting a role for NOTCH1 in the early phase of tumor development in OOSCC in general. Unfortunately, among all the studies of NOTCH1 expression in OOSCC that we identified, none compared NOTCH1 expression with T-stage.[19,127-131,159] However, one study reported increasing NOTCH1 expression with increasing stage group in oral tongue cancer.[129] However, we did not observe any association between NOTCH1 and stage group, in the entire case series or after stratification by tumor site or HPV status (data not shown). Two studies reported higher expression of NOTCH1 in node-positive compared with node-negative oral tongue cancer.[129] However, we observed no association between NOTCH1 and lymph node metastasis in all cases combined, or after stratification by tumor site (oral vs. oropharyngeal) and HPV status. Unfortunately, due to small sample size, we were unable to separate tongue cancer from other

oral cancers. Therefore, our results for NOTCH1 and nodal status may not be directly comparable to prior reports.[129]

4.5.3 Lifestyle Risk Factors for OOSCC and Markers of Tumor Angiogenesis

We also provide as part of this study a report on the association between lifestyle risk factors for OOSCC and tumor biology using a standardized questionnaire. While it is established that tobacco smoking and alcohol drinking are etiological factors in OOSCC,[56] it is unclear what relationship, if any, these exposures have with tumor angiogenesis. In our study we observed higher expression of EGFR in alcohol drinkers compared with non-drinkers. We are aware of only two studies reporting on this association in OOSCC, both showing a null result.[150,152] However, both studies used small sample sizes and may not have been powered to detect a difference in EGFR expression between drinkers and non-drinkers.[150,152] Furthermore, these studies relied on medical records for assessment of lifestyle factors, and such data may not be reliably recorded in the medical record. The lack of association between EGFR expression and increasing consumption (drinks/day) or years drinking in our study argues against a biological association, and the mechanism through which alcohol drinking might increase expression of EGFR in OOSCC is unclear. The relationship between cigarette smoking and EGFR signaling is better defined, however. Specifically, exposure to cigarette smoke increases expression of EGFR and its ligand transforming growth factor (TGF)-alpha in oral mucosa,[149] and smoking is strongly associated with OOSCC etiology in epidemiological studies.[56] In addition, Bergler, et al.[160] demonstrated that EGFR expression was nearly non-existent in mucosa from non-smoking and non-drinking patients undergoing tonsillectomy, frequent in healthy volunteers with a regular smoking or drinking habit, and ubiquitous in oral cancer patients. In addition, smoking has been

associated with higher EGFR expression in IHC studies of oral, pharyngeal, and largyneal cancer.[150,151] While we detected higher EGFR expression in smokers compared with non-smokers in our case series, this difference was not statistically significant. At least two other studies show null or inverse[151,152] relationships between smoking and EGFR expression. These results appear counter to what is known about the effect of cigarette smoke on EGFR and its ligands,[9] the importance of smoking in OOSCC etiology,[56] and the ubiquitous expression of EGFR in these tumors. It is possible that the associations reported in these studies are a result of bias related to missing data, possibly due to reliance on the medical record for assessment of smoking status.[151,152] Finally, we detected an inverse association between body size and VEGF expression. Cases who were categorized as obese within one year of their diagnosis (BMI > 30kg/m^2) had statistically significantly lower expression of VEGF compared with cases who were not obese (i.e., overweight, normal weight, or underweight). To our knowledge, we are the first to report an association between BMI and VEGF expression in OOSCC. The direction of the association we observed supports the frequent observation in epidemiological studies that higher BMI is associated with reduced risk of OOSCC[112] and suggests impaired angiogenesis as a potential mechanism that might reduce the risk of developing frank tumors. Indeed, obesity is associated with a wide variety of vascular impairments in patients without a cancer diagnosis, including reduced microvessel density in skeletal muscle and skin, as well as development of peripheral vascular disease, which is characterized by poor perfusion of tissue in the limbs.[161,162] We are unaware of any studies examining the effect of obesity on expression of VEGF in human oral tissues, however. In addition, higher serum levels of VEGF have been detected in obese persons compared with non-obese persons and it is unclear what meaning this has in the context of OOSCC pathogenesis.[163]

4.5.4 Strengths and Limitations

Our results are accompanied by strengths in several areas. First, assessment of protein staining scores was performed by an expert head and neck pathologist who was blinded to tumor HPV status, thus reducing the possibility of biased assessment of protein expression according to tumor HPV status. We were also able to verify that variation in specimen handling did not effect our results (Appendix C). Antibody quality can also effect measurement of protein expression in IHC studies. To mitigate this, we used commercially available antibodies--including two monoclonal antibodies (to NOTCH1 and EGFR), which are less likely to exhibit background staining than polyclonal antibodies--and we tested these antibodies with positive controls. Finally, HPV positivity was more common among the five cases missing one or more markers compared with cases in whom all markers were assessed. If HPV-positive cases missing EGFR were those that under-expressed EGFR, then our results may under-estimate the true difference in EGFR expression comparing HPV-positive and HPV-negative OOSCC. However, we cannot ignore the possibility that the HPV-positive cases for whom EGFR data were missing may represent the minority of HPV-positive cases that express high levels of EGFR. In this case, our results would overestimate the true difference in EGFR expression comparing HPV-positive and HPV-negative OOSCC. However, we are able to point to internal consistencies within our results that strengthen our observations. Specifically, our observation of lower EGFR expression in HPV-positive OOSCC, and therefore possibly lower angiogenic potential in HPV-positive OOSCC, is consistent with the tendency we observed for these tumors to be associated with smaller size, as indicated by clinical T-stage. Although this association was not statistically significant, this may have been due to sample size. In addition, we also noted a tendency for HPV-positive tumors to be node-positive. This may suggest a predilection for HPV-positive

tumors to metastasize through the lymph system and is consistent with our observation that the EGFR-VEGF association, which is relevant to angiogenesis rather than lymphangiogenesis, was not present in HPV-positive tumors, suggesting hematogenous metastasis may be less important in HPV-positive tumors.

Our study is also accompanied by several limitations. In particular, we studied angiogenesis in HPV-positive and HPV-negative OOSCC using a relatively small sample and we conducted many statistical tests without correction for Type I error. However, as our study is one of the first to contribute data on expression of angiogenesis markers in HPV-positive and HPV-negative OOSCC using IHC, we view our results primarily as hypothesis-generating. In addition, we must consider the external validity of our results because the ultimate goal of this research is to provide impetus for further investigation that will lead to translational applications in OOSCC on a population level. The primary reason for exclusion of cases from our study was missing tumor HPV status. When comparing cases enrolled in our study to those who would have been eligible if HPV status were known, we observed that cases in our study were more likely to represent oropharyngeal cancer and node-positive disease (Appendix A). However, we do not see this as limiting the external validity of our study as these are factors associated with HPV-positive OOSCC,[7] and we specifically selected cases for our study such that HPV status would be over-represented. Finally, perhaps the greatest limitation of our study is that our results can only speak to associations between biological factors and cannot describe mechanisms through which these associations are produced. However, this is not the purpose of our research. Rather, our data provide the impetus to conduct future studies that evaluate such mechanisms, and that might inform translational discoveries such as biomarker development, novel combinations of

existing therapies, development of new therapies, or addition of biological endpoint assessments in clinical trials.

4.5.5 Conclusions

In summary, our study suggests that HPV-positive OOSCC may have lower angiogenic potential than HPV-negative OOSCC as evidenced by lower expression of EGFR in HPV-positive tumors, a lack of association between EGFR and VEGF in HPV-positive OOSCC, and a tendency for smaller tumor size in HPV-positive OOSCC. In addition, we showed that the NOTCH pathway may play a role in tumor growth in OOSCC in general, and that the NOTCH pathway may be associated with angiogenesis in HPV-negative OOSCC while possibly playing a different role in HPV-positive OOSCC. Our study represents an early investigation into differences in angiogenesis in HPV-positive and HPV-negative OOSCC. Therefore, our results require replication. Further study of this topic may prove worthwhile in determining which patients are most likely to respond to anti-angiogenesis therapies, as well as help to identify potential biomarkers of treatment response, or new therapeutic targets. Finally, while our results suggest HPV-positive tumors may be less angiogenic than HPV-negative tumors, our data also reiterate the existence of a unique subgroup of HPV-positive tumors that expresses high levels of EGFR, which are known to have poor prognosis.[120] Whether poor prognosis in this subgroup is related to differences in angiogenesis is unclear from our data and this should be explored in future studies as these cases may represent a subgroup of OOSCC that are good candidates for anti-angiogenesis therapies. In conclusion, we believe that our research reveals biological differences in HPV-positive and HPV-negative OOSCC that have potential to be translated into impactful

interventions for OOSCC patients, and that our results will serve as the launching point for future translational investigations in OOSCC.

4.6 TABLES

Table 6. Antibodies Used for Immunohistochemistry

Staining Order	Protein	Antibody	Origin and Binding Site	Dilution	Positive Control	Localization
1	VEGF	Santa Cruz Biotechnology SC-152	• Rabbit polyclonal antibody to the N-terminus of VEGF-A • Detects the 189, 165 (predominant), and 121 amino acid sequence isoforms of VEGF-A	1:400 for 60 minutes	Normal kidney	Cytoplasm and nuclei
2	NOTCH1	Cell Signaling Technologies 3608	• Rabbit monoclonal antibody to proline 2439 • Recognizes the whole (in-tact) NOTCH1 protein or the transmembrane/intracellular region	1:400 for 45 minutes	Lung cancer	Membrane and cytoplasm
3	EGFR	Sigma Chemical E3138	• Mouse monoclonal antibody to the intracellular domain of the receptor	1:7,500 for 60 minutes	Head and neck cancer	Membrane and cytoplasm

Table 7. Analytic Cohort for Immunohistochemistry

	All (N=67)	HPV Negative (N=40)	HPV Positive (N=27)	
	n (%)	n (%)	n (%)	P-Value*
Age				0.31
<50	19 (28.4)	8 (20.0)	11 (40.7)	
50-59	27 (40.3)	17 (42.5)	10 (37.0)	
60-69	16 (23.9)	11 (27.5)	5 (18.5)	
>=70	5 (7.5)	4 (10.0)	1 (3.7)	
Sex				0.15
Male	50 (74.6)	27 (67.5)	23 (85.2)	
Female	17 (25.4)	13 (32.5)	4 (14.8)	
Race				0.64
Non-White/Unknown	4 (6.0)	3 (7.5)	1 (3.7)	
White	63 (94.0)	37 (92.5)	26 (96.3)	
Tumor Site				< .001
Oral Cavity	28 (41.8)	26 (65.0)	2 (7.4)	
Oropharynx	39 (58.2)	14 (35.0)	25 (92.6)	
Year of Diagnosis				< .01
2007	5 (7.5)	3 (7.5)	2 (7.4)	
2008	15 (22.4)	6 (15.0)	9 (33.3)	
2009	26 (38.8)	12 (30.0)	14 (51.9)	
2010	21 (31.3)	19 (47.5)	2 (7.4)	
Procedure Type				0.43
Biopsy	21 (31.3)	11 (27.5)	10 (37.0)	
Resection/excision	46 (68.7)	29 (72.5)	17 (63.0)	
T clinical				0.09
1/2	40 (59.7)	22 (55.0)	18 (66.7)	
3/4	25 (37.3)	18 (45.0)	7 (25.9)	
X	2 (3.0)	0 (0.0)	2 (7.4)	
N clinical				0.27
Negative	18 (26.9)	13 (32.5)	5 (18.5)	
Positive	49 (73.1)	27 (67.5)	22 (81.5)	
M clinical				> 0.99
0	65 (97.0)	38 (95.0)	27 (100.0)	
1	1 (1.5)	1 (2.5)	0 (0.0)	
X	1 (1.5)	1 (2.5)	0 (0.0)	

Table 7 Continued

	All (N=67)	HPV Negative (N=40)	HPV Positive (N=27)	
	n (%)	n (%)	n (%)	P-Value*
Ever Smoked				0.78
No	17 (25.4)	11 (27.5)	6 (22.2)	
Yes	50 (74.6)	29 (72.5)	21 (77.8)	
Ever Drank Alcohol				0.10
No	12 (17.9)	10 (25.0)	2 (7.4)	
Yes	55 (82.1)	30 (75.0)	25 (92.6)	
Childhood Passive Smoke				0.77
No	14 (20.9)	9 (22.5)	5 (18.5)	
Yes	53 (79.1)	31 (77.5)	22 (81.5)	
BMI 1 year pre-diagnosis				0.60
<30 kg/m^2	45 (67.2)	28 (70.0)	17 (63.0)	
>=30 kg/m^2	22 (32.8)	12 (30.0)	10 (37.0)	
*Fisher's exact test				

Table 8. Results of Immunohistochemistry: Analysis of Protein Expression and HPV Status

Marker	HPV Status	N	Positive n (%)*	Score†#	Percent Staining	Intensity
EGFR	All	67	56 (83.6)	20.0 (0-300)	15.0 (0-100)	1.0 (0-3)
	Negative	40	37 (92.5)	30.0 (0-300)	22.5 (0-100)	1.0 (0-3)
	Positive	27	19 (70.4)	7.5 (0-200)	7.5 (0-100)	1.0 (0-2)
	P-value‡			0.006	0.004	0.03
VEGF	All	67	64 (95.5)	70.0 (0-200)	60.0 (0-100)	1.0 (0-2)
	Negative	40	38 (95.0)	60.0 (0-200)	60.0 (0-100)	1.0 (0-2)
	Positive	27	26 (96.3)	70.0 (0-180)	70.0 (0-100)	1.0 (0-2)
	P-value‡			0.82	0.97	0.87
NOTCH1	All	66	58 (87.9)	40.0 (0-240)	20.0 (0-100)	2.0 (0-3)
	Negative	40	35 (87.5)	42.5 (0-240)	20.0 (0-100)	2.0 (0-3)
	Positive	26	23 (88.5)	40.0 (0-160)	22.5 (0-80)	1.5 (0-3)
	P-value‡			0.68	1.0	0.29

EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor; NOTCH1=notch receptor 1; HPV=human papillomavirus.

*Positive is defined as having greater than 0% of cells staining
† Numbers are median (min-max)
#Score represents the product of Percent Staining and Intensity
‡p-value is from a Wilcoxon Rank Sum test comparing HPV-positive and HPV-negative tumors

Table 9. Spearman Correlations For Markers Detected by Immunohistochemistry

All Cases

	NOTCH1	EGFR	VEGF
NOTCH1		0.11 0.37 66	0.22 0.08 66
EGFR	0.11 0.37 66		0.33 0.007 67
VEGF	0.22 0.08 66	0.33 0.007 67	

HPV-Positive Cases

	NOTCH1	EGFR	VEGF
NOTCH1		0.32 0.11 26	-0.12 0.57 26
EGFR	0.32 0.11 26		0.25 0.20 27
VEGF	-0.18 0.57 26	0.25 0.20 27	

HPV-Negative Cases

	NOTCH1	EGFR	VEGF
NOTCH1		-0.04 0.79 40	0.40 0.01 40
EGFR	-0.04 0.79 40		0.40 0.01 40
VEGF	0.40 0.01 40	0.40 0.01 40	

Numbers are:

Rho (correlation coefficient)
P-value
N

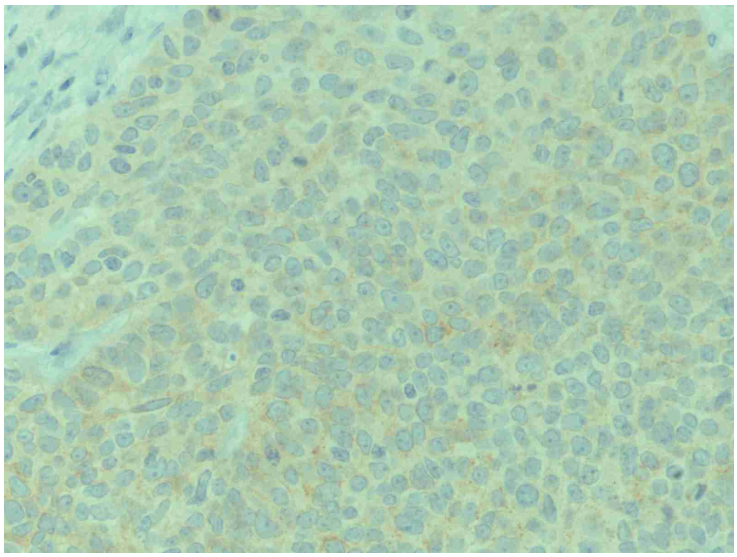
Table 10. Demographic and Lifestyle Factors Associated With Protein Expression

	EGFR	VEGF	NOTCH1
	N, median (min-max)	N, median (min-max)	N, median (min-max)
Age			
<50	19, 30.0 (0-80)	19, 40.0 (0-160)	19, 40.0 (5-180)
50-59	27, 20.0 (0-300)	27, 60.0 (0-180)	27, 20.0 (0-240)
60-69	16, 60.0 (0-200)	16, 100 (0-140)	15, 60.0 (0-160)
>=70	5, 10.0 (0-300)	5, 100 (20-200)	5, 30.0 (0-120)
P-value	0.50	0.27	0.77
Sex			
Female	17, 60.0 (0-300)	17, 90.0 (0-150)	17, 40.0 (0-195)
Male	50, 20.0 (0-300)	50, 60.0 (0-200)	49, 40.0 (0-240)
P-value	0.08	0.46	0.69
Race			
White	63, 20.0 (0-300)	63, 70.0 (0-200)	62, 40.0 (0-240)
Non-White/Unknown	4, 60.0 (0-180)	4, 60.0 (30-100)	4, 50.0 (20-100)
P-value	0.49	0.95	0.58
Ever Smoked			
Yes	50, 22.5 (0-300)	50, 70.0 (0-200)	49, 40.0 (0-195)
No	17, 15.0 (0-80)	17, 50.0 (0-140)	17, 60.0 (0-240)
P-value	0.32	0.71	0.41
Childhood Passive Smoke			
Yes	53, 20.0 (0-300)	53, 70.0 (0-200)	52, 40.0 (0-240)
No	14, 17.5 (0-180)	14, 60.0 (0-160)	14, 45.0 (0-160)
P-value	0.46	0.61	> 0.99
Ever Drank Alcohol			
Yes	55, 25.0 (0-300)	55, 70.0 (0-200)	54, 40.0 (0-180)
No	12, 7.5 (0-60)	12, 35.0 (0-150)	12, 50.0 (5-240)
P-value	0.03	0.25	0.57
BMI 1 year pre-diagnosis			
<30 kg/m^2	45, 25.0 (0-300)	45, 80.0 (0-200)	45, 40.0 (0-240)
>=30 kg/m^2	22, 15.0 (0-200)	22, 32.5 (0-150)	21, 60.0 (0-195)
P-value	0.20	0.03	0.15

EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor; NOTCH1=notch receptor 1; HPV=human papillomavirus. Numbers (N, median [min-max]) refer to the protein staining score. P-values are from the Wilcoxon rank sum test (for dichotomous variables) or the Kruskal-Wallis test (for multi-level nominal variables).

4.7 FIGURES

EGFR Weak Intensity



EGFR Strong Intensity

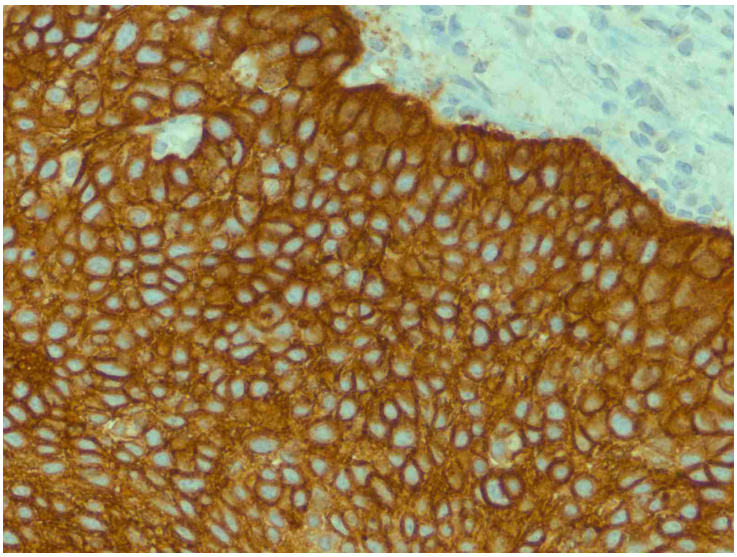
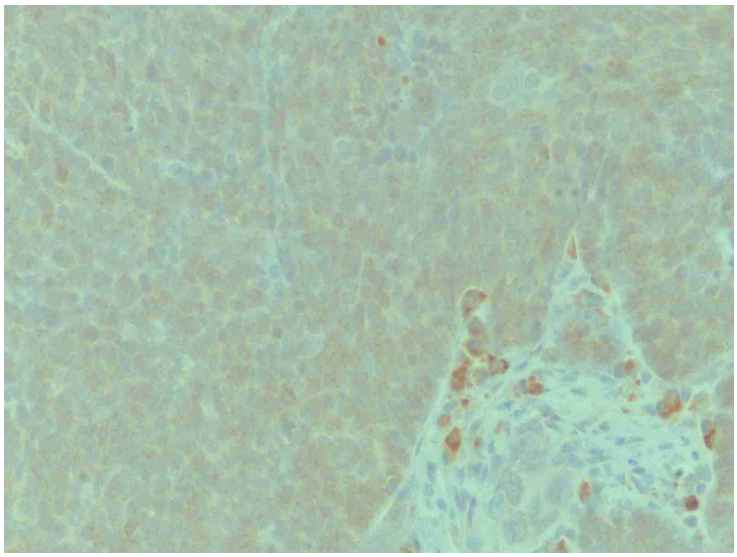


Figure 1. Representative Weak and Strong Intensity IHC Stains for EGFR (400x)

VEGF Weak Intensity



VEGF Strong Intensity

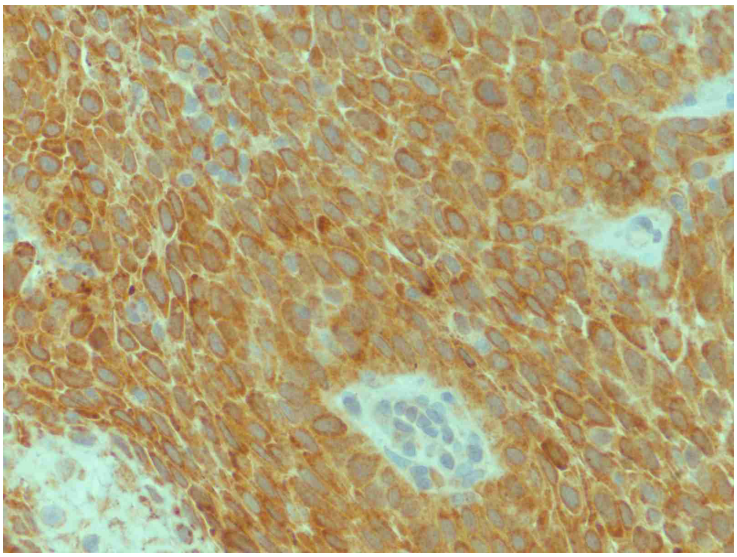


Figure 2. Representative Weak and Strong Intensity IHC Stains for VEGF (400x)

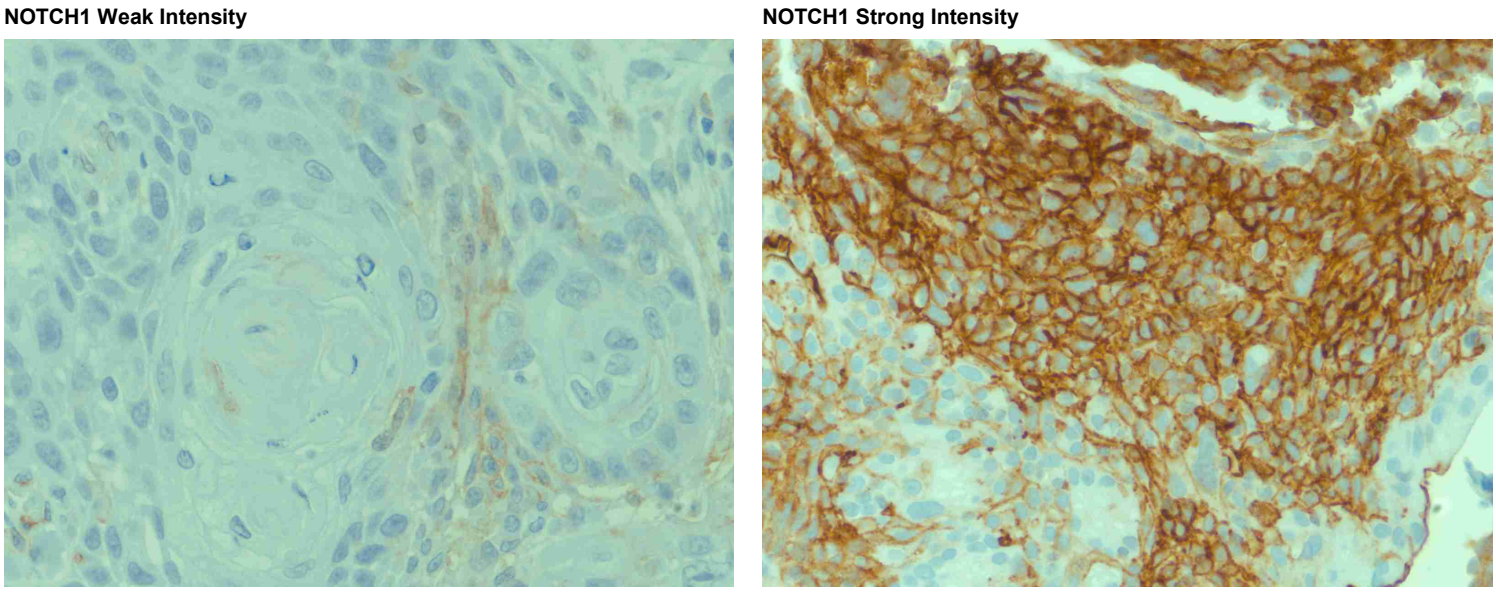


Figure 3. Representative Weak and Strong Intensity IHC Stains for NOTCH1 (400x)

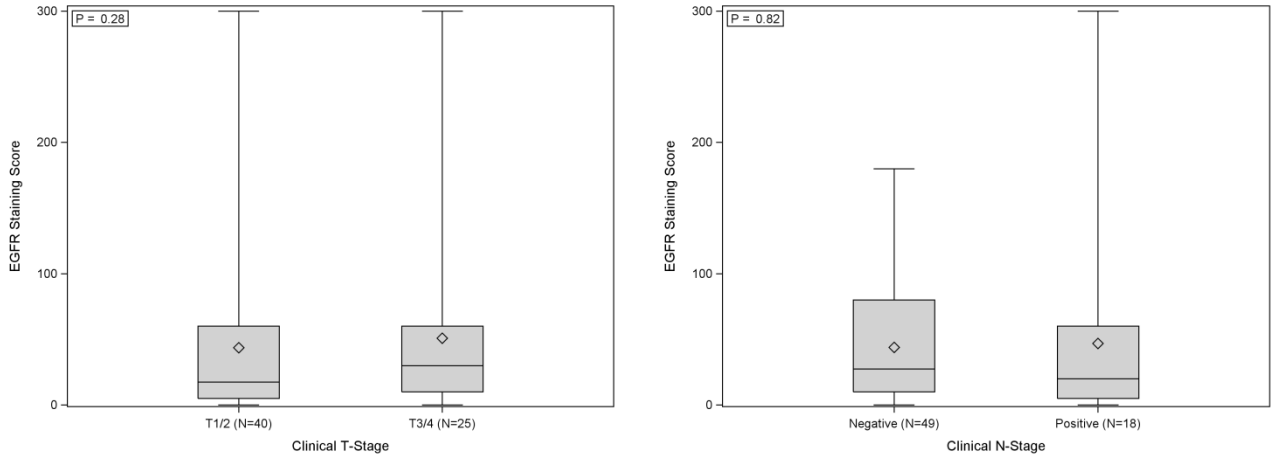


Figure 4. EGFR Expression According to Clinical T- and N-Stage

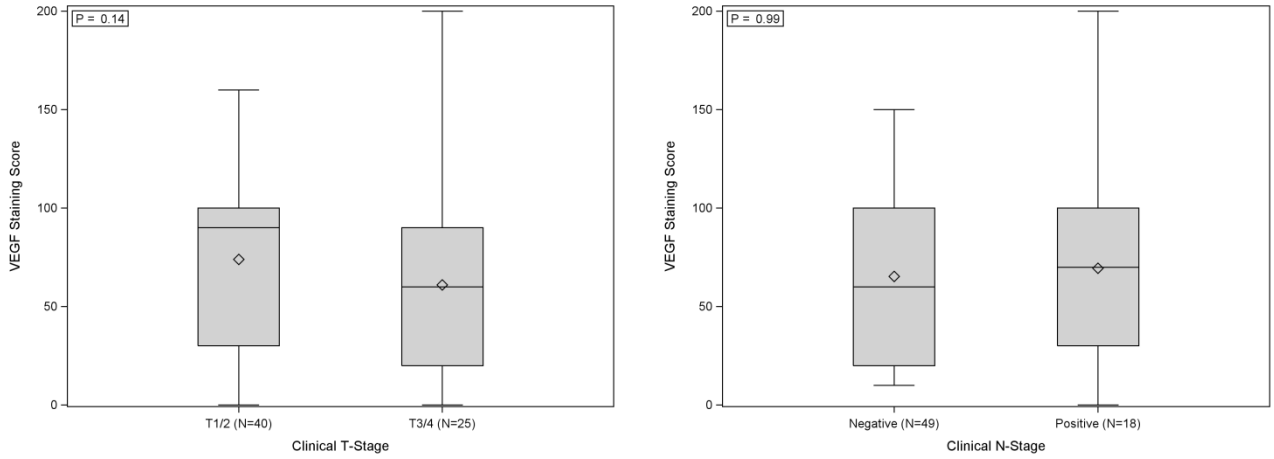


Figure 5. VEGF Expression According to Clinical T- and N-Stage

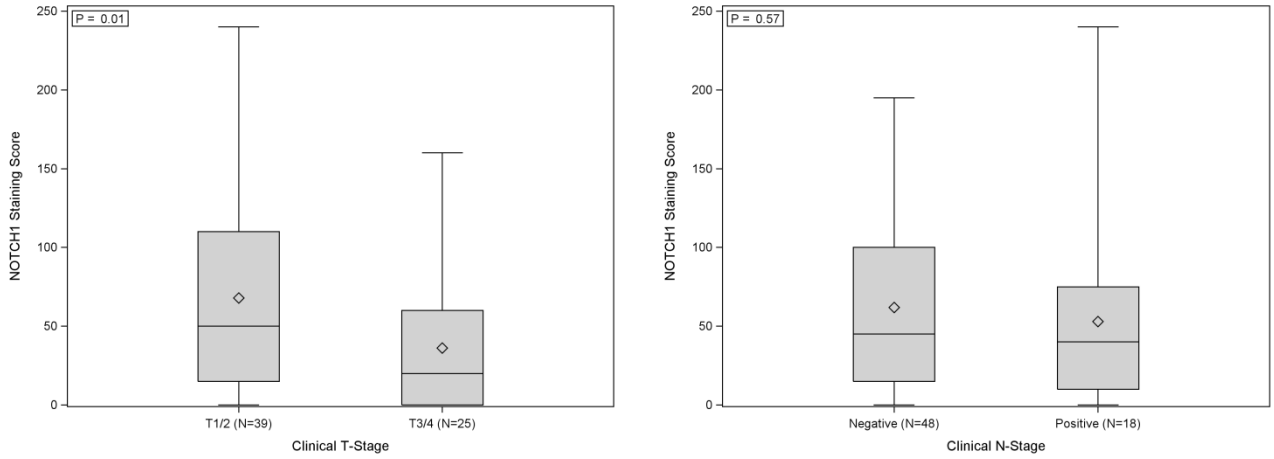


Figure 6. NOTCH1 Expression According to Clinical T- and N-Stage

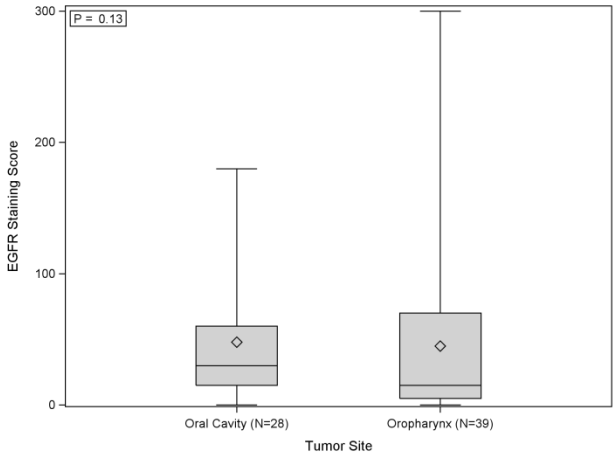


Figure 7. EGFR Expression Stratified By Tumor Site

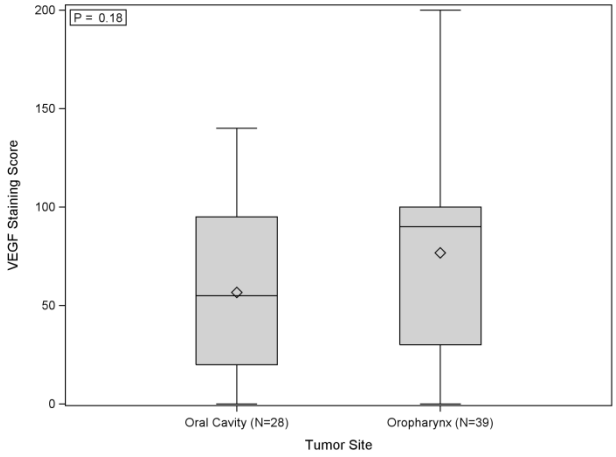


Figure 8. VEGF Expression Stratified By Tumor Site

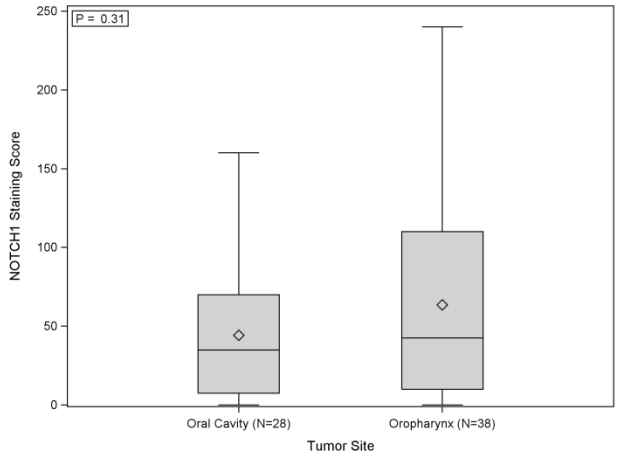


Figure 9. NOTCH1 Expression Stratified By Tumor Site

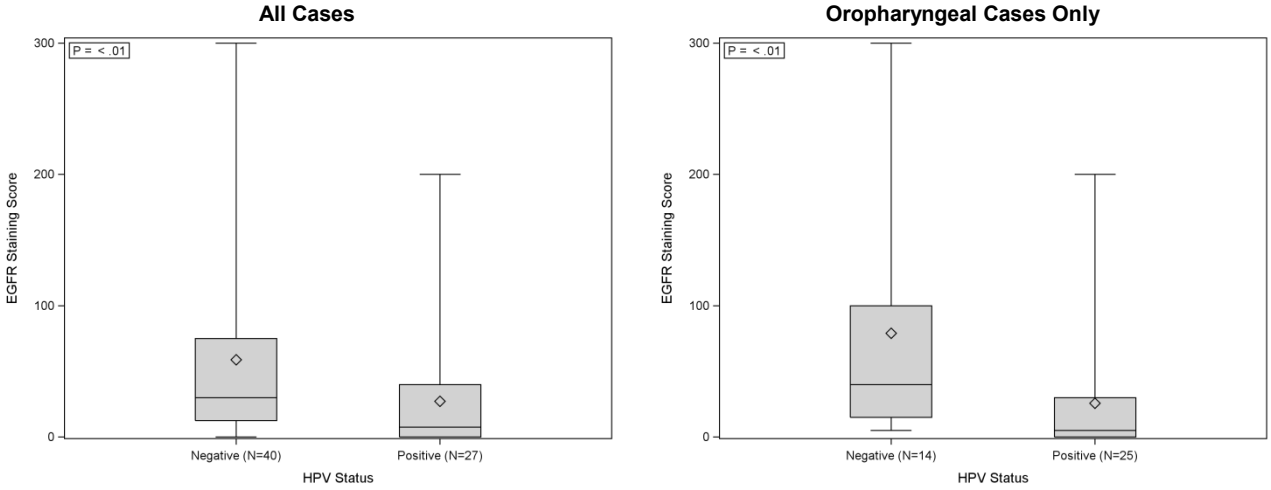
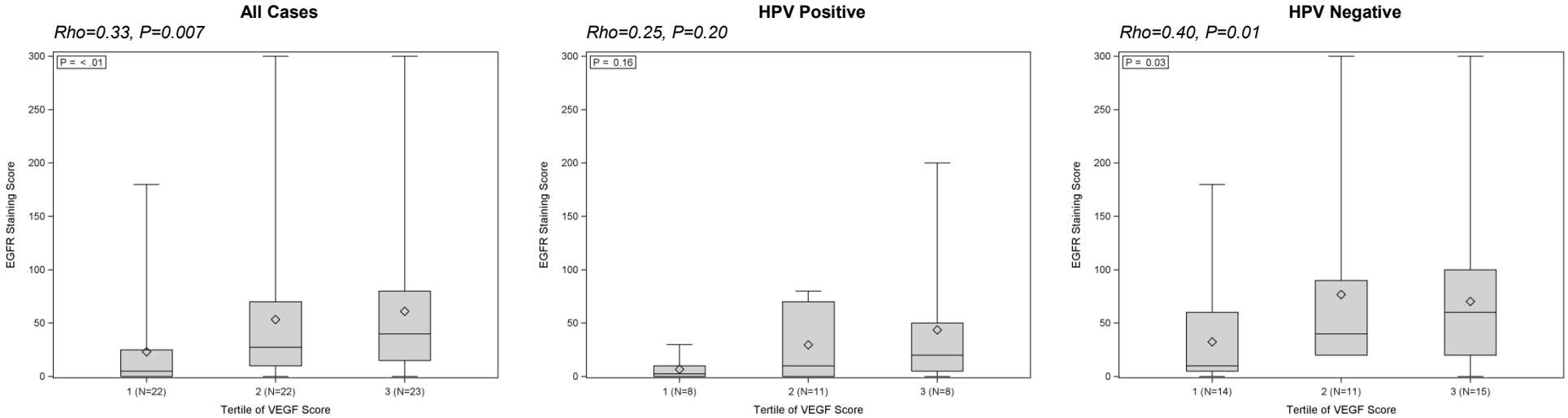
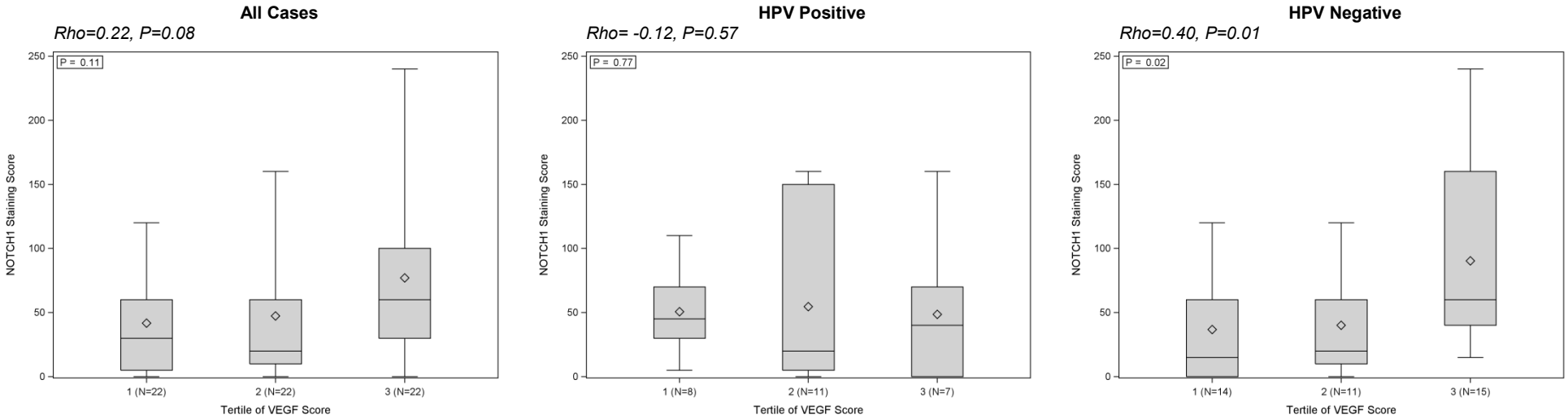


Figure 10. EGFR Expression According to HPV Status in All Cases and Oropharyngeal Cases



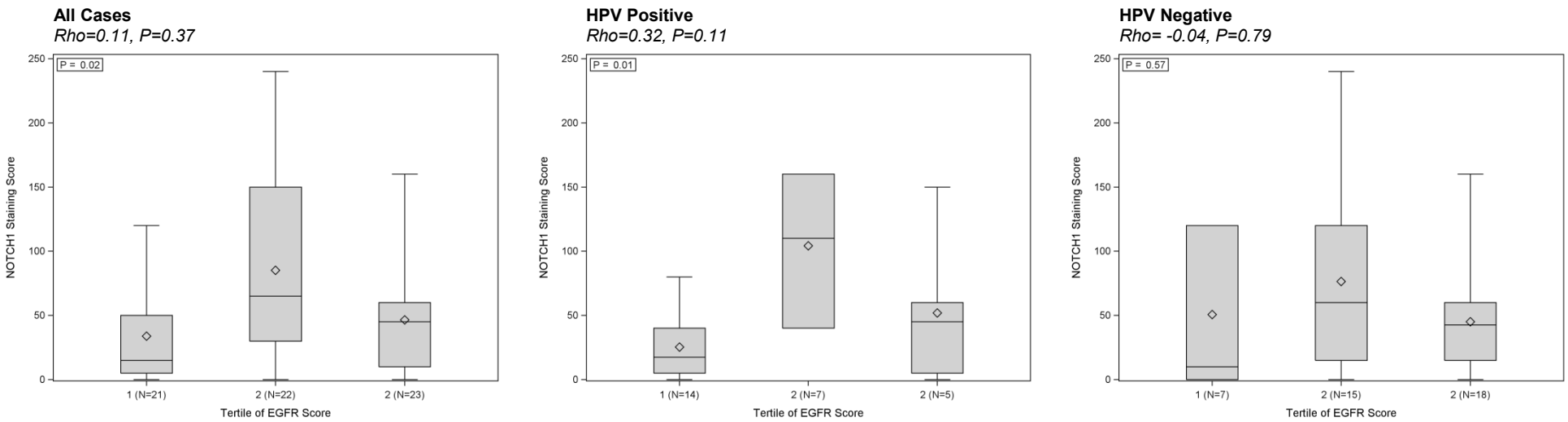
HPV=human papilloma virus; EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor. Whiskers on the box plots indicate the range of protein staining score. The lower and upper boundaries of the box indicate the 25th and 75th percentiles, respectively. The solid line in the center of the box indicates the median value, and the diamond indicates the mean. P-valves shown on box plots are from the Kruskall-Wallis test. Rho=Spearman correlation coefficient.

Figure 11. Association Between EGFR and VEGF in All Cases and Stratified By Tumor HPV Status



HPV=human papilloma virus; NOTCH1=NOTCH receptor 1; VEGF=vascular endothelial growth factor. Whiskers on the box plots indicate the range of protein staining score. The lower and upper boundaries of the box indicate the 25th and 75th percentiles, respectively. The solid line in the center of the box indicates the median value, and the diamond indicates the mean. P-valves shown on box plots are from the Kruskall-Wallis test. Rho=Spearman correlation coefficient.

Figure 12. Association Between NOTCH1 and VEGF in All Cases and Stratified By Tumor HPV Status



HPV=human papilloma virus; NOTCH1=NOTCH receptor 1; EGFR=epidermal growth factor receptor. Whiskers on the box plots indicate the range of protein staining score. The lower and upper boundaries of the box indicate the 25th and 75th percentiles, respectively. The solid line in the center of the box indicates the median value, and the diamond indicates the mean. P-valves shown on box plots are from the Kruskall-Wallis test. Rho=Spearman correlation coefficient.

Figure 13. Association Between NOTCH1 and EGFR in All Cases and Stratified By Tumor HPV Status

5.0 ARTICLE 2: CHILDHOOD PASSIVE SMOKE EXPOSURE IS ASSOCIATED WITH ADULT HEAD AND NECK CANCER

To be submitted for publication

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Key words: adolescent, child, head and neck neoplasms, infant, oropharyngeal neoplasms, tobacco smoke pollution

5.1 ABSTRACT

Introduction. Passive smoke is carcinogenic but its association with head and neck squamous cell carcinoma (HNSCC) is uncertain. **Methods.** We conducted a case-control study of childhood passive smoke exposure (CPSE) and HNSCC in N=862 cases (262 with human papillomavirus [HPV] status) and N=806 frequency-matched controls using an interviewer-administered questionnaire. Odds ratios (OR) and 95% confidence intervals (CI) were estimated with logistic regression controlling for smoking, and in never-smokers (N=186 cases, N=415 controls). Adjusted population attributable risk percent for CPSE was estimated. In addition, CPSE was studied in oropharyngeal separately from other HNSCC, and HPV-positive separately from HPV-negative HNSCC, using polytomous logistic regression. Finally, HPV-CPSE interaction was assessed in case-only analyses. **Results.** CPSE was associated with HNSCC (OR=1.28, 95% CI: 1.01,1.63) controlling for smoking, but not in never-smokers (OR=1.22, 95% CI: 0.83,1.81). However, CPSE was associated with oropharyngeal (OR=2.04, 95% CI: 1.02,4.08) and not other HNSCC in never-smokers (OR=1.08, 95% CI: 0.71,1.66) (P-for-heterogeneity=0.08). CPSE was not associated with HPV-positive (OR=1.60, 95% CI: 0.91, 2.81) or HPV-negative HNSCC (OR=1.34, 95% CI: 0.89,2.01) (P-for-heterogeneity=0.57), and CPSE was unrelated to HPV in never-smoking cases (P=0.37). Assuming causality, 16.9% (95% CI: 0.8%,29.4%) of HNSCC would not occur without CPSE. **Conclusions.** These data suggest limiting CPSE may reduce HNSCC risk.

5.2 INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) occur in the oral cavity, pharynx, and larynx,[46] and are a significant contributor to the worldwide burden of cancer.[1] Historically, these tumors are linked with tobacco and alcohol and use, but declining smoking rates have revealed an epidemic of oropharyngeal tumors caused by the human papilloma virus (HPV).[2,6,7,54,56] Although this epidemic is occurring in patients with little or no smoking history, most patients were born when smoking rates remained high and may have had childhood passive smoke exposure (CPSE).[2,54] CPSE is associated with increased risk of childhood infections.[26] However, little is known about CPSE and risk of HNSCC as an adult, and whether this risk differs for HPV-positive or HPV-negative HNSCC.

Passive smoke is a human carcinogen and is associated with increased risk of lung cancer in never-smokers.[25] However, we are aware of only one study that examined CPSE and HNSCC, showing no association with larynx cancer after adjustment for smoking.[31] One other study reported on risk of smoking-related cancers associated with CPSE in former and never-smokers, but did not report results separately for HNSCC.[99] Furthermore, no studies have examined CPSE and tumor HPV status in HNSCC.[31,99] Finally, reports of CPSE and nasopharyngeal carcinoma[28,98] may not be relevant to HNSCC, as nasopharyngeal carcinoma is histologically and etiology distinct from HNSCC.[29,30]

Therefore, we conducted a case-control study of CPSE and HNSCC in a United States population of N=862 cases (186 never-smokers) and N=806 controls (415 never-smokers). CPSE and adult risk factors for HNSCC were collected using an interviewer-administered questionnaire. Tumor HPV status was available for N=262 cases (58 never-smokers). Our objectives were to evaluate the association between CPSE and HNSCC after controlling for

smoking, to investigate CPSE separately in oropharyngeal and other HNSCC in never-smokers, and to explore the role of CPSE in HPV-positive and HPV-negative HNSCC. To our knowledge, ours is the first report of CPSE and HNSCC in a United States population.

5.3 METHODS

5.3.1 Study Population

Between August 4, 2004 and December 31, 2010, N=907 HNSCC cases and N=807 cancer-free controls were recruited from the University of Pittsburgh Medical Center otolaryngology clinics for a case-control study of HNSCC etiology. Cases were age 18-79 at diagnosis with pathologically verified HNSCC within 1 year of interview (primary tumors [excluding *in situ* cancer] of the lip, oral cavity, pharynx [including base of tongue, soft palate, and uvula], larynx, nasal cavity, and paranasal sinuses). Controls were age 18-80 at enrollment, had no history of HNSCC (verified by clinical examination) and were frequency matched to cases on age, sex, race and enrollment time period. Participants completed an interviewer-administered questionnaire, collecting demographic, personal/family cancer history, tobacco/alcohol use, anthropometry, diet, usual oral care habits, and history of CPSE. Eligibility was verified and informed consent was obtained prior to enrollment.

5.3.2 Eligibility for the Present Analysis

We selected cases from the parent study who were diagnosed with squamous cell carcinoma (SCC) of the oral cavity, pharynx, or larynx representing, to the best of our knowledge, the patient's first-ever HNSCC (history of cancer at other sites was allowed) and who provided data on CPSE. Beginning with the N=907 cases in the parent study, we excluded 9 lip, 6 nasal cavity/middle ear, 8 sinus tumors, 11 tumors with ill-defined/overlapping sites, 6 unknown primaries, and 2 cases of *in situ* disease. Finally, we excluded 3 cases with missing CPSE data. Therefore, we included N=862 cases in our analysis. We excluded only 1 control due to missing CPSE data, leaving N=806 controls for analysis. Excluded cases did not differ from included cases with respect to age, sex, race, smoking, drinking, Body Mass Index (BMI) one year before diagnosis, or CPSE ($P > 0.20$ for all).

5.3.3 Exposure Variables

Our primary interest was CPSE: exposure to passive cigarette smoke in the home up to age 18 (yes/no). We also defined the following for exposed persons: father smoked, mother smoked, sibling(s) smoked, and other person(s) in the household smoked (yes/no for each), number of household smokers (continuous), years of CPSE (maximum duration of smoking among all household smokers, up to 18 years), number of cigarettes/day smoked in the household (sum of cigarettes/day for each household smoker), and pack-years of CPSE (product of cigarettes/day smoked in household [divided by 20] and years of CPSE). The frequency-matched factors were defined as: age (continuous), sex, race (White, non-White/unknown), and enrollment time period (early [2004,2005], middle [2006-2008], and late [2009,2010]). We also defined other variables

to explore confounding and interaction: smoking status (ever/never, where ever-smoking was defined as smoking at least one cigarette/day for six months or longer), drinking status (ever/never, where ever-drinking was defined as drinking at least one drink/month for one year or longer), BMI at reference (one year prior to diagnosis [for cases] or ascertainment [for controls]) (continuous [kg/m^2] and using WHO categories: underweight [<18.5], normal [18.5-24.9], overweight [25.0-29.9], and obese [>=30]), level of education (grade school, high school, vocational, or college), cigar smoking (ever/never, defined as smoking at least one cigar per week for six months or longer), pipe smoking (ever/never, defined as smoking at least one pipe per day for six months or longer), smokeless tobacco (ever/never, defined as using tobacco/chew/snuff at least once a day for three months or longer), servings (continuous) of fruit or vegetables (separately) consumed per day, United States vs. non-United States birthplace, times/week teeth were brushed (continuous), times/week mouthwash was used (continuous), personal history of any cancer (yes/no), and history of HNSCC in a blood relative (natural parents, brothers, sisters, or children) (yes/no). Finally, tumor HPV status (positive/negative, determined by *in situ* hybridization) was available from pathology reports.

5.3.4 Statistical Analysis

First, descriptive statistics were calculated for the entire sample and comparison of risk factors by tumor site within the case series was performed using Fisher's exact test. Then, univariable logistic regression was used to explore factors associated with CPSE in the control group. Crude comparisons of cases and controls were performed using logistic regression adjusted for the frequency-matched factors. Factors associated with both CPSE and HNSCC (alpha=0.10) were entered simultaneously into a base model that included the frequency matched factors, smoking

status, drinking status, and CPSE. Next, we identified other important main effects by testing factors (one-at-a-time) known to be associated with HNSCC, or that might be associated with cancer risk in general. All significant (alpha=0.10) factors were entered into the model simultaneously. We then removed factors with the highest p-value (one at a time) until all remaining factors were part of our base model or were significant at alpha=0.05. No first-order interactions were observed between any of the remaining factors. The final model included the frequency matched factors, smoking status, drinking status, personal history of cancer, education, and CPSE. The same covariates were used in polytomous logistic regression comparing oropharyngeal and other cases [oral cavity, other pharynx, or larynx] with controls, or comparing HPV-positive and HPV-negative cases with controls. All models were run with control for smoking and in subgroup analyses of never-smokers. Odds Ratios (OR) were interpreted as risk estimates. Finally, we performed a case-only analysis of CPSE and HPV status in never-smokers using contingency table methods to evaluate interaction between CPSE and HPV in HNSCC risk.

Model fit was evaluated using the Hosmer-Lemeshow test. Delta-deviance plots and sensitivity analyses were used to verify there were no influential covariate patterns. Statistical significance was evaluated using the likelihood ratio chi-square test. Categorical variables were treated as indicators. Continuous variables were separated into categories according to their distribution in the control group. Tests for trend in log-odds of HNSCC (interpreted as a trend in risk) were performed among participants with the factor of interest by entering a continuous variable into the model, or by treating ordinal variables as continuous. Interactions were treated as indicators except for those involving two continuous variables. Formal tests of heterogeneity of ORs in polytomous models were done using the Wald chi-square test. Adjusted population

attributable risk percent (aPAR-p) for CPSE was calculated using results from our dichotomous response models.[164]

Analyses were performed using SAS 9.2 (SAS Institute, Cary, NC), except for aPAR-p, which was performed using *aflogit* in Stata 11 (StataCorp, College Station, TX).

5.4 RESULTS

A total of N=862 cases and N=806 controls were included in this analysis (Table 11). The median age of participants was 59 years (range: 18-80). The majority were male (67.9%) and White (95.3%). A total of 64.0% of participants reported ever smoking as an adult. CPSE was reported by 69.0% of participants. The case series represented primarily oral cavity (44.4%), laryngeal (26.9%), and oropharyngeal (23.1%) tumors, and included few hypopharyngeal (4.1%) and nasopharyngeal (1.5%) tumors (Appendix D). Differences between oropharyngeal and other cases were consistent with prior reports[7] (Appendix D). N=516 (64.0%) controls reported CPSE (Table 12). Older age, birth cohort, ever-smoking, ever-drinking, and history of cancer in a blood relative, were associated with CPSE in controls ($P < 0.05$ for all) (Table 12).

Table 11 shows crude comparisons between cases and controls. CPSE was associated with a 59% increased risk of HNSCC (OR=1.59, 95% CI: 1.29, 1.97). Ever-smoking (OR=3.60, 95% CI: 2.89, 4.48), ever-drinking (OR=1.69, 95% CI: 1.32, 2.17), United States birthplace (OR=4.55, 95% CI: 2.02, 10.22), use of smokeless tobacco (OR=1.53, 95% CI: 1.10, 2.12), personal history of cancer (OR=1.89, 95% CI: 1.40, 2.55), and were also associated with increased risk of HNSCC. Higher education and higher BMI were generally associated with reduced risk of HNSCC (P-trend < 0.0001 for both). Ever use of pipes (OR=0.82, 95% CI: 0.54,

1.24) and cigars (OR=1.30, 95% CI: 0.90, 1.87) and history of cancer in blood relatives (OR=1.05, 95% CI: 0.85, 1.29) were not associated with HNSCC.

5.4.1 Childhood Passive Smoke Exposure And Head And Neck Cancer After Adjustment For Smoking

Table 13 shows results of multivariable logistic regression modeling among 858 cases and 806 controls with data available on confounding factors. CPSE was associated with a 28% increased risk of HNSCC after adjustment for smoking (OR=1.28, 95% CI: 1.01, 1.63). Based on this model, we estimated aPAR-p for CPSE to be 16.9% (95% CI: 0.8%, 29.4%). We did not observe any trends in HNSCC risk with respect to years of exposure, cigarettes/day smoked in the household, pack-years of exposure, number of household smokers, or the presence of maternal, paternal, sibling, or other household smokers ($P > 0.05$ for all). Polytomous logistic regression showed similar associations of CPSE with oropharynx (OR=1.44, 95% CI: 0.99, 2.10) and other HNSCC (OR=1.25, 95% CI: 0.97, 1.62) (P-for-heterogeneity=0.47). Finally, because United States birthplace was associated with high risk of HNSCC we repeated our analyses restricted to subjects born in the United States, but results were unchanged (data not shown).

5.4.2 Childhood Passive Smoke Exposure In Never-Smokers And Risk Of Head And Neck Cancer

As shown in Table 14, CPSE was unrelated to HNSCC in never-smokers after multiple adjustment (OR=1.19, 95% CI: 0.80, 1.76). However, among exposed persons, having siblings who smoked was associated with increased risk of HNSCC (OR=3.46, 95% CI: 1.28, 9.39), and

risk among the exposed increased with the number of household smokers (P=0.04). Years of exposure, cigarettes/day smoked in the household, pack-years, or smoking by the mother, father, or other household members were unrelated to HNSCC (P > 0.05 for all).

Polytomous logistic regression in never-smokers (Table 15) showed CPSE was associated with oropharynx (OR=2.02, 95% CI: 1.01, 4.06) but not other HNSCC (OR=1.04, 95% CI: 0.68, 1.60), although this difference was not significant (P-for-heterogeneity=0.08). However, we noted increasing risk of oropharynx cancer with an increasing number of cigarettes/day smoked in the household (P=0.01), with > 20 cigarettes/day associated with substantially higher risk than <= 20 cigarettes/day (OR=3.78, 95% CI: 1.40, 10.22). We noted a similar results for pack-years of CPSE. Risk of oropharynx cancer also increased with the number of household smokers (P=0.01). The presence of two or more smokers was associated with twice the risk of oropharynx cancer vs. one household smoker (OR=2.15, 95% CI: 1.03, 4.46). Duration of CPSE, and having a mother, father, sibling, or other household members who smoked were unrelated to oropharynx cancer (P > 0.05 for all). In contrast to these results, risk of HNSCC at sites other than the oropharynx was, with the exception of having siblings who smoked (OR=3.99, 95% CI: 1.42, 11.22), generally unrelated to CPSE (Table 15).

5.4.3 Childhood Passive Smoke Exposure And HPV In Head And Neck Cancer

HPV status was available for N=262 cases (30.5%) included in multivariable regression models (Appendix E). Cases with known HPV status were younger and more often male than cases whose HPV status was unknown, but HPV testing was unrelated to smoking, drinking, or CPSE (data not shown). Risk of HPV-positive (OR=1.60, 95% CI: 0.91, 2.81) and HPV-negative (OR=1.34, 95% CI: 0.89, 2.01) HNSCC were similar after adjustment for smoking (P-for-

heterogeneity=0.57). In never-smoking cases, CPSE was more frequent among HPV-positive (40.5%) compared with HPV-negative (25.0%) cases, although this difference was not statistically significant (P=0.37).

5.5 DISCUSSION

In this large, single-institution case-control study, CPSE was associated with increased risk of HNSCC after adjustment for smoking. Assuming a causal association, we estimate 17% of HNSCC would be prevented if CPSE were eliminated, thus making CPSE an important contributor to HNSCC risk. In addition, our data are highly suggestive of an association between CPSE and oropharynx cancer in never-smokers. However, CPSE was not specifically associated with HPV-positive or HPV-negative HNSCC, and we did not observe statistically significant interaction between CPSE and HPV in HNSCC among never-smokers.

Studies of CPSE and cancer in the head and neck are few in number and have included histologies other than SCC,[27,28,98] which are etiologically and histologically distinct from HNSCC.[29,30] We identified only two studies that examined CPSE and HNSCC. The first was a case-control study showing no association between CPSE and larynx cancer after adjustment for smoking.[31] This result is consistent with our observation that CPSE is not a strong risk factor for non-oropharyngeal HNSCC. The second study was a prospective cohort study of smoking-related cancers (tumors at several sites, including HNSCC) in former and never-smokers.[99] Although no association was reported between CPSE and smoking-related cancers, it is difficult to interpret these results for HNSCC alone.[99]

Despite the small number of corroborating reports, our results are bolstered by certain strengths. We enrolled incident cases of HNSCC and frequency-matched controls to cases based on age and time period, helping insure controls were representative of the exposure experience of the non-diseased source population from which the cases arose. CPSE temporally preceded the cancer under study, and we noted a strong and statistically significant association for cancer at a specific anatomical site (the oropharynx) in never-smokers. In addition, we noted trends in risk wherein various patterns of CPSE was associated with oropharynx cancer but not other HNSCC in never-smokers. Using our extensive questionnaire, we were able to explore confounding and interaction with a variety of other lifestyle factors related to HNSCC or cancer in general. In addition, HPV status was available for a subset of our case series, enabling us evaluate the joint role of CPSE and HPV in HNSCC.

Passive smoke consists of sidestream smoke (emitted from the cigarette) and mainstream smoke (exhaled by the smoker) and contains at least fifty carcinogens, including polycyclic aromatic hydrocarbons (PAH) and N-nitrosamines, which are found in the bloodstream of non-smokers exposed to passive smoke.[26] The United States Surgeon General has concluded there is sufficient evidence to demonstrate a causal relationship between passive smoke and lung cancer in lifetime never-smokers.[26] Active cigarette smoking causes cancer through a variety of mechanisms, including formation of DNA adducts, disruption of DNA repair and cell cycle control, and activation of cytoplasmic signaling networks relevant to cell growth and proliferation.[165] The carcinogenic mechanisms of passive smoke exposure are likely similar.[26] However, our observation of increased risk of oropharyngeal cancer in never-smokers is particularly compelling given the frequent HPV-related etiology of these tumors.[7] Therefore, it is natural to ask whether and how CPSE and HPV act together to promote oropharynx cancer. Our

results indicate CPSE is associated with HNSCC regardless of tumor HPV status, and we did not observe strong evidence of interaction between CPSE and HPV in never-smoking HNSCC cases. However, we were unable to explore CPSE and HPV in never-smoking *oropharynx* cases due to small sample size. However, such investigation might not be fruitful. While oral[166-169] and oropharyngeal[168,170] HPV infection is detectable in children, these infections are transient[171] and unrelated to parental smoking.[166,167] Therefore, it seems unlikely that childhood HPV infection and interaction with CPSE promote adult HNSCC. However, this does not rule out long-term effects of CPSE on the immune system that may increase susceptibility to, or facilitate persistence of, HPV later in life. CPSE is associated with increased risk of respiratory and middle ear infections in children, likely through a broad range of effects on the immune system.[172] It is unclear whether such immunological effects last when CPSE is removed, and whether they effect risk of HPV infection or clearance later in life. However, at least 1 lasting immunological consequence of CPSE is known: asthma.[172]

Our results are accompanied by several limitations. First, the exposure in our study was measured via questionnaire and it was not possible to obtain biological evidence of exposure. We asked participants to recall exposures that happened several decades in the past. Therefore, it is possible that recall was different in cases and controls, particularly in never-smokers who might place blame on others for their diagnosis with a typically smoking-related illness. However, the specificity of our findings within the never-smoking subgroup, i.e., increased risk of oropharynx cancer and not other HNSCC, refutes the possibility of recall bias. If such bias were present, we might expect it to affect our risk estimates for HNSCC at all anatomic sites.

Our study used clinic controls and this is known to result in bias if the controls' disease is associated with the exposure, as this yields a control group with a different prevalence of

exposure than would be observed in the source population.[173] However, our controls had a variety of non-malignant conditions of the head and neck not known to be related to CPSE (data not shown). Finally, we frequency matched controls to cases on age and enrollment time period, avoiding selection of controls based on birth cohort, which is associated with CPSE (Table 12).

Residual confounding is also possible. In particular, we did not have data on adult passive smoke exposure (APSE) although this appears to be a weak risk factor for head and neck tumors. A study of maxillary sinus cancer in Japan showed a positive association with APSE in the home among never-smoking women and noted increasing risk with an increasing number of household smokers.[95] In the United States, a case-control study of never-smokers observed an association between APSE in the home and the workplace and cancer of the oral cavity, pharynx, larynx, or sinus.[96] However, APSE was not related to cancer of the oral cavity, pharynx, larynx, sinus, lip, salivary glands, and esophagus among never-smokers in another United States study.[97] Furthermore, there was no association between lifetime passive smoke exposure and larynx cancer in German never-smokers.[31] Finally, a prospective study that examined risk of HNSCC associated with APSE at home and in the workplace observed no association between passive smoke exposure and pharyngeal or laryngeal cancers among never smokers.[99] It must be recognized, however, that these null results may derive from the combination of tumors weakly related to passive smoke with tumors that are strongly related to passive smoke, thus masking an association such as the one we observed by studying oropharyngeal tumors separately from other HNSCC. Finally, residual confounding is possible due to other environmental exposures related to cancer risk. In particular, the Pittsburgh, Pennsylvania area has a history of industrial steel production that created various chemical pollutants associated with respiratory cancer risk.[174] We are unaware of our study participants' occupation, length of residence in areas affected by

industrial pollution, or their extent of exposure, and this information might be relevant in the study of cancer risk in never-smokers.

In summary, our results support an etiologic role for CPSE in HNSCC. In particular, CPSE may be carcinogenic in the oropharynx of never-smokers. The biological mechanism through which this occurs is uncertain and may involve direct effects of carcinogens in passive smoke, or disruptions in immunological development effecting response to HPV infection later in life. Pooled analyses of CPSE and tumor HPV status in never-smoking oropharyngeal cancers may improve understanding of this mechanism. Finally, despite declining smoking rates,[2] children in the United States continue to suffer ill effects of passive smoke exposure.[175] Our data suggest these effects include an increased risk of HNSCC. This should be considered in the making of public policy that protects that health of children through restricting the opportunity for CPSE.

5.6 TABLES

Table 11. Crude Analysis of Factors Associated With Head and Neck Cancer

	All Participants (N=1,668)	Cases (N=862)	Controls (N=806)	
	n(%)*	n(%)*	n(%)*	OR (95% CI)†
Age				
<50	322 (19.3)	153 (17.7)	169 (21.0)	-
50-59	562 (33.7)	277 (32.1)	285 (35.4)	-
60-69	507 (30.4)	279 (32.4)	228 (28.3)	-
>=70	277 (16.6)	153 (17.7)	124 (15.4)	-
Sex				
Male	1,133 (67.9)	630 (73.1)	503 (62.4)	-
Female	535 (32.1)	232 (26.9)	303 (37.6)	-
Race				
White	1,589 (95.3)	821 (95.2)	768 (95.3)	-
Non-White/Unknown‡	79 (4.7)	41 (4.8)	38 (4.7)	-
Recruitment Period				
Early	281 (16.8)	138 (16.0)	143 (17.7)	-
Middle	887 (53.2)	421 (48.8)	466 (57.8)	-
Late	500 (30.0)	303 (35.2)	197 (24.4)	-
Birth Cohort				
<=1920	66 (4.0)	33 (3.8)	33 (4.1)	1.05 (0.50-2.19)
1930	305 (18.3)	172 (20.0)	133 (16.5)	1.15 (0.76-1.74)
1940	524 (31.4)	285 (33.1)	239 (29.7)	1.00
1950	508 (30.5)	251 (29.1)	257 (31.9)	0.74 (0.50-1.09)
1960	190 (11.4)	96 (11.1)	94 (11.7)	0.71 (0.36-1.41)
>=1970	75 (4.5)	25 (2.9)	50 (6.2)	0.36 (0.11-1.18)
Childhood Passive Smoke				
No	517 (31.0)	227 (26.3)	290 (36.0)	1.00
Yes	1,151 (69.0)	635 (73.7)	516 (64.0)	1.59 (1.29-1.97)
Ever Smoked				
No	601 (36.0)	186 (21.6)	415 (51.5)	1.00
Yes	1,067 (64.0)	676 (78.4)	391 (48.5)	3.60 (2.89-4.48)
Ever Drank Alcohol				
No	397 (23.8)	159 (18.5)	238 (29.5)	1.00
Yes	1,269 (76.2)	701 (81.5)	568 (70.5)	1.69 (1.32-2.17)

Table 11 continued

	All Participants (N=1,668)	Cases (N=862)	Controls (N=806)	
	n(%)*	n(%)*	n(%)*	OR (95% CI)†
BMI 1 year pre-diagnosis				
<18.5	25 (1.5)	20 (2.3)	5 (0.6)	3.75 (1.36-10.32)
18.5-24.9	480 (28.8)	270 (31.5)	210 (26.1)	1.00
25.0-29.9	606 (36.4)	292 (34.0)	314 (39.0)	0.64 (0.50-0.82)
>=30	553 (33.2)	276 (32.2)	277 (34.4)	0.71 (0.55-0.92)
Highest Level of Education				
Grade school	42 (2.5)	39 (4.5)	3 (0.4)	5.61 (1.71-18.42)
High school	778 (46.7)	533 (62.0)	245 (30.4)	1.00
Vocational	100 (6.0)	48 (5.6)	52 (6.5)	0.47 (0.31-0.73)
College	746 (44.8)	240 (27.9)	506 (62.8)	0.22 (0.17-0.27)
Birth Country				
Outside United States	36 (2.2)	8 (0.9)	28 (3.5)	1.00
United States	1,632 (97.8)	854 (99.1)	778 (96.5)	4.55 (2.02-10.22)
Ever Smoked Cigars				
No	1,527 (91.5)	775 (89.9)	752 (93.3)	1.00
Yes	141 (8.5)	87 (10.1)	54 (6.7)	1.30 (0.90-1.87)
Ever Used Smokeless Tobacco				
No	1,479 (88.7)	742 (86.1)	737 (91.4)	1.00
Yes	189 (11.3)	120 (13.9)	69 (8.6)	1.53 (1.10-2.12)
Ever Smoked Pipe				
No	1,563 (93.7)	805 (93.4)	758 (94.0)	1.00
Yes	105 (6.3)	57 (6.6)	48 (6.0)	0.82 (0.54-1.24)
Personal History of Cancer				
No	1,433 (85.9)	707 (82.0)	726 (90.1)	1.00
Yes	235 (14.1)	155 (18.0)	80 (9.9)	1.89 (1.40-2.55)
Blood Relative Had Cancer				
No	642 (38.8)	322 (37.7)	320 (40.1)	1.00
Yes	1,011 (61.2)	533 (62.3)	478 (59.9)	1.05 (0.85-1.29)

OR=odds ratio; CI=confidence interval.

*Number may not sum to total due to missing values for some variables.

†Odds ratios and 95% confidence intervals were calculated using logistic regression models adjusted for the frequency matched factors (age, sex, race, and recruitment period). Estimates are not shown for the frequency matched factors.

‡Non-White participants included 69 African Americans (36 cases, 33 controls), 6 Asians (3 cases, 3 controls), 1 American Indian/Eskimo control, 2 Other races (1 case, 1 control), and 1 case who did not report a race.

Table 12. Factors Associated With Childhood Passive Smoke Exposure in the Control Group

	Exposed N=(516)	Unexposed (N=290)		
	n(%)*	n(%)*	OR (95% CI)†	P-Value‡
Age				<.001
<50	92 (17.8)	77 (26.6)	1.00	
50-59	194 (37.6)	91 (31.4)	1.78 (1.21-2.64)	
60-69	163 (31.6)	65 (22.4)	2.10 (1.38-3.19)	
>=70	67 (13.0)	57 (19.7)	0.98 (0.62-1.57)	
Birth Cohort				0.002
<=1920	18 (3.5)	15 (5.2)	0.46 (0.22-0.96)	
1930	77 (14.9)	56 (19.3)	0.52 (0.34-0.82)	
1940	173 (33.5)	66 (22.8)	1.00	
1950	169 (32.8)	88 (30.3)	0.73 (0.50-1.07)	
1960	56 (10.9)	38 (13.1)	0.56 (0.34-0.93)	
>=1970	23 (4.5)	27 (9.3)	0.33 (0.17-0.61)	
Sex				0.07
Male	334 (64.7)	169 (58.3)	1.00	
Female	182 (35.3)	121 (41.7)	0.76 (0.57-1.02)	
Race				0.43
White	494 (95.7)	274 (94.5)	1.00	
Non-White/Unknown	22 (4.3)	16 (5.5)	0.76 (0.39-1.48)	
Ever Smoked				0.02
No	250 (48.4)	165 (56.9)	1.00	
Yes	266 (51.6)	125 (43.1)	1.40 (1.05-1.88)	
Ever Drank Alcohol				<.001
No	131 (25.4)	107 (36.9)	1.00	
Yes	385 (74.6)	183 (63.1)	1.72 (1.26-2.34)	
BMI 1 year pre-diagnosis				0.35
<18.5	2 (0.4)	3 (1.0)	0.44 (0.07-2.66)	
18.5-24.9	127 (24.6)	83 (28.6)	1.00	
25.0-29.9	202 (39.1)	112 (38.6)	1.18 (0.82-1.69)	
>=30	185 (35.9)	92 (31.7)	1.31 (0.91-1.91)	
Highest Level of Education				0.56
Grade school	1 (0.2)	2 (0.7)	0.25 (0.02-2.82)	
High school	163 (31.6)	82 (28.3)	1.00	
Vocational	33 (6.4)	19 (6.6)	0.87 (0.47-1.63)	
College	319 (61.8)	187 (64.5)	0.86 (0.62-1.18)	

Table 12 continued

	Exposed N=(516)	Unexposed (N=290)		
	n(%)*	n(%)*	OR (95% CI)†	P-Value‡
Birth Country				0.45
Outside United States	16 (3.1)	12 (4.1)	1.00	
United States	500 (96.9)	278 (95.9)	1.35 (0.63-2.89)	
Ever Smoked Cigars				0.65
No	483 (93.6)	269 (92.8)	1.00	
Yes	33 (6.4)	21 (7.2)	0.88 (0.50-1.54)	
Ever Used Smokeless Tobacco				0.63
No	470 (91.1)	267 (92.1)	1.00	
Yes	46 (8.9)	23 (7.9)	1.14 (0.67-1.92)	
Ever Smoked Pipe				0.93
No	485 (94.0)	273 (94.1)	1.00	
Yes	31 (6.0)	17 (5.9)	1.03 (0.56-1.89)	
Personal History of Cancer				0.35
No	461 (89.3)	265 (91.4)	1.00	
Yes	55 (10.7)	25 (8.6)	1.26 (0.77-2.08)	
Blood Relative Had Cancer				0.01
No	189 (36.9)	131 (45.8)	1.00	
Yes	323 (63.1)	155 (54.2)	1.44 (1.08-1.94)	

*Numbers may not sum to total due to missing values for some variables

†Odds ratios and 95% confidence intervals were calculated using univariable logistic regression models.

‡P-values represent likelihood ratio Chi-square tests comparing the factor of interest with the null model. All variables were treated as indicators.

Table 13. Childhood Passive Smoke Exposure and Head and Neck Cancer, Adjusted for Smoking

	Cases (N=858)*	Controls (N=806)		
	n (%)†	n (%)†	OR (95% CI)‡	P-trend#
Childhood Passive Smoke				0.04
No	225 (26.2)	290 (36.0)	1.00	
Yes	633 (73.8)	516 (64.0)	1.28 (1.01-1.63)	
Years Exposed				0.38
<18	110 (17.5)	126 (24.6)	1.00	
18	519 (82.5)	386 (75.4)	1.17 (0.84-1.62)	
Cigarettes/Day				0.80
<=10	72 (13.1)	69 (14.9)	1.00	
11-20	147 (26.8)	135 (29.2)	0.86 (0.54-1.38)	
21-40	187 (34.1)	148 (32.0)	0.97 (0.61-1.52)	
>40	142 (25.9)	110 (23.8)	0.96 (0.60-1.55)	
Pack-Years				0.82
<=15	109 (20.0)	113 (24.6)	1.00	
>15-25	132 (24.2)	107 (23.3)	0.96 (0.63-1.47)	
>25-40	169 (31.0)	137 (29.8)	0.99 (0.66-1.47)	
>40	136 (24.9)	103 (22.4)	1.02 (0.67-1.56)	
Number of Household Smokers				0.84
1	356 (56.3)	307 (59.5)	1.00	
2	235 (37.2)	188 (36.4)	0.97 (0.73-1.28)	
>=3	41 (6.5)	21 (4.1)	1.13 (0.61-2.09)	
Mother Smoked				0.47
No	314 (49.7)	249 (48.3)	1.00	
Yes	318 (50.3)	267 (51.7)	0.90 (0.69-1.19)	
Father Smoked				0.53
No	103 (16.3)	103 (20.0)	1.00	
Yes	529 (83.7)	413 (80.0)	1.12 (0.79-1.59)	
Sibling(s) Smoked				0.55
No	579 (91.6)	487 (94.4)	1.00	
Yes	53 (8.4)	29 (5.6)	1.18 (0.69-2.01)	
Other Household Members Smoked				0.70
No	610 (96.5)	493 (95.5)	1.00	
Yes	22 (3.5)	23 (4.5)	0.88 (0.44-1.73)	

OR (odds ratio) and CI (confidence interval) are from logistic regression models

*4 cases (2 oropharynx, 1 oral cavity, and 1 larynx) were dropped from this analysis due to missing data on alcohol drinking status or education.

†Numbers may not sum to total due to missing values for some variables.

‡Odds ratios and 95% confidence intervals are adjusted for age, sex, race, recruitment period, alcohol drinking status, active smoking status, personal history of cancer, and education.

#P-values represent likelihood ratio Chi-square tests of continuous variables representing a 1-unit change in the factor of interest.

Table 14. Childhood Passive Smoke Exposure and Head and Neck Cancer in Never-Smokers

	Cases (N=184)	Controls (N=415)		
	n (%)*	n (%)*	OR (95% CI)†	P-trend‡
Childhood Passive Smoke				0.38
No	64 (34.8)	165 (39.8)	1.00	
Yes	120 (65.2)	250 (60.2)	1.19 (0.80-1.76)	
Years Exposed				0.74
<18	30 (25.4)	74 (30.0)	1.00	
18	88 (74.6)	173 (70.0)	1.21 (0.69-2.11)	
Cigarettes/Day				0.25
<=10	18 (18.2)	41 (18.6)	1.00	
11-20	20 (20.2)	65 (29.4)	0.62 (0.28-1.41)	
21-40	37 (37.4)	63 (28.5)	1.29 (0.60-2.75)	
>40	24 (24.2)	52 (23.5)	1.07 (0.48-2.40)	
Pack-Years				0.24
<=15	26 (26.5)	65 (29.5)	1.00	
>15-25	16 (16.3)	47 (21.4)	0.65 (0.29-1.45)	
>25-40	33 (33.7)	59 (26.8)	1.26 (0.63-2.51)	
>40	23 (23.5)	49 (22.3)	1.14 (0.55-2.39)	
Number of Household Smokers				0.04
1	70 (58.8)	163 (65.2)	1.00	
2	40 (33.6)	83 (33.2)	1.06 (0.63-1.78)	
>=3	9 (7.6)	4 (1.6)	6.76 (1.82-25.06)	
Mother Smoked				0.54
No	57 (47.9)	129 (51.6)	1.00	
Yes	62 (52.1)	121 (48.4)	1.17 (0.72-1.90)	
Father Smoked				0.84
No	24 (20.2)	56 (22.4)	1.00	
Yes	95 (79.8)	194 (77.6)	1.06 (0.58-1.96)	
Sibling(s) Smoked				0.01
No	108 (90.8)	241 (96.4)	1.00	
Yes	11 (9.2)	9 (3.6)	3.46 (1.28-9.39)	
Other Household Members Smoked				0.66
No	114 (95.8)	237 (94.8)	1.00	
Yes	5 (4.2)	13 (5.2)	0.77 (0.24-2.50)	

OR (odds ratio) and CI (confidence interval) are from logistic regression models
*Numbers may not sum to total due to missing values for some variables.
†Odds ratios and 95% confidence intervals are adjusted for age, sex, race, recruitment period, drinking status, personal history of cancer, and education.

‡P-values represent likelihood ratio Chi-square tests of continuous variables representing a 1-unit change in the factor of interest..

Table 15. Childhood Passive Smoke Exposure as a Risk Factor for Oropharyngeal and Other Head and Neck Cancers in Never-Smokers

	Controls N=(415)	Oropharyngeal Case (N=52)			Other Case (N=132)*		
	n(%)	n (%)	OR (95% CI)†	P-trend‡	n (%)	OR (95% CI)†	P-trend‡
Childhood Passive Smoke				0.05			0.84
No	165 (39.8)	13 (25.0)	1.00		51 (38.6)	1.00	
Yes	250 (60.2)	39 (75.0)	2.02 (1.01-4.06)#		81 (61.4)	1.04 (0.68-1.60)#	
Years Exposed				0.99			0.72
<18	74 (30.0)	10 (25.6)	1.00		20 (25.3)	1.00	
18	173 (70.0)	29 (74.4)	1.39 (0.60-3.24)		59 (74.7)	1.16 (0.62-2.16)	
Cigarettes/Day				0.01			0.78
<=20	106 (48.0)	6 (20.0)	1.00		32 (46.4)	1.00	
>20	115 (52.0)	24 (80.0)	3.78 (1.40-10.22)		37 (53.6)	1.15 (0.64-2.08)	
Pack Years				0.01			0.79
<=20	111 (50.5)	7 (23.3)	1.00		31 (45.6)	1.00	
>20	109 (49.5)	23 (76.7)	3.62 (1.40-9.36)		37 (54.4)	1.33 (0.74-2.41)	
Number of Household Smokers				0.01			0.28
1	163 (65.2)	18 (46.2)	1.00		52 (65.0)	1.00	
>=2	87 (34.8)	21 (53.8)	2.15 (1.03-4.46)		28 (35.0)	1.03 (0.59-1.80)	
Mother Smoked				0.12			0.76
No	129 (51.6)	13 (33.3)	1.00		44 (55.0)	1.00	
Yes	121 (48.4)	26 (66.7)	1.86 (0.86-4.03)		36 (45.0)	0.92 (0.53-1.58)	
Father Smoked				0.32			0.86
No	56 (22.4)	7 (17.9)	1.00		17 (21.3)	1.00	
Yes	194 (77.6)	32 (82.1)	1.64 (0.62-4.34)		63 (78.8)	0.94 (0.48-1.85)	
Sibling Smoked				0.53			0.01
No	241 (96.4)	37 (94.9)	1.00		71 (88.8)	1.00	
Yes	9 (3.6)	2 (5.1)	1.71 (0.32-9.12)		9 (11.3)	3.99 (1.42-11.22)	
Other Smoked				0.50			0.99
No	237 (94.8)	38 (97.4)	1.00		76 (95.0)	1.00	
Yes	13 (5.2)	1 (2.6)	0.47 (0.05-4.22)		4 (5.0)	1.01 (0.29-3.46)	

OR (odds ratio) and CI (confidence interval) are from polytomous logistic regression models
*Other Cases include squamous cell cancers of the oral cavity, hypopharynx, nasopharynx, and larynx.
†Odds ratios and 95% confidence intervals are adjusted for age, sex, race, recruitment period, drinking status, personal history of cancer, and education (high school or less vs beyond high school).
‡Wald P-value for the model parameter predicting the log-odds of oropharynx (or other HNSCC) obtained by entering a continuous variable, representing a 1-unit change, into the polytomous model.
#P-for-heterogeneity=0.08 comparing odds ratios for oropharyngeal and other cases.

Table 16. Childhood Passive Smoke Exposure as a Risk Factor for HPV-Positive and HPV-Negative Head and Neck Cancers, Adjusted for Active Smoking

	Controls N=(806)	HPV Negative Case (N=180)*			HPV Positive Case (N=82)*		
	n(%)	n (%)	OR (95% CI)†	P-trend‡	n (%)	OR (95% CI)†	P-trend‡
Childhood Passive Smoke				0.16			0.10
No	290 (36.0)	48 (26.7)	1.00		20 (24.4)	1.00	
Yes	516 (64.0)	132 (73.3)	1.34 (0.89-2.01)#		62 (75.6)	1.60 (0.91-2.81)#	
Years Exposed				0.10			0.62
<18	126 (24.6)	18 (13.6)	1.00		14 (22.6)	1.00	
18	386 (75.4)	114 (86.4)	1.60 (0.89-2.91)		48 (77.4)	1.01 (0.51-2.03)	
Cigarettes/Day				0.22			0.69
<=20	204 (44.2)	35 (31.5)	1.00		16 (32.7)	1.00	
>20	258 (55.8)	76 (68.5)	1.42 (0.87-2.33)		33 (67.3)	1.25 (0.63-2.47)	
Pack Years				0.17			0.63
<=20	212 (46.1)	35 (31.5)	1.00		18 (36.7)	1.00	
>20	248 (53.9)	76 (68.5)	1.61 (0.98-2.64)		31 (63.3)	1.20 (0.61-2.35)	
Number of Household Smokers				0.99			0.79
1	307 (59.5)	72 (54.5)	1.00		37 (59.7)	1.00	
>=2	209 (40.5)	60 (45.5)	0.99 (0.64-1.54)		25 (40.3)	0.75 (0.42-1.35)	
Mother Smoked				0.73			0.43
No	249 (48.3)	58 (43.9)	1.00		28 (45.2)	1.00	
Yes	267 (51.7)	74 (56.1)	0.92 (0.59-1.45)		34 (54.8)	0.79 (0.44-1.42)	
Father Smoked				0.09			0.79
No	103 (20.0)	18 (13.6)	1.00		14 (22.6)	1.00	
Yes	413 (80.0)	114 (86.4)	1.68 (0.92-3.08)		48 (77.4)	0.91 (0.45-1.82)	
Sibling Smoked				0.75			0.74
No	487 (94.4)	125 (94.7)	1.00		58 (93.5)	1.00	
Yes	29 (5.6)	7 (5.3)	0.86 (0.33-2.21)		4 (6.5)	1.22 (0.38-3.98)	
Other Smoked				0.18			0.51
No	493 (95.5)	130 (98.5)	1.00		58 (93.5)	1.00	
Yes	23 (4.5)	2 (1.5)	0.35 (0.08-1.64)		4 (6.5)	1.50 (0.45-5.05)	

OR (odds ratio) and CI (confidence interval) are from polytomous logistic regression models
*HPV status is determined by in-situ hybridization
†Odds ratios and 95% confidence intervals are adjusted for age, sex, race, recruitment period, drinking status, smoking status, personal history of cancer, and education (high school or less vs beyond high school).
‡Wald P-value for the model parameter predicting the log-odds of oropharynx (or other HNSCC) obtained by entering a continuous variable, representing a 1-unit change, into the polytomous model.
#P-for-heterogeneity=0.57 comparing odds ratios for HPV-positive and HPV-negative cases.

Table 17. Case-Only Analysis of Childhood Passive Smoke Exposure and Tumor HPV Status in Never-Smokers

	Childhood Passive Smoke	
	Exposed (N=42)	**Unexposed (N=16)**
	n (%)	**n (%)**
HPV Positive Case	17 (40.5)	4 (25.0)
HPV Negative Case	25 (59.5)	12 (75.0)
$P_{Fisher's\ exact}$=*0.37* Empirical odds ratio (95% CI)= 2.04 (0.56-7.39)		

6.0 ARTICLE 3: POLYMORPHISMS IN NAT2 AND GSTP1 ARE ASSOCIATED WITH SURVIVAL IN ORAL AND OROPHARYNGEAL CANCER

To be submitted for publication

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Key words: head and neck neoplasms, NAT2, GSTP1, polymorphism single nucleotide, SNP

6.1 ABSTRACT

Background. Polymorphisms in Phase I/II enzymes that metabolize tobacco/alcohol and chemotherapy may be determinants of survival in oral and oropharyngeal squamous cell carcinoma (OOSCC). **Methods.** N=159 OOSCC cases treated during 2000 -2004 were genotyped for eight Phase I/II enzymes. Overall and disease-specific survival were analyzed using Kaplan-Meier plots. Hazard ratios (HR) and 95% confidence intervals (CI) were calculated using Cox regression. **Results.** N-acteyltransferase-2 (*NAT2*) fast acetylators had improved survival (vs. slow acetylators) when treated with surgery alone (HR, 0.26; 95% CI, 0.10-0.66) but not chemoradiotherapy (HR, 1.21; 95% CI, 0. 54-2.73) or radiotherapy (HR, 0.67; 95% CI, 0.31-1.59) after adjustment for tumor site and stage (P-for-NAT2/treatment-interaction=0.04). Reduced activity glutathione S-transferase pi-1 (*GSTP1*) was associated with improved disease-specific survival in men only (HR, 0.12; 95% CI, 0.02-0.91; women: HR, 2.29; 95% CI, 0.41-12.69; P-for-interaction=0.02). **Conclusions.** Metabolic enzyme genotype is associated with OOSCC survival and may inform selection of therapy.

6.2 INTRODUCTION

Oral and oropharyngeal squamous cell carcinomas (OOSCC) represent the world's 10th most common cancer and 7th most common cause of cancer death,[46] and 5-year relative survival remains low despite therapeutic advances.[23,176] Although tobacco and alcohol use are responsible for the majority of OOSCC, the risk of OOSCC associated with these behaviors is modified by genetic variation in xenobiotic metabolism, which is accomplished by a 2-phase enzyme system

that detoxifies (Phase I) xenobiotics and forms hydrophilic compounds (Phase II) to facilitate excretion.[33-35,56,83,132] For example, polymorphisms in *GSTM1*, *CYP1A1*, and *meH* modify OOSCC risk in the presence of cigarette smoking.[33-35] However, it is unclear whether polymorphisms in tobacco/alcohol metabolizing enzymes are associated with OOSCC survival. This may be of concern for patients who continue to smoke during treatment, and given the additional role of some tobacco/alcohol metabolizing enzymes in metabolism of chemotherapy used to treat OOSCC,[40] as well as dietary and environmental carcinogens such as heterocyclic amines in cooked meat,[42] and polycyclic aromatic hydrocarbons (PAH) produced by burning fossil fuels.[41] In fact, polymorphisms in Phase I/II enzymes are associated with survival in lung, colorectal, and ovarian cancer.[40] However, data on genetic variation in Phase I/II enzymes and OOSCC survival is scant. Reduced disease-free survival has been associated with the *CYP1A2*1C* polymorphism,[139] reduced overall survival was associated with non-null *GSTT1*,[143] and an elevated risk of second primary tumors was associated with non-null *GSTM1*.[142] However, at least one study showed no association between *GSTM1* or *GSTP1* and overall or disease-specific survival.[143]

To further investigate the association between polymorphisms in Phase I/II enzymes and OOSCC survival, we selected 8 genes associated with metabolism of tobacco, alcohol, or cancer chemotherapies (*NAT2*, *mEH*, *MPO*, *CYP1A1*, *CYP2E1*, *GSTP1*, *GSTT1*, and *GSTM1*) and explored polymorphisms in these genes in relation to survival in OOSCC. *NAT2* activates carcinogenic heterocyclic amines such as those found in cigarette smoke and roasted meat.[42] At least 60 *NAT2* polymorphisms are grouped into "slow" and "fast" acetylator phenotypes and have been associated with cancer risk.[177,178] An association between *NAT2* and survival was observed in colorectal[179] and gastric cancer,[180] but not in several other cancers.[181-184] We are unaware of

any reports of NAT2 and survival in OOSCC. The *mEH* gene encodes a Phase I enzyme expressed in the oral cavity and oropharynx and activates PAHs.[35] Polymorphisms in *mEH* have been associated with overall survival in breast cancer patients.[185] *MPO* is expressed in neutrophil lysosomes and activates PAHs and heterocyclic amines.[186] A common polymorphism in *MPO* (463G>A) is associated with decreased enzyme expression[186] and has also been linked to improved survival in breast cancer patients.[187] We are unaware of any studies of *mEH* or *MPO* and survival in OOSCC. *CYP2E1* is responsible for 10% of ethanol metabolism and activates benzene. Both *CYP2E1* and *CYP1A1* activate PAHs.[132,133] *CYP2E1* and *CYP1A1* were not associated with known prognostic factors, including tumor stage and nodal status, in a German oral, pharyngeal, and laryngeal cancer case series,[138] although *CYP2E1* was associated with higher tumor stage in a Brazilian case series.[139] We are unaware of any data on OOSCC survival associated with polymorphisms *CYP2E1* or *CYP1A1*. Homozygous deletion of two glutathione S-transferases (GST) --*GSTT1* and *GSTM1*--that metabolize chemotherapies used to treat OOSCC is common and results in absence of protein expression and therefore lack of enzyme function.[40,140] In addition, polymorphisms in the *GSTP1* enzyme, also a chemotherapy-metabolizing GST, result in four different forms of this protein with different levels of enzymatic activity.[40,141] To date, there are a small number of reports on these GSTs and OOSCC survival and evidence of an association is equivocal.[142,143]

To clarify the prognostic relevance of polymorphic forms of tobacco, alcohol, or chemotherapy metabolizing enzymes in OOSCC, we conducted a study of germline variation in *NAT2*, *mEH*, *MPO*, *CYP1A1*, *CYP2E1*, *GSTP1*, *GSTT1*, *GSTM1* and overall and disease-specific survival in an OOSCC case series with a history of smoking and/or drinking, and who were treated with surgery, radiotherapy, or chemoradiotherapy. All cases completed an interviewer-

administered questionnaire soliciting tobacco and alcohol use, anthropometry, diet, and oral care habits.[188] Our objective was to determine whether polymorphisms in these genes are associated with survival in OOSCC, to evaluate interaction between genotype and treatment, and to explore gene/environment interactions using our standardized questionnaire.

6.3 MATERIALS AND METHODS

6.3.1 Patients

OOSCC cases (*N*=203) were recruited at University of Pittsburgh Medical Center otolaryngology clinics during 2000-2004 for participation in a case-control study of OOSCC etiology, including polymorphisms in tobacco and alcohol metabolizing enzymes.[188] Cases were enrolled during 2000-2004, were age 18-79 at diagnosis with biopsy-verified primary lip, oral cavity (mouth or anterior tongue) or oropharyngeal (base of tongue, tonsil fossa, or soft palate) squamous cell carcinoma within 1 year of interview (excluding *in-situ* cancer), white race only, and were self-reported smokers or drinkers (smoked >= 1 cigarette per day for >= 6 months or consumed >= 1 drink/month for >= 1 year). Surgery was the standard primary therapy for oral cancer whereas radiotherapy or chemoradiotherapy was used for oropharyngeal cancer. All cases completed an interviewer-administered questionnaire that included data on tobacco/alcohol use, anthropometry, and diet.[188]

In our analysis, we included oral and oropharyngeal cases only, treated at our institution for their first-ever OOSCC, and who consented to follow-up. We excluded 44 (22%) of the original 203 cases: 6 lip cancers, 5 cases later found ineligible for the original study (3 with *in*

situ and 2 with recurrent disease), 22 cases who did not consent to follow-up, 4 cases not treated at our institution, 3 cases with undocumented tumor site, 1 case with unknown diagnosis date, and 3 cases treated at our institution for a second primary tumor or recurrence. This left 159 cases (92 oral cavity and 67 oropharyngeal) for analysis. Excluded cases were more likely to be underweight (22.7%) than included cases (2.5%) ($P < 0.001$) and more often had wild type *CYP1A1* (95.5%) than included cases (78.6%) (P=0.03).

All cases consented to the use of their genotype, questionnaire, and follow-up information. This study was approved by the University of Pittsburgh Institutional Review Board.

6.3.2 Genotype Assays

Genotyping of this case series has been described in detail previously.[188] Briefly, polymorphisms in *CYP1A1*, *CYP2E1*, *MPO*, *GSTP1,* and *mEH* were identified by polymerase chain reaction (PCR) and restriction fragment length polymorphism; homozygous deletions of *GSTT1* and *GSTM1* were identified by differential PCR; and *NAT2* phenotype was predicted using international consensus criteria after genotyping 13 SNPs using a Nanogen NanoChip Molecular Biology Workstation and algorithmic gametic phasing check.[178,188]

6.3.3 Exposure Variables

The following variables were of primary interest: CYP1A1 (wild type [*1/*1] vs. mutant), CYP2E1 (wild type [G/G, C/C] vs. mutant), mEH (slow, normal, and rapid), MPO463G>A (wild type [G/G] vs. mutant), GSTP1 (normal activity diplotype [*A/*A, *A/*B, *A/*D] vs. reduced

activity diplotype [*A/*C, *B/*B,*B/*C, *B/*D, *C/*C, *C/*D, and *D/*D] where *A, *B, *C, and *D refer to conventional Ile105Val-Ala114Val haplotypes as follows: *A=Ile-Ala (wild type), *B=Val-Ala, *C=Val-Val, and *D=Ile-Val),[141] GSTT1 and GSTM1 (homozygous null vs. any non-null), and NAT2 (fast vs. slow acetylator). We also defined: sex, tumor stage (I/II, III/IV), age at diagnosis (continuous), tumor site (oral cavity or oropharynx), cigarette smoking (ever vs. never), alcohol drinking (ever vs. never), BMI [kg/m^2] 1 year before diagnosis (underweight [<18.5], normal [18.5-24.9], overweight [25.0-29.9], and obese [>=30]), education (grade school, high school, vocational, or college), servings/day (continuous) of fruit and vegetables (separately), eating habits at interview unchanged compared with 3-5 years ago (yes/no), United States vs. non-United States birthplace, teeth brushing frequency (continuous; times/day), personal history of cancer (yes/no), and cancer in a blood relative (yes/no). For smokers, we defined: maximum number of cigarettes smoked/day (continuous), duration of smoking (continuous), pack-years (continuous; product of maximum number of cigarettes/day and duration), and years since quitting (continuous). Finally, treatment was defined as surgery only, radiotherapy (with or without surgery), or chemoradiotherapy (with or without surgery).

6.3.4 Survival Endpoints and Outcome Ascertainment

We designated 5-year survival as a clinically relevant primary endpoint. Overall survival time was calculated from the procedure date (the date of primary treatment [surgery or first radio- or chemoradiotherapy]) to the date of death from any cause. Disease-specific survival time was calculated from the procedure date to the date of death from OOSCC. Deaths were ascertained by monthly analysis of an electronic patient registry and verified using the Social Security Death Index. Cause of death was assigned using information recorded at the time of death or last

contact prior to death. Cases were censored if they were not known to have died during the study period (all analyses) or if they died of causes other than OOSCC (disease-specific survival). We considered follow-up through December 31, 2010.

6.3.5 Statistical Analysis

Descriptive statistics were calculated for clinicopathological characteristics and germline polymorphisms. Subgroup comparisons were performed using the Wilcoxon rank sum test or Fisher's exact test. Associations between clinicopathological factors and survival were assessed using the Kaplan-Meier method. We also applied the Kaplan-Meier method to screen each of 8 genes for associations with survival, selecting genes with log-rank *P*-values <= 0.10 for regression modeling. For each selected gene, Cox proportional hazards regression was used to identify the best model to predict the risk of death associated with that gene. The gene of interest, as well as tumor site, stage, and treatment, were forced into the model. Other main effects were tested 1 at a time, with the final model including all significant (alpha=0.20) main effects identified by this process. Continuous first-order interactions between the gene of interest and other predictors were tested 1 at a time. Tests for statistical significance were conducted using the likelihood ratio Chi square test. Tests for trend were conducted only among cases with the factor of interest by adding a continuous variable (symbolizing a 1-unit change) to the final model. All statistical tests based on the final model used a 2-sided alpha=0.05. The proportional hazards assumption was verified graphically and no violations were observed.

Due to overlap in substrate specificity of glutathione S-transferases (GST),[189] we examined the joint impact of *GSTP1*, *GSTT1*, and *GSTM1* on disease-specific survival using Cox proportional hazards regression. First, we explored univariable associations between these genes

and OOSCC death. Then, we summed the number of conjugation-reducing mutations per patient (i.e., *GSTM1*-null, *GSTT1*-null, and reduced activity *GSTP1*) and modeled this as a continuous predictor of OOSC death.

Analyses were performed with PROC LIFETEST and PROC PHREG in SAS 9.2 (SAS Institute, Cary, NC).

6.4 RESULTS

The 159 cases included in this study (Table 18) were predominantly male (77.4%), between the ages of 50-69 (61.0%), stage III/IV (69.0%), and represented primarily oral cancer (57.9%). All cases were either ever-smokers or ever-drinkers, with the majority of cases (76.1%) reporting a history of both. A total of 95 (60.1%) cases had a blood relative with cancer while only 16 (10.1%) reported a personal cancer history. Details of treatments administered by tumor site and stage are shown in Table 19. Among cases receiving chemotherapy, 17 (29.8%) received a single platinum agent, 5 cases (8.8%) received platinum with 5-fluorouracil, 28 (49.1%) received platinum with a taxane, and type of chemotherapy was undocumented for 7 (12.3%) cases. Median follow-up was 5.3 years (range: 0.1-10.8). A total of 79 (49.7%) cases died, including 40 deaths from OOSCC.

6.4.1 Overall Survival

Kaplan-Meier analysis of clinicopathological factors (Table 18) showed older age (P=0.02), combined smoking/drinking (P=0.05), radiotherapy (P=0.02), and higher stage (P=0.09) were

associated with reduced overall survival. Analysis of polymorphisms (Table 20) showed *NAT2* fast acetylators experienced a 19.7% higher 5-year survival rate than slow acetylators (P=0.03) and this association was similar in oropharynx and oral cancer (Figure 14, Figure 15, Figure 16). No other polymorphisms were associated with outcome. *NAT2* phenotype was unrelated to other polymorphisms or clinicopathological factors ($P > 0.10$ for all; data not shown). Improved survival associated with the *NAT2* fast acetylator phenotype was no longer significant after multiple adjustment (HR, 0.64; 95% CI, 0.40-1.04) (Table 21). However, we noted statistically significant interaction with treatment (P=0.04), in which a survival benefit was evident among cases receiving surgery alone (HR, 0.26; 95% CI, 0.10-0.66) but not radiotherapy (HR, 0.67; 95% CI, 0.31-1.59) or chemoradiotherapy (HR, 1.21; 95% CI, 0.54-2.73) after controlling for tumor site and stage. No trends were observed for duration of smoking, cigarettes/day, pack-years, or years since quitting ($P >0.05$ for all). In addition, our results were unchanged after further adjustment for level of education, BMI, daily servings of fruit or vegetables, consistency of eating habits, United States vs. non-United States birthplace, number of times per day teeth were brushed, and personal or blood relative cancer history ($P > 0.10$ for all). Finally, we did not observe any interaction between *NAT2* and age, gender, smoking status, and daily servings of fruit or vegetables ($P > 0.20$ for all).

6.4.2 Disease-Specific Survival

Late stage (P=0.04) and radiation treatment (P=0.01) were associated with worse disease-specific survival in our Kaplan-Meier analysis (Table 18). In addition, normal activity *GSTP1* was associated with a significant 19.2% reduction in 5-year disease-specific survival (P=0.04) (Table 20; Figure 17). However, *GSTP1* was not significantly associated with disease-specific

survival in a multivariable model (Table 22) (HR, 0.33; 95% CI, 0.10-1.06) and *GSTP1* did not interact with treatment (*P*=0.12) but we did observe significantly different associations between *GSTP1* and OOSCC death by sex (*P*=0.02). Reduced-activity *GSTP1* was associated with an 88% reduction in risk of OOSCC death among men (HR, 0.12; 95% CI, 0.02-0.91). However, women did not experience such a benefit (HR, 2.29; 95% CI, 0.41-12.69). Additional adjustment for education, BMI, daily servings of fruit or vegetables, consistent eating habits, United States vs. non-United States birthplace, teeth brushing frequency, and personal or blood relative cancer history did not alter these results ($P > 0.10$ for all).

We also analyzed the combined effects of GST polymorphisms on OOSCC death. There were no associations between the GST polymorphisms themselves, and each was unrelated to polymorphisms in the other genes we studied ($P > 0.20$ for all). When considering the total number of conjugation-reducing polymorphisms in each patient, we noted each additional polymorphism was associated with a 35% reduction in risk of OOSCC death (HR, 0.65; 95% CI, 0.43-0.98). Results were unchanged after controlling for gender (HR, 0.64; 95% CI, 0.43-0.97) and treatment (HR, 0.67; 95% CI, 0.45-1.0), and no interaction was observed with either factor (gender: *P*=0.78; treatment: *P*=0.70).

6.5 DISCUSSION

During 700 person-years of follow-up among 159 cases, we observed improved overall survival among *NAT2* fast acetylators treated with surgery alone, and improved disease-specific survival among men with reduced activity *GSTP1*.

NAT2 fast acetylators experienced a 36% reduction in all-cause mortality after adjustment for age, gender, smoking history, treatment, and tumor site. However, this benefit was strongest in cases treated with surgery alone, where fast acetylators experienced a 74% reduced risk of death. The interaction of *NAT2* phenotype with treatment remained significant after additional control for tumor stage, suggesting aspects of advanced disease did not produce the pattern we observed. In addition, *NAT2* phenotype was unrelated to treatment or any other clinicopathological factors. We were unable to identify previous reports of *NAT2* polymorphisms and survival in OOSCC, and *NAT2* is not strongly associated with survival in other cancers.[179-184] Our positive finding for NAT2 might be explained by an improvement in *NAT2* phenotype prediction based on 13 SNPs. In addition, we were able to adjust for clinicopathological factors associated with survival and explore gene-environment interaction using a standardized questionnaire.

The mechanism through which *NAT2* might effect survival is unclear. *NAT2* is a phase II enzyme expressed primarily in the liver and its substrates are commonly found in the environment, including heterocyclic and aromatic amines in cigarette smoke, diesel exhaust, and roasted meat.[42] Therefore, our observation of improved survival among fast acetylators treated with surgery alone may reflect an impact of *NAT2* on environmental exposures in patients unencumbered by treatments that otherwise overwhelm the benefits of fast acetylation. In addition, radiation and platinum chemotherapies are not substrates of *NAT2* and their impact on survival is not expected to be modified by *NAT2*. Provided *NAT2* modifies the effects of environmental exposures on survival, it seems reasonable that the *NAT2*-associated risk of death would become apparent only after prolonged exposure. Indeed, we observed survival curves did not separate until 2 years after cases underwent their first medical procedure. Examples of

prolonged exposures might include continued smoking and dietary patterns. While we did not detect significant modification of the *NAT2*-survival association by fruit or vegetable consumption, our questionnaire did not directly measure the major dietary source of *NAT2* substrates--roasted meat.[42] While smoking status post-diagnosis was not recorded in our study, other reports show 20%-40% of head and neck cancer patients continue to smoke after their diagnosis.[36-39]

Our Kaplan-Meier analysis also showed *GSTP1* was associated with disease-specific survival, but this association did not persist after adjustment for gender and treatment, and we observed no interaction with treatment. Instead, we were surprised to observe benefits of reduced activity *GSTP1* in men only. *GSTP1* is a Phase II enzyme expressed throughout the body and is known to detoxify platinum chemotherapies used in our case series.[189,190] Previous research shows an association between reduced activity *GSTP1* and improved response to chemotherapy in head and neck cancer,[137] as well as improved survival in lung, colorectal, and ovarian cancers.[40] We are aware of only 1 prior report of *GSTP1* and overall or disease-specific survival that included OOSCC, and this report showed no association between reduced activity *GSTP1* and disease-specific survival among 190 oral, pharyngeal, and laryngeal cancer cases.[143] However, only 19% of cases in this study received chemotherapy.[143] In our study, 35.8% of cases received chemotherapy and platinum agents were used extensively. We likely failed to observe interaction between *GSTP1* and treatment due to small subgroup sizes defined by 6 combinations of *GSTP1* activity and treatment. Finally, a more comprehensive assessment of GST activity may be a better indicator of survival than any single GST, as shown by our observation of progressively improving survival with decreasing ability to conjugate substrates to glutathione.

The association between reduced activity *GSTP1* and improved survival was restricted to men in our case series. We noted that a low proportion of women (19.4%) in our case series received chemotherapy compared with men (40.7%) (*P*=0.02). However, we cannot ignore that our estimate of this interaction was based on a small number of female deaths. Despite this, differences in survival between sexes with the same *GSTP1* polymorphism seem plausible as *GSTP1* conjugates DNA-reactive catechol estrogens to glutathione[191] and women have higher levels of lifetime endogenous estrogen exposure than men. Unfortunately, we were not able to explore exogenous estrogen exposures as these data were not available for the women in our case series.

Our results are accompanied by limitations. If genetic variants of Phase I/II enzymes not included in our study are inherited with *NAT2* or *GSTP1* polymorphisms, this may confound our results through associations with *NAT2* or *GSTP1* and survival. In addition, measurement of *NAT2* alone may not adequately classify acetylator phenotype as this enzyme shares substrates with *NAT1*.[42] Our method of vital status ascertainment may have resulted in failure to record deaths during the study period. However, this should not impact our results as cases were known to be alive when censored at last contact. In addition, we observed similar survival comparing oral and oropharyngeal cases. While oropharyngeal tumors are often associated with improved survival due to a more frequent HPV-related etiology, our results apply largely to smoking-related OOSCC as smokers were specifically selected for our study. Finally, our results are based on a small sample and we estimate our study provided only 60% power to detect the main effect of *NAT2* on overall survival that we observed (details not shown).

In summary, we observed a benefit of *NAT2* fast acetylation on overall survival in OOSCC, which we believe has not been reported previously, and improved disease-specific

survival associated with reduced activity *GSTP1*. Our results for *NAT2* may reflect interaction with lifestyle or other environmental exposures post-diagnosis and future studies of *NAT2* and survival in OOSCC should assess such factors. Our observation that women did not benefit from reduced activity *GSTP1* may reflect interaction with estrogens and future studies should collect such information in a larger sample of women. We observed an association between the cumulative number of null genotypes at *GSTP1*, *GSTT1*, and *GSTM1* and *OOSCC* survival. This observation is consistent with a commonly held notion that a reduced capacity to metabolize chemotherapy can result in better cancer survival.[40] Therefore, measurement of germline polymorphisms in GSTs may inform the decision to opt for standard therapy in OOSCC patients likely to benefit, or conversely, to identify ideal candidates for clinical trials of novel therapies.

6.6 TABLES

Table 18. Clinicopathological Characteristics and Outcome Among N=159 Oral and Oropharyngeal Cases

			Overall Survival			Disease-Specific Survival		
	N	%	Deaths	% Surviving 5 Years*	P-value†	Deaths	% Surviving 5 Years*	P-value†
Age					0.02			0.59
< 50	39	24.5	15	66.2		8	79.5	
50-59	57	35.8	24	65.7		18	68.8	
60-69	40	25.2	22	57.5		11	71.2	
>=70	23	14.5	18	35.2		3	76.4	
Gender					0.19			0.15
Female	36	22.6	15	66.6		6	82.1	
Male	123	77.4	64	57.4		34	70.3	
Race					0.19			0.15
White	159	100.0	79	59.5		40	73.1	
Site					0.78			0.54
Oral cavity	92	57.9	44	58.3		21	74.6	
Oropharynx	67	42.1	35	61.1		19	70.9	
Stage‡					0.09			0.04
Stage I/II	49	31.0	21	72.4		8	84.9	
Stage III/IV	109	69.0	58	53.4		32	67.3	
Smoking/Drinking					0.05			0.76
Ever drinker only	33	20.8	11	72.7		8	75.5	
Ever smoker only	5	3.1	2	80.0		1	80.0	
Ever drank & ever smoked	121	76.1	66	54.8		31	71.9	
BMI 1 year pre-diagnosis					0.75			0.70
<18.5	4	2.5	2	75.0		0	100.0	
18.5-24.9	67	42.1	37	50.3		18	70.4	
25-29.9	52	32.7	24	67.8		13	75.2	
>=30	36	22.6	16	63.0		9	71.8	
Blood Relative Had Cancer[e]					0.71			0.56
Yes	95	60.1	48	61.1		22	75.6	
No	63	39.9	31	56.3		18	68.9	

Table 18 continued

			Overall Survival			Disease-Specific Survival		
	N	%	Deaths	% Surviving 5 Years*	P-value†	Deaths	% Surviving 5 Years*	P-value†
Treatment					0.02			0.01
Radiotherapy	39	24.5	27	44.8		15	60.0	
Chemoradiotherapy	57	35.8	27	59.6		16	69.3	
Surgery Only	63	39.6	25	68.6		9	84.9	
Personal History of Cancer‖					0.22			0.40
Yes	16	10.1	10	43.8		5	64.3	
No	143	89.9	69	61.3		35	74.0	

*Kaplan-Meier survival estimate

†Log-rank test

‡Stage is missing for 1 case

$32 cases (82.1%) who received radiotherapy also received surgery and 21 cases (36.8%) who received chemoradiotherapy also received surgery. A total of 39 (61.9%) of cases treated with surgery only, 8 (20.5%) of cases treated with radiotherapy, and 2 (3.6%) of cases treated with chemoradiotherapy were diagnosed as Stage I/II.

‖Personal and blood relative cancer history is missing for 1 case

Table 19. Treatment of Oral and Oropharyngeal Cancer Cases (N=159)

		Oral cavity	Oropharynx	Stage 1/2	Stage 3/4	Unknown Stage
Radiotherapy (w/ or w/out surgery) N=39	**n (%)**	20 (51.3)	19 (48.7)	8 (20.51)	31 (79.49)	
Chemoradiotherapy (w/ or w/out surgery) N=57	**n (%)**	19 (33.3)	38 (66. 7)	2 (3.51)	54 (94.74)	1 (1.75)
Surgery Only N=63	**n (%)**	53 (84.1)	10 (15.9)	39 (61.90)	24 (38.10)	

Table 20. Genotype and Outcome Among N=159 Oral and Oropharyngeal Cancer Cases

			Overall Survival			Disease-Specific Survival		
	N*	%	Deaths	% Surviving 5 Years†	P-value‡	Deaths	% Surviving 5 Years†	P-value‡
NAT2					0.03			0.06
Fast	79	53.7	34	68.1		14	80.6	
Slow	68	46.3	39	48.4		22	64.1	
GSTP1					0.14			0.04
Reduced Activity	28	17.7	11	75.0		3	88.7	
Normal Activity	130	82.3	67	56.5		37	69.5	
CYP1A1					0.74			0.67
*1/*1	125	82.2	62	62.5		31	74.2	
non-1/1	27	17.8	14	46.1		8	64.7	
CYP2E1					0.16			0.20
G/G C/C	140	92.1	71	59.2		36	72.8	
non-G/G C/C	12	7.9	3	83.3		1	90.9	
MEH					0.64			0.69
Slow/Very Slow	61	38.6	33	57.1		18	69.3	
Normal	71	44.9	33	60.3		16	76.3	
Rapid	26	16.5	12	65.4		6	74.2	
MPO463G>A					0.18			0.57
Variants	60	37.7	33	58.8		16	72.9	
Wild Type	99	62.3	46	59.9		24	73.2	
GSTT1					0.30			0.37
Non-null	101	64.3	52	56.9		28	70.2	
Null	56	35.7	25	66.5		12	77.9	
GSTM1					0.92			0.40
Non-null	57	36.1	26	64.1		17	69.8	
Null	101	63.9	52	57.4		23	74.9	

*Genotype is missing for some cases due to insufficient blood volume or assay failure
†Kaplan-Meier survival estimate
‡Log-rank test

Table 21. Results of Cox Proportional Hazards Regression: Predicted NAT2 Phenotype and Risk of Death from Any Cause

	N	Deaths	Person-Years	HR (95% CI)*
NAT2 Phenotype†				
Slow	68	39	285.6	1.00
Fast	78	34	407.3	0.64 (0.40-1.04)
Radiation				
Slow	21	14	76.0	1.00
Fast	16	11	70.4	0.67 (0.31-1.59)
Chemoradiotherapy				
Slow	21	10	102.5	1.00
Fast	32	16	159.0	1.21 (0.54-2.73)
Surgery Only				
Slow	26	15	107.1	1.00
Fast	30	7	177.9	0.26 (0.10-0.66)

HR, hazard ratio from Cox proportional hazards regression model, CI=confidence interval.
NAT2 phenotype is inferred from genotype as described under *Materials and Methods*.
*Adjusted for gender, continuous age, smoking status (ever vs. never), treatment (radiation, chemoradiotherapy, or surgery only), tumor site (oropharynx vs. oral cavity), and tumor stage (stage III/IV vs. stage I/II).
†The interaction between NAT2 and treatment is significant (*P*=0.04)

Table 22. Results of Cox Proportional Hazards Regression: Predicted GSTP1 Activity and Risk of Head and Neck Cancer Death

Genotype	N	Deaths	Person-Years	HR (95% CI)*
GSTP1†				
Normal Activity‡	130	37	591.4	1.00
Reduced Activity‡	28	3	156.8	0.33 (0.10-1.06)
Men‡				
Normal Activity	103	33	439.0	1.00
Reduced Activity	19	1	121.5	0.12 (0.02-0.91)
Women				
Normal Activity	27	4	152.4	1.00
Reduced Activity	9	2	35.3	2.29 (0.41-12.69)

HR, hazard ratio from Cox proportional hazards regression model, CI=confidence interval.
*Adjusted for gender, treatment (radiotherapy, chemoradiotherapy, or surgery only), tumor site (oropharynx vs. oral cavity), and tumor stage (stage III/IV vs. stage I/II).
†The interaction between GSTP1 and gender is significant (P=0.02).
‡Normal activity GSTP1 genotypes are: A/A, A/B, A/D. Reduced activity genotypes are: B/B, B/C, C/C, C/D, D/D, A/C, B/D.

6.7 FIGURES

Figure 14. Kaplan-Meier Plot of *NAT2* and Overall Survival in All Cases

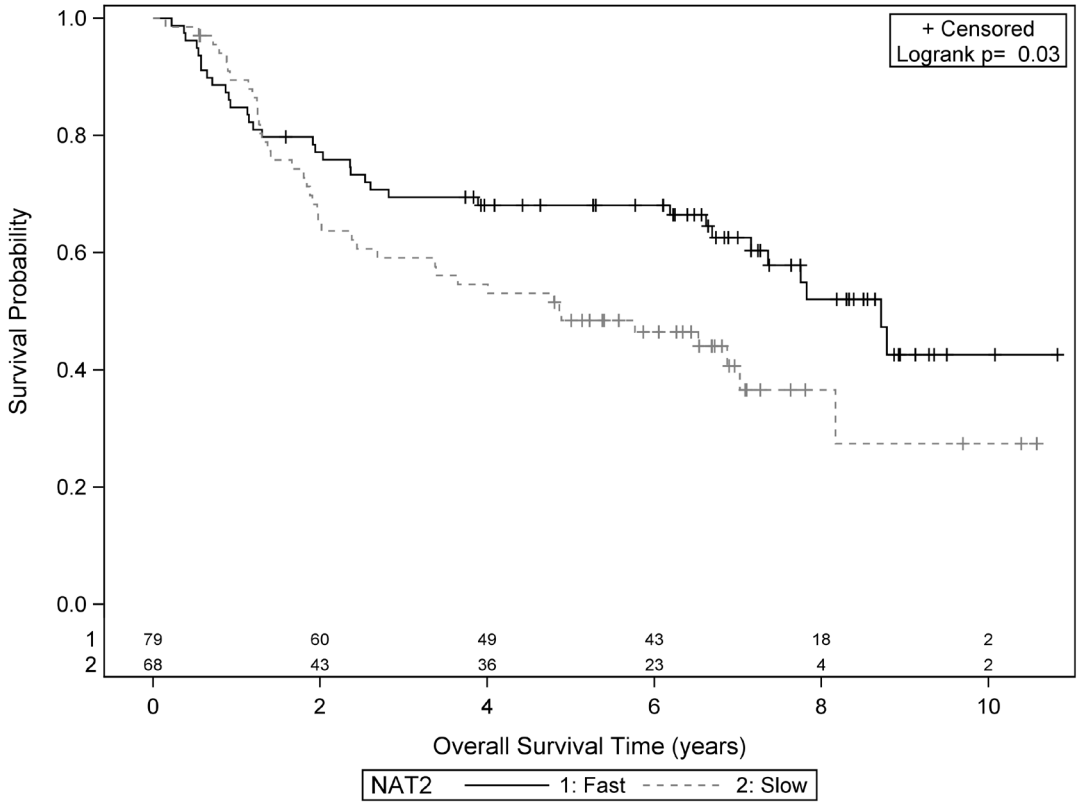


Figure 15. Kaplan-Meier Plot of NAT2 and Overall Survival in Oral Cavity Cases

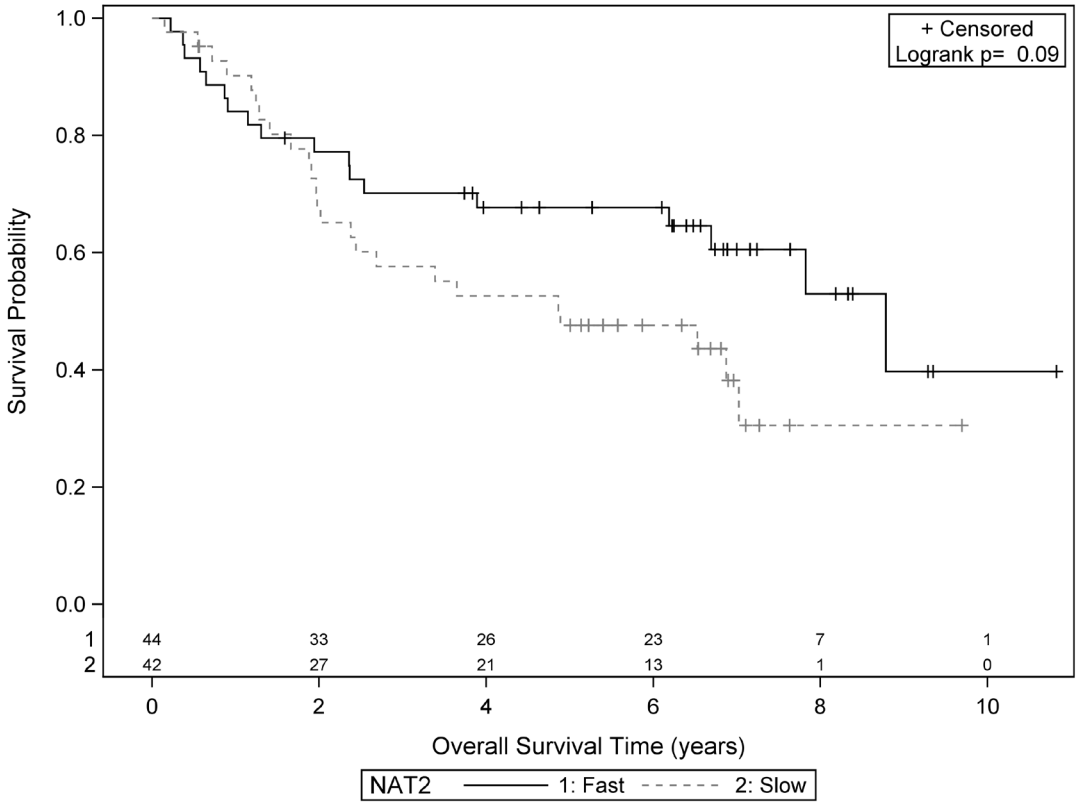


Figure 16. Kaplan-Meier Plot of *NAT2* and Overall Survival in Oropharyngeal Cases

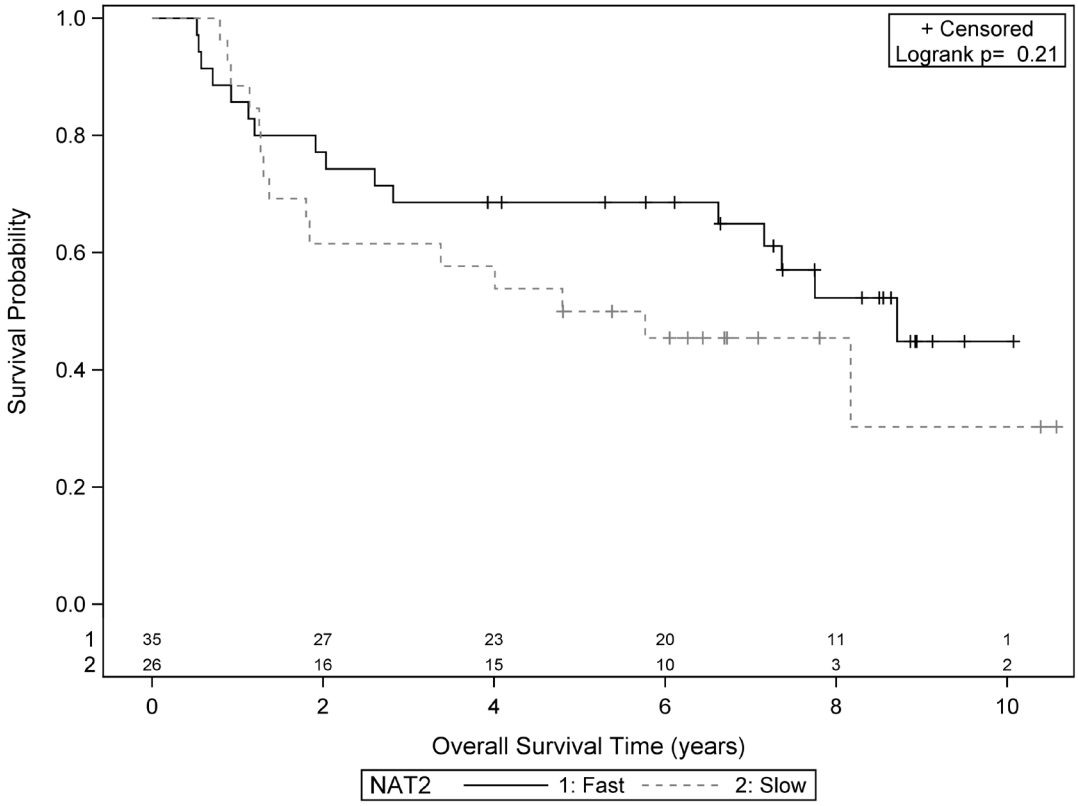
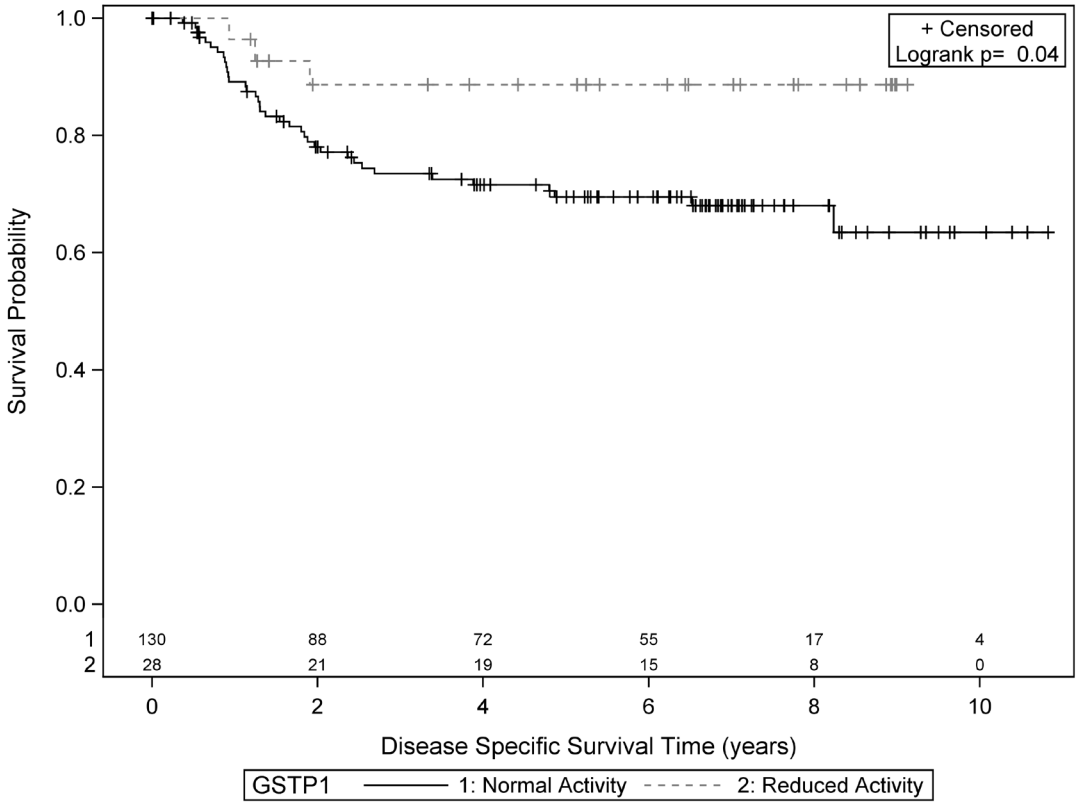


Figure 17. Kaplan-Meier Plot of *GSTP1* and Disease-Specific Survival



7.0 CONCLUSIONS AND PUBLIC HEALTH SIGNIFICANCE

HNSCC are the sixth most common cancers in the world and are the eighth leading cause of cancer death worldwide, representing approximately 600,000 new cases and 300,000 deaths annually.[23] Molecular differences in HNSCC,[9] the emergence of a new virus-associated subtype of HNSCC,[7] and wide variation in survival across patient subgroups[32] emphasize the importance of considering disease heterogeneity in the quest to reduce HNSCC morbidity and mortality. Therefore, it was the objective of this research to apply epidemiology, in the translational context, to make basic discoveries related to the heterogeneity of HNSCC that would reveal candidate applications for HNSCC therapy and prevention.[147] Specifically, this research investigated heterogeneity in HNSCC with respect to tumor angiogenesis, risk of HNSCC associated with CPSE, and HNSCC survival associated with germline variation in metabolic enzymes.

Angiogenesis is the process by which new, tumor-infiltrating blood vessels develop from existing vasculature.[8] Angiogenesis is required for tumor growth and also contributes to metastasis, which is the primary cause of cancer death.[8,116] However, it remains unclear whether angiogenesis is a universally consistent process across all HNSCC.[9-13,124] This research represents one of the first attempts to address this concern through a comprehensive investigation of differences in expression of angiogenesis markers in HPV-positive and HPV-negative HNSCC. Specifically, this research demonstrated that: 1) there are biological differences in

angiogenesis comparing HPV-positive and HPV-negative HNSCC, with HPV-positive tumors exhibiting lower potential for angiogenesis as indicated by reduced expression of EGFR, a lack of association between EGFR and VEGF, and a tendency for smaller size compared with HPV-negative tumors; 2) the NOTCH pathway is associated with angiogenesis in HPV-negative HNSCC; and 3) the NOTCH pathway may be associated with tumor development through other means, possibly unrelated to angiogenesis, in HPV-positive HNSCC. These results represent an initial investigation into differences in expression of angiogenesis markers in HPV-positive and HPV-negative HNSCC and should be replicated in larger samples. Nonetheless, these results provide impetus for studying the mechanisms of angiogenesis *in vitro* using HPV-positive and HPV-negative cell lines. Furthermore, clinical studies of anti-angiogenesis therapies should assess response to therapy separately in HPV-positive and HPV-negative cases.

This research also sought to characterize the relationship between CPSE and risk of HNSCC, and explore heterogeneity in this association according to tumor site and HPV status. A case-control study design was used to achieve this goal, and results showed that CPSE is associated with an increased risk of HNSCC, even after control for adult smoking. In addition, this research identified CPSE as a significant contributor to HNSCC risk in the population, with as much as 17% of HNSCC being attributable to CPSE (assuming causality). Furthermore, this study demonstrated a strong and specific association between CPSE and cancer of the oropharynx in never-smokers. These results add to the literature demonstrating the carcinogenic effects of cigarette smoke in never-smokers and provides further impetus for aggressive public policy efforts to reduce environmental tobacco smoke pollution.[26] In addition, this research further underscores the particular vulnerability of children to environmental tobacco smoke exposures, and demonstrates that these exposures may have effects that last into adulthood.[26]

Finally, this research demonstrated an intriguing possibility in HNSCC etiology. Specifically, this research suggests CPSE may interact with adult HPV infection to promote HNSCC in never-smokers. Given the small sample size that this observation is based on, results of this research are inconclusive in this regard but warrant further exploration in larger case series in which both CPSE and tumor HPV status are available. Pooled studies are likely to be necessary to achieve this.

Finally, this research sought to identify genetic determinants of survival in HNSCC by exploring germline variation in enzymes that metabolize toxicants associated with HNSCC pathogenesis,[33-35] drugs used to treat HNSCC,[40] and toxic substances found in the environment that are detrimental to health.[42] This research provides the only data available on *NAT2* phenotype and survival in HNSCC. Specifically, this research demonstrated substantially improved overall survival in cases with the *NAT2* fast acetylator phenotype compared with the *NAT2* slow acetylator phenotype that persisted after multiple adjustment. Furthermore, this research showed the greatest benefit of the *NAT2* fast acetylation on survival among cases treated with surgery only, even after adjustment for tumor stage. These results may reflect interaction between *NAT2* and post-diagnosis lifestyle in HNSCC cases unencumbered by caustic treatments such as chemotherapy and radiation. Future studies of *NAT2* and survival in HNSCC should incorporate measures of tobacco/alcohol use and dietary patterns post-diagnosis. Given that 20-40% of HNSCC patients continue to smoke after their cancer diagnosis,[36-39] knowledge of metabolic phenotype and lifestyle choices during treatment may inform interventions aimed at improving outcomes for HNSCC patients. This research also showed that reduced activity *GSTP1* was associated with improved disease-specific survival relative to normal activity *GSTP1* in men only. Because *GSTP1* is involved in conjugation of DNA-reactive estrogen metabolites to

glutathione,[191] and women have higher levels of estrogen exposure than men, a reduction in *GSTP1* activity may produce a greater burden of toxins in women--both treatment-related and estrogen-related--that could overwhelm survival. Future studies of *GSTP1* and HNSCC survival should assess markers of endogenous and exogenous estrogen exposure (e.g., parity, age at menarche, use of hormone replacement therapy, etc.) in a larger sample of women. Such research may have implications in considering HNSCC therapy in the context of concomitant medication use.

The etiology of HNSCC is based in lifestyle factors[56,83] and infectious disease.[7] As a result, public health action--e.g., behavioral intervention, legislation, taxation, or vaccination--has the potential to ameliorate some burden of the disease. At the same time, new therapies are needed for patients suffering from HNSCC,[23] and these therapies should address HNSCC subtypes, especially HPV-positive and HPV-negative HNSCC, and should consider the likelihood of individual patients to respond to therapies. This research applies epidemiology to address each of these concerns and contributes significantly to public health by: 1) providing insight into biological differences between subgroups of HNSCC that implicate disease subtypes amenable to specific therapeutic approaches; 2) providing scientific evidence supporting changes in public policy to protect children's' health through limiting environmental tobacco smoke exposure, and thereby reducing the incidence of HNSCC in the population; and 3) identifying heritable characteristics that might modify survival after HNSCC diagnosis and therefore enable selection of therapy most appropriate for individual patients.

APPENDIX A

DERIVATION OF SAMPLE FOR ARTICLE 1

The following describes the process used to identify our analytic sample of N=71 cases for whom tumor blocks were retrieved and submitted for study using IHC. As shown in Figure 18, N=1,170 head and neck cancer cases were enrolled between 2000-2010.[‡] Starting in 2007 head and neck tumors treated at our institution were commonly assayed for HPV using ISH and this procedure became standard practice in 2009. Because we were interested in comparing characteristics of HPV-positive and HPV-negative tumors, we began by restricting our sampling frame to the N=616 cases diagnosed during 2007 and later. Among the N=616 cases diagnosed during 2007 and later, N=400 were listed in the organ-specific database (OSD).[§] At this point, identification of eligible cases for our analysis branched into two separate efforts. The first effort focused on identifying eligible cases among the N=400 in OSD. The second effort focused on identifying eligible cases among the remaining N=216 cases not found in OSD.[**]

[‡] This is based on a snapshot of the study database from May 24, 2011

[§] The organ specific database (OSD) includes detailed pathological and clinical information on head and neck cancer cases who sought treatment or advice from our institution. The greatest level of detail is available on cases whose diagnosis and treatment was initiated with us, and it is these cases for whom archival tumor specimens are most likely to be available for research.

[**] Although the OSD now attempts to capture information on all cases who visit our institution, requirements for entry of a case into the OSD have evolved over time. Therefore, some cases who were diagnosed in the distant past

Beginning with the N=400 cases in OSD (Figure 18), N=331 self-reported no prior history of cancer. Detailed information on the cancer diagnosis was available for N=317 of these cases, but only N=316 were found to have SCC of the head and neck. These cases represented N=305 single primary cancers and N=11 cases with multiple primary cancers. Among the cases with multiple primaries, N=4 had tumors in the same anatomical site and could confidently be classified as oral or oropharyngeal cases. However, HPV status was unavailable for all of these cases and they were therefore excluded. From the remaining N=305 cases with a single primary tumor, N=205 represented oral and oropharyngeal cancer. However, only N=64 of these had known tumor HPV status (37 HPV-positive [2 oral, 35 oropharyngeal] and 27 HPV-negative [15 oral, 12 oropharyngeal]) and were included in our request for tumor blocks.

The N=216 cases not in OSD (Figure 19) represented primarily recently diagnosed cases (58.0%, 19.9%, 13.4%, and 8.8% diagnosed in 2010, 2009, 2008, and 2007 respectively). Tumor site was retrieved from study records for these cases by study staff. We determined HPV status for these cases through a manual review of pathology reports in the electronic medical record system. A total of N=177 self-reported no prior history of cancer and N=113 of these represented oral and oropharyngeal cancer. Among these, N=39 represented cases with a single primary tumor, or the index tumor from cases with multiple primaries, for which tumor HPV status was recorded in the pathology report. Therefore, through manual review we were able to identify N=39 extra cases (13 HPV-positive [1 oral, 17 oropharyngeal]) and 26 HPV- [21 oral, 5 oropharyngeal]) for whom we would request tumor blocks.

As shown in Figure 20, we requested tumor blocks for N=103 cases (50 HPV-positive [3 oral, 47 oropharyngeal] and 53 HPV-negative [36 oral, 17 oropharyngeal]). This request was

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may not appear in the database. In addition, there is a natural lag in data entry due to quality control procedures and therefore recently diagnosed cases may not be found in the database when a query is issued.

based on N=322 cases, all of whom would have been eligible for our study if HPV status were available: N=209 identified in OSD (205 with single primaries and 4 with multiple primaries; Figure 18), plus N=113 identified through our manual review of cases not in OSD (Figure 19). Therefore, we excluded (322-103=219)/322=68.0% of otherwise eligible cases due to missing HPV status. Based on past experience of other investigators, we expected to retrieve 70% of the tumor blocks requested, or N=72. Ultimately, tumor blocks were retrieved for N=50 cases from the Iron Mountain storage facility, and blocks were retrieved for N=21 cases from other projects. Therefore, we obtained tumor blocks for N=71 cases, nearly exactly the number we expected to identify. After obtaining tumor blocks, we were notified by another investigator (Brenda Diergaarde, PhD) that the tumor HPV status for one oropharyngeal case had been reviewed by the pathology department at her request and the original classification of HPV-negative had been changed to HPV-positive. Therefore, we also treated this case as HPV-positive.

Our final analytic sample, shown in Figure 20, included N=71 cases of OOSCC, representing N=30 HPV-positive (3 oral, 27 oropharyngeal) and N=41 HPV-negative (27 oral, 14 oropharyngeal) cases.

A.1 REPRESENTATIVENESS OF RECOVERED TUMOR BLOCKS

To determine the degree to which our analytic sample reflected the universe of cases eligible for our study, we compared selected characteristics of cases in our sample (N=71) with those who were eligible but not enrolled (N=251 [32 cases for whom tumor blocks were not recovered plus 219 cases excluded for missing HPV status]) (Table 23). Our analytic sample included cases with more recent diagnoses than excluded cases ($P < 0.001$), consisted of more oropharyngeal

tumors (57.7%) than excluded cases (37.5%) ($P < 0.01$), and more often represented node-positive disease than excluded cases, whether assessed clinically (71.8% vs. 45.7%; $P < 0.001$) or pathologically (38.6% vs. 30.3%; P=0.02). No differences were observed with respect to clinical and pathological T- or M-stage, age, sex, race, ever-smoking, childhood passive smoke exposure, alcohol drinking, or BMI one year prior to diagnosis ($P > 0.05$ for all).

A.2 FACTORS ASSOCIATED WITH TUMOR BLOCK RECOVERY

Table 24 shows comparisons between cases with recovered and unrecovered tumor blocks. Due to small sample sizes involved in these comparisons, we considered either a >10% difference in frequency or P-value < 0.05 indicative of a potentially important difference between recovered and unrecovered tumor blocks. Recovered tumor blocks (71.8%) were more likely to represent cases diagnosed during 2009 and 2010 than unrecovered blocks (53.2%) (P=0.15). Recovered tumor blocks were less likely to represent oropharyngeal tumors (57.7%) than unrecovered tumor blocks (71.9%) (P=0.19). Tumor block recovery was also significantly associated with HPV status (P=0.03), with only 42.3% of recovered tumors HPV-positive whereas 65.6% of unrecovered tumors were HPV-positive. Recovered tumors were less likely to have indeterminate pathological T-stage (X) (31.4%) compared with unrecovered tumors (45.2%) than recovered tumors (P=0.45). Inability to assess pathological N-stage was also less common among recovered (35.7%) than unrecovered tumors (48.4%) (P=0.41). In addition, recovered tumors more often represented persons who were not classified as obese within one year of diagnosis (66.2%) than unrecovered tumors (53.1%) (P=0.27) and the frequency of childhood passive smoke exposure was higher among recovered tumors (80.3%) than unrecovered tumors

(62.5%) (P=0.08). We did not observe any differences between recovered and unrecovered tumor blocks with respect to clinical or pathological assessment of distant metastases (M-stage), age, sex, race, cigarette smoking, and alcohol drinking (difference in frequency <10% and P > 0.05 for all).

A.3 TABLES

Table 23. Characteristics of Included and Excluded Cases

	Included (N=71)	Excluded* (N=251)	
	n (%)†	n (%)†	P-Value‡
Age			0.42
<50	20 (28.2)	58 (23.1)	
50-59	29 (40.8)	89 (35.5)	
60-69	17 (23.9)	73 (29.1)	
>=70	5 (7.0)	31 (12.4)	
Sex			0.25
Male	53 (74.6)	167 (66.5)	
Female	18 (25.4)	84 (33.5)	
Race			0.11
Non-White/Unknown	4 (5.6)	5 (2.0)	
White	67 (94.4)	246 (98.0)	
Year of Diagnosis			< .001
2007	5 (7.0)	67 (26.7)	
2008	15 (21.1)	85 (33.9)	
2009	29 (40.8)	54 (21.5)	
2010	22 (31.0)	45 (17.9)	
Tumor Site			< .01
Oral Cavity	30 (42.3)	157 (62.5)	
Oropharynx	41 (57.7)	94 (37.5)	
T clinical			> 0.99
1/2	44 (62.0)	138 (61.9)	
3/4	25 (35.2)	77 (34.5)	
X	2 (2.8)	7 (3.1)	
in situ#	0 (0.0)	1 (0.4)	
N clinical			< .001
Negative	20 (28.2)	119 (53.4)	
Positive	51 (71.8)	102 (45.7)	
X	0 (0.0)	2 (0.9)	

Table 23 continued

	Included (N=71)	Excluded* (N=251)	
	n (%)†	n (%)†	P-Value‡
M clinical			0.77
0	69 (97.2)	198 (98.0)	
1	1 (1.4)	1 (0.5)	
X	1 (1.4)	3 (1.5)	
T path			0.58
1/2	34 (48.6)	118 (53.4)	
3/4	14 (20.0)	50 (22.6)	
X	22 (31.4)	52 (23.5)	
in situ	0 (0.0)	1 (0.5)	
N path			0.02
Negative	18 (25.7)	98 (44.3)	
Positive	27 (38.6)	67 (30.3)	
X	25 (35.7)	56 (25.3)	
M path			0.92
0	35 (50.0)	101 (50.8)	
1	0 (0.0)	1 (0.5)	
X	35 (50.0)	97 (48.7)	
Ever Smoked			> 0.99
No	18 (25.4)	66 (26.3)	
Yes	53 (74.6)	185 (73.7)	
Childhood Passive Smoke			0.22
No	14 (19.7)	69 (27.5)	
Yes	57 (80.3)	182 (72.5)	
Ever Drank Alcohol			0.52
No	13 (18.3)	57 (22.9)	
Yes	58 (81.7)	192 (77.1)	
BMI 1 year pre-diagnosis			0.89
<30 kg/m^2	47 (66.2)	168 (67.2)	
>=30 kg/m^2	24 (33.8)	82 (32.8)	

Table 23 continued

*Excluded cases are those who were otherwise eligible but for whom tumor blocks were not requested due to missing human papilloma virus (HPV) status (N=219), and those for whom tumor blocks were requested but not returned (N=32).
†Number may not sum to total due to missing values for some variables.
‡Fisher's exact test
#SID 62803-9 was diagnosed with clinical stage T2 and pathological T-stage *in situ*. SID 63061-6 was diagnosed as clinical T-stage *in situ* and pathological T1. Tumor blocks were not requested for either case due to missing HPV status.

Table 24. Factors Associated With Tumor Block Recovery Among N=103 Requested Tumor Blocks

	Recovered (N=71)	Unrecovered (N=32)	
	n (%)	n (%)	P-Value*
Age			0.93
<50	20 (28.2)	7 (21.9)	
50-59	29 (40.8)	14 (43.8)	
60-69	17 (23.9)	9 (28.1)	
>=70	5 (7.0)	2 (6.3)	
Sex			0.81
Male	53 (74.6)	25 (78.1)	
Female	18 (25.4)	7 (21.9)	
Race			0.31
Non-White/Unknown	4 (5.6)	0 (0.0)	
White	67 (94.4)	32 (100.0)	
Year of Diagnosis			0.15
2007	5 (7.0)	2 (6.3)	
2008	15 (21.1)	13 (40.6)	
2009	29 (40.8)	7 (21.9)	
2010	22 (31.0)	10 (31.3)	
Tumor Site			0.19
Oral Cavity	30 (42.3)	9 (28.1)	
Oropharynx	41 (57.7)	23 (71.9)	
HPV Status			0.03
Negative	41 (57.7)	11 (34.4)	
Positive	30 (42.3)	21 (65.6)	
T clinical			0.73
1/2	44 (62.0)	19 (61.3)	
3/4	25 (35.2)	10 (32.3)	
X	2 (2.8)	2 (6.5)	
N clinical			0.35
Negative	20 (28.2)	12 (38.7)	
Positive	51 (71.8)	19 (61.3)	
M clinical			> 0.99
0	69 (97.2)	30 (100.0)	
1	1 (1.4)	0 (0.0)	
X	1 (1.4)	0 (0.0)	

Table 24 continued

	Recovered (N=71)	Unrecovered (N=32)	
	n (%)	n (%)	P-Value*
T path			0.45
1/2	34 (48.6)	13 (41.9)	
3/4	14 (20.0)	4 (12.9)	
X	22 (31.4)	14 (45.2)	
N path			0.41
Negative	18 (25.7)	8 (25.8)	
Positive	27 (38.6)	8 (25.8)	
X	25 (35.7)	15 (48.4)	
M path			0.66
0	35 (50.0)	13 (43.3)	
X	35 (50.0)	17 (56.7)	
Ever Smoked			0.35
No	18 (25.4)	11 (34.4)	
Yes	53 (74.6)	21 (65.6)	
Childhood Passive Smoke			0.08
No	14 (19.7)	12 (37.5)	
Yes	57 (80.3)	20 (62.5)	
Ever Drank Alcohol			0.43
No	13 (18.3)	8 (25.8)	
Yes	58 (81.7)	23 (74.2)	
BMI 1 year pre-diagnosis			0.27
<30 kg/m^2	47 (66.2)	17 (53.1)	
>=30 kg/m^2	24 (33.8)	15 (46.9)	
*Fisher's exact test			

A.4 FIGURES

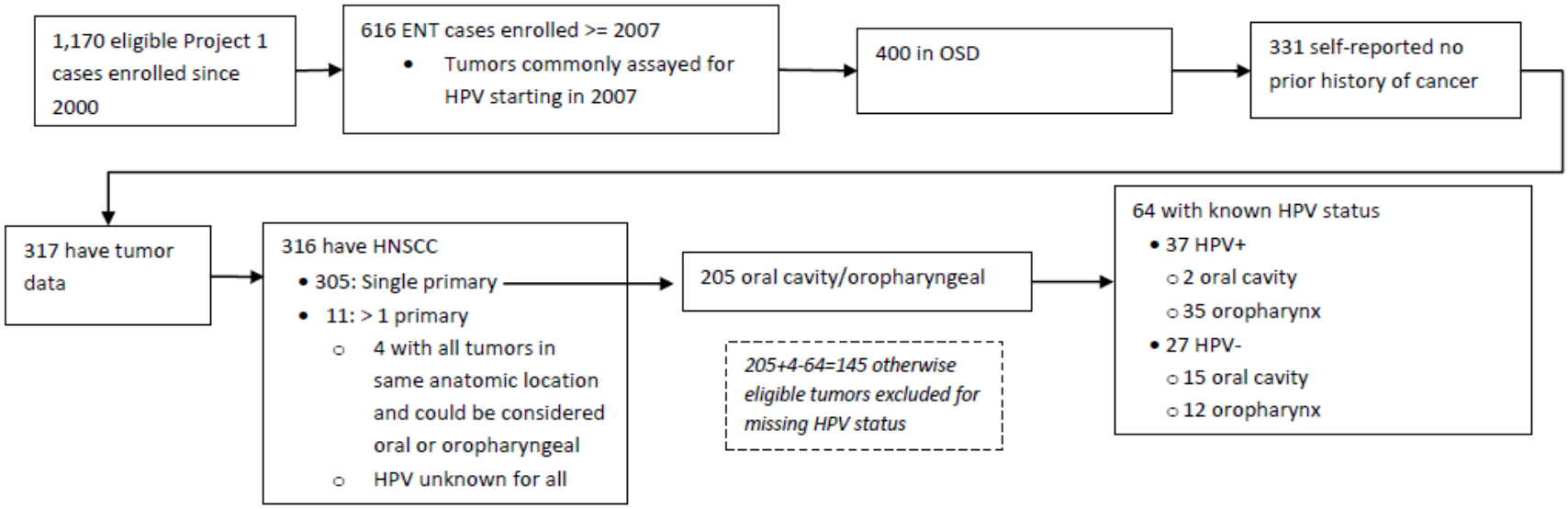

Figure 18. Derivation of Study Sample for Specific Aim 1: First Step, Based on Database Snapshot from 24-MAY-2011

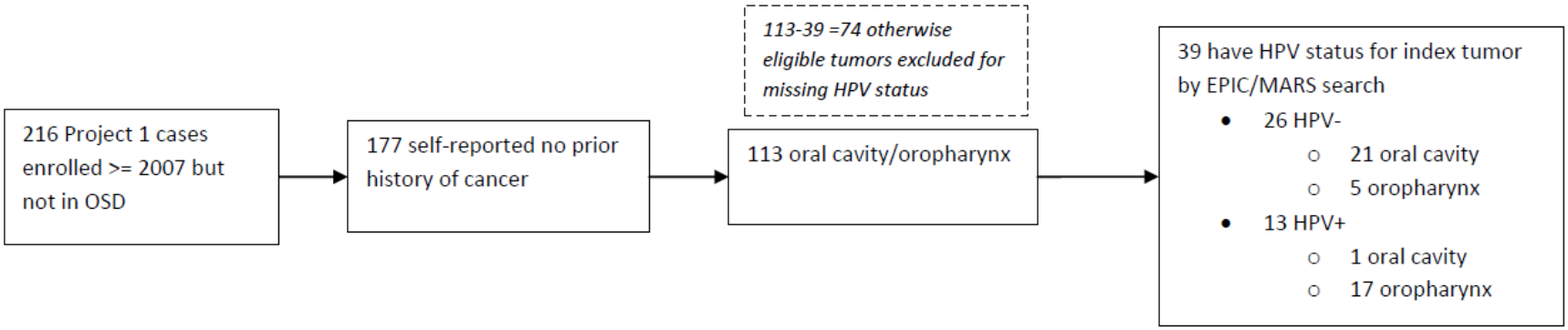

Figure 19. Derivation of Study Sample for Specific Aim 1: Second Step, Manual Search for More Cases

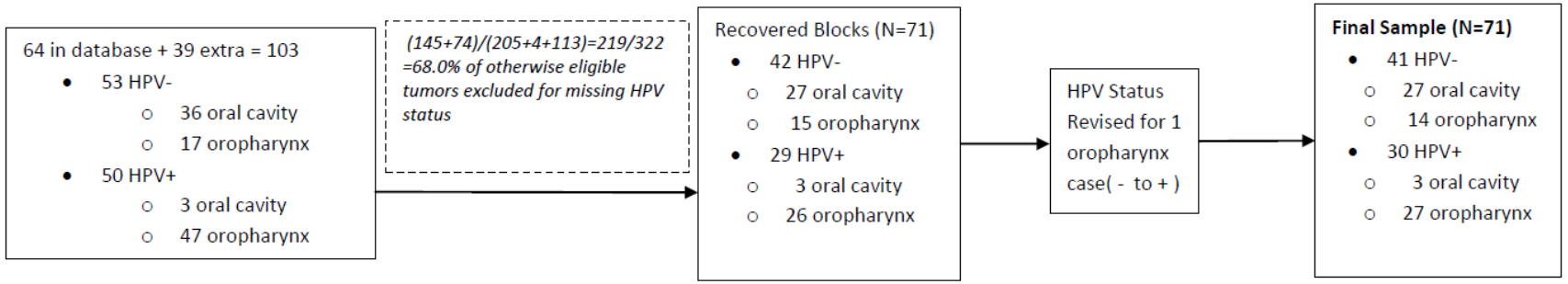

Figure 20. Derivation of Study Sample for Specific Aim 1: Third Step, Assembly of Final Sample

APPENDIX B

MISSING DATA FOR IMMUNOHISTOCHEMISTRY IN ARTICLE 1

Five of the N=71 cases for whom tumor blocks were recovered had insufficient amounts of tumor available for staining of one or more markers (Supplementary Table 3). Four cases did not have enough tumor for staining of any of the markers and one case could not be stained for NOTCH1. Four of the five cases (80%) missing one or more markers were HPV-positive, whereas 26/66 (39.4%) cases in whom all markers were assessed were HPV-positive. Four of the five cases missing data (80%) were diagnosed in 2009, and the most common surgical procedure among cases missing data was resection/excision (also 4/5, or 80%). Missing one or more markers did not appear to be associated with the day of week or month of the surgical procedure, or tumor site.

Table 25. Tabulation of Missing Immunohistochemistry Data (N=5 cases)

Case	EGFR	VEGF	NOTCH1	HPV Status	Tumor Site	Procedure Type	Procedure Day of Week	Procedure Month	Procedure Year
1				Positive	Oropharynx	Biopsy	Friday	October	2009
2				Positive	Oral cavity	Resection/excision	Wednesday	January	2010
3				Positive	Oropharynx	Resection/excision	Wednesday	September	2009
4	X	X		Positive	Oropharynx	Resection/excision	Saturday	May	2009
5				Negative	Oral cavity	Resection/excision	Wednesday	December	2009

EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor; NOTCH1=notch receptor 1.
*Cells marked with an X indicate non-missing data. Blank cells indicate missing data.
†Fisher's exact test.

APPENDIX C

PROTEIN EXPRESSION AND TUMOR HPV STATUS IN RELATION TO SPECIMEN HANDLING IN ARTICLE 1

Specimens used in our study were collected as part of routine clinical practice and were not processed according to a protocol. Therefore, we were concerned that variation in specimen handling, e.g., elapsed time to fixation or time in fixative, might have biased our results.[192] To address this concern, we examined the association between surrogate markers of variation in specimen handling and results of our IHC experiments, as well as tumor HPV status. Overall, our results are strongly suggestive of consistent specimen handling. Specifically, we observed no evidence of systematic differences in protein expression according to the month and day of week that the surgical procedures were conducted (Table 26), and tumor HPV status was unrelated to these factors as well (Table 27). While we did observe an association between HPV status and year of diagnosis in our sample (Table 27), we believe this reflects evolving practices at our institution. In particular, HPV testing became common in 2007-2008 and specimens were likely selected for testing that were felt to have a strong possibility of being HPV-positive based on histological assessment. Subsequently, in 2009-2010, a policy was instituted requiring all tumors to be tested for HPV pending availability of tissue.

Table 26. Surrogate Markers of Variation in Specimen Handling and Expression of Markers Detected Using Immunohistochemistry Among N=67 Cases

	EGFR	VEGF	NOTCH1
	N, median (min-max)	N, median (min-max)	N, median (min-max)
Year of Diagnosis			
2007	5, 20.0 (0-180)	5, 80.0 (10-90)	5, 60.0 (0-120)
2008	15, 15.0 (0-300)	15, 90.0 (0-200)	15, 45.0 (0-195)
2009	26, 30.0 (0-300)	26, 65.0 (0-140)	25, 40.0 (0-240)
2010	21, 20.0 (0-180)	21, 60.0 (0-160)	21, 40.0 (0-160)
P-value	0.63	0.80	0.94
Procedure Type			
Biopsy	21, 20.0 (0-300)	21, 80.0 (10-200)	21, 15.0 (0-240)
Resection/excision	46, 25.0 (0-200)	46, 60.0 (0-150)	45, 50.0 (0-195)
P-value	0.58	0.07	0.09
Procedure Year			
2007	3, 20.0 (0-40)	3, 80.0 (10-90)	3, 80.0 (60-120)
2008	17, 15.0 (0-300)	17, 80.0 (0-200)	17, 40.0 (0-195)
2009	23, 30.0 (0-300)	23, 70.0 (5-140)	22, 40.0 (0-240)
2010	23, 20.0 (0-180)	23, 40.0 (0-160)	23, 40.0 (0-160)
2011	1, 20.0 (20-20)	1, 60.0 (60-60)	1, 5.0 (5-5)
P-value	0.67	0.76	0.43
Procedure Month			
January	5, 20.0 (0-300)	5, 60.0 (40-200)	5, 20.0 (0-100)
February	6, 12.5 (0-70)	6, 80.0 (0-180)	6, 10.0 (0-240)
March	6, 30.0 (0-120)	6, 45.0 (10-100)	6, 50.0 (0-160)
April	5, 15.0 (0-40)	5, 90.0 (8-100)	5, 100 (40-150)
May	3, 70.0 (10-200)	3, 90.0 (60-100)	2, 103 (45-160)
June	2, 7.5 (5-10)	2, 10.0 (0-20)	2, 60.0 (0-120)
July	6, 45.0 (5-100)	6, 80.0 (0-160)	6, 50.0 (0-180)
August	8, 30.0 (0-180)	8, 55.0 (10-100)	8, 60.0 (10-120)
September	6, 6.3 (0-300)	6, 82.5 (10-150)	6, 17.5 (5-195)
October	12, 27.5 (5-80)	12, 37.5 (10-120)	12, 20.0 (0-60)
December	8, 45.0 (0-100)	8, 95.0 (5-140)	8, 55.0 (10-160)
P-value	0.65	0.76	0.19

Table 26 continued

	EGFR	VEGF	NOTCH1
	N, median (min-max)	**N, median (min-max)**	**N, median (min-max)**
Procedure Day of Week			
Monday	2, 210 (120-300)	2, 80.0 (60-100)	2, 50.0 (20-80)
Tuesday	13, 60.0 (0-180)	13, 70.0 (0-100)	13, 40.0 (0-150)
Wednesday	11, 10.0 (0-300)	11, 40.0 (10-200)	11, 40.0 (0-160)
Thursday	29, 20.0 (0-100)	29, 70.0 (0-180)	29, 30.0 (0-160)
Friday	10, 15.0 (0-180)	10, 55.0 (0-160)	10, 115 (0-240)
Saturday	2, 140 (80-200)	2, 100 (100-100)	1, 20.0 (20-20)
P-value	0.04	0.81	0.10

EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor; NOTCH1=notch receptor 1; HPV=human papillomavirus. Numbers (N, median [min-max]) refer to the protein staining score. P-values are from the Wilcoxon rank sum test (for dichotomous variables) or the Kruskal-Wallis test (for multi-level nominal variables).

Table 27. Surrogate Markers of Variation in Specimen Handling and Tumor HPV Status Among N=67 Cases

	All (N=67)	HPV Negative (N=40)	HPV Positive (N=27)	
	n (%)	n (%)	n (%)	P-Value*
Year of Diagnosis				< .01
2007	5 (7.5)	3 (7.5)	2 (7.4)	
2008	15 (22.4)	6 (15.0)	9 (33.3)	
2009	26 (38.8)	12 (30.0)	14 (51.9)	
2010	21 (31.3)	19 (47.5)	2 (7.4)	
Procedure Type				0.43
Biopsy	21 (31.3)	11 (27.5)	10 (37.0)	
Resection/excision	46 (68.7)	29 (72.5)	17 (63.0)	
Procedure Year				< .01
2007	3 (4.5)	2 (5.0)	1 (3.7)	
2008	17 (25.4)	7 (17.5)	10 (37.0)	
2009	23 (34.3)	10 (25.0)	13 (48.1)	
2010	23 (34.3)	20 (50.0)	3 (11.1)	
2011	1 (1.5)	1 (2.5)	0 (0.0)	
Procedure Month				0.47
January	5 (7.5)	4 (10.0)	1 (3.7)	
February	6 (9.0)	3 (7.5)	3 (11.1)	
March	6 (9.0)	4 (10.0)	2 (7.4)	
April	5 (7.5)	2 (5.0)	3 (11.1)	
May	3 (4.5)	0 (0.0)	3 (11.1)	
June	2 (3.0)	2 (5.0)	0 (0.0)	
July	6 (9.0)	4 (10.0)	2 (7.4)	
August	8 (11.9)	6 (15.0)	2 (7.4)	
September	6 (9.0)	2 (5.0)	4 (14.8)	
October	12 (17.9)	8 (20.0)	4 (14.8)	
December	8 (11.9)	5 (12.5)	3 (11.1)	
Procedure Day of Week				0.84
Monday	2 (3.0)	2 (5.0)	0 (0.0)	
Tuesday	13 (19.4)	7 (17.5)	6 (22.2)	
Wednesday	11 (16.4)	8 (20.0)	3 (11.1)	
Thursday	29 (43.3)	16 (40.0)	13 (48.1)	
Friday	10 (14.9)	6 (15.0)	4 (14.8)	
Saturday	2 (3.0)	1 (2.5)	1 (3.7)	
*Fisher's exact test				

APPENDIX D

DETAILS OF N=862 CASES OF HEAD AND NECK CANCER INCLUDED IN ARTICLE 2

Table 28. Head and Neck Cancer Cases (N=862)

	N	%
Oral Cavity	383	44.4
Larynx	232	26.9
Oropharynx	199	23.1
Hypopharynx	35	4.1
Nasopharynx	13	1.5

Table 29. Factors Associated With Oropharyngeal Cancer Among 862 Cases of Head and Neck Cancer

	Oropharyngeal Cases (N=199)	Other Cases (N=663)		
	n(%)*	n(%)*	OR (95% CI)†	P-Value‡
Age				<.0001
<50	49 (24.6)	104 (15.7)	1.00	
50-59	84 (42.2)	193 (29.1)	0.92 (0.60-1.41)	
60-69	54 (27.1)	225 (33.9)	0.51 (0.32-0.80)	
>=70	12 (6.0)	141 (21.3)	0.18 (0.09-0.36)	
Birth Cohort				<.0001
<=1920	2 (1.0)	31 (4.7)	0.19 (0.05-0.83)	
1930	13 (6.5)	159 (24.0)	0.25 (0.13-0.46)	
1940	71 (35.7)	214 (32.3)	1.00	
1950	75 (37.7)	176 (26.5)	1.28 (0.88-1.88)	
1960	33 (16.6)	63 (9.5)	1.58 (0.96-2.60)	
>=1970	5 (2.5)	20 (3.0)	0.75 (0.27-2.08)	
Sex				0.004
Male	161 (80.9)	469 (70.7)	1.00	
Female	38 (19.1)	194 (29.3)	0.57 (0.39-0.84)	
Race				0.33
White	192 (96.5)	629 (94.9)	1.00	
Non-White/Unknown	7 (3.5)	34 (5.1)	0.67 (0.29-1.55)	
Childhood Passive Smoke				0.53
No	49 (24.6)	178 (26.8)	1.00	
Yes	150 (75.4)	485 (73.2)	1.12 (0.78-1.62)	
Ever Smoked				0.05
No	53 (26.6)	133 (20.1)	1.00	
Yes	146 (73.4)	530 (79.9)	0.69 (0.48-1.00)	
Ever Drank Alcohol				0.16
No	30 (15.2)	129 (19.5)	1.00	
Yes	168 (84.8)	533 (80.5)	1.36 (0.88-2.09)	
BMI 1 year pre-diagnosis				0.59
<18.5	3 (1.5)	17 (2.6)	0.66 (0.19-2.33)	
18.5-24.9	57 (28.8)	213 (32.3)	1.00	
25.0-29.9	72 (36.4)	220 (33.3)	1.22 (0.82-1.82)	
>=30	66 (33.3)	210 (31.8)	1.17 (0.79-1.76)	

Table 29 continued

	Oropharyngeal Cases (N=199)	Other Cases (N=663)		
	n(%)*	n(%)*	OR (95% CI)†	P-Value‡
Highest Level of Education				0.01
Grade school	4 (2.0)	35 (5.3)	0.42 (0.14-1.19)	
High school	115 (58.1)	418 (63.1)	1.00	
Vocational	8 (4.0)	40 (6.0)	0.73 (0.33-1.60)	
College	71 (35.9)	169 (25.5)	1.53 (1.08-2.16)	
Ever Smoked Cigars				0.81
No	178 (89.4)	597 (90.0)	1.00	
Yes	21 (10.6)	66 (10.0)	1.07 (0.64-1.79)	
Ever Used Smokeless Tobacco				0.06
No	163 (81.9)	579 (87.3)	1.00	
Yes	36 (18.1)	84 (12.7)	1.52 (0.99-2.33)	
Ever Smoked Pipe				0.03
No	192 (96.5)	613 (92.5)	1.00	
Yes	7 (3.5)	50 (7.5)	0.45 (0.20-1.00)	
Personal History of Cancer				0.03
No	173 (86.9)	534 (80.5)	1.00	
Yes	26 (13.1)	129 (19.5)	0.62 (0.39-0.98)	
Blood Relative Had Cancer				0.001
No	95 (47.7)	227 (34.6)	1.00	
Yes	104 (52.3)	429 (65.4)	0.58 (0.42-0.80)	

*Numbers may not sum to total due to missing values for some variables

†Odds ratios and 95% confidence intervals were calculated using univariable logistic regression models.

‡P-values represent likelihood ratio Chi-square tests comparing the factor of interest with the null model. All variables were treated as indicators.

APPENDIX E

TUMOR HPV STATUS AMONG N=858 CASES INCLUDED IN MULTIVARIABLE LOGISTIC REGRESSION MODELS IN ARTICLE 2

Among the 858 cases included in our multivariable logistic regression analysis of CPSE and HNSCC, HPV status was determined by *in situ* hybridization in 262 cases (30.5%) (Table 30). Evaluation of tumor HPV status was more common among recently diagnosed cases (Table 30), in accordance with evolving practices at our institution. As shown in Table 31, the 262 evaluated cases consisted of 82 HPV-positive (69 oropharyngeal, 13 other sites) and 180 HPV-negative (22 oropharyngeal, 158 other sites) tumors. Cases that were evaluated for HPV tended to be younger (median=57.4 years) than unevaluated cases (median=61.0 years) (P <.0001) and were more often male (79.0%) than unevaluated cases (70.6%) (P=0.01). No difference was observed comparing evaluated and unevaluated cases with respect to active smoking (P=0.79), alcohol drinking (P=0.34), or CPSE (P=0.93).

Table 30. HPV Evaluation By Year of Diagnosis in the Case Series (N=858)

Year of Diagnosis	Evaluated, n/N (%)
2003	0/1 (0.0)
2004	0/50 (0.0)
2005	6/103 (5.8)
2006	26/120 (21.7)
2007	35/144 (24.3)
2008	60/167 (35.9)
2009	62/148 (41.9)
2010	73/125 (58.4)
All Years	262/858 (30.5)
N=858 represents the number of cases used for multivariable modeling, which are a subset of the entire (N=862) case series	

Table 31. HPV Status by Tumor Site Among N=858 Cases Included in Multivariable Logistic Regression Models

		HPV Status		
		Missing/Not Evaluated (N=596)	Negative (N=180)	Positive (N=82)
Oropharyngeal case (N=197)	**n**	106	22	69
	Row %	53.8	11.2	35.0
	Column %	17.8	12.2	84.1
Other case (N=661)	**n**	490	158	13
	Row %	74.1	23.9	2.0
	Column %	82.2	87.8	15.9

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