HHS Public Access

Author manuscript

*Oral Oncol*. Author manuscript; available in PMC 2017 April 01.

Published in final edited form as:

*Oral Oncol*. 2016 April ; 55: 1–5. doi:10.1016/j.oraloncology.2016.02.004.

# Salivary secretory leukocyte protease inhibitor (SLPI) and head and neck cancer: The Cancer Prevention Study II Nutrition Cohort

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## Abstract

**Objectives—**Secretory leukocyte protease inhibitor (SLPI) is an innate-immunity protein displaying antimicrobial and anti-inflammatory properties that is found in high concentrations in saliva. The role of extracellular salivary SLPI in head and neck squamous cell carcinoma (HNSCC) remains unclear. Thus, we aimed to evaluate the association between SLPI and HNSCC risk in the Cancer Prevention Study II Nutrition Cohort.

**Materials and Methods—**Among 53,180 men and women with no history of cancer who provided an oral rinse between 2001 and 2002, 60 were subsequently diagnosed with incident HNSCC between specimen collection and June 2009. In this nested case-control study, archived oral supernatants were evaluated using the Human SLPI Quantikine ELISA Kit for all 60 cases and 180 controls individually matched on gender, race, date of birth, and date of oral rinse collection. Conditional logistic regression was used to estimate HNSCC risk.

**Results—**Overall, pre-diagnostic salivary SLPI was associated with a non-statistically significant higher risk of HNSCC (OR=1.6, 95% CI=0.9–3.0). Among never smokers, high SLPI was associated with a non-statistically significant lower risk (OR=0.5, 95% CI=0.1–1.9), whereas among ever smokers, high SLPI was associated with a statistically significant higher risk (OR=2.1, 95% CI=1.0–4.3) of HNSCC, compared to low SLPI.

**Conclusion—**While results from this study suggest that higher concentrations of salivary SLPI might increase the risk of HNSCC among ever smokers, more research is needed to verify these findings and define the mechanisms by which SLPI and smoking influence the etiology of HNSCC.

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**Conflict of Interest:** ARG receives research funding from Merck Sharp & Dohme Corp. and is a consultant of Merck Sharp & Dohme Corp. for HPV vaccines. None of the other authors have conflicts of interest to report.

**Keywords**

secretory leukocyte protease inhibitor; SLPI; innate immunity; head and neck cancer; oral disease; mouthwash

## Introduction

Head and neck cancer (HNC) is the eighth most common cancer worldwide [1], arising in the epithelial lining of the oral cavity, pharynx, and larynx. Lifestyle risk factors include tobacco and alcohol consumption, although human papillomavirus (HPV) infection has recently emerged as a contributing factor, especially at the oropharynx [2, 3]. Although early-stage cancers have a favorable prognosis, there are no reliable methods for the early detection of HNC. Over half of all HNCs present with advanced disease, highlighting the need for studies to investigate molecular biomarkers indicative of HNC risk.

Secretory leukocyte protease inhibitor (SLPI) is a defensin-like innate immunity-associated protein with broad antimicrobial and anti-inflammatory properties [4-6] that has been identified as a potential diagnostic and prognostic biomarker in HNC [7]. It is found in various bodily secretions, but is present in particularly high concentrations in the saliva [8]. The role of salivary SLPI in reducing transmission of HIV in the oral cavity has been well studied [9, 10], and there is growing evidence that SLPI may protect against oral HPV infection [11, 12]. Results from tumor studies suggest that SLPI may be associated with HNC carcinogenesis [7], metastasis [11, 13], and prognosis [14], although the exact mechanisms by which SLPI protects against HNC development and progression remain unclear. SLPI is believed to protect mucosal surfaces against proteolysis and epithelial tissue damage [15] by inhibiting neutrophil elastase, a serine protease produced by tumor and inflammatory cells that is known to induce tumor cell proliferation [16].

The association between extracellular salivary SLPI and HNC risk has yet to be investigated in an epidemiologic study. Using a nested case-control analysis of data and biospecimens collected in a large, prospective cohort study in the US, we examined the association between salivary SLPI measured in oral rinses collected prior to cancer diagnosis and the risk of developing HNC.

## Material and Methods

### Study population

Men and women included in this analysis were among the 184,194 participants of the American Cancer Society (ACS) Cancer Prevention Study II Nutrition Cohort (CPS-II NC) [17], a prospective study of cancer incidence established in the US in 1992. The goal of the CPS-II NC was to follow participants for incident cancers and deaths and obtain extensive information on dietary, lifestyle, and other cancer risk factors. At enrollment, CPS-II NC participants ranged in age from 40 to 93 years and the majority were white. Follow-up questionnaires were sent to participants in 1997 and every two years thereafter to ascertain newly diagnosed cancer cases and update risk factor data. Self-reported cancer diagnoses were verified through medical records or state cancer registries. Additional details of the

CPS-II NC cohort are described elsewhere [17]. Between 2001 and 2002, CPS-II NC participants were invited to provide a buccal cell sample by rinsing the oral cavity with mouthwash. All participants provided informed consent at the time of oral rinse collection. The Emory University (Atlanta, GA, US) Human Investigations Committee approved the CPS-II study, and Liberty IRB Human Subjects Committee (Tampa, FL, US) approved the current study.

## Case and control selection

Of 70,004 CPS-II NC participants who provided an oral rinse, we excluded 16,664 who had a previous cancer diagnosis, 158 whose oral rinse specimens were exhausted, and two whose gender data were missing. Among 53,180 eligible participants, 60 were diagnosed between the time of oral rinse collection and June 2009 with a primary incident head and neck squamous cell carcinoma (HNSCC), including the oral cavity, oropharynx, hypopharynx, and larynx.

For each case, three controls were randomly selected among individuals who provided oral rinse specimens, were alive, and had no history of cancer on the diagnosis date of the case. Controls were individually matched to cases on gender, race (white, black, or other/unknown), date of birth (±6 months), and date of oral rinse collection (±30 days).

## Oral rinse collection and processing

Each CPS-II NC participant was mailed an introductory letter, consent form, oral rinse collection kit, and detailed instructions. In the collection kit, participants were provided with a 44 mL bottle of Scope mouthwash (Proctor & Gamble, Cincinnati, OH, US) and two 15mL sterile collection cups. Once before bedtime and again upon waking the following morning before brushing their teeth, participants were instructed to swish 10 mL of mouthwash vigorously in the mouth for 60 seconds, and then spit the mouthwash into the collection cup. Postage-paid envelopes were provided for returning specimens.

Oral rinse specimens were received, processed, and stored at a central biospecimen repository in Rockville, MD, US. For each participant, the two oral rinse collection cups were pooled into a 50 mL centrifuge tube and spun for 15 minutes at 2700 rpm. Using a sterile pipette, 1.5 mL of supernatant was aliquoted into a sterile cryovial for long-term storage in liquid nitrogen, and the remaining supernatant was discarded.

## SLPI measurement

SLPI was analyzed using the Human SLPI Quantikine ELISA Kit (DP100, R&D Systems, Minneapolis, MN, US) according to the manufacturer's instructions, with modifications made for mouthwash specimens [18]. Briefly, archived buccal cell supernatants were diluted 1:200 using the provided RD5T calibrator diluent. Diluted samples and standards (100 μL each) were added to a 96-well plate and run in triplicate. A standard curve was created for each run, and the optical density was used to interpolate SLPI concentrations of diluted samples (pg/mL), which were then used to estimate concentrations of salivary SLPI in the original oral rinses (ng/mL). As the coefficient of variation for this assay was <10%, triplicate measurements were averaged to derive a single value per specimen.

### Statistical analyses

As SLPI concentrations were positively skewed, non-parametric statistical methods were used. Median SLPI concentrations (ng/mL) and interquartile ranges (IQR) were calculated and compared between cases and controls using the Wilcoxon Rank Sum-Mann Whitney test. Associations between SLPI and participant characteristics were calculated separately for cases and controls using the Kruskal-Wallis test for overall differences and the Jonckheere-Terpstra trend test, after excluding missing data.

SLPI was analyzed as a continuous and categorical variable. As the distribution of continuous SLPI was highly skewed, values were transformed using log base10 to attenuate the influence of outliers. A categorical variable was also created by dichotomizing continuous SLPI at the median based on the distribution among controls (low <151.57 ng/mL, high ≥151.57 ng/mL).

As cases were individually matched to controls, conditional logistic regression (CLR) was used to estimate odds ratios (OR) and 95% confidence intervals (CI) for the association between salivary SLPI and risk of HNSCC. ORs were calculated separately for categorical SLPI (high vs. low) and continuous SLPI (per one unit increase in log SLPI). Univariate and multivariable conditional logistic regression models were developed to assess confounding and potential effect modification. An interaction between smoking status (never, ever cigarette smoking) and SLPI was investigated by adding a product term to a model containing the two main effects.

Statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA, US) and SAS version 9.4 (SAS Institute, Cary, NC, US). All statistical tests were two-sided and deemed significant at $\alpha$=0.05.

## Results

Included in this analysis were 60 HNSCC cases and 180 matched controls. The anatomical distribution of tumors was as follows: larynx, n=27; oral cavity, n=19; oropharynx, n=12; and hypopharynx, n=2 (Table 1). All participants were white. Compared to cases, controls were more likely to be well educated and never smokers. The median age of participants was 71 years (range: 59–87) at the time of oral sample collection. On average, oral samples were collected 3.5 years (SD: 2.1; range: 0.2–8.2) prior to cancer diagnosis.

Concentrations of salivary SLPI detected in oral rinse specimens ranged from 0.3–4975.8 ng/mL, with a mean concentration of 291.5 ng/mL (SD: 428.4) and median of 168.3 ng/mL (IQR: 37.9–450.1). Median SLPI concentrations were higher among cases (243.9 ng/mL) than controls (151.6 ng/mL; p=0.058), though this difference was of borderline statistical significance. SLPI concentrations were lowest among individuals with cancer of the oropharynx, followed by the larynx, oral cavity, and hypopharynx (200.3, 201.2, 339.3, and 628.9 ng/mL, respectively; p=0.429).

Among cases, SLPI concentrations were significantly associated with cigarette smoking (Table 1), with current smokers having values nearly 1.5-fold higher than former smokers

and six-fold higher than never smokers (351.7, 238.5, and 61.7 ng/mL, respectively; p=0.035; $p_{trend}$=0.011). SLPI was not significantly associated with any participant characteristics among controls.

High SLPI concentrations were associated with a non-statistically significant higher risk of developing HNSCC in unadjusted conditional logistic regression (OR: 1.6; 95% CI: 0.9–3.0; Table 2). After adjusting for smoking status, the odds ratio for the association between SLPI and HNSCC risk was slightly attenuated (OR: 1.5; 95% CI: 0.8–2.9). Furthermore, when an interaction term between SLPI and smoking status was included in the model, differences in the direction of the odds ratios for ever and never smokers suggested potential effect-measure modification by smoking, with a marginally significant interaction noted (p=0.064). Among never smokers, high concentrations of salivary SLPI were associated with a non-statistically significant lower risk of HNSCC (OR: 0.5; 95% CI: 0.1–1.9) compared to low SLPI concentrations. In contrast, among ever smokers, high SLPI concentrations were associated with a statistically significant higher risk of developing HNSCC (OR: 2.1; 95% CI: 1.0–4.3) compared to low concentrations of SLPI. Similar results were observed with continuous log SLPI models.

## Discussion

In this small nested case-control study of incident HNSCC, we evaluated whether extracellular salivary SLPI concentration measured in oral rinse specimens collected prior to cancer diagnosis was associated with the subsequent risk of HNSCC. We showed that pre-diagnostic salivary SLPI concentration was elevated among those who developed HNSCC compared to those who did not, although this difference only reached borderline statistical significance. Furthermore, we demonstrated that current and former smokers with high salivary SLPI were at a significant increased risk of developing HNSCC compared to those with low concentrations of SLPI. Conversely, we observed that never smokers with high salivary SLPI appear to be at decreased risk of HNSCC, however, the difference in risk was not statistically significant.

Increasing evidence indicates that SLPI may play an important role in the development, metastasis, and prognosis of HNC. In a recent study of oral squamous cell carcinoma (OSCC), SLPI concentration was found to be five-fold lower in the non-cancerous tissue of OSCC cases and 25-fold lower in the cancerous tissue of cases, compared to the oral epithelium of healthy normal controls [7]. Furthermore, *in vitro* experiments using oral premalignant cell lines showed that SLPI inhibits NF-kB transcriptional activity [7], thereby controlling the expression of pro-inflammatory cytokines and preventing the subsequent development of cancer. SLPI is also known to inhibit several enzymes known to promote tumor cell invasion and tumor progression [19, 20]. Through its inhibition of neutrophil elastase, SLPI protects the epithelial cell surface against tissue degradation [15] and air trapping [20], which prolongs epithelial cell exposure to carcinogens such as tobacco and alcohol. SLPI is also thought to have a substantial protective effect on HNSCC metastases. In patients with HNSCC, a strong, statistically significant association was found between lower SLPI protein expression and increased risk of lymph node metastases [13]. It has been theorized that through the inhibition of leukocyte elastase and cathepsin [21] and

suppression of matrix metalloproteinases [13], SLPI prevents the breakdown of extracellular matrix proteins surrounding the tumor, thereby preventing tumor cell proliferation and invasion [16]. Furthermore, high SLPI has been independently associated with increased survival in OSCC patients [14].

Our finding that SLPI may be differentially associated with HNSCC risk depending on smoking status is consistent with published findings on the effect of cigarette smoking on SLPI production [11, 22, 23]. Nicotine stimulates SLPI expression in the nasal and oral mucosa, and also promotes the proliferation of cancer cells [24]. In a study of non-HPV driven HNSCC, SLPI gene and protein expression (i.e., intracellular SLPI) was more than 100-fold higher among smokers than non-smokers when evaluated in HNSCC tumor tissue, 20-fold higher among smokers when measured in non-neoplastic tissue of HNSCC patients, and 10-fold higher among smokers when measured in non-HNSCC patients [23]. Similarly, in our study of HNSCC we found current smokers to have significantly higher levels of SLPI than never smokers. Thus, we hypothesized that the magnitude and direction of the association between SLPI and HNSCC would differ depending on smoking status, and as a result, included cigarette smoking as an effect modifier in our statistical models.

To our knowledge, this is the first epidemiologic study to examine the association between pre-diagnostic salivary SLPI and subsequent HNSCC risk. Utilizing a case-control study design nested within a prospective cohort study allowed us to evaluate the temporal relationship between salivary SLPI and HNSCC, as SLPI was measured up to eight years prior to cancer diagnosis. However, with only 60 incident HNSCC cases, power was limited to detect statistically significant associations, particularly when stratified on smoking status. While it would have been interesting to examine the role of oral HPV, too few cases (n=7; 12%) and controls (n=6; 3%) were positive for high-risk HPV. It is possible that the use of an alcohol-based mouthwash may have contributed to protein degradation; however, SLPI is a protein rich in disulfide bonds [25] that render the molecule resistant to denaturation [26]. Furthermore, degradation would have been non-differential, equally effecting cases and controls. Lastly, there are no known clinically relevant cutoff values for SLPI, as few studies have described extracellular SLPI *in vivo* [27]. High SLPI levels were hypothesized to protect against HNSCC, thus SLPI was analyzed as high versus low concentrations.

## Conclusion

These findings suggest that higher concentrations of salivary SLPI might increase HNSCC risk among ever smokers; however, larger studies are needed to better understand the association between SLPI and HNSCC risk. In particular, future studies should focus on the relationship between SLPI, tobacco use, and other factors, including HPV infection, on the anatomic subsite-specific risk of HNSCC, as well as metastasis. Saliva-based biomarkers represent a promising, inexpensive, and noninvasive approach to the diagnosis of oral and systemic diseases [28], including the early detection of HNC [29].

## Acknowledgments

CMPC was supported through postdoctoral fellowships from the National Cancer Institute (R25T CA147832) and the American Cancer Society (PF-13-222-01 - MPC). The American Cancer Society funds the creation,

maintenance, and updating of the Cancer Prevention Study II Nutrition Cohort. The authors would like to acknowledge the contribution to this study from central cancer registries supported through the Centers for Disease Control and Prevention's National Program of Cancer Registries and cancer registries supported by the National Cancer Institute's Surveillance Epidemiology and End Results Program. The authors would also like to thank Dr. Ernst Schonbrunn for the use of the plate reader.

**Role of the funding sources**: Pilot funds for this project were supplied from the parent NCI grant R25T CA147832.

## Abbreviations

| | |
|---|---|
| **SLPI** | secretory leukocyte protease inhibitor |
| **HPV** | human papillomavirus |
| **HNC** | head and neck cancer |
| **HNSCC** | head and neck squamous cell carcinoma |

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## Highlights

1. Pre-diagnostic salivary SLPI was higher among HNSCC cases than controls
2. Among cases, salivary SLPI was positively associated with cigarette smoking
3. Among never smokers, high salivary SLPI was not associated risk of HNSCC
4. Among smokers, high SLPI was associated with a significantly higher risk of HNSCC

*Oral Oncol*. Author manuscript; available in PMC 2017 April 01.

**Table 1**

Participant characteristics and median SLPI concentrations among HNSCC cases and matched controls, by participant characteristics; ACS Cancer Prevention Study II Nutrition Cohort, 2001–2002

| | Overall HNSCC | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Cases (n=60) | | | | | Controls (n=180) | | | | |
| | n | %[a] | Median SLPI, ng/mL | IQR | P-value[b] | n | %[a] | Median SLPI, ng/mL | IQR | P-value[b] |
| Age at oral rinse collection, years | | | | | 0.129 | | | | | 0.088 |
| <65 | 11 | 18.3 | 522.7 | 122.3–701.8 | | 32 | 17.8 | 202.5 | 57.8–636.2 | |
| 65–69 | 16 | 26.6 | 99.2 | 23.6–301.0 | | 49 | 27.2 | 134.7 | 19.8–365.5 | |
| 70–74 | 13 | 21.7 | 238.5 | 68.0–479.3 | | 39 | 21.7 | 173.8 | 74.6–468.5 | |
| 75+ | 20 | 33.3 | 270.7 | 109.5–515.0 | | 60 | 33.3 | 90.1 | 26.6–302.5 | |
| Gender | | | | | 0.570 | | | | | 0.086 |
| Male | 44 | 73.3 | 243.9 | 78.5–506.7 | | 132 | 73.3 | 167.1 | 39.3–441.4 | |
| Female | 16 | 26.7 | 181.5 | 32.4–572.3 | | 48 | 26.7 | 91.9 | 18.7–310.2 | |
| Education[c] | | | | | 0.901 | | | | | 0.505 |
| High school graduate or less | 23 | 38.3 | 194.8 | 68.0–521.5 | | 52 | 28.9 | 102.5 | 30.8–304.0 | |
| Some college | 21 | 35.0 | 287.9 | 46.1–569.9 | | 43 | 23.9 | 167.4 | 37.4–470.5 | |
| College graduate | 16 | 26.7 | 281.7 | 52.7–500.2 | | 84 | 46.7 | 168.0 | 33.0–405.3 | |
| Body mass index (kg/m$^2$)[d] | | | | | 0.339 | | | | | 0.750 |
| <22.5 | 8 | 13.3 | 122.4 | 2.8–323.4 | | 15 | 8.3 | 173.8 | 74.4–426.1 | |
| 22.5–<25 | 13 | 21.7 | 339.3 | 118.3–521.5 | | 45 | 25.0 | 138.0 | 20.4–384.7 | |
| 25–<30 | 22 | 36.7 | 121.1 | 31.8–538.0 | | 79 | 43.9 | 151.0 | 36.3–374.8 | |
| 30+ | 10 | 16.7 | 236.7 | 178.6–372.4 | | 29 | 16.1 | 134.7 | 18.6–413.4 | |
| Mouthwash use[c] | | | | | 0.563 | | | | | 0.661 |
| Yes | 32 | 53.3 | 269.2 | 72.5–523.2 | | 99 | 55.0 | 151.0 | 31.4–357.5 | |
| No | 27 | 45.0 | 234.9 | 46.1–521.5 | | 80 | 44.4 | 153.6 | 35.5–467.9 | |
| Mouthwash use, times per week[c] | | | | | 0.368 | | | | | 0.845 |
| None | 27 | 45.0 | 234.9 | 46.1–521.5 | | 80 | 44.4 | 153.6 | 35.5–467.9 | |
| 1–2 | 9 | 15.0 | 118.3 | 76.9–410.6 | | 24 | 13.3 | 115.2 | 31.7–317.8 | |
| 3–6 | 7 | 11.7 | 351.7 | 31.4–1121.5 | | 31 | 17.2 | 171.6 | 41.3–366.9 | |

| | Overall HNSCC | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Cases (n=60) | | | | | Controls (n=180) | | | | |
| | n | %[a] | Median SLPI, ng/mL | IQR | P-value[b] | n | %[a] | Median SLPI, ng/mL | IQR | P-value[b] |
| 7+ | 11 | 18.3 | 339.3 | 249.2–633.5 | | 31 | 17.2 | 153.7 | 30.3–440.2 | |
| Smoking status[e] | | | | | **0.035** | | | | | 0.347 |
| Never | 10 | 16.7 | 61.7 | 21.7–287.9 | | 83 | 46.1 | 130.8 | 30.3–366.9 | |
| Former | 33 | 55.0 | 238.5 | 46.1–508.5 | | 94 | 52.2 | 151.6 | 36.3–425.8 | |
| Current | 17 | 28.3 | 351.7 | 194.8–633.5 | | 3 | 1.7 | 483.9 | 81.9–1171.7 | |
| Alcohol consumption, drinks per day[d] | | | | | 0.731 | | | | | 0.290 |
| Non-drinker | 12 | 20.0 | 301.0 | 150.6–539.2 | | 40 | 22.2 | 128.8 | 36.4–220.5 | |
| <1 | 22 | 36.7 | 301.1 | 31.4–538.0 | | 78 | 43.3 | 134.4 | 28.8–413.4 | |
| 1+ | 17 | 28.3 | 339.3 | 210.0–521.5 | | 36 | 20.0 | 200.1 | 54.7–538.7 | |
| Anatomic subsite of cancer | | | | | 0.429 | | | | | |
| Oropharynx | 12 | 20.0 | 200.3 | 23.1–529.7 | | -- | -- | -- | -- | |
| Oral cavity | 19 | 31.7 | 339.3 | 55.5–522.7 | | -- | -- | -- | -- | |
| Larynx | 27 | 45.0 | 201.2 | 63.5–491.8 | | -- | -- | -- | -- | |
| Hypopharynx | 2 | 3.3 | 628.9 | 504.9–753.0 | | -- | -- | -- | -- | |

Notes: -- Not applicable; SLPI, salivary leukocyte protease inhibitor; IQR, interquartile range.

[a] Columns that do sum to 100% reflect missing data.

[b] Non-parametric Kruskal Wallis test assessing differences in median SLPI by participant characteristics among cases and controls, separately

[c] As reported on the 1982 CPS-II baseline survey

[d] As reported on the 1999 CPS-II NC follow up survey

[e] As reported on the last survey returned prior to oral rinse collection (1999 or 2001).

**Table 2**

Associations between SLPI concentration[a] and risk of HNSCC, ACS Cancer Prevention Study II Nutrition Cohort, 2001–2002

| | Overall HNSCC | | |
|---|---|---|---|
| | Cases (n=60) | Controls (n=180) | |
| | n | n | OR (95% CI) |
| **Unadjusted**[b] | | | |
| Low SLPI | 23 | 90 | 1.0 |
| High SLPI | 37 | 90 | 1.6 (0.9–3.0) |
| Per 1 unit SLPI (log ng/mL) | | | 1.2 (1.0–1.4) |
| **Adjusted**[c] | | | |
| Low SLPI | 23 | 90 | 1.0 |
| High SLPI | 37 | 90 | 1.5 (0.8–2.9) |
| Per 1 unit SLPI (log ng/mL) | | | 1.1 (0.9–1.4) |
| **Interaction**[d,e] | | | |
| Never smokers | | | |
| Low SLPI | 7 | 42 | 1.0 |
| High SLPI | 3 | 41 | 0.5 (0.1–1.9) |
| Per 1 unit SLPI (log ng/mL) | | | 0.9 (0.6–1.4) |
| Ever smokers | | | |
| Low SLPI | 16 | 48 | 1.0 |
| High SLPI | 34 | 49 | **2.1 (1.0–4.3)** |
| Per 1 unit SLPI (log ng/mL) | | | 1.2 (1.0–1.5) |

[a] Models run separately for categorical and continuous SLPI, with categorical SLPI dichotomized at the median among controls (low: <151.57 ng/mL, high: ≥151.57 ng/mL).

[b] Unadjusted model: conditional logistic regression model conditioned on matched set, therefore adjusting for the matching factors, race, gender, date of oral rinse collection and birth date.

[c] Adjusted model: conditional logistic regression model conditioned on matched set, therefore adjusting for the matching factors, race, gender, date of oral rinse collection and birth date, and additionally adjusted for smoking status (never, ever cigarette smoker).

[d] Interaction model: conditional logistic regression model conditioned on matched set, therefore adjusting for the matching factors, race, gender, date of oral rinse collection and birth date, and additionally adjusted for smoking status (never, ever cigarette smoker) and an interaction term between SLPI and smoking status.

[e] P-value for the interaction between smoking status and categorical SLPI=0.0635; P- value for the interaction between smoking status and continuous SLPI=0.2116.