

Title: The Association between Inflammatory Biomarkers and Statin Use among Head and Neck Squamous Cell Carcinoma Patients

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Abstract

Background: Tumor-infiltrating Lymphocytes (TILs) and cytokines are associated with prognosis among head and neck squamous cell carcinoma (HNSCC) patients. Statins (cholesterol-lowering drugs) may improve HNSCC prognosis, particularly in human papillomavirus (HPV) positive cases, but the mechanism remains unclear.

Methods: Statin use was collected from medical records for HNSCC cases (2008-2014). TILs were counted in tumor tissue, and a total weighted score (TILws) was created. Cytokines were measured in blood. The associations between statins and biomarkers were estimated using logistic (biomarker categories: <median, ≥median) and linear regression models (log-transformed continuous biomarkers) adjusted for age, smoking, and comorbidities.

Results: We observed a positive association between statins and TILs among HPV-positive patients (TILws Odds Ratio (OR)=2.80; 95%CI=1.03-7.61), but no association among HPV-negative patients. We observed no association between statins and cytokines.

Conclusions: Statins may influence TILs in HPV-positive patients. This may be the mechanism through which they improve prognosis in HPV-positive HNSCC patients.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a debilitating cancer that can be found in the mucosal lining of the aerodigestive tract with prominent sites including the nasopharynx, oral cavity, oropharynx, hypopharynx and larynx.¹⁻³ The main risk factors associated with HNSCC development can be delineated into two subgroups: patients with human papillomavirus (HPV)-positive tumors and those with HPV-negative tumors, whose risk for disease is often attributed to smoking and alcohol drinking.¹ Etiology of disease, underlying tumorigenesis, patient characteristics, site of disease, treatment and prognosis often differ between patients with HPV-positive versus HPV-negative tumors.⁴

Statins are a class of cholesterol-lowering medications that are often utilized to prevent the development or progression of heart disease.⁵ In addition to their cholesterol lowering attributes statins possess anti-inflammatory and immunomodulatory actions that may inhibit the development or progression of cancer.⁶ Research has established a protective association between the use of statin drugs and the incidence and mortality of cancer at numerous sites,^{7,8} including HNSCC.⁹⁻¹¹ Although the relationship between statin use, cancer development and cancer related outcomes has been investigated across various cancer sites with promising findings the potential mechanisms by which statins may be exerting their protective effect remains unclear, particularly for HNSCC. However, their effects on inflammation and immunomodulation that may be responsible for factors that influence the progression and development of cancer are largely unknown.¹²

Recent research findings suggest that there is an inverse association between number of tumor infiltrating lymphocytes (TILs) and HNSCC death and recurrence.^{13,14} Another inflammatory marker that potentially influences HSNCC outcomes is the level of circulating cytokines. Previous research has identified that higher levels of the pro-inflammatory cytokine interleukin-6 (IL-6) has been found to be positively associated with recurrence and death among patients with HNSCC.¹⁵

Given the established relationship between HNSCC outcomes and both TILs and circulating cytokines, as well as the known effects of statins on inflammation and immune modulation, this study

aimed to identify the association between statin use and these inflammatory biomarkers. Through this research we determined whether statins may be influencing HNSCC outcomes through these inflammatory processes. Because patients with HPV-positive tumors may have a different immune response and are different etiologically than HPV-negative tumors, we explored whether HPV status may modify the association between statin use and the presence and quantity of TILs and circulating cytokines.

Materials and Methods

Study Population

Subjects in this study were recruited to participate in the University of Michigan Head and Neck Cancer Specialized Program of Research Excellence II (SPORE II). The SPORE II cohort consists of incident HNSCC patients who were diagnosed and/or treated at the University of Michigan Rogel Cancer Center from 2008-2014. In order to be eligible to participate in the study patients had to be 18 years or older, their cancer could not have been previously treated, and their disease could not be a recurrence of disease (i.e. they had to be free of disease for 5 years prior to their current diagnosis). A more detailed description of the cohort and information about the percent of patients who agree to participate has been published previously.^{15,16} The SPORE II cohort consist of 1,042 participants; complete TILs and statin use information was available for 475 participants and circulating cytokines were measured in 205 participants.

Inflammatory Marker Measurements

Tumor infiltrating lymphocytes (TILs)

Tumor tissue was collected from previously untreated patients who had tissue available from biopsies. Details of the TILs measurement procedure and creation of variables has been previously published.^{13,14} Briefly, triplicate 0.7mm diameter cores for each patient sample were selectively punched/extracted and transferred to a recipient tissue array block. Immunohistochemical staining was completed on a DAKO autostainer using LSAB+ (liquid streptavidin biotin horseradish peroxidase) and DBA (DAKO labeled avidin-biotin-peroxidase kits) as chromogens. Deparaffinized

sections were stained with six monoclonal antibodies at the following titrations: cluster of differentiation 4 (CD4)-1:250 (Abcam Ab846); cluster of differentiation 8 (CD8)-1:40 (Nova Castra VP-C320); forkhead box P3 (FoxP3) -1:200 (Abcam Ab20034); cluster of differentiation 104 (CD104) -1:50 (Beta-4 integrin, eBioscience 439-9b). Appropriate negative (without primary antibodies) and positive (tonsillar tissue and various carcinomas) controls were stained concurrently on the same slides. The stained TMA slides were assessed by a technician blinded to patient clinical status and treatment outcome. The whole TMA slides were digitally imaged, scanned, and retrieved with Aperio ImageScope v.12 software. Grid software (Measure, C Thing Software 2.01) was used to overlay each tissue core image prior to counting cells. CD104 staining (beta-4 integrin) for each core was examined first to locate and confirm the extent and location of carcinoma within the tissue cores. TILs were manually counted within tumor parenchyma on tissue microarrays and presented as number of cells per millimeters squared; in addition, a total weighted score (TILws) was created combining data across the three types of TILs.¹⁴ TILs were measured for CD4 (N=481), CD8 (N=481), FoxP3 (N=485) and TILws (N=475).

Circulating Cytokines

Circulating cytokines were measured from blood samples collected from participants at diagnosis, prior to treatment. A detailed explanation of the procedure to measure the circulating cytokines was previously published.^{15,17} Briefly, cytokines were measured at the University of Michigan Cancer Center Immune Monitoring Core using commercially available ELISA (Enzyme-Linked Immunosorbent Assay) kits. Blood samples were stored at 4° C for less than 2 hours after collection when they were centrifuged, and serum was separated in 2ul aliquots for storage at -80° C. When samples were selected for cytokine measurement, they were thawed and incubated overnight at 4° C on microtiter plates pre-coated with monoclonal antibodies specific to each cytokine. All samples were measured in duplicate and blinded duplicates of study samples for two participants were included as quality control samples. After washing away unbound substances, biotin-linked polyclonal antibodies for each cytokine were introduced. After incubation for two hours

at room temperature, the plates were washed and incubated with streptavidin-HRP for an hour. After a final wash, substrate solution was added; color development was stopped after 25 minutes. Colorimetric densities were measured for each sample from a standard curve using a microplate reader. The cytokines measured were interferon gamma (IFN- γ), interleukin-6 (IL-6), interleukin-8, (IL-8), interleukin-10 (IL-10), interleukin-17 (IL-17), growth related oncogene (GRO), hepatocyte growth factor (HGF), tumor necrosis factor alpha (TNF- α), vascular endothelial growth factor (VEGF) and transforming growth factor (TGF). Samples that exceeded the limit of detection were assigned the maximum value.

Confounding Variables

Variables that may confound the association between statin use and biomarkers were collected in various ways. Clinical variables such as age (continuous), and comorbidities (none, mild, moderate, or severe) measured through the Adult Comorbidity Evaluation 27 score (ACE-27) were collected through medical record review whereas potential confounders associated with behavior such as smoking status (never, former, current) were collected through a baseline health survey. Individuals that were missing the listed potential confounders, specifically smoking status (N=20) and ACE-27 (N=4) were assigned to the most common category for the TILs analysis; no individuals were missing confounding variables for the cytokines analysis.

Statin Measurement

Statin use was collected through a retrospective medical record review by trained research personnel. Study personnel identified the patient's initial encounter at the University of Michigan hospital for the patient's HNSCC diagnosis and medications were recorded from that encounter. If the patient did not have medications recorded at the initial encounter the next closest encounter was checked. Medications were recorded from the closest encounter to initial diagnosis prior to treatment initiation. If a participant was identified as using a statin at diagnosis s/he was considered a statin user. Data were collected by two reviewers and achieved an inter-rater reliability coefficient, Kappa

of 95%. Information that was not concordant across reviewers was reconciled after comparison. Data was stored in a Research Electronic Data Capture (REDCap) database.¹⁸

Statistical Analysis

Basic descriptive statistics were calculated to identify if demographic and clinical characteristics were different for statin users compared to non-statin users at diagnosis. TIL and cytokine variables were operationalized continuously and categorically. Due to the highly skewed distribution of the data toward zero, TILs and cytokine values were log transformed to achieve a more normal distribution. In addition to analyzing this association linearly, based on the highly skewed distribution, individual TIL measures and cytokine measures were categorized as low or high (i.e. < median or \geq median). Since TILs and cytokines were examined continuously and dichotomously (< median vs. \geq median), linear and logistic regression models were conducted. Logistic regression models calculated odds ratios (OR). Multivariable-adjusted (MV-adj) models included the following variables that may confound the association between statin use and TILs; age, smoking status, and ACE-27. Because statin use appeared to be associated with HPV status and disease site for participants who had cytokines measured, we conducted models adjusting for those variables as well.

Based on our previous research we observed an association between statin use and HNSCC outcomes, but only among patients whose disease was HPV-positive.¹⁹ We therefore wanted to assess if effect modification by HPV status was present (HPV-positive, HPV-negative, HPV status invalid/missing) for the statin-inflammatory marker associations. Statistical interaction was evaluated using the likelihood ratio test.

HPV status was missing for 125 participants who had at least 1 of the TIL biomarkers measured and 30 participants who had the circulating cytokines measured. In order to identify if these missing values influenced the association of statin use and the TIL measures we utilized various methods to analyze the HPV status as a potential effect modifier. Initially participants who had HPV status missing were excluded from the analysis. We also conducted the analysis by

including the participants with HPV missing as a separate category. Lastly, we utilized inverse probability weighting (IPW) by generating weights to emulate a population where no participants are missing HPV status. We created the weights by calculating the probability of having HPV status missing by conducting a logistic regression model consisting of variables (year enrolled in the study, age at diagnosis, gender, smoking status, marital status, stage of disease at diagnosis, disease site, ACE-27, drinking status and body mass index (BMI)); this is the denominator of the weight. Participants with missing values for the predictors in the logistic regression model were dropped from the analysis. The weights were then applied to a logit model with a binomial distribution and robust 95% confidence intervals, excluding participants who were missing HPV status. This approach was also utilized to assess the potential effect modification of HPV status on the association between statin use and the circulating cytokine measures.

All analyses were conducted using SAS version 9.4 (Cary, NC). All tests were two-sided, and results were considered statistically significant if $p < 0.05$.

Results

Descriptive statistics

Presented in Tables 1 and 2 are the distribution of baseline demographic characteristics by statin use for the participants included in the TILs and circulating cytokines analyses, respectively. For the TILs analysis, participants taking a statin at diagnosis (29.32%) appear to be older, more likely to have a higher BMI, higher ACE-27 and are less likely to be current smokers compared to non-statin users at baseline. The distribution of characteristics among statin users (36.10%) and non-users was slightly different for participants who had circulating cytokines measured at baseline. Statin users were older, less likely to have larynx or oral cavity as their primary site of disease, more likely to have HPV status missing, higher ACE-27 and less likely to be current smokers compared to participants who were not using a statin at diagnosis.

Multivariable analysis

When assessing the relationship between statin use and TILs, operationalizing the outcome as high vs. low TIL counts, there did not appear to be a clear association between statin use and TILs. After adjusting for confounders: age, smoking status and ACE-27, those who were taking a statin had a slightly higher odds of having higher TILs but this association was not statistically significant for any of the measured TIL values (TILws: [MV-adj OR=1.15; 95% CI= (0.74, 1.78)]; FoxP3: [MV-adj OR=1.13; 95% CI= (0.74, 1.73)] ; CD4: [MV-adj OR=1.23; 95% CI= (0.80, 1.90)]; CD8: [MV-adj OR=1.10; 95% CI= (0.72, 1.69)]. Similar null findings were observed when TILs were log-transformed and examined as a continuous variable (Table 3). The association between statin use and circulating cytokines was also null (Supplemental Table 1).

Effect modification

We observed a statistically significant interaction with HPV status such that there was a statistically significant positive association between statin use and having a high number of FoxP3 (HPV-positive: [MV-adj OR=4.15; 95% CI= (1.55, 11.14)]; HPV-negative: [MV-adj HR= 0.81; 95% CI= (0.43, 1.52)], p for interaction = 0.003 (Table 4)). There was a marginally significant association observed for TILws (above the median) among statin users whose tumors were HPV-positive (TILws: HPV-positive [MV-adj OR=2.80; 95% CI= (1.03, 7.61)]; HPV-negative [MV-adj HR= 1.07; 95% CI= (0.57, 2.02)], p for interaction = 0.1 (Table 4)), which may be driven by FoxP3. Interactions were suggestive, but not statistically significant for the association between statin use and CD4 and CD8.

When we examined the association between statin use and circulating cytokines, we observed no statistically significant interaction between statin use at diagnosis and HPV status for any of the individual circulating cytokines except HGF. Patients who were HPV-positive and on a statin at diagnosis had higher odds of having a high level of HGF compared to those who had HPV-positive tumors and were not taking a statin, whereas those who were HPV-negative and were taking a statin appear to have an inverse relationship with HGF. However, neither of the stratum-specific associations were statistically significant (HPV-positive: [MV-adj OR=2.27; 95% CI= (0.82,

6.27)]; HPV-negative: [MV-adj HR= 0.47; 95% CI= (0.17, 1.31)], p for interaction = 0.03 (Supplemental Table 2)).

Similar findings were observed when the HPV status missing category was included and when using the IPW method, although the associations observed using the IPW method were slightly stronger they did not appear to be meaningfully different for both the individual TIL and circulating cytokine measures (Supplemental Tables 3 & 4).

Discussion

In this study we observed that HNSCC patients taking a statin at the time of diagnosis had higher lymphocyte infiltration in their tumors than non-users, but only for HPV-positive patients. The strongest association was observed for FoxP3. Our recently published study found that statins were protective for HNSCC outcomes, but this protective association was observed only among patients whose tumors were HPV-positive.¹⁹ The findings from this manuscript support that the inverse association between statin use and HPV-positive HNSCC previously reported by our group may be due to an effect of statins on TILs in patients with HPV-positive tumors.¹⁹

The inflammatory and immunomodulatory effects of cancer are not clearly directional by cell type. This effect often depends on the type of cancer, whether there is a presence of inflammatory markers and what combination of these markers are beneficial or harmful to cancer prognosis.²⁰ Research has established that FoxP3 influences cancer prognosis but the directionality of this association differs by cancer type, with certain cancers such as breast, cervical, pancreatic and melanoma observing a positive association between FoxP3 infiltration and death whereas other cancer sites such as HNSCC, colorectal and esophageal cancers observe an inverse association.^{13,14,21} FoxP3 infiltration appears to have varying impacts on cell development and proliferation but the explanation behind why its influences differ by cancer sites is not clearly established.²²

Although to our knowledge this is the first study to investigate an association between statin use and TILs among HNSCC patients, a study investigating this association among patients with

colorectal cancer identified similar results. Al-Husein et al. identified a positive association between statin use and FoxP3 among patients with colorectal cancer and determined this association was modified by stage of disease.²³ Another study by Lee et al. found that statins were associated with the increased production of T-cells (“FoxP3 transcription factor”) in mice and lung tumor cell lines.²⁴

Our finding that the statin-TIL association may be limited to patients with HPV-positive tumors is plausible given that patients with HPV-positive tumors often have a different immune response and may be less immunosuppressed than HNSCC patients with HPV-negative disease.^{25,26} HPV-positive HNSCC have been shown to have a stronger immune response, particularly stronger T-cell infiltration, than patients with HPV-negative disease^{27,28} One possible explanation is that HPV-positive HNSCC is usually found in the oropharynx, (specifically the tonsils).^{28,29} Tonsils are made of lymphatic tissue which is rich in various immunological processes.³⁰ Therefore, one possible explanation for our finding of a statin-TIL association only among HPV-positive patients is that the stronger immune response of HNSCC patients with HPV-positive disease works synergistically with the anti-inflammatory and immunomodulatory actions of statins. Statins may, therefore, improve cancer related outcomes specifically in HPV-positive patients.

Although our findings were relatively null for the association between statin use and circulating cytokines, other studies have reported statin-cytokine associations, but the specific cytokines reported to be associated with statin use were not consistent across studies. One previous study identified an inverse association between pro-inflammatory cytokines in tissue and serum among patients with colorectal cancer.³¹ Two studies investigated this association in participants with hypercholesterolemia finding a reduction in the pro-inflammatory cytokines IL-6 and TNF for participants on a statin.^{32,33} In a study that investigated the association among a random sample of Swiss adults, the authors reported lower C-reactive protein (CRP) concentrations among participants using a statin.³⁴ To our knowledge our study is the first to investigate this association in HNSCC patients. It is possible our findings differ from those reported previously because cytokine levels may not be as affected by statin use in HNSCC patients. It is also possible that the results are

different due to the design of the studies. The studies that found an association between statin use and circulating cytokine levels utilized a more experimental design in which they administered statins to participants and then measured circulating cytokine levels pre and post medication administration. Our study uses an observational approach which may make it more difficult to identify the true effect of statins on circulating cytokines, particularly if the effect is modest.

Strengths and Limitations

To our knowledge this study is the first to assess the association between inflammatory biomarkers and statin use among patients with HNSCC. A strength of this study is the amount data we have for each patient and the opportunity we have to integrate biomarker data with behavioral, epidemiological and clinical data. This allowed us to test and identify confounders and should mitigate bias that may arise due to lack of information from participants.

Although this study has many strengths there are some notable limitations that should be addressed in future studies. The sample size for this study is quite small. Not all participants within the SPORE II cohort had tumor tissue from biopsy available to measure TILs or provided a blood sample at baseline to measure circulating cytokines. This may lead to a reduction in power especially when investigating the interaction between statin use and HPV status. Since the effect size of the association particularly for FoxP3 was relatively large this may not be an issue but as noted by the wide confidence interval the point estimate may not be precise. Future studies investigating this association among a larger study population is necessary. This may also help to explain why there did not appear to be a clear relationship between circulating cytokines and statin use among HPV-positive patients.

There is the potential for selection bias as well. Since not all participants provided specimen for biomarker measurement it is possible that the patients who provided specimen were different from those who did not, with regard to the relationship between statin use and these biomarker measures. This does not appear to be an issue for the analytic sample who have TILs measured. The frequency of participants who were using a statin at diagnosis and distribution of the

demographic and clinical characteristics between statin users and non-users was very similar to what was observed in the entire study population. There did appear to be some differences between the sample of participants who had baseline circulating cytokines measured compared to the complete study population. There appeared to be slightly more males and patients with higher stages of disease, but other factors that may bias the associations observed are similarly distributed in the total study population and the analytic sample who have baseline circulating cytokines measured. We additionally used IPW to emulate a population had no SPORE participants had TILs or circulating cytokines missing. This would provide participants who are similar to those who are missing to have larger weights. After including these weights, the association between TILs and statin use at diagnosis does not meaningfully change (Supplemental Table 5). The point estimates for the association between circulating cytokines and statin use do slightly change but the findings still remain relatively null (Supplemental Table 6). It is possible that selection bias may be an issue for the circulating cytokine measures.

Another limitation of this study is that the data is cross-sectional. Both the medication information and the inflammatory markers were measured at diagnosis prior to cancer treatment. This can possibly lead to reverse causation specifically with the cytokine measures. If inflammation and high cholesterol are associated we may observe a positive association between inflammation and statin use if those who have higher levels of pro-inflammatory cytokines were taking a statin because of risk factors associated with high cholesterol such as coronary heart disease and obesity, but this association is not clearly defined.³⁵⁻³⁷ It would be very difficult to identify this bias because we do not have information on when statins were initiated. We also do not have biomarker information from patients (blood and tumor tissue samples) prior to their HNSCC diagnosis. Since we did not appear to observe an association between statin use and circulating cytokines or an interaction between HPV status and statin use for the majority of the studied cytokines it is possible this limitation did not affect our study. This limitation should not be an issue with TILs because this measurement is based on inflammatory markers that are found within the tumor tissue. We would

assume that TILs would not be influenced by other comorbidities that the patient may have at diagnosis.

Conclusion

Our findings suggest that one mechanism by which statins may influence prognosis in HNSCC patients is through an effect on TILs, particularly FoxP3. This association appears to be restricted to HPV-positive patients. Future research investigating this association may shed light on the role of type, dose of statin, and duration of use, with TILs in HNSCC tumors. Additional studies are needed to examine other immune and inflammatory markers that predict HNSCC outcomes to further elucidate this potential mechanism by which statins may be protecting against poor outcomes in HPV-positive HNSCC patients.

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Data Availability Statement: The data that supports the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement: Patients recruited through SPORE II provided written informed consent, and the medical record review and SPORE study was approved through the University of Michigan Medical School's Institutional Review Board.

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Table 1: Demographic and Clinical Characteristics by Statin Use (TILs Participants)

	Statin User (N=156, 29.32%)	Non-Statin User (N=376, 70.68%)	P-value
Age at Diagnosis, years	66.89	58.91	<0.0001
Sex (Male)	67.95%	71.81%	0.37
Race			0.45
White	92.95%	91.49%	
Not white	3.21%	5.59%	
Missing	3.85%	2.93%	
Disease Site			0.78
Larynx	14.10%	15.16%	
Oral Cavity	47.44%	49.20%	
Oropharynx	30.13%	28.99%	
Hypopharynx	3.21%	3.72%	
Other	5.13%	2.93% ¹	
Stage at Diagnosis			0.997
0 and 1	13.46%	13.30%	
2	14.74%	15.16%	
3	14.74%	14.10%	
4	57.05%	57.45%	
HPV status			0.73
Negative	48.08%	48.94%	
Positive	26.28%	28.46%	
Invalid/Missing	25.64%	22.61%	
ACE-27 Score*			<0.0001
None	11.54%	29.26%	
Mild	50.00%	47.07%	
Moderate	21.15%	17.55%	
Severe	15.38%	5.85%	
Missing	1.92%	0.27%	
Highest Education			0.56
Less than high school	3.21%	6.65%	
High school/GED	22.44%	24.20%	
Some College	25.64%	23.67%	
4 year degree	8.97%	9.04%	
More than 4-year degree	10.90%	7.71%	
Missing	28.85%	28.72%	
BMI			0.01
Underweight/Normal Weight	24.36%	39.36%	
Overweight/Obese 1	58.33%	47.07%	
Obese 2/Obese 3	12.82%	9.84%	
Missing	4.49%	3.72%	
Smoking Status			<.0001
Current	26.28%	48.67%	
Former	46.79%	25.80%	
Never	21.79%	22.34%	
Missing	5.13%	3.19%	
Drinking Status			0.13
Current	53.21%	64.10%	
Former	28.85%	21.81%	
Never	12.82%	10.64%	
Missing	5.13%	3.46%	

* Fisher's exact test p-value, for variables that had cell sizes smaller than 5

Abbreviations: ACE-27= Adult Comorbidity Evaluation 27, BMI= Body Mass Index, GED=General Educational Development, HPV= Human Papillomavirus, TILs=Tumor Infiltrating Lymphocytes

Table 2: Demographic and Clinical Characteristics by Statin Use (Cytokines Participants)

	Statin User (N=74, 36.10%)	Non-Statin User (N=131, 63.90%)	P-value
Age at Diagnosis, years	63.34	57.55	<0.0001
Sex (Male)	78.38%	82.44%	0.48
Race			0.75*
White	95.95%	93.89%	
Not white	4.05%	6.11%	
Disease Site			0.05*
Larynx	16.22%	22.90%	
Oral Cavity	24.32%	31.30%	
Oropharynx	44.59%	40.46%	
Hypopharynx	1.35%	2.29%	
Other	13.51%	3.05%	
Stage at Diagnosis			0.97
0 and 1	8.11%	6.87%	
2	8.11%	6.87%	
3	16.22%	16.03%	
4	67.57%	70.23%	
HPV status			0.0015
Negative	35.14%	53.44%	
Positive	39.19%	38.17%	
Invalid/Missing	25.68%	8.40%	
ACE-27 Score			0.0005*
None	13.51%	34.35%	
Mild	51.35%	41.98%	
Moderate	20.27%	20.61%	
Severe	14.86%	3.05%	
Highest Education			0.53*
Less than high school	4.05%	6.87%	
High school/GED	25.68%	32.06%	
Some College	27.03%	28.24%	
4 year degree	14.86%	7.63%	
More than 4-year degree	14.86%	15.27%	
Missing	13.51%	9.92%	
BMI			0.77
Underweight/Normal Weight	29.73%	33.59%	
Overweight/Obese 1	59.46%	58.02%	
Obese 2/Obese 3	10.81%	8.40%	
Smoking Status			0.08
Current	29.73%	45.80%	
Former	43.24%	32.82%	
Never	27.03%	21.37%	
Drinking Status			0.63
Current	64.86%	70.99%	
Former	27.03%	21.37%	
Never	8.11%	7.63%	

* Fisher's exact test p-value, for variables that had cell sizes smaller than 5

Abbreviations: ACE-27= Adult Comorbidity Evaluation 27, BMI= Body Mass Index, GED=General Educational Development, HPV= Human Papillomavirus

Table 3: Multivariable Models-TILs

	Crude Model Median TILs OR (95% CI)	Adjusted Model* Median TILs OR (95% CI)	Crude Model (Log Transformed TILs)	P	Adjusted Model* (Log Transformed TILs)	P
TILws (N=475)			0.06 (0.14)	0.67	0.09 (0.14)	0.54
Non-Statin User	1	1				
Statin User	1.20 (0.80, 1.78)	1.15 (0.74, 1.78)				
FoxP3 (N=485)			0.01 (0.16)	0.95	0.09 (0.17)	0.59
Non-Statin User	1	1				
Statin User	1.01 (0.69, 1.50)	1.13 (0.74, 1.73)				
CD4 (N=481)			0.06 (0.19)	0.76	0.14 (0.20)	0.50
Non-Statin User	1	1				
Statin User	1.08 (0.73, 1.59)	1.23 (0.80, 1.90)				
CD8 (N=481)			0.19 (0.18)	0.31	0.12 (0.19)	0.55
Non-Statin User	1	1				
Statin User	1.19 (0.80, 1.77)	1.10 (0.72, 1.69)				

* Adjusted for age at diagnosis, smoking status and ACE-27

Abbreviations: CD4=Cluster of Differentiation 4, CD8= Cluster of Differentiation 8, CI= Confidence Interval, FoxP3= Forkhead box P3, OR= Odds Ratio, TILs=Tumor Infiltrating Lymphocytes, TILws=Total Weighted Score

Supplemental Table 1: Multivariable Models-Cytokines

N=205	Crude Model Median Cytokines OR (95% CI)	Adjusted Model* Median Cytokines OR (95% CI)	Fully Adjusted Model† Median Cytokines OR (95% CI)	Crude Model (Log Transformed Cytokines)	P	Adjusted Model* (Log Transformed Cytokines)	P	Fully Adjusted Model† (Log Transformed Cytokines)	P
IFN-γ				0.12 (0.46)	0.79	0.09 (0.51)	0.85	0.07 (0.54)	0.89
Non-Statin User	1	1	1						
Statin User	1.17 (0.66, 2.06)	1.19 (0.64, 2.22)	1.15 (0.60, 2.22)						
IL-6[§]				0.11 (0.32)	0.73	0.17 (0.35)	0.63	0.08 (0.37)	0.82
Non-Statin User	1	1	1						
Statin User	0.79 (0.45, 1.39)	0.79 (0.43, 1.48)	0.76 (0.39, 1.46)						
IL-8				0.21 (0.18)	0.25	0.21 (0.20)	0.30	0.15 (0.21)	0.47
Non-Statin User	1	1	1						
Statin User	1.74 (0.97, 3.09)	1.62 (0.87, 3.03)	1.46 (0.75, 2.83)						
IL-10[§]				0.20 (0.39)	0.61	0.32 (0.42)	0.44	0.18 (0.44)	0.68
Non-Statin User	1	1	1						
Statin User	0.93 (0.53, 1.65)	1.01 (0.54, 1.88)	0.97 (0.50, 1.88)						
IL-17[‡]				0.77 (0.42)	0.07	0.87 (0.45)	0.06	0.67 (0.48)	0.16
Non-Statin User	1	1	1						
Statin User	1.71 (0.89, 3.30)	1.91 (0.91, 3.97)	1.64 (0.76, 3.56)						
GRO				0.07 (0.26)	0.80	0.05 (0.28)	0.84	0.01 (0.29)	0.97
Non-Statin User	1	1	1						
Statin User	1.17 (0.66, 2.06)	1.21 (0.65, 2.28)	1.09 (0.56, 2.13)						
HGF				0.31 (0.12)	0.01	0.24 (0.13)	0.08	0.22 (0.14)	0.13
Non-Statin User	1	1	1						
Statin User	1.07 (0.61, 1.90)	0.99 (0.53, 1.86)	1.01 (0.52, 1.96)						
TNF-α[‡]				0.38 (0.36)	0.30	0.42 (0.39)	0.28	0.34 (0.42)	0.41
Non-Statin User	1	1	1						
Statin User	1.05 (0.58, 1.90)	1.04 (0.55, 1.98)	1.01 (0.51, 1.98)						
VEGF				0.07 (0.23)	0.77	0.21 (0.25)	0.40	0.23 (0.26)	0.38
Non-Statin User	1	1	1						
Statin User	1.51 (0.85, 2.67)	1.37 (0.73, 2.57)	1.31 (0.67, 2.55)						
TGF				-0.05 (0.07)	0.49	0.006 (0.08)	0.94	-0.001 (0.08)	0.99
Non-Statin User	1	1	1						
Statin User	0.76 (0.43, 1.35)	0.94 (0.50, 1.78)	0.86 (0.44, 1.68)						

* Adjusted for age at diagnosis, smoking status and ACE-27

† Adjusted for age at diagnosis, smoking status, ACE-27, HPV status and disease site

‡ Due to excess zeros a median split of the data results in a zero vs non-zero split

§ Low cytokines category \leq median

Abbreviations: CI= Confidence Interval, GRO= Growth Related Oncogene, HGF= Hepatocyte Growth Factor, IFN- γ = Interferon Gamma, IL-6= Interleukin-6, IL-8= Interleukin-8, IL-10= Interleukin-10, IL-17= Interleukin-17, OR=Odds Ratio, TGF= Transforming Growth Factor, TNF- α = Tumor Necrosis Factor Alpha, VEGF= Vascular Endothelial Growth Factor

Table 4: HPV-Stratified TILs Models (dropping missing HPV status)

	# of Events	Non-Statin User	# of Events	Statin User
		OR (95% CI)*		OR (95% CI)*
TILws				
HPV status w/o missing				
HPV-Positive	64	1 (REF)	29	2.80 (1.03, 7.61)
HPV-Negative	72	1 (REF)	31	1.07 (0.57, 2.02)
<i>P for interaction</i>	0.1			
FoxP3				
HPV status w/o missing				
HPV-Positive	59	1 (REF)	32	4.15 (1.55, 11.14)
HPV-Negative	81	1 (REF)	27	0.81 (0.43, 1.52)
<i>P for interaction</i>	0.003			
CD4				
HPV status w/o missing				
HPV-Positive	62	1 (REF)	29	2.42 (1.00, 5.86)
HPV-Negative	73	1 (REF)	32	1.42 (0.75, 2.69)
<i>P for interaction</i>	0.32			
CD8				
HPV status w/o missing				
HPV-Positive	63	1 (REF)	29	1.84 (0.75, 4.55)
HPV-Negative	64	1 (REF)	30	1.10 (0.58, 2.07)
<i>P for interaction</i>	0.34			

* Adjusted for age at diagnosis, smoking status and ACE-27

Abbreviations: CD4=Cluster of Differentiation 4, CD8= Cluster of Differentiation 8, CI= Confidence Interval, FoxP3= Forkhead box P3, HPV=Human Papillomavirus, OR= Odds Ratio, TILs=Tumor Infiltrating Lymphocytes, TILws=Total Weighted Score

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Supplemental Table 2: HPV-Stratified Cytokines Models (dropping missing HPV status)

	Non-Statin User		Statin User	
	# of Events	OR (95% CI)*	# of Events	OR (95% CI)*
IFN-γ				
HPV status w/o missing				
HPV-Positive	24	1 (REF)	15	1.32 (0.50, 3.49)
HPV-Negative	34	1 (REF)	14	1.30 (0.50, 3.42)
<i>P for interaction</i>	1			
IL-6				
HPV status w/o missing				
HPV-Positive	22	1 (REF)	14	1.32 (0.50, 3.51)
HPV-Negative	38	1 (REF)	13	0.77 (0.29, 2.02)
<i>P for interaction</i>	0.42			
IL-8				
HPV status w/o missing				
HPV-Positive	21	1 (REF)	17	1.96 (0.73, 5.27)
HPV-Negative	33	1 (REF)	14	1.14 (0.44, 2.99)
<i>P for interaction</i>	0.43			
IL-10				
HPV status w/o missing				
HPV-Positive	26	1 (REF)	16	1.27 (0.48, 3.39)
HPV-Negative	35	1 (REF)	11	0.80 (0.30, 2.13)
<i>P for interaction</i>	0.50			
IL-17				
HPV status w/o missing				
HPV-Positive	9	1 (REF)	11	3.95 (1.26, 12.38)
HPV-Negative	11	1 (REF)	5	1.29 (0.36, 4.67)
<i>P for interaction</i>	0.17			
GRO				
HPV status w/o missing				
HPV-Positive	25	1 (REF)	16	1.39 (0.52, 3.72)
HPV-Negative	33	1 (REF)	12	0.95 (0.36, 2.53)
<i>P for interaction</i>	0.58			
HGF				
HPV status w/o missing				
HPV-Positive	20	1 (REF)	16	2.27 (0.82, 6.27)
HPV-Negative	38	1 (REF)	11	0.47 (0.17, 1.31)
<i>P for interaction</i>	0.03			
TNF-α				
HPV status w/o missing				
HPV-Positive	16	1 (REF)	12	1.69 (0.62, 4.64)
HPV-Negative	26	1 (REF)	8	0.73 (0.26, 2.05)
<i>P for interaction</i>	0.23			
VEGF				
HPV status w/o missing				
HPV-Positive	22	1 (REF)	19	2.46 (0.90, 6.78)
HPV-Negative	32	1 (REF)	11	0.76 (0.28, 2.02)
<i>P for interaction</i>	0.09			
TGF				
HPV status w/o missing				
HPV-Positive	28	1 (REF)	14	1.13 (0.41, 3.08)
HPV-Negative	34	1 (REF)	12	0.88 (0.32, 2.42)
<i>P for interaction</i>	0.73			

* Adjusted for age at diagnosis, smoking status and ACE-27

Abbreviations: CI= Confidence Interval, GRO= Growth Related Oncogene, HGF= Hepatocyte Growth Factor, HPV= Human Papillomavirus, IFN- γ = Interferon Gamma, IL-6= Interleukin-6, IL-8= Interleukin-8, IL-10= Interleukin-10, IL-17= Interleukin-17, OR=Odds Ratio, TGF= Transforming Growth Factor, TNF- α = Tumor Necrosis Factor Alpha, VEGF= Vascular Endothelial Growth Factor

Supplemental Table 3: HPV Stratified Analysis using various strategies to account for missing data on HPV status (TILs)

Outcomes	Non-Statin User		Statin User	
	No. of Events	OR (95% CI)*	No. of Events	OR (95% CI)*,†
TILws				
HPV status with missing				
HPV-Positive	64	1 (REF)	29	2.60 (0.96, 7.02)
HPV-Negative	72	1 (REF)	31	0.93 (0.50, 1.74)
HPV-Missing/Invalid	29	1 (REF)	13	0.87 (0.37, 2.03)
HPV status IPW				
HPV-Positive	57	1 (REF)	27	3.16 (1.06, 9.47)
HPV-Negative	70	1 (REF)	31	1.02 (0.51, 2.05)
FoxP3				
HPV status with missing				
HPV-Positive	59	1 (REF)	32	3.83 (1.44, 10.18)
HPV-Negative	81	1 (REF)	27	0.74 (0.40, 1.37)
HPV-Missing/Invalid	32	1 (REF)	12	0.81 (0.35, 1.88)
HPV status IPW				
HPV-Positive	52	1 (REF)	30	5.51 (1.91, 15.86)
HPV-Negative	79	1 (REF)	26	0.66 (0.34, 1.30)
CD4				
HPV status with missing				
HPV-Positive	62	1 (REF)	29	2.14 (0.89, 5.14)
HPV-Negative	73	1 (REF)	32	1.20 (0.65, 2.22)
HPV-Missing/Invalid	33	1 (REF)	12	0.74 (0.32, 1.72)
HPV status IPW				
HPV-Positive	57	1 (REF)	27	2.47 (0.98, 6.22)
HPV-Negative	69	1 (REF)	31	1.29 (0.64, 2.62)
CD8				
HPV status with missing				
HPV-Positive	63	1 (REF)	29	1.85 (0.75, 4.55)
HPV-Negative	64	1 (REF)	30	1.06 (0.57, 1.97)
HPV-Missing/Invalid	39	1 (REF)	16	0.69 (0.31, 1.55)
HPV status IPW				
HPV-Positive	57	1 (REF)	27	2.22 (0.82, 6.01)
HPV-Negative	62	1 (REF)	29	1.12 (0.56, 2.22)

*Adjusted for age at diagnosis, smoking status and ACE-27

† Inverse probability weighted model

Abbreviations: CD4=Cluster of Differentiation 4, CD8= Cluster of Differentiation 8, CI= Confidence Interval, FoxP3= Forkhead box P3, HPV= Human Papillomavirus, IPW=Inverse Probability Weight, OR= Odds Ratio, TILs=Tumor Infiltrating Lymphocytes, TILws=Total Weighted Score

Supplemental Table 4: HPV Stratified Analysis Using Various Strategies to Account for Missing Data on HPV Status (Cytokines)

Outcomes	Non-Statin User		Statin User	
	No. of Events	OR (95% CI)*	No. of Events	OR (95% CI) [†]
IFN-γ				
HPV status with missing				
HPV-Positive	24	1 (REF)	15	1.20 (0.46, 3.17)
HPV-Negative	34	1 (REF)	14	1.29 (0.50, 3.33)
HPV-Missing/Invalid	6	1 (REF)	10	0.997 (0.22, 4.55)
HPV status IPW				
HPV-Positive	24	1 (REF)	15	1.35 (0.49, 3.72)
HPV-Negative	34	1 (REF)	14	1.23 (0.46, 3.28)
IL-6				
HPV status with missing				
HPV-Positive	22	1 (REF)	14	1.32 (0.50, 3.49)
HPV-Negative	38	1 (REF)	13	0.83 (0.32, 2.17)
HPV-Missing/Invalid	8	1 (REF)	7	0.22 (0.04, 1.15)
HPV status IPW				
HPV-Positive	22	1 (REF)	14	1.48 (0.56, 3.94)
HPV-Negative	37	1 (REF)	13	1.03 (0.36, 2.90)
IL-8				
HPV status with missing				
HPV-Positive	21	1 (REF)	17	1.87 (0.70, 4.98)
HPV-Negative	33	1 (REF)	14	1.19 (0.46, 3.07)
HPV-Missing/Invalid	6	1 (REF)	13	1.68 (0.35, 7.95)
HPV status IPW				
HPV-Positive	21	1 (REF)	17	2.17 (0.80, 5.92)
HPV-Negative	32	1 (REF)	14	1.50 (0.54, 4.16)
IL-10				
HPV status with missing				
HPV-Positive	26	1 (REF)	16	1.18 (0.44, 3.12)
HPV-Negative	35	1 (REF)	11	0.78 (0.30, 2.05)
HPV-Missing/Invalid	5	1 (REF)	9	1.26 (0.27, 5.84)
HPV status IPW				
HPV-Positive	26	1 (REF)	16	1.31 (0.47, 3.63)
HPV-Negative	35	1 (REF)	11	0.81 (0.30, 2.17)
IL-17				
HPV status with missing				
HPV-Positive	9	1 (REF)	11	3.69 (1.20, 11.38)
HPV-Negative	11	1 (REF)	5	1.39 (0.40, 4.84)
HPV-Missing/Invalid	6	1 (REF)	6	0.41 (0.09, 1.99)
HPV status IPW				
HPV-Positive	9	1 (REF)	11	3.82 (1.17, 12.51)
HPV-Negative	11	1 (REF)	5	1.69 (0.42, 6.78)
GRO				
HPV status with missing				
HPV-Positive	25	1 (REF)	16	1.32 (0.49, 3.54)
HPV-Negative	33	1 (REF)	12	0.99 (0.38, 2.59)
HPV-Missing/Invalid	6	1 (REF)	11	1.21 (0.26, 5.71)
HPV status IPW				
HPV-Positive	25	1 (REF)	16	1.41 (0.51, 3.92)
HPV-Negative	33	1 (REF)	12	1.02 (0.37, 2.80)
HGF				
HPV status with missing				
HPV-Positive	20	1 (REF)	16	2.11 (0.78, 5.73)
HPV-Negative	38	1 (REF)	11	0.49 (0.18, 1.33)
HPV-Missing/Invalid	7	1 (REF)	11	0.65 (0.14, 3.15)
HPV status IPW				
HPV-Positive	20	1 (REF)	16	2.23 (0.83, 5.95)

HPV-Negative	37	1 (REF)	11	0.64 (0.20, 2.06)
TNF-α				
HPV status with missing				
HPV-Positive	16	1 (REF)	12	1.59 (0.58, 4.35)
HPV-Negative	26	1 (REF)	8	0.74 (0.27, 2.04)
HPV-Missing/Invalid	6	1 (REF)	8	0.64 (0.14, 2.95)
HPV status IPW				
HPV-Positive	16	1 (REF)	12	1.67 (0.58, 4.86)
HPV-Negative	25	1 (REF)	8	0.88 (0.29, 2.73)
VEGF				
HPV status with missing				
HPV-Positive	22	1 (REF)	19	2.32 (0.85, 6.32)
HPV-Negative	32	1 (REF)	11	0.74 (0.28, 1.95)
HPV-Missing/Invalid	7	1 (REF)	12	0.998 (0.21, 4.81)
HPV status IPW				
HPV-Positive	22	1 (REF)	19	2.82 (1.07, 7.44)
HPV-Negative	31	1 (REF)	11	0.64 (0.22, 1.84)
TGF				
HPV status with missing				
HPV-Positive	28	1 (REF)	14	1.14 (0.42, 3.05)
HPV-Negative	34	1 (REF)	12	0.99 (0.37, 2.66)
HPV-Missing/Invalid	7	1 (REF)	8	0.40 (0.08, 1.91)
HPV status IPW				
HPV-Positive	28	1 (REF)	14	1.25 (0.47, 3.35)
HPV-Negative	34	1 (REF)	12	0.80 (0.30, 2.16)

*Adjusted for age at diagnosis, smoking status and ACE-27

† Inverse probability weighted model

Abbreviations: CI= Confidence Interval, GRO= Growth Related Oncogene, HGF= Hepatocyte Growth Factor, HPV= Human Papillomavirus, IFN- γ = Interferon Gamma, IL-6= Interleukin-6, IL-8= Interleukin-8, IL-10= Interleukin-10, IL-17= Interleukin-17, IPW= Inverse Probability Weight, OR=Odds Ratio, TGF= Transforming Growth Factor, TNF- α = Tumor Necrosis Factor Alpha, VEGF= Vascular Endothelial Growth Factor

Supplemental Table 5: Multivariable Models: TILs using IPW to Account for Missing Values

	Crude Model Median TILs OR (95% CI)	Adjusted Model* Median TILs OR (95% CI)
TILws (N=427)		
Non-Statin User	1	1
Statin User	1.06 (0.67, 1.68)	1.08 (0.65, 1.80)
FoxP3 (N=437)		
Non-Statin User	1	1
Statin User	1.08 (0.69, 1.70)	1.23 (0.75, 2.01)
CD4 (N=433)		
Non-Statin User	1	1
Statin User	1.06 (0.67, 1.67)	1.21 (0.74, 1.98)
CD8[†] (N=433)		
Non-Statin User	1	1
Statin User	1.11 (0.70, 1.75)	1.05 (0.64, 1.74)

* Adjusted for age at diagnosis, smoking status and ACE-27

† Low TILs category ≤ median

Abbreviations: CD4=Cluster of Differentiation 4, CD8= Cluster of Differentiation 8, CI= Confidence Interval, FoxP3= Forkhead box P3, IPW= Inverse Probability Weight, OR= Odds Ratio, TILs=Tumor Infiltrating Lymphocytes, TILws=Total Weighted Score

Supplemental Table 6: Multivariable Models: Cytokines Using IPW to Account for Missing Values

	Crude Model Median Cytokines OR (95% CI)	Adjusted Model* Median Cytokines OR (95% CI)
IFN-γ (N=204)		
Non-Statin User	1	1
Statin User	0.78 (0.41, 1.51)	0.76 (0.37, 1.56)
IL-6 (N=204)[†]		
Non-Statin User	1	1
Statin User	0.76 (0.40, 1.45)	0.88 (0.45, 1.75)
IL-8 (N=204)		
Non-Statin User	1	1
Statin User	1.73 (0.89, 3.38)	1.87 (0.91, 3.82)
IL-10 (N=204)[†]		
Non-Statin User	1	1
Statin User	0.72 (0.38, 1.38)	0.80 (0.38, 1.68)
IL-17 (N=204)[‡]		
Non-Statin User	1	1
Statin User	1.36 (0.64, 2.89)	1.63 (0.70, 3.77)
GRO (N=204)		
Non-Statin User	1	1
Statin User	0.86 (0.45, 1.66)	0.84 (0.40, 1.75)
HGF (N=204)		
Non-Statin User	1	1
Statin User	0.84 (0.44, 1.62)	0.81 (0.40, 1.66)
TNF-α (N=204)[‡]		
Non-Statin User	1	1
Statin User	0.74 (0.38, 1.45)	0.74 (0.34, 1.60)
VEGF (N=204)		
Non-Statin User	1	1
Statin User	1.28 (0.66, 2.48)	1.29 (0.64, 2.61)
TGF (N=204)		
Non-Statin User	1	1
Statin User	0.91 (0.47, 1.76)	1.02 (0.49, 2.14)

* Adjusted for age at diagnosis, smoking status and ACE-27

[†] Median split is \leq median, =0; $>$ median=1

[‡] Due to excess zeros a median split of the data results in a zero vs non-zero split

Abbreviations: CI= Confidence Interval, GRO= Growth Related Oncogene, HGF= Hepatocyte Growth Factor, IFN- γ = Interferon Gamma, IL-6= Interleukin-6, IL-8= Interleukin-8, IL-10= Interleukin-10, IL-17= Interleukin-17, IPW= Inverse Probability Weight, OR=Odds Ratio, TGF= Transforming Growth Factor, TNF- α = Tumor Necrosis Factor Alpha, VEGF= Vascular Endothelial Growth Factor