Mutational Landscape of Recurrent/Metastatic Head and Neck Squamous Cell Carcinoma and Association with Immune Checkpoint Inhibitor Outcomes

Patricia McCoon[1], Ying Wang[1], Zhongwu Lai[1], Qu Zhang[2], Weimin Li[2], Sophie Wildsmith[3], Nassim Morsli[3], Rajiv Raja[2], Nicholas Holoweckyj[2], Jill Walker[3], Melissa de los Reyes[2], Ricard Mesía[4], Lisa Licitra[5], Robert L. Ferris[6], Jérôme Fayette[7], Dan P. Zandberg[6], Lillian L. Siu[8], and Robert Haddad[9]

ABSTRACT

Purpose: Understanding the mutational landscape of recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC) is important in identifying biomarkers to determine which patients may benefit from immune checkpoint inhibitors (ICI).

Experimental Design: The HAWK (NCT02207530), CONDOR (NCT02319044), and EAGLE (NCT02369874) studies evaluated R/M HNSCC treatment with durvalumab or durvalumab–tremelimumab. Tumor tissue samples pooled from HAWK/CONDOR ($n = 153$) and plasma cell–free DNA samples from EAGLE ($n = 285$) were analyzed to identify somatic alterations and association with survival.

Results: The mutational landscape was similar in tissue and plasma. Compared with the wild type, *TP53* mutations were associated with significantly shorter overall survival (OS; HR; 95% confidence interval) with standard of care (SoC; EAGLE: 2.12; 1.20–3.78) and ICIs (HAWK/CONDOR: 1.49; 1.05–2.12 and EAGLE: 1.44; 0.99–2.10). In EAGLE, patients with *TP53* mutations had significantly longer OS with durvalumab–tremelimumab versus SoC ($P = 0.045$). *KMT2D* mutations were associated with a trend toward longer OS (HR; 95% confidence interval) versus the wild type in HAWK/CONDOR (0.81; 0.56–1.19) and a trend toward longer OS with ICIs versus SoC in EAGLE. For both mutations, a European Cooperative Oncology Group performance status of 1 was associated with worsened OS, and PD-L1 positivity was associated with improved OS.

Conclusions: This is the first large-scale study to show the mutational landscape of R/M HNSCC and its association with clinical outcomes in patients treated with ICIs or SoC. The *TP53* mutation was a negative prognostic marker; however, treatment with durvalumab–tremelimumab significantly improved survival over SoC. Further investigation of *KMT2D* as a predictive biomarker for immunotherapy in R/M HNSCC is warranted.

Introduction

Recurrent or metastatic head and neck squamous cell carcinoma (R/M HNSCC) is associated with poor prognosis and survival, with objective response rates of 4% to 14% and median overall survival (OS) of 4.3 to 6.7 months, following second-line treatment with chemotherapeutics (methotrexate, cetuximab, or paclitaxel; ref. 1).

Immune checkpoint inhibitors (ICI) targeting PD-1 and PD-L1 have demonstrated significant survival benefit for various tumor types when compared with standard therapies in prospective randomized clinical trials (2–4). Pembrolizumab (anti–PD-1) is approved by the FDA and European Medicines Agency (EMA) for the first-line treatment of metastatic or unresectable, recurrent HNSCC in combination with platinum and 5-fluorouracil [by the EMA for patients whose tumors express PD-L1 with a combined positive score (CPS) ≥1] or as monotherapy for patients whose tumors express PD-L1 with a CPS ≥1 (5, 6). Pembrolizumab and nivolumab (anti–PD-1) are approved by the FDA and EMA as monotherapy for R/M HNSCC with disease progression on/after platinum-based chemotherapy (pembrolizumab is approved by the EMA for patients whose tumors express PD-L1 with a tumor proportion score ≥50%; refs. 5–8), and in this setting, durvalumab (anti–PD-L1) with/without tremelimumab (anti–cytotoxic T-lymphocyte–associated antigen 4) has demonstrated variable antitumor activity (9–11).

Although treatment with ICIs can result in significant clinical benefit, many patients with R/M HNSCC experience limited efficacy; thus, biomarkers to identify patients most likely to benefit from ICI treatment are of increasing interest (9–13). Genomic complexity, intratumoral genetic heterogeneity, and high instability of HNSCC genomes may explain this treatment resistance and the tendency for locoregional recurrence (14, 15). PD-L1 has shown potential as a biomarker (13), with PD-L1 status used to guide treatment selection for R/M HNSCC [pembrolizumab monotherapy is recommended for untreated HNSCC with PD-L1 CPS ≥1 (FDA and EMA) and in patients with disease progression on/after

[1]AstraZeneca, Waltham, Massachusetts. [2]AstraZeneca, Gaithersburg, Maryland. [3]AstraZeneca, Cambridge, United Kingdom. [4]Catalan Institute of Oncology, B-ARGO Group, IGTP, Badalona, Spain. [5]Head and Neck Medical Oncology, IRCCS Istituto Nazionale Tumori Milan and University of Milan, Milan, Italy. [6]UPMC Hillman Cancer Center, University of Pittsburgh, Pittsburgh, Pennsylvania. [7]Centre Léon Bérard, Lyon, France. [8]Princess Margaret Cancer Centre, Toronto, Canada. [9]Dana-Farber Cancer Institute, Boston, Massachusetts.

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Corresponding Author: Patricia McCoon, Translational Medicine, Oncology R&D, AstraZeneca, 35 Gatehouse Dr, Waltham, MA 02451. E-mail: patricia.mccoon@astrazeneca.com

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Translational Relevance

In many patients with recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC), the efficacy of immune checkpoint inhibitors (ICI) is limited. This retrospective molecular analysis of data from the HAWK, CONDOR, and EAGLE studies, which investigated durvalumab with/without tremelimumab, seeks to further understand the mutational landscape of R/M HNSCC and identify biomarkers that can help ascertain which patients with R/M HNSCC with disease progression on/after platinum-based chemotherapy may benefit from treatment with ICIs. The *TP53* mutation was found to be a negative prognostic biomarker in R/M HNSCC, yet durvalumab plus tremelimumab performed significantly better than standard-of-care chemotherapy for patients with the *TP53* mutation. *KMT2D* mutations were also associated with sensitivity to ICIs, and further investigation of *KMT2D* as a predictive biomarker for immunotherapy is warranted.

chemotherapy with PD-L1 and a tumor proportion score ≥50% (EMA); refs. 5, 6]. A recent study demonstrated that the tumor mutational burden (TMB) is predictive of survival with second-line ICI treatment versus chemotherapy in platinum-resistant R/M HNSCC (16). Analysis of plasma-derived ctDNA is a noninvasive method that has shown potential for guiding treatment selection in some tumor types, including HNSCC (16–18).

Furthermore, clinical differences exist between HNSCCs: oropharyngeal cancers and human papillomavirus (HPV)–positive HNSCCs are associated with better clinical outcomes compared with non–oropharyngeal cancers and HPV-negative HNSCCs, respectively, with oropharyngeal cancers also being more frequently HPV-positive (64%) compared with non–oropharyngeal cancers (6%), although regional variations in HNSCC etiology should be taken into account (15, 19, 20). Understanding the molecular landscape of HNSCCs may illuminate the mechanisms underlying differential clinical outcomes and allow targeted treatment selection.

In this retrospective study, we conducted a comprehensive molecular analysis of data from the HAWK (9), CONDOR (10), and EAGLE (11) studies investigating durvalumab with/without tremelimumab in R/M HNSCC with disease progression on/after platinum-based chemotherapy and investigated the associations of identified mutations with survival after ICI treatment. These data further define the mutational landscape in R/M HNSCC and provide preliminary evidence for biomarkers that may be predictive of clinical benefit from ICI therapy.

Materials and Methods

Patients

Patients aged ≥18 years with histologically or cytologically (EAGLE only) confirmed R/M HNSCC of the oral cavity, oropharynx, larynx, or hypopharynx were included (9–11). In HAWK, only patients with PD-L1–high expression [tumor cells (TC) ≥25%] were included, whereas CONDOR only included those with PD-L1–low or –negative expression (TCs < 25%). Patients were not amenable to curative therapy and had tumor progression or recurrence during/after systemic treatment with one platinum-based regimen for R/M disease (HAWK/CONDOR/EAGLE) or progression within 6 months of the last dose of platinum therapy given as part of multimodality therapy with curative intent (EAGLE; refs. 9–11). All studies were conducted in accordance with ethical principles originating from the Declaration of Helsinki and consistent with International Conference on Harmonization and Good Clinical Practice guidelines, applicable regulatory requirements, and sponsor's policy on Bioethics and Human Biological Samples. All patients provided written informed consent before performing any protocol-related procedures (9–11).

Study designs

Study designs from HAWK, CONDOR, and EAGLE have been previously published (9–11). The phase II HAWK (NCT02207530; single-arm) and CONDOR (NCT02319044; randomized) and the phase III EAGLE (NCT02369874; randomized) studies were international, multicenter studies investigating second-line treatment with durvalumab monotherapy [10 mg/kg i.v. every 2 weeks (HAWK, CONDOR, and EAGLE)] and/or tremelimumab monotherapy [10 mg/kg i.v. every 4 weeks for seven doses, then every 12 weeks for two doses for up to 12 months (CONDOR)] and/or their combination [durvalumab 20 mg/kg every 4 weeks plus tremelimumab 1 mg/kg every 4 weeks for four cycles followed by durvalumab 10 mg/kg every 2 weeks (CONDOR and EAGLE)] in patients with R/M HNSCC (9–11). In CONDOR, the randomization ratio was 2:1:1 for combination therapy, durvalumab monotherapy, and tremelimumab monotherapy arms, respectively. In EAGLE, patients were randomized 1:1:1 to durvalumab monotherapy, durvalumab plus tremelimumab in combination, or investigator's choice of single-agent standard of care (SoC), administered according to local regulations.

Sample collection for biomarker analysis was a prespecified exploratory objective of the HAWK, CONDOR, and EAGLE studies. In HAWK and CONDOR, either newly acquired (preferred) or archival tumor tissue (<3 years old) was collected (9, 10). In EAGLE, plasma samples were collected at baseline. Tumor samples from the phase II HAWK/CONDOR studies were pooled and used for signal finding and proof of concept. ctDNA analysis of plasma samples from the randomized phase III EAGLE study was used to assess whether mutations were predictive biomarkers of immunotherapy.

Mutational profiling of tumor tissue and plasma-derived ctDNA

In the HAWK/CONDOR studies, paired formalin-fixed, paraffin-embedded tumor (recent or archival tissue <3 years old) and peripheral blood mononuclear cell samples (germline controls) were evaluated by whole-exome sequencing, with 100 million reads at 200× coverage, using the HiSeq 4000 system (Illumina) for 2 × 100 paired-end reads. Somatic alterations were identified using VarDict (AstraZeneca; ref. 21). Only single-nucleotide variants (SNV) and small insertions and deletions (indels; <20 bp) with allele frequencies <80% and ≥5%, respectively, were retained in the mutation-calling pipeline.

In EAGLE, baseline plasma samples containing ctDNA were profiled to identify somatic alterations, including SNVs, indels, and copy-number amplifications, using the GuardantOMNI platform (Guardant Health), a targeted next-generation sequencing platform using a 500-gene panel with a 2 Mb DNA footprint (1 Mb coding regions only). Copy-number losses are typically hard to assess from ctDNA; hence, only amplifications derived from plasma are reported here.

The number of somatic mutations per patient sample was used to assess the mutational burden of tumors. Genes for investigation were selected based on historical evidence around HNSCC-associated mutations (15). Oncogenic and likely oncogenic mutations were annotated using information from ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Catalogue of Somatic Mutations in Cancer (https://cancer.sanger.ac.uk/cosmic), and OncoKB (https://www.oncokb.org/; ref. 22).

Evaluation of other biomarkers

PD-L1 expression status was determined using the VENTANA PD-L1 (SP263) Assay (Ventana Medical Systems), with high PD-L1 expression defined as patients whose tumors express PD-L1 in ≥25% of TCs (23). HPV was assessed locally using any method (HAWK, CONDOR, and EAGLE), or centrally using p16 IHC (HAWK and CONDOR).

Statistical analysis

The Wilcoxon rank-sum test was used to compare continuous variables, and the Fisher exact test was used to compare binary data. All *P* values were two-sided. Analyses were performed using SAS (version 9.4, SAS Institute) and R (version 4.1.2, R Foundation). Survival analyses were run using the R "survival" package (24, 25). The Kaplan–Meier method was used to calculate univariate survival estimates for OS and progression-free survival (PFS). A Cox proportional hazard model was used to define the association between the mutational status of genes with HRs for OS and PFS. *P* values were assessed using the log-rank test. Multivariate Cox proportional hazard model analyses were used to compute HRs and 95% confidence intervals for *TP53* and *KMT2D* mutations in the EAGLE dataset, using risk factors that included mutation presence, treatment arm, HPV status, PD-L1 status, smoking status, TMB category, European Cooperative Oncology Group (ECOG) performance status, and mutation × treatment arm interaction. Schoenfeld residuals by time were checked for proportional hazard (26). Statistical measures of biomarker data and association with clinical outcomes are descriptive only and considered exploratory.

Data availability

Data underlying the findings described in this article may be obtained in accordance with AstraZeneca's data sharing policy described at: https://astrazenecagrouptrials.pharmacm.com/ST/Submission/Disclosure. Data for studies directly listed on Vivli can be requested through Vivli at www.vivli.org. Data for studies not listed on Vivli could be requested through Vivli at https://vivli.org/members/enquiries-about-studies-not-listed-on-the-vivli-platform/. The AstraZeneca Vivli member page is also available outlining further details: https://vivli.org/ourmember/astrazeneca/.

Results

Patient demographics and clinical characteristics

Tissue samples were available for analysis from 153 of 378 patients from the HAWK ($n = 50/111$) and CONDOR ($n = 103/267$) studies, and plasma samples were obtained from 285 of 736 patients in the EAGLE study. Between HAWK/CONDOR and EAGLE, patients' baseline clinical characteristics were generally similar, except for ECOG performance status and HPV status (**Table 1**). Objective response rates in the HAWK/CONDOR and EAGLE studies were also summarized (**Table 1**).

Somatic mutational patterns in second-line R/M HNSCC tumor tissue and plasma-derived ctDNA

In both datasets, significantly more somatic mutation counts (number of somatic SNVs/indels) were detected in samples from patients with versus without smoking history (**Fig. 1A** and **B**). No association was observed between somatic mutation counts and PD-L1 or HPV status in tissue or plasma samples (**Fig. 1C–F**). There was no statistical difference in the number of somatic mutation counts between HPV-positive or HPV-negative patients with oropharyngeal cancer and a history of smoking, although a numerically higher number of mutations were seen in patients with smoking history, regardless of HPV status (Supplementary Fig. S1).

Mutational landscape similarities between tumor tissue and plasma-derived ctDNA

A similar mutational landscape was observed in the HAWK/CONDOR tissue and EAGLE plasma samples across demographics and tumor locations (**Fig. 1G** and **H**). Mutational prevalence was significantly correlated between tissue and plasma samples (Supplementary Fig. S2; Spearman $\rho = 0.456$, $P = 0.001$). Six genes had mutational prevalence of ≥20% in EAGLE [*TP53*, *FAT1* (promotor mutations found in EAGLE plasma samples only), *NOTCH1*, *PIK3CA*, *KMT2D*, and *TERT*; **Fig. 1H** and **Table 2**]. *TP53* was the most frequently mutated gene in both tissue (64%) and plasma (78%) samples; other tumor-suppressor gene mutations had moderate prevalence (*NF1*, 16% and 9%; *IGF2R*, 13% and 6%; *PTCH1*, 13% and 7%, respectively). Frequent mutations were identified in several epigenetic regulation and DNA damage response and repair (DDR) genes, with comparable prevalence found between tissue and plasma samples (**Fig. 1G** and **H**; **Table 2**).

A significantly higher prevalence of *TP53* mutations was observed in the HPV-negative versus HPV-positive subgroup in both tissue (78% vs. 28%, $P = 1.4e{-}08$) and plasma (85% vs. 51%, $P = 3.9e{-}07$) samples (**Table 2**). The prevalence of *PIK3CA* mutations was numerically greater in the HPV-positive versus HPV-negative subgroup for tissue samples (24% vs. 14%, $P = 0.16$); however, for plasma samples, the prevalence of *PIK3CA* mutations was numerally lower in the HPV-positive versus HPV-negative subgroup (16% vs. 22%, $P = 0.36$). *NOTCH1* mutations had similar prevalence between HPV subgroups for tissue samples but were more prevalent in the HPV-negative subgroup for plasma samples. In plasma samples, *TERT* promoter mutations were significantly more prevalent in the HPV-negative versus HPV-positive subgroup (25% vs. 9%, $P = 0.007$). Among a total of 70 observed *TERT* mutations, 59 mutations occurring in 57 patients were in the promoter and were likely oncogenic [based on OncoKB (22)] recurrent mutations, −124 C>T, $n = 34$ and −146 C>T, $n = 11$.

Copy-number alteration landscape in R/M HNSCC

Amplifications were identified in 98 genes from 145 of 285 (51%) patients in EAGLE plasma samples; for patients with amplifications, a median of 3 was found (**Fig. 2**). *CCND1* on 11q13 was the most frequently amplified gene, amplified more frequently in HPV-negative than HPV-positive tumors (29% vs. 11%, $P = 0.005$). *FGF3*, *FGF19*, *PIK3CA*, and *PIK3CB* had recurrent amplifications in >10% of plasma samples (**Fig. 2**). Notably, *CCND1*, *FGF3*, and *FGF19* on 11q13 were co-amplified in samples from most patients, as were amplifications in *PIK3CA* and *PIK3CB*. Recurrent amplifications in several receptor tyrosine kinases, such as *EGFR* and *ERBB2*, predominated in HPV-negative tumors.

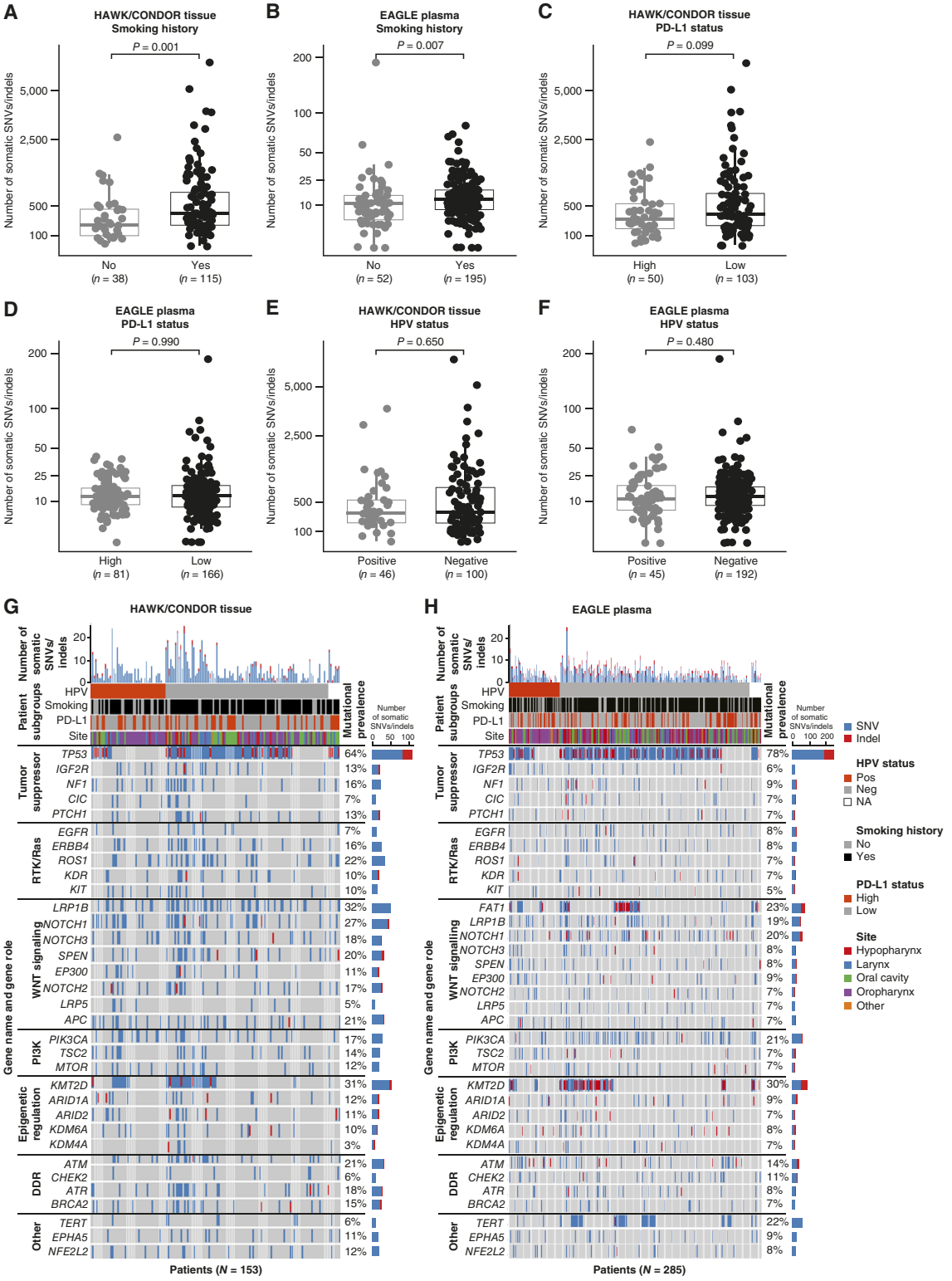

Figure 1.
Somatic SNVs/indels and somatic mutational patterns derived from tumor tissue and plasma samples from patients with R/M HNSCC. Somatic SNV/indel counts detected in the HAWK/CONDOR tissue and EAGLE plasma samples by smoking history (**A** and **B**, respectively), PD-L1 status (**C** and **D**, respectively), and HPV status (**E** and **F**, respectively). The somatic mutational patterns across demographics and tumor sites in the HAWK/CONDOR tissue samples (**G**) and the EAGLE plasma samples (**H**). NA, not applicable; Neg, negative; Pos, positive; RTK, receptor tyrosine kinase.

Table 1. Patient demographics and clinical characteristics in the HAWK/CONDOR tissue and EAGLE plasma cohorts.

	HAWK/CONDOR (*N* = 153[a])	EAGLE (*N* = 285)
Median (range) age, years	60 (23–84)	60 (23–84)
Age <65 years	106 (69.3)	128 (44.9)
Sex, male	114 (74.5)	241 (84.6)
Race, White	132 (86.3)	211 (74.0)
ECOG performance status = 1	116 (75.8)	194 (68.1)
Smoking history		
Yes	115 (75.2)	219 (76.8)
No	38 (24.8)	66 (23.2)
Primary tumor site		
Oral cavity	44 (28.8)	70 (24.6)
Oropharynx	66 (43.1)	113 (39.6)
Hypopharynx	18 (11.8)	53 (18.6)
Larynx	24 (15.7)	40 (14.0)
Other	1 (0.7)	9 (3.2)
PD-L1 high	50 (32.7)	94 (33.0)
HPV positive	46 (30.1)	57 (20.0)
Oral cavity	7 (4.6)	6 (2.1)
Oropharynx	30 (19.6)	38 (13.3)
Hypopharynx	3 (2.0)	5 (1.8)
Larynx	6 (3.9)	4 (1.4)
Other	0 (0)	4 (1.4)
Objective response		
CR	0 (0)	7 (2.5)
PR	17 (11.1)	51 (17.9)
SD	34 (22.2)	75 (26.3)
PD	96 (62.7)	143 (50.2)
NE	6 (3.9)	9 (3.2)

Data are *n* (%) unless otherwise specified.
Abbreviations: CR, complete response; NE, not evaluated; PD, progressive disease; PR, partial response; SD, stable disease.
[a]50 tissue samples were obtained from patients enrolled in HAWK, and 103 tissue samples were obtained from patients enrolled in CONDOR.

TP53 mutations and resistance to immunotherapy and SoC chemotherapy in R/M HNSCC

TP53 mutations were identified in 64% of patients in HAWK/CONDOR and 78% of patients in EAGLE (**Fig. 1G** and **H**; **Table 2**). Compared with the wild type (WT), *TP53* mutations were associated with shorter OS in HAWK/CONDOR (ICI, $P = 0.024$; **Fig. 3A**) and in EAGLE (SoC, $P = 0.009$; **Fig. 3B** and ICI, $P = 0.055$; **Fig. 3C**). Similar results were observed for PFS (Supplementary Fig. S3A–S3C), supporting *TP53* mutation as a negative prognostic marker.

In EAGLE, the 18-month OS rate of ICI-treated patients whose tumors harbored *TP53* mutations was roughly four times that of patients treated with SoC (23–25% vs. 6%, respectively; **Fig. 3D**). In patients with *TP53* mutations, OS HRs were <1, favoring ICIs over SoC (**Fig. 3D**). Based on this observation, a multivariate Cox proportional hazard model analysis of the EAGLE dataset was undertaken which showed significantly longer OS for patients with *TP53* mutations who were treated with durvalumab plus tremelimumab ($P = 0.045$) and a trend toward improved OS for patients treated with durvalumab versus SoC (**Fig. 3E**). OS HRs were not confounded by TMB category [even though *TP53* mutations were significantly correlated with increased somatic mutation numbers, a surrogate of the TMB (Supplementary Fig. S3D)], HPV status, or smoking history; however, ECOG performance status and PD-L1 status were strongly associated with OS (**Fig. 3E**).

In EAGLE, in patients whose tumors harbored *TP53* mutations, durvalumab significantly improved OS versus SoC in patients with non–oropharyngeal cancer ($P = 0.012$), and durvalumab and durvalumab plus tremelimumab both numerically improved 18-month OS rates versus SoC in patients with non–oropharyngeal cancer and oropharyngeal cancer (**Fig. 3F** and **G**).

Predictability of mutations for immunotherapy outcomes

The TMB was greater in tissue and plasma samples with *KMT2D* mutations compared with WT *KMT2D* (**Fig. 4A** and **B**). Compared with the WT, a trend of longer OS was found in patients with *KMT2D* mutations in HAWK/CONDOR (**Fig. 4C**), whereas shorter OS was found in the EAGLE SoC arm (**Fig. 4D**). In EAGLE, patients whose tumors harbored *KMT2D* mutations showed significantly longer OS with ICIs versus SoC (**Fig. 4E**). A multivariate Cox proportional hazard model analysis of the EAGLE dataset showed a trend toward longer OS for patients with *KMT2D* mutations treated with durvalumab compared with patients treated with SoC (**Fig. 4F**). OS HRs were not associated with TMB category, HPV status, or smoking history; however, ECOG performance status and PD-L1 status were strongly associated with OS, similar to results for *TP53* mutations (**Fig. 4F**). In patients with non–oropharyngeal cancer, OS was significantly longer for durvalumab plus tremelimumab, and a trend for longer OS for durvalumab, versus SoC. In patients with oropharyngeal cancer, OS versus SoC trended longer in both ICI arms (**Fig. 4G** and **H**; limited statistical power due to small sample size).

In EAGLE, *TERT* promoter mutations correlated with significantly worse OS and PFS, compared with WT *TERT*, for patients treated with durvalumab plus tremelimumab (HR, 3.32; log-rank

Table 2. Mutational prevalence across demographics and tumor locations of select genes in the HAWK/CONDOR tissue samples and EAGLE plasma samples.[a]

	HAWK/CONDOR (tissue)						
	All pts (*N* = 153[c])	**HPV−[b] (*N* = 100)**	**HPV+[b] (*N* = 46)**	**White (*N* = 132)**	**Others (*N* = 21)**	**OPC (*N* = 66)**	**Non-OPC (*N* = 87)**
TP53	98 (64%)	78 (78%)	13 (28%)	88 (67%)	10 (48%)	31 (47%)	67 (77%)
FAT1[b]	0	0	0	0	0	0	0
NOTCH1	42 (27%)	27 (27%)	13 (28%)	36 (27%)	6 (29%)	19 (29%)	23 (26%)
PIK3CA	26 (17%)	14 (14%)	11 (24%)	21 (16%)	5 (24%)	10 (15%)	16 (18%)
KMT2D	47 (31%)	33 (33%)	13 (28%)	41 (31%)	6 (29%)	19 (29%)	28 (32%)
TERT[d]	9 (6%)	7 (7%)	1 (2%)	8 (6%)	1 (5%)	3 (5%)	6 (7%)
	EAGLE (plasma)						
	All pts (*N* = 285)	**HPV−[b] (*N* = 216)**	**HPV+ (*N* = 57)[b]**	**White (*N* = 211)**	**Others (*N* = 74)**	**OPC (*N* = 113)**	**Non-OPC (*N* = 172)**
TP53	221 (78%)	183 (85%)	29 (51%)	164 (78%)	57 (77%)	78 (69%)	143 (83%)
FAT1[b]	66 (23%)	45 (21%)	14 (25%)	45 (21%)	21 (28%)	25 (22%)	41 (24%)
NOTCH1	57 (20%)	48 (22%)	7 (12%)	44 (21%)	13 (18%)	18 (16%)	39 (23%)
PIK3CA	61 (21%)	48 (22%)	9 (16%)	47 (22%)	14 (19%)	24 (21%)	37 (22%)
KMT2D	85 (30%)	66 (31%)	13 (23%)	63 (30%)	22 (30%)	27 (24%)	58 (34%)
TERT[d]	63 (22%)	55 (25%)	5 (9%)	36 (17%)	27 (37%)	17 (15%)	46 (27%)

Data are *n* (%) unless otherwise specified.
Abbreviations: OPC, oropharyngeal cancer; pts, patients; WES, whole exome sequencing.
[a]Table contains the genes in which more than 20% prevalence was reported.
[b]HPV testing was carried out on all HNSCC locations.
[c]50 tissue samples were obtained from patients enrolled in HAWK, and 103 tissue samples were obtained from patients enrolled in CONDOR.
[d]*FAT1* and *TERT* promoter mutations are included in the GuardantOMNI panel used for plasma sequencing of EAGLE samples; however, promotor mutations are not available from WES data, resulting in lower reported frequencies of *FAT1* and *TERT1* mutations in HAWK/CONDOR.

$P < 0.001$ and HR, 2.19, $P = 0.003$, respectively) or SoC (HR, 1.91, $P = 0.010$ and HR, 2.45, $P = 9.5e{-}04$, respectively; Supplementary Fig. S4A–S4D); in the durvalumab arm, there was a trend for poorer OS but no significant difference in either OS or PFS (Supplementary Fig. S4E and S4F). *CCND1* amplifications correlated with a significantly worse outcome in the EAGLE durvalumab arm only (OS HR, 2.12, $P = 0.002$; Supplementary Fig. S5A–S5C). *PIK3CA* and *PIK3CB* amplifications were associated with significantly shorter OS in both the durvalumab and SoC arms (Supplementary Fig. S5D–S5F).

Discussion

These results suggest that for R/M HNSCC, the mutational landscape derived from sequencing plasma or tumor samples is comparable. Consistent with the understanding that carcinogens in tobacco could cause DNA damage and gene mutations (27), samples in this analysis from patients with smoking history showed numerically higher somatic mutation counts than those without smoking history. No association was observed between somatic mutation counts and PD-L1 or HPV status, similar to previous reports in treatment-naïve patients (15, 28).

TP53 was the most frequently mutated gene, in concordance with reports from primary tumors (15) and other studies in R/M HNSCC (29). Consistent with other studies, and with the known pathogenesis of HPV-associated HNSCC, *TP53* was more frequently mutated in the HPV-negative subgroup (15, 29–31). Recurrent mutations in the *KMT2D* epigenetic regulation gene in R/M HNSCC are prevalent (32). Notably, *KMT2D* showed numerically increased mutation frequency compared with HNSCC tumors from the treatment-naïve The Cancer Genome Atlas (TCGA) cohort (31% in tissue/32% in plasma vs. 18% in TCGA), as did other genes involved in epigenetic regulation or DDR, and *TERT* (15, 33). *LRP1B* and *NOTCH1* had higher mutation frequency, and *PIK3CA* had lower mutation frequency, in HAWK/CONDOR tissue samples compared with TCGA cohort. However, these genes and *FAT1* showed similar mutation frequency between EAGLE plasma samples and TCGA cohort (15, 33). This suggests that the somatic mutational landscape in R/M HNSCC may be modified in comparison with treatment-naïve HNSCC. Alternatively, the differences in mutation prevalence may be driven by differences in patient population (HAWK/CONDOR and EAGLE examined R/M HNSCC only, whereas TCGA HNSCC cohort included all stages) or sample type (sequencing of plasma ctDNA in EAGLE captured primary and metastatic sites, whereas sequencing of tissue in HAWK/CONDOR and TCGA cohort captured only a single tumor location) or differences in the proportion of HPV-related versus nonrelated tumors between cohorts. Additional studies are required to explore the impact of first-line treatment on these genes. Tumor-suppressor p53 is critical for maintaining genomic stability and preventing oncogenesis; functional mutations of *TP53* confer resistance to chemotherapies in multiple tumor types (34). In the EAGLE SoC arm, *TP53* mutations were associated with significantly shorter OS compared with WT *TP53,* with a similar trend observed for PFS, suggesting *TP53* mutations in R/M HNSCC confer resistance to chemotherapies, consistent with results in other tumor types (34). However, patients whose tumors harbored *TP53* mutations showed significantly longer OS for durvalumab plus tremelimumab, and a trend for longer OS for durvalumab, versus SoC. Although HPV-

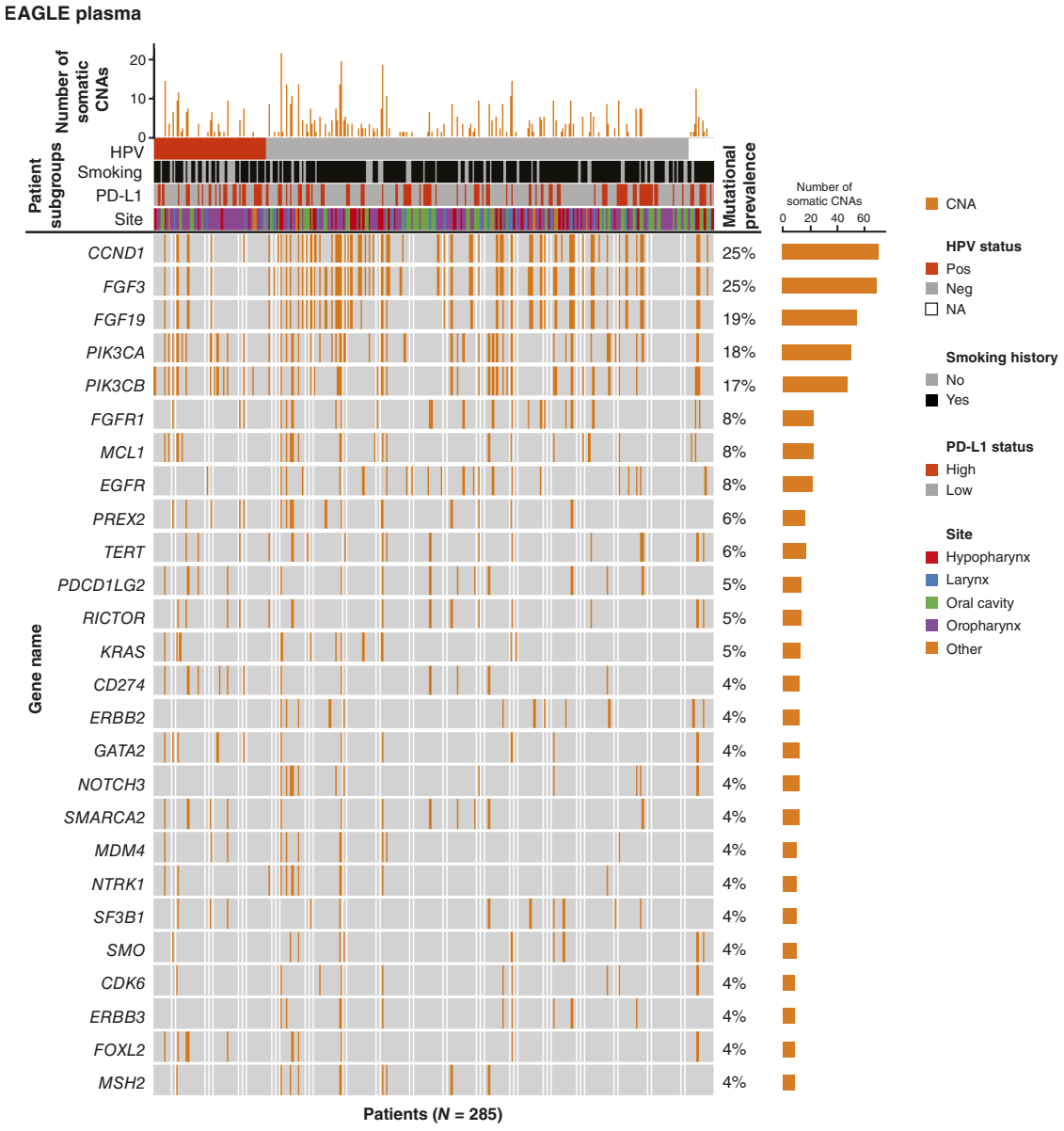

Figure 2.

CNA mutational landscape across demographics and tumor locations in plasma samples from patients with R/M HNSCC from the EAGLE study. Mutations with prevalence ≥4% are shown. CNA, copy-number alteration; NA, not applicable; Neg, negative; Pos, positive.

negative tumors more frequently harbored *TP53* mutations, OS HRs were not significantly different in HPV-positive and HPV-negative patients. In contrast to previous studies which showed longer OS for patients with low-risk or WT *TP53* mutations versus high-risk *TP53* mutations in patients with HNSCC or HPV-negative HNSCC (35, 36), application of the same classification system to the analysis of OS and PFS in HAWK, CONDOR, and EAGLE did not differentiate high-risk versus low-risk *TP53* mutations in patients or the HPV-negative subgroup. In patients whose tumors harbored *TP53* mutations, the 18-month survival rate was approximately four times higher with ICIs versus SoC, highlighting the predictive value of *TP53* mutations for durvalumab therapies. In order to evaluate the influence of *TP53* mutational status on disease-free survival of patients with locally advanced HNSCC, the phase II ECOG-ACRIN 3132 trial utilizes *TP53* mutational status as an integral biomarker for stratification of patients before randomization to adjuvant radiotherapy with or without cisplatin (37). Future studies of ICIs may be similarly designed to determine whether *TP53* mutational status could be used as a prognostic or predictive biomarker. *TP53* mutations were significantly correlated with somatic mutation numbers, suggesting *TP53* mutations may result in accumulation of mutations leading to better outcomes with ICI treatment; however, *TP53* mutations were predictive of outcomes independent of the TMB.

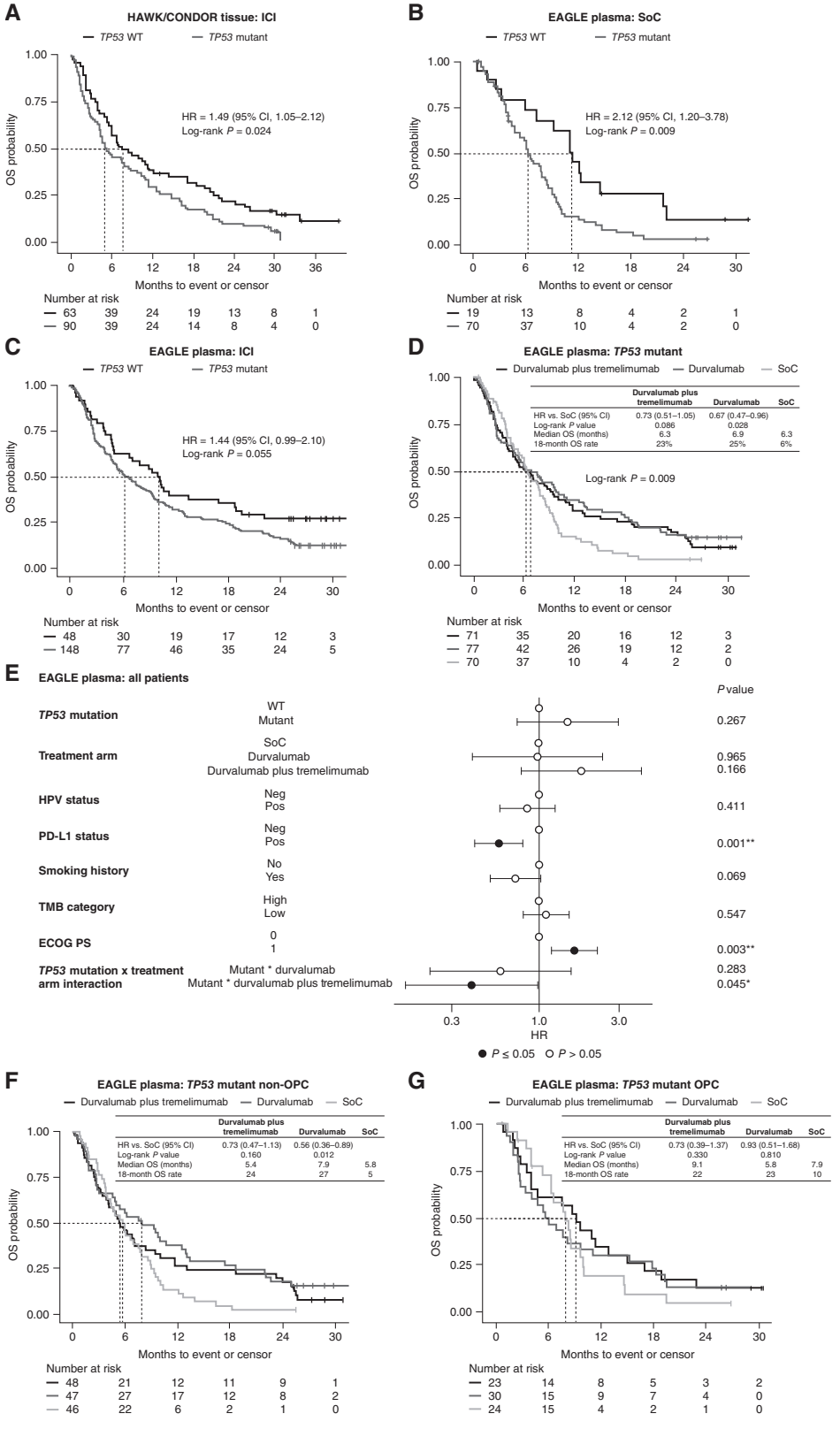

Figure 3.

TP53 mutations and resistance to immunotherapy and SoC chemotherapy in R/M HNSCC assessed by OS. Kaplan–Meier plots of OS in the *TP53* WT and mutant subgroups for patients treated with ICIs in the HAWK/CONDOR study (**A**), SoC in the EAGLE study (**B**), or ICIs in the EAGLE study (**C**). Kaplan–Meier plots of OS in the SoC, durvalumab, or durvalumab plus tremelimumab treatment subgroups for patients with *TP53* mutations in the EAGLE study (**D**). Multivariate Cox proportional hazard model analysis showing OS HRs according to individual risk factors in the EAGLE study (**E**). Kaplan–Meier plots of OS in the SoC, durvalumab, or durvalumab plus tremelimumab treatment subgroups for patients with *TP53* mutations and R/M HNSCC non–OPC (**F**) or OPC (**G**) in the EAGLE study. CI, confidence interval; Neg, negative; OPC, oropharyngeal cancer; Pos, positive; PS, performance status.

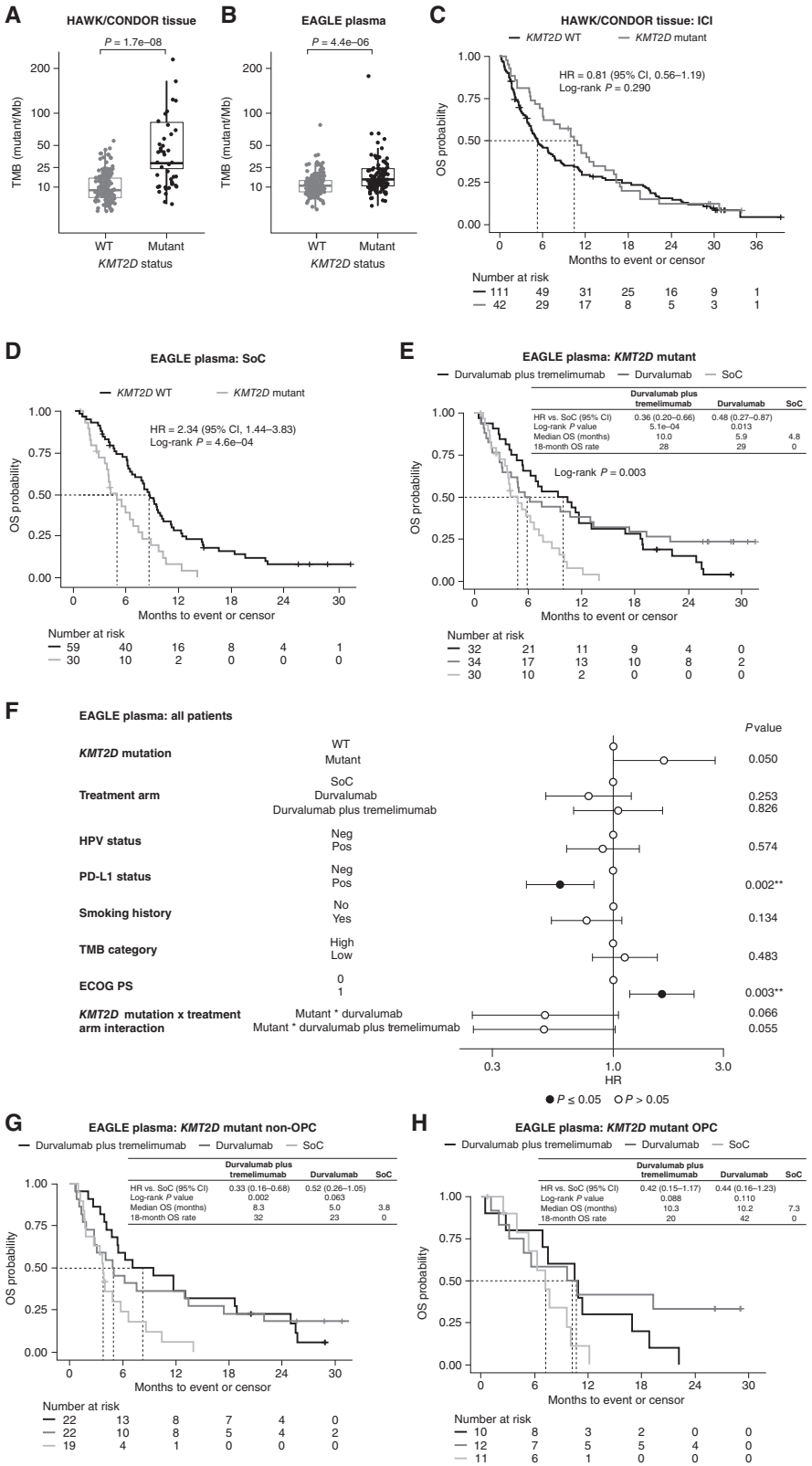

Figure 4.

KMT2D mutations and response to immunotherapy and SoC chemotherapy in R/M HNSCC. The TMB by *KMT2D* mutation status detected in the HAWK/CONDOR study tissue samples (**A**) and EAGLE study plasma samples (**B**). Kaplan–Meier plots of OS in the *KMT2D* WT and mutant subgroups for patients treated with ICIs in the HAWK/CONDOR study (**C**) and SoC in the EAGLE study (**D**). Kaplan–Meier plots of OS in the SoC, durvalumab, or durvalumab plus tremelimumab treatment subgroups for patients with *KMT2D* mutations in the EAGLE study (**E**). Multivariate Cox proportional hazard model analysis showing OS HRs according to individual risk factors in the EAGLE study (**F**). Kaplan–Meier plots of OS in the SoC, durvalumab, or durvalumab plus tremelimumab treatment subgroups for patients with *KMT2D* mutations and R/M HNSCC non-OPC (**G**) or OPC (**H**) in the EAGLE study. CI, confidence interval; Neg, negative; OPC, oropharyngeal cancer; Pos, positive; PS, performance status.

Similar to results in esophageal squamous cell carcinoma (ref. 38), R/M HNSCC tumors harboring *KMT2D* mutations were associated with a higher mutational burden compared with the *KMT2D* WT. In tumor models, *KMT2D* mutations have been shown to sensitize tumors to ICIs (32) and have been significantly correlated with longer OS in esophageal squamous cell carcinoma (38). Consistent with these observations, a trend for longer OS was found in patients whose tumors harbored *KMT2D* mutations versus the *KMT2D* WT in HAWK/CONDOR. In EAGLE, patients whose tumors harbored *KMT2D* mutations showed a trend for longer OS when receiving durvalumab compared with SoC, suggesting *KMT2D* mutations may be a predictive biomarker for durvalumab. Further validation in R/M HNSCC with a larger sample size is warranted.

Consistent with previous reports, *TERT* promoter mutations were significantly correlated with worse PFS and OS with both ICIs and SoC, suggesting a potentially prognostic effect (39). Amplification of *CCND1* was correlated with worse outcomes in the EAGLE durvalumab arm, consistent with reports from a meta-analysis across multiple tumor types (40). As oncogenic drivers, *PIK3CA* and *PIK3CB* amplifications were associated with shorter OS with durvalumab or SoC, suggesting their amplification may be prognostic for poor outcomes.

Limitations of our study include that the HAWK/CONDOR (9, 10) studies did not include a SoC arm; therefore, these findings need further validation. The HAWK (9), CONDOR (10), and EAGLE (11) studies investigated ICI treatment in patients with R/M HNSCC with progression on/after platinum-based chemotherapy; thus, these findings may not be applicable to first-line ICI treatment. The fact that patients were undergoing second-line treatment for HNSCC may also account for the high frequency of *TP53* mutations observed across HPV-positive samples. It is also conceivable that a plasma-based analysis based on p53 mutations may be confounded by the detection of ctDNA and mutations attributable to clonal hematopoiesis (41). However, it is unlikely that these are major confounders in the present analysis, as mutation frequency is broadly similar in both tissue and plasma samples, and the prognostic impact is observed in tissue-only analyses in the HAWK and CONDOR studies. Furthermore, the same analytical assays were used for both p16/HPV-positive and p16/HPV-negative populations. Although p16 status is used as a surrogate marker for HPV, a proportion of p16-positive patients have been shown to be HPV negative; therefore, it is possible that some HPV-negative patients were classified as HPV positive in the present study (42). Additionally, given that durvalumab and tremelimumab are not used in clinical practice in HNSCC, it is important to establish the clinical relevance of these potential biomarkers in patients receiving current ICI SoC. Some tissue samples analyzed from HAWK/CONDOR (9, 10) were archival (up to 3 years old, potentially including diagnostic samples from treatment-naïve patients) and therefore may not have been representative of the disease at the time of treatment. Plasma samples were taken at baseline in EAGLE (11). Different assays were also used for each sample type (whole-exome sequencing in tissue vs. next-generation sequencing with a targeted 500-gene panel in plasma), though similar results were observed. Finally, evaluable samples were not available for all patients, potentially due to ascertainment bias. Overall, we consider the study population to be representative of patients with second-line HNSCC; cohorts were equivalent in terms of patient and tumor characteristics, with the exception of PD-L1 status. However, it is important to note the inherent difficulty of accurately profiling the genotype of a heterogeneous tumor using limited tissue samples (43).

To the best of our knowledge, this is the first large-scale study to show the mutational landscape of R/M HNSCC with progression on/after platinum-based chemotherapy and its association with ICI efficacy. Tumor tissue samples and liquid biopsies demonstrated similar mutational landscapes, supporting liquid biopsy as an option for mutation profiling of R/M HNSCC. There were frequent mutations in DDR and epigenetic regulation genes, possibly induced by previous treatment. Mutations in *TP53* were a negative prognostic biomarker, but patients with *TP53* mutations performed better with immunotherapy than SoC chemotherapy. *KMT2D* mutations are also associated with sensitivity to immunotherapies; further investigation and prospective validation of *KMT2D* as a predictive biomarker for immunotherapy in R/M HNSCC is warranted. Overall, future studies involving immune checkpoint blockade may benefit through further investigation of these findings.

Authors' Disclosures

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Authors' Contributions

P. McCoon: Conceptualization, formal analysis, supervision, writing–original draft, project administration, writing–review and editing. **Y. Wang:** Formal analysis, writing–original draft, writing–review and editing. **Z. Lai:** Formal analysis, writing–original draft, writing–review and editing. **Q. Zhang:** Conceptualization, formal analysis, writing–original draft, writing–review and editing. **W. Li:** Conceptualization, data curation, formal analysis, supervision, writing–original draft, project administration, writing–review and editing. **S. Wildsmith:** Conceptualization, data curation, supervision, writing–original draft, project administration, writing–review and editing. **N. Morsli:** Conceptualization, formal analysis, writing–original draft, writing–review and editing. **R. Raja:** Data curation, formal analysis, writing–original draft, writing–review and editing. **N. Holoweckyj:** Data curation, writing–original draft, project administration, writing–review and editing. **J. Walker:** Conceptualization, data curation, supervision, writing–original draft, writing–review and editing. **M. de los Reyes:** Conceptualization, formal analysis, writing–original draft, project administration, writing–review and editing. **R. Mesía:** Data curation, formal analysis, writing–original draft, writing–review and editing. **L. Licitra:** Data curation, formal analysis, writing–original draft, writing–review and editing. **R.L. Ferris:** Data curation, formal analysis, writing–original draft, writing–review and editing. **J. Fayette:** Data curation, formal analysis, writing–original draft, writing–review and editing. **D.P. Zandberg:** Data curation, formal analysis, supervision, writing–original draft, writing–review and editing. **L.L. Siu:** Data curation, formal analysis, supervision, writing–original draft, writing–review and editing. **R. Haddad:** Conceptualization, data curation, formal analysis, supervision, writing–original draft, writing–review and editing.

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