MAPK Pathway–Activating Alteration and Immunotherapy Efficacy in Squamous Cell Lung Carcinoma: Results from the Randomized, Prospective SQUINT Trial

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ABSTRACT

Background: The role of activating alterations in the MAPK pathway in predicting immunotherapy efficacy in patients with lung squamous cell carcinoma (LSCC) is largely unknown. The aims of the randomized, phase II SQUINT trial were to assess the efficacy of nivolumab plus ipilimumab (NI) versus platinum-based chemotherapy plus nivolumab (N-CT) and to identify clinically available biomarkers of response to immunotherapy in patients with advanced or metastatic LSCC.

Patients and Methods: SQUINT was an open-label, randomized, parallel, noncomparative, phase II trial of NI versus N-CT in chemo-naïve adult patients with metastatic or recurrent LSCC. The study was conducted across 15 Italian centers from September 2017 to February 2022 (ClinicalTrials.gov ID: NCT03823625).

Results: Forty-five patients were included in the NI arm and 46 patients in the N-CT arm. At 12 months, the overall survival (OS) rate was 62% in the NI arm and 50% in the N-CT arm. In total, 74 patients were included in the analyses for individual biomarkers. In patients with mutations or copy-number variations of genes involved in the *MAPK* pathway, we observed higher response to immunotherapy (43% vs. 15%), longer progression-free survival ($P = 0.03$), and OS ($P < 0.001$). A higher density of $CD8^+PD1^+$ T cells ($P = 0.04$) among *MAPK*-altered tumors versus that in wild-type tumors, together with an increased $CD8^+PD1^+$/FOXP3 ratio ($P = 0.047$), was observed. In the validation cohort of patients not exposed to immunotherapy, OS was similar between *MAPK1/3*-mutant and wild-type LSCC.

Conclusions: We showed for the first time that MAPK pathway–activating alterations influence the outcome of LSCC treated with immunotherapy, highlighting the relevance of gene profiling.

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Introduction

Immunotherapy alone or in combination with chemotherapy is the current first-line standard of care in advanced non–small cell lung cancer without targetable molecular alterations (1, 2). The PD-L1 axis inhibitors pembrolizumab, cemiplimab, or atezolizumab are now approved for patients with high (≥50%) PD-L1 expression levels, whereas the same agents in combination with platinum-based chemotherapy have demonstrated efficacy regardless of PD-L1 expression (3–7). Other effective strategies include the possibility of combining different immunotherapeutic agents with cytotoxic chemotherapy. The CheckMate 9LA was a randomized phase III study comparing the combination of the anti–PD-1 nivolumab plus the anti–cytotoxic T lymphocyte–associated protein 4 antibody ipilimumab and two cycles of platinum-based chemotherapy versus chemotherapy alone. The study showed a significant survival benefit in the favor of the experimental arm regardless of PD-L1 expression (8). Of note, in the recent update of this study, the median overall survival (OS) for patients receiving immunotherapy combination plus chemotherapy were 17.8 and 14.5 months in nonsquamous and squamous histology, respectively (9), suggesting a clearer benefit from the addition of anti–cytotoxic T lymphocyte–associated protein 4 inhibition in nonsquamous histology. Consistently, in the POSEIDON trial, progression-free survival (PFS) and OS benefits of durvalumab + tremelimumab + chemotherapy versus chemotherapy alone seemed more prominent in the subgroup with nonsquamous histology (10).

Translational Relevance

This study provides critical insights into the role of MAPK pathway–activating alterations as predictive biomarkers for immunotherapy efficacy in lung squamous cell carcinoma (LSCC). The findings from the SQUINT trial demonstrate that patients with LSCC with activating mutations or copy-number variations in MAPK-related genes exhibit a higher response rate, improved progression-free survival, and overall survival when treated with immunotherapy, compared with those without these alterations. Additionally, the study identified immunologic differences, such as increased $CD8^+PD1^+$ T-cell density and a higher $CD8^+PD1^+$/FOXP3 ratio, in MAPK-altered tumors, suggesting enhanced immune activation in these patients. These results underscore the importance of molecular profiling in tailoring immunotherapy strategies, offering a potential pathway for more personalized and effective treatment approaches in LSCC. The ability to identify patients who may benefit from immunotherapy based on MAPK pathway alterations could significantly impact clinical decision-making and improve patient outcomes.

However, although immunotherapy has improved the outcomes of advanced non–small cell lung cancer, a large fraction of patients rapidly progress, with only 15% to 20% experiencing long-term survival (11). In addition, for patients with squamous histology, survival is invariably shorter compared with patients with nonsquamous histology. Limited therapeutic options, together with a distinct biology of the disease, could account for this difference (8, 12).

In the past decade, several predictors for immunotherapy efficacy have been extensively investigated, including PD-L1 expression, microsatellite instability, and tumor mutational burden (TMB). In the United States, TMB ≥10 is used as a selection criterion for pembrolizumab monotherapy across all cancer types according to Q8 FDA and NCCN recommendations; in Europe, PD-L1 expression is the only biomarker used in clinical practice to guide treatment selection for immunotherapies (13). However, PD-L1 inadequately predicts the benefit from immunotherapy as PD-L1–negative tumors may respond to the treatment, whereas among patients with high PD-L1 expression, primary resistance is seen in >45% of cases. TMB also failed to demonstrate clinical utility for patient selection across different cancer types, including lung squamous cell carcinomas (LSCC) in which the smoking-related mutational load is predominant (14). In addition, although several molecular alterations have been evaluated, many events, such as *STK11* or *SMARCA4* mutations, are typically associated with nonsquamous histology, making them unsuitable in guiding LSCC therapy (15–18). The MAPK pathway regulates the EGF- and IFN-γ–induced PD-L1 expression in lung adenocarcinoma, and additional findings suggested that activating alterations in the MAPK pathway, including the amplification of *KRAS*, *BRAF*, and *RAF1* genes and truncations of *NF1*, could modify sensitivity to immunotherapy (19–21). However, the role of MAPK-activating alterations as potential molecular predictors of immunotherapy efficacy in patients with LSCC is still in need of investigation.

The aims of the randomized, phase II SQUINT trial were to assess the efficacy of nivolumab plus ipilimumab (NI) versus platinum-based chemotherapy plus nivolumab (N-CT) and to identify molecular determinants that are able to predict sensitivity to immunotherapy in patients with advanced or metastatic LSCC.

Patients and Methods

Study design and patients

SQUINT was an open-label, randomized, parallel, noncomparative, phase II trial of nivolumab plus ipilimumab versus platinum-based chemotherapy plus nivolumab in chemo-naïve adult patients with metastatic or recurrent LSCC. The study was conducted across 15 Italian centers from September 2017 to February 2022 (see Appendix I for the centers' list). Inclusion criteria were a histologically confirmed diagnosis of stage IIIB LSCC ineligible for treatment with definitive chemoradiation, stage IV LSCC, or recurrent LSCC (according to the seventh edition of the lung cancer stage classification), availability of tumor tissue, Eastern Cooperative Oncology Group performance status ≤1, adequate bone marrow (absolute neutrophil count $\geq 1.5 \times 10^9$/L, platelets $\geq 100 \times 10^9$/L, and hemoglobin >9 g/dL) and liver functions (bilirubin <G2 and transaminases no more than 3 × ULN/<5 × ULN in the presence Q9 of liver metastases), and normal level of alkaline phosphatase and creatinine. Adjuvant/neoadjuvant chemotherapy was allowed if therapy was completed at least 6 months before trial inclusion. Prior palliative radiotherapy for non–central nervous system lesions must have been completed at least 2 weeks prior to treatment. Patients with tumor negative for p63 (or p40) or positive for TTF1, untreated brain metastases, diagnosis of any other malignancy during the last 2 years (except for *in situ* carcinoma of cervix uteri and cutaneous squamous cell carcinoma), and pregnant or in breastfeeding stage were excluded. The study was performed in line with Good Clinical Practice guidelines and the Declaration of Helsinki, and the study protocol was approved by independent ethics committees at each site. This study is registered with ClinicalTrials.gov, number NCT03823625. Written informed consent was obtained from the patients.

Treatment and procedures

All eligible patients were randomly assigned to the NI arm or the N-CT arm. Nivolumab was administered at the standard dosage of 360 mg every 3 weeks. Ipilimumab was administered at the dosage of 1 mg/kg every 6 weeks. Platinum-based chemotherapy was chosen by the investigator from the following regimens: cisplatin 80 mg/mq (i.v.) on day 1 and gemcitabine 1,250 mg/m^2 (i.v.) on days 1 and 8 for every 21 days; carboplatin AUC 5 mg/mL minutes (i.v.) on day 1 and gemcitabine 1,000 mg/m^2 (i.v.) on days 1 and 8 for every 21 days; and carboplatin AUC 6 mg/mL minutes (i.v.) on day 1 and paclitaxel 200 mg/m^2 (i.v.) on day 1 for every 21 days. In both arms, immunotherapy was given until disease progression, toxicity, or patient refusal and, in any case, for up to 24 months. Platinum-based chemotherapy was administered for up to six cycles.

Disease assessment was performed every 8 weeks with a confirmatory evaluation in all patients with response or disease stabilization no less than 4 weeks after the response assessment, according to RECIST version 1.1 criteria. A complete disease staging was performed in the case of progressive disease and, in every case, at the time of patient withdrawal from the trial. Following completion of protocol therapy, patients were followed up for survival every 12 weeks. Toxicity was evaluated according to NCI Common Ter- Q10 minology Criteria for Adverse Events version 4.0.

Biomarkers analyses

SQUINT cohort

Archive tumor tissues were collected for studies on biomarkers and efficacy in the SQUINT study. Patient specimens from 87 individuals were used to generate libraries using the hybrid capture-based OncoKey SL 525 Library Preparation Kit (Vela Diagnostics Germany GmbH) following the manufacturer's protocol. A total of 87 DNA and 87 RNA libraries were sequenced on a NovaSeq (Illumina). The fastQ files were processed with Vela SentosaSQ Reporter software developed by Vela Diagnostics. A small variants variant call file was generated, including SNPs, indels, frameshifts, truncations, and a JSON file including fusions and copy-number variations (CNV). The variant call and JSON files were subsequently imported to Vela Analytics software for interpretation. Actionable variants were identified for each patient. Patients with TMB ≥50 mutations/Mb were excluded from the subsequent biomarker analysis, which was considered to fail the quality control of the sequencing. Genes with actionable variants were used to perform pathway analysis by Reactome (https://reactome.org/PathwayBrowser/#/DTAB=MT&TOOL=AT). Fifteen submitted genes were found mapping to 23 Reactome genes in the MAPK1/MAPK3 signaling pathway. This gene list was selected *a priori* based on the biological hypothesis that activating MAPK alterations can associate with an immune-inflamed tumor microenvironment and therefore potentially improved outcomes to immunotherapy. Those genes were *BRAF*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR2*, *FGFR3*, *JAK3*, *KRAS*, *MET*, *NF1*, *NRAS*, *NRG1*, *PIK3CA*, *PIK3R1*, and *RAF1*. Patients with oncogenic mutations or CNVs in these 15 genes in the MAPK1/MAPK3 signaling pathway were included in the genomic analysis.

- PD-L1 assessment

The PD-L1 tumor proportion score was assessed by trained pathologists with the help of IHC using validated monoclonal anti–PD-L1 antibodies: E1L3N (Cell Signaling Technology), 22C3 (Dako North America Inc.), and 28-8 (Epitomics Inc.).

- Tumor genomic profiling

Targeted exome next-generation sequencing (NGS) was performed using the validated OncoPanel assay in the Center for Cancer Genome Discovery at the Dana-Farber Cancer Institute (DFCI) for 277 (POPv1), 302 (POPv2), or 447 (POPv3) cancer-associated genes, as previously described and reported (PMID: 28557599, PMID: 36775193, and PMID: 34740862). Tumor DNA was prepared and hybridized to custom RNA bait sets (Agilent SureSelectTM) and sequenced using Illumina HiSeq 2500 with 2 × 100 paired-end reads. Sequence reads were aligned to reference sequence b37 edition from the Human Genome Reference Consortium, using bwa, and further processed via Picard (version 1.90; http://broadinstitute.github.io/picard) and Genome Analysis Toolkit (version 1.6-5-g557da77). Single-nucleotide variants were called using MuTect v1.1.46, and insertions and deletions were called using Genome Analysis Toolkit Indelocator. CNVs and structural variants were called using the internally developed algorithms RobustCNV and BreaKmer. For each gene, the absolute copy number was estimated based on the tumor purity (p) and the weighted average of segmented $\log_2$ ratios across the gene (L) using the following formula: absolute copy number $= [2^{\wedge}((I+1))-2(1-p)]/p$. TMB, defined as the number of somatic mutations per Mb of the genome examined (including coding, base substitution, and indels), was determined using OncoPanel as previously described (22).

Multiplexed immunofluorescence (ImmunoProfile)

Multiplexed immunofluorescence was performed on samples from the DFCI by staining 4 or 5-micron formalin-fixed, paraffin-embedded whole-tissue sections with standard, primary antibodies sequentially and paired with a unique fluorochrome followed by staining with nuclear counterstain 4′,6-diamidino-2-phenylindole (DAPI). All samples were stained for PD-L1 (clone E1L3N, Cell Signaling Technology), PD-1 [clone EPR4877(2)], CD8 (clone 4B11, Thermo Fisher Scientific), FOXP3 (clone D608R, Thermo Fisher Scientific), and cytokeratin (clone AE1/AE3, Thermo Fisher Scientific). Regions of Interest (ROI) were defined for each image, and only these regions were used for quantitative image analysis. InForm Image Analysis software (PerkinElmer/Akoya) was run within each ROI to phenotype and score cells based on biomarker expression. A custom script quantified the number/percentage of positive cells for relevant biomarkers in specific tissue regions. The cell count was calculated per ROI, averaged across ROIs, and reported as counts per millimeter squared ±SE. Tumor cells were defined as cells with DAPI^{+}CK (AE1/AE3, Thermo Fisher Scientific) staining. Nontumor (inflammatory) cells were defined as cells without CK staining.

Analyses of individual biomarkers and genes

Quality sequencing outputs with high coverage were selected for survival analysis. Stratification analysis was used to estimate the association between clinical parameters and OS and PFS by analyzing the parameters in subgroups. For subgroup analyses, patients were stratified according to TMB status (TMB high: ≥10 mutations/Mb and TMB low: <10 mutations/Mb), PD-L1 expression (<1%, <50%, and ≥50%), and MAPK1/3 genomic status (presence vs. absence of mutations or copy number alterations). Comparisons between arms were performed using the stratified log-rank test. $P < 0.05$ was considered statistically significant. Statistical analyses were conducted using JMP, version 16, SAS Inc.

Study endpoints

The primary study endpoint was the OS rate at 12 months. Secondary endpoints included (i) the median OS, response rate (RR), median PFS, median time-to-treatment response, duration of response in both NI and N-CT arms, and median OS in patients with or without bone metastases and (ii) correlation of OS and PFS with gene deregulations identified by NGS, including TMB status, PD-L1 expression, and *MAPK1*/*3* genomic status. Subsequent treatments, safety, and toxicity were also assessed. A key exploratory endpoint included the OS in different subgroups on the basis of patient characteristics.

Statistical analysis

The study was initially sized according to the crude PFS at 12 months using the Fisher exact test, resulting in 170 patients, drop-out included. It was later amended in a noncomparative trial with OS at 12 months as the primary endpoint. A sample size of 45 patients was needed to reject the null hypothesis of an OS rate at 12 months of 40% under the hypothesis of a 60% OS rate at 12 months. The treatment was considered active if at least 25 patients were alive at 12 months.

The Kaplan–Meier method was used to estimate the survival curves. OS was calculated from the date of randomization to the date of death due to any cause. OS was censored on the last date the subject was known to be alive. PFS was calculated from the date of randomization to the first documented evidence of progressive disease or death due to any cause (whichever occurs first). Subjects who died without a reported progression were considered to have progressed on the date of their death. Subjects who did not progress or die were censored on the date of their last evaluable tumor assessment.

The RR was defined as the number of subjects with the best overall response (BOR) of complete response or partial response divided by the number of randomized subjects for each treatment arm. BOR was defined as the best response designation recorded between the date of randomization and the date of objectively documented progression per RECIST 1.1, the date of initiation of palliative local therapy, or the date of subsequent anticancer therapy, whichever occurs first. All available response designations contributed to the BOR determination for subjects without documented progression or palliative local therapy or subsequent anticancer therapy. For subjects who continued on study medication beyond progression, the BOR was determined based on response designations recorded at the time of the initial RECIST 1.1–defined progression. For subgroup analyses, patients were stratified according to bone metastases (yes vs. no). Safety was measured by the incidence of adverse events (AE), serious AEs, discontinuation due to AEs, and deaths and laboratory abnormalities. Statistical analyses were conducted using IBM SPSS Statistics for Windows, version 28.0. Armonk, NY e R v.4.1.2 (www.R-project.org). The P value < 0.05 was considered statistically significant.

In the multivariate analysis, event–time distributions were estimated using Kaplan–Meier methodology. Log-rank tests were used to test for differences in event–time distributions, and Cox proportional hazards models were fitted to obtain estimates of HRs in multivariable analysis after testing for Cox regression assumptions. Clinically relevant variables including age, sex, smoking history, PD-L1 expression, and TMB were chosen as covariates to adjuvant clinical outcomes.

Data availability

The datasets generated during and/or analyzed during the current study, including clinical outcomes, genomic, and immunophenotypic data, are available from the corresponding author on reasonable request.

Results

A total of 91 patients with advanced LSCC were included in the study; 45 were assigned to the NI arm and 46 to the investigator's choice of the N-CT arm. Patient characteristics were balanced between the two arms (Supplementary Table S1). The majority of patients were males (84.4% and 73.9% in NI and N-CT arms, respectively) and current or former smokers (95.6% and 95.7%, respectively; Supplementary Table S1). Brain, liver, or bone metastases were present in 4.4%, 8.9%, and 22.2% of patients in the NI arm and in 8.7%, 15.2%, and 30.4% of patients in the N-CT arm, respectively (Supplementary Table S1). PD-L1 expression was ≥1% in most patients in both arms (62.2% in the NI arm and 73.9% in the N-CT arm; Supplementary Table S1). Carboplatin or cisplatin in combination with gemcitabine was the most common chemotherapy regimen (98.0%; 70.0% carboplatin and 28.0% cisplatin-based chemotherapy).

Clinical activity

OS

At 12 months, the OS rate was 62% in the NI arm and 50% in the N-CT arm (**Fig. 1A**). The median OS was numerically in the favor of the NI arm [14.7 months; 95% confidence interval (CI), 11.1–18.3 months] compared with that in the N-CT arm (12.0 months; 95% CI, 10.1–13.9 months). Exploratory subgroup analyses of OS found that current smoker status (HR = 0.45; 95% CI, 0.21–0.98) and the presence of bone metastases at the baseline (HR = 0.19; 95% CI, 0.04–0.86) were factors favoring the NI combination treatment (**Fig. 1B**). The risk of death was also decreased in patients with PD-L1 <1% treated with NI (HR = 0.54; 95% CI, 0.18–1.61; **Fig. 1B**).

In the NI arm, the median OS of patients with bone metastases was not reached in the study period, whereas it was 14.0 months (95% CI, 10.7–17.3) in patients without bone metastases (Supplementary Fig. S1). Among N-CT patients, the presence of bone metastases did not have any impact on the median OS (Supplementary Fig. S1).

The RR (Supplementary Table S2) and related OS, PFS (Supplementary Fig. S2), time to response, and duration of response (Supplementary Fig. S3) are presented in the supplementary material, as well as subsequent treatment (Supplementary Table S3), safety, and toxicity data are also provided (Supplementary Table S4).

OS and PFS for individual biomarkers and genes

A total of 74 of 91 intention-to-treat patients were included in the analyses for individual biomarkers and genes. We report the analysis in the aggregate form without distinction between NI and N-CT arms, considering the similar outcomes of the two populations. Analyses on clinical outcomes in the two treatment arms were provided according to PD-L1, TMB, and MAPK status with an explorative intent only.

PD-L1 and TMB

PD-L1 expression was evaluable in 69 patients. As expected, patients with PD-L1–positive (≥1%) expression had significantly longer PFS and numerically longer OS than PD-L1–negative (<1%) patients, as illustrated in Supplementary Fig. S4. Supplementary Figure S5 reports an exploratory analysis of the impact of NI versus N-CT on clinical outcomes according to the PD-L1 status.

Overall, 53 patients presented with high TMB (≥10 mutations/Mb; see Appendix II for details). The full list of analyzed genes and related mutational rates are reported in Supplementary Fig. S6. In patients with high TMB, the median OS was significantly longer [14.0 months (95% CI, 13.1–20.5)] compared with that in low TMB patients [11.3 months (95% CI, 4.1–12.1); $P = 0.038$; **Fig. 2A**]. The TMB status did not impact the PFS in our cohort (**Fig. 2B**). Clinical outcomes were explored according to TMB alterations in each treatment arm. Improved outcomes were observed among NI patients (Supplementary Fig. S7).

MAPK1/MAPK3 signaling pathway

MAPK1/MAPK3 signaling pathway status was available for 74 patients (Supplementary Table S5). Among them, 68.9% ($n = 51$) presented with at least one mutation and/or oncogenic CNV in genes belonging to the MAPK1/MAPK3 signaling pathway. Clinical characteristics of MAPK1/MAPK3-positive and -negative patients were balanced (Supplementary Table S5), with a predominance of males in the MAPK1/MAPK3-positive subgroup. *NF1* gene mutation was the most common event (14.9%, $n = 11$), followed by

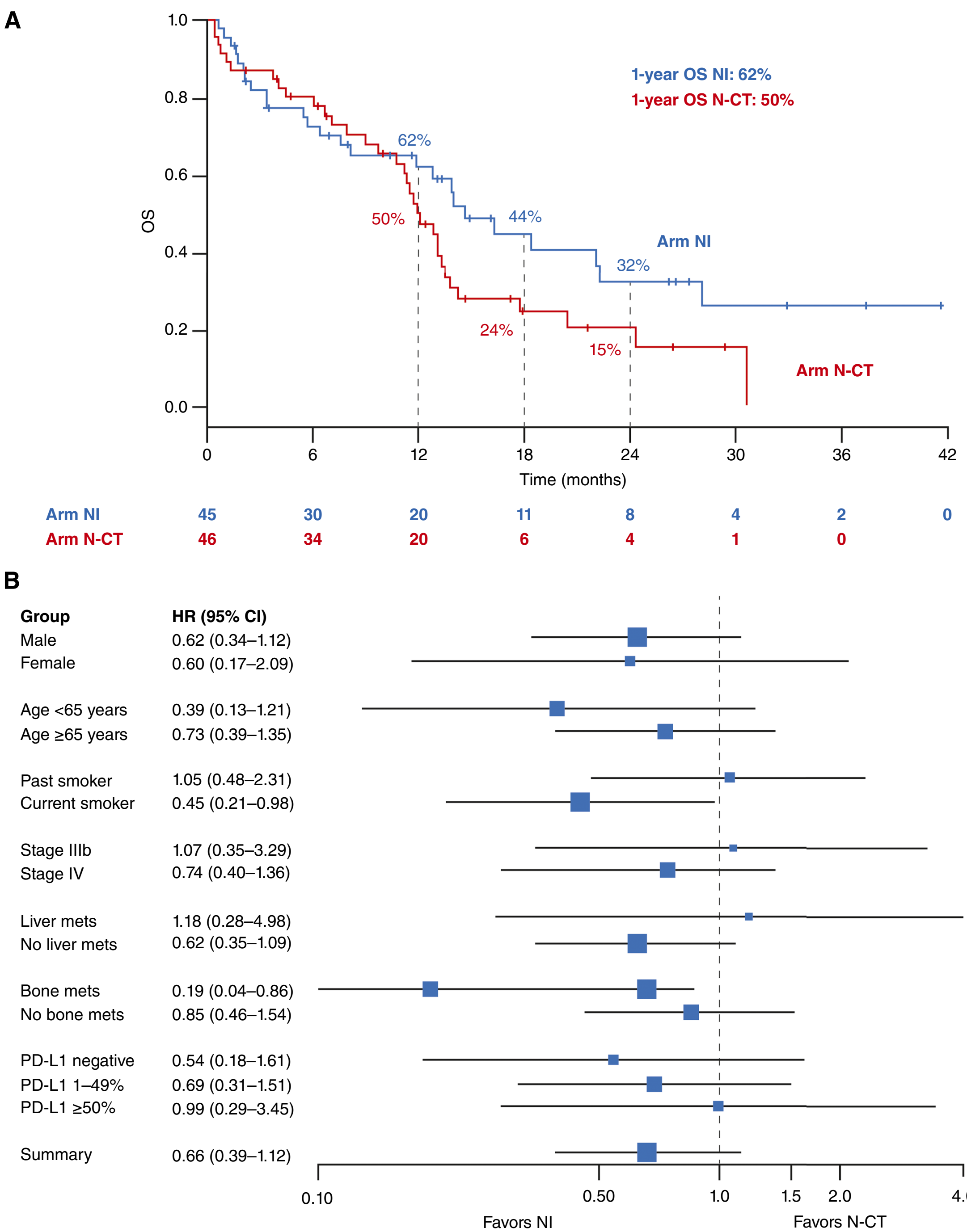


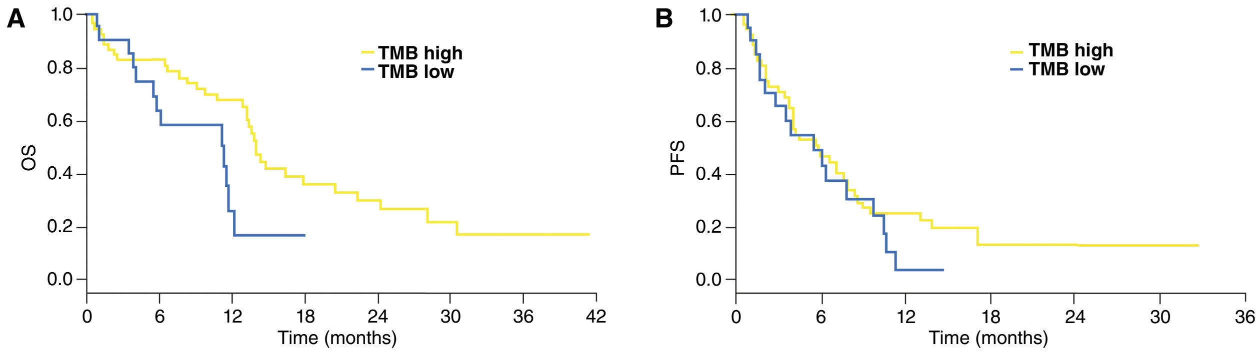
Figure 1.

Q19 Overall survival. **A,** OS rates at 12, 18, and 24 months. **B,** Exploratory subgroup analyses of OS. Mets, metastases.

mutations in *PI3KCA* (8.1%, $n = 6$) and *KRAS* (6.8%; $n = 5$) genes (Supplementary Fig. S8). Patients positive for mutation and/or CNV in MAPK1/MAPK3 signaling pathway genes had significantly longer OS [16.3 months (95% CI, 13.5–30.6) vs. 10.7 months (95% CI, 6.1–13.3); $P < 0.001$] and PFS [6.3 months (95% CI, 4.1–10.5) vs. 4.2 months (95% CI, 3.0–8.6); $P = 0.03$] than individuals without any MAPK1/MAPK3 mutation or CNV (**Fig. 3**).

Clinical outcomes were explored according to MAPK alterations in each treatment arm. Improved outcomes were reported in both groups. However, the magnitude of this effect was significant among N-CT patients (Supplementary Fig. S9).

We further evaluated whether excluding patients with CNV in genes belonging to the MAPK1/MAPK3 pathway ($n = 18$) affected immunotherapy outcomes. Again, a significantly longer PFS (HR = 2.48,



Group	n	Median OS (months)	Lower 95% CI	Upper 95% CI	P value (log-rank test)
TMB high (≥10 mut/Mb)	53	14.0	13.1	20.5	0.038
TMB low (<10 mut/Mb)	21	11.3	4.1	12.1	

Note: Patient with low coverage and high TMB excluded (n = 11).

Group	n	Median PFS (months)	Lower 95% CI	Upper 95% CI	P value (log-rank test)
TMB high (≥10 mut/Mb)	53	6.0	4.0	7.9	0.350
TMB low (<10 mut/Mb)	21	6.1	2.0	9.7	

Note: Patient with low coverage and TMB ≥50 was excluded (n = 11).

Figure 2.
Median OS (**A**) and PFS (**B**) according to TMB status. High TMB (≥10 mut/Mb; yellow line, n = 53) versus low TMB (<10 mut/Mb; blue line, n = 21). mut, mutations.

P = 0.001) and OS (HR = 2.45, P = 0.005) were observed in patients with a MAPK1/MAPK3 mutation compared with those in individuals without any MAPK1/MAPK3 mutation (Supplementary Fig. S10). In addition, RRs to the therapy were higher in MAPK1/MAPK3-positive versus those in MAPK1/MAPK3-negative patients (43% vs. 15%).

Lastly, we conducted an exploratory analysis of the survival outcomes according the most common alterations reported in the MAPK1/MAPK3 signaling pathway, including *NF1*, *EGFR* family, and *FGFR* family gene mutations. We reported a stronger association between *NF1* and *FGFR* family mutations and improved outcomes with immunotherapy. By contrast, there was no association between *EGFR* alterations and outcomes (Supplementary Fig. S11).

- Validation cohort

To further confirm the predictive value of MAPK-activating alteration, we analyzed the clinical outcomes of an independent cohort of 63 patients with advanced LSCC treated at DFCI (see supplementary material and Supplementary Table S6 for details). Among them, 29 (46.0%) presented with mutation and/or CNV in genes belonging to the MAPK1/MAPK3 signaling pathway. In this independent validation cohort, patients positive for mutation or CNV in *MAPK1/MAPK3* genes had longer OS [23.4 months (95% CI, 11.9–41.2) vs. 9.1 months (95% CI, 5.9–26.0); P < 0.001] than individuals without any *MAPK1/MAPK3* mutation or CNV (**Fig. 4**).

This result was also observed in the combined SQUINT and DFCI cohort, including 80 (58.3%) patients with *MAPK1/MAPK3* mutation or CNV who achieved longer OS [16.3 months (95% CI, 13.9–30.6)] compared with 57 (41.7%) patients without *MAPK1/MAPK3* mutations or CNV [10.7 months (95% CI, 6.1–13.3); P = 0.0005; **Fig. 5A**]. Comparable results were observed after excluding patients with CNV [16.3 (95% CI, 13.8–41.2) vs. 11.7 (95% CI, 9.1–14.3) months; P = 0.006; **Fig. 5B**].

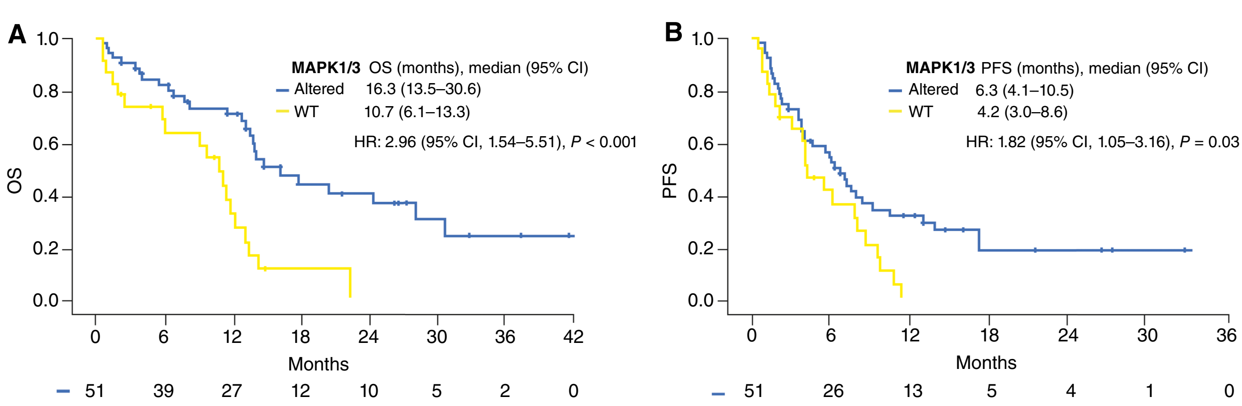


Figure 3.
Median OS (**A**) and PFS (**B**) according to MAPK status. MAPK1/3 WT (yellow line; n = 23) versus MAPK1–3 altered (blue line; n = 51). WT, wild-type.

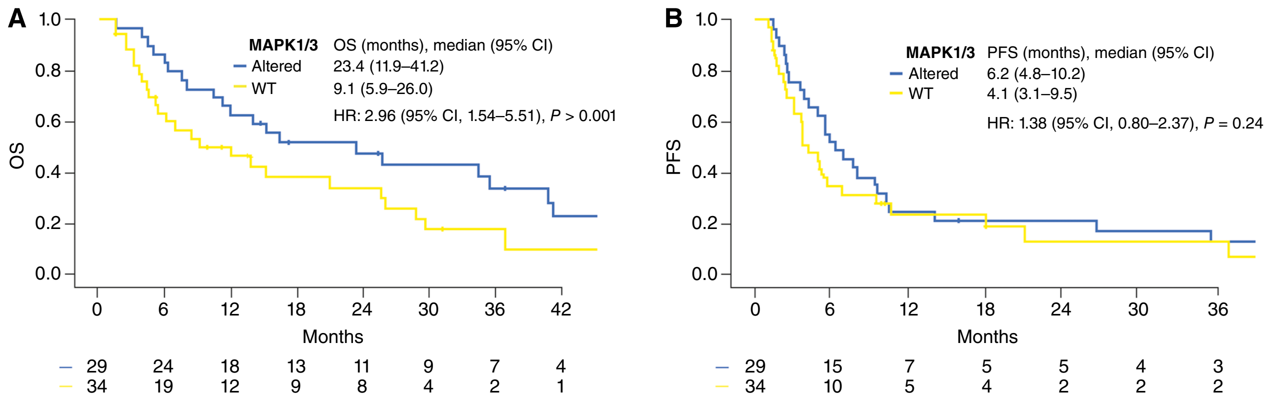


Figure 4.
Median OS (**A**) and PFS (**B**) according to MAPK status in the DFCI validation cohort. MAPK1/3 WT (yellow line; $n = 34$) versus MAPK1/3 altered (blue line; $n = 29$). WT, wild-type.

Multivariable analysis for OS and PFS in the SQUINT and combined SQUINT + DFCI cohorts supported these results and reported a significant decrease in the risk of death in patients with *MAPK1*/*MAPK*3 mutation or CNV versus that in *MAPK1*/*MAPK*3 wild-type patients (adjusted HR = 4.02, $P < 0.001$; Supplementary Fig. S12A–S12D).

To further discriminate the predictive versus the prognostic value of *MAPK1*/*MAPK*3 alterations, we analyzed an independent, control cohort of 79 patients with advanced LSCC who did not receive PD-L1–based therapies at DFCI. As illustrated in Supplementary Fig. S13, there was no difference in the median OS between patients with versus without *MAPK1*/*MAPK*3 alterations, supporting the predictive rather than simply prognostic role of such molecular events [HR = 0.74 (95% CI, 0.43–1.27); $P = 0.28$].

Multiplexed immunofluorescence

We lastly examined whether MAPK1/3 pathway alterations were associated with tumor microenvironment parameters. Among 60 tumor samples, 28 (46.7%) had oncogenic alterations in the MAPK1/3 pathway, including mutations and CNV (Supplementary Fig. S14). We noted a significantly higher density of CD8$^+$PD1$^+$ T cells ($P = 0.04$; **Fig. 6A**) and an increased CD8$^+$PD1$^+$/FOXP3 ratio ($P = 0.047$; **Fig. 6A**) among MAPK-altered tumors versus those of MAPK wild-type tumors. After excluding samples with only oncogenic CNV in the MAPK1/3 pathway, we confirmed that MAPK-altered squamous cancers have a higher density of CD8$^+$ and CD8$^+$PD1$^+$ T cells (**Fig. 6B**) compared with MAPK wild-type cancers. Although these data are exploratory and should be interpreted cautiously, given the small sample size, they suggest that MAPK1/3 alterations may be associated with a distinct immunophenotype.

Other mutations

TP53 mutations were the most frequently reported mutations (55.4% of patients; Supplementary Fig. S4). Mutational events associated with failure of immunotherapy in nonsquamous histology (e.g., *STK11* or *KEAP1* mutations) were not reported in our cohort given the differences in prevalence and context (e.g., association with *KRAS* mutation) among LSCC cases. OS and PFS were not statistically different among *TP53*-positive and -negative patients (OS: $P = 0.826$; PFS: $P = 0.343$; Supplementary Fig. S15).

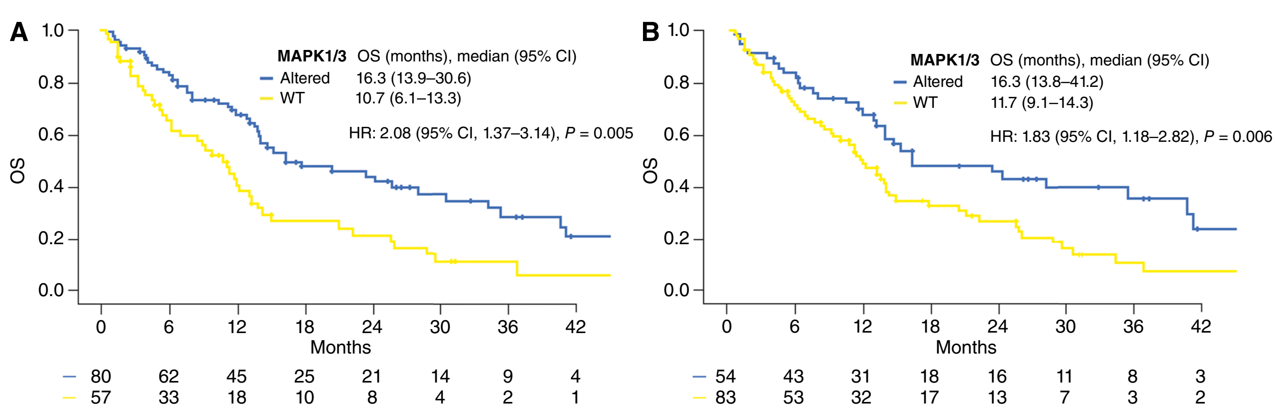


Figure 5.
Median OS according to MAPK status in the combined SQUINT + DFCI cohort. **A,** MAPK1/3-positive patients including patients with CNV. MAPK1/3 WT (yellow line; $n = 57$) versus MAPK1/3 altered (blue line; $n = 80$). **B,** MAPK1/3-altered patients excluding patients with CNV. MAPK1/3 WT (yellow line; $n = 83$) versus MAPK1/3 altered (blue line; $n = 54$). OS, WT, wild-type.

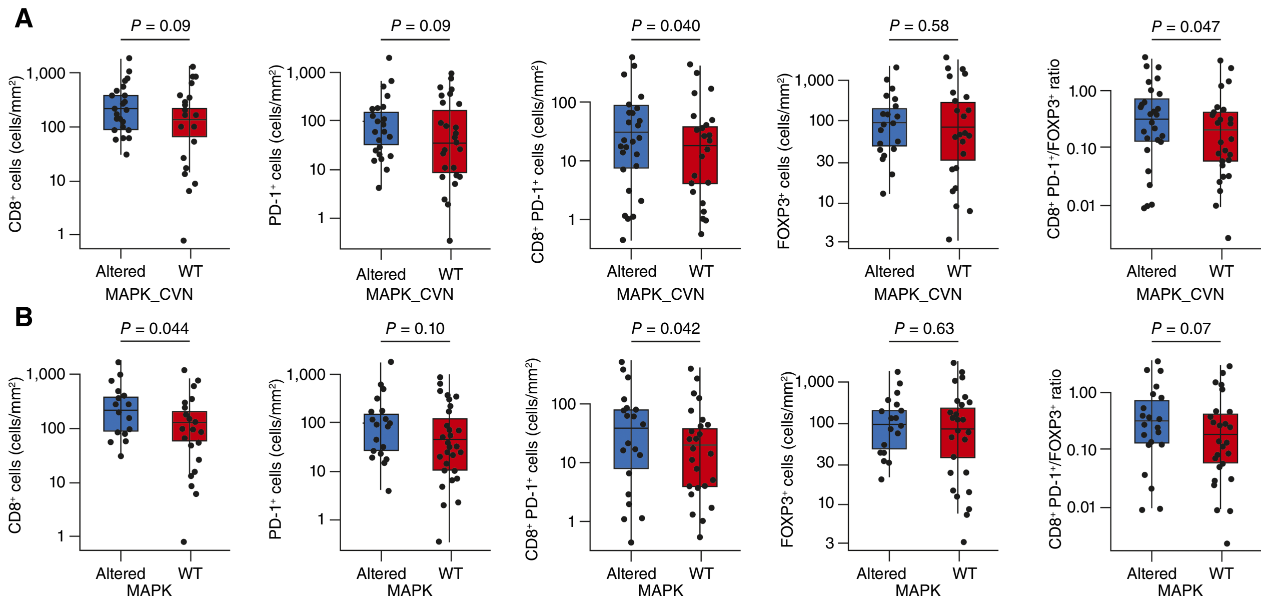


Figure 6.

Representative cases of MAPK-altered including CNV (**A**) and excluding CNV (**B**) and WT LSCC which underwent multiplexed immunofluorescence. WT, wild-type.

Discussion

In the present study, we observed that activating alterations of genes involved in the MAPK pathway identify a group of patients with LSCC particularly sensitive to checkpoint inhibitors. In patients with mutations or CNV of *MAPK* genes who respond to the therapy, PFS and OS were increased than those in the counterpart lacking MAPK alterations, and these findings were confirmed in independent cohorts.

In patients with nonsquamous histology, tumor drivers or specific mutations, such as *STK11* or *SMARCA4*, predict a lack of immunotherapy efficacy. Such molecular events are rare in patients with squamous histology, with an urgent need for predictive factors (15–18). To identify potential biomarkers of ICI response, we conducted an extensive NGS analysis specifically focusing on MAPK-activating alterations. The MAPK pathway is a key signaling hub integrating extracellular signals to control cell proliferation, survival, differentiation, and drug resistance (23, 24). Previous studies showed that head and neck cancers harboring MAPK alterations are $CD8^+$ T-cell inflamed, positively impacting immunotherapy efficacy. Recent bioinformatic analysis in patients with head and neck squamous cell carcinoma showed that *MAPK*-mutant head and neck squamous cell carcinoma tumors, irrespective of human papillomavirus status, have elevated $CD8^+$ T-cell and dendritic cell infiltrations suggestive of active cytolytic $CD8^+$ T cell–inflamed status in these tumors (25). In our study, we analyzed the outcome in terms of RR, PFS, and OS in patients with or without MAPK alterations, and individuals harboring *MAPK* mutations or CNV had the best outcome for all clinical endpoints. In addition, we noted a significantly higher density of $CD8^+PD1^+$ T cells among MAPK-altered tumors versus those in wild-type tumors, together with an increased $CD8^+PD1^+$/FOXP3 ratio in samples with mutations and/or CNV as well as in samples with mutations only, providing a mechanistic explanation on the higher immunotherapy efficacy. To confirm our findings and to clarify the predictive versus the prognostic effect of MAPK-activating alterations, we analyzed two independent cohorts of patients with LSCC followed at DFCI. In the cohort of patients exposed to immunotherapy, we confirmed that MAPK mutations and CNV were significantly associated with longer survival. In the cohort of LSCCs not exposed to immunotherapy, survival was similar between *MAPK1*/*MAPK3*-mutant and wild-type LSCCs, suggesting that these alterations are predictive rather than prognostic factors. These findings have important implications. First, our results suggest that tumor genomic profiling may also play a role in LSCC. Differently from lung adenocarcinoma, actionable driver mutations are exceedingly uncommon in LSCC and tumor genomic profiling is not routinely offered to these patients. This study indicates that MAPK1/3 pathway alterations may be easily identified using targeted exome sequencing panels, and these alterations may possibly inform clinical decisions. Second, our findings suggest that *MAPK1*/*MAPK3* alterations can be independent predictive factors of ICI efficacy; thus, further study can be planned to define whether future ICI clinical trials should employ stratification measures to ensure that treatment arms are well balanced in terms of *MAPK1*/*MAPK3* oncogenic events to avoid treatment outcomes affected by imbalances in these events. Lastly, as adjuvant and neoadjuvant PD-L1 blockade is increasingly being used in clinical practice, we still do not have biomarkers that help identify patients at high risk of relapse. Our data highlight the importance of also exploring the impact of MAPK1/MAPK3 pathway–activating alterations in patients with early-stage LSCC.

In this study, with the limitation of the noncomparative design of the trial, no difference in terms of efficacy was observed among individuals treated with NI versus N-CT combination. This finding agrees with all available clinical data showing no substantial difference in PFS or OS between immunotherapy and chemo-immunotherapy combinations (8, 10, 26). No unexpected toxicities potentially impacting study results were observed.

Certainly, our study has some limitations, including the exploratory nature of biomarker analysis and the relatively small sample size. Lastly, we were powered to detect a class effect of MAPK1/MAPK3 alterations on outcomes of ICI. However, whether specific genes are more associated with ICI efficacy than others remain to be determined in larger studies.

Conclusions

In conclusion, our study showed for the first time that MAPK-activating alterations influence the outcome of LSCC treated with immunotherapy, highlighting the relevance of gene profiling in individuals with squamous histology.

Authors' Disclosures

F. Cappuzzo reports other support from Bristol Myers Squibb during the conduct of the study, as well as personal fees from Roche, AstraZeneca, Bristol Myers Squibb, Pfizer, Takeda, Eli Lilly and Company, Bayer, Amgen, Sanofi, Pharmamar, Novocure, Mirati, Galecto, OSE, ILLUMINA, Thermo Fisher Scientific, BeiGene, and MSD outside the submitted work. B. Ricciuti reports personal fees from AstraZeneca, Regeneron, Amgen, and Bayer outside the submitted work. L. Bonanno reports grants, personal fees, and other support from AstraZeneca, personal fees and other support from MSD, Roche, and Bristol Myers Squibb, personal fees from Novartis, and other support from Pharmamar outside the submitted work. G. Minuti reports personal fees from Bristol Myers Squibb, AstraZeneca, Novartis, MSD, Roche, Amgen, Johnson & Johnson, Gilead, Sanofi, and Daiichi Sankyo outside the submitted work. J.V. Alessi reports personal fees from Bristol Myers Squibb, MSD, AstraZeneca, and Janssen outside the submitted work. A. Di Federico reports personal fees from Novartis, Hanson Wade, and IQVIA outside the submitted work. S. Rodig reports receiving research funding from Bristol Myers Squibb, KITE/Gilead, and Coherus Oncology for research unrelated to this work and serving as a member of the scientific advisory board of Immunitas Therapeutics. M.M. Awad reports grants and personal fees from Genentech, Bristol Myers Squibb, AstraZeneca, Eli Lilly and Company, and Amgen and personal fees from Merck, Mirati, Gritstone, EMD Serono, Regeneron, Janssen, Affini-T, Novartis, Coherus, D3Bio, Synthekine, Gilead, Seagen, and AbbVie outside the submitted work. G. Metro reports grants from Takeda and personal fees from Amgen and Daichii Sankyo outside the submitted work. I. Attili reports personal fees from Bristol Myers Squibb during the conduct of the study. S. Pilotto reports grants and personal fees from AstraZeneca and personal fees from Regeneron, Roche, MSD, Sanofi, Takeda, Pfizer, Amgen, Novartis, and Daichii Sankyo outside the submitted work. S. Buglioni reports personal fees from Roche, Novartis, Johnson & Johnson, Eli Lilly and Company, AstraZeneca, Amgen, and Bayer outside the submitted work. L. Landi reports personal fees from Pfizer, AstraZeneca, Roche, Takeda, Bristol Myers Squibb, MSD, Eli Lilly and Company, Regeneron, Amgen, Novartis, Sanofi, AbbVie, and Johnson & Johnson outside the submitted work. No disclosures were reported by the other authors. Q15

Authors' Contributions

F. Cappuzzo: Conceptualization, supervision, writing–original draft, writing–review and editing. **B. Ricciuti:** Conceptualization, writing–original draft, writing–review and editing. **A. Delmonte:** Writing–review and editing. **L. Bonanno:** Writing–review and editing. **X. Wang:** Writing–review and editing. **W.K. Lye:** Writing–review and editing. **A. Görtz:** Writing–review and editing. **K. Andrikou:** Writing–review and editing. **A. Dal Maso:** Writing–review and editing. **G. Minuti:** Writing–review and editing. **M. Papi:** Writing–review and editing. **J.V. Alessi:** Writing–review and editing. **A. Di Federico:** Writing–review and editing. **S. Rodig:** Writing–review and editing. **M.M. Awad:** Writing–review and editing. **G. Metro:** Writing–review and editing. **I. Attili:** Writing–review and editing. **F. Vitiello:** Writing–review and editing. **S. Pilotto:** Writing–review and editing. **S. Gori:** Writing–review and editing. **G. Rossi:** Writing–review and editing. **S. Buglioni:** Writing–review and editing. **D. Giannarelli:** Writing–review and editing. **L. Landi:** Writing–review and editing. Q16

Ethics Approval

The study was conducted within the protocol approved by independent ethics committees at each site.

Consent to Participate

All the participants signed an informed consent form.

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Note

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