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Phase II Studies with Refametinib or Refametinib plus Sorafenib in Patients with *RAS*-mutated Hepatocellular Carcinoma

Running title: Refametinib or Refametinib + Sorafenib in *RAS*-mutated HCC

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Authors' Contributions

All authors contributed to the collection and analysis of the data and to the writing of the manuscript, and approved the final version before submission. H.Y. Lim and J.M. Llovet: study design; coordinating investigators for the review; approved the clinical study reports; had full access to all the data in the studies; vouch for the integrity of the data analyses; had final responsibility for the decision to submit. K. Roth: statistical analysis. M. Teufel: study design, interpretation of the biomarker results. D. Reis, B.H. Childs, and H. Krissel: study design.

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Translational Relevance

The frequency of *RAS* mutations in hepatocellular carcinoma (HCC) is reported to be ~4%. In this study, we report on the use of a liquid biopsy to prospectively screen patients with HCC for *RAS* mutations using circulating tumor DNA for treatment with the MEK inhibitor refametinib in monotherapy or in combination with sorafenib. The low prevalence of *RAS* mutations in HCC was confirmed (4.4% of patients). *RAS* mutational status was confirmed by next-generation sequencing using circulating tumor DNA, which allowed for the determination of the mutational landscape in patients with HCC. The most frequently detected mutations were in *TERT*, *TP53*, and β-catenin, confirming data reported in The Cancer Genome Atlas. This is the first study using a liquid biopsy for large-scale mutational testing, which offers the opportunity for comprehensive mutational analysis using a non-invasive approach.

Abstract

Purpose: Refametinib, an oral MEK inhibitor, has demonstrated antitumor activity in combination with sorafenib in patients with *RAS*-mutated hepatocellular carcinoma (HCC). Two phase II studies evaluated the efficacy of refametinib monotherapy and refametinib plus sorafenib in patients with *RAS*-mutant unresectable or metastatic HCC.

Methods: Eligible patients with *RAS* mutations of cell-free circulating tumor DNA (ctDNA) determined by beads, emulsion, amplification, and magnetics technology received twice-daily refametinib 50 mg ± sorafenib 400 mg. Potential biomarkers were assessed in ctDNA via next-generation sequencing (NGS).

Results: Of 1318 patients screened, 59 (4.4%) had a *RAS* mutation, of whom 16 received refametinib and 16 received refametinib plus sorafenib. With refametinib monotherapy, the objective response rate (ORR) was 0%, the disease control rate (DCR) was 56.3%, overall survival (OS) was 5.8 months, and progression-free survival (PFS) was 1.9 months. With refametinib plus sorafenib, the ORR was 6.3%, the DCR was 43.8%, OS was 12.7 months, and PFS was 1.5 months. In both studies, time to progression was 2.8 months. Treatment-emergent toxicities included fatigue, hypertension, and acneiform rash. Twenty-seven patients had ctDNA samples available for NGS. The most frequently detected mutations were in *TERT* (63.0%), *TP53* (48.1%), and β-catenin (*CTNNB1*; 37.0%).

Conclusions: Prospective testing for *RAS* family mutations using ctDNA was a feasible, non-invasive approach for large-scale mutational testing in HCC patients. A median OS of 12.7 months with refametinib plus sorafenib in this small population of *RAS*-mutant patients may indicate a synergistic effect between sorafenib and refametinib – this preliminary finding should be further explored.

132 **Word count:** 249

Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide (1,2), and the prognosis for HCC remains extremely poor (2,3). The recommended standard of care in advanced HCC is treatment with the multikinase inhibitor sorafenib (3-5). Lenvatinib has been shown to be non-inferior to sorafenib in a recent phase III trial (6). In second-line, the multikinase inhibitor regorafenib has been approved after showing significantly improved survival versus placebo in patients who had disease progression on sorafenib (7). The kinase inhibitor cabozantinib has also demonstrated promising survival improvements versus placebo as second-line therapy in a phase III trial (8). Immunotherapy has shown promise in HCC, with the immune checkpoint inhibitor nivolumab recently approved for the second-line treatment of advanced HCC based on durable responses observed in a phase I/II trial (9). Treatment with the monoclonal antibody ramucirumab has shown survival improvement versus placebo in patients progressing to sorafenib with alpha-fetoprotein >400 ng/ml (10). Poor prognosis and a lack of treatment options highlight a need for additional viable treatment regimens in the advanced setting.

Refametinib (BAY 86-9766; Bayer AG, Berlin, Germany) is an oral, potent, non-adenosine triphosphate competitive inhibitor targeting MEK 1 and 2 (11), which play a central role in the RAS signal transduction cascade. RAS-MAPK signaling has been implicated in tumor progression and dissemination in HCC (2). A phase I study of the combination of refametinib with sorafenib in patients with advanced malignancies including HCC demonstrated a favorable safety profile and pharmacokinetic profile at a maximum tolerated dose of refametinib 50 mg twice daily in combination with sorafenib 400 mg twice daily (12).

The analysis of cell-free circulating tumor DNA (ctDNA) using beads, emulsion, amplification, and magnetics technology (BEAMing; Sysmex Inostics GmbH, Hamburg,

Germany) enables tumor genotyping at the time of treatment and offers a viable, non-invasive approach to identifying clinically relevant mutations (13,14). BEAMing may therefore be a feasible tool to support the need for the identification of predictive biomarkers in HCC (3), through proof-of-concept studies. Previous proof-of-concept studies of kinase inhibitors in other cancer types have successfully detected predictive mutations, such as vemurafenib in patients with inoperable melanoma with a *BRAF*^{V600} mutation (15) and crizotinib in patients with non-small-cell lung cancer with *EML4-ALK* fusion (16).

A retrospective analysis in a phase II study evaluating refametinib plus sorafenib in Asian patients with HCC found that patients with *RAS* mutations exhibited a robust clinical response compared with patients with wild-type *RAS* (objective tumor response rate [ORR]: 3/4 patients [75.0%] compared with 1/65 patients [1.5%], respectively) (17). Here we describe the first proof-of-concept studies based on mutations conducted in patients with HCC. Two phase II studies prospectively evaluated the efficacy of refametinib monotherapy (NCT01915589) and refametinib plus sorafenib (NCT01915602) in patients with unresectable or metastatic HCC with mutated *RAS*, as determined by BEAMing of ctDNA.

Patients and Methods

Study design

These were two phase II, prospective, single-arm, multicenter, uncontrolled, open-label studies. The primary objective was to evaluate the efficacy of refametinib alone or in combination with sorafenib in patients with *RAS*- (*KRAS*- or *NRAS*-) mutated unresectable or metastatic HCC. The primary efficacy variable was the central radiologic assessment of ORR (complete response [CR] plus partial response [PR]) according to modified Response Evaluation Criteria in Solid Tumors (mRECIST) (18). The secondary objective was safety,

and additional objectives included evaluation of biomarkers aiming to identify biomarkers or biomarker signatures which could correspond to therapy response. Secondary efficacy variables included centrally assessed ORR according to RECIST version 1.1, investigator-assessed ORR according to mRECIST and RECIST version 1.1, overall survival, disease control rate, time to radiographic tumor progression, duration of response, and progression-free survival.

Fifteen patients with *RAS* mutations were planned to be included in the first stage of each study. The second stage was to be initiated if five or more of these patients had a confirmed objective response according to mRECIST.

Important protocol amendments

In the refametinib monotherapy study, the protocol was amended once, with changes implemented globally. Prior cytotoxic chemotherapy was added as an exclusion criterion to omit a population of overtreated patients who may have been different from patients conventionally treated with sorafenib; this change affected eligibility criteria for *RAS* mutation testing and treatment exclusion criteria. An exclusion criterion was also added regarding women of childbearing potential to reduce the time gap between the pregnancy evaluation and the beginning of treatment.

In the refametinib plus sorafenib study, the protocol was amended twice. Changes were implemented globally and included the following amendments: patients with a corrected QT interval >480 ms at the time of screening were excluded from the study because of the potential for QT prolongation with sorafenib; the exclusion criterion regarding women of childbearing potential was amended to reduce the time gap between the pregnancy evaluation and the beginning of treatment; the exclusion criterion regarding systemic anticancer therapy was clarified, as patients with prior systemic anticancer therapy were not eligible for this

study. In addition, a dose-modification scheme for hepatotoxic events was included, because hepatotoxicity is an "identified risk" for the refametinib–sorafenib combination.

Amendments to the statistical analysis plan for the refametinib plus sorafenib study included the collection of survival data to be continued until the last patient's last visit instead of until 12 weeks after the last patient's first treatment, or earlier if all patients had withdrawn from the study. A data rule was also added regarding tumor assessment by centralized blinded reading; for cases with missing adjudication for patients who had completed or withdrawn from treatment at the time of primary analysis, the worst-case approach was to be applied.

Patients

Written, informed consent was obtained from all patients. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the institutions' human research committees.

Eligibility criteria for *RAS* mutational testing included: age ≥18 years with unresectable or metastatic HCC, confirmed histologically (mandatory for non-cirrhotic patients) or by non-invasive radiologic criteria; Eastern Cooperative Oncology Group performance status of 0 or 1; and life expectancy ≥12 weeks. Prior use of targeted agents, experimental therapy, or systemic anticancer treatment was not allowed, although prior sorafenib treatment was permitted in patients who received refametinib only.

Treatment eligibility criteria included: *KRAS* or *NRAS* mutation based on BEAMing plasma test; Child-Pugh class A liver function status; at least one uni-dimensional measurable lesion by computed tomography or magnetic resonance imaging; and Eastern Cooperative Oncology Group performance status of 0 or 1. Treatment exclusion criteria included: any cancer curatively treated less than 3 years before study entry (except cervical carcinoma *in situ*,

treated basal cell carcinoma, and superficial bladder tumors); eligibility for surgery, liver transplantation, ablation, or transarterial chemoembolization for hepatocellular carcinoma; renal failure requiring hemo- or peritoneal dialysis; a history of cardiac disease; or uncontrolled hypertension.

Treatment

In both studies, eligible patients harboring *RAS* mutations received refametinib 50 mg twice daily in 21-day cycles. In the refametinib plus sorafenib study, patients also received standard sorafenib (400 mg twice daily), starting with a dose of 600 mg daily (200 mg in the morning plus 400 mg in the evening) in cycle 1, escalating to the standard sorafenib dose in cycle 2 if no hand-foot skin reaction, fatigue, or gastrointestinal toxicities of grade ≥2 occurred. Patients received treatment on a continuous basis until radiologic disease progression, clinical progression, or other criteria for discontinuation of treatment were met.

Assessments

ctDNA from plasma samples collected in the pre-treatment period was centrally evaluated for *RAS* mutational status using BEAMing technology (13), with a limit of detection at 0.02% mutant allele. Tumor assessments were performed at screening and every 6 weeks. Treatment response was centrally assessed according to mRECIST for the primary endpoint, and was also investigator-assessed according to mRECIST (18). Safety, including adverse events (AEs) and concomitant medications, was monitored throughout the studies. Creatine phosphokinase (CPK) increase of grade ≥3 was considered an AE of special interest and was to be reported as a serious AE (SAE). Plasma samples for biomarker analysis were collected at screening, at cycle 1, days 1 and 15, and at cycle 2, day 15. Peripheral whole-blood samples from patients with mutated *RAS* were analyzed for detection of genomic alterations using FoundationACT® (Foundation Medicine®, Cambridge, MA, USA), a targeted next-

generation sequencing (NGS)-based ctDNA assay (19). The detection limit of FoundationACT® is specified at 0.1% mutant allele frequency, i.e. a lower sensitivity than BEAMing. FoundationACT® is a hybrid-capture-based assay that is designed to interrogate 62 genes, identifying all classes of alterations including base substitutions, insertions and deletions, copy number variations, and rearrangements/fusions through computational algorithms (20).

Statistical analysis

In each study, it was estimated that approximately 350 patients were needed to be tested via BEAMing to identify 15 patients with mutated *RAS* in stage 1, and that approximately 2300 patients would need to be tested via BEAMing to identify a sufficient number of patients with mutated *RAS* to be treated in stage 2.

Descriptive statistics were calculated for the presented endpoints.

Results

Patient disposition, demographics, and baseline characteristics

In the refametinib monotherapy study, 498 patients were enrolled at 58 study centers in 17 countries across Asia, Europe, and the USA from September 2013 to June 2014. *RAS* mutational testing was performed in 493 patients (Fig. 1A); 32 (6.5%) had a *RAS* mutation. In the refametinib plus sorafenib study, 820 patients were enrolled at 80 study centers in 21 countries across Asia, Europe, and the USA from September 2013 to April 2015. *RAS* mutational testing was performed in 815 patients (Fig. 1B); 27 (3.3%) had a *RAS* mutation. Overall, 4.4% of HCC patients screened (59/1318) had a *RAS* mutation determined by BEAMing. Of those, 32/59 patients received treatment, either refametinib monotherapy

(*n* = 16) or refametinib plus sorafenib (*n* = 16). Reasons for patients not receiving treatment are summarized in Fig. 1.

In the refametinib monotherapy study, the median age was 69 years and the median time since initial HCC diagnosis was 72.1 weeks (Table 1). Nine patients (56.3%) had received prior first-line sorafenib treatment. In the refametinib plus sorafenib study, the median age was 67 years and the median time since initial HCC diagnosis was 32.2 weeks (Table 1). Demographics and baseline characteristics were similar between patients irrespective of *RAS* mutational status in both studies.

Efficacy

Of the 16 patients treated with refametinib monotherapy, no patient had a CR or PR when centrally assessed according to mRECIST, and the ORR was 0% (Table 2). One patient (6.3%) achieved an unconfirmed PR and eight (50.0%) achieved stable disease; the disease control rate was 56.3%. ORR was 0% by independent assessment according to RECIST version 1.1: no patients had a CR or PR, 10 (62.5%) had stable disease, two (12.5%) had disease progression, and four (25.0%) were not evaluable. The investigator-assessed ORR was 0% according to mRECIST (Supplementary Table S1) and RECIST version 1.1: no patients had a confirmed or unconfirmed CR or PR, 10 (62.5%) had stable disease, two (12.5%) had disease progression, and four (25.0%) were not evaluable.

Of the 16 patients treated with refametinib plus sorafenib, one patient (6.3%) achieved a confirmed PR when centrally assessed according to mRECIST, and the ORR was 6.3% (Table 2). Two patients (12.5%) achieved unconfirmed PRs (confirmatory computed tomography scan showed progression) and four (25.0%) had stable disease; the disease control rate was 43.8%. Independent assessment according to RECIST version 1.1 reported an ORR of 6.3%: one patient (6.3%) had a confirmed PR, six (37.5%) had stable disease,

five (31.3%) had disease progression, and four (25.0%) were not evaluable or had data missing. Investigator-assessed ORR was 6.3% according to mRECIST (one patient [6.3%] had a confirmed PR) (Supplementary Table S1). The investigator-assessed ORR was also 6.3% according to RECIST version 1.1: one patient (6.3%) had a PR, one (6.3%) had an unconfirmed PR, five (31.3%) had stable disease, six (37.5%) had disease progression, and three (18.8%) had missing data.

In the refametinib monotherapy study, four patients (25.0%) had radiologic progression and the median time to progression was 2.8 months (Fig. 2A). Seven patients (43.8%) in the refametinib plus sorafenib study had radiologic progression and the median time to progression was 2.8 months (Fig. 2B).

Duration of response could not be calculated in the refametinib monotherapy study because no patient achieved a CR or PR. Duration of response based on central assessment in the refametinib plus sorafenib study was 1.4 months for the one patient who achieved a confirmed PR; this patient had a $KRAS^{G35A}$ point mutation. Duration of response was 2.7 months for the one patient who was investigator-assessed as achieving a confirmed PR; this patient had a $KRAS^{G38A}$ mutation.

In the refametinib monotherapy study, nine patients (56.3%) had disease progression or died and median progression-free survival was 1.9 months (Fig. 2C). Eight patients (50.0%) died and median overall survival was 5.8 months (Fig. 2E). In the refametinib plus sorafenib study, 10 patients (62.5%) had disease progression or died and median progression-free survival was 1.5 months (Fig. 2D). Nine patients (56.3%) died and median overall survival was 12.7 months (Fig. 2F).

Exposure and dose modifications

With refametinib monotherapy, the median duration of treatment (including interruptions) was 7.14 weeks. The mean (± standard deviation) daily dose of refametinib (excluding interruptions) was 90.01 ± 13.88 mg.

With refametinib plus sorafenib, the median durations of treatment (including interruptions) for refametinib and sorafenib were 8.21 weeks and 6.43 weeks, respectively. The mean (± standard deviation) daily doses (excluding interruptions) of refametinib and sorafenib were 85.06 ± 16.07 mg and 514.24 ± 124.23 mg, respectively. The majority of patients experienced treatment-emergent AEs (TEAEs) of hand-foot skin reaction, fatigue, or gastrointestinal toxicities of grade ≥2 in cycle 1, so only three patients (18.8%) received the full dose of sorafenib (800 mg/day) following cycle 1. One patient remained on treatment at the time of data-cut off and has been ongoing for approximately 2 years.

TEAEs led to dose modification (interruption or reduction) in 14 patients (87.5%) receiving refametinib monotherapy (Table 3) and were considered drug-related in 13 patients (81.3%). Treatment was permanently discontinued because of TEAEs in four patients (25.0%) and were considered drug-related in three patients (18.8%).

With refametinib plus sorafenib, dose modifications were reported in 11 patients (68.8%) with refametinib and 11 patients (68.8%) with sorafenib. TEAEs led to dose modification in 15 patients (93.8%) (Table 3); events were considered refametinib-related in 13 patients (81.3%) and sorafenib-related in 14 patients (87.5%).

Safety

At least one TEAE was reported in all 16 patients (100%) receiving refametinib monotherapy (Table 3). The most common TEAEs of worst grade 3 were fatigue and increased CPK

(three patients each [18.8%]). Grade 5 TEAEs occurred in five patients (31.3%): sepsis, death not otherwise specified, multi-organ failure, lung infection, and heart failure. The causes of death were progressive disease (one patient) and AE associated with clinical disease progression and AE not associated with clinical disease progression (two patients each). Drug-related TEAEs occurred in 14 patients (87.5%) (Supplementary Table S2). In most patients (75.0%), the worst grade of drug-related TEAEs was grade 3, and one patient (6.3%) had a drug-related TEAE of grade 4 (increased serum amylase). Twelve patients (75.0%) experienced SAEs (Supplementary Table S3), of which the most common worst grade was grade 3 (43.8%). SAEs were refametinib-related in seven patients (43.8%), most commonly increased CPK (three patients [18.8%]). All other refametinib-related SAEs were reported in one patient each (6.3%) (Supplementary Table S3).

TEAEs occurred in all 16 patients (100%) receiving refametinib plus sorafenib (Table 3). Hand-foot skin reaction was reported in two patients (12.5%). The most common TEAEs of worst grade 3 were hypertension (10/16 [62.5%]) and increased aspartate aminotransferase and increased CPK in five patients each (31.3%). Seven TEAEs of worst grade 4 were reported in three patients (18.8%): increased aspartate aminotransferase, increased CPK, decreased platelet count, investigations - other, hypophosphatemia, and hyperuricemia. Grade 5 TEAEs included general disorders and administration site conditions - other and dyspnea (one patient each); the cause of death was AE associated with clinical disease progression and progressive disease (one patient each). Refametinib- and sorafenib-related TEAEs were reported for all 16 patients (100%) (Supplementary Table S2). Nine patients (56.3%) had refametinib-related TEAEs of grade 3 and three patients (18.8%) had refametinib-related TEAEs of grade 4 (Supplementary Table S2). Twelve patients (75.0%) had sorafenib-related TEAEs of worst grade 3 and three patients (18.8%) had sorafenib-related TEAEs of worst grade 4. One patient (6.3%) had a grade 5 TEAE considered related

to both refametinib and sorafenib (general disorders and administration site conditions - other). SAEs were reported in 13 patients (81.3%) (Supplementary Table S3), most commonly increased CPK in six patients (37.5%; five grade 3, one grade 4). Refametinib-related SAEs were experienced by 12 patients (75.0%), most frequently worst grade 3 (7/16 [43.8%]). Increased CPK was the most commonly reported refametinib-related SAE (5/16 [31.3%]; four grade 3, one grade 4). Sorafenib-related SAEs occurred in 10 patients (62.5%); seven patients (43.8%) had events of worst grade 3 and one patient (6.3%) experienced worst grade 4 (increased CPK). One SAE of grade 5 was considered refametinib-related and sorafenib-related (general disorders and administration site conditions - other).

Biomarker analyses

To identify potential genomic biomarkers which might be associated with resistance to refametinib monotherapy or combination therapy, NGS (FoundationACT®) was performed on available ctDNA from 27 patients (refametinib monotherapy, $n = 15$; refametinib plus sorafenib, $n = 12$). *RAS* mutations were not called by NGS in over 60% of the samples with a mutant allele frequency of between 0.02% and 0.1% as determined by BEAMing. *RAS* mutational status was confirmed by NGS in 12 patients (44.4%), all with a mutant allele frequency above 0.1%. The *RAS* somatic aberration detected was concordant with BEAMing results in 11 patients (91.7%). Excluding *RAS*, the most frequently detected mutation was in the promoter region of telomerase reverse transcriptase (*TERT*; 17/27 [63.0%]), followed by *TP53* (13/27 [48.1%]), and β-catenin (*CTNNB1*; 10/27 [37.0%]) (Fig. 3). Actionable mutations were rare (<10%) and included oncogenes such as *EGFR*, *JAK2*, *BRAF*, *FLT3*, *PIK3CA*, and *cKIT*.

Discussion

These two phase II proof-of-concept studies prospectively evaluated the efficacy and safety of refametinib monotherapy or refametinib plus sorafenib in patients prospectively screened for *RAS*-mutant unresectable or metastatic HCC based on evaluation of mutational status in ctDNA. The previous phase II BASIL trial in a separate population of Asian patients with HCC receiving refametinib plus sorafenib demonstrated that the majority of patients who responded to this regimen had mutant *RAS* tumors, with an ORR of 75% in patients with *RAS*-mutant HCC compared with 1.5% in HCC patients with no *RAS* mutation (17).

In these studies, prospective testing for *RAS* mutations using ctDNA isolated from plasma was a feasible, non-invasive approach for large-scale mutational testing in HCC patients. The current findings support a previous report of the use of ctDNA to detect *KRAS* mutations via BEAMing in a small study of patients with refractory colorectal carcinoma treated with regorafenib (21), although *KRAS* mutational frequency was notably higher in the colorectal carcinoma population (~40%) compared with that reported here (~5%). Overall, 59/1318 (4.4%) of the HCC patients screened had a *RAS* mutation. The *RAS* mutation rates reported here are consistent with previous reports in this patient population (~5%) (22-25). It should therefore be noted that the low *RAS* mutational frequency in this population suggests that identifying *RAS*-mutant patients may be challenging in practice.

The primary efficacy variable was not met in the refametinib monotherapy study, with no patient with mutated *RAS* achieving a CR or PR. In the refametinib plus sorafenib study, one patient with mutated *RAS* achieved a PR, resulting in an ORR of 6.3%, which is broadly similar to the ORR of 6.9% reported in the BASIL trial (17).

The target for the first stage of the trials (≥5/15 patients with a CR or PR) was not reached; therefore, these studies did not proceed to the planned evaluation of refametinib monotherapy or combination therapy in a larger number of patients. Further exploration would be required to understand the lower ORR with refametinib plus sorafenib in this study compared with previous reports (17). These results suggest that the use of *RAS* mutational status as a prognostic biomarker for treatment response to refametinib monotherapy or in combination with sorafenib was unsuccessful, and targeting MEK with refametinib in this *RAS*-mutant patient population did not lead to a significant proportion of objective responses. However, the low number of patients treated should be taken into account, and the low proportion of responses observed may reflect random error – these results should therefore be interpreted with caution. Additional molecular events may explain the limited responses seen using mutated *RAS* as a prognostic biomarker for targeted MEK inhibition in these studies. It is possible that with intra-tumor heterogeneity, mutations occurring in low-frequency subclonal tumor cell populations may have acquired mutations that conferred resistance to refametinib, which was targeted to progenitor cells expressing truncal driver mutations in *RAS*, negatively affecting clinical outcomes (26). Evaluation of non-truncal mutations, together with longer-term evaluations of changes in allele frequency, were not planned in this study, although may provide useful insights into the development of resistance to refametinib in patients with HCC.

Median overall survival was 5.8 months with refametinib monotherapy and 12.7 months with refametinib plus sorafenib, with over half of events occurring during the study period. *KRAS* mutation is generally associated with poorer outcomes in most cancers, although there are no established data in HCC due to the lack of robust testing in large studies of advanced disease (23). In our study, the effect of refametinib monotherapy on overall survival can be considered insignificant, since the expected outcome of placebo at first or second line is 7–8

436 months (27,28). In fact, this survival of under 6 months might indicate that advanced *RAS*-
437 positive HCC tumors have a poor natural history. It should also be noted that 56% of patients
438 in the monotherapy arm had received prior sorafenib, possibly contributing to the poor
439 survival seen. However, the approximately 13-month survival outcome with refametinib plus
440 sorafenib treatment is more intriguing, considering the expected median survival with first-
441 line sorafenib monotherapy alone is 11 months (29). This result may indicate a synergistic
442 effect between sorafenib and refametinib, which is relevant as tumors harboring *RAS*
443 mutations remain some of the most challenging to treat because of the paucity of successful
444 drugs targeting the RAS pathway (30). However, this finding should be interpreted with
445 caution because of the heterogeneity in baseline liver function and tumor factors, which could
446 affect response to treatment. Also, patients in the refametinib plus sorafenib study had a
447 much shorter median time from initial diagnosis to study treatment compared with patients in
448 the monotherapy study (32.1 weeks vs. 72.1 weeks, respectively). The overall survival
449 findings in the combination study support those described for patients receiving refametinib
450 plus sorafenib in the BASIL trial (17), although median overall survival was increased in our
451 study (12.7 months vs. 9.5 months, respectively).

452 Median time to progression was the same across the refametinib monotherapy and
453 refametinib plus sorafenib studies (2.8 months), with similar median progression-free
454 survival observed between the studies (1.9 months and 1.5 months, respectively). However,
455 progression-free survival times were lower than previous reports (17).

456 Drug exposure was similar between both studies and similar to the median refametinib dose
457 observed in the BASIL study (17). The majority of patients in the refametinib plus sorafenib
458 study experienced AEs during cycle 1 that prevented sorafenib dose escalation to 800 mg per
459 day, which was also observed in the BASIL study (17). The majority of patients across both

studies experienced AEs leading to dose modifications, which may have caused insufficient drug exposure, potentially leading to reduced efficacy of both refametinib regimens. Overall, median duration of treatment was relatively short in both trials (7 weeks and 8 weeks, respectively), similar to that reported for the BASIL study (8 weeks) (17).

Overall, refametinib was tolerated as monotherapy and combination therapy, and the majority of TEAEs were manageable in both studies. In patients receiving refametinib monotherapy, the most common TEAEs of limb edema, fatigue, nausea, and vomiting were consistent with the safety profile previously reported in a phase I study of refametinib (31). The high overall incidence of grade 3 TEAEs irrespective of causality in both studies (68.8%) was similar to that reported in the BASIL trial (60.0%) (17). Generally, the observed incidence and severity of refametinib-related TEAEs observed with refametinib monotherapy were comparable with data from the previous refametinib phase I study (31). Refametinib-related SAEs were less frequent with refametinib monotherapy than with refametinib plus sorafenib (43.8% vs. 75.0%, respectively). Increased CPK grade ≥3 was the most common refametinib-related SAE reported in both studies, consistent with reports of increased CPK as a class effect of MEK inhibitors (32-34).

Compared with the known safety profile of sorafenib monotherapy (29,35), a higher incidence of liver and gastrointestinal toxicities and rash was observed in patients who received refametinib plus sorafenib. However, alopecia and hand-foot skin reaction were less common compared with those reported for sorafenib monotherapy (14% vs. 12.5% and 21% vs. 6.3%, respectively) (29), possibly due to the reduced exposure to sorafenib in the majority of patients in our study.

Biomarker analysis of ctDNA analyzed by NGS showed the observed mutational landscape to be consistent with published data for HCC (36). The most common mutation was in the

promoter region of *TERT*, supporting previous observations in patients with HCC and combined HCC-cholangiocarcinoma (37,38). Few actionable mutations were found, with none appearing to explain the resistance to refametinib alone or in combination with sorafenib, and few of the detected mutations are feasible for targeting with existing drugs. It therefore remains inconclusive from our results as to whether somatic mutations in oncogenes affect the efficacy of refametinib in monotherapy or combination therapy. Although analyses were planned to evaluate the role of biomarkers in the response to treatment, due to limited sample size and early study termination it was not possible to fully address the role of intra-tumor heterogeneity (26). In addition, although the two study populations included only Child-Pugh A patients, these patients were heterogeneous for various factors that may be prognostic for treatment response, such as a history of ascites (in four patients overall [12.5%]) (39), alpha-fetoprotein (>400 µg/L in 12 patients [37.5%]) (40), microvascular invasion (in 11 patients [34%]) (41), extrahepatic spread (in 16 patients [50%]) (41), and hepatitis C (in seven patients [21.9%]) (41). However, no formal analysis of lung function status and tumor factors as prognostic markers for treatment response was planned in these studies.

In these studies, *RAS* mutational status as determined by BEAMing was confirmed in 44% of samples using NGS, all with mutant allele frequencies of 0.1% or higher. Although BEAMing technology is highly sensitive (42), the newly developed NGS from ctDNA approach has demonstrated high concordance, confirming nearly all mutations identified by BEAMing, and offers the additional advantage of providing the mutational landscape based on ctDNA. A comparison of sensitivity between both assays is difficult due to the different detection limit of each method (0.02% for BEAMing vs. 0.1% for NGS), which did not allow for the detection of *RAS* mutational status by NGS in over 60% of samples with mutant allele frequency between 0.02% and 0.1%. Nonetheless, our results demonstrated that NGS

appears to be a promising non-invasive approach to determine the landscape of somatic mutations, particularly for patients in whom a biopsy is not an option (19).

Despite the poor ORR in patients with *RAS* mutations, a median overall survival of 13 months in the small population included in the refametinib plus sorafenib study may indicate a synergistic effect between refametinib and sorafenib that should be further explored in a larger patient population that is not stratified by *RAS* mutational status, taking into account other prognostic factors based on patient heterogeneity and intra-tumor heterogeneity. The analysis of mutational status using ctDNA isolated from plasma as a liquid biopsy was a feasible, non-invasive technique in patients with unresectable or metastatic HCC, although *RAS* mutational frequency was low. Further analysis of this technique is warranted for discovery of predictive biomarkers in HCC and other cancers.

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Table 1. Demographics and baseline characteristics of patients receiving refametinib monotherapy or refametinib plus sorafenib

	Refametinib monotherapy (*n* = 16)	**Refametinib plus sorafenib (*n* = 16)**
Male, *n* (%)	13 (81.3)	12 (75.0)
Race, *n* (%)		
White	9 (56.3)	9 (56.3)
Asian	7 (43.8)	6 (37.5)
Black or African American	0	1 (6.3)
Median age, years (range)	69 (37–84)	67 (53–82)
Median body mass index, kg/m^2 (range)	23.7 (20.5–31.8)	23.6 (16.4–34.8)
Baseline ECOG PS, *n* (%)		
0	7 (43.8)	10 (62.5)
1	9 (56.3)	6 (37.5)
Medical history, *n* (%)[a]		
Hepatic cirrhosis	14 (87.5)	11 (68.8)
Ascites	3 (18.8)	1 (6.3)
Gastroesophageal reflux disease	3 (18.8)	1 (6.3)
Abdominal pain[b]	2 (12.5)	2 (12.5)
Esophageal varices	2 (12.5)	1 (6.3)
Portal hypertension	1 (6.3)	2 (12.5)
Confirmation of liver cirrhosis, *n* (%)		
Histologic	4 (25.0)	3 (18.8)
Clinical	10 (62.5)	5 (31.3)
Histologic and clinical	0	3 (18.8)
Missing	2 (12.5)	5 (31.3)

Etiology of HCC, *n* (%)		
Alcohol use	6 (37.5)	3 (18.8)
Alcohol use/genetic/metabolic	0	1 (6.3)
Alcohol use/hepatitis B	2 (12.5)	0
Alcohol use/hepatitis C	0	1 (6.3)
Hepatitis B	2 (12.5)	5 (31.3)
Hepatitis C	3 (18.8)	4 (25.0)
Non-alcoholic steatohepatitis	1 (6.3)	1 (6.3)
Unknown	2 (12.5)	1 (6.3)
Overall Child-Pugh A score, *n* (%)		
5	8 (50.0)	10 (62.5)
6	8 (50.0)	6 (37.5)
BCLC stage, *n* (%)		
A (early)	0	2 (12.5)
B (intermediate)	2 (12.5)	2 (12.5)
C (advanced)	14 (87.5)	12 (75.0)
Presence of macrovascular invasion, *n* (%)	4 (25.0)	7 (43.8)
Presence of extrahepatic spread, *n* (%)	10 (62.5)	6 (37.5)
Alpha-fetoprotein >400 µg/L, *n* (%)	9 (56.3)[c]	3 (18.8)
Bilirubin, mg/dL, median (range)	0.9 (0.4–2.3)	0.7 (0.3–1110.9)
Albumin, g/dL, median (range)	3.9 (2.8–4.3)	3.9 (3.2–38.0)
Prothrombin INR, median (range)	1.1 (0.9–1.3)	1.1 (0.9–1.2)
Median time since initial diagnosis, weeks (range)	72.1 (5.9–262.3)	32.2 (8.1–342.7)
Median time since most recent progression, weeks (range)	8.6 (1.1–57.0)	8.6 (3.1–21.0)
Prior anticancer therapies and procedures, *n* (%)		

Surgical therapeutic procedure	6 (37.5)	9 (56.3)
Systemic anticancer therapy (sorafenib)	9 (56.3)	0
Local anticancer therapy	6 (37.5)	8 (50.0)
Number of target lesions (mRECIST), *n* (%)		
1	2 (12.5)	4 (25.0)
2	10 (62.5)	10 (62.5)
3	4 (25.0)	1 (6.3)
4	0	1 (6.3)
Number of non-target lesions (mRECIST), *n* (%)		
0	4 (25.0)	6 (37.5)
1	9 (56.3)	8 (50.0)
2	2 (12.5)	0
3	1 (6.3)	1 (6.3)
4	0	1 (6.3)

[a]In two or more patients overall; [b]Includes upper and lower abdominal pain in one patient each in the combination study; [c]Baseline data missing for one patient.

Abbreviation: BCLC, Barcelona Clinic Liver Cancer; ECOG PS, Eastern Cooperative Oncology Group performance status; INR, international normalized ratio.

Table 2. Response evaluation by central assessment using mRECIST in patients receiving refametinib monotherapy or refametinib plus sorafenib

***n* (%) [95% CI]**	**Refametinib monotherapy (*n* = 16)**	**Refametinib plus sorafenib (*n* = 16)**
Best overall response		
Complete response	0	0
Confirmed partial response	0	1 (6.3) [0.16–30.23]
Unconfirmed partial response	1 (6.3) [0.16–30.23]	2 (12.5) [7.27–52.38]
Stable disease	8 (50.0) [24.65–75.35]	4 (25.0) [7.27–52.38]
Disease progression	3 (18.8) [4.05–45.65]	5 (31.3) [11.02–58.66]
Not evaluable	0	1 (6.3) [0.16–30.23]
Missing	4 (25.0) [7.27–52.38]	3 (18.8) [4.05–45.65]
Objective tumor response rate	0	1 (6.3) [0.16–30.23]
Disease control rate[a]	9 (56.3) [29.88–80.25]	7 (43.8) [19.75–70.12]

[a]Includes unconfirmed complete and partial responses ≥6 weeks from baseline assessment.

Abbreviation: CI, confidence interval.

Table 3. Summary of safety and incidence of treatment-emergent adverse events (by worst CTCAE grade) occurring in three or more patients receiving refametinib monotherapy or refametinib plus sorafenib

***n* (%)**	**Refametinib monotherapy (*n* = 16)**	**Refametinib plus sorafenib (*n* = 16)**
Any TEAE[a]	16 (100)	16 (100)
Worst grade		
3	11 (68.8)	11 (68.8)
4	0	3 (18.8)
5 (death)	5 (31.3)	2 (12.5)
Serious adverse events	12 (75.0)	13 (81.3)
Led to dose modification	14 (87.5)	15 (93.8)
Led to permanent discontinuation	4 (25.0)	5 (31.3)
Incidence of TEAEs (any grade) occurring in ≥10% of the total population		
Limb edema	7 (43.8)	3 (18.8)
Fatigue	6 (37.5)	12 (75.0)
Nausea	6 (37.5)	2 (12.5)
Vomiting	6 (37.5)	5 (31.3)
Increased creatine phosphokinase	5 (31.3)	8 (50.0)
Diarrhea	5 (31.3)	10 (62.5)
Acneiform rash	5 (31.3)	8 (50.0)
Increased aspartate aminotransferase	4 (25.0)	8 (50.0)
Maculo-papular rash	4 (25.0)	6 (37.5)
Hypertension	4 (25.0)	13 (81.3)
Anemia	3 (18.8)	2 (12.5)

Abdominal pain	3 (18.8)	2 (12.5)
Ascites	3 (18.8)	3 (18.8)
Anorexia	3 (18.8)	4 (25.0)
Hypoglycemia	3 (18.8)	0
Back pain	3 (18.8)	1 (6.3)
Dyspnea	3 (18.8)	2 (12.5)
Dry skin	3 (18.8)	2 (12.5)
Skin and subcutaneous tissue disorders - other, specify	3 (18.8)	0
Oral mucositis	2 (12.5)	5 (31.3)
Hypoalbuminemia	2 (12.5)	4 (25.0)
Decreased platelet count	2 (12.5)	4 (25.0)
Constipation	2 (12.5)	3 (18.8)
Investigations - other, specify	2 (12.5)	3 (18.8)
Hyperglycemia	2 (12.5)	3 (18.8)
Increased alanine aminotransferase	1 (6.3)	3 (18.8)
Malaise	1 (6.3)	3 (18.8)
Skin infection	1 (6.3)	3 (18.8)
Headache	0	3 (18.8)
Increased lipase	0	3 (18.8)

[a]Number (%) of patients with the specified event starting or worsening between the start of treatment and 30 days after the end of treatment.

Abbreviation: CTCAE, Common Terminology Criteria for Adverse Events.

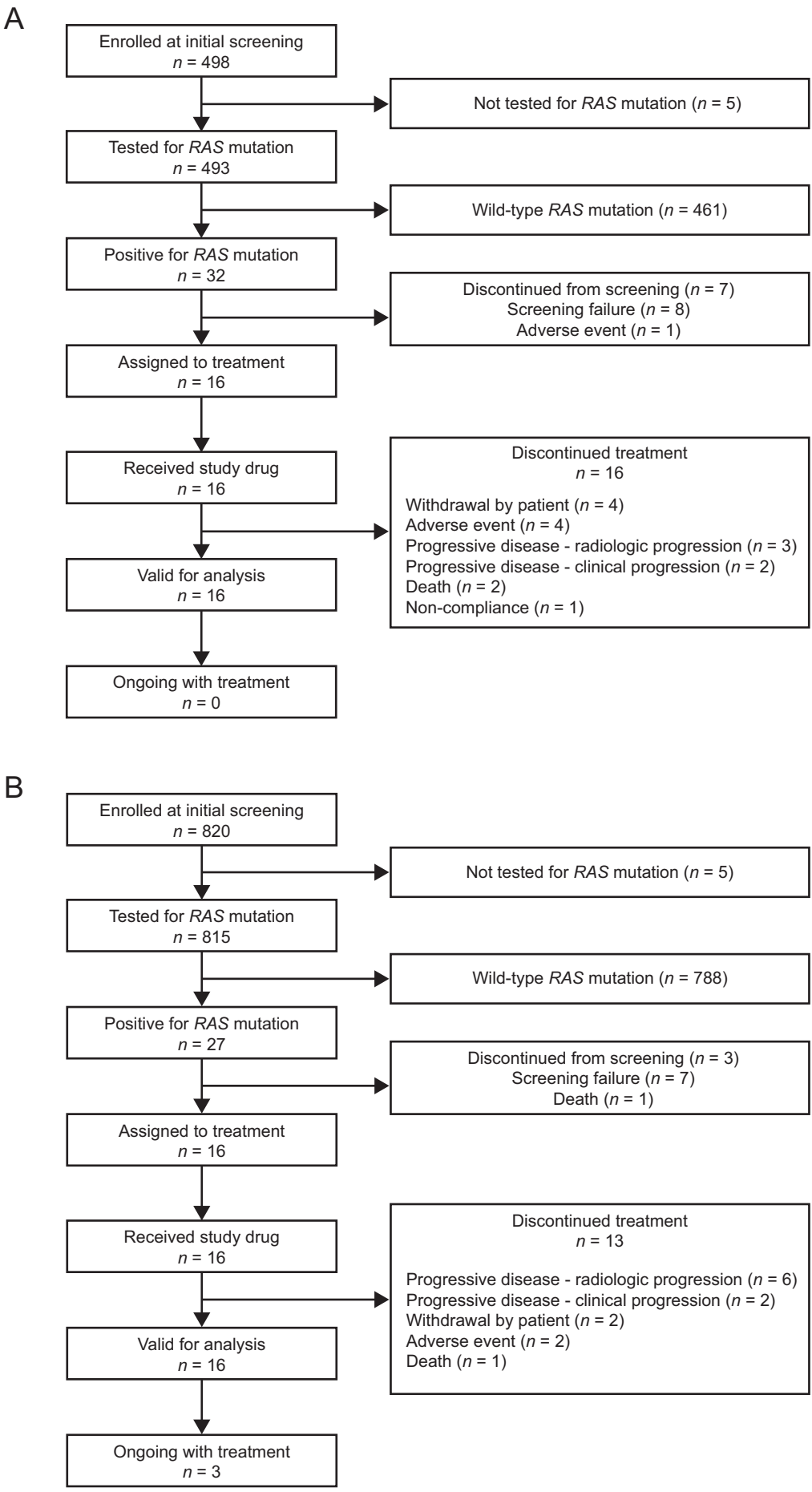
Figure legends

Figure 1. Patient disposition in the two phase II studies. (A) Refametinib monotherapy study. (B) Refametinib plus sorafenib study.

Figure 2. Kaplan-Meier curves of TTP, PFS, and OS in the two phase II studies. (A) TTP in patients who received refametinib monotherapy. (B) TTP in patients who received refametinib plus sorafenib. (C) PFS in patients who received refametinib monotherapy. (D) PFS in patients who received refametinib plus sorafenib. (E) OS in patients who received refametinib monotherapy. (F) OS in patients who received refametinib plus sorafenib. Abbreviations: CI, confidence interval; NE, not estimable due to censored data; OS, overall survival; PFS, progression-free survival; TTP, time to progression.

Figure 3. Somatic aberrations of patients with *RAS* mutations as detected in circulating tumor DNA. Abbreviations: r, rearrangement; s, short variant.

Figure 1



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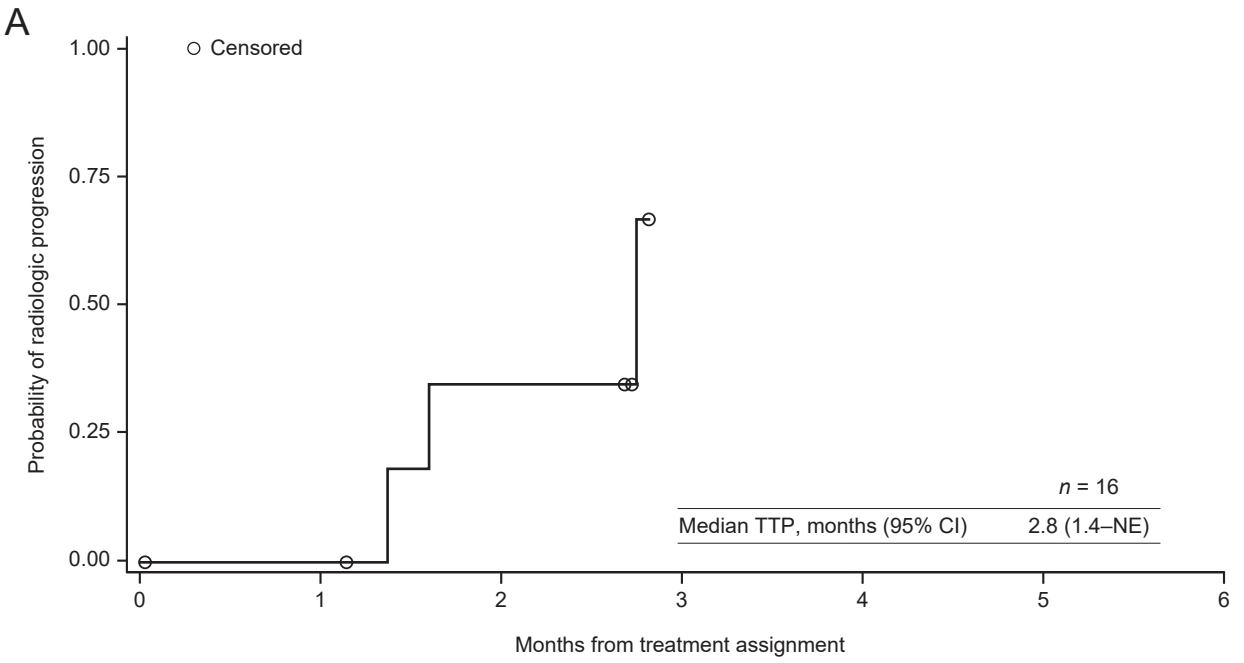

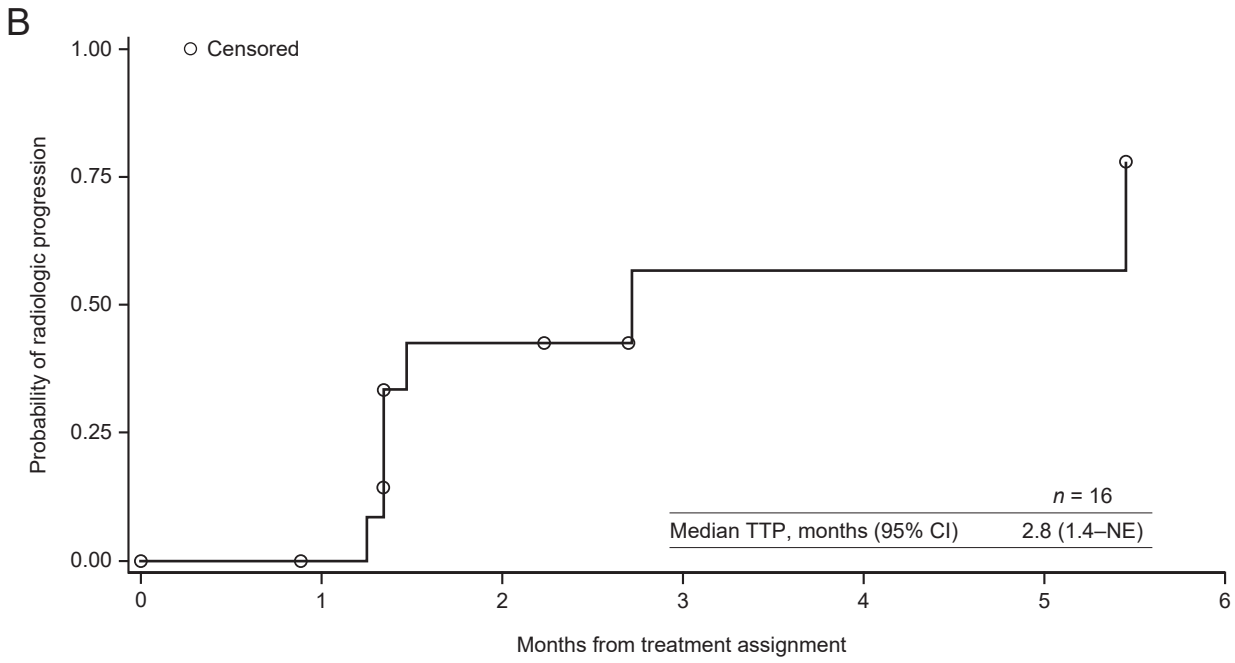

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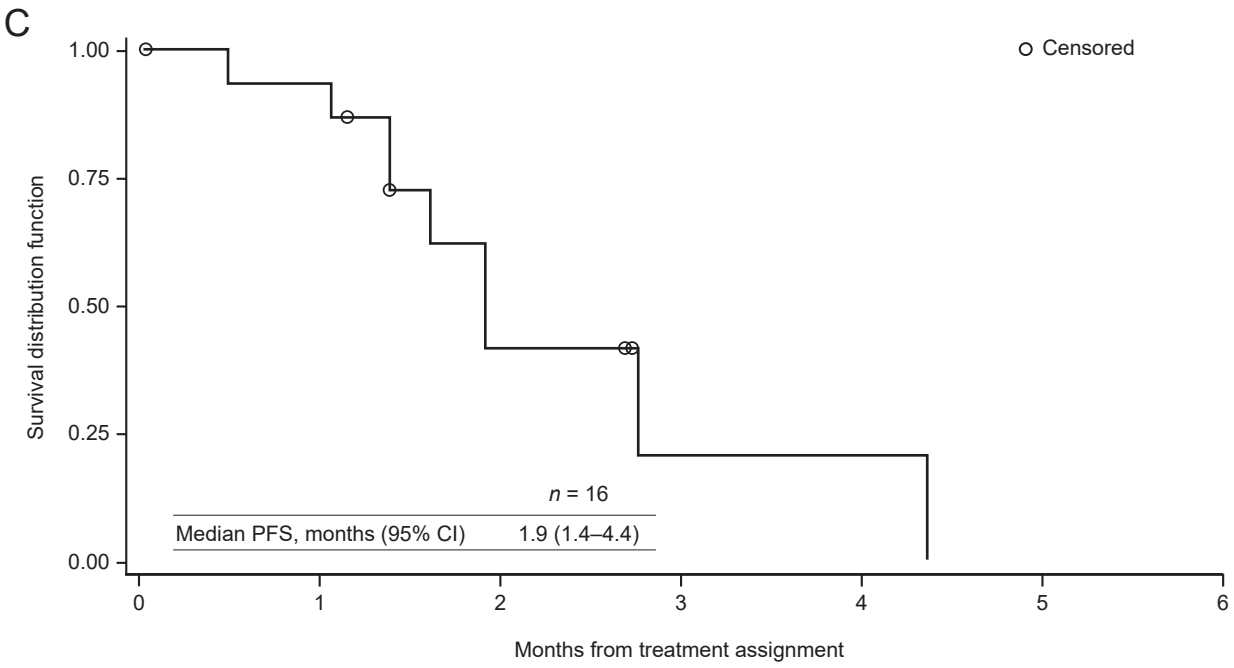

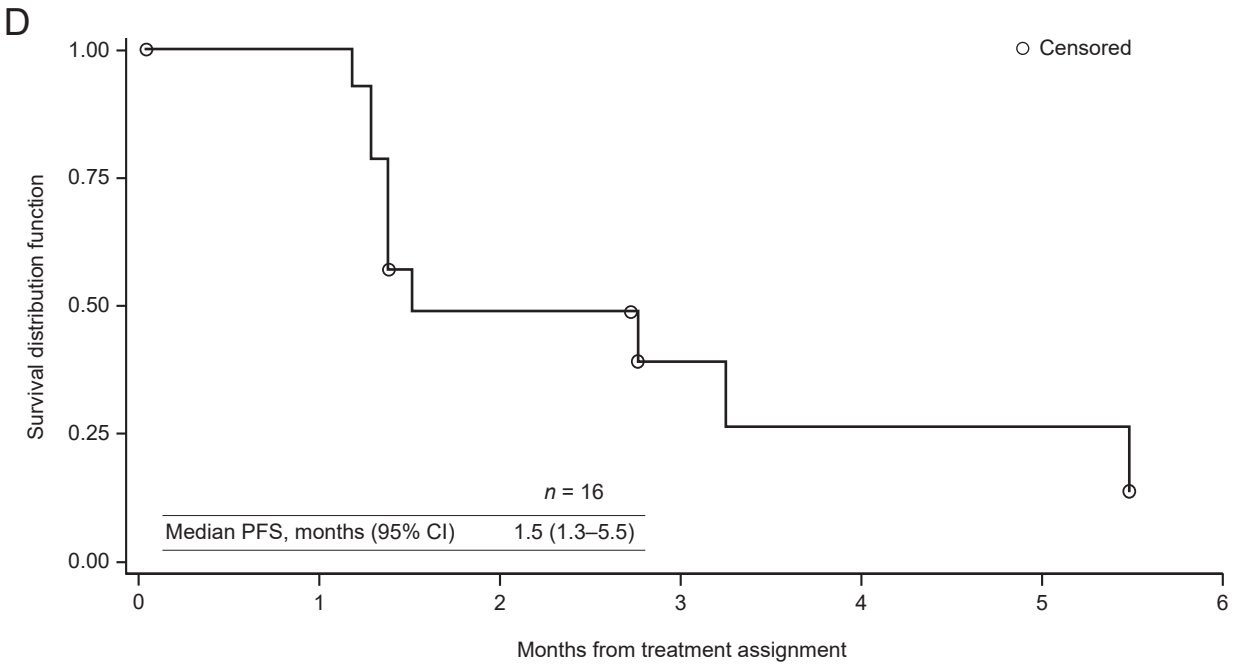

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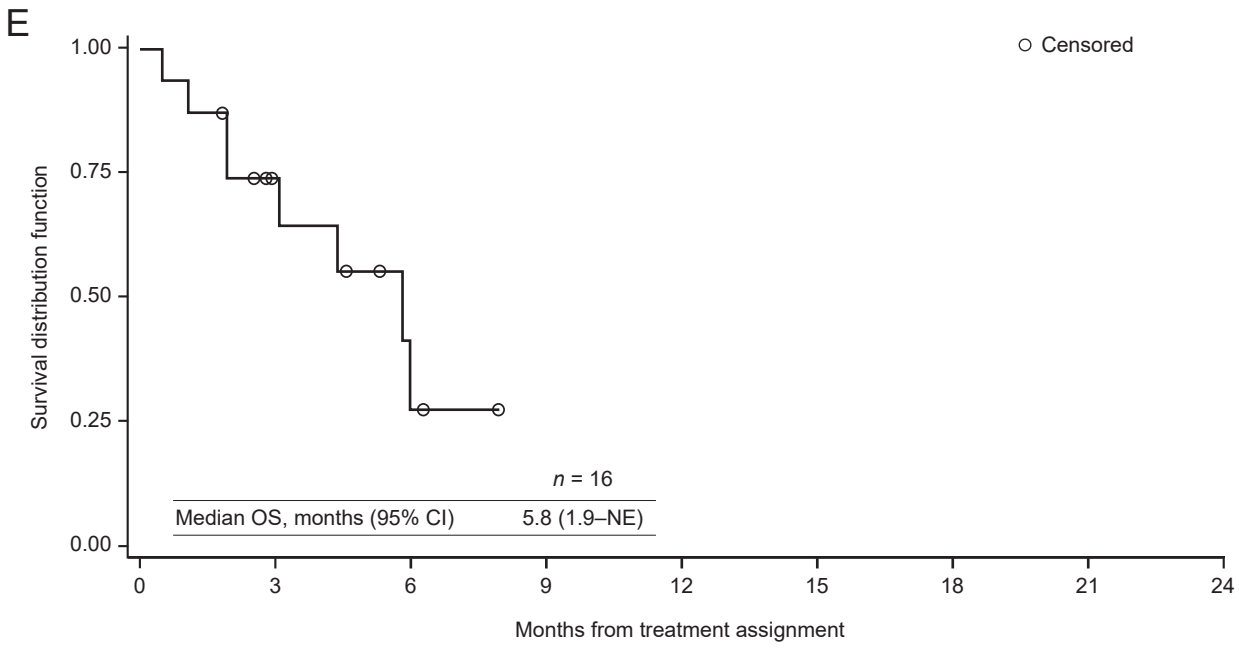

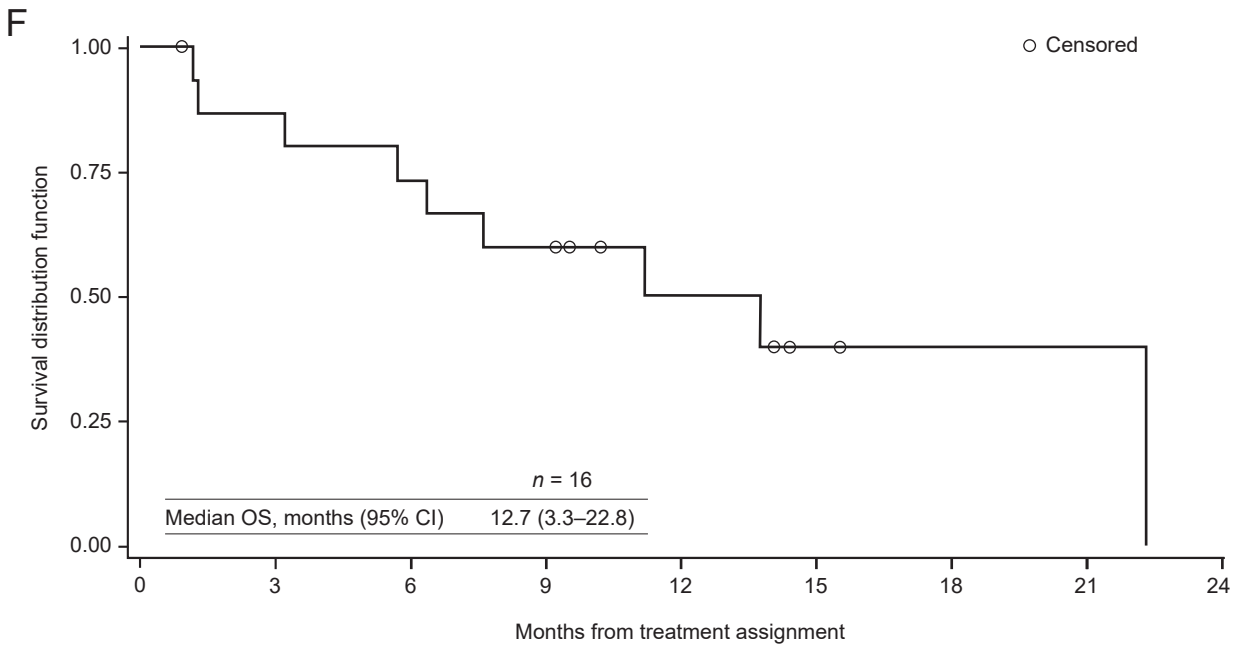

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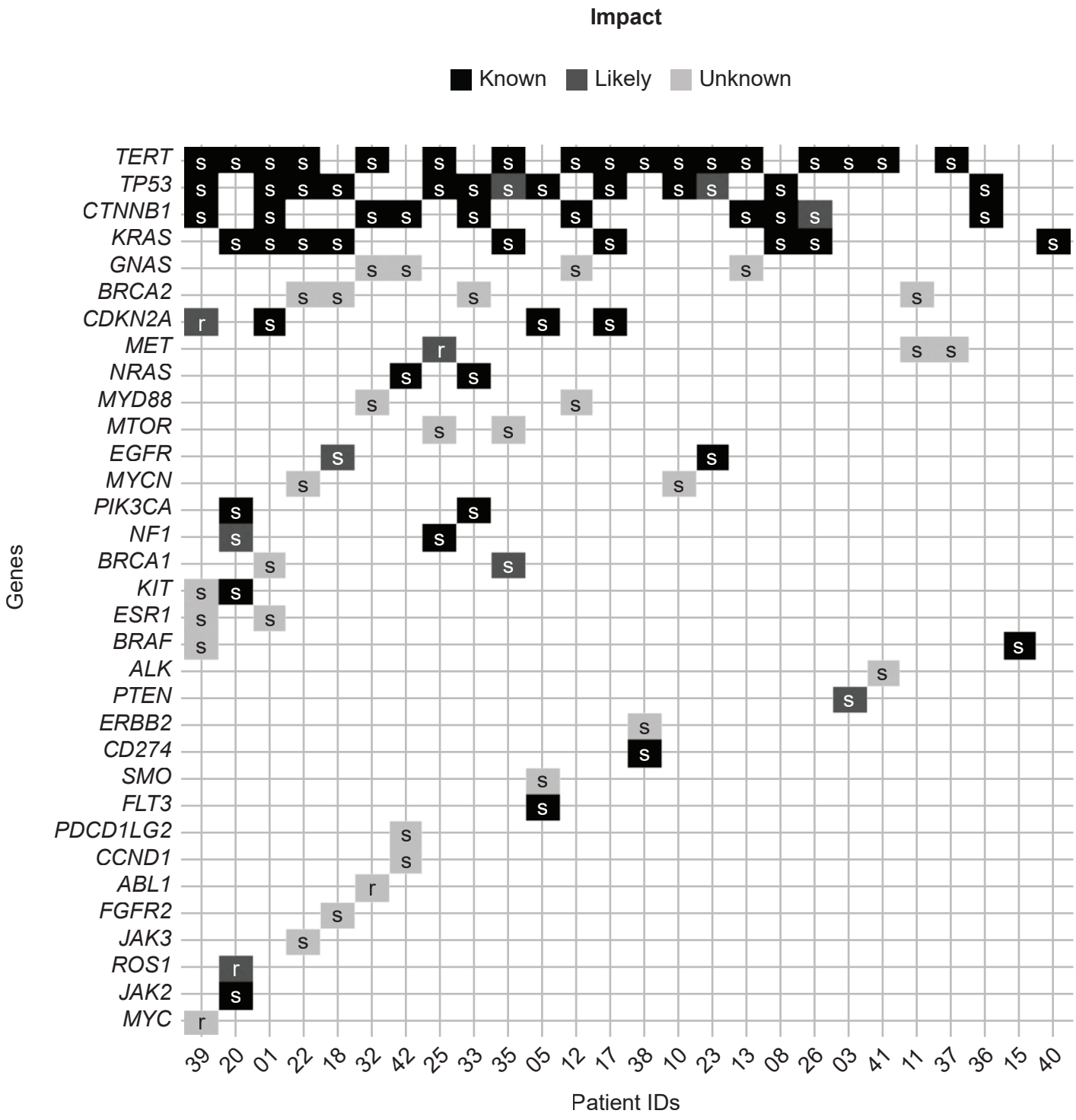
Figure 3



AACR American Association for Cancer Research

Clinical Cancer Research

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