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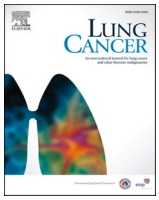

BRAF mutational status is associated with survival outcomes in locally advanced resectable and metastatic NSCLC

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Abbreviations: cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; CGW, Clinical Genomics Workspace; CH, Clonal Hematopoiesis; CH-IO, Chemo-immunotherapy; ESP, Exon Sequencing Project; FFPE, Formalin-Fixed Paraffin-Embedded; ICI, Immune Checkpoint Inhibitor; INDELs, Insertion and Deletions variants; MAF, Mutant Allele Frequency; MAPK, Mitogen-Activated Protein Kinase; Mb, Megabase; NGS, Next-Generation Sequencing; NSCLC, Non-Small-Cell Lung Cancer; ORR, Overall Response Rate; OS, Overall Survival; pCR, Pathological Complete Response; PFS, Progression-Free Survival; RECIST, Response Evaluation Criteria in Solid Tumors; TMB, Tumor Mutation Burden; UDG, Uracil-DNA Glycosylasa.

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ABSTRACT

Background: Immunotherapy-based treatments have demonstrated high efficacy in patients with advanced and locally advanced non-small-cell lung cancer (NSCLC). *BRAF* mutations affect a small but significant fraction of NSCLC. The efficacy of these therapies in this subgroup of patients is unknown.
Materials and methods: Plasma and tissue samples from 116 resectable stage IIIA/B NSCLC patients, included in NADIM and NADIM II clinical trials (NADIM cohort), and from a prospective academic cohort with 84 stage IV NSCLC patients (BLI-O cohort), were analyzed by next-generation sequencing.
Results: The p.G464E, p.G466R, p.G466V, p.G469V, p.L597Q, p.T599I, p.V600E (n = 2) *BRAF* mutations, were identified in four (3.45 %) samples from the NADIM cohort, all of which were cases treated with neoadjuvant chemoimmunotherapy (CH-IO), and four (4.76 %) samples from the BLI-O cohort, corresponding to cases treated with first-line immunotherapy (n = 2) or CH-IO (n = 2). All these patients were alive and had no evidence of disease at data cut-off. Conversely, patients with *BRAF* wild-type (wt) tumors in the BLI-O cohort had a median progression-free survival (PFS) of 5.49 months and a median overall survival (OS) of 12.00 months (P-LogRank = 0.013 and 0.046, respectively). Likewise, PFS and OS probabilities at 36 months were 60.5 % and 76.1 % for patients with *BRAF*-wt tumors in the NADIM cohort. The pathological complete response (pCR) rate after neoadjuvant CH-IO in patients with *BRAF*-positive tumors (n = 4) was 100 %, whereas the pCR rate in the *BRAF*-wt population was 44.3 % (RR: 2.26; 95 % CI: 1.78–2.85; P < 0.001).
Conclusion: BRAF mutations may be a good prognostic factor for advanced and locally advanced NSCLC patients undergoing immunotherapy-based treatments.

1. Introduction

Immunotherapy-based treatments have emerged as highly effective therapies for patients with advanced and locally advanced non-small-cell lung cancer (NSCLC) [1].

Among the diverse molecular alterations encountered in NSCLC, *BRAF* mutations occur in a modest yet significant subset of tumors. Specifically, *BRAF* mutations account for approximately 1–5 % of NSCLC cases [2,3]. *BRAF* encodes a protein that is involved in the mitogen-activated protein kinase (MAPK) signaling pathway, regulating cell proliferation and differentiation. Dysregulation of these pathways is a hallmark of various solid cancers [4,5]. The majority of *BRAF* mutations are classified as class I, occurring at codon 600, with *BRAF* p.V600E being the most frequent mutation in solid tumors which serves as an important biomarker that identifies patients who may benefit from *BRAF* inhibitors [6]. Class II mutations, defined as non-V600 mutations, activate *BRAF* signaling as a RAS-independent dimer, and Class III mutations have low/absent kinase activity and require additional upstream signaling.

While *BRAF* mutations are well-characterized in other cancers, their impact on treatment outcomes in NSCLC has received limited attention, primarily due to the low prevalence of *BRAF*-positive NSCLCs. Consequently, the efficacy of immune checkpoint inhibitors (ICIs) in *BRAF*-positive NSCLC has been scarcely investigated, and doubts persist regarding the suitability of immunotherapy-based treatments versus targeted therapies as the preferred approach for treating tumors with class I *BRAF* mutations. In this manner, a French study showed an ORR (Overall Response Rate) of 24 % and a median PFS (Progression-Free Survival) of 3.1 months in previously treated *BRAF*-mutant NSCLC [7].

On the other hand, it is well-established that ICIs are an effective treatment option for melanoma patients harboring *BRAF* V600 mutations [8,9]. Recent studies have also suggested potential sensitivity to ICIs in *BRAF*-positive NSCLC [10]. A retrospective study analyzing three independent cohorts of NSCLC patients with oncogene alterations (total n = 4189) showed PFS benefit from ICIs in patients whose tumors harbored the p.V600E *BRAF* mutation [11].

The current study aims to investigate the prognostic implications of *BRAF* mutations in locally advanced NSCLC patients who underwent neoadjuvant chemoimmunotherapy (CH-IO), using data from the NADIM [12,13] and NADIM II [14] clinical trials. Furthermore, this study extends its scope to encompass an academic cohort of NSCLC patients, with advanced-stage who received first-line immunotherapy or CH-IO (BLI-O cohort).

2. Materials and methods

2.1. Study cohort

In this study, two different patient cohorts were used, namely the NADIM cohort and the BLI-O cohort. The NADIM cohort included 116 patients with resectable stage IIIA/B NSCLC included in NADIM (NCT03081689; n = 46) and NADIM II (NCT03838159; experimental arm, n = 46; control arm, n = 24) clinical trials (**Supplementary Fig. 1**). Patients in the NADIM trial and in the experimental arm of the NADIM II trial were treated with neoadjuvant nivolumab plus platinum-based chemotherapy followed by adjuvant nivolumab. Clinical outcomes of patients included in NADIM and NADIM II have been published elsewhere [12–14]. Specifically, we have analyzed 99 baseline plasma samples and 70 formalin-fixed, paraffin-embedded (FFPE) pre-treatment biopsy samples in the NADIM cohort (Supplementary **Table 1**). The BLI-O academic cohort included 84 plasma samples from stage IV NSCLC patients, who were candidates to receive immunotherapy-based treatments. For the BLI-O cohort tumors testing positive for *EGFR* or *ALK* mutations or rearrangements were excluded. Samples were collected before first-line treatment initiation with immunotherapy or CH-IO (Supplementary Figs. 1, 2 and Supplementary **Table 1**). In the BLI-O cohort, the choice of therapy was left to the discretion of the physician.

Written consent was obtained from all patients, and the study was conducted in accordance with the precepts of the Code of Ethics of the World Medical Association (Declaration of Helsinki). Treatment response was assessed as per Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 criteria. For resectable tumors (NADIM cohort), pathological complete response was defined as the absence of viable tumor cells in the resected lung specimen and lymph nodes. When the resection of the tumor was not possible, patients were counted as having an incomplete response. PD-L1 expression was evaluated in FFPE tumor samples.

2.2. Sample collection and processing

Peripheral whole-blood samples were collected in 8.5-mL PPT™ tubes (Becton Dickinson, Franklin Lakes, NJ, USA) before treatment initiation. Plasma was separated from the cellular fraction by two consecutive centrifugations at 1600 g for 10 min and at 6000 g for 10

min. Both centrifugations were performed at room temperature, and plasma samples were frozen at −80 °C until processing. The cell-free DNA (cfDNA) was isolated from plasma samples using the QIAamp Circulating Nucleic Acid Kit (QIAgen, Valencia, CA, USA) following the manufacturer's instructions.

Nucleic acids from FFPE tumor diagnostic samples were extracted by the truXTRAC® FFPE total Nucleic Acid (Covaris) kit according to the commercial protocol. Specifically, three paraffin sections of 10 μm minimum thickness were used for the isolation. The presence of tumor tissue was first evaluated by expert pathologists in a representative slide stained with hematoxylin and eosin. Only samples with at least 20 % of tumor were considered valid for the analysis.

cfDNA and FFPE-derived DNA were quantified using the Qubit 1 × dsDNA HS Assay Kit (ThermoFisher, Palo Alto, CA, USA ThermoFisher Scientific®) in a Qubit 2.0 Fluorometer (ThermoFisher Scientific).

2.3. Library preparation and next-generation sequencing

Libraries for cfDNA obtained from BLI-O (N = 84) cohort samples and NADIM (N = 43) samples were prepared using the Oncomine™ Pan-Cancer Cell-Free Assay kit (ThermoFisher, Palo Alto, CA, USA), according to the manufacturer's protocol. The panel covers 52 genes (Supplementary Table 2). For library purification, AMPureXP magnetic beads (Beckman Coulter, Inc., Brea, CA, USA) were used. The individual libraries were then quantified using the Ion Library TaqMan® Quantitation Kit (ThermoFisher, Palo Alto, CA, USA) in a StepOnePlus™ qPCR machine (ThermoFisher, Palo Alto, CA, USA), and subsequently diluted to 50 pM. Libraries were prepared in batches of eight samples and stored at −20 °C up to a maximum of two weeks. Eight samples were pooled for chip loading in an Ion 550™ Chip. Templating and chip loading were carried out with an Ion Chef™ System (ThermoFisher, Palo Alto, CA, USA). Finally, an Ion GeneStudio™ S5 plus Sequencer (ThermoFisher, Palo Alto, CA, USA) was used to sequence loaded Ion 550™ chips. Raw sequencing data was analyzed using the Torrent Suite Software (v5.14) and the CoverageAnalysis (v.5.14) plugin was used for sequencing coverage analysis (ThermoFisher, Palo Alto, CA, USA). Raw reads were aligned to the human reference genome hg19. Variant calling and annotation were performed on the Ion Reporter (v5.14) platform using the OncomineTagSeq Pan-Cancer Liquid Biopsy workflow (v2.1), which detects and annotates low frequency variants including SNPs/insertions and deletions (InDels) from targeted nucleic acid libraries. An improved internal pipeline was used to filter variants of potential interest from non-filtered tsv files downloaded from the Ion reporter platform (**Supplementary Fig. 3**).

For NADIM II plasma samples, libraries were prepared using the hybrid capture-based TruSight Oncology 500 ctDNA next-generation sequencing (NGS) assay following the manufacturer's instructions. The panel covers 1.94 megabases (Mb) across 523 genes (Supplementary Table 2). Libraries were then pooled, denatured and diluted to the appropriate concentration for sequencing on a NovaSeq 6000 system (Illumina). 24 samples were sequenced per run in an S4 flow cell. Data were analyzed with the DRAGEN TSO 500 ctDNA Analysis Software v1.2 using the TSO 500 pipeline. Reads were mapped to the hg19 genome. Variant calling was performed using PierianDx Clinical Genomics Workspace (CGW) v6.23. Germline variants were filtered out using the information of the public database GnomAD. In addition, a post-database filtering strategy that uses allele frequency information and variants proximity to coding sequence was conducted. Mutations in *TET2, DNMT3A*, and *CBL* were excluded as they were catalogue as clonal hematopoiesis (CH) derived mutations. Specific information regarding variant filtering is shown in **Supplementary Figure 4**.

DNA obtained from FFPE samples was treated with heat-labile uracil-DNA glycosylase (UDG), following the manufacturer's recommendations, to remove deaminated bases before target amplification. Briefly, 20 ng of FFPE DNA was incubated with UDG for 2 min at 37 °C followed by an incubation at 50 °C for 10 min. Library preparation was carried out in an Ion Chef™ System (ThermoFisher Scientific), using 20 ng of input DNA and the Oncomine Tumor Mutation Load Assay (ThermoFisher Scientific), which covers 1.65 Mb across 409 cancer genes (**Supplementary Table 2**). The final barcoded libraries were pooled and adjusted to a final concentration of 50 pM. Eight samples were loaded onto an Ion 540™ chip. Template preparation and chip loading were performed in an Ion Chef™ System, and Ion 540™ chips were finally sequenced in an Ion S5™ Sequencer (ThermoFisher, Palo Alto, CA, USA). Reads were aligned to hg19 using Torrent Suite 5.14 and BAM files were transferred to Ion Reporter 5.14 for variant calling. Non-filtered tsv files downloaded from Ion Reporter 5.14 were subsequently analyzed to identify mutations of potential clinical significance. Particularly, variant filtering was performed using an internal pipeline. Detailed information of variant filtering conditions is available in **Supplementary Figure 5.** Additionally, the tumor mutation burden (TMB) of the sequenced samples was computed according to the manufacturer's pipeline using the TMB filter chain and the TMB algorithm 3.0 (ThermoFisher Scientific). Briefly, germline variants were filtered out using a germline filter-chain based on population databases: 1000 Genome Project, NHLBI GO Exome Sequencing Project (ESP), and ExAC. TMB, as determined by the Oncomine™ Tumor Mutation Load Assay is defined as the number of nonsynonymous variants (missense and nonsense single nucleotide variants, plus InDels detected per Mb of exonic sequence).

Only pathogenic or likely pathogenic variants were considered for the analysis.

2.4. Publicly available data

For the analysis of the prevalence of *BRAF* pathogenic and likely pathogenic variants in NSCLC patients, two different public datasets were used. Particularly, the pan-lung cancer dataset [15], containing whole-exome sequencing data of 660 lung adenocarcinomas and 484 lung squamous cell carcinomas tumor/normal pairs, and the metastatic non-small cell lung cancer (MSK) dataset [16], which includes targeted sequencing data of circulating tumor DNA (ctDNA) samples drawn from 1127 patients with metastatic NSCLC using the Resolution ctDx Lung platform, were downloaded from cBioportal [17,18].

2.5. Statistical analysis

The statistical analysis was performed using Stata Statistical Software version 15 (StataCorp LLC, College Station, TX, USA) and R software version v.4.1.2. Categorical and numeric variables are summarized as frequencies or mean/median, respectively. Potential association between the pCR and *BRAF* mutational status was evaluated using the Fisher exact test. Association between *BRAF* mutational status and clinicopathological characteristics (sex, age, ECOG, smoking status, histology, stage, and PD-L1 expression) was also evaluated.

Overall survival (OS) was defined as the time from treatment initiation to death from any cause or the date of the last follow-up for patients. PFS was defined as the time between the treatment initiation and disease progression, evaluated by RECIST criteria, or death from any cause. Median follow-up time and data maturity were estimated as previously described [12]. Survival analysis was performed using the Kaplan-Meier method to evaluate differences between groups. The proportional hazard assumption in the cox model was checked by comparing the log–log survival curves using R. P values < 0.05 were considered statistically significant. The data cutoff dates were March 2021 for NADIM, November 2022 for NADIM II, and June 2023 for the BLI-O cohort.

3. Results

3.1. Identification of BRAF oncogenic mutations

We identified eight patients whose tumors harbored an oncogenic *BRAF* mutation (Table 1). Specifically, four of these samples (3.45 %) tested positive in NADIM trials, in all cases, patients were treated with neoadjuvant CH-IO (experimental arm). Similarly, four samples tested positive (4.76 %) in the BLI-O cohort. Among these, two cases corresponded to patients treated with CH-IO, while the remaining two cases corresponded to patients treated with immunotherapy alone. Specifically, we found seven unique mutations, namely, p.V600E (class I mutation), p.G464E, p.G469V, p.L597Q, p.T599I (Class II mutations), p.G466R and p.G466V (class III mutations). These mutations are located in the P-loop region or the activation loop of the protein, which regulate the kinase activity of the protein (Fig. 1). Seven of these variants were identified in liquid biopsy samples, with mutant allele frequency (MAF) ranging from 0.11 % to 0.34 %. Additionally, two *BRAF* mutations were identified in the tumor biopsies (Table 1).

To validate the prevalence of oncogenic *BRAF* mutations in NSCLC, we evaluated the presence of these alterations in publicly available datasets. The prevalence of *BRAF* mutations was consistent across all datasets (Supplementary Table 3). Among the most prevalent mutations observed in public datasets, we found p.V600E, p.G466V, which were also identified in our cohorts (Supplementary Table 4).

In addition, we evaluated the presence of concomitant pathogenic and likely pathogenic mutations in *BRAF*-positive NSCLC. With the exception of one tumor (patient B, see Table 1), all tumors harbored at least one co-mutation alongside the *BRAF* mutation. *TP53* was the most frequent co-mutated gene, with mutations present in 5 out of 8 cases (62.5 %), followed by *SMAD4* mutated in 2 out of 8 cases (25.0 %). We also found alterations in *KIT*, or *MAP2K1*, among others (**Supplementary Figure 6**).

PD-L1 data were available in 171 cases of the NADIM cohort and BLI-O cohort (Table 2**,** Supplementary Figures 7A and 7B**).** The PD-L1 expression according to *BRAF* status is presented in Supplementary Figures 7A and 7B. As shown, no significant differences were observed in PD-L1 status and *BRAF* mutation status, although the two cases harboring the p.V600E mutation had a PD-L1 expression $\geq$ 50 % (**Supplementary Figure 7B).**

TMB analysis was available for the NADIM cohort (n = 64). No significant differences were seen between *BRAF*-wt (n = 61) and *BRAF*-positive tumors (n = 3), but we observed that two out of three *BRAF*-positive tumors (66.67 %) had a TMB $\geq$ 20 mutations (mut) per Mb, while this proportion was 13.11 % in *BRAF-wt* tumors (**Supplementary Figure 7C).**

3.2. Prognostic value of BRAF mutations

The demographic and clinical characteristics of the patients included in the study are summarized in Table 2. According to our data, patients with *BRAF*-positive tumors had similar clinicopathological features compared to those whose tumors were *BRAF*-wt. No significant associations were found between any of the analyzed variables and *BRAF* mutation status, including sex, age, ECOG, smoking status, histology, stage, TMB, and PD-L1 expression.

For the analysis of the prognostic value of *BRAF* mutations in the NADIM cohort, patients from the control arm of the NADIM II trial were excluded due to the absence of *BRAF* mutations in this subset of patients (n = 24) and since these patients were treated with neoadjuvant chemotherapy alone.

Among the subset of patients treated with neoadjuvant CH-IO (n = 92), the median follow-up was 34.62 months (interquartile range: 29.06 – 38.73 months), with 99.87 % data maturity at 12 months. At the time of data cut-off, 32 patients (34.78 %) presented with disease progression, and 19 (20.65 %) deaths were recorded.

Median PFS and OS were not reached for either *BRAF*-wt or *BRAF*-positive NSCLC patients in the NADIM cohort. However, Kaplan Meier curves showed that patients whose tumors harbored oncogenic *BRAF* mutations were with no evidence of disease and alive at data cutoff (Fig. 2 **A, B**), whereas the probability of being with no evidence of disease and alive at 36 months in patients with *BRAF-wt tumors* was 60.5 % and 76.1 %, respectively (Supplementary table 5). Likewise, oncogenic *BRAF* mutations were significantly associated with a pathological complete response (pCR) after neoadjuvant treatment with CH-IO (p = 0.044) (Fig. 3). Specifically, the pCR rate after neoadjuvant CH-IO for patients whose tumors were *BRAF*-positive (n = 4) was 100 %, whereas the pCR rate in the *BRAF-wt* population was 44.3 % (RR: 2.26; 95 %CI 1.78–2.85; P < 0.001).

The median follow-up for patients included in the BLI-O cohort was 17.92 months (interquartile range: 12.82–22.09 months), with a data maturity at 12 months of 94.62 %. During the study, 60 patients (71.43 %), were diagnosed as having progressive disease and 46 (54.76 %) patients were deceased. No significant differences in survival outcomes were observed according to the type of treatment received (immunotherapy alone or CH-IO) (**Supplementary Figure 8**).

The median PFS and the median OS in BLI-O patients whose tumors were *BRAF-wt* were 5.49 and 12 months, respectively, while the median PFS and OS in patients with *BRAF* mutant tumors were not reached (LogRank p-value of 0.013 for PFS and 0.046 for OS) (Fig. 2 **C, D**). Of note, all patients with tumors carrying oncogenic *BRAF* mutations were with no evidence of disease progression and alive at data cutoff, while the probability of being with no evidence of disease progression at 12 months in patients without oncogenic *BRAF* mutations was 30.5 %, with a 12-month overall survival probability of 48.7 % (Supplementary table 5).

Table 1
BRAF pathogenic and likely pathogenic variants identified.

	Cohort	Variant	Reference transcript	MAF BL	MAF FFPE
patient A	BLI-O	p.G466R	NM_004333.6	0,195	NA
patient B	BLI-O	p.V600E	NM_004333.6	0,25	NA
patient C	BLI-O	p.G466V	NM_004333.6	0,327	NA
patient D	BLI-O	p.T599I	NM_004333.6	0,112	NA
patient E	NADIM	p.L597Q	NM_004333.6	0,344	18,449
patient F	NADIM	p.G469V	NM_004333.6	0,111	ND
patient G	NADIM	p.V600E	NM_004333.6	0,135	NA
patient H	NADIM	p.G464E	NM_004333.6	NA	37,121

Abbreviations: NA, not available; ND, not detected.

4. Discussion

In this study, we examined two independent cohorts of NSCLC patients to explore the impact of *BRAF* mutations on immunotherapy-based treatment survival outcomes. In the NADIM cohort, all *BRAF*-positive NSCLC patients were alive and with no evidence of disease at data cutoff, and all of them achieved a pCR after neoadjuvant treatment with CH-IO. Noteworthy, it has been clearly demonstrated that having pCR after neoadjuvant CH-IO highly correlates with prolonged survival [14]. Of note, these results are provided in the context of an extensive follow-up period and with a high degree of data maturity.

We believe this information is of significance as, up until now, there have been no publications supporting the neoadjuvant use of the CH-IO in NSCLC patients with *BRAF*-positive tumors. On the contrary, currently a trend leans towards the exploration of new adjuvant or neoadjuvant therapies with targeted therapy, a trend which may be driven by the impressive outcomes demonstrated by adjuvant osimertinib in the ADAURA trial [19], as well as the fact of the exclusion of patients with known *EGFR* or *ALK* mutations from neoadjuvant trials

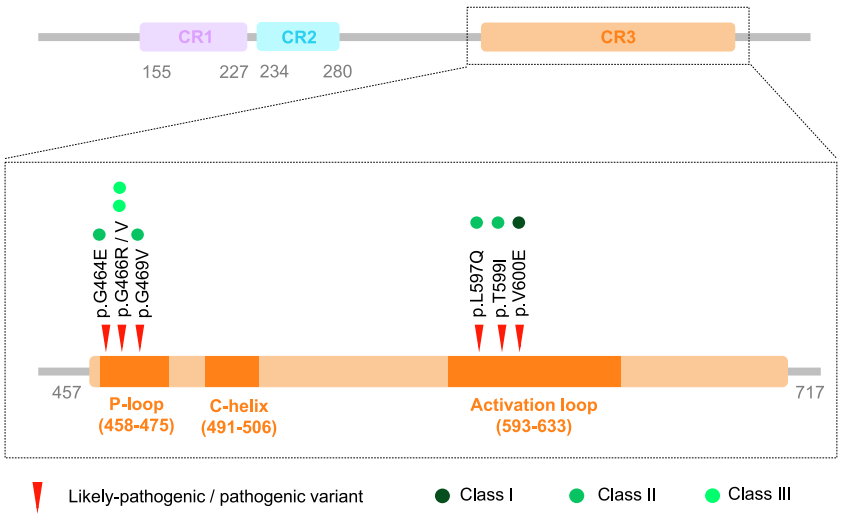

Fig. 1. Schematic representation of *BRAF* protein structure showing pathogenic and likely pathogenic identified variants. Highly conserved domains CR1, CR2 (regulatory domains) and CR3 (kinase domain) are represented in violet, blue and orange, respectively. P-loop, C-helix and activation loop regions are displayed in dark orange. Aminoacid positions are also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Demographic and Clinical Characteristics of the Patients at Baseline.

	BLI-O COHORT		NADIM COHORT				
			NADIM		NADIM II Experimental arm		Control arm
Clinical features	***BRAF mut***	***BRAF wt***	***BRAF mut***	***BRAF wt***	***BRAF mut***	***BRAF wt***	***BRAF wt***
N	(n = 4)	(n = 80)	(n = 2)	(n = 44)	(n = 2)	(n = 44)	(n = 24)
Age, mean (SD), years	70.5 (1.73)	65.01 (10.37)	56.00 (18.38)	63.45 (8.53)	63.06 (2.40)	63.06 (9.63)	62.32 (11.02)
Sex, No. (%)							
Female	0 (0 %)	22 (27.50 %)	1 (50 %)	11 (25.00 %)	0 (0 %)	16 (36.36 %)	12 (50.00)
Male	4 (100 %)	58 (72.50 %)	1 (50 %)	33 (75.00 %)	2 (100 %)	28 (63.64 %)	12 (50.00)
Smoking, No. (%)							
Never smokers	0 (0 %)	9 (11.25 %)	0 (0 %)	0 (0 %)	0 (0 %)	4 (9.09 %)	0 (0.00)
Former smokers	2 (50 %)	32 (40.00 %)	0 (0 %)	25 (56.82 %)	2 (100 %)	17 (38.64 %)	6 (25.00)
Active smokers	2 (50 %)	39 (48.75 %)	2 (100 %)	19 (43.18 %)	0 (0 %)	23 (52.27 %)	18 (75.00)
ECOG-PSa, No. (%) with data							
0	2 (50 %)	18 (23.08 %)	2 (100 %)	23 (52.27 %)	2 (100 %)	23 (52.27 %)	12 (50.00)
1	2 (50 %)	47 (60.26 %)	0 (0 %)	21 (47.73 %)	0 (0 %)	21 (47.73 %)	12 (50.00)
2	0 (0 %)	13 (16.67 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0.00)
Histologyb, No. (%) with data							
Adenocarcinoma	2 (66,67 %)	59 (78.67 %)	2 (100 %)	24 (54.55 %)	1 (50 %)	18 (40.91 %)	9 (37.50)
Squamous cell carcinoma	0 (0 %)	14 (18.67 %)	0 (0 %)	16 (36.36 %)	0 (0 %)	19 (43.18 %)	13 (54.17)
Large-cell carcinoma	1 (33,33 %)	2 (2.67 %)	0 (0 %)	0 (0 %)	0 (0 %)	2 (4.55 %)	1 (4.17)
NOS / Undifferenciated	0 (0 %)	0 (0 %)	0 (0 %)	4 (9.09 %)	1 (50 %)	4 (9.09 %)	0 (0.00)
Other	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	1 (2.27 %)	1 (4.17)
Expression PD-L1c, No. (%) with data							
< 1 %	0 (0 %)	16 (20.25 %)	0 (0 %)	10 (38.46 %)	1 (50 %)	15 (37.50 %)	5 (26.32)
≥ 1 %	3 (100 %)	63 (79.75 %)	2 (100 %)	16 (61.54 %)	1 (50 %)	25 (62.50 %)	14 (73.68)
< 50 %	1 (33,33 %)	42 (53.16 %)	0 (0 %)	15 (57.69 %)	1 (50 %)	24 (60 %)	13 (68.42)
≥ 50 %	2 (66,67 %)	37 (46.84 %)	2 (100 %)	11 (42.31 %)	1 (50 %)	16 (40 %)	6 (31.58)
Treatment, No. (%) with data							
Immunotherapy (IO)	2 (50 %)	36 (45.00 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
CH-IO	2 (50 %)	44 (55.00 %)	2 (100 %)	44 (100 %)	2 (100 %)	44 (100 %)	0 (0 %)
Chemotherapy (CH)							24 (100.00)
Stage¥, No. (%) with data							
IIIA	0 (0 %)	0 (0 %)	2 (100 %)	44 (100 %)	2 (100 %)	33 (75 %)	21 (87.50)
IIIB	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	11 (25 %)	3 (12.50)
IVA	2 (50 %)	33 (42.31 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0.00)
IVB	2 (50 %)	33 (42.31 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0.00)
IV (not specified)	0 (0 %)	14 (17.50 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0.00)
Median follow-up, months (IQR)	17.92 (12.82–22.09)		33.73 (27.85–37.15)				

[a]2 patients from BLI-O cohort without information; [b]6 patients from BLI-O cohort without information; * 1 patient with adenosquamous carcinoma in BLI-O cohort and 1 in NADIM II; [c]2 patients from BLI-O cohort, 18 patients from NADIM, 4 patients from NADIM II experimental arm, and 5 patients from control without information.
¥ Patients from NADIM study were classified using the 7th Edition of the TNM Classification for Lung Cancer. Patients from BLI-O cohort and NADIM II study were classified using the 8th Edition of the TNM Classification for Lung Cancer.
Abbreviations: ECOG-PS: Eastern Cooperative Oncology Group Performance Status; IQR: Interquartile range.

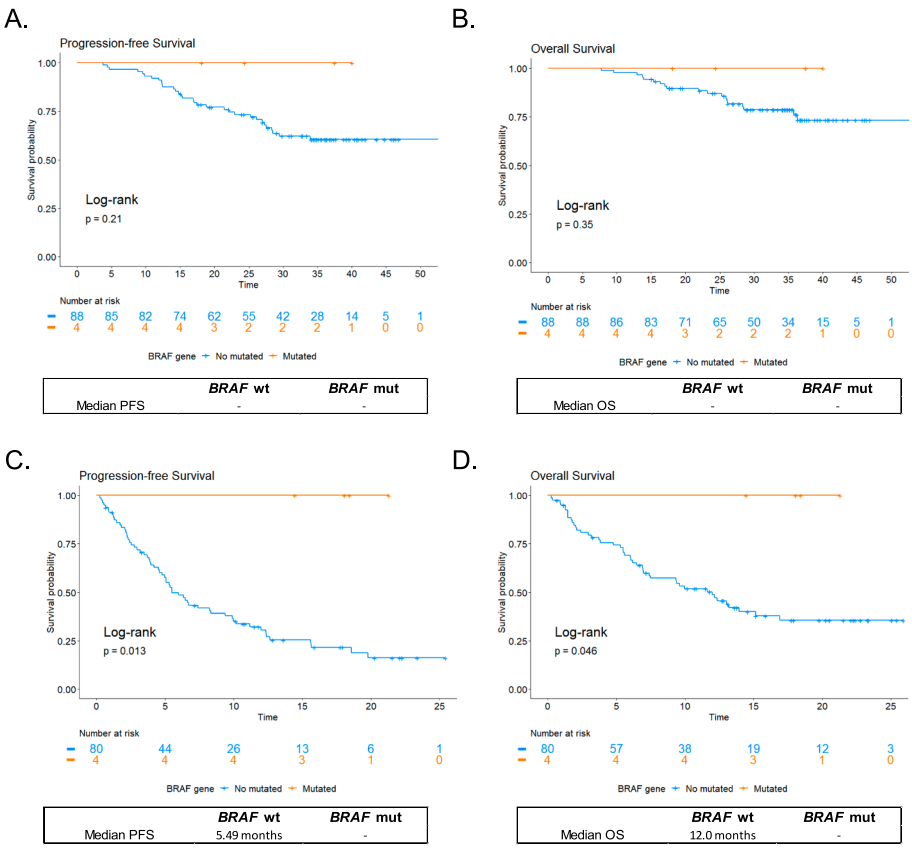

Fig. 2. Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) according to *BRAF* mutational status. PFS (A) and OS (B) in NADIM cohort (stage IIIA/B patients treated with neoadjuvant CH-IO). PFS (C) and OS (D) in BLI-O cohort (stage IV patients treated with a CH-IO or immunotherapy alone).

assessing the efficacy of CH-IO combinations [14,20].

In the metastatic setting, first-line therapy options for patients with *BRAF* pV600E mutated NSCLC include dabrafenib plus trametinib as standard treatment [21,22]. Other targeted therapeutic approaches against *BRAF* V600-Mutant NSCLC have also displayed meaningful clinical benefit [23]. On the other hand, the potential benefits that immunotherapy holds for *BRAF*-positive NSCLC patients have been relatively underexplored. In a study by Dudnik E *et al.*, although a significant association was found between *BRAF*-positive NSCLC and a high level of PD-L1 expression, *BRAF* mutation status was not associated with the response probability (ORR) nor PFS [24]. Similarly, Guisier F *et al.* did not find any improvement in ICIs efficacy in *BRAF*-positive NSCLC [25], albeit in a setting where patients had undergone a median of at least one prior treatment line before ICI administration. Therefore, overall, data regarding immunotherapy efficacy in *BRAF*-positive NSCLC stems from retrospective observational studies, with limited sample size. Similarly, there is little information available regarding the efficacy of CH-IO in *BRAF*-positive NSCLC. Several retrospective studies have suggested some efficacy of CH-IO in metastatic NSCLC [26] with similar survival outcomes (unweighted median OS for CH-IO 17.7 months) [27] to the previously reported clinical trial data of dabrafenib plus trametinib [28] (median OS 17.3 months for first-line setting).

V600-*BRAF*-mutant NSCLC has been associated with high TMB and PD-L1 expression suggesting that this subset of tumors would be more sensitive to immunotherapy-based treatments [11]. Although we were unable to demonstrate a significant association between PD-L1 expression and *BRAF* mutation status, a trend was observed. We also found that two out of three *BRAF*-positive tumors had a TMB $\geq$ 20, while this proportion was 13.11 % in *BRAF*-wt tumors. However, these are small numbers and it is not possible to draw any conclusions. Moreover, TMB was not associated with pCR, PFS, or OS in any of the NADIM trials highlighting the limited utility of this biomarker for CH-IO.

In our study, it can be criticized that, even though the prevalence of *BRAF* mutations was very similar to that reported in public databases or previous studies [29], the sample size remained modest and did not allow for establishing definitive conclusions, with only two cases with the p.V600E mutation identified. However, findings were consistent across two completely independent cohorts (NADIM and BLI-O). Regarding non-V600 mutations, some researchers have suggested that some of them are responsive to *MEK* and *BRAF* inhibitors [3]. Likewise, it can be hypothesized that non-V600 mutations could also identify NSCLC patients with prolonged OS when undergoing immunotherapy-based treatments. Indeed, dramatic and prolonged response has been documented in a patient with a *BRAF* p.G469A mutated NSCLC when treated with second-line treatment nivolumab [30].

Furthermore, the observed survival outcomes are congruent with outcomes seen in metastatic melanoma. In this way, in the DREAMseq trial, advanced *BRAF*-positive melanoma patients were randomized to receive either a combination nivolumab/ipilimumab or dabrafenib/trametinib (targeted therapy) as first-line treatment, and after disease progression patients were switched to the other treatment combination. Of note, the 2-year OS was significantly higher in patients treated first with immunotherapy compared to patients treated first with the targeted therapy combination (72 % vs 52 %), demonstrating superior efficacy of immunotherapy over *BRAF* targeted therapy [31] and highlighting the association of *BRAF*-positive tumors with improved survival outcomes when treated with immunotherapy-based treatments.

5. Conclusions

Our data clearly suggest that immunotherapy-based treatments may benefit patients with both locally advanced and advanced *BRAF*-positive NSCLC. The extraordinary outcomes observed in the NADIM cohort with CH-IO treatment, including a 100 % pCR rate further reinforce the

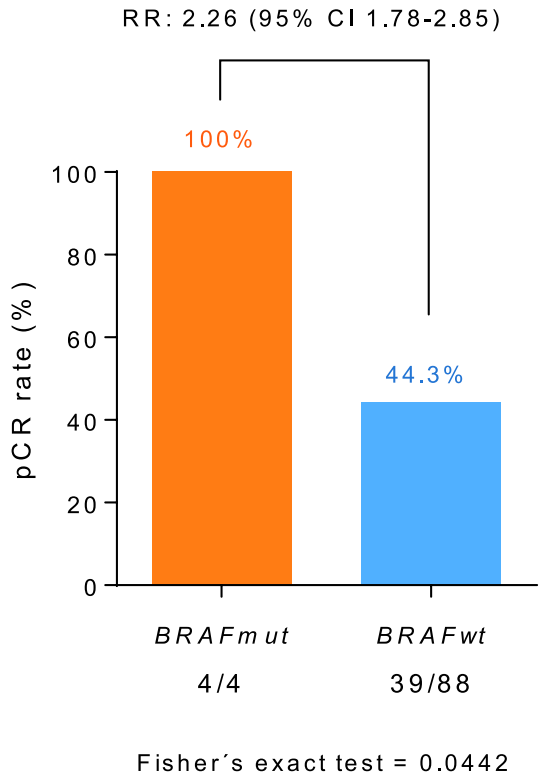

Fig. 3. Association of *BRAF* mutational status with pathological complete response, after neoadjuvant CH-IO, in stage III NSCLC patients (NADIM cohort). Only patients treated with neoadjuvant chemo-immunotherapy were considered in this analysis. A pathological complete response was defined as 0% residual viable tumor cells in both the primary tumor (lung) and sampled lymph nodes. Patients who did not undergo surgery were considered to have not had a response.

therapeutic merit of the neoadjuvant CH-IO approach in NSCLC patients.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: **M. Provencio** declares consulting fees, payment for lectures, presentations or speakers bureaus and support for attending meetings and/or travel from BRISTOL-MYERS SQUIBB, Astra Zeneca, MSD, Roche, Takeda. This author has also declared that he have a pending patent (n° EP 22 382 701.5). **R. Serna-Blasco** declares to be in possession of a pending patent (n° EP 22 382 701.5). **E. Nadal** declares Grants or contracts from Roche, Pfizer, Bristol-Myers Squibb and Merck Serono; consulting fees from Roche, Bristol Myers Squibb, Merck Sharp Dohme, Merck-Serono, Sanofi, Pfizer, Lilly, Janssen, AMGEN, Daiichi-Sankyo, Boehringer-Ingelheim, AstraZeneca, Takeda, Sanofi, Pierre Fabre, Qiagen, and Bayer; payment for lectures, presentations or speakers bureaus from Roche, Bristol Myers Squibb, Merck Sharp Dohme, Merck-Serono, Sanofi, Pfizer, Lilly, Janssen, Amgen, Boehringer-Ingelheim, AstraZeneca, Takeda, Sanofi, Pierre Fabre and Qiagen; support for attending meetings and/or travel from Roche, Takeda and Pfizer; and Participation on data safety Monitoring board or advisory board from Roche, Apollomics and MSD. **M.P. Diz Taín** declares Payment or honoraria for lectures, presentations or speakers bureaus from BRISTOL-MYERS SQUIBB, Astra-Zeneca, Roche, MSD, Takeda, Pfizer and Amgen; support for attending meetings and/or travel from BRISTOL-MYERS SQUIBB, Roche, MSD and Takeda; Participation on a Data Safety Monitoring Board or Advisory Board from BRISTOL-MYERS SQUIBB, Astra-Zeneca, and Boehringer Ingelheim; and other financial or non-financial interests from Astra-Zeneca, Roche, Mirati, and Pfizer. **B. Massuti** declares consulting fees and support for attending meetings and/or travel from Bristol-Myers Squibb, Takeda, Merck Sharpe and Dohme. **J.L. González Larriba** declares consulting fees from MSD, Janssen Cilag, Bristol Myers Squibb, Boehringer Ingelheim, and AMGEN; Payment or honoraria for lectures, presentations or speakers bureaus from MSD, Astra Zeneca, Roche, Pfizer, Janssen-Cilag, Novartis, Astella Pharma, and Bristol-Myers Squibb; Support for attending meetings and/or travel from MSD, Takeda, Bristol-Myers Squibb, Roche, Pfizer, and Janssen-Cilag. **A. Insa** declares Payment or honoraria for lectures, presentations or speakers bureaus from AMGEN, Bristol-Myers Squibb, Roche, and Takeda; Support for attending meetings and/or

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Appendix A. Supplementary data

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