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THE RAS-LUNG PROJECT: IMPLEMENTING BLOOD AND TISSUE GENOTYPING IN KRAS-POSITIVE NON-SMALL CELL LUNG CANCER TREATMENT

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Abstract

Background: The frontline management of non-oncogene addicted non-small cell lung cancer (NSCLC) involves immunotherapy (ICI) alone or combined with chemotherapy (CT-ICI). As therapeutic options expand, refining NSCLC genotyping gains paramount importance. The dynamic landscape of KRAS-positive NSCLC presents a spectrum of treatment options, including ICI, targeted therapy, and combination strategies currently under investigation.

Methods: The two-year RASLUNG project, featuring both retrospective and prospective cohorts, aimed to analyze the predictive and prognostic impact of KRAS mutations on tumor tissue and circulating DNA (ctDNA). Secondary objectives included assessing the roles of co-mutations and longitudinal changes in KRAS mutant copies concerning treatment response and survival outcomes. An external validation study confirmed the prognostic or predictive significance of co-mutations.

Results*:* In the prospective cohort (n=24), patients with liver metastases exhibited significantly elevated ctDNA levels(p=0.01), while those with >3 metastatic sites showed increased Allele Frequency (AF) (P=0.002). Median overall survival (OS) was 7.5 months, progression-free survival (PFS) was 4.0 months, and the objective response rate (ORR) was 33.3%. Higher AF correlated with an increased risk of death (HR 1.04, p = 0.03), though not progression. Notably, a reduction in plasma DNA levels was significantly associated with objective response(p=0.01). In the retrospective cohort, KRAS and STK11 mutations co-occurred in 14/21 patients (p=0.053). STK11 mutations were independently detrimental to OS (HR 1.97, p=0.025) after adjusting for various factors. KRAS tissue AF did not correlate with OS or PFS. Within the validation dataset, STK11 mutations were significantly associated with an increased risk of death in univariate (HR 2.01, p<0.001) and multivariate models (HR 1.66, p=0.001) after adjustments.

Conclusion: The RAS-Lung Project, employing innovative genotyping techniques, underscores the significance of comprehensive NSCLC genotyping. Tailored next-generation sequencing (NGS) and ctDNA monitoring may offer potential benefits in navigating the evolving landscape of KRAS-positive NSCLC treatment.

Introduction

Lung cancer is the leading cause of cancer death, regardless of gender, worldwide. In 2020, there were over 2.000.000 new cases of lung cancer and over 1.700.000 deaths worldwide[1].

Determining the appropriate therapeutic pathway for cancer patients requires a crucial first step - histological typing followed by biomolecular characterization. In cases of unresectable advanced NSCLC, various systemic therapies like chemotherapy, targeted therapies, and immunotherapy are available for treatment[2,3]. Identifying predictive biomarkers of response to targeted therapies involves exploring various mutations that are drivers of the oncogene process. As of today, effective targeted treatments for advanced Non-Small Cell Lung Cancer (NSCLC) with mutation of EGFR (15-20 %), ALK (3-5 %), ROS1 (1-3 %), BRAF V600E (2 %) and NTRK (<1 %) are widely approved[2]. Approximately 25 % of lung adenocarcinomas have an activating mutation in the KRAS gene, historically an 'orphan' of targeted therapy until recent trials of effective drugs[4].

The therapeutic approach for advanced NSCLC relies on the dichotomization of patients based on predictive biomarkers and approved targeted therapies for oncogene or non-oncogene addiction[2,3,5].

The current first-line therapy for advanced non-oncogene addicted NSCLC involves monoclonal antibodies targeting immune checkpoint receptors (ICIs). These ICIs have demonstrated greater efficacy than conventional chemotherapy regimens and can be administered as a solo treatment for patients with elevated PD-L1 expression levels (≥50%) or in tandem with chemotherapy, irrespective of PD-L1 expression [3,6,7]. For the patient population with PD-1 ≥50% there are currently no studies comparing single-agent and combination regimens with chemotherapy [7].

Moreover, although the results have been considerable regarding improved median survival of patients and favorable toxicity profile, the percentage of patients responding to such treatments usually does not exceed 40-45%, depending on the studies and line of treatment. Thus, identifying factors that potentially reduce the efficacy of immunotherapy is a research topic of great interest. Several studies have evaluated KRAS mutation's predictive and prognostic role during immunotherapy

treatment. However, results are heterogeneous, as co-mutations in genes such as STK11, KEAP1, TP53, and SMARCA4 may influence prognosis during immunotherapy [8–11]. Furthermore, phase 1-2 studies have documented for the first time the efficacy of KRAS p.G12C mutation inhibitors, sotorasib, and, even more recently, adagrasib in a population of pretreated patients [12,13]. The Codebreak200 phase 3 randomized trial results indicate that Sotorasib exhibits superior clinical efficacy over Docetaxel in KRAS G12C patients who have experienced progression post-ICI treatments[14].

As the therapeutic options expand and prognostic biomarkers emerge, optimizing NSCLC genotyping is crucial to enhance patient care and economic sustainability. In particular, the revolution hitting the KRAS-positive NSCLC will lead to a growing armamentarium of therapeutics ranging from immunotherapy to targeted therapy and combination strategies that are currently under evaluation[15].

In this context, choosing the molecular findings may be pivotal to setting the appropriate upfront or sequential strategy. Next-generation sequencing techniques (NGS) have shown to be cost-effective[16], even if the available panels are different in terms of the number and type of analyzed genomic alterations. On the other hand, monitoring of KRAS mutations in circulating tumor DNA (ctDNA) using highly sensitive techniques such as digital droplet PCR aimed at allele frequency (AF) determination of mutant alleles (e.g. G12C) may find application in the dynamic evaluation of therapeutic response[17].

In the present study, we report the outcomes of a translational project designed to enhance KRAS mutant NSCLC genotyping in a real-world context by utilizing lab-developed NGS and allele-specific blood monitoring of ctDNA.

Genotyping in NSCLC: present and future perspective

With the emergence of several predictive biomarkers, extensive genotyping in NSCLC has become necessary, with several alterations considered mandatory at diagnosis for advanced stages.

Cancer tissue represents the best specimen for molecular testing, even if small biopsies or cytological samples are frequently encountered in clinical practice for advanced NSCLC diagnosis[18]. NGS is a molecular assay for testing that simultaneously analyzes multiple biomarkers for different patients[19]. The NGS

workflows follow four steps: library preparation, fragment amplification, massively parallel sequencing, and data analysis[19].

Choosing the right gene panels is critical when conducting NGS analysis. The panels selected will directly impact the accuracy and relevance of the results. Various commercial panels are available, including narrow gene panels (10-15 genes), broad panels (up to 50 genes), tumor comprehensive panels (up to 150 genes), and human cancer comprehensive panels (up to 400 genes)[20].

While DNA-based techniques excel at identifying point mutations, insertions, and deletions, they are not as effective when detecting gene fusions. For this purpose, RNA sequencing is a preferred alternative despite the pre-analytical challenges posed by RNA instability[21].

Nevertheless, the rate of molecular testing is suboptimal, with less than two-thirds of patients effectively screened at diagnosis in a recent international survey. The suboptimal molecular coverage may be caused by cost, prolonged turnaround time, availability of technologies, and scarce awareness of specialists[22]. Adopting a reflex strategy may help increase the number of tested patients, decrease costs, and improve the turnaround time[23]. Delayed molecular testing can worsen outcomes in NSCLC patients[24]. An effective solution is to use single gene analysis alongside NGS[25], which can still work even if NGS fails due to pre-analytical tissue characteristics. Nevertheless, the NGS-based approach can be more cost-effective than single-gene testing[16,26] but should be weighted with other variables such as reimbursement policy, laboratory network organization, and treatment availability[27].

The liquid biopsy procedure is a minimally invasive technique that offers an alternative means of detecting genomic alterations in tumors[28]. Through liquid biopsy, various body fluids, including blood, urine, malignant pleural effusion, and ascites, can be analyzed providing a reliable way to detect, analyze, and monitor cancer[29]. This innovative method allows for real-time monitoring of cancer molecular alterations and is becoming increasingly popular as it overcomes the limitations of traditional methods in NSCLC [30]. Although the technique employed to identify mutations in ctDNA boasts a high degree of specificity, the sensitivity levels are less satisfying, with a maximum of 70%[28]. This deficiency is primarily attributed to the extensive dilution of ctDNA in the bloodstream, resulting from the

substantial presence of non-tumoral cell-derived cell-free DNA[31]. Allele-specific amplification and emulsion PCR assays are utilized in PCR-based platforms to identify mutant DNA, which may take up to 3 days to produce outcomes [32]. Conversely, plasma-testing platforms based on NGS may have a turnaround time of around two weeks [33].

Pivotal mutations in non-oncogene addicted NSCLC: KRAS, STK11, KEAP1, P53, and SMARCA4

The acronym KRAS stands for Kirsten rat sarcoma viral oncogene homologue. It is a frequently mutated oncogene in NSCLC, with a prevalence rate of around 25-30%[34]. KRAS mutations are more commonly observed in lung adenocarcinoma and patients with a cigarette smoking history [35]. In contrast to other oncogenic driver alterations, KRAS mutant NSCLCs express higher PD-L1 and mutational load, which may determine a greater sensitivity to ICI[36,37].

The most frequent isoform is G12C, accounting for around 40% of KRAS mutant NSCLCs, followed by G12V, G12D, G12A, or others such as Q61[38]. Specific isoforms have been associated with peculiar clinical and genomic characteristics that may influence ICI efficacy[38].

However, recent studies have demonstrated that KRAS mutant NSCLCs are highly heterogenous, and tumors with concurrent mutations in STK11 and KEAP1 genes generally resist immunotherapies, highlighting the importance of developing novel therapeutic approaches for this patient population.

The serine/threonine kinase 11 (STK11) is an onco-suppressor gene coding for a protein involved in cellular metabolism, proliferation, and growth by activating AMPK pathway[39–41]. About 15% of nonsquamous NSCLC present STK11 inactivation with a significant co-occurrence with KRAS mutations in up to 30% of patients. STK11 mutated NSCLC presents a peculiar tumor microenvironment (TME) characterized by low T CD8, high intra-tumor neutrophils, and low PD-L1 expression[40,42]. Moreover, STK11 mutated NSCLC had low dendritic-cell prevalence, NK-T cells, and macrophages[43,44]. The co-occurrence of KRAS/STK11 mutation had a reflection on TME composition, with greater tumor-associated neutrophils, T-reg, and lower T CD8 alongside decreased PD-L1 expression[10].

The Kelch-like ECH-associated protein 1 (KEAP1) is involved in the cellular regulation of oxidative stress and its dysfunction may promote carcinogenesis[45–48]. Furthermore, the increased activity in detoxification may contrast with the cytotoxic effect of chemotherapeutic agents[45]. KEAP1-NRF2 interaction leads to an immunomodulatory effect via the secretion of cytokines and chemokines, thus influencing the infiltration of TME[49]. KEAP1 mutant NSCLC, even squamous or adenocarcinoma histology, is characterized by low lymphocyte infiltration up to the 'immune desert' phenotype[8,50].

The presence of KEAP1 mutation was associated with impaired survival outcomes of advanced NSCLC treated with platinum-based chemotherapy in several observational experiences[51,52]. Even if the prognostic role seemed to be independent of other pivotal mutations[53], the co-existence of KEAP1/KRAS mutation negatively impacted the outcomes under chemotherapy[54].

The predictive role under immunotherapy for KEAP1 mutant NSCLC is still controversial. Retrospective findings evidenced a possible positive impact of KEAP1 mutation for ICI-treated patients[55,56]. Exploratory analyses within the prospective KEYNOTE 042 trial suggested the efficacy of single-agent pembrolizumab over chemotherapy for KEAP1 mutated patients[57]. Despite this evidence, other KEAP1 positive cohorts experienced detrimental outcomes under ICI[58,59]. Remarkably, the co-occurrence with other pivotal mutations such as STK11, PBRM1 and SMARCA4 leads to dismal results in a large dataset analysis[8].

The SMARCA4 gene transcribes for the BRG1protein, which is involved in the chromatin remodeling complex[60]. Around 10% of NSCLCs are deficient for SMARCA4 with an unclear prognostic relevance[60]. Globally, the SMARCA4 deficiency seems to be related to poor outcomes under chemotherapy or immunotherapy, even if the high rate of co-occurring mutation, such as KEAP1, STK11, or oncogenic drivers as EGFR, should be taken into account[61,62].

TP53 is an onco-suppressor gene encoding for a protein involved in cellular metabolism, proliferation, apoptosis, and aging mechanisms[63]. The negative prognostic value is widely recognized among various malignancies, including NSCLC, which exhibits a TP53 inactivation rate of up to 50% in diagnosed cases. The physiological impact of TP53 and the consequences of its inactivation on the

immune system have been extensively studied, rendering it an intriguing factor in the context of immunotherapy efficacy[64,65].

Materials and method

The RASLUNG project was an academic, no-profit, observational study with translational analysis conducted between November 2021 and September 2023 at the IRCCS Azienda Ospedaliera Universitaria of Bologna, Italy. The study included both a retrospective and a prospective cohort.

The prospective cohort consisted of patients with advanced KRAS G12C mutant NSCLC eligible for first-line immunotherapy-based treatments (single agent or combined with histology-driven chemotherapy) or subsequent lines of KRAS inhibitors.

The retrospective cohort included patients affected by advanced, non-oncogene addicted, nonsquamous NSCLC who received first-line immunotherapy-based treatment (single agent or combined with histology-driven chemotherapy) .In this cohort, all patients received at diagnosis an NGS panel (Oncomine Focus Assay, 52 genes) per clinical practice

The study collected information from electronic and paper-based medical records. The variables recorded encompassed age, gender, tumor histology, biomolecular characteristics, antineoplastic treatments, Eastern Cooperative Oncology Group (ECOG) performance status (PS) at the start of the study, radiological findings at the beginning and throughout the study, number of metastatic sites, biomolecular characterization, the most recent follow-up, reason for death, and date of death.

The primary objective was to analyze the predictive and prognostic impact of AF levels of the KRAS mutation detected on tumor tissue and circulating DNA.

The project had two secondary objectives. The first secondary objective was to determine the predictive or prognostic role of co-mutations, such as STK11, KEAP1, TP53, and SMARCA4, with treatment response and survival outcomes (*retrospective cohort*).

The second secondary objective was to establish the predictive and prognostic role of longitudinal changes in KRAS G12C mutant copies/ml and AF levels on ctDNA, with treatment response and survival outcomes (*prospective cohort*).

To ascertain the prognostic or predictive significance of co-mutations, we conducted an external validation study utilizing clinical and blood-based NGS data extracted from the OAK/POPLAR randomized trials dataset[66]. In the external validation cohort, we included advanced nonsquamous NSCLC patients treated with docetaxel or atezolizumab.

The following analyses have been performed after obtaining informed consent to participate in the study.

Within the prospective cohort, we performed the dosage of KRAS G12C mutant alleles on circulating DNA with ddPCR at three preplanned time points: baseline (T0), after 3 months(T1), and at subsequent disease progression(T2).

Within the observational cohort, we analyzed additional genomic mutations (KEAP1, STK11, TP53, and SMARCA4) performed through a lab-developed NGS panel on archival tumor tissue.

Laboratory methods - tissue NGS

The next-generation sequencing (NGS) analysis was performed using a multi-gene panel developed in the Molecular Pathology Laboratory of IRCCS Azienda Ospedaliera Universitaria of Bologna. The panel allows the analysis of the entire coding regions (CDS) or hot-spot regions of 15 genes for a total of 423 amplicons (about 43.05 kb, human reference sequence hg19/GRCh37), starting from archived formalin-fixed and paraffin-embedded (FFPE) tissues. The entire CDS of the following genes was analyzed: BRAF, CDH1, CDK4, CTNNA1, HRAS, KEAP1, KRAS, MITF, NRAS, PTPN11, SMARCA4, SMARCB1, SMARCE1, STK11, and TP53. NGS was performed using the Gene Studio S5 Prime sequencer (ThermoFisher Scientific Inc). About 30ng of DNA were used per each panel for the amplicon library preparation, performed with the AmpliSeq Plus Library Kit 2.0. Templates were prepared with an Ion Chef Machine and sequenced using an Ion 530 chip. Sequences were analyzed with the IonReporter tool (v. 5.18 – Thermo Fisher Scientific). Only nucleotide variations detected in both strands and at least

5% of the total number of reads analyzed were considered for the mutational calls[67]. The pathogenicity of each mutation was assessed using the Varsome tool[68].

Laboratory methods - liquid biopsy

Blood samples (10 mL) were collected in EDTA tubes at specific time points. The blood samples were processed within 3 h. Blood was centrifuged at 2000 g for 10 min at 4 °C to extract plasma. The extracted plasma was stored at -80 °C. cfDNA was then extracted from 4 ml of plasma with QIAamp Circulating Nucleic Acid Kit (Qiagen) following the manufacturer's instructions and stored at -20 °C.

For the detection and quantification of wild-type and G12C mutated KRAS a specific kit was purchased from Bio-Rad (ddPCR KRAS G12 Screening Kit #12001094) and the samples were partitioned into a mean of 15,000 droplets by using QX200 Droplet Generation (Bio-Rad). Then PCR reaction was prepared according to the manufacturer's instructions and the droplets were analyzed using the QX200 Droplet Reader (Bio-Rad), to provide absolute quantification of the wt or mutated KRAS. The results were analyzed with the QuantaSoft Analysis Pro Software (v1.0 Bio-Rad). A cut-off of three droplets was used to call a sample mutant, according to Poisson's law of small numbers (as reported in the manufacturer's instructions). According to this limit, we could detect a minimum amount of 2.7 copies per ml of plasma. The mutant allele fraction (AF) was calculated as the number of mutated droplets / (wt + mutated droplets). The kit used had a detection limit of AF of 0.2% as reported in the manufacturer's instructions.

Statistical methods

The clinical and laboratory findings were presented as continuous and categorical variables, and median values and proportions were used to summarize them. The normality distribution was verified through the Shapiro test. Means and proportions were compared by performing T-test (ANOVA, Pearson correlation test, or Kruskal-Wallis test if required) and chi2-test (or Fisher's exact test if required). The overall survival was defined as the duration between the treatment start and death due to any cause, while progression-free survival was defined as the duration between the treatment start and radiological or clinical progression or death due to any cause. The objective response rate was expressed as the percentage of patients who achieved a partial or complete response according to the RECIST 1.1 criteria assessed by physicians.

The Kaplan-Meier method was used to estimate OS and Log-rank Test to compare OS curves according to score prognostic assessment. The relationship between variables and survival outcome was explored through a univariate and multivariate analysis using a Cox model regression. A p-value ≤ 0.05 was considered statistically significant. Statistical analyses were accomplished with R-Studio free software, version 2023.06.2, utilizing the following packages: 'dplyr', 'prodlim', 'survminer', 'survMisc',' finalfit', 'CI', 'ggplot2'.

Results

Prospective cohort

From November 2021 to August 2023, 26 consecutive patients have been enrolled. One patient was excluded due to misdiagnosis (KRAS G13C), while one patient was excluded for not confirmed advanced disease.

Thus, 24 patients were included in the final analysis. The most frequent baseline characteristics were: nonsquamous histology (23/24, 95.8%), male gender (15/24,62.5%), ECOG PS 0-1 (19/24, 79.2%), less than three metastatic sites (13/24, 54.2%). Regarding the treatment, 18 out of 24 patients (75%) were treated with first-line therapy. Among them, 13 (54.2%) received histology-driven CT-ICI, and 5 patients (20.8%) received ICI single-agent. 6 patients (25%) were treated with Sotorasib in a subsequent line **(Table 1)**.

		Total (%)
Age	<70	14 (58.3)
	≥70	10 (41.7)
Histology	nonsquamous	23 (95.8)
	squamous	1 (4.2)
Sex	female	9 (37.5)
	male	15 (62.5)
ECOG PS	<2	19 (79.2)
	≥2	5 (20.8)
Number of met. sites	<3	13 (54.2)
	≥3	11 (45.8)
Brain met.	no	16 (66.7)
	yes	8 (33.3)
Liver met.	no	20 (83.3)
	yes	4 (16.7)
Lung met.	no	10 (41.7)
	yes	14 (58.3)
Bone met	no	13 (54.2)
	yes	11 (45.8)
Line of treatment	First	18 (75)
	Subsequent	7 (25)
Type of treatment	chemo-immunotherapy	13 (54.2)
	immunotherapy	5 (20.8)
	sotorasib	6 (25.0)
Objective response	no	16 (72.7)
	yes	6 (27.3)

Table 1. Baseline characteristics of patients enrolled in the prospective cohort.

17/24 patients (70.8%) had detectable circulating DNA at time 0 (t0) dosage. The median plasma DNA concentration was 6.81 cp/ml (IQR 0-56.7). Patients with baseline liver metastases (4/24,16.7%) had a significantly increased mean T0 DNA concentration (1157.4 cp/ml) in comparison with patients without (27.8 cp/ml) (p=0.01) **(figure 1)**. The median T0 AF was 1.2 % (IQR, 0-9.9). The presence of at least 3 metastatic sites significantly increased mean T0 AF (17.4%) compared to 1-2 metastatic sites (1.2%) (p=0.002) **(figure 2)**.

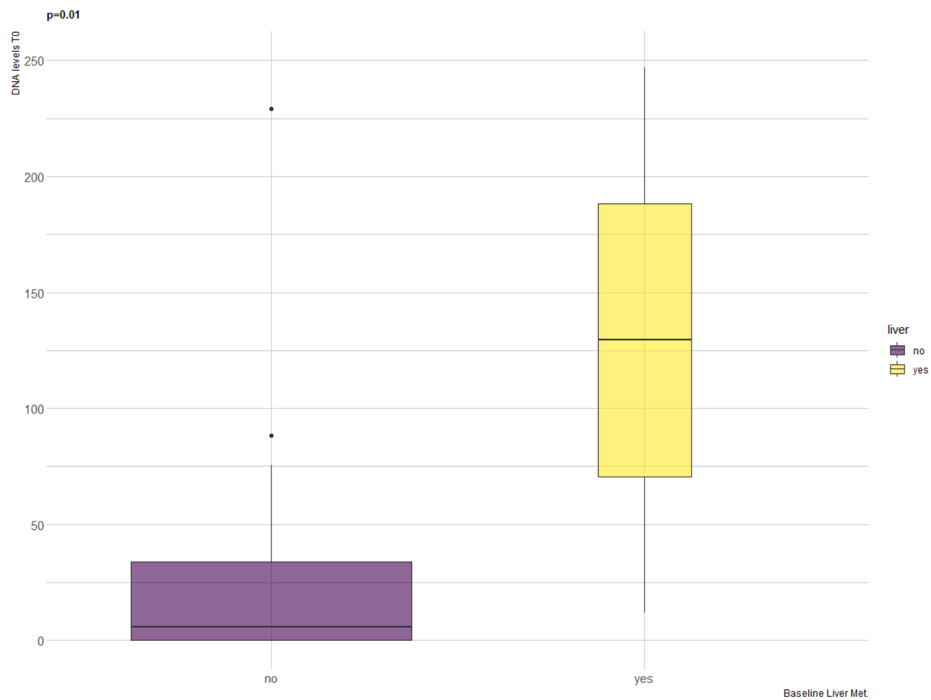

Figure 1. Plasma DNA levels (cp/ml) at T0 according to the presence of baseline liver metastasis.

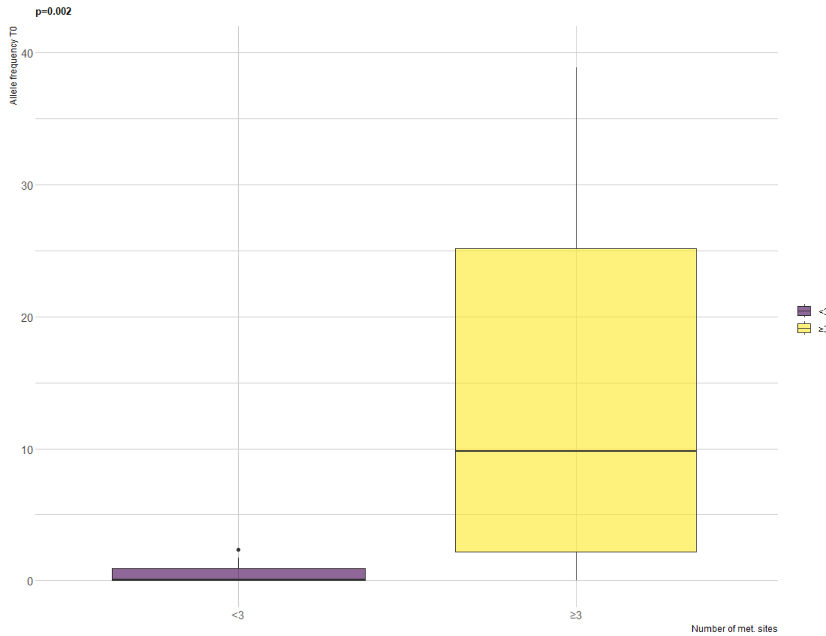

Figure 2. Plasma allele frequency (AF) at T0 according to the baseline number of metastatic sites.

16/24 patients (66.6%) underwent T1 dosage after three months of treatment per protocol. Before 90 days from treatment start, 6 out of 24 patients (25%) died. One patient did not receive the T1 dosage due to worsening clinical conditions and being discharged to home. One patient refused to undergo T1 dosage. The mean plasma DNA concentration was 19.7 cp/ml (IQR 0-21.2) at T1. 4/16 patients (25%) had increased plasma DNA levels compared to T0, of them 2 had undetectable plasma DNA at T0. The median T1 AF was 0.2% (IQR 0-6.0). 8/16 patients (50%) presented decreased DNA levels compared to T0, and 5 had a complete plasma clearance at T1. 4/16 patients (25%) had undetectable plasma DNA at T0 and T1. One patient underwent liquid biopsy at three-time points. Specifically, at T0 the plasma DNA level was 50.2 cp/ml, and AF was 38.8%. At T1, we registered a decrease in plasma DNA level (5.8 cp/ml) and AF (0.5%) with a concomitant radiological partial response. At T2, according to the protocol for PD recognition, we observed an increase in DNA levels (19 cp/ml) and AF (3.1%) **(Figure 3)**.

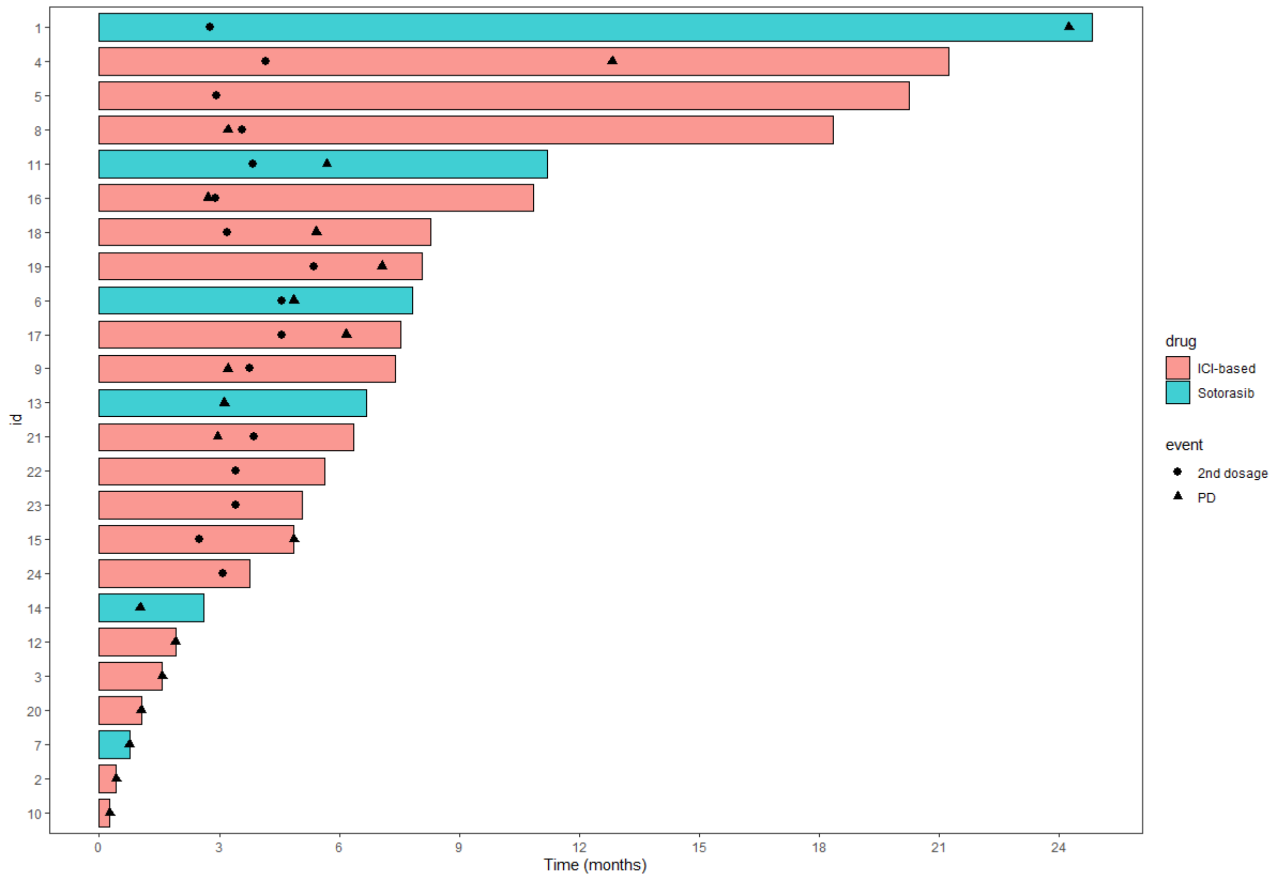

Figure 3. Swimmer plot of patients enrolled in the prospective cohort.

The median overall survival (OS) was 7.5 months (95% CI, 4.8-NR), and the median progression-free survival (PFS) was 4.0 months (95% CI, 2.9-7.0).

When analyzed as a continuous variable, AF was associated with an increased risk of death (HR 1.04, 95% CI, 1.0-1.08, p = 0.03) but not progression (HR 1.02, 95% CI, 0.97-1.06, p = 0.1) in the univariate regression model. We used the median T0 AF (1.2%) to dichotomize patients in high-AF and low-AF groups. The median OS was 7.5 months (95% CI, 1.91-NR) in high-AF group and 11.3 months (95% CI, 6.6-NR) in low-AF group (p = 0.38). The median PFS was 2.9 months (95% CI, 1.05 – NR) in the high-AF group and 4.8 (95% CI, 3.1-NR) in the low-AF group, respectively (p= 0.58).

Patients with T0 detectable DNA copies had a not reached OS (95% CI, 4.8 – NR), while those with undetectable DNA copies had 7.2 months of m OS (95% CI, 2.6-NR) (p= 0.65). Analogously, median PFS did not significantly differ according to T0 DNA detectability (3.2 vs. 5.4 months, p = 0.35).

The objective response rate (ORR) was 33.3% (95% CI, 17% - 53%). The mean plasma T0 DNA levels were 171.7 cp/ml among responders and 258.2 cp/ml among non-responders (p=0.36) **(figure 4)**. The mean T0 AF was 10.9 among responders and 8.3 among non-responders (p=0.27) **(figure 5)**. A decrease in plasma DNA levels from T0 to T1 was significantly associated with objective response (p=0.01).

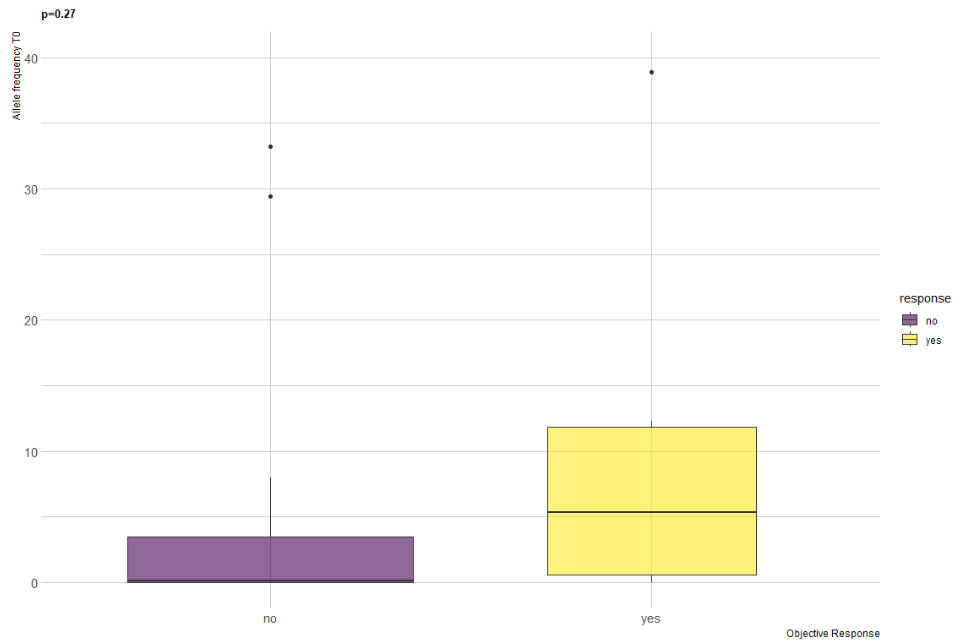

Figure 4. Plasma Allele frequency (AF) at T0 according to objective response

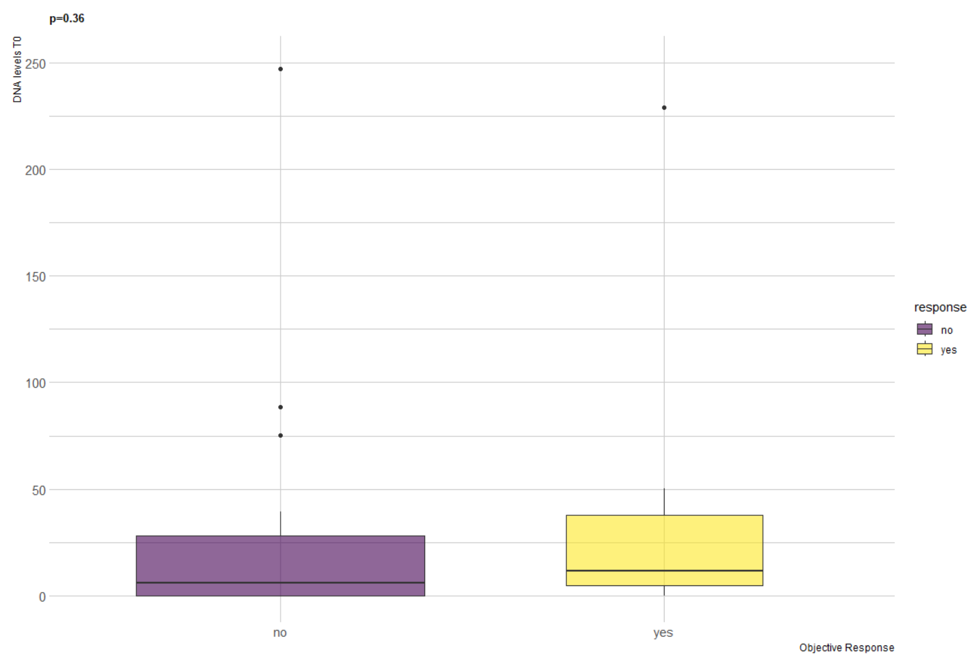

Figure 5. Plasma DNA levels (cp/ml) at T0 according to objective response

Retrospective cohort
Internal cohort

In the internal cohort, 145 patients have been enrolled. Most patients were male (59.7%), former smokers (61.1%), with ECOG PS 0-1 (84%), and received first-line CT-ICI (58.6%).

44.8% had a mutation in KRAS, 21.4% in KEAP1, 50.3% in TP53, 13.1% in SMARCA4, and 14.4% in the STK11 gene. KRAS mutation was associated with female sex (p=0.004), smoking habit (p=0.03), and TP53 co-occurrence p=0.038) **(Table 2)**.

		KRAS Mut (%)	KRAS Wild (%)	Total (%)	p value
Age	<70	32 (49.2)	41 (51.2)	73 (50.3)	0.940
	≥70	33 (50.8)	39 (48.8)	72 (49.7)	
Sex	Female	35 (53.8)	23 (28.8)	58 (40.0)	0.004
	Male	30 (46.2)	57 (71.2)	87 (60.0)	
Smoking status	current smoker	17 (26.6)	25 (31.2)	42 (29.2)	0.030
	former smoker	45 (70.3)	43 (53.8)	88 (61.1)	
	never smoker	2 (3.1)	12 (15.0)	14 (9.7)	
ECOG PS	0-1	55 (85.9)	66 (82.5)	121 (84.0)	0.741
	2	9 (14.1)	14 (17.5)	23 (16.0)	
PD-L1 expression	1 to 49	2 (3.2)	5 (6.2)	7 (4.9)	0.560
	50 to 100	25 (39.7)	35 (43.8)	60 (42.0)	
	absent	36 (57.1)	40 (50.0)	76 (53.1)	
Num. of metastatic sites	>3	17 (27.0)	21 (26.6)	38 (26.8)	1.000
	≤3	46 (73.0)	58 (73.4)	104 (73.2)	
Brain met	no	47 (73.4)	59 (74.7)	106 (74.1)	1.000
	yes	17 (26.6)	20 (25.3)	37 (25.9)	
Liver met	no	58 (90.6)	66 (83.5)	124 (86.7)	0.321
	yes	6 (9.4)	13 (16.5)	19 (13.3)	
Type of treatment	CT-ICI	40 (61.5)	45 (56.2)	85 (58.6)	0.636
	ICI	25 (38.5)	35 (43.8)	60 (41.4)	
LIPI score	intermediate-high	24 (53.3)	33 (61.1)	57 (57.6)	0.565
	low	21 (46.7)	21 (38.9)	42 (42.4)	
STK11	mut	14 (21.5)	7 (8.8)	21 (14.5)	0.053
	wild	51 (78.5)	73 (91.2)	124 (85.5)	
KEAP1	mut	17 (26.2)	14 (17.5)	31 (21.4)	0.289
	wild	48 (73.8)	66 (82.5)	114 (78.6)	
TP53	mut	26 (40.0)	47 (58.8)	73 (50.3)	0.038
	wild	39 (60.0)	33 (41.2)	72 (49.7)	
SMARCA4	mut	7 (10.8)	12 (15.0)	19 (13.1)	0.615
	wild	58 (89.2)	68 (85.0)	126 (86.9)	

Table 2. Baseline characteristics of internal cohort according to KRAS mutational status. Abbreviations: Mut, Mutated; Num, number; met, metastasis; CT, chemotherapy; ICI, immune-checkpoint inhibitors; LIPI, lung immune prognostic index

In the whole cohort, the median OS was 13.2 months (95% CI, 8.6-19.6), while the median PFS was 6.5 months (95% CI, 4.8-8.9).

The median OS was 15.9 months (95% CI, 8.7-27.0) among KRAS wild patients and 9.2 months (95% CI, 6.5- 19.6) among KRAS mutant patients (p = 0.27) **(Figure 6).** Dissecting the survival outcomes for the type of KRAS mutations, the KRAS-G12C group had a median OS of 11.13 months (95% CI, 2.9 – NR), while the KRAS-other group had a median OS of 9.24 months (95% CI, 6.5- NR) (p = 0.61).

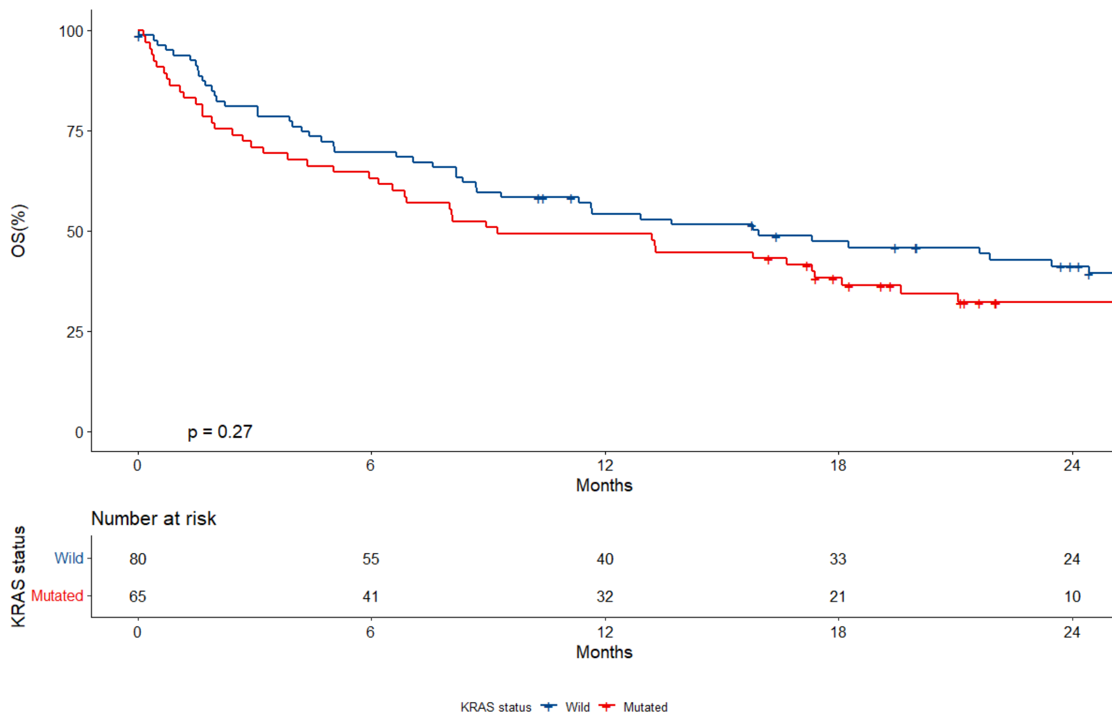

Figure 6. Overall survival (OS) according to KRAS mutational status

The median PFS was 7.1 months (95% CI, 4.9 - 11.1) among KRAS wild patients and 5.9 months (95% CI, 2.8-8.9) among KRAS mutant patients (p = 0.27) **(Figure 7).** Dissecting the survival outcomes for the type of KRAS mutations, the KRAS-G12C group had a median PFS of 5.9 months (95% CI, 2.5 – 13.9), while the KRAS-other group had a median PFS of 5.8 months (95% CI, 2.8- 8.9) (p = 0.74).

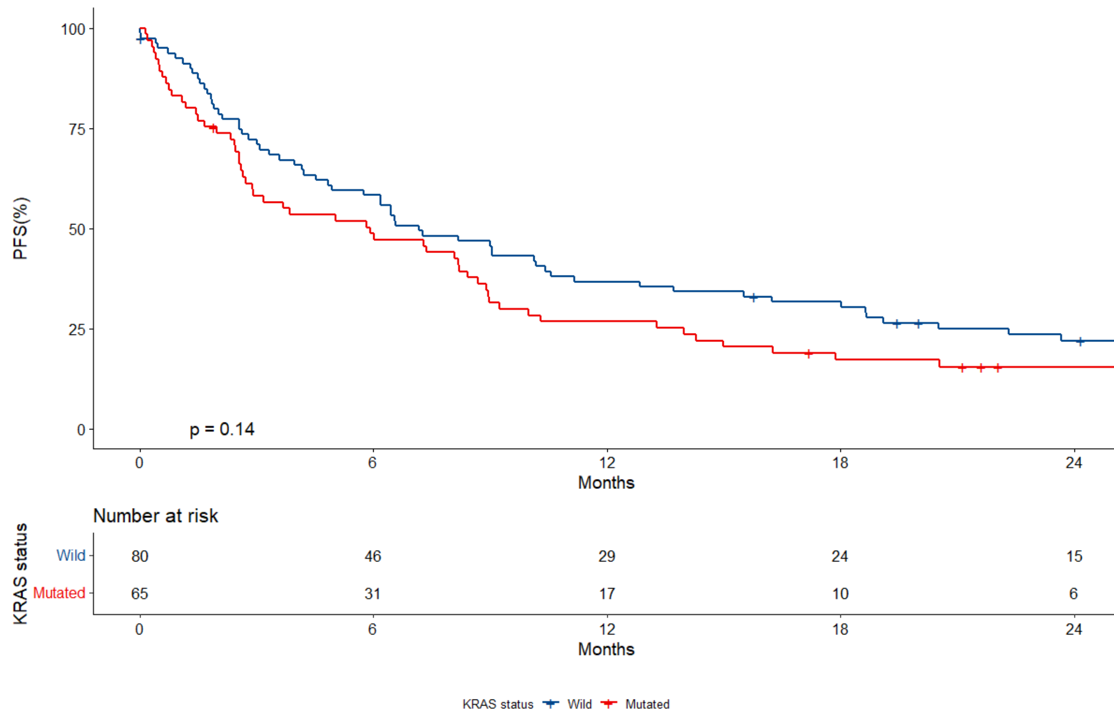

Figure 7. Progression-free survival (PFS) according to KRAS mutational status.

The KRAS tissue AF, analyzed as continuous variable, was not related to OS (p 0.30) or PFS (p=0.29).

The median OS was 8 months (95% CI, 5-16.7) for STK11 mutated patients and 17.3 months for STK11 wild type (95% CI, 8.9-24.4) (p=0.038) (figure 8). The presence of STK11 mutation was not associated with impaired PFS (6.4 vs. 6.5 months, p = 0.1).

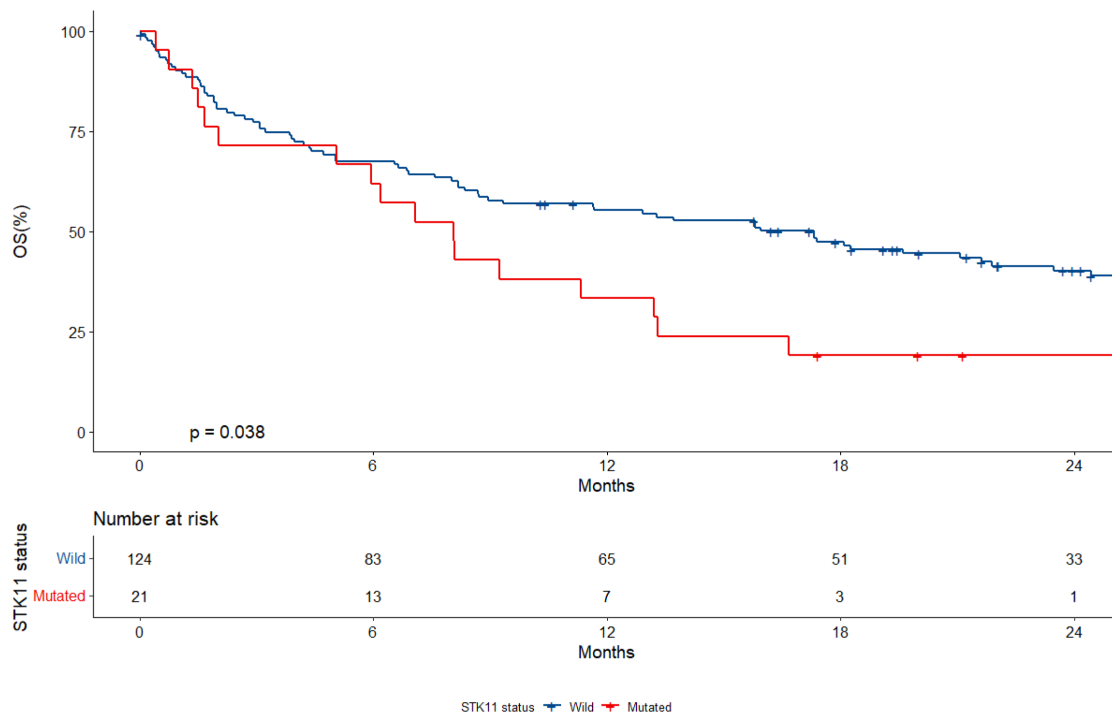

Figure 8. Overall survival (OS) according to STK11 mutational status.

Patients harboring TP53 (8.3 vs. 17.3 months, p=0.2) and KEAP1 (8.9 vs. 15.9 months, p = 0.2) mutation experienced a trend for dismal median OS but no PFS impairment. SMARCA4 status had no impact on survival outcomes.

STK11 mutations were detrimental to OS in the univariate (HR 1.74, 95% CI, 1.02-2.97, p=0.041) and multivariate model (HR 1.97, p=0.025) after adjusting for sex, age, ECOG PS, treatment (ICI vs CT-ICI), KRAS, KEAP1, TP53, SMARCA4 status. In the same model, ECOG PS predicted increased death risk (multivariate HR 2.82, 95% CI, 1.67-4.75, p<0.001) **(Table 3)**.

Concerning the regression analyses for PFS, the ECOG PS was the unique prognostic factor of increased progression risk in the univariate (HR 2.15, 95% CI, 1.34-3.45, p=0.001) and multivariate model (HR 2.46, 95% CI, 1.50-4.05). Notably, genomic alterations did not impact the median progression risk in our cohort **(Table 3)**.

		All (%)	PFS HR (univariable)	HR (multivariable)	OS HR (univariable)	HR (multivariable)
Sex	Female	58 (40.0)	-	-	-	-
	Male	87 (60.0)	0.81 (0.56-1.17, p=0.267)	0.74 (0.50-1.10, p=0.139)	0.96 (0.64-1.46, p=0.861)	0.88 (0.57-1.38, p=0.585)
Age	<70	73 (50.3)	-	-	-	-
	≥70	72 (49.7)	1.33 (0 .93-1.92, p=0.11)	1.54(1.03-2.31, p= 0.033)	1.19 (0.79-1.79, p=0.39)	1.27 (0.82-1.98, p=0.27)
Type of treatment	CT-ICI	85 (58.6)	-	-	-	-
	ICI	60 (41.4)	0.81 (0.56-1.19, p=0.283)	0.75 (0.50-1.13, p=0.172)	0.99 (0.65-1.51, p=0.972)	0.98 (0.62-1.54, p=0.923)
ECOG PS	0-1	121 (84.0)	-	-	-	-
	2	23 (16.0)	2.15 (1.34-3.45, p=0.001)	2.46 (1.50-4.05, p<0.001)	2.64 (1.61-4.32, p<0.001)	2.87 (1.70-4.86, p<0.001)
KRAS	wild	80 (55.2)	-	-	-	-
	mut	65 (44.8)	1.32 (0.91-1.89, p=0.140)	1.21 (0.82-1.79, p=0.339)	1.26 (0.84-1.89, p=0.273)	1.28 (0.82-2.00, p=0.270)
STK11	wild	124 (85.5)	-	-	-	-
	mut	21 (14.5)	1.41 (0.86-2.31, p=0.171)	1.55 (0.90-2.68, p=0.117)	1.74 (1.02-2.97, p=0.041)	1.95 (1.08-3.54, p=0.028)
KEAP1	wild	114 (78.6)	-	-	-	-
	mut	31 (21.4)	1.23 (0.80-1.90, p=0.341)	1.03 (0.64-1.65, p=0.901)	1.32 (0.82-2.13, p=0.261)	0.98 (0.57-1.67, p=0.938)
TP53	wild	72 (49.7)	-	-	-	-
	mut	73 (50.3)	0.87 (0.60-1.24, p=0.436)	0.87 (0.59-1.27, p=0.462)	1.31 (0.87-1.97, p=0.199)	1.33 (0.87-2.05, p=0.187)
SMARCA4	wild	126 (86.9)	-	-	-	-
	mut	19 (13.1)	1.33 (0.80-2.20, p=0.266)	1.35 (0.80-2.27, p=0.256)	1.05 (0.57-1.92, p=0.884)	1.24 (0.66-2.33, p=0.510)

Table 3. Univariate and multivariate analysis for progression-free survival (PFS) and overall survival (OS) within the Internal cohort.

Validation cohort

818 patients have been included in the validation cohort. The clinical characteristics were balanced, as per protocol definition. 54.6% of patients were male, 75.2% were younger than 70, and 66.9% had a maximum of 3 metastatic sites at study randomization. 7.3 % of patients had STK11 mutation, 7.9% KRAS, 32.2% TP53, 11% KEAP1, 7.6% SMARCA4.

KRAS mutant patients had an increased rate of tumor mutational burden (TMB)>16 mut/mb (41.5% vs. 22.7% among KRAS wild type, p = 0.002). KRAS mutations significantly co-occur with TP53 (47.7 vs. 30.8% among KRAS wild type, p = 0.008) and SMARCA4 (15.4% vs. 6.9% among KRAS wild type, p 0.025). In this cohort, no KRAS G12C patients have been included. The most frequent KRAS subtype was G12V (41.5%, 27/65 patients), followed by G61X (16.9%, 11/65 patients).

The presence of STK11 mutation was associated with an increased rate of TMB>16 mut/mb (43% vs. 22.7% among STK11 wt, p = 0.001), co-occurring KEAP1 (41.7% vs. 8.6% among STK11 wt, p< 0.001) and SMARCA4 mutations (18.3% vs. 6.7% among STK11 wt, p=0.003). Noteworthy, patients with TP53(p<0.001), KEAP1(p<0.001) and SMARCA4 (p <0.001) mutations had more frequently TMB > 16 mut/mb. There was no significant imbalance according to the genomic features across the two arms of treatment **(Table 4)**.

		KRAS Mut (%)	KRAS Wild (%)	Total (%)	p value
Sex	Female	32 (49.2)	339 (45.0)	371 (45.4)	0.600
	Male	33 (50.8)	414 (55.0)	447 (54.6)	
Age	<70	56 (86.2)	559 (74.2)	615 (75.2)	0.047
	≥70	9 (13.8)	194 (25.8)	203 (24.8)	
TMB	<16 mut/mb	38 (58.5)	412 (77.3)	450 (75.3)	0.002
	≥16 mut/mb	27 (41.5)	121 (22.7)	148 (24.7)	
Treatment	Docetaxel	32 (49.2)	378 (50.2)	410 (50.1)	0.984
	Atezolizumab	33 (50.8)	375 (49.8)	408 (49.9)	
Num. of metastatic sites	>3	21 (32.3)	250 (33.2)	271 (33.1)	0.992
	≤3	44 (67.7)	503 (66.8)	547 (66.9)	
STK11	mut	6 (9.2)	54 (7.2)	60 (7.3)	0.717
	wild	59 (90.8)	699 (92.8)	758 (92.7)	
TP53	mut	31 (47.7)	232 (30.8)	263 (32.2)	0.008
	wild	34 (52.3)	521 (69.2)	555 (67.8)	
KEAP1	mut	10 (15.4)	80 (10.6)	90 (11.0)	0.332
	wild	55 (84.6)	673 (89.4)	728 (89.0)	
SMARCA4	mut	10 (15.4)	52 (6.9)	62 (7.6)	0.025
	wild	55 (84.6)	701 (93.1)	756 (92.4)	

Table 4. Baseline characteristics of validation cohort according to KRAS mutational status. Abbreviations: Mut, Mutated; TMB, tumor mutational burden; Num, number.

The median OS was 13.1 months (95% CI, 11.9-14.4) among KRAS wild patients and 7.8 months (95% CI, 5.9- 13) among KRAS mutant patients (p = 0.0033). The median PFS was 3.7 months (95% CI,2.8 - 4.1) among KRAS wild patients and 2.7 months (95% CI, 1.5-4) among KRAS mutant patients (p = 0.059).

The median OS was 6.4 months (95% CI, 4.8-9.7) for STK11 mutated patients and 17.3 months for STK11 wild type (95% CI, 12-14.5) (p< 0.0001) **(Figure 9)**.

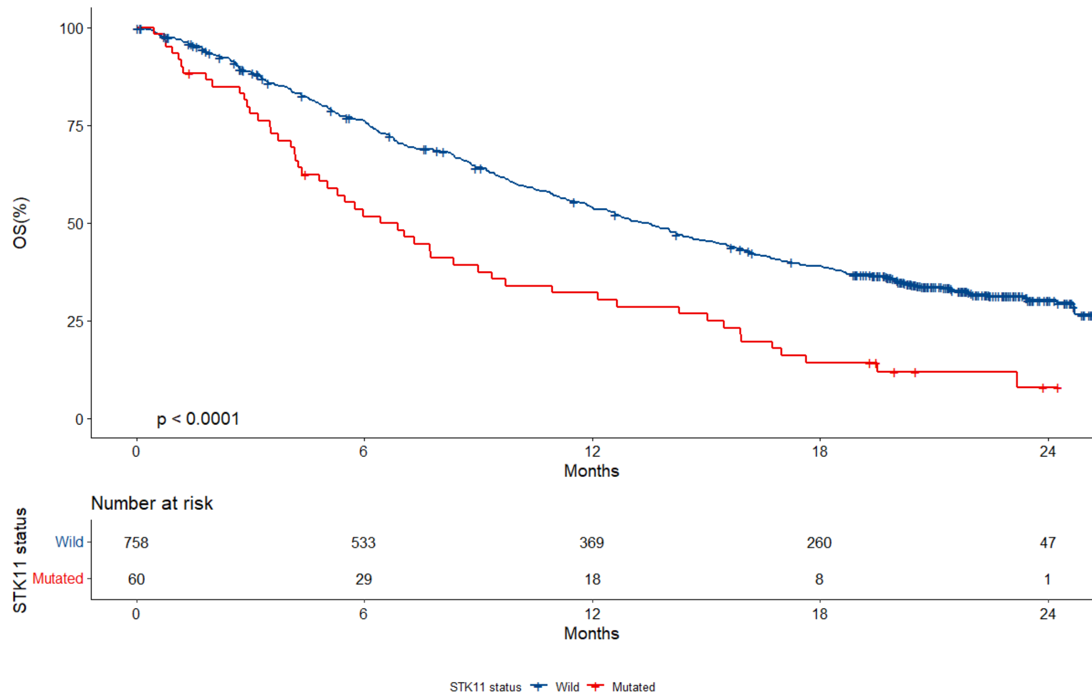

Figure 9. Overall survival (OS) according to STK11 mutational status.

KRAS (HR 1.55, p=0.004), STK11 (HR 1.65, p=0.001) and KEAP1 (HR 1.74, p<0.001) mutations independently increased the death risk in the multivariate model after adjusting for sex, age, treatment (CT vs. ICI) and co-mutation status. In the same model, having more than 3 metastatic sites was independently associated with death risk (multivariate HR 1.79, p < 0.001). Conversely, Atezolizumab treatment independently reduced death risk in the univariate and multivariate models (multivariate HR 0.68, p<0.001) **(table 5)**.

The number of met. Sites (>3) and KEAP1 mutations were detrimental to PFS in the univariate (HR 1.55, p<0.001 and HR 1.64 p>0.001, respectively) and multivariate model (HR 1.50, p<0.001) after adjusting for sex, age, treatment (IO vs CT), KRAS, STK11, TP53, SMARCA4 status. In the same model, TP53 and STK11 predicted increased progression risk in the univariate but not multivariate model **(table 5)**.

			PFS		OS	
		All (%)	HR (univariable)	HR (multivariable)	HR (univariable)	HR (multivariable)
Sex	Female	371 (45.4)	-	-	-	-
	Male	447 (54.6)	1.10 (0.95-1.28, p=0.196)	1.04 (0.89-1.21, p=0.623)	1.17 (0.99-1.39, p=0.073)	1.08 (0.91-1.29, p=0.364)
Age	<70	615 (75.2)	-	-	-	-
	≥70	203 (24.8)	0.89 (0.75-1.06, p= 0.194)	0.94 (0.79-1.12, p=0.546)	1.03 (0.84-1.76, p=0.76)	1.2 (0.99-1.48, p= 0.06)
Type of treatment	Docetaxel	410 (50.1)	-	-	-	-
	Atezolizumab	408 (49.9)	0.99 (0.85-1.14, p=0.844)	0.98 (0.84-1.14, p=0.788)	0.72 (0.61-0.85, p<0.001)	0.68 (0.57-0.81, p<0.001)
Num. of metastatic sites	≤3	547 (66.9)	-	-	-	-
	>3	271 (33.1)	1.55 (1.33-1.81, p<0.001)	1.50 (1.28-1.76, p<0.001)	1.85 (1.55-2.20, p<0.001)	1.79 (1.49-2.14, p<0.001)
STK11	wild	758 (92.7)	-	-	-	-
	mut	60 (7.3)	1.49 (1.13-1.96, p=0.004)	1.32 (0.99-1.75, p=0.056)	2.01 (1.50-2.69, p<0.001)	1.65 (1.22-2.24, p=0.001)
KRAS	wild	753 (92.1)	-	-	-	-
	mut	65 (7.9)	1.29 (0.99-1.67, p=0.061)	1.24 (0.94-1.62, p=0.122)	1.55 (1.15-2.08, p=0.004)	1.55 (1.15-2.09, p=0.004)
TP53	wild	555 (67.8)	-	-	-	-
	mut	263 (32.2)	1.20 (1.03-1.40, p=0.020)	0.98 (0.83-1.16, p=0.838)	1.43 (1.19-1.71, p<0.001)	1.07 (0.89-1.30, p=0.478)
KEAP1	wild	728 (89.0)	-	-	-	-
	mut	90 (11.0)	1.64 (1.30-2.05, p<0.001)	1.44 (1.14-1.84, p=0.003)	2.20 (1.72-2.81, p<0.001)	1.74 (1.34-2.26, p<0.001)
SMARCA4	wild	756 (92.4)	-	-	-	-
	mut	62 (7.6)	1.15 (0.88-1.52, p=0.312)	0.96 (0.73-1.28, p=0.805)	1.57 (1.16-2.12, p=0.004)	1.18 (0.87-1.62, p=0.289)

Table 5. Univariate and multivariate analysis for progression-free survival (PFS) and overall survival (OS) within the Validation cohort.

Discussion

In our translational study conducted in a single center, we endeavored to analyze the genomic makeup of a population of non-oncogene addicted advanced NSCLC patients. We employed liquid biopsy and an integrative NGS panel to achieve this, particularly focusing on KRAS-positive disease. The primary objective was to demonstrate an association between KRAS AF and response to immunotherapy-based or targeted therapy. Our analyses confirmed the primary research hypothesis only within the prospective cohort, considering continuous AF as a predictor of death risk. In addition, our study has demonstrated the crucial role of longitudinal DNA monitoring in accurately predicting the response to a treatment. Moreover, the integration of baseline NGS analysis with a lab-developed panel has allowed us to ascertain the presence of pivotal genes such as STK11, which showed significant prognostic impact, regardless of the type and line of treatment.

The prognostic evaluation of AF from baseline ctDNA was investigated in several studies, including advanced solid malignancies. In a prospective investigation including BRAF-V600E advanced colorectal patients, the prognostic role of plasma AF was explored through digital-droplet PCR[69]. Patients with higher baseline AF (2% threshold) reported a significantly decreased PFS and OS. Furthermore, the authors found that a baseline high AF predicts sensitivity to encorafenib-binimetinib-cetuximab compared to standard doublets[69].

A phase 2 clinical trial was conducted to study the effectiveness of Pembrolizumab in treating advanced solid tumors, including a group of patients with NSCLC[70]. The trial also explored the potential of personalized ctDNA analysis based on tissue whole exome sequencing findings as a predictive tool[70]. Variant AF were determined for each of the 16 target mutations. The plasma ctDNA levels were assessed by normalizing AF per plasma volume. The authors found that lower level ctDNA at baseline was associated with improved survival outcomes (OS, PFS)[70].

A large study explored the association between plasma NGS-based AF and survival outcomes among patients with KRAS or EGFR-positive NSCLC[71]. The study included 488 patients with stage I-IV NSCLC. In the KRAS cohort with 286 patients, a significant correlation was found between plasma AF and OS, regardless of whether it was considered a categorical variable with different thresholds (1% or

10%) or a continuous variable[71]. The various settings of patients included and the lack of data regarding the treatment administered limit the fitting of this model in the context that we investigated.

Remarkably, the EGFR-positive disease may be considered the paradigm of oncogene-addicted NSCLC, for which the AF detected at baseline or during targeted therapy is a clear predictor of response and survival outcomes[72,73].

The present findings provide support for the utilization of AF ctDNA as a baseline prognostic factor, which serves as a surrogate marker of disease burden or aggressiveness.

The variation of ctDNA has been related to disease response or progression in our prospective cohort. The previously discussed study by Bratman et al., demonstrated that the identification of gene targets through tissue biopsy may be used to personalize the liquid biopsy monitoring during the treatment[70]. The authors concluded that ctDNA kinetics strongly correlated with treatment response, and particularly ctDNA clearance was associated with long-term benefit from pembrolizumab[70].

The LungBEAM study was a prospective multicenter project aimed at assessing the predictive value of EGFR plasma monitoring under first or second-generation EGFR-inhibitors[74]. The rise of plasma AF was associated with a significantly increased risk of disease progression. Interestingly, as confirmed in our results, the AF decreases rapidly, anticipating the radiological response, and then increases progressively until the radiological progression[74].

Another single-center study enrolled retrospectively 97 patients treated with single-agent immunotherapy for whom frozen plasma samples were available at two time points (baseline vs. 1 month)[75]. Liquid biopsy was performed through a 36-gene NGS panel, finding a correlation between survival outcomes and early ctDNA variations[75].

Analogously, Ricciuti et al. investigated the role of ctDNA changes in 62 NSCLC patients treated with upfront pembrolizumab, either as a single agent or in combination with platinum doublets[76]. Tumor response and longer survival outcomes were significantly associated with plasma AF decrease at the first timepoint (median of 21 days from therapy start).

Recently, Paweletz et al. conducted an exploratory analysis of ctDNA changes in a phase 2 trial of Adagrasib activity in KRAS G12C advanced NSCLC[77]. Interestingly, plasma samples were analyzed at longitudinal time points with NGS and ddPCR, and the results were presented as AF. They found an excellent correlation between NGS and ddPCR analysis, paving the way for the use of this method as a faster and more economical plasma analysis for ctDNA in KRAS positive NSCLC. Notably, the clearance of ctDNA at cycle 2 or 4 of treatment was associated with improved ORR and survival outcomes (OS, PFS), respectively. In our analysis, we proposed a ctDNA analysis performed using ddPCR at baseline and after 4 cycles of treatment (3 months), demonstrating a correlation with tumor response. Our research, consistent with prior studies, indicates that plasma ddPCR can serve as a dynamic method for monitoring treatment and playing an essential role in identifying early treatment failure in conjunction with radiological findings. In plenty of competitive treatments, ddPCR plasma genotyping can help adjust treatment intensity to reduce toxicity or prevent failure.

In this optic, recently, the results of the adaptative phase 2 BR.36 trial have been published[78]. The stage 1 of the trial enrolled 50 patients with advanced NSCLC who received pembrolizumab per clinical practice with the aim to assess the right timing and association with radiological response. Remarkably, they identified 4 molecular response patterns according to clearance timing (2 or 3 cycles), max AF reduction over 85% and ctDNA persistence[78]. They found a significant association between ctDNA kinetics and ORR, PFS and OS, but not between baseline AF and OS. The upcoming stage 2 of the BR.36 trial will use early ctDNA detection during pembrolizumab monotherapy for identifying high-risk patients with advanced NSCLC and PD-L1 ≥50%, who will be randomized to receive pembrolizumab plus chemotherapy or continue with pembrolizumab.

In our retrospective internal cohort, we found that KRAS patients exhibited a trend of dismal survival outcomes under first-line immunotherapy or chemo-immunotherapy. No significant differences were found according to mutation subtype (G12C vs. others). In the validation cohort, KRAS mutation was independently associated with dismal OS regardless of treatment received (ICI vs. CT), other clinical variables, and genomic features.

A systematic review and meta-analysis were conducted, including 25 randomized controlled trials to assess the prognostic factors associated with response to immunotherapy or chemo-immunotherapy[37]. Notably, KRAS mutation was associated with improved overall survival under chemo-immunotherapy versus chemotherapy, even if no differences were found through meta-regression indirect comparison between immunotherapy and chemo-immunotherapy[37].

In a large multicenter study including 2327 patients affected by KRAS-positive NSCLC, the KRAS G12D patients were less exposed to smoking and had lower PD-L1 expression levels than KRAS G12C patients[38]. When comparing the survival outcomes according to the treatment administered, KRAS G12D patients experienced worse ORR, PFS, and OS with single-agent immunotherapy than KRAS G12C patients.

As previously mentioned, the co-occurrence of pivotal co-mutations with KRAS may impact survival outcomes. Remarkably, our retrospective investigation confirmed the negative prognostic role of STK11 mutation in both internal and validation cohorts regardless of treatment received (ICI, ICI-CT, CT) and concurrent mutations (KRAS, KEAP1, SMARCA4, TP53).

STK11 inactivation leads to a peculiar subset of KRAS-positive lung adenocarcinoma characterized by a 'cold' tumor microenvironment and low PD-L1 expression[10,39]. In a clinical setting of advanced KRAS-mutant NSCLC, the survival outcomes and antitumor response under single agent ICI were significantly hampered by the co-occurrence of STK11 mutation[9]. Intriguingly, a bi-centric cohort included 1261 patients affected by advanced lung adenocarcinoma and treated with ICI[79]. The STK11 and KEAP1 mutations within this cohort were associated with impaired PFS and OS. This datum was confirmed in KRAS mutant patients but not in KRAS wild-type populations, suggesting a dependency from KRAS mutational status[79]. The authors found that STK11 and KEAP1 mutant NSCLC showed a different transcriptomic profile and tumor microenvironment infiltration according to KRAS mutation[79]. Nevertheless, the STK11 and KEAP1 mutational status were analyzed separately in a multivariable model of KRAS mutant or KRAS wild patients, limiting the conclusions' generalizability. In addition, 69.2% of patients received a second or subsequent line of treatment, 10.2% had an EGFR alteration, and 9.8% had another oncogenic driver mutation.

Boeschen et al. performed a comprehensive bioinformatic analysis of public datasets intending to assess the prognostic value of KRAS, STK11, and KEAP1 mutations as triplet, doublets, or single gene[80]. Patients expressing the triple mutation, STK11 plus KEAP1, or KRAS plus KEAP1 had a significantly shorter OS in the proportional hazard model, including other combinations or single gene alterations[80]. Notably, the impact on survival was not related to immunotherapy treatment, reinforcing the prognostic rather than predictive value of these gene alterations. The aforementioned evidence corroborates our research findings that tumors expressing STK11, KEAP1, KRAS, or TP53 mutations display an elevated biological aggressiveness and unfavorable prognosis, irrespective of the treatment regimen.

With the emergence of anti-KRAS G12C targeted therapies, this data needs to be reanalyzed. In the phase2 codebreak100 trial, the efficacy of Sotorasib was investigated in a population of pretreated KRAS G12C mutant patients[12]. In a descriptive-only explorative analysis, the impact of STK11, KEAP1, and TP53 mutation on antitumor activity was evaluated. Antitumor activity was encountered across the molecular subgroups, even if the presence of KEAP1 alterations seemed to reduce the ORR.

Analogously, the KRISTAL1 trial demonstrated the activity of the KRAS G12C inhibitor Adagrasib[13]. The exploratory biomarker assessment evidenced a negative impact on antitumor response in patients with KEAP1 mutant but STK11 wild type. Notably, no inferential analyses have been provided in the prospective trials above discussed[12,13]. A multicenter observational study assessed the clinical outcomes of 105 patients treated with Sotorasib for advanced KRAS G12C NSCLC[81]. The presence of KEAP1 co-mutation was associated with significantly reduced PFS and OS. Otherwise, STK11 mutation did not impact survival outcomes in this cohort[81]. These findings suggest that KRAS inhibitors may be effective alone or in combination with other treatments, such as ICI or chemotherapy[15], in the context of STK11 co-mutant disease.

Finally, the SMARCA4 deficiency has been associated with dismal prognosis under immunotherapy from limited case data, even if other experiences did not find any significant impact[60–62]. In both internal and validation subgroups, our retrospective

cohort failed to evidence any prognostic influence regardless of the treatment received.

The present work has several limitations worthy of discussion. Firstly, the sample size was globally limited, including the prospective (26 participants) and the retrospective cohort (145 participants for internal subgroup). In the prospective cohort, the survival analyses were predominantly limited by the sample size. In addition, in the retrospective cohort we evidenced a non-significant trend of hampered survival outcomes for KEAP1, KRAS, TP53 mutations. The use of OAK/POPLAR dataset allowed to explore the prognostic rather than predictive value of pivotal mutations, but we need to underline that a different setting of treatment (first vs. subsequent line) and NGS methodic (tissue vs. liquid biopsy) may limit the comparability of two cohorts. It is important to note that the application of liquid biopsy in the validation cohort may have been subject to certain biases, particularly due to the poor prognosis of cancer that may have resulted in a greater shedding of circulating ctDNA. In addition, the OAK/POPLAR dataset did not include G12C mutants, which decreases the external validity of the findings for KRAS-positive patients.

Additionally, due to partial data collection and physician-based radiological assessment and evaluation, the retrospective nature of the study itself constituted another limitation.

Nevertheless, the RAS-Lung project has the great strength to be an independent, academic, translational project aimed to improve the genotyping of non-oncogene addicted advanced NSCLC with a focus on KRAS-positive disease. The implementation of a tailored genomic approach allows to improve the prognostic prevision within the daily clinical practice. The choice of a narrow NGS panel including oncogenic driver mutations should also involve clinically relevant mutations such as KEAP1, STK11 and TP53. With its rapid turnaround time, a tailored liquid biopsy using ddPCR may be a crucial source of baseline and dynamic information derived from tissue genomic data. This sequential approach could be effectively applied in clinical practice, and further investigations are warranted to validate its cost-effectiveness.

Conclusion

The RAS-Lung Project presents an innovative approach to diagnosing and treating KRAS-positive NSCLC. The project utilized lab-developed NGS and allele-specific blood monitoring of ctDNA to augment KRAS mutant NSCLC genotyping in a real-world context. The study's outcomes demonstrate the importance of comprehensive genotyping in NSCLC, with several alterations considered mandatory at diagnosis for advanced stages.

NGS techniques have shown to be cost-effective, while ctDNA monitoring of KRAS mutations using highly sensitive techniques such as digital droplet PCR could be used to evaluate therapeutic response.

In conclusion, the RAS-Lung Project's findings highlight the importance of comprehensive genotyping in NSCLC and the potential benefits of innovative approaches such as tailored NGS and ctDNA monitoring. As the therapeutic options expand and prognostic biomarkers emerge, optimizing NSCLC genotyping is crucial to enhance patient care and economic sustainability.

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