

# Metformin Enhances Cisplatin-Induced Apoptosis and Prevents Resistance to Cisplatin in Co-mutated *KRAS/LKB1* NSCLC

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## ABSTRACT

**Introduction:** We hypothesized that activating *KRAS* mutations and inactivation of the liver kinase B1 (*LKB1*) oncosuppressor can cooperate to sustain NSCLC aggressiveness. We also hypothesized that the growth advantage of *KRAS/LKB1* co-mutated tumors could be balanced by higher sensitivity to metabolic stress conditions, such as metformin treatment, thus revealing new strategies to target this aggressive NSCLC subtype.

**Methods:** We retrospectively determined the frequency and prognostic value of *KRAS/LKB1* co-mutations in tissue specimens from NSCLC patients enrolled in the TAILOR trial. We generated stable *LKB1* knockdown and *LKB1*-overexpressing isogenic H1299 and A549 cell variants, respectively, to test the in vitro efficacy of metformin. We also investigated the effect of metformin on cisplatin-resistant CD133<sup>+</sup> cells in NSCLC patient-derived xenografts.

**Results:** We found a trend towards worse overall survival in patients with *KRAS/LKB1* co-mutated tumors as compared to *KRAS*-mutated ones (hazard ratio: 2.02, 95% confidence interval: 0.94–4.35,  $p = 0.072$ ). In preclinical experiments, metformin produced pro-apoptotic effects and enhanced cisplatin anticancer activity specifically in *KRAS/LKB1* co-mutated patient-derived xenografts. Moreover, metformin

prevented the development of acquired tumor resistance to 5 consecutive cycles of cisplatin treatment (75% response rate with metformin-cisplatin as compared to 0% response rate with cisplatin), while reducing CD133<sup>+</sup> cells.

**Conclusions:** *LKB1* mutations, especially when combined with *KRAS* mutations, may define a specific and more aggressive NSCLC subtype. Metformin synergizes with

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Drs. Moro, Caiola, and Ganzinelli contributed equally to this work.

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cisplatin against *KRAS/LKB1* co-mutated tumors, and may prevent or delay the onset of resistance to cisplatin by targeting CD133<sup>+</sup> cancer stem cells. This study lays the foundations for combining metformin with standard platinum-based chemotherapy in the treatment of *KRAS/LKB1* co-mutated NSCLC.

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**Keywords:** Non-small cell lung cancer; Metformin; *KRAS/LKB1*; Cisplatin resistance; Cancer stem cells

## Introduction

Lung cancer is the most frequent cause of cancer-related death worldwide, accounting for more than 1.4 million deaths per year.<sup>1</sup> NSCLC accounts for approximately 85% of all lung cancers, and is poorly sensitive to most of available treatment options, with response rates ranging between 10% and 25% with standard therapies.<sup>2</sup> For the majority of patients with advanced NSCLC, cytotoxic chemotherapy (ChT) remains the cornerstone treatment. In recent years, inhibitors of constitutively active EGFR, ALK receptor tyrosine kinase (ALK) or ROS1 kinases dramatically improved tumor responses and clinical outcomes of patients with mutated tumors.<sup>3</sup> Furthermore, pembrolizumab has recently replaced platinum-containing ChT as the first-line treatment for tumors expressing programmed death ligand 1 in more than 50% of cancer cells.<sup>4</sup> When considered together, targeted therapies and pembrolizumab represent approximately 30% of all first-line treatments, whereas platinum-based ChT remains the preferred first-line option for the remaining patients.

Another frequently altered gene in NSCLC is *KRAS*, whose mutations are usually mutually exclusive with *EGFR*, *ALK* and *ROS1* mutations, and are found in approximately 25% of all NSCLCs.<sup>5</sup> Decades of intense research to effectively inhibit constitutively active *KRAS*, or to target its major downstream effectors, led to unsatisfactory clinical results in NSCLC patients.<sup>6,7</sup> So far, no predictive or prognostic role for *KRAS* mutations have been consistently documented.<sup>8-10</sup> Some evidences suggest that specific subgroups of *KRAS*-mutated tumors with co-mutations in other crucial genes may display specific clinical behavior, such an intrinsically aggressive clinical course or exquisite response/resistance to therapies.<sup>11-13</sup> NSCLCs with activating *KRAS* mutations frequently harbor other concurrent mutations in liver kinase B1 (*LKB1*), long non-coding RNAs (*p53*), cyclin dependent kinase 4/6 (*CDK4/6*) genes. In particular, approximately 10% of all NSCLC are co-mutated for both

*KRAS* and *LKB1*.<sup>14,15</sup> *LKB1* is a tumor suppressor kinase that is involved in regulating cell growth, metabolism, and survival. Its major direct target is the protein kinase AMP-activated catalytic subunit alpha 1 (AMPK), which is activated in conditions of metabolic stress and orchestrates an adaptive metabolic response that aims to spare energy units and nutrients.<sup>16</sup> For example, AMPK phosphorylates and inhibits acetyl-CoA carboxylase alpha (ACC1) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) enzymes, which are involved in fatty acid and cholesterol biosynthesis, two energy-consuming processes. Moreover, by phosphorylating TSC complex subunit 2 (TSC2), AMPK inhibits the mammalian target of rapamycin (mTOR) kinase, with the consequent inhibition of protein synthesis (Supplementary Fig. 1).<sup>17</sup> Recent studies have revealed that *KRAS/LKB1* co-mutated pancreatic cancer cells display a “hypermetabolic state” characterized by enhanced glycolysis and serine-glycine-one carbon metabolism.<sup>18</sup> Another preclinical study published by Shackelford et al.<sup>11</sup> has shown that the biguanide phenformin produces meaningful in vitro and in vivo antitumor effects in *KRAS/LKB1* co-mutated NSCLC. In particular, the authors of this paper suggested that *LKB1* inactivation makes NSCLC cells unable to halt energy and anabolite-consuming processes even in conditions of metabolic stress caused by phenformin, while constitutively active *KRAS* forces cells to duplicate their DNA and other intracellular structures, thus accelerating energy depletion, damage to intracellular components, and induction of cell apoptosis.

In this study, we hypothesized that the metabolic frailty of *KRAS/LKB1* co-mutated NSCLC cells could be exploited pharmacologically by combining drugs that affect cell metabolism with compounds that increase intracellular stress by interfering with DNA replication and repair, such as platinum compounds. To induce metabolic stress, we used the antidiabetic drug metformin, which has revealed interesting antitumor activity in both in vitro and in vivo preclinical studies. Metformin has been reported to be active against ChT-resistant cancer stem cells (CSCs) in different cancer types.<sup>19,20</sup> Moreover, metformin use in diabetic patients has been associated with reduced risk of several cancers, as well as a better prognosis in patients with advanced malignancies, including NSCLC. The proposed direct anti-cancer mechanism of metformin likely relies on the inhibition of complex I of the electron transport chain, with the consequent reduction of intracellular adenosine triphosphate levels and the induction of an energetic crisis in tumor cells.<sup>21,22</sup> However, the antitumor in vitro effects of metformin vary across different cancer types.<sup>23</sup>

By taking advantage of the outcome data obtained in the TAILOR trial, we retrospectively assessed frequency and prognostic value of *KRAS/LKB1* co-mutation in

advanced NSCLC. Moreover, we evaluated the efficacy of metformin to induce energetic crisis in NSCLC cell lines with different *KRAS/LKB1* mutational status. We generated stable *LKB1* knockdown and *LKB1* overexpressing isogenic H1299 and A549 cells variants, respectively, and tested the hypothesis that *KRAS/LKB1* co-mutated tumors are not able to counteract metformin-induced energy crisis, thus undergoing enhanced apoptosis after in vitro treatment. Finally, we exploited NSCLC patient-derived xenografts (PDXs), which closely mimic patients tumors, to investigate therapeutic activity of metformin plus cisplatin combination in the context of *LKB1/KRAS* co-mutated tumors.<sup>24</sup>

## Materials and Methods

### Clinical Data

TAILOR ([ClinicalTrials.gov](http://ClinicalTrials.gov), trial number NCT00637910) was a nonprofit multicenter, open label, randomized trial, funded by the Italian Regulatory Agency AIFA and conducted by 52 Italian hospitals. The clinical results of the TAILOR trial have been published.<sup>10</sup> Patients with *EGFR* wild-type (WT) and known *KRAS* mutational status progressing after platinum-based chemotherapy were randomized in a phase 3 trial to receive either docetaxel or erlotinib as their second-line therapy. The primary endpoint of the trial was overall survival (OS) after second-line treatment. Secondary endpoints included progression-free survival (PFS) and objective response rate. A pre-specified analysis was conducted also for the first-line outcomes.<sup>25</sup> The protocol was approved by the human investigational review board at each participating site, and voluntary written informed consent was obtained from each patient. All experiments were performed in accordance with the Declaration of Helsinki. The remaining fractions of tissue samples collected at patient enrollment in the TAILOR study were used for the present one.

### Statistical Analysis

Only *KRAS*-mutated tumors were considered for the present analysis. The chi-squared test was used to analyze the associations between *LKB1* status and different clinical variables. PFS was defined as the time from the randomization up to the date of progression or death from any cause, whichever came first. OS was defined as the time from randomization up to the date of death from any cause. Subjects who had not progressed or died while on study were censored at the last disease assessment date. Survival curves were estimated with the Kaplan-Meier method, and statistical significance between survival curves was assessed through the log-rank test. Cox proportional hazards models were used to assess at univariate and multivariable (adjusted for

Eastern Cooperative Oncology Group performance status [ECOG-PS], sex, histotype, smoking history, and treatment arm) analysis the association of specific mutational profiles with patient PFS and OS. Results were expressed as hazard ratios (HRs) and their corresponding 95% confidence intervals (95% CIs). All statistical tests were two-sided and a  $p < 0.05$  was considered statistically significant. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, North Carolina).

### Cell Lines and Treatments

The human NSCLC cell lines A549 (*KRAS*<sup>G12S</sup>/*LKB1*<sup>del</sup>) and H1299 (*KRAS*<sup>WT</sup>/*LKB1*<sup>WT</sup>) were obtained from the American Type Culture Collection. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Lonza, Basel, Switzerland), supplemented with 10% (volume/volume) fetal bovine serum (Lonza) and a solution of 100 U/mL penicillin and 100 U/mL streptomycin (Lonza). For cellular assays see [Supplementary Material and Methods](#)

### Patient-Derived Xenograft Treatments

Patient-derived xenografts (PDXs) were established as previously described.<sup>26</sup> The experimental protocol was approved by the C.E.S.A. (Ethical Committee for Animal Experimentation, of the National Cancer Institute Foundation), and animal experimentation was performed following guidelines drawn-up by C.E.S.A. according to the guidelines for the welfare and use of animals in cancer research.<sup>27</sup> PDXs were implanted, starting from a previously established NSCLC PDXs platform in 5-week-old severe combined immune deficiency (SCID) mice.<sup>24</sup> Groups of four mice, bearing a PDX sample in each flank for a total of  $n = 8$  tumors per group were treated once a week for 3 weeks with intraperitoneal injection of 5 mg/kg cisplatin (Teva, Petah Tikva, Israel) and/or daily with 100 mg/kg or 800 mg/kg metformin (per gavage, 2 doses of 50 mg/kg or 400 mg/kg every 10 to 14 hours). Tumor growth was followed by caliper once a week and results were analyzed using GraphPad Prism software (La Jolla, California). Response rate was calculated as previously described.<sup>24</sup> Mice were weighed twice a week and no weight loss was observed ([Supplementary Fig. 2](#)). Serial transplantation experiment was performed as previously reported.<sup>28</sup>

## Results

### *LKB1* Mutations as Prognostic Factors

To investigate if *KRAS/LKB1* co-mutated tumors represent a distinct subgroup of NSCLC endowed with higher clinical aggressiveness, we retrospectively analyzed data from 222 patients enrolled in the TAILOR trial between October 2007 and March 2012. Of these,

134 tumor tissue specimens were available for further molecular analyses (Supplementary Fig. 3). The main characteristics of our cohort of patients are summarized in Table 1. In particular, 47 patients (35.1%) had *KRAS*-mutated tumors, whereas *LKB1* mutations were found in 21 (15.7%) patients (Supplementary Tables 1 and 2).

Thirteen patients (9.7% of all patients and 27.7% of *KRAS*-mutated patients) harbored mutations in both *KRAS* and *LKB1* genes. Among different variables explored, we only found a positive association between double *KRAS/LKB1* co-mutations and low-grade tumors ( $p = 0.004$ ) (Table 1). At a median follow-up of 63 months, 46 patients had progressed (44 died and 2 experienced disease progression without death). Among patients with *KRAS*-mutated tumors, median PFS for *LKB1*-mutated and *LKB1*-WT patients was 2.7 and 3.4 months, respectively (Fig. 1A). *LKB1* status was nonsignificantly associated with PFS at both univariable analysis (HR: 1.62, 95% CI: 0.82–3.19,  $p = 0.163$ ) and multivariate analysis, which adjusted for ECOG-PS, sex, histotype, smoking history, and treatment arm (HR: 1.77, 95% CI: 0.85–3.68,  $p = 0.125$ ) (Table 2). Differences in median OS were more discernible: 4.4 and 10.5 months for *KRAS/LKB1* co-mutated and *KRAS*-mutated *LKB1*-WT patients, respectively (Fig. 1B). As in the case of PFS, the association between *LKB1* status and patient OS was non-statistically significant at both univariable and multivariable analysis (HR: 1.78, 95% CI: 0.90–3.52,  $p = 0.095$ ; HR: 2.02, 95% CI: 0.94–4.35,  $p = 0.072$  for univariate and multivariate analysis, respectively) (Table 2). Kaplan Meier curves for PFS and OS are depicted in Figures 1A and B, respectively.

### Effects of Metformin Treatment in NSCLC Cell Lines

Having established that *LKB1/KRAS* co-mutation has no significant prognostic value in advanced NSCLC patients, we investigated the ability of metformin to selectively target *LKB1/KRAS* co-mutated NSCLC cells in vitro. To this aim, we treated A549 (a model of *LKB1/KRAS* co-mutated NSCLC) and H1299 (a model of *LKB1*<sup>WT</sup>/*KRAS*<sup>WT</sup> NSCLC) cells with increasing metformin concentrations and assessed cell growth rate. Metformin inhibited growth to a similar extent in both cell lines, irrespective of the mutational status of *KRAS* and *LKB1*. At high metformin concentration (250 mmol/L), growth was almost completely inhibited, and the lowest concentration that showed a partially inhibitory effect was 50 mmol/L (Fig. 2A). Metformin also reduced mitochondrial membrane potential in both cell lines (Supplementary Fig. 4A). We then investigated if metformin induced differential pro-apoptotic effects depending on *KRAS* and *LKB1* mutational status. To do so, we determined the percentage of annexin V positivity

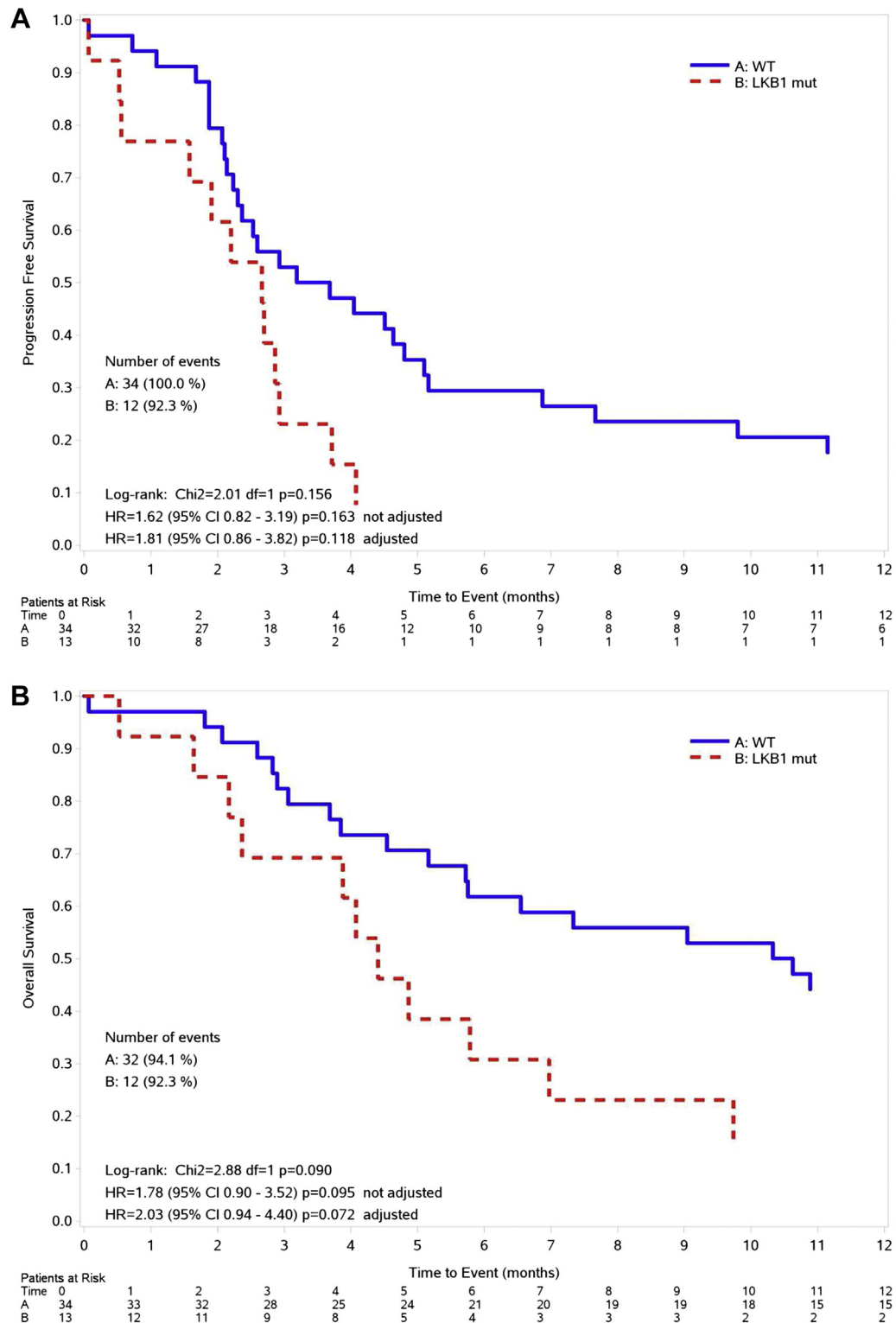
Table 1. Patient Characteristics

	<i>KRAS</i> mut/ <i>LKB1</i> wt n = 34	<i>KRAS</i> mut/ <i>LKB1</i> mut n = 13	Overall N = 47
<b>Age (y)</b>			
Mean (SD)	63.5 (7.6)	64.1 (8.6)	63.6 (7.8)
Median (Q1 - Q3)	63.9 (59.0- 68.5)	66.9 (58.2- 70.7)	64.0 (58.2- 70.1)
Min - Max	48.0 - 78.5	50.2 - 76.4	48.0 - 78.5
<b>Sex</b>			
Male	24 (70.6)	9 (69.2)	33 (70.2)
Female	10 (29.4)	4 (30.8)	14 (29.8)
<b>Performance status</b>			
0/1	32 (94.1)	13 (100)	45 (95.7)
2	2 (5.9)	0 (0.0)	2 (4.3)
<b>Smoking history</b>			
Never smoker	4 (11.8)	2 (15.4)	6 (12.8)
Former smoker	18 (52.9)	6 (46.2)	24 (51.1)
Current smoker	12 (35.3)	5 (38.5)	17 (36.2)
<b>Stage at diagnosis</b>			
I - IIIA	14 (41.2)	3 (23.1)	17 (36.2)
IIIB/IV	20 (58.8)	10 (76.9)	30 (63.8)
<b>Stage at randomization</b>			
IIIA-IIIB	5 (14.7)	1 (7.7)	6 (12.8)
IV	29 (85.3)	12 (92.3)	41 (87.2)
<b>Grade</b>			
G1	0 (0.0)	3 (33.3)	3 (8.8)
G2	9 (36.0)	2 (22.2)	11 (32.4)
G3	16 (64.0)	4 (44.4)	20 (58.8)
Missing	9	4	13
<b>Histology</b>			
Adenocarcinoma	29 (85.3)	12 (92.3)	41 (87.2)
Squamous + NOS	3 (8.8)	1 (7.7)	4 (8.5)
Other	2 (5.9)	0 (0.0)	2 (4.3)
<b>First-line treatment</b>			
pem/cis	11 (32.4)	6 (46.2)	17 (36.2)
gem/cis	6 (17.6)	4 (30.8)	10 (21.3)
nav/cis	6 (17.6)	1 (7.7)	7 (14.9)
gem/carbo	3 (8.8)	1 (7.7)	4 (8.5)
pem/carbo	3 (8.8)	0 (0.0)	3 (6.4)
nav/carbo	2 (5.9)	0 (0.0)	2 (4.3)
cis/gem/bev	1 (2.9)	0 (0.0)	1 (2.1)
nav	1 (2.9)	0 (0.0)	1 (2.1)
Other	1 (2.9)	0 (0.0)	1 (2.1)
Unknown	0 (0.0)	1 (7.7)	1 (2.1)
<b>Randomization arm</b>			
docetaxel	16 (47.1)	10 (76.9)	26 (55.3)
erlotinib	18 (52.9)	3 (23.1)	21 (44.7)

Values are presented as n (%) unless otherwise indicated.

G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; NOS, not otherwise specified; pem, pemetrexed; gem, gemcitabine; nav, navelbine; cis, cisplatin; carbo, carboplatin; bev, bevacizumab; SD, standard deviation; Q1, first quartile; Q3, third quartile; Min, minimum; Max, maximum; wt, wild type; mut, mutation.

in untreated and in metformin-treated cells. Interestingly, 50 mmol/L metformin caused higher apoptosis rates in A549 cells (10-fold increase in metformin-treated cells compared to control cells) than in H1299



**Figure 1.** Kaplan Meier curves for progression-free survival (A) and overall survival (B) in KRAS<sup>MUT</sup>/LKB1<sup>WT</sup> versus KRAS<sup>MUT</sup>/LKB1<sup>MUT</sup> patients enrolled within the TAILOR trial. Hazard ratio: univariate (unadjusted) and multivariate (adjusted for ECOG-PS, sex, histotype, smoking history and treatment arm). WT, wild-type; LKB1, liver kinase B1; HR, hazard ratio; Chi2, chi squared; CI, confidence interval; ECOG-PS, Eastern Cooperative Oncology Group performance status.

cells (7.6-fold increase) (Fig. 2B), whereas cell cycle arrest in the G1 phase was only induced in H1299 cells (Supplementary Fig. 4B). This difference in the apoptotic

rate among the two cell lines was further exacerbated with a metformin concentration of 250 mmol/L (46.2-fold increase and 18.5-fold increase in A549 and

**Table 2.** Cox Models – Multivariate Analysis for PFS and OS

	PFS			OS		
	HR	95% CI	p Value	HR	95% CI	p Value
LKB1 (mut vs. WT) <sup>a</sup>	1.81	0.86-3.82	0.118	2.03	0.94-4.40	0.072
Sex (female vs. male)	1.17	0.56-2.48	0.674	0.61	0.28-1.31	0.206
Smoking habits (Current smokers vs. never/former)	1.93	0.94-3.94	0.072	2.82	1.32-6.06	0.008
Performance status (2 vs. 0/1)	4.71	0.62-35.57	0.133	4.54	0.45-45.69	0.199
Histology (reference adenocarcinoma)						
Squamous	0.64	0.15-2.76	0.547	0.45	0.07-3.01	0.413
Other	3.03	0.60-15.26	0.179	0.87	0.18-4.21	0.857
Randomization arm (docetaxel vs. erlotinib)	0.73	0.36-1.47	0.377	0.89	0.43-1.84	0.754
Stage at random (IV vs. IIIA/IIIB)	0.85	0.31-2.32	0.751	0.94	0.37-2.42	0.899

<sup>a</sup>Adjusted on the initial stage.

PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; OS, overall survival; mut, mutation; WT, wild-type.

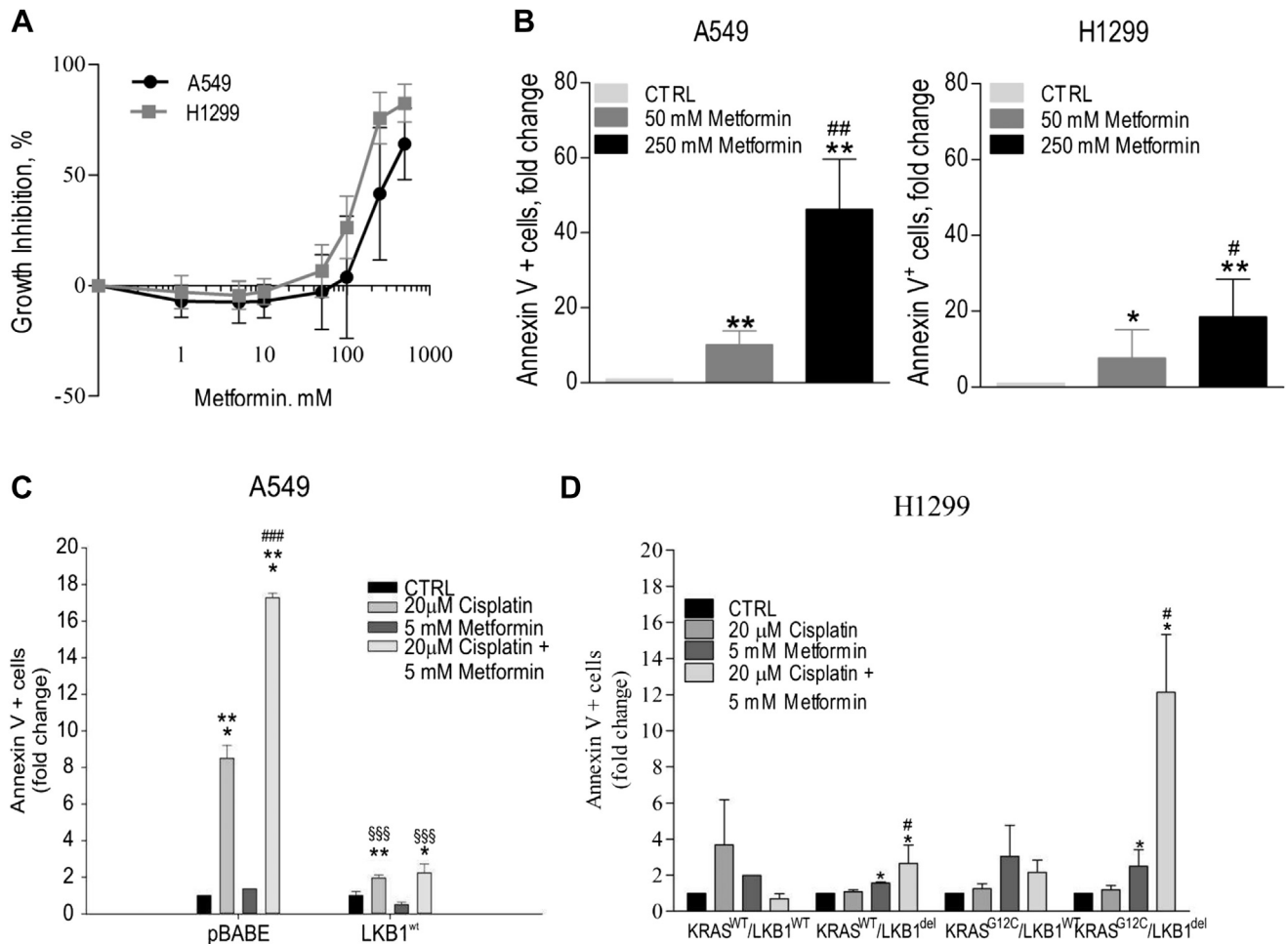
H1299, respectively) (Fig. 2B). To investigate if this differential sensitivity to metformin-induced apoptosis actually depends on the mutation status of *KRAS* and *LKB1* genes, we generated A549 *LKB1*<sup>WT</sup> cells and H1299 cells with different *KRAS* and *LKB1* status (*KRAS*<sup>WT</sup>/*LKB1*<sup>WT</sup>, *KRAS*<sup>G12C</sup>/*LKB1*<sup>WT</sup>, *KRAS*<sup>WT</sup>/*LKB1*<sup>del</sup>, or *KRAS*<sup>G12C</sup>/*LKB1*<sup>del</sup>). However, because metformin concentrations used in previous experiments are highly super-physiological and cannot be reached in vivo without causing potentially lethal toxicities, we explored lower metformin concentrations in combination with cisplatin, the reference compound for the first-line treatment of advanced NSCLC. In parental A549 cells, relatively low metformin concentration (5 mmol/L) significantly enhanced cisplatin-induced apoptosis (9-fold and 17-fold annexin V positivity increase compared to controls in cisplatin-treated and cisplatin plus metformin-treated cells, respectively), whereas this effect was absent in A549 *LKB1*<sup>WT</sup> cells (Fig. 2C). Similar results were obtained with H1299 isogenic cell lines, where 5 mmol/L metformin enhanced cisplatin-induced apoptosis specifically in double mutated H1299-*KRAS*<sup>G12C</sup>/*LKB1*<sup>del</sup> cells (1.2-fold and 12.1-fold annexin V positivity increase compared to controls in cisplatin treated and cisplatin and metformin treated cells, respectively). Noticeably, A549 *LKB1*<sup>WT</sup> cells showed a fold change in annexin V positive cells similar to that observed in H1299<sup>WT/WT</sup> cells (2- and 1.2-fold increase, respectively) and A549 cells showed a fold change comparable to that of H1299<sup>G12C/del</sup> cells (17- and 12.1-fold increase, respectively) (Fig. 2D) when treated with the combination of cisplatin and metformin. Moreover, metformin was able to counteract cisplatin-induced increase of CD133<sup>+</sup> CSCs in both A549 and H1299 cell lines (48% and 44% average prevention of cisplatin-induced enrichment, respectively (Supplementary Fig. 4C).

Altogether, these data indicate a role for functional *LKB1* in preventing metformin-induced apoptosis and enhancement of cisplatin cytotoxic effects, especially in a *KRAS*-mutated background.

### Role of *LKB1* in the In Vivo Response to Cisplatin and Metformin Cotreatment in NSCLC PDXs

To assess the in vivo effects of *LKB1* inactivation on metformin-induced apoptosis in *KRAS*-mutated NSCLC, we treated the following two PDXs models: PDX111 (*KRAS*<sup>G12V</sup>/*LKB1*<sup>WT</sup>) and PDX73 (*KRAS*<sup>G12V</sup>/*LKB1*<sup>K287X</sup>). Low metformin dosages (100 mg/kg daily) partially inhibited tumor growth in *LKB1*<sup>WT</sup> PDX111 (50.5 ± 14.8% maximal growth inhibition [MGI]), but not in *LKB1*-mutated PDX73 (MGI of 21.8 ± 19.9%) (Fig. 3A). No apoptosis induction was appreciable in PDX111 tumors, whereas metformin increased the levels of cleaved caspase 3 in the *LKB1*/*KRAS* co-mutated PDX73 model (Fig. 3B) despite the lack of meaningful tumor shrinkage. Moreover, loss of *LKB1* in PDX73 was associated with increased tumor necrosis following metformin treatment, suggesting an impaired capacity of *LKB1*-deficient tumor to adapt to the metabolic stress caused by biguanide therapy (Fig. 3B).

When we increased metformin dosage to 800 mg/kg daily, tumor growth was inhibited comparably to the 100 mg/kg dosage in PDX111 (49.0 ± 25.5% MGI), whereas dose-dependent decrease in tumor volume was appreciable in PDX73 (41.1 ± 11.7% MGI) (Fig. 3C). Increasing metformin dosages was associated with progressive and dose-dependent decrease in Ki67 expression in PDX111, and a dramatic increase in cleaved caspase 3 positivity in PDX73 model (Fig. 3B). Moreover, a more intense expression of pAMPK in PDX111 compared with PDX73 tumors after 800 mg/kg metformin treatment was appreciable (Supplementary Fig. 5), suggesting the involvement of AMPK in the



**Figure 2.** Effects of metformin on A549 (*KRAS*<sup>G12S</sup>/*LKB1*<sup>del</sup>) and H1299 (*KRAS*<sup>WT</sup>/*LKB1*<sup>WT</sup>) NSCLC cell line. (A) Dose-effect curve shows that both cell lines are affected by biguanide treatment in a similar way in terms of number of live cells after 24 hours of treatment. (B) Annexin V staining shows that 50 mmol/L metformin treatment for 24 hours induces a 10-fold increase and 7.6-fold increase of apoptosis induction in A549 and H1299 cells, respectively. These differences are exacerbated with 250 mmol/L metformin treatment for 24 hours where the biguanide induces a 46.2-fold increase and 18.5-fold increase in A549 and H1299 cells, respectively. \**p* < 0.05; \*\**p* < 0.01 compared to controls; #*p* < 0.05; ##*p* < 0.01 compared to 50 mM metformin. (C) Metformin increases apoptosis induced by cisplatin in A549 transfected with control plasmid (pBABE) but not in A549 cells with ectopic expression of wild-type LKB1 (*LKB1*<sup>WT</sup>). \**p* < 0.05; \*\**p* < 0.01; compared to untreated control. \$\$\$*p* < 0.001 *LKB1*<sup>WT</sup> compared to pBABE cells. ###*p* < 0.001 cisplatin-treated compared to cisplatin and metformin treated. (D) Metformin increases apoptosis induced by cisplatin treatment only in isogenic H1299 *KRAS*<sup>G12C</sup>/*LKB1*<sup>del</sup> cell line. \**p* < 0.05; compared to control cells #*p* < 0.01; cisplatin-treated compared to cisplatin-and-metformin treated cells. pBABE, control plasmid; LKB1, liver kinase B1.

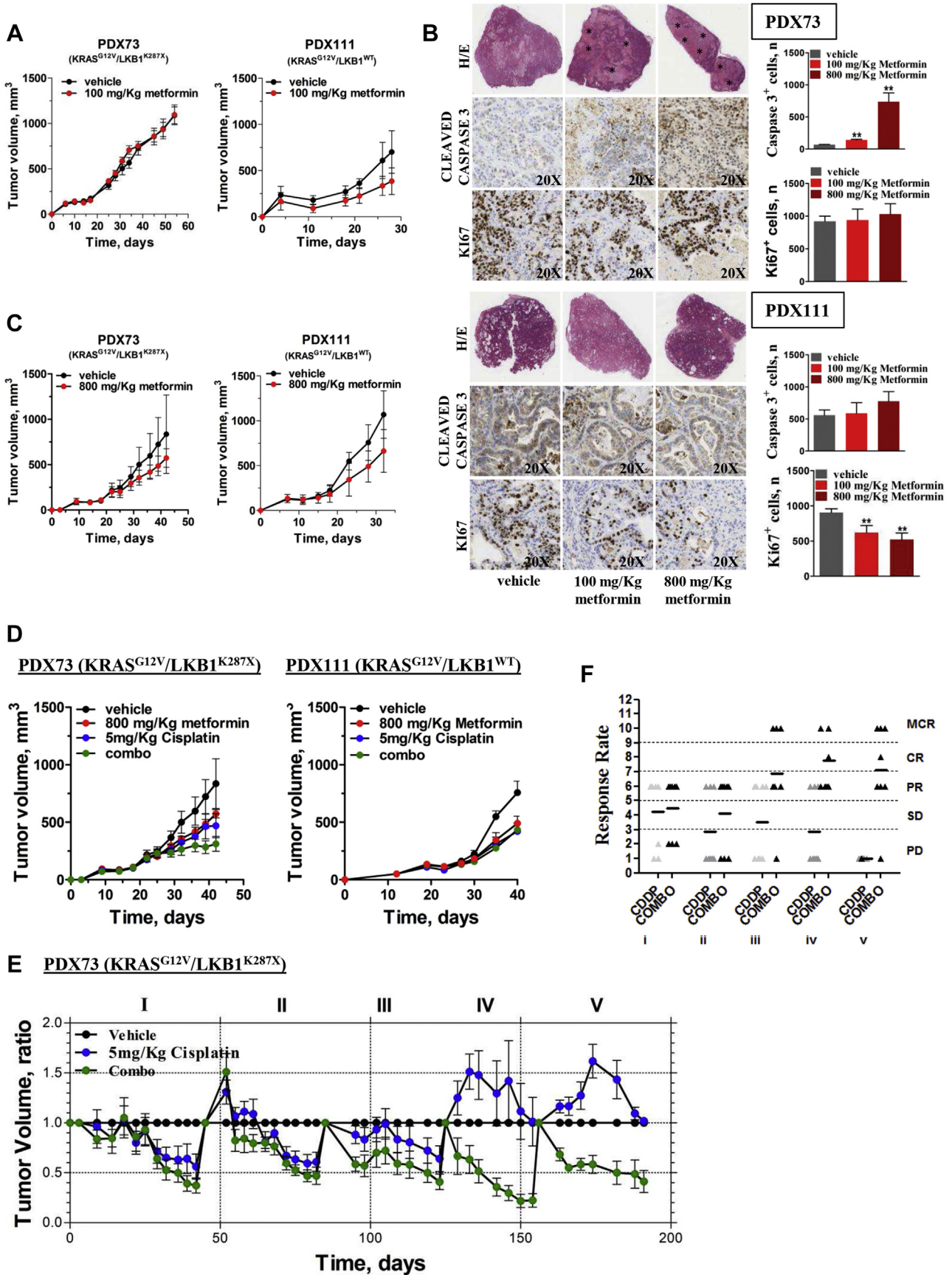
response of tumors to the biguanide. Finally, metformin synergized with cisplatin to reduce tumor size specifically in the PDX73 model (Fig. 3D and Supplementary Table 3), thus recapitulating results obtained with in vitro experiments.

Altogether, these data suggest that metformin differentially affects NSCLC cell growth and survival in a way that depends on *LKB1* and *KRAS* mutational status: in particular, high-dose metformin halts tumor growth by inhibiting cancer cell proliferation in *KRAS*-mutated *LKB1*-WT NSCLC, while it promotes apoptosis of *LKB1*/*KRAS* co-mutated NSCLC cells. Moreover, *LKB1*/*KRAS*

co-mutated tumors are specifically sensitive to the cisplatin-metformin combination.

### Serial Transplantation Experiments

Primary or acquired resistance to cytotoxic ChT is a common and apparently insurmountable problem for patients with advanced NSCLC. To investigate if metformin affects tumor sensitivity throughout subsequent cycles of cisplatin treatment, we performed a serial transplantation experiment. After five serial transplantations and 5 cycles of cisplatin treatment, PDX73 (*LKB1*<sup>mut</sup>/*KRAS*<sup>mut</sup>) became almost completely resistant





to ChT. On the contrary, 5 cycles of metformin and cisplatin combination produced long-lasting effects in terms of tumor volume shrinkage (Fig. 3E and Supplementary Table 4). The response rate of tumors treated with cisplatin progressively decreased after each serial transplantation cycle (50% of cases underwent progression of disease (PD) and 50% partial response at first cycle, whereas 100% of tumors showed PD at the fifth cycle). Conversely, the metformin-cisplatin combination progressively increased tumor response rate across subsequent treatment cycles (50% PD and 50% partial response at first cycle versus 12.5% PD and 37.5% PR; 12.5% complete response and 37.5% maintained complete response at fifth cycle) (Fig. 3F).

These data suggest that metformin is able not only to acutely boost the anticancer effects of cisplatin, but also to progressively reduce the occurrence of chemoresistance in a *KRAS/LKB1* co-mutated preclinical model.

### CSC Involvement in the In Vivo Response to Metformin

Based on our in vitro findings in A549 and H1299 cells, we hypothesized that metformin synergizes with cisplatin to kill the population of chemoresistant CSCs, which can repopulate the tumor by progressively increasing the number of chemoresistant cells. To test this hypothesis, we analyzed the content of CD133<sup>+</sup> cells in PDX73 *LKB1/KRAS* co-mutated tumors during serial transplantation experiments. Fluorescence-activated cell sorting analysis of tumors after the first cycle of treatment showed enrichment of CD133<sup>+</sup> cells in cisplatin-treated as compared to vehicle-treated tumors. This enrichment was partially reverted by metformin-cisplatin (MC) combination (0.94%, 1.95%, and 1.25% CD133<sup>+</sup> cells in vehicle-, cisplatin-, and MC-treated tumors, respectively). CSC enrichment in cisplatin-treated tumors, as well as CSC decrease in MC-treated ones, was also observed after the second treatment cycle

(0.84%, 2.22%, and 0.47% CD133<sup>+</sup> cells in vehicle-, cisplatin-, and MC-treated tumors, respectively). After four treatment cycles, that is, when tumors treated with cisplatin alone had become almost completely cisplatin-resistant, we no longer detected any CD133<sup>+</sup> enrichment in cisplatin-treated tumors, whereas a decrease in CD133<sup>+</sup> CSCs was still appreciable in MC-treated ones (0.91%, 0.78%, and 0.38% CD133<sup>+</sup> cells in vehicle-, cisplatin-, and MC-treated tumors, respectively) (Figs. 4A and B). Moreover, the time needed by tumors to reach the target volume of 150 mm<sup>3</sup> (lag time) progressively increased in tumors treated with the MC combination compared to the lag time of vehicle- or cisplatin-treated tumors (Fig. 4C). A similar effect of metformin in reducing CSCs was also appreciable in *LKB1*<sup>WT</sup> PDX111, although it was less pronounced (33.7% decrease of the 2,2-fold cisplatin-induced CSCs enrichment and 69.3% decrease of the 2.1-fold CSCs enrichment observed in PDX111 and PDX73, respectively) (Fig. 4D).

Altogether, these data suggest that the MC combination can specifically target CD133<sup>+</sup> cells in *KRAS/LKB1* co-mutated NSCLC PDXs, thus preventing or delaying resistance to cisplatin.

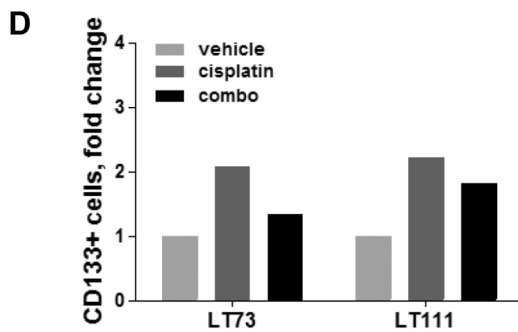
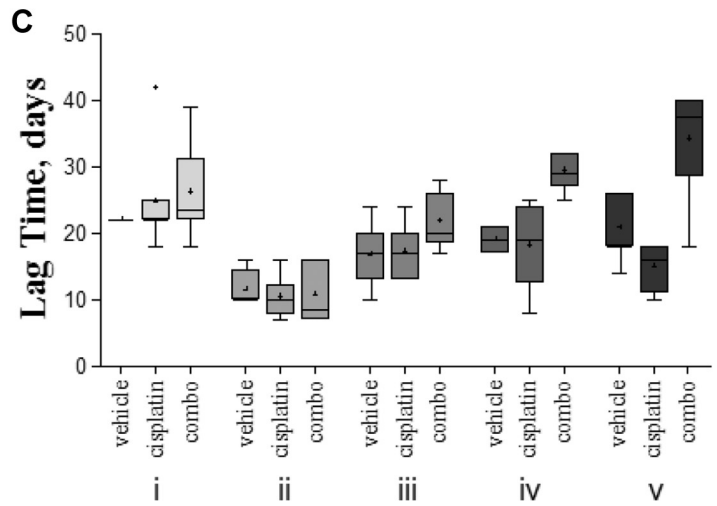
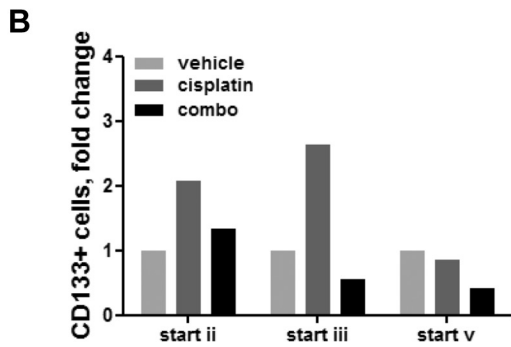
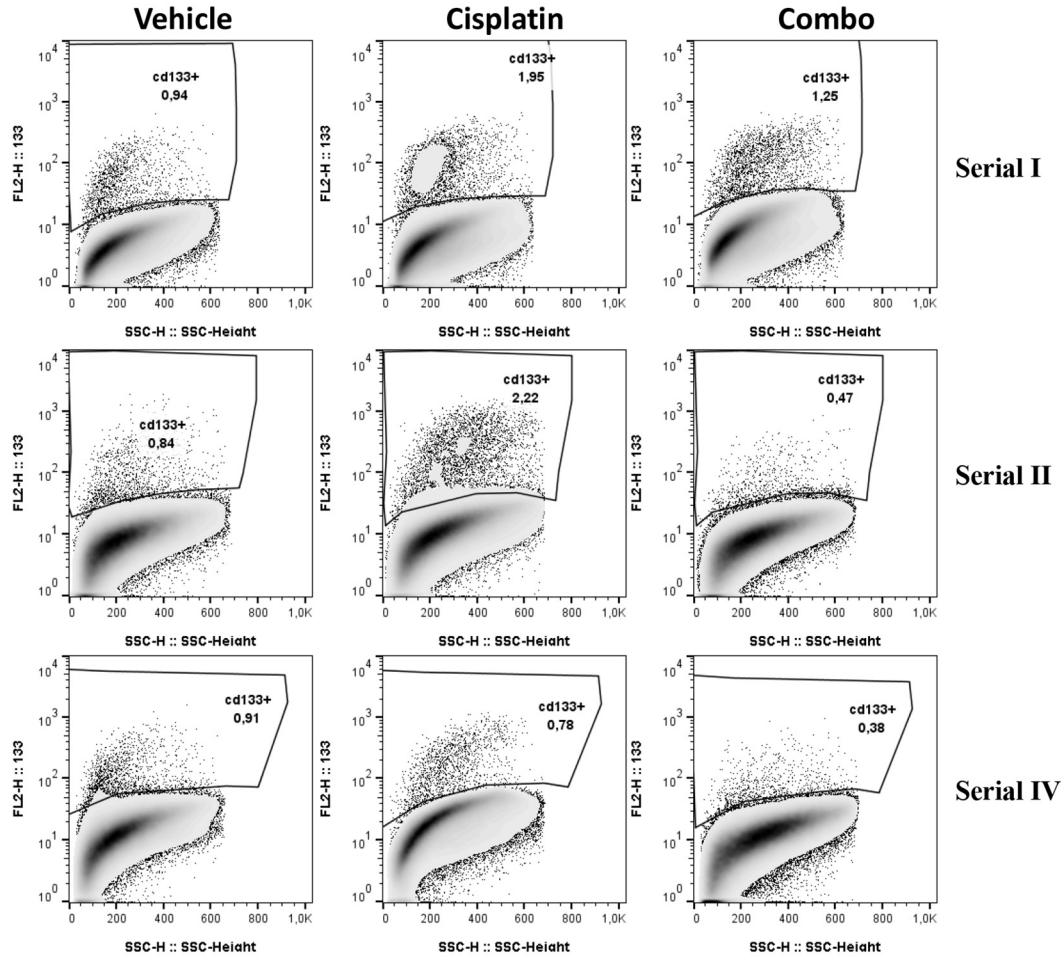
### Discussion

*KRAS* oncogenic mutations are found in approximately 20% to 25% of NSCLCs, and are implicated in the stimulation of cancer cell growth, proliferation, and metabolic reprogramming.<sup>5,29</sup> However, despite decades of intense research, no truly active therapies to target constitutively active *KRAS* have been found yet.<sup>6,7</sup> The prognostic role of *KRAS* mutations remains debatable, and the different genetic variants described so far are not clearly associated with specific clinical behaviors.<sup>8-10,30,31</sup>

Our working hypothesis is that genetic alterations co-occurring with *KRAS* activating mutations may define NSCLC subgroups characterized by different prognosis

**Figure 3.** In vivo treatments of PDX111 (*KRAS*<sup>G12V</sup>/*LKB1*<sup>WT</sup>) and PDX73 (*KRAS*<sup>G12V</sup>/*LKB1*<sup>K287X</sup>). (A) Metformin induces a slight decrease in tumor growth at 100 mg/kg daily dosage in PDX111, and has no effects on tumor growth at 100 mg/kg daily dosage in PDX73. (B) Immunohistochemistry analysis cleaved caspase 3 and Ki-67 expression of treated PDXs. Cleaved caspase 3 expression in PDX111 remains stable upon metformin treatments both at 100 mg/kg (average of 600 positive cells/quadrant) and at 800 mg/kg (750 positive cell/quadrant). On the contrary, Ki-67 expression gradually decreases with increasing metformin doses (920, 615, 550 positive cells/quadrant in untreated, 100 mg/kg and 800 mg/kg metformin-treated tumors, respectively). In the *KRAS/LKB1* co-mutated PDX73, metformin induces an increase of apoptosis, as shown by a gradual increase in cleaved caspase 3 with increasing metformin dosage (80, 150, 750 positive cells/quadrant in untreated, 100 mg/kg and 800 mg/kg metformin-treated tumors, respectively); whereas Ki-67 expression remains at levels comparable with those of untreated tumors (950, 950, 1000 positive cells/quadrant in untreated, 100 mg/kg and 800 mg/kg metformin-treated tumors, respectively). Hematoxylin-eosin staining shows necrotic areas (highlighted with an asterisk [\*]) only in double-mutated PDX73 upon metformin treatment. (C) Increasing metformin dosage to 800 mg/kg daily, a dose-dependent effect of biguanide treatment on PDX growth is appreciable only in *KRAS/LKB1* co-mutated PDX73. (D) Metformin treatment (800 mg/kg) ameliorates cisplatin effects only in *KRAS/LKB1* co-mutated PDX73, whereas no differences between metformin alone, cisplatin alone, or a combination of the two are appreciable in *LKB1* wild-type PDX111. (E) Serial transplantation and serial treatment cycles of cisplatin or cisplatin and metformin combination in *KRAS/LKB1* co-mutated PDX73. Cisplatin-treated tumors progressively become resistant to treatments, whereas effects of the combination of cisplatin and metformin last for 5 cycles with a progressive increase in the response rate (F). *LKB1*, liver kinase B1.

**A** PDX73 (KRAS<sup>G12V</sup>/LKB1<sup>K287X</sup>)



and/or response to specific treatments, possibly opening up new possibilities of therapies targeted to specific populations of *KRAS*-mutated NSCLC patients. For instance, mutations in *LKB1*, *p53*, and *CDK4/6* co-occur with *KRAS* mutations in a clinically meaningful percentage of NSCLCs, and a negative prognostic role of *KRAS/LKB1* co-mutations in NSCLC has been recently reported.<sup>13-15</sup> Consistently with these findings, we found that NSCLC patients with tumors bearing *KRAS/LKB1* co-mutations, and receiving second-line treatment within the TAILOR trial, had a trend towards poorer PFS and OS when compared to patients with *KRAS*-only mutated tumors.<sup>10</sup> Based on our results, *LKB1* mutations may define a more aggressive subset of *KRAS*-mutated NSCLC, as previously reported.<sup>32,33</sup> Higher aggressiveness of *LKB1*-mutated tumors could result from the loss of *LKB1* oncosuppressive function, which is involved in regulating cancer cell growth and proliferation on the basis of nutrient availability.<sup>17</sup> *LKB1* loss, as caused by inactivating mutations or deletions of the *LKB1* gene, could make cancer cells unresponsive to nutrient starvation and energetic stress, thus accelerating their growth even when nutrients (e.g., glucose, amino acid) are scarce. When combined with activating mutations in oncogenes, such as *KRAS*, which stimulates unrestrained cell growth, proliferation and energy-requiring anabolic processes, *LKB1* inactivation may confer a particularly aggressive clinical phenotype associated with reduced patient survival.

In our preclinical experiments, we tested the hypothesis that *LKB1* mutations, which are associated with aggressive growth phenotype in the absence of an effective anticancer treatment, may confer metabolic frailty to tumors with *KRAS*-induced deregulation of cell growth and proliferation. We showed that supra-physiological dosages of metformin, which causes energetic stress in cancer cells by interfering with mitochondrial activity, selectively induce apoptosis in *KRAS/LKB1* co-mutated models (A549 cells and PDX73), but not in *KRAS*<sup>WT</sup>/*LKB1*<sup>WT</sup> cells (H1299) or in the *KRAS*<sup>MUT</sup>/*LKB1*<sup>WT</sup> PDX (PDX111), where the activation of the *LKB1*/AMPK signaling pathway reduces cell proliferation, thus reducing metabolic requirements and preventing metabolic crisis in cancer cells.<sup>21</sup> This result was not surprising because recently published data have shown a meaningful pro-apoptotic effect of the

biguanide phenformin, a more potent analog of metformin, specifically in *KRAS/LKB1* co-mutated NSCLC cells.<sup>11</sup> However, both in Shackelford et al.<sup>11</sup> and in our study, the biguanide dosages used are by far too high to be reached in the plasma of cancer patients.

In the perspective of future clinical applications, our main finding is that metformin is capable of enhancing cisplatin-induced *in vitro* pro-apoptotic and *in vivo* antitumor effects specifically in *KRAS/LKB1* co-mutated tumors. Moreover, metformin prevented secondary resistance to cisplatin and actually progressively enhanced cisplatin-induced antitumor effects specifically against *KRAS*<sup>G12V</sup>/*LKB1*<sup>K287X</sup> NSCLC *in vivo* models.

The possibility that the safe and low-cost drug metformin prevents or substantially delays the onset of secondary resistance to platinum-based ChT is highly relevant from a clinical point of view and is worth being explored in the clinical setting. In the clinical setting, cisplatin-based combinations (with pemetrexed or gemcitabine) are the standard first-line options to treat NSCLCs lacking specific oncogenic addiction or strong programmed death ligand 1 expression. Although in this study we did not explore the effects of metformin in combination with platinum-based doublet chemotherapy, our findings are significant because cisplatin represents the compounds with the most relevant anti-cancer activity, and is actually the backbone of these combinations. Based on our findings, metformin could prevent cisplatin-induced increase of CD133<sup>+</sup> cells, which we previously reported to represent a highly tumorigenic, cisplatin-resistant cell subpopulation enriched of cancer initiating cells with high disseminating potential.<sup>28,34</sup> These findings suggest a potential mechanism by which metformin could prevent acquired resistance to cisplatin in *KRAS/LKB1* co-mutated NSCLC.

A limitation of these *in vivo* experiments consists in the high metformin dosages used. Indeed, 800 mg/kg metformin/d approximately correspond to a daily dosage of 3891 mg for a 60-kg patient, whereas the maximum safe dose of metformin is 2550 mg/d in diabetic patients, and could be lower in combination with cisplatin in NSCLC patients.<sup>35</sup> Thus, to envisage a clinical application of metformin for cancer treatment, further studies will be needed to assess either efficacy of lower dosages or safety of short periods of higher dosage treatment. In one preclinical study, Morgillo et al.<sup>36</sup>

**Figure 4.** Fluorescence-activated cell sorting (FACS) analysis of tumor samples from *KRAS/LKB1* co-mutated PDX73 tumors treated with cisplatin or cisplatin and metformin during the serial transplantation experiment. (A, B) CD133<sup>+</sup> CSCs percentage increases at each passage in the cisplatin-treated tumors and this CSCs enrichment is counteracted in the cisplatin-and-metformin-treated tumors. In the last serial passage, when tumors became completely resistant to treatment, cisplatin effects are no more appreciable in terms of CSCs enrichment, whereas in the cisplatin-and-metformin-treated tumors a decrease of CSCs percentage is appreciable. (C) Tumors lag time (time tumors need to reach 150 mm<sup>3</sup> volume after implant) gradually increases in cisplatin-and-metformin-treated tumors compared to both controls and cisplatin-treated tumors. (D) A similar effect on counteracting cisplatin-induced CSCs enrichment is appreciable also in *KRAS*<sup>G12V</sup>/*LKB1*<sup>WT</sup> PDX111. *LKB1*, liver kinase B1.

showed that metformin sensitizes NSCLC cells to gefitinib independently from *EGFR* and *KRAS* mutational status, and recently published preliminary clinical data have shown acceptable tolerability and promising anti-cancer activity with the metformin-erlotinib combination in *EGFR*<sup>WT</sup> NSCLCs, which are typically poorly responsive to erlotinib monotherapy.<sup>37</sup> The synergistic anti-proliferative and pro-apoptotic effects of metformin and gefitinib are limited to NSCLC cells with active LKB1, whereas *LKB1*-mutated models do not benefit from this combination.<sup>36</sup> Although these data seem to contradict our findings of an exquisite sensitivity of *LKB1*-mutated cells to the MC combination, they could be reconciled by considering the different mechanism of action of gefitinib/erlotinib, which specifically inhibit EGFR, and cisplatin, which induces DNA intra-strand and inter-strand links that result in single- and double-strand DNA breaks. The combination of metformin and gefitinib/erlotinib could inhibit two crucial signal transduction pathways, namely the MAPK (via EGFR inhibition by gefitinib/erlotinib) and mTOR (via metformin-induced activation of AMPK, which requires the presence of active LKB1) cascade, thus resulting in synergistic antiproliferative and pro-apoptotic effects in *LKB1*<sup>WT</sup> NSCLC. Conversely, metformin-induced metabolic stress does not result in AMPK activation and mTOR inhibition when LKB1 is inactive (*LKB1*<sup>MUT</sup>). If *KRAS* mutations are contemporarily present, cells go on proliferating even in conditions of metformin-induced energetic and anabolic stress, which can expose them to increased cisplatin-induced DNA damage and less efficient DNA repair, an energy- and anabolite-requiring process.

In addition to the ongoing study on erlotinib-metformin combination in *EGFR* WT NSCLC, metformin is being tested in combination with first-line, platinum-based ChT in unselected NSCLC patient populations (NCT 00637910). On the basis of our findings, as well as of results from other groups, only specific NSCLC subgroups may benefit from adding metformin to standard ChT. We therefore expect that the ongoing studies could fail to show any improvement from adding metformin to cytotoxic ChT, and that careful patient selection on biomarker expression will be essential to optimize clinical results in future studies.

In conclusion, *LKB1* mutations may define more aggressive NSCLC subtypes even in the absence of other common oncogene mutations, such as *KRAS* mutations. However, the co-occurrence of *LKB1/KRAS* mutations could define a specific subgroup of NSCLCs characterized by exquisite sensitivity to the combination of metformin-induced metabolic stress and cisplatin-induced replication stress and DNA damage. In particular, the MC combination could specifically target the population of

CD133<sup>+</sup> cells that are involved in the development of secondary resistance to cisplatin. Despite some limitations of our study, including the relatively small number of patients analyzed and the high metformin dosage needed to produce meaningful biological effects in both in vitro and in vivo experiments, targeting *LKB1/KRAS* co-mutated tumors with the MC combination may represent a novel therapeutic strategy for the treatment of this aggressive subgroup of NSCLC. Based on excellent safety profile and low costs of metformin, we believe that our results provide sufficient evidence to encourage the conduction of clinical trials to assess the antitumor activity of cisplatin-pemetrexed-metformin triple therapy as first-line treatment of patients with *LKB1/KRAS* co-mutated NSCLCs who are not candidate to receive pembrolizumab.

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## Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at [www.jto.org](http://www.jto.org) and at <https://doi.org/10.1016/j.jtho.2018.07.102>

## References

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015;136:E359-E386.
2. Ettinger DS, Akerley W, Bepler G, et al. Non-small cell lung cancer. *J Natl Compr Canc Netw*. 2010;8:740-801.
3. Gerber DE, Gandhi L, Costa DB. Management and future directions in non-small cell lung cancer with known activating mutations. *Am Soc Clin Oncol Educ B*. 2014;15:353-365.
4. Garon EB, Rizvi NA, Hui R, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. *N Engl J Med*. 2015;372:2018-2028.
5. Herbst RS, Heymach JV, Lippman SM. Lung cancer. *N Engl J Med*. 2008;359:1367-1380.

6. Jänne PA, Shaw AT, Pereira JR, et al. Selumetinib plus docetaxel for KRAS-mutant advanced non-small-cell lung cancer: a randomised, multicentre, placebo-controlled, phase 2 study. *Lancet Oncol.* 2013;14:38-47.
7. Heavey S, O'Byrne KJ, Gately K. Strategies for co-targeting the PI3K/AKT/mTOR pathway in NSCLC. *Cancer Treat Rev.* 2014;40:445-456.
8. Meng D, Yuan M, Li X, et al. Prognostic value of K-RAS mutations in patients with non-small cell lung cancer: a systematic review with meta-analysis. *Lung Cancer.* 2013;81:1-10.
9. Cuffe S, Bourredjem A, Graziano S, et al. A pooled exploratory analysis of the effect of tumor size and KRAS mutations on survival benefit from adjuvant platinum-based chemotherapy in node-negative non-small cell lung cancer. *J Thorac Oncol.* 2012;7:963-972.
10. Garassino MC, Martelli O, Broggin M, et al. Erlotinib versus docetaxel as second-line treatment of patients with advanced non-small-cell lung cancer and wild-type EGFR tumours (TAILOR): a randomised controlled trial. *Lancet Oncol.* 2013;14:981-988.
11. Shackelford DB, Abt E, Gerken L, et al. LKB1 inactivation dictates therapeutic response of non-small cell lung cancer to the metabolism drug phenformin. *Cancer Cell.* 2013;23:143-158.
12. Konstantinidou G, Ramadori G, Torti F, et al. RHOA-FAK is a required signaling axis for the maintenance of KRAS-driven lung adenocarcinomas. *Cancer Discov.* 2013;3:444-457.
13. Calles A, Sholl LM, Rodig SJ, et al. Immunohistochemical Loss of LKB1 is a biomarker for more aggressive biology in KRAS-mutant lung adenocarcinoma. *Clin Cancer Res.* 2015;21:2851-2860.
14. Skoulidis F, Byers LA, Diao L, et al. Co-occurring genomic alterations define major subsets of KRAS-mutant lung adenocarcinoma with distinct biology, immune profiles, and therapeutic vulnerabilities. *Cancer Discov.* 2015;5:861-878.
15. Sanchez-Cespedes M, Parrella P, Esteller M, et al. Inactivation of LKB1 / STK11 is a common event in adenocarcinomas of the lung. *Cancer Res.* 2002;62:3659-3662.
16. Hardie DG, Alessi DR. LKB1 and AMPK and the cancer-metabolism link – ten years after. *BMC Biol.* 2013;11:1-11.
17. Garcia D, Shaw RJ. AMPK: Mechanisms of cellular energy sensing and restoration of metabolic balance. *Mol Cell.* 2017;66:789-800.
18. Kottakis F, Nicolay BN, Roumane A, et al. LKB1 loss links serine metabolism to DNA methylation and tumorigenesis. *Nature.* 2016;539:390-395.
19. Courtois S, Durán RV, Giraud J, et al. Metformin targets gastric cancer stem cells. *Eur J Cancer.* 2017;84:193-201.
20. Tudies S. REVIEW metformin and cancer. *Rev Diabet Stud.* 2013:228-235.
21. Owen MR, Doran E, Halestrap AP. Complex 1 of the mitochondrial respiratory chain. 2000;614:607-614.
22. El-Mir M, Nogueira V, Fontaine E, et al. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem.* 2000;275:223-228.
23. Hawley SA, Ross FA, Chevztzoff C, et al. Use of cells expressing  $\gamma$  subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab.* 2010;11:554-565.
24. Moro M, Bertolini G, Caserini R, et al. Establishment of patient derived xenografts as functional testing of lung cancer aggressiveness. *Sci Rep.* 2017;7:6689.
25. Rulli E, Marabese M, Torri V, et al. Value of KRAS as prognostic or predictive marker in NSCLC: results from the TAILOR trial. *Ann Oncol.* 2015;26:2079-2084.
26. Moro M, Bertolini G, Tortoreto M, Pastorino U, Sozzi G, Roz L. Patient-derived xenografts of non small cell lung cancer: resurgence of an old model for investigation of modern concepts of tailored therapy and cancer stem cells. *J Biomed Biotechnol.* 2012;2012:568567.
27. Workman P, Aboagye EO, Balkwill F, et al. Guidelines for the welfare and use of animals in cancer research. *Br J Cancer.* 2010;102:1555-1577.
28. Moro M, Bertolini G, Pastorino U, Roz L, Sozzi G. Combination treatment with all-trans retinoic acid prevents cisplatin-induced enrichment of CD133+ tumorigenic cells and reveals heterogeneity of cancer stem cell compartment in lung cancer. *J Thorac Oncol.* 2015;10:1027-1036.
29. Brunelli L, Caiola E, Marabese M, Broggin M, Pastorelli R. Comparative metabolomics profiling of isogenic KRAS wild type and mutant NSCLC cells in vitro and in vivo. *Sci Rep.* 2016;6:1-6.
30. Marabese M, Ganzinelli M, Garassino MC, et al. KRAS mutations affect prognosis of non-small-cell lung cancer patients treated with first-line platinum containing chemotherapy. *Oncotarget.* 2015;6:34014-34022.
31. Piva S, Ganzinelli M, Garassino MC, et al. Across the universe of K-RAS mutations in non-small-cell-lung cancer. *Curr Pharm Des.* 2014;20:3933-3943.
32. Shen Z, Wen XF, Lan F, Shen ZZ, Shao ZM. The tumor suppressor gene LKB1 is associated with prognosis in human breast carcinoma. *Clin Cancer Res.* 2002;8:2085-2090.
33. Bonanno L, De Paoli A, Zulato E, et al. LKB1 expression correlates with increased survival in patients with advanced non-small cell lung cancer treated with chemotherapy and bevacizumab. *Clin Cancer Res.* 2017;23:3316-3324.
34. Bertolini G, Roz L, Perego P, et al. Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proc Natl Acad Sci U S A.* 2009;106:16281-16286.
35. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J.* 2007;22:659-661.
36. Morgillo F, Sasso FC, Della Corte CM, et al. Synergistic effects of metformin treatment in combination with gefitinib, a selective EGFR tyrosine kinase inhibitor, in LKB1 wild-type NSCLC cell lines. *Clin Cancer Res.* 2013;19:3508-3519.
37. Morgillo F, Fasano M, Della Corte CM, et al. Results of the safety run-in part of the METAL (METformin in Advanced Lung cancer) study: a multicentre, open-label phase I-II study of metformin with erlotinib in second-line therapy of patients with stage IV non-small-cell lung cancer. *ESMO Open.* 2017;2:e000132.