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ORIGINAL ARTICLE

The pathway alteration load is a pan-cancer determinant of outcome of targeted therapies: results from the Drug Rediscovery Protocol (DRUP)

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Background: Many patients with cancer exhibit primary or rapid secondary resistance to targeted therapy (TT). We hypothesized that a higher number of altered oncogenic signaling pathways [pathway alteration load (PAL)] would reduce the benefit of TT which only intervenes in one pathway. This hypothesis was tested in the Drug Rediscovery Protocol (DRUP).

Patients and methods: DRUP is a prospective, pan-cancer, non-randomized clinical trial (NCT02925234) that treats patients with therapy-refractory metastatic cancer and an actionable molecular profile using matched off-label targeted and immunotherapies. All patients treated with TT with available clinical outcomes and whole genome sequencing were included. PAL was determined based on driver gene alterations and correlated with clinical benefit rate (CBR), progression-free survival (PFS) and overall survival (OS). Outcomes were validated in the independent Hartwig Medical database of metastatic cancers.

Results: In 154 patients treated with TT, the median PAL was 3. Patients with a PAL below median ($n = 60$) demonstrated a higher CBR (41.7% versus 25.5%, odds ratio 0.48, $P = 0.051$), longer PFS [median 4.7 versus 2.9 months, adjusted hazard ratio (aHR) 1.70, $P = 0.020$] and OS (median 13.7 versus 5.6 months, aHR 3.80, $P < 0.001$) compared with those with PAL $\geq$3. Two hundred and fifty-eight patients in the Hartwig database showed similar results for CBR (54.2% versus 36.7%, odds ratio 2.04, $P = 0.009$) and PFS (7.0 versus 4.2 months, aHR 1.55, $P = 0.009$).

Conclusions: In our population, PAL emerged as a pan-cancer determinant of outcome to TT. Our findings support refined patient selection for TT and highlight the rationale for combinatorial treatment strategies in patients with multiple affected pathways.

Key words: targeted therapy, oncogenic signaling pathways, metastatic cancer

INTRODUCTION

By specifically inhibiting molecular signaling mechanisms essential for tumorigenesis and proliferation, targeted therapies (TTs) have significantly improved response rates and survival across several solid tumor types.[1-4] However, still a considerable amount of patients who are molecularly eligible for targeted treatment do not benefit from this treatment modality, either by showing primary resistance or rapid progression after initial response, indicative of development of secondary resistance mechanisms.[5]

While TTs, like most anticancer drugs, are traditionally developed for a specific tumor type or histology, broad sequencing techniques increasingly demonstrate that the molecular alterations for which they were originally approved also occur outside the registered indication. In the Dutch Drug Rediscovery Protocol (DRUP), patients are treated with registered targeted and immunotherapies matched to their molecular profile, but outside of their label indication.[6] To date, ~1500 patients with therapy-refractory metastatic cancer have received treatment in the trial, with clinical benefit rates (CBRs), defined as objective response (OR) or stable disease (SD) >4 months,

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of 34% and 33% in the first 215 and 500 treated patients, respectively.[6,7] While these findings are significant in the context of last-line treatment, they also clearly highlight an unmet need in two-thirds of patients and stress the need to improve current practice.

The premise of precision medicine is optimal patient selection for (targeted) therapy to improve outcomes, reduce futile treatment and spare patients from unnecessary toxicity. This is especially important in late-stage disease where the trade-off between quality of life and the potential likelihood of benefit from a drug is paramount. Furthermore, considering the continuously increasing costs associated with targeted anticancer drugs, it is imperative to employ them responsibly. Therefore, to improve patient selection and optimize targeted treatment strategies, identifying biomarkers of early resistance to therapy is critical.

One of the mechanisms that has been suggested as a primary explanation of treatment ineffectiveness is the existence of parallel activated oncogenic signaling pathways that support tumor survival.[5,8] If one or more of such pathways remain untargeted, this may hamper the efficacy of TT that acts upon only one specific gene or pathway. Particularly in the setting of advanced, systemically pretreated cancers, genomic tumor evolution following therapeutic pressure may result in such parallel pathway activation.[9-12] Recent studies in non-small cell lung cancer (NSCLC) have demonstrated that the presence of concomitant driver alterations is negatively associated with responses to TT.[13-18] This could also explain the disappointing outcomes of many other studies with single-agent targeted treatment. However, although this hypothesis appears plausible, limited studies have been carried out to demonstrate its validity in a broader biologic and histologic context and explore its use as a potential biomarker. We hypothesized that clinical benefit (CB) of TT directed against a single pathway in DRUP is influenced by the number of altered oncogenic signaling pathways [pathway alteration load (PAL)]. Subsequently, we compared our key findings with those in a pan-cancer real-world dataset encompassing clinical outcomes to TT and whole genome sequencing (WGS) data from patients with metastatic disease.

PATIENTS AND METHODS

DRUP discovery cohort

DRUP is a Dutch, multicenter, prospective, non-randomized, pan-cancer platform trial that encompasses a basket and umbrella framework in which patients with advanced or metastatic solid tumors are treated with approved TTs or immune checkpoint blockade (ICB) outside their registered indications, based on their tumor molecular profile.[6] Eligible patients have exhausted all regular treatment options and are required to have measurable disease according to RECIST[19] or RANO criteria,[20] an Eastern Cooperative Oncology Group (ECOG) performance status of 0-2 and acceptable organ function. Furthermore, a tumor genetic or protein-expression test, carried out outside the context of the trial, must have revealed a potentially actionable molecular variant for which matching treatment is available within the trial. Treatment is administered until disease progression or unmanageable toxicity and may not be combined with any other anticancer treatment outside of the study.

Primary endpoints of DRUP include CB, defined by confirmed OR or SD for at least 16 weeks, and safety. Secondary endpoints include progression-free survival (PFS) and overall survival (OS), as well as explorative biomarker analyses. To this end, all patients undergo a mandatory pretreatment tumor biopsy for WGS. As per protocol, patients who receive less than one full treatment cycle are considered non-assessable and are replaced. Details on trial design, available drugs and eligible molecular alterations, as well as results from individual cohorts have been previously reported.[6,7,18,21-23]

DRUP (ClinicalTrials.gov: NCT02925234) was approved by the Medical Ethical Committee at the Netherlands Cancer Institute in Amsterdam, The Netherlands, and was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki's ethical principles for medical research. Patients provided written informed consent upon enrollment.

Validation cohort

To validate the findings in our DRUP discovery cohort, we made use of a pan-cancer, real-world dataset generated by Hartwig Medical Foundation (Amsterdam, The Netherlands). Patients diagnosed with advanced or metastatic cancer who were eligible for systemic treatment were enrolled in the Hartwig database through participation in the CPCT-02 (NCT01855477) and DRUP clinical studies. The CPCT-02 aimed to identify potential molecular targets for personalized treatment by sequencing tumor DNA and using this information to guide therapy decisions. It collected comprehensive real-world data, including genetic and clinical profiles, to enhance understanding of treatment outcomes and cancer progression. Like DRUP, CPCT-02 was approved by the medical ethical committees of the University Medical Center Utrecht and the Netherlands Cancer Institute, was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki and obtained written informed consent from all included patients. Establishment of this database as well as outcomes from several studies that made use of its data have been previously described.[24-26]

Endpoints and study objectives

The primary aim of this analysis was to explore the association between PAL and the endpoints of DRUP: CB, PFS and OS. From the DRUP cohort, patients for whom clinical outcomes and WGS data were available were included. Patients who were non-assessable for the study's primary endpoint according to protocol definition, and patients for whom the genomic alteration on which study treatment was based could not be confirmed through WGS, were excluded. For the primary analysis, we selected all patients

who were treated with targeted monoclonal antibodies, small molecule inhibitors or poly (ADP-ribose) polymerase inhibitors, administered as monotherapy or as a registered combination focused on a single pathway, hereafter referred to as TT. The findings from the primary analysis in the DRUP cohort were subsequently validated in the Hartwig cohort. To this end, only patients who were treated with TT as previously specified were selected. Patients who were concomitantly treated with any other form of anticancer therapy or did not have sufficient clinical follow-up data available to be evaluated according to the DRUP clinical outcome metrics were excluded. To avoid duplicative use of data, DRUP patients in the Hartwig database were excluded from the validation cohort.

As an exploratory endpoint for the DRUP cohort, we conducted a similar analysis on the patients treated with ICB in DRUP. This analysis was done to assess if any correlation between PAL and outcomes remained consistent across different treatment types, signifying a potential prognostic value, or if it could serve as a predictive biomarker specifically for response to TT.

Whole genome sequencing and driver identification

Sequencing of tumor tissue, together with matching blood sample to correct for germline variants, was carried out by Hartwig as previously described.[24-27] In accordance with pre-specified thresholds for accurate variant calling, only biopsies with a pathologist-estimated tumor cellularity $\geq$30% and a sequencing-estimated tumor purity $\geq$20% were included in this analysis. WGS was conducted at median depths of approximately 100$\times$ and 40$\times$ for tumor and normal samples, respectively. The analysis involved an optimized pipeline based on open-source tools accessible on GitHub (https://github.com/hartwigmedical/pipeline5).

Genomic drivers were identified using PURPLE, as described previously.[24] Within PURPLE, mutation-specific calculations of clonality and biallelic status are integrated and mutations occurring in known hotspots are flagged. For the current analysis, to distinguish functionally relevant 'driver' mutations from 'passenger' events, the threshold for high driver probability, driver likelihood $>$0.8, was applied. To identify significant amplifications and homozygous deletions, the thresholds were a minimal copy number greater than three times the sample ploidy and a minimal copy number below 0.5, respectively. For fusions, only well-established fusion pairs, generated by combining all known fusions from COSMIC, CIViC, CGI, and OncoKB, were considered drivers in this analysis.

Pathway classification

After driver identification, the drivers present were assigned to 10 canonical signaling pathways, as previously defined for 335 genes by Sanchez-Vega et al.: (i) cell cycle, (ii) Hippo signaling, (iii) Myc signaling, (iv) Notch signaling, (v) oxidative stress response/Nrf2, (vi) phosphoinositide 3-kinase (PI3K) signaling, (vii) receptor tyrosine kinase (RTK)/RAS/MAP-kinase signaling, (viii) transforming growth factor-β signaling, (ix) p53 and (x) β-catenin/Wnt signaling.[28] An overview of the classified genes and corresponding pathways is presented in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2024.104112. A pathway was considered altered if at least one pathway-associated gene contained a driver alteration as previously defined, and PAL was defined as the sum of altered pathways. Of note, for this analysis not all identified drivers were classified within an oncogenic signaling pathway. Consistent with the approach reported by Sanchez-Vega et al., alterations in DNA repair pathways, epigenetic modifiers, splicing, and other cellular processes commonly found to be disrupted in cancer were excluded due to their primary role in promoting genomic instability, rather than directly affecting the cell's proliferative potential.[28]

Therapeutic actionability

Therapeutic actionability was annotated by Hartwig on 18 August 2023 by mapping the genomic driver alterations to the CKB and ICLUSION databases. For the current study, only level A and B variants that predict responsiveness were considered, which can be mapped to the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) criteria[29] as previously described.[24] In brief, 'level A on-label' equals ESCAT tier IA + B, 'level A off-label' equals ESCAT tier IC, 'level B on-label' equals ESCAT tier IIA + B and 'level B off-label' equals ESCAT tier IIIA.

Statistical analyses

Patient characteristics were summarized using descriptive statistics. Difference in PAL according to CB was analyzed using the Kruskal—Wallis test. Difference in CBR between groups was calculated using Fisher's exact test. Kaplan—Meier methods were used to estimate PFS (from the first day of treatment to date of progression or death from any cause, censoring patients alive without progression) and OS (calculated from the first day of treatment administration to date of death from any cause, censoring patients who were alive at last follow-up). Initially, dichotomization based on the population's median PAL was employed to achieve equal groups and give an unbiased indication of the influence of PAL on outcomes. Subsequently, analyses were repeated with the population dichotomized at all possible PAL cut-offs as well as with PAL as a continuous variable, to ensure that any potential association was not caused by overfitting. Additionally, the influence of each individual pathway alteration on patient outcomes, as well as the effects of all possible pathway pairs, was assessed. False discovery rates were controlled using Benjamini—Hochberg correction for multiple testing.

In multivariate Cox proportional hazards modeling, for the DRUP cohort we included the following covariates: PAL (applied both dichotomous and continuous), major drug classes (received by $\geq$10% of the study population versus 'other'), number of previous systemic therapy lines (continuous), ECOG performance status (continuous), tumor mutational burden (TMB, log 10 transformed and

continuous) and tumor ploidy (continuous). Major tumor types (occurring in ≥10% of the study population versus 'other') were employed as a stratification factor. For the Hartwig validation cohort, ECOG performance status was unavailable and therefore not included in the Cox proportional hazards model. The number of previous systemic therapy lines was replaced by previous systemic treatment ('yes' or 'no') as this was the information available. Since drugs in the Hartwig cohort were principally administered for on-label indications, resulting in a strong correlation between drugs and tumor type, correction was only applied for tumor type (as a stratification factor) and here no longer for drug category. All statistical tests were two-sided and a *P* value of <0.05 was deemed to indicate statistical significance. All analyses were carried out using R version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Altered signaling pathways, clinical outcomes and therapeutic actionability in patients treated with targeted therapy in the DRUP discovery cohort

Between September 2016 and May 2023, 154 response-assessable patients were treated with TT in DRUP and had available WGS confirming their inclusion target (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2024.104112). Their baseline characteristics are reported in Table 1. In these 154 patients, a total of 830 driver alterations were identified in 217 different genes. The five most frequently altered genes were *TP53* (*n* = 85, 55.2%), *CDKN2A* (*n* = 45, 29.2%), *ERBB2* (*n* = 38, 24.7%), *FGFR2* (*n* = 23, 14.9%) and *PIK3CA* (*n* = 22, 14.3%); the median number of driver alterations per patient sample was 5 (range 1-15, Figure 1A and C). Based on the drivers present, most altered signaling pathways were RTK–RAS (*n* = 129, 83.8%), TP53 (*n* = 103, 66.9%) and cell cycle (*n* = 75, 48.7%) (Figure 1B). The median PAL per sample was 3 (range 0-6, Figure 1D). Notably, of the 217 genes that were altered with a high driver likelihood at least once in this population, 62 (28.6%) were included in the pathway classification. However, this accounted for 498 of the identified 830 driver alterations (60.0%). An overview of drivers observed per patient sample, organized according to the 10 oncogenic signaling pathways, is presented in Supplementary Figure S2 and Table S2, available at https://doi.org/10.1016/j.esmoop.2024.104112.

We found CB to be significantly associated with a lower PAL (*P* = 0.004, Figure 2A and B). After dichotomization based on the median PAL of 3, 25 out of 60 patients with a PAL <3 experienced CB [41.7%, 95% confidence interval (CI) 29.1% to 55.1%], while among patients with PAL ≥3, 24 out of 94 did (25.5%, 95% CI 17.1% to 35.6%). This resulted in an odds ratio of 0.48 (95% CI 0.23-1.02, *P* = 0.051). Of the 105 patients without CB, 8 (7.6%) initially experienced OR which remained unconfirmed. Kaplan–Meier analyses demonstrated that the subgroup characterized by a lower PAL (<3) exhibited a longer PFS in comparison to the group with a PAL of ≥3 [median 4.7 months (95% CI 3.5-6.8 months) versus 2.9 months (95% CI 1.9-3.6 months), Figure 2C]. Unadjusted hazard ratio (HR) for progression was 1.58 (95% CI 1.10-2.26, *P* = 0.013) in the PAL ≥3 group compared with the group with PAL <3, which remained statistically significant after multivariate adjustment (HR 1.70, 95% CI 1.09-2.65, *P* = 0.020, Figure 2E).

For OS, similar results were found; the subgroup comprising patients with PAL <3 demonstrated a significantly longer OS compared with patients with PAL ≥3, with a median OS of 13.7 months (95% CI 10.1-19.6 months) versus 5.6 months (95% CI 4.8-7.7 months), respectively (Figure 2D). Unadjusted HR for death was 2.32 (95% CI 1.59-3.39, *P* < 0.001) in the PAL ≥3

Table 1. Baseline characteristics of the targeted therapy population in the DRUP discovery cohort

	Patients *n* = 154
Age (approximately) at consent, median (IQR)	63 (52-70)
Sex, *n* (%)	
Male	77 (50)
Female	77 (50)
Tumor type, *n* (%)	
Non-small cell lung carcinoma	40 (26.0)
Colorectum	20 (13.0)
Biliary tract	14 (9.0)
Prostate	10 (6.5)
Breast	8 (5.2)
Gastrointestinal stromal tumor	8 (5.2)
Endometrium	7 (4.5)
Ovarium	7 (4.5)
Thyroid gland	6 (3.9)
Salivary gland	4 (2.6)
(Adeno)carcinoma of unknown primary	3 (1.9)
Soft tissue sarcoma	3 (1.9)
Other[a]	24 (15.8)
ECOG performance status, *n* (%)	
0	52 (33.8)
1	89 (57.8)
2	10 (6.5)
Missing	3 (1.9)
Previous systemic treatment lines, *n* (%)	
0	10 (6.5)
1-2	61 (39.6)
3-5	61 (39.6)
≥6	22 (14.3)
Previous radiotherapy, *n* (%)	
Yes	77 (50)
No	77 (50)
Study treatment, *n* (%)	
Trastuzumab + pertuzumab	35 (22.7)
Lenvatinib	31 (20.1)
Olaparib	22 (14.3)
Panitumumab	15 (9.7)
Crizotinib	13 (8.4)
Trametinib	11 (7.1)
Vemurafenib + cobimetinib	11 (7.1)
Nilotinib	9 (5.8)
Palbociclib	4 (2.6)
Other[b]	3 (2.2)

DRUP, Drug Rediscovery Protocol. ECOG, Eastern Cooperative Oncology Group; IQR, interquartile range.

[a]Two patients were included with anal carcinoma, cervical carcinoma, esophageal carcinoma, neuroendocrine carcinoma, small intestinal carcinoma, urothelial carcinoma, uterine sarcoma. For the following tumor types, one patient was included: glioblastoma multiforme, hypopharyngeal carcinoma, ocular melanoma, pancreatic carcinoma, small-cell lung carcinoma, gastric carcinoma, thymic carcinoma, urachal carcinoma, urethral carcinoma, vaginal carcinoma.

[b]The following drugs were received by only one patient: dabrafenib monotherapy, dabrafenib + trametinib, sunitinib.

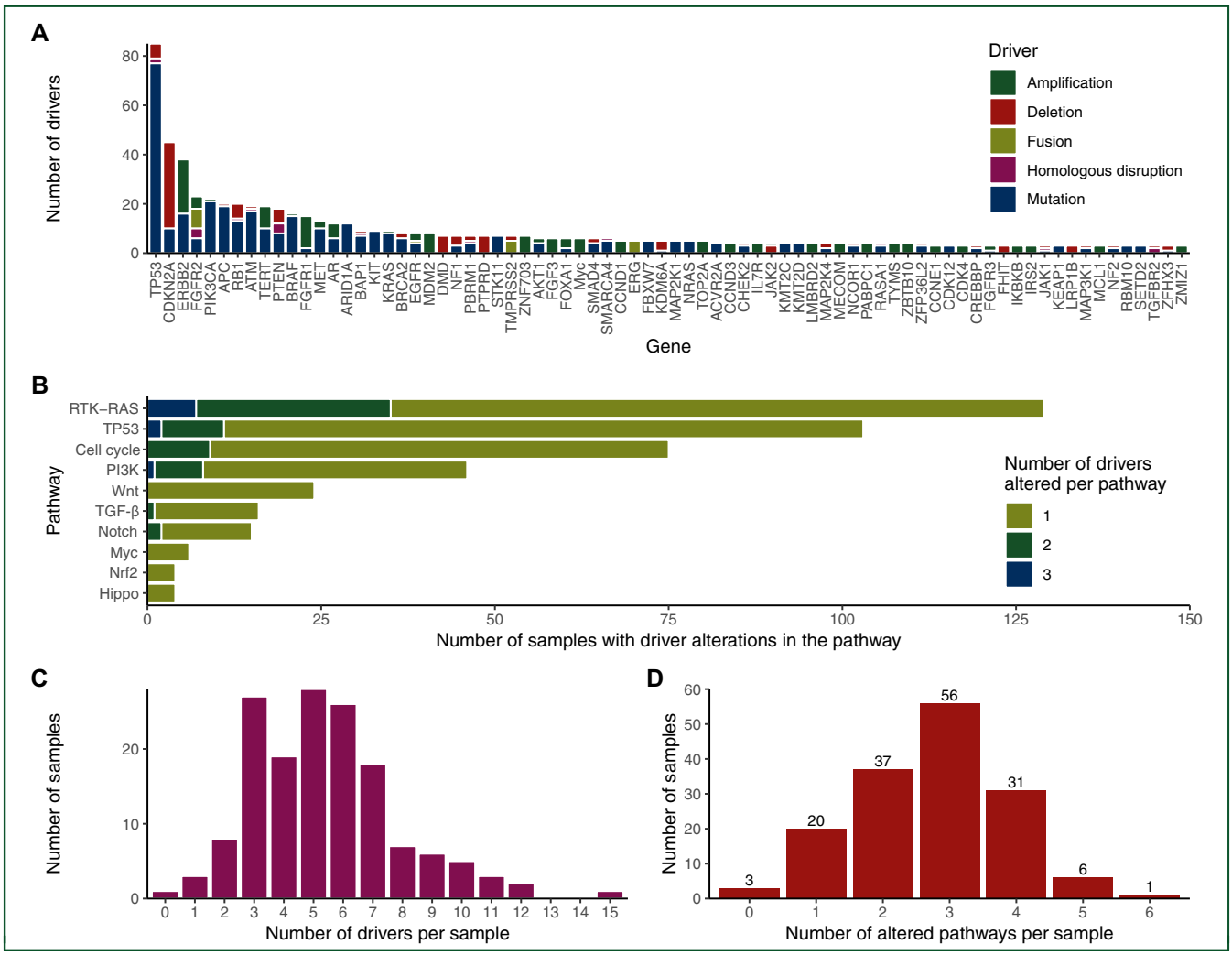


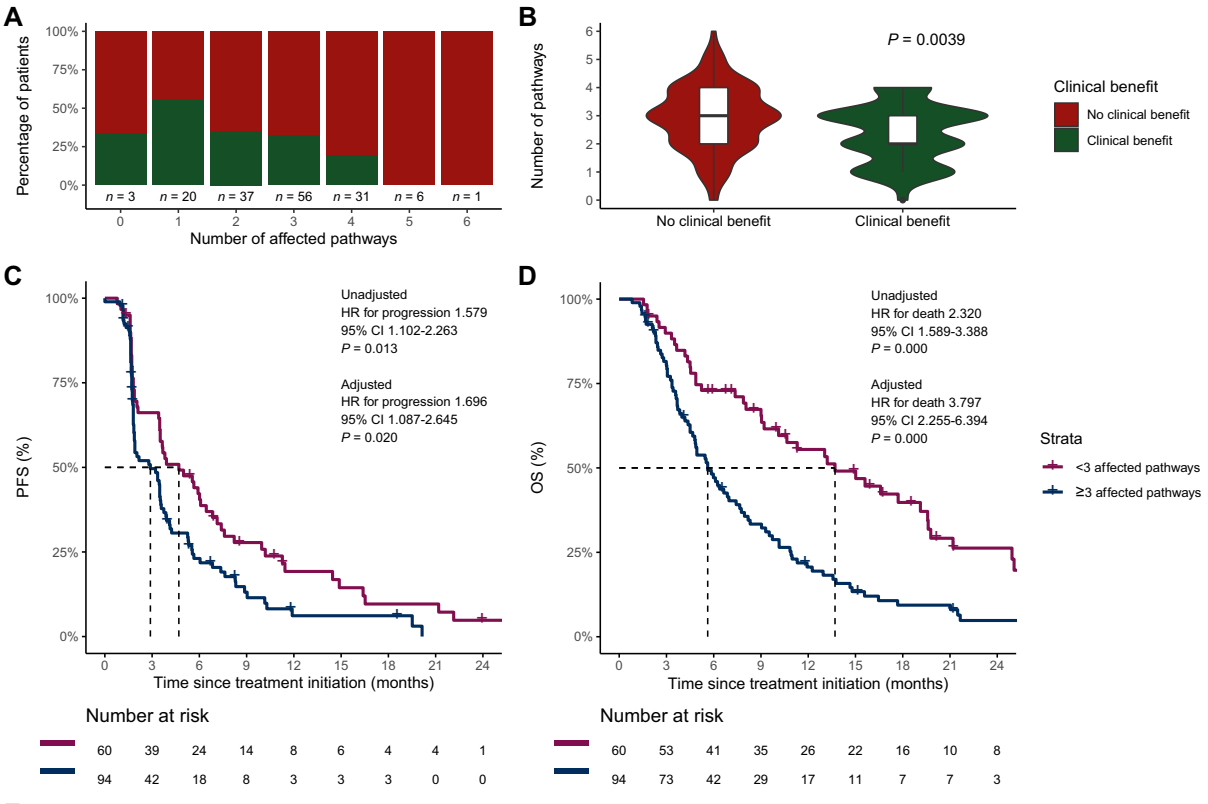
Figure 1. Molecular characteristics of the targeted therapy population in the DRUP discovery cohort. (A) Overview of the most frequent genomic drivers in the population that received targeted therapy. (B) The number of driver events per oncogenic signaling pathway. (C) Breakdown of the number of driver events per tumor sample. (D) Breakdown of the number of altered oncogenic signaling pathways per sample.
DRUP, Drug Rediscovery Protocol; PI3K, phosphoinositide 3-kinase; TGF-β, transforming growth factor-β.

group compared with the PAL <3 group. After multivariate adjustment, the HR was 3.80 (95% CI 2.26-6.39, $P < 0.001$, Figure 2F). Interestingly, in multivariate analysis, the number of previous systemic therapy lines was negatively associated with both PFS and OS (HR for progression 1.09, 95% CI 1.02-1.17, $P = 0.015$; HR for death 1.17, 95% CI 1.08-1.26, $P < 0.001$). Furthermore, also a higher ECOG performance score was identified as an independent negative predictor for OS (HR 2.05, 95% CI 1.42-2.97, $P < 0.001$).

Upon further population subdivision, applying PAL continuously rather than dichotomously, these observations persisted with even more significant association [adjusted HR (aHR) for progression 1.49, 95% CI 1.22-1.82, $P < 0.001$; aHR for death 1.76, 95% CI 1.42-2.18, $P < 0.001$, Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2024.104112]. Moreover, the application of cut-offs other than the median PAL yielded comparable results (Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2024.104112). Of note, despite a small sample size of only seven patients, none with PAL ≥5 exhibited CB, and all had poor PFS and OS.

Analysis of individual pathway alterations showed a trend toward reduced benefit from TT across most pathways (Supplementary Table S4, available at https://doi.org/10.1016/j.esmoop.2024.104112). However, after adjusting for confounders using Cox proportional hazards modeling and correcting for multiple testing with Benjamini–Hochberg, only cell cycle and PI3K pathways were significantly associated with increased HR for both progression and death. Additionally, pathway pair analysis revealed that co-alteration of the cell cycle and Notch pathways had a detrimental effect in eight patients (HR >7 for both progression and death), while cell cycle and TP53 co-alteration was linked to HR >3 for progression and death in 47 patients (Supplementary Table S5, available at https://doi.org/10.1016/j.esmoop.2024.104112).

Subsequently, we investigated the therapeutic actionability of the identified drivers and pathways. In total, 361 of



E

Covariate	Level	HR (95% CI)	*P* value
Drug	Other	<reference>	
	Lenvatinib	0.73 (0.41-1.30)	0.285
	Olaparib	0.69 (0.38-1.23)	0.203
	Trastuzumab + pertuzumab	1.03 (0.61-1.73)	0.914
Previous treatment lines	<continuous>	1.09 (1.02-1.17)	0.015
ECOG	<continuous>	1.21 (0.87-1.68)	0.257
TMB (log 10)	<continuous>	1.30 (0.77-2.19)	0.331
Tumor ploidy	<continuous>	1.11 (0.87-1.42)	0.419
Pathway alteration load	<3 affected pathways	<reference>	
	≥3 affected pathways	1.70 (1.09-2.65)	0.020

The model was stratified by tumor (other, CRC, NSCLC).

F

Covariate	Level	HR (95% CI)	*P* value
Drug	Other	<reference>	
	Lenvatinib	0.89 (0.49-1.62)	0.698
	Olaparib	1.07 (0.58-1.98)	0.831
	Trastuzumab + pertuzumab	1.01 (0.59-1.74)	0.961
Previous treatment lines	<continuous>	1.17 (1.08-1.26)	0.000
ECOG	<continuous>	2.05 (1.42-2.97)	0.000
TMB (log 10)	<continuous>	0.70 (0.42-1.19)	0.187
Tumor ploidy	<continuous>	1.06 (0.82-1.36)	0.661
Pathway alteration load	<3 affected pathways	<reference>	
	≥3 affected pathways	3.80 (2.26-6.39)	0.000

The model was stratified by tumor (other, CRC, NSCLC).

Figure 2. Response to targeted therapy stratified by median PAL in the DRUP discovery cohort. (A) Clinical benefit of targeted therapy according to the number of altered signaling pathways. (B) Violin plot depicting the number of signaling pathways for the patient group that experienced clinical benefit from therapy versus no clinical benefit. (C) PFS, group stratified at median PAL. (D) OS, group stratified at median PAL. (E and F) Forest plots presenting the influence of all pre-specified covariates on the probability of disease progression (E) and death from any cause (F) according to the multivariate Cox model. Each square represents a covariate HR, with the horizontal lines indicating the 95% CI. The models were stratified for largest tumor type subgroups (i.e. CRC, NSCLC and 'other'). Due to missing ECOG performance status data, three patients were excluded from this analysis.

CI, confidence interval; CRC, colorectal cancer; DRUP, Drug Rediscovery Protocol; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; NSCLC, non-small cell lung cancer; OS, overall survival; PAL, pathway alteration load; PFS, progression-free survival; TMB, tumor mutational burden.

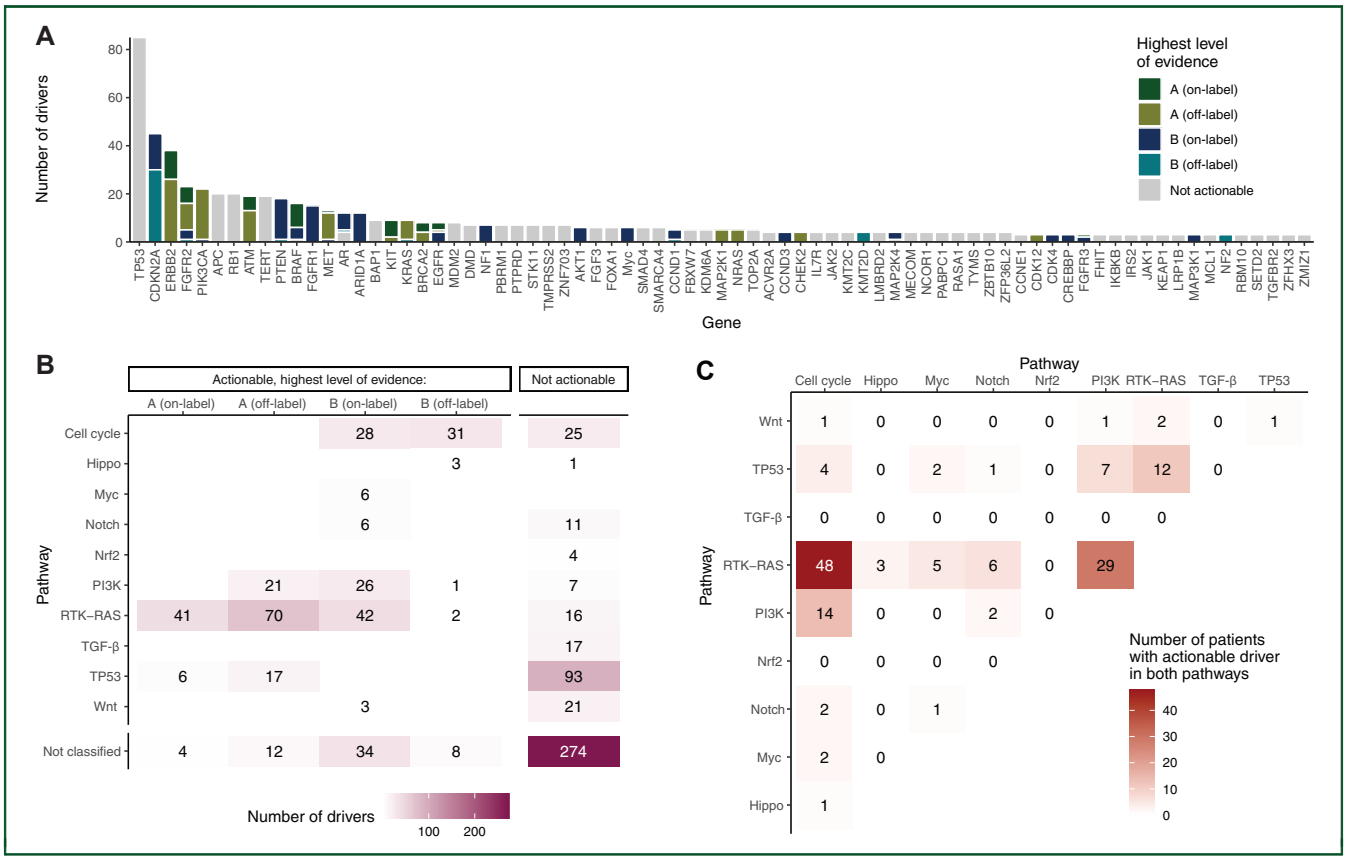


Figure 3. Actionability of the identified drivers and altered pathways in the targeted therapy population in the DRUP discovery cohort. (A) Actionability of most frequent genomic drivers in the population that received targeted therapy. Level A refers to biomarkers that are associated with an approved therapy or established clinical guidelines, while level B refers to biomarkers supported by strong biological evidence or clinical trials suggesting they are actionable. 'On-label' refers to treatments that are officially approved by regulatory authorities for a specific tumor type, whereas 'off-label' refers to treatments that are approved for other tumor types but used in an unapproved context for the given tumor type. (B) Actionable drivers clustered according to the oncogenic signaling pathways. (C) Co-occurrence of actionable pathways.
DRUP, Drug Rediscovery Protocol; PI3K, phosphoinositide 3-kinase; TGF-β, transforming growth factor-β.

the 830 identified drivers (43.5%) were therapeutically actionable, of which 51 (6.1%) were level A on-label, 120 (14.5%) level A off-label, 145 (17.5%) level B on-label and 45 (5.4%) level B off-label (Figure 3A, Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2024.104112). Most of the actionable drivers belonged to the RTK—RAS pathway (155 of 361, 42.9%, Figure 3B). Of the 154 patients, 57 (37.0%) had actionable drivers in two pathways, 27 (17.5%) in three pathways and 1 (0.6%) in four pathways. RTK—RAS and cell cycle were the most frequent co-targetable pathways (48 of 154 patients, 31.2%), followed by RTK—RAS and PI3K (29 of 154 patients, 18.8%, Figure 3C).

Exploring the link between altered oncogenic signaling pathways and benefit from ICB in DRUP

In total, 160 patients with a WGS-confirmed molecular target received ICB, based on mismatch repair deficiency or WGS-derived high tumor mutational load (baseline characteristics are reported in Supplementary Table S6, available at https://doi.org/10.1016/j.esmoop.2024.104112). As expected, the median number of driver alterations and median PAL in this group, 9 (range 1-18) and 4 (range 0-6), respectively, were higher than those of the patient population treated with TT (Supplementary Figure S4A, available at https://doi.org/10.1016/j.esmoop.2024.104112). In contrast to our findings for the population treated with TT, patients who experienced CB from ICB had a significantly higher PAL compared with those who did not ($P = 0.039$, Supplementary Figure S4B, available at https://doi.org/10.1016/j.esmoop.2024.104112).

The median PFS and OS appeared considerably different between patients with PAL <3 and PAL ≥ 3, in favor of higher PAL [PFS 3.3 months (95% CI 2.0-11.1 months) versus 7.8 months (95% CI 4.0-30.8 months); OS 12.6 months (95% CI 6.5-24.8 months) versus 16.2 months (95% CI 11.9-44.8 months), Supplementary Figure S4C and D, available at https://doi.org/10.1016/j.esmoop.2024.104112]. However, these differences did not attain statistical significance (unadjusted HR for PFS 0.70, 95% CI 0.45-1.09, $P = 0.111$; OS 0.72, 95% CI 0.45-1.14, $P = 0.155$). In fact, after multivariate analysis, TMB emerged as a strong significant predictor of survival (PFS: HR 0.25, 95% CI 0.12-0.50, $P < 0.001$; OS: HR 0.37, 95% CI 0.18-0.78, $P = 0.009$) (Supplementary Figure S4E and F, available at https://doi.org/10.1016/j.esmoop.2024.104112).

Altered oncogenic signaling pathways and benefit of targeted therapy in the Hartwig validation cohort

We next continued to validate our findings in the Hartwig database. In total, 4506 non-DRUP patients with metastatic cancer were included in this database. Of these, 258 patients received TT with documented WGS and outcome data that were evaluable according to the DRUP protocol definition (comprehensive accrual reported in Supplementary Figure S5, available at https://doi.org/10.1016/j.esmoop.2024.104112). Baseline characteristics of this cohort are reported in Supplementary Table S7, available at https://doi.org/10.1016/j.esmoop.2024.104112. Importantly, in contrast to our primary analysis, here a considerably larger proportion of patients (45.3%) had received no prior systemic therapy at time of enrollment.

The median number of drivers per patient was 4 (range 0-17), with a median PAL of 2 (range 0-6, Figure 4A). Similar to our primary analysis, patients with CB had a lower PAL compared with those who did not have CB ($P = 0.043$, Figure 4B). After dichotomization between PAL <3 and PAL ≥ 3, CBR in patients with PAL <3 was 54.2% (95% CI 46.3% to 61.9%, $n = 91/168$) versus 36.7% (95% CI 26.8% to 47.5%, $n = 33/90$) in patients with PAL ≥ 3 (odds ratio 2.04, 95% CI 1.17-3.58, $P = 0.009$). Along those lines, patients with PAL <3 demonstrated a significantly longer PFS in comparison to those with PAL ≥ 3 [median 7.0 months (95% CI 5.9-8.5 months) versus 4.2 months (95% CI 3.4-5.1 months), Figure 4C]. Unadjusted HR for progression was 1.87 (95% CI 1.40-2.48, $P < 0.001$) and remained statistically significant after multivariate adjustment (HR 1.55, 95% CI 1.12-2.16, $P = 0.009$, Figure 4E). Likewise, median OS was longer for patients with PAL <3 than it was for patients with PAL ≥ 3 [14.7 months (95% CI 12.6-20.1 months) versus 8.2 months (95% CI 7.1-11.9 months), Figure 3D]. Unadjusted HR for death was 2.02 (95% CI 1.46-2.79, $P < 0.001$), but this significance was not maintained after multivariate adjustment (HR 1.37, 95% CI 0.93-2.00, $P = 0.111$, Figure 4F).

Again, applying PAL continuously demonstrated similar results: aHR for progression 1.14 (95% CI 1.01-1.30, $P = 0.037$), aHR for death 1.13 (95% CI 0.97-1.30, $P = 0.116$, Supplementary Figure S6, available at https://doi.org/10.1016/j.esmoop.2024.104112).

DISCUSSION

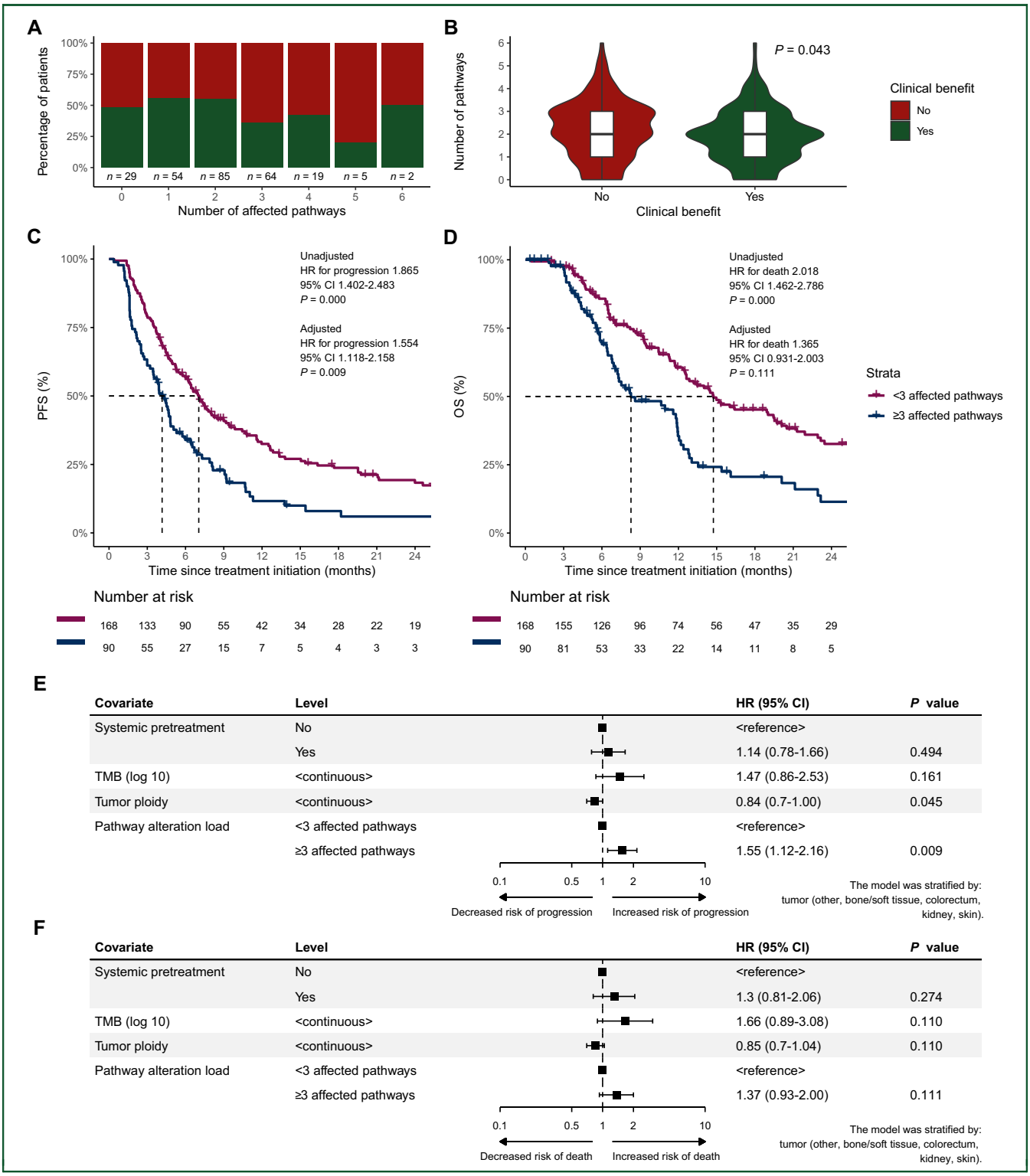
Results of this study demonstrate that in patients with treatment-refractory cancer treated with TT outside their registered indication, a lower PAL was significantly associated with a higher CBR to TT, as well as longer PFS and OS. These associations remained robust even after accounting for multiple potential confounding factors and across different cut-offs for high versus low PAL. With each added altered pathway, resistance to TT appeared to increase further.

To validate our findings, we employed the Hartwig database. While the population in this database did not fully resemble the population in DRUP given the larger proportion of non-pretreated patients with metastatic cancer (45.3% versus 6.5%), administered TTs that were on-label and not necessarily matched to the tumor molecular profile and the absence of data on ECOG performance status, we here also established clear associations between PAL and therapeutic benefit. In the Hartwig database, however, differences in benefit did not lead to significantly improved OS in the subgroup harboring lower PAL. This outcome may be attributed to the possibility that patients in the Hartwig database had more remaining lines of therapy after enrollment compared with the DRUP population, thereby potentially influencing OS outcomes.

Signals similar to our findings have been reported, albeit with a restricted focus on the presence of co-occurring driver alterations (as a total, but also specifically focused on particular co-occurring alterations), rather than the PAL.[15,30] While the findings may not appear entirely unexpected considering the biological rationale, to our knowledge, our study is the first in evaluating the relationship between PAL and TT response in this population, receiving molecularly matched therapies in the last-line setting. The lack of prior studies on this topic may be attributed to the fact that extensive molecular profiling still is, for many tumor types, not part of routine diagnostics. The DRUP study, which integrates standardized clinical outcomes with molecular characterization of tumors, therefore presented a unique opportunity to address this.

Our findings may have several implications for molecularly targeted treatment. Firstly, they raise the question whether patient selection should be refined, by excluding those with limited chance of benefit. Especially in the end-stage setting of disease, this choice can be of great importance as (unnecessary) exposure to potential toxicity may significantly impair a patient's quality of life. Results of this study argue that a higher PAL limits the chance of benefit to treatment. In our population, no benefit of TT was observed in the subgroup harboring PAL ≥ 5. However, as this group encompassed only seven patients, the precise cut-off required to identify the patient group most or least likely to benefit from personalized treatment at this stage warrants further research and may vary per tumor type, drug or drug category. Of note, the use of PAL as a biomarker does not necessarily require WGS, as most genes in the classification are included in broad next-generation sequencing panels. Furthermore, circulating tumor DNA may play a valuable role in enabling longitudinal monitoring.

A second consideration is how we can use these observations to improve TT strategies. The observed inverse association between the number of previous systemic therapy lines and therapeutic benefit suggests potential advantage of moving the administration of these therapies to an earlier treatment stage. Furthermore, considering the observation that co-altered pathways decreased chances of benefit to therapy targeting a single pathway and that 55% of patients had two or more targetable pathways, for this group it may be hypothesized that intensifying treatment by



E

Covariate	Level	HR (95% CI)	*P* value
Systemic pretreatment	No	<reference>	
	Yes	1.14 (0.78-1.66)	0.494
TMB (log 10)	<continuous>	1.47 (0.86-2.53)	0.161
Tumor ploidy	<continuous>	0.84 (0.7-1.00)	0.045
Pathway alteration load	<3 affected pathways	<reference>	
	≥3 affected pathways	1.55 (1.12-2.16)	0.009

The model was stratified by: tumor (other, bone/soft tissue, colorectum, kidney, skin).

F

Covariate	Level	HR (95% CI)	*P* value
Systemic pretreatment	No	<reference>	
	Yes	1.3 (0.81-2.06)	0.274
TMB (log 10)	<continuous>	1.66 (0.89-3.08)	0.110
Tumor ploidy	<continuous>	0.85 (0.7-1.04)	0.110
Pathway alteration load	<3 affected pathways	<reference>	
	≥3 affected pathways	1.37 (0.93-2.00)	0.111

The model was stratified by: tumor (other, bone/soft tissue, colorectum, kidney, skin).

Figure 4. Response to targeted therapy stratified by median PAL in the Hartwig validation cohort. (A) Clinical benefit of targeted therapy according to the number of altered signaling pathways. (B) Violin plot depicting the number of signaling pathways for the patient group that experienced clinical benefit from therapy versus no clinical benefit. (C) PFS, group stratified at median PAL. (D) OS, group stratified at median PAL. (E and F) Forest plots presenting the influence of all pre-specified covariates on the probability of disease progression (E) and death from any cause (F) according to the multivariate Cox model. Each square represents a covariate HR, with the horizontal lines indicating the 95% CI. The models were stratified for largest tumor type subgroups (i.e. bone/soft tissue sarcoma, colorectal cancer, kidney cancer, skin cancer and 'other').

CI, confidence interval; HR, hazard ratio; OS, overall survival; PAL, pathway alteration load; PFS, progression-free survival; TMB, tumor mutational burden.

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simultaneously targeting multiple pathways may overcome resistance. Additionally, novel targeted approaches that do not rely solely on inhibiting specific molecular signaling pathways, such as antibody–drug conjugates and radioligand therapies, could provide alternative options with the potential for improved outcomes.

Notably, with our current dataset, we cannot exclude the possibility that PAL contributes to tumor aggressiveness, acting more as a prognostic marker (similar to, for instance, the Royal Marsden Hospital score[31]) than a predictive one. This aligns with the findings by Zhou et al., who reported an association between higher PAL and increased recurrence in early-stage resected NSCLC.[32] However, in DRUP patients treated with ICB, we observed the opposite trend: higher PAL was associated with increased CB, though not with significantly improved PFS or OS. This suggests that high PAL does not universally predict poorer outcomes across all treatment types. Nevertheless, without a study that randomizes TT-eligible patients across PAL subgroups to receive either TT or non-TT, definitive conclusions remain uncertain. Yet, despite this uncertainty, our findings clearly indicate that patients with high PAL experience suboptimal benefit from TT as currently administered, emphasizing the need for improved patient selection or intensified treatment approaches in this subgroup.

Additional limitations of our study include that despite being well established and extensively applied before, driver alterations were assessed according to the Hartwig WGS pipeline that, inherent to bioinformatics approaches, has its own characteristics. It could therefore be that certain algorithmically insignificant, but clinically relevant, molecular events were missed. Likewise, despite selection of only events with a driver likelihood of $\geq$0.8, some clinically irrelevant events could have unjustly passed this threshold. Moreover, all types of driver alterations—mutations, fusions, amplifications, deletions and homologous disruptions—were treated as equally important, which may not reflect their differing clinical and therapeutic relevance. Secondly, for replicability purposes, we specifically opted to employ an established pathway classification system.[28] The trade-off was that a considerable portion of driver alterations identified were not included in this classification. For example, although alterations in homologous recombination repair genes are clinically significant and define eligibility for treatment in DRUP, they are not classified within an oncogenic signaling pathway. This is understandable, as these genes are not directly involved in proliferative signaling but rather contribute to genomic instability. Nonetheless, this limitation highlights the need for further research to explore the role of these pathways in cancer therapy and their potential impact in the context of the signaling pathway classification used in this study. Lastly, as DRUP is a multi-drug, pan-cancer trial, the decision to analyze all data in aggregate may, despite careful correction, have obscured certain drug-specific or tumor-specific signals or associations.

In conclusion, in patients with heavily pretreated metastatic cancer, we demonstrate that PAL is a strong, pan-cancer predictor of outcome to mono-pathway-directed TT. Patients with a poor chance of response may benefit from intensified treatment, such as combinatorial regimens that target multiple pathways, or TT at an earlier treatment stage. However, it may also be necessary to protect a particular subgroup from futile, yet potentially toxic, therapy. Furthermore, these data underscore the crucial need to incorporate biomarker analyses into clinical precision oncology trials to enhance our understanding of how to maximize therapeutic benefit.

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DISCLOSURE

EEV is the founder and current member of the supervisory board of the Hartwig Medical Foundation, independent non-executive director of Sanofi, co-founder of Mosaic Therapeutics and board member and founder of the Center for Personalized Cancer Treatment. He has received clinical study grants from Amgen, AstraZeneca, BI, BMS, Clovis, Eli

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