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Quantifying the impact of immunotherapy on RNA dynamics in cancer

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ABSTRACT

Background Checkpoint inhibitor (CPI) immunotherapies have provided durable clinical responses across a range of solid tumor types for some patients with cancer. Nonetheless, response rates to CPI vary greatly between cancer types. Resolving intratumor transcriptomic changes induced by CPI may improve our understanding of the mechanisms of sensitivity and resistance.

Methods We assembled a cohort of longitudinal pre-therapy and on-therapy samples from 174 patients treated with CPI across six cancer types by leveraging transcriptomic sequencing data from five studies.

Results Meta-analyses of published RNA markers revealed an on-therapy pattern of immune reinvigoration in patients with breast cancer, which was not discernible pre-therapy, providing biological insight into the impact of CPI on the breast cancer immune microenvironment. We identified 98 breast cancer-specific correlates of CPI response, including 13 genes which are known IO targets, such as toll-like receptors *TLR1*, *TLR4*, and *TLR8*, that could hold potential as combination targets for patients with breast cancer receiving CPI treatment. Furthermore, we demonstrate that a subset of response genes identified in breast cancer are already highly expressed pre-therapy in melanoma, and additionally we establish divergent RNA dynamics between breast cancer and melanoma following CPI treatment, which may suggest distinct immune microenvironments between the two cancer types.

Conclusions Overall, delineating longitudinal RNA dynamics following CPI therapy sheds light on the mechanisms underlying diverging response trajectories, and identifies putative targets for combination therapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Explorations of molecular dynamics following checkpoint inhibitor (CPI) therapy within small cohorts have shed light on the biological activity of CPI-modulated immune responses such as early T-cell turnover and expansion. However, an understanding of the impact of CPI on transcriptomic changes and similarities and differences in mechanisms of response to CPI between solid tumor types remains limited.

WHAT THIS STUDY ADDS

⇒ This study integrates longitudinal pre-therapy and on-therapy transcriptomic data across solid tumor types to identify meaningful transcriptomic dynamics on-therapy, enabling a deeper insight into the mechanisms of CPI response and resistance and how these may differ across melanoma and breast cancer.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Findings from this study provide a rational for further investigation of promising putative targets for combination therapy with CPI to enhance response rates in breast cancer.

INTRODUCTION

A molecular understanding of immune checkpoints, and their aberrant activation in developing tumors, has led to the recent clinical development of immune checkpoint inhibitors (CPIs). Over the last decade, CPI agents have demonstrated durable responses across a range of cancer types and are FDA-approved for multiple indications, including as a first-line therapy option for metastatic disease.[1] However, only a minority of patients achieve clinical responses ranging from objective response rates of 45% in advanced melanoma cases,[2] to 5% to 10% in metastatic triple-negative breast cancers (TNBCs).[3 4]

Due to variability in CPI response rates between patients, numerous studies have attempted to identify molecular predictors of response. Using single timepoint data, collected prior to CPI treatment (pre-therapy), factors that have been linked to CPI sensitivity have been derived from tumor genomics and tumor microenvironmental (TME) studies.[5] Notably, baseline PD-L1 expression and tumor mutational burden are both FDA-approved as predictive biomarkers to stratify patients

for CPI therapy. Furthermore, numerous transcriptomic signatures have been developed, predominantly within melanoma, as putative RNA-based predictors of CPI sensitivity including signatures of interferon-gamma (IFNγ) signaling, cytolytic activity, and antigen presentation.[6–13]

Nevertheless, biomarkers of immune response, based on pre-therapy molecular profiles in discovery cohorts have displayed varying performance in large validation cohorts (n>1000 patients), particularly in a pan-cancer setting.[14 15] These markers implicitly assume that baseline molecular features are sufficient to encapsulate the dynamic impact of CPI on-therapy, including resculpting the cellular composition and functional states within the tumor microenvironment. Moreover, these factors do not account for the well-recognized interindividual variation in baseline immune status.

Paired longitudinal analyses of patients receiving CPI agents, with pre-therapy and on-therapy tumor biopsies, have the potential to address these limitations and decipher important factors driving an effective immune response. However, these analyses are complicated by the logistical challenges of tumor re-biopsy on treatment. To date, small exploratory cohorts (n≤20 patients) of this kind have reported a phenomenon termed T-cell "reinvigoration,"[16] whereby early T-cell turnover and expansion or persistence of high-frequency T-cell clonotypes are associated with CPI response,[17 18] and improved survival outcomes.[19 20] Other longitudinal CPI studies have demonstrated clonal replacement of T-cell clones on-therapy, which were not present within responding tumors pre-treatment.[21] In addition, other studies have demonstrated mechanisms of CPI treatment failure, such as defective antigen presentation,[22 23] impaired IFNγ signaling,[22] or neoantigen loss.[24] In metastatic TNBC, on-therapy increases in T-cell receptor (TCR) clonality and T-cell infiltration have been shown to be associated with response to CPI.[25] Furthermore, similar shifts in eosinophils were enriched in patients responding to CPI, possibly due to their role in enhancing CD8+ T-cell activation.[26]

Despite the small numbers of patients in these longitudinal studies, profiling molecular dynamics following CPI therapy has enabled the biological activity of CPI-modulated immune responses to be defined. In turn, this has improved our understanding of the mechanisms underpinning sensitivity or resistance. To build on this body of work, we established the CPI-Dynamics ("CPIΔ") multi-cancer cohort of 174 patients across six datasets, leveraging the power of paired longitudinal (pre-treatment and early on-treatment samples) to investigate transcriptomic changes during CPI therapy. The objectives of our approach were to better understand why some cancer types respond more favorably to CPI treatment than others, specifically melanoma compared with TNBC, by identifying correlates which underlie the biological mechanisms of response.

METHODS

Human clinical data

The CPIΔ cohort uses transcriptomic sequencing data from the following studies:

1. Voorwerk *et al*,[25] the metastatic TNBC anti-PD-1 (nivolumab) treated "breast" cohort.
2. Riaz *et al*,[27] the advanced melanoma anti-PD-1 (nivolumab) treated "melanoma (a)" and "melanoma (b)" cohorts.
3. Yang *et al*,[28] the pan-cancer anti-PD-1 (pembrolizumab) treated "pan-cancer" cohort.
4. Powles *et al*,[29] the advanced urothelial anti-PD-L1 (atezolizumab) treated "urothelial (a)" cohort.
5. Dijk *et al*,[30] the advanced urothelial anti-PD-1 (nivolumab) and anti-CTLA-4 (ipilimumab) treated "urothelial (b)" cohort.

Across all selected studies, patients provided written consent before enrollment into respective trials, furthermore, trial approval was obtained from each institutions medical-ethical committee/board, as described previously.[25 27–30] Inclusion criteria for the CPIΔ cohort were (1) treatment with CPI (anti-PD-1/PD-L1 or anti-CTLA4); (2) availability of longitudinal transcriptomic data at both pre-therapy and on-therapy timepoints; and (3) availability of clinical outcome data (overall survival (OS), radiological response and/or pathological response). These criteria yielded a total of 174 CPI-treated patients with paired samples across six cancer types. The individual cohorts comprising the CPIΔ study are henceforth referred to as the "breast" cohort (n=25 patients with TNBC), "melanoma (a)" cohort (n=23 patients with melanoma, CPI-naïve), "melanoma (b)" cohort (n=19 patients with melanoma, prior lines of CPI therapy), "pan-cancer" cohort (head and neck carcinoma n=3, TNBC n=3, high-grade serous ovarian cancer n=6, melanoma n=6, rare solid tumors n=12), "urothelial (a)" cohort (n=64), and "urothelial (b)" cohort (n=13). A breakdown of sample numbers for each study/cancer type and treatment course is contained in table 1 and online supplemental figure 1. The majority of our cohorts included patients with either metastatic or advanced disease, many of whom had other lines of systemic therapy prior to CPI. The details of those previous lines of therapy are included where available. For the "breast" cohort, patients had received one to three lines of prior systemic non-CPI treatment (chemotherapy) with disease progression on the last treatment regiment; however, all patients were CPI naïve prior to entering the trial (online supplemental figure 1A). For the "melanoma (a)" and "melanoma (b)" cohorts, patients with unresectable stage III or IV melanoma who were refractory, intolerant, or refused standard therapy for treatment of metastatic disease were enrolled. For the "melanoma (a)" cohort all patients had not received previous lines of CPI (online supplemental figure 1B), whereas patients within the "melanoma (b)" cohort had progressed following anti-CTLA-4 treatment prior to entering the trial (online supplemental figure 1C). For the "pan-cancer" cohort, patients had either

Table 1 CPIΔ cohort overview

Cohort	Cancer type	Pre-therapy (n)	Pre-therapy data type	On-therapy (n)	On-therapy data type	Paired longitudinal samples (n)	Longitudinal data type
Breast	Metastatic TNBC	44	RNA-seq	25	NanoString	25	NanoString
Melanoma (a)	Metastatic melanoma	29	RNA-seq	23	RNA-seq	23	RNA-seq
Melanoma (b)	Metastatic melanoma	19	RNA-seq	19	RNA-seq	19	RNA-seq
Pan-cancer	Metastatic solid tumors	30	RNA-seq	30	RNA-seq	30	RNA-seq
Urothelial (a)	Muscle-invasive urothelial cancer	64	RNA-seq	64	RNA-seq	64	RNA-seq
Urothelial (b)	Locoregionally advanced urothelial cancer	13	RNA-seq	13	RNA-seq	13	RNA-seq

CPI, immune checkpoint inhibitor ; RNA-seq, RNA sequencing; TNBC, triple-negative breast cancer.

failed prior systemic non-CPI treatment, had no standard non-CPI therapy options, or were not appropriate for standard non-CPI options. All patients in this cohort were CPI naïve prior to entering the trial (online supplemental figure 1D). For both the "urothelial (a)" and "urothelial (b)" cohorts, patients who refused neoadjuvant cisplatin-based chemotherapy or where neoadjuvant cisplatin-based therapy was not deemed appropriate, were enrolled onto the respective trials. Patients within both cohorts were CPI naïve prior to entering the trials (online supplemental figure 1E,F). Detailed treatment history and demographic data regarding, age, sex, and race were either limited or unavailable for selected cohorts and hence not used within our analyses.

Clinical endpoints and stratification

Across the CPIΔ cohorts, four had both OS and RECIST (Response Evaluation Criteria in Solid Tumors) radiological response available; the remaining two cohorts had pathological complete response (pCR) data available. Radiological clinical responses were harmonized across the studies to ensure consistency in this outcome measurement, with "responder" defined as complete response or partial response and "non-responder" as stable disease or progressive disease, as conducted previously.[14] Furthermore, for pathological response, "responder" was defined as pCR or major pathological response (MPR), and "non-responder" as no pCR or MPR. These definitions of response criteria were used for the stratification of samples within the CPIΔ cohorts into CPI-responding and CPI-non-responding tumors, per cohort, throughout the analyses. Additional stratification was applied using OS, splitting tumors into groups of either favorable or poorer clinical outcomes by a median split of OS per cohort. No other filters were used here to stratify patients, as the last follow-up time was not available for the majority of cohorts.

Longitudinal bulk transcriptomic data

Preprocessed transcriptomic data were accessed for every patient-tumor within the CPIΔ study. All cohorts included transcriptomic data from tumor biopsies collected from two specific timepoints; pre-therapy (baseline sample prior to CPI treatment) and on-therapy (after at least one cycle of CPI, ranging from 2 to 9 weeks). Across the CPIΔ cohorts, longitudinal transcriptomic information was available in the form of either RNA sequencing (n=6) or NanoString (n=1) data. For the "breast" cohort, transcriptomic data in the form of RNA sequencing was available pre-therapy only (n=44), whereas paired pre-therapy and on-therapy transcriptomic data were only available in the form of NanoString data, and hence these data were used for the majority of analyses. For the "breast" cohorts, a total of n=25 patients had NanoString data from both pre-therapy and on-therapy timepoints available. The melanoma cohorts ("melanoma (a)" and "melanoma (b)") had transcriptomic data available in the form of RNA sequencing. In total, all n=19 "melanoma (b)" patients had sequencing data available across both timepoints. Only 23/29 'Melanoma a' patients had sequencing data available from both timepoints. For both the urothelial cohorts, RNA sequencing was available across both timepoints for n=64 ('urothelial a') and n=13 ('urothelial b') patients. For analysis, all transcriptomic data, in the form of transcript per million (TPM), was transformed using log2(TPM+1). The exception to this was the RNA sequencing data from the pan-cancer cohort, where data was already log2 transformed and batch normalized for n=30 patients across both pre- and on-therapy timepoints.

CPI1000+ study samples

Transcriptomic data in the form of TPM were leveraged from the CPI1000+ study[14] consisting of pre-therapy RNA-sequencing data across four "melanoma" CPI-naïve cohorts: Liu *et al* (n=66), an advanced melanoma anti-PD-1

treated cohort[31] ; Snyder *et al* (n=20), an advanced melanoma anti-CTLA-4 treated cohort[32]; Hugo *et al* (n=26), an advanced melanoma anti-PD-1 treated cohort[6] ; Van Allen *et al* (n=39), an advanced melanoma anti-CTLA-4 treated cohort.[33] One melanoma CPI-pretreated cohort referred to as "melanoma (c)," Liu *et al* (n=55), an advanced melanoma anti-PD-1 treated cohort.[31] Two bladder cohorts: Snyder *et al* (n=21), an advanced urothelial cancer anti-PD-L1 treated cohort[34]; Mariathasan *et al* (n=331), an advanced urothelial cancer anti-PD-L1 treated cohort.[11] One renal cohort, McDermott *et al* (n=39), a metastatic renal cell carcinoma anti-PD-L1 treated cohort.[12] One lung cohort, Shim *et al* (n=195), an advanced non-small-cell lung cancer (NSCLC) anti-PD-L1 treated cohort.[35] One gastric cohort, Kim *et al* (n=55), an advanced gastric cancer anti-PD-1 treated cohort.[36] Unless otherwise stated, all data were log2(TPM+1) transformed.

Pre-therapy bulk transcriptomic data

For the purpose of comparing pre-therapy expression across both "melanoma (a)" and "breast" CPIΔ cohorts, pre-therapy RNA sequencing data for these cohorts were obtained from the CPI1000+ study.[14] Here, data were processed from raw sequencing reads, and standardized processing/quality control procedures were executed as described previously.[14]

Derivation of published signatures

The following published transcriptomic signatures/marker genes were tested for associations with response to CPI therapy, including 15 pre-therapy-derived signatures/markers: *CD8A*,[37] *CD274*(PD-L1),[38] *CXCL9*,[39] both MHC I and MHC II,[31] CD8 T-cell effector referred to as T-Effector,[12] and the CD8 T-cell effector signature from the POPLAR trial referred to as POPLAR,[40] stroma-EMT,[41]12-chemokine referred to as Chemokine,[42] T-cell inflamed signature referred to as IFNγ,[7] TGFβ pan fibroblast referred to as Pan-F-TBRS,[11] IMPRES,[8] cell proliferation,[43] 15 β-catenin target genes referred to as BCTGs,[44] and cytolytic score referred to as CYT.[45] An addition 17 signatures/markers derived at the on-therapy timepoint were evaluated including, CD8 exhaustion,[46] *CD27*, *CD69*, *CD8B*, *GZMA*, *GZMK*, *HAVCR2*, *ICOS*, *ENTPD1*, *EOMES*, *LAG3*, *PDCD1*, *CTLA4*, *TBX21*, *TIGIT*, *ZAP70*,[16] and *CXCR6*.[47]

TCGA analysis

The Cancer Genome Atlas (TCGA) data across two cancer types (breast cancer and melanoma) were acquired using R software (V.4.0.2). Gene expression data, Workflow Type: HTSeq-Counts and clinical data were downloaded from Genomic Data Commons Data Portal using the R/Bioconductor package TCGAbiolinks V.2.16.4.[48] Clinical data were used to select for CPI-naïve tumor samples and for patients with OS of at least 1 day, yielding 712 breast cancer and 67 melanoma cases. Gene expression counts were normalized using DESeq2 V.1.28.1[49] variance stabilizing transformation function and data were filtered for genes of interest. For survival analyses, OS was estimated from clinical data using "days_to_last_followup" and "days_to_death" and an OS event was defined from "vital_status" (Dead/Alive).

Sampling bias

Sampling bias analyses evaluating genes identified to change in expression on-therapy were conducted using two CPI-naïve-multi-region cohorts, including the TRACERx lung cohort (lung, TRACERx, n=797 regions from 278 patients)[50] and the ADAPTeR cohort (renal, ADAPTeR, n=26 regions from 5 patients).[18] Spatial RNA variation in these treatment-naïve cohorts were calculated as RNA intratumor heterogeneity (ITH) scores as previously performed.[51] Briefly, per tumor, the SDs of expression values for a particular gene across tumor regions were calculated, generating a gene-specific, patient-specific measure of variance. This variance was then summarized across all tumors (median). Generated scores, per given gene, were used to compare the spatial variance to longitudinal variance. Longitudinal variance was captured between longitudinal tumor samples (SD) and summarized across all tumors (median), generated using selected CPIΔ longitudinal cohorts per analysis. Both RNA-ITH and longitudinal variance scores were then compared. This analysis was conducted separately using CPIΔ CPI-responding tumors and CPIΔ CPI-non-responding tumors where appropriate.

Statistic selection

The statistic for capturing RNA expression change between the pre-therapy and on-therapy timepoints:

"Hedge's g"

$$delta = \left(mB - mA \right) / SD * d$$

Where m*A* is the mean expression of a given gene at the pre-therapy timepoint across selected tumor samples and m*B* is the mean expression on-therapy. *SD*d* is the pooled and weighted *SD* across tumor samples, accounting for biased estimates of effect size in small sample sizes.[52]

Statistics and reproducibility

The statistical tests used are indicated in the accompanying figure legends and are two sided, where applicable, unless otherwise stated. All findings were considered significant at a p value threshold of 0.05. Significant p values are indicated within the figures. Plots and graphs were generated with R Studio software (V.4.0.2). Ilustrations were created using BioRender (https://biorender.com).

RESULTS

Assembling the CPI-Dynamics ("CPIΔ") cohort

To assemble the CPIΔ cohort, we leveraged molecular data from six longitudinal CPI studies (table 1; online supplemental figure 1; Methods section). The individual cohorts comprising the CPIΔ study are henceforth referred to as the "breast" cohort (patients with TNBC),

"melanoma (a)" cohort (patients with melanoma, CPI-naïve), "melanoma (b)" cohort (patients with melanoma, prior lines of CPI therapy), "pan-cancer" cohort (head and neck carcinoma, TNBC, high-grade serous ovarian cancer, melanoma, rare solid tumors), "urothelial (a)" cohort, and a "urothelial (b)" cohort.

To explore known biological factors associated with CPI sensitivity, established published transcriptomic signatures and marker genes of CPI response were evaluated. Although the majority of selected signatures encompassed genes associated with immune response and were derived across a variety of cancer types, their pan-cancer applicability was unknown. First, 15 signatures previously derived as informative from pre-therapy samples were evaluated, consisting of both single- and multi-gene markers/signatures with several overlapping genes between signatures. To explore the pan-cancer utility of these signatures, we employed pre-therapy RNA sequencing data from the CPIΔ cohort in addition to 10 pre-therapy cohorts from the CPI1000+ dataset[14] (Methods section). Across the "CPIΔ plus CPI1000+" cohort (16 cohorts, n=1046 patients), each signature was evaluated individually and the generated effect sizes, OR based on RECIST response or pCR (Methods section), and generated SEs were combined in a meta-analysis across cohorts (figure 1A; Methods section).

Sixty percent (9/15) of pre-therapy derived signatures were significantly associated with CPI response in the pre-therapy meta-analysis (figure 1A; online supplemental table 1). However, at the individual cohort-level, for the "breast" pre-therapy CPIΔ cohort, no significant associations were observed (figure 1A; online supplemental table 2). Furthermore, most signatures did not validate in the cancer types in which they had been derived, specifically noted across melanoma (1/7), NSCLC (0/3), urothelial (1/2), and renal (0/1) derived signatures (figure 1A; online supplemental table 2). These data indicate that the majority of established signatures were not informative of CPI response in this breast cancer cohort, and that overall, a minority of RNA signatures are associated with response in more than one cohort.

Next, 17 signatures previously derived as informative of CPI response from on-therapy samples were evaluated using the on-therapy sequencing data from the CPIΔ cohorts. Eighty-eight percent (15/17) of on-therapy derived transcriptomic signatures were significantly associated with response within the on-therapy meta-analysis (figure 1B; online supplemental table 3). In the "breast" CPIΔ cohort, contrary to pre-treatment where no signatures were significantly associated with response, 50% (8/16) of assessable on-therapy derived signatures were significantly associated with response (figure 1B; online supplemental table 4), including markers of immune activation (*ZAP70*, p=0.046) and exhausted T-cell phenotypes (*GZMA*, p=0.020) among other immune checkpoint genes (*LAG3*, p=0.01; *CTLA*-4, p=0.02; TIGIT, p=0.02). These signals of immune reinvigoration could be detected on-therapy in CPI-treated breast tumors, which was not discernible in pre-therapy signature and samples, suggesting that on-therapy gene expression may provide additional biological insight into the impact of CPI on the breast cancer immune microenvironment.

Dynamics of published RNA markers following CPI therapy

Since in the "breast" CPIΔ cohort, the on-therapy derived RNA signatures demonstrated more informative signals on-therapy, we next used expression data for all evaluated gene signatures (both pre- and on-therapy derived) to explore the impact of CPI on transcriptomic expression changes between pre- and on-therapy timepoints at both the cohort- and patient-level, within the "breast" CPIΔ cohort (n=25).

Using this approach, the *CD8A* marker demonstrated a normal distribution of expression pre-therapy, which became flatter on-therapy, suggesting that tumors in this cohort exhibited either no change, an increase, or a decrease in *CD8A* expression after CPI (figure 2A, top). At the patient-level, a wide diversity in RNA trajectories were revealed following CPI therapy for the *CD8A* gene, whereby some tumors with the lowest pre-therapy *CD8A* expression shifted to the highest on-therapy expression levels, and vice versa (figure 2A, bottom). When assessing the dynamic changes in *CD8A* expression on-therapy compared with pre-therapy, we observed a striking association of dynamically increasing *CD8A* with improved favorable survival which would not be possible to determine using pre-treatment *CD8A* expression levels only (figure 2A, bottom). Lastly, correlating on-therapy expression shifts with clinical outcomes demonstrated a significant enrichment (p=0.0048) for favorable survival outcomes (OS>median, Methods section) within tumors with on-therapy increases in *CD8A* expression (77% (10/13)), while 75% (9/12) of tumors with decreased *CD8A* expression had poorer survival outcomes within the "breast" CPIΔ cohort (figure 2A, bottom). Across the remaining transcriptomic signatures, similar dynamics were observations (figure 2B–E). Furthermore, 76% (19/25) of other signatures were significantly enriched for favorable survival outcomes within breast tumors with dynamic on-therapy expression increases (figure 2E).

For the other CPIΔ cohorts, significant enrichments for favorable survival outcomes within tumors with expression increases were observed within the "melanoma (a)" for 13% of signatures (4/32) and the "urothelial (b)" cohort for (6%) of signatures (2/32) (online supplemental figure 2A,B). No other significant differences were observed (online supplemental figure 2C–E). Taken together these results highlight the importance of delineating changes which occur on-CPI as they may hold key biological information about the impact of CPI on the tumor microenvironment. Furthermore, these data emphasize the need to move beyond established gene signatures and examine RNA dynamics across all genes, which may aid in deciphering patterns of response or resistance to CPI in breast cancer.

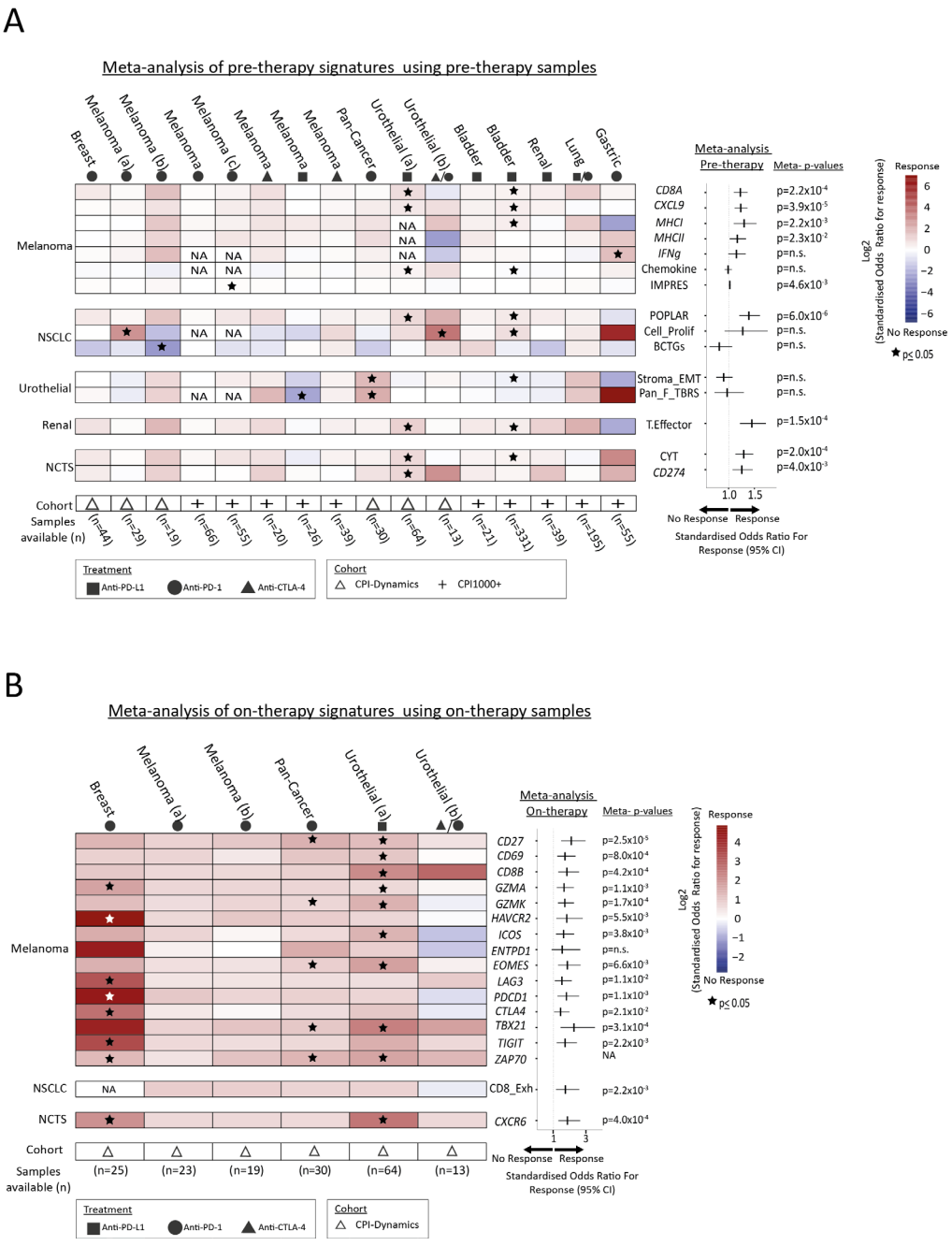

Figure 1 Meta-analysis of RNA signatures associated with CPI response. (A) Pre-therapy meta-analysis. Pre-therapy-derived signatures are shown as rows and individual cohorts within the "CPIΔ plus CPI1000+" cohorts as columns; studies in which each cohort was included are indicated (Δ=CPIΔ; +=CPI1000+; bottom of the heatmap). (B) On-therapy meta-analysis. Here on-therapy-derived signatures are shown as rows and individual cohorts within the CPIΔ cohort as columns. The heatmaps indicates the effect size of each signature in each cohort, measured using the log2 odds ratio (OR) for response versus non-response (RECIST criteria), derived from logistic regression. Red represents an association with response, blue an association with non-response, and significance indicated with an asterisk. Both cohort size (bottom of the heatmap) and drug received (top of the heatmap) are indicated. Signatures are split into the cancer type in which they were derived (left of heatmaps; melanoma, non-small-cell lung cancer (NSCLC), urothelial; renal; NCTS (non-cancer-type-specific)). The forest plots on the right display the overall effect size and significance (p values) of each signature in the meta-analysis across all studies, based on the effect sizes and SEs from each individual cohort. A random-effects model was used for the meta-analysis to account for different cancer types .

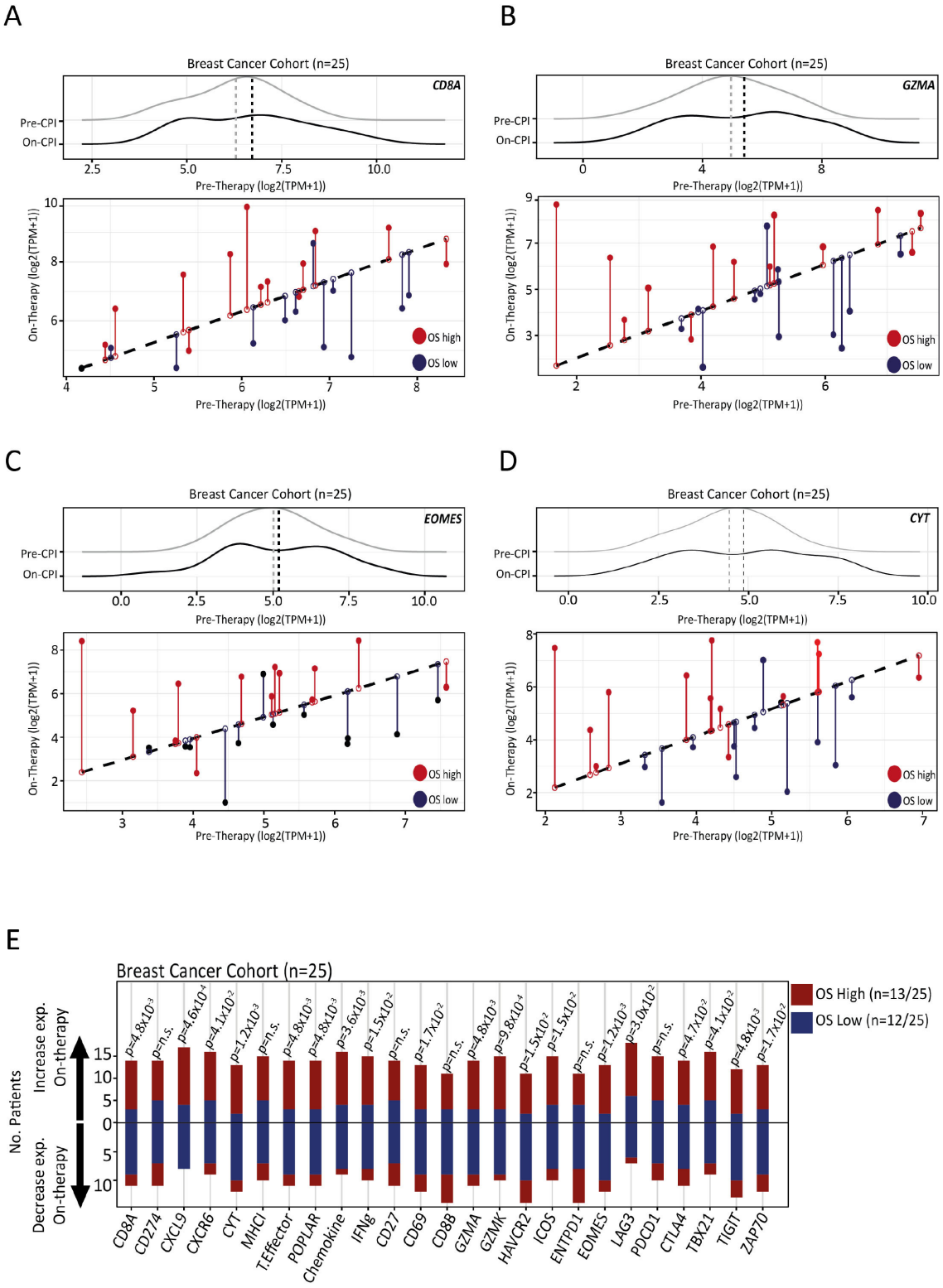

Figure 2 Dynamics of RNA signatures pre-therapy and on-therapy. (A–D) Longitudinal expression of (a) *CD8A*, (b) *GZMA*, (c) *EOMES*, (d) CYT marker genes within the "breast" CPIΔ cohort. Top density plots display pre-therapy (gray curve) and on-therapy (black curve) expression across the breast cancer cohort with longitudinal data available (n=25). Bottom residual plot demonstrates pre-therapy expression (log2(TPM+1)) plotted along the diagonal line (x-axis) and on-therapy (y-axis) expression which is joined to paired patient-level pre-therapy expression with a vertical line. Red indicates cohort stratification into patient-tumors with high overall survival (OS; >median (OS)) and dark blue indicates patient-tumors with low OS (OS<median (OS)). (E) Summary plot of all available signatures (n=25) within the breast cancer cohort. Signatures are depicted on the x-axis and number of patient tumors which either increase (top arrow) or decrease (bottom arrow) in expression for each signature on the y-axis. Bars in red (OS high) and dark blue (OS low) depict patient-tumor stratification based on OS. Significance was tested using a Fisher's exact test and displayed. CPI, checkpoint inhibitor.

RNA dynamics induced by CPI in breast cancer

Previous work has focused on delineating response signals by comparing responding and non-responding breast tumors using static pre-therapy or on-therapy timepoints separately. In light of the dynamic expression changes observed on-therapy, we next explored transcriptional changes associated with CPI treatment across the 778 immune-related genes from the immune NanoString panel, to achieve further insight into the biological mechanisms underpinning response in breast cancer. Here, we sought to quantify RNA dynamics from bulk pre-therapy and on-therapy transcriptomic data by applying the hedges' g statistic (Methods section) and identifying genes that either increase ("positive delta"), decrease ("negative delta") or do not change ("neutral delta") in expression on-therapy within individual CPI response groups (CPI-responding tumors and CPI-non-responding tumors; Methods).

To delineate expression modules with shared or diverging longitudinal patterns following CPI treatment, genes displaying dynamic expression shifts on-therapy were compared between the CPI response groups (figure 3A, diagram left; Methods section), yielding four classifications of genes (figure 3A, diagram right; online supplemental table 5). Two groups of genes were characterized by either positive or negative dynamic shifts in expression in CPI-responding tumors: "Response Pos" with n=135 genes and "Response Neg" with n=7 genes from the "breast" CPIΔ cohort (figure 3A, diagram right; online supplemental table 5). In addition to two groups of genes with either positive or negative dynamic shifts in expression in CPI-non-responding tumors only; "Resistance Pos" with n=7 genes and "Resistance Neg" with n=6 genes from the "breast" CPIΔ cohort (figure 3A, diagram right; online supplemental table 5). Classification of genes was conducted across all CPIΔ cohorts, subsetted to 778 immune genes within the immune NanoString panel. Within the breast cancer cohort 79% of genes (107/135) identified within the "Response Pos" group (figure 3B), 86% of genes (6/7) within the "Response Neg" and "Resistance Pos" (figure 3C; online supplemental figure 3A and tables 6 and 7), and 83% of genes (5/6) within the "Resistance Neg" group demonstrated dynamic expression shifts on-therapy in the "breast" CPIΔ cohort only (online supplemental figure 3B and tables 6 and 7).

Next, to address the limitation of single-region biopsies, which may lead to some identified genes within the "breast" CPIΔ cohort being confounded by tumor sampling bias, longitudinal RNA variation following CPI therapy was compared with spatial RNA variation in treatment-naïve tumors (multi-region samples; table 2) for each gene across CPI-responding and CPI-non-responding tumors (RECIST radiological response) within the CPIΔ "breast" cohort (Methods section). Ninety-eight percent (105/107) of the "Response Pos" genes, 83% (5/6) of the "Response Neg" genes, 50% (3/6) of the "Resistance Pos" genes, and 20% (1/5) of the "Resistance Neg" genes were not confounded by sampling bias, whereby spatial RNA variation underlying tumor sampling bias was surpassed by the changes in RNA expression during CPI therapy within the "breast" CPIΔ cohort (figure 3D).

CPI dynamics and clinical outcomes in breast cancer

After filtering out genes which were confounded by sampling bias (figure 3D), we explored whether the expression changes on-CPI of the remaining 114 genes were associated with clinical outcomes. Patient-level deltas (on-therapy – pre-therapy) expression change values were calculated for each individual gene, and both RECIST radiological response and OS clinical endpoints were evaluated within the "breast" CPIΔ cohort. Data were also leveraged from CPI-naïve-tumors from the TCGA breast cancer datasets, and OS was evaluated to distinguish CPI response signals from the prognostic signals.

Using these last filtering steps, 90/114 genes were significantly associated with favorable clinical outcomes (clinical outcome being OS) in patients with breast cancer treated with CPI (figure 3E, left; online supplemental table 8). Genes comprising this group included those expressed on macrophages (*C1QA* and *C1QB*), T-cells (*CD3G*), myeloid cell expressed genes (*FCGR1A*), chemokine receptors and ligands (*CCR5*, *CXCL10*, *CXCL11*, *XCL2*), IFNγ -induced genes associated with immune infiltration (*GBP1* and *GBP4*), crucial regulators of T-cell and NK-cell proliferation and differentiation (*IL21R*), genes involved in antigen processing (*PSMB9* and *B2M*), and genes expressed by NK cells and other cytotoxic immune cells such as *KLRK1* (*NKG2D*). Furthermore, 13/90 identified genes were also known IO targets (*CD38*, *CD7*, *CD80*, *CD27*, *SLAMF7*, *TIGIT*, *CD274*, *ICOS*, *TNFRSF9*, *JAK2*, *TLR1*, *TLR1*, and *TLR8*).

Crucially, 8/114 genes were identified as significantly associated with CPI response (RECIST radiological response; figure 3E, right; online supplemental table 8). These eight genes included, *FCGR3A*, the low-affinity Fc gamma receptor associated with cytotoxic lymphocytes and critical for antibody-dependent cytotoxicity, *REN*, an aspartic protease that is part of the renin–angiotensin–aldosterone system, *NRAS*, an oncogene associated with poor clinical outcomes in breast cancer, *PIK3R2*, a lipid kinase associated with tumor progression, *CCL3* a chemokine ligand which mediates macrophage chemotaxis and enhancing differentiation of T cells into effector T cells, *COL4A5*, a collagen type 4 alpha 5 chain associated with response to chemotherapy in breast cancer, *AQP9*, an aquaporin family member, and *H2AX*, a variant histone (figure 3E, right; online supplemental table 8). The remaining 16/114 genes were either not associated with clinical outcomes in patients with breast cancer treated with CPI or associated with clinical outcomes in CPI-naïve patients with breast cancer. Together these results suggest that genes identified to be associated with clinical outcomes (RECIST radiological response and OS) may play roles in response to CPI in breast cancer.

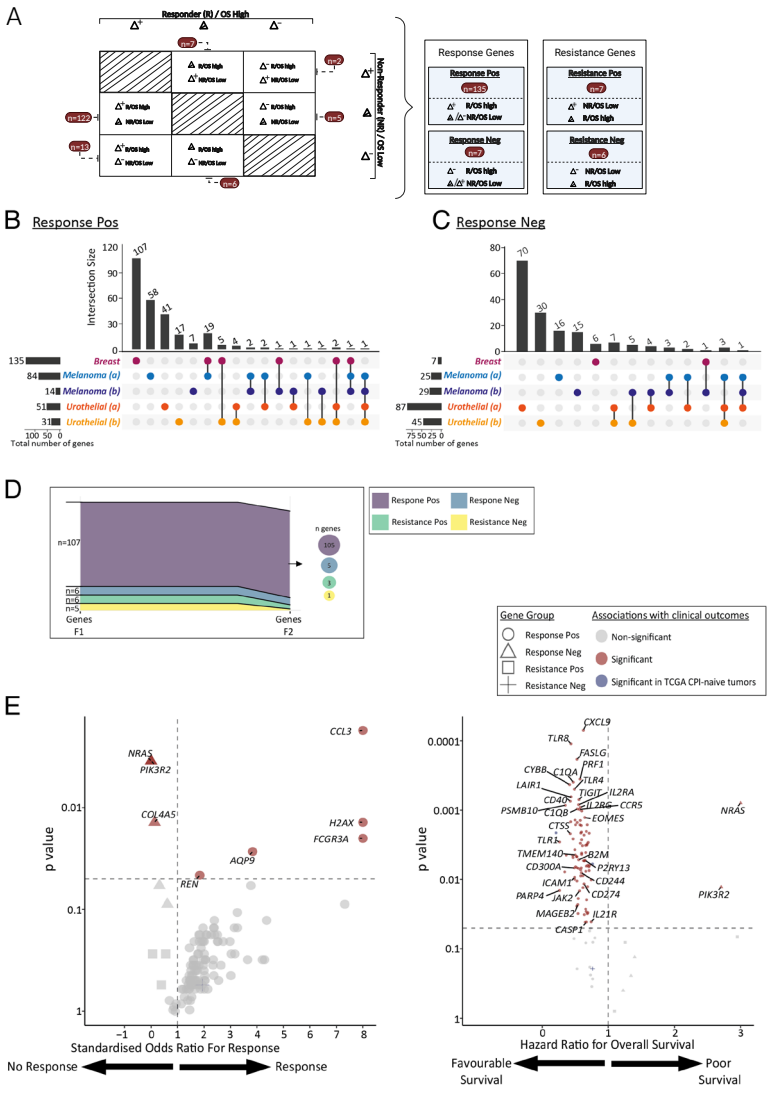

Figure 3 CPI induced gene expression dynamics in breast cancer. (A) Left diagram depicting RNA dynamics (Δ+ "increase," Δ– "decrease," Δ neutral expression change on-therapy) observed across tumors stratified into CPI-responding-tumors (RECIST Responder(R)/OS high (>median OS)) and CPI-non-responding-tumors (RECIST non-responder (NR)/OS low (<median OS)) within the "breast" CPIΔ cohort, with number of genes highlighted in red. Diagram on the right demonstrates the classification of genes into four categories, "Response Pos" (Δ+ in CPI-responding-tumors), "Response Neg" (Δ– in CPI-responding-tumors), "Resistance Pos" (Δ+ in CPI-non-responding-tumors), and "Resistance Neg" (Δ– in CPI-non-responding-tumors) genes, with total genes displayed. The illustrations were created using BioRender (https://biorender.com). (B, C) Upset plots of genes comprising the (B) "Response Pos" and (C) "Response Neg" categories across six of the CPIΔ cohorts, subsetted for 778 genes within the "breast" CPIΔ immune NanoString panel. For each upset plot, the left bar plots indicate the number of genes identified in each gene category per CPIΔ cohort, including "breast" (pink), "melanoma (a)" (light blue), "melanoma (b)" (dark blue), "urothelial (a)" (orange), "urothelial (b)" (yellow). The top bar plot indicates the total number of genes which are unique to each cohort and the number of shared genes between cohorts, with interactions highlighted in the bottom dot plot. (D) Proportion plots depicting gene identified to "increase" or "decrease" in expression on-therapy across gene categories ("Response Pos," purple; "Response Neg," blue; "Resistance Pos," green; "Resistance Neg," yellow) in the "breast" CPIΔ cohort only. Y axis indicates the number of genes identified per gene category at F1 (filter one), followed by the number of genes identified per gene category to not be confounded by sampling bias F2 (filter 2; Methods section) within the "breast" CPIΔ cohort. Analysis was conducted on 778 genes comprising the immune NanoString panel. (E) Scatter plot left, depicting the overall effect size (OR; x-axis) and significance (p value; y-axis) of identified genes (each point) identified within one of the four gene categories ("Response Pos," circle; "Response Neg," triangle; "Resistance Pos," square; "Resistance Neg," cross) and not confounded by sampling bias (results from figure 3D). Associations with clinical outcomes were assessed using RECIST radiological response across the "breast" CPIΔ cohort. Gray indicates genes with no association with CPI response, in red a significant association with CPI response, and in blue associated with clinical outcomes in CPI-naïve tumors within the TCGA dataset. Scatter plot right, depicting the hazard ratio (HR; x-axis) and significance (p value; y-axis) of identified genes. Associations with clinical outcomes were assessed using OS across the "breast" CPIΔ cohort. CPI, checkpoint inhibitor; OS, overall survival; RECIST, Response Evaluation Criteria in Solid Tumors.

Table 2 Treatment-naïve multi-region datasets

Cohort	Cancer type	Patients (n)	Regions (n)	Data type
TRACERx Lung	NSCLC	278	797	RNA-seq
ADAPTeR	Renal	7	26	RNA-seq

Gene-level explorations of differing mechanisms of CPI dynamics between breast cancer versus melanoma

To evaluate differences in biological mechanisms of CPI response between breast cancer and melanoma, we compared pre-therapy expression of the crucial genes identified to be associated with CPI-response (RECIST/OS) in breast cancer (n=98 genes) across both breast and CPI-naïve melanoma tumors ("melanoma (a)" CPIΔ cohort). To objectively compare gene expression data between these two cohorts and limit any variability which may arise due to varied data processing, here pre-therapy RNA sequencing data from these two cohorts were leveraged from the CPI1000+ study, whereby data were processed through the same pipeline (Methods section). In melanoma, 16 genes identified to increase in expression in CPI-responding breast tumors after treatment were expressed significantly higher within the melanoma tumors compared with breast tumors pre-therapy (figure 4A). Furthermore, 4/16 genes are known IO targets (*TLR8*, *TLR4*, *TLR1*, *JAK*). Likewise, two genes, *COL4A5* and *PRK3R2*, which were identified to decrease in expression in CPI-responding breast tumors after treatment, presented significantly lower expression in melanoma compared with breast tumors pre-therapy (figure 4B). These results suggest that patients with melanoma potentially respond better to CPI due to crucial genes already either being highly or lowly expressed pre-therapy and suggest that RNA dynamics following CPI treatment may differ between breast and melanoma tumors, which may partially inform the difference in response rates to CPI.

Next, exploring differences in RNA dynamics between pre-therapy and on-therapy timepoints between breast tumors and melanoma, the same approach used in the "breast" CPIΔ cohort was applied to the melanoma CPI-naïve ("melanoma (a)") CPIΔ cohort, evaluating the 778 genes from the immune NanoString panel. Of the genes identified to only change in expression within the melanoma cohort (genes from the "Response Pos," "Response Neg," "Resistance Pos," and "Resistance Neg" categories) a total of 64/95 genes were not confounded by sampling bias (figure 4C). Twenty-two percent of these genes (14/64) were associated with either response or non-response to CPI therapy, including seven genes which decreased in expression on-therapy in CPI-responding tumors and were significantly associated with non-response (figure 4D). These seven genes included *PMS2*, an essential gene in DNA mismatch repair, and *SOX10*, a melanocyte-inducing transcription factor, *BAMBI*, a TGFβ pseudo-receptor, two genes involved in cancer metabolism, *SLC16A*, a solute carrier family member that plays a role in the transport of lactate and pyruvate, and *LDHA*, a lactate dehydrogenase which converts pyruvate to lactate, and *SNCA*, which codes for alpha-synuclein, that has been associated with enhancing cell survival in melanoma (figure 4D).

Furthermore, three genes increased in expression in CPI-non-responding melanoma tumors and were associated with non-response, including *RAD51*, involved in homologous recombination and DNA repair, and has been linked to immune dysregulation, *MAGEA1*, the melanoma-associated antigen 1, and *BLM*, a helicase family member which plays a role in cellular metabolic processes (figure 4D). Lastly, three genes increased in expression on-CPI in CPI-responding melanoma tumors and were significantly associated with response to CPI in melanoma, including *RORC*, coding for a DNA-binding transcription factor (figure 4D). These genes were not observed to significantly change in expression on-therapy in breast tumors. Taken together, these results demonstrate varied response patterns between CPI-treated melanoma and breast tumors, which warrant further investigation as potential targets for combination therapy for patients with breast cancer.

DISCUSSION

CPIs have been shown to be effective in treating several types of cancer, including melanoma. Nonetheless, response rates in many other cancer types, most notably breast cancer which remains one of the most prevalent cancer worldwide, are far inferior. This differential efficacy could reflect different mechanisms of response to treatment within breast cancer and the complexities of the immune system's response to the disease. Here, we explored the transcriptomic response of cancers to CPI treatment through the analysis of our CPIΔ cohort. Our analysis of both pre- and on-therapy-derived published signatures demonstrated that most cancer-type-derived signatures did not inform clinical outcomes in the cancer type they were derived from. This lack of reliability highlights the potential differences in signals of response across tumor types. In the breast cancer cohort, unlike pre-therapy signatures, 50% of on-therapy signatures were associated with response. On-therapy signals within the breast cancer cohort were consistent with signals of immune reinvigoration of T-cell exhausted phenotypes on-therapy, as suggested by genes such as *CTLA-4*, *HAVCR2* (*TIM-3*), and *GZMA*, which have been shown to increase in expression following anti-PD-1 in melanoma.[16]

By exploring transcriptomic changes on CPI in the "breast" CPIΔ cohorts, we identified key breast cancer-specific correlates of CPI response. We identified eight genes which change in expression on-therapy and may define CPI response within breast cancer, including *FCGR3A*, an Fc gamma receptor, for which high expression on NK cells has been functionally shown to cause higher

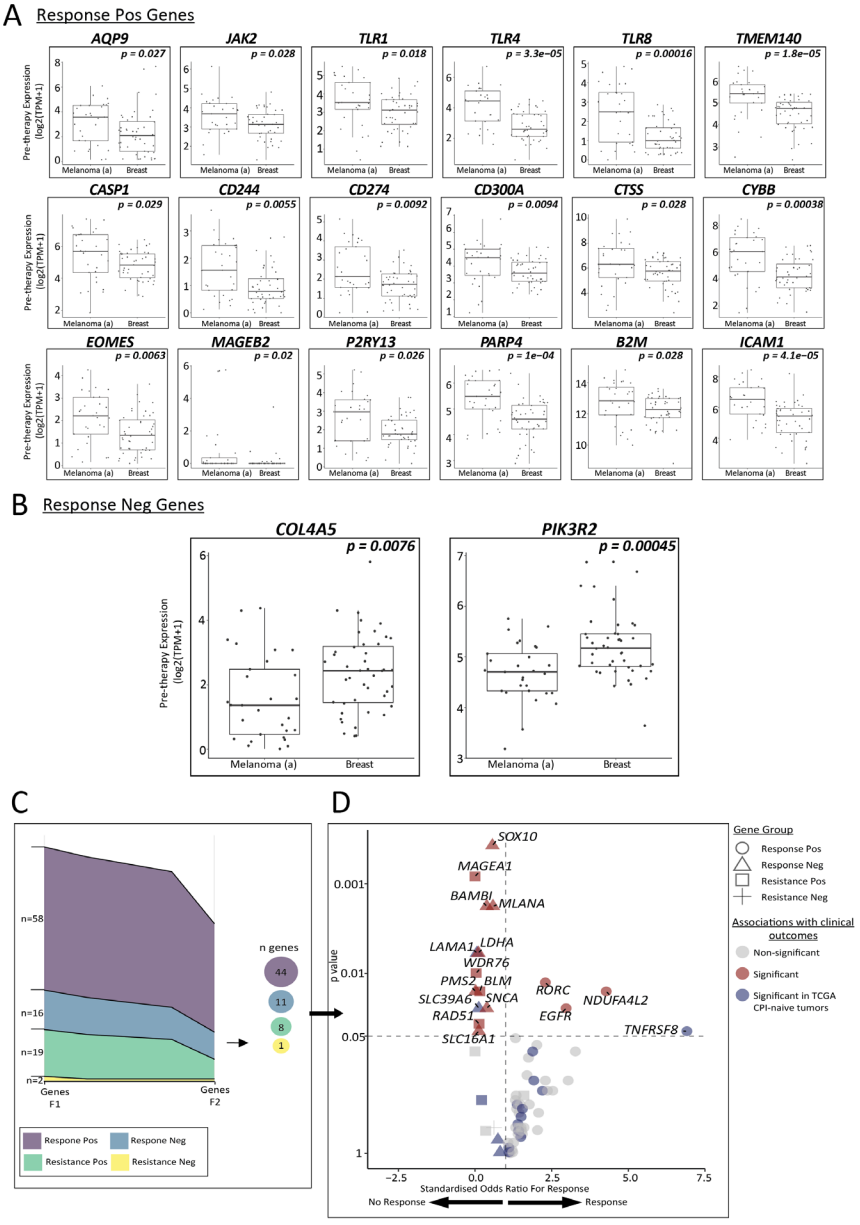

Figure 4 Differential mechanisms of CPI dynamics breast cancer versus melanoma. (A) Box plots comparing pre-therapy expression (y-axis) of genes identified to increase in expression on-therapy (Response Pos genes) and be associated with clinically favorable response to CPI in breast cancer (figure 2E, right), within the "melanoma (a)" and "breast" CPIΔ cohort (x-axis). This analysis used RNA sequencing data processed from FASTq to TPM matrix through a standardized pipeline allowing for comparison across both cohorts. Significance was tested using a Wilcoxon test and displayed. (B) Box plots comparing pre-therapy expression (y-axis) of genes identified to decrease in expression on-therapy (Response Neg genes) and observed to be associated with no response to CPI breast cancer (figure 2D, right), within the "melanoma (a)" and "breast" CPIΔ cohort (x-axis). Data used here were RNA sequencing across both cohorts processed from FASTq to TPM matrix through a standardized pipeline allowing for comparison. Significance was tested using a Wilcoxon test and displayed. (C) Proportion plots depicting gene identified to "increase" or "decrease" in expression across gene categories ("Response Pos," purple; "Response Neg," blue; "Resistance Pos," green; "Resistance Neg," yellow) on-therapy across the "melanoma (a)" CPIΔ cohort only. Y axis indicates the number of genes identified per gene category at F1 (filter one), followed by the number of genes identified per gene category to not be confounded by sampling bias F2 (filter 2; Methods section) within the "melanoma (a)" CPIΔ cohort. This analysis was conducted on 778 genes comprising the immune NanoString panel. (D) Scatter plot depicting the overall effect size (OR; x-axis) and significance (p value; y-axis) of identified genes (each point) identified within one of the four gene categories ("Response Pos," circle; "Response Neg," triangle; "Resistance Pos," square; "Resistance Neg," cross) and not confounded by sampling bias (results from figure 3D). Associations with clinical outcomes were assessed using RECIST radiological response across the "Melanoma (a)" cohort. Gray symbols indicates genes with no association with CPI response, in red a significant associations with CPI response, and in blue associated with clinical outcomes in CPI-naïve tumors within the TCGA dataset. CPI, checkpoint inhibitor; RECIST, Response Evaluation Criteria in Solid Tumors.

anti-PD-L1 antibody-dependent cellular cytotoxicity lysis of tumor cells.[53] Also, *CCL3*, a chemokine ligand, overexpression of which in colorectal cancer mouse models led to the rapid regression of tumors, increased proliferation, and functionality of tumor-infiltrating T cells and accelerated tumor-regression on CPI (anti-PD-1).[54] These genes may play roles in response to CPI in breast cancer and are putative targets for combination therapy, which in turn could aid in the delivery of immuno-oncology and enhancing CPI efficacy within breast cancer.

In parallel, we also identified 90 genes in breast cancer associated with favorable clinical outcomes in patients with breast cancer treated with CPI including *CCL5*, XCL1, *XCL2*, *KLRK1*, and *GZMB*, associated with the presence of functional cytotoxic NK cells.[55–58] Of these genes, *KLRK1* (*NKG2D*) has been reported not only to enhance the cytotoxicity of NK cells, aiding in the elimination of tumor cells, but also facilitate the activation of breast-resident vδ1 cells[57 59–62] and has been associated with good prognosis in breast cancers.[58] Together these results suggest the presence of functional cytotoxic NK cell phenotypes and other cytotoxic immune cells within the breast cancer TME may play a critical role in response to CPI in breast cancer.

We identified 18 genes which significantly increase in expression on-CPI in breast cancer responders but were already highly expressed in melanoma pre-therapy. These 18 genes included *B2M*, a component in MHC-I antigen processing and presentation,[63] and 3 Toll-like receptor genes (*TLR1*, *TLR4*, and *TLR8*), which have been suggested as potential IO targets.[64] Importantly, multiple TLR agonists have been investigated in combination with CPI to enhance efficacy across various cancer types.[65–68] Furthermore, of the genes identified to decrease in expression on-CPI in breast cancer, *PIK3R2* was already lowly expressed in melanoma tumors pre-therapy and has been associated with repressed cell proliferation, invasion, epithelial–mesenchymal transition, and cell apoptosis in melanoma cells,[69] which could hold potential applicability in breast cancer. These genes warrant further investigation and may be potential targets for combination therapy in IO refectory cancers like TNBC, particularly genes such as *TLR8*, *TLR4*, and *TLR1*, which are known IO targets.

We also identified 14 genes associated with either CPI response or non-response within the melanoma CPIΔ cohort, which did demonstrate significant expression changes on-CPI within the breast cancer cohort, including *SOX10*, *SLC16A*, *SNCA*, *BAMBI*, and *RAD51*. Of the genes identified to decrease in expression in melanoma responding tumors, *SOX10* has been shown to hinder immunogenicity in melanoma cell lines through the *IRF4–IRF1* axis and regulate PD-L1 expression.[70] Furthermore, suppression of *SOX10* has demonstrated increased efficacy to combination therapy including anti-PD-1 in melanoma.[70] *SLC16A*, has been associated with lower OS across cancer types,[71 72] and in breast cancer *SLC16A* has been associated with more aggressive phenotypes.[73] Knockout of *SNCA* in melanoma cells has been associated with higher rates of apoptosis in xenografts,[74] suggesting that lower expression observed in melanoma tumors treated with CPI may aid in cancer cell elimination, whereas knockout of *BAMBI*, has been associated with growth inhibition in TNBC cell lines.[75] Of the genes identified to increase in expression in CPI-non-responding tumors, *RAD51*, has been associated with poor survival in breast cancer and other cancer types, as well as with increased genomic instability and immune dysregulation.[76] These results highlight key genes associated with response to CPI in melanoma, which warrant further study to investigate their biological effect on immune response in the context of CPI, in addition to providing additional targets for combination therapy, which may prove to also be beneficial in the treatment of breast cancer.

Our study is not without limitations. The biggest challenge faced in longitudinal CPI response analyses is the difficulty in obtaining paired data from CPI-treated patients, resulting in small cohorts for such analyses, in addition to varied timing of biopsies on-treatment across cohorts, which may influence the biological composition of the TME following CPI therapy. Despite this, our study is the largest meta-analysis of its kind and provides valuable insights into the biology of CPI-mediated immune response. A further complication in leveraging data from multiple studies is the lack of standardized reporting of covariates such as patient demographics and detailed treatment histories. However, our study aimed to mitigate this limitation where possible by selecting cohorts with metastatic and advanced disease only, in addition to restricting more detailed comparisons exclusively between cohorts of patients reported as CPI-naïve prior to entering the trials. We explored data from various cohorts with RNA sequencing and NanoString data which were not unified in a single pipeline, which could potentially affect the comparability of the data. Nevertheless, the aforementioned limitation was addressed where possible by leveraging RNA sequencing data processed through a unified pipeline when conducting head-to-head comparisons of gene expression of identified genes. Furthermore, our meta-analyses still provide important insights into the biology of CPI-mediated immune response and contributes to an advancement of the field. However, the use of transcriptomic data generated from different platforms may lead to limitations in conclusions that could be drawn, as analyses were limited to 778 genes from the NanoString panel for comparison between cohorts as opposed to genome-wide, which may restrict our analysis. Although much of our data were obtained from a single sampled biopsy, which could introduce sampling bias, this limitation was mitigated by validating our findings in orthogonal multi-regional datasets to ensure that the intra-sample longitudinal variability was larger than the spatial treatment-naïve variability. However, we acknowledge the limitations introduced by the lack of concordant cancer type and stage-matched multi-region datasets

used. Nonetheless, our study creates a wealth of opportunities for further research to validate and expand on these findings, aiming to improve personalized treatment options for patients with cancer .

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Data availability statement Data are available in a public, open access repository. Data are available upon reasonable request. The tumor region RNA sequencing data (in each case from the TRACERx study) used during this study has been deposited at the European Genome–phenome Archive (EGA), which is hosted by The European Bioinformatics Institute and the Centre for Genomic Regulation under the accession code under the accession code EGAS00001006517 (RNA sequencing); access is controlled by the TRACERx data access committee. Details on how to apply for access are available on the linked page. Details of all published datasets obtained from third parties used in this study are as follows. Data are available on reasonable request. Multi-region RNA sequencing data from the ADAPTeR trial (NCT02446860) are available at EGA (EGAD00001008163). For the "breast" cohort, from the TONIC trial (NTC02499367), RNA sequencing data have been deposited in the European Genome phenome Archive (EGA) under accession number EGAS0001003535 and will be made available from the corresponding author on reasonable request. Data requests for RNA sequencing and NanoString data will be reviewed by the institutional review board of the Netherlands Cancer Institute (NKI) and applying researchers will need to sign a data access agreement with the NKI after approval. RNA sequencing from the "urothelial (a)" cohort from the ABACUS trial (NCT02662309) is available at EGA (EGAC00001001602) and from the "urothelial (b)" cohort from the NABUCCO trial (NCT03387761) is available at EGA (EGAS00001001648). Data are available in a public, open access repository. RNA sequencing data from the "melanoma (a)" and "melanoma (b)" cohorts are available at the Gene Expression Omnibus under GSE91061. "Pan-cancer" cohort transcriptomic and clinical data from the INSPIRE trial (NCT02644369) were downloaded as SourceData_Fig4.zip from Yang et al (https://doi.org/10.1038/s41467-021-25432-7). The dataset for the 2 TCGA cancer cohorts can be downloaded from the genomic data commons data portal (https://portal.gdc.cancer.gov/).

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