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Rationale and feasibility of a rapid integral biomarker program that informs immune-oncology clinical trials: the ADVISE trial

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ABSTRACT

Background ADVISE (ADaptiVe biomarker trial that InformS Evolution of therapy) (NCT03335540) was a biomarker-adapted feasibility clinical trial of immunohistochemistry (IHC) to inform combination immuno-oncology (I-O) treatment.

Methods To inform I-O combination selection, messenger RNA expression analyses from The Cancer Genome Atlas evaluated associations between programmed death 1/programmed death ligand 1 (PD-1/PD-L1) and other I-O-associated genes. Tumor tissue blocks of melanoma, non-small cell lung cancer, renal cell carcinoma, urothelial carcinoma, squamous cell carcinoma of the head and neck, and gastroesophageal junction/gastric cancer were stained by IHC to assess expression of CD8, colony-stimulating factor 1 receptor, glucocorticoid-induced tumor necrosis factor receptor (GITR), indoleamine 2,3-dioxygenase 1, lymphocyte-activation gene 3, NKp46, forkhead box P3, and PD-L1. These results facilitated an I-O treatment selection algorithm where patient biopsy results dictated allocation into combinations of nivolumab with cabiralizumab, urelumab, linrodostat mesylate, relatlimab, BMS-986156 (anti-GITR), lirilumab, ipilimumab, or irradiation. The primary endpoint was the proportion of patients with qualified baseline tumor biopsy specimens where decision-enabling biomarker analysis was completed within 12 business days to select an I-O combination therapy.

Results Correlation of PD-1/L1 and I-O-associated genes varied across the spectrum of T-cell-inflamed versus non-inflamed tumors; however, tumors with low/intermediate *PD-L1* expression demonstrated distinct upregulation of immune markers grouped by cell type (T cell, macrophage, etc). IHC analyses of I-O naïve tumors corroborated these findings with distinct immune target upregulation in low-to-intermediate inflamed tumors and significant associations between IHC-detected markers and T-cell inflammation score across most markers. In the clinical trial, 20/23 (87%) of eligible patients were successfully allocated and started on treatment within the 12-day window, meeting the primary endpoint. The safety profile appeared to generally align with those reported for the individual combinations from other trials. No treatment responses occurred. Most patients were allocated to the cabiralizumab treatment arm.

Conclusions Actualization of a patient-specific I-O combination treatment selection strategy is feasible, however, determination of de novo integral biomarker thresholds of novel I-O targets to facilitate effective treatment of PD-1-refractory cancer remains fraught. These data emphasize the difficulty of integral biomarker development for I-O in translating from immunotherapy treatment-naïve biospecimens to the selection of patients in the PD-1-refractory state.

Trial registration number NCT03335540.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Robust biomarkers for selection of immuno-oncology (I-O) combinations in the anti-programmed death-1 (PD-1)-resistant setting remain elusive. The ADaptiVe biomarker trial that InformS Evolution of therapy (ADVISE) study investigates the feasibility of identifying biomarkers from tumor tissue to inform combination I-O therapy choice in patients with selected solid tumors within a 12-day window.

WHAT THIS STUDY ADDS

⇒ The ADVISE study confirmed the use of real-time biomarker analyses to inform the selection of combination I-O therapies was feasible. However, this approach did not result in treatment responses, but instead highlighted the challenges of translating pretreatment tumor assay validation into clinically relevant biomarkers for successful immunotherapy of PD-1-refractory cancer.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Results from the ADVISE trial indicate that future studies validating I-O combination treatment guided by specific biomarkers would benefit from the use of a larger patient population, as well as optimized biomarker cut-offs that are tailored toward the specific tumor type and prior I-O therapy experience.

BACKGROUND

Immuno-oncology (I-O) has revolutionized cancer treatment paradigms and

improved outcomes,[1] yet most patients do not benefit. Tumor microenvironment (TME) analysis has suggested multiple complementary mechanisms for I-O combination therapy to counteract tumor-induced immunosuppression.[2] Biomarkers (eg, programmed death ligand 1 (PD-L1), microsatellite instability status, and tumor mutational burden) allow identification of patients who may be more likely to benefit from anti-programmed death 1 (PD-1)/PD-L1-based therapy,[3–5] however, robust selection markers for I-O combinations in the PD-1-resistant setting remain elusive. Integration of tumor and immune-cell profiling via real-time tumor biomarker data might advance the potential for personalized I-O combinations. Previous clinical trials, such as the BATTLE (Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination)[6] and NCI MATCH (National Cancer Institute Molecular Analysis for Therapy Choice)[7] protocols, demonstrated the feasibility of adaptive trials for targeted therapies. However, these studies required only modest tissue for DNA sequencing. In contrast, assessment of I-O biomarkers requires substantially larger biopsies for evaluation of the TME. The feasibility of identifying multiple biomarkers from tumor tissue on a per-patient level to inform the selection of combination I-O agent therapies in a clinical trial setting has not been previously determined.

The ADaptiVe biomarker trial that InformS Evolution of therapy (ADVISE; NCT03335540) was a multicenter, open-label, adaptive phase 1 study designed to assess the clinical feasibility of using immunohistochemistry (IHC) results from a pretreatment biopsy to direct personalized I-O combination treatments in patients with selected solid tumors within a defined and rapid turnaround time frame.

Here, the translational rationale and design of the ADVISE trial are described, along with feasibility and clinical outcomes of the study. These data emphasize the upregulation of a distinct set of immune-related therapeutic targets in tumors with low-to-intermediate levels of T-cell inflammation, supporting the hypothesis that targeted combination immunotherapies may provide clinical benefit and inform the development of a biomarker-based treatment decision algorithm. The feasibility of applying a patient-specific, IHC-derived, biomarker-based platform to inform the selection of combination I-O therapies was confirmed. However, this approach did not result in treatment response, emphasizing the complexity of translating pretreatment tumor assay validation into clinically relevant integral biomarkers for successful immunotherapy of PD-1-refractory cancer.

METHODS

Translational analyses using messenger RNA (mRNA) data from The Cancer Genome Atlas (TCGA) and IHC and mRNA data generated from commercially acquired formalin-fixed, paraffin-embedded (FFPE) tumor blocks formed the basis for the ADVISE study design. The study protocol is available in supplemental file 2.

RNA expression analyses

TCGA samples were clustered into T-cell-inflamed cohorts using a defined T-cell-inflamed gene signature, as previously published.[8] As a marker of T-cell inflammation, samples were stratified by quartiles of *PD-L1* expression (low, low/intermediate, intermediate/high, high) and associated with individual gene transcript expression for the potential combination therapies to be administered as combination therapies with nivolumab in the ADVISE trial. In the TCGA melanoma cohort (n=354), correlations were evaluated between PD-L1 and other immune biomarkers (glucocorticoid-induced tumor necrosis factor receptor (GITR), forkhead box P3 (FOXP3), indoleamine 2,3-dioxygenase 1 (IDO1), lymphocyte activation gene-3 (LAG-3), colony-stimulating factor 1 receptor (CSF-1R), T-cell immunoglobulin mucin 3, cytotoxic T-lymphocyte antigen-4 (CTLA-4), and killer cell immunoglobulin-like receptor 3DL1).

Gene expression was measured in a subset of the commercially obtained subset of FFPE blocks (n=128) using the HTG EdgeSeq system Oncology and Immuno-Oncology Biomarker Panels (HTG Molecular, Tucson, Arizona, USA). Data were transformed into log2 trimmed mean of M-values normalized counts per million before analysis, based on manufacturer's instructions.[9] Gene expression data were used to generate T-cell-inflamed signature profiles.[8 10 11] *PD-1* was selected as the relevant I-O target because nivolumab disrupts PD-L1/programmed death ligand 2 binding to PD-1, thus identifying co-expression patterns of *PD-1* and other I-O therapy targets that might inform potential therapeutic combinations.

Gene expression profiling analyses supporting ADVISE trial design

The immune markers assessed in this study were direct or hypothesized targets of specific I-O therapies with mechanisms of action considered complementary to nivolumab to enhance or restore an innate or adaptive antitumor immune response. These immune markers targeted CTLA-4,[12] LAG-3,[12 13] KIR2D,[12 14] IDO1,[15–18] CSF-1R,[19 20] and GITR.[21] Agents that modulate these immunologically relevant targets included ipilimumab (anti-CTLA-4 monoclonal antibody (mAb)), relatlimab (anti-LAG-3 mAb), lirilumab (anti-KIR2D mAb), linrodostat mesylate (an IDO1 inhibitor), cabiralizumab (anti-CSF-1R mAb), and BMS-986156 (anti-GITR mAb), which have demonstrated tolerability when dosed in combination with nivolumab.[3 22–31]

Tumor samples used for validation of I-O target expression

Cohorts of solid tumors were analyzed by IHC including urothelial carcinoma (UC/bladder, Avaden); gastroesophageal junction (GEJ)/gastric cancer (GC) (Avaden, Bristol Myers Squibb (BMS)); melanoma, non-small cell

lung cancer (NSCLC), or squamous cell carcinoma of the head and neck (SCCHN) (Avaden, CHTN, or Sofia Bio); or renal cell carcinoma (RCC, Avaden, CHTN, NDRI, Sofia Bio).

Quantitative immunohistochemistry analysis of tumor samples

FFPE blocks of tumors including melanoma (n=40), NSCLC (n=41), RCC (n=43), UC (n=40), SCCHN (n=40), or GEJ/GC (n=41) were profiled by IHC. Tumor sections were stained using antibodies directed against potential therapeutic decisional markers IDO1 (BMS 1E7, Leica Bond Rx), LAG-3 (LS Biosciences 17B4, Leica Bond Rx), FOXP3 (Abcam 236A/E7, Dako Link 48), GITR (BMS 6G10, Leica Bond Rx), CSF-1R (BMS 31F, Leica Bond Rx), NKp46 (Innate Pharma 8E5B, Dako Link 48), PD-L1 (Dako 28–8, Dako Link 48), and cluster of differentiation 8 (CD8) (Dako CD8/144B, Dako Link 48), and for PD-1 using the Leica Biosystems BOND Rx or Dako Link 48 platforms (online supplemental table S1). Biomarker expression was assessed semiquantitatively by digital image analysis of whole slide images. The primary readout was the total number of cells expressing a biomarker of interest divided by the total number of cells in the tumor and tumor-associated stroma expressed as a percentage. Data were generated by percent positivity in the tumor and TME using image analysis algorithms. Aperio (Leica Biosystems) Nuclear V9 was used for the LAG-3, FOXP3, GITR, and CD8 image analysis algorithm and HALO (Indica Labs) cytonuclear with nuclear localization module was used for the IDO, CSF-1R, and NKp46 algorithm development. Each result was reviewed by the team pathologist to confirm the region of interest (ROI) annotation and algorithm performance. If the image algorithm failed quality control (eg, cell segmentation, cell-level annotation as positive/negative within the specified ROI), the specimen was scored by visual review of the digital image. Tumor cell PD-L1 as measured by tumor proportion score (TPS) was scored manually. Assays were validated following College of American Pathologists guidelines: ≥40 specimens for each tumor type were stained and analyzed for each marker encompassing a broad dynamic range of expression; interday and intraday precision data were generated.

PD-L1 as measured by TPS and CD8 expression as measured by immune cell IHC was used to assess overall tumor inflammation, with levels ≥1% considered positive for both markers. The dynamic range of expression for each biomarker generated from the assay validations was utilized to define categorical cut-offs for low, medium, and high expression. The high expression cut-offs were set to capture specimen outliers, consistent with the underlying study hypothesis that a high level of biomarker expression indicates a greater tumor dependency on that pathway in impairing the immune response, and will predict efficacy from the respective treatment in combination with nivolumab. Tertile cut-offs (low, medium, high) for each of the six tumor types (UC, GEJ/GC, melanoma, NSCLC, SCCHN, RCC) were averaged to create one set of combined cut-offs across tumors per marker.

ADVISE clinical trial design for assessing the feasibility of real-time biomarker analyses

In the ADVISE study, previously treated patients (aged≥18 years) with ≥2 lesions with measurable disease (per Response Evaluation Criteria in Solid Tumors V.1.1) and selected solid tumors (melanoma, NSCLC, RCC, UC, SCCHN, or GEJ/GC) were enrolled in one of seven nivolumab-based I-O treatment arms based on the biomarker analysis results from baseline tumor biopsy (for full patient selection criteria, see online supplemental appendix A1). Of the seven proposed treatment arms, six investigated nivolumab in combination with other I-O agents (figure 1).[1 11–16 18–21 27 29–33] The combination of nivolumab and stereotactic body radiation therapy (SBRT) was proposed for patients with tumors with low expression of PD-L1 and CD8.

Tissue sampling and feasibility assessment across clinical study sites

The feasibility of using real-time IHC-derived, biomarker-based evaluation of a fresh biopsy from patients was assessed across three sites (University of Chicago, Johns Hopkins, and UPMC Hillman Cancer Center) in the ADVISE trial. Mandatory biopsies were collected at baseline. Evaluable biopsies were defined as those comprizing at least four core needle biopsies or incisional/excisional biopsy of approximately equivalent size of four core needle biopsies, with ≥30% tumor content. Sufficient stromal content to assess the immune infiltrate and associated biomarkers was also required.

During the assay validation process and for the first part of the study, when patients were enrolled only at the University of Chicago, some IHC data underwent peer review by BMS pathologists, during which time a predefined reconciliation process was not in place. Beginning with patient enrollment at Johns Hopkins and UPMC, all results underwent peer review by external pathologists who had received training on the study and biomarker assessment methodology. Interpretive differences were reconciled between peer review pathologists and primary study pathologists through a consensus meeting.

The primary endpoint was the proportion of patients with qualified tumor biopsy specimens at baseline with decision-enabling biomarker analysis completed within 12 business days from tumor biopsy. If ≥66% of patients had biopsies collected with the required IHC assays performed and the results provided to the investigator within 12 business days after the biopsy was taken, then the study approach was determined to be adequate.

Statistical analyses

In the TCGA and gene expression analyses, correlations between *PD-1* and other immune biomarkers were assessed by Pearson correlation coefficient. Unsupervised

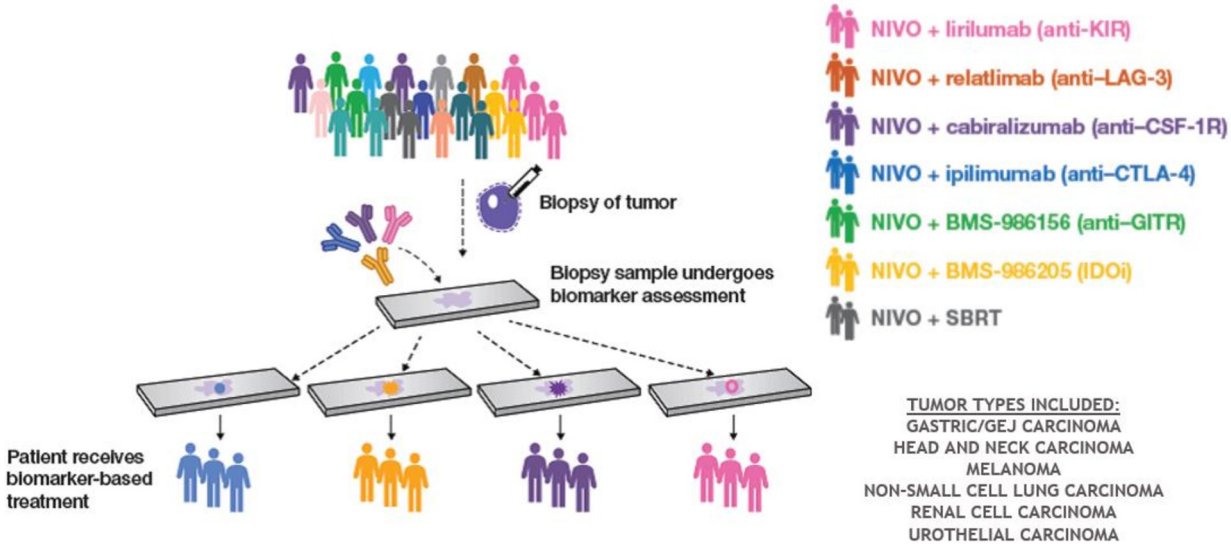


Figure 1 ADVISE trial schema. ADVISE, ADaptiVe biomarker trial that InformS Evolution of therapy; CSF-1R, colony-stimulating factor 1 receptor; CTLA-4, cytotoxic T-lymphocyte antigen-4; GEJ, gastroesophageal junction; GITR, glucocorticoid-induced tumor necrosis factor receptor; IDO1, indoleamine 2,3-dioxygenase 1; KIR, killer cell immunoglobulin-like receptor; LAG-3, lymphocyte activation gene-3; NIVO, nivolumab; SBRT, stereotactic body radiation therapy.

clustering was performed using the Ward method and Euclidean distance.[34] For supervised clustering results, samples were presorted by low to high *PD-L1* expression. The 10-gene interferon-gamma (IFN-γ) signature (*CCR5, CXCL10, CXCL11, CXCL9, GZMA, HLA-DRA, IDO1, IFNG, PRF1, STAT1*) was described and published previously.[10]

The IFN-γ signature score was calculated by the arithmetic mean of 13 gene expression levels (*CD8A*, *CCL2*, *CCL3*, *CCL4*, *CXCL9*, *CXCL10*, *ICOS*, *GZMK*, *IRF1*, *HLA-DMA*, *HLA-DMB*, *HLA-DOA*, and *HLA-DOB*).[2 10 35]

In the IHC analyses, unsupervised clustering was performed using Ward's method with scaling based on *z*-score. Tertiles of CD8 expression in melanoma, NSCLC, RCC, SCCHN, and GEJ/GC, or IFN-γ expression in UC samples (N≈38 each) were added to the figures for reference. IFN-γ signature score[10] was compared with expression of decisional biomarkers by IHC using the Wilcoxon rank-sum test. For supervised clustering results, samples were ordered by CD8 expression (low to high), with the remaining markers ordered based on high-to-low Spearman correlation to CD8 status.

RESULTS

Characterization of the relationship between individual immune relevant gene expression and T-cell-inflamed status in the TME

Pan-tumor RNAseq data from TCGA and tumor specimen IHC analysis were used to assess the complexity and diversity of the relationship between a panel of immune-related or cancer-related genes and overall T-cell-inflamed gene expression. Using this approach, TCGA samples were categorized as inflamed, non-inflamed, and intermediate based on a previously published T-cell inflamed gene signature.[8 11 36] A collection of immune-related and cancer-related genes, curated from the literature[2] and commercially relevant gene panels,[37] was correlated with *PD-1* across levels of T-cell inflammation. Figure 2A,B comprehensively present the analysis of more than 1567 genes and a selection of approximately 100 highest and lowest association genes with PD-1 expression and PD-1 expression in T cells that are inflamed or non-inflamed. Notable genes strongly associated with *PD-1* expression in inflamed tumors beyond those relevant to the ADVISE trial (*CD8*, *CD274 (PD-L1)*, *CTLA4*, *LAG-3*, *IDO1*, *CSF-1R*, *GITR*, and *KIR* genes) included but were not limited to immune-checkpoints such as *TIGIT*, transcription factors associated with antigen presentation such as *BATF3*, and type I IFN-associated pattern recognition receptors such as *TLR9*.[38] Ingenuity pathway analysis of the genes identified as strongly associated with *PD-1* identified pathways included Th1 and Th2 activation pathway, crosstalk between dendritic cells and natural killer cells, and antigen presentation pathway. Conversely, notable genes inversely correlated with *PD-1* expression include but are not limited to those with known mechanisms of immune exclusion such as WNT/β-catenin signaling (*CTNNB1* and *GSK3B*), hedgehog signaling (*SMO* and *PTCH1*), and PI3K signaling (*PIK3CA* and *EIF4B*).[8]

Co-expression of genes with *PD-1* was then stratified by T-cell-inflamed status (inflamed, intermediate, or non-inflamed). A weaker correlation was observed between the expression of *PD-1* and other immune genes from the strongly correlated gene cluster (399 genes) in non-T-cell-inflamed tumors compared with those that were T-cell-inflamed (p=0.00017, two-sided Wilcoxon signed-rank test; figure 2B). These results suggest potentially distinct profiles of immune gene signatures in non-T-cell-inflamed versus T-cell-inflamed tumors.

To refine observations made across all tumors of TCGA, the metastatic melanoma cohort was more specifically

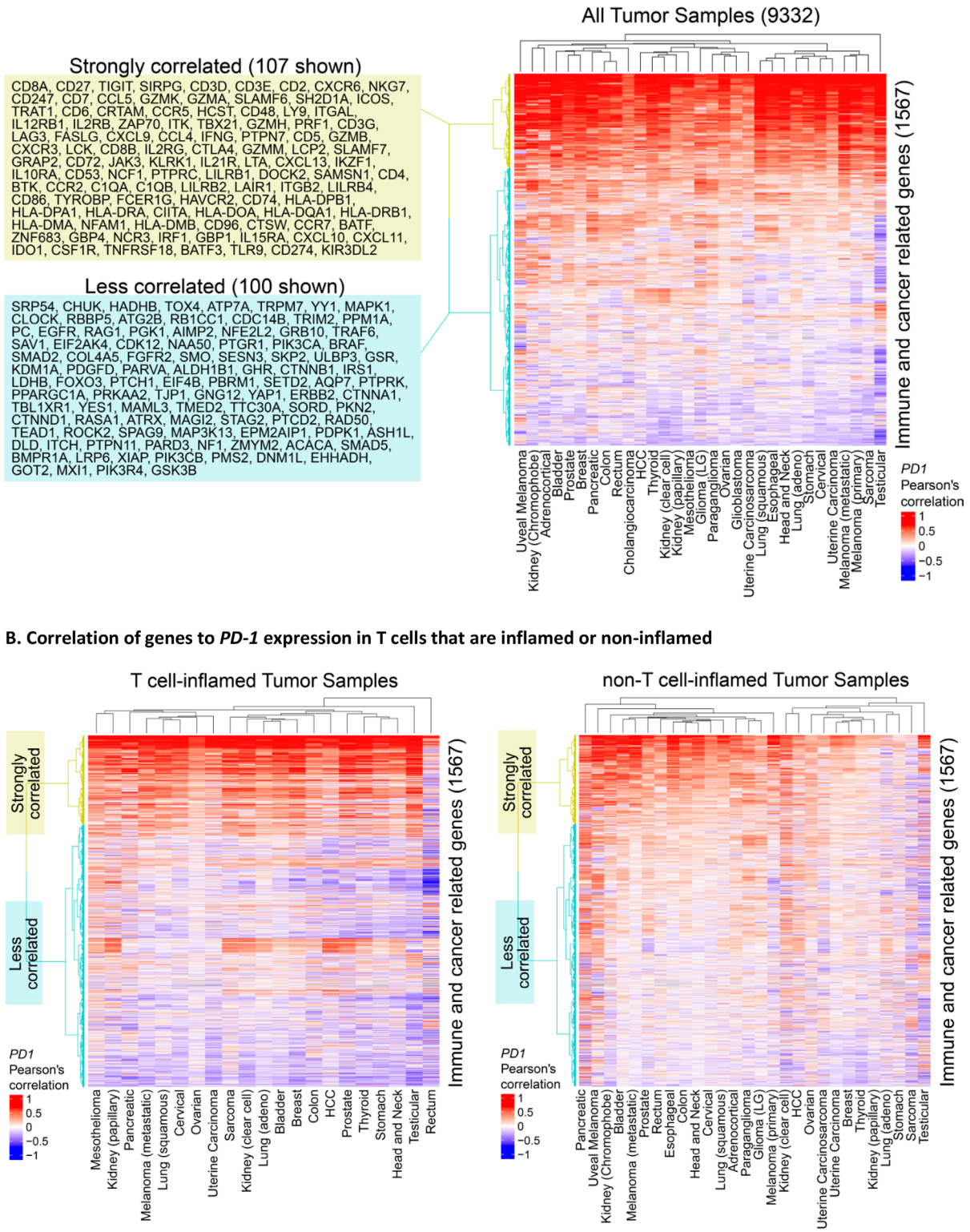


Figure 2 Characterization of the relationship between immune and cancer-related gene expression and tumor inflammation status in the TME from TCGA samples. (A) *PD-1* expression correlated with expression of 1567 genes across tumor types across all samples regardless of inflammation status. Two clusters of genes are shown. (B) Correlation of genes to PD-1 expression in T cells that are inflamed or non-inflamed tumors is presented here. Less correlation between the expression of *PD-1* and other immune genes from the strongly correlated gene cluster (399 genes) was observed in the tumors classified as non-inflamed compared with inflamed (p=0.00017, two-sided Wilcoxon signed-rank test). The most strongly (light yellow box) and less correlated genes (light blue box) are shown in panels A and B of all TCGA tumor samples. Genes were selected from the literature and clinically relevant gene expression panels. HCC, hepatocellular carcinoma; PD-1, programmed death 1; TCGA, The Cancer Genome Atlas; TME, tumor microenvironment.

analyzed to identify the distribution of potentially relevant drug targets for combination I-O approaches. Based on prior analysis demonstrating high correlations between immune-relevant molecules and *PD-1* in highly inflamed tumors, it was hypothesized that melanoma samples with low/intermediate overall T-cell inflammation may display individualized immune gene expression profiles that may suggest specific I-O treatment combinations. Using IFN-γ dependent *PD-L1* mRNA expression as a marker of T-cell inflammation, samples were stratified by quartiles of *PD-L1* expression (low, low/intermediate, intermediate/high, high) and associated with individual gene transcript expression for other I-O targets. Supervised and unsupervised clustering are shown in online supplemental figure S1; results of the latter are described in online supplemental appendix A2. As expected, these genes become more commonly expressed as the expression of *PD-L1* increased. Tumors expressing low/intermediate levels of *PD-L1* demonstrated distinctly upregulated immune markers that might be targeted for combination therapy (online supplemental figure S1).

To extend these gene expression analyses to an individual patient level, the relative expression of immune target genes relevant for the drug combinations to be tested in the trial was assessed. Particularly in tumors expressing low/intermediate levels of *PD-L1*, substantial interpatient variability of individual immune gene expression was observed, with some genes notably higher in expression relative to others analyzed (see expanded view of quartile 2, arrows, online supplemental figure S1). The increased expression of these individual genes, in individual samples, suggests the possibility of particular mechanistic dependence as mechanisms of resistance to anti-PD-1 and might suggest specific vulnerabilities that could inform combination I-O treatment approaches.

IHC validation of TCGA analyses in clinical tumor samples

To further explore and potentially validate that these transcriptional patterns manifest as protein expression in tumors, samples from six tumor types (ie, melanoma, NSCLC, RCC, UC, SCCHN, or GEJ/GC) were stained by IHC for I-O protein expression.

To determine whether IHC profiling would identify outlier biomarker expression as observed in the melanoma TCGA data, we conducted both unsupervised hierarchical clustering and supervised analysis by CD8 IHC. Unsupervised clustering of samples by tumor type revealed subgroups of tumors spanning the full dynamic range of CD8 expression, as a surrogate of T-cell inflammation, across tumor types (online supplemental figure S2). In line with observations from the TCGA melanoma transcriptional data, increased expression of multiple I-O targets was observed in tumors with high T-cell inflammation (as defined by CD8 (NSCLC or RCC) or IFN-γ (UC) expression), and supervised clustering also highlighted tumors with outlier biomarker expression in the low and intermediate inflammation groups (figure 3A–C).

Samples were also organized by IFN-γ gene signature score[10] confirming that tumors with high T-cell inflammation scores also displayed high levels of I-O biomarker expression on IHC, while tumors with low inflammation signature concordantly had lower I-O protein expression. Upregulation of distinct immune marker targets was observed in low-to-intermediate inflammation groups, supporting the hypothesis that the IHC could be applied to identify tumors with distinct biomarker profiles. As shown in online supplemental figure S3, IHC profiles correlate with both CD8 and IFN-γ IHC T-cell inflammation gene signatures across the validation cohort.

To further assess the relationship between expression levels of the target biomarkers and overall tumor inflammation, the correlation between IHC expression of the individual biomarkers and the inflammation signature score was analyzed across UC, GEJ/GC, SCCHN, melanoma, NSCLC, and RCC tumors. Similar to the data from TCGA, significant associations were identified between IHC biomarker expression level and T-cell inflammation signature score across most markers (online supplemental table S2). Data for LAG-3 are shown in figure 4 for all tumor types combined (4A) and by individual tumor type (4B).

Generation of a treatment selection algorithm in the ADVISE trial

The primary objective of the trial was to determine whether biomarker profiles could be analyzed and communicated within 12 business days across multiple clinical sites (figure 5A). Biopsies were deemed adequate for analysis if at least four cores were collected with >30% tumor cellularity. As data from TCGA and the IHC analyses suggested some tumors with low/intermediate levels of *PD-L1* or tumor inflammation may have distinctly upregulated immune markers, an algorithm was designed that defines a tumor as inflamed if IHC PD-L1 tumor cell and/or CD8 immune cell expression are ≥1% positive. Tumor samples were initially assessed for expression of PD-L1 and CD8 as a screen of T-cell-inflamed status and feasibility of immune combination therapy. If PD-L1 (TPS) and CD8 tumor cell IHC expression showed <1% staining, but one or more biomarkers had a categorical expression of moderate or high, then patients were assigned to combination therapy including nivolumab with the I-O marker with the highest expression (CTLA-4, LAG-3, IDO1, CSF-1R, GITR and NKp46; figure 5B). Where ≥2 IHC markers showed equivalent categorical expression, a randomization process defined the therapy given in combination with nivolumab. If PD-L1 and CD8 expression were <1% with all other decisional biomarkers falling into the lowest expression tertile, then the tumor was considered non-inflamed, and the patient would be assigned nivolumab and SBRT.

As an example, a patient in their 60s was diagnosed with NSCLC in June 2017, and treated with pembrolizumab from July 2017 to April 2018. After consent, biopsy from this patient demonstrated high categorical expression

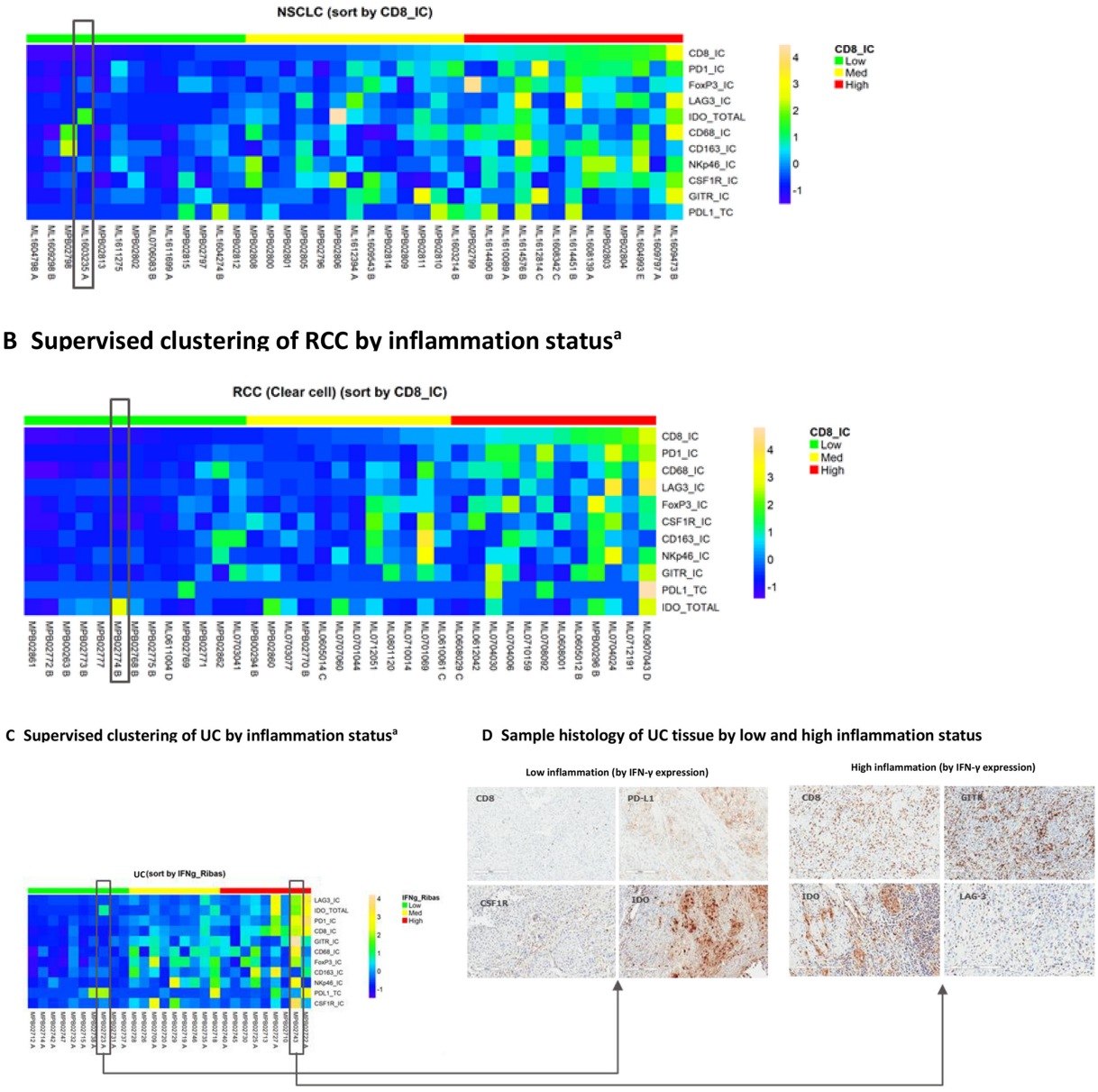


Figure 3 Supervised clustering of IHC data identified discrete inflammation profiles in low/intermediate groups from commercially acquired tumor samples. IHC samples shown are sorted by inflammation status (CD8 IHC or IFN-γ expression signature with low, medium and high tertiles indicated) in individual tumors: (A) NSCLC, (B) RCC, and (C) UC, as well as (D) sample histology of UC with low and high inflammation by IFN-γ gene expression. Examples of tumors with generalized high inflammation or with outlier biomarker expression are marked by vertical rectangles in the heat map. [a]Samples were ordered by low-to-high inflammatory marker expression (CD8 for NSCLC and RCC, IFN-γ for UC), with the rest of the markers ordered based on highest to lowest Spearman correlation to CD8 or IFN-γ. CD, cluster of differentiation; CSF-1R, colony-stimulating factor 1 receptor; FoxP3, forkhead box P3; GITR, glucocorticoid-induced tumor necrosis factor receptor; IC, immune cell; IDO1, indoleamine 2,3-dioxygenase 1; IFN-γ, interferon-gamma; IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3; NSCLC, non-small cell lung cancer; PD-1, programmed death 1; PD-L1, programmed death ligand 1; RCC, renal cell carcinoma; TC, tumor cell; UC, urothelial carcinoma.

of CSF-1R and FOXP3 (figure 5C). Based on a randomization process between CSF-1R and FOXP3, the patient was assigned to receive nivolumab+cabiralizumab (anti-CSF-1R) combination therapy, demonstrating the clinical feasibility of real-time, biomarker-driven treatment selection (figure 5C).

Online supplemental table S3 shows the tumor types and the anatomical locations of biopsies for the initial 21 patients enrolled in the trial, highlighting that the biopsies were obtained from multiple sites and organs. The workflow from sample acquisition through initiation of treatment is outlined in figure 6A, and each patient's

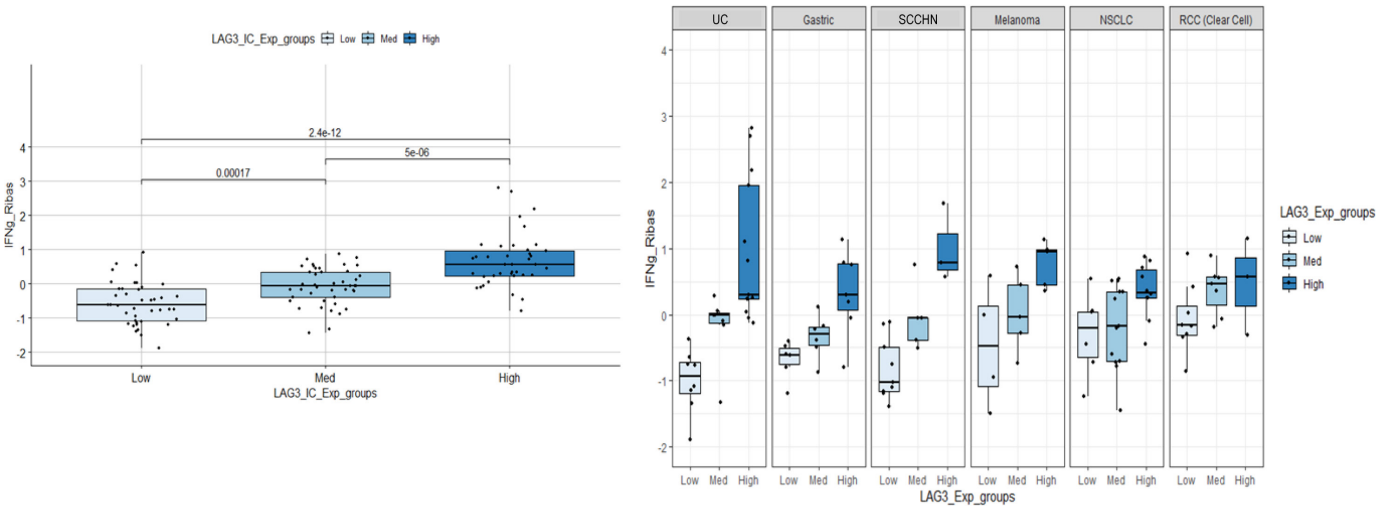


Figure 4 LAG-3 expression by IHC correlates with tumor inflammation in validation samples. (A) LAG-3 expression by tertiles in all tumors (B) LAG-3 expression by tertiles in individual tumors. IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; SCCHN, squamous cell carcinoma of the head and neck; UC, urothelial carcinoma.

timeline is shown in figure 6B. 23 patients were enrolled. Two patients were deemed to be screen failures by clinical eligibility criteria and one was deemed not evaluable due to no tumor being present in the biopsy. The 20 remaining patients all received biopsy results that led to treatment assignment, with 19 successfully receiving biopsy results within the 12-day window (and one at 15 days), indicating that the primary endpoint of the trial was met. The patient who was not evaluated within the 12-day window had an improperly embedded biopsy that necessitated re-embedding, requiring additional time. Five of the 20 patients did not proceed to treatment due to rapid disease progression or patient choice, with 15 initiated on combination I-O treatment.

Clinical outcomes

Demographic characteristics in all treated patients are summarized in online supplemental table S4. At baseline, 12 (80%) patients were I-O refractory and 3 (20%) were I-O naïve. The safety profile observed in the ADVISE trial appeared to be generally aligned with safety described for each agent in combination with nivolumab from other trials.[3 23 24 26–29 31 39] A summary of adverse events by grade, severity, and relatedness is shown in table 1. The investigator-reported best overall response achieved was stable disease in 2 patients (1 patient each in the linrodostat and ipilimumab plus nivolumab combinations) and progressive disease in 11 patients; best overall response was not evaluable in 2 patients.

Translational and practical observations from pretreatment biopsies obtained in the ADVISE trial

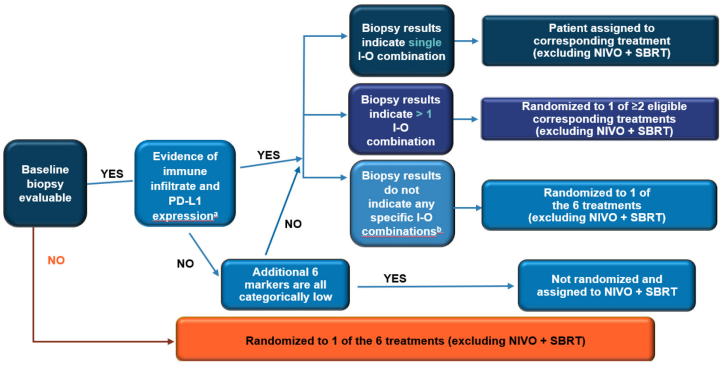
During the trial, a few notable learnings lessons emerged as related to tumor sample analysis. Most of the patients enrolled had progressed on one or more immunotherapies before enrollment. Despite clinical progression, biopsied lesions frequently showed a tumor morphology contexture that was distinct from features observed in the validation samples and was consistent with some degree of antitumor response. Tumors from I-O-experienced patients frequently showed marked evidence of inflammation (lymphocytic and tumor-associated macrophages) in the background of proliferative fibrosis and neovascularization that surrounded tumor islands (online supplemental figure S4). In contrast, I-O-naïve tumors exhibit classic tumor parenchyma with large dense nodules, intervening tumor stroma, and minimal immune infiltrate. This unique contexture morphology required adjusting the criteria for defining the ROIs analyzed, including features consisting of smaller clusters of tumor cells interspersed with proliferative fibrosis, neovascularization, and an inflamed TME. CSF-1R expression by IHC is consistently elevated in the in the post-I-O setting. The heatmap of a subset of patients shows that CSF-1R at baseline tumor samples is consistently elevated compared with other immune markers (online supplemental figure S5). Since the marked myeloid inflammation observed included CSF-1R+tumor-associated macrophages, this impacted the dynamic range of CSF-1R relative to the levels noted in the I-O-naïve validation samples, with resultant upward skewing of the dynamic range of CSF-1R expression.

The impact of this apparent overexpression of CSF-1R resulted in 35% (7/20) of patients being assigned to receive the combination of nivolumab and cabiralizumab (online supplemental figure S5).

DISCUSSION

The ADVISE trial tested the primary hypothesis that I-O-relevant selection biomarkers can be identified from

A Decision matrix algorithm used for ADVISE study treatment selection



[a]CD8 & PD-L1 expression >1%; [b]All 6 biomarker levels are low

B Decisional IHC biomarkers

Arm	Biomarker	Cell type scored
N/A	CD8	IC
N/A	PD-L1	TC
Relatlimab	LAG3	IC
Cabiralizumab	CSF1R	IC
BMS-986205	IDO	All cell types
Lirilumab	NKp46	IC
Ipilimumab	FoxP3	IC
BMS-986156	GITR	IC

C Case study of IHC biomarker results in a patient with NSCLC

IHC assay (analyte)	Expression (low/medium/high)
PD-L1	≥ 1%
CD8	≥ 1%
NKp46	Low
LAG-3	Medium
CSF-1R	High
FOXP3	High
IDO	Low
GITR	Medium

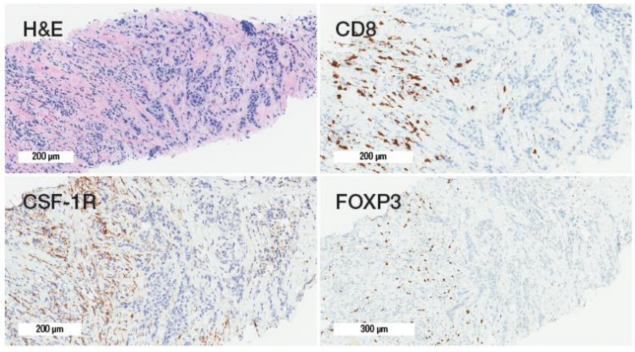


Figure 5 Decision matrix for combination therapy selection. (A) Schema, (B) decisional IHC biomarkers, (C) case example of IHC biopsy results in patient with NSCLC informed I-O combination treatment selection in ADVISE (image presented previously at European Society for Medical Oncology (ESMO) 2018; Poster 1135PD. Munich, Germany).[36] (Panel A notes) [a]CD8 and PD-L1 expression >1%. [b]All six biomarker levels are low. ADVISE, ADaptiVe biomarker trial that InformS Evolution of therapy; CD8, cluster of differentiation 8; CSF-1R, colony-stimulating factor 1 receptor; FOXP3, forkhead box P3; GITR, glucocorticoid-induced tumor necrosis factor receptor; IC, immune cell; IDO, indoleamine 2,3-dioxygenase; IHC, immunohistochemistry; I-O, immune-oncology; LAG-3, lymphocyte-activation gene 3; NIVO, nivolumab; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1; SBRT, stereotactic body radiation therapy; TC, tumor cell.

tumor biopsies via IHC, and reported back to the clinical site for treatment selection within a 12-day feasibility window. This was demonstrated as feasible in multicenter fashion and has the potential to facilitate biomarker-adapted clinical trials moving forward. However, in the initial experience of this study, biomarker cut points for I-O target selection in combination therapy with nivolumab did not generate treatment responses.

As a lead-up to the trial, evaluation of the TME through transcriptome and IHC analyses demonstrated heterogeneity of the immune profile across tumor types and demonstrated the potential to identify subsets of tumors with individual immune biomarkers that are more highly expressed than would be predicted based on T-cell-inflamed gene expression in general. It was observed across individual tumor specimens that mRNA and protein levels were closely matched, and that subsets of tumors with individual I-O target gene expression could be found across tumor types, especially at low-to-intermediate inflammation levels. This suggests that the IHC-derived (and possibly transcript level profiling) biomarker-based treatment-selection algorithm is a rational approach to test for I-O combination therapy selection for patients with advanced cancer.

The results from ADVISE suggest that using real-time biomarker analyses and patient biopsy results for prospective biomarker selection is clinically feasible to inform the selection of combination I-O therapies, even with the inclusion of the pathologist peer review step to confirm IHC results. In current clinical practice, a median of 21–24 days has been reported between initial consultation and result reporting.[40 41] It should be noted that 5 of the 20 patients with a tumor biopsy in ADVISE did not proceed to treatment due to disease progression or patient choice, suggesting that patients with aggressive disease require a shorter time to result reporting. Nevertheless, these results demonstrate that it is possible to obtain biomarker analysis of baseline tumor biopsy samples, perform quality control steps, and report the analysis results to the treating clinician within 12 business days, resulting in treatment decisions within 1 day and initiation of treatment within 1 week of receipt of the biopsy results. Despite demonstrating the clinical feasibility of using real-time biomarker analyses and patient biopsy results to

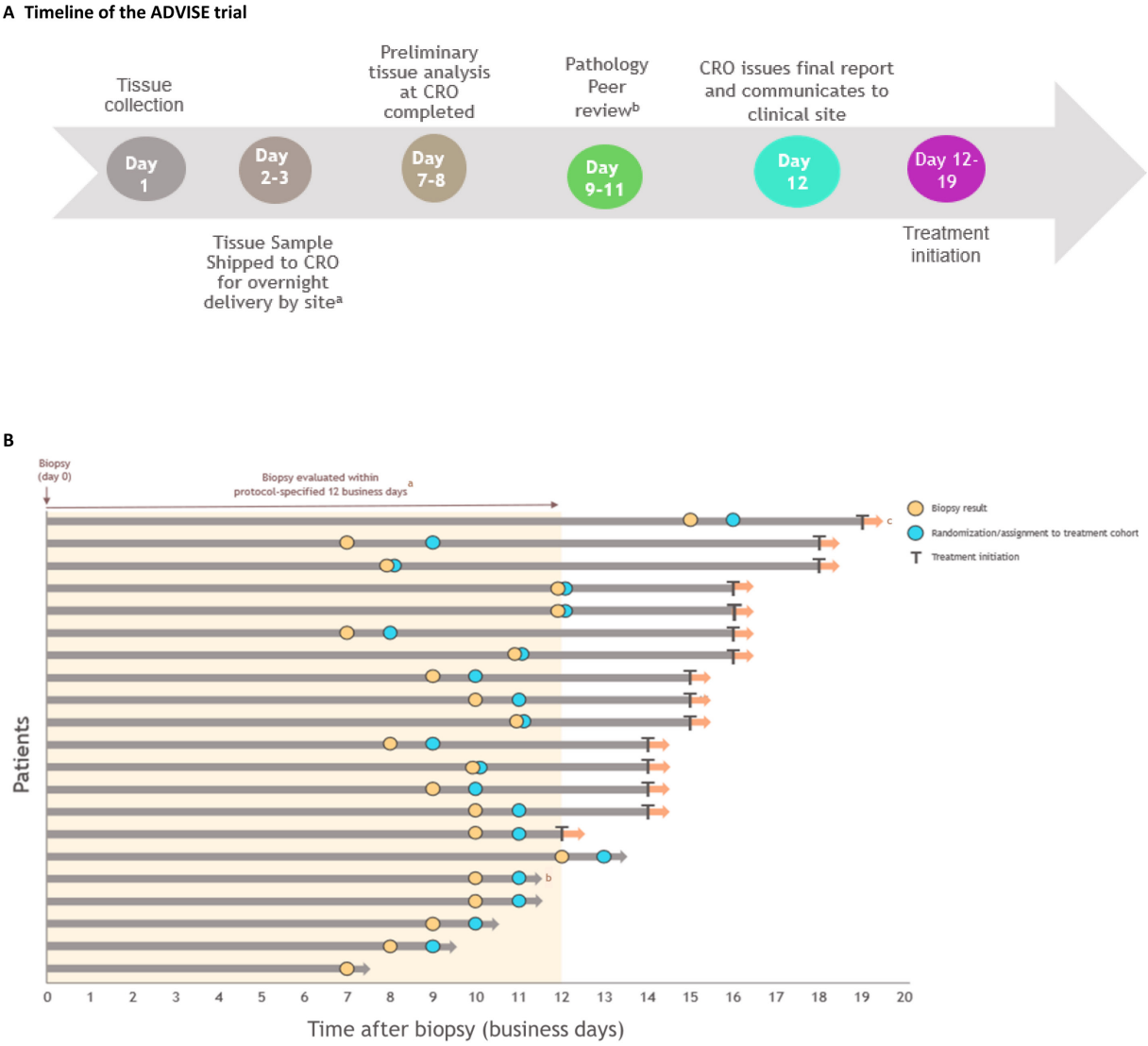


Figure 6 Assessment of the feasibility of real-time biomarker analysis in the ADVISE trial. (A) Planned timeline. (B) 21 patients received biopsy results (orange circles), 20 of whom were successfully assigned to combination therapy using the ADVISE algorithm (blue circles), with 19 of them receiving that assignment within 12 days of initial biopsy, and 15 initiating treatment. (Panel A notes) Steps involved with 12 business days timeline from biopsy collection to results communicated to clinical site. [a]Depending on whether fixation time was 24 or 48 hours. [b]Pathology peer review was initiated beginning with patient #16. (Panel B notes) [a]Equivalent to 16–18 calendar days depending on the day of the week the biopsy was obtained. [b]Patient withdrew consent for treatment due to decline in status. [c]Biopsy was evaluated within 15 business days. Screen failed patients due to no tumor biopsy. ADVISE, ADaptiVe biomarker trial that InformS Evolution of therapy; CRO, clinical research organization.

inform the selection of combination I-O therapies, most of the 15 treated patients had progressive disease. A small patient population and a number of challenges observed over the course of the study may have impacted treatment response, including challenges related to (1) biomarker validation, (2) image analysis algorithm development, and (3) consistently elevated CSF-1R expression across tumor types (online supplemental table S5).

First among these challenges, recent studies of biopsy tissue from patients after I-O therapy have revealed marked features of immune activation and wound healing/tissue repair (moderate to high levels of tumor-infiltrating lymphocytes and plasma cells, neovascularization, and proliferative fibrosis) that are not found in I-O-naïve clinical biopsy specimens.[42–44] These differences may impact the scoring and appearance of IHC samples obtained from I-O-experienced patients when validation parameters are developed using biopsy samples from treatment-naïve patients. ROI annotation parameters determined for IHC image analysis algorithms may be impacted, leading to suboptimal performance of the assay. Of note, most of the enrolled patients had a prior history of receiving I-O therapy, whereas the tumors used for I-O target biomarker cut points were I-O naïve. Because of this, annotation criteria for ROIs were continuously refined with a focus on tumor and proximally associated

Table 1 Summary of adverse events by grade, severity, and relatedness are described (all treated patients)

Event, n (%)	All treated patients (n=15)
All-causality adverse events	15 (100)
Grade 3–4	6 (40.0)
Grade 5	4 (26.7)
Drug-related adverse events	10 (66.7)
Grade 3–4	4 (26.7)
Grade 5	0
All-causality adverse events leading to discontinuation	3 (20.0)
Drug-related adverse events leading to discontinuation	2 (13.3)
All-causality serious adverse events	8 (53.3)
Drug-related serious adverse events	1 (6.7)
Grade 3–4	1 (6.7)
Grade 5	0

stroma, which may help mitigate potential differences in the TME and morphologic features between post-I-O and I-O-naïve tumors. Furthermore, the TME is now understood to be a more complex structure involving cancer cells surrounded by diverse TME host cells (eg, immune cells, pericytes, endothelial cells, and cancer-associated fibroblasts) that can differ by patient characteristics and organ sites.[45] Further complexity involving the spatial context of molecular and cellular interactions within the TME in intact tissues is also beginning to emerge.[45 46] Based on this level of heterogeneity, the use of one decisional biomarker is likely insufficient to predict treatment response.

Second, algorithms for image analysis were developed from individual marker cut-offs based on qualitative interpretations of the dynamic range across I-O-naïve tumor types. As noted above, immune marker dynamic ranges varied across tumor types and by prior I-O experience, with patients enrolled in the trial often having different treatment histories from the patients who had provided tissues for the validation samples. The hypersegmentation and hyposegmentation errors in the algorithm may have impacted the percentage of positive cells. Therefore, the biomarker cut-offs likely were not optimal. In the future, the biomarker cut-offs could be further optimized based on I-O experienced samples and refined by specific tumor types. Thus, a visual evaluation of immune cell markers was required to improve and confirm categorical assessment. To mitigate this, pathology peer review was instituted. However, 15 patients were enrolled at the University of Chicago during the enrollment process, during which time some IHC data underwent peer review by BMS pathologists without the predefined reconciliation process. As such, patient stratification and the time from tumor biopsy to generation of decision-enabling biomarker analysis may have been impacted in a subset of these 15 patients without additional independent pathologist review and reconciliation.

Third, this biomarker cut-off threshold very likely contributed to CSF-1R being the most highly expressed marker in 60% of cases with the result of patients being assigned to receive nivolumab and cabiralizumab combination therapy. This scenario is likely to have been compounded through the use of only one decisional biomarker per I-O-combination treatment, without use of confirmatory markers. Whether this might be the optimal combination regimen then is open for questioning, and it is also worth noting that minimal efficacy of targeting CSF-1R in the post-I-O treatment setting has been observed.[47]

Finally, as part of a protocol amendment on May 13, 2019, study treatment with nivolumab in combination with lirilumab or BMS-986156 (anti-GITR agonist) was discontinued, based on termination of these development programs. Therefore, the lack of treatment response may also be due to the therapies assigned, not just the biomarker-based treatment-selection algorithm.

In conclusion, ADVISE was a practical clinical trial demonstrating that a 12-day turnaround for I-O biomarker assessment is feasible. However, the study did not demonstrate clinical utility, leaving open the question of whether more efficient precision medicine-based identification and implementation of personalized, rational I-O combination treatment paradigms is possible. Future studies validating I-O combination treatment guided by specific biomarkers will require a larger patient population and use of optimized biomarker cut-offs that are tailored toward the specific tumor type and prior I-O therapy experience.

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Contributors JJL: conception or design, data acquisition, data analysis, data interpretation. KB: data acquisition, data interpretation. FSH: conception or design, data interpretation. JT, AM, DY, JN, RT, GL, AG, SD, and PMS: data analysis, data interpretation. RB: data acquisition, data analysis, data interpretation. TR: conception or design, data acquisition, data analysis, data interpretation. All authors drafted or critically reviewed the manuscript. JJL is the guarantor. AM, DY, GL, AG, PMS, TR: at the time the study was conducted.

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Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by UPMC Hillman Cancer Center (Principal Investigator: JJL). IRB approval number: #19-078. University of Chicago Comprehensive Cancer Center (Principal Investigator: Dr R Sweis). IRB approval number: #17-0732. Johns Hopkins University (Principal Investigator: KB). IRB approval number: #19-2075. The study was conducted in accordance with the Declaration of Helsinki and in compliance with theprotocol. All patients provided written informed consent before enrollment.

Provenance and peer review Not commissioned; externally peer reviewed.

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