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# Development and validation of a serum proteomic test for predicting patient outcomes in advanced non-small cell lung cancer treated with atezolizumab or docetaxel

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## ABSTRACT

**Background** Programmed cell death-ligand 1 (PD-L1) expression is used in treatment decision-making for patients with advanced non-small cell lung cancer, determining if immune checkpoint inhibitors (ICI) are recommended. Patient selection for ICI treatment can be improved by incorporating the host response. We developed and carried out multiple independent validations of a blood-based test designed to stratify outcomes for patients treated with atezolizumab.

**Methods** A mass spectrometry-based test was developed from a cohort of patients treated with atezolizumab and validated in two clinical trials (n=269, 823) comparing atezolizumab with docetaxel. The test classifies patients as Good or Poor indicating better or worse outcomes, respectively. The prognostic and predictive power of the test was assessed and evaluated within PD-L1 subgroups. Protein enrichment methods were used to investigate the association of test classification with biological processes.

**Results** Approximately 50% of patients were assigned to each classification in all three cohorts. When treated with atezolizumab, the Good subgroup had superior outcomes in all cohorts. Overall survival (OS) HR (95% CI) for Good patients in each cohort was: 0.23 (0.12 to 0.44), 0.32 (0.21 to 0.51), and 0.52 (0.41 to 0.66) and persisted in all PD-L1 subgroups. The test was predictive of differential OS and progression-free survival in one cohort, but not in the other. Enrichment techniques indicated the test was associated with acute inflammatory response, acute phase response, and complement activation.

**Conclusions** Aspects of host immune response to disease can be assessed from the circulating proteome and provide outcome stratification for patients treated with atezolizumab. Combining this information with PD-L1 measurements improves prediction of outcomes.

### WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Biomarkers that could improve prediction of patient outcomes when treated with immune checkpoint inhibitors (ICI), such as atezolizumab, would be useful for patient–physician discussions, treatment selection, and development of new immunotherapeutic regimens. Programmed cell death-ligand 1 (PD-L1) is currently the standard biomarker used for ICI treatment decision.

### WHAT THIS STUDY ADDS

⇒ A blood-based test assessing aspects of host biology from mass spectrometry of serum effectively stratifies outcomes for patients with advanced non-small cell lung cancer treated with atezolizumab and provides information complementary to patient baseline characteristics and PD-L1 status.

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Host response data determined from liquid biopsy could be added to tumor-based data to improve prediction of outcomes and inform treatment decisions.

## INTRODUCTION

Binding of programmed cell death-ligand 1 (PD-L1) to the programmed cell death protein-1 (PD 1) and B7.1 receptors on antigen presenting cells and T cells can inhibit immune response to a tumor via suppression of cytotoxic T-cell activity, T-cell proliferation, and cytokine production.[1 2] Atezolizumab, a humanized monoclonal antibody against PD-L1, can alleviate this immune response inhibition by blocking the interactions of this ligand with both the PD-1 and the B7.1 receptors.[3] The US Food and Drug Administration has approved atezolizumab for the treatment of melanoma, small cell lung cancer, hepatocellular carcinoma, and non-small cell lung cancer (NSCLC).[3]

The efficacy of atezolizumab monotherapy as treatment for patients with advanced NSCLC who have already progressed on platinum-based chemotherapy was

demonstrated in the POPLAR Phase 2 (NCT01903993)[4 5] and OAK Phase 3 (NCT02008227)[5–7] randomized clinical trials, both of which compared single-agent atezolizumab with docetaxel. In the final intent-to-treat analysis of the POPLAR trial, median overall survival (mOS) was 12.6 months for the 144 patients randomized to atezolizumab compared with 9.7 months for the 143 randomized to docetaxel (HR (95% CI): 0.76 (0.58 to 1.00)).[5] Results were similar in the OAK trial, with mOS of 13.3 months for the 613 patients randomized to atezolizumab versus 9.8 months for the 612 patients randomized to docetaxel.[5] Although both trials demonstrated superior progression-free survival (PFS) and OS of atezolizumab over docetaxel independent of PD-L1 expression; subgroup analyses from both trials demonstrated that higher PD-L1 expression predicted increased benefit of atezolizumab.[4–6] However, it was noted that when treated with atezolizumab, outcomes were better and the benefit of atezolizumab compared with docetaxel was greater in patients with higher levels of PD-L1 expression.[5] However, some patients with high levels of PD-L1 expression experienced early progression (more than 30% of patients with high PD-L1 expression (≥50% of tumor cells (TCs) and/or ≥10% of tumor-infiltrating immune cells (ICs)) progressed by 1.5 months,[5] while some patients with low levels of PD-L1 expression demonstrated durable responses when treated with atezolizumab (objective response rate (ORR)=7.7% with median duration of response 23.9 months for patients with PD-L1 expression of <1% TCs or ICs[5]). Although atezolizumab effectively blocks binding of PD-L1 to the PD 1 and B7.1 receptors,[3] it is known that tumors can evade the host immune response by multiple alternative mechanisms.[8 9] Hence, it is reasonable to expect that the efficacy of atezolizumab therapy depends on several factors, in addition to the PD-L1 expression of tumor and tumor-infiltrating ICs.

One promising avenue for the discovery of other predictive and prognostic markers that complement atezolizumab specifically, or checkpoint inhibitors in general, is to assess the circulating proteome.[10 11] It is known that around 24% of classical plasma proteins are associated with immune response.[12] The abundances of these proteins are indicative of multiple physiological processes related to the adaptive and innate immune systems, including complement activation, wound healing, angiogenesis, and extracellular matrix remodeling.[11 13 14] It has been shown that proteomic tests based on mass spectrometry of pretreatment serum are able to stratify outcomes for patients with melanoma treated with the checkpoint inhibitors nivolumab, pembrolizumab, or ipilimumab[15 16] and for patients with advanced NSCLC treated in second and higher line with nivolumab.[17] The results of these tests were found to be associated with biological processes related to host immune response, but not to be associated with tumor PD-L1 expression.[15–17] Therefore, it is hypothesized that atezolizumab efficacy complementary to PD-L1 expression is related to the abundance of certain proteins in pretreatment serum samples. The aim of this work was to investigate this hypothesis using the same techniques that have been successfully applied in other indications. Specifically, a matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectrometry method designed to allow deep probing of the serum proteome (Deep MALDI mass spectrometry[18]) paired with a machine learning approach called the Diagnostic Cortex platform.[19 20] We developed a serum proteomic test designed to stratify patients with advanced NSCLC into two groups differentiated by OS outcome with single-agent atezolizumab. The test performance was validated in two independent cohorts that were blinded to clinical data. Additional goals included assessing the predictive potential of the test to determine the relative benefit of atezolizumab over docetaxel and the ability to combine test results with PD-L1 expression of the tumor to better predict outcomes and benefit from atezolizumab therapy for patients with advanced NSCLC.

## METHODS

### Patients

The test was developed in a single cohort consisting of patients with advanced NSCLC who participated in a Phase I trial assessing the safety, tolerability, and pharmacokinetics of atezolizumab in locally advanced or metastatic cancers (NCT01375842)[21 22] and had available pretreatment serum samples. Patients received intravenous atezolizumab 1–20 mg/kg or 1200 mg every 3 weeks.

The test was subsequently validated in two cohorts consisting of the phase 2 POPLAR trial and the phase 3 OAK trial. The POPLAR cohort included patients randomized to receive atezolizumab or docetaxel in a Phase 2 study (NCT01903993)[4 5] who had pretreatment serum samples available for testing. The OAK cohort included patients who had been randomized to receive atezolizumab or docetaxel in a Phase 3 study (NCT02008227)[5–7] with pretreatment serum samples available for testing. Inclusion criteria for both studies specified Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 or 1, one or two prior lines of cytotoxic chemotherapy for advanced disease, measurable disease, and adequate hematological and organ function. Atezolizumab was administered in 1200 mg doses every 3 weeks until unacceptable toxicity or disease progression. Continuation of atezolizumab beyond disease progression was permitted if the investigator determined that the patient was receiving clinical benefit. Docetaxel was administered intravenously at 75 mg/m$^2$ every 3 weeks until unacceptable toxicity or disease progression.

All three clinical trials were carried out in full accordance with the guidelines for Good Clinical Practice and the Declaration of Helsinki. Protocol approval was obtained from independent ethics committees for each site.[4 6 21]

### Samples

Serum samples from patients in the development cohort, the POPLAR cohort, and OAK cohort, were collected from patients prior to initiation of therapy (at screening for the development cohort and on Cycle 1 day 1 for POPLAR and OAK) and were stored at −80°C. A total of 149 serum samples were used for protein enrichment analyses collected from patients with NSCLC and from cancer-free individuals and purchased from Oncology Metrics (Fort Worth, Texas, USA), Discovery Life Sciences (Huntsville, Alabama, USA), AdeptBio (Memphis, Tennessee, USA) and ProMedDx (Norton, Massachusetts, USA).

### Acquisition and processing of mass spectra

Mass spectra were generated from serum samples using the DeepMALDI method[18] on a SimulTOF 100 mass spectrometer (SimulTOF Systems, Marlborough, Massachusetts, USA). The resulting spectra were processed to facilitate comparison across samples. Sample preparation, spectral acquisition, and spectral processing methods have been described in detail previously[15 16] and changes to this protocol are specified in the Supplement (online supplemental tables 1 and 2). A set of 357 mass spectral regions, or *features*, was defined excluding peaks known to be irreproducible or related to hemolysis. The feature value of each feature for a mass spectrum was defined as the intensity of the processed spectrum integrated across the region defining the feature.

### Test development by machine learning

Spectral data was generated from the development cohort in February 2016. Test development was carried out using processed mass spectral data and associated clinical data with the Diagnostic Cortex machine learning platform. This method is designed to generate classifiers where the number of measured attributes (features or proteoforms for this study) matches or exceeds the number of samples available for test development.[19 20] Test development only used data from the development cohort. Reliable assessment of test performance could be generated from this cohort using out-of-bag estimates[23] of the ensemble averages[24] employed in the machine learning approach.[19]

### Evaluation of the association of test classification with biological processes

The biological underpinnings of the test were assessed via previously described protein enrichment analysis assessment,[25 26] where the peaks identified in the classifier were tested for association with 23 biological processes in 149 serum samples for which protein panel data (SomaLogic, Boulder, Colorado, USA) and DeepMALDI mass spectra were available. False discovery rates (FDRs) for the multiple comparisons over protein sets were generated using the method of Benjamini and Hochberg.[27]

### Test validation

Test validation was carried out blinded to all clinical and outcome data. Validation was performed in March 2017 for the POPLAR cohort and in February through March 2021 for the OAK cohort.

### Measurement of tumor PD-L1 expression

Tumor PD-L1 expression was measured using the Ventana SP142 immunohistochemistry assay (Ventana Medical Systems, Tucson, Arizona, USA) in TCs and ICs. PD-L1 status was categorized according to the percentage of PD-L1 expressing TCs (TC3 (≥50%), TC2 (<50% and ≥5%), TC1 (<5% and ≥1%), and TC0 (<1%)) and to the percentage of tumor area occupied by PD-L1 expressing ICs (IC3 (≥10%), IC2 (<10% and ≥5%), IC1 (<5% and ≥1%), and IC0 (<1%)).[21 22 28] Four subgroups were predefined: TC3 or IC3, TC2/3 or IC2/3, TC1/2/3 or IC1/2/3 and TC0 and IC0. For multivariate analyses, PD-L1 was binarized as negative (TC0 and IC0) or positive (TC1/2/3 or IC1/2/3).

### Statistical analysis

Test performance was assessed in terms of OS, PFS, and best response to therapy. Outcomes were defined the same as the original trial protocols. Response was evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) V.1.1 and PFS was defined as time from randomization to the first occurrence of RECIST V.1.1-defined investigator-assessed disease progression. Time-to-event outcomes were summarized using the Kaplan-Meier method. Cox proportional hazard models were used to assess differences in time-to-event outcomes between patient subgroups defined by PD-L1 status and/or proteomic test classification. The ability of the test to predict differential benefit between atezolizumab and docetaxel was evaluated using a Cox proportional hazards model with treatment–test classification interaction both adjusted and unadjusted for baseline covariates (PD-L1 status, ECOG PS, histology, smoking status, number of prior lines of therapy). Association between categorical variables was assessed using Fisher's exact test and between a categorical variable and a continuous variable by the Mann-Whitney test. Statistical analyses were performed with Enterprise Guide V.8.2 using SAS V.9.4 (SAS Institute, Cary, North Carolina, USA) and with PRISM (GraphPad, La Jolla, California, USA).

## RESULTS

### Generation of mass spectral data for machine learning

High quality mass spectral data were generated for the 77 samples in the development cohort with associated clinical data shown in figure 1. Baseline characteristics for the patients in this cohort are shown in table 1.

### Test development using the development cohort

The test was trained using data from 50 of the 77 development cohort samples. This subset was chosen to exclude never smokers and be enriched in current smokers, as it has been hypothesized that smoking may be indicative of improved outcomes with immunotherapies.[29] However,

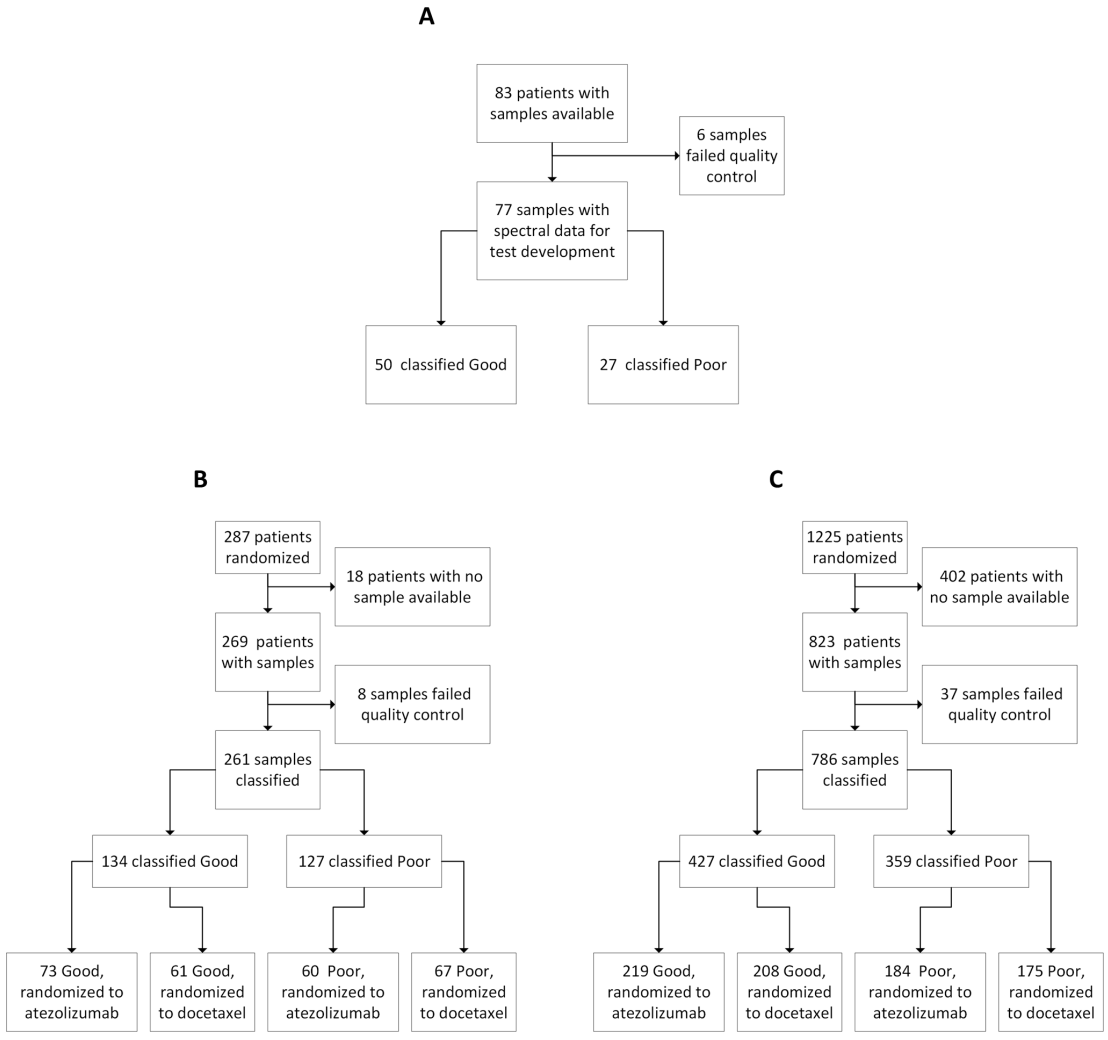


**Figure 1** Consort diagrams for (A) Development, (B) POPLAR, and (C) OAK cohorts.

all results for the development set are given for the full development cohort of 77 samples. An iterative refinement approach was used for classifier development.[20] This method has been successful in projects where the training classes corresponding to the underlying phenotypes of interest are not obvious a priori.[15–17] A bagged feature deselection method,[30] was used at each iterative refinement step to discard features that provided no useful information for stratification of survival. 93 features, shown in online supplemental table 3, were used to generate the final test classifier. Figure 2 shows a heatmap of the 93 feature values in the 77-sample development cohort. More details of test generation methods and parameters are contained in the online supplemental file 2.

The test stratifies patients into "Good" and "Poor" groups corresponding to better and worse outcomes respectively, when treated with atezolizumab. The reproducibility of the test was assessed by rerunning the final test on the development cohort, starting from sample preparation of frozen aliquots to final classification generation. Classification concordance was 96%.

## Test performance on the development cohort

The test classified 27 samples (35%) as Poor and 50 (65%) as Good. Baseline patient characteristics by test classification for the development cohort are shown in table 1. OS and PFS by test classification are shown in figure 3A,B.

The test successfully stratified both OS and PFS for the development cohort. mOS was 6.4 months (95% CI: 4.8 to 14.2 months) for the Poor group and 25.1 months (95% CI: 17.1 months-undefined) in the Good group (HR=0.23 (95% CI: 0.12 to 0.44)). Survival at 1 year was 39% in the Poor group, compared with 78% in the Good group. Median PFS (mPFS) was 1.4 months (95% CI: 1.2 to 3.0 months) for the Poor group and 8.5 months (95% CI: 2.8 to 11.6) for the Good group (HR=0.52 (95% CI: 0.31 to 0.88)). The percentage of patients progression-free at 6 months was more than twice as high in the Good group as in the Poor group (56% vs 26%). ORR was 13% in the Poor group and 30% in the Good group, while disease control rate (DCR) was 35% in the Poor group versus 64% in the Good group (online supplemental table 4). Test classification remained a significant predictor of OS and PFS when

**Table 1** Patient characteristics for each cohort (by treatment arm) and by test classification (separate file)

| | Dev | POPLAR | | OAK | | Dev | | POPLAR | | | OAK | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A (N=77) | A (N=133) | D (N=128) | A (N=403) | D (N=383) | Poor (N=27) | Good (N=50) | Poor (N=127) | Good (N=134) | | Poor (N=359) | Good (N=427) | |
| Age | 60 (24–84) | 61 (42–82) | 62 (36–84) | 63 (25–84) | 64 (34–85) | 66 (47–84) | 58 (24–82) | 61 (36–84) | 63 (37–82) | | 64 (34–85) | 63 (25–85) | |
| Gender | | | | | | | | | | | | | |
| Female | 31 (40) | 45 (34) | 57 (45) | 149 (37) | 146 (38) | 13 (48) | 18 (36) | 48 (38) | 54 (40) | | 123 (34) | 172 (40) | |
| Male | 46 (60) | 88 (66) | 71 (55) | 254 (63) | 237 (62) | 14 (52) | 32 (64) | 79 (62) | 80 (60) | | 236 (66) | 255 (60) | |
| Histology | | | | | | | | | | | | | |
| Non-squamous | 60 (78) | 89 (67) | 83 (65) | 302 (75) | 289 (75) | 20 (74) | 40 (80) | 83 (65) | 89 (66) | | 247 (69) | 344 (81) | * |
| Squamous | 17 (22) | 44 (33) | 45 (35) | 101 (25) | 94 (25) | 7 (26) | 10 (20) | 44 (35) | 45 (34) | | 112 (31) | 83 (19) | |
| Smoking history | | | | | | | | | | | | | |
| Ever | 62 (81) | 106 (80) | 104 (81) | 338 (84) | 332 (87) | 23 (85) | 39 (78) | 106 (83) | 104 (78) | | 320 (89) | 350 (82) | * |
| Never | 15 (19) | 27 (20) | 24 (19) | 65 (16) | 51 (13) | 4 (15) | 11 (22) | 21 (17) | 30 (22) | | 39 (11) | 77 (18) | |
| ECOG PS | | | | | | | | | | | | | |
| 0 | NA | 43 (32) | 41 (32) | 145 (36) | 145 (38) | NA | NA | 32 (25) | 52 (39) | † | 104 (29) | 186 (44) | * |
| 1 | NA | 90 (68) | 87 (68) | 258 (64) | 238 (62) | NA | NA | 95 (75) | 82 (61) | | 255 (71) | 241 (56) | |
| Line of therapy | | | | | | | | | | | | | |
| 1 | 14 (18) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 4 (15) | 10 (20) | 0 (0) | 0 (0) | | 0 (0) | 0 (0) | |
| 2 | 16 (21) | 87 (65) | 87 (68) | 306 (76) | 292 (76) | 8 (30) | 8 (16) | 87 (69) | 87 (65) | | 270 (75) | 328 (77) | |
| 3 or higher | 47 (61) | 46 (35) | 41 (32) | 97 (24) | 91 (24) | 15 (56) | 32 (64) | 40 (31) | 47 (35) | | 89 (25) | 99 (23) | |
| PD-L1 status | | | | | | | | | | | | | |
| TC0 and IC0 | NA | 47 (35) | 35 (27) | 162 (41) | 170 (45) | NA | NA | 41 (32) | 41 (31) | | 147 (41) | 185 (44) | |
| TC1/2/3 or IC1/2/3 | NA | 86 (65) | 93 (73) | 237 (59) | 212 (55) | NA | NA | 86 (68) | 93 (69) | | 211 (59) | 238 (56) | |
| TC3 or IC3‡ | NA | 21 | 20 | 45 | 53 | NA | NA | 19 | 22 | | 48 | 50 | |
| TC2/3 or IC2/3‡ | NA | 44 | 50 | 100 | 117 | NA | NA | 44 | 50 | | 103 | 114 | |
| KRAS mutation | | | | | | | | | | | | | |
| No | NA | 25 (19) | 15 (12) | 91 (23) | 80 (21) | NA | NA | 17 (13) | 23 (17) | | 83 (23) | 88 (21) | † |
| Yes | NA | 14 (11) | 11 (9) | 36 (9) | 29 (8) | NA | NA | 11 (9) | 14 (10) | | 21 (6) | 44 (10) | |
| NA | NA | 94 (71) | 102 (80) | 276 (68) | 274 (72) | NA | NA | 99 (78) | 97 (72) | | 255 (71) | 295 (69) | |
| EGFR mutation | | | | | | | | | | | | | |
| T790M | NA | 1 (1) | 0 (0) | nr | nr | NA | NA | 1 (1) | 0 (0) | | nr | nr | |
| Yes | NA | 10 (8) | 7 (5) | 30 (7) | 25 (7) | NA | NA | 6 (5) | 11 (8) | | 24 (7) | 31 (7) | |
| No | NA | 66 (50) | 66 (52) | 308 (76) | 298 (78) | NA | NA | 63 (50) | 69 (51) | | 269 (75) | 337 (79) | |
| NA | NA | 56 (42) | 55 (43) | 65 (16) | 60 (16) | NA | NA | 57 (45) | 54 (40) | | 66 (18) | 59 (14) | |

Continued

**Table 1** Continued

| | Dev | POPLAR | | OAK | | Dev | | POPLAR | | OAK | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A (N=77) | A (N=133) | D (N=128) | A (N=403) | D (N=383) | Poor (N=27) | Good (N=50) | Poor (N=127) | Good (N=134) | Poor (N=359) | Good (N=427) |

n(%) are shown for all categories, except for age, where median (range) is presented.
*0.0001≤p<0.005, with NA categories not included.
†0.005≤p<0.05.
‡Subsets of TC1/2/3 or IC1/2/3.
A, atezolizumab; D, docetaxel; Dev, Development; ECOG PS, Eastern Cooperative Oncology Group performance status; IC, immune cell; NA, not available; nr, not reported; PD-L1, programmed cell death-ligand 1; TC, tumor cell.

adjusted for gender, histology, smoking status and line of therapy (online supplemental table 5).

### Association of test classification with biological processes

The test was applied to mass spectra generated from reference sets of 100 and 49 serum samples for which protein abundance data was available for panels of 1,305 and 1,116 proteins, respectively. Protein set enrichment analysis using these data[25 26] indicated that test classification was associated with acute inflammatory response, complement activation, and acute phase response (p for association <0.05 and FDR<0.20), table 2.

### Test performance in validation cohorts

#### Validation set 1: POPLAR cohort

Spectra which passed all quality control metrics were acquired for 261 samples (97.0% of patients with samples available in the cohort) with 133 from the atezolizumab arm and 128 from the docetaxel arm, figure 1; 127 (49%) classified as Poor and 134 (51%) classified as Good. ECOG PS was the only baseline characteristic associated with test classification (table 1).

Within the atezolizumab arm, the test validated well, with patients classified as Good having superior OS and PFS compared with patients classified as Poor (OS HR=0.32 (95% CI: 0.21 to 0.51) and PFS HR=0.49 (95% CI: 0.34 to 0.71)), solid curves in figure 3C,D. mOS was 7.3 months for the Poor group (95% CI: 5.7 to 10.1 months) and not reached in the Good group (95% CI: 15.1 months to undefined). One-year survival in the Good group exceeded twice that of the Poor group (69% vs 33%). mPFS was 1.4 months for patients classified as Poor (95% CI: 1.3 to 2.7 months) compared with 4.5 months for those classified as Good (95% CI: 2.9 to 8.2 months). The progression-free percentage at 6 months in the Good group was more than twice that in the Poor group (45% vs 21%). The Poor group had an ORR of 7.5%, with 61% of patients having progressive disease at the time of response assessment. Within the Good group, ORR was 23%, and only 30% of patients had a best response of progressive disease. DCR was 39% in the Poor group compared with 70% in the Good group (online supplemental table 6).

Multivariate analysis within the atezolizumab arm showed that test classification remained a significant predictor of OS and PFS when adjusted for possible confounding factors, table 3 Analysis 1.

Test classification did not stratify outcomes for patients in the POPLAR cohort treated with docetaxel (OS HR=0.87 (95% CI: 0.59 to 1.28) and PFS HR=1.03 (95% CI: 0.71 to 1.49)), dashed curves in figure 3C,D. mOS was 9.1 months (95% CI: 6.9 to 11.9 months) for patients classified as Poor and 11.6 months (95% CI: 9.2 to 14.7 months) for patients classified as Good. 1-year survival was 37% in the Poor group and 48% in the Good group. mPFS was 3.0 months (95% CI: 2.4 to 4.2 months) for patients classified as Poor compared with 4.1 months (95% CI: 2.8 to 5.6 months) for those classified as Good. The percentage of patients progression-free at

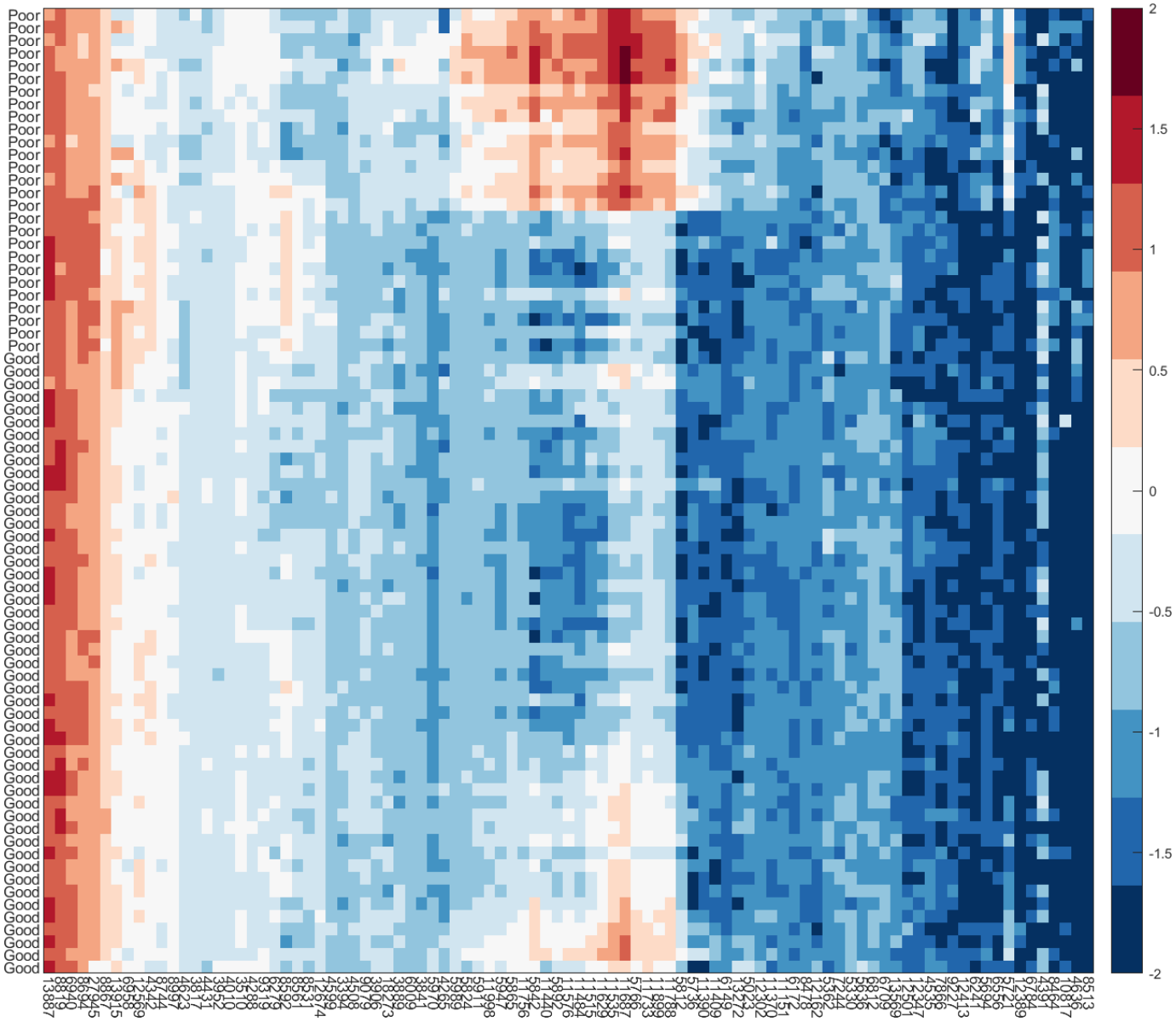


**Figure 2** Heatmap of the $\log_{10}$-transformed feature values from the development cohort clustered by sample and feature. Samples are labeled by their test classification and the features are labeled by their mass/charge location.

6 months was 29% in the Poor subgroup and 34% in the Good subgroup. Results were qualitatively similar when adjusted for possible confounding factors, online supplemental table 7. Best responses to docetaxel were also similar between test classifications, online supplemental table 6.

Test classification was predictive of differential benefit between atezolizumab and docetaxel for OS (interaction p unadjusted for baseline clinical factors=0.001) and PFS (interaction p unadjusted for baseline clinical factors=0.004). For patients classified as Poor, survival was similar for either treatment (HR=1.17 (95% CI: 0.79 to 1.73)), whereas patients classified as Good had better OS on atezolizumab than on docetaxel (HR=0.40 (95% CI: 0.26 to 0.63)). Results were qualitatively similar for PFS. The Poor group had similar PFS when receiving either treatment (HR=1.40 (95% CI: 0.97 to 2.02)), while the Good group had better PFS when treated with atezolizumab (HR=0.65 (95% CI: 0.45 to 0.94)). The test remained predictive of differential treatment benefit when adjusted for baseline clinical factors, table 3, Analysis 3.

### Validation set 2: OAK cohort

Mass spectra passing quality control were acquired for 786 samples (95.5% of patients with available samples) collected for the OAK cohort, with 403 patients in the atezolizumab arm and 383 patients in the docetaxel arm. 359 (46%) were classified as Poor and 427 (54%) as Good. ECOG PS, histology, smoking status and KRAS mutation status were associated with test classification in this larger cohort (table 1).

When applied to patients receiving atezolizumab, the test stratified both OS (HR=0.52 (95% CI: 0.41 to 0.66) and PFS (HR=0.76 (95% CI: (0.62 to 0.94)), solid curves

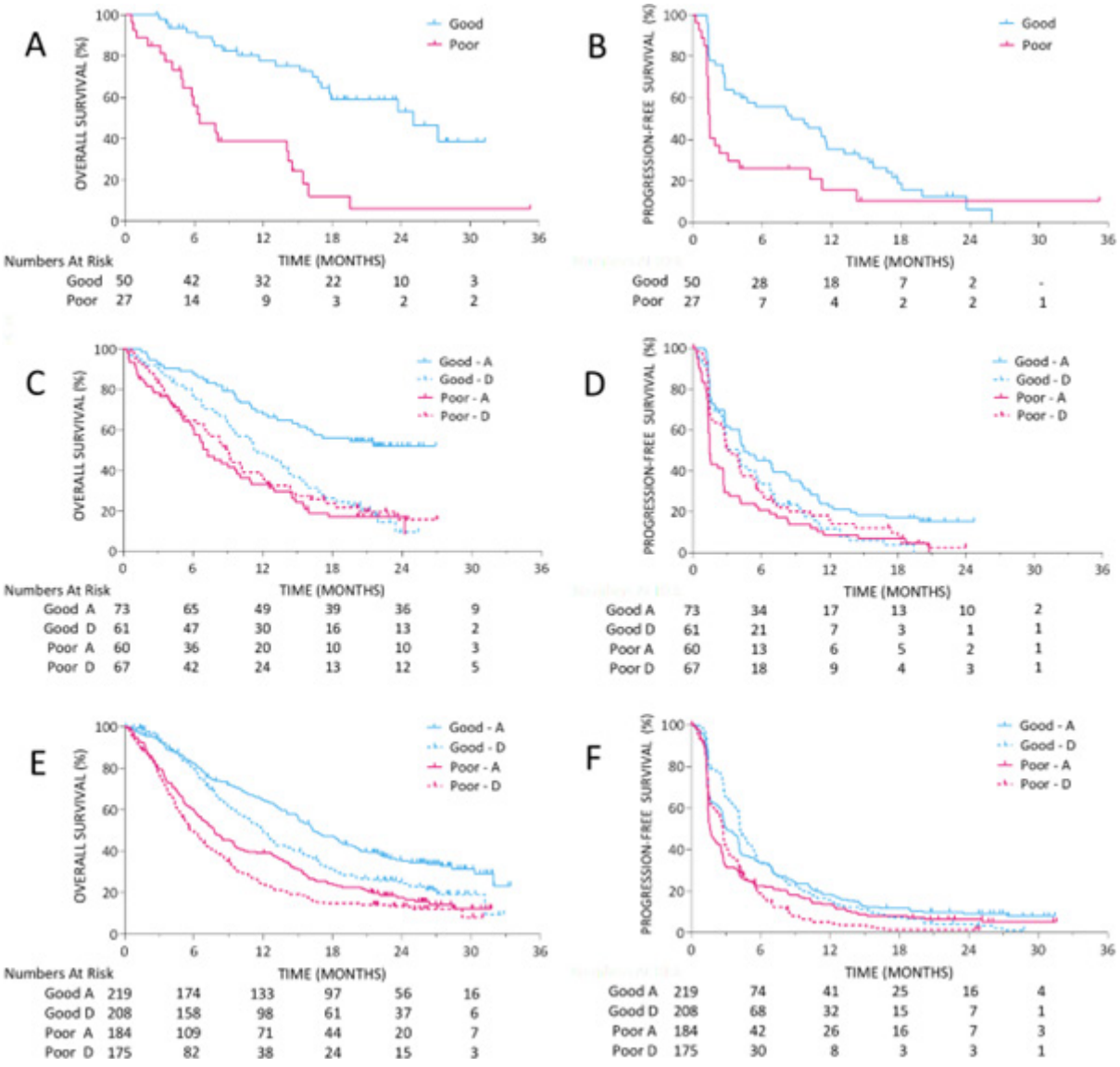


**Figure 3** Kaplan-Meier plots of OS and PFS by test classification (Good vs Poor) and treatment (A=atezolizumab, D=docetaxel) for the Development cohort (A and B), POPLAR cohort (C and D), and OAK cohort (E and F). OS, overall survival; PFS, progression-free survival.

in figure 3E,F. For patients treated with atezolizumab, mOS was 7.8 months (95% CI: 6.3 to 9.5 months) for the Poor subgroup and more than twice that, 16.3 months (95% CI: 15.0 to 19.3 months) for the Good subgroup. 1-year survival was 39% for patients classified as Poor, compared with 64% for those classified as Good. Results were qualitatively similar for PFS for the atezolizumab arm. mPFS was 1.6 months (95% CI: 1.5 to 2.2 months) for patients classified as Poor and 3.0 months (95% CI: 2.7 to 4.1 months) for patients classified as Good. PFS percentage at 6 months was 23% versus 34% in the Poor and Good subgroups, respectively. ORRs and DCRs were 12% versus 16% and 40% versus 57% in the Poor and Good subgroups, respectively, online supplemental table 8. Multivariate analysis within the atezolizumab arm showed that test classification remained a significant predictor of OS and PFS when adjusted for baseline characteristics, table 3, Analysis 2.

In this second validation cohort, the test also stratified OS and PFS for patients treated with docetaxel (OS HR=0.54 (95% CI: 0.43 to 0.68) and PFS HR=0.59 (95% CI: 0.48 to 0.73)), dashed curves in figure 3E,F. These results remained qualitatively similar in multivariate analysis (online supplemental table 9). Within the docetaxel arm mOS was twice as long for the Good group as for the Poor group (12.0 months (95% CI: 10.3 to 13.0 months) vs 5.8 months (95% CI: 4.9 to 7.1 months)). 1-year survival was also twice as high (49% vs 23%). mPFS for patients treated with docetaxel was 2.7 months (95% CI: 2.2 to 2.9 months) for the Poor group compared with 4.2 months (95% CI: 4.1 to 4.9 months) for the Good group. Progression-free percentage at 6 months was 19% (Poor) versus 34% (Good). ORR to docetaxel was 10% in the Poor group and 17% in the Good group, and DCR exceeded 50% in both test classification groups (54% for Poor and 69% for Good), online supplemental table 8.

**Table 2** Association of the test with various biological processes

| Biological process | P value of association | False discovery rate |
|---|---|---|
| Acute inflammatory response | 0.020 | <0.20 |
| Complement activation | 0.021 | <0.20 |
| Acute phase response | 0.024 | <0.20 |
| Cytokine production involved in immune response | 0.041 | <0.30 |
| Immune tolerance and suppression | 0.045 | <0.30 |
| Cellular component of morphogenesis | 0.069 | <0.30 |
| Type 2 immune response | 0.161 | <0.60 |
| Type 17 immune response | 0.183 | <0.60 |
| B cell mediated immunity | 0.271 | <0.70 |
| Response to hypoxia | 0.333 | <0.70 |
| Innate immune response | 0.382 | <0.70 |
| Angiogenesis | 0.421 | <0.70 |
| Extracellular matrix organization | 0.435 | <0.70 |
| Nk cell mediated immunity | 0.435 | <0.70 |
| Wound healing | 0.457 | <0.70 |
| T cell mediated immunity | 0.469 | <0.70 |
| Chronic inflammatory response | 0.502 | <0.70 |
| Glycolysis | 0.829 | <1.00 |
| Interferon type 1 | 0.841 | <1.00 |
| Type 1 immune response | 0.841 | <1.00 |
| Behavior | 0.843 | <1.00 |
| Epithelial-mesenchymal transition | 0.889 | <1.00 |
| Interferon γ signaling and response | 0.912 | <1.00 |

Nk, natural killer.

OS was better for atezolizumab-treated patients than for patients receiving docetaxel in both Good (HR=0.69 (95% CI: 0.55 to 0.87) and Poor groups (HR=0.79 (95% CI: 0.63 to 0.99)), while PFS was similar for both treatments for both Good (HR=1.01 (95% CI: 0.82 to 1.23)) and Poor groups (HR=0.94 (95% CI: 0.75 to 1.17)). Hence, in contrast to the results from the POPLAR cohort, in the OAK cohort the test was not predictive of differential treatment benefit for either OS (interaction p=0.743) or PFS (interaction p=0.391). This conclusion remained unchanged when the analysis was adjusted for possible confounding factors, table 3, Analysis 4.

### Analysis of benefit of atezolizumab compared with docetaxel within PD-L1 status and test classification subgroups of the OAK cohort

The large size of the OAK cohort allowed an analysis of test performance within PD-L1 defined groups. OS by treatment arm and test classification group is shown for the PD-L1 groups TC0 and IC0 (PD-L1 negative), TC1/2/3 or IC1/2/3, TC2/3 or IC2/3, and TC3 or IC3 in figure 4. The corresponding plots for PFS are shown in online supplemental figure 1.

Patient outcome depends on treatment, PD-L1 status, and test classification. Patients classified as Good with high PD-L1 had the best outcomes and patients classified as Poor with low PD-L1 expression had the worst outcomes when treated with atezolizumab. However, patients classified as Good and PD-L1 negative (TC0 and IC0) had numerically better OS than patients classified as Poor and positive PD-L1 status (TC1/2/3 or IC1/2/3) (TC0 and IC0, Good: mOS=14.9 months (95% CI: 10.6 to 16.4 months); TC1/2/3 or IC 1/2/3, Poor: mOS=9.5 months (95% CI: 7.4 to 14.3 months); HR=0.77 (95% CI: 0.55 to 1.07)). Relative benefit of atezolizumab and docetaxel may depend on test classification and PD-L1 status. Patients with low PD-L1 (TC0 and IC0) trended to benefit from atezolizumab compared with docetaxel if they are classified as Good (HR=0.74 (95% CI: 0.53 to 1.04)) but had similar outcomes on the two therapies if they are classified as Poor (HR=1.12 (95% CI: 0.79 to 1.59)), figure 4E right-hand forest plot.

## DISCUSSION

We developed a test based on information in the pretreatment circulating proteome stratifying patients into two groups, Good and Poor, with better or worse outcomes, respectively when treated with atezolizumab. This test validated well in two independent cohorts of patients drawn from a Phase 2 and a Phase 3 clinical trial and demonstrated reproducibility in excess of 95%. Test classification remained an independent predictor of OS and PFS on atezolizumab therapy when adjusted for other prognostic factors, including PD-L1 status, indicating the test provides additional information on patient outcome.

Our results demonstrate that it is possible to improve prediction of patient outcomes on immunotherapy by combining information gleaned from the tumor with measurements of the circulating proteome. In particular, proteins related to complement activation, acute phase response, acute inflammatory response, and the response of the host to the tumor were associated with test classification in this study. It is now understood that systemic inflammation can affect cancer prognosis by supplying bioactive molecules to the tumor microenvironment which may sustain proliferation, limit cell death, and facilitate invasion and angiogenesis.[8 9 31–33] Circulating innate immunity components, such as acute-phase reaction and complement activation, have also been found to impact cancer prognosis and the response to cancer therapy via their direct effects on TCs and their support of a cancer-aiding microenvironment.[8 9 11 34 35] Our results show that the influence on the outcome of test classification derived from assaying the circulating proteome is of similar order of magnitude to that of PD-L1 status; OS of patients assigned to the Good subgroup whose tumors were negative for PD-L1 expression was longer than that of patients receiving a Poor classification with PD-L1 positive tumors. Hence, reliance on only tumor-based information to assess patient prognosis fails to incorporate

**Table 3** Multivariate analyses of OS and PFS in the POPLAR and OAK cohorts. Analyses 1 and 2 are within the atezolizumab arms and analyses 3 and 4 include both treatment arms and a treatment–test interaction

| | | | OS | | PFS | |
|---|---|---|---|---|---|---|
| | Cohort | Covariate | HR (95% CI) | p | HR (95% CI) | p |
| 1. | POPLAR | Poor versus Good | 0.35 (0.22 to 0.56) | <0.001 | 0.52 (0.35 to 0.76) | 0.001 |
| | | ECOG PS 0 versus 1 | 1.51 (0.90 to 2.55) | 0.122 | 1.44 (0.94 to 2.21) | 0.094 |
| | | 1 versus 2 prior lines of therapy | 1.17 (0.75 to 1.83) | 0.499 | 0.89 (0.61 to 1.31) | 0.570 |
| | | PD-L1 status negative versus positive | 0.66 (0.42 to 1.03) | 0.069 | 0.77 (0.52 to 1.13) | 0.184 |
| | | Ever smoker versus never smoker | 0.72 (0.38 to 1.35) | 0.303 | 1.61 (1.00 to 2.58) | 0.049 |
| | | Squamous versus non-squamous | 0.66 (0.41 to 1.06) | 0.083 | 0.72 (0.49 to 1.07) | 0.108 |
| 2. | OAK | Poor versus Good | 0.55 (0.43 to 0.70) | <0.001 | 0.76 (0.62 to 0.95) | 0.013 |
| | | ECOG PS 0 versus 1 | 1.60 (1.24 to 2.07) | <0.001 | 1.30 (1.04 to 1.62) | 0.022 |
| | | 1 versus 2 prior lines of therapy | 0.94 (0.71 to 1.24) | 0.642 | 0.75 (0.59 to 0.97) | 0.027 |
| | | PD-L1 status negative versus positive | 0.62 (0.49 to 0.79) | <0.001 | 0.78 (0.64 to 0.97) | 0.024 |
| | | Ever smoker versus never smoker | 1.03 (0.74 to 1.44) | 0.876 | 1.59 (1.20 to 2.12) | 0.001 |
| | | Squamous versus non-squamous | 0.84 (0.64 to 1.10) | 0.208 | 1.01 (0.79 to 1.30) | 0.925 |
| 3. | POPLAR | Poor versus Good | 0.88 (0.60 to 1.31) | 0.535 | 1.05 (0.73 to 1.52) | 0.778 |
| | | Docetaxel versus atezolizumab | 1.16 (0.78 to 1.73) | 0.467 | 1.41 (0.97 to 2.05) | 0.072 |
| | | Interaction | 0.37 (0.20 to 0.68) | 0.001 | 0.47 (0.27 to 0.79) | 0.005 |
| | | ECOG PS 0 versus 1 | 1.34 (0.96 to 1.86) | 0.089 | 1.25 (0.93 to 1.67) | 0.145 |
| | | 1 versus 2 prior lines of therapy | 1.10 (0.80 to 1.50) | 0.557 | 0.97 (0.73 to 1.27) | 0.806 |
| | | PD-L1 status negative versus positive | 0.88 (0.64 to 1.20) | 0.424 | 0.94 (0.71 to 1.25) | 0.669 |
| | | Ever smoker versus never smoker | 0.66 (0.43 to 1.01) | 0.055 | 1.02 (0.71 to 1.44) | 0.935 |
| | | Squamous versus non-squamous | 0.75 (0.55 to 1.03) | 0.072 | 0.74 (0.56 to 0.99) | 0.041 |
| 4. | OAK | Poor versus Good | 0.58 (0.46 to 0.73) | <0.001 | 0.65 (0.52 to 0.80) | <0.001 |
| | | Docetaxel versus atezolizumab | 0.73 (0.58 to 0.92) | 0.008 | 0.89 (0.71 to 1.11) | 0.288 |
| | | Interaction | 0.97 (0.70 to 1.35) | 0.859 | 1.17 (0.87 to 1.57) | 0.298 |
| | | ECOG PS 0 versus 1 | 1.73 (1.45 to 2.07) | <0.001 | 1.37 (1.17 to 1.60) | <0.001 |
| | | 1 versus 2 prior lines of therapy | 0.96 (0.79 to 1.17) | 0.686 | 0.81 (0.68 to 0.97) | 0.019 |
| | | PD-L1 status negative versus positive | 0.76 (0.64 to 0.90) | 0.001 | 0.86 (0.74 to 1.00) | 0.051 |
| | | Ever smoker versus never smoker | 1.08 (0.85 to 1.37) | 0.550 | 1.47 (1.20 to 1.81) | <0.001 |
| | | Squamous versus non-squamous | 0.81 (0.67 to 0.98) | 0.029 | 0.97 (0.81 to 1.16) | 0.717 |

ECOG PS, Eastern Cooperative Oncology Group performance status; OS, overall survival; PD-L1, programmed cell death-ligand 1; PFS, progression-free survival.

key markers that strongly impact patient outcomes and are easily accessible from a simple blood draw. Only a few microliters of serum are required to perform the test and serum can be shipped at ambient temperature dried on a serum collection card.

Other circulation-derived biomarkers that may be of use in predicting outcomes for patients with advanced NSCLC treated with immunotherapies or alternative treatments have been investigated. These markers include some related to the tumor, such as blood-based tumor mutation burden (bTMB)[36–38] and those designed to assess host immunity or inflammation, such as neutrophil-to-lymphocyte ratio (NLR)[39 40] and Lung Immune Prognostic Index.[41 42] bTMB showed the ability to stratify outcomes on atezolizumab and to predict differential benefit of atezolizumab versus docetaxel for PFS but not OS within a cohort of patients drawn from the OAK study with the cut-off defined using a cohort of patients from the POPLAR study.[36] However, it failed to provide a significant stratification of PFS in a prospective study in the first line advanced NSCLC setting[38] and further work is necessary to refine the assay if bTMB is to become a standard of care biomarker in NSCLC.[38] Recent research, also using a cohort of patients from the OAK study, showed promise for the utility of pretreatment NLR as a prognostic and predictive biomarker for treatment with atezolizumab[40] and future studies may elucidate the potential of this marker in the first-line setting. In another study, a test similar to ours was developed for advanced, anti-PD-1-treated melanoma where test classification was

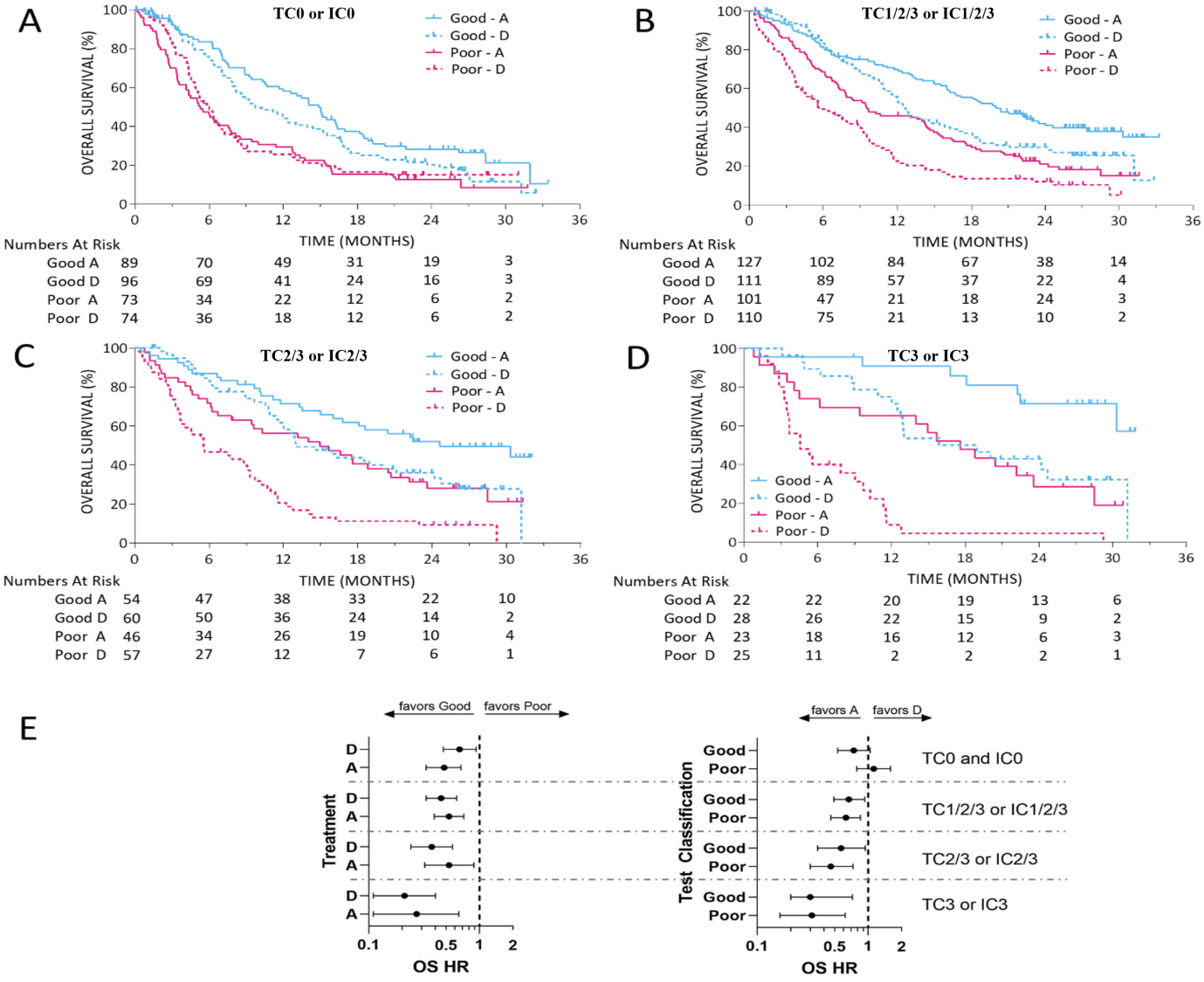


**Figure 4** Kaplan-Meier plots of OS by treatment arm and test classification within PD-L1 defined subgroups of the OAK cohort. (A) TC0 and IC0, (B) TC1/2/3 or IC1/2/3, (C) TC2/3 or IC2/3, (D) TC3 or IC3. (E) Forest plots of OS HRs between test classifications by PD-L1 and treatment subgroup (left) and between treatments by PD-L1 and test classification subgroup (right). A, atezolizumab; D, docetaxel; IC, immune cell; OS, overall survival; PD-L1, programmed cell death-ligand 1; TC, tumor cell.

complementary to NLR and together the two immune/inflammation-based markers improved outcome stratification.[11 16] Given the complex nature of cancer progression and mechanisms of action of cancer therapies,[8 9 31] it seems likely that multiple markers spanning tumor, tumor microenvironment,[21 43] circulation, and possibly microbiome,[44 45] may need to be combined to assess patient prognosis and response to therapy most accurately.

Assessments of the ability of the test to predict differential benefit of atezolizumab over docetaxel were inconsistent between the two cohorts. Whereas the test provided little stratification of outcomes for patients treated with docetaxel and a corresponding large predictive ability (interaction p≤0.005) for both OS and PFS in the POPLAR cohort, within the OAK cohort the test stratified OS and PFS similarly in the atezolizumab and docetaxel treatment arms, with no indication of any significant predictive power (interaction p≥0.3). Examination of differences in patient characteristics between the two apparently similar cohorts did not lead to any satisfactory explanation of these inconsistent results. It is possible that at least some of the discrepancy could be due to the benefit of atezolizumab compared with docetaxel depending on a non-trivial, not simply additive, combination of PD-L1 status and test classification, and possibly also other factors such as PS. Supporting this hypothesis, the results of subgroup analysis of the OAK cohort do indicate that while patients with tumors negative for PD-L1 expression receive similar benefit from docetaxel and atezolizumab when assigned a Poor test classification, those classified as Good may benefit more from atezolizumab than from docetaxel. This observation requires validation in additional studies.

Although the current study allowed blinded validation of the developed test in two independent cohorts from

randomized clinical trials, these analyses were retrospective in nature. Prospective studies designed specifically for validation of this test would be desirable. A major limitation of the current study is that immunotherapy has now moved into the first-line treatment of advanced NSCLC for patients with tumors without a targetable genetic aberration.[46] It would be interesting to explore the utility of the test in the first-line setting for patients with high PD-L1 receiving atezolizumab monotherapy and for patients, regardless of PD-L1 status, treated with the combination of atezolizumab and platinum-based chemotherapy.[3 47 48] Another direction for future research would be to examine the ability of the test to stratify outcomes for patients with early-stage cancer (stage II–IIIA) who may receive atezolizumab as adjuvant therapy following surgery with curative intent.[3 49] Possible utility of the test in other cancer types where atezolizumab has shown efficacy[3] would also be of interest, as would an exploration of the specificity of the test to atezolizumab or anti-PD-L1 and anti-PD-1 antibodies.

In conclusion, we developed and validated a novel blood-based test capable of stratifying the outcomes of previously treated patients receiving atezolizumab. The ability of the test to stratify patient responses was complementary to PD-L1 expression and commonly used clinical factors. Furthermore, by combining the measurement of the circulating proteome and tumor characteristics such as PD-L1, survival stratification can be refined to accurately identify patients likely to benefit from atezolizumab rather than with docetaxel. Overall, this study suggests that the combined use of blood-based measurement of circulating proteins and PD-L1 can aid treatment selection and identification of patients likely to benefit from anti-PD-(L)1 therapies.

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