



Circulating Biomarkers of Immunotherapy Response and Immune-Related Events

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**Scholarly Report submitted in partial fulfillment of the MD Degree
at Harvard Medical School**

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Scholarly Report Title: Circulating Biomarkers of Immunotherapy
Response and Immune-Related Events

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ABSTRACT

Title: Circulating Biomarkers of Immunotherapy Response and Immune-Related Events.

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Background: Therapeutic inhibition of programmed cell death receptor-1 (PD-1) has revolutionized the treatment of melanoma. Immune checkpoint inhibitors (ICI) target specific interactions in the immune cascade, ultimately activating tumor-specific T cells to promote tumor destruction. This mechanism of action gives rise to a potential immune response against self-antigens that can lead to the emergence of immune-related adverse effects (irAEs). There is growing evidence that patients who suffer from a subset of tissue-specific irAEs may derive greater benefit from therapy. We hypothesize that there is a subset of immune cells that is responsible for organ-specific toxicities and that is distinct from immune populations that are responsible for ICI-induced tumor response in certain patients.

Methods: A retrospective analysis of 50 melanoma patients treated at the Massachusetts General Hospital has been previously performed, generating detailed response and toxicity clinical data. In parallel, proteomic data from plasma of these patients via a proximity extension array (Olink®) was generated, examining ~1000 markers of protein expression simultaneously. Samples were analyzed prior to treatment, as well as 6 weeks and 6-month following treatment initiation.

Results: Out of the 50 patients in this cohort, 60% (n=30) experienced at least one irAE of any grade. At 6 weeks and 6 months post treatment, NOS3 expression was increased among patients who did vs. did not experience an irAE. A large breadth of irAE classes were documented including gastrointestinal, musculoskeletal, cutaneous, endocrine, pulmonary, renal and hepatitis. Based on our hypothesis that a distinct group of immune cells is responsible for organ-specific toxicities, we examined the proteomic profiles of three groups of patients: (1) cutaneous and musculoskeletal toxicities (hypothesized shared mechanism of toxicity and response), (2) colitis (hypothesized distinct mechanism of toxicity), and (3) patients with no toxicities. While there were no markers that were statistically significant, a number of markers revealed trends over multiple iterations of analyses, most notably NOS3. Analysis of expression over time and correlations with other markers was also performed.

Discussion: Unbiased proteomic profiling and correlation with tissue-specific irAEs can yield insights into shared and exclusive markers of toxicities. The translational melanoma infrastructure at the MGH allows retrospective analysis of patients treated with ICI. This project importantly demonstrates the feasibility of retrospective analysis of irAEs using this data-set. Further investigation of protein markers of toxicities is warranted.

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GLOSSARY OF ABBREVIATIONS

AE	Adverse event
CD	Cluster of differentiation
CTLA-4	Cytotoxic T-lymphocyte-associated protein
DEP	Differentially expressed proteins
GI	Gastrointestinal
MSK	Musculoskeletal
ICI	Immune checkpoint inhibitors
IFN	Interferon
IL	Interleukin
irAE	Immune-related adverse event
LDH	Lactate dehydrogenase
MGH	Massachusetts General Hospital
NCI	National Cancer Institute
NCCN	National Comprehensive Cancer Network
NOS3	Nitric oxide synthase 3
PD-L1	Programmed cell death ligand-1
PD-1	Programmed cell death receptor-1
RECIST	Response Evaluation Criteria in Solid Tumors
US FDA	United States Food and Drug Administration

SECTION 1: INTRODUCTION

1.1 Immunotherapy in the Setting of Melanoma

Invasive cutaneous melanoma is estimated to be the fifth most common cancer in the United States, accounting for approximately 96,000 new cases and 7,230 deaths in 2019 (1). Early identification of suspicious skin lesions is critical for prognosis, as surgical resection is the mainstay of treatment. For patients with lesions less than 1mm in thickness and no nodal involvement (stage I), 5-year survival is excellent (2). In the setting of localized lesions greater than 1mm (stage II), 5-year survival depends on Breslow thickness, ulceration, and mitotic rate but can approach 90% (2-4). Prognosis is historically poor in patients with advanced disease either in regional lymph nodes (stage III) or distant metastatic sites such as the lung, brain or bone (stage IV) (5). Specifically for stage IV patients with brain metastases, median survival was historically 4 to 5 months (6, 7). Elevated lactate dehydrogenase (LDH) (8) and *BRAF* genetic mutations (9) are associated with worse outcomes among all advanced melanoma patients.

Therapeutic inhibition of programmed cell death receptor-1 (PD-1) has revolutionized the treatment of metastatic melanoma (10). While conventional chemotherapy nonspecifically target rapidly dividing cells, immune checkpoint inhibitors (ICI) target specific interactions in the immune cascade. Physiologically, inhibitory receptors on T cells such as PD-1 downregulate effector cell function (11). This interaction is critical in maintaining immunologic self-tolerance and minimizing damage to healthy tissue during a pathologic response. Certain tumors, particularly melanoma, are thought to take advantage of this system by overexpressing PD-L1 (12), preventing generation of an effective tumor-specific immune response. Monoclonal

antibodies against T cell receptor PD-1 or its ligand PD-L1 block this interaction, thus activating tumor-specific T cells to promote tumor destruction (11). Indeed, ICIs are now the first-line treatment for a number of tumors, including metastatic melanoma, non-small cell lung cancer, and clear cell, and renal cell carcinoma.

As of August 2019, three ICIs (ipilimumab, nivolumab, and pembrolizumab) have been approved by the US Food and Drug Administration for metastatic melanoma based on a series of clinical trials that showed a survival benefit. Ipilimumab, a monoclonal antibody that blocks immune checkpoint receptor CTLA-4, has been shown to significantly improve progression-free survival (PFS) and overall (OS) in patients with unresectable stage III/IV melanoma compared to placebo (13). However, up to 20% of patients experience grade 3 or 4 toxicities. The KEYNOTE-006 study compared adjuvant pembrolizumab (an anti-PD1 antibody) to ipilimumab in advanced melanoma patients. Pembrolizumab not only prolonged PFS and OS (74.1% vs. 58.2% 12-month survival rates, $p < 0.0001$), but it was also associated with fewer high-grade adverse events than ipilimumab (14). Pembrolizumab has also demonstrated prolonged recurrence-free survival (RFS) in patients with resected stage III melanoma (15). Nivolumab, another anti-PD-1 antibody, has shown similar efficacy and safety to pembrolizumab (16). Monotherapy with nivolumab or pembrolizumab are now National Comprehensive Cancer Network (NCCN) Category 1 recommendations for metastatic or unresectable disease (17).

1.2 Immune-Related Adverse Events among Melanoma Patients

The mechanism of action of ICI therapy gives rise to a potential immune response against self-antigens that can lead to the emergence of immune-related adverse effects (irAEs) (18). These are unique and generally acceptable side effects. This unique profile of toxicities can resemble autoimmune disorders, likely caused the reactivation of cellular immunity against self-antigens.

A recent systematic review by Wang et al. of 125 clinical trials assessed the incidence irAEs among different drugs and cancer types, examining the current landscape of treatment-related adverse events (19). Approximately 2 in 3 patients treated with PD-1 or PD-L1 inhibitors had at least one adverse event, and 1 in 7 patients experienced at least one grade 3 or higher adverse event. Melanoma patients had the highest incidence of all-grade adverse events (1.72%; 95% CI, 1.45%-2.27%). The most common irAEs were endocrine dysfunctions, specifically hypothyroidism (6.07%; 95% CI, 5.35%-6.85%) and hyperthyroidism (2.82%; 95% CI, 2.40%-3.29%). Hyperglycemia, thyroiditis, adrenal insufficiency, type 1 diabetes, and hypopituitarism were present but less common. However, these adverse events were more likely to be severe. Approximately 20% to 35% of these rare adverse events were grade 3 or higher, as opposed to about 2% for thyroid dysfunction. Treatment-related death due to ICI was an rare complication (0.04%), most commonly related to pneumonitis (19).

There is growing evidence that melanoma patients who suffer from certain irAEs may derive greater benefit from therapy. Even in early immunotherapy clinical trials, the development of vitiligo during melanoma treatment was associated with improved survival (20-22). This is hypothesized to result from immune-mediated destruction of melanocytes through recognition of

antigens shared by normal melanocytes and tumor cells (23). Therefore, development of vitiligo could represent robust activation of melanoma-specific T cells, thus serving as a surrogate marker for anti-tumor efficacy. Several retrospective analysis of ICI-treated melanoma patients have showed improved outcomes associated with vitiligo (24-26). In one retrospective study limited to treatment with pembrolizumab, a complete or partial response to treatment was associated with a higher occurrence of vitiligo (71% vs. 28%; $p=0.002$ (27)). However, vitiligo is a not a common side effect, and its occurrence does not preclude disease progression (24). Furthermore, it is unclear if other irAEs could be associated with improved outcomes.

High-dose glucocorticoids are often used to manage irAEs. However, there is a concern that since immune checkpoint blockade works by increasing antitumor immunity, systemic immunosuppression may reverse any therapeutic benefit (28, 29). Recently, the Boland lab at Massachusetts General Hospital (MGH) has shown that treatment with high-dose glucocorticoids may mitigate the better clinical outcomes predicted by irAEs (Bai XE et al., submitted). A retrospective analysis of 142 patients treated at MGH was performed, 55% of whom had irAEs. They demonstrated that the presence of a subset of irAEs correlated with longer progression-free survival (PFS) and overall survival (OS). Interestingly, they also demonstrated that the use of high-dose glucocorticoids (>30mg/day) impaired, but did not entirely offset, the improved therapeutic outcomes associated with a subset of irAEs in melanoma. Specifically, treatment of cutaneous and musculoskeletal toxicities but not colitis with high-dose glucocorticoids was associated with worse prognosis (Supplementary Figure 1).

There is growing evidence that certain organ-specific toxicities should be managed carefully to not offset antitumor immunity. Currently, there are no appropriate clinical tools available to assess which patients would benefit from treatment of irAEs. Furthermore, it is unclear whether there exist blood-based biomarkers of response and/or toxicity, and if so whether these immune populations are shared or distinct across various irAEs (30).

1.3 Project Goals

Unbiased proteomic profiling and correlation with tissue-specific irAEs may yield insight into shared and exclusive markers of toxicities. We hypothesize that a distinct subset of immune cells is responsible for organ-specific toxicities. We are examining proteomic profiles of three groups of patients: (1) cutaneous and musculoskeletal toxicities (hypothesized shared mechanism of toxicity and response), (2) colitis (hypothesized distinct mechanism of toxicity), and (3) patients with no toxicities. This is supported by the findings that patients with cutaneous and musculoskeletal toxicities who were treated with immunosuppression had worse outcomes (Supplementary 1). The immune cells responsible for toxicity could be critical for anti-tumor response. In contrast, patients with colitis who were treated with immunosuppression appear to have similar outcomes to those who were not treated (Bai XE et al., submitted).

Our hypothesis is that the identification of blood-based biomarkers of disease burden and immune competence can be used to predict responses and toxicities, and ultimately be utilized to manage patients with irAEs in the clinic. This project will leverage a longitudinal biobank of melanoma patients undergoing ICI treatment at MGH to identify potential blood-based biomarkers of toxicity. The Boland lab has previously mined the electronic medical record to

identify a cohort of melanoma patients treated with ICI with key data on their clinical response and irAEs.

This project takes advantage of the Olink® Multiplex Protein Extension Immunoassay. This platform enables high-throughput, quantitative analysis of protein expression, allowing for analysis of approximately 1000 markers simultaneously. Previous work in the Boland lab used this platform to assess for plasma proteomic predictors of clinical response among melanoma patients treated with PD-1 immunotherapy (Arnav et al., unpublished). Preliminary data identified 38 differentially expressed proteins (DEPs) between anti-PD1 responders and non-responders, many of which reflect immune and resistance pathways. Interestingly, increased protein expression of IL-6 prior to treatment was found non-responders vs. responders, suggesting that baseline inflammation may impeded immunotherapy efficacy (Supplementary Figure 2). These data demonstrated the feasibility of this platform within this patient population.

By examining multiple clinical variables and circulating biomarkers in parallel, we aim to assess the proteomic profile in melanoma patients before and after development of irAEs. This will help the field potentially establish blood-based biomarkers for the emergence of irAEs. Early detection and management of toxicities will be particularly important, as the field expands utilization of ICI into the adjuvant setting and create clinical guidelines for irAE management.

SECTION 2: STUDENT ROLE

The overall project was conceived by myself in collaboration with Arnav Mehta, David Lieb, Nir Hacohen, Keith T. Flaherty, Ryan J. Sullivan, and Genevieve M. Boland. Retrospective annotation of patients regarding demographics, treatment plan, and immune-related adverse events had been previously performed by Xue Bai, Dennie T. Frederick, Gyulnara Kasumova and Michelle Kim. The plasma proteomic data via a proximity extension array (Olink®) was generated by Marijana Rucevic, Markus Sallman-Almen, and Lina Hultin Rosenberg.

I consolidated, processed and analyzed the proteomic data related to toxicity. Analysis was reviewed by Arnav Mehta and Genevieve M. Boland. The writing is my own.

SECTION 3: METHODS

3.1 Patients

Patients with stage III or IV melanoma treated with anti-PD-1 antibody monotherapy (nivolumab or pembrolizumab) were identified at Massachusetts General Hospital Cancer Center (MGH, n=169). Patients who initiated anti-PD-1 monotherapy between Sept 2009 and Aug 2018 were included. Those treated both within and outside clinical trial settings were included in this study. The last date for follow-up of irAEs was May 2019. Detailed clinical data including baseline demographics, melanoma subtype, mutational status, clinical stage, oncologic treatment and survival was collected and reported elsewhere (Bai XE et al., submitted). Samples were available from 50 patients at three timepoints to perform initial downstream proteomic analysis.

IrAEs were graded based on clinical descriptions gathered from medical notes, clinical trial data, and/or by the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 when objectively based. Effectiveness of anti-PD-1 monotherapy was determined by local radiologists using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1.

This study was been conducted in compliance with local Institutional Review Board policies.

3.2 Plasma proteomic data (Olink®)

Peripheral whole blood for proteomic analysis was collected at baseline (prior to initiation of anti-PD-1 antibody monotherapy), as well as at 6 weeks and 6 month follow-up visits. Plasma was separated from whole blood, aliquoted and stored at -80°C using standard techniques.

Samples were analyzed using the Olink® Multiplex Protein Extension Immunoassay in June 2019. Olink® is a high-throughput, multiplex immunoassay that enables the analysis of ~1000 protein biomarkers simultaneously (Olink Bioscience, Uppsala, Sweden). The platform utilizes antibodies labeled with DNA oligonucleotides. In the presence of binding between the marker of interest and the Olink antibody, the DNA oligonucleotides hybridize. Microfluidic qPCR is then used to amplify and quantify this DNA barcode (Supplementary Figure 3).

Data was collected for the following panels: Cardiometabolic (v. 3602), Cardiovascular III (v. 6112), Immune Response (v. 3201), Immuno-Oncology (v. 3101), Neurology (v. 8011), Oncology II (v. 7202), Oncology III (v. 4001), and Inflammation (v. 3021). Note that the panels are overlapping, such that there are protein markers that are seen in multiple panels. Protein levels were expressed as Normalized Protein eXpression (NPX) values, an arbitrary unit on log₂-scale, to account for interplate variation and background marker expression levels. This was performed using the built-in quality control system. Further information on markers in each of the panels, assay validation and data normalization can be found on the manufacturer's website (www.olink.com/downloads).

3.3 Statistical Analysis

Protein expression markers with >10% data measurements below the limit of detection were excluded from further analysis. For the remaining assays, samples with measurements below the detection limit were replaced by the lowest value present after normalization.

All statistical tests were two-sided, and $p < 0.05$ was considered to be of statistical significance. The differences between the groups were analyzed using the non-parametric Wilcoxon signed-ranked test. Given the limited number of samples, comparisons were performed with and without correcting for multiple comparisons. As the primary goal of the study was to generate hypotheses to be tested in other settings, power analysis was not performed to determine the number of patients necessary to detect difference. All analyses were performed using JMP Version 15.

SECTION 4: RESULTS

4.1 Breadth of irAEs Among by Melanoma Patients Treated with Immunotherapy

Patients with unresectable stage III or IV melanoma treated with anti-PD-1 antibody monotherapy (nivolumab or pembrolizumab) at MGH were evaluated. Detailed response and toxicity clinical data was collected. Baseline characteristics of the entire cohort have been previously described (Bai XE et al., submitted; Supplementary Figure 4). In parallel, proteomic data from plasma via a proximity extension array (Olink®) was generated. Samples were collected from 50 patients prior to treatment, as well as 6 weeks and 6 months following treatment initiation.

Out of the 50 patients in this cohort, 60% (n=30) experienced at least one irAE of any grade (Figure 1). 30% (n=15) and 18% (n=9) of the patients experienced at least two or three irAEs, respectively. The majority of irAEs occurred within 12 weeks of treatment initiation, with the latest irAE occurring at the six month follow-up.

For the 30 patients who experienced any irAE, we further classified the adverse event. Eight classes of irAE were documented based on the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. These include (1) gastrointestinal (colitis, diarrhea, gastritis), (2) hepatitis, (3) pulmonary (pneumonitis), (4) musculoskeletal (arthritis, arthralgias), (5) renal (nephritis), (6) cutaneous (vitiligo, dermatitis), (7) endocrine (central adrenal insufficiency, hypothyroidism, hypophysitis), as well as other noncategorized events. The most common first irAE class was endocrine (30%, n=9; Figure 2). The next most common classes

were musculoskeletal (n=6), gastrointestinal (n=5), pulmonary (n=4) and cutaneous (n=3). Hepatitis and nephritis were rare complications and were only experienced by one patient each. Additionally, one person experienced an ulcer on the tongue, which did not fit into any of the above classifications. Interestingly, the patient who experienced five complications had exclusively endocrine and musculoskeletal complications.

4.2 Differentially Expressed Proteins Among Patients Who Did vs. Did Not Experienced

Any irAE

We then analyzed the proteomic data generated from the Olink® proximity extension array (Figure 3). Data was collected for eight different panels. After review of the genes in the each of the panels, we focused on the immuno-oncology and immune response panels, as this would best cover the hypothesized markers of interests. After exclusion of failed assays, there were 146 multiplex subject-timepoints. Protein expression markers with >10% data measurements below the limit of detection were excluded from further analysis. In total, 77 unique protein markers were available for analysis (Figure 3). 16 markers were excluded (IL-2, IL-22, RA1, IL-13, IL-33, IFN-g, IL-2RB, IL-1a, TSLP, PD-L1, IL-24, ARTN, TNF, IL-20, IL-4, LIF and NRTN).

We first examined differentially expressed proteins (DEPs) among patients who experienced any irAE (n= 30) compared to those who experienced none (n=20; Figure 4). Prior to treatment, there were no DEPs that meet the statistically threshold of significance. At 6 weeks after treatment initiation, median protein expressions of NOS3 and PDCD1 were significantly increased in patients who experienced any irAE, while CCL4 was decreased (Figure 4A). At 6 months,

NOS3, TNFSF14, CCL20, and CD27 were significantly increased in patients who experienced any irAE, while CX3CL1 was decreased (Figure 4B).

NOS3 is an enzyme involved in the generation of free radicals and can be a surrogate marker of inflammation (31). Given that it was the only marker significantly increased at both 6 weeks and 6 months, we further investigated its expression among patients who had experienced multiple irAEs (Figure 5). For example, we compared NOS3 expression among patients who experienced two or more irAEs compared to those who experienced fewer than two, as well as three or more irAEs vs. fewer than three. There was increased median expression among patients who experienced increasing numbers of irAEs. There was also a trend of increased expression of increased NOS3 expression among patients who experienced exclusively three vs. two irAEs, though it was not statistically significant.

4.3 Differentially Expressed Proteins Among Patients Who Experienced Different Classes of irAEs

Based on our hypothesis that a distinct group of immune cells is responsible for organ-specific toxicities, we examined the proteomic profiles of three groups of patients: (1) cutaneous and musculoskeletal toxicities (hypothesized shared mechanism of toxicity and response), (2) colitis (hypothesized distinct mechanism of toxicity), and (3) patients with no toxicities. Out of the 50 patients, 34 were in one of these three categories: Group A (n=9), Group B (n=5), and Group C (n=20; Figure 6). Remaining 16 subjects did not fit in any designated class.

Likely given the limited number of subjects, all of the comparison between any two groups across all of the protein markers were not statistically significant. In order to determine if there were any markers suggested some trend across the different hypothesized classes of irAEs, we assessed the top markers that approached statistical significance (Figure 7). At six months following treatment, the top five markers that approached significance were NOS3, CD27, DCN, CXCL5, and CCL20. Interestingly, at 6 weeks and baseline, these five markers were among the top ten that approached significance.

In order to increase power, we performed a second analysis, where we included patients who experienced other, less common irAEs into group B. These subjects experienced rare irAEs with few subjects in each group, such that there was no statistical difference in therapeutic outcomes regarding the use of immunosuppression. However, it was difficult to tell if this was a true effect reflecting a distinct mechanism of toxicity and response, or the result of low power.

Thus, we examined the proteomic profiles of three new groups of patients: (1) cutaneous and musculoskeletal toxicities (hypothesized shared mechanism of toxicity and response, n=9), (2) all other irAEs (hypothesized distinct mechanism of toxicity, n=21), and (3) patients with no toxicities (n=20; Figure 8). Given the increased power, we focused on markers that had a comparison between any two groups that was statistically significant. At six months, there were two markers that had differential expression across any two groups that was statistically significant: NOS3, CD27, and CCL20 (Figure 9). Specifically, for all three markers there was increased expression in Group A vs. C, and a trend towards increased expression in B vs. C.

At six weeks, there were two of the three markers had some comparison between any two groups that was statistically significant: NOS3 and CD27.

4.4 Further Investigation of Hypothesized Markers of Interests

Previous data has explored differential protein expression over time among all patients in this cohort, which is thought to reflect changes in response to treatment. Here, we further investigated the protein markers that could reflect difference across classes of irAEs. We focused on NOS3 and CD27, as these protein markers appeared in both sets of analyses using the limited and larger cohorts (Figures 6 and 8). Additionally, they both approach statistical significant at 6 weeks and 6 months (Figures 7 and 9).

We first looked at differential expression of these two markers at the three timepoints (Figure 10). For both protein markers, there was no change in expression over time that was statistically significant and followed a trend. This was also true, when analysis was performed in the different hypothesized classes of irAEs.

Additionally, we performed multivariate analysis to assess what other proteins were associated with NOS3 and CD27 (Figure 11). Three markers that correlated with NOS3 expression across all patients (absolute value Pearson's $r > 0.5$, $p < 0.01$) were IL6 ($r = 0.6$), TNFRSF9 ($r = 0.5$), CD4 ($r = 0.5$). Ten markers fit this criteria for CD27, including five that were highly correlated (absolute value Pearson's $r > 0.8$, $p < 0.01$): DCN, ICOSLG, CXCL3, VEGF-A, and PD-L2.

SECTION 5: DISCUSSION

Therapeutic inhibition of programmed cell death receptor-1 (PD-1) has revolutionized the treatment of many cancer types. While conventional chemotherapy nonspecifically target rapidly dividing cells, immune checkpoint inhibitors target specific interactions in the immune cascade. This ultimately allows activated tumor-specific T cells to promote tumor destruction. Indeed, these immune checkpoint inhibitors are now the first-line treatment for a number of tumors, including metastatic melanoma, non-small cell lung cancer, clear cell renal cell carcinoma.

In this study, we performed a retrospective analysis of melanoma patients treated with PD-1 inhibitors, specifically investigating proteomic markers of different classes of irAEs via a proximity extension array (Olink®). Out of the 50 patients in this cohort, 60% (n=30) experienced at least one irAE of any grade. A large breadth of irAE classes were documented including gastrointestinal, musculoskeletal, cutaneous, endocrine, pulmonary, renal and hepatitis. We examined the proteomic profiles of three groups of patients: (1) cutaneous and musculoskeletal toxicities (hypothesized shared mechanism of toxicity and response), (2) colitis (hypothesized distinct mechanism of toxicity), and (3) patients with no toxicities. While there were no markers that were statistically significant, a number of markers revealed trends over multiple iterations of analyses, most notably NOS3 and CD27.

This study had a number of limitations. First, we were clearly limited by the small number of patients. This problem was exacerbated as we divided the cohort into three groups, based on the hypothesized class of irAEs. We increased our power by expanding the inclusion criteria, but as stated above, this arguably made the groups less clean. Second, although a key advantage of the

Olink® platform is that we were able to look at a number of markers simultaneously, we had to adjust for multiple comparisons in our analysis. This increased the statistical threshold for significance. Third, although 169 patients were assessed in the initial retrospective analysis of detailed clinical response, only 50 patients from this group were analyzed, potentially leading to selection bias. Fourth, samples were analyzed prior to treatment, as well as 6 weeks and 6-month following treatment initiation. These timepoints do not reflect when the actual irAE occurred. Ideally, in order to assess proteomic markers of toxicity, it would be important to look at plasma shortly after the irAE was documented.

While it is difficult to make any conclusions, this study generated a number of hypothesis that could be tested in different settings. Interestingly, NOS3 protein expression was significantly increased in patients who experienced any irAE compared to those with no irAEs at 6 months following treatment (Figure 4). Furthermore, there was a trend towards increased NOS3 expression among those with hypothesized shared vs. distinct mechanism of toxicity in both the selective and expanded cohorts (Figures 7 and 9). There was no evidence that expression increased across all groups as a function of time (Figure 10).

NOS3, also known as endothelial nitric oxide (eNOS), is a reactive oxide species that plays an important role in inflammation and apoptosis, critical components of carcinogenesis (31-33). Furthermore, upregulation of NOS3 is associated with lymphangiogenesis and angiogenesis in a number of oncologic settings including breast, lung, and gastric cancer (34-36). Its role in toxicity to ICI treatment is unclear, but increased expression in patients who experience irAE could reflect antitumor immunity. Future experiments could use flow cytometry to test the

hypothesis that NOS3 is increased in patients who experience irAEs. This platform could also assess which cells are responsible for its production, as there is evidence that endothelial cells and macrophages are responsible for NOS3 production in other settings (33). Also, flow cytometry could assess coexpression at cellular level of markers that correlated with NOS3 expression. Limited samples prevented multiparameter flow cytometry analysis using a typical panel that assess CD4⁺ and CD8⁺ T cell function. This Olink analysis supports future efforts to utilize a curated flow cytometry panel to assess immune markers of toxicity.

A number of studies have investigated genomic and transcriptomic markers of PD1 response (30, 37-39). Preliminary analysis in the Boland lab using the Olink platform and this patient cohort identified 38 differentially expressed proteins between anti-PD1 responders and non-responders, many of which reflect immune and resistance pathways (Mehta A et al., unpublished). Few studies have investigated genomic, transcriptomic or proteomic markers of toxicity.

Interestingly, one study showed increased expression of inflammatory cytokines (such as IL-1a, IL-2, IFN α 2) in patients who experienced at baseline and early during treatment (40). There is also evidence for the predictive value of autoimmune antibodies at baseline (41). Future studies using this platform could use a larger cohort to assess for cytokines associated with inflammation and antibody maturation.

Currently, the monitoring and management of irAEs focuses on organ dysfunction (18, 42). For example, thyroid and liver function monitoring are now standard of care during treatment .

Counseling for early patient reporting of diarrhea and dyspnea may prevent fatal complications from colitis or pneumonitis. However, these signs are non-specific and may cause premature

termination of oncologic treatment (42). This study is an important step in understanding if there are patients that are more susceptible to irAEs at baseline, and if there exist circulating biomarkers that would predict which types of irAEs would best respond to immunosuppression without sacrificing antitumor immunity.

This study highlights the unique research that can be performed with the translational melanoma infrastructure at MGH. There is remarkable access to a highly-annotated retrospective cohort of ICI patients with response data, organ-specific toxicities, and steroid treatment information. Additionally, there is a large volume of prospective patients initiating therapy with PD1 who could be included in future studies. This project importantly demonstrated the feasibility of retrospective analysis of irAEs using this data-set. Further investigation of protein markers of toxicities is warranted.

SECTION 6: ACKNOWLEDGEMENTS

I would like to first and foremost thank my mentor Dr. Genevieve Boland for her support not only for this research project but also for my career. She has been an excellent role model in the paving the path of a surgeon-scientist. With her support, I know that I have the best chance of becoming a scientist who will be able to contribute to academic surgery as well as caring surgeon.

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Finally, I would like to acknowledge my family who has supported me through medical school and helped me prepare for the exciting journey ahead of general surgery residency.

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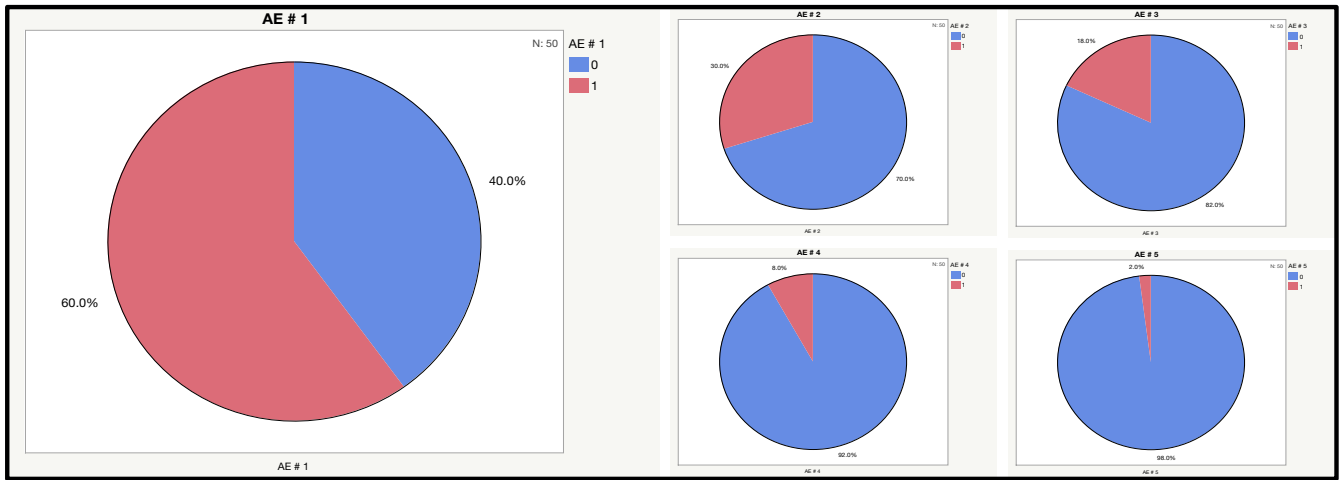


Figure 1: Patients by number of immune-related adverse events (irAE) experienced (n=50 for each pie). Red (1) represents documented irAE. Each pie represents total number of patients who experienced up to 5 irAE. Note that 40% of all patients experience no AE.

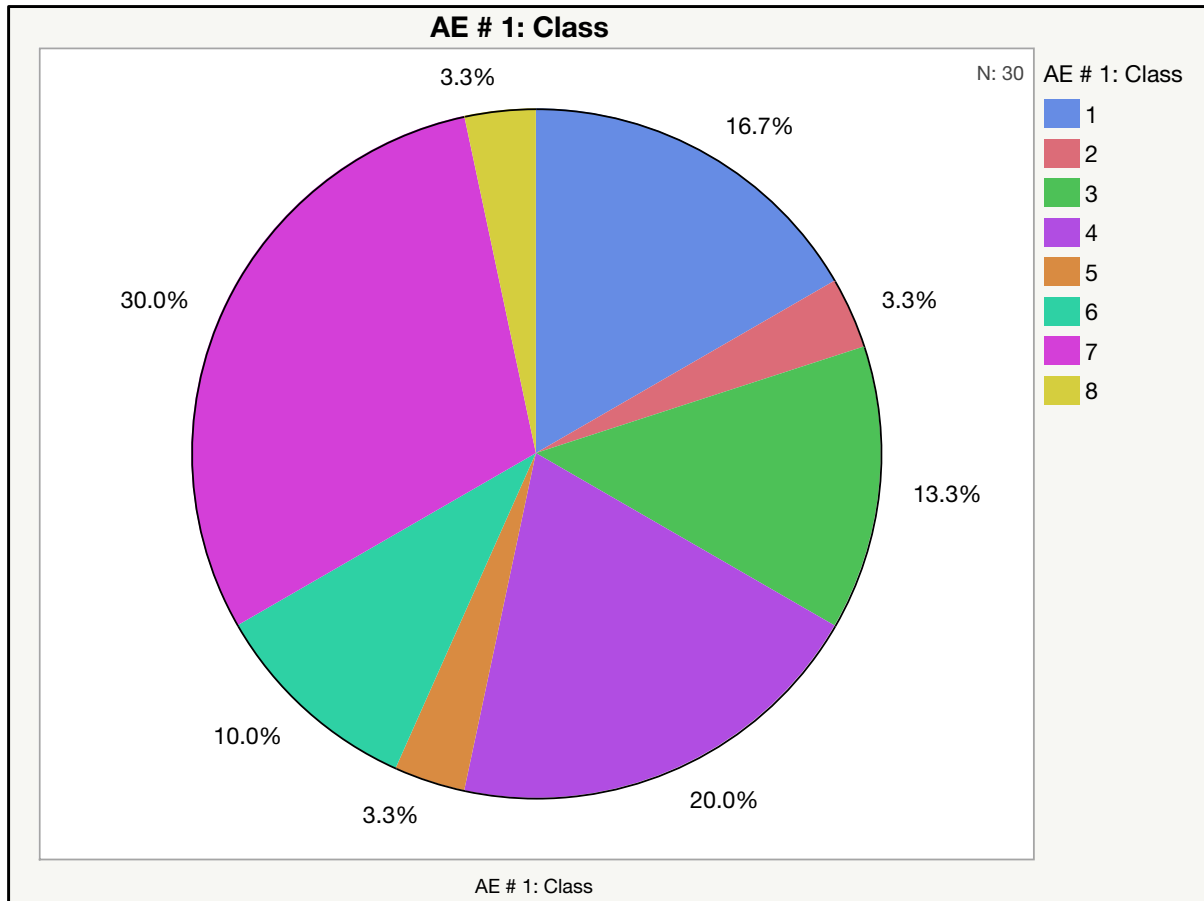


Figure 2: All patients who experienced at least one immune-related adverse events (irAE) [N = 30]. This shows the class of the first or only AE they experienced. 1 = GI (colitis/diarrhea/gastritis); 2 = hepatitis/transaminitis; 3 = pneumonitis; 4 = MSK (arthritis/arthralgia); 5 = nephritis; 6 = Skin (pruritis/dermatitis/rash); 7 = Endocrine (central adrenal insufficiency/hypothyroidism/hypophysitis/SIADH); 8 = Other (ulcer on tongue)

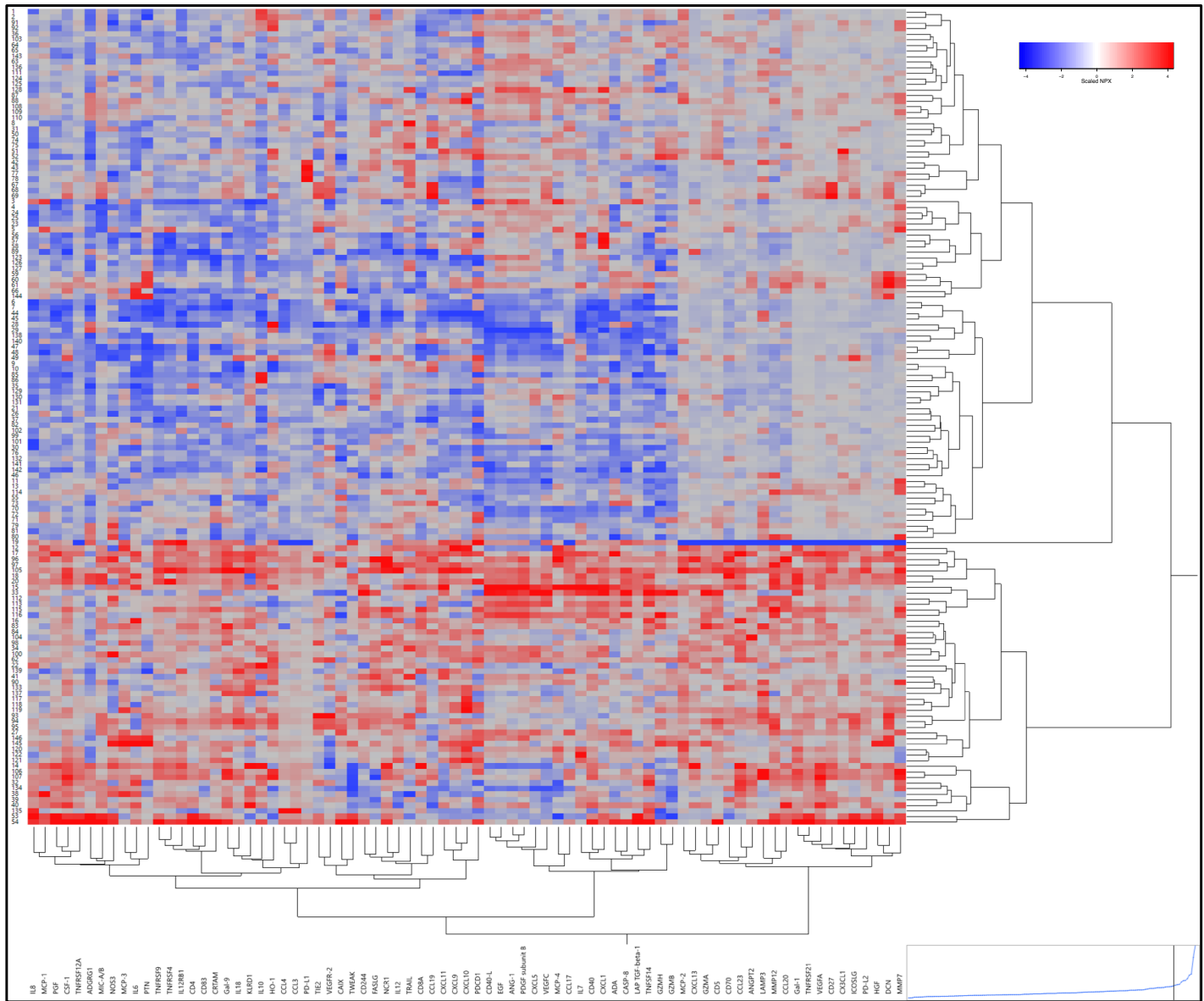


Figure 3: Multivariate analysis of 146 sample-timepoint and 77 markers. Heatmap shows data after processing of 50 patients, and 93 markers, and 3 timepoints (pre-treatment, 6 weeks, and 6 months). Expression is represented as Normalized Protein Expression (NPX), an arbitrary unit on \log_2 -scale. It is center around the mean for each marker to depict relative high (red) vs. low (blue) expression.

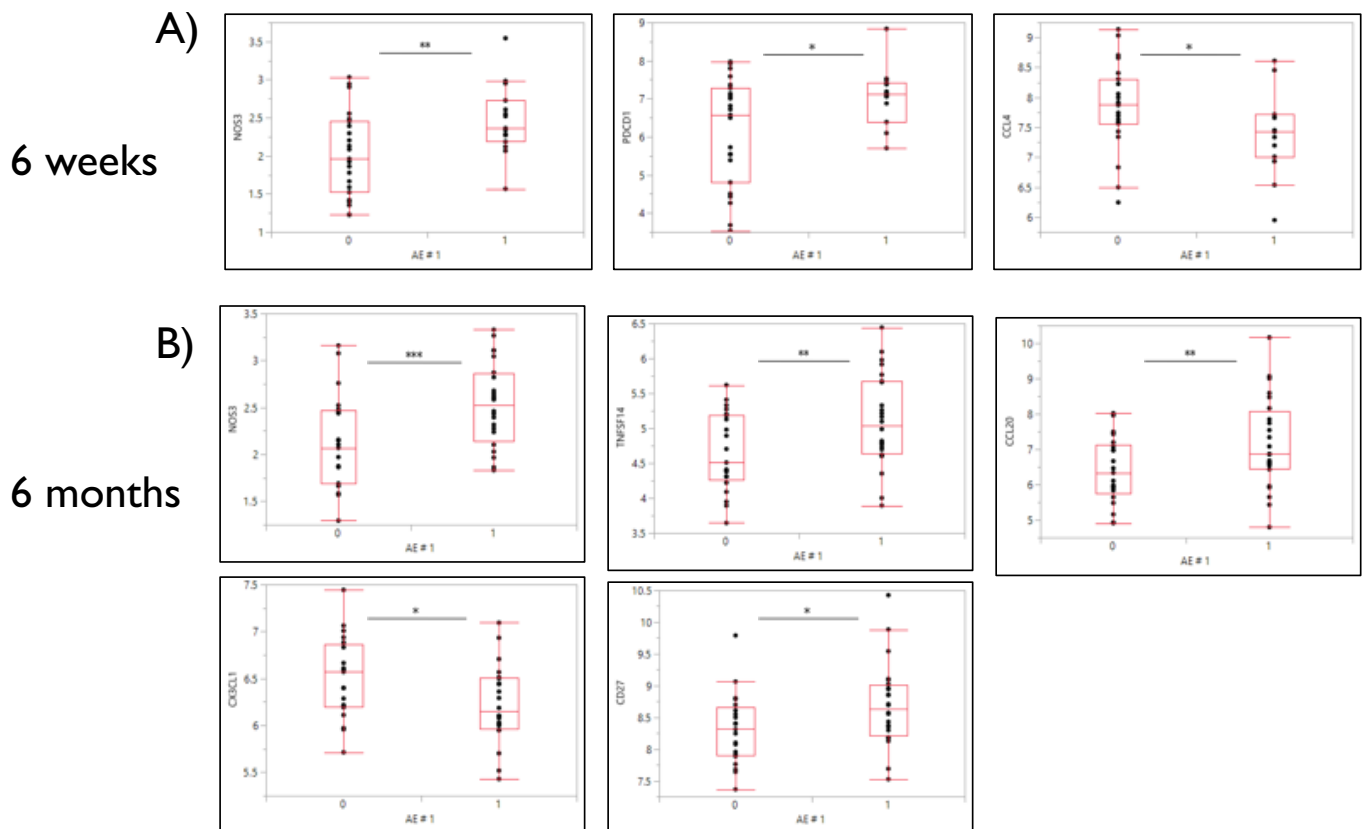


Figure 4: Differentially expressed proteins among patients who did vs. not experience any AE at (A) 6 weeks, (B) 6 months after starting treatment with immunotherapy. Y axis displays Normalized Protein Expression (NPX), an arbitrary unit on \log_2 -scale. It is center around the mean for each marker. P values displayed reflect two-sided t tests, assuming unequal variances but not accounting for multiple tests. Box plots show median, 25% and 75% quartiles.

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

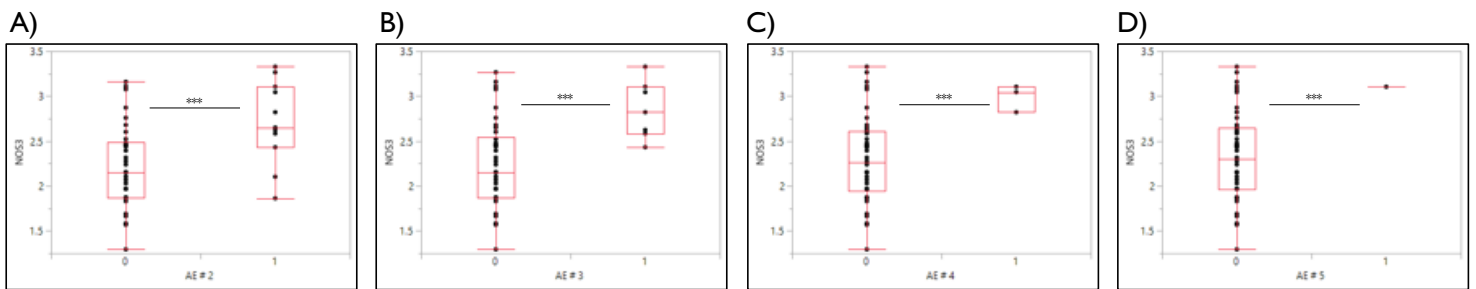


Figure 5: NOS3 protein expression at 6 months among patients who did vs. not experience (A) ≥ 2 , (B) ≥ 3 , (C) ≥ 4 , or (D) ≥ 5 irAEs. Box plots show median, 25% and 75% quartiles. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

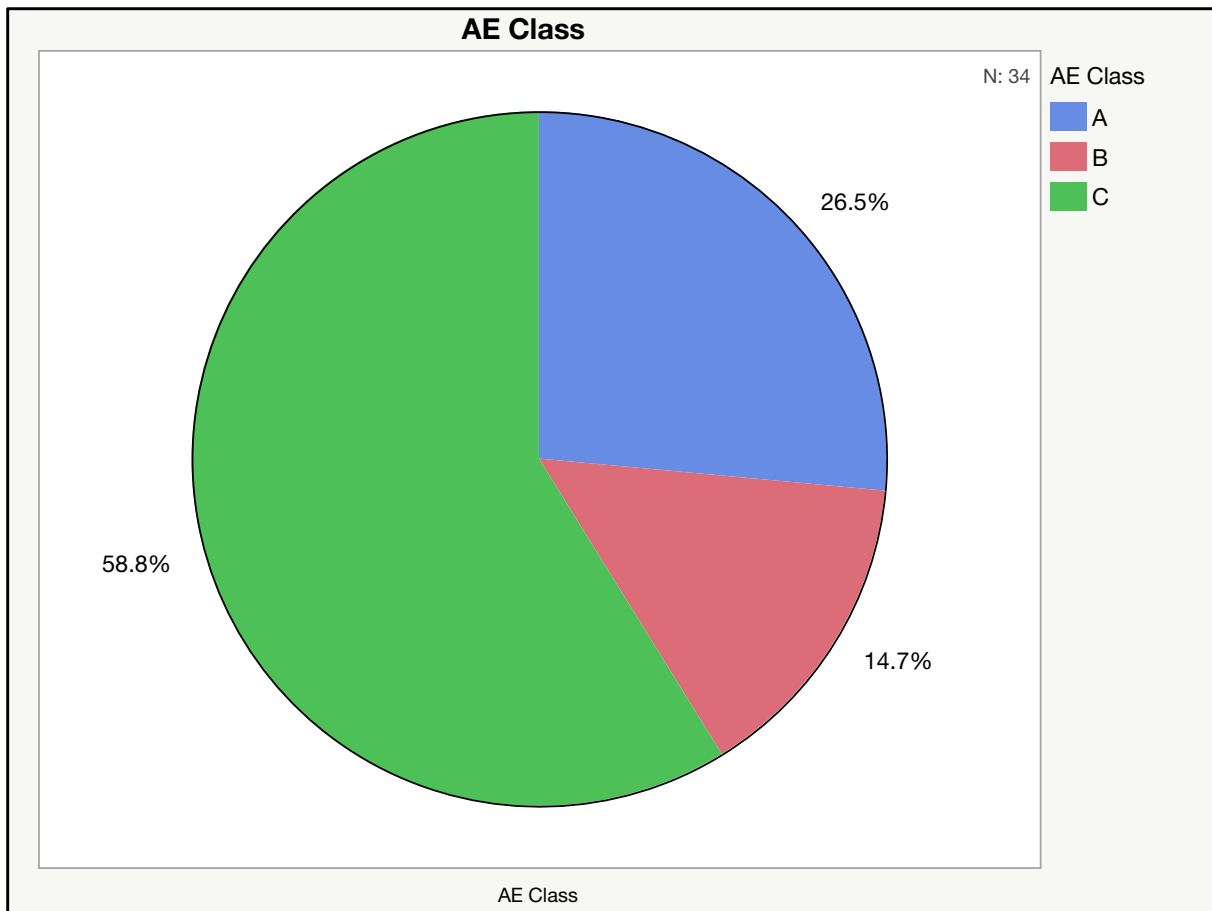


Figure 6: Patients displayed by class of first documented irAE (n=34). Group A = hypothesized shared mechanism (cutaneous and musculoskeletal). Group B = hypothesized distinct mechanism (colitis). Group C = no irAE. Note that remaining 16/50 patients do not fit in one of these three categories

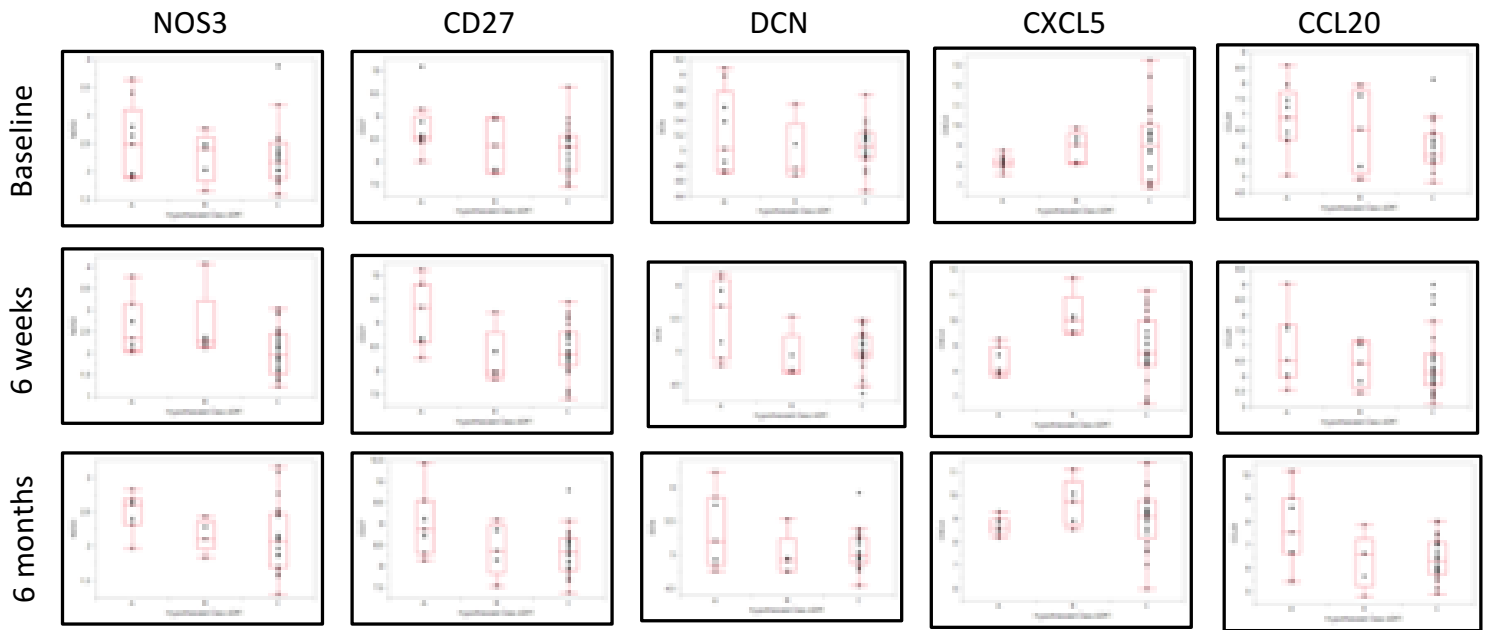


Figure 7: Differentially expressed proteins across three hypothesized classes of irAEs. Top markers that approached statistical significance are shown. Expression shown at baseline, 6 weeks, and 6 months following treatment initiation. Box plots show median, 25% and 75% quartiles.

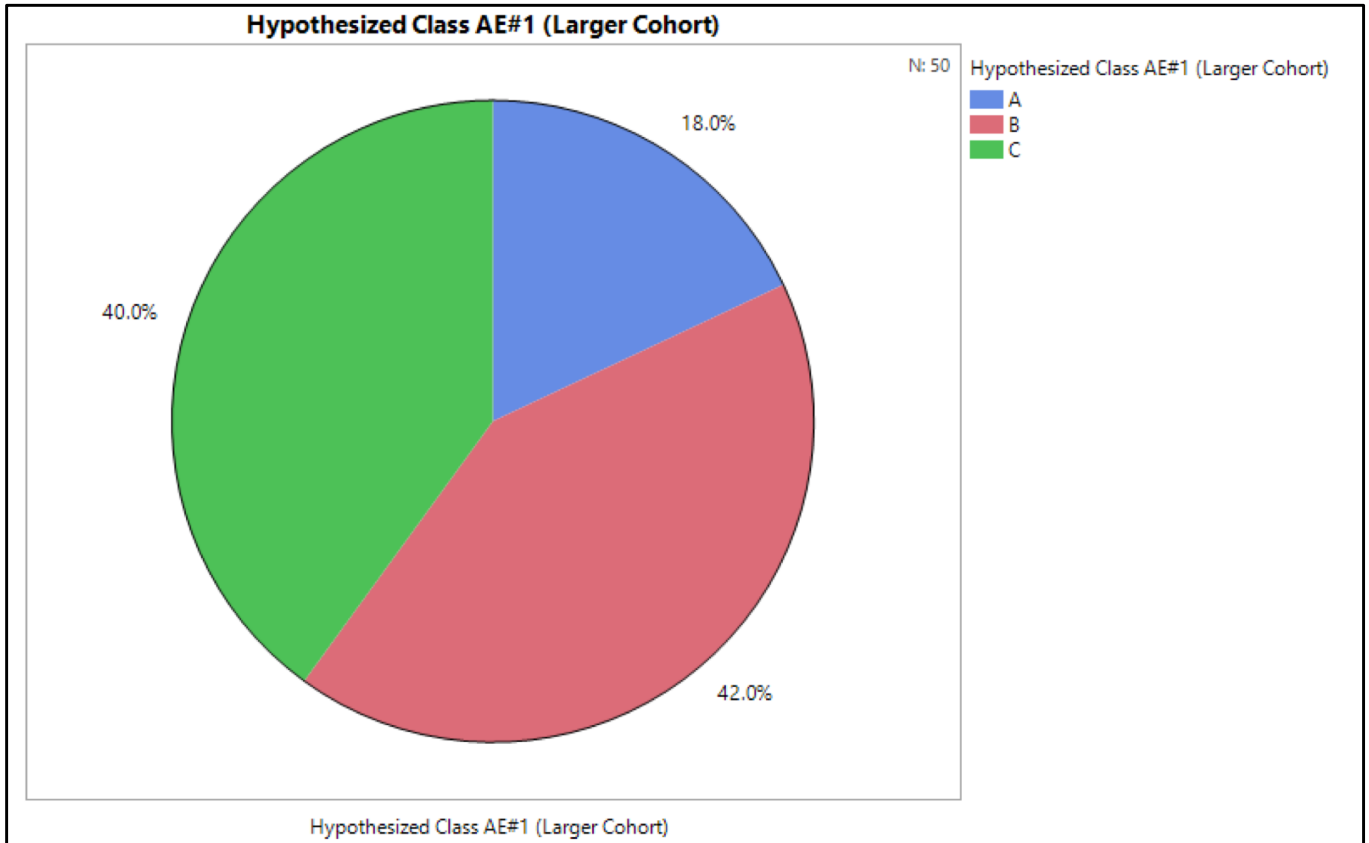
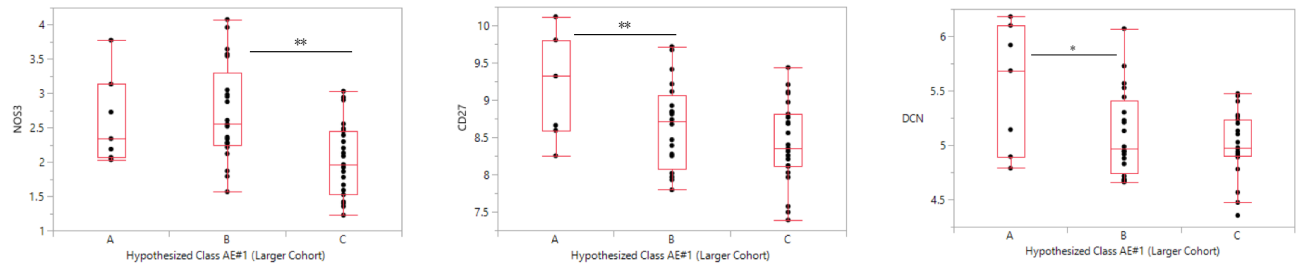


Figure 8: Patients displayed by class of first documented irAE using larger cohort (n=50). Group A = hypothesized shared mechanism (cutaneous and musculoskeletal). Group B = hypothesized distinct mechanism (colitis and all other irAE). Group C = no irAE.

Baseline

None

6 weeks



6 months

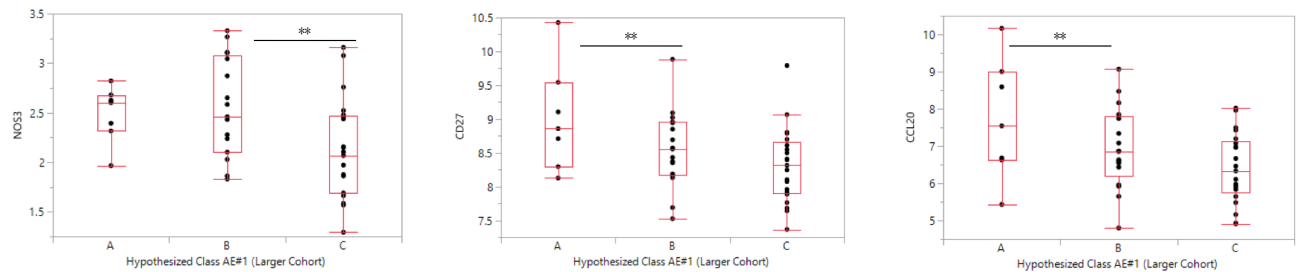


Figure 9: Differentially expressed proteins among three hypothesized classes of irAEs, using the larger cohort depicted in Figure 8. Group A: shared mechanism, Group B: distinct mechanism, Group C: no irAEs. Here, only markers with a two-sided t test that was statistically significant are shown. Expressions at baseline, 6 weeks, and 6 months shown. Box plots show median, 25% and 75% quartiles. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

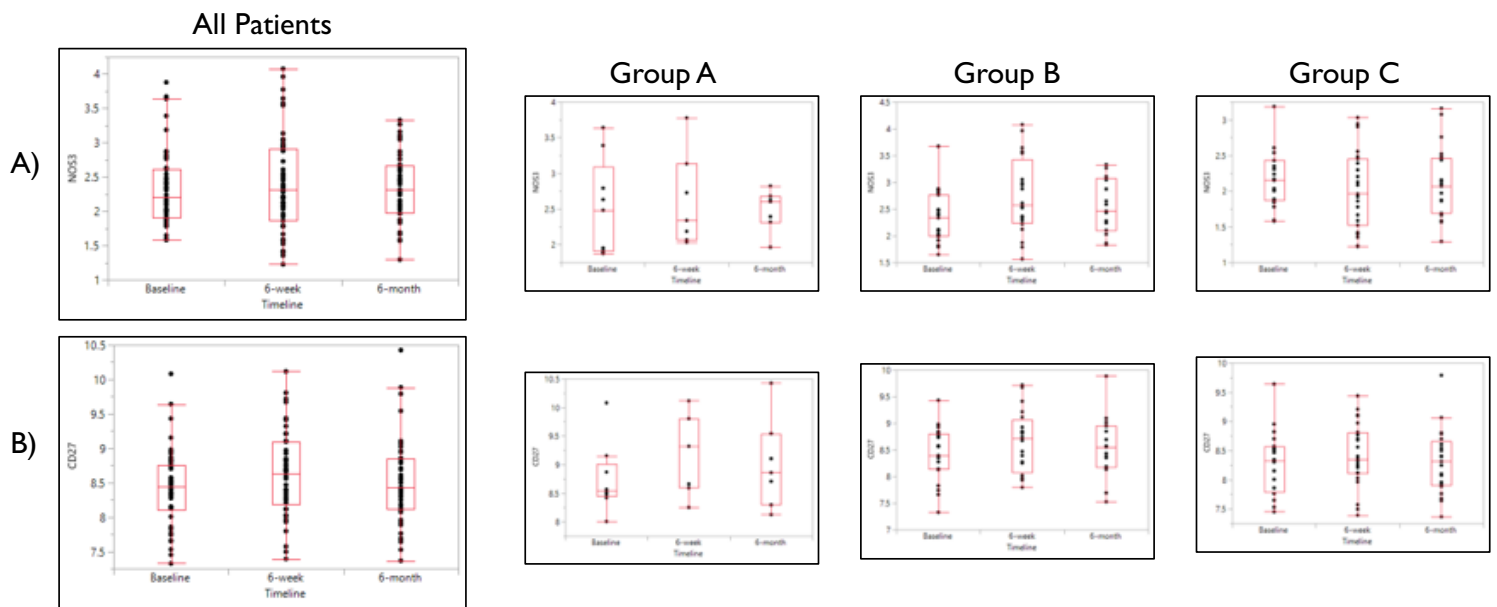


Figure 10: (A) NOS3 and (B) CD27 protein expression as a function of time at baseline, 6 weeks, and 6 months post treatment initiation. Data is shown with all patients, as well as broken up into groups (n=50). Groups A, B, and C represent the division using the larger cohort as depicted in Figure 8. Box plots show median, 25% and 75% quartiles. No two-sided t tests were significant.

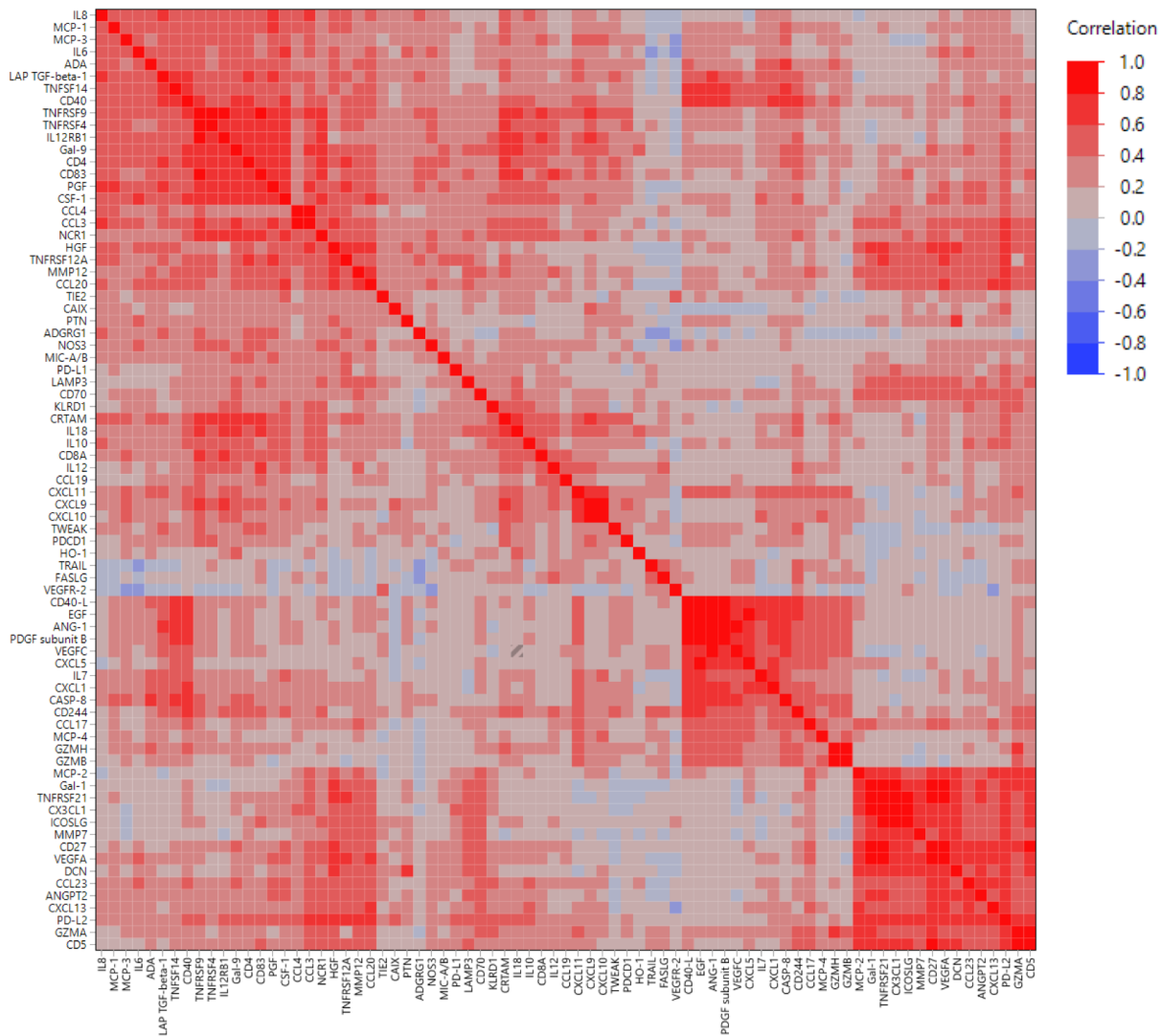
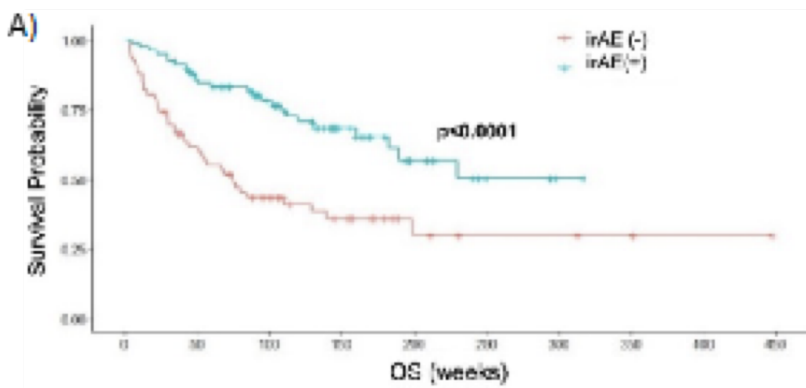


Figure 11: Heatmap depicting correlations of all 77 proteins to each other. Color legend reflect correlation coefficients, Pearson's R with a range -1 (blue) to +1 (red).

Supplementary Figures

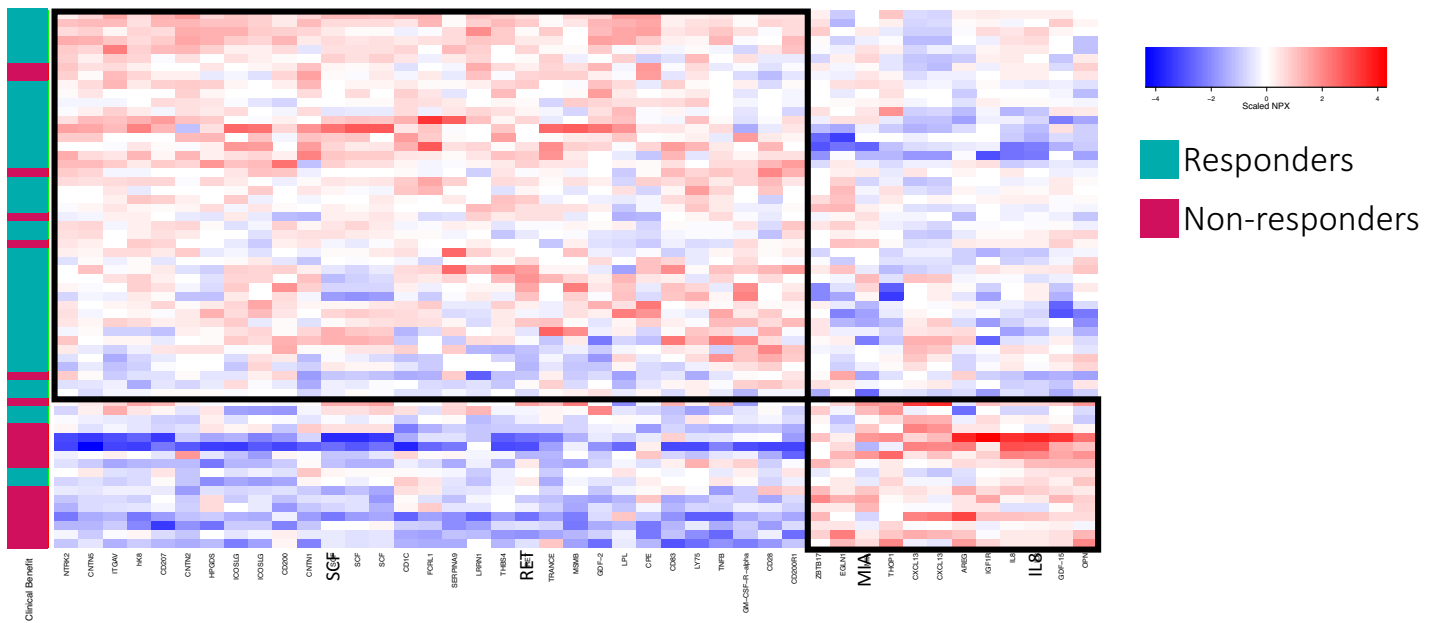


B)

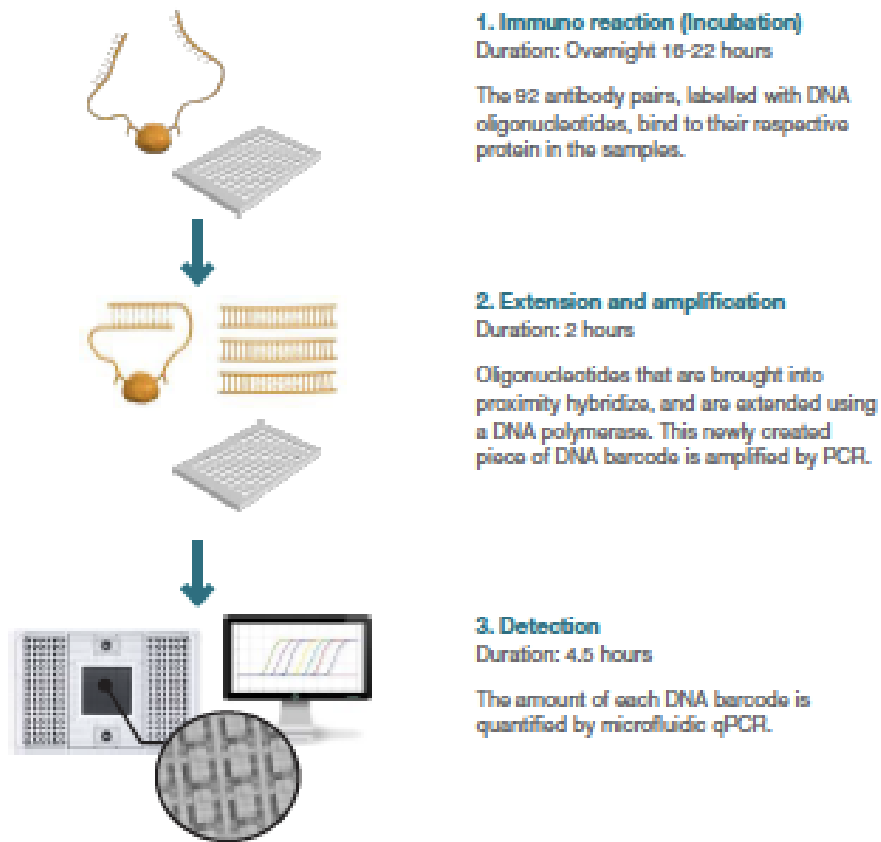
Toxicity	ORR	PFS	OS
Cutaneous	0.016	0.022	0.031
Musculoskeletal	<0.001	<0.001	0.001
Endocrine	0.066	0.048	0.138
GI	0.036	0.08	0.221
Pulmonary	0.406	0.577	0.298
Hepatic	0.497	-0.452	0.936
Rare	0.366	0.364	0.747

Supplementary Figure 1: (A) Overall survival (OS) in melanoma patients who did and did not experience immune-related adverse events (irAEs). (B) Correlation of tissue-specific irAEs with objective response rate (ORR), progression free survival (PFS) and overall survival (OS).

Taken with permission from Bai XE et al., submitted.



Supplementary Figure 2: Serum proteomic markers differentiate anti-PD1 responders and non-responders. 38 differentially expressed proteins were identified between anti-PD1 responders and non-responders, many of which reflect immune and resistance pathways. Taken with permission from Arnav M et al., unpublished.



Supplementary Figure 3: Technology description of Olink® Multiplex Protein Extension

Immunoassay platform. Taken from Olink ® User Manual available at:

<https://www.olink.com/content/uploads/2019/02/Olink-User-Manual-v2.0.pdf>

Characteristics	Number (%)	
	MGH	VUMC
Median age (range) (year)	67 (20-97)	65 (22-88)
Sex		
Female	56 (33)	91 (37)
Male	113 (67)	155 (63)
Subtype		
Cutaneous	133 (79)	187 (76)
Mucosal	7 (4)	11 (4)
Acral	0	11 (4)
Ocular	12 (7)	9 (4)
Unknown primary	17 (10)	28 (11)
M stage	(n=168)	
M1a	25 (15)	64 (26)
M1b	26 (15)	52 (21)
M1c	59 (35)	101 (41)
M1d	49 (29)	29 (12)
M0 (unresectable stage III)	9 (5)	0
LDH	(n=156)	(n=238)
normal	90 (58)	140 (59)
elevated	66 (42)	98 (41)
Mutation	(n=158)	(n=224)
BRAF	58 (37)	92 (41)
NRAS	41 (26)	40 (18)
NF1	12 (8)	7 (3)
Triple-negative	47 (30)	84 (38)
Prior treatment		(n=243)
I.O. (including IL-2 & ipilimumab)	76 (45)	126 (52)
MAPKi	27 (16)	66 (27)
Other TT (mTORi,CKiTi, etc.)	4 (2)	9 (4)
Chemo	5 (3)	26 (11)
None	86 (51)	91 (37)
Anti-PD-1 antibody		
Pembrolizumab	147 (87)	214 (87)
Nivolumab	22 (13)	32 (13)
IrAE occurrence		
Yes	90 (53)	101 (41)
No	79 (47)	145 (59)

Supplementary Figure 4: Baseline characteristics of patients in the MGH and VUMC cohorts (MGH n=169, VUMC n=246). MGH = Massachusetts General Hospital. VUMC = Vanderbilt University Medical Center. Taken with permission from Bai XE et al., submitted.