developed a novel workflow combining the single molecule and single cell visualization capabilities of the RNAscope in situ hybridization (ISH) assay with the highly multiplexed spatial profiling capabilities of the GeoMx™ Digital Spatial Profiler (DSP) RNA assays. Using these methods, we sought to spatially profile and compare gene expression signatures of tumor niches with high and low CTNNB1 expression.

Methods After screening 120 tumor cores from multiple tumors for CTNNB1 expression by the RNAscope assay, we identified melanoma as the tumor type with the highest CTNNB1 expression while prostate tumors had the lowest expression. Using the RNAscope Multiplex Fluorescence assay we selected regions of high CTNNB1 expression within 3 melanoma tumors as well as regions with low CTNNB1 expression within 3 prostate tumors. These selected regions of interest (ROIs) were then transcriptionally profiled using the GeoMx DSP RNA assay for a set of 78 genes relevant in immuno-oncology. Target genes that were differentially expressed were further visualized and spatially assessed using the RNAscope Multiplex Fluorescence assay to confirm GeoMx DSP data with single cell resolution.

Results The GeoMx DSP analysis comparing the melanoma and prostate tumors revealed that they had significantly different gene expression profiles and many of these genes showed concordance with CTNNB1 expression. Furthermore, immunoregulatory targets such as ICOSLG, CTLA4, PDCD1 and ARG1, also demonstrated significant correlation with CTNNB1 expression. On validating selected targets using the RNAscope assay, we could distinctly visualize that they were not only highly expressed in melanoma compared to the prostate tumor, but their expression levels changed proportionally to that of CTNNB1 within the same tumors suggesting that these differentially expressed genes may be regulated by the WNT-β-catenin pathway.

Conclusions In summary, by combining the RNAscope ISH assay and the GeoMx DSP RNA assay into one joint workflow we transcriptionally profiled regions of high and low CTNNB1 expression within melanoma and prostate tumors and identified genes potentially regulated by the WNT-β-catenin pathway. This novel workflow can be fully automated and is well suited for interrogating the tumor and stroma and their interactions. GeoMx Assays are for RESEARCH ONLY, not for diagnostics.

http://dx.doi.org/10.1136/jitc-2020-SITC2020.0004
Luminex. To identify biomarkers of checkpoint inhibition, mice transferred with a defined population of ovalbumin (OVA)-specific T cells were challenged with OVA antigen or EG7 tumour. Activation and proliferation of antigen-specific T cells was determined and Nanostring gene expression analysis performed. Flow cytometry staining panels for human immune markers including CD4, CD14, CD25 and FOXP3 were established pre-clinically. As part of the assay validation process for a clinical trial, whole blood SEB activation was performed in normal donors, with Luminex analysis of IL-2, IL-17, IFN-gamma and TNFalpha.

**Results** Immune checkpoint inhibitors resulted in increased IL-2 and IFN-gamma secretion in human PBMC stimulated with SEB. In the murine PD model, anti-PD-L1 caused upregulation of CD25, IFN-gamma and granzyme B by antigen-specific CD8 T cells. Gene expression analysis of murine tumours elucidated changes in response to a vaccine. Flow cytometry panel staining determined the frequencies of human Treg and monocytes, which are common targets of immune-modulating therapies. Fit-for-purpose validation was performed for a human SEB activation assay resulting in robust changes in cytokine production.

**Conclusions** The experiments here show the flow of experiments that can be performed to identify a PD biomarker for use in first in man trials; the pre-clinical human PBMC SEB screening assay provides a simple assay demonstrating that a therapy can enhance T cell function and would be translatable to the clinic. The murine PD model provides a platform to screen for biomarkers of T cell function and monitor gene expression modulation. Biomarkers identified in the murine setting provide a good starting point for exploratory assessment in early phase clinical trials, where inclusion of exploratory PD biomarker endpoints can confirm proof of mechanism and improve study success rates.

**Ethics Approval** Human tissues used in this study were collected with ethical approval from UK Research Ethics Committee South West, Bristol (UK), approval number 15/SW/0029.

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**Abstract 7 Figure 1** Time to death - Kaplan-Meier survival plot

**Background** Immune checkpoint inhibitors (ICI) have changed the paradigm of advanced malignant melanoma (MM). Several prognostic factors, mostly linked to inflammation, have been under scope to better select patients for such therapies. We aimed to build and apply a prognostic score in this setting.

**Methods** Baseline characteristics and outcomes on 147 patients with advanced MM treated with an anti-PD1 (nivolumab or pembrolizumab) in monotherapy, between Jan-2016 and Oct-2019, in the 1st, 2nd or 3rd line setting were collected from two centres in Portugal. Data cut-off for follow-up was May-2020. Cox proportional hazards regression was used to identify independent prognostic factors for OS.

**Results** With a median FU of 28.93 months (95% CI [22.52–33.54]), mOS for the whole cohort was 14.75 months (95% CI, [10.80–18.71]). Overall, 43 and 104 patients were treated with nivolumab and pembrolizumab, respectively. We identified four adverse prognostic factors that were independent predictors of bad prognosis: number of metastatic sites >2 (p<0.001), baseline PS-ECOG =1 (p<0.001), presence of baseline lymphopenia (over lower limit of normal) (p=0.002) or very high baseline LDH (>2x upper limit of normal) (p<0.001). Patients were separated into three risk categories according to the number of risk factors present: favourable prognosis (no risk factors; n=34), intermediate prognosis (one risk factor; n=65) and poor prognosis (two or more risk factors; n=48). mOS was 43.41 (95% CI [32.13–54.69], 14.39 (95% CI [6.78–22.01]) and 6.53 months (95% CI [3.61–9.44]), for favourable, intermediate, and poor prognosis group, respectively (p<0.001; figure 1). AUC of ROC curve for OS was 0.737 (95% CI [0.654–0.819], p<0.001).

**Conclusions** Using easily accessible parameters from our daily practice, we propose the MELImmune prognostic score for advanced MM patients treated with anti-PD1 in monotherapy that could be incorporated to the daily clinical practice and clinical trials. We further aim to validate this score in an independent larger sample.

**Ethics Approval** The study was approved by both institutions’ Ethics Committee.