



Spatial Profiling Identifies Prognostic Features of Response to Adjuvant Therapy in Triple Negative Breast Cancer (TNBC)

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Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer that has few effective treatment options due to its lack of targetable hormone receptors. Whilst the degree of tumour infiltrating lymphocytes (TILs) has been shown to associate with therapy response and prognosis, deeper characterization of the molecular diversity that may mediate chemotherapeutic response is lacking. Here we applied targeted proteomic analysis of both chemotherapy sensitive and resistant TNBC tissue samples by the Nanostring GeoMx Digital Spatial Platform (DSP). By quantifying 68 targets in the tumour and tumour microenvironment (TME) compartments and performing differential expression analysis between responsive and non-responsive tumours, we show that increased ER-alpha expression and decreased 4-1BB and MART1 within the stromal compartments is associated with adjuvant chemotherapy response. Similarly, higher expression of GZMA, STING and fibronectin and lower levels of CD80 were associated with response within tumour compartments. Univariate overall-survival (OS) analysis of stromal proteins supported these findings, with ER-alpha expression (HR=0.19, p=0.0012) associated with better OS while MART1 expression (HR=2.3, p=0.035) was indicative of poorer OS. Proteins within tumour compartments consistent with longer OS included PD-L1 (HR=0.53, p=0.023), FOXP3 (HR=0.5, p=0.026), GITR (HR=0.51, p=0.036), SMA (HR=0.59, p=0.043), while EPCAM (HR=1.7, p=0.045), and CD95 (HR=4.9, p=0.046) expression were associated with shorter OS. Our data provides early insights into the levels of these markers in the TNBC tumour microenvironment, and their association with chemotherapeutic response and patient survival.

Keywords: TNBC (Triple negative breast cancer), spatial proteomics, adjuvant chemotherapy, tumor microenvironment, spatial transcriptomics

INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed form of cancer and is the leading cause of cancer related mortality in women worldwide (1). The molecular subtypes of BC have distinct pathological features and are characterised by amplification of their hormone receptors, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2), or the absence thereof in triple-negative BC (TNBC) (2, 3). Treatment strategies for ER/PR positive and HER2-positive breast cancer include hormone targeted therapies like tamoxifen (4, 5), aromatase inhibitors (6) and HER-2 specific monoclonal antibody, trastuzumab (7) which are often used in combination with chemotherapeutic agents like paclitaxel (8).

TNBC, however, which accounts for 15% of diagnosed breast cancers, lacks these key targets, and as such remains difficult to treat with current generations of therapies (9). Standard treatment for TNBC include anthracycline, taxane and platinum-based regimens (2). In patients with advanced TNBC and chemotherapy resistant tumours, PARP inhibition and immunotherapy have been gaining traction (10), while patients with metastatic TNBC with *BRCA1/2* deficiency, can respond well to PARP inhibitors including Olaparib and Talazoparib (11, 12).

Methods to stratify TNBC patients are currently lacking, however elevated levels of tumour infiltrating lymphocytes (TILs) have been shown to indicate favourable response to chemotherapy, particularly for TNBC and HER-2 positive patients (13, 14). TNBC tumours are considered to be the most immunogenic BC subtype, and display expression of programmed cell death ligand 1 (PD-L1) on both tumour and immune cells, and increased TIL infiltration (15, 16). TNBC tumours also display a higher burden for non-synonymous mutations that are capable of inducing T-cell anti-tumour responses (17). Anti PD-1 antibody pembrolizumab has received accelerated approval from the FDA for PD-L1 positive TNBC patients in combination with chemotherapy (18). Clinical trials with existing immunotherapies in combination with approved chemotherapies and targeted therapies, like PARP inhibitors, and those investigating novel immunotherapy agents are currently in progress (16).

To garner insight into the properties of TNBC that may benefit therapy stratification, a greater understanding of the TIL repertoire within the tumour microenvironment is necessary. The cellular composition of, and the interactions that occur within this niche between cancer cells and the host immune components are likely to influence disease progression. In this study we have used the Nanostring GeoMX Digital Spatial Profiler (DSP) to gain tumour and stroma compartment specific protein expression profiles of TNBC tumours. Our data indicates the utility of such multiplex approaches to discover the properties of primary tumours that may lead to their propensity to progress despite treatment, thereby distinguishing those patients most likely to respond.

MATERIALS AND METHODS

This study has Queensland University of Technology Human Research Ethics Committee Approval (UHREC #200000494).

A tissue microarray (TMA) containing twenty-four triple negative breast cancer specimens with clinicopathological findings was obtained in collaboration with Tristar Technology Group (USA). The cohort was comprised of patients with histological grade 3 invasive ductal carcinoma who received first-line FEC (5-fluorouracil, epirubicin and cyclophosphamide) adjuvant chemotherapy. Patients were defined by complete response (CR), where no evidence of disease was found during subsequent follow up, while progressive disease (PD) patients were refractory to treatment and subsequently succumbed to disease within the follow-up period.

Nanostring GeoMX Digital Spatial Profiling (DSP)

The TNBC TMA slides were stained and analysed on the Nanostring GeoMx[®] Digital Spatial Profiling (DSP) platform by the Systems Biology and Data Science Group at Griffith University (Gold Coast, Australia). Visualisation markers consisted of Pan-cytokeratin and CD45. Protein panel consisted of 68 antibodies including human immune cell core panel, immuno-oncology (IO) drug target, immune activation, immune cell typing, pan-tumour, cell death, and PI3K/AKT panels. Slides were processed as per manufacturer's instructions and tumour/stroma demarcated by masking on PanCK⁺ or PanCK⁻ regions, respectively. Antibody barcodes were counted on Ncounter[®] platform as per manufacturer's instructions and External RNA Controls Consortium (ERCC) QC performed in DSP analysis suite prior to outputting data for bioinformatic analysis.

Bioinformatic Analysis

Data analysis was performed by Queensland Cyber Infrastructure Foundation (QCIF, Qld, Australia). Data was evaluated by principal component analysis and coefficients of variation were assessed to determine suitability of the RUV-III normalisation method (19, 20). Differential analysis was performed within Limma packages (21) and sparse partial least squares-discriminant analysis (sPLS-DA) was performed within mixOmics package (22). The Kaplan-Meier survival analysis and Cox proportional hazards models was constructed within R studio (23) using Survival package (24) and plots generated by ggplot2 (25).

RESULTS

TNBC Cohort

To identify proteins that influence disease progression in response to chemotherapy, we evaluated a cohort of TNBC tissue samples from women undergoing adjuvant chemotherapy in collaboration with TriStar Technology Group (USA). Our annotated tissue microarray (TMA) with 8.5-year follow-up data included 15 cases without relapse (responders) following therapy versus 9 cases with tumour relapse (non-responders) (Table 1). All non-responsive patients subsequently succumbed to metastatic disease following

surgery within the follow-up period time, while responders remained alive (**Figure 1A**). One non-responsive patient with progressive disease was censored at final follow up time point.

Identification of Differentially Expressed Proteins by the Nanostring GeoMX DSP Assay

We compared the two groups of patient chemotherapy responders and non-responders by performing multiplex proteomics using the Nanostring GeoMx[®] Digital Spatial Profiling (DSP) platform. We identified key deregulated proteins within the tumour and stromal compartments independently by masking on PanCK+/PanCK- regions, respectively. (**Figures 1B, C**). Analysis of the PanCK- stroma within each core indicated higher expression of estrogen receptor alpha (ER α) in responsive patients, with concurrent decreased

levels 41BB and MART1 (**Figures 2A, B**). Differential analysis of tumour compartments indicated the enrichment of GZMA, STING and fibronectin in responsive patients, while CD80 levels were higher within refractory tumours (**Figures 2C, D**).

Prognostic Benefit of Proteins Between Responder/Non-Responder Patient Groups

We next sought to investigate the associations between our protein expression data and overall survival (OS). Cox regression indicated DE protein ER α (HR=0.19, p=0.0012) and GTR (HR=0.24, p=0.043) within the stroma to be associated with better prognosis, while DE protein MART1 (HR=2.3, p=0.035) indicated poorer outcome (**Figure 3A**). Interestingly, stromal PD-L1 (HR=0.46, p=0.059) expression demonstrated a trend for improved outcome.

Notably, evaluation of tumour compartments showed little consistency with DE results, with the expression of PD-L1 (HR=0.53, p=0.023), FOXP3 (HR=0.5, p=0.026), GTR (HR=0.51, p=0.036), SMA (HR=0.59, p=0.043) implicated in improved outcome. Conversely, tumour EPCAM (HR=1.7, p=0.045), and CD95 (HR=4.9, p=0.046) were associated with poorer outcome.

To further investigate factors which influenced disease progression, we utilised a multivariate analysis by sparse partial least-squares discriminant analysis (sPLSDA) to identify a minimal signature that distinguishes TNBC therapy response. sPLSDA is a statistical method to identify directionally weighted features that collectively discriminate groups of samples. Signatures within tumour (**Figure 4A**) and stroma (**Figure 4B**) compartments were able to effectively stratify patients by response to therapy. Our tumour compartment specific

TABLE 1 | Clinicopathological details of the TNBC patient cohort.

	Responders (n=15)	Non-responders (n=9)
Age	53 (34,77)	55 (28,76)
Grade	3	3
T Stage		
1	8	5
2	7	3
3	–	–
4	–	1
Nodal Met		
1	15	7
2	–	2
Survival (Days)	2747 (2374,3226)	1989 (365,2892)
Metastasis (Liver, Lung)	0	9

*Data are mean with range in brackets.

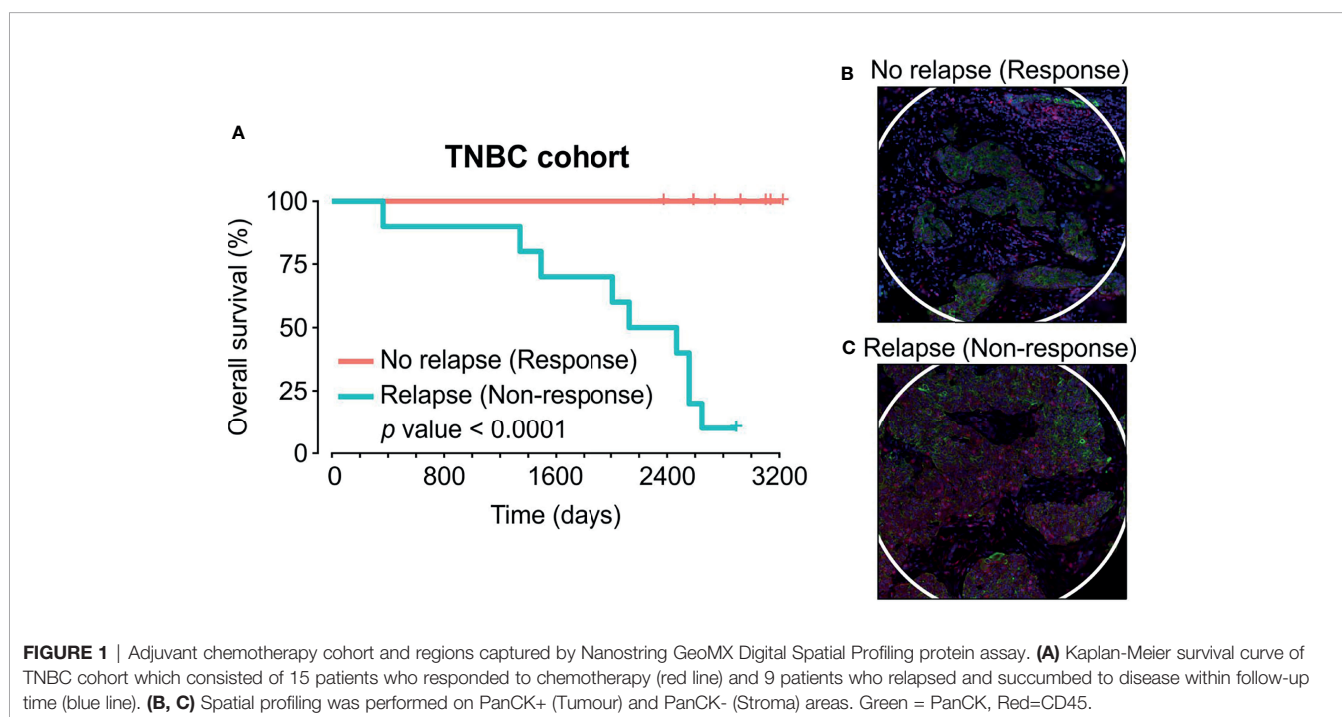
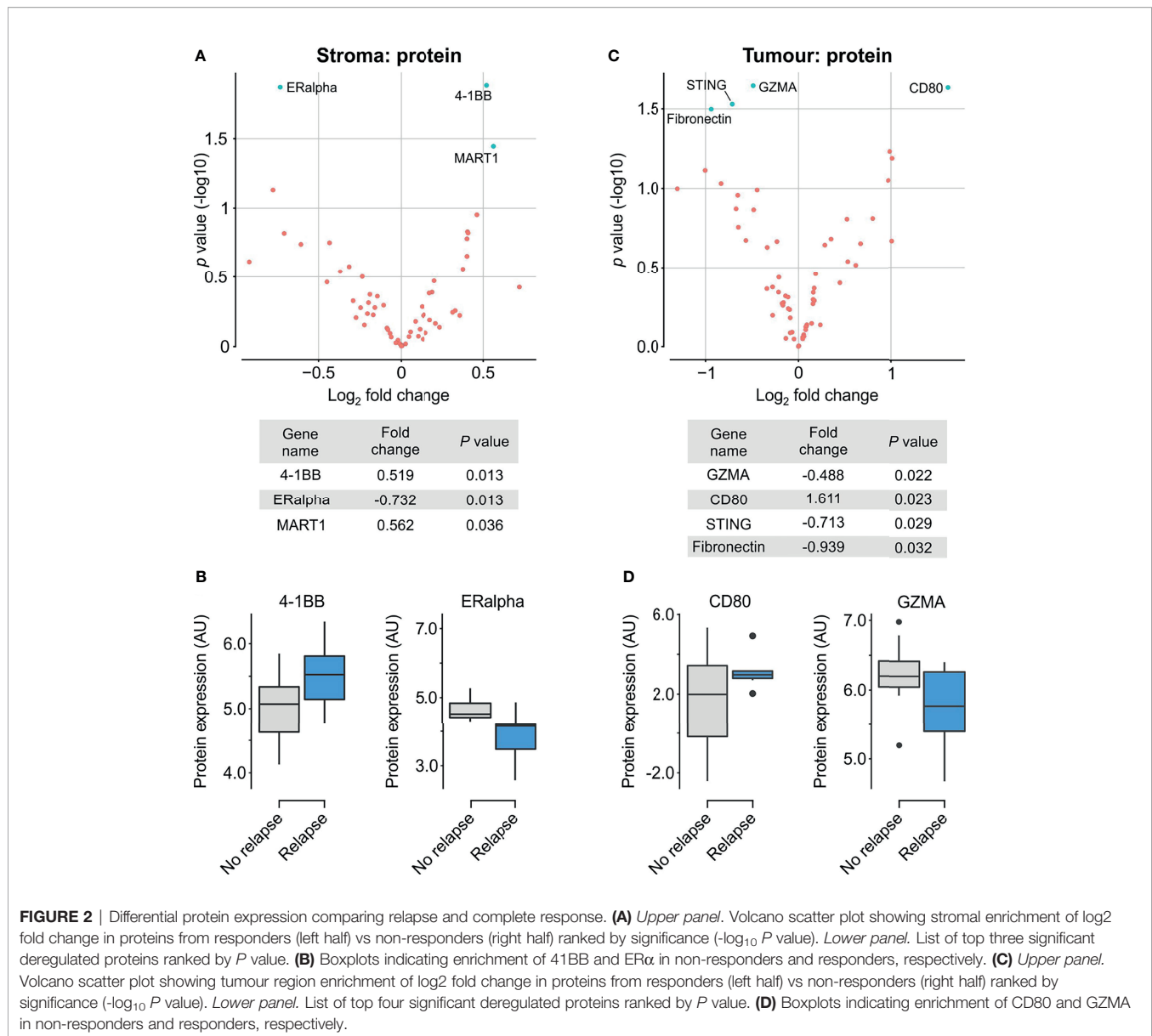


FIGURE 1 | Adjuvant chemotherapy cohort and regions captured by Nanostring GeoMX Digital Spatial Profiling protein assay. **(A)** Kaplan-Meier survival curve of TNBC cohort which consisted of 15 patients who responded to chemotherapy (red line) and 9 patients who relapsed and succumbed to disease within follow-up time (blue line). **(B, C)** Spatial profiling was performed on PanCK+ (Tumour) and PanCK- (Stroma) areas. Green = PanCK, Red=CD45.



multivariate signature for response was largely consistent with the DE results (**Figure 4C**), comprising higher levels of fibronectin, GZMA and STING and lower EPCAM and CD80 (AUC = 0.875) (**Figure 4E**). Interestingly, NF1, a negative regulator of the RAS/MAPK pathway almost solely comprised a second tumour signature, indicative of therapy response (AUC=1) (**Figure 4F**). Similarly, a stromal signature composed solely of ERα was indicative of response (**Figure 4D**) (AUC=0.925) (**Figure 4G**). A second multivariate stromal signature composed of T cell costimulatory agent 41BB as well as CD8, CD45, CD3, CD4 with apoptotic regulators BAD and BCLXL also discriminated response (AUC=0.95) (**Figure 4H**). Our discovery data suggest that we have identified a potential unique multi-protein signature to identify those TNBC tumours most likely to respond to adjuvant chemotherapy.

DISCUSSION

Breast cancer is a heterogenous disease, wherein the TNBC subtype demonstrates a particular refractory response to treatment due to lack of canonical hormone directed targets. TIL infiltration in TNBC has been shown to be a key predictor of response to neoadjuvant chemotherapy (13), in addition to forming the basis for efficacy of current generations of immune checkpoint inhibitors that reinvigorate anti-tumour immune activity. Despite this, little is known regarding relative levels of TIL subsets, and the greater immune composition in TNBC tumours. Thus, an opportunity to investigate these properties and their relationship with clinical outcomes exists. Here we evaluated a preliminary cohort of TNBC tumours from patients that responded to adjuvant chemotherapy and compared them

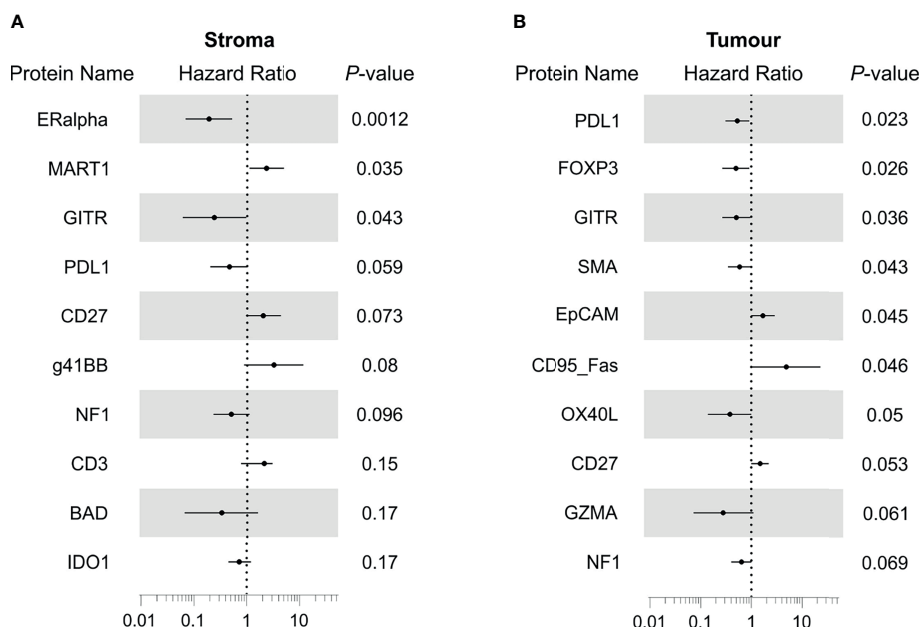


FIGURE 3 | Identification of proteins with prognostic associations for treatment of TNBC with adjuvant chemotherapy. **(A)** Forest plot showing hazard ratio and 95% confidence interval for stromal proteins that possessed overall survival associations. **(B)** Forest plot showing hazard ratio and 95% confidence interval for tumour proteins that possessed overall survival associations. HR > 1 indicates association with poorer outcome.

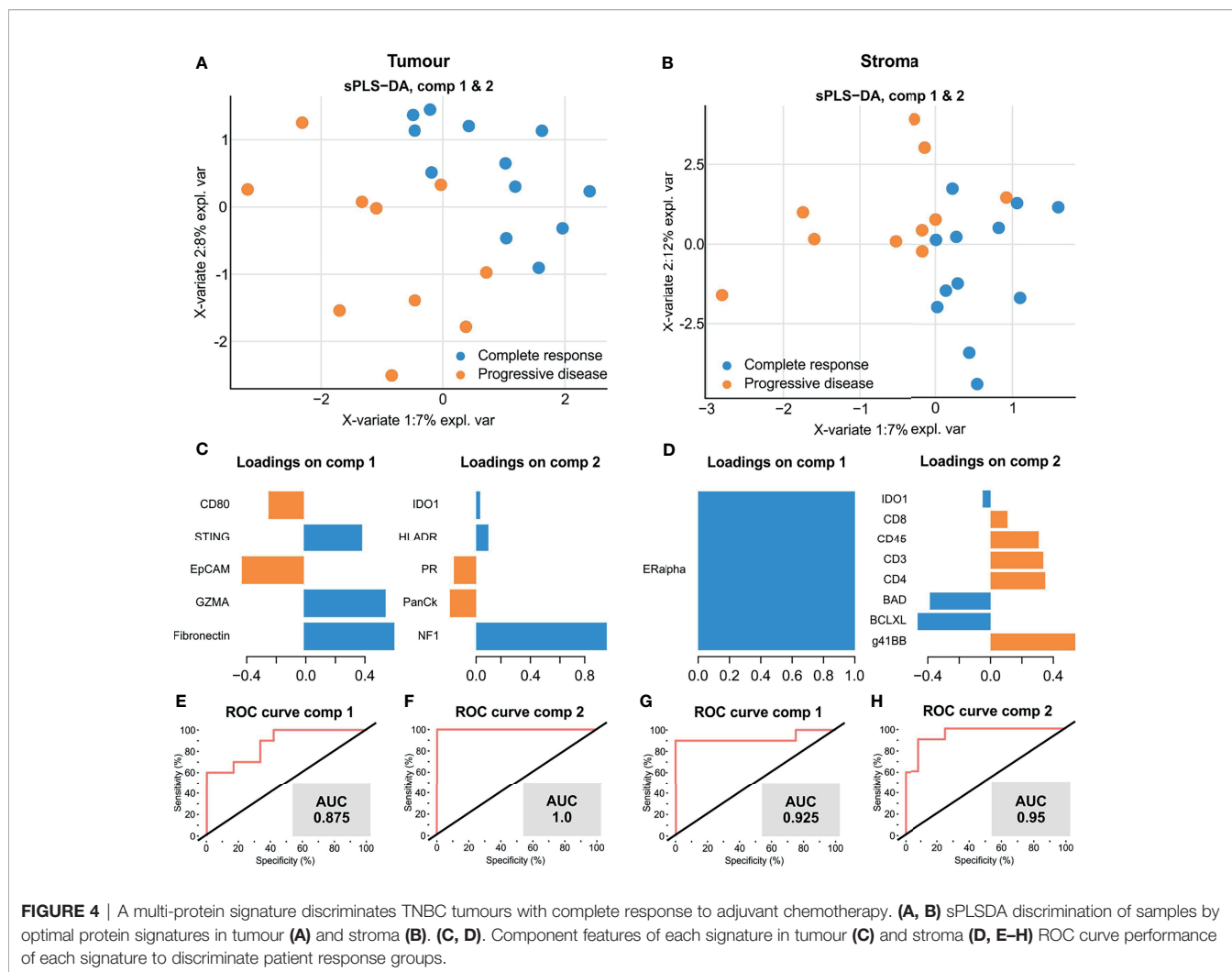
to those that exhibited treatment resistance resulting in progressive disease.

To the best of our knowledge, this is the first study employing proteomic DSP to examine the TNBC tumour microenvironment to discern chemotherapy treatment outcomes. Herein, we extend earlier spatial studies (26) by identifying differentially expressed proteins and protein signatures that associate with prognosis and treatment response. However, our study has a number of limitations. For example, our findings require validation in larger independent retrospective cohorts of TNBC tumours, and in future, trialling within prospectively collected tissues. Moreover, our findings could be further examined in cohorts of neoadjuvant chemotherapy, an established therapy modality in TNBC, and associated with pathological complete response (pCR) (27–29). Future investigations should also utilise orthogonal approaches (immunohistochemistry) to qualify abundance of TIL infiltration and validate experimental findings from the DSP platform (30). Furthermore, biological characterisation and validation of putative targets within immunocompetent murine models is required to delineate their cellular roles in chemotherapeutic response. Nonetheless, our findings putatively identify tumour and stroma compartment-specific proteins that may have prognostic and therapy outcome implications in archival FFPE tissue taken routinely during cancer treatment.

Differential analysis implicated increased stromal ER α and decreased MART1 and 41BB to be associated with response. ER α is typically expressed by luminal breast epithelial cells and forms an axis of hormone directed therapy. Its role within human

stromal cells is relatively poorly defined (31), however ER α expression in cancer associated fibroblasts (CAFs) has been observed (32), and its activation has been implicated in the promotion of pro-tumorigenic angiogenesis in breast tumours (33). Its stromal upregulation in responding patients (**Figure 2**) and association with a favourable therapy response (**Figures 3, 4**) might indicate that such angiogenesis also benefits chemotherapy delivery, thereby improving drug efficacy. 4-1BB, TNF receptor superfamily 9 (TNFRSF9) is responsible for effector CD8 T cell function and survival, and its suppression in responding patients in our data suggests some moderation of T cell activity (34). Consistently, our findings identified the association of stromal GITR expression with favourable therapy response (**Figure 3**). Activation of this TNFR superfamily member is co-stimulatory for T cell proliferation and anti-apoptotic cell survival (35, 36). Our findings suggest that expression of these factors, at least within the stroma, participate in subtle modulation of T cell activity to impact TNBC therapy response.

Our study additionally identified that within the tumour compartments, responding patients exhibited higher levels of GMZA, STING and fibronectin and reduced levels of CD80 (**Figure 3**). Furthermore, our analysis demonstrated that each of these proteins contributed to a multi-protein signature discerning between chemotherapy responsive and nonresponsive tumours (**Figure 4**). Of these proteins, granzyme A (GZMA) is a key cytolytic protease for cytotoxic activity of CD8+ T cells and NK cells (37), while STING acts as a cytosolic DNA sensor that induces production of interferon α/β . Accordingly, recent clinical



evaluation of the DNA damage Immune Response (DDIR) Signature, which is representative of cGAS-STING activation (38), demonstrated that in chemotherapy treated TNBC, DDIR positivity associated with improved overall survival and disease free survival (39). Taken together, our findings are consistent with increased immune cell recruitment and activation, promoting immuno-reactive functions that potentially synergise with chemotherapy, resulting in enhanced response.

Further to our proteomics analysis of tumour compartment proteins, it is worth noting that the survival-associated proteins appeared independent of those proteins found through our differential analysis. This is unlike the proteins enriched within the stromal compartment of patient responders that were largely associated with longer OS. Within the tumour compartment, those tumours with increased levels of PD-L1, FOXP3 and GITR expression were associated with longer OS (**Figure 3**). These findings point to a potential beneficial role for T-regs and infiltration of GITR expressing TILs in modulating immune activity for patient survival. Similarly, our observation that tumour cell PD-L1 expression in TNBC is positively associated

with improved OS is consistent with published findings (15, 40). Furthermore, PD-L1 positive disease displayed improved OS in response to anti-PD-L1 immunotherapy in phase III Impassion130 trial (41), and has subsequently shown improved outcome in response to combination therapies (18).

In addition to our study, other deep profiling methods to evaluate TIL subtypes and cellular markers associated with therapy response in TNBC have included multiplex imaging (42), bioinformatic deconvolution of bulk public mRNA data (43, 44), and RNAseq of tumours stratified by CD8⁺ TIL abundance (45, 46). Our data here provides an additional layer of insight into TNBC tumour composition. Future investigations may benefit from larger TNBC cohorts stratified by TIL abundance to further delineate immune cell roles in therapy response.

In summary, we have shown the utility for spatially mapping the tumour microenvironment in TNBC to identify putative markers associated with response and resistance to adjuvant chemotherapy. Whilst preliminary, the data supports further investigation and validation of the identified biomarkers which

can be of prognostic utility for TNBC. The integration of spatial proteomic datasets, using approaches as described in this manuscript, are highly novel and like to lead to the identification of novel biomarkers for TNBC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Queensland University of Technology Human Research Ethics Committee Approval (UHREC #2000000494). The patients/participants provided their written informed consent to participate in this study.

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AUTHOR CONTRIBUTIONS

Design/concept: AK and KO'B. Experimental: AK, JM, and MA. Analysis: all authors. Writing and review: all authors. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: NM is employed by QCIF Bioinformatics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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