**Single-cell immunoprofiling and spatial analysis of hormone receptor subtypes in HER2+ and HER2low breast tumors using multiplexed immunofluorescence**

**Background:** Breast cancer is characterized by distinct molecular subtypes based on expression of the ER and PR hormone receptors, and epidermal growth factor receptor 2 (HER2). To investigate presence and location of multiple distinct immune cell populations on the single cell level we utilized the multiplexed immunofluorescence (mIF) platform MultiOmyx™ to investigate the tumor immune microenvironment (TME) in HER2+ breast cancer. We have been growing data implicating distinct immunophenotypes in the breast cancer TME with breast cancer outcomes by profiling 1) prevalence, 2) location (e.g. intratumoral, stromal), 3) phenotype (e.g. activated, exhausted) of infiltrating immune cells.

**Methods:** We optimized a custom 26-marker MultiOmyx panel interrogating HER2, immunofluorescence (IF) expression (HER2+), HER2 signaling, stromal, and immune markers. This mIF platform leverages serial IF image capture to allow concurrent profiling of all 26 markers in a high-throughput setting at single cell resolution. We applied the 26-marker panel to a tissue microarray of 208 unique patients with matched tumor/normal tissue cores (15 cores/patient; total 333 tumor and 307 normal cores). HER2-positive was defined via ASCO/CAP guidelines; HER2-low was defined as HER2 immunohistochemistry (IHC) 1+/2x but HER2 in situ hybridization (ISH) negative.

**Image Analysis Workflow**

We developed a novel machine-learning system to quantify single-cell HER2 IF expression.

**Sample-level Cluster Analysis**

Figure 2. Immune lineagespecific and functional markers were interrogated as single markers and established immune cell phenotypes based on combinations of markers, with number of positive cells per mm² (mean per patient for multiple cores). Patterns of patient-level immunophenotypes were interrogated via unsupervised hierarchical clustering, which identified four main clusters: immune-high, immune-intermediate, immune-low/tumor PDL1 high, immune-low/PDL1 low. Example image provided.

**Spatial Cluster Analysis by HR Subtype**

Figure 3. Prevalence of immune clusters by breast subtype (A) Tumor subtypes measured for immune clusters and disease progression at 2 years post surgery. (B) The number of immune clusters that showed significant association with HER2 status. (C) The number of immune clusters that showed significant association with hormone receptor status. (D) The number of immune clusters that showed significant association with triple-negative breast cancer.

**Key Take-Aways**

- We developed a novel machine-learning system to quantify single-cell HER2 IF expression and translate this expression data into a clinically relevant HER2 scoring system.
- Patient-level immunophenotype based on 23 distinct immune cell types reveals unique subsets associated with outcome.
- Among hormone receptor (HR)-negative, HER2+ tumors there was a significant association with immune clusters and RFS.