# Immune Heterogeneity in Breast Cancer Natural History: Benign Breast Disease to Breast Cancer

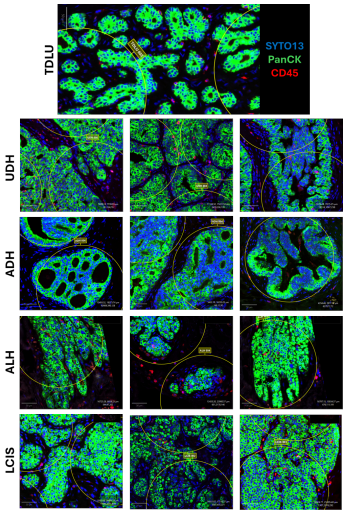
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## BACKGROUND & STUDY GOALS

- Benign breast disease (BBD) is common and associated with variable future breast cancer risk.
- Most BBD lesions do not progress, making risk prediction challenging.
- Reliable biomarkers to identify lesions likely to progress are currently lacking.
- Spatial immune protein expression is a promising tool for risk stratification.
- The stability of immune markers within individuals and their ability to distinguish risk states remain unclear.
- We aimed to evaluate predictability of immune protein expression of the breast microenvironment as a biomarker
- Intra-individual and inter-individual variability in expression of immune-related proteins were assessed and tested for association with breast cancer risk

## METHODS




**Abbreviation Key**

- ROI = Region of Interest
- TDLU = Terminal Ductal Lobular Unit
- UDH = Usual Ductal Hyperplasia
- ADH = Atypical Ductal Hyperplasia
- ALH = Atypical Lobular Hyperplasia
- LCIS = Lobular Carcinoma in situ
- ER = Estrogen Receptor

- Immune protein expression was quantified using GeoMx DSP (Digital Spatial Profiler)
- Regions of interest (ROIs) were selected from BBD (**Figure 1**, left) and tumor specimens
- ConsensusCluster Plus and k-means clustering identified stable global immune clusters
- Intra-lesion heterogeneity: pairwise Euclidean distances were calculated (summing squared differences in protein expression values and square rooting the sum)

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## TABLES AND FIGURES

**Table 1.** Demographic characteristics of study participants in Carolina Breast Cancer Study and two benign breast disease (BBD) studies (UNC and Mayo).

| | Cancer Cohort (CBCS, n=58, # of ROIs = 193) | UNC BBD (n =20, # of ROIs = 217) | Mayo Clinic BBD (n=177, # of ROIs = 1131) |
|---|---|---|---|
| | # (%) | # (%) | # (%) |
| **Age** | | | |
| < 50 years | 30 (52%) | 6 (30%) | 81 (46%) |
| ≥ 50 years | 28 (48%) | 14 (60%) | 96 (54%) |
| **Race** | | | |
| Black | 27 (47%) | 3 (15%) | 0 (0%) |
| Non-Black | 31 (53%) | 17 (85%) | 177 (100%) |
| **CBCS Tumors** | | | |
| ER Positive | 24 (41%) | - | - |
| ER Negative | 34 (59%) | - | - |
| Stage I-II | 11 (19%) | - | - |
| Stage III | 25 (43%) | - | - |
| **BBD Histologic Summary** | | | |
| Atypia, High Risk Findings (LCIS/ADH/ALH) | - | 11 (55%) | 16 (9%) |
| Low Risk Findings (UDH/TDLU/Other) | - | 9 (45%) | 159 (90%) |

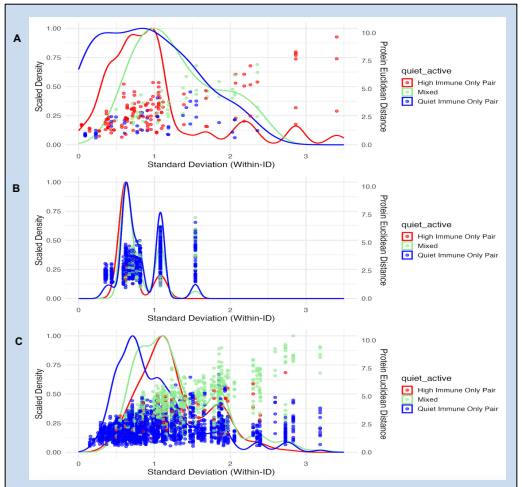



**Figure 3. Intra-lesional heterogeneity is higher in BBD than in tumors. (A–C)** Within-lesion heterogeneity was quantified using SD **(x-axis)** and scaled density plots of within-individual protein expression variability **(primary y-axis)** overlaid with scaled Euclidean distances between ROI pairs **(secondary y-axis)** for: **(A)** CBCS, **(B)** UNC BBD, and **(C)** Mayo BBD cohorts.

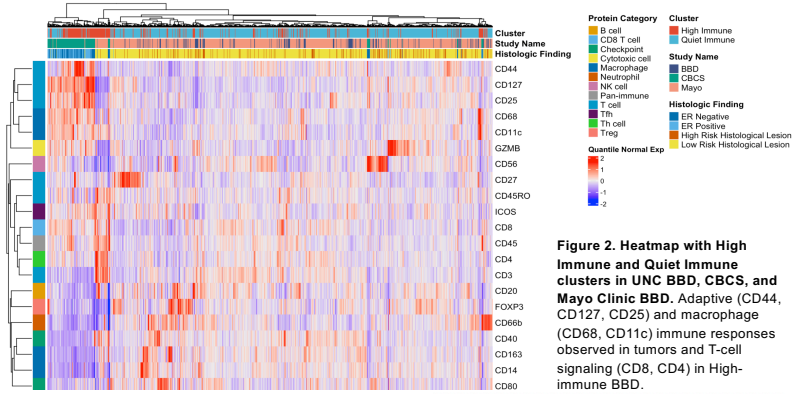



**Figure 2. Heatmap with High Immune and Quiet Immune clusters in UNC BBD, CBCS, and Mayo Clinic BBD.** Adaptive (CD44, CD127, CD25) and macrophage (CD68, CD11c) immune responses observed in tumors and T-cell signaling (CD8, CD4) in High-immune BBD.

**A**

| | High Immune | | Quiet Immune | |
|---|---|---|---|---|
| **CBCS** | ROI n (%) | Participant n (%) | ROI n (%) | Participant n (%) |
| ER Negative | 72 (38%) | 11 (19%) | 38 (20%) | 23 (40%) |
| ER Positive | 30 (16%) | 14 (24%) | 52 (27%) | 10 (17%) |
| Odds Ratio (95% confidence interval) p-value | 3.28 (1.34 - 8.04) 0.0093 | 2.92 (0.99 - 8.65) 0.0521 | REF | REF |
| **UNC BBD** | | | | |
| Histological Findings | | | | |
| High-Risk Findings (LCIS, ADH, ALH) | 29 (13%) | 3 (15%) | 33 (15%) | 7 (35%) |
| Low-Risk Findings (UDH, TDLU) | 45 (20%) | 3 (15%) | 110 (50%) | 7 (35%) |
| Odds Ratio (95% confidence interval) p-value | 1.68 (0.57 - 4.97) 0.3436 | 1.00 (0.15 - 6.70) 0.2195 | REF | REF |
| **Mayo Clinic BBD** | | | | |
| Histological Findings | | | | |
| High-Risk Findings (ADH, ALH) | 11 (1%) | 23 (13%) | 24 (2%) | 14 (8%) |
| Low-Risk Findings (UDH, TDLU, Other) | 461 (40%) | 97 (55%) | 635 (56%) | 43 (24%) |
| Odds Ratio (95% confidence interval) p-value | 1.58 (0.66 - 3.77) 0.2993 | 0.72 (0.34 - 1.54) 0.4106 | REF | REF |

**B**

| Immune Class | High Immune # of ROIs (%) | Quiet Immune # of ROIs (%) |
|---|---|---|
| **Cancer Cohort (CBCS)** (ROIs N=192; Participants N =58) | | |
| ROI assignment | 147 (77%) | 45 (23%) |
| ≥ 50% of ROIs High immune | 47 (81%) | 11 (19%) |
| ≥ 1 ROI High immune | 52 (90%) | 6 (10%) |
| **UNC Benign Breast Disease Cohort (BBD)** ROIs N = 217, Participants N = 20) | | |
| ROI assignment | 62 (29%) | 155 (71%) |
| ≥ 50% of ROIs High immune | 7 (35%) | 13 (65%) |
| ≥ 1 ROI High immune | 12 (60%) | 8 (40%) |
| **Mayo Clinic BBD Cohort** (ROIs N = 1130, Participants N = 177) | | |
| ROI assignment | 472 (42%) | 659 (58%) |
| ≥ 50% of ROIs High immune | 46 (26%) | 131 (74%) |
| ≥ 1 ROI High immune | 170 (96%) | 7 (4%) |

**Table 2. Immune Class Distributions and Clinical Correlates Across Tumor and Benign Cohorts. A)** Associations between participant- and ROI-level High vs Quiet Immune class assignments and clinical or histologic features across CBCS, UNC BBD, and Mayo BBD cohorts. **B)** ROI- and participant-level immune class distributions across cohorts comparing tumor immunity to benign breast disease.

## SUMMARY

- Consensus Clustering identified two global immune clusters: High Immune and Quiet Immune.
- Intra-lesional heterogeneity in tumors show left shifted density function for High Immune ROI pairs, while BBD cohorts show broad, multi-peaked distributions and wider spread in standard deviation
- Intra-individual heterogeneity show higher % of BBD participants with Mixed Immune clustering (UNC BBD: 42%, Mayo BBD: 54%) compared to tumors (CBCS: 32%)
- Using different thresholds of defining patients as High Immune, tumors met stricter thresholds more consistently, whereas BBD participants showed large gaps between definitions
- Spatial immune protein profiles in BBD display high intra-individual variability, limiting their utility as stable biomarkers of risk