Molecular Landscapes of Breast Cancer Subtypes: Data Independent Acquisition and Identification of Potential Targets for Stromal Reprogramming

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Introduction

Breast cancer is a highly complex and heterogenous disease with multiple subtypes associated with distinct patient outcomes and treatment strategies. It is therefore instrumental to comprehend the 'molecular landscape' of human breast cancer subtypes by determining what dynamic changes occur within the tumor in comparison to disease-free tissue to improve patient outcomes by developing new biomarkers and potential therapies.

Breast Carcinoma Subtypes & Survival Rates

Cancer Subtype	Abbreviation	5 Year Survival Rate
Metaplastic	Meta	55%
Triple Negative	TN	77%
HER2 Positive	HER2	83%
Luminal B	Lum B	89%
Luminal A	Lum A	92%

Source: North American Association of Central Cancer Registries (NAACCR), 2019.
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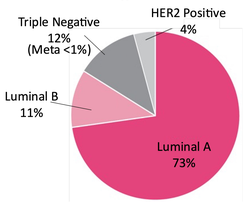
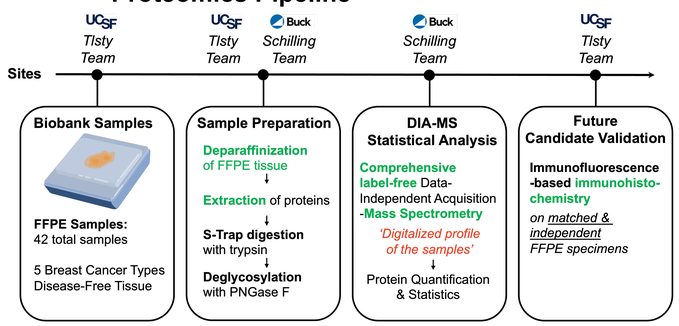
Distribution of Female Breast Cancer Subtypes US 2012 – 2016



Proteomics Pipeline

We demonstrate the use of a robust proteomic pipeline which utilizes expertise from multiple labs to gain insights into molecular tissue remodeling during breast cancer by analyzing human **formalin-fixed, paraffin-embedded** (FFPE) breast tissue. Our work uses a novel, high-resolution **ZenoTOF 7600** mass spectrometer with a Waters M-class microflow chromatography system is used to acquire proteomic data from FFPE human tissues with **Data-Independent Acquisition** (DIA), a label-free quantification method to obtain a digitalized profile of the samples for protein quantification and statistics. Proteins identified as prospective biomarker candidates will be validated with immunohistochemistry.



Extracellular Matrix Proteins are Remodeled in Breast Cancer

Disruption to the stroma and extracellular matrix (ECM) is an important aspect of cancer cell dedifferentiation, as it reflects the degree to which the stroma/ECM may instruct cancer cells to drift from the specialized state seen in healthy tissue. More differentiated cancers tend to be associated with less alterations in the surrounding stroma and ECM compared to more aggressive and invasive, less differentiated cancers in the context of a greater remodeling of the normal tissue architecture and ECM.

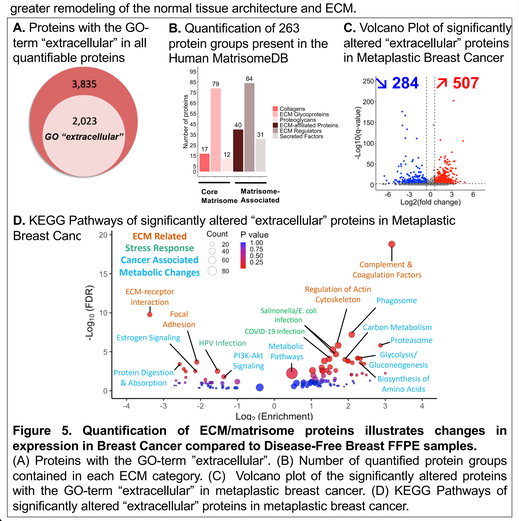


Figure 5. Quantification of ECM/matrisome proteins illustrates changes in expression in Breast Cancer compared to Disease-Free Breast FFPE samples. (A) Proteins with the GO-term "extracellular". (B) Number of quantified protein groups contained in each ECM category. (C) Volcano plot of the significantly altered proteins with the GO-term "extracellular" in metaplastic breast cancer. (D) KEGG Pathways of significantly altered "extracellular" proteins in metaplastic breast cancer.

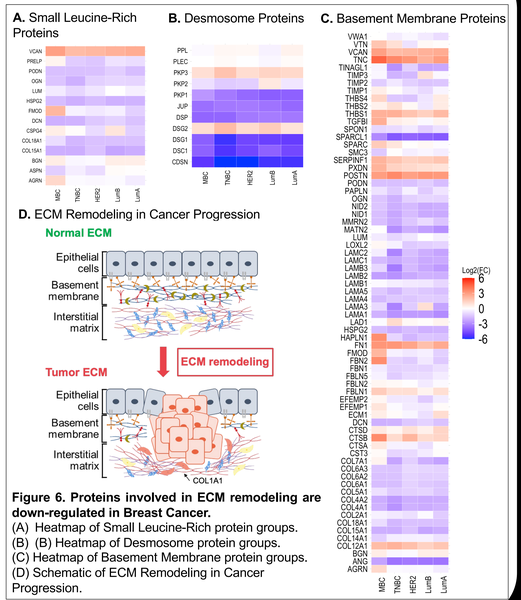


Figure 6. Proteins involved in ECM remodeling are down-regulated in Breast Cancer.
(A) Heatmap of Small Leucine-Rich protein groups.
(B) (B) Heatmap of Desmosome protein groups.
(C) Heatmap of Basement Membrane protein groups.
(D) Schematic of ECM Remodeling in Cancer Progression.

Proteome Remodeling in Breast Cancer

Our optimized quantitative proteomics pipeline for FFPE Breast Cancer tissue samples resulted in **5,858 protein group identifications and quantifications with ≥ 2 unique peptides** per protein group, measured by **Zeno SWATH DIA on the ZenoTOF 7600 system** and quantified using **Spectronaut v16**. Significantly altered protein groups between each breast cancer subtype (n = 7 per subtype) and the disease-free samples (n = 7) showed strong remodeling of the proteome.

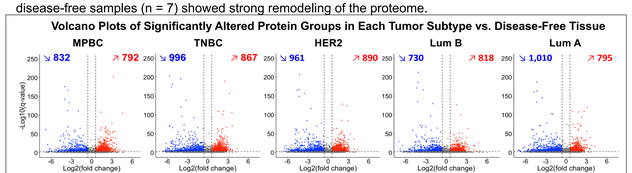


Figure 1. Breast Cancer Subtype Proteomes reflect dramatic tissue remodeling. Volcano plots showing proteome remodeling in breast cancer subtypes. More than 26% of all protein groups are significantly altered in comparisons with human breast cancer subtypes vs. disease-free tissue. **Applying q-value ≤ 0.001 and $|Log_2(FC)| \geq 0.58$.**

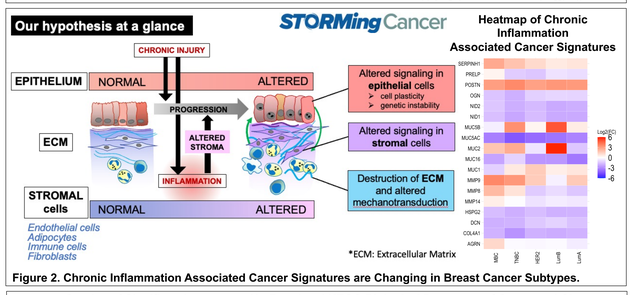


Figure 2. Chronic Inflammation Associated Cancer Signatures are Changing in Breast Cancer Subtypes.

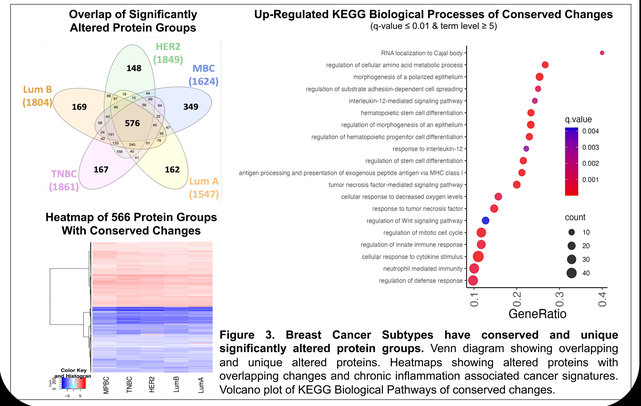
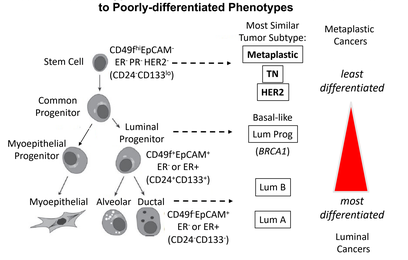


Figure 3. Breast Cancer Subtypes have conserved and unique significantly altered protein groups. Venn diagram showing overlapping and unique altered proteins. Heatmaps showing altered proteins with overlapping changes and chronic inflammation associated cancer signatures. Volcano plot of KEGG Biological Pathways of conserved changes.

Proteome Remodeling in Metaplastic Breast Cancer

Metaplastic breast cancer is the rarest breast cancer subtype. Metaplastic breast cancer has been characterized as the least differentiated and most aggressive form of breast cancer and has the lowest survival rate of all breast cancer subtypes. Metaplastic breast cancer has the most characteristics common to other chronic inflammation associated cancers, including aggressiveness and increased levels of metastasis. There are **349 protein groups quantified with ≥ 2 unique peptides** that are uniquely altered in the comparison between metaplastic breast cancer (n = 7) and disease-free tissue (n = 7). Changes unique to Metaplastic breast cancer are associated with changes in the extracellular matrix, a dominant and dynamic contributor to cancer aggressiveness. Other unique changes in metaplastic breast cancer are associated with promoting cancer cell invasion, migration, and metastasis.



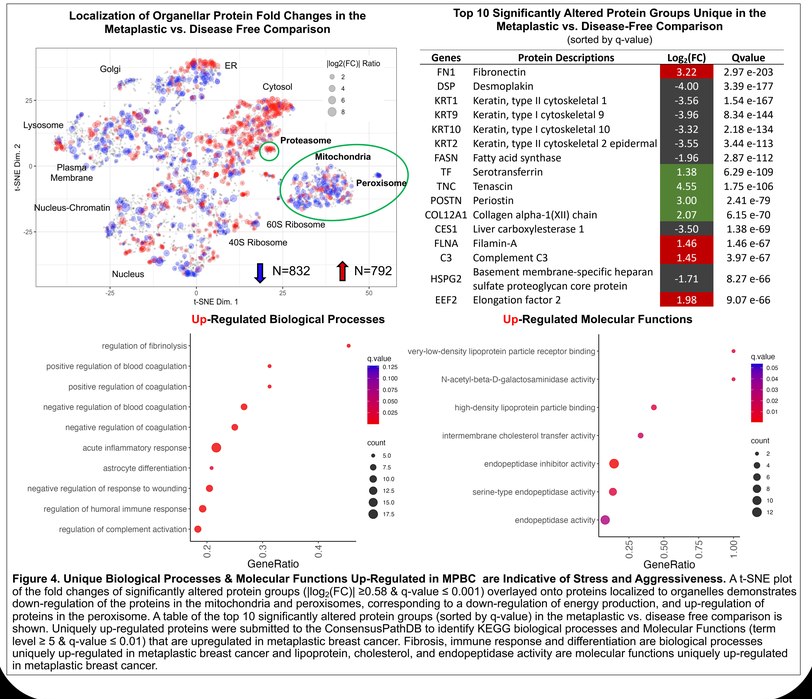
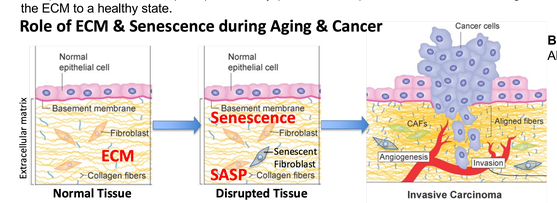


Figure 4. Unique Biological Processes & Molecular Functions Up-Regulated in MPBC are Indicative of Stress and Aggressiveness. A t-SNE plot of the fold changes of significantly altered protein groups ($|log_2(FC)| \geq 0.58$ & q-value ≤ 0.001) overlayed onto proteins localized to organelles demonstrates down-regulation of the proteins in the mitochondria and peroxisomes, corresponding to a down-regulation of energy production, and up-regulation of proteins in the peroxisome. A table of the top 10 significantly altered protein groups (sorted by q-value) in the metaplastic vs. disease free comparison is shown. Uniquely up-regulated proteins were submitted to the ConsensusPathDB to identify KEGG biological processes and Molecular Functions (term level ≥ 5 & q-value ≤ 0.01) that are upregulated in metaplastic breast cancer. Fibrosis, immune response and differentiation are biological processes uniquely up-regulated in metaplastic breast cancer and lipoprotein, cholesterol, and endopeptidase activity are molecular functions uniquely up-regulated in metaplastic breast cancer.

Observed Changes in Senescence Associated Secretory Phenotype Markers

The incidence of cellular senescence, a state of permanent cell cycle arrest, increases during healthy aging. The secretion of cytokines, matrix metalloproteinases, and growth factors by senescent cells, known as the senescence-associated secretory phenotype (SASP), can contribute to the disruption of the stroma and ECM. Stiffening of the ECM, as a result of ECM reorganization, makes tissues more prone for cancer. We compared SASP and ECM profiles across breast cancer subtypes, including highly aggressive metaplastic breast cancer (MBC), to identify potential therapeutics aimed at remodeling the ECM to a healthy state.

Role of ECM & Senescence during Aging & Cancer



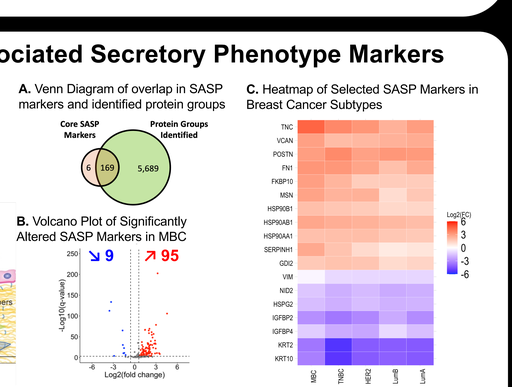


Figure 7. Senescent Cells accumulate during aging and elevated SASP markers are associated with ECM Reorganization. Breast Cancer Subtypes Show an Up-Regulation of SASP Markers Compared to Disease-Free Samples. (A) Venn diagram of the overlap in core SASP markers and protein groups identified with ≥ 2 unique peptides. (B) Volcano plot of significantly altered SASP markers in MBC. (C) Higher expression of SASP markers in Breast Cancer with the worst 5-year survival.

Conclusion

Our **FFPE** tissue proteomics pipeline is robust and can be applied to cohorts of patients with known outcomes.

- Almost **6,000** protein groups were quantified with 2 or more unique peptides.
- Over **25% of quantified protein groups were significantly altered** in each breast cancer subtype vs. disease-free breast tissue (q-value ≤ 0.001 and $|Log_2(FC)| \geq 0.58$).
  - Conserved changes in all breast cancer subtypes were observed in 566 protein groups.
  - There were 10 proteins that have conserved changes in all but one breast cancer subtype.

ECM/matrisome reorganization was observed across breast cancer subtypes.

Altered proteomic expression profiles in breast cancer subtypes were associated with **ECM** reorganization, stress response, and metabolic changes, reminiscent of SASP phenotypes.

We identified a potential correlation between expression of **SASP** markers and lowest survival rate among breast cancer patients.

Acknowledgments

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