The Microbiome of Sterile Human Breast Tissue in Varied Disease States

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Disclosures

- No relevant financial disclosures
Breast Cancer

- Globally breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females
  - 23% of total cancer cases
  - 14% of cancer deaths
Breast Cancer – Risk Factors

- Female Sex
- Increasing Age
- Radiation
- Early menarche/late menopause
- Age FP/nulliparity
- HRT
- Obesity
- Alcohol

- Personal and/or Family History
- Genetic Predisposition
  - BRCA1/2, PALB2, ATM, PTEN, CHEK2, RAD50, MRE11A, NBN, CDH1
Breast Cancer – Risk Factors

- Most cases etiology unknown

>70%
Mayo BBD Group

Mission

• Understand how breast cancer develops
• Identify women at elevated risk
• Develop optimal strategies for risk reduction and cancer prevention
Breast Cancer Risk – The Challenge
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Breast cancer risk prediction will be improved by study of the breast tissue at risk using the platform of Benign Breast Disease (BBD)
Mayo Benign Breast Disease (BBD) Cohort

The NEW ENGLAND JOURNAL of MEDICINE

Benign Breast Disease and the Risk of Breast Cancer

Lynn C. Hartmann, M.D., Thomas A. Sellers, Ph.D., Marlene H. Frost, Ph.D., Wilma L. Lingle, Ph.D., Amy C. Degnim, M.D., Karthik Ghosh, M.D., Robert A. Vierkant, M.S., Shaun D. Maloney, B.A., V. Shane Pankratz, Ph.D., David W. Hillman, M.S., Vera J. Suman, Ph.D., Jo Johnson, R.N., Cassann Blake, M.D., Thea Tlsty, Ph.D., Celine M. Vachon, Ph.D., L. Joseph Melton III, M.D., and Daniel W. Visscher, M.D.

- 1967-2001
- 13,652 women benign breast biopsy
- Median 16 years follow-up
- 1,231 cancers
Mayo Clinic BBD Cohort

- Unique platform for improving BC risk prediction
- Unique resource for identification of novel biomarkers of future BC risk
- 13,560 women, age 18-85 at biopsy, benign breast biopsy 1967-2001
- BBD categorized after review by a pathologist
- Tissue blocks are available on >95% of the cohort
Breast Cancer Risk Prediction
Epithelial Proliferation Increases Risk

- RR 1.3
  Non-proliferative
  FA LOBULE

- 1.9
  Proliferative
  UDH

- 4.2
  Atypical Hyperplasia
  ADH

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Hartmann et al. NEJM 2005
Degnim et al. JCO 2007
Lobular Involution (Age-Related Regression)

None ---------------------------------- Partial -------------------------- Complete
Breast Cancer Risk Prediction
Age-Related Lobular Involution and Histology

![Graph showing relative risk for different categories of involution and histology.](image)
Breast Cancer Risk Prediction
Number of Foci of Atypia

Degnim et al. JCO 2007; 25(19)
Breast Cancer Risk and Number of Foci of Atypia

Figure 2. Cumulative Incidence of Breast Cancer after a Diagnosis of Atypical Hyperplasia.

Hartmann et al. NEJM 2015; 372:78-89
New BBD-BC Model

- C-statistic 0.63 vs 0.47 with BCRAT

Pankratz et al. JCO 2015; 33
BC Risk and ERβ

ERβ has tumor suppressor properties: it inhibits BC cell proliferation in culture and is expressed more highly in normal breast cells than in cancer.

ERβ expression in 171 women with atypia

- ERβ expression was lower in atypical tan normal lobules
- High ERβ expression in the atypia lesions increased BC risk
- High ERβ expression in normal lobules increased BC risk.

Incidence of BC in women with atypia aged<45, stratified for ERβ expression.
Healthy human breast tissue contains immune effectors in breast lobules, with cytotoxic T lymphocytes and dendritic cells intimately associated with the breast epithelium.
Microbial Populations

World Microbes: $5,000,000,000,000,000,000,000,000,000,000,000,000,000 \times 10^{30}$

People: $6,770,000,000 \times 10^9$

Stars: $1,000,000,000,000,000,000,000,000,000,000,000,000,000 \times 10^{24}$
Microbial Populations

- Microbes make up the majority of biomass, diversity, species, and organisms
Human Microbiome

- Within the human body ~10x more microbial cells than human cells
- Microbes carry out metabolic reactions **not encoded in the human genome** which are **necessary for human health**
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- Within the human body ~10x more microbial cells than human cells
- Microbes carry out metabolic reactions not encoded in the human genome which are necessary for human health
- Microbial dysbiosis implicated in various malignant diseases states including gastric cancer and lymphoma and colon cancer
- Most analysis to date on stool samples
Microbe Distribution by Body Site

15 to 18 body sites sampled
In the HMP (based on analysis of 242 of targeted 300 humans) the diversity and abundance of each habitat’s signature microbes to varied widely even among healthy subjects, with strong niche specialization both within and among individuals.

Background & Rationale

- Breast = complex microenvironment including epithelium, stroma, and a mucosal immune system
- Mucosal immune systems develop as a direct result of microbial exposure, thus the finding of immune effectors suggests a breast microbiome
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- Mucosal immune systems develop as a direct result of microbial exposure, thus the finding of immune effectors suggests a breast microbiome
- Bacteria have been cultured from intraoperatively-obtained breast tissues
- Lack of data on microbiome of sterile human breast tissue by a culture-independent molecular genetic approach
Breast Microbiome

- The breast milk microbiome appears to differ from other human niches.
- Less diverse bacterial community observed in breast milk from obese mothers indicating a breast-related microbiome varies with a known risk factor for breast cancer.
Hypothesis

- Breast tissue obtained via sterile collection contains an endogenous microbiome
- Systematic and demonstrable differences between benign and malignant disease states
- These variants in the breast microbiome may contribute to the development of breast cancer
Microbe Distribution by Body Site

Breast?
Study Design & Methods

- Sterile intraoperative samples from patients with benign disease and patients with malignant disease
- Excluded patients taking systemic steroids, PPIs, antibiotics
- At enrollment
  - Buccal swab
  - Stool sample
Study Design & Methods

- In the operating room after frozen section pathology confirms clear margins
  - 1 cc$^2$ adjacent uninvolved breast tissue
  - skin tissue strip from incision edge
  - skin swab
  - ± lesional tissue (sterile frozen section accessioning)
Study Design & Methods

- Patient, procedure, pathology data
Study Design & Methods

- Whole tissue DNA extraction
- 2-step pCR to amplify V3-V5 region of 16SrRNA
- 16SrRNA hypervariable tag sequencing (Illumina MiSeq)
- Sequence reads aligned with our custom multiple alignment tool (IM-TORNADO) that merges paired end reads into a single multiple alignment and obtains taxa calls
- QIIME for visualization and statistical analysis
16S rDNA
Computational Methodology
Taxon Organization from RNA Dataset Operations (TORNADO v2)

Bioinformatics Core Ready

QIIME
Patients

- 46 breast-conserving surgery patients
  - 21 benign disease
  - 25 malignant disease
    - ER+ 100%
    - HER2+ 29%
    - Grade I/II 94%
    - LN negative 100%
Patients

- Preop Hibiclens bathing (95%)
- Chloraprep skin prep (98%)
- Preoperative Cefazolin (98%)
### Analysis of 46 Patient Sample Sets

**Patient & Specimen Features**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Benign (N=21)</th>
<th>Malignant (N=25)</th>
<th>Total (N=46)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Age Median (Range)</td>
<td>51 (33-80)</td>
<td>66 (44-84)</td>
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<td>Distance of specimen from nipple (cm)</td>
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Analysis of Patient Sample Sets

UniFrac distance

PC2 (14.8%)

PC1 (21%)
Skin Tissue versus Breast Tissue
Alpha-diversity analysis

- Breast tissue has greater species richness (OTU #) and overall diversity (evenness) than skin tissue
Alpha-diversity analysis

- The increased species richness of the breast tissue microbiota is confirmed by the heat map.
- These OTUs are mostly of low abundance.
Beta-diversity analysis

- Beta-diversity reveals that the breast tissue has a unique microbiota (MiKRAT omnibus $p < 0.001$, combining UniFrac [+/-], weighted UniFrac [abundance], BC distance [taxa dissimilarity])
- Individual MiKRAT test $p < 0.001$ for all three distance measures, indicating a large community difference
Microbiome regression-based kernel association test (MiRKAT)

- Directly regresses the outcome on the microbiome profiles via the semi-parametric kernel machine regression framework
- Allows for easy covariate adjustment and extension to alternative outcomes while non-parametrically modeling the microbiome through a kernel that incorporates phylogenetic distance
- Uses a variance-component score statistic to test for the association with analytical p value calculation
- Allows simultaneous examination of multiple distances, alleviating the problem of choosing the best distance
Differential Taxa Breast Tissue v Skin Tissue

Permutation Testing
Differential Taxa Breast Tissue v Skin Tissue

Taxa with p<0.05 at Family and Genus Level
Heatmap based on Boruta selected taxa

- **Firmicutes**
- **Actinobacteria**
- **Bacteroidetes**
- **Proteobacteria**
- **Fusobacteria**

*Enriched in Breast Tissue*
Breast Tissue from Benign versus Malignant Disease States
Benign Non-Atypia vs Malignant Invasive Alpha-diversity analysis

- No significant difference ($P > 0.4$)
Beta-diversity analysis

• Beta-diversity reveals that malignant and benign state breast tissue microbiota is significantly different (MiKRAT omnibus $P = 0.016$, combining UniFrac, weighted UniFrac, BC distance)

• Individual MiKRAT test $P < 0.05$ for all three distance measures (P=0.01, 0.04 and 0.03), indicating that the difference lies in both abundant and rare lineages
Breast from Benign Non-Atypia versus Invasive Malignant Disease States
Benign versus Malignant Disease

- Adjacent histologically normal breast tissue
  - 13 patients with non-atypia BBD
  - 15 patients with invasive cancer
Benign Non-Atypia vs Invasive Malignant

- Similar findings regarding
  - Alpha diversity (not significantly different)
  - Beta diversity (significantly different)
Benign Non-Atypia vs Invasive Malignant

Permutation Testing
Benign Non-Atypia vs Invasive Malignant
Benign Non-Atypia vs Invasive Malignant

- Breast tissue from patients with invasive cancer most significantly enriched (genus level)
  - Fusobacteria Fusobacterium
  - Actinobacteria Actopobium
  - Proteobacteria Gluconacetobacter
  - Euryarchaeota Methanothermococcus (Hydrogenophilus)
  - Firmicutes Lactobacillus
Functional Analysis
Functional Analysis
Benign Non-Atypia vs Invasive Malignant
Functional Analysis
Benign Non-Atypia vs Invasive Malignant

- Cysteine and methionine metabolism most significant
- Methionine associated with cancer risk and progression
- Recently methionase (to upregulate methionine metabolism) introduced as potential novel way to treat cancer

KEGG pathways with p<0.05
PICRUST
Conclusions

- The microbiome of sterile human breast tissue is distinct from sterile breast skin tissue.
- Both are distinct from the buccal and skin swab microbiome.
- The human breast microbiome (in adjacent putatively normal breast tissue) varies with disease states.
Next Steps

- Further analysis of existing samples within this pilot sample set
- Analysis of larger sample set including lesional tissue under uniform conditions with replicates and controls
- Microbial community composition vs single pathogenic bacteria
- Associations with other –omics including the estrogen metabolism
Next Steps

- Risk prediction signature
- Cancer prevention vaccine
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**Immunology**
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**Microbiology**
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**Pathology**
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Thank you
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