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14. ABSTRACT

Patients diagnosed with breast cancer within 10 years of a completed pregnancy are at ~3 fold higher risk of subsequently developing metastatic disease. This increased risk is independent of ER, PR or Her-2 status, and is not observed in age matched breast cancer patients who have never had a child nor in women initially diagnosed during their pregnancy, highlighting an unappreciated role of postpartum reproductive biology in breast cancer metastases in young women. Importantly, the only reported site of increased metastases in postpartum women is the liver. This specificity for liver metastasis is the foundation for proposing that the mammary gland and liver are functionally linked postpartum, leading to increased risk for breast cancer metastasis to the liver. To address this novel hypothesis, we turned to rodent models and identified a previously unreported biology, weaning-induced liver involution. In our rodent models we discovered that like the breast, the liver expands to meet the nutrient and metabolic demands of lactation and upon weaning undergoes a developmental program to return to a pre-pregnant like state through an active tissue remolding process characterized by hepatocyte apoptosis, catabolic metabolism, immune cell influx and ECM remodeling. Further, using our rodent models we found liver involution supports enhanced establishment of metastatic tumors compared to livers of nulliparous hosts. Since programmed cell death is functionally linked to immune tolerance in a variety of biologic contents, we hypothesis that PPBC liver metastases will be characterized by active immune tolerance and responsivity to checkpoint inhibitors.

15. SUBJECT TERMS
None listed.

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Contents

1. INTRODUCTION:

Metastasis of breast cancer to the liver has a catastrophic outcome, and liver metastasis occurs at high frequency in younger, childbearing age patients. In rodent models, we have demonstrated a functional link between the liver and the breast during pregnancy and lactation that transiently transforms the liver into rich "soil" for breast cancer establishment. In addition, our lab has the first-ever evidence that similar post-partum liver biology is occurring in women as well (revised manuscript under-review). In this grant, we will expand our molecular understanding of weaning-induced liver involution in mice; investigate how liver involution influences the immune milieu of breast cancer metastasis to the postpartum liver (MLBC) in both rodents and women, and employ rodent models to interrogate immunomodulatory agents to target MLBC.

KEYWORDS: Young women's breast cancer, pregnancy, postpartum breast involution, postpartum breast cancer, metastatic niche, liver growth, tumor microenvironment

2. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1. Define the immune milieu of healthy, involuting rodent livers compared to nulliparous host livers.

A. Preform RNAseq on livers from non-tumor bearing animals
   - Year 1, months 1-6: Isolation and Quality control of RNA: *100% complete*
   - Year 1, months 7-9: Library preparation: *100% complete*
   - Year 1, months 9-15: Two rounds of sequencing: *100% complete*
   - Year 1, months 16-18: Computational alignment and normalization of data: *100% complete*
   - Year 1, months 19-30: Analysis of RNA expression results: *50% complete*
   - Year 2, manuscript preparation: *5% complete*

B. Multiplex IHC of non-tumor bearing mouse livers across postpartum developmental spectrum, n = 6 for each time point, Nulliparous, Lactation, Days 2, 4, 6, 8 and 4wks post weaning
   - Year 1, months 1-6: Antibody validation [MDSC panel *95% complete*; lymphoid panel *25% complete*.
   - Year 1, months 7-12, Year 2, months 1-12, Year 3, months 1-2: Multiplex IHC image acquisition: *0% complete*
   - Year 3, months 3-5: Image alignment, region selection, cell segmentation: *0% complete*
   - Year 3, months 5-10: Image analysis: *0% complete*

Aim 2. Identify immune milieu of PPBC and non-PPBC liver metastases in rodents and humans.

A. Perform RNA-Seq on FFPE from postpartum and non-postpartum breast cancer liver metastases human samples (n =16): *0% complete*
B. Multiplex IHC of Human Breast Cancer Liver Metastasis from 16 Non-postpartum and 9 Postpartum and 5 antibody control FFPE specimens.
   - Year 1, months 1-6: Antibody reagent purchase, lot validation and aliquot: *50% complete*
   - Year 1, months 7-12: Multiplex IHC staining, image acquisition for 30 cases for 25 analytes: *50% complete*
   - Year 2, months 1-6: Image Alignment, Region Selection and Cell Segmentation: *50% complete*
   - Year 2, months 6-12: Single Cell Image Cytometry Analysis: *25% complete*
   - Year 3, months 1-12: Data Analyses and manuscript preparation: *15% complete*
C. Multiplex IHC on Murine Postpartum and Nulliparous Liver metastases (6 postpartum, 6 nulliparous)
   - Year 1, months 1-6: Antibody reagent purchase, lot validation and aliquot: *50% complete*
   - Year 1, months 7-12: Multiplex IHC image acquisition for 12 slides for 25 analytes: *50% complete*
   - Year 2, months 1-3: Image Alignment, Region Selection and Cell Segmentation: *50% complete*
   - Year 2, months 4-6: Singe Cell Image Cytometry Analysis: *50% complete*

Year 2, month 6: Completion of immune characterization of murine breast cancer liver metastasis from postpartum and non-postpartum murine models: *15% complete*

Aim 3. Identify efficacy and specificity of immunotherapy for PPBC liver metastasis.

A. Portal vein implantation of breast cancer cell lines in nulliparous and involution animals with therapeutic administration immunotherapeutics

Year 1, months 1-3: Balb/c Animal Ordering, Breeding and nursing: *0% complete*

Year 1, months 4-5: Portal Vein implantation of D2A1 breast cancer cell line and tumor out growth: *0% complete*

Year 1, month 6: Administration of immunomodulatory agents and tumor growth: *0% complete*

Year 1, month 7: Collect final tissues – Measure Liver Metastatic incidence and burden: *0% complete*

Year 1, months 8-12, Year 2, months 1-3: Repeat for Balb/c mice and D2OR breast cancer cell line: *0% complete*

Year 2, months 4-11: Repeat for C57BL6 mice and EO771 breast cancer cell line: *10% complete*

Year 2, month 1, Year 3, months 1-7: Repeat for C57BL6 mice and PY230 breast cancer cell line: 1*0% complete*

Year 2, month 8-12:– Monitor metastatic tumor growth in response to immunomodulatory agents, collect samples for flow cytometry, flash frozen, and FFPE tissue banks: *0% complete*

B. Determination of tumor specific T cell response to therapy by flow cytometric analysis of liver draining lymph nodes and spleen – (640 samples)

Year 1, month 7: Antibody reagent purchase, lot validation and aliquot: *60% complete*

Months 7, 15, 23, 31: Perform Flow cytometry

a. Develop flow panel: *60% complete*
b. Perform flow analysis on study samples: *0% complete*

Year 3, months 8-10: Detailed Analysis of Flow Cytometric Data and manuscript preparation: *0% complete*

Year 3, month 12: Milestone(s) Achieved: Completion of first therapeutic postpartum liver metastasis mouse model for two background and 4 breast cancer cell lines with determination of the influence of checkpoint blockade and COX-2 inhibition on cytotoxic T cell number and manuscript: *0% complete*

C. Immune focused Multiplex IHC on livers from tumor injected mice comparing involution experienced mice with and without immunomodulatory treatment based upon response to treatment across 2 strains and 4 cell lines. (48 slides)

Year 1, months 1-6: Antibody reagent purchase, lot validation and aliquot: *60% complete*

Year 1-3 Rolling: Multiplex IHC image acquisition for 48 slides for 25 analytes, as specimens become available: *0% complete*

Year 1-3 Rolling: Image Alignment, Region Selection and Cell Segmentation: *0% complete*

Year 1-3 Rolling: Singe Cell Image Cytometry Analysis and manuscript preparation: *0% complete*

Year 3, month 12: Milestone(s) Achieved: Completion of immune characterization of multiplex – IHC immune profiling of checkpoint responsive and non-responsive breast cancer liver metastasis from 4 postpartum breast cancer mouse models, and a bank of frozen and FFPE tissues: *0% complete*

What was accomplished under these goals?

Aim 1 Objective: Define the immune milieu of healthy, involuting rodent livers compared to nulliparous host livers, using RNAseq and mIHC methodologic approaches.

Major Activities Overview

- RNAseq approach: Isolated RNA from snap frozen livers from various reproductive cycle time points for RNA sequencing to capture global changes to the liver
    - Identified major pathways that change during a reproductive cycle.
- mIHC approach: Established a murine lymphoid panel for multiplex IHC to investigate changes to liver lymphoid populations and their activation states might change with reproductive stage.

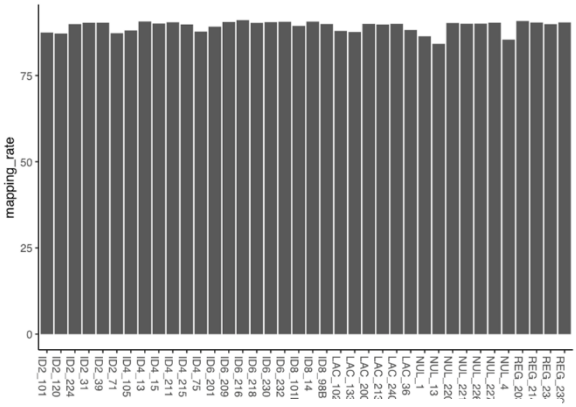

Fig 1. RNA quality check showed mapping rate of sequences was consistent across groups.

Aim 1: Significant Results and Key Conclusions

Aim 1A. RNAseq to define the immune milieu of healthy, involuting rodent livers compared to nulliparous host livers.

In order to better understand how liver involution is contributing to increased metastasis in postpartum breast cancer cases, we sought to capture a global view of how the liver changes between reproductive stages. In order to do this we employed RNA sequencing (RNAseq). For this analysis, RNA was isolated from snap frozen livers by combining mice from three different studies, encompassing mice from two different vendors. We successfully isolated liver RNA from 37 mice across a complete lactation/involution cycle including nulliparous (n=7), lactation (n=6), involution day 2 (n=6), day 4 (n=5), day 6 (n=6), day 8 (n=3), and regression (n=4) reproductive stages. We obtained high quality RNA from each animal (**Fig 1**), permitting all cases to move towards RNAseq. Initial analysis of the RNAseq data using principal component analysis revealed that the largest driver of gene expression differences, as reported in principal component 1 (PC1) was based on vendor differences (**Fig 2**).

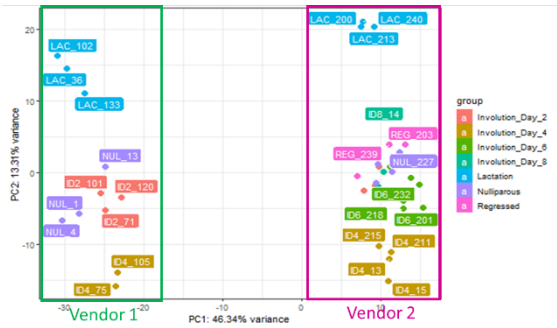

Fig 2. Principal component analysis revealed the primary driver of gene expression differences was attributed to the vendor from which the mice were purchased.

To address these vendor differences, we took two approaches. First, we analyzed the livers from the different vendors separately. This principal component analysis showed samples from each reproductive group now clustered together, as predicted if RNA expression in the liver is dependent on reproductive state (**Fig 3**). While this data showed there are uniquely expressed genes at each reproductive cycle, we now faced loss of statistical power due a decrease in sample size. To account for this, we used batch correction methods to restore our sample sizes to original study design levels. We applied batch correction by using the CombatSeq program, a tool developed for R, to the first vendor data set **(Fig 3A)**. Principal component analysis of the batch corrected data yielded similar results to the second vendor **(Fig 3B)**, showing samples now exclusively clustering by their reproductive group (**Fig 4**). As expected, nulliparous and regressed groups clustered similarly, a result predicted based on the premise that weaning-induced liver involution is a transient event designed to return the liver to its baseline, pre-pregnant functioning state. Interestingly, lactation liver had the most distinct expression pattern, with involution days 2, 4, and 6 all showing expression patterns

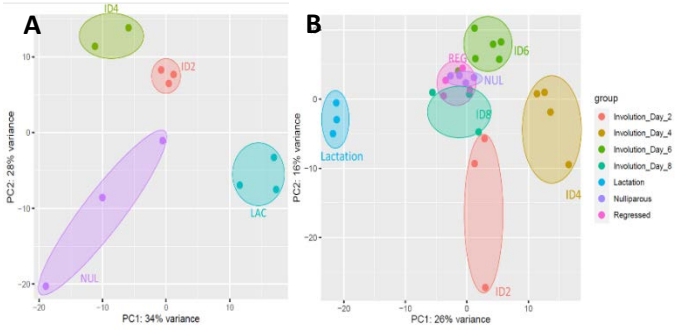

Fig 3. Principal component analysis done on each set of livers based on vendor showed samples separate by reproductive group.

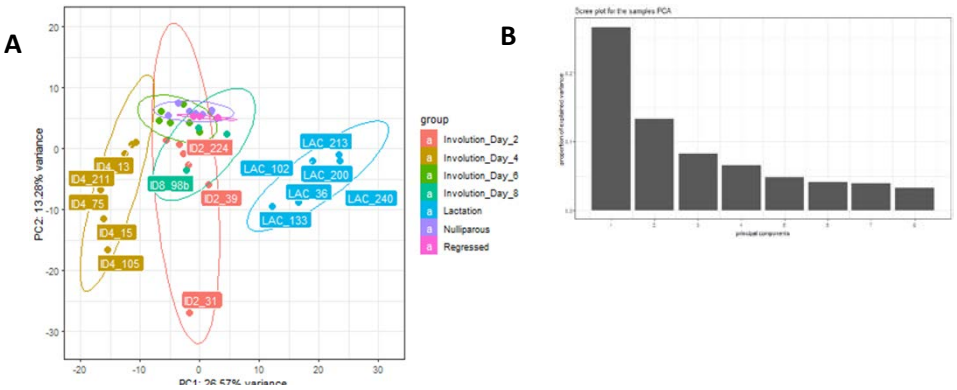

Fig 4. A) Principal component analysis done on batch corrected data yielded samples that clustered by their reproductive group. **B)** Principal component 1 is the main driver of difference, with each successive component having less power on clustering.

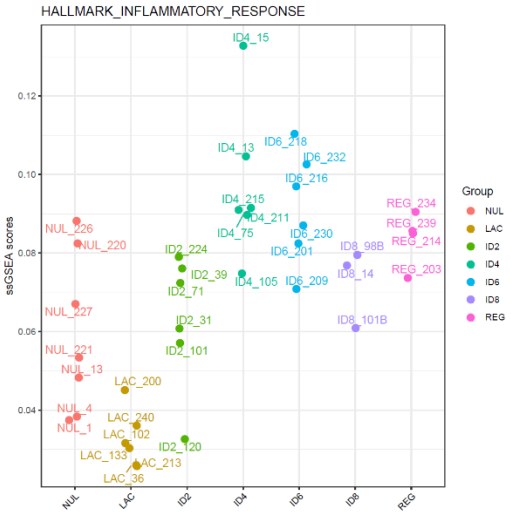

Fig 5. ssGSEA analysis shows enrichment of inflammatory response pathway genes during involution days 4 and 6.

distinct from lactation and nulliparous livers. The largest differences between 2 reproductive stages was observed between involution day 4 and lactation samples.

We hypothesized expression profiling will reveal discrete waves of immune cell influx and polarization during liver involution, consistent with the three phases of wound healing (inflammatory, proliferative, maturation phases). We used single sample Gene Set Enrichment Analysis (ssGSEA) to identify immune pathways that were enriched or downregulated during reproductive cycles. Our initial analysis utilizes the Broad Institute's hallmark pathway gene signatures. Our analyses revealed an enrichment in the hallmark inflammatory response pathways at involution days 4 and 6 (**Fig 5**). Other hallmark pathway analysis revealed an enrichment for interferon gamma, interferon alpha, and an increase in complement signatures with a similar pattern, being enriched at involution days 4 and 6 (data not shown). These initial analyses indicate differential immune response during liver remodeling, supporting our initial hypothesis.

We followed up on these analyses by using ssGSEA to input other curated pathway signatures, in order to investigate specific immune cell signatures. We did not detect a change in the CD8 signatures across a reproductive cycle (**Fig 6A**). We did observe a rather dramatic decrease in the T-regulatory cell signature during lactation, with a slight enrichment at involution D4 (**Fig 6B**). Changes in myeloid signatures across a cycle were more evident, with loss of an M1 signal during lactation (**Fig 6C**), and an enrichment in M2 signatures during involution (**Fig 6D**). The increase in T-regulatory and M2 macrophage signatures are often correlated with an immune suppressive phenotype.

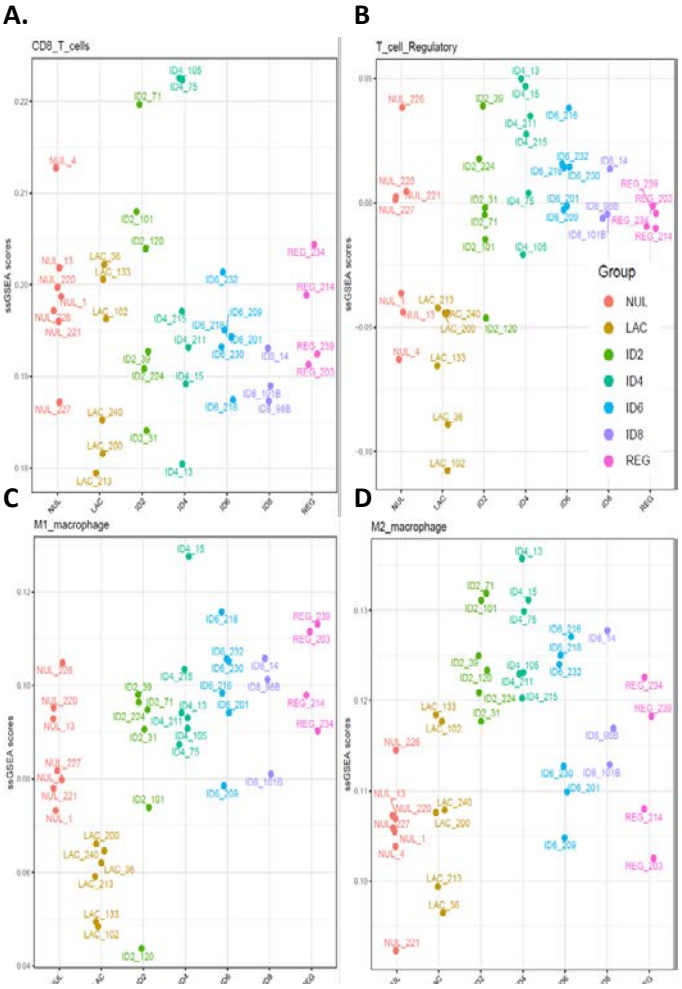

Fig 6. ssGSEA analysis of mouse livers isolated at different reproductive time points. Immune cell gene signature pathways, derived from public data bases, were analyzed for CD8 T cells **(A)**, T-reg cells **(B)**, M1 macrophages **(C)**, and M2 macrophages **(D)** were analyzed.

Aim 1B. Multiplex IHC of non-tumor bearing mouse livers across postpartum developmental spectrum, n = 6 for each time point, Nulliparous, Lactation, Days 2, 4, 6, 8 and 4wks post weaning.

To follow up on these biochemical findings from Aim 1A, we will employ multiplex immunohistochemistry (mIHC) to guide us towards identify specific immune cell populations, their tissue context, and how these populations change due to reproductive state. To this end, we have generated a tissue microarray (TMA) of liver samples from mice throughout a full reproductive cycle (n=6 per group) that we are using to optimize our mIHC antibody panels. We will use this TMA as in internal control on each mIHC study slide. Two mIHC panels are in the works: a myeloid derived suppressor cell (MDSC) panel, and a lymphoid panel. The lymphoid panel has been successfully worked up, and tissues are ready for staining (**Table 1**), the myeloid panel is ~50% developed. Similar to flow cytometry, we can use mIHC to analyze different cell populations by combining several immune markers, and will use the following gating scheme similar to the one displayed in **Fig 7**, which depicts the gating scheme used to identify different functional markers for CD4 T-cells. A similar gating scheme will be used to identify CD8 T-cells.

Table 1. Lymphoid panel to delineate activation state of CD4 and CD8 T cells.

Lineage	Identification
Epithelium	$CD45^-CK18^+$
$CD4^+$ T lymphocytes	$CD45^+CD3^+CD4^+CD8^-$
Regulatory T cells (Treg)	$CD45^+CD3^+CD8^-Foxp3^+$
$CD8^+$ T lymphocytes	$CD45^+CD3^+CD8^+$
Myeloid cells	$CD45^+CD3^-CD11b^+$
Functional cell state	**Marker**
'Exhaustion'	Tox, Tim3
Checkpoint	PD-1, TIM3
Proliferation	Ki67
Cytotoxicity	Granzyme B^+
Death	CC3

Aim 2 Objective: Identify immune milieu of PPBC and non-PPBC liver metastases in rodents and humans

Aim 2 Major Activities Overview:

- We have obtained demographic and clinical data for 30 breast cancer patients who had metastases to the liver. Of this patient cohort, 18 patients are considered non-PPBC and 12 were identified as PPBC. Staying consistent with our previous human studies, we are defining PPBC as patients who were diagnosed with breast cancer within 10 years after their last pregnancy.
- We have evaluated the "differentiation and ki67 status of each of these metastatic lesions.
- Used our previously published ECM liver proteomics database to interrogate ECM proteins associated with PPBC and no-PPBC MLBC (via mIHC), and association with differentiation and Ki67 status.

Table 2. Demographics of patients with liver metastases and primary breast cancer.

	N (%)
Age at diagnosis	
<45	14 (50.0%)
45-60	12 (42.9%)
>60	2 (7.1)
PPBC	
Yes	10 (35.7%)
No	18 (64.3%)
Race	
White	26 (92.9%)
Black	0 (0%)
Asian	1 (3.6%)
Hispanic	1 (3.6%)
Estrogen	
Positive	22 (78.6%)
PR+	17 (77.3%)
PR-	5 (22.7%)
Negative	5 (17.9%)
Unknown	1 (3.6%)

Aim 2: Significant Results and Key Conclusions

Aim 2A. Perform RNA-Seq on FFPE from PPBC and non-PPBC breast cancer liver metastases human samples (n =16). We have obtained samples liver metastases from breast cancer patients, both postpartum and non-postpartum. We currently have 28 cases, 18 non-postpartum cases, and 10 postpartum (**Table 2**). Her2 data are currently being collected. We will perform RNAseq on FFPE tissue samples from these samples. We are currently in the process of updating our IRB to get approval for genetic sequencing. Once this is completed, we will proceed with isolating RNA from the samples currently in our lab.

Aim 2B. Multiplex IHC of Human Breast Cancer Liver Metastasis from 16 Non-postpartum and 9 Postpartum and 5 antibody control FFPE specimens. We have begun initial staining of the samples mentioned in Aim 2A. Upon initial analysis of the samples by H&E staining, we identified varying states of differentiation (**Fig 8**).

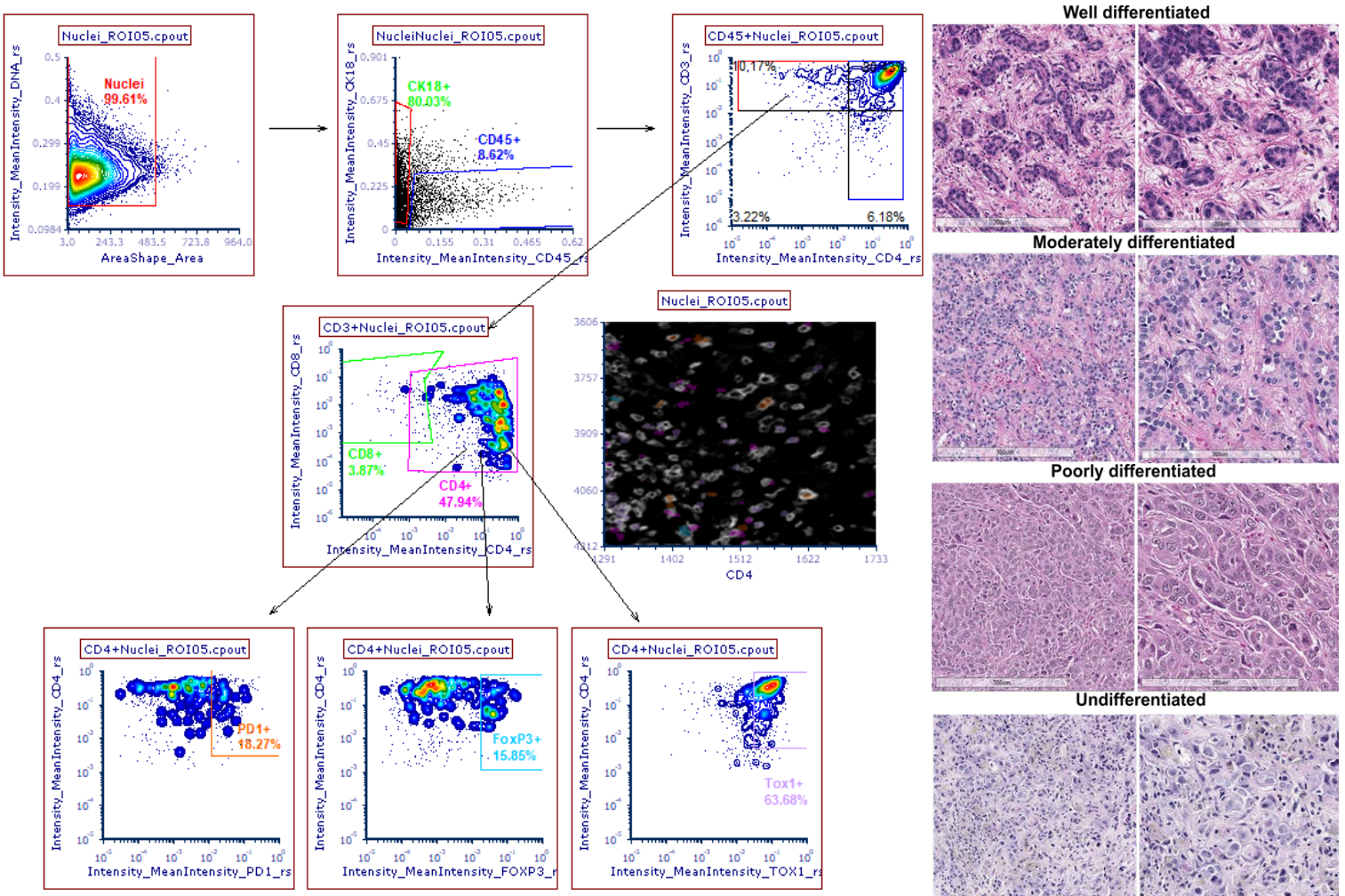

Fig 7. Gating scheme to look at activation state of lymphoid cells by mIHC with an example image. Positivity of markers is confirmed using intensity readout and corresponding images. The image shown shows CD4+ cells displayed in pink, and activation state markers, PD1 (orange), FoxP3 (blue), and Tox1 (lavender) are shown overlaid on CD4+ cells.

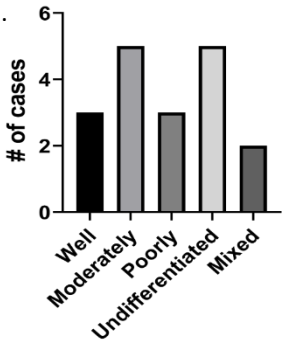

Fig 8. Differentiation states of breast cancer metastatic lesions in liver. Quantitation for the first 18 cases analyzed.

Interestingly, these metastatic lesions look very similar to breast cancer at the primary tissue, with many lesions being highly differentiated (as measured by presence of mammary duct-like structures). We next developed and mIHC panel to stain for tumor cell markers and host liver cells, which we refer to as the Discovery panel (**Table 3**). We developed this panel to better delineate tumor cells from liver parenchyma, and to interrogate associations between tumor differentiation state, and tumor growth patterns in the liver (i.e., pushing, replacement) as well as tumor cell proliferation (Ki67). Staining for this panel has been completed, and analyses are currently underway. Initial results show that these MLBC lesions are dominantly cytokeratin 7 and 18 positive and CK5 negative, consistent with luminal breast cancer (**Fig 9**). These MLBC are also dominantly ER+, suggesting the liver as a preferred site of metastasis for ER+ cancers. This initial mIHC analysis also revealed a close association between mammary tumor cells and the ECM protein collagen IV. Thus, a second panel was developed to look at the ECM milieu with higher definition. This ECM panel is shown in **Table 4**. Staining for ECM proteins is of further interest because there is a strong relationship between immune cells and stromal composition[1]. Staining for the ECM panel has been completed, and analyses are in progress. Initial analysis has been on determining the percent positive stained area for each antibody within the tumor region. We next employed the use of TWOMBLI, a Fiji macro program designed to analyze ECM patterns, to further evaluate for associations between ECM and MLBC tumor characteristics. Using this program we found interesting correlations between ECM density and Ki67 staining for Tenascin-C (TNC). More poorly differentiated tumors, and tumors with high Ki67 percentages, correlated with increased TNC branch points (**Figure 10**).

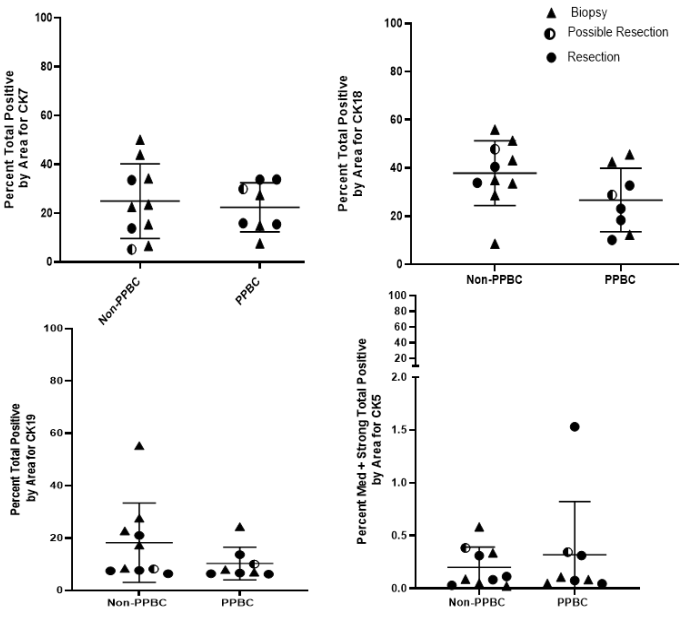

Fig 9. The first 18 cases we analyzed showed MLBC lesions were stained for CK7, CK18, CK19, and CK5 by

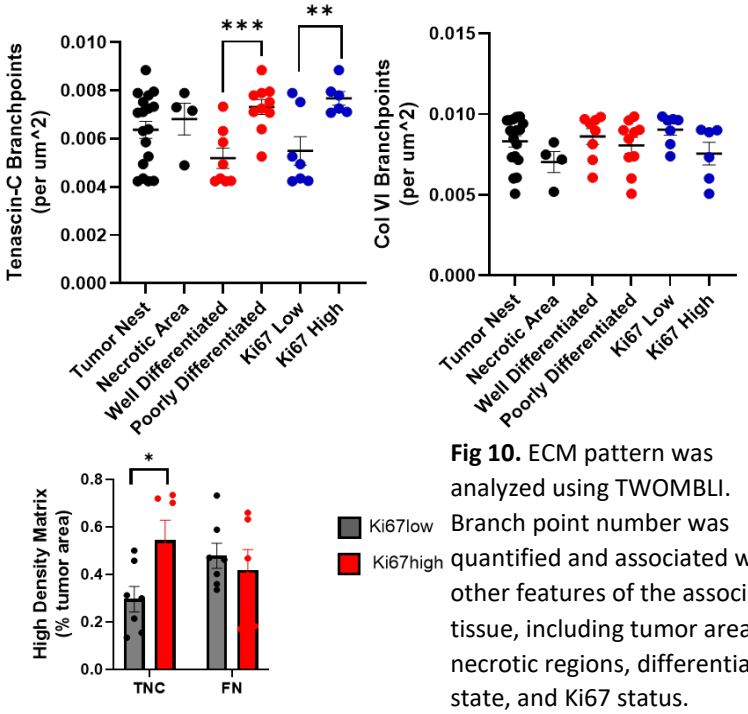

Fig 10. ECM pattern was analyzed using TWOMBLI. Branch point number was quantified and associated with other features of the associated tissue, including tumor area, necrotic regions, differentiation state, and Ki67 status.

Further analyses of this data will include correlating immune cell abundance with density and pattern of ECM within the tumor, as well as assessing each ECM biomarker by parity status.

To directly address how immune cell populations change in breast cancer patients by parity status, we will next stain these tissues with our worked up lymphoid panel (**Table 1**). Concurrently, we will begin staining with our myeloid derived suppressor cell (MDSC) panel, which is nearly optimized (**Table 5**).

Table 3. The Discovery Panel is composed of a variety of markers to look at native liver cell types, as well as markers for breast cancer cells.

Marker	Purpose
CK18	Luminal-origin breast cells, and hepatocytes
Gata3	Luminal-origin breast cells
Aquaporin 1	Bile duct cells, endothelial cells
ER	Estrogen receptor status in liver metastases
CK7	Luminal-origin breast cells, bile duct cells
CK5	Basal-orgin breast cells
Ki67	Assess cell proliferation
Col IV	Basement membrane
Heppar	Hepatocytes
E-cad	Junctional complex associated with polarized cells
aSMA	Fibroblast marker

Table 4. The ECM panel is composed of a variety of ECM and ECM-associated proteins.

Marker	Purpose
CD45	Immune cells
TNC	Glycoprotein expressed in ECM, associated with inflammation, infection, tumorigenesis
FN1 (insoluble)	Glycoprotein of ECM that binds integrins, collagen, fibrin. Produced by fibroblasts.
Col I	Fibrillar ECM protein
Col VI	Interacts with perlecan, stabilizes ECM of skeletal muscle, inhibits apoptosis.
TGM2	Binds to FN
CK18	Luminal-origin breast cells, and hepatocytes
Gata3	Luminal-origin breast cells
Pepsin	Cleaves collagen fibrils to assist with remodeling
Col IV	Sheet forming collagen, associated with fibrosis

Aim 2C. Multiplex IHC on Murine Postpartum and Nulliparous Liver metastases (6 postpartum, 6 nulliparous). We have developed a lymphoid panel (**Table 1**) for mIHC on tissues collected from our mouse liver metastatic model. Initial studies showed that abundance of CD8 cells did not change when measured by parity status (**Fig 11A**), so we sought to investigate the functionality of these CD8 cells. We used the panel in **Table 1** to look at the types of T cells and their activation state using mIHC analysis. Hierarchical clustering analysis showed samples grouped by parity status (**Fig 11B**). We found evidence for immune activation, as indicated by Ki67, PD1, or Tox1, in two out of seven involution mouse tumors. We also found involution tumors have a lower CD4: CD8 ratio compared to nulliparous mice, and that involution tumors have increased F480+ macrophages. These murine PPBC findings were recently published (Bartlett, et al. Cancers, 2021), and will further inform our immune biomarker selection for our human studies. .

Table 5. MDSC panel was developed to investigate the different myeloid immune cell populations that may be changing by parity status.

Cell	Identification
T cells	CD45+CD3+
B cells	CD45+CD3-CD20+
NK	CD45+CD3-CD20-CD56+
Neutrophil	CD45+CD3-CD20-CD56-CD66b+S100A-
DC	CD45+CD3-CD20-CD56-CD66b+S100A-CD11c+
Macrophage	CD45+CD3-CD20-CD56-S100A+CD68+
PMN-MDSC	CD45+CD3-CD56-HLA-S100A+CD66b-CD11b+CD33+CD68-CD14-CD15+
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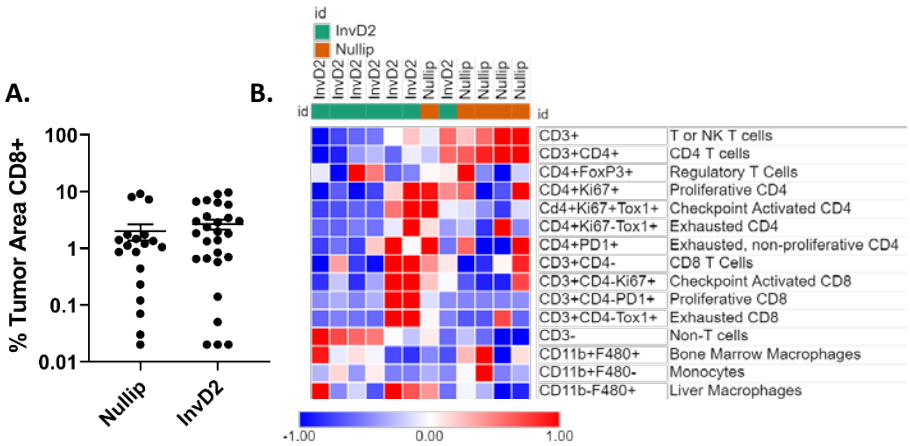

Fig 11. A) CD8 cell number did not appear to be different based on parity status, as assessed by IHC. **B)** Hierarchical clustering of mIHC analysis of T-cell phenotype and activation state showed grouping by parity status.

The murine data also implicate the importance of myeloid derived cells. We intend to develop a murine myeloid panel, similar to the human panel we currently have (**Table 5**). The development of this panel is currently underway.

Aim 3 Objective: Identify efficacy and specificity of immunotherapy for PPBC liver metastasis

Major Activities Overview:

- Completed the first liver metastatic study in C57BL6 mice.
- Currently working up two C57BL6 mouse breast cancer cell lines in our postpartum breast cancer model.
- Trained new lab personnel to complete the portal vein injection surgeries
- Development of CD4 and CD8 flow immune cell panels

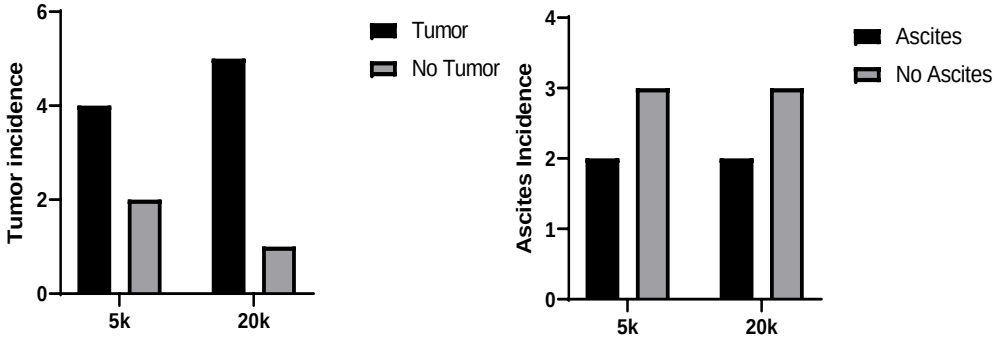

Fig 12. A) EO771 tumor incidence as assessed by gross analysis. **B)** Ascites incidence of EO771 cells.

Aim 3: Significant Results and Key Conclusions

Our lab has primarily used BALB/C mice in our postpartum breast cancer studies. BALB/C mice have a Th2 skewed environment,

whereas C57/B6 have a Th1 skew. As we investigate the importance of immune cell changes in postpartum breast cancer metastasis, we feel it is important to also investigate our model in C57/B6 mice. We have conducted our first study in these mouse strains using two C57/B6 breast cancer lines, EO771 and PY230, to optimize cell concentration and study endpoints. In our EO771 pilot study, we injected two different cell concentrations, 5k and 20k, into postpartum mice. While this work and analysis is still ongoing, gross analysis at time of sacrifice revealed tumor incidence does not vary greatly based on cell number (**Fig 12A**). We have also found an interesting phenotype in our EO771 cells, identifying that about 40% of the mice develop ascites (**Fig 12B**). This is a phenotype we have not seen in our other breast cancer cell models. This work is ongoing, and current analysis includes quantification of tumor burden by histology. The results from these pilot studies will be used to inform aims listed in our SOW.

Table 6. CD4 (left) and CD8 (right) panels developed for flow cytometry.

Marker	Identification
CD45	General immune cells
CD3	T-cells
CD4	CD4 helper T-cells
PD1	Activated or exhausted T-cells
FoxP3	T-regulatory
RORγT	Th17
Tbet	Th1
Gata3	Th2
Live/Dead	-

Marker	Identification
CD45	General immune cells
CD3	T-cells
CD8	Cytotoxic T-cells
PD1	Activated or exhausted T-cells
CTLA4	Activation
CD49b	NK cells
CD11b	Leukocyte and NK maturation
Live/Dead	-

This work led to the training of the lab's new graduate student, Michelle Ozaki, as the primary surgeon for the portal vein surgeries, and the lab's new research assistant, AeSoon Bensen, as the assistant surgeon. Ms. Ozaki and Ms. Bensen will complete the animal surgeries necessary for the remainder of this work.
In addition to these animal studies, we have developed two flow cytometry panels to identify different CD4 and CD8 activation states and phenotypes (**Table 6**). These panels will be used to identify how immune cells respond to immunotherapies in future studies.

What opportunities for training and professional development has the project provided?

Alex Quackenbush: Dr. Quackenbush is a recently graduated PhD candidate who has contributed a significant body of work under this award. Many of the projects she worked on led to substantial training and professional development opportunities

- Developed bench skills in multiplex IHC, flow cytometry, animal handling, tissue staining, and analysis
- Learned about and implemented IRB and DoD regulatory requirements, including document drafting and revision
- Honed data presentation skills across monthly presentations in lab meeting
- Developed professional network and awareness of the field through participation in conferences including:
    - Society for Immunotherapy of Cancer Annual Meeting. November 11-14, 2020.
    - San Antonio Breast Cancer Symposium. December 8-12, 2020.
    - AACR Virtual Special Conference: The Evolving Tumor Microenvironment in Cancer Progression: Mechanisms and Emerging Therapeutic Opportunities. January 11-12, 2021.
    - American Society of Matrix Biology Biennial Meeting. September 15, 2021.
- Much of the first year of work on this grant was included in her dissertation, ""The liver is a dynamic organ shaped by reproduction with implications for young women's breast cancer". Cancer Biology Dissertation Defense, May 27, 2021
- Gained mentoring experience as a preceptor for undergraduate summer research interns
- Developed written communication ability through process of writing two first-author manuscripts

Michelle Ozaki: Ms. Ozaki is a PhD candidate who has been working on the aims of this award. This award has allowed tremendous training opportunities as she has transitions into the lab.

- Developed bench skills in multiplex IHC, flow cytometry, animal handling, tissue staining, and analysis

- Trained in the unique portal vein surgery to model liver metastasis, and expanded the Schedin lab's expertise to include two new murine mammary tumor cell lines required for the completion of this grant.
- Honed data presentation skills across monthly presentations in lab meeting
- Developed written communication skills through the preparation of monthly plans and study summaries, completion of her qualifying exam, and preparation of three grants including the NSG GRFP, an internal departmental grant, and the NDSEG
- Honed presentation skills through completion of the oral portion of her qualifying exam, monthly presentations at lab meetings, and presentations at joint department lab meetings
- Expanded knowledge of the field by attending the American Society for Matrix Biology conference

How were the results disseminated to communities of interest?

Pepper Schedin:

National/International Speaking Invitations:

- Young Women's Breast Cancer: Bench to Population Science and Back Again. Genome Science Institute, Graduate Program in Genetics and Genomics, Boston University, Boston MA, September 23, 2000
- Proximity to Pregnancy Determines Outcomes in Young Women's Breast Cancer. McGill University, Montreal Quebec, February 8, 2021
- Proximity to Pregnancy Determines Outcomes in Young Women's Breast Cancer. ICR Institute of Cancer Research, London, UK April 28, 2021

Local Speaking Invitations:

- Breast cancer extracellular matrix in the human liver. SMMART Stroma in Cancer Mini-Symposium, Knight Cancer Institute, April 6, 2021
- COX2 biology in breast cancer, Faculty Forum Lunch, CDCB, May 26, 2021
- Risk factors for postpartum breast cancer: developing a model for early detection. CRUK/OHSU ACED Symposium Presentation, June 4, 2021

Alex Quackenbush:

- Poster presentation: Natural killer cells restrict the growth of liver metastases in nude hosts. Society for Immunotherapy of Cancer Annual Meeting. November 11-14, 2020.
- Poster presentation: Evidence for reproductive control of liver size with implications for risk of liver metastases in postpartum breast cancer patients. San Antonio Breast Cancer Symposium. December 8-12, 2020.
- Lightning talk and poster presentation: Immune suppression established by postpartum liver involution promotes liver metastasis. AACR Virtual Special Conference: The Evolving Tumor Microenvironment in Cancer Progression: Mechanisms and Emerging Therapeutic Opportunities. January 11-12, 2021.
- Invited talk: "Multiplex IHC for Extracellular matrix proteins". American Society of Matrix Biology Biennial Meeting. September 15, 2021.
- Dissertation: "The liver is a dynamic organ shaped by reproduction with implications for young women's breast cancer". Cancer Biology Dissertation Defense, May 27, 2021
- Manuscripts:
    - Bartlett, A.Q.; Pennock, N.D.; Klug, A.; Schedin, P. Immune Milieu Established by Postpartum Liver Involution Promotes Breast Cancer Liver Metastasis. Cancers 2021, 13, 1698. https://doi.org/10.3390/cancers13071698
    - Alexandra Q Bartlett, Kimberly K Vesco, Jonathan Q Purnell, Melanie Francisco, Erica Goddard, Andrea DeBarber, Michael C Leo, Eric Baetscher, William Rooney, Willscott Naugler, Alex Guimaraes, Patrick Catalano, and Pepper Schedin. "Pregnancy and weaning regulate human maternal liver size and function". Revision under review at PNAS.

What do you plan to do during the next reporting period to accomplish the goals?

- Next steps for Aims (additional detail above in yearly report)
    - Aim 1 RNAseq data-perform hallmarks of cancers and transcription factor regulon analyses
    - Aim 1 mIHC-advance to myeloid and lymphoid panels in normal liver

- Aim 1 regulatory work-submit IRB modification
- Aim 2 mIHC-advance to myeloid and lymphoid panels in PPBC tumors
- Aim3-rodent studies-begin therapeutic interventions

3. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

- Our studies are paradigm changing for the metastasis field, which is founded on metastasis being an intrinsic attribute of tumor cells rather than dependet on reproductive biology of the host. Our data shows how a common physiological process, weaning-induced liver involution, remodels the liver microenvironment and makes it susceptible to metastasis.

- Our studies have advanced the early onset breast cancer community be delineating between pregnancy associated breast cancer, a rare and not poor-prognostic cancer, and postpartum breast cancer, a common and deadly cancer. This last year, a group of world thought leaders came together to write an opinion piece updating the filed on PPBC: Amant F, Lefrère H, Borges VF, Cardonick E, Lambertini M, Loibl S, Peccatori F, Partridge A, Schedin P. The definition of pregnancy-associated breast cancer is outdated and should no longer be used. Lancet Oncol. 2021 Jun;22(6):753-754. doi: 10.1016/S1470-2045(21)00183-2, PMID:34087122

What was the impact on other disciplines?

- Our studies on breast cancer metastasis to the liver have revealed an entire new biology, weaning induced liver involution, which may have implications for both maternal and infant health. This work has led to a new concept paper lining lactating insufficiency to liver biology: Betts, CB, Quackenbush, A, Anderson, W, Marshall, N, Schedin, P. Mucosal immunity and liver metabolism in the complex condition of lactation insufficiency. Journal of Human Lactation, Aug 14, 2020. PMID: 32795211.

- Further, we have the first-ever data suggesting that liver growth during pregnancy and liver volume loss postpartum are associated with a normal, healthy pregnancy, whereas lack of this growth patter associated with poorer maternal health including hypertension (Alexandra Q Bartlett, Kimberly K Vesco, Jonathan Q Purnell, Melanie Francisco, Erica Goddard, Andrea DeBarber, Michael C Leo, Eric Baetscher, William Rooney, Willscott Naugler, Alex Guimaraes, Patrick Catalano, and Pepper Schedin. “Pregnancy and weaning regulate human maternal liver size and function”. Revision under review at PNAS).

What was the impact on technology transfer?

Nothing to report

What was impact on society beyond science and technology?

- Improving medical providers knowledge of young women’s breast cancer (Lancer Oncology 2021 opinion piece)
- Improving public knowledge- the following is from an email ft Pepper Schedin, received in August of 2021, from a breast cancer advocate: *“Don’t know if you’ve heard, but I’ve just retired from the KCI Scientific Research Advocate group. I will especially miss the opportunity to continue to work with you. I remember when the MBCA Landscape Report came out. I looked for the names of any OHSU researchers – and there was your name. Shortly after that I heard your lecture – in the old Hatfield conference room. Your work wasn’t like anything I’d heard before. Over the years I’ve learned more about your exciting research. It’s not that you think outside of the box. It’s more like you’re saying, “What’s a box?” You’ve investigated an area full of myths and shown how the breast and other organs adapt to pregnancy and then return to normal. I know you inspire others. It’s been a pleasure to know you. Thank you for the work you do.”*

4. Challenges/Problems

Changes in approach and reasons for change

OHSU and the State of Oregon had some of the country's most restrictive COVID containment policies, resulting in no/or only partial access to the labs from March2020 through May 2021. Even now, as PI of the lab, I am considered non-essential and asked to work 100% remotely. After one year of working remotely, 50% of my lab staff elected to take positions elsewhere. I have returned to working 80% in the lab to better mentor the remaining lab staff and trainees. Thus, this first year of DoD BC191620 funding has been a difficult year to stay on track. None-the-less, I feel the lab members have pivoted well, and in-the-end have been highly productive given the circumstances. While we have not made changes to our original grant aims, we have changed how we have prioritized these aims. Specifically, we have performed fewer animal studies than expected and performed more multiplex IHC and RNA seq analyses. We expect to be able to refocus on animal experiments in year 2 of funding.

Actual or anticipated problems or delays and actions or plans to resolve them

See above.

Changes that had a significant impact on expenditures

We have had personnel loss this last year, and are in the process of actively recruiting new lab members.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

No changes/nothing to report

5. Products

Publications, conference papers, and presentations

Journal publications:

- Betts, CB, Quackenbush, A, Anderson, W, Marshall, N, Schedin, P. Mucosal immunity and liver metabolism in the complex condition of lactation insufficiency. Journal of Human Lactation, Aug 14, 2020. PMID: 32795211.
- Bartlett, A.Q.; Pennock, N.D.; Klug, A.; Schedin, P. Immune Milieu Established by Postpartum Liver Involution Promotes Breast Cancer Liver Metastasis. Cancers 2021, 13, 1698. https://doi.org/10.3390/cancers13071698
- Alexandra Q Bartlett, Kimberly K Vesco, Jonathan Q Purnell, Melanie Francisco, Erica Goddard, Andrea DeBarber, Michael C Leo, Eric Baetscher, William Rooney, Willscott Naugler, Alex Guimaraes, Patrick Catalano, and Pepper Schedin. Pregnancy and weaning regulate human maternal liver size and function. Revision under review at PNAS.

Books or other non-periodical, one-time publications: Nothing to report

Other publications, conference papers, and presentations:

Conference presentations:

- Young Women's Breast Cancer: Bench to Population Science and Back Again. Genome Science Institute, Graduate Program in Genetics and Genomics, Boston University, Boston MA, September 23, 2000
- Proximity to Pregnancy Determines Outcomes in Young Women's Breast Cancer. McGill University, Montreal Quebec, February 8, 2021
- Proximity to Pregnancy Determines Outcomes in Young Women's Breast Cancer. ICR Institute of Cancer Research, London, UK April 28, 2021
- Invited talk: "Multiplex IHC for Extracellular matrix proteins". American Society of Matrix Biology Biennial Meeting. September 15, 2021, presented by graduate student Alex Quackenbush-Bartlett.

Website(s) or other internet site(s)

Nothing to Report

Technologies or techniques
Nothing to report

Inventions, patent applications, and or/licenses
Nothing to report

Other products
Nothing to report

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Pepper Schedin*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0003-4244-987X
Nearest person month worked:	*1.20*
Contribution to Project:	*Dr. Schedin led all aspects of the project, including scientific focus, experimental design, data analysis, data integrity, budget management, human and animal regulatory aspects, and manuscript writing.*
Funding Support:	

Name:	*Weston Anderson*
Project Role:	*Scientific Writer*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.49*
Contribution to Project:	*Mx. Anderson worked on all manuscript writing associated with this project.*
Funding Support:	

Name:	*AeSoon Bensen*
Project Role:	*Animal Husbandry*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.50*
Contribution to Project:	*Ms. Bensen worked with Ms. Michelle to complete the animal surgeries and tissue collection done for Aim 3.*

Funding Support:	

Name:	*Andrea Calhoun*
Project Role:	*Histotechnician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4.65*
Contribution to Project:	*Ms. Calhoun completed TMA development for Aim 1B, completed panel development, sectioning, staining, and analysis of Aim 2.*
Funding Support:	

Name:	*Grace Kim*
Project Role:	*Computational analysis*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4.82*
Contribution to Project:	*Ms. Kim completed much of the RNAseq analysis for the batch correction and principal component analysis.*
Funding Support:	

Name:	*Michelle Ozaki*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	0000-0002-3356-9071
Nearest person month worked:	*12.00*
Contribution to Project:	*Ms. Ozaki carried out analysis and manuscript preparation for Aim 1, performed analysis for Aim 2, coordinated the animal studies, collected tissues, and worked on data analysis for Aim 3.*
Funding Support:	NSF GRFP

Has there been a change in the active other support of the PD/PI(S) or senior key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

7. SPECIAL REPORTING REQUIREMENTS

Nothing to report

8. APPENDICES

N/A