AWARD NUMBER: W81XWH-15-1-0547

TITLE: Genetic and Functional Analysis of the Role of Clonal Tumor-infiltrating Leukocytes in Breast Cancer Pathogenesis and Therapy

PRINCIPAL INVESTIGATOR: Jorge S. Reis-Filho

CONTRACTING ORGANIZATION: Sloan Kettering Institute for Cancer Research
New York NY 10065-6007

REPORT DATE: December 2020

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick,

Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE
December 2020

2. REPORT TYPE
Final

3. DATES COVERED
30 Sep 2015 - 29 Sep 2020

4. TITLE AND SUBTITLE

Genetic and Functional Analysis of the Role of Clonal Tumor-infiltrating Leukocytes in Breast Cancer Pathogenesis and Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH- 15-1-0547

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S

Jorge S. Reis-Filho

E-Mail: reisfilj@mskcc.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Sloan Kettering Institute for Cancer
Research 1275 York Avenue
New York NY 10065-6007

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT: Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

We hypothesize that some leukocytes infiltrating breast cancers possess oncogenic mutations which affect breast cancer growth and metastasis. We have investigated this hypothesis in 3 different patient groups: 1) patients with newly diagnosed breast cancer and high amounts of tumor infiltrating leukocytes, 2) patients who developed secondary leukemias after treatment for breast cancer, and 3) breast cancer patients with clonal hematopoiesis (CH) incidentally found on genomic screens but with no apparent hematologic disorders. We have over 6,500 breast cancer patients who have undergone genomic testing of their tumors and peripheral lymphocytes. Of these patients, ~25% have CH. We have clinical information from these patients and have sought to define whether key clinical features influence CH. For those CH patients who have primary tumor tissue available, we have also sequenced the TILs in the primary breast cancer to evaluate for mutations associated with CH. 4) We have also prospectively collected blood samples from patients pre/post neoadjuvant therapy and pre/post surgery to assess how chemotherapy and primary tumor presence may affect CH. From a functional standpoint, we have made progress in evaluating models to assess how mutant hematopoietic cells impact tumor growth. Specifically, we evaluated two genes, Dnmt3A and Tet2, in select models and have expanded our studies to assess the functional role of Tet2 in a second transgenic model. Additionally, we have set up our assays to investigate first, whether select leukocytes produce inflammatory cytokines which impact breast cancer progression, second, whether Tet2 expression defines response to chemotherapy, and third, whether hypomethylating agents alone or in combination with chemotherapy lead to increased therapeutic efficacy.

15. SUBJECT TERMS
Characterization of the genomic landscape of tumor infiltrating hematopoietic cells in breast cancer. Evaluation of the functional interaction between mutant leukocytes and breast cancer cells.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	24	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents **Page**

1.	**Introduction**	**4**
2.	**Keywords**	**4**
3.	**Accomplishments**	**5**
4.	**Impact**	**19**
5.	**Changes/Problems**	**21**
6.	**Products**	**22**
7.	**Participants & Other Collaborating Organizations**	**22**
8.	**Special Reporting Requirements**	**25**
9.	**Appendices**	**25**

INTRODUCTION:

This proposal was designed to identify a unique way in which the tumor microenvironment drives breast cancer growth and to determine novel ways to treat breast cancer based on these insights. Specifically, this proposal sought to lay the foundation for understanding whether the acquisition of somatic mutations in cancer genes, in particular those related to leukemogenesis, within tumor-infiltrating leukocytes contributes to breast tumor initiation and/or progression. This study was based on our previous work which identified leukemia associated mutations within tumor infiltrating leukocytes in primary breast cancers. The mutations found in infiltrating leukocytes were not identified in the peripheral blood or epithelial cells of the same breast cancer patients. Our hypothesis was that breast cancers intimately interact with mutated leukocytes and that this interaction may contribute to breast cancer metastasis. To test this hypothesis clinically, we investigated mutated leukocytes in 3 different patient groups: 1) patients with newly diagnosed breast cancer and high amounts of tumor infiltrating leukocytes, 2) patients who developed secondary leukemias after treatment for breast cancer, and 3) breast cancer patients with clonal hematopoiesis incidentally found on genomic screens but with no apparent hematologic disorders. From a functional standpoint, we developed select models to determine whether mutations within hematopoietic cells can impact tumor growth and metastasis, and utilized these models to interrogate how breast cancer cells and mutant white blood cells interact and, importantly, how this affects therapeutic response. Despite efforts to develop targeted therapies against breast cancer cells and decrease metastatic growth, breast cancer metastasis continues to drive mortality. This work has provided a fundamental step in transforming breast cancer treatment for patients with newly diagnosed breast cancer, including those at risk for and with metastatic disease. Our studies constitute a springboard from which we can develop novel therapeutic approaches, in which dual targeting of cancer cells and of tumor-associated leukocytes improves outcomes for breast cancer patients.

KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Breast cancer, tumor infiltrating, leukocytes, mutations, *TET2*, *DNMT3A*, myeloid leukemia, secondary hematological malignancies, clonal hematopoiesis, inflammation, cytokines

ACCOMPLISHMENTS:

1. What were the major goals of the project?

Specific Aim 1: To evaluate the mutational spectrum of tumor infiltrating leukocytes in 150 samples of primary triple-negative breast cancers. We will follow these patients and in those patients who develop metastasis, we will compare the mutational spectrum of tumor-infiltrating leukocytes in primary tumors to those found in metastatic lesions. We will also assess the mutation spectrum found in tumor-infiltrating leukocytes in 10 de novo metastatic breast cancer patients.

 Major Task 1: Recruitment of 150 triple-negative patients of 2.5 years with newly diagnosed, locally advanced (non-metastatic) breast cancer.

Subtask	Month	Completion
Request for Approval for Use of Human Subjects	1-3	100%
Accrual of 150 triple-negative newly diagnosed patients	4-48	~100%. We expanded our efforts to include not only triple-negative breast cancer patients but also hormone receptor positive and HER2+ breast cancer patients. To date, 150 blood samples and 99 tumor samples were collected from breast cancer patients of all subtypes. We also collected samples from 46 breast cancer patients who received neoadjuvant chemotherapy. We collected blood samples at each time point of chemotherapy as well as their pre-chemo biopsy and any remaining tissue at the time of surgery. We are in final stages of reviewing the sequencing data from the leukocytes in these samples.
Obtain samples from 10 patients with de novo metastatic disease with intact primary breast cancer	4-15	10%. We re-focused our efforts on accruing biospecimens from patients with metastatic disease independently of their treatment status.
Obtain samples from patients who developed metastatic disease within 2.5 years	8-48	0% In our original cohort, only 1 patient developed presumed metastatic disease and she elected against a biopsy and further treatment. As part of a separate cohort, we are tracking patients with clonal hematopoiesis and metastatic disease.

 Major Task 2: Targeted sequencing analysis of tumor infiltrating leukocyte, germline, peripheral blood, and tumor DNA.

Subtask	Month	Completion/Status

Perform targeted sequencing on tumor- infiltrating leukocytes purified from primary and metastatic sites	4-48	DNA samples from tumor infiltrating leukocytes, germline, peripheral blood, and tumor DNA have been submitted for sequencing analysis. We have performed the processing of all samples. Owing to the delays imposed by the pandemic in 2020, we will be performing the final sequencing analysis of these samples and curating the final results in 2021.
Validation of identified mutations in tumor- infiltrating leukocytes (including sequence analysis of peripheral blood and micro-dissected tumor cells)	18-48	In the original cohort of 27 patients, we did not identify somatic variants in sorted TILs from these patients. Whilst variants were present, the quality of the sequencing was insufficient to consider these mutations for verification by secondary analyses. We suspect this was due to limitations in our ability to sort and capture sufficient leukocytes in the samples processed. Moving forward, we have developed superior single cell sequencing analysis capacity such that identifying key mutations will be more rigorous. We have 72 samples that are being sequenced using superior methods, specifically Tapestri by Mission Bio. Owing to the delays imposed by the pandemic in 2020, we will be subjecting these samples to MissionBio processing and single cell sequencing in 2021.
Perform computational analysis of sequencing results and compare variants identified in tumor- infiltrating leukocytes to metastatic, peripheral blood, and tumor sequencing results	6-36	We are actively working with Mission Bio to optimize the dual DNA-sequencing and oligonucleotide-based antibody barcoding for immunophenotyping approach. The availability of MissionBio reagents has been severely affected by the pandemic. We are final stages of optimization and anticipate the completion of this subtask within the next 9 months.

Milestone #1: *Identification of somatic mutations in tumor-infiltrating leukocytes in primary and metastatic disease (4-36).*

Specific Aim 2: To determine whether mutations within hematopoietic cells can impact tumor growth and metastasis.

 Major Task 3: Analysis of the impact of mutated hematopoietic cells on tumor growth and metastasis in mouse models of breast cancer.

Subtask	**Month**	**Completion/Status**
Request Approval for Use of Animals	1-3	100%

Assess the effect of *Tet2* mutated hematopoietic cells on tumor growth and metastasis in the E0771 breast cancer model	4-10	100%
Assess the effect of *Bcor* and *Tet2* mutated hematopoietic cells on tumor growth and metastasis in the transgenic MMTV/*neu* model of breast cancer	10-48	50%. We have completed an evaluation of *Tet2* loss of function in the MMTV-PyMT orthotopic (Py8119) model, however the experiments to evaluate the impact of *Bcor* mutations have not been performed. Although no differences in tumor latency between *Tet2* KO and WT mice have been observed, there are significant changes in the composition of the TME and the functional expression of polarization markers by TAMs we are considering alternative models to determine if Tet2, Dnmt3a, Asxl1 or Trp53 deficiency alters tumor growth and metastatic potential as well as response to therapy, despite showing minimal difference in time to disease onset using MMTV-PyMT breast cancer models. Specifically, we are working with different types of metastasis models including TVI injections and delaying the onset implanting 3D cultured mammospheres from the orthotopic models described above.

Milestone #2: *Determining the functional role of mutations in tumor-infiltrating leukocytes on tumor growth and metastasis using murine breast cancer models (4-48)*

Specific Aim 3: To interrogate functional interactions between breast cancer cells and white blood cells with somatic mutations and its relevance to therapeutic response

 Major Task 4: Elucidate inflammatory cytokine production from tumor-infiltrating leukocytes in murine models of breast cancer and human breast cancer.

Subtask	**Month**	**Completion/Status**
Assess the inflammatory cytokine production profile from specific hematopoietic CD45-positive subpopulations in the E0771 and MMTV/*neu* breast cancer models	10-24	10%. Since the submission of the last report, have assessed the cytokine secretion potential of macrophages isolated from tumor-bearing wt or *Tet2* KO mice and macrophages from mammary fat pads of healthy mice (in collaboration with Dan Landau, Cornell). Instead of using E0771 and MMTV/*neu* breast cancer models we have used PY230 and

		PY8819 orthotopic breast cancer models given that we have already robust and established CH models in the C57BL/6 background.
Interrogation of the therapeutic potential of hypomethylating agents and JAK inhibitors in murine models of breast cancer, alone and in combination with breast cancer therapy	16-48	10% (see pitfalls).
Determine secretomic profiles of tumor- infiltrating leukocytes in human breast cancers	12-48	0%

Milestone #3: *Mapping the deep functional phenotypes in major hematopoietic cell lineages in breast tumors using single cell cytokine profiling in mouse models and primary tumors of breast cancer (10-30)*

□ **Major Task 5:** Present findings in national meetings and publish in peer-reviewed journals.

Subtask	**Month**	**Completion/Status**
Present at national meetings including the American Association for Cancer Research, American Society of Clinical Oncology, and the San Antonia Breast Cancer Symposium.	12-24	Not applicable
Subtask 2: Prepare work for publication in peer- reviewed journals	12-36	Not applicable
Subtask 3: Publish our work in peer-reviewed journals	12-48	As a result of the support from this grant, we published our work on secondary hematologic malignancies in breast cancer patients. Comen EA, Bowman RL, Selenica P, Kleppe M, Farnoud NR, Pareja F, Weigelt B, Hill CE, Alon A, Geyer FC, Akturk G, Reis-Filho JS, Norton L, Levine RL. Evaluating clonal hematopoiesis in tumor infiltrating leukocytes in breast cancer and secondary hematologic malignancies. J Natl Cancer Inst. 2019 Aug 27

Milestone #4: *To present out work in national meetings and to publish our findings in scientific journals in order to present out work to the breast cancer community, to build future collaborations, and to work towards the development of novel therapeutic approaches for breast cancer patients. (12-36).*

2. What was accomplished under these goals?

Specific Aim 1: To evaluate the mutational spectrum of tumor infiltrating leukocytes in 150 primary triple-negative breast cancer cases.

Major Activities:

1. Patient accrual and tumor tissue collection in newly diagnosed breast cancer patients

Specific Objective: To characterize the genomic landscape breast cancer and its tumor immune microenvironment.

Results:
A. Patient identification and peripheral blood specimens collection: To test our hypothesis that select subsets of white blood cells harbor unique mutations, we expanded our screen search to include patients who were HER2+ and ER+. Because many patients with tumors >1cm get neoadjuvant therapy, we collected blood samples from patients undergoing neoadjuvant chemotherapy. Specifically, blood samples from patients pre-, during and post-neoadjuvant therapy. These samples will allow us to evaluate for mutations associated with clonal hematopoiesis (CH) within peripheral blood samples. These results will be compared to the sequencing of TILs within biopsy and residual tumors (at time of surgery). We submitted the blood of 46 breast cancer samples (23 pre/post-neoadjuvant and 23 pre/post surgery)for sequencing. We detected CH in 15 of these patients. As a collaborative effort, other members of our Breast Medicine Service Team have also collected blood samples on neoadjuvant breast cancer patients at comparable time points. We are now pooling our efforts together to increase the power of the analysis and assess CH over time in breast cancer patients exposed to chemotherapy.

Standard screening workflow (breast): Once we identify a potentially eligible patient, H&E slides are then reviewed by our breast pathologist (Dr. Hannah Wen, MSKCC) and scored for the level of tumor-infiltrating leukocytes by a breast pathologist within the Reis-Filho Lab (Dr. Fresia Pareja, MSKCC). Patients eligible for the study are then approached by Dr. Comen to obtain informed consent. In an effort to obtain more samples, Dr. Wen has involved the entire breast pathology service within the Department of Pathology such that if any pathologist reviews a case with increased tumor-infiltrating leukocytes, we are notified and then try in reverse to consent the patient prior to planned surgery. Additionally, many tumors over 1>cm are funneled to neoadjuvant chemotherapy. To be able to include sample analysis on tumors <1cm, we are now employing a more streamlined tumor dissociation protocol as described below and single cell sequencing on a MissionBio Tapestri device. This more sensitive technique has allowed us to collect not only samples from triple-negative breast cancer patients, but also smaller samples from ER+ and HER2+ patients. This approach has resulted in the collection of a total of now 41 tumor samples over the course of the project representative of all clinical subtypes defined by ER and

HER2 status, and irrespective of tumor size. Furthermore, TIL yield has been improved using the new platform that does not require a priori cell enrichment or sorting.

Standard sample collection workflow: For each patient, Dr. Pablo Sanchez Vela obtains fresh tumor specimens, peripheral blood and saliva samples at the time of their primary surgery. Tumor tissue, including stromal cells and tumor-infiltrating leukocytes, are dissociated from the primary tumor using the Miltneyi GentleMACS dissociator utilizing anenzymatic dissociation. After generating a single cell suspension, they are viably frozen. We have optimized the freezing and thawing processing such that viably frozen cells can be batch processed by single cell DNA-seq paired with antibody-oligonucleotide barcoding for subpopulation identification.

Lung and colon: are also collected samples from lung and colon cancer patients as well, including primary tumors, saliva and blood samples. We will perform the same analysis as described above to investigate the presence of mutations in TILs in lung and colon cancer patients. So far, we have successfully collected 31 lung cancer cases and 25 colon cancer cases. Our aim is to get 50 lung cancer cases and 50 colon cancer cancers to have enough cases with CH and their appropriate controls. The rationale for this expansion was the recognition that CH and mutated TILs could be present in a variety of solid tumors, and our aim is to define the generalizability of the findings in breast cancer samples. Our pilot collection with lung and colon cancer has been successful, and tumor size, a barrier experienced in the context of the accrual of breast cancer samples, has proven not to be an impediment in the case of lung and colon cancers. \ In addition due to our interest in studying the evolution of mutant TILs in response to therapy and in advanced disease, we have collected up to 15 metastasis samples (brain and lung) from these primary subtypes.

B. Targeted capture sequencing: To date, we have accrued a total of 150 blood samples from breast cancer patients. The complete set of samples from 46 of these patients have been processed, and we were successful in the purification and extraction of sufficient amounts of DNA for downstream sequencing analysis in 34 out of these 46 patients. In our first batch analysis,15 samples (see below), were identified as harboring CH.

C. Analysis of sequencing data from the blood of breast cancer samples: To date, we submitted DNA from blood, from 34 breast cancer patients for sequencing analysis. To generate mutational profiles, sequencing was performed using a targeted panel covering 156 myeloid genes at an average depth of 600x on Illumina HiSeq 4000 (~100 bp paired-end reads). The raw sequence data was aligned to GRCh37 reference genome using BWA-MEM algorithm (v. 0.7.12-r1039). The data quality was assessed using FastQC (v. 0.11.5). Candidate substitutions and insertions/deletions were identified using our validated and benchmarked pipeline, which also includes post-hoc filters that remove systematic sequencing artifacts as well as artifacts that arise from mapping errors. All candidate mutations were compared to COSMIC, ExAC, and 1000 Genomes and gnomAD databases to provide further annotation that would help to exclude common mutations in normal populations and identify somatic mutations. Each identified variant was manually visualized using Integrated Genomics Viewer (v. 2.3.92) to ensure the high quality of the variant at the sequence level. We compared variants found in peripheral blood before and after treatment interventions (neoadjuvant chemotherapy or surgery).

Pitfalls and Alternative Approaches:

1. Sample Size: We have significantly circumvented the issues with sample collection we had previously faced. By broadening the breast cancer spectrum, including breast cancers of any estrogen receptor and HER2 status, and smaller tumors, we have been able to collect a total of 150 samples blood samples and 99 tumor samples. Our sequencing methods as per above were not sufficiently sensitive in our original group of 27 patients to detect with confidence select mutations. As we have improved our sample collection, we will run our newly acquired samples through the MissionBio platform. We have also established a better integration with the Precision Pathology Biobanking Center and the Department of Pathology. In addition, based on the infrastructure available at MSKCC, we have now appointed a dedicated research study assistant to identify cases for pathology review on a daily basis.

2. Sequencing Analysis: We have strong circumstantial evidence to suggest that the inadequate sequencing quality of the first batch of 27 patients was largely due to limitations in our ability to sort and capture sufficiently sized populations of leukocytes. We have now implemented a superior single cell sequencing analysis capacity such that identifying key mutations will be more rigorous. We have recently optimized a platform that will allow for single cell DNA sequencing across a panel of genomic loci often mutated in CH and breast cancer. The MissionBio Tapestri platform has a greater detection capacity than that of traditional sequencing technologies, and provides a better approach for identifying rare CH clones within a tumor. This single cell sequencing system is fully implemented in both the Reis-Filho and the Levine laboratories; the methods, protocols and standard operating procedures have been fully optimized for both bood samples and dissociated tumor samples. After generating a single-cell suspension, these cells, were subsequently cryopreserved, will be sequenced using the Mission Bio Tapestri platform. We are currently optimizing oligonucleotide barcoding of antibodies for concurrent sequencing-based immunophenotyping paired to single cell DNA mutation detection. By utilizing this single cell technology, we are confident to circumvent what has been the greatest limiting factor in our success to date, isolating quality DNA from limited cell quantities for bulk sequencing. Furthermore, since we are not limited by sorting only 4 to 8 immune populations on a FACS Aria, we can now resolve the cells that harbor the CH mutations with substantially greater precision and resolution. We anticipate processing our samples within the next few months.

2. Sequencing analysis of tumor infiltrating leukocytes from breast cancer patients with incidental clonal hematopoiesis

Specific Objectives: To assess whether clonal hematopoietic cells are enriched in primary breast tumors.

A. Approach and Results: The Levine laboratory and other groups have demonstrated that a subset of older individuals have clinically inapparent, CH characterized by recurrent somatic mutations in genes previously associated with myeloid cancer. Our original plan at the beginning of this proposal lead to the identification of 7 breast cancer patients with incidentally diagnosed CH. The specific mutations associated with CH had already been identified through sequencing analysis of the peripheral blood samples from these patients. Breast cancer tumor blocks for these patients were also retrieved, reviewed and processed. Originally, laser capture microdissection was performed on representative tissue sections to isolate tumor infiltrating leukocytes. The original plan was to then use AmpliSeq sequencing to test for the absence/presence of the mutations

identified in the peripheral blood in the tumor infiltrating leukocytes. We have modified our approach to this project given the tremendous advance in assessing CH at MSKCC. MSKCC has now sequenced over 6,500 breast cancer patients tumors and peripheral blood. Moreover, over ~25% of these patients were found to have CH. We have access to all the clinical information for the aforementioned patients and the clonal hematopoietic clone is already known for each patient. From the patients with incidental CH, the Jorge Reis-Filho and Levine laboratories are now collaborating together to reviewed TILs in select breast cancer patient breast tumor blocks. Owing to the limitations imposed by the pandemic since March 2020, this process has been severely delayed. We are also working to optimize CH assays so that we can evaluate larger numbers of breast cancer patients. Despite the delays, we are confident that we will complete the correlative analyses proposed, and commit to communicating these findings as presentations in national or international meetings and publish the results in peer reviewed journals.

Pitfalls and alternative approaches:
Given that we will retrospectively work with breast cancer tumor blocks, we cannot micro-dissect specific hematopoietic subsets. Hence, the implementation of the Mission BioTapestri device has been proven instrumental to circumvent the pitfalls we experienced earlier in the course of this project. We aim, however, to address the question of whether mutated leukocytes are found within the tumors and, more importantly, if these cells are enriched in the tumor as compared to peripheral blood. Efforts to understand the role of mutated TILs in breast cancer patients align with our murine modelling efforts as described in this report.

3. Sequencing analysis of tumor infiltrating leukocytes from breast cancer patients with secondary hematological malignancies

Specific objectives: To investigate whether mutations found in the hematopoietic cells of patients with secondary hematological malignancies following breast cancer are already present in white blood cells infiltrating primary breast cancers.

Approach and Results: We have recently demonstrated that some breast cancer patients may be at increased risk for secondary leukemias based on the presence of oncogenic mutations in infiltrating white cells, which pre-exist before systemic therapy, and are likely subsequently selected for by cytotoxic chemotherapy. Our retrospective study investigated whether breast cancer patients harbor leukemia associated TILs years before their leukemia diagnosis. We screened for patients with secondary hematological malignancies following breast cancer using the following criteria: >20% blast count (bone marrow or PBMC), access to viably frozen MNC cells or bone marrow aspirate slides (diagnosis) and breast tumor blocks, and suitable for laser-capture microdissection. Using this approach, 12 cases were identified, of which seven samples yielded sufficient tumor infiltrating leukocytes and tumor cells for sequence analysis. First, we sequenced the matching secondary leukemia sample using a targeted sequencing panel of 585 genes commonly mutated in leukemia, lymphoma, and solid tumors and. In parallel, we subjected the DNA samples extracted from laser-capture microdissected and isolated TILs and tumor cells using a targeted amplicon sequencing to define whether the somatic genetic alterations found the leukemia samples would be present in the TILs and tumor cell samples retrieved from the breast cancer specimens from the respective patients. Our analysis led to a recent publication: **Comen EA, Bowman RL**, Selenica P, **Kleppe M**, Farnoud NR, Pareja F, Weigelt B, Hill CE, Alon A,

Geyer FC, Akturk G, **Reis-Filho JS**, Norton L, Levine RL. Evaluating clonal hematopoiesis in tumor infiltrating leukocytes in breast cancer and secondary hematologic malignancies. J Natl Cancer Inst. 2019 Aug 27. Specifically, we demonstrated that in four out of the seven patients (Patients #2, 4, 6 and 7), mutations which were present in the leukemia were also present in the TILs of the breast cancer samples (**Figures A and B**, below). In patient #2, we observed mutations in *TGFBR2* (p.E150K) and *DNMT3A* (p.S638P) in the pre-treatment breast cancer TILs (VAF of 3.10%, 10 mutant /32 total reads and 1.00%, 9 mutant /902 total reads respectively), which were enriched relative to tumor cell samples (VAF of 2.87%, 6 mutant /209 total reads and 0.21%, 1 mutant /477 total reads respectively). In patient #4, the breast cancer TILs were found to harbor *TET2* (p.C1263Y) and *DNMT3A* (p.L639H) mutations, which were clonal in the post-treatment leukemia, at VAFs of 5.87% 1297 mutant /22082 totals reads and 2.88%,12 mutant /417 total reads respectively, whereas these mutations were detected at a much lower proportion (VAFs of 0.73%, 156 mutant /21450 total reads and 0.56%, 5 mutant /894 total reads respectively) in the tumor cell samples. In Patient #6, we identified the known oncogenic *IDH2* R140Q mutation in the TILs, which was clonally present in the therapy-related leukemia. This alteration was detected at a VAF of 1.27% (100 mutant /7862 totals reads) in the TILs versus 0.49% (49 mutant /9962 totals reads) in tumor cell samples. In patient #7, we found that TILs had an increased VAF for the *NRAS* p.G12A mutation, which was present in the post-treatment leukemia. The *NRAS* mutation was present at a MAF of 0.72% in the TILs (2 mutant /277 totals reads), by contrast none of the sequencing reads from microdissected tumor cells harbored the mutation (229 total reads). See Figure 1 A/B below.

Figure 1 A/B: Mutations in secondary hematologic malignancies are present in the tumor infiltrating leukocytes.
A) Mutation heat map showing variants found in the leukemia samples that were included in the targeted sequencing panel for the breast cancer samples. Percentages in the boxes reflect the VAF observed in the respective tumor infiltrating leukocytes L samples. **B)** Scatter plot showing the variant allele fraction (VAF) in the AML sample versus in the tumor infiltrating leukocytes samples. Data points are annotated with the gene name and colored to match patients in panel B.

These analyses provide direct evidence that CH is not limited to peripheral blood leukocytes but rather can be detected in tumor-infiltrating hematopoietic cells. In patients who subsequently develop leukemia, TILs in the primary tumor harbored mutations that were present in the leukemic clone years later. This is consistent with the notion that pre-leukemic clones are present at the time of a diagnosis of a solid tumor, pre-therapy, and precede the subsequent development of leukemia.

Pitfalls and alternative approaches:

1. **Processing of patient tumor specimen and approach to mutation identification:**

One limitation of the study was that blood samples at the time of each patient's breast cancer diagnosis were not available. Therefore, we were not able to sequence blood samples to assess for peripheral CH associated mutations in the peripheral blood at the time of breast cancer diagnosis. We have, however, previously shown that CH mutations were preferentially enriched in breast cancer TILs compared to peripheral blood samples. Furthermore, despite the limited number of patients analyzed in this study, we were able to detect the presence of the mutations present in the leukemic clone in the breast cancer TILs in four of the seven patients analyzed. Despite the use of laser capture microdissection, the presence of a small proportion of TILs in the tumor cell samples cannot be ruled out in breast cancers where leukocytes are admixed with tumor cell clusters.

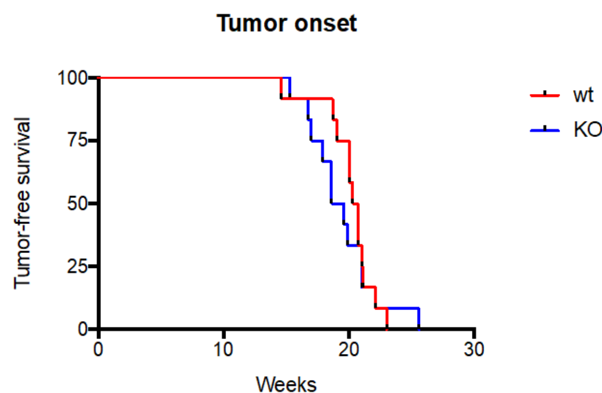
2. Patient accrual

A. To date, it is not known when during the exposure to chemotherapy, CH evolves. This is particularly important to understand in light of those women who may be at risk for secondary hematologic malignancies as a result of receiving chemotherapy. To help elucidate this process, we are collecting blood samples among women undergoing neoadjuvant chemotherapy. Blood samples are taken pre-chemotherapy, during each cycle of chemotherapy and at the completion of chemotherapy. We will analyze these samples for CH and track the potential evolution of CH clones therein.

B. As a result of our work evaluating the TILs in seven breast cancer patients who developed secondary hematologic malignancies, we sought to increase our sample size analyze of patients who developed secondary hematologic malignancies. Since the previous report, we identified 36 epithelial cancer patients who developed t-MN, from whom clinical and genomic profiling data at the time of the leukemia diagnosis are available. In addition to the original untreated solid tumor samples from which we can isolate tumor cells and tumor-admixed hematopoietic cells, we have blood samples from 6 of the 36 patients within seven weeks or less of their solid tumor diagnosis. The availability of these samples has allowed us to investigate whether the clone harboring the genetic alterations present in the t-MN is present in peripheral leukocytes compared to infiltrating solid tumor leukocytes at solid tumor diagnosis and to ascertain whether there is enrichment for mutant clones within the tumor compared to the peripheral hematopoietic compartment. By defining the presence and characteristics of mutated infiltrating leukocytes among solid tumor patients who developed secondary leukemias, we will address whether the identification of CH mutations in solid tumors can help risk stratify those at risk for leukemia. Although the sequencing analysis of these samples was planned for 2020, given the restrictions imposed by the SARS-CoV-2 pandemic, we are performing the sequencing experiments utilizing the materials from these 36 patients in 2021.

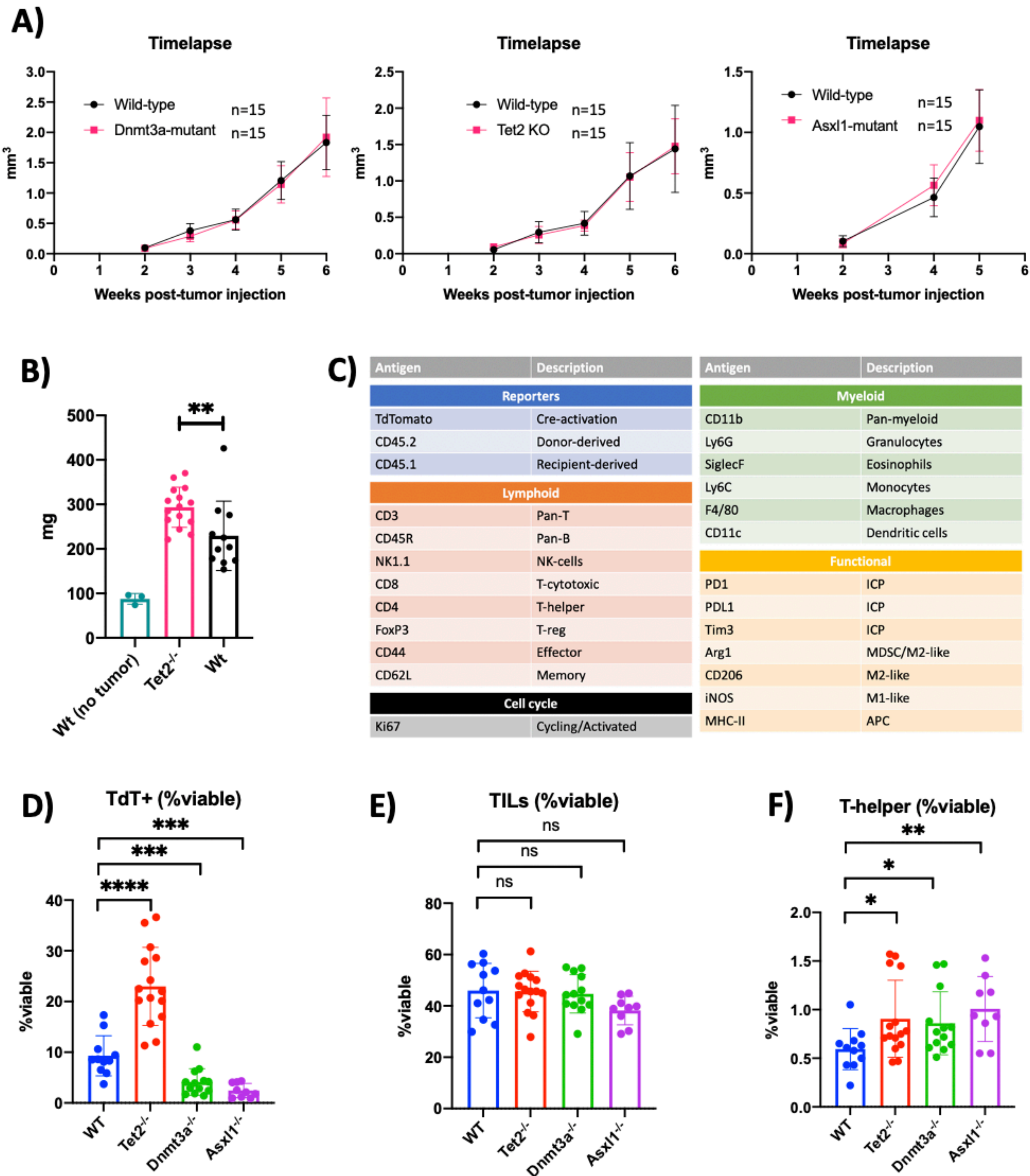
Specific Aim 2: To determine whether mutations within hematopoietic cells can impact tumor growth and metastasis.
Major Activities:
1. Effect of *Tet2* mutated hematopoietic cells on tumor growth and metastasis in different breast cancer models
Specific objective: Determine if hematopoietic deletion of TET2 alters time to disease onset in a Erbb2 driven model.

Approach and Results: Here, we bred the *Tet2* Vav:Cre mice to the MMTV:Neu/Erbb2 model of mammary tumorigenesis. To accelerate tumor formation, we synchronized female mice by inducing superovulation and pregnancy. This has been shown previously to accelerate the Erbb2 models and, critically, reduce the variability in disease onset and penetrance. We next monitored mice for the onset of disease and found no difference between *Tet2* KO and WT mice with regard to the time until tumors emerged (data shown to the right). We will continue to evaluate whether these tumors possess altered immune composition and patterns of metastatic outgrowth.



Although no differences in tumor latency between *Tet2* KO and WT mice have been observed, we considered alternative models to determine if Tet2-deficiency alters tumor growth and metastatic potential as well as response to therapy, despite showing minimal difference in time to disease onset. Specifically, we have tried to orthotopically implant various breast cancer cell lines (MMTV-PyMY driven) in C57BL/6 with the results as follows:BRC-P cell line: Inconsistent engraftment and slow growth with only one of the nineteen mice showing tumor formation after four months; Py8119 undifferentiated mesenchymal cell line: Full engraftment even at low cell number; Tumors develop after only one monthPy230 epithelial-like cell line: High engraftment. Tumors develop more slowly than Py8119, after two months. Due to these results we decided to focus on the use of Py8119 and develop the following models of concurrent CH and breast cancer.

A) Models of CH:
HSPCs derived from our C57BL/6J CH models (SCL:CreERT2 mediated deletion of *Dmnt3a*, *Tet2*, and *Asxl1* with a TdTomato reporter) and Cre-negative controls were transplanted at a WT (CD45.2, TdTomato negative) to mutant (CD45.2 TdTomato positive) ratio of 1:10 into lethally irradiated B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) host mice. This mutant to WT ratios recapitulated human disease, as CH mutations are most commonly seen at relatively low Variant Allele Frequencies (VAFs).



Antigen	Description
Reporters	
TdTomato	Cre-activation
CD45.2	Donor-derived
CD45.1	Recipient-derived
Lymphoid	
CD3	Pan-T
CD45R	Pan-B
NK1.1	NK-cells
CD8	T-cytotoxic
CD4	T-helper
FoxP3	T-reg
CD44	Effector
CD62L	Memory
Cell cycle	
Ki67	Cycling/Activated

Antigen	Description
Myeloid	
CD11b	Pan-myeloid
Ly6G	Granulocytes
SiglecF	Eosinophils
Ly6C	Monocytes
F4/80	Macrophages
CD11c	Dendritic cells
Functional	
PD1	ICP
PDL1	ICP
Tim3	ICP
Arg1	MDSC/M2-like
CD206	M2-like
iNOS	M1-like
MHC-II	APC

Figure 1: CH effects on the growth and composition of breast cancer TME.: A) Growth observed in the murine models of concurrent CH (*Dnmt3a* KO, *Tet2* KO and *Asxl1* KO) and orthotopic breast cancer (Py8119); B) Difference in spleen weight at 6 weeks post-tumor injection; C) Structure of the 25-color multispectral panel used to analyze the composition of the TME in these CH/Breast cancer models; D) Percent of KO derived clones in the TME at 6 weeks post-tumor injections. Mice were transplanted 4 weeks before tumor injections at a 1:10 mutant (TdT+) to WT(TdT-) ratio. The WT TdT+ represent a control cohort were 10% of the cells are TdT+, but no gene was excised; E) Percentage of cells in the tumor representing TILs (CD45+) among the different cohorts; F) Percent of cells in the tumor representing CD4 T-helper cells.

B) Models of breast cancer:
To investigate the effects of CH in solid tumor progression and metastasis, 4 weeks after transplantation recipient mice were injected orthotopically into the fourth mammary fat pad with the breast cancer line Py8119 derived from C57BL/6J females with a mammary tumor virus promoter driven Polyoma middle T-antigen (MMTV-PyMT). Tumor growth was monitored using a caliper. Six weeks after implantation, mice were euthanized, and their tumors were surgically removed and processed through a 25-color multispectral flow cytometry panel after single-cell dissociation (Figure 1C).

Although there were no differences in tumor growth in these experiments (Figure 1A), we observed clear differences in the expansion of the different alleles in the TME (Figure 1B) and in the extent of splenomegaly (Figure 1C). In addition, the presence of these KO even at a low VAF, was enough to modify the composition of the TME by increasing the number of CD4 helper T-cells (not dependent on the mutant clone) (Figure 1F) while keeping the total number of TILs unchanged (Figure 1E).

In addition to these changes in the composition of the TME, we observed changes in the expression of functional markers in the surface of tumor associated macrophages (TAM) carrying functional loss of different CH alleles. Specifically, Dnmt3a loss was associated with an increase in PDL1 TAM expression, supporting a potential role of *Dnmt3a* mutant macrophages in mediating T-cell exhaustion (Fig 2A and B).

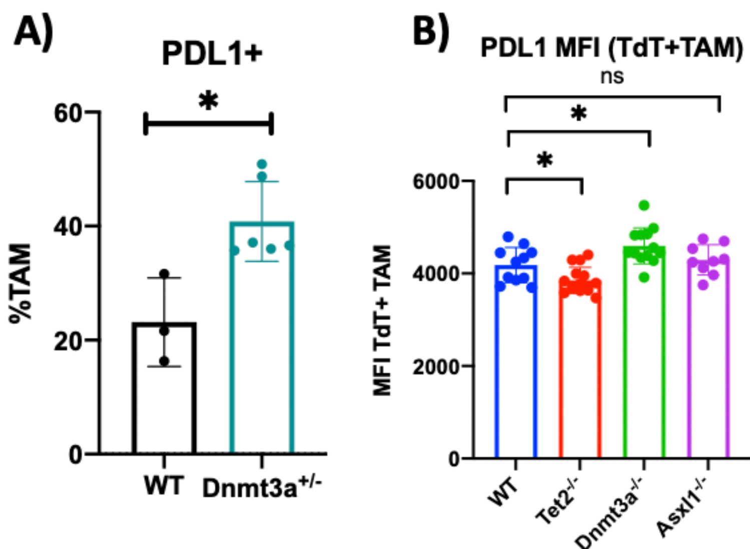


Figure 2: CH mutations modify the expression of Pdl1 in tumor associated macrophages with *Dnmt3a* loss-of-function. A) Protein expression of Pdl1 in TAM from the TME of a concurrent CH/breast cancer model. This model was a non-competitive transplant of *Dnmt3a* heterozygous loss of function HSPC; B) Pdl1 expression expressed as the MFI of TdT+ TAM. Mice were transplanted in a competitive setting at a 1:10 mutant (TdT+) to WT(TdT-) ratio. The WT TdT+ represent a control cohort were 10% of the cells are TdT+, but no gene was excised.

Specific Aim 3: To interrogate the functional interactions between breast cancer cells and white blood cells with somatic mutations and its relevance to therapeutic response

Major Activities:

1.Interrogation of the therapeutic potential of hypomethylating agents and JAK inhibitors in murine models of breast cancer, alone and in combination with breast cancer therapy.

Approach and Results: Given our difficulty in generating orthotopic breast tumors, we opted to evaluate JAK1 inhibition *in vitro* while we optimized tumor take *in vivo*. Here we directly evaluated JAK1 inhibition in its capacity to affect *Tet2* mutant hematopoietic cell self-renewal. We found that prolonged treatment with the JAK1 inhibitor, INCB052793, led to decreased colony formation compared to no treatment alone.

Pitfalls and alternative approaches: The most significant pitfall thus far is our inability to generate orthotopic breast cancer models reliably. One alternative approach is to use genetic mouse models including both the MMTV/Neu and MMTV/PyMT model. Whilst we have employed these models, limited differences have been identified when crossed to a *Tet2* hematopoietic specific knockout mouse. The reasons for this may stem from 1) the kinetics of these models are either too fast or too asynchronous to identify a specific role for *Tet2* mutant cells or 2) the sheer number of mutant cells in the genetic mouse may overwhelm any subtle effects of *Tet2* mutations, 3) a purely genetic system in the hematopoietic compartment may mask any potentially subclonal interactions between *Tet2* mutant and WT immune cells, and lastly 4) *Tet2*-mutant cells may simply not play a role in tumor progression. To evaluate the first three possibilities fully we feel it is critical to implement orthotopic models so that we can control tumor burden, time of tumor initiation and implant tumors in mixed chimeras where the *Tet2* allele burden can also be controlled. In prior years, Dr Kleppe used transgenic mouse models in a BALBc strain background (MMTV:Neu/Erbb2 driven). As an alternative, this year we are expressly working out the best orthotopic breast cancer model in a c57bl/6 mouse strain background (the same that our lab has implemented for CH and leukemia models). We have also tried different injections techniques (open breast surgery under anesthesia and percutaneous intramammary injections). Percutaneous injections have proven to be faster and have shown similar (if not better) results compared to those of an open surgery. Given the limitations imposed by the response to the SARS-CoV-2 pandemic, we will seek to utilize this model we have established and initiate the *in vivo* trials with the JAK1 inhibitor as part of our future work.

What opportunities for training and professional development has the project provided?
For Dr. Comen, this project has offered unique opportunities to solidify fruitful collaborative efforts across the breast medicine and experimental pathology services, in addition to developing new collaborations with members of the hematologic and other solid tumor services. Specifically, Dr. Comen has now ongoing collaborative research endeavors with not only her leukemia colleagues but also physicians and scientists in the colon and lung cancer teams at MSKCC. This work is part of larger collaborative efforts at MSKCC to evaluate CH in solid tumor patients. Dr. Comen is leading these efforts on the breast medicine service. As such, recognition for this work has also contributed to her being recently promoted. For Dr. Jorge Reis-Filho this approach has helped develop better bioinformatics pipelines for the detection of subclonal mutations in cancer and the detection of CH in tumor specimens; in turn, this resulted in the development of new collaborative endeavors with members of the Breast Medicine Service to study the impact of TILs harboring leukemogenic mutations in the biology and clinical behavior of human cancers.

How were the results disseminated to communities of interest?
Nothing to report.

IMPACT:

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

Our work has generated substantial interest in our leukemia and solid tumor colleagues for several reasons. First, tumor infiltrating leukocytes are not unique to breast cancers but are known to influence prognosis in a variety of solid tumors. Recent research and the successful development of immunotherapy approaches across many solid tumors underscore the essential role the immune system plays in surveillance, tumor initiation and progression. We have previously demonstrated that many of these white blood cells, while appearing morphologically normal, actually have acquired mutations in known cancer genes. These mutations were either not found or present at a lower frequency in peripheral white blood cells, tumor cells themselves or other normal cells in the body. Moreover, many of these mutations were associated with leukemia. This suggests that mutant infiltrating white blood cells may interact with cancer cells, which has significant clinical implications for tumor development and response to treatment. Our colleagues are interested in investigating this phenomenon not only in breast cancer patients but in a variety of other solid tumors, including cancers associated with *BRCA1, BRCA2* and *CHEK2* mutations. Second, it has long been believed that secondary leukemias after treatment for breast cancer are uniquely related to chemotherapy. Moreover, patients with a variety of solid tumors are at risk for future secondary leukemias. Third, roughly 25% of breast cancer patients harbor CH mutations. As a result of our preliminary data, we are now investigating additional groups of breast patients: 1) we are sequencing peripheral blood cells from newly diagnosed breast cancer patients before receiving neoadjuvant therapy and after treatment completion. This will provide insight as to whether CH mutations are present in newly diagnosed breast cancer patients and whether chemotherapy modifies the mutations in peripheral blood cells. This is particularly important since CH mutations in solid cancer patients are associated with adverse clinical outcomes; 2) we are sequencing peripheral blood cells from breast cancer patients (with no chemotherapy exposure) before and after surgical resurrection. We will compare the mutational landscape of peripheral cells in patients both pre and post-surgical resection. We believe that the presence of the tumor cells may effect the "fitness" of mutant white blood cells and in turn that mutated white blood cells may affect the behavior of tumors. 3) Our work has generated significant interest in how we genotype tumors. At present, when a mutation is identified in a given cancer the assumption is that this mutation is in the cancer cells themselves; we posit that in some instances the identifying mutation may actually be in an infiltrating white blood cell. This has important implications for targeted therapies which are matched to select mutations. 4) In a group of breast cancer patients with a history of secondary lethal leukemias, we have provided direct evidence of the presence of the leukemic clone in the TILs present in the breast cancer sample years before the leukemia diagnosis. To date, there is no ability to predict who is at risk for therapy-related/ therapy-induced neoplasms. Absent a predictive biomarker for secondary hematologic malignancies, clinicians blindly counsel patients on chemotherapy risks without an ability to refine treatment based on risk. Determining which early stage breast cancer patients are at highest risk for secondary hematologic malignancies is a crucial unmet medical need. Moreover, CH can increase the risk for other epithelial cancer as well as increase the risk of other co-morbidities including heart disease. Identifying patients early on in treatment who may be at risk for t-MN, other solid tumor malignancies as well as heart disease may help refine treatment rendered and screening for additional malignancies and disease. Alongside the clinical efforts, our models of disease provide a powerful platform to identify therapeutic avenues tailored to tumors with mutated immune cells. This fits a critical unmet need

in the field, especially given the large percentage of patients anticipated to possess mutant immune cells in their tumors, and the growing interest in immune-targeted therapy.

Fundamentally, we are confident that our findings have provided a strong basis for subsequent studies seeking to reveal whether mutated leukocytes contribute to tumor growth and metastasis. We also believe that mutated leukocytes may be clinically relevant in patients with CH and those patients who may develop secondary leukemias after treatment for breast cancer.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS:

I. Changes in approach and reasons for change.

We acknowledge that the parts of the sequencing and single cell sequencing work proposed in the non-cost extension phase of this project were delayed due to the restrictions imposed by the measures to mitigate the SARS2-CoV-2 pandemic. We commit to complete the experiments in the course of 2021 and after, as described in the respective sections above.

Changes that had a significant impact on expenditures

Nothing to report.

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

Nothing to report.

PRODUCTS: List any products resulting from the project during the reporting period. Examples of products include:
Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS I. Individuals who have worked on the project (>1 calendar month):

Name:	Elizabeth Comen
Project Role:	Partnering PI
Researcher Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	2.28
Contribution to Project:	Dr. Comen oversees all aspects of the protocol related to human subjects, and in particular human sample collection and screening daily. Dr. Comen tracks eligible patients, reviews their study eligibility and follows patient's clinical course accordingly. Dr. Comen ensures that tissue/blood samples are obtained and processed appropriately. Dr. Comen participates with data analysis and interpretation. Dr. Comen also helps identify patients with histories of breast cancer and secondary hematologic malignancies.
Funding Support:	Funding support for Dr. Comen is provided by the present grant as well as philanthropic funds. She also has a grant from the Breast Cancer Research Foundation for work unrelated to this project.

Name:	Jorge S Reis-Filho
Project Role:	Partnering PI
Researcher Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	2
Contribution to Project:	Dr. Reis-Filho oversees the pathology, genomics, and solid tumor-based single cell MissionBio Tapestri sequencing, as well as the bioinformatic analyses of breast cancer samples. Dr. Reis-Filho also coordinated and/or performed the pathology review of breast cancer specimens included in this project.
Funding Support:	During the period of this report, Dr. Reis-Filho was supported in part by this grant as well as by a Breast Cancer Research Foundation fellowship, a STARR Cancer Consortium grant and the NIH 1 P50 CA247749 01, unrelated to this project.

Name:	Damian Kim
Project Role:	Research Study Assistant
Researcher Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	6
Contribution to Project:	Damian Kim functions as the clinical research study assistant for this project. He assists with patient screening, accrual and sample transport.
Funding Support:	Damian Kim is supported by funds through the Breast Medicine Service, MSKCC.

Name	Robert Bowman
Project Role:	Postdoctoral researcher
Researcher Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	2
Contribution to project:	Dr. Bowman is in charge of the bioinformatic analysis of the samples. He also supports Dr. Sanchez Vela work and is one of the points of contact between the Levine Lab and clinical collaborators including Dr. Comen and Dr. Reis-Filho.
Funding Support:	Dr. Bowman's salary is covered by the Damon-Runyon cancer research foundation.

Name	Dr. Pablo Sanchez Vela
Project Role:	Postdoctoral researcher
Researcher Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	4
Contribution to project:	Dr. Sanchez Vela is responsible for the supervision, processing of patient samples, DNA extraction and sample preparation for downstream analysis including flow cytometry and sequencing. He is also responsible for the

	murine studies and perform techniques such as tail vein injections, bone marrow harvests and mammary fat pad injections to support. Dr. Sanchez Vela is also responsible for cell culture techniques and mouse husbandry to maintain experimental cell lines and mouse models.
Funding Support:	Dr. Sanchez Vela salary is covered by the Levine laboratory at MSKCC.

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

No.

III. What other organizations were involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS: COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.