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PRINCIPAL INVESTIGATOR: Holly Harris, ScD

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center, Seattle, WA

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Holly Harris, ScD

E-Mail: hharris@fredhutch.org

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Fred Hutchinson Cancer Research Center
1100 Fairview Ave N, Seattle, WA 98109

Michigan State University
17 Michigan Street NE, Grand Rapids, MI 49503

Brigham and Women's Hospital, Inc.
221 Longwood Ave, Boston, MA 02115

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14. ABSTRACT

We conducted a study to examine the association between inflammatory markers in peritoneal fluid and driver mutations and markers of cell proliferation and invasiveness in endometriosis tissue. We will also evaluated whether inflammation-related epidemiologic factors and systemic inflammation (e.g., CRP, IL-6 plasma levels) are associated with inflammatory markers in peritoneal fluid. Data and specimens for this study have been previously collected from A2A cohort, a longitudinal cohort of women oversampled for those surgically diagnosed with endometriosis. This project consists of selecting appropriate individuals with peritoneal fluid, endometriosis tissue, blood, and epidemiologic data, accessing samples for biospecimen assays, and conducting analyses. Targeted sequencing was used to identify our genes of interest, ELISA was used to measure inflammatory biomarkers in peritoneal fluid and blood, and RNA Seq to assess the tissue expression of selected markers. In our final analytic sample sets we had 52 endometriosis tissue samples all from superficial peritoneal endometriosis, and 101 samples in our inflammation relate exposures analysis. We observed a similar recurring variant in three samples (5'UTR in PTEN) and additionally observed that mutational load was associated with peritoneal fluid inflammatory markers. Further, inflammatory markers in peritoneal fluid were significantly correlated with each other, indicating an overall pro-inflammatory environment in some participants. There were limited associations with inflammatory related exposures and inflammatory markers, however, there was the suggestion of lower levels of inflammation among those with more frequent analgesic use.

15. SUBJECT TERMS

endometriosis, ovarian cancer, cancer driver mutations, peritoneal fluid, inflammation, epidemiology, risk factors

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Introduction

We have conducted this study to examine the association between inflammatory markers in peritoneal fluid and driver mutations and immunohistochemical (IHC) markers of cell proliferation and invasiveness in endometriosis tissue. We also evaluated whether inflammation-related epidemiologic factors and systemic inflammation (e.g., CRP, IL-6 plasma levels) are associated with inflammatory markers in peritoneal fluid. Data and specimens for this study had been previously collected from A2A cohort, a longitudinal cohort of women oversampled for those surgically diagnosed with endometriosis. This project consisted of selecting appropriate individuals with peritoneal fluid, endometriosis tissue, blood, and epidemiologic data, accessing samples for biospecimen assays, and conducting analyses. Targeted sequencing was used to identify our genes of interest, ELISA is being used to measure inflammatory biomarkers in peritoneal fluid and blood, and RNASeq to assess the tissue expression of selected markers.

Keywords

endometriosis, ovarian cancer, cancer driver mutations, peritoneal fluid, inflammation, epidemiology, risk factors

Accomplishments

What were the major goals of this project? What was accomplished under these goals?

Goal 1. Specimen identification and processing

Specific objectives: Identify and locate suitable existing samples for study inclusion, create master surgical and participant characteristics database. Pilot test methods.
Key outcomes: RNA quality was verified. We identified the 59 participants with tissue, peritoneal, and blood samples (Aim 1) and 101 total participants (43 additional from Aim 1) with peritoneal and blood samples (Aim 2).
Status: 100% complete

Goal 2. Assays

Specific objectives: DNA sequencing and gene expression profiling, RNA seq, and peritoneal and blood inflammatory assays. Biomarker data merged with covariate data.
Key outcomes: IL1-beta, CRP, IL10, IL6, and TNF-alpha receptor 2 were assayed in the blood and peritoneal fluid of all participants. Sequencing was conducted on 59 endometriosis tissue samples and paired (normal) blood samples.
Status: 100%

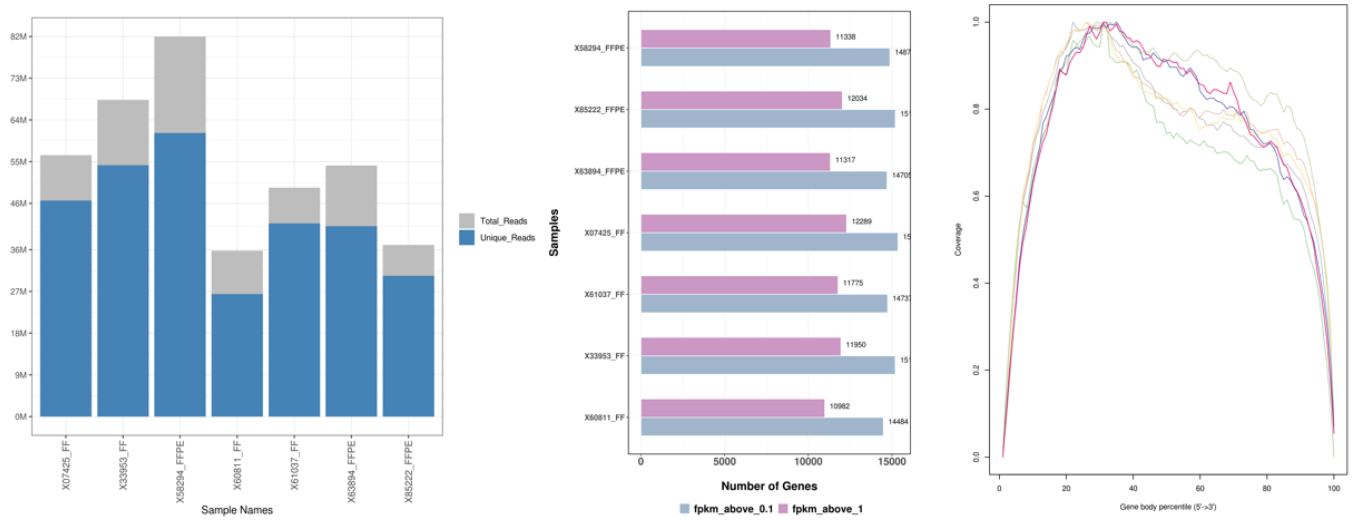
Endometriosis tissue DNA/RNA extractions and sequencing pilot

We have successfully extracted RNA from 8 superficial endometriosis tissue samples (4 fresh frozen samples and 4 formalin-fixed paraffin embedded (FFPE) samples) at the Dana-Farber Cancer Institute Molecular Pathology Core Laboratory and successfully generated RNA sequencing data on 7 samples (4 fresh frozen and 3 FFPE samples) working with the Molecular Biology Core Facilities at Dana-Farber Cancer Institute. As shown in **Table 1**, we observed successful RNA extraction with yielded RNA concentrations between 10 and 45 ng/uL for the 3 FFPE samples and between 10 and 512 ng/uL for 4 fresh frozen samples. All 4 fresh frozen samples had percentage of fragments greater than 200 nucleotides (DV200) > 70% and 3 of the 4 FFPE samples had DV200 ≥ 50%.

We performed RNA sequencing on RNA extracted from 4 fresh frozen and 3 FFPE samples. As shown in **Figure 1**, we were able to detect about 15,000 genes regardless of sample type and observed good read counts and 5' to 3' coverage for both fresh frozen and FFPE samples. Preliminary analyses of these data were conducted further examining the number of genes that were mapped and detailed quality control metrics of the generated data, and potential batch effect by basic covariates. We did not observe clear batch effect by basic covariates including sample type (i.e. fresh frozen, FFPE), age, year at surgery, hospital type, or body mass index at surgery, further supporting the feasibility of this assay method.

Table 1. Quality control metrics of the extracted RNA samples from 8 SPE tissue samples							
		RiboGreen Quant			BioAnalyzer RNA QC		
Sample Type	ID	RNA Con.c [ng/uL]	RNA Volume [uL]	RNA Yield [ng]	RNA Con.c [pg/uL]	RIN#	DV200
Fresh Frozen	**X60811**	14.24	25	356.00	1997	N/A	>70%
Fresh Frozen	**X33953**	512.10	25	12802.50	24109	5.3	/
Fresh Frozen	**X61037**	25.28	25	632.00	2767	7.3	/
Fresh Frozen	**X07425**	9.97	25	249.25	657	8.2	/
FFPE scroll	**X17281**	0.75	25	18.75	59	1.2	<30%
FFPE scroll	**X63894**	44.93	25	1123.25	2575	2.2	30-50%
FFPE scroll	**X85222**	10.31	25	257.75	392	2.4	50-70%
FFPE scroll	**X58294**	19.00	25	475.00	1431	2.1	50-70%

Figure 1. Quality control metrics on RNAseq data from 4 fresh frozen and 3 FFPE ectopic endometriosis samples.



Furthermore, we were able to successfully generate exome sequencing data using DNA extracted from 4 fresh frozen and 2 FFPE samples. More than 20,000 variants were identified in both fresh frozen and FFPE samples, demonstrating that the exome sequencing quality does not differ between these different sample type (**Table 2**). Identified variants included missense variants, frameshift variants, structural interaction variants. Of the 8 cancer driver mutations that were hypothesized in this application, there were up to 3 somatic mutations detected in these superficial endometriosis tissue samples (**Table 3**). We are planning to sequence germline DNA samples which will allow us to detect copy number variants and identify somatic mutations with more precision.

Table 2. Summary of variants detected

	Number of variants				
Sample	**Raw**	**PASS filters**	**On target unfiltered (multiallelic split)**	**PASS on target**	**PASS panel**
20201215_FFPE_X58294_KT8112_S187	21,348	5,132	21,741	5,132	2
20201215_FFPE_X85222_KT8112_S186	18,673	4,924	18,976	4,924	1
20201215_FF_X07425_KT8112_S189	24,940	5,623	25,640	5,623	1
20201215_FF_X33953_KT8112_S191	29,137	4,893	30,153	4,893	2
20201215_FF_X60811_KT8112_S188	21,753	4,949	22,290	4,949	0
20201215_FF_X61037_KT8112_S190	26,080	4,844	26,940	4,844	3

Table 3. Number of somatic mutations detected

Sample	# of somatic variants (out of the 8*)	Type of somatic mutation
FFPE_X58294	2	ARID1A, PTEN
FFPE_X85222	1	PPP2R1A
FF_X07425	1	PTEN
FF_X33953	2	KLLN, ERBB2
FF_X60811	0	
FF_X61037	3	ARID1A, PTEN, KRAS

*ARID1A, BRAF, CTNNB1, ERBB2, KRAS, PIK3CA, PPP2R1A, PTEN

Goal 3. Data analysis and manuscript preparation

Specific objectives: This task includes data cleaning, analyses, and preparation and submission of manuscripts for two areas:
1) Examine the association between inflammatory markers (IL1-beta, CRP, IL10, IL6, and TNF-alpha receptor 2) in peritoneal fluid and cancer driver mutations (*ARID1A*, *PIK3CA*, *PPP2R1A*, *CTNNB1*, *PTEN*, *KRAS*, *BRAF*, *ERBB2*) and markers of cell proliferation in endometriosis tissue.
2) Evaluate whether inflammation-related factors (e.g., ovulatory cycles, NSAID use, BMI, dysmenorrhea, IUD use, pelvic infections) and systemic inflammation (IL1-beta, CRP, IL10, IL6, and TNF-alpha receptor 2 plasma levels) are associated with inflammatory markers in peritoneal fluid.

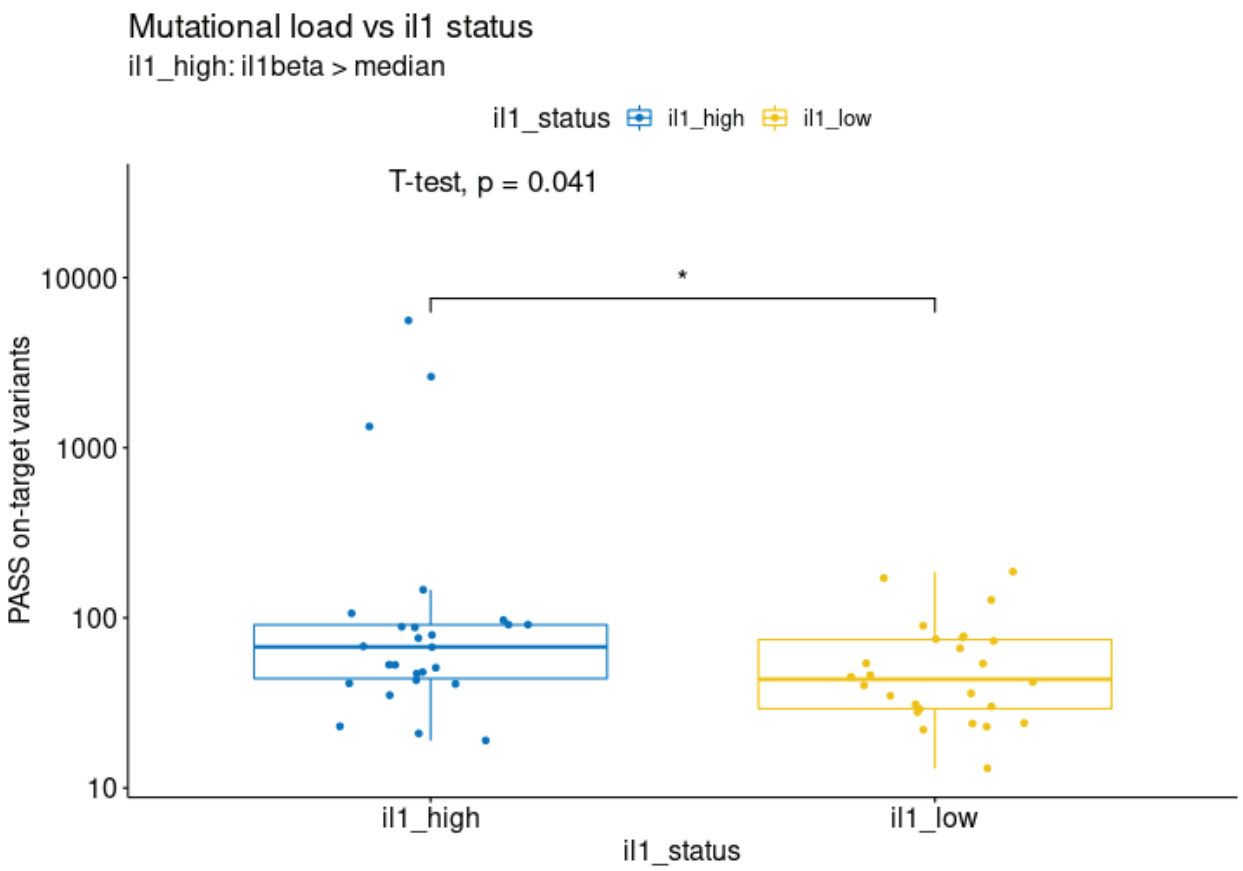
Key outcomes: Data cleaning was done on epidemiologic data, blood and peritoneal biomarker assays, including calculating CVs and identifying outliers for the inflammatory biomarkers.
For Aim 1 we conducted sequencing on 59 endometriosis tissue samples and paired normal (blood) samples. 52 of these samples passed quality control (QC) checks. This project was focused on cancer driver mutations in the following genes: *ARID1A*, *PIK3CA*, *PPP2R1A*, *CTNNB1*, *PTEN*, *KRAS*, *BRAF*, *ERBB2*, both their presence in superficial endometriosis samples and their correlation peritoneal inflammatory markers.
For Aim 2 we examined associations between inflammatory-related exposures, inflammatory markers in the peritoneal fluid and blood, and endometriosis tissue related characteristics (e.g. cancer driver mutations).
Manuscripts are being prepared for submission.
Status: 100%

All endometriosis tissue samples were superficial peritoneal endometriosis which, to our knowledge, have not been previously examined in relation to cancer driver mutations. Among the 52 samples the following variants in the cancer drive mutations described above were observed:

- Recurring variant in two samples, with a third sample with a very close variant): 5'UTR in PTEN (unknown in COSMI or Gnomad)
- In one sample KRAS missense in X89599 – COSMIC variant

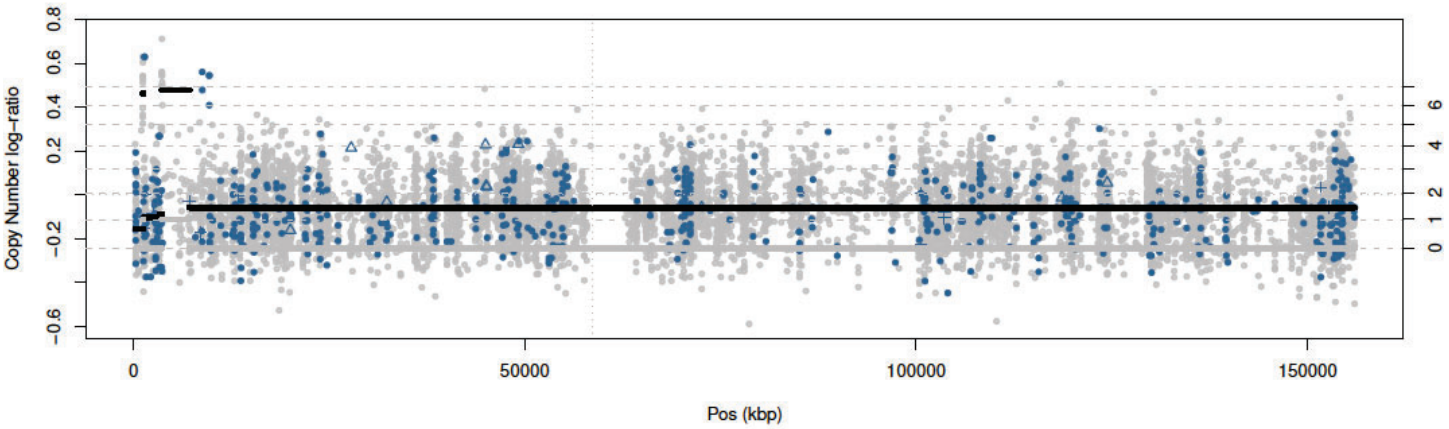
As there were few cancer driver related mutations in the superficial peritoneal endometriosis tissues that were examined, which is of itself a new finding, we then looked at overall mutational burden and copy number alterations, thus expanding beyond only examining cancer driver mutations. Three samples had a high mutational load and mutational load across the samples was associated with peritoneal fluid inflammatory markers. **Figure 2** presents the results for IL-1 beta. Results were similar for IL-6 and IL-10, while there was less of an impact of CRP and TNF-alpha.

Figure 2. Mutation load by IL-1 beta status



For the copy number analyses we observed alterations in chromosome X in some samples (**Figure 3**).

Figure 3. Copy number variation in chromosome X



In aim 2 we examined the correlation between inflammatory markers in the peritoneal fluid and blood. AS seen in **Table 4**, peritoneal fluid levels of all inflammatory markers were statistically significantly associated with each other (all $p<0.05$) with the strongest correlation between IL-6 and IL-10. Only TNF-alpha receptor levels were significantly correlated across peritoneal fluid and blood, demonstrating the utility of examining inflammatory markers in the peritoneal fluid as a measure of local inflammation that may not be reflected in blood and supporting our hypothesis that peritoneal fluid levels are driven by both local and systemic factors.

Table 4. Spearman correlations and corresponding p-values for associations between inflammatory makers in the peritoneal fluid versus blood among 103 A2A participants

	Peritoneal IL-1 beta	Peritoneal IL-6	Peritoneal IL-10	Peritoneal TNF-alpha	Blood IL-1 beta	Blood IL-6	Blood IL-10	Blood TNF-alpha
Peritoneal IL-1 beta	1.00	**0.30 (p<0.0001)**	**0.44 (p<0.0001)**	**0.32 (p=0.001)**	0.19 (p=0.05)	**0.34 (p<0.0004)**	0.05 (p=0.61)	-0.02 (p=0.82)
Peritoneal IL-6		1.00	**0.72 (p<0.0001)**	**0.38 (p<0.0001)**	0.19 (p=0.05)	0.13 (p=0.18)	0.06 (p=0.56)	0.04 (p=0.69)
Peritoneal IL-10			1.00	**0.42 (p<0.0001)**	0.30 (p=0.002)	0.12 (p=0.23)	0.02 (p=0.81)	0.04 (p=0.69)
Peritoneal TNF-alpha				1.00	0.06 (p=0.56)	**0.20 (p=0.04)**	0.12 (p=0.23)	**0.27 (0.006)**
Blood IL-1 beta					1.00		0.12 (p=0.23)	0.16 (p=0.10)
Blood IL-6						1.00	0.23 (p=0.02)	0.23 (p=0.02)
Blood IL-10							1.00	**0.27 (p=0.005)**
Blood TNF-alpha								1.00

When we examined the associations between inflammatory-related exposures and inflammatory markers in the peritoneal fluid and blood there were few significant associations observed. We observed an inverse association between BMI and blood levels of IL-6 and peritoneal fluid levels of CRP where a higher BMI (overweight/obese) was associated with lower levels of inflammation compared to those with a BMI that was <25 (**Table 5**). In addition, there was the suggestion of lower levels of inflammatory markers in the peritoneal fluid for most markers with more frequent analgesic use but those results did not reach statistical significance (**Table 6**).

Table 5. Odds ratios for associations between BMI and peritoneal and blood inflammatory markers among 101 A2A participants

	Peritoneal fluid	Blood
IL-1 beta	0.82 (0.40-1.66)	1.54 (0.76-3.13)
IL-6	0.90 (0.44-1.82)	**0.46 (0.22-0.93)**
IL-10	1.56 (0.77-3.18)	1.48 (0.73-3.01)
TNF-alpha	1.14 (0.56-2.32)	1.27 (0.62-2.57)
CRP	**0.30 (0.14-0.63)**	

Table 6. Odds ratios for associations between analgesic use and peritoneal inflammatory markers among 101 A2A participants

	No analgesic use	<2 days/week	2+ days/week
IL-1 beta	1.00 (reference)	0.96 (0.31-2.97)	0.80 (0.39-1.65)
IL-6	1.00 (reference)	0.64 (0.20-1.98)	0.63 (0.30-1.29)
IL-10	1.00 (reference)	0.85 (0.27-2.62)	0.60 (0.29-1.23)
TNF-alpha	1.00 (reference)	0.71 (0.23-2.21)	0.74 (0.36-1.53)
CRP	1.00 (reference)	4.45 (1.39-14.31)	1.28 (0.62-2.64)

What opportunities for training and professional development has the project provided?
Nothing to report.

How were the results disseminated to communities of interest?
Results will be disseminated once manuscripts are published.

What do you plan to do during the next reporting period to accomplish these goals?
This is the final report.

Impact
What was the impact on the development of the principal disciplines of this project?

To our knowledge, we are the first to examine superficial endometriosis lesions for the presence of cancer driver mutations. Our findings that few of these lesions have cancer driver mutations and that the mutational burden of these lesions was generally low which adds to our knowledge of natural history of endometriosis. In addition, the recurring (same variant in two samples, third sample with very close variant) 5'UTR PTEN may be a new discovery and could provide insight into the 2-3% of women with endometriosis who later develop ovarian cancer. In addition, we observed that local inflammation (i.e. inflammation in the peritoneal fluid) was associated with a higher mutational burden highlighting that inflammatory pathways could be important in the endometriosis to ovarian cancer transition and some samples showed copy number variation alterations.

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

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

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

Changes/Problems
Changes in approach and reasons for change.
None.

Actual or anticipated problems or delays and actions or plans to resolve them.
The COVID-19 pandemic and subsequent laboratory shut-downs caused delays to our study but once laboratories re-opened we are were able to complete our project.

Changes that had a significant impact on expenditures.
Nothing to report.

Significant changes in use or care of human subjects.
Nothing to report.

Significant changes in use or care of vertebrate animals.
n/a

Significant changes in biohazards and/or select agents.
Nothing to report.

Products
Publications, conference papers, and presentations
Manuscripts are in process.

Websites or other internet sites
Nothing to report.

Technologies or techniques
Nothing to report.

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

Other products
Nothing to report.

Participants & Other Collaborating Organizations

What individuals have worked on this project?

Name	Degree	Project Role	Researcher Identifier	Person Months	Contribution to Project	Funding Support
Harris, Holly	ScD	Principal Investigator	0000-0002-2572-6727	1	PI responsible for all facets of the study	N/A

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

Yes, Dr. Harris' other support has changed. The 3 new grants below have been added to her other support.

No Number 11/1/2020 – 4/30/2022 1.2 CM
Cascadia Data Alliance (Bashashati, Harris Huntsman, Morgan)

Pathology AI for a Federated Quality Assurance Program: Ovarian Cancer Pilot

Our vision is to establish an international network for AI-based pathology quality assurance across different sites, which can operate without explicitly sharing any patient data. In this project, as a proof of concept, we propose to develop and deploy an ML-based ovarian cancer histopathology classifier, using privacy-preserving data sharing mechanisms.

RFP #75N92019R0030 (Anderson) 2/1/2021 – 1/31/2026 .48 CM
NIH

Women's Health Initiative (WHI) – Clinical Coordinating Center

The primary objectives of the WHI Clinical Coordinating Center are: To maximize the knowledge gained from the WHI resource to impact public health, particularly of older women, by conducting high quality studies examining the epidemiology, biologic mechanisms, and health outcomes among postmenopausal women; To maintain and expand the WHI resource to address these public health concerns through additional active and passive follow-up (2020-2025) and collection of high quality outcomes data; To provide an efficient service infrastructure to support the governance of the WHI and facilitate the use of this resource by the broader research community; To engage and facilitate access to researchers not previously associated with WHI, particularly early career and under-represented minority investigators.
Role: Co-Investigator

R01 CA248288 1/13/2021 – 12/31/2025 1.2 CM
NIH

Relating Molecular Subgroups of Endometriosis-Associated Ovarian Cancers to Survival and Risk

Our approach uses integrated analysis of multiple layers of information, including relationships between risk factors and genomic and prognostic associations, to provide powerful biological and mechanistic insight into ovarian cancer biology with potential to point to novel targeted therapeutic options.
Role: Co-Investigator

What other organizations were involved as partners?

Organization Name: Brigham and Women's Hospital, Inc.
Location of Organization: Boston, MA 02115
Partner's Contribution: Collaboration

Organization Name: Michigan State University
Location of Organization: Grand Rapids, MI
Partner's Contribution: Collaboration

Special Reporting Requirements

n/a

Appendices

n/a