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14. ABSTRACT To develop a much-needed cure for endometriosis, it is first necessary to understand the pathogenesis and evolution of the disease. Endometrial stem/progenitor cells have been implicated as well as multiple genetic variations, each contributing a small effect but combined may have as yet unknown biological roles. A single polygenic risk score (PRS) can be used to categorize this genetic load of the risk variants in an individual. In this project, we will assess the biological role of a high PRS in endometriosis patients versus a low PRS in normal women. Specifically, we will assess changes in gene expression in endometrial cells of eutopic endometrium at the single cell level, functional behavior of endometrial stem/progenitor cells in organoid assays, including co-culture with stromal cells and localization of risk gene variants, and key gene signatures in relation to stem/progenitor cell locations. We have completed all required approvals, developed new tools, questionnaire, databases, assays and protocols, recruited 9 women and collected samples for all 3 aims in spite of significant challenges faced with the timing of HRPO approval and COVID-19 impacts on gynecological surgeries. As surgeries recommence following easing of 8 months COVID-19 restrictions in Melbourne, we have participants waiting for their delayed surgeries and we are ready to accelerate progress in Aims 1 and 2 in the second reporting period.					
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1. Introduction

Endometriosis is defined as the growth of endometrial-like tissue (glands and stroma) outside the uterus. Endometriosis is a serious, chronic disorder affecting millions of women worldwide and is estimated to cost \$70 billion per year in the US (Simoens et al. 2012). A recent analysis of longitudinal data in Australia estimates the cumulative prevalence of endometriosis at 11.4% with a peak age-specific incidence of 6 per 1000 person-years at age 30-34 years (Rowlands et al. 2020). The cause and pathogenesis of endometriosis is unknown, diagnosis takes at least 10 years due to lack of a non-invasive diagnostic test and current treatments are often ineffective as the disease frequently recurs. To develop a much-needed cure for endometriosis, it is first necessary to understand the pathogenesis and evolution of the disease. Only stem/progenitor cells are sufficiently long-lived and capable to establish endometriotic growths in the pelvis. Our studies have characterised rare populations of stem/progenitor cell types of the uterine lining, as well as numerous gene variants of endometriosis risk. The purpose of this work is to determine the biological role of endometriosis risk, and its effect on stem/progenitor cell function. Specifically, for endometrial stem/progenitor cells from women with a defined endometriosis risk score we will (1) obtain gene expression signatures at the single cell level, (2) determine the contribution of the endometrial niche and gene transcription in survival of these cells in the pelvis and initiation of endometriosis and (3) determine the temporal and spatial relationships between the risk gene variants, key gene signature genes and stem/progenitor cell locations. Recent somatic mutation analysis of epithelium of endometriosis lesions and individual endometrial glands suggest a role for endometrial epithelial progenitor cells in initiating endometriosis. We hypothesise that endometriosis genetic risk factors collectively confer selective advantage to endometrial epithelial progenitor cells in initiating growth of endometriotic lesions and that endometrial mesenchymal stem cells promote lesion growth in women with endometriosis.

2. Keywords

Endometriosis, endometrial stem/progenitor cells, genetic risk, endometriosis pathogenesis, prognostics, endometrial epithelial organoids, precision medicine.

3. Accomplishments

Several events have impacted our progress and these have been shown schematically in Figure 1. Delays in obtaining HPRO approval and the COVID-19 pandemic have interacted and severely curtailed our ability to obtain clinical samples and conduct the research outlined in the approved SOW (and the revised SOW). These are shown schematically for easy reference to the following sections of this technical report.

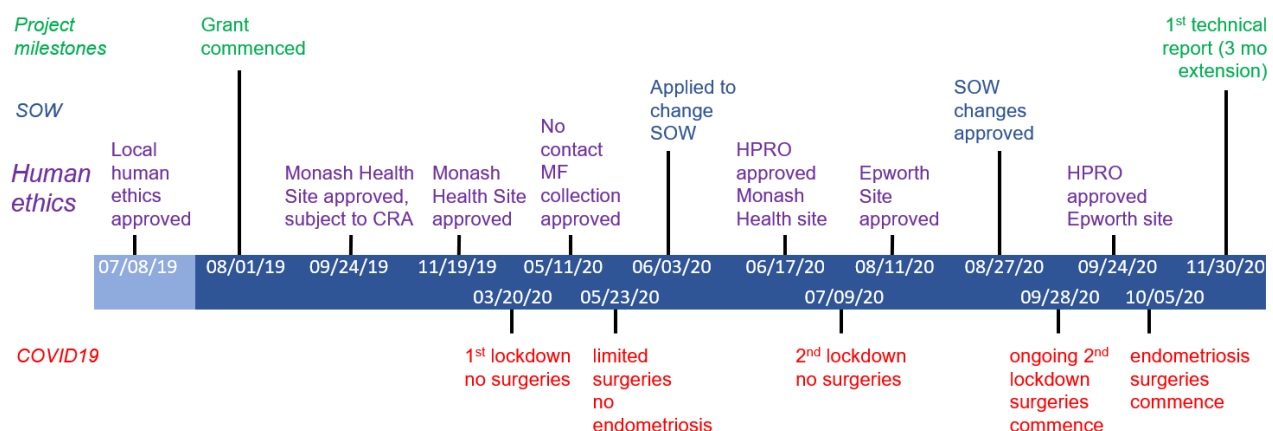


Figure 1 – Timeline showing the impact of timing of ethics approval and COVID-19 on our ability to collect human tissue samples for our experimental work during stage 3 and ongoing stage 4

lockdown to contain the pandemic. In total there was 7 weeks of tissue collection allowable in the first 14 months, and 3 weeks had very limited and no endometriosis surgeries. We are reporting on 5 available weeks for tissue collection in this first technical report. Also shown is the time of application and subsequent approval to modify the SOW with the goal of commencing Aim 2 earlier by bringing forward the ability to collect menstrual fluid and undertake subsequent experiments (Aim 2). This was to mitigate the lack of surgeries undertaken during COVID-19 restrictions. MF, menstrual fluid

Milestone 1a Monash Health Human Research Ethics Committee approval was obtained by **PI Gargett** on 8 July 2019 and Monash Health Site Specific approval on 19 Nov 2019 on schedule (Fig 1). This met the milestone 1a timeline. HPRO approval for the Monash Health site was granted on 17 June 2020, 6 months after submission, exceeding the timeline stated in the Statement of Work. Biosafety approvals were completed by June 20, 2020.

Staff Recruitment and Training: **PI Gargett** and Key Personnel Filby have recruited and trained two new team members. A Research Nurse has been trained in gynecological research patient recruitment, consent, and sample collection, including in use of the new research database (see below) and specific training in recruitment for studies involving genomics research. The Research Nurse has also been trained in collection of human tissue samples according to COVID-19 safety guidelines. A Research Assistant was recruited and trained in tissue processing, fluorescent activated cell sorting, organoid culture and RNA isolation and preparation. Both, together with Dr Filby have been trained in the use of the new web-based database developed at IMB specifically for this project (see below).

Patient Questionnaire: Partnering **PI Montgomery** and his group in collaboration with PIs Gargett and Rombauts developed a patient questionnaire for this study. There are a number of patient questionnaires for endometriosis research including the World Endometriosis Research Foundation (WERF EPHeCT) questionnaires (Rahmioglu et al. 2014). These questionnaires collect clinical and co-variate phenotype data including patient symptoms. One disadvantage of existing questionnaires is they ask general questions about symptom and clinical data. However, many patients can have more than one surgery. We would like to know about symptoms and clinical data relevant to the surgery when patient samples for this study are collected. We therefore developed a questionnaire, based on the WERF EPHeCT that asks symptom and clinical data, but structured to capture this data for the specific surgical episode when samples are collected. The patients can answer the same questions for at least two previous treatment episodes (if relevant) so we can estimate if symptom and clinical data for the patients has changed since previous treatments. A web-based version of the questionnaire has been developed and approved by the Monash Health Human Research Ethics Committee allowing participants to complete the questionnaires at home at their own pace improving study compliance and direct data capture, eliminating input error.

Research Database: Partnering **PI Montgomery** works closely with the Program on Complex Trait Genetics (PCTG) and Human Studies Unit at the Institute for Molecular Bioscience (IMB) at the University of Queensland (UQ). The HSU have developed a purpose-built research database to capture and store participant data and track biological samples for research projects including research on motor neuron disease. We worked with HSU who developed dedicated infrastructure to support our project including collection and storage of our on-line questionnaire and clinical data, sample tracking for collection and receipt of blood samples and DNA extractions involving both Hudson and IMB sites.

Regular team meetings and surgeons' meetings: **PI Gargett** and Partnering **PI Montgomery** and their teams have held fortnightly ZOOM meetings from the 3 months prior to and since commencement of the DoD grant on 1 August 2019, working from a prepared Agenda for each meeting and generating minutes and action points. These meetings have provided opportunity to plan our research, solve

problems and develop strategies to ensure maximal progress on the grant given the extenuating circumstances that prevailed during this first 15 months of the grant. It has enabled us to fully prepare for when surgeries slowly recommenced by optimizing many of our methods, particularly as technologies have rapidly developed since the writing of the original application. We also initiated monthly surgeon meetings involving our associate PIs and their surgical teams. These were initiated by **PI Gargett**, commencing on December 4 2019 and have been held monthly since. These planning meetings deal with the ethics and logistics of tissue collection from their surgeries and with the collection and curating of data collected in the surgical questionnaires used by the surgeons. Since August 2020, Partnering PI Montgomery and his team have participated in these meetings to streamline the collection of patient and surgical questionnaire information in the Research database they have developed at IMB. These regular meetings of the clinical and scientific teams have placed us in a strong position to maximize collection of patient samples and demographic data under the difficult circumstances imposed by the pandemic.

Prior to the pandemic, **PIs Gargett and Montgomery** and their teams held 2 face-to-face meetings, one in Melbourne at the Hudson on 1 August 2019 and the second at UQ on 12 November 2019, which included hands on training in use of the purpose-designed Research Database for Hudson research staff and a research planning meeting with the scientific staff of both teams. A third face-to-face meeting was held in Melbourne on 10 October 2019, specifically focused on Research Database planning. These face-to-face meetings will resume on an annual basis once the state of Victoria emerges from lockdown and the borders open between our two states.

a) Major Goals and Accomplishments

AIM 1: To investigate the biological effects of risk gene variants in the causation of endometriosis by determining gene-expression signatures of N-cadherin+ eEP and SUSD2+ eMSC from women with defined Polygenic Risk Scores for endometriosis risk gene variants

i. Major Activities

Major activities for **Initiating PI Gargett** focused on preparing and obtaining human ethics approval (8 July 2019; ahead of schedule) and Monash Health Site approval (24 September 2019; on schedule but was subject to collaborative research agreement which was dependent on finalization of the award, 1 August 2019) for this project which was fully signed on 12 November 2019. Human ethics approval for the Monash Health site was granted on 19 November 2019. HRPO approval was then sought (11 December 2019) and obtained for Monash Health site (17 June, 2020) (Fig 1). Delayed HRPO approval coincided with a lack of gynecological surgeries in our approved Monash Health hospital site due to COVID-19 which has severely hampered our progress (see Figure 1 and point 5). Therefore, HRPO approval at a private hospital site at Epworth HealthCare was sought and approved (24 September 2020) to increase our recruitment during restrictions to elective surgery during the COVID-19 pandemic. We have started screening, recruitment, consent and data collection as surgeries commenced during COVID-19 lockdown (Fig 1) The intervening time was used to establish a database at UQ (see above) for managing patient demographic data on samples collected from Monash/Hudson, sample tracking of Hudson samples shipped to UQ and to optimize methodology as described below. In depth training of appropriate recruitment for genetic studies and data entry into the database was also undertaken. Experimental methodologies were optimized and with commencement of endometriosis surgeries in the final month of the 15-month reporting period, we have commenced recruiting, collecting and processing samples for experimental work. At 31 October 2020, 9 women were consented to the study.

Major activities for **Partnering PI Montgomery** focused on preparing and implementing data collection tools to accompany sample collection and improve phenotyping, as well as the optimization of

sample processing for genotyping, tracking and data analysis for designated experiments. Human research ethics approval for the use and processing of samples at UQ originally collected through the initiating PI Gargett was prepared and obtained (09 August 2019) and HPRO approval prepared and obtained (05 March 2020). The purpose-built research database has strict privacy and security functionality to ensure data protection for participant data and tracking of biological samples through the sample processing pipeline for multiple collection and processing sites. Additional data collection tools through an online patient questionnaire was developed to improve the collection of accompanying phenotypic data and was digitized to provide easier, contactless completion and enhance protocol completion. Automated data collection also reduces the potential for secondary data entry errors.

AIM 2 To determine the association between endometrial cell survival and the genetic risk profile of endometriosis cases and controls

i. Major Activities

Major activities for **Initiating PI Gargett** on Aim 2 focused on developing a safe non-contact protocol for collecting Menstrual Fluid samples from consented women during COVID-19 lockdown. We also obtained Human Ethics approval to collect menstrual fluid in a COVID-19 safe manner as well as saliva rather than peripheral blood for genotyping purposes (Fig 1). In the 5 weeks available to us (taking HPRO approval dates and severe lack of surgeries into account, Fig 1), we have collected 3 samples, processing the Menstrual Fluid for organoid cultures and stored saliva samples to be shipped in batches to IMB for genotyping. Protocols developed by AI Filby (Hudson) have been provided to AI McKinnon (UQ) with remote training for the establishment and maintenance of organoid cultures.

Major activities for **Partnering PI Montgomery** focused on establishing conditions for the successful and consistent sterile culture of organoids that will be applied for co-culture experiments. All reagents and consumables have been sourced (as above) to align with those used in the original organoids developed at the Hudson Institute. Analysis has been performed on the existing endometrial stromal cells and available whole genome genotyping data to create a polygenic risk score (PRS) for each cell preparation. Endometrial PRS scores based on the latest meta-analysis data of endometriosis GWAS has been used and has generated a comprehensive list of the relative PRS for each available preparation.

AIM 3: To map the temporal and spatial relationship of N-cadherin+ eEP and SUSD2+ eMSC in eutopic (from cases) and normal endometrium, and in ectopic lesions in relation to important proteins identified in endometriosis risk gene pathways

i. Major Activities

As indicated in Aim 1 and shown in Figure 1, our ability to collect patient samples was severely affected. In the 5 weeks available to us for collecting tissue samples during this reporting period, we consented and collected 2 eutopic endometrium and 2 lesions for Aim 3.

ii. **Specific Objectives Table 1** (as outlined in the Statement of Work) showing completions, progress, outstanding and plans for action

SUBTASK Project commenced 08/01/19	TIMELINE Months	SITE 1	SITE 2	COMPLETED	PROGRESS	OUTSTANDING	PLAN FOR ACTION
AIM 1							
1A: Institutional HREC, AEC & Biosafety approvals	1-3	Gargett	Montgomery	MH IRB 07/08/19 MH site 11/19/19 EH site 08/11/20 UQ IRB 09/08/19 Biosafety 06/20/20		Monash AEC only required for 27-36 mo	Apply for AEC in Nov 2020
1B: HRPO and ACURO approvals	2-4	DoD	DoD	HPRO for MH site 06/17/20 EH site 09/24/20 UQ 03/05/2020		ACURO	Apply in April 2021
1C: submit minor amendments	as required	Gargett	Montgomery	MA#1 addition of staff 01/28/20 MA#2 electronic survey 09/08/20			
1D: submit local HREC, AEC and Biosafety Annual Reports	annually	Gargett	Montgomery	MH approved 06/01/20 Biosafety approval renewed 06/20/20	HPRO submitted 06/25/20		
2: Patient recruitment and hysterectomy/ endometrial biopsy tissue (n=40)	2-30	Gargett		9 women recruited	06/22/20- 07/08/20, 09/28/20 (break due to COVID-19 restrictions)	31	Collecting menstrual fluid (MF) during restrictions
4: 6 way FACS sort single cell suspensions from tissue samples	4-30	Gargett		Single cell organoids protocol optimized	Waiting for recruited participants now COVID-19 restrictions on surgery has lifted	15	
5: Barcoding FACS sorted cells using 10X Genomics machine, ship single cell cDNA libraries to IMB (n=8/gp, 2 gps, 6 cell types/gp)	3-24	Gargett		Tags for pooling cell populations validated	Waiting for recruited participants now COVID-19 restrictions on surgery has lifted	15	

SUBTASK Project commenced 08/01/19	TIMELINE Months	SITE 1	SITE 2	COMPLETED	PROGRESS	OUTSTANDING	PLAN FOR ACTION
6: Full genotyping blood cells of patient samples (n=8/gp/2 gps)	7-30		Montgomery	Developed PRS method for 42 risk genes	Awaiting sufficient samples to genotype in batches	16	
7: single cell RNA sequencing (n=8/gp, 2 gps, 6 cell types/gp) in pooled batches of cells	7-24		Montgomery		Waiting for recruited participants now COVID-19 restrictions on surgery has lifted	16	
10: establish organoid cultures for 4 epithelial cell types (n=8/gp, 2 gps), passage 1-3 times and freeze	4-30	Gargett		Organoid isolation, and culture method established	Waiting for recruited participants now COVID-19 restrictions on surgery has lifted	16	Developed organoids from MF (Aim 2, subtask3b)
11: Cell proliferation studies on organoids using IncuCyte (n=8/gp, 2 pgs)	12-33	Gargett			Waiting for recruited participants now COVID-19 restrictions on surgery has lifted	16	
AIM 2							
1: Subject recruitment for menstrual fluid and peripheral blood (n=24/gp, 2 gps)	9-33	Gargett			Started 09/28/20 9 recruited, 3 collected	45	Continue with COVID safe collection protocol
2: Full genotyping peripheral blood samples (n=8/gp, 4 gps) for all risk variants (currently 44)	9-33		Montgomery		3 saliva samples at Hudson, waiting to be shipped in batches	45	Genotype in batches as batch number reached
3a: MACS sort suspensions of dissociated menstrual blood samples	9-31	Gargett		Method established	3 completed	45	Request change to SOW to increase time for subtask
3b: organoid cultures of epithelial, stromal and epithelial/stromal cells passage 1-3 and freeze (n=8/gp, 2 gps, 3 culture types/gp)	9-36	Gargett	Montgomery	Method for menstrual blood organoid cultures developed	Multicellular organoid experiments planned	Organoid cultures on samples collected through current project	Request change to SOW to increase time for subtask
4: 2D and 3D organoid cultures of epithelial and stromal cells passage 1-3 (n=8/gp, 2 groups, 2 cell types)	9-18		Montgomery	high / low PRS stromal cell cultures identified	Procurement of reagents, established conditions for culture.	Co-culture of organoid cultures with the stromal high and low PRS scores	

SUBTASK Project commenced 08/01/19	TIMELINE Months	SITE 1	SITE 2	COMPLETED	PROGRESS	OUTSTANDING	PLAN FOR ACTION
5: RNA sequencing on 2D and 3D organoid cultures (n=8/gp, 4 gps, 2 cell types/gp)	13-24		Montgomery	Setting up and planning of organoid cultures	Organoid culture under development	RNA-sequencing to be performed once co-cultures established.	
6: Processing, Quality control and mapping of RNAseq data and bioinformatics analysis (n=8/gp, 4 gps, 2cell type/group)	13-30		Montgomery	Bioinformatic pipelines established for RNA-seq analysis	Continual refinement of pipeline with new methods	Awaiting generation of RNA-seq data	
7: use thawed organoids to assess progesterone effect on organoid growth, apoptosis, fibronectin ligands (n=8/gp, 2 gps)	9-18	Gargett			Delayed by inability to collect samples due to COVID-19	16	Request change to SOW to delay start of this subtask
AIM 3							
1: Collection of eutopic endometrium and endometriotic lesions over 3 stages of the menstrual cycle.	4-34	Gargett		2 eutopic and 2 lesions collected	Waiting for recruited participants (6) now COVID-19 restrictions on surgery has lifted	Ongoing collection	Request change to SOW to extend subtask
2: full genotyping patient DNA samples (n=8/gp, 2 gps) for all risk variants (currently 44).	6-34		Montgomery	Bioinformatic pipeline for quality control and analysis of Global screening array chips established.	Waiting for recruited participants now COVID-19 restrictions on surgery has lifted	Genotyping will be performed in batches across the project timeline.	Request change to SOW to extend subtask. Perform in batches as batch number reached

iii. Significant Results, outcomes, developments, conclusions

Subtask 1A Human ethics approval – Ethics approved by local Human Ethics Research Committee and HPRO for two hospital sites to collect human tissues (17 June and 24 September 2020).

Subtask 1B - Animal ethics Approval - ACURO advised **Initiating PI Gargett** on 29th May 2019 that approval should be sought 3 months prior to animal experiments (due to commence October 2021).

Subtask 1C – Human Ethics - Non-substantive Minor Amendments were submitted by **Initiating PI Gargett** and approved for additional staff (28 January 2020) and for an online modified version of the WERF questionnaire (08 September 2020). Additionally, we sought to add Epworth HealthCare, another hospital site on 26 June 2020. Approval by Epworth HealthCare was obtained on 3 July 2020, followed by Collaborative Research Agreement approval on 11 August 2020 and HRPO approval on 24 September 2020. We expect that recent access to private surgical samples at Epworth HealthCare will substantially build our capacity for sample collection during the pandemic as more surgeries will most likely be scheduled in private hospitals (Epworth HealthCare) than in public hospitals (Monash Health) as restrictions are eased. The public system is required to maintain capacity to respond to subsequent waves of the pandemic.

Subtask 1D – An Annual Ethics Report was submitted by **Initiating PI Gargett** to Monash Health HREC on 30 April 2020 which was approved 1 June 2020. This approved annual report was then submitted to HPRO on 25 June 2020 (HPRO acknowledged receipt on 27 June 2020), and approval is pending.

Subtask 2 – Patient screening and recruitment by the **Initiating PI Gargett** team occurred at Monash Health during a 3-week window (17 June –9 July 2020) between HPRO approval and Melbourne COVID-19 restrictions which did not permit category 3 surgeries (hysterectomies, endometrial biopsies) and a second 4-week window (5 – 31 October 2020) between when easing of Melbourne COVID-19 restrictions allowed recommencement of category 3 surgeries and the end of the reporting period (Fig 1). During this time, 14 participants showed interest in the study, and of those 9 were recruited and completed the questionnaire. Prior to HPRO approval, protocols for blood sample collection, plasma fraction and shipping to IMB for storage and genotyping were developed and tested to ensure their integrity, using funds and ethical approvals from other funded projects.

Subtasks 3 was removed from the revised SOW approved by the US Department of Defense as it was subsumed into Subtask 6.

Subtask 4 – 6-way FACS sorting single cells suspensions from patient samples.

Initiating PI Gargett has identified specific markers for a mesenchymal stem cell and 3 epithelial progenitor populations in human endometrium, which can be used to purify these rare cell populations from endometrial cell suspensions by multicolor fluorescent activated cell sorting (FACS). Purifying these rare subpopulations enables us to combine them together with mature cells from the same sample in similar proportions for each cell type. This provides greater opportunity to assess the cellular heterogeneity of the rarer populations at a higher resolution by single cell RNA sequencing (subtask 7). It will also enable the investigation of changes in gene expression modified by the risk genes in the various stem/progenitor populations, pinpointing those most relevant and opening further investigation into the precise pathogenesis of endometriosis.

During the 6-month wait for HRPO approval, the **Initiating PI Gargett** team optimized a 6-way multicolor fluorescent activated cell sorting (FACS) protocol for single cell suspensions derived from endometrial tissues using samples obtained under another human ethics approval and funds from other

grants. Briefly, endometrial tissue is dissociated into single cells which are then stained with a cocktail of fluorochrome-conjugated antibodies to lineage (CD45 and CD31), epithelial (EpCAM, SSEA-1, N-cadherin) and stromal (CD140b, SUSD2) markers, as well as a viability marker (propidium iodide) allowing fractionation of 6 populations of endometrial stem/progenitor cells and their progeny (Figure 2). As endometriosis surgeries have gradually recommenced since October 5 2020 (Fig 1), we have sorted 1 sample, barcoded and generated single cell cDNA libraries using the 10X Genomics at MU/Hudson for shipping to IMB (Subtask 5) for sequencing (Subtask 7) and deconvolution of scRNAseq data and bioinformatic analysis (Subtask 8).

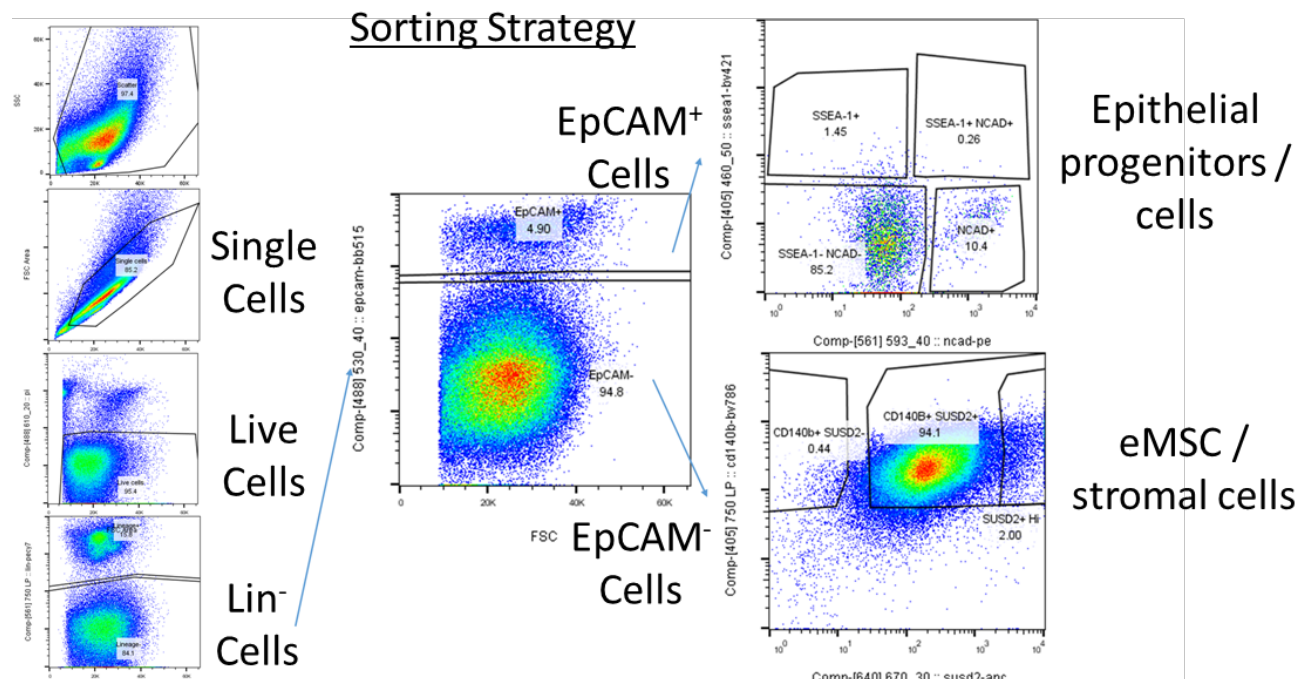


Figure 2: 6-way flow sorting strategy for endometrial stem/progenitor cells. Single, live, lineage negative (CD45⁻, CD31⁻) endometrial cells are sorted into stem/progenitors of epithelial origin (EpCAM⁺) based on expression of SSEA-1 or N-Cadherin and of stromal (CD140b⁺) origin-based expression of SUSD2.

Subtask 5 – Tagging and Barcoding FACS sorted cells to generate single cell cDNA libraries (contingent on Subtask 2 and 4).

Tagging of FACS sorted cells from individual samples (Subtask 4) allows pooling of samples for scRNA sequencing runs and subsequent bioinformatic demultiplexing, thereby reducing costs. Since writing the application the proposed supplier withdrew their product from market. We have validated the expression of tags from a new supplier in the 6 populations we will be sequencing (Table 2). Although more expensive, use of the tags still greatly reduces sequencing costs. These studies were performed using samples obtained under another ethics approval and funds from other grants held by **PI Gargett**. Since 5 October 2020, we have tagged one FACS sorted and recombined single cell suspensions and generated single cell cDNA libraries.

Table 2: Two Hashtags to be used in the study are expressed abundantly on all subpopulations of endometrial stem/progenitor cells and mature cells to be assessed in the study

Sorted Cell Subpopulation	% Hashtag 1	% Hashtag 2
EpCAM+NCAD+	100 %	100 %
EpCAM+SSEA-1+	100 %	100 %
EpCAM+NCAD+SSEA-1+	100 %	100 %
EpCAM+NCAD-SSEA-1-	100.0 %	100 %
CD140b+SUSD2+	100.0 %	97.3 %
CD140b+ SUSD2-	99.9 %	100.0 %

Subtask 6 Genotyping DNA from patient samples

In the original Statement of Work, we proposed designing and validating a multiplex PCR assay for rapid analysis of 14 endometriosis risk variants to categorize samples into high and low endometriosis risk based on these 14 genotypes. Since writing the application **Partnering PI Montgomery** and his group have continued to collaborate with the International Endometriosis Genetics Consortium on a new meta-analysis with 60,674 endometriosis cases and 701,926 controls. Results from these analyses have now identified 42 genome-wide significant loci for overall endometriosis risk, with 31 novel associations. The new risk variants will provide much greater precision to categorize samples into high and low endometriosis risk. We also expect risk prediction to improve as the likely causal genetic variants in these risk regions are better defined. Any custom designed genotyping panel will become rapidly outdated and we will therefore genotype all samples using Illumina Infinium Global Screening Array (GSA) BeadChips in preference to a custom assay with limited content. Illumina GSA BeadChips are run regularly on the Illumina BeadArray at the IMB, UQ. Samples can be analyzed as they are received without delaying risk prediction estimates for samples or delaying experiments.

Subtask 7 – Single cell RNA sequencing

Single-cell RNA sequencing (scRNA-seq) is a powerful method to generate RNA sequence data at a cellular level providing greater resolution than “bulk” level analyses and allowing greater understanding of cellular heterogeneity. Every cell has a distinct ontogeny informed by both autonomous and non-autonomous factors. These factors induce subtle alterations, influencing phenotype and potentially predispose subsets of cells to aberrant behaviour. The extent of cellular phenotype heterogeneity within cells of similar origin and presumed to be equivalent is becoming increasingly clear through the integration of genome-wide analysis and Next Generation Sequencing (NGS) at massively parallel single cell levels (scRNA-seq).

Analysis of individual cells also provides the opportunity to observe and classify subtle temporal variations in cell transitions through fate determination. Many distinct cell types evolve from common precursors evolving over the course of cell fate determination that occurs during development. Influences on cell fate determination can be both cell-autonomous (within the cell) such as regulatory modifications of gene expression, or non-autonomous (outside the cell) that may not always be consistent and induce subtle variations. Non-synonymous transitions for individual cells within larger groups may contribute to disease pathogenesis, progression, the creation of subtypes or how pathological cells respond to treatment and is worthy of further study.

Since writing the application we have conducted additional single cell RNA-sequencing (scRNA-seq) on endometrial stromal cell cultures to investigate the possible role of endometrial stromal cells in

endometriosis and cellular heterogeneity in endometrial stromal cell cultures. Cell lines were derived from patient samples collected with collaborators in Switzerland with other funding available to Partnering **PI Montgomery**. We include results from these experiments as further proof of principal for the single-cell RNA-seq experiments planned for this project which have been delayed as indicated above and in Fig 1. We generated scRNA-seq data on stromal cell cultures analysing 33,758 individual primary cultured endometrial stromal cells from 19 women with and without endometriosis measuring the expression of 20,950 genes. Sequencing was conducted in four libraries each with pooled individuals (see below). Using this dataset, we assessed variation between individual cells, identified cell groups within each sample and assessed the relationship between sub-groups of cells for *in vitro* behaviour in cell culture and clinical characteristics of the patients.

Identification of distinct fibroblast cluster in cultured endometrial stromal cells:

Between-cell gene expression was normalized using scTransform (Hafemeister and Satija 2019) prior to Louvain clustering of cells with the first 50 principal components. Initial examination of the clustering results revealed between-pool batch effects, identified as clusters containing cells that were predominantly from a single sequencing pool. This effect was corrected using the filtered, non-normalized data with Harmony (Korsunsky et al. 2019), a program that uses PCA for reducing the dimensionality within each iteration, which was renormalized and clustered using a parameter sweep across multiple resolutions between 0.01 and 1.0. Cluster stability was assessed using clustree (Zappia and Oshlack 2018) (**Figure 3A**), and clustering information from resolutions 0.1 and 0.6 were retained. This allowed us to examine endometrial stromal cell heterogeneity at both broad (3 clusters) and fine resolutions (21 clusters) (**Figure 3B and C**), while also preventing over-clustering and retaining the 'true' biology underlying the data. The number of cells from each pool in each cluster was stable (**Figure 3D**) giving confidence they were based on biology and not on batch effects induced by pooling of cells.

Cell phenotype classification: To establish which cell types were present in the primary cell cultures and to determine the extent of cellular heterogeneity, we classified individual cells into cell types based on their gene expression profiles. We applied the Single cell RNA-seq cell recognition package SingleR (Aran et al. 2019), superimposing the transcriptomic signatures of cells derived from the Human Primary Cell Atlas, curated from publicly available microarray datasets of Human Primary Cells Atlas (HPCA) (Mabbott et al. 2013) and the Blue+Encode reference dataset. The analysis revealed the distinct signature of fibroblast, mesenchymal stem cell and smooth muscle cell populations, all cells derived from a mesoderm and mesenchymal lineage (**Figure 4A**). SingleR assigned each cell in our dataset to 1 of 5 different cell types (**Figure 4B and D**), the most prevalent of which were fibroblasts, representing 84.78% of the total population. MSC were the next most commonly identified cell type making up 13.66% of the total cells, followed by smooth muscle cells that represented 1.21% of the population. The final two cell types identified included induced pluripotent stem (IPS) cells at 0.24% and tissue stem cells at 0.11%.

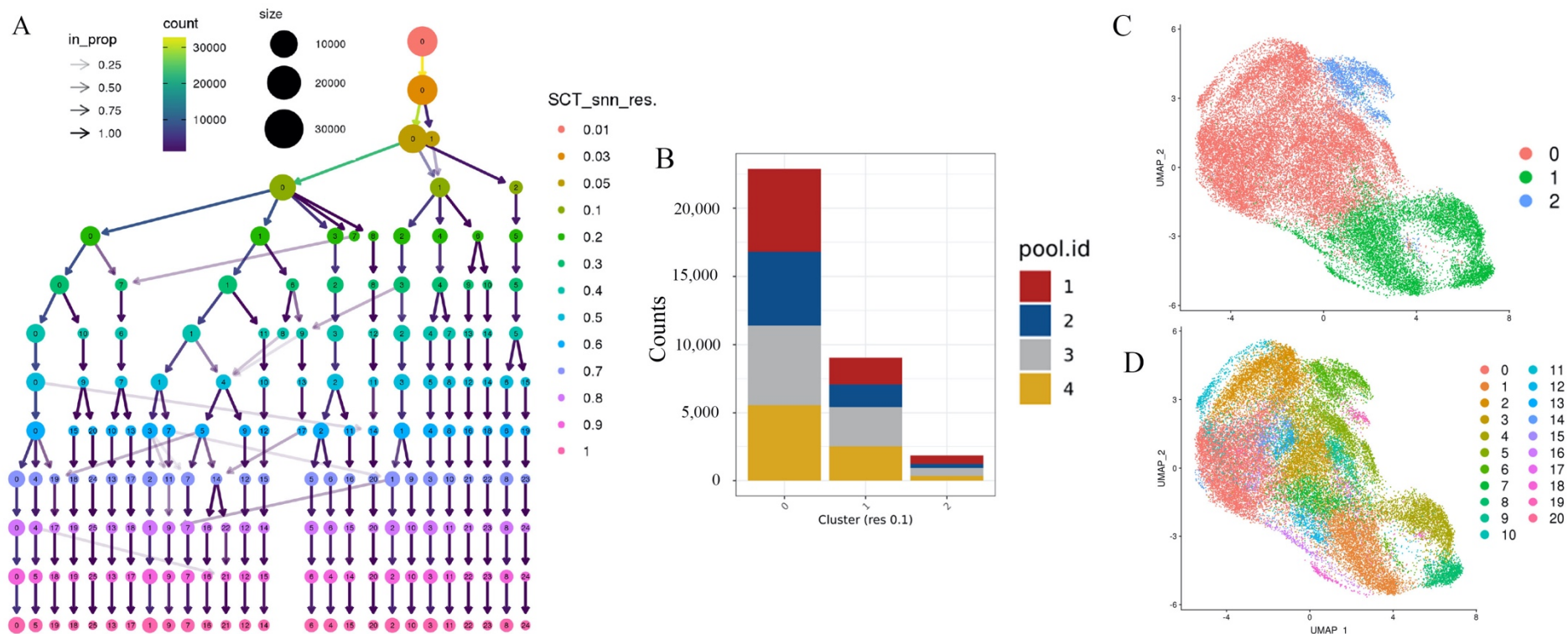


Figure 3: Cluster of cells with similar transcriptomes in endometrial stromal cell cultures. (A) *Clustree* analysis was performed to determine the stability of bioinformatically defined cellular relationships at increasing resolution and to identify the level of resolution relevant to biological variability. Cluster information was retained at both **(B)** 0.1 and **(C)** 0.6 resolution. **(D)** At cluster resolution cell numbers from each pool remained stable providing confidence the clusters were based on real biology.

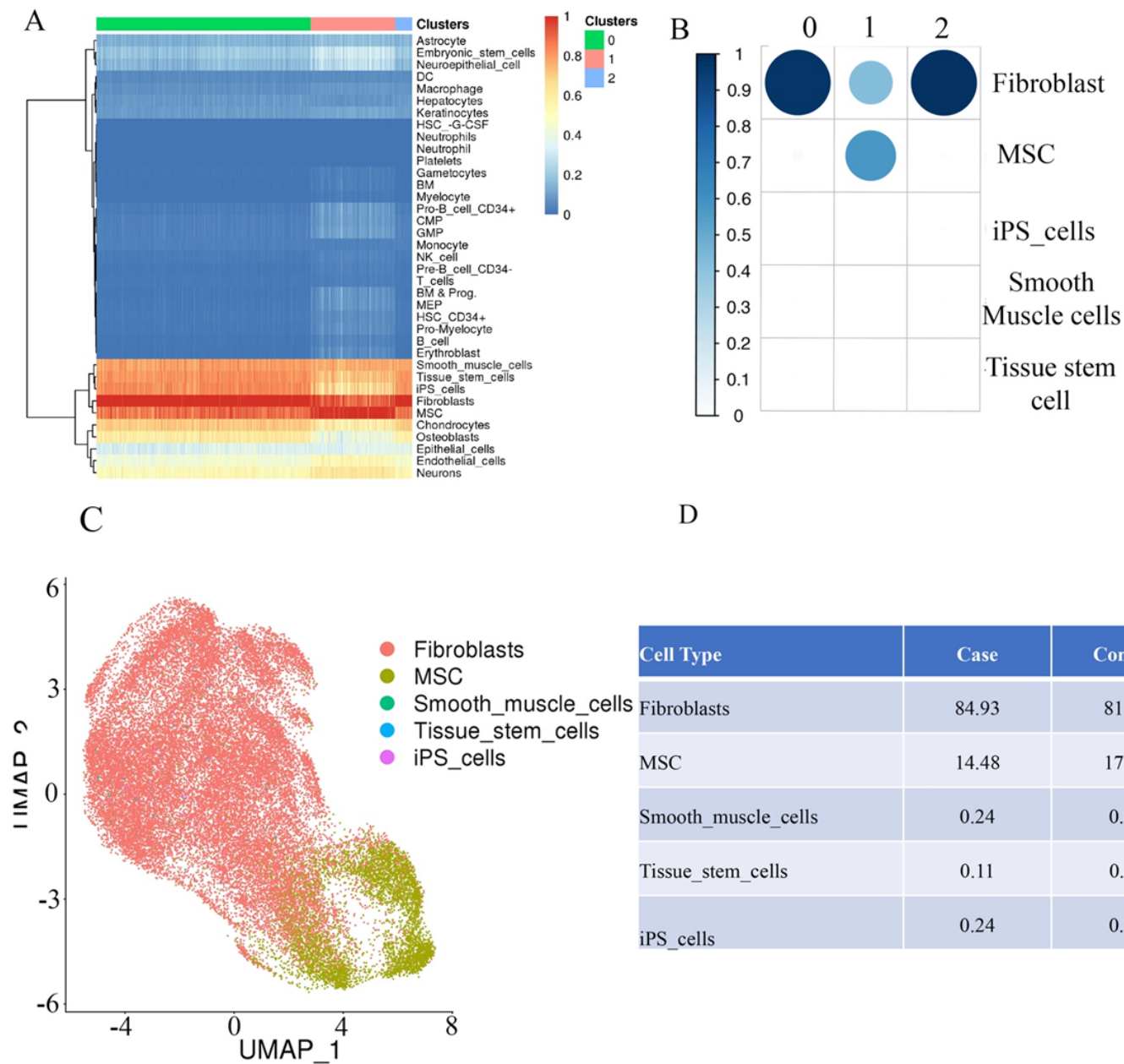


Figure 4: Cell and cluster phenotype identification.

Comparison to cells in the **(A)** Human Primary Cell Atlas revealed the distinct signature of **(B)** Fibroblasts, mesenchymal Stem cells, IPS cells, smooth muscle cells and tissue stem cells. **(C)** Overlay of cell types of the clusters revealed fibroblasts were the dominant cell in cluster 0 and 2. Cluster 1 was a mix of fibroblast and MSC with a dominant MSC phenotype. **(D)** Comparison of cell phenotypes between endometriosis cases and controls did not identify a significant difference between any of the cell types.

Our results show purified stromal cell cultures consist of mesenchymal stem cells and two distinct fibroblasts groups. These two-subgroups of fibroblasts were indicative of distinct temporal states of mesenchymal cell maturation. Significantly, one fibroblast sub-population was predominantly derived (85%) from women with endometriosis. Cell differentiation trajectory analysis indicated this cell type showed an incomplete maturation. These results identify the variation in primary cell cultures, highlighting a source of variation that may significantly influence *in vitro* experimental results and identifies a novel subset of endometrial stromal cell with an altered growth profile and associated with endometriosis. This study highlights the potential for ontogenetic variations during cell lineage fate determination to contribute to disease risk.

Subtask 10 – Establish Organoid Cultures (contingent on Subtask 2 and 4)

3D organoid cultures were recently described for human endometrial epithelium (EMOs) (Turco et al 2018, Boretto et al 2018), enabling long term culture of endometrial epithelial cells. These original EMOs were derived from fragments of freshly isolated endometrial epithelium. Large numbers of organoids can be generated per patient sample and can be used for multiple analyses. The organoids more closely represent endometrial glandular tissue than 2D culture models, can be passaged multiple times including at the clonal level and frozen for future use. The organoid model enables assessment of expression quantitative trait loci (eQTL) for the individual patient samples for each of the 42 gene regions associated with endometriosis risk, explore molecular pathways identified in scRNAseq of freshly isolated epithelial subpopulations of known endometriosis risk alleles, and assessing *in vivo* effects of identified molecular pathways on endometrial tissue growth in a xenograft model of endometriosis lesion development. The organoid cultures and xenografts provide powerful tools to determine the effect of genotype on the function of molecular pathways involved in the development of endometriosis lesions.

While waiting for HPRO approval to commence sample collection, the **Initiating PI Gargett** team has developed for the first time a human endometrial epithelial organoid culture protocol using our FACS sorted epithelial progenitor cell populations (Figure 5). The samples used were obtained under another human ethics approval using funds from other grants held by PI Gargett. Of particular importance is that these organoids were developed from single sorted endometrial epithelial progenitor cells. Our data shows that numerous organoids develop from single fresh N-cadherin+ epithelial cells, considered the most primitive endometrial epithelial progenitor (Nguyen et al 2017) and from co-expressing SSEA-1+N-cadherin+ progenitors, which we consider are the next cell type in the endometrial epithelial lineage. The SSEA-1+N-cadherin- cells however produced far fewer organoids, confirming their more mature state. (Figure 5). As gynecological surgeries have recently recommenced, we will begin generating the epithelial organoids from women with and without endometriosis as described in our original application.

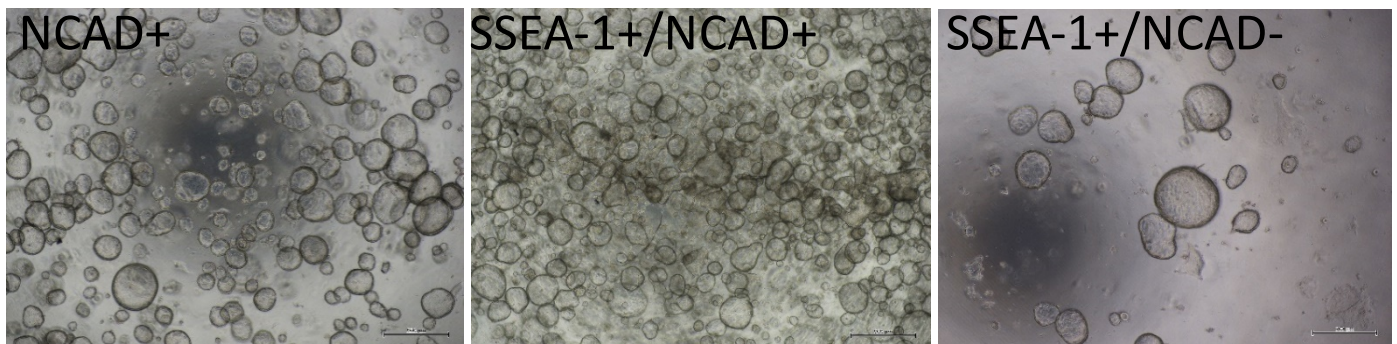


Figure 5: Single cell derived human endometrial epithelial organoids cultured from defined epithelial progenitor subpopulations

Subtask 11 – Cell Proliferation studies on organoids using IncuCyte

This has not yet commenced because we have only been able to collect tissues since 28 September 2020 and endometriosis tissue since 5 October 2020 (see Fig 1).

Aim 2 Subtasks and Milestones

To mitigate delays due to COVID-19 lockdown and HPRO approval, we applied to DoD on 3 June 2020 to bring forward subtasks 1, 2, 3a, 3b, 4, 5, 6 and 7 to enable us to collect menstrual fluid in a non-contact, COVID-19 safe manner (Fig 1) and commence organoid culture studies from these samples. Our modified SOW was accepted on 27 August 2020 (Fig 1).

Subtask 1 Subject recruitment for menstrual fluid and peripheral blood

Initiating PI Gargett team developed a non-contact COVID-19-safe method for collecting menstrual fluid from women on the surgical waiting list for endometriosis surgery, including collecting DNA for polygenic risk score determination using a saliva kit instead of peripheral blood (ethically approved). In the time available and in collaboration with endometriosis gynecologist **PI Rombauts** and his colleagues, we have recruited 9 women from surgical waiting lists from the approved 2 hospital sites. To date we have received 3 donated menstrual fluid samples.

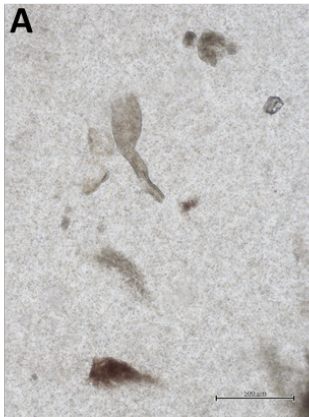
Subtask 2. Genotyping peripheral blood samples for all endometriosis risk variants

Saliva samples (n=3) will be shipped to **Partnering PI Montgomery** at IMB in batches for genotyping and polygenic risk score determination during the study period. Genotyping will commence in the second reporting period.

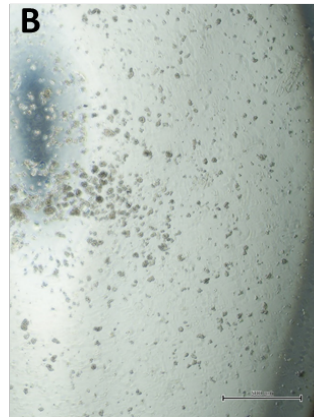
Subtasks 3a and 3b. MACS sort suspensions of dissociated menstrual blood samples; Organoid cultures of epithelial, stromal and epithelial/stromal cells and freezing

The **Initiating PI Gargett** team have developed methods for harvesting endometrial cells from tissue fragments in menstrual fluid using enzymatic dissociation and purification using Dynal magnetic bead sorting with EpCAM and SUSD2 antibodies respectively. This protocol was developed on menstrual fluid samples collected under another human ethics approval and with another source of funding. Subsequently, following HPRO approval and DoD approval of the modified SOW, we have obtained 3 samples and using menstrual fluid fragments (Fig 6A) generated menstrual blood organoids from purified EpCAM+ cells (Figure 6B and C), and collected EpCAM- cells, SUSD2+ eMSC and SUSD2- stromal cells from menstrual fluid cells.

Glands, stromal cells
Pre-Digest



EpCAM+ Glands
Post-Digest



EpCAM+ MBO
P0 (Day 7)

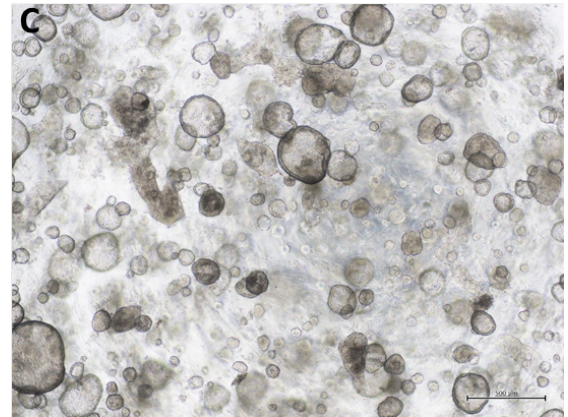


Figure 6: EpCAM+ menstrual blood organoids derived from menstrual fluid endometrial glands (A) enzymatically digested and purified using EpCAM magnetic beads (B) and seeded into a novel menstrual blood organoid (MBO) assay (C). Bar 500µm

Subtasks 4-7 have not commenced due to the delays and serious impact of COVID-19.

Aim 3 Subtasks and Milestones

Subtask 1 – Collection of eutopic endometrium and endometriotic lesions over 3 stages of the menstrual cycle

In the 5 weeks available for us, (Fig 1), there were only 3 weeks when endometriosis surgeries were scheduled, and most of these women did not meet our inclusion criteria (were on hormones). We were able to collect the following samples including peripheral blood for polygenic risk score determination: 2 lesions (Bladder peritoneum, Right ovarian cyst), 2 normal endometrium from a total of 3 participants.

However, prior to HRPO approval we have collected the following fixed samples under our existing Tissue Bank Ethics (using other funds) during the reporting period:

Endometriosis cases with matched fixed ectopic and eutopic	11
Endometriosis cases with fixed eutopic only	16
Endometriosis cases with fixed ectopic only	12
Controls with fixed eutopic endometrium	24
Other cases (e.g. adenomyosis, Asherman's, fixed control cysts, etc)	11

Because we were not able to recruit these women under the study ethics due to HRPO delays, these samples lack genomic data for PRS determination (a key component of the study).

iv. Other achievements

An invited review (**PI Gargett**) entitled "Cellular Origins of Endometriosis: towards novel diagnostics and therapeutics" by CE Filby, L Rombauts GW Montgomery, LC Giudice and CE Gargett has been accepted for publication on 04/29/20 in Seminars in Reproductive Medicine (appendix A).

We have submitted several abstracts containing data from other projects related to this SOW. The following were

1. Presented at the Society for Reproductive Biology (August 2019)
 - a. Endometrial Stem/Progenitor Cells: Cellular Origins of Endometriosis? C Filby, **Invited Oral Presentation**
 - b. Quantification and clonogenicity of stem/progenitor cells in menstrual blood from healthy women, C Gargett, S Suke, C Filby, K Wyatt, J Evans, 18-21 August, 2019 **Oral presentation**
 - c. Expression quantitative trait loci in endometrial stromal cells isolated from women with and without endometriosis B McKinnon, S Mortlock, J Crawford, M Mueller, G Montgomery **Poster**
2. Gargett CE New Concepts in the Etiology of Endometriosis. Asian Research and Training Institute for Skill Transfer (ARTIST) Knowledge Café Webinar 19 June 2020. **Invited presentation.**
3. Gargett CE, Filby CE, Cousins FL 1 in 10 women are affected by endometriosis. Why does it take so long to diagnose? The Conversation August 2020. <https://theconversation.com/1-in-10-women-are-affected-by-endometriosis-so-why-does-it-take-so-long-to-diagnose-141803>
4. Accepted for presentation at the 14th World Congress on Endometriosis (initially May 2020, delayed to February 2021)
 - a. Menstrual fluid cellular and protein content – a potential minimally invasive screening tool for adenomyosis and endometriosis, C Filby, K Wyatt, J Evans, C Gargett, **Oral Session #04 - Molecular Targets For Diagnosis and Therapy**
 - b. Single cell gene expression analysis reveals endometrial stromal cells harbor subsets with gene expression signatures indicative of precursor endometrial mesenchymal stem cells. B McKinnon, S Lukowski, S Mortlock, J Crawford, M Mueller, G Montgomery **Seminar presentation**, Session #09, Genetics, epigenetics and transcriptomics

b. Opportunities for Training and Development

The research nurse and scientific team at Hudson/Monash underwent “in house” training in use of the purpose-designed Research Database at UQ for inputting and storing participant data, the questionnaire and sample tracking for collection and dispatch of biological samples. These occurred at 2 face-to-face meetings, one at the Hudson in Melbourne for planning on 10 October 2019 and the second at UQ on 12 November 2019 for practical hands on training for Hudson team members.

The research team at Hudson/Monash attended the following free/member symposiums related to developing expertise in the project:

- Organoids Are Us, hosted by the Doherty Institute, June 25 2019
- Emerging Technologies in Organoid Research, hosted by the Monash BDI Epithelial Regeneration Laboratory, November 26, 2019

- Applying Organoids: The 3D Frontier, Parts 1-4 weekly webinars hosted by the International Society for Stem Cell Research, 8, 14, 21 and 29 October, 2020

The World Congress of Endometriosis 2020 conference was postponed until 24-27 Feb 2021, due to the COVID-19 pandemic.

c. Dissemination of Results

Nothing to report.

d. Plan for Accomplish Goals in Next Reporting Period

- i. **Milestone 1a.** Local AEC and ACURO approvals will be sought in the next reporting period as the animal studies will commence in FY2021/2022.
- ii. **Sample collection.**
We plan to undertake concurrent work on Aims 1 and 2 during the next reporting period as surgeries have now recommenced as Victoria slowly emerges from a second COVID-19 wave, albeit below normal volume. With recent HPRO approval at a second hospital site (Epworth HealthCare) we anticipate substantially increasing our tissue collection rate over the next reporting period.
- iii. **Graduate student recruitment**
To increase productivity to overcome delays, we are currently recruiting a PhD student to work on this project, who is awaiting the outcome of a scholarship application to Monash University. We have advertised our project at recent student Open Days for Monash University and are attracting a lot of interest from students seeking to do a one-year Honours research year. We have recruited a Bachelor Medical Science student to work on this project (may lead to their enrolling into PhD program).

4. Impact

a. Impact on Principal Discipline

Generating organoids from defined human endometrial epithelial progenitor populations will provide the endometrial biology field an approach to investigate the biology of these stem/progenitor cells and their capacity to differentiate. They could also be used for drug screening for endometrial disorders of endometrial epithelial cells including endometriosis.

Endometriosis recurrence is common with up to 60% of women experiencing a new episode of endometriosis within 5 years after surgical removal of the lesions. Moreover, each subsequent episode of endometriosis will present differently both in term of symptoms and physical presentation. If the connection between specific symptoms and particular episodes of endometriosis are not clearly delineated during data collection the data can be compromised. Currently available data collection tools do not take into account the potential of women experiencing multiple episodes of endometriosis and the likelihood that each episode will be different. The easily accessible online data collection tool we have development in the form of a patient questionnaire has been designed to ensure symptom details are linked to the tissue collected. In addition, symptomatic details of previous episodes of endometriosis, their onset and progression are captured. It is expected this will significantly improve data accuracy and linkage for this project and additional projects for endometriosis.

b. Impact on Other Disciplines

Nothing to report

c. Impact on Technology Transfer

Nothing to report

d. Impact on Society Beyond Science and Technology

Nothing to report

5. Changes/Problems

i. Delays in approvals

The local institutional human ethics approval was obtained ahead of schedule. Additionally, the Monash Health Site specific approval was submitted (August 29 2019) and approved (September 24 2019) subject to a fully executed research agreement within expected timeframes. However, there were delays in the collaborative research agreement which could not be initiated until the grant was awarded (1 August 2019 –first day of the project) and therefore was not finalized until November 19 2019 and fully executed until December 4 2019. HRPO approval was sought (December 11 2019) and took 6 months for approval (17 June 2020) (Fig 1), resulting in >5 months of lost time to recruit participants, collect samples, and perform experiments.

ii. COVID-19 pandemic

HRPO approval coincided with a lack of gynecological surgeries in our approved hospital site due to COVID-19 which has hampered our progress in the 19 weeks of reportable time between HRPO approval and the end of Year 1 (17 June 2020 to 30 November 2020), including the 3-month extension granted on August 27, 2020 (Fig 1). To date, there has been a combination of complete suspension and significant reduction in category 3 gynecological surgeries, and stage 3 and 4 (July 8 2020 and on-going at the time of writing) restrictions in Victoria (Figure 1). Those urgent scheduled surgeries did not include women with endometriosis and did not meet the study inclusion/exclusion criteria until October 5 2020, when endometriosis surgeries recommenced. We anticipate that COVID-19 will continue to have an impact on surgical recruitment until mid-2021. For these reasons we have started on Aim 2, utilizing menstrual fluid, earlier than originally planned.

b. Changes in Approach

i. COVID-19

In anticipation of an expected delay in surgical samples for Aims 1 and 3, we requested modifications to the SOW on June 3 2020, which was granted on 27 August 2020 (Fig 1) to enable us to proceed with collection of menstrual fluid samples in Aim 2 earlier while we waited for less frequent surgical samples. To facilitate the collection of menstrual fluid during the pandemic we also sought and were granted (11 May 2020) an exemption by Monash Health to collect Menstrual Fluid during the pandemic. We developed a non-contact method for recruitment, consent, collection of samples and data, including generation of an electronic version of the patient survey approved 9 August 2020 (see Accomplishments). These non-substantive changes to the protocol will minimize the spread of COVID-19. We anticipate that numerous women with endometriosis will undergo surgery in the coming months due to the backlog of delayed surgeries spanning at least 6 months, especially in the private health system at Epworth HealthCare.

We have actively sought a good working relationship with the surgeons who work in the private hospital system, having monthly meetings and email/phone contact in between. Epworth Credentialing has been granted to Jenny Ryan Research nurse, so she has access to the Epworth operating theatres where most of the surgeries will be performed.

ii. Methodological

1. Our original application specified the use of a cell barcoding technology (Aim 1 Subtask 5) supplied by BD Biosciences. A significant increase in cost and the availability of similar technology from another supplier (Biolegend) for a lower cost meant we have slightly modified our methodological approach without an anticipatable change in project outcome.
2. **Risk score determination** data generated by the International Endometriosis Consortium of which Partnering PI Montgomery contributes has identified 42 endometriosis risk regions, as opposed to the original 14. Continued analysis is likely to identify additional regions. Any custom panel is likely to become obsolete. Inclusion of additional regions will significantly strengthen risk prediction therefore we will generate genome wide genotyping data on all samples using GSA chips and imputation. This will enable risk scores are generated with the current 42 regions, as well as the potential to include additional regions once identified.

c. Actual/anticipated problems/delays and actions/plans for resolution

i. Limited Surgical Samples

We anticipate there will be a slower rate of recruitment and consent for surgical samples in the short-term (Aims 1 and 3) due to COVID-19, particularly in the public system. We requested and have DoD approved for a 12 month no-cost extension to allow extra time to collect these samples. Our DoD application requesting modification to SOW timelines for Aim 2 to enable earlier collection of menstrual fluid samples while surgeries are less frequent has also been accepted (August 27 2020) (Fig 1).

ii. Reduced Participation due to COVID-19

It is possible that women are less likely to participate in research during the pandemic due to the excessive burden women are experiencing during COVID-19 restrictions, or from fear of exposure to COVID-19. To alleviate these and other concerns, we are recruiting and consenting prior to the day of surgery to give participants time to consider participation and to seek reassurance that the risk is no greater whether or not they choose to participate.

iii. Access to Participating Institutions

1. Monash Health

While research nurse access to Monash Health is not limited by the pandemic, the number of surgeries scheduled has reduced. These women who are waiting for their surgery are prime candidates for recruitment to Aim 2 (menstrual fluid collection).

2. Epworth HealthCare

We expect the number of surgeries to begin to increase rapidly at Epworth HealthCare following release from current Stage 4 restrictions as the 6-month backlog of surgeries are scheduled in the coming months. Our initial recruitment from this site which commenced in early October 2020 appears promising.

3. Monash University

During stage 4 restrictions, access to Monash University was not permitted. However, this coincided with suspended category 3 gynecological surgeries. While access to Monash University for flow sorting and single cell library preparation has resumed, we will have to sort 6 populations sequentially instead of concurrently, due to a lack of lab space for the hashtagging. We believe this will pose no additional barrier to achieving those aims.

iv. Collaborative Work/Meetings

Originally planned face-to-face collaborative meetings have been and will continue to be hosted by ZOOM (online). We seek permission to delay expenditure for these meetings to a later reporting period and flexibility to use these funds in other ways.

It was originally proposed for key personnel in Brisbane to travel to Melbourne and learn organoid culture techniques in person from the Initiating PI Gargett Laboratory. Currently limited sample collection and travel restrictions between Brisbane and Melbourne, due to COVID-19 has made in-person training difficult and sample availability limited.

Alternative arrangements have been made to establish organoid cultures in Brisbane prior to restrictions lifting. Initiating PI Gargett laboratory has provided protocols, guidance and aliquots of the exact reagents used in Melbourne. Additionally, live streaming discussion during culturing procedures will be used to assist protocol consistency across sites. Samples from other research projects collected with ethical approval will be used for optimization steps only until sample collected under the current project are available. Once travel restrictions between Brisbane and Melbourne have been lifted key personnel in Brisbane will travel to Melbourne to ensure compatibility and consistency between organoid models developed at both sites.

d. Changes with Significant Impact on Expenditure

Reduction in Consumables Spending As we have not been able to recruit the expected number of samples due to HRPO delays and COVID-19.

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

There were no significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects. We have developed a non-contact method for menstrual fluid collection detailed in 5.b.ii.

Animal work has not yet commenced for this study.

6. Products

a. Publications, conference papers, and presentations

- i. Caitlin E. Filby, Luk Rombauts, Grant W. Montgomery, Linda C. Giudice, Caroline E. Gargett; Cellular Origins of Endometriosis: Towards Novel Diagnostics and Therapeutics; Seminars in Reproductive Medicine; Vol. 38: 2020; 1–15; (appendix A).

b. Website(s) or other Internet site(s)

- i. The Conversation article on delayed diagnosis for women with endometriosis
<https://theconversation.com/1-in-10-women-are-affected-by-endometriosis-so-why-does-it-take-so-long-to-diagnose-141803>

c. Technologies or techniques

- i. 6-way sort technique for endometrial surgical samples: A protocol has been developed and optimized for the sorting of endometrial surgical samples into 6 populations including 4 stem/progenitor populations using multicolor fluorescence-activated cell sorting (FACS) (see accomplishments Figure 2 for details).
- ii. Endometrial organoids derived from single epithelial progenitor cells i.e. N-cadherin+, SSEA-1+N-cadherin+, SSEA-1+N-cadherin- cells (accomplishments (Figure 5).
- iii. EpCAM+ menstrual blood organoids

These protocols will not be shared outside of the collaborative research project until publication of the techniques and results.

d. Inventions, patent applications, and/or licenses

Nothing to Report.

e. Other Products

Nothing to report

7. Participants & Other Collaborating Organizations

a. Individuals working on the project

Name:	<i>Caroline Gargett</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0002-3590-2077</i>
Nearest person month worked:	<i>3.25-person months</i>
Contribution to Project:	<i>Lead PI for this collaborative project.</i>
Funding Support:	<i>Funding support provided from this award.</i>

Name:	<i>Caitlin Filby</i>
Project Role:	<i>Post-Doctoral Researcher</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0003-2272-5902</i>
Nearest person month worked:	<i>10.85-person months</i>
Contribution to Project:	<i>Obtained ethics approvals (HREC, SSA, CRA and HRPO), developed assays and optimized protocols (6-way FACS Sort, Hastagging, single cell organoids, menstrual blood organoids), recruited and trained research staff, generated and analysed data, contributed to modified participant questionnaire, reporting (ethics, HRPO and DoD technical report)</i>
Funding Support:	<i>Funding support provided from this award.</i>

Name:	<i>Katherine Wyatt</i>
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	<i>11.38-person months</i>
Contribution to Project:	<i>Optimized protocols, generated and analysed data, ordering and management of reagents.</i>
Funding Support:	<i>Funding support provided from this award.</i>

Name:	<i>Jennifer Ryan</i>
Project Role:	Clinical Research Nurse
Researcher Identifier (e.g. ORCID ID):	<i>NMW0001370460 (Australian Health Practitioner Regulation Agency registration number)</i>
Nearest person month worked:	<i>7.13-person months</i>
Contribution to Project:	<i>Screened and recruited participants, collected clinical samples and medical data.</i>
Funding Support:	<i>Funding support provided from this award.</i>

Name:	<i>Grace Heo</i>
Project Role:	Clinical Research Nurse
Researcher Identifier (e.g. ORCID ID):	<i>NMW0001621910 (Australian Health Practitioner Regulation Agency registration number)</i>
Nearest person month worked:	<i>1-person months</i>
Contribution to Project:	<i>Coordinated research nurse staffing and training, participant screening and recruitment, and handling of medical data.</i>
Funding Support:	<i>Internal funds</i>

Name:	<i>Luk Rombauts</i>
Project Role:	<i>Co-investigator; Clinical Collaborator</i>
Researcher Identifier (e.g. ORCID ID):	<i>MED0001174149 (Australian Health Practitioner Regulation Agency registration number)</i>
Nearest person month worked:	<i>1-person month</i>

Contribution to Project:	<i>Provided clinical knowledge and facilitated patient screening and recruitment.</i>
Funding Support:	<i>Medical Practitioner</i>

Name:	<i>Jim Tsaltas</i>
Project Role:	<i>Associate Investigator; Clinical Collaborator</i>
Researcher Identifier (e.g. ORCID ID):	<i>MED0001131319 (Australian Health Practitioner Regulation Agency registration number)</i>
Nearest person month worked:	<i>1-person month</i>
Contribution to Project:	<i>Provided clinical knowledge and facilitated patient screening and recruitment.</i>
Funding Support:	<i>Medical Practitioner</i>

Name:	Brett McKinnon
Project Role:	<i>Post-Doctoral Researcher</i>
Researcher Identifier (e.g. ORCID ID):	0000-0002-9881-1252
Nearest person month worked:	<i>7.5-person months</i>
Contribution to Project:	<i>Obtained ethics approvals (HREC UQ, HRPO, UQ), designed and prepared online patient questionnaire, assisted with implementation into database, developed and, optimized single cell sequencing analysis protocols,</i>
Funding Support:	<i>Funding support provided from this award.</i>

Name:	Dr Sally Mortlock
Project Role:	<i>Post-Doctoral Researcher</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1.25-person month</i>
Contribution to Project:	<i>Performed and optimized single cell sequencing data analysis</i>
Funding Support:	<i>Funding support provided from this award.</i>

Name:	Prof Montgomery
Project Role:	<i>Partnering Principal Investigator</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>2.5-person months</i>
Contribution to Project:	<i>Partnering PI for the collaborative project</i>
Funding Support:	<i>Funding support provided from this award.</i>

b. Change in Active Support of PI / Key Personnel

None to Report.

c. Partnering Organizations

i. Organization Name:

Hudson Institute of Medical Research

ii. Location of Organization: (if foreign location list country)

27-31 Wright Street, Clayton VIC 3168, AUSTRALIA

iii. Partner's contribution to the project (identify one or more)

- Initiating PI and her team
- Coordinated local Institutional HREC, AEC & Biosafety approvals, including minor amendments.
- Designed and commenced patient recruitment and consent, and sample collection.
- Developed and executed experimental planning as per Statement of Work.
- Location of laboratories and platforms for experimental work

iv. Financial support;

Funding support provided from this award W81XWH1910365 PR180827

v. In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);

Laboratory equipment and infrastructure required for experimental work
Intellectual property of optimized experimental processes

vi. Facilities (e.g., project staff use the partner's facilities for project activities);

Office and research facilities utilized for conductance of project activities.

vii. Collaboration (e.g., partner's staff work with project staff on the project);

Key project staff are situated permanently at this partnering organization.

viii. Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and

Project staff use the contracting and partnering organization facilities.

ix. **Other.** None to Report.

i. **Organization Name:**

Monash University

ii. **Location of Organization: (if foreign location list country)**

Wellington Road, Clayton, Victoria 3800, AUSTRALIA

iii. **Partner's contribution to the project (identify one or more)**

- Administering Institution
- Location of platforms for experimental work

iv. **Financial support;**

None to Report. Administers funds on behalf of Hudson Institute of Medical Research.

v. **In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);**

Administrative support

vi. **Facilities (e.g., project staff use the partner's facilities for project activities);**

Research facilities utilized for conductance of project activities.

vii. **Collaboration (e.g., partner's staff work with project staff on the project);**

Partner staff provide services to project staff at this partnering organization.

viii. **Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and**

Project staff use the contracting and partnering organization facilities.

ix. **Other.** None to Report.

i. **Organization Name:** Monash Health

ii. **Location of Organization: (if foreign location list country)**

246 Clayton Road, Clayton, VIC 3168, AUSTRALIA

iii. **Partner's contribution to the project (identify one or more)**

- Responsible institutional HREC for Victorian Sites.
- Primary site for clinical collaborators, allowing for screening and recruitment of participants, and collection of samples.

iv. **Financial support;**

None to Report.

v. **In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);**

Monash Health clinical collaborators donate their time, expertise and access to ethically approved patient information to facilitate participant screening and recruitment, and sample collection.

- vi. **Facilities (e.g., project staff use the partner's facilities for project activities);**
Hospital databases utilized to facilitate participant screening and recruitment, and surgical theatres utilized for sample excision and collection.
- vii. **Collaboration (e.g., partner's staff work with project staff on the project);**
Clinical collaborators work with project staff on project.
- viii. **Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and**
Project staff use the partnering organization facilities (surgical theatres, medical databases)
- ix. **Other.** None to Report.
 - i. **Organization Name:** Epworth Health
 - ii. **Location of Organization: (if foreign location list country)**
320 Victoria Parade, East Melbourne VIC 3002, AUSTRALIA
89 Bridge Rd Richmond, VIC 3121, AUSTRALIA
 - iii. **Partner's contribution to the project (identify one or more)**
 - Secondary site for clinical collaborators, allowing for screening and recruitment of participants and collection of samples.
 - iv. **Financial support;**
None to Report.
 - v. **In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);**
Epworth Health clinical collaborators donate their time, expertise and access to ethically approved patient information to facilitate participant screening and recruitment, and sample collection.
 - vi. **Facilities (e.g., project staff use the partner's facilities for project activities);**
Hospital databases utilized to facilitate participant screening and recruitment, and surgical theatres utilized for sample excision and collection.
 - vii. **Collaboration (e.g., partner's staff work with project staff on the project);**
Clinical collaborators work with project staff on project.
 - viii. **Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and**
Project staff use the partnering organization facilities (surgical theatres, medical databases)
 - ix. **Other.** None to Report.
 - i. **Organization Name:** Institute for Molecular Bioscience, The University of Queensland
 - ii. **Location of Organization: (if foreign location list country)**
The University of Queensland,
306 Carmody Rd,
St Lucia

iii. Partner's contribution to the project (identify one or more)

Partnering PI and his team

Co-ordinated local institutional HREC approvals for UQ site

Designed and implemented patient questionnaire

Design and built dedicated database for secure patient data storage and co-ordination across sites

iv. Financial support;

Funding support provided from award (W81XWH-19-1-0364)

In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);

Laboratory equipment and infrastructure required for experimental work

Intellectual property of optimized experimental processes

v. Facilities (e.g., project staff use the partner's facilities for project activities);

Project staff has been able to use the facilities both laboratory and office, for experimental work, data collection and processing.

vi. Collaboration (e.g., partner's staff work with project staff on the project);

Partner's staff collaborate with project staff on the project.

vii. Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and

Project staff has had access to data collection tools developed by the partnering organization

viii. Other.

None to report

8. Special Reporting Requirements – Collaborative Award

Initiating and Partnering PIs are submitting a duplicative technical report which clearly outlines the responsible PI and research site for the various tasks as outlined in our identical Statement of Work.

9. Appendices

Appendix A: Publication Proof for Paper i): Caitlin E. Filby, Luk Rombauts, Grant W. Montgomery, Linda C. Giudice, Caroline E. Gargett; Cellular Origins of Endometriosis: Towards Novel Diagnostics and Therapeutics; Seminars in Reproductive Medicine; Vol. 38: 2020; 1–15.