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PRINCIPAL INVESTIGATOR: Caroline Gargett

CONTRACTING ORGANIZATION: Monash University, Clayton VIC 3800 Australia

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6. AUTHOR(S)
Professor Caroline Gargett, Dr Caitlin Filby
Professor Grant Montgomery, Dr Brett McKinnon
Ms Katherine Wyatt
E-Mail: caroline.gargett@hudson.org.au

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Monash University
Level 1, Chancellery Building D,
Clayton Campus
26 Sports Walk
Clayton VIC 3800 Australia

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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 eutoptic 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.

15. SUBJECT TERMS
Endometriosis, endometrial stem/progenitor cells, genetic risk, endometriosis pathogenesis, prognostics,

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TABLE OF CONTENTS

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.

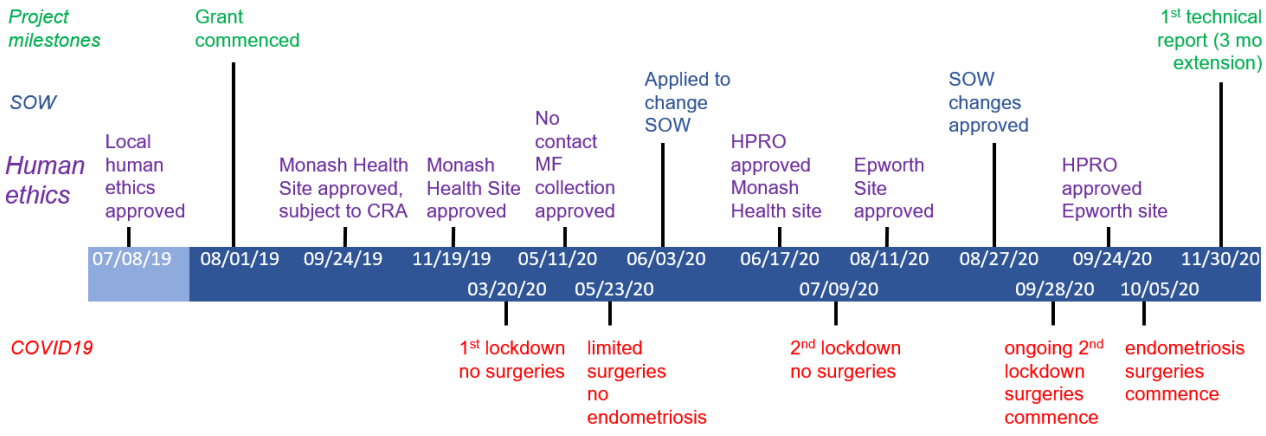


Figure 1 – Timeline showing the impact of timing of ethics approval and COVID-19 on our ability to collect human tissue samples for our experimental work during stage 3 and ongoing stage 4

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 accompanying 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 27June 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).

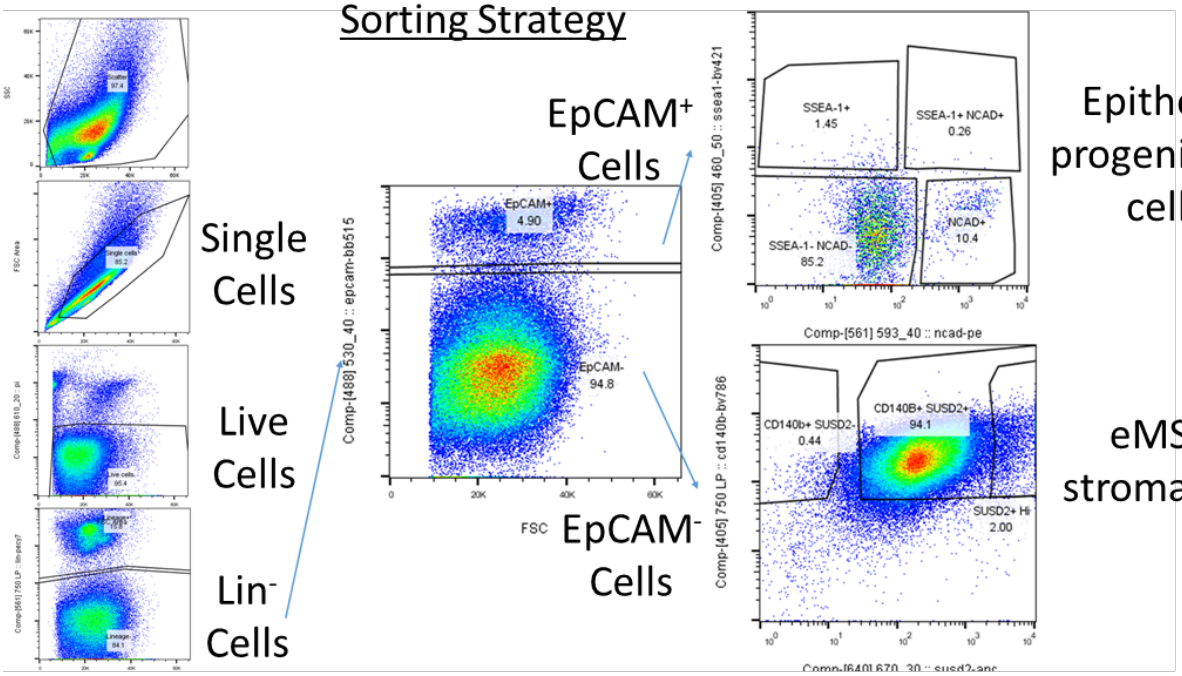


Figure 2: 6-way flow sorting strategy for endometrial stem/progenitor cells. Single, live, lineage negative (CD45-, CD31-) endometrial cells are sorted into stem/progenitors of epithelial origin (EpCAM+) based on expression of SSEA-1 or N-Cadherin and of stromal (CD140b+) origin-based expression of SUSD2.

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%.

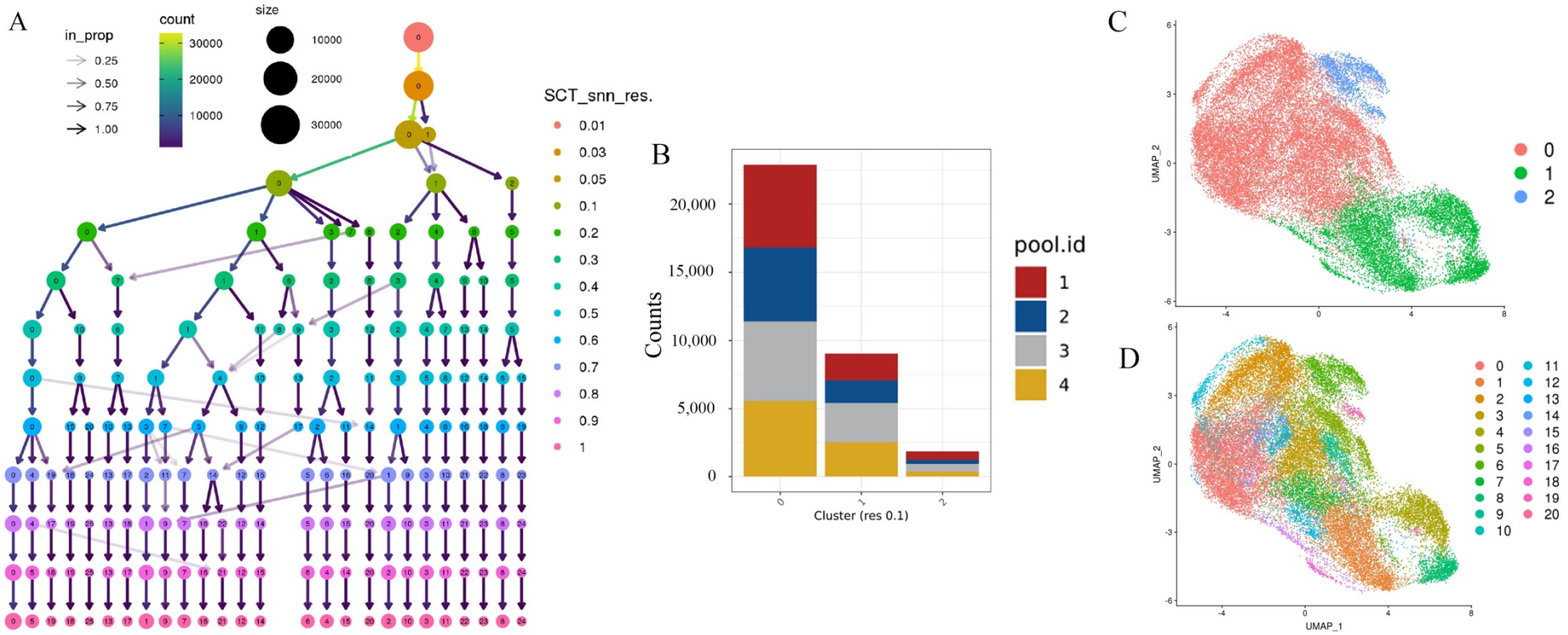
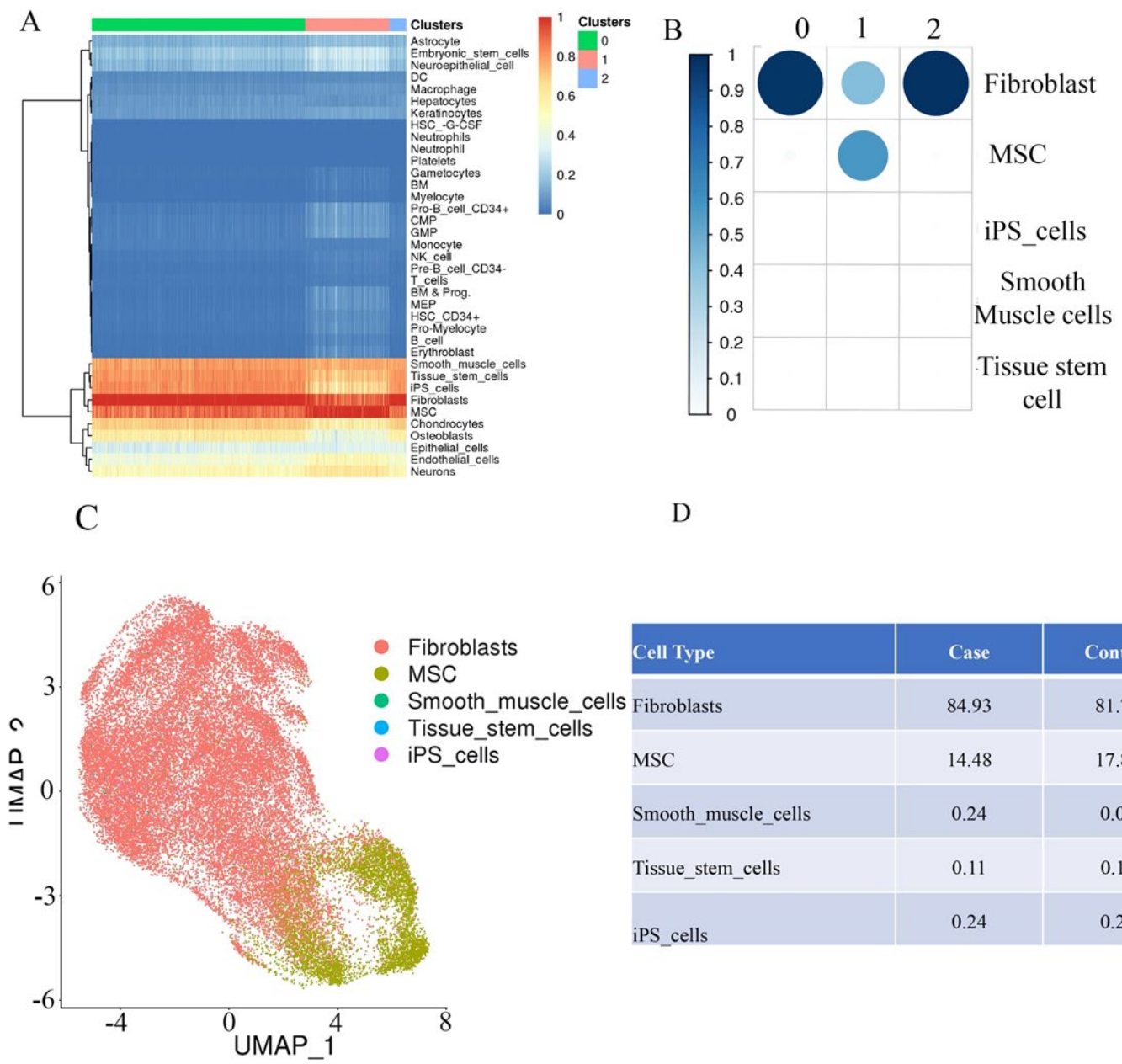


Figure 3: Cluster of cells with similar transcriptomes in endometrial stromal cell cultures. (A) *Clustree* analysis was performed to determine the stability of bioinformatically defined cellular relationships at increasing resolution and to identify the level of resolution relevant to biological variability. 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.



Cell Type	Case	Control	p VALUE
Fibroblasts	84.93	81.74	
MSC	14.48	17.83	
Smooth_muscle_cells	0.24	0.08	0.0773
Tissue_stem_cells	0.11	0.11	
iPS_cells	0.24	0.25	

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.

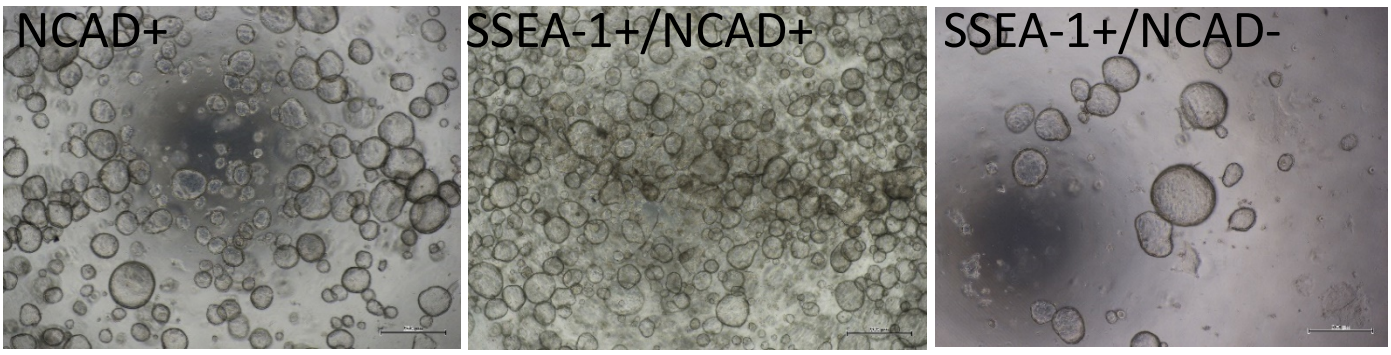


Figure 5: Single cell derived human endometrial epithelial organoids cultured from defined epithelial progenitor subpopulations

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.

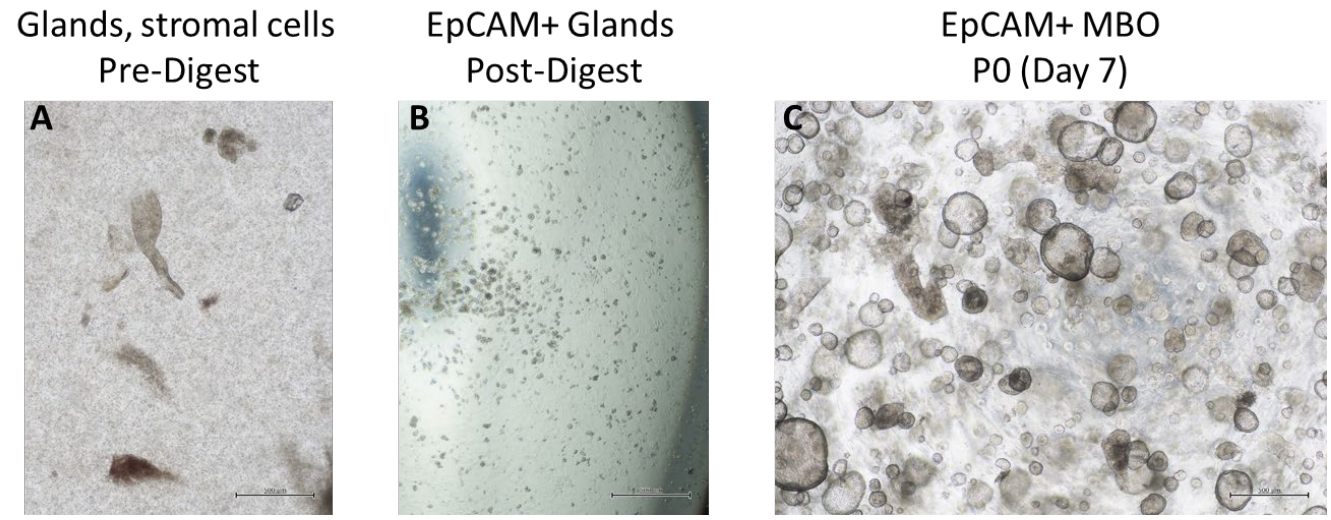


Figure 6: EpCAM+ menstrual blood organoids derived from menstrual fluid endometrial glands (A) enzymatically digested and purified using EpCAM magnetic beads (B) and seeded into a novel menstrual blood organoid (MBO) assay (C). Bar 500µm

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

Name:	*Caitlin Filby*
Project Role:	*Post-Doctoral Researcher*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-2272-5902*
Nearest person month worked:	*10.85-person months*
Contribution to Project:	*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)*
Funding Support:	*Funding support provided from this award.*

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

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

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

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

Contribution to Project:	*Provided clinical knowledge and facilitated patient screening and recruitment.*
Funding Support:	*Medical Practitioner*

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

Name:	Brett McKinnon
Project Role:	*Post-Doctoral Researcher*
Researcher Identifier (e.g. ORCID ID):	0000-0002-9881-1252
Nearest person month worked:	*7.5-person months*
Contribution to Project:	*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,*
Funding Support:	*Funding support provided from this award.*

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

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

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 W81XWH1910364 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
Brisbane Queensland 4072, AUSTRALIA

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 facilitates 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.

Cellular Origins of Endometriosis: Towards Novel Diagnostics and Therapeutics

Caitlin E. Filby, PhD[1] Luk Rombauts, MD, PhD[2] Grant W. Montgomery, PhD[3]
Linda C. Giudice, MD, PhD[4] Caroline E. Gargett, PhD[1]

[1] The Ritchie Centre, Hudson Institute of Medical Research, Department of Obstetrics and Gynaecology, Monash University, Melbourne, Victoria, Australia
[2] Department of Obstetrics and Gynaecology, Monash University, Reproductive Medicine at Women's Health, Monash Health, Monash IVF, Melbourne, Victoria, Australia
[3] UQ Genome Innovation Hub, Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland, Australia
[4] Department of Obstetrics, Gynecology and Reproductive Sciences, Center for Reproductive Sciences, University of California, San Francisco, California

Address for correspondence Caroline E. Gargett, PhD, The Ritchie Centre, Hudson Institute of Medical Research, TRF L5, 27-31 Wright Street, Clayton, VIC 3168, Australia (e-mail: caroline.gargett@hudson.org.au).

Semin Reprod Med 2020;38:1–15

Abstract

Endometriosis remains an enigmatic disease of unknown etiology, with delayed diagnosis and poor therapeutic options. This review will discuss the cellular, physiological, and genomic evidence of Sampson's hypothesis of retrograde menstruation as a cause of pelvic endometriosis and as the basis of phenotypic heterogeneity of the disease. We postulate that collaborative research at the single cell level focused on unlocking the cellular, physiological, and genomic mechanisms of endometriosis will be accompanied by advances in personalized diagnosis and therapies that target unique subtypes of endometriosis disease. These advances will address the clinical conundrums of endometriosis clinical care—including diagnostic delay, suboptimal treatments, disease recurrence, infertility, chronic pelvic pain, and quality of life. There is an urgent need to improve outcomes for women with endometriosis. To achieve this, it is imperative that we understand which cells form the lesions, how they arrive at distant sites, and what factors govern their ability to survive and invade at ectopic locations. This review proposes new research avenues to address these basic questions of endometriosis pathobiology that will lay the foundations for new diagnostic tools and treatment pathways.

Keywords
- endometriosis
- retrograde menstruation
- pathogenesis
- stem/progenitor cells
- endometrial waves

Endometriosis is a chronic, inflammatory disease affecting between 6 and 11% of girls and women,[1–3] where cells of endometrial histology form lesions throughout the pelvic cavity and in rare cases at distant sites. There are three main types of endometriosis lesions: (1) endometriomas, which form within the ovaries; (2) deep infiltrating endometriosis (DIE) lesions, which invade the bowel and other organs; and (3) superficial lesions, which adhere to the surface of organs and the peritoneum. Endometriosis lesions can cause debilitating pain and their infiltration into pelvic organs results in further disease and infertility. The current diagnostic standard is histologically confirmed presence of endometrial stromal fibroblasts and epithelial cells in endometriosis lesions excised during laparoscopic surgery. Presently, the delay to diagnosis is a staggering $>$ 7 years due in part to the lack of a noninvasive diagnostic test, the wide range in symptoms experienced by women, and in some cases a lack of awareness and education of endometriosis among

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both the community and healthcare providers. During this diagnostic delay, girls and women suffer without optimal care and risk progression of their disease and its sequelae. There is no cure for endometriosis, and debilitating endometriosis-associated pain impedes a woman's potential to thrive personally, professionally, and financially. This silent, long-neglected disease costs €9.95b in the United Kingdom,[4] USD 53.76b[4] in the US and AUD 9.67b[5] in Australia annually. Endometriosis is also a major cause of infertility, affecting the female partner of 10 to 40% of couples undergoing *in vitro* fertilization. Many women with endometriosis undergo numerous operations to remove lesions (often with limited success and 30–60% recurrence within 12 months[6]) to overcome the pain and increase chances of pregnancy. Current drug treatments for endometriosis are hormonally based (thus precluding pregnancy), often ineffective, and can only be used short term due to severe adverse effects in young women. Defining the *cellular origins of endometriosis*, their genomic program, physiological mechanisms, and the developmental context when cells establish endometriotic lesions are anticipated to enable development of novel diagnostic tools that reduce the time to diagnosis, a single definitive surgery based on etiology and prognosis, and personalized therapies that take into account the heterogeneous nature of this disease.

Pathogenesis of Endometriosis

The causes of endometriosis are unknown, although several theories with a strong scientific basis have been invoked, including (1) retrograde menstruation whereby endometrial cells and tissue fragments, shed during menstruation, reflux through the fallopian tubes into the pelvic cavity, escape immune surveillance, and initiate a neuroangiogenic and proinflammatory response; (2) coelomic metaplasia whereby the coelomic lining (developmental origin of Müllerian ducts and uterus) transforms from one differentiated cell type (coelomic) to another (endometrial)[7]; (3) embryonic rests whereby Müllerian remnants give rise to endometrial cells; and (4) lymphovascular metastasis—all of which have been reviewed elsewhere.[8,9] More recently, the genetic/epigenetic theory[10] proposes that changes in the genome or epigenome underlie all four theories.[11] The focus of this review is on the cellular origins of endometriosis involving the physiological process of retrograde menstruation, the predominant pathogenesis theory of endometriosis[12] and associated genomic programs, and developmental context. We will discuss how advances in these areas of endometriosis pathobiology will likely form the basis of new screening/diagnostic tools and therapeutics.

Cellular Origins of Endometriosis

Antegrade and Retrograde Menstruation

Human endometrium comprises the basalis and functionalis layers. The basalis contains the basal boundary of the endometrial glands and dense stroma and occasional lymphoid nodules. The functionalis layer emanates from the basalis; contains glandular epithelium, stroma, and a variety of immune cells; and is covered by a luminal epithelium (►**Fig. 1a**). The functionalis is shed during *antegrade (normal) menstruation* resulting in *vaginal bleeding*. Menstrual blood consists of erythrocytes, leukocytes, endometrial cells (epithelial, stromal, and immune cells), and secreted factors such as proteins.[13] Human menstruation occurs in a piecemeal fashion, with regions of unshed, shed, and repaired endometrial functionalis coexisting at the same time. Scanning electron microscopy (SEM) studies show shedding of the functionalis containing both glandular and luminal epithelium and stroma, leaving behind glandular stumps,[14] which we propose are the source of cells that reepithelialize and regenerate the endometrium. Two recent breakthrough findings have the potential to propel new understanding of this process that has been hampered by previous studies in animal and *in vitro* culture models: (1) the capacity of cells derived from endometrial biopsies[15–18] and endometriosis lesions[19] to form endometrial organoids (EMOs) in culture and (2) discovery of the menstruating spiny mouse which also sheds in a piecemeal fashion.[20,21] These new tools provide a platform to expedite endometriosis research, generating an enhanced understanding of human menstruation and its role in endometriosis in the near future.

Retrograde menstruation is the physiological process whereby menstrual blood flows backward via the fallopian tubes into the pelvic cavity, resulting in peritoneal bleeding. Sampson's theory postulates that shed endometrial fragments present in menstrual blood initiate endometriosis lesions by adhering to the surfaces of organs and the peritoneum, eliciting an inflammatory and proangiogenic response. We have proposed previously that endometrial fragments refluxed into the peritoneal cavity contain endometrial stem/progenitor cells that are the cells of origin of endometriosis lesions.[22–24] Ourselves[25,26] and more recently others[27] have further proposed a two-stem/progenitor cell hypothesis, whereby both epithelial and stromal compartments are believed necessary for the formation of *typical* endometriosis lesions containing glandular epithelium surrounded by stroma and that *atypical* stromal endometriosis which contains no epithelial glands is a variant of the disease seeded only by stromal cells.[28,29]

Stem/Progenitor Cells of the Endometrium

The amazing capacity of the endometrium to renew each cycle led to the hypothesis that adult stem/progenitor cells must exist in the endometrium.[30,31] There is now evidence of both epithelial progenitor cells (eEPCs) and mesenchymal stem cells (eMSCs) in human endometrium[32] and their identities have been confirmed.[15,33–35] The locations, cell surface markers, gene expression, and function of *human* endometrial stem/progenitor cells and their relevance to endometriosis pathogenesis and detection will be discussed here.

Endometrial Epithelial Progenitor/Stem Cells

Within the endometrial epithelium, a hierarchy of eEPC has been proposed[24]—with distinct cell populations expressing

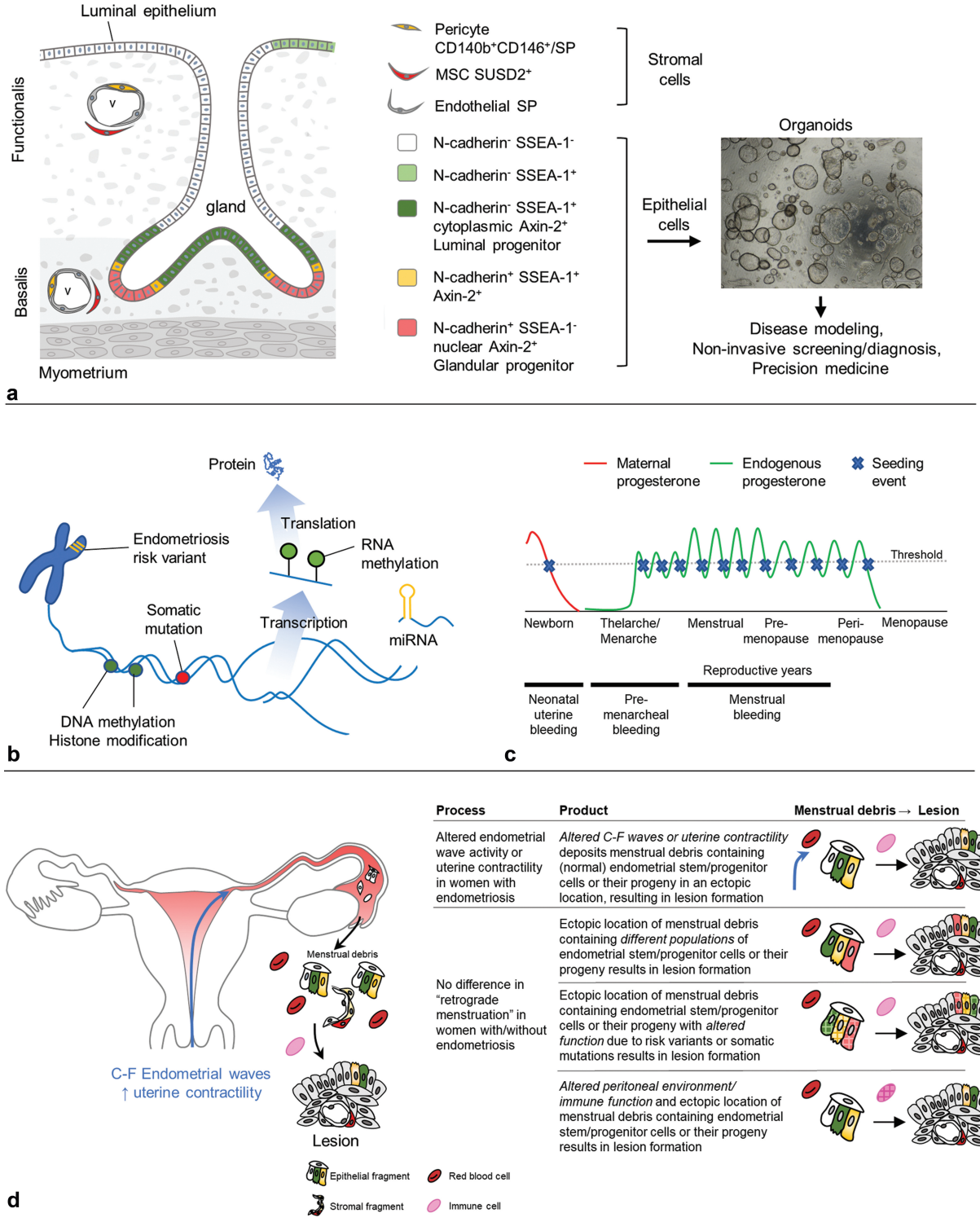

Fig. 1 Interplay of factors involved in the pathogenesis of endometriosis. (**a**) Cellular origins of endometriosis lesions. The endometrium contains eMSC, eEPC, and their differentiated progeny. Endometrial organoids generated from eEPC in eutopic and ectopic endometrium may be used for disease modeling, screening/diagnosis, and precision medicine. V, vessel. (**b**) Genomic program affecting cellular, physiological, and developmental aspects of endometriosis lesion formation, including risk variants, DNA/RNA methylation, histone modification, and miRNAs. (**c**) Developmental timing of cellular origin seeding events (blue crosses) over a woman's lifetime in relation to maternal and endogenous progesterone (red and green lines, respectively). (**d**) Physiological processes and products of retrograde menstruation leading to lesion establishment. Physiological processes include altered endometrial waves and uterine contractility, while products of retrograde menstruation include red blood cells as well as epithelial and stromal components of menstrual debris. (Parts of Panels **A** and **D** have been adapted from our publications with permission from Elsevier[24] and Oxford University Press.[124])

different cell surface markers including N-cadherin, stage-specific embryonic antigen-1 (SSEA-1/CD15), or leucine-rich repeat-containing G protein-coupled receptor (LGR5). Intracellular markers also describe human eEPC populations, including SOX9, AXIN-2,[36] OCT4, NOTCH1/NUMB, TNAP1, and telomerase.[37] In mice, evidence of a FoxA2$^+$ bipotent epithelial stem/progenitor in the intersectional zone of the luminal and glandular epithelium[38] and an Axin2$^+$ glandular eEPC[39] require further characterization to identify their cell surface markers, to enable prospective isolation and subsequent functional characterization of their human counterparts.

N-cadherin$^+$ eEPC are located deep within the basalis glands and are largely quiescent *in vivo*. However, *in vitro* they are the most clonogenic endometrial epithelial cells described to date.[15] The expression profile of N-cadherin$^+$ eEPC is yet to be described and may reveal novel effectors of eEPC function (►**Fig. 1b**). N-cadherin$^+$ cells can form glandular-like structures in 3D culture[15] indicating progenitor activity. SSEA-1$^+$ epithelial cells are located in luminal epithelium, the basalis glandular epithelium, and occasionally in the lower functionalis glandular epithelium.[15] SSEA-1$^+$eEPC show low levels of proliferation *in vivo*, despite greater telomerase activity and capacity to form spheroids in 3D cultures,[34] possibly reflecting activation by soluble factors in culture media. A rare subset of N-cadherin$^+$ eEPC is also SSEA-1$^+$ which may represent a transitional state between the two distinct eEPC populations.[15] The N-cadherin$^+$/SSEA-1$^+$ cells are located just below the "junction" of the basalis and functionalis and thus are likely located near the rough edge of the gland stumps seen on day 2 of menstruation by SEM.[14] These N-cadherin$^+$/SSEA-1$^+$ double positive cells are ideally placed to produce SSEA-1$^+$ luminal progenitors that may regenerate/reepithelialize the denuded surface following menstruation. The SSEA-1$^+$ and SOX9$^+$ luminal epithelial cells may have progenitor activity.[40]

LGR5 marks a population of epithelial stem cells in the colon and more recently mouse endometrium[41]; however, controversy surrounds its role as a human endometrial stem/progenitor cell marker.[37] This may be due in part to the lack of a reliable LGR5 antibody. However, the likely role of LGR5 in luminal eEPC[42] as determined by *in situ* hybridization and evidence that LGR5$^+$ cells in the adult mouse endometrium are short-lived[41] indicates that LGR5 is unlikely to mark a primitive human eEPC population.

Endometrial Mesenchymal Progenitor/Stem Cells

While a hierarchy of eMSCs is yet to be defined, several different populations can be isolated by cell surface markers as platelet-derived growth factor receptor β (PDGFRb)$^+$ CD146$^+$ pericytes[35] and sushi domain containing-2 SUSD2; previously W5C5$^+$ perivascular eMSC. Both populations are located around blood vessels in both the basalis and functionalis (►**Fig. 1a**), are highly clonogenic, and have multilineage mesodermal and decidual differentiation potential.[23,43] SUSD2$^+$ eMSC are capable of both clonal expansion and reconstituting endometrial stroma when transplanted under the mouse-kidney capsule, indicative of a more primitive stem/progenitor cell population,[33,35] although this capacity has not yet been assessed for PDGFRb$^+$ CD146$^+$ eMSC.

Initiating Cells of Endometriosis Lesions

Menstrual Blood Contains Putative Cellular Origins of Endometriosis

Endometriosis lesions are defined histologically by the presence of both epithelial glands and mesenchymal stroma and have recently been shown to contain SSEA-1$^+$ [34] and clonogenic stromal cells.[44,45] New evidence[46] indicates that both epithelial and mesenchymal progenitors are involved in lesion formation, rather than a single cell of origin capable of producing both epithelial and mesenchymal cell types. Extending Sampson's theory of retrograde menstruation, it seems logical that menstrual blood would contain the initiating cells of endometriosis. Indeed, menstrual tissue contains both eMSC and eEPC (Masuda H, MD, PhD, Schwab KE, PhD, Filby CE, PhD, Gargett CE, PhD, data 2020; and Wyatt KA, BBiomedSci(Hons), Filby CE, PhD, Gargett CE, PhD, data 2020) and endometrial stromal fibroblast cells with defective decidualization and downregulation of ALDH1A1. Menstrual tissue forms lesions in primate[29] and rodent models[47] of endometriosis. In humans, more basalis-like glandular structures are shed during menstruation in women with endometriosis than those without the disease.[48] Women with endometriosis shed the endometrium more vigorously due to retrograde[49] (cervix to fundus) endometrial waves of increased frequency,[50] compared with women with-out disease resulting in sloughing of both functionalis *and* basalis tissue. Alternatively, functionalis tissue of women with endometriosis may contain more primitive stem/progenitor cells, thus increasing the likelihood of endometriosis lesion formation each month. A greater capacity for lesion formation may be due to a combination of genetic risk factors, somatic mutations, and concurrent deposition of epithelial and stromal endometrial cells into the pelvic cavity.

Dual Cell of Origin: Ectopic Epithelial and Mesenchymal Cells Are Clonally Distinct

Endometriosis is a benign disease; however, it shares many hallmarks with cancer including evasion of immune system detection/clearance, adhesion and aberrant proliferation of cells at an ectopic site, and local invasion into the surrounding tissue. Indeed, benign endometriosis lesions acquire somatic mutations in several cancer-associated genes indicating that endometriosis may sit within a gray area between benign and malignant phenotypes. The presence of somatic mutations implicated in tumorigenesis (KRAS, BRAF, and others[46,51]) in eutopic epithelial glands and enriched in ectopic epithelial glands in women with endometriosis, but near absent from the stroma,[46,52] also supports our two-stem/progenitor cell hypothesis for the formation of endometriosis lesions.

The aforementioned evidence strongly suggests that endometriosis requires cells from at least two cellular origins, rather than a single multipotent cell of origin which usually initiates a cancerous tumor. The different stem/progenitor cellular origins of endometriosis likely contribute in different

ways—those with epithelial potential containing somatic mutations may show enhanced proliferation, while those with stromal potential may show angiogenic properties. The low level of somatic mutations in the stromal compartment likely reflects contaminating epithelial cells accidentally collected during laser capture of the stromal compartment,[53] rather than true positive stromal somatic mutations. Together these findings indicate that the epithelial component of endometriosis lesions is clonal in origin, with a cell of origin distinct from lesion stroma and that somatic mutations and/or an altered peritoneal environment may favor survival of endometrial epithelial cells.

Epithelial Cellular Origin of Endometriosis: Candidates and Heterogeneity

Currently described stem/progenitor cell candidates for the cellular origin of endometriosis include N-cadherin$^+$ and SSEA-1$^+$ cells. Indeed, there are increased numbers of SSEA1$^+$/SOX9$^+$ progenitor cells in the functionalis of eutopic endometrium of women with endometriosis,[40] and ectopic lesions contain clonogenic stromal cells,[44,45] SSEA-1$^+$ cells,[34] and N-cadherin$^+$ cells localized to the thin, highly proliferative glands at the leading/invasive edge of endometriosis lesions.[54] We have found N-cadherin$^+$ cells in both menstrual blood and peritoneal fluid of menstruating women with endometriosis (Masuda H, MD, PhD, Schwab KE, PhD, Filby CE, PhD, Gargett CE, PhD, data 2020). Similarly, others have found both epithelial (55% cases; 75% controls) and stromal (67% cases and 38% controls) cells in peritoneal fluid are obtained during the menstrual phase[55]; however, the markers used in that study (EpCAM and CD10) are neither specific to endometrium or stem/progenitor cells. Future studies that quantify the proportions of endometrial stem/progenitor cells in menstrual blood and peritoneal fluid, their stem/progenitor capacity, and ability to form lesions *in vitro* and *in vivo* will clarify the involvement of endometrial stem/progenitor cells in endometriosis caused by retrograde menstruation.

The number of endometrial epithelial progenitor/stem cells in each gland is unknown. However, vertical glands containing somatic mutations of both high variant allele frequency and a unique signature are consistent with a single ancestor.[46] There may be an epithelial stem/progenitor cell that gives rise to several clustered glands in eutopic and ectopic endometrium with a common mutational signature.[46,56] Alternatively, enrichment of different somatic mutations in glandular, DIE, and incisional endometriosis (from caesarean section[56]) may suggest different epithelial progenitors for different endometriosis subtypes. Future studies that combine somatic mutation analyses with spatial organization *in situ* are needed to clarify these points.

Mesenchymal Cellular Origin and the Role of Epithelial-Mesenchymal Transition

Currently, the primary stem cell candidate for the mesenchymal cell of origin is the SUSD2$^+$ eMSC. Whether a hierarchy of more/less primitive eMSC, potentially based on their basalis/functionalis location, exists (as for eEPC) remains to be determined. Elucidation of the subtypes of eMSC populations may reveal novel causes of endometriosis heterogeneity and pathogenesis. Their location and identity suggest they have a role in angiogenesis, required for endometrial regeneration each month and key to endometriosis lesion establishment and progression.

Epithelial-mesenchymal transition (EMT) has been proposed as a mechanism involved in the pathogenesis of endometriosis. However, the stromal compartment of lesions harbors a unique mutational profile distinct to the epithelial compartment,[52,53] indicating that the contribution of cells that have undergone EMT is likely to be small. EMT may still play a role, particularly if it precedes MET, in which case the cells would return to their original epithelial fate. Indeed, such cells may act as a vehicle for collective cell migration of a multicellular origin of endometriosis. Evidence of multiple cells of origin is supported by collective cell migration,[54] whereby a group of stem/progenitor cells and their niche cells held together by intact cell junctions migrate and invade other tissues such as the bowel as a unit. Collective cell migration and invasion is a key mechanism of metastasis for ovarian cancer and several other epithelial cancers.[57]

Bone Marrow–Derived Stem Cells

While outside the scope of this review addressing Sampson's Hypothesis, we note evidence for the role of bone marrow–derived stem cells (BMDSCs) in endometrial biology and pathology. There is much conjecture in this field.[58] While there are reports in mice and human of BMDSC engrafting into the endometrium and preexisting ectopic endometriosis lesions,[59] the engraftment rate is very low and their identity is unconfirmed. Therefore, it is unlikely that BMDSCs are a putative cell of origin for endometriosis, but they may be implicated as bystanders[60] that contribute via cell fusion.[61] Indeed, BMDSC may explain the development of endometriosis in males.[62] Studies that identify which of the three BMDSCs (hematopoietic stem cells, mesenchymal stem cells, or endothelial progenitor cells) are involved and determine whether BMDSCs also carry the somatic mutations present in eutopic and ectopic endometrium of women with endometriosis[46] are necessary to elucidate this complex issue. Furthermore, the frequency of somatic mutations in the coelomic epithelium may inform alternate theories of endometriosis initiation (e.g., coelomic metaplasia).

Genomic Programs Implicated in Endometriosis

Involvement of alterations to DNA in endometriosis etiology includes germline risk variants passed onto offspring, somatic mutations acquired by individual cells over time, and induced epigenetic modifications altering cellular programming. It is likely that there is interplay between these factors and they can together regulate gene expression (miRNA, mRNA) as well as protein function and influence cellular states. There is genetic regulation of methylation signals in the endometrium and altered methylation signals are located in regions of endometriosis risk (GREB, C11orf46, NR2C1, KDR, WNT4) identified from the genome-wide association studies (GWAS).[63] As such, alterations in genomic regulation

have the potential to affect cellular behavior and the physiological mechanism and developmental context of cells initiating endometriosis lesions (►**Fig. 1b**).

Genetics

Endometriosis is a complex disease with about half of the variation due to genetic risk factors. GWAS have reported 27 genomic regions robustly associated with an elevated risk for endometriosis.[64–66] The gene regions implicated thus far have been reviewed elsewhere.[66] Many of the key risk variants are located near genes involved in estrogen signaling (*ESR1*, *FSHB*, *GREB1*) or cellular adhesion, proliferation, or migration (*CDC42*, *CDKN2B-AS1*, *KDR*). The endometriosis risk variants are located in noncoding regions of the genome and likely play a role in gene regulation. They do not affect protein structure and function, and do not provide direct evidence of the genes or pathways implicated in increased disease risk. Furthermore, it is unknown whether the endometriosis risk alleles are selectively active in different subsets of cells—for example, stem/progenitor cells, the likely cells of origin of endometriosis. Investigation of the functional effects of endometriosis risk alleles at the single cell level will be necessary to elucidate this issue.

In addition, there may be different combinations of risk alleles in different subtypes of the disease, each with their own contribution to disease phenotype. The risk of endometriosis for a woman with a known family history may be as high as five times that of the general population.[67] GWAS studies provide evidence for stronger genetic effects for severe invasive endometriosis.[68] Thus, this subgroup represents a high priority for both early diagnosis and for research into the pathogenesis of endometriosis.

Environment

Inherited genetic factors account for approximately 50% of endometriosis risk, and the remaining variation is thought to be due to environmental factors which includes somatic mutations or changes in methylation.[66] Chemical pollutants implicated in endometriosis include polychlorinated biphenyls (PCBs) such as dioxins in pesticides, phthalates, and bisphenol-A in plastics, trace metals, and others.[69] Although banned in the 1990s, PCBs are resistant to degradation and persist in the environment, accumulating in the food chain. They are known endocrine disrupters, with estrogenic and antiandrogenic effects including delayed time to pregnancy.[70] Exposure to environmental toxins during the prenatal period is thought to have the greatest effect on reproduction and endometriosis,[71] and elevated serum phthalates in women are associated with endometriosis.[72,73] However, our search could not locate any publications on the specific effect of environmental pollutants or other environmental factors on endometrial stem/progenitor cells. Environmental pollutants have persistent effects on other cell fates and differentiation of other stem cells such as those from the mammary gland.[74] Clearly, the effect of environmental factors on endometrial stem/progenitor cell function is an area needing further research.[10]

Genomic Regulation of Cellular Pathways Involved in Endometriosis Pathogenesis

It is likely that changes to the genome influence numerous cellular pathways and mediators implicated in the pathogenesis of endometriosis.[75] Briefly, they include members of the Wnt signaling pathway,[19,76,77] kinase signaling pathway,[78] micro-RNAs and long noncoding RNAs,[79] as well as cell adhesion molecules such as N-cadherin[54] and Claudin 11.[80] Furthermore, cellular functions such as enhanced cell survival (resistance to apoptosis; Tetraspanin-30), cell migration, angiogenesis, cellular proliferation,[81] and innervation[82,83] are thought to contribute to establishment of lesions. Cellular functions can also be modified by reproductive hormones[84] and conditions such as hypoxia,[85] resulting in an adhesive, and angiogenic phenotype. Furthermore, immunomodulatory factors may affect immune cell evasion and establishment of lesions at ectopic sites. Indeed, there are reduced numbers[86] and activity[87] of natural killer cells in endometriosis. Additionally, exosomes produced by endometriosis MSC result in reduced phagocytic activity of macrophages *in vitro*.[88] Immunomodulatory factors may also be a result of lesion establishment, as suggested by greater numbers of circulating PD-1^{+} T and B cells in advanced endometriosis.[89]

Physiological Mechanisms of Retrograde Menstruation

Products and Processes of Retrograde Menstruation

When studying retrograde menstruation, it is important to distinguish between the *products* and *process* of retrograde menstruation (►**Table 1**). The *products* of menstruation consist of menstrual fluid, which is distinct from peripheral blood, and contains endometrial tissue fragments and individual cellular components, including epithelial cells, stromal fibroblasts, their progenitors, blood vessel components, blood cells, and menstrual blood plasma proteins.[13,90] The physiological *process* of menstruation likely involves changes in endometrial wave direction and uterine contractility responsible for transporting the cells from the uterine cavity to the pelvic cavity.

It is also important to acknowledge that methods used to establish the presence of *products* of retrograde menstruation in the pelvic cavity and/or define laparoscopically confirmed controls necessitates the introduction of several confounding factors. These include potential iatrogenic retrograde menstruation or iatrogenic bleeding due to surgery or insufflation, peritoneal dialysis/coexisting kidney disease, and reference groups that consist mostly of women with pelvic pain and/or infertility and therefore may have subclinical/undetected endometriosis. These studies therefore may overestimate the prevalence of retrograde menstruation in confounded reference groups. On the other hand, evidence supporting the physiological *process* of retrograde menstruation involves minimally invasive procedures (pressure catheter recordings or transvaginal ultrasound [TV US]) less likely to cause iatrogenic retrograde menstruation and more likely to have control groups without another underlying condition

Table 1 Evidence of the products and processes of retrograde menstruation

		Reference group	Endometriosis group	Estimated incidence of retrograde menstruation	Limiting factors	Reference
Products	**Blood**	Blood-stained PF from patients with end-stage renal failure		Not determined	Patients with end-stage renal failure; effect of peritoneal dialysis	125
		Blood-stained PF obtained from laparoscopy for tubal ligation or infertility/pelvic pain	Blood-stained PF obtained from laparoscopy for endometriosis	90% of all women	Controls with infertility/pelvic pain (57%); effect of surgery and insufflation; no cellular evidence	126
		Increased erythrocyte number, haemoglobin and haematocrit in PF obtained from laparoscopy performed during menstrual phase for infertility/pelvic pain	Increased erythrocyte number, hemoglobin and hematocrit in PF obtained from laparoscopy performed during menstrual phase for infertility/pelvic pain	Not determined; controls and cases analysed together	Controls with infertility/pelvic pain (100%); effect of surgery and insufflation; did not analyse cases vs controls	127
	Menstrual debris	Epithelial (75%), stromal (37.5%), or concurrent epithelial and stromal (37.5%) cells in PF obtained from laparoscopy for infertility/pelvic pain	Epithelial (55%), stromal (67%), or concurrent epithelial and stromal (44%) in PF obtained from laparoscopy for infertility/pelvic pain	37.5% of controls, 44% of women with endometriosis	Controls with infertility/pelvic pain (100%); effect of surgery and insufflation	55
		Epithelial ($EpCAM^+$ 0%; $Cytokeratin^+$ 0–100%) and stromal/mesothelial cells in PF obtained from laparoscopy performed during menstrual phase for infertility/pelvic pain	Epithelial ($EpCAM^+$ 40%; $Cytokeratin^+$ 20–60%) and stromal/mesothelial cells in PF obtained from laparoscopy performed during menstrual phase for infertility/pelvic pain	0% of controls, 40% of women with endometriosis	Controls with infertility/pelvic pain (100%); effect of surgery and insufflation	127
		Epithelial ($BW495/36^+$ 50%) cells in PF obtained from laparoscopy performed during menstrual phase for infertility	Epithelial ($BW495/36^+$ 62.5%) cells in PF obtained from laparoscopy performed during menstrual phase for infertility	50% of controls, 62.5% of women with endometriosis	Controls with infertility (100%); effect of surgery and insufflation	128
		No difference in PF endometrial cells that are native (11%), but lower levels of iatrogenic endometrial PF cells following uterine irrigation (42%)	No difference in PF endometrial cells that are native (19%), but higher levels of iatrogenic endometrial PF cells following uterine irrigation (76%)	11% of controls, 19% of women with endometriosis	Controls with infertility (100%); effect of surgery and insufflation; cycle stage not documented	129
Processes	**Retrograde** (C–F) endometrial waves or transport	Retrograde waves present during menstrual phase of the cycle in 4.8% of controls	Retrograde waves present during menstrual phase of the cycle in 87.5% of women with endometriosis	4.8% of controls, 87.5% of women with endometriosis	Controls were not laparoscopically defined	49
		Lower transport of radioactive particles into fallopian tube and peritoneal cavity (~5%) during menstrual phase	Increased transport of radioactive particles into fallopian tube and peritoneal cavity (~15%) during menstrual phase	Not determined		93
	Uterine Contractions corroborated by evidence of blood and cells	Lower frequency (11.1 ± 3.3/10 min) and amplitude of contractions (6.77 ± 2.8mm Hg) and lower baseline pressure (24.7 ± 6.1mm Hg). Blood stained PF uncommon (9%) and no viable endometrial stromal and epithelial cells (0%) could be cultured *in vitro*.	Greater frequency (22.7 ± 5.7/10 min) and amplitude of contractions (20.8 ± 3.9mm Hg) and greater baseline pressure (50.1 ± 16.3mm Hg). Blood stained PF (73%) and viable endometrial stromal and epithelial cells from many women (45%) could be cultured *in vitro*.	Not determined for uterine contractility; 0% of controls, 45% of women with endometriosis for viable cells	Controls with infertility; pressure recordings but not laparoscopy was performed on the peak day of bleeding (day 2); endometrial cell-specific markers not used.	94

Notes: Blood-stained peritoneal fluid (PF) is considered evidence of retrograde bleeding and is purported to occur in 90% of all women, however it does not demonstrate the presence of menstrual debris containing the cellular origins of endometriosis or retrograde transport of these cellular origins. Existence of endometrial epithelial/stromal cells in PF is purported to occur in 0–37.5% of control women and 40–44% of women with endometriosis and is evidence of the products of retrograde menstruation but is not evidence of the process of retrograde menstruation. Caudal-Fundal (C–F) endometrial waves, increased uterine pressure, and contractile frequency and amplitude during the menstrual phase in women with endometriosis, but near-absence in women without endometriosis is evidence of a pathophysiological process of retrograde menstruation in women with endometriosis.

or subclinical endometriosis. These studies are therefore less likely to overestimate the prevalence of retrograde menstruation in controls, and this is supported by a lower incidence of retrograde menstruation in physiological studies that document the *process* rather than the *products* of retrograde menstruation (►**Table 1**).

Retrograde flow of blood, which may or may not contain menstrual debris, may be a passive process, while retrograde menstruation, through which menstrual debris containing putative cellular origins of endometriosis is refluxed into the pelvic cavity, may be an active process involving endometrial waves and uterine contractions. There is a clear need to characterize the *cellular composition* and *endometrial/progenitor origin* of peritoneal fluid of women with/without endometriosis during the *menstrual phase*.

The nature of the physiological *process* of how menstrual debris is transported into the pelvis is a topic worthy of further research, as it may improve our understanding of the pathophysiology of endometriosis, as well as reveal novel therapeutic targets which prevent retrograde transport without interrupting the ovarian/endometrial cycle. Endometrial cells in the peritoneal cavity and blood within the pelvis during/after menstruation are evidence in support of the *physiological process* of retrograde menstruation. How, when, or why blood or cells arrived in the pelvis have been considered in numerous studies. There are reports of increased blood-stained peritoneal fluid containing cells of endometrial origin, increased uterine pressure, and contractile frequency and amplitude[50] during the menstrual phase in women with versus without endometriosis.[50] Furthermore, a predominance of retrograde cervical-fundal endometrial wave direction determined by video recordings of TV US during the menstrual phase of women with disease[49] indicates a difference in the *process* of retrograde menstruation in women with and without endometriosis (►**Table 1**).

Endometrial waves largely involve the subendometrial unit (Müllerian in origin) consisting of the endometrium and inner myometrium (stratum subvasculare) which is densely packed with circular smooth muscle fibers that demonstrate cyclic estrogen and progesterone receptor expression.[91] The subendometrial unit is thought to contain interstitial Cajal-like cells,[92] pacemaker cells that likely regulate endometrial wave function. In contrast, uterine contractions involve the outer myometrial layers (non-Müllerian in origin) consisting of the mesh-like stratum vasculare and the stratum supravasculare which contains longitudinal smooth muscle fibers (with stable estrogen and progesterone receptor expression[91]). Current methods of determining transport of menstrual debris have their limitations, including invasiveness (catheters), subjectivity, and laborious (analysis of TV US recordings to determine wave direction)[49,91] and health risks (hysterosalpinoscintigraphy[93]).[94] Recently, electrohysterography[95] has shown promise in monitoring uterine contractions during pregnancy and could be adapted for the menstruating uterus. Pathways underlying these contractions could be mined for possible therapeutic targets that mitigate the uterine contractions in retrograde menstruation.

The outer myometrium and subendometrial layer appear to have different activities during the menstrual phase in normal women[96] and investigation of whether this is altered in women with endometriosis may provide key clues on how, *how much*, and *what type of* menstrual debris enters the pelvic cavity (►**Fig. 1d**). Clearly, there is a need to develop noninvasive, objective, safe, and reliable methods for detection of endometrial wave direction and nonpregnant uterine contractility. These studies could be combined with cellular evidence of cells of endometrial origin in the peritoneal fluid. Determining whether cervical-fundal endometrial waves during menstruation are the cause or effect of endometriosis has important ramifications for potential interventions and fertility management, respectively.

Developmental Context of Endometriosis Pathogenesis

There are three proposed developmental time points when refluxed endometrial cells seed endometriosis lesions: (1) those shed during the neonatal period—a phenomenon defined as neonatal uterine bleeding,[97,98] (2) those shed in the premenarchal period,[99] and (3) those shed during menstruation after menarche.[12] In all cases, progesterone withdrawal is key in driving menstrual shedding, although the proposed methods of refluxed menstrual debris are distinct. During the neonatal period, female babies are removed from the effects of maternal progesterone following birth, while in the premenarcheal and reproductive (postmenarcheal) periods the progesterone is endogenous (►**Fig. 1c**). In the newborn, retrograde menstruation is facilitated by the long, obstructed cervical canal,[97,98] and this may also play a part in the premenarcheal period. Age at first symptoms can occur in prepubertal, perimenarcheal girls with proposed developmental time periods 1 and 2, but only after menarche with time period 3.

After menarche, retrograde menstruation is likely due to altered endometrial wave activity,[49,50] which may be enhanced by a narrow (< 4.5 mm) cervical os.[100] These distinct seeding stages (►**Fig. 1c**) may be responsible for the differing clinical presentations, severity,[98] and patterns of disease progression and therefore may determine the phenotypic classification of and subsequent personalized therapy for endometriosis.

Toward Novel Diagnostics and Therapeutics

Limitations of Current Clinical Management

While much can be achieved in the clinical management of endometriosis through addressing individual symptoms, large improvements in patient outcomes are likely to depend on a greater understanding of the basic mechanisms of lesion formation and how the disease causes pain and infertility. Most of the current guidelines for the management of endometriosis conclude that significant investment in innovative research that uncovers basic disease pathophysiology is required to improve outcomes for women with endometriosis. We propose that improved scientific knowledge of the cells that initiate endometriosis lesions, and an understanding of the genomic, physiological, and developmental

context, will lead to new avenues to address the main clinical conundrums including prevention, diagnostic delay, inability to identify all lesions noninvasively, high recurrence rate/spread, lack of a cure, heterogeneity in patient response to treatments, mechanisms underlying pain, fertility affected by the disease and its treatments, and psychological and other impacts, and the wide variation in clinical care (►**Table 2**). Greater understanding of disease mechanisms will lead to new avenues for a personalized medicine approach for treating endometriosis.

Reducing Diagnostic Delay

Currently, 6 to 11% of reproductive-age women are diagnosed with endometriosis.[1–3] However, with a current diagnostic delay of 7 to 15 years,[101] it is likely that the proportion of women affected by endometriosis is much larger. While increased education of women, health care professionals, and the general public will gradually aid in preventing unnecessary diagnostic delay, it is unlikely to significantly reduce the time to diagnosis, as there is currently no single imaging technique or laboratory test that can diagnose all forms of endometriosis without surgery. Repeat surgeries[102] are undesirable, and thus new strategies must be developed.

Currently, detection of endometriosis utilizes TV US performed by a gynecological sonographer trained in the detection of DIE. Specificity and detection are heavily influenced by the skill of the sonographer.[103] Visualization of DIE by *specific* TV US has had a major impact on clinical management of endometriosis in the past 10 years, although this is controversial. Some, but not all, advanced laparoscopists experience improved preoperative planning and can engage other surgical specialists as consultants before surgery. It can allow for improved preoperative counseling and planning and may reduce the need for a two-stage approach to surgical management of women with higher stage endometriosis.[104,105] This approach detects endometriomas and DIE lesions; however, even the most skilled sonographer cannot detect superficial lesions, thought to represent early stage disease. Hence, there is a need to find alternative screening and diagnostic approaches for endometriosis that can detect all forms of the disease. Some of the proposed strategies for reducing the time to diagnosis reviewed here include menstrual or peripheral blood screening, EMOs, extending the use of TV US, repurposing other imaging technologies, and a combinational tool.

Blood Screening

The utility of biomarkers in blood, urine, and peritoneal fluid have long been investigated for the screening and diagnosis of endometriosis and have been reviewed extensively elsewhere.[55,106–108] These include miRNAs, lncRNAs, adhesion molecules, glycoproteins, immunological/inflammatory markers, and soluble factors in peripheral blood, soluble receptors, and matrix metalloproteinases in urine and growth factors and immunological/inflammatory markers in peritoneal fluid.[106–108] Some have shown admissible biomarkers requiring further validation.[109] Progress in this area has been complicated by small-scale studies with non-standardized procedures as well as the heterogeneity of endometriosis. Future understanding of endometriosis phenotypes may later reveal subtype-specific utility of some of these markers.

An often overlooked bodily fluid with excellent potential for screening/diagnostic is menstrual blood, which can easily be noninvasively collected using a menstrual cup.[110,111] Menstrual blood contains unique soluble factors,[90] exosomes,[13] single cells and clumps of endometrial cells,[111] a distinct immune cell profile,[110] and blood clots. Menstrual blood can be noninvasively and easily collected by women themselves and samples taken to existing pathology collection centers located throughout the community. We have shown the presence of both eEPC and eMSC in menstrual fluid (Masuda H, MD, PhD, Schwab KE, PhD, Filby CE, PhD, Gargett CE, PhD, data 2020). Future research is needed using this overlooked bodily fluid as a diagnostic medium.

Endometrial Organoids and Precision Medicine

Endometrium contains N-cadherin$^+$ eEPCs which can produce EMOs in culture.[15] EMOs can also be generated from mixed cell populations from both eutopic and ectopic endometrium.[16,17,19] Single-cell RNA sequencing reveals EMO contain five distinct populations.[18] EMOs can be cultured for multiple passages, showing features of endometrial glands and EMO from ectopic endometrium form lesions in mice.[19] Patient-derived organoids can be used for drug screening both to understand pathogenesis of endometriosis and to develop precision medicine therapies that account for the heterogeneity in endometriosis presentation.

Precision medicine is used widely in cancer treatment and accounts for heterogeneity in phenotype, disease progression, and developmental, genomic, and environmental factors, to tailor the best therapy for the individual. The heterogeneous nature of multiple risk alleles,[64] multiple somatic mutations,[46] several environmental influencers, different clinical presentations and age of symptom onset, variable progesterone resistance,[112] and heterogeneity of lesions indicate endometriosis is likely to benefit from a personalized medicine approach. EMO could simultaneously be used to detect known somatic mutations which may predict lesion establishment,[46] as well as endometriosis risk alleles by GWAS,[64] deficiencies in decidualization, and altered proportions of NK cells.[86] Furthermore, the ability to generate EMO allows researchers to perform high-throughput screening of small molecules to determine drug sensitivity/resistance *before* treatment begins, allowing selection of the most suitable therapeutic strategy for each individual.

Imaging

The use of ultrasound contrast agents (UCAs) may overcome the inability of TV US to detect superficial lesions which lack echogenicity/contrast to the surrounding tissue. UCAs allow visualization of low contrast regions in the vasculature, kidney, liver, and in ovarian cancer. Microbubbles typically contain perfluorocarbon or nitrogen gas[113] surrounded by a lipidic or protein shell. When injected intravenously, they

Table 2 Clinical conundrums of endometriosis: advances in scientific knowledge required to improve patient outcomes

Clinical conundrum		Scientific knowledge needed to produce a change in practice/outcome	Role of clinicians	Role of scientists	Other improvements needed to produce a change in practice/outcome
Diagnosis	Diagnostic delay >7 y	Defining the cellular origins of the lesions, and/or their secreted factors or their associated genomic profile will enable development of new or modify existing screening/diagnostic tools	Facilitate collection of clinically annotated samples (biopsies, lesions, menstrual fluids, and peripheral blood) for basic research	Develop assays to determine lesion-forming capacity of endometrial cell types and their associated secreted factors and genomic profiles	Increase awareness and education of endometriosis for women, general public, GPs, other health care providers to reduce time to presentation and time to specialist care
	Superficial lesions can only be identified laparoscopically	Use an improved understanding of unique cellular origins, mechanisms, and secreted factors of superficial lesions to develop new or modify existing screening/diagnostic tools	Use novel cell surface markers to test feasibility of targeted contrast-enhanced imaging to detect superficial lesions	Classify the cell surface markers, signaling molecules, and genomic program specific to superficial lesions	Develop new screening tools to predict superficial lesions based on a combinational tool of all available tests/assays
			Conduct high-quality clinical trials to test therapeutics that are tolerable to women "suspected" of endometriosis	Develop therapeutics that are safe and with minimal side effects/low risk that are suitable and satisfactory for women "suspected" of endometriosis	
Treatment	High recurrence rate/spread to other organs	Model lesion establishment, recurrence, and spread in *in vivo* and *in vitro* systems using known/proposed cellular origins and associated genomic and physiological programs	Facilitate collection of clinically annotated samples (biopsies, lesions, menstrual fluids, and peripheral blood) for basic research	Use *in vitro* and *in vivo* lesion-forming assays to test capacity of endometrial cells and their associated secreted factors and genomic profile	Develop minimum standards so that women with endometriosis undergo a single definitive surgery by a highly skilled surgical team
		Develop therapeutics that target the physiological processes involved in seeding of cells of origin (e.g., retrograde menstruation or invasion/spread)	Perform clinical research that investigates the timing, nature, genomic program, and action of seeding of cellular origins e.g. studies on cellular composition of menstrual fluid, neonatal uterine bleeding, peritoneal fluid, and physiological evidence of retrograde uterine activity. Use this information to develop targeted tailored therapies that prevent *retrograde* menstruation		
	No cure	Use an improved understanding of the pathogenesis of endometriosis to develop a cure—i.e., what cells form the lesions and how can we specifically target them	Work collaboratively with scientists, imaging specialists, and consumer groups to facilitate basic research that has a high potential for translation and clinical impact	Determine the underlying genomic program, physiological mechanism, developmental context and cellular origins to develop a treatment that prevents lesion formation	Significant worldwide financial investment in high-quality collaborative research that seeks to understand the underlying causes of endometriosis and how to prevent its initiation and treat its progression
	Patients respond differently to treatments	Use an improved understanding of the heterogeneity (biological, genomic, etc.) of endometriosis pathogenesis, paired with clinical data to understand the physiological basis of responders/nonresponders allowing personalized care plans	Facilitate collection of clinically annotated samples (biopsies, lesions, menstrual fluids, and peripheral blood) for basic research	Classify distinct phenotypes of the disease based on cellular composition of eutopic and ectopic endometrium, genomic profile, clinical annotation, etc.	Provision of endometriosis clinical care and research nurses to facilitate sample and data collection
Infertility	Fertility is often compromised by the disease and its treatments	Use an improved understanding of the mechanisms of endometrioma formation to develop preventative therapies that avoid growth and subsequent resection of lesions on the ovaries	Facilitate collection of clinically annotated endometrioma samples for basic research	Develop an *in vitro* model of endometrioma to study this particular disease subtype	Provide education to women, general public, GPs, other health care providers about the unique symptoms and fertility consequences of endometriosis
			Develop early screening tools to predict the risk of endometrioma formation, enabling counseling and preemptive fertility advice		
		Use an improved understanding of how lesions affect endometrial receptivity to improve fecundity	Conduct high-quality clinical trials to test therapeutics that improve fecundity of women with endometriosis	Develop an *in vitro* model of endometrial receptivity to study the effects of endometriosis signals	
		Use improved knowledge on etiology to develop nonsurgical treatments that have long-term safety in young women and that are compatible with fertility and pregnancy (e.g., IUD is not recommended for young women)		Use knowledge of basic cellular signaling pathways and *in vitro* culture systems (organoids) to screen for novel targets	
Quality of life	Patients often suffer	Use improved knowledge of how endometriosis	Conduct clinical research trials on only the best	Use knowledge of basic cellular signaling	Develop/expand centers of excellence for

Table 2 (*Continued*)

Clinical conundrum		Scientific knowledge needed to produce a change in practice/outcome	Role of clinicians	Role of scientists	Other improvements needed to produce a change in practice/outcome
	pain, fatigue, psychological and other impacts even after the best available treatments	causes pain, fatigue, and psychological effects to develop new preventative strategies/therapies that target the effectors in addition to symptoms	targets to ensure translation of safe, effective novel therapies	pathways and *in vivo* models (e.g., spiny mice) to develop new therapies	endometriosis management that treat the whole patient, not just the lesions
Quality of clinical care	Patients receive a wide variation in the quality of clinical care	Use nation-wide or international datasets on clinical outcomes to identify underperforming providers needing further training and accreditation	Develop minimum standards of training and competency in GPs, sonographers, specialists, etc., and ensure only those who meet these standards receive accreditation and federal rebates/funding	Collect longitudinal data that identify key modifiable factors that determine variations in quality of clinical care	Develop/expand centers of excellence for endometriosis management—particularly in regional areas

improve the echogenicity (500–1,000×) of blood perfusing the organ, tumor, or lesion of interest by changes in backscatter. Microbubbles are 1 to 4 µm, rapidly cleared by the lungs[114] and can be conjugated to an antibody against a molecular marker of interest, a technique called targeted contrast-enhanced ultrasound imaging. Identification of the cellular of origins of endometriosis and composition of endometriosis lesions, and their cell surface markers, could assist in targeted contrast-enhanced imaging of endometriosis lesions. Before and after images following high-frequency oscillation (which bursts the microbubbles) can be used to distinguish between targeted and free floating bubbles.[115] This strategy has been employed in cancer screening, ultrasound-guided biopsy, and targeted therapy where a targeted microbubble-encapsulated drug is released at the region of interest.[113] UCA can detect early vascularization of ovarian tumors[116] and increase contrast of avascular tumors such as pancreatic cancer.[117] Since vascularization is a feature of superficial lesions,[118] microbubbles may offer a potential method for their detection and treatment.

Magnetic resonance imaging (MRI) is a more sophisticated imaging modality and has high specificity for endometriosis lesions, although is limited by low sensitivity, reproducibility, and cost.[119] Magnetic resonance spectroscopy (MRS), where MRI is combined with spectroscopy of cellular metabolites, remains unexplored for endometriosis lesions, which produce both lactate and N-acetylaspartate[120] and have elevated levels of lactate dehydrogenase A.[121] MRS may substantially increase the sensitivity and reproducibility of detecting endometriosis by MRI.

Clinical Diagnosis of Endometriosis

Recently, there has been a call to action to develop a gold standard for clinical diagnosis of endometriosis.[122] Until new screening and detection strategies are devised, a combination of approaches using machine learning algorithms may result in diagnostic classifiers of endometriosis and perhaps staging of disease. This may provide a screening strategy to prioritize those at highest risk for early intervention which may include a single definitive surgery.

Conclusions

Much progress has been made toward understanding the cellular origins, genomic program, physiological processes, and developmental context of endometriosis. There is now access to state-of-the art tools and highly skilled researchers, imaging specialists, and clinicians. Future research on this enigmatic disease has received global attention.[123] Major areas of focus include (1) disease heterogeneity suggesting possible subtypes or a syndrome with different phenotypes, (2) collaborative approaches across disciplines to understand the complex interplay of multiple factors, and (3) prompt action at the current time when governments and funding agencies have begun to appreciate the enormity of the problem. Underpinning the concept of improved outcomes for women with endometriosis is an understanding of the cellular causes of the disease, studied concurrently with the associated genomic, physiological, and developmental programs. Investment in collaborative, multidisciplinary research aiming to use enhanced knowledge of the pathophysiology of endometriosis, to reduce the time to diagnosis and provide novel therapeutic approaches, and to treat early disease and prevent progression and recurrence is required. Importantly, clinical trials that test new treatments need to be conducted with transparency and data sharing, and based on evidence of efficacy in preclinical studies, preferably using patient-derived xenografts. Teams of general physicians, gynecologists, neurologists, urologists, psychiatrists, immunologists, radiologists, and pelvic floor therapists together with researchers from multiple disciplines (immunology, stem cell biology, cell biology, pain, infertility, epidemiologists) are ideally placed to translate basic scientific discoveries of the cellular origins of endometriosis into improved patient outcomes for the more than 176 million women worldwide who are affected by this insidious disease.

Authors' Contributions

C.E.F. and C.E.G. defined the scope of the review. L.C.G., G.M., and L.R. advised on the scope of the review and contributed to the interpretation of the literature. L.C.G. and L.R. provided input from a clinical perspective. G.M. provided input on

the genomics sections. C.E.F. drafted the review. All authors edited and approved the final manuscript.

Conflicts of Interest

L.R. reports personal fees from Monash IVF Group, grants from Ferring Australia, personal fees from Ferring Australia, nonfinancial support from Merck Serono, nonfinancial support from MSD, and nonfinancial support from Guerbet, outside the submitted work, and is a minority shareholder and the group medical director for Monash IVF Group, a provider of fertility preservation services. Dr. Giudice reports a patent on diagnosing endometriosis using endometrial gene products. Nothing else to disclose relevant to this publication. The disclosures were for peer-reviewed research grants from organizations that had no influence on the writing of the manuscript. G.M. has nothing to disclose.

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