**Protocol Document**

**Understanding the Pathogenesis of Endometriosis and the Influence on Treatment Response**

Version 1.4, March 2021

Akwasi Amoako1,2, Akram Khalil1, Grant Montgomery3, Brett Mckinnon3, David Baartz1, Joanna Crawford3, Sally Mortlock3, Margaret Cummings4, Keisuke Tanaka1,2, Matthew Smith1, Bart Schmidt1, Hayden Homer1,5, Dr Quan Nguyen3, Sugarniya Subramaniam3, Lauren Walkley3, Stacey Andersen6, Sohye Yoon6, Di Xia6, Jun Ma6, Jon Xu6, Jenny Fung7

1. Department of Obstetrics & Gynaecology, Womens’ and Newborn Services, Royal Brisbane and Women’s Hospital, Brisbane, Australia
2. Discipline of Obstetrics & Gynaecology, Faculty of Medicine, University of Queensland
3. Institute for Molecular Bioscience, University of Queensland
4. Pathology Queensland
5. University of Queensland Centre for Clinical Research
6. Genome Innovation Hub, University of Queensland
7. School of Biomedical Science, University of Queensland

**Functional consequences of genetic variation in endometriosis;**

1. **Project Team roles and Responsibilities**

Department of Obstetrics & Gynaecology, Royal Brisbane and Women’s Hospital (RBWH) - Brisbane, Australia:

Dr Akwasi Amoako Chief Investigator

* Staff Gynaecologist, Department of Obstetrics & Gynaecology, RBWH
* Senior Lecturer, Faculty of Medicine, University of Queensland

Responsible for Project Management, Tissue collection and data analysis and regulatory compliance

Dr Bart Schmidt Investigator

* Staff Gynaecologist, Department of Obstetrics & Gynaecology, RBWH

Responsible for the collection of surgical tissue

Dr Akram Khalil Investigator

* Staff Gynaecologist, Department of Obstetrics & Gynaecology, RBWH

Responsible for the collection of surgical tissue

Dr David Baartz Investigator

* Staff Gynaecologist, Department of Obstetrics & Gynaecology, RBWH

Responsible for the collection of surgical tissue

Dr Keisuke Tanaka Investigator

* Staff Gynaecologist, Department of Obstetrics & Gynaecology, RBWH

Responsible for the collection of surgical tissue and analysis.

Dr Matthew Smith Investigator

* Staff Gynaecologist, Department of Obstetrics & Gynaecology, RBWH

Responsible for the collection of surgical tissue and analysis.

Institute for Molecular Bioscience, University of Queensland - Brisbane, Australia:

Professor Grant Montgomery Chief Investigator

* Group Leader

Responsible for tissue processing, data collection and analysis and regulatory compliance at UQ site.

Dr Brett McKinnon Investigator

* Visiting Academic

Responsible for tissue processing, experimental procedures and data analysis

Dr Sally Mortlock Investigator

* Postdoctoral Researcher

Responsible for Data processing and analysis

Joanna Crawford Investigator

* Lab Manager

Supervision of tissue processing, experimental procedures and data production

Dr Quan Nguyen

* Group leader Investigator

Supervision and analysis of transcriptomic data

Sugarniya Subramaniam Investigator

* Research technician

Tissue processing and experimental procedures

Lauren Walkley Investigator

* Master’s student

Tissue processing and experimental procedures

University of Queensland Centre for Clinical Research (UQCCR) – Brisbane, Australia

Professor Hayden Homer Investigator

* Christopher Chen Chair in Reproductive Medicine

Will assist in sample processing and storage at the University of Queensland Centre for Clinical Research (UQCCR) site

Pathology Queensland Royal Brisbane and Women’s Hospital – Brisbane, Australia

Associate Professor Margaret Cummings Investigator

* Pathologist

Perform or supervise analysis of both eutopic and ectopic tissue

Genome Innovation Hub, University of Queensland – Brisbane, Australia

Stacey Andersen Investigator

* Researcher

Experimental procedures

Sohye Yoon Investigator

* Researcher

Experimental procedures

Di Xia Investigator

* Researcher

Experimental procedures

Jun Ma Investigator

* Researcher

Experimental procedures

Jon Xu investigator

* Researcher

Experimental procedures

School of Biomedical Science, University of Queensland

Jenny Fung

* Researcher Investigator

Tissue processing and experimental procedures

* 1. **Site Information**

Royal Brisbane and Women’s Hospital

Department of Obstetrics & Gynaecology

Butterfield St, Herston

Brisbane 4029

Queensland,

Australia

 Institute of Molecular Bioscience

 University of Queensland

 306 Carmody Rd,

Brisbane 4067

Queensland

Australia

University of Queensland Centre for Clinical Research – Brisbane, Australia

University of Queensland

Building 71/918

RBWH Herston

Brisbane 4029

Queensland

Australia

Pathology Queensland

Block 7, Level 9

RBWH

Herston Road

Brisbane 4029

Queensland

Australia

1. **Resources**

*2.1 Resources*

* + Sample collection and processing equipment required for initial collection and the temporary storage of collected samples
	+ Sample processing and storage space provided for biological samples and derivative material by both the UQ, Clinical Research Center and the Institute for Molecular Bioscience
	+ Research laboratory in the Institute for Molecular Bioscience, University of Queensland and all associated equipment

*2.2 Funding*

* + Funding available from Metro North Collaborative Research Grant, ($47,635.00). Awarded to Chief Investigator Akwasi Amoako and Grant Montgomery as a start up fund for collaborative research projects.
	+ Funding available to Prof Montgomery from University of Queensland for experimental procedures
	+ Funding available from the Department of Obstetrics & Gynaecology for tissue collection
	+ Additional funding will be sought from the NHMRC and other external funding bodies.
1. **Literature Review;**

Endometriosis is extremely prevalent and heterogenic disease, affecting approximately 10% of women of reproductive age leading to significant pelvic pain, subfertility and has been associated with an increased chance of developing ovarian cancer later in life (1). Endometriosis is currently managed either medically via hormonal modulation, or surgically by laparoscopic excision. At present there are no non-invasive diagnostic tests and hormonal modulation is an inadequate option for women wishing to retain fertility (2). Surgical excision is difficult, is accompanied by a high rate of recurrence, with up to 30% of patients experiencing recurrence within 5 years, and places an extraordinary strain on health care systems (3). The critical need for more research to improve health care options, both now and in the future, has been recognized by the federal government with the launch of a national action plan to tackle endometriosis treatment (4).

Endometriosis is the growth of endometrial epithelial and stromal cells outside the uterine cavity. These lesions are currently split into three subcategories based on presentation and location; superficial peritoneal lesions (SUP), ovarian endometriosis lesions (OMA) and deeply infiltrating endometriosis (DIE). DIE is the most severe form and are characterized by growth of more than 5mm into the underlying tissue.

Although originally proposed in 1928 Sampson’s theory of retrograde menstruation, in which viable endometrial epithelial and stromal fibroblast cells are refluxed back into the peritoneal cavity (5), is still the most commonly accepted theory. However, up to 90% of women experience retrograde menstruation (6) thus there must be additional factors that predispose some women to an increased risk of pathogenesis. Previous research has implicated a wide variety of biological mechanisms in multiple cell types. In reality, pathogenesis is likely to result from a combination of these effects. Inherent differences have been observed in both the endometrial epithelial and stromal cells of women with endometriosis that lead to favorable growth (7). Endometrial mesenchymal stem cells (eMSC), the precursors cell of the stromal cells (8) are present in the menstrual efflux (9) and have also been proffered as a source of cells that initiate lesions, as have the epithelial progenitor cells (10) that are the precursors to the endometrial epithelial cells.

Endometriotic lesion growth is also accompanied by an inflammatory response that contributions to lesion progression. In women with endometriosis macrophages (11) and dendritic cells (12) have an altered phagocytosis and natural killer (NK) cells show reduced cytotoxic function (13). Increased levels of neutrophils (15) and cytotoxic T cells (16) in women with endometriosis compared to those without have also been reported. The unresolved pathogenesis and heterogeneous presentation with endometriosis suggest all these cells (both endometrial and immune cells) may be relevant and contribute incrementally to the disease. What underpins the aberrant activity of each cell type and how it contributes to endometriosis progression is not yet clear.

Endometriosis is a complex genetic disease with research showing genetics variants contribute approximately 51% (17) to endometriosis susceptibility. Through the Genomics of Reproductive Disorders laboratory Professor Montgomery has led international efforts to apply genome wide association studies (GWAS) to map genetic risk factors for endometriosis (18)(19)(20). His group led a study with results from eleven case-control data sets (17,045 endometriosis cases and 191,596 controls) which replicated and extended results for nine previously reported genomic regions and identified five novel regions significantly associated with endometriosis risk (18). Another study now available on BioRxiv extends this towards 27 regions (20) and new, yet to be published data, with 61,077 cases and 711,319 controls will extend the number of these regions associated with endometriosis risk to 44 (*personal communication – Prof Montgomery*).

In addition to inherited genetic variants, somatic mutations may also play a role. In a recent landmark paper in the New England Journal of Medicine (21) the researchers identified somatic changes in cells from DIE lesions that could contribute to lesion growth. In this study exome sequencing identified somatic mutations in 79% of the 24 patients studied. The number of mutations in each lesion was variable and lesions from five patients (21%) harbored known somatic cancer driver mutations in *ARID1A*, *PIK3CA*, *KRAS*, and *PPP2R1A*. More detailed experiments on samples from three other patients revealed *KRAS* mutations in two patients; one with two different activating *KRAS* mutations, and the other with the same somatic *KRAS* mutation in three separate lesions. Importantly, even though lesions contain multiple cell types, microdissection of the lesions and separation of epithelial and stromal cells found the cancer driver mutations of KRAS, ARID1A and PPP2R1A were in the epithelial cells, but not in the stroma. This underlines the importance not only of the genetic signature within the lesion, but also of the differing contribution of various cell types in giving rise to endometriosis pathogenesis.

The challenge now is to understand how these genetic variants influence cellular function and contribute to endometriosis pathogenesis, progression and response to treatment. Genetic variants are increasingly being shown to influence gene expression. Genetic variants in regions known as expression quantitative trait loci (eQTL) influence between 15-100% of gene expression in some tissue (22)(23). These expression differences will significantly influence endometriotic lesions behaviour both in terms of disease progression and response to treatment. The underlying genetic architecture of the patients, and how this influences expression and cellular function, should therefore should be taken into consideration when elucidating the mechanism of pathogenesis, as well as the potential to target current treatments and assess novel therapeutics.

Current endometriosis treatments function primarily at a systemic level to reduce the circulating estrogen concentrations (24). Such a systemic wide effect can be accompanied by multiple unwanted side effects and in many cases, fails to alleviate primary symptoms. The underlying genetic signature of individuals is increasingly being recognized as a significant predictive factor in response to treatment. At present the clinical Pharmacogenetics Implementation Consortium provides clinical guidelines for the use of 35 different medications. An understanding of the role of genetic variants in hormonal treatments may also be warranted. Recently, the presence of a genetic variant in a progesterone metabolizing enzyme, a significant hormone in endometriosis pathogenesis was found to correlate with an increased rate of progesterone breakdown and reduced effectiveness of progesterone-based treatments.

While an improved targeting of current therapeutics is a short-term goal, ultimately novel, targeted non-hormonal treatments are still needed to avoid the problems associated with systemic hormone modulation. A number of biological mechanisms, including the inflammatory response and the endocannabinoid system (ECS) have been identified to play a role in endometriosis and could potentially be targets for non-hormonal treatments.

The ECS is made up of endogenous phospholipid-based ligands, that are synthesised intracellularly on demand and then exported to activate membrane receptors, which mediate a cellular response. Their bioavailability is limited by cellular reuptake and degradation by catabolising enzymes (25). The ECS has previously been shown to affect mechanisms critical to endometriosis establishment and maintenance including cell migration, proliferation, survival and inflammation (26) and has been reported to be significantly influenced by genetic variants (27)(28)(29). Revealing how both the germline and somatic genetic architecture influence the endocannabinoid and inflammatory systems in women with and without endometriosis and with a knowledge of the underlying genetic architecture will be important for assessing the potential of targeting systems influenced by genetics as non-hormonal targeted therapeutics.

Finally, understanding the consequences of genetic variants on biological and cellular behavior and in response to treatments requires well-characterized patient tissue specifically associated with the disease. Numerous cell types, including endometrial epithelial and stromal cells, the underlying mesothelial cells, peritoneal immune cells and peripheral sensory neurons contribute to endometriosis pathogenesis and symptoms and evidence supports a cell-specific influence of genetic variants (30) on cellular behavior. Therefore, to understand the consequence of genetic variants on biological and cellular behavior and the role these variants play in treatment response, there is a need to isolate and characterize multiple tissue types and sources to study each cell type in detail and to create *in vitro* models that can be used for laboratory-based testing.

It is vital therefore to collect tissue from women with and without endometriosis to assess cellular behavior, including gene and protein expression, epigenetic changes and functional changes in all cells that have been implicated in endometriosis pathogenesis. Hence, we wish to establish a tissue collection protocol at the Royal Brisbane Women Hospital (RBWH) to undertake a study designed to identify the pathogenesis and treatment response of various compounds in relevant endometriosis tissue.

The Department of Obstetrics and Gynaecology, RBWH currently performs laparoscopic surgery for the removal of endometriosis tissue approximately 5/week, producing a potentially invaluable resource for endometriosis research. Through the expertise of the IMB and CCR, UQ research groups, this tissue can be successfully processed and stored to enable accurate and efficient implementation of the aims outlined below.

1. **Research question**

**Aims:**

* Collect sufficient, high-quality tissue involved in endometriosis pathogenesis from women both with and without endometriosis for subsequent clinical and laboratory-based studies to understand both pathogenesis and cellular response to specific compounds
* Characterize the genetic variants related to endometriosis pathogenesis and treatment response in the variety of cell types from relevant tissue
* Identify gene and protein expression differences resulting from genetic variants in endometriosis related biological material which may relate to endometriosis pathogenesis and inform treatment approaches
* Isolate and propagate pure populations of endometriosis associated cells with known germline and somatic genetic variants
* Identify the consequence of germline and somatic genetic variations in endometriosis related cells from women with and without endometriosis

**Objectives:**

Identify underlying genetic differences in women with and without endometriosis, determine their relationship to biological variations and functional activity and identify their role in disease progression, symptoms and treatment response.

**Hypothesis:**

Genetic variants in endometriosis associated cells will lead to biological variations that increase susceptibly to endometriosis pathogenesis and influence symptoms and treatment response.

**Expected Outcomes:**

It is expected that this project will establish a large, professionally processed and curated tissue resource with linked clinical and surgical information. These samples and information will greatly contribute to both current and potential future endometriosis projects. In the context of this project it will allow investigation into the germline and somatic mutations in endometrial tissue. It will provide an understanding of 1) how both somatic and germline genetic variants influence gene expression and the functional activity of individual cell types associated with endometriosis, 2) how these genetic, biological and functional variations contribute to endometriosis pathogenesis and lastly, 3) how these genetic, biological and functional variations lead to differences in symptoms and treatment response.

1. **Project Design:**
* ***Research project setting***

The project will be run as collaboration between the Department of Obstetrics & Gynaecology (RBWH), Pathology Queensland, the Genomics of Reproductive disorders laboratory (IMB, UQ) and the UQ CCR. Sample collection will be performed at the Department of Obstetrics & Gynaecology, RBWH. Histopathological analysis will be performed at Pathology Queensland. Tissue and data collected will be processed on site at the UQ CCR laboratory and subsequently at the IMB, UQ site. Patient and surgical data collected will stored in locked, linked databases housed behind University/Hospital firewalls at IMB, UQ. Experimental procedures and data analysis will be performed at the UQ IMB laboratory. Tissue collected and processed as described below will be stored in locked University sites accessible only through identifiable entry.

* ***Methodological approach***

Relevant biological samples from peripheral blood, peritoneal fluid / washing and tissue implicated in endometriosis pathogenesis, including endometrium and endometriotic lesions will be collected from women undergoing laparoscopic surgery and endometrial biopsies indicated as part of their designated treatment regime. Collected blood will be separated into its derivatives (serum, plasma and buffy coat). Peritoneal fluid will be clarified of immune cells with the resulting immune cells stored separately from the remaining peritoneal fluid. Serum, plasma and peritoneal fluid will be used for inflammation and metabolomic analysis. DNA, or live immune cells will be extracted from Buffy coats to identify germline genetic variants. The various tissue collected will be processed to isolate either RNA, DNA, cell lysate or live cells, or to be formalin fixed and paraffin embedded (FFPE). Tissue DNA will be used to identify somatic genetic variants, RNA for gene expression and cell lysate for protein expression. Live cells will be utilized for primary cell cultures or gene expression analysis. FFPE tissue will be used for both gene and protein expression.

It is intended for recruitment procedures to continue for three years to establish a database of sufficient samples. Engagement for the patient will include time required to complete the initial questionnaire, as well as the potential to contact the patient at least twice (at 6-month intervals) after the completion of surgery to provide details on the subsequent result of the treatment, as well as additional details that might be required for this or other ethically approved projects.

* ***Participants***

***Number of patients inclusion and exclusion criteria***

Inclusion criteria will be women already selected for laparoscopic surgery for suspected endometriosis. Women of all age, ethnicity and sociodemographic background will be eligible for inclusion. Exclusion criteria will be patients suffering from another or pre-existing inflammatory disease, pregnancy, malignancy or if surgery is performed due to an emergency. It is currently estimated the Department of Obstetrics & Gynaecology, RBWH is able to enroll up to 10 endometriosis surgeries patients per week. We will aim to recruit 300 participants per year for 3 years.

* ***Participant recruitment strategies and timeframes***

***First patient contact:*** First patient contact will occur during an initial consultation in which the treating physician and patients will become known to each other. After the initial consultation the treating physician, under the supervision of Chief Investigator Dr Amoako, will identify women that match the inclusion criteria for the study and that have been indicated and scheduled for laparoscopic surgery at the Department of Obstetrics & Gynaecology, RBWH.

***Second patient contact:*** Once potential patients have been identified the treating physician will organize subsequent contact via a phone call, or other approved modes of telehealth contact via either the treating physician, or a dedicated research nurse. This may occur as part of either the patients planned telehealth treatment, or as an additional phone call. The treating physician, or dedicated research nurse will explain the project in detail and will ask the patient if they would like additional information. If the patient agrees to be provided with more information about the study a patient information sheet (Attachment 1) Consent form (Attachment 2) and withdrawal of participation sheet (Attachment 3) will be sent to the patient.

**Third Patient contact;** a follow up phone call between the study doctor and the patient will be arranged to discuss the information that was provided. During this phone call the study doctor, or study nurse can go through the patient information sheet and informed consent forms and allow the patient to ask any questions they may have. If they agree the patient will be asked to sign the PICF, using an electronic signature and date that is considered acceptable to QLD Health.

Any electronically signed consent forms must be printed out on receipt by the study doctor or research nurse. The study doctor or research nurse is to write a file note on consent form on the process consent was obtained, sign and date the form. A copy of the completed consent form will be returned electronically to the patient for their records.

**Forth Patient contact;** Recruited and consented participants will be sent either a link to an online questionnaire (Paper version, Attachment xx), or, if requested, a hardcopy version that has been based on the World Endometriosis Research Foundation (WERF) questionnaire. The online Questionnaire data will be collected using a "LimeSurvey" interface. This software is freeware but allows a user to install a version behind a secure firewall and does not rely on cloud based storage. All data will be held independently within the Human Studies Unit (HSU) Endometriosis database with the local site investigator approving access to site specific staff. Access is through personalised logins and passwords for the research team. Collection of this data is done under informed consent allowing indefinite storage and use with other information collected and / or generated. Withdrawal of a participant may necessitate destruction of the data. This can be done unless data has already been used in prior analyses. This questionnaire is to be completed in the participants own time and prior to surgery. As endometriosis surgery is scheduled as category 2, which is recommended to performed within 90 days, they are estimated to have approximately 60-90 days to perform the questionnaire.

**Fifth patient contact:** The women will arrive at the Department of Obstetrics & Gynaecology, RBWH for the scheduled surgical procedure. Patients will hand in their completed WERF questionnaire. During surgical preparation and prior to the administration of anaesthesia, peripheral blood will be collected. Remaining biological samples, including eutopic endometrial biopsies, ectopic endometriotic lesions and peritoneal fluid / washing will be collected when clinically indicated during surgery and processed as described. Patient will receive the required standard of care and involvement in this study will not have an influence on the quality of treatment provided.

***Third patient contact:*** Six months post treatment the patients will be sent an additional questionnaire that records information on the response to treatment, including the reduction in symptoms as well as the presence of side effects (**Attachment 5),** along with a cover letter (**Attachment 6**) and a pre-addressed envelope asking them to complete the survey and return it through the post. The questionnaire will include the study ID of the patient and no other identifiable data.

***Fourth patient contact:*** Twelve months post treatment the patients will again be sent the same questionnaire via post with the cover letter and pre-addressed envelope to capture information on their continued response to treatment including the reduction, or not of symptoms, as well as side effects.

* **Approach to provision of information to participants and consent**

The recruitment will be continuous until the close of the study and under the direction of the project leader. Once patients have been identified as eligible for the study the main purpose and design of the study will be discussed directly with the patient via a research nurse or the treating physician at their original consultation. Once the project has been verbally explained by the treating physician, the patient will also be provided with the participant information sheet and consent form that further outlines the details of the study and the procedures involved. If they choose to participate in the study, they will be asked to sign the consent form. They will be provided with a copy of the signed consent form for reference of the details of the study, the requirements of their involvement, as well as the phone number of the Department of Obstetrics & Gynaecology that can be used if further information is sought.

Both the participant information sheet and the consent form will state that participation is voluntary, and consent can be withdrawn at any time and that a decision to not participate will not affect treatment in anyway. The information sheet will outline how their data will be managed to maintain confidentiality and also detail the risks and benefits of participation in the study. If at any stage the patient withdraws consent any biological material and patient information previously collected will be destroyed. No remuneration will be provided to the patients who participate in the study.

* **Research Activities**

*Participant commitment*

The participant commitment will include the completion of a series of validated questionnaires both pre and post-surgery and the removal of biological material for experimental studies during laparoscopic surgery already planned for their predesignated treatment.

*Collection of relevant clinical and phenotypical data*

Patients that agree to the study and have provided informed consent will be asked to complete a questionnaire that will capture data relevant for the study and include information on symptoms related to endometriosis via the previously tabulated WERF Endometriosis phenome and biobanking harmonization project (EPhect) questionnaire. Additional questionnaires 6 and 12 months after treatment will elicit information regarding treatment response.

*Collection of Biological tissue*

Blood samples will be collected from every participant on the day of surgery when intravenous access is sited. The remaining biological tissue, including peritoneal fluid, endometrial biopsies and ectopic lesions will be collected as clinically indicated during surgery. Endometrial biopsies will be performed only when clinically indicated. Ectopic lesions will be removed to surgically treat endometriosis, peritoneal fluid is removed and peritoneal cavity is washed with normal saline as a routine procedure.In the context of the surgery no additional surgical action is performed and therefore there is no additional risk to the patient. The treatment received is not altered by participation in the study.

Biological material collected will be stored securely at appropriate storage facilities, either at the participating UQCCR or IMB UQ laboratory. In all cases, only approved research personnel will have access to the specimens.

*Project duration*

The total duration of the project is 4 years. 3 years of tissue collection followed by an additional year to complete tissue and data analysis.

*Participant follow-up*

Follow-up questionnaire to be provide to participants at 6 and twelve months post surgery. These questionnaires capture details of the individual’s response to treatment and will be mailed to individual participants by personal authorized to access the address of the patients (treating physician, or study nurse).

* **Data collection/Gathering:**

*Information that will be collected*

*Patient information:* Data will be collected using the WERF questionnaire and an additional response to treatment questionnaire. Once patients have provided the signed consent form they will be provided with a questionnaire containing their study ID. No other identifiable data will be contained within the questionnaire.

*Clinical data:* Clinical data relevant to endometriosis pathology and symptoms, including but not limited to age, weight, BMI, gravidity and parity, ethnicity, previous use of medication and other gynaecological disorders will be collected through the completion of the WERF EPHect project, surgical phenotype data collection form (**Attachment 7**) that are to be completed by the surgeon at the time of surgery.

*Whole blood:* Whole blood will be collected into a total ofone 20ml evacuated tube per patient and separated into plasma, serum and buffy coat. Buffy coats will be used either for immune cell isolation and culture, or DNA isolation to identify germline genetic variants.

*Endometrial biopsy:* Primarily samples will be saved in RNAlater for subsequent analysis of somatic mutations, gene or protein expression. Any remaining sample will be flash frozen in liquid nitrogen or stored in cyro-protective media and used for gene or protein expression and the isolation and culture of endometrial and immune cells contained within endometrial biopsies. Any further remaining sample will be stored in *tissue tek* for the potential use of frozen sections.

*Peritoneal fluid / washing:* On arrival in the laboratory peritoneal fluid / washing will be immediately centrifuged to remove any immune cell content. The immune cell pellet will be kept in cyro-protective media for subsequent culture of immune cells, and the supernatant aliquoted for the analysis of pure peritoneal fluid. The aliquoted fluid will be stored at 70°C to allow batch analysis of immune, hormonal, endocannabinoids or metabolomic components related to endometriosis pathogenesis and progression.

*Ectopic lesions:* Endometrial ectopic tissue collected from either the SUP, OMA or DIE lesions will be carefully dissected from any surrounding tissue and both the lesion and surrounding tissue collected in either RNAlater for somatic mutation, gene or protein expression analysis, or either flash frozen or stored in cyro-protective media for subsequent isolation and culture of ectopic endometrial cells, or formalin fixed for production of slides that will be used for haemotoxylin and eosin staining, histological confirmation of endometriosis and protein analysis through immunohistological analysis.

*Cell isolation and culture:* The eutopic, ectopic and immune cells isolated from women undergoing surgery will be used for the isolation and culture of cells to produce *in vitro* models. Standard isolation procedures including size exclusion and fluorescence activated cell sorting (FACS) will be utilised to isolate cell of interest and establish *in vitro* models.

*Genetic assays:* To determine genetics variants that could be related to endometriosis risk DNA will be extracted from and assessed on genomic arrays to determine genome wide genetic variants. These arrays are developed to identify single nucleotide polymorphisms that commonly occur randomly across the population. To increase the number of assayed single nucleotide variants (SNP) imputation using data derived from the 1000 genomes project will be used to inferred SNP data that is within linkage disequilibrium (LD) with the assayed SNPs.

*Somatic mutational analysis:*

Selected tissue, or isolated cells will be used for somatic mutational screening via either targeted sequencing assays, whole exome or whole genome sequencing with next generation sequencing dependent on available budget.

*Induced genetic mutational for in vitro analysis of somatic cells:*

Each cell will contain millions of common SNP’s. Assessing the influence of individual SNP, or combination of individual SNP by comparing their activity in cells from different patients is difficult as subtle influences may be masked by the natural genetic variation at other locations. To assess the influence of individual, or multiple SNPs associated with endometriosis in the context of consistent genetic backgrounds in isolated endometrial cells these SNP can be induced in the cells via CRISPR-cas9 technology. We will induce this SNP in the *in vitro* cell models generated from isolated endometrial cells. We will determine their influence on gene expression and cell function determined with methods described below. These somatic cells will be used for in vitro experiments only.

*Gene expression:*

RNA will be isolated using standard procedures and either targeted gene expression via Real-time PCR, multiplexed targeting via Nanostring technology, or genome wide gene expression via RNA-seq or single cell RNA-sequencing, depending on available budget will be performed to determine gene and transcript expression levels.

*Protein expression:*

Cellular protein concentrations of the isolated tissue will be performed by both quantitative and semi-quantitative methods including Western blot analysis and immunohistochemistry. Secreted proteins will be measured via enzyme linked immune-absorbent assay using the standard manufacturers protocol.

*Metabolomic profiling:*

A non-targeting metabolomic profiling will be performed with Ultra high-performance liquid chromatography high resolution mass spectrometry (UHPLC-HRMS).

*Functional Assay:*

The influence of observed biological variations on the cellular functional behaviour either as individual or as dual cell cultures will be determined by numerous cellular assays that will include, but not be limited to viability, proliferation, apoptosis, migration and invasion.

*Impact of participant withdrawal*

Patients may withdraw their consent at any time during the project by contacting either the Department of Gynaecology, or their treating physician whose details are provided on the patient information sheet. Any biological sample already collected, as well as health related data will be removed from the project and the laboratory. Subject data not included in the prior analysis will not be affected by the withdrawal from the study.

* **Data management**

Each patient identified for inclusion in the study will be assigned a unique study identification number (Study ID). This study number will be assigned upon collection of the signed consent at the Department of Obstetrics & Gynaecology, RBWH and matched with a hospital derived patient identification number (PID). This file will represent the only link between the study number and PID number and will remain accessible only to authorized members of the RBWH clinical team in a secure database with an audit trial function and accessible only via a username and password. Clinical and surgical data collected through the completion of the surgeons reporting questionnaire and the patient questionnaire respectively will be entered into a secure database by authorized staff that is stored behind on a secure hospital firewall.

The biological material and the health-related data required for analysis will be provided to the UQ laboratory attached to the study ID only and all subsequent experimental data generated during the project will be stored attached to the study ID on secure servers behind a University firewall. This data will be considered anonymous to laboratory staff. Only authorized staff for the project will have access to the data. Transfer of any data will be via secure transfer protocols. Data storage will be for the duration of the project, as well as and for subsequent projects based on information or material derived from this project.

* **Data analysis**

Data analysis derived from collected health related data and experimental work will be undertaken at both the IMB, UQ and at the RBWH. This will make use of the best, high performance computing clusters and local expertise available at both sites. Additional analysis by other groups may occur with the appropriate governance and human research ethics approval. Data shared across sites will be via the study ID only and contain re-identifiable data with the linkage key stored only at the RBWH, Department of Gynaecology.

*Matching and sampling strategies*

Each patient identified for inclusion in the study will be assigned a unique study identification and all data and biological material collected eligible for inclusion in the study. Only once full histopathological analysis of the excised endometriotic samples is complete and the diagnosis reported to the physician will the patients will be allocated to either the control (endometriosis frees) or case (endometriosis) groups. The physician will inform the laboratory of the final endometriosis status via the study ID only.

*Accounting for potential bias, confounding factors and missing information*

To avoid bias between groups of women with and without endometriosis only once final diagnosis from the histopathological analysis of tissue removed during surgery has been performed and reported will the women be designated to either the endometriosis case, or control group.

*Statistical power calculation*

Samples will come from women both with or without endometriosis and this project is designed to establish continuous collection procedures and store relevant tissue for multiple comparisons between women with and without endometriosis, as well as between different genotypes. As such, a statistical power analysis will be performed as part of the design for each individual experiment prior to beginning the experiment. The use of biological materials for each individual experiments and will go ahead only once it has been confirmed sufficient power is available.

* **Data linking: What linkage are planned or anticipated**

Genetic information generated during this project and forming the basis of publications may be provided to international, not-for-profit databases which collect and store information about disease, with all information anonymized. This is essential to ensure maximal effectiveness of patient donated material in addressing research questions.

By following the WERF EPHect guidelines for both the collection of health-related data in the questionnaires and the adherence to biological processing standards outlined in the EPHect project we anticipate the potential to become registered as a Center for WERF Ephect tools. This will provide the opportunity to collaborate directly with other registered centers. This will enable multi-center collaborations. Any data shared through this program will be strictly anonymized.

1. **Results outcomes and future plans**:
* **Plans for return of results of research participants**

In this study it is the results and analysis of the whole cohort of samples that is expected to produce significant data and contribute towards improvement in the treatment of future patients. The genotyping assay that is planned will not examine regions known to be causative of specific diseases and thus produce clinically relevant data. The use of either whole or exome genome sequencing of the endometriotic lesions is to identify somatic mutations relevant to these benign lesions and thus it is very unlikely incidental findings relevant to the individual participant will be identified. It is extremely unlikely therefore that the data at this stage of the project will be meaningful to share with individual patients. In the rare case, or possible future cases, where results of the health or genetic analysis could result in an incidental finding that could be potentially beneficial to the participant it will be relayed back to the treating physicians of the original study using the study ID.

It will be possible for the physicians to then re-identify this patient through the linkage data stored at the RBWH, Brisbane. The treating physician will then have the opportunity to investigate the validity of the finding using accredited diagnostic laboratories and subsequently have the capacity to decide on whether this information should be passed onto their patient.

* **Plans for dissemination and publication:**

Research results will be published in peer-reviewed journals and the results presented at conferences where appropriate. If requested by the journals, de-identified results may be submitted to recognized data repositories with controlled access policies to provide open access for research purposes.

* **Other potential usage of data:**

Re-identifiable data will be stored in secure electronic storage facilities as described above and may be provided to addition projects if deemed relevant and approved by the relevant human research ethics committees.

* **Project closure procedure:**

The collection of patient samples is initially requested for 3 years. The project will include the production of *in vitro* models potentially useful to future projects and other researchers and thus will not be destroyed at the completion of the project. These models will be made conditionally available, subject to the receipt of appropriate ethical approval has been received. 3rd party researchers will only have access to non-identifiable data and will not be able to match these cell models with previous patients.

* **Sharing and future use:**

It is likely there will be remaining tissue after the analysis performed for this project. It is possible this tissue could be useful for future studies related to endometriosis. Any use of this remaining tissue would be dependent on the project acquiring relevant ethics approval. It is envisioned that the *in vitro* models produced during this project will be viable models for subsequent studies. Established cell cultures will be shared on the understanding that they will remain de-identified.

**List of attachments;**

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| --- | --- | --- | --- |
| Attachment 1 | Participant information sheet |  |  |
| Attachment 2 | Consent form |  |  |
| Attachment 3 | Form for withdrawal of participation |  |  |
| Attachment 4 | WERF EPhect – Clinical data standard questionnaire |  |  |
| Attachment 5 | Response to treatment questionnaire |  |  |
| Attachment 6 | Mail out Cover letter |  |  |
| Attachment 7 | WERF EPHect - Surgical questionnaire |  |  |

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