### Study Protocol

### Project Title: Genetic and Biochemical Predictors of Pelvic Organ Prolapse

Named investigators:

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Patient recruitment, sample collection and expertise in pelvic organ prolapse diagnosis and treatment

Abstract:

Pelvic organ prolapse (POP), the descent of one or more of the pelvic organs into, or out from the vagina. It is a common condition in women but can vary greatly in terms of severity and age of onset. It results in incontinence and discomfort. Treatment options which include physiotherapy, pessaries and surgery have significant limitations. Data from overseas suggests there is a genetic component to POP risk, with certain genetic variants either increasing or decreasing a woman’s risk of POP. Here we aim to find further risk factors for the development of POP - both genetic and biochemical - in New Zealand women, and understand how genotype influences POP risk in the context of other factors such as parity and weight. We are also aiming to understand how particular genetic factors alter the biochemical properties of both the cells and the connective tissues of the pelvic floor. We are aiming to link genetic and biochemical factors with risk of developing POP, with a view to guiding the management and also developing therapies for women with this condition.

Aims:

To identify genetic and biochemical markers which are associated with pelvic organ prolapse with a view to developing predictive biomarkers for this condition.

Hypotheses:

1. Women experiencing a severe POP will carry a burden of rare, genetic variants, when compared to control women, that predispose them to the condition
2. These rare, genetic variants will affect genes that encode proteins critical to the function of pelvic floor connective tissue
3. Women that carry certain rare, genetic variants will have measureable differences in the composition of pelvic floor connective tissue that makes them more susceptible to extreme prolapse

Methods:

A three step process will be adopted to identify genetic and biochemical biomarkers for this condition:

1. Ascertain a cohort of women with the condition from a variety of clinical contexts including primary and secondary care clinical environments. A variety of clinical data will be gathered including the type and degree of prolapse, age at surgery, parity and delivery method, weight and ethnicity.
2. Biological samples will be obtained from the participants including blood, urine and a sample of anterior vaginal wall from women undergoing pelvic surgery for either POP or non-POP related reasons (control group). These samples will be used to:

* Extract nucleic acid for genomic or transcriptomic analysis
* Urine will be analysed for biochemical indices of connective tissue turnover
* Pelvic floor fascia will be analysed to assess the diversity, activity and abundance of extracellular connective tissue proteins and glycoproteins and for the assessment of gene expression

1. This biological data will be collected together with current and past health data, including age at surgery, parity and delivery method, weight and ethnicity. A control group will be ascertained to facilitate comparative analyses relating to the genetic and biochemical data as described above.

Research design:

1. Background

Pelvic organ prolapse is an umbrella term for a range of different types of organ misplacement within the pelvic region, including cystocele where the bladder prolapses into the vagina, rectocele where the rectum prolapses into the vagina and uterine prolapse.1 POP can cause significant discomfort, loss of sexual function, urinary incontinence, urinary tract infection, constipation and pain as well as substantial shame and embarrassment.

Pelvic organ prolapse affects all women to some degree as they age and 19% will require a surgical repair by the time they are 80.2 All pelvic prolapses result from a weakening of connective tissue, such as ligaments and fascia, that secure the pelvic organs in position within the cavity of the pelvis and they can be graded by severity.3 A number of treatment options are available however almost all use mechanical methods alone to repair or support the connective tissue, rather than addressing the underlying biological mechanism of tissue failure. Furthermore the use of surgical mesh to support prolapse repairs is now no longer an option in New Zealand because of significant issues such as permanent nerve damage and long-lasting pain in large groups of women,.4 Consequently there are no off-the-shelf surgical devices available in New Zealand to support the repair of POP.

Rare, human genetic diseases offer unique insights into the function of genes during development and over the lifespan. Work carried out by the applicant, as well as others globally, has discovered the underlying cause of rare connective tissue disorders that cause severe inherited herniation and prolapse.5,6 Furthermore, common genetic variation associated with related genes has been shown to increase an individual’s susceptibility to POP.7 The hypothesis is that women with rare, coding genetic variants in genes previously implicated in POP, or in genes in which loss of function leads to a severe, syndromic disease, will be more susceptible to and will present clinically with POP. An example of this is *EFEMP1,* a gene in which homozygous loss-of-function variants cause a severe failure of the fascia5,6 but also a gene in which heterozygous loss-of-function and missense variants cause increased risk of hernia or prolapse.7

While genome-wide association studies have associated certain genotypes with risk of POP and other connective tissue disorders, these approaches typically find low-effect variants in studies that require large numbers of individuals and have not looked specifically at the New Zealand population. The goal of this study is to look for protein-changing variation within a designated subset of genes in a cohort of New Zealand women with a severe POP phenotype. This extreme phenotype is defined as pre-menopausal women (<55) with a prolapse score of 3 or 4 as defined by the POP-Q scale8 (personal communication, gynecological surgeon, Dr Michael Stitely).

This defined group of women may have a genetic cause underlying their prolapse. In this study, a gene-collapsing burden analysis of genetic variation in a defined set of genes will be performed in the experimental group as compared to the control group (pre-menopausal women, para ≥ 1, POP-Q score 0 or 1). This method allows for the discovery of significant contributions of gene-associations to the POP phenotype.9 The contributing variants may be rare, or even unique to families, therefore using a nominated panel of genes that are (a) implicated in POP (b) other connective tissue disorders and/or (c) are expressed in pelvic organ fascia, will narrow the search space and enhance the chance that these variants have a direct influence on the development of POP.

This study will also go beyond discovering risk genotypes and take steps to understand how changes in the genome influence the protein content and turnover of pelvic connective tissues. This work will therefore link genotype to phenotype and reveal potential biological therapeutic options for further study and development.

1. Methods and experimental design

Study objective:

This study aims to collect genetic information and tissue from women over an extended period of time. The aim is to understand if women experiencing pelvic organ prolapse are more likely to carry rare, protein coding variants in genes associated with fascial strength. Furthermore, how these genes contribute to the tissue composition and break-down of fascia in the pelvic region, will be investigated.

Study design:

This is a comparative study involving women undergoing surgery for pelvic organ prolapse repair or non-prolapse related gynaecological surgery (e.g. hysterectomy) as a control group. Recruitment targets will be women scheduled for their first pelvic surgery for prolapse repair or for abdominal hysterectomy.

The inclusion criteria are:

1. Aged between 18 and 55
2. Able to give informed consent for participation
3. For the surgical group only: undergoing planned pelvic prolapse repair surgery or a planned abdominal hysterectomy.

Exclusion criteria:

1. Taking anti-coagulant medications
2. For the surgical group only: any medical condition that, in the assessment of the operating surgeon, contraindicates taking a tissue biopsy
3. Diagnosed primary genetic disorder affecting connective tissue or a secondary disorder that affects connective tissue such as autoimmune diseases

No similar studies have been conducted linking the contribution of genetic variation to fascial tissue composition and strength in the pelvic floor, in women susceptible to severe pelvic organ prolapse.

Māori women may be disproportionately affected by pelvic organ prolapse. Compared to other New Zealanders, Māori have more pregnancies with a greater proportion of vaginal deliveries. Both of these are risk factors for POP. Our research aims to directly benefit Māori. To ensure this the following steps have been taken:

1. Undertaken consultation with Hine Forsyth, Ōtākou Runaka through the Māori Community Consultation at the Otago Medical School and the Ngāi Tahu Research Committee at the University of Otago. This communication has confirmed that pelvic organ prolapse is an issue for Māori women and Māori women often do not seek medical care for these issues.
2. Begun an ongoing relationship with Māori healthcare workers, coordinated through Wendi Raumati (kaiawhina – Dunedin hospital) allowing us to tune research questions and feedback findings to the community. Together with physiotherapists and surgeons, we will hold Hui to enable discussion of pelvic organ prolapse with local women, and the services and treatments available. This forum will also allow us to feedback findings from this study to the affected community.
3. Specifically considered the consent process with Māori participants in mind. Informed consent will be sought at a pre-surgical appointment in the weeks leading up to the surgery. Consent forms will be available in Te Reo Māori. Potential participants will have the option to have a kaiawhina present during the consent process. Six months after surgery a follow-up will be undertaken with the kaiawhina and a researcher to ensure ongoing confidence with the research involvement.
4. Based on the He Tangata Kei Tua Guidelines we have implemented Tikanga around sampling and tissue/data storage. Māori tissues will be stored in freezers dedicated to the storage of human tissues only. The Laboratory for Genomic Medicine (LGM), where this research will take place, holds regular enoi, led by Ben Te Aika (Research and Enterprise, University of Otago) at the dispatch or disposal of Māori samples or tissues. This process will be introduced at the consent stage and participants can opt in. Members of the team in the LGM, who handle human tissue specimens in the laboratory, have undertaken level three courses in Tikanga at Te Wānanga o Aotearoa.
5. Ensured that the participants themselves will have ultimate ownership of their samples and the data generated. We recognise that members of the LGM are custodians of these samples and data while it is in our hands. Participants will be followed up six months post-surgery to ensure ongoing comfort with sample storage and data generation. Participants will have the ability to withdraw at any stage after the consent process. At withdrawal, participants will be able to have their sample and/or data returned if possible, or opt for destruction as laid out in point 4. Videos of karakia spoken at sample destruction can be provided back to participants if they desire.
6. Undertaken the responsibility to put the collected data into an accessible format to be accessed by study participants if they so wish. Data will be explained to study participants by an LGM researcher, if the participant wishes. Raw, genome sequencing data can be returned to a participant if they desire but researchers will be unable to discuss details of genetic findings, beyond the ones of interest in this study. At the completion of data collection on a particular sample, the participant will be notified allowing them to provide feedback to a researcher.

Study methodology:

Recruitment will be ongoing over a 10 year period. This length of time is necessary to recruit an appropriately sized cohort. There are two parts to this study:

1. Women undergoing surgical repair of a pelvic organ prolapse or an abdominal hysterectomy (control group) will have a blood, urine and tissue sample collected alongside their surgery. They will have their genome sequenced, and the biochemistry and proteins of the urine and tissue will be analysed.
2. Women with or without prolapse (control group) will be identified in the primary care setting and have only a blood sample taken and a genome sequencing performed.

Women will be recruited by gynaecological surgeons working in the Southern region: Michael Stitely in Invercargill and Elliot Mackenzie in Dunedin. Approximately 25 POP repair surgeries will be carried out each year across the two cities, therefore we anticipate a maximum of 250 women recruited into our experimental group and 250 recruited into the control group. A further 350 will be recruited from the primary care setting to contribute to the clinical and genetics limbs of this study only.

We understand that many women do not seek surgery for these pathologies, however we anticipate that by including community engagement in this study we may encourage more women to seek appropriate treatment. If a woman decides to undergo surgery as a result of our community outreach, she will have no obligation to be a part of this study.

Women will also be recruited in a primary care setting and/or at routine cervical screening appointments. These women will not be required to give a tissue sample, these women will be enrolled in the genome analysis part of the study only. Women aged between 18 and 55 will be identified in the primary care setting by one of three routes:

1. Nurses at participating general practices and hospital clinics will identify women with POP-Q grade 0 or 1 (controls), or grade 2-4 pelvic organ prolapse whilst undertaking routine cervical screening
2. GPs will identify potential participants with prolapse during routine primary care appointments for the purpose of symptoms of prolapse. A POP-Q grade 2-4 prolapse will be confirmed by the GP
3. Physiotherapists at participating physiotherapy clinics will identify women who are seeking help for a medically diagnosed POP-Q grade 2-4 pelvic organ prolapse

The criteria for the prolapse group versus the control group are as follows:

Prolapse group:

* Women (including all people with female reproductive anatomy [but no history of male hormone therapy]) between 18 and 55
* Current or previous prolapse of POP-Q grade 2-4

Control group:

* Women (including all people with female reproductive anatomy [but no history of male hormone therapy]) between 30 and 55
* Para 1 or higher (pregnancy carried to at least 24 weeks)
* POP-Q grade 0 or 1
* No first degree relatives that fit into the prolapse group

The study will be briefly introduced to the potential participant by the primary care provider or the surgeon. A flyer will be provided to the woman and their email address and/or telephone number will be collected with the woman’s permission. A study researcher or the primary care provider (depending on the wishes of the primary care provider) will conduct a follow-up phone call a week later to clearly explain the study and state what will be involved.

A face-to-face meeting will be arranged if the potential participant is interested. At the face-to-face meeting with a researcher, and with a research coordinator from the University of Otago in the initial stages of recruitment, the woman will be taken through the participant information sheet and be given the opportunity to ask any questions. Consent will be requested for the collection of medical history (via researcher interview), blood sample, genome sequencing, tissue extraction if applicable, as well as for provision of ethnicity. The potential participant can choose to give informed consent and answer medical history questions at this appointment, or another appointment will be arranged if she would like more time to consider. A blood test form will be provided to the woman recruited from the primary care setting, allowing her to give a blood sample at Southern Community Laboratories (SCL). Blood samples can be collected by any SCL branch and couriered to Dunedin. DNA extraction will take place in the Laboratory for Genomic Medicine. The study may be expanded to other regions and arrangements will be made for blood collection in those regions.

A medical history will be collected from the participants as follows:

General

- Age

- Weight

- Smoker/smoked

- Ethnicity/ancestry

Pregnancy/prolapse related

- Number pregnancies

- Number births

- Method of birth (vaginal/caesarean/instrumental)

- Injuries during birth

- Position of baby

- Method of contraception

- Prolapse grade (if a prolapse case)

- Treatment for previous prolapse (details, treatment sought, age and severity)

- Family history of prolapse

- Personal or family history of joint laxity/blood vessel disorders

For the surgical cohort, waste blood and urine will be collected during the surgery for use in this study (rather than discarded). Women will either be undergoing pelvic organ prolapse repair or abdominal hysterectomy (control group), however in both cases, the same tissue will be biopsied. The operating surgeon will take a 5 mm punch biopsy of anterior vaginal wall after the repair or hysterectomy has been performed, prior to closure. The tissue will be removed using a sterile punch and forceps then placed in a vial containing sterile saline. These tissues are routinely removed and normally discarded during these procedures, the removal of these tissues will have no detrimental effect on the woman’s recovery. An independent safety monitor will review clinical notes to ascertain if any excess in adverse events are occurring in study subjects.

The samples will be collected by a Laboratory for Genomic Medicine researcher and the woman will be notified that her sample has been received. This is the last time that the participant will be contacted by a researcher unless they have consented to or requested more contact.

Women recruited into the study will be de-identified and assigned a random participant number. A master database will be kept, allowing the re-identifying of samples if they are required to be destroyed or results requested by the participant. Day-to-day, researchers working in the LGM will not be able to access this information. The database will be accessible to one, trained member of the LGM and will be kept on a password protected computer, in a locked office.

All handling of biological specimens will take place in a class II biological safety cabinet until biological risk is removed.

Blood

A 10-20 mL sample of peripheral blood will be collected into an EDTA tube. The samples may be stored in a dedicated specimen fridge for up to 16 hrs. Blood will be processed on the day of collected in a class II biological safety cabinet for extraction of genomic DNA using the Wizard DNA extraction kit (Promega, A1120). DNA will be quantified and stored in a freezer dedicated to human DNA in the LGM (Hercus Building, Dunedin School of Medicine).

DNA will be subject to whole genome sequencing at AgResearch, Dunedin, New Zealand, using their Illumina Instrument.

Data will be returned to the LGM via secure server to server encrypted exchange and stored a high capacity, secure server (University of Otago) accessed only by members of the LGM. Alignment to reference sequence and variant calling will take place using established pipelines developed by the Broad Institute (Cambridge, MA, USA) and used routinely by the LGM. Protein-altering variants (missense, splice, frameshift or stop-loss or gain) in a pre-curated list of genes will be characterised.

The curated list of target genes will be created undertaking proteomic analysis of pelvic floor tissue of six control women. This method will identify extracellular proteins that are highly abundant in the tissue of the pelvic floor. The hypothesis is that protein-coding, variants in genes that encode these proteins will be more likely to have an effect on the integrity of the pelvic floor tissue. Genes implicated in pelvic organ prolapse risk via genome wide association studies, or through rare disease, will also be included in this list.

We estimate a list of approximately 100 genes. This defined search space means a discovered protein coding variant will more likely have an effect on pelvic floor tissue, but also reduces the chances of discovering incidental findings that may be clinically actionable. Using this list, two analyses will be performed. Firstly, there will be a search for genetic variants which unequivocally destroy the function of the gene (i.e. loss-of-function). A power calculation suggests we need 168 POP samples versus 168 control samples, to find a significant effect, based on 6% of women having a loss-of-function variant in one of the 100 genes (assumptions: mean allele frequency of loss-of-function variants: 0.06%, alpha = 0.05, power = 90%). Secondly, a Variant Burden Analysis will be performed by comparing the number of rare variants (AF < 1%), that affect the protein, in the genes of interest between POP and control samples.9 Tools which can test the variance of genetic effects will be used to cope with both trait-increasing and –decreasing variants.10 A larger cohort of women (up to 350) will be recruited for genome sequencing only to generate enough power to undertake these two studies. These analyses together will then allow the development of a genetic risk score, based on the average number of loss-of-function and/or rare variation present in women with POP.

Handling of incidental findings discovered during analysis.

Broadly based screening methodologies such as exome and genome sequencing will result in the discovery of a large number of genetic variants. For this research much of this variation will be computationally filtered from datasets as part of the analysis to find the relevant variants of interest. These approaches however still have the potential to uncover genetic variants that are of direct relevance to the health care of the research participant or their family.

International experience has shown that the chance of this occurring using the analytical approaches applied here are well less than 1%. Since the aim of this research is to solely address the contribution of variation in a defined set of genes to the incidence of POP, the chance is even lower.

A list of 70 genes has been outlined by the American College of Medical Geneticists as representing a gene set that if a variant is identified within them then that variant is deemed potentially medically actionable. One of the researchers is a clinical geneticist and he will be available to interpret such findings and determine if they should be acted upon in terms of informing the participant of the finding, explaining its significance and arranging the necessary health care referrals. The consent form and information sheet make it clear that it will be our policy to return such findings to the families/participants and if participants decline to agree to this plan, this will represent an exclusion criterion for the study.

Urine

A sample of urine will be collected from study participants on the morning of their surgery and collected immediately by a LGM researcher. Urine will be processed in a class II biological safety cabinet on return to the LGM for the quantification of hydroxyproline (marker of collagen degradation) and of desmosine (marker of mature elastin degradation).11 The two biomarkers of interest will be quantified via enzyme-linked immunosorbent assay (ELISA) using commercially available kits.

Briefly, for hydroxyproline measurement, a 100 µL sample of urine will be hydrolysed with hydrochloric acid, neutralised with activated charcoal and then allowed to dry on a well of a 96-well test plate. The sample will be re-hydrated with buffer and reagent from the kit (hydroxyproline assay kit, Sigma Aldrich, MAK008) and the well absorbance will be measured at 560 nm on an appropriate plate reader, alongside known standards.

For the quantification of desmosine, 100 µL of urine will be added to sample wells of the desmosine ELISA kit (MY BioSource, MBS730011), followed by balance solution and conjugate solution provided with the kit. The samples will then be washed and substrate A and B (provided by the kit) will be added. The samples are incubated and the absorbance is measured at 450 nm on an appropriate plate reader alongside known standards.

Level of protein degradation products in the urine will compared with numbers of loss-of-function or rare variants in the genomes. If there is significant positive correlation between number of variants (or presence of a loss-of-function variant), high levels of degradation products, and POP, this will be a useful, non-invasive biomarker to predict POP.

Pelvic tissue

Harvested pelvic tissue will be collected by a LGM researcher during surgery and returned immediately to the LGM. The tissue will be weighed, washed with saline and dissected.

Pelvic tissue will be biochemically analysed using different methods to quantify protein expression, enzymatic activity and proteoglycan content. Initially, a proteomic analysis will be undertaken on eight POP and eight control samples to curate the list of genes-of-interest needed for the genomic analysis. Further proteomic analysis will be undertaken to allow direct comparison between genetic variation and effects on protein content. Briefly, anterior vaginal wall tissue will be processed for mass spectrometry to quantify the extracellular (EC) proteins using a specialist kit to obtain the extracellular protein fraction.12 The insoluble EC proteins will be submitted to the Centre for Protein Research (CPR) (Department of Biochemistry, University of Otago). The CPR will undertake protein quantification, identifying ~80 proteins in the samples. Data will be processed using Proteome Discoverer software, by searching against the SWISS-PROT amino acid sequence database for protein identification. Further pieces of tissue biopsy will subject to other tests, for example protease activity will be quantified using commercially available plate-reader assays and for proteoglycan content will be assessed using anion-exchange HPLC as described previously.13

The genetic variants found by genomic analysis can be put into context by undertaking quantification of the protein content and enzymatic activity of the pelvic floor tissue. After analysis, any remaining biopsy tissue will be destroyed. All tissue analysis will take place in the LGM or New Zealand based-laboratories.

1. Significance

This research ultimately aims to improve outcomes for individuals experiencing the common, life-altering condition of pelvic organ prolapse. This comparative study will allow us to understand if certain women are more at risk of POP at a younger age, and may one day provide methods to identify these women and offer preventative procedures. Furthermore, by understanding key protein alterations in pelvic tissue during prolapse, we can begin to develop novel biological treatments. The data generated will be of interest to clinicians, women’s health advocates, and other health professionals. Our findings will be transferred to these groups through local and international meetings and general interest and specialist medical publications. We also aim to publish in international medical journals and collaborate with industry partners to develop novel therapies.

References

1. Australia, C.F.o.,Series Pelvic organ prolapse 2015.

2. Smith, F.J., C.D.A.J. Holman, R.E. Moorin, et al., Lifetime Risk of Undergoing Surgery for Pelvic Organ Prolapse. Obstetrics & Gynecology, 2010. **116**(5).

3. Arian, A., Z. Ghanbari, M. Deldar Pasikhani, et al., Agreement of Manual Exam (POP-Q) with Pelvic MRI in Assessment of Anterior Pelvic Organ Prolapse. Iran J Radiol, 2017. **14**(4): p. e38542.

4. Medsafe,Series Regulatory action on surgical mesh products 2018.

5. Driver, S.G.W., M.R. Jackson, K. Richter, et al., Biallelic variants in EFEMP1 in a man with a pronounced connective tissue phenotype. Eur J Hum Genet, 2020. **28**(4): p. 445-452.

6. Bizzari, S., L. El-Bazzal, P. Nair, et al., Recessive marfanoid syndrome with herniation associated with a homozygous mutation in Fibulin-3. European Journal of Medical Genetics, 2020. **63**(5): p. 103869.

7. Olafsdottir, T., G. Thorleifsson, P. Sulem, et al., Genome-wide association identifies seven loci for pelvic organ prolapse in Iceland and the UK Biobank. Communications biology, 2020. **3**(1): p. 129-129.

8. Persu, C., C.R. Chapple, V. Cauni, et al., Pelvic Organ Prolapse Quantification System (POP-Q) - a new era in pelvic prolapse staging. Journal of medicine and life, 2011. **4**(1): p. 75-81.

9. Cirulli, E.T., S. White, R.W. Read, et al., Genome-wide rare variant analysis for thousands of phenotypes in over 70,000 exomes from two cohorts. Nature Communications, 2020. **11**(1): p. 542.

10. Lee, S., G.R. Abecasis, M. Boehnke, et al., Rare-variant association analysis: study designs and statistical tests. American journal of human genetics, 2014. **95**(1): p. 5-23.

11. Luisetti, M., S. Ma, P. Iadarola, et al., Desmosine as a biomarker of elastin degradation in COPD: current status and future directions. European Respiratory Journal, 2008. **32**(5): p. 1146.

12. Au - Naba, A., K.R. Au - Clauser, and R.O. Au - Hynes, Enrichment of Extracellular Matrix Proteins from Tissues and Digestion into Peptides for Mass Spectrometry Analysis. JoVE, 2015(101): p. e53057.

13. Mizumoto, S. and K. Sugahara, *Glycosaminoglycan Chain Analysis and Characterization (Glycosylation/Epimerization)*, in *Proteoglycans: Methods and Protocols*, F. Rédini, Editor. 2012, Humana Press: Totowa, NJ. p. 99-115.