Investigation of the Effect of the Female Urinary Microbiome on Incontinence

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RESEARCH PROTOCOL

Specific Aims

Overactive bladder (OAB) syndrome is characterized by the symptom complex of urinary urgency, usually with associated frequency and nocturia, with or without urgency urinary incontinence in the absence of infection or other pathology [9]. OAB affects approximately 31% of women over the age of 65 [10]. Vaginal estrogen, a well-documented treatment for OAB in hypoestrogenic women, has been shown to improve symptoms of frequency, urgency and urgency urinary incontinence (UUI) [7, 11, 12]. Several theories have been proposed to explain the mechanism underlying estrogen's effect on lower urinary tract symptoms (LUTS) [13, 14]. We propose that estrogen treatment influences bacterial communities (microbiomes) in the vagina and bladder and alters urothelial and vaginal (AMPs); thereby improving OAB symptoms in hypoestrogenic women.

Long-standing medical dogma has been replaced by clear evidence that a female urinary microbiome (FUM) exists [3, 4]. Our research team recently reported that the FUM in women without OAB is less diverse than the FUM of women with OAB [3]. We soon will report that FUM status stratifies women with OAB into treatment response groups and women with less diverse FUMs are more likely to respond to anti-cholinergic OAB therapy (Thomas-White et al., in prep). This suggests that the FUM is a factor in lower urinary tract symptoms (LUTS) and that FUM diversity contributes to LUTS and treatment response, like the vaginal microbiome and its contribution to vaginal symptoms [5, 6].

In hypoestrogenic women, the vaginal microbiome shifts from low diversity communities, commonly dominated by *Lactobacillus*, to more diverse communities dominated by anaerobes [5]; this change can be reversed with estrogen treatment [6-8]. Since the FUM of women with OAB includes bacteria similar to those of the vaginal microbiome (e.g. *Lactobacillus*, *Gardnerella*, and diverse anaerobes) [3], we reason the FUM would respond similarly to estrogen and become less diverse. Although transvaginal medications likely alter nearby bacterial niches (e.g. the bladder), no study has reported the urinary microbiomic response to vaginal estrogen.

While almost nothing is known about urinary/vaginal microbiome interplay, even less is known about immune response modulation in the bladder and vagina. However, estrogen reduces the subsequent UTI rate in hypoestrogenic women affected by recurrent UTI [9, 10], and estrogen induces urothelial antimicrobial peptide (AMP) expression [15]. Since AMPs exhibit microbicidal activity, stimulate inflammation, and facilitate epithelial barrier homeostasis [11], estrogen may work through AMPs as mediators to optimize microbial equilibrium.

We hypothesize that, following estrogen treatment of hypoestrogenic women with OAB, symptom improvement will be associated with 1) reduced FUM diversity, 2) alteration of other FUM characteristics and 3) increased AMP levels. We propose two specific aims:

Aim 1: To compare pelvic floor microbiome (PFM) diversity and AMP levels before and after estrogen treatment in hypoestrogenic women with OAB symptoms. We will enroll 20 hypoestrogenic patients with bothersome OAB prior to clinically indicated vaginal estrogen treatment as part of a pilot study. We will collect catheterized urine, voided urine, perineal swabs, vaginal swabs and vaginal lavage at baseline and 12 weeks post treatment. We will assess the associated PFMs (i.e. urinary and vaginal microbiomes) using two complementary approaches: *(i)* Expanded Quantitative Urine Culturing (EQUC) and/or *(ii)* 16S rRNA amplicon sequencing. We will quantify candidate AMPs by quantitative PCR and protein analysis [12, 13]. We will compare and contrast each PFM and its associated AMP profiles. Relevant questions include: Do associations exist amongst adjacent PFMs and between PFMs and AMPs? Do PFMs and AMPs change in response to estrogen? Does an improved (less diverse) PFM correlate with an optimized AMP profile?

Aim 2: Determine if FUM characteristics correlate with OAB symptoms.
At baseline and 12 weeks, we will use the overactive bladder questionnaire (OAB-q) [14] to evaluate symptoms and determine if PFM characteristics (e.g., composition, diversity, abundance, specific organisms) and AMP levels (gene expression and protein levels) correlate with patient symptoms. Relevant questions include: Does response to estrogen associate with pre-treatment PFM and/or AMP characteristics? Do PFM and/or AMP changes correlate with treatment response?

Significance: The medical field is beginning to adopt treatments that alter an individual's microbiome and AMP profile to improve patient health; however, this approach has not been adopted for LUTS treatment. Here, we propose the first step in development of such a therapy. If our hypothesis is correct, we could validate estrogens a first line of treatment in hypoestrogenic women. It could also propel the use of an individual's PFM and/or AMP characteristics to develop future personalized therapies to improve treatment response and/or prevent LUTS.

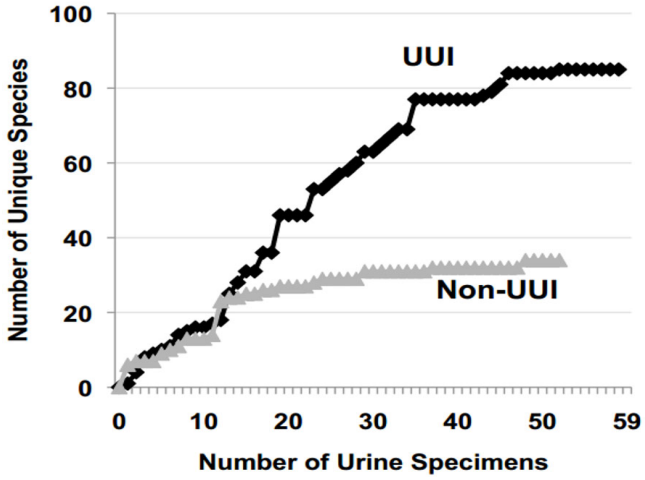
Estrogen improves OAB symptoms: There is compelling evidence to suggest that vaginal estrogen therapy relieves symptoms of OAB including frequency, urgency and UUI in postmenopausal women [7, 8, 11, 12]. The evidence is sufficient to list the application of vaginal estrogen alone as a treatment option for OAB in the clinical practice guidelines for postmenopausal women with urogynecological complaints [18]. The mechanism of vaginal estrogen's effect on OAB symptoms is poorly understood. Vaginal estrogen has the ability to modify the vaginal microbiome and reduce recurrent urinary tract infections in postmenopausal women [16]. We reason that vaginally applied estrogen would also modify the FUM, which appears to play a role in OAB [1].

The Bladder is Not Sterile. Current treatments and preventive strategies are based upon the dogma that the bladder is a sterile environment. Compelling new evidence challenges this long-held belief. Detection of bacterial DNA and isolation of live bacteria from urine taken directly from the bladders of women with and without urinary symptoms has been reported by us [1-3], and by others [4, 5]. However, the role of the female urinary microbiome (FUM) in urinary health and disease is incompletely understood.

FUM diversity correlates with symptoms. Recently, we sequenced and cultured urines from individuals with urgency urinary incontinence (UUI) and continent controls using two complementary methods, 16S rRNA sequencing and expanded quantitative urine culturing (EQUC) [1]. We observed a striking difference in the diversity between the two cohorts (**Figure 1**). Continent controls had a markedly less diverse FUM than did the UUI cohort (1 [interquartile range (IQR) = 1 to 2] versus 3 [IQR = 1-7], p=0.0001). This suggests that a healthy FUM is a less diverse FUM. By decreasing FUM diversity, we might be able to improve patient symptoms.

Vaginal and bladder microbiomes are similar. The vaginal microbiome is characterized by five dominant genera or species; these groups are called community state types (CSTs). Four of the five CSTs are low diversity microbiomes dominated by different *Lactobacillus* species, making *Lactobacillus* the most commonly found organism in the vagina. The final CST (CST IV) is a diverse mixed community dominated by anaerobes such as *Gardnerella*, *Prevotella*, and *Aerococcus* [19]. The organisms found in the bladder tell a very similar story. The FUM of the majority of individuals is dominated by *Lactobacillus*, with another group dominated by one or more anaerobes, such as *Gardnerella*, *Prevotella*, and *Aerococcus* [1]. This similarity in bacterial composition and the anatomical proximity of these two biological niches, lead us to reason that bacteria of bladder and vagina might behave similarly. A major factor in determining the health of the vaginal microbiota is estrogen.

Fig 1: UUI communities are more diverse than non-UI controls. A rarefaction analysis showing the number of unique species of bacteria isolated (using EQUC) with each new patient sampled. More unique species of bacteria are seen in the UUI population than the non-UI controls.



Diversity of the vaginal microbiome changes in response to estrogen. The majority of healthy pre-menopausal women have low diversity vaginal microbiomes dominated by *Lactobacillus* [19]. A small percentage of women have a higher diversity microbiome dominated by anaerobes [19]. Following menopause, many women lose *Lactobacillus* and their vaginal microbiomes become more diverse and anaerobic. However, this change can be reversed with estrogen treatment. Since the vagina and bladder have

similar organisms, we anticipate that estrogen would alter the FUM as it does the vaginal microbiome, reducing its diversity. Since high FUM diversity correlates with UUI and low FUM diversity with a lack of UUI, we reason that estrogen treatment will improve LUTS.

Methodology

Power Calculation/Sample Size: Based on the difference in diversity between UUI and non-UI controls (Fig 1), we were able to do a power analysis and determine that a sample size of 60 will give us sufficient power (0.816), using the Wilcoxon-Mann-Whitney test, to detect the difference between patients whose urinary microbiomes retain high diversity compared to those that change to a low diversity state. Initially, we plan to conduct a pilot study of 20 patients to inform a future, larger funded study.

Recruitment*:* Women will be recruited from the ambulatory urogynecology clinic at Loyola University Medical Center. A total of 60 hypoestrogenic, post-menopausal women will be enrolled as part of a pilot study. The PFM and AMPs will be measured prior to, and following 12 weeks of, vaginal estrogen treatment. Each patient will act as her own control, with her own baseline prior to estrogen treatment. Subjects who meet eligibility criteria and agree to research participation will indicate their consent by signing the research consent form. Subjects will indicate their consent for biorepository storage of leftover urine for potential future IRB-approved studies by signing the biorepository consent form. Consistent with prevalent research regulations and policies, a signed copy of the research and biorepository consent documents will be given to the subject and the investigator will keep the original research consent documents. Participation in the research will not depend upon participation in the biorepository (**Appendix 1**). Please see the ethics and patient consent section for details of the consent process.

Inclusion Criteria:

- Women who present with symptoms of OAB, defined as a condition characterized by urgency, with or without urgency incontinence, usually with frequency and nocturia in the absence of obvious pathology or infection [9], with atrophic vaginitis.
- Postmenopausal:
    - by history, defined as twelve months or greater since last menstrual period
    - surgical menopause with removal of bilateral ovaries
    - age over 55 if they have had a previous hysterectomy alone without removal of bilateral ovaries.
- English language skills sufficient to complete questionnaires
- Clinical indication for vaginal estrogen use (hypoestrogenic findings on physical examination)
- Patients not currently receiving vaginal estrogen therapy

Exclusion Criteria:

- Patients currently on systemic hormone replacement therapy (HRT) or who have been on HRT within the past three months
- Patients with current diagnosis or history of estrogen dependent malignancies (breast, endometrial)
- Contraindication or allergy to local estrogen therapy
- Insufficient language skills to complete study questionnaires
- Women with active, standard culture positive urinary tract infection at baseline assessment or Patients with a urine dip positive for leukocytes and nitrates on straight catheterized sample.
- Patients who have received antibiotics within the past two weeks
- Patients with stage 3 or 4 pelvic organ prolapse based on the pelvic organ prolapse quantitation system (POP-q)
- Patient unwilling to use vaginal estrogen preparation
- Patients currently on anticholinergic medication or who have received anticholinergic medication within the past three months

- Patients who have previously failed two medications for treatment of OAB or have previously received more advanced treatment for OAB including intra-vesicle botulinum toxin injections, posterior tibial nerve stimulation or implantation of sacral neuromodulator
- Patients wishing to start anticholinergic medication at the initial encounter
- Undiagnosed abnormal genital bleeding
- Active DVT, PE, or a history of these conditions
- Active arterial thromboembolic disease (for example, stroke and MI), or a history of these conditions
- Known liver dysfunction or disease
- Known protein C, protein S or antithrombin deficiency or other known thrombophilic disorders

Patient Data Collection:

Visit 1 (baseline): After enrollment and informed consent is obtained, patients will be asked to complete the OAB-q, a validated questionnaire, (**Appendix 3**) to measure OAB-related symptoms and quality of life [17]. Voided urines will be collected in a hat for 16SrRNA, PCR and protein analysis. A perineal swab will then be collected and assessed by 16S rRNA sequencing. Then a 30 mL vaginal lavage will be collected from the anterior wall of the vagina using sterile water for PCR and protein analysis. A vaginal swab will also be collected from the posterior fornix prior to the catheterized urine collection and assessed by 16S rRNA sequencing. Following the collection of the vaginal swab, a post void residual specimen will be collected by transurethral catheter, which is part of our standard practice for new patients. This specimen will be sent for standard clinical microbiology culture, EQUC and 16S rRNA gene evaluation. The specimens destined for the Radek lab will be stored on ice until pick up and transport. The swabs and urine for culture will be stored at room temperature until processed. The urine for sequencing will be refrigerated.

Demographic information and clinical characteristics will be collected, including age, parity, medical illness, previous hysterectomy, previous incontinence surgery, post void residual volume and body mass index (**Appendix 4**).

Physical examination will be completed, including evaluation for pelvic organ prolapse with the POP-q examination, which is part of a standard initial clinical evaluation.

Patients will be counseled on the evidence-based, first-line options for the treatment of OAB, including behavioral modifications, physical therapy, and vaginal estrogen per standard practice in our clinic. All study participants will be provided vaginal estrogen Premarin Cream® 0.625 mg conjugated estrogen/gram and instructed to use 0.5 grams twice weekly with the applicator. This is based on the clinical practice guidelines for vaginal estrogen use in postmenopausal women with urogynecological complaints developed by the Society of Gynecological Surgeons Systematic Review Group [18]. Patients will receive compensation at visit one with a parking voucher.

Patients will be given a medication diary for the use of vaginal estrogen to assess compliance (**Appendix 5**).

Visit 2 (12 weeks): Patients will be scheduled to return at twelve weeks from the initial visit for follow up which is part of our standard practice. They will be called a few days prior to the scheduled appointment as a reminder of the appointment and reminder to bring the medication diary. At the follow up, patients will repeat the OAB-q to reassess symptoms and quality of life measures. Again, voided and transurethral catheterized urine samples will be collected for analysis as described in visit 1. The perineal swab, vaginal lavage sample, and vaginal swab samples will also be collected at this visit and processed as described in visit 1. The patient will be screened for vaginal bleeding or spotting. The patient will be compensated with a second parking voucher at the conclusion of the study participation.

At this point, based on the patients' response to treatment, they may continue using vaginal estrogen. The symptoms of OAB will be reassessed and the next step in treatment will be based on the clinician's usual practice.

Adverse Event Reporting: We do not anticipate adverse events with this protocol; however as with all clinical protocols, it is feasible that a variety of unforeseen adverse events could occur. The risk of serious adverse event from the study participation in this protocol is extremely low and could include allergic reaction to the estrogen preparations, which would be considered reportable.

We would also report privacy breeches. There is also an unlikely risk of privacy violation despite diligent adherence to protection of all identified human data, using the Loyola secure server and in compliance with our institutional policies.

Sample Collection and Preparation: Consenting participants will contribute 2 catheterized urine samples and two voided urine samples: at the first visit and 12 weeks later. Urine samples obtained at the first visit are part of routine standard care and thus will be sent to the Clinical Microbiology Lab for standard urine culture. The Clinical Microbiology Lab also will assess the catheterized urine sample by EQUC. The Genomics Facility will perform 16S rRNA gene sequencing on the voided and catheterized urine samples and the Radek lab with do PCR and protein analysis. Both urine samples obtained at 12 weeks will be assessed by 16S rRNA gene sequencing, PCR and protein analysis. Catheterized urine samples will also be assessed by EQUC.

Immediately after collection, the human subjects-certified research team personnel will refrigerate and de-identify each urine sample, removing all protected health information prior to submission to any non-clinical investigators. Only the study ID will be available to individuals who are not involved in human subject recruitment, retention and oversight. Within 4 hrs, 1 aliquot of the catheterized urine sample will be sent to the clinical microbiology lab for standard culture and EQUC. A 2nd aliquot of the voided and catheterized urine sample will be sent to the genomics facility, where it will be inventoried and maintained in 10% DNA preservative (e.g., AssayAssure) at -80°C until bacterial DNA isolation and sequence analysis. After these analyses, leftover samples will remain stored in this biorepository for potential future IRB-approved analyses. A 3rd set of samples will be sent to the Radek lab for analysis of candidate AMPs (e.g. RNAse 7, cathelicidin, β-defensins, s100A7) in urinary, vaginal and perineal epithelial cells (obtained from urine and vaginal lavage) and perineal/vaginal swabs.

Sequencing: Genomic DNA (gDNA) will be extracted from urine with the Qiagen DNeasy Blood and Tissue kit as described previously. We will amplify the V4 region of 16S rRNA, add Illumina sequencing adapters and dual-index barcodes, purify the resulting amplicons, quantify, and sequence using the Illumina MiSeq System. To assess potential DNA contamination, an extraction (no urine) negative control will be processed with the samples and sequenced in every run. To ensure reproducibility, each sample will be independently extracted and sequenced at least twice. This is the protocol used for our recent publications [1, 2].

Bioinformatics Analysis: Quality control and de-multiplexing of sequence data are done with onboard MiSeq Control software and MiSeq Reporter (current version: 2.1.43). The *mothur* pipeline, specifically formatted for its use with MiSeq-generated data will combine paired end reads and remove contigs of incorrect length (<285 bp, >300 bp), contigs containing ambiguous bases and chimeric sequences. Within *mothur*, sequences will be assigned to OTUs and taxonomically classified using a naïve Bayesian classifier and the *mothur*-customized RDP training set v9. Alpha diversity metrics (inverse Simpson index, Shannon index), beta diversity metrics (PCoA plots), mean sequence abundance, and dendrograms will be generated using *mothur*, METAGENassist and R. This protocol was used for our recent publications [1, 2].

Standard and Expanded Urinary Quantitative Culture (EQUC) Procedures*:* Catheterized urine samples are processed with standard and/or EQUC methods. Standard urine culture involves inoculation of 0.001 mL of urine onto 5% sheep blood agar plate (BAP) and MacConkey agar plate, incubated aerobically at 35°C for 24h. Thus, the level of detection for standard culture is 10^3 CFU/mL, represented by 1 colony of growth on either plate. In contrast, EQUC involves inoculation of 100X (0.1mL) more urine onto diverse types of media (BAP, chocolate agar, colistin and nalidixic acid (CNA) agar, CDC anaerobe 5% BAP) with incubation in more environments and temperatures (5% CO2 at 35°C for 48 h, aerobic conditions at 35°C & 30°C for 48 h, Campy gas mixture (5% O2, 10% CO2, 85% N) or anaerobic conditions at 35°C for 48 h). The level of detection for EQUC is 10 CFU/mL, represented by 1 colony of growth on any plate. Each morphologically distinct colony type in both standard and EQUC procedures will be counted and isolated on a different plate of the same media to prepare a pure culture to be used for identification with Matrix-Assisted Laser Desorption/Ionization Time-of Flight (MALDI-TOF) mass spectroscopy. This is the protocol used for our recent publications [1, 2].

Biolog Assay: Characterization of bacterial strains isolated from urine obtained from the female bladder by transurethral catheter will facilitate our understanding of the role that these bacteria play in the bladder. For

example, commensal bacteria of the gut are known to inhibit the growth of pathogenic bacteria by outcompeting the pathogen for available nutrients, a process called colonization resistance.
The Biolog assay is de rigeur for determining bacterial growth characteristics. It requires a device that the Loyola team does not possess, but Kimberly-Clark does. Thus, the isolates will be sent to Kimberly-Clark for Biolog characterization.
The urinary bacterial isolates received by Kimberly-Clark will be undergo phenotypic analysis of their growth curves and biochemical utilization profiles. To determine growth rates isolates will be grown in a nutrient rich broth and a nutrient limited broth. Bacterial growth will measured kinetically using a spectrophotometer under the appropriate atmospheric conditions. Biochemical utilization profiles of the isolates will be determined using a series of Biolog plates, either endpoint or kinetic growth will be measured.

Data Storage and Confidentiality: Following HIPPA guidelines, patient identifiable data will be coded to protect each patient's identity. The data obtained from the patients will be entered onto electronic forms in the secure Redcap database. We are familiar with using this technology from other studies. It is efficient to complete in a clinical setting and minimizes data entry errors. The data will be presented in peer reviewed manuscripts and other public presentations at the group level only. No individual patients will be identified.

Statistical Analysis Plan: All data will be maintained on the Loyola secure, HIPAA-compliant research server. Only human-subjects certified team members will have access to identified data. Laboratory assessment will be performed using anonymized data and unique study numbers for each subject.
Statistical analyses of the microbiome data (EQUC and 16S rRNA) will be performed using SAS software version 9.4. Descriptive statistics for demographics, clinical characteristics, and microbiome data will be calculated including means with standard deviations, medians with interquartile ranges, or counts and percentages. Medians and interquartile ranges will be presented for OAB-q subscales at each time point as well as a change score, and Wilcoxon signed-rank tests assessed statistical significance of change in OAB-q. Each diversity measure will be modeled as a separate dependent variable in a linear mixed-effects regression, with independent variables including time, OAB-q symptom severity, physical therapy completed, weeks of estrogen compliance, and body mass index, and random intercepts for subjects. Spearman's rho will be calculated for change in Lactobacillus levels associated with change in the OAB-q symptom severity (12-week minus baseline values). For AMP data analyses, Spearman's rho will be used to test associations between change in relative abundance and change in area of bacterial growth inhibition.
Expectations: Since the FUM is more diverse in women with UUI than in women without UUI, since the bacteria of the FUM are similar to those of the vagina, and since estrogen treatment can reverse the increased diversity of the vaginal microbiome associated with menopause, we predict that estrogen treatment will make the FUM less diverse and improve LUTS. On the basis of our recently published study [1], we expect that hypoestrogenic women with UUI, on average, will have 3 different bacterial species as detected by EQUC. We predict that estrogen treatment will reduce this average number towards the average of 1 bacterial species as detected by EQUC in the continent controls from our previous study [1]. In that study, the difference between women with UUI and women without UUI was highly significant ($p=0.0001$). In the proposed study, we anticipate a smaller difference (since all women will have UUI) and will consider significance at $p<0.05$. Since 16S sequencing and EQUC provide complementary views of the FUM [1], we anticipate that sequencing will provide similar results; however, sequencing can identify bacteria that are not cultured, even by EQUC, and thus will provide us with a broader view of FUM composition. We predict that AMP levels will correlate with certain microbiota profiles, and anticipate that increased AMPs *via* estrogen supplementation will correlate with improved frequency and severity of OAB symptoms.

Possible Limitations to the study: We do not anticipate any problems recruiting and retaining patients, as we have a long track record of similar studies. We anticipate some loss to follow-up (~7%). The timing of the first sample, taken at the first visit, will be necessarily variable but will be about 12 weeks from the commencement of estrogen treatment.

We do not anticipate any problems in the transfer of samples from the Ambulatory Clinic to the Clinical Microbiology Lab or the Genomics Facility, respectively, for culture and sequencing, as we have an established

and successful protocol for such transfer. For similar reasons, we also do not anticipate problems with the culture or sequence analyses.

We know that some urinary bacteria do not grow, even under EQUC conditions. We also know that some bacteria are not readily sequenced. However, the strengths and weaknesses of EQUC and sequencing are complementary and thus permit a broad and relatively unbiased view of the bacterial composition of the FUM.

The study will also exclude patients with severe urgency urinary incontinence as mentioned in the exclusion criteria. We reasoned that these patients might benefit from an anticholinergic medication prescribed at visit one. There is some evidence from our unpublished data that anticholinergic medications affect the FUM, making estrogen's effect on the FUM less clear if given concomitantly. These patients may represent a unique FUM that will not be studied in this project. In future studies, we plan to include patients with severe UUI receiving anticholinergic medications along with estrogen.

Ethics and Patient Consent

Patients will be identified when they present to the Loyola Urogynecology outpatient clinic for management of their OAB symptoms. Co-investigators (Brincat, Taege) will identify and recruit patients for participation. The consent form will be read to the patient; it will describe the nature of the trial and potential associated risks and benefits. Potential participants will be informed that neither participation nor refusal will influence the care received at Loyola University Medical Center. A copy of the consent form will be given to them. Participation is completely voluntary and they may discontinue participation in the study at any time

Patient Compensation: Patients will receive a three months' supply of Premarin vaginal cream at no cost. Patients will receive a five dollar parking voucher at both visit one and two for their participation in the study.

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