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Associations between improvement in genitourinary symptoms of menopause and changes in the vaginal ecosystem

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Abstract

Objective: The aim of the study was to identify associations between improvement in genitourinary symptoms of menopause (GSM) and vaginal microbiota, vaginal glycogen, and serum estrogen.

Methods: Thirty postmenopausal women enrolled in a hot flash treatment trial (oral estradiol vs venlafaxine vs placebo) who reported GSM and provided vaginal swabs at 0, 4, and 8 weeks were studied. Bacterial communities were characterized using deep sequencing targeting the 16S rRNA gene V3-V4 region. Participants selected a most bothersome genitourinary symptom (dryness, discharge, pain, itch/burn, or inability to have sex) and rated severity on a 10-point scale at baseline and 8 weeks. Vaginal glycogen and serum estradiol and estrone were measured at enrollment and 8 weeks. Comparisons according to improvement in most bothersome symptom (MBS) were made using χ^2, Wilcoxon signed-rank test, or Hotelling's t test.

Results: Of 30 participants, 21 (70%) had improvement in MBS over the 8-week study and 9 (30%) had no improvement or worsening of MBS. A higher proportion of women receiving estradiol or venlafaxine reported improvement in MBS (88%, 78%) compared with placebo (54%; $P = 0.28$). MBS improvement was associated with *Lactobacillus*-dominant vaginal microbiota at enrollment (57% vs 22%, $P = 0.08$). Vaginal glycogen, serum estradiol, and estrone significantly increased in women whose MBS improved.

Conclusions: A larger proportion of women whose MBS improved had a *Lactobacillus* dominant microbiota at enrollment than those who had no improvement during the trial, though this difference was not statistically significant. Larger trials are needed to determine whether vaginal microbiota modify or mediate treatment responses in women with GSM.

Key Words: Genitourinary syndrome of menopause – Serum estradiol – Vaginal glycogen – Vaginal microbiome.

Genitourinary syndrome of menopause (GSM) is reported in at least 45% of postmenopausal women and can cause significant distress.[1,2] The most commonly reported symptoms are vaginal dryness, pain with intercourse, and vulvovaginal itching/irritation.[3] Little is known about the etiology of these symptoms, beyond the association with decreased serum estrogen levels. Atrophy of the genitourinary tissue is a common finding after menopause,

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but physical examination findings of atrophy do not correlate consistently with participant report of symptoms.[4,5] In many studies, both symptoms and physical examination findings improve with systemic or local estrogen therapy.[6,7] Recently, studies showing an association between vulvovaginal atrophy or dryness on examination and decreased vaginal detection of *Lactobacillus* bacteria have suggested that vaginal colonization with lactobacilli may mediate the development of genitourinary symptoms.[8,9] Our recent evaluation of participant-reported symptoms and vaginal *Lactobacillus* colonization in a cross-sectional analysis of postmenopausal women experiencing hot flashes did not, however, identify an association between GSM and vaginal detection of or dominance by *Lactobacillus* species.[10]

After menopause, vaginal colonization with *Lactobacillus* species is much less common than in premenopausal women.[11,12] Treatment with systemic hormone therapy is associated with increased detection of lactobacilli in the vagina.[13,14] In Chinese women, those with both GSM and examination findings of atrophy (atrophic vaginitis) had significantly lower abundance of vaginal *Lactobacillus* species than women without symptoms or atrophy.[15] After treatment of atrophic vaginitis with low-dose oral estrogen, abundance of vaginal lactobacilli increased and symptoms improved. Abundance of *Lactobacillus* was significantly negatively associated with symptom scores, but there was no discussion of symptom prevalence or severity in the six women who did not have a *Lactobacillus*-dominant vaginal microbiota after 4 weeks of estrogen therapy.

We performed a longitudinal study of the association between changes in vaginal symptoms and microbiota in postmenopausal women in a placebo-controlled trial of oral estradiol or venlafaxine for hot flashes. We hypothesized that women whose symptoms improved would have an increase in the detection, quantity, and dominance of *Lactobacillus* species, either because colonization is a marker of a healthy vaginal environment, or because colonization drives postmenopausal vaginal health.

METHODS

Study participants

This study used samples and data from a subset of women who participated in a three-arm double-blind, 8-week randomized trial of oral estradiol, venlafaxine, or placebo for the alleviation of menopausal hot flashes. The study was conducted at three Menopause Strategies: Finding Lasting Answers for Symptoms and Health (MsFLASH) network sites (Boston, Philadelphia, and Seattle). Details about the MsFLASH Research Network,[16] study design, methods, and main trial results have been reported elsewhere.[17] Women aged 40 to 62, in the menopausal transition ($\leq$12 months since last menstrual period) or in menopause (>12 months since last period), who reported $\geq$14 hot flashes per week and met other inclusion criteria participated. The protocol was approved by the appropriate institutional review board at each site. All participants provided written informed consent. Women who agreed to the ancillary vaginal microbiome study signed a second consent form. The parent study enrolled 339 women between November 2011 and October 2012. Enrollment in the vaginal health substudy was offered between June and October 2012 and 93 of 117 (79%) women who enrolled in the parent study during that time also enrolled in the vaginal health substudy. Of those, 30 women with vaginal symptoms at enrollment who also agreed to provide multiple vaginal samples over the course of the 8-week trial were included in a longitudinal analysis described here.

Demographic and symptom measures

Baseline demographic characteristics were assessed by questionnaire and included smoking status, menopause status (menopausal transition, postmenopausal), and health status. Height and body weight were measured at baseline and were used to calculate body mass index (BMI). At enrollment, participants completed the Patient Health Questionnaire (PHQ9)[18] to screen for depression, and the Generalized Anxiety Disorder Scale (GAD-7)[19] to evaluate anxiety. Self-reported vaginal symptom measures included (1) selection of the most bothersome symptom (MBS) for the participant, and rating of severity on a 10-point scale and (2) the presence and severity of individual symptoms (vaginal dryness, vulvovaginal itch/burn, vaginal discharge, vaginal pain with intercourse) on a 5-point scale. Choices for MBS were limited to vaginal dryness, vaginal discharge, vulvovaginal pain, vulvovaginal itch/burn, or inability to have sex. Women had blood drawn at enrollment and at 8 weeks. Women self-collected vaginal swabs at 0 and 8 weeks in clinic, and at 4 weeks at home and sent those swabs through mail to the Fredricks Lab in Seattle.

Characterization of the vaginal microbiota

After receipt, vaginal swabs were stored at -80°C until processed. Swabs were eluted in 400 μL sterile, filtered saline, and centrifuged at 14,000 rpm for 10 minutes. DNA was extracted from the cell pellet using the MoBio Bacteremia extraction kit (MoBio, Carlsbad, CA) as previously described.[20] The bacterial 16S rRNA gene was amplified using primers for the V3-V4 hypervariable regions, libraries were created using barcoded primers, and amplicons were sequenced using the Roche 454 Titanium platform (Roche, CT).[21] Negative controls included sham digests that were processed in the same way as samples to assess contamination from DNA extraction or PCR reagents. Sequences were filtered for length (minimum 250 bp) and quality score (minimum 30), and reads originating from contaminants in PCR controls were removed. Sequence reads were classified using the *pplacer* phylogenetic placement tool and a curated reference set of vaginal bacterial sequences.[21] Sequence reads have been deposited to the National Center for Biotechnology Information Short Read Archive (SRP100779). Based on the dominant bacterial genus, participants were categorized into two groups: *Lactobacillus* dominant (>50% of sequences from *Lactobacillus* species) and non-*Lactobacillus* dominant. For quantification of *L. crispatus*

and *L. iners*, species-specific qPCR using a TaqMan-based assay was performed as previously described.[20,22]

Vaginal glycogen assay

A second vaginal swab was processed as described above, and supernatant from that eluate was further diluted in saline (1:5), and 50 μL of the diluted fluid was used in a fluorometric assay (BioVision, Milpitas, CA) to measure glycogen levels.

Serum estrogen measurements

At each clinic visit blood was drawn and serum stored at −80°C until processing. An ultrasensitive stable isotope dilution liquid chromatography/selected reaction monitoring/mass spectrometry (LC/SRM/MS) assay was used to measure total and unconjugated estradiol and estrone.[23] The limit of detection for each estrogen using 0.5 mL of serum was 0.156 pg/mL and linear standard curves were obtained up to 20 pg/mL. Serum samples were not available for all participants at all time points.

Statistical analysis

Improvement in MBS was defined as a greater than 1 point decrease in the MBS severity score between enrollment and 8 weeks. Comparisons of participant characteristics between women whose MBS improved or did not used Student's *t* test, Kruskal-Wallis test, χ^2, or Fisher's exact test, as appropriate. Wilcoxon signed-rank test was used to compare laboratory values between visits within each group. Detection of *Lactobacillus* species by qPCR was compared between visits using McNemar's test. Log_{10}-transformed qPCR values at 0 and 8 weeks, as well as the change in value between week 8 and enrollment, were compared between groups using Student's *t* test. Differences in Shannon diversity index were compared between groups and time points using either linear regression (adjusted for treatment arm) or Wilcoxon rank-sum test. Differences in Bray-Curtis dissimilarity at each time point were compared between groups using MiRKAT.[24] Taxon-level associations with improvement in symptoms were assessed across time points using Hotelling's *T*-squared test,[25] adjusted for multiple comparisons using Benjamini-Hochberg equations.[26]

RESULTS

Of the 30 women included in this longitudinal analysis, 21 (70%) had improvement in their MBS over the course of the study and 9 (30%) had no improvement or worsening of the MBS. A higher proportion of women receiving estradiol or venlafaxine reported improvement in MBS (88%, 78%) compared with placebo (54%; $P = 0.28$). The mean change in MBS for the entire group was a decrease of 2 points (SD of ±3), with a range of decrease by 8 points to an increase of 3 points. There were no significant differences in age, ethnicity, treatment arm, enrollment laboratory values, or which symptom was the most bothersome between the two groups (Table 1). Most women reported the same MBS at enrollment and at follow-up, but eight women reported a different MBS at the follow-up visit. In all eight, the original MBS decreased in severity and the new MBS was reported as the same or less severe than at the original visit, so these women are classified as ''MBS improved.'' There was no association between improvement in vulvovaginal MBS and number of hot flashes per day at enrollment, or improvement in hot flashes over the course of the trial. Women whose MBS did not improve had significantly higher depression and anxiety scores at baseline, though the median values in this group were just at or slightly above the cutoff for ''mild'' anxiety or depression.[18,19]

Of the 21 women whose MBS improved, 12 (57%) had a *Lactobacillus*-dominant microbiota at enrollment, compared with 2 (22%) of those whose MBS did not improve (Table 1; $P = 0.08$). At 8 weeks, 11 (52%) women whose MBS improved had *Lactobacillus*-dominant community compared with 3 (33%) in the group that did not improve ($P = 0.22$) (Fig. 1A). In the group whose symptoms improved, one woman lost *Lactobacillus* dominance (placebo treatment group), whereas among women whose symptoms did not improve, two women gained *Lactobacillus* dominance over 8 weeks (one placebo, one estradiol), though in both women this was primarily *L. iners* (Fig. 1C), In unadjusted analysis, several non-*Lactobacillus* bacterial species had different mean prevalence across all three time points between women whose MBS improved and those whose did not, largely due to a higher prevalence in women who did not improve (Fig. 2A). After adjustment for multiple comparisons none of these differences was significant. When comparing alpha diversity, there was a trend to a higher Shannon diversity index in women whose MBS did not improve, especially when adjusting for treatment assignment, but differences were not statistically significant (Fig. 2B). Using MiRKAT, there was no association between beta diversity and symptom improvement at enrollment ($P = 0.58$) or 8 weeks ($P = 0.64$). There was a trend to lower detection of *L. crispatus* at enrollment in the group whose MBS did not improve, but this did not reach statistical significance (Table 1). There were no differences between groups in mean log_{10} gene copies/swab of *L. crispatus* as measured by qPCR at enrollment (MBS improved 4.3 ± 0.6 vs not improved 2.9 ± 0.7; $P = 0.22$), 8 weeks (4.2 ± 0.7 vs 3.2 ± 0.9; $P = 0.43$), or change in quantity over time (-0.1 ± 0.4 vs 0.3 ± 0.3; $P = 0.48$). The same was true for log_{10}-transformed quantity of *L. iners* measured by qPCR at 0 weeks (MBS improved 5.0 ± 0.7 vs not improved 4.6 ± 1.1; $P = 0.76$), 8 weeks (4.7 ± 0.7 vs 5.4 ± 1.2; $P = 0.57$), or in change between 0 and 8 weeks (-0.3 ± 0.2 vs 0.8 ± 0.7; $P = 0.06$) (Fig. 2C).

Vaginal-free glycogen significantly increased over the 8 weeks in women whose MBS improved ($P = 0.03$), and showed a trend to decrease between enrollment and week 8 in women whose MBS did not improve, though this did not reach statistical significance (Fig. 3). Serum unconjugated and total estradiol significantly increased in the women whose MBS improved, and although there was a trend to increase in those with no improvement, the magnitude was smaller (Fig. 3). Of the 12 women with an increase in total serum estradiol, 5

TABLE 1. *Demographic, laboratory, and symptom characteristics at enrollment*

	MBS improved ($n = 21$)	MBS did not improve ($n = 9$)	P^a
Age (y, mean ± SD)	53 ± 4	54 ± 4	0.82
BMI (kg/m^2, mean ± SD)	30 ± 7	27 ± 4	0.31
Ethnicity			0.77
White	9 (43%)	5 (56%)	
African American	10 (48%)	3 (33%)	
Hispanic	1 (5%)	1 (11%)	
Other	1 (5%)	0	
Study arm			0.22
Placebo	7 (33%)	6 (67%)	
Venlafaxine	7 (33%)	2 (22%)	
Estradiol	7 (33%)	1 (11%)	
Menopause status			0.84
LMP <1 y	4 (19%)	2 (22%)	
LMP >1 y	17 (81%)	7 (78%)	
Female sexual function index (median, IQR)	21 (12-27)	20 (18-23)	0.82
PHQ-9 (median, IQR)	2 (0-4)	6 (3-10)	0.008
GAD-7 (median, IQR)	2 (0-3)	5 (2-10)	0.02
Vasomotor symptoms			
Hot flashes/d at week 0 (median, IQR)	8 (6-9)	6 (5-11)	0.39
>50% decrease in hot flashes/d at week 8	7 (33%)	3 (33%)	1.0
Baseline laboratory values			
Vaginal glycogen (pg/mL, median, IQR)[b]	5.3 (2.7-8.3)	7.1 (4.3-166)	0.12
Estradiol (pg/mL, median, IQR)[c]			
Total	22 (4-30)	14 (0.5-20)	0.31
Unconjugated	3 (0.5-7)	5 (0.5-6)	0.99
Estrone (pg/mL, median, IQR)[c]			
Total	195 (146-314)	173 (122-229)	0.27
Unconjugated	26 (22-40)	27 (12-35)	0.61
Lactobacillus-dominant vaginal microbiota	12 (57%)	2 (22%)	0.08
L. crispatus (+qPCR)	10 (48%)	2 (22%)	0.11
L. iners (+qPCR)	14 (67%)	5 (56%)	0.79
Moderate-severe symptoms:			
Vaginal dryness	8 (38%)	5 (56%)	0.27
Vulvar itch/burn	6 (29%)	4 (44%)	0.33
Vaginal itch/burn	3 (14%)	1 (11%)	0.89
Vaginal discharge	6 (29%)	4 (44%)	0.40
Pain	5 (24%)	1 (11%)	0.58
Most bothersome symptom (MBS)			
Vaginal dryness	7 (33%)	4 (44%%)	0.27
Vulvar itch/burn	7 (33%)	1 (11%)	
Vaginal itch/burn	2 (10%)	0	
Vaginal discharge	0	1 (11%)	
Pain	1 (5%)	2 (22%)	
Inability to have sex	3 (14%)	1 (11%)	
Severity of MBS (median, IQR)	6 (4-7)	3 (2-5)	0.14

BMI, body mass index; GAD-7, Generalized Anxiety Disorder Scale; LMP, last menstrual period; MBS, most bothersome symptom; PHQ-9, Patient Health Questionnaire; qPCR, quantitative polymerase chain reaction.
[a]*P* value calculated by χ^2 or Fisher's exact test for categorical data, Student's *t* test or Kruskal-Wallis for continuous data, as appropriate.
[b]Missing values for 4 in the improved group and 2 in the not improved group.
[c]Missing values for 6 in the improved group.

(42%) were in the estradiol treatment group, 5 (42%) were in the venlafaxine group, and 2 (16%) were in the placebo group. Comparisons between treatment arms were significantly confounded by race, as 7/8 (88%) women from the estradiol treatment group were African American compared with 3/13 (23%) in the placebo group and 3/9 (33%) in the venlafaxine group (Table 2). Serum estrogens increased most, as expected, in the oral estradiol treatment group (Table 2).

DISCUSSION

Our pilot analysis of this small cohort of postmenopausal women recruited from a randomized placebo-controlled trial evaluating systemic therapy for hot flashes showed an association between decreasing severity of vulvovaginal symptoms and increased serum estrogen and vaginal glycogen levels. In addition, this small pilot study suggested that vaginal *Lactobacillus* dominance might predispose to a better treatment response, though we did not see a shift to greater *Lactobacillus* presence or dominance in women whose symptoms improved. This suggests that the presence of vaginal lactobacilli may be more a marker of a healthy vagina than a driver of vulvovaginal symptom severity.

In premenopausal women, the absence of vaginal *Lactobacillus* colonization is associated with bacterial vaginosis (BV), a condition that can present with vaginal discharge and odor. Half of women with BV are, however, asymptomatic. Mechanisms underlying vulvovaginal symptoms are not well understood. One study linked detection of *Gardnerella* and report of pain through the metabolite

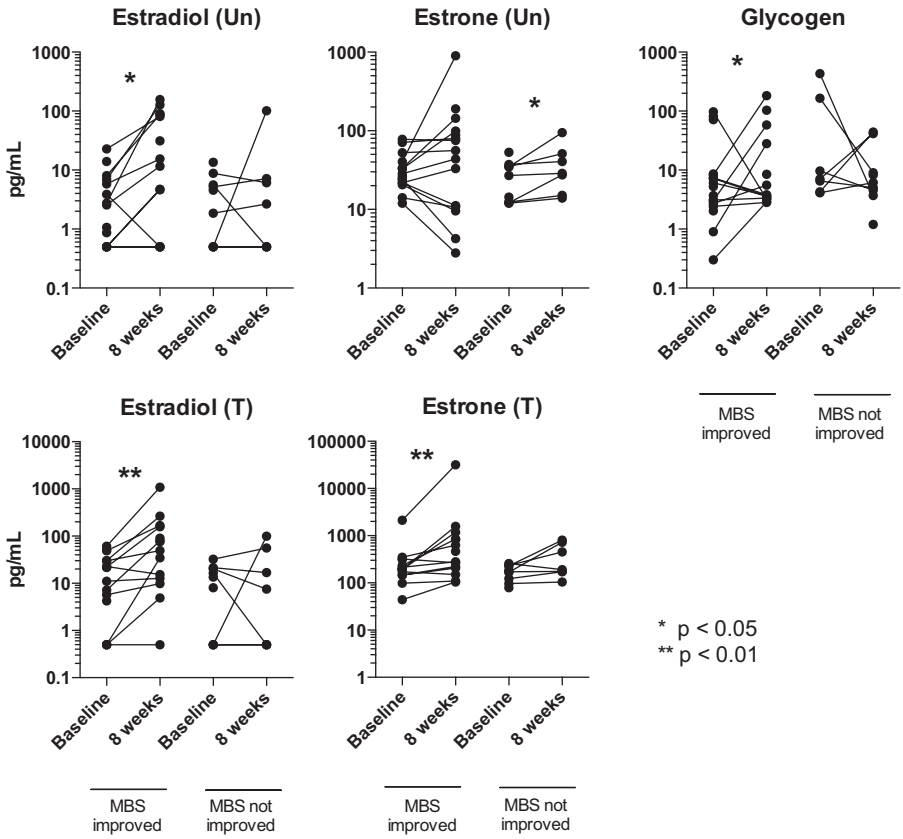

FIG. 3. Change in serum estrogen levels and vaginal glycogen levels over 8 weeks in women whose symptoms did and did not improve. Serum estrogen values were missing for nine participants in the MBS improved group and two from the not improved group. Significant change ($^{*}P < 0.05$, $^{**}P < 0.001$) within a group between values at enrollment and at 8 weeks.

diethylene glycol.[27] Another study linked bacteria such as *Prevotella timonensis*, *Leptotrichia amnionii* (now *Sneathia amnii*), *Eggerthella*, and *Parvimonas micra* with vaginal discharge.[21] A study of postmenopausal, Chinese women with atrophic vaginitis showed a negative correlation between abundance of vaginal Lactobacillus and severity of vaginitis symptoms.[15]

Vaginal colonization with lactobacilli in postmenopausal women has been associated with vaginal fluid glycogen, which is presumed to be driven by serum estrogen levels.[28] Although quantities of vaginal-free glycogen and vaginal lactobacilli seem to be correlated, no association was, however, seen between serum estradiol and vaginal glycogen or lactobacilli in premenopausal women[28] or in our cross-

TABLE 2. *Comparison of ethnicity, symptom improvement, and change in biologic measurements over 8 weeks by treatment arm*

	Placebo ($n = 13$)	Venlafaxine ($n = 9$)	Estradiol ($n = 8$)	P^a
Ethnicity				0.02
Black	3 (23%)	3 (33%)	7 (88%)	
Nonblack	8 (77%)	6 (67%)	1 (12%)	
MBS improved	7 (54%)	7 (78%)	7 (88%)	0.28
Median change (IQR) in:				P^b
Glycogen	0.6 (−3.7 to 1.7)	2.1 (−1.0 to 32.7)	1.2 (−2.5 to 4.6)	0.58
Estradiol (T)[c]	−4.7 (−12.8 to 19.5)	38 (1.7-99)	119 (23-235)	0.03
Estradiol (Un)	1.4 (−2.1 to 3.1)	10 (9-100)	58 (4.2-79)	0.13
Estrone (T)	50 (7.8-220)	65 (−22 to 553)	616 (578-1376)	0.05
Estrone (Un)	1.8 (−6.3 to 5.8)	11.1 (−10.7 to 59.9)	15.4 (3.5-66.5)	0.17

T, total; Un, unconjugated; MBS, most bothersome symptom.
[a]Fisher's exact test.
[b]Kruskal-Wallis.
[c]Serum estrogen values not available for six participants from placebo group, two from venlafaxine group, and three from estradiol group.

sectional analysis of postmenopausal women.[10] We did see an increase in vaginal-free glycogen in women whose MBS improved, without a corresponding change in *Lactobacillus* detection or quantity. Our glycogen measurements were significantly lower than in other reports[28]; thus the magnitude of change may have been too small to induce changes in the microbiota. It is also possible that we did not follow women long enough to see a change in *Lactobacillus*; however, this observation also supports the hypothesis that lactobacilli are a marker of health, rather than a driver of health.

The most commonly reported MBS in our cohort was vaginal dryness, followed by vulvar itch/burn. Postmenopausal Canadian women with vaginal dryness on examination were found to have increased expression of genes for inflammatory cytokines in the vaginal mucosa.[9] We have shown that in premenopausal women vaginal colonization with *Lactobacillus* is associated with lower levels of the proinflammatory cytokine IL1b.[29] Preliminary data suggest that estrogen decreases neutrophil activity in the vaginal mucosa by altering cytokine gradients,[30] and inhibits Th17 differentiation and proliferation.[31] A separate study, however, suggested that vaginal proinflammatory cytokines were not associated with GSM.[32] Our pilot results suggest that decrease in symptom severity is associated more with increased serum estrogen than change in vaginal *Lactobacillus* colonization. However, treatment with low-dose vaginal preparations of estradiol that do not significantly increase serum estrogen has also been shown to decrease genitourinary symptoms of menopause, suggesting that other mechanisms may also be important. It is possible that changes in the hormonal environment facilitate changes in *Lactobacillus* function that would not be captured by the quantitative methods used in this study.

Our study did show greater improvement in MBS in the two groups of women treated with an active agent compared with placebo. Oral estradiol has been shown to improve GSM in many studies.[6,33,34] MBS improvement with venlafaxine correlates with MsFLASH trial findings from all 335 women when vaginal dryness was assessed as part of the MENQOL questionnaire. At baseline, 37.9%, 37.5%, and 41.7% of women in the estradiol, venlafaxine, and placebo groups, respectively, reported vaginal dryness. At 8 weeks, this decreased to 26.4%, 18.2%, and 35.6%, respectively. In the larger cohort, only the venlafaxine group had statistically significant improvement in the proportion of women reporting vaginal dryness, compared with placebo ($P = 0.006$).[35]

This pilot study is limited by its small size, which precludes more complex analyses controlling for or stratifying by treatment arm or other participant characteristics. The sample size was, however, adequate to show a change in serum estrogens and vaginal glycogen levels over 8 weeks. Women were recruited from a trial for which the inclusion criteria were based on hot flashes, not genitourinary symptoms, thus symptom severity was moderate, which may also have limited our ability to assess associations between symptoms and microbiota. We did, however, ask about a wide range of genitourinary symptoms, as opposed to the single question about dryness present in the MENQOL.

CONCLUSION

Many studies of GSM use examination findings of atrophy, vaginal pH, vaginal maturation index, or other examination findings to measure response to treatment. The true goal of treating GSM is improving quality of life for postmenopausal women. Results from this pilot analysis suggest that laboratory-based evaluation of treatment responses may not correlate as expected with change in symptom severity, highlighting our lack of understanding of the underlying cause of GSM, and the need for mechanistic studies in this area. These results support the possibility that vaginal microbiota affect changes in GSM in response to treatment. Larger trials are, however needed to determine whether vaginal microbiota modify or mediate treatment responses in women with GSM.

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