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Article

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Breast cancer therapy-induced menopause rapidly and persistently disrupts vaginal health and microbial ecology

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Abstract

Breast cancer therapies that induce iatrogenic menopause expose premenopausal women to abrupt estrogen deprivation and frequently result in genitourinary syndrome of menopause (GSM), yet the timing and durability of symptom onset and associated changes in vaginal health and ecology remain poorly understood. In this multicentre prospective longitudinal study, 60 premenopausal women with stage I–IIIC breast cancer were followed from before treatment initiation through iatrogenic menopause across four study visits. At each visit, vulvovaginal health, GSM manifestations, vaginal microbiome composition, menopausal status, and general and sexual wellbeing were assessed using gynecologic examination, 16S rRNA amplicon sequencing, serum estradiol measurements and validated questionnaires. Within three months of menopause onset, participants exhibited significant deterioration in vulvovaginal health and an increased prevalence of sexual dysfunction, particularly among those receiving endocrine therapy. Menopause onset was accompanied by a rapid loss of *Lactobacillus* dominance and an increase in vaginal microbial diversity, with more pronounced changes in endocrine therapy recipients. These microbial shifts persisted for up to 33 months, including among patients who later regained premenopausal estrogen levels, suggesting limited resilience of the vaginal ecosystem. Our findings provide robust longitudinal evidence that iatrogenic menopause rapidly and durably disrupts vaginal health and microbial ecology, underscoring the need for early intervention and supporting the exploration of microbiome-modulating strategies to support survivorship care.

Introduction

Breast cancer is the most common cancer among women globally, with approximately 2.3 million new cases annually and nearly one in eight women affected during their lifetime in the Western countries.[1] While most diagnoses occur after menopause, a substantial minority (31%) of patients are premenopausal and experience abrupt, iatrogenic menopause as a result of systemic breast cancer treatments such as cytotoxic chemotherapy or ovarian function suppression (OFS) with a gonadotropin-releasing hormone agonist (GnRH).[2,3] This iatrogenic menopausal transition is characterized by a rapid decline in circulating estrogen levels, which differs from a natural menopausal transition where different trajectories of FSH and estradiol are observed.[4] In patients with hormone receptor-positive breast cancer, adjuvant endocrine therapy with aromatase inhibitors or tamoxifen is prescribed for at least five years and can exacerbate menopausal symptoms.[5] Aromatase inhibitors further reduce circulating estrogen levels by blocking the peripheral conversion of androgens into estrogens, thereby intensifying estrogen depletion and

its physiological effects.[6] This abrupt iatrogenic hormonal transition in premenopausal patients is associated with more severe and sudden menopausal symptoms, particularly in women before the age of 45. The genitourinary syndrome of menopause (GSM) is a burdensome consequence of estrogen deficiency on the female genitourinary tract. GSM encompasses a spectrum of symptoms, including dryness, irritation, burning, dyspareunia, and recurrent urinary complaints (such as increased urinary urgency, frequency, incontinence, and recurrent urinary tract infections). This can have a profound effect on sexuality, intimate relationships, physical and psychosocial wellbeing.[7–9] Despite its substantial impact on daily functioning, sexual wellbeing, and intimate relationships of up to70–80% of breast cancer survivors, GSM remains widely underreported, underdiagnosed, and undertreated. More than 75% of affected women with GSM do not seek or receive adequate care due to shame, limited awareness or insufficient clinical discussion.[10–14] Vaginal moisturizers and, when appropriate, low-dose vaginal estrogen therapy can alleviate symptoms of GSM. However, longstanding concerns about oncologic safety have historically limited their use in breast cancer survivors, despite emerging evidence supporting their safety. [15–20]

Moreover, growing evidence suggests that the vaginal microbiome may influence the development and severity of GSM. However, the temporal relationship between vaginal microbiome changes, estrogen depletion, and GSM development remains poorly understood, particularly in the context of iatrogenic menopause.[21–25] During the natural menopausal transition, estrogen depletion has been associated with increasing vaginal pH and reduced epithelial shedding of glycogen, which is a key nutrient source for lactobacilli in the vagina.[26] Experimental and clinical studies have demonstrated that estrogen promotes vaginal epithelial maturation and glycogen accumulation, supporting the growth and persistence of lactobacilli.[27,28] These observations identify estrogen as a major ecological driver of the vaginal ecosystem. The loss of estrogen during menopause is therefore thought to contribute to the consistently observed shift in the vaginal microbiome compared to women in their reproductive stage, characterized by a reduced *Lactobacillus* abundance and increased anaerobic diversity such as *Gardnerella*, *Prevotella*, and *Anaerococcus*.[21,22,29,30] Similar microbiome patterns have been reported in breast cancer patients receiving aromatase inhibitors,[25] though available evidence remains limited and inconsistent, originating primarily from cross-sectional studies.[25,31] Iatrogenic menopause provides a uniquely tractable framework to investigate the consequences of abrupt estrogen depletion. Unlike natural menopause, which is diagnosed retrospectively and therefor has an inherently uncertain onset, iatrogenic menopause occurs at a defined timepoint, enabling prospective evaluation of hormonal, microbial and clinical changes over time. In this multicentre

prospective longitudinal study, we followed premenopausal breast cancer patients from before treatment initiation through iatrogenic menopause to characterize onset of GSM, the temporal dynamics of the vaginal microbiome, and changes in general and sexual wellbeing. By integrating serum estradiol, vaginal microbiome profiling, clinical assessment, and patient reported outcomes, we aimed to define the timing, magnitude and persistence of vaginal ecosystem changes following abrupt estrogen deprivation and to evaluate their relationship with GSM severity and sexual health outcomes. To the best of our knowledge this is the first prospective longitudinal study to follow women through menopausal transition.

Results

Study cohort and treatment pathways

A total of 60 premenopausal women with stage I-IIIC breast cancer were enrolled and followed prospectively from treatment initiation through iatrogenic menopause. Participants had a mean age of 42.9 years (SD 6.9 y; range = 26 to 58y) at baseline (V1) and were all premenopausal. Follow-up assessments were performed approximately 3 months (V2), 6 months (V3), and 13–33 months (V4) after menopause induction (Fig. 1). All participants reached menopausal estradiol levels (estradiol < 20 ng/L) at least once during follow-up. Premenopausal estradiol levels were detected unexpectedly in a minority of participants during V2 (5/55, 9.1%) and V3 (1/52, 1.9%). At long-term follow-up (V4), 8/38 (21.1%) participants exhibited premenopausal estradiol levels, reflecting ovarian function recovery after chemotherapy (n = 2), non-adherence to endocrine therapy (n = 5), or 1 patient with an isolated unexpected premenopausal estradiol range despite GnRH treatment. Treatment pathways varied according to tumor characteristics. Following baseline assessment at V1, 43/60 (71.7%) participants started (neo-)adjuvant chemotherapy, whereas 17/60 (28.3%) participants started endocrine therapy without prior chemotherapy. Of those receiving chemotherapy, 32 subsequently received endocrine therapy. Consequently, participants experienced diverse standard of treatment trajectories adjusted to individual characteristics while undergoing iatrogenic menopause. Detailed patient, tumor, treatment, and menopausal-status characteristics are provided in Supplementary Information Part 1A-C.

Recovery of functional and general well-being contrasting increasing sexual dysfunction

Physical functioning according to the Eastern Cooperative Oncology Group score (ECOG) deteriorated significantly during active treatment (V2 and V3, both $P < 0.001$) but recovered by long-term follow-up (V4) (Table 1). General wellbeing remained largely stable over time and was significantly higher at V4 compared with baseline ($P = 0.047$). Relationship quality among

participants with a partner did not change significantly during the study (Table 1). Among sexually active participants (*n*=56; 93.3% at baseline), mean sexual function and sexual distress did not differ significantly across study visits. However, the proportion of participants meeting the criteria for a sexual dysfunction (i.e., Female Sexual Function Index (FSFI) below 26.55 and Female Sexual Distress Scale-revised (FSDS-r) above 11) increased significantly following menopause induction, highlighting a growing burden of sexual health impairment in contrast to recovery in overall wellbeing and functional status (Table 1).

Rapid onset of clinical signs of GSM and symptom severity

A rapid deterioration in vulvovaginal health was observed following iatrogenic menopause, reflected by significant increases in both vaginal and vulvar composite scores compared with baseline (all $P < 0.001$; Fig. 2). Vaginal composite scores significantly increased further between V2 and V3 and remained elevated at V4, whereas no significant difference was observed between V3 and V4, indicating that the most pronounced vaginal changes occurred within the first six months after menopause onset. In contrast, vulvar composite scores showed a more gradual trajectory, with a significant increase between V2 and V4 but not between V2 and V3 or V3 and V4 (Fig. 2). At V4, participants receiving ongoing endocrine therapy had significantly higher vulvar composite scores than those without active therapy ($P = 0.015$; Suppl. I. part 2), suggesting that vulvar manifestations of GSM may be exacerbated by continued estrogen suppression beyond menopause onset.

Higher vaginal and vulvar composite scores were associated with greater patient-reported symptom burden, including sexual distress (FSDS-r), and pain during examination. (Suppl. I. part 3). Among patients receiving endocrine therapy, treatment exposure was positively associated with vaginal sensitivity ($r = 0.42$), vaginal dryness ($r= 0.22$), dyspareunia ($r= 0.39$), vulvar pain ($r = 0.24$), and vulvar irritation ($r = 0.15$). In contrast, among participants not receiving ongoing therapy, negative associations with these clinical symptoms were observed.-No meaningful associations were observed among patients receiving chemotherapy (Fig. 3). Together, these findings show that clinical manifestations of GSM are reflected in patient-reported symptoms and suggest an additional adverse effect of ongoing endocrine therapy on vulvovaginal symptom burden.

Loss of *Lactobacillus* dominance and persistent microbial diversification after iatrogenic menopause

Given the rapid onset of GSM and vulvovaginal changes following menopause induction, we next investigated whether these clinical alterations were accompanied by shifts in the vaginal

microbiome using 16S rRNA sequencing. Specifically, we assessed whether abrupt estrogen deprivation coincided with loss of *Lactobacillus* dominance and increased microbial diversity. Of the collected samples, 58/60 (97%) passed quality control at V1, 54/55 (98%) at V2, 45/52 (86%) at V3 and 33/38 (86%) at V4. Longitudinal analyses were conducted on 44 participants with microbiome data available at V1-V3, and on 28 participants with data available at all four study visits.

Before treatment initiation, the vaginal microbiome was predominantly *Lactobacillus*-dominated, with *Lactobacillus* on average accounting for 65% of total relative abundance and dominating 70.6% of samples. The most abundant taxa belonged to the *L. crispatus* (33%) and *L. iners* (24%) groups, with smaller contributions from *L. (para)gasseri and L. jensenii/L. mulieris* (Fig. 4A-B). Following menopause induction, *Lactobacillus* dominance declined rapidly, from 70.6% of samples at baseline to 40.8%, 22.2%, and 18.2% at V2, V3, and V4, respectively (Fig. 4B). This decline was accompanied by enrichment of anaerobic taxa, including the *Prevotella* group*, Gardnerella vaginalis (Bifidobacterium vaginale* group in the Genome Taxonomy Database*),* and *Anaerococcus* (Fig. 4A).

All alpha diversity metrics indicated a rapid shift towards a more diverse vaginal microbial community following menopause onset. Observed richness (obs index) increased significantly over time (Friedman's test: W = 0.197, P = 0.00086), whereas community dominance decreased significantly (Berger–Parker index) (W = 0.348, P = 1.96×10^{-6}), reflecting loss of *Lactobacillus* dominance (Suppl. I. part 4). Similarly, Shannon diversity increased significantly across study visits (W = 0.296, P = 1.65×10^{-5}) (Fig. 4C). Post-hoc analyses showed that these differences were driven by significant changes between baseline and all subsequent visits, whereas no significant differences were observed between V2, V3, and V4. This indicates a rapid and sustained restructuring of the vaginal ecosystem following iatrogenic menopause.

Menopausal status is the primary determinant of vaginal microbiome composition

Among all clinical, treatment-related, and patient-reported variables examined, menopausal status was the only factor consistently associated with vaginal microbiome composition (Fig. 5). Menopause was negatively associated with *Lactobacillus spp.* abundance across participants, highlighting the robust and persistent impact of abrupt estrogen depletion on the vaginal ecosystem. Menopause was positively associated with Gram-negative anaerobic *Dialister spp*. and Gram-positive *Varibaculum spp,* Treatment modality (chemotherapy vs. endocrine therapy) had no significant associations with individual microbial taxa. Similarly, vaginal and vulvar composite scores, sexual function, and sexual distress were not significantly associated with

vaginal microbiome composition (Fig. 5). Notably, among the eight participants who regained premenopausal hormonal levels at V4, the vaginal microbiome did not revert to its premenopausal composition, suggesting that these menopause-associated ecological changes may persist beyond restoration of ovarian function (Fig 6).

Discussion

This longitudinal study demonstrates that iatrogenic menopause in breast cancer patients rapidly and durably disrupts both vulvovaginal health and microbial ecology. Within three months after iatrogenic menopause, participants exhibited marked deterioration in vulvovaginal symptoms of GSM, increased sexual dysfunction, loss of *Lactobacillus* dominance, and increased microbial diversity. These changes persisted throughout follow-up and remained evident, indicating that the consequences of iatrogenic menopause extend beyond the initial period of hormonal change. There are no significant differences in vaginal health or microbial ecology between chemotherapy or endocrine therapy-based ovarian suppression. Among the eight participants who later regained premenopausal hormonal levels, the vaginal microbiome did not revert to its premenopausal composition, underpinning the limited resilience of the vaginal ecosystem following abrupt estrogen deprivation.

To the best of our knowledge this is a first longitudinal study to follow women from a premenopausal state through iatrogenic menopause while simultaneously integrating hormonal measurements, vaginal microbiome profiling, clinical assessment of GSM, psychosexual and sexual health outcomes. The findings suggest that abrupt estrogen deprivation may induce a ecological transition within the vaginal ecosystem that persists beyond the acute menopausal onset and in participants who regain ovarian function. This study is not only relevant to breast cancer survivorship, it provides uniquely informative model to study iatrogenic menopause and abrupt estrogen deprivation, since natural menopause can only be diagnosed retrospectively following twelve months of amenorrhea, where the timing of onset of estrogen decline is unknown. This study allows unprecedented temporal resolution of biological and clinical changes occurring during the menopausal transition. This methodology allowed us to demonstrate that clinical and microbial changes emerge remarkably rapidly following iatrogenic menopause.

The rapid deterioration of vaginal and vulvar health observed following menopause onset coincided with pronounced changes in the vaginal microbiome, consistent with the central role of estrogen maintaining the vaginal ecosystem. Estrogen withdrawal reduces glycogen availability within the vaginal epithelium, thereby limiting a key nutrient source for *Lactobacillus* spp. and

promoting increase in the vaginal pH.[26] These changes contribute to destabilizing the low-diversity, *Lactobacillus*-dominated microbial communities that characterize vaginal health during the reproductive years.[26] Consistent with this model, we observed a rapid loss of *Lactobacillus* dominance accompanied by increased microbial diversity following iatrogenic menopause. Increased diversity within the vaginal ecosystem is generally associated with less favorable vaginal health outcomes, in contrast to other body sites.[32] Our findings extend observations from the Isala cohort[29] and other cross-sectional studies[21–25,30,31] demonstrating associations between menopausal status and increased vaginal microbial diversity, in women with and without a history of breast cancer. Our study enabled direct observation of temporal dynamics within the same individual across the menopausal transition, in contrast to previous studies with cross-sectional study-design. These cross-sectional studies designed to capture microbial patterns during natural menopause compare samples from premenopausal and postmenopausal women with or without symptoms of GSM. [21–25,30,31] They largely rely on static comparisons that infer, rather than demonstrate, temporal change. Importantly, our unique longitudinal design enabled us to observe these dynamics within the same individuals across the menopausal transition, providing temporal evidence that has previously been lacking.

Particularly interesting was the apparent lack of microbiome recovery in eight women who recovered from menopause. Reduced *Lactobacillus* dominance and increased microbial diversity persisted over time despite normalization of circulating estradiol levels, suggesting that abrupt estrogen deprivation may induce an ecological transition that is not readily reversed following restoration of ovarian function. This finding extends previous cross-sectional observations in menopause research by suggesting that the effects of menopause on the vaginal ecosystem may be more durable than previously appreciated.[21–24,30] Together, our findings indicate that menopausal status, more than chronological age, is a major determinant of vaginal microbial composition and that menopause-associated ecological restructuring may persist beyond normalization of circulating estradiol levels.

Notably, menopausal status was the only factor consistently associated with vaginal microbiome composition. In contrast, GSM severity, sexual function, sexual distress, and treatment modality were not significantly associated with microbial composition. These findings suggest that hormonal status may exert a stronger influence on vaginal microbial ecology than symptom burden itself and highlights the complexity of host-microbiome interactions in GSM. Although depletion of *Lactobacillus* species is frequently linked to vulvovaginal symptoms in cross-sectional studies, our findings indicate that microbial alterations alone may not fully explain the clinical manifestations of GSM. Host factors, including epithelial integrity, immune responses,

and local estrogen signaling are likely to contribute substantially to symptom development and persistence. This hypothesis is supported by previous studies[21,23–25,30] reporting discordance between vaginal microbial composition and GSM symptom severity. However, epithelial integrity and inflammatory markers were beyond the scope of the present study: due to the clinical framework of his investigation, we chose to involve only clinical parameters for the evaluation of GSM, as used in routine clinical practice in Belgium.

Menopausal women frequently experience genitourinary discomfort, particularly those with a history of breast cancer who receive aromatase inhibitors.[6,19,25,33] The rapid onset of GSM observed in this cohort has important clinical implications. Significant deterioration in vulvovaginal health was already evident within three months of menopause induction, emphasizing the need for early assessment and proactive management of GSM at initiation of breast cancer treatment rather than waiting until symptoms become severe. The need for such proactive management is illustrated by the substantial proportion of patients in our cohort who were prescribed a vaginal moisturizer or vaginal estrogen therapy during the study period (Suppl.I, Part 1B). Early recognition and treatment of vulvovaginal symptoms may improve quality of life and sexual wellbeing, particularly among patients receiving long-term endocrine therapy. At the end of our study, five participants had discontinued GnRH agonists due to intolerable adverse side effects including vulvovaginal discomfort and impaired sexual functioning. This exemplifies the need for proactive patient counseling and supportive care to optimize treatment adherence.[14,34–40]

The persistent microbial alterations observed in this cohort further suggest that current symptom-directed approaches to treat vulvovaginal symptoms of GSM may not fully address menopause-associated changes in the vaginal ecosystem. Future studies should investigate whether microbiome-modulating interventions, alongside established therapies for GSM, can promote restoration of vaginal health and improve long-term survivorship outcomes.

A key advantage of this study is the use of iatrogenic menopause as a precisely timed model of abrupt estrogen deprivation. This enabled longitudinal integration of hormonal, microbial, anatomical, and psychosexual outcomes across the menopausal transition, providing temporal insight that is rarely achievable in studies of natural menopause. Taken together, our findings suggest that menopause may represent not only a hormonal transition but also a potentially long-lasting ecological transition of the vaginal ecosystem. Future studies should determine whether this ecological restructuring can be prevented or reversed and whether doing so improves vaginal health and survivorship outcomes.

Conclusion

This longitudinal study demonstrates that iatrogenic menopause in breast cancer patients rapidly and durably reshapes both vaginal health and microbial ecology. Within three months of menopause onset, participants exhibited marked deterioration in vulvovaginal health, increasing symptom burden, loss of *Lactobacillus* dominance, and increased microbial diversity. These alterations persisted over time, even among women who later regained premenopausal hormonal levels, suggesting limited resilience of the vaginal ecosystem following abrupt estrogen deprivation. By integrating hormonal, microbial, anatomical, and psychosexual outcomes, we provide the first longitudinal evidence of how iatrogenic menopause restructures the vaginal ecosystem across the menopausal transition. Beyond its implications for breast cancer survivorship, this work establishes iatrogenic menopause as a unique human model for studying the biological consequences of abrupt estrogen withdrawal. These findings support proactive assessment of GSM early during breast cancer treatment and motivate future development of interventions aimed at preserving or restoring vaginal ecosystem health.

Table 1

Table 1| Mean scores for general mental well-being, functional status, partner relation quality, sexual function, sexual distress and proportion of participants meeting the criteria for sexual dysfunction per study visit.

Outcome	V1 (n=60)	V2 (n=55)	V3 (n=52)	V4 (n=38)
General wellbeing: WHO-5[41](mean, (SD))	51.85, (18.36)	49.75, (19.27)	55.43, (17.93)	62.30, (16,48)
p-value pairwise comparison; compared to V1		1.000	1.000	.047(*)
Functional status: ECOG (mean, SD)	.13, (.47)	.96, (.80)	.94, (.73)	.41, (.59)
p-value pairwise comparison; compared to V1		<.001(*)	<.001(*)	.234
Relational wellbeing: DAS (mean, SD)	120.10, (12.22)	118.71, (12.63)	116.79, (12.66)	115.51, (16.38)
p-value pairwise comparison; compared to V1		1.000	1.000	1.000
Sexual function: FSFI (mean, SD)	21.70, (6.06)	18.32, (8.04)	18.54, (7,20)	18.55, (8.53)
p-value pairwise comparison; compared to V1		.274	.377	.274
Sexual distress: FSDS-r (mean, SD)	12.00, (7.70)	16.42, (8.90)	16.35, (8.70)	16.58, (8.9)
p-value pairwise comparison; compared to V1		.187	.214	.230
Sexual dysfunction[$]: (proportion)	.33	.52	.58	.49
p-value paired sample t-test; compared to V1		.037(*)	.037(*)	.211

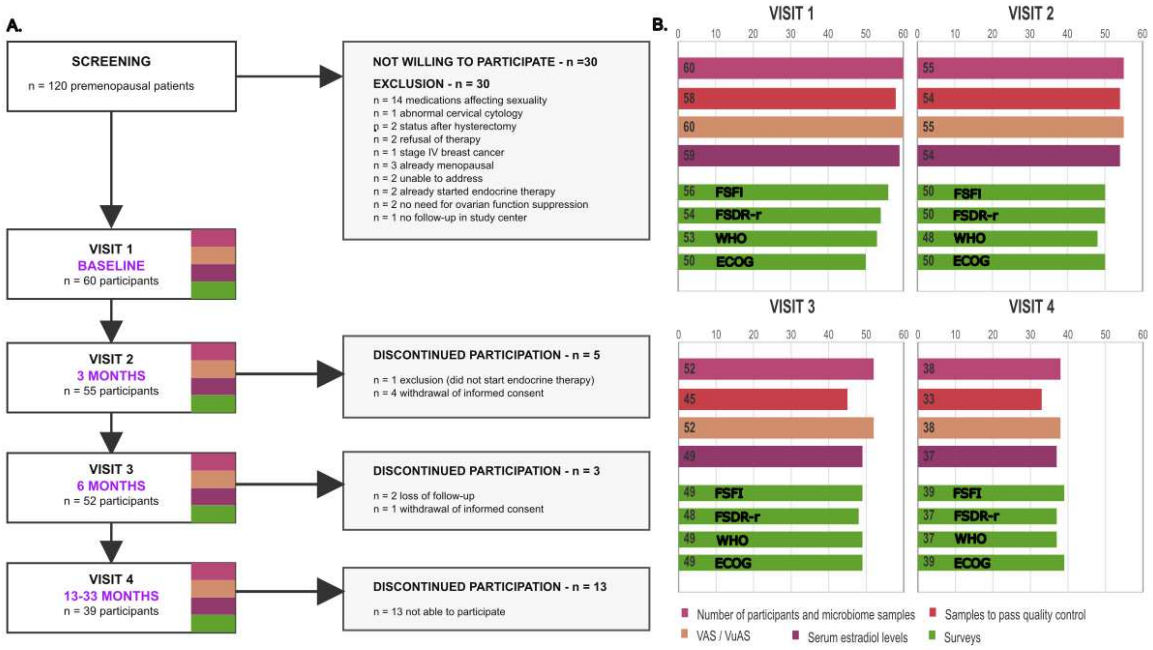
V1 = visit 1, V2 = visit 2, V3 = visit 3, V4 = visit 4, WHO-5 = World Health Organization-5 index, [41] SD = Standard Deviation, ECOG = Eastern Cooperative Oncology Group score, [42] DAS = Dyadic Adjustment Scale, [43] FSFI = Female Sexual Function Index, [44] FSDS-r = Female Sexual Distress Scale – revised) [44]

[$] *Sexual dysfunction is defined as a combination of low sexual function (FSFI < 26.55) and high sexual distress (FSDS-r >11)*

(*) indicates results that reached significance

Figure 1

Figure 1| Study design overview: CONSORT flowchart with timeline and study specific participation rates



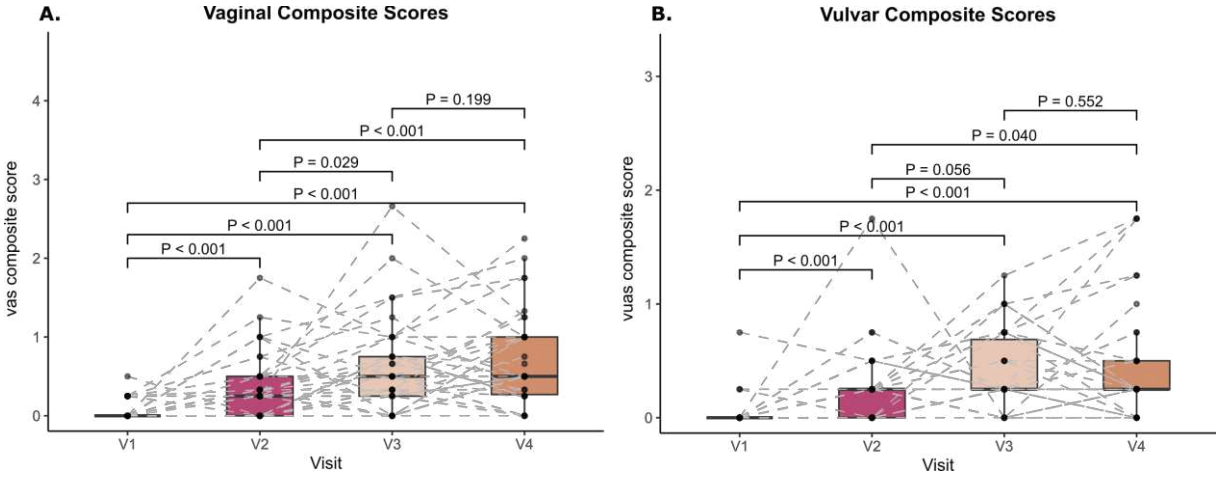
VAS = vaginal assessment scale, VuAS = Vulvar assessment scale, FSFI = female sexual function index, FSDS-r = female sexual distress scale – revised, WHO-5 = world health organization - 5 index, ECOG = eastern cooperative oncology group score

***A)** CONSORT diagram: 120 premenopausal patients were screened to include 60 participants. During this investigation, 5 participants withdrew informed consent, 1 patient was excluded after V1, and 2 patients were lost to follow-up. A total of 39 participants were able to attend V4.*

***B)** Study specific participation rates: At each evaluation, genitourinary symptoms of menopause (GSM) were evaluated during gynecological examination. The validated vaginal and vulvar assessment scale (VAS/VuAS) was used.[45] During gynecologic examination, both a pH sample and an eNAT® (Copan) swab for microbiome sampling were collected. Serum estradiol levels were measured; menopausal status was reached <20 ng/ml.[4,46] Psychosocial outcomes were measured using validated questionnaires: sexual function (FSFI), sexual distress (FSDS-r), general wellbeing (WHO-5) and functional status (ECOG).* [4,41–44,47–49]

Figure 2

Figure 2| Vaginal and vulvar composite scores per study visit



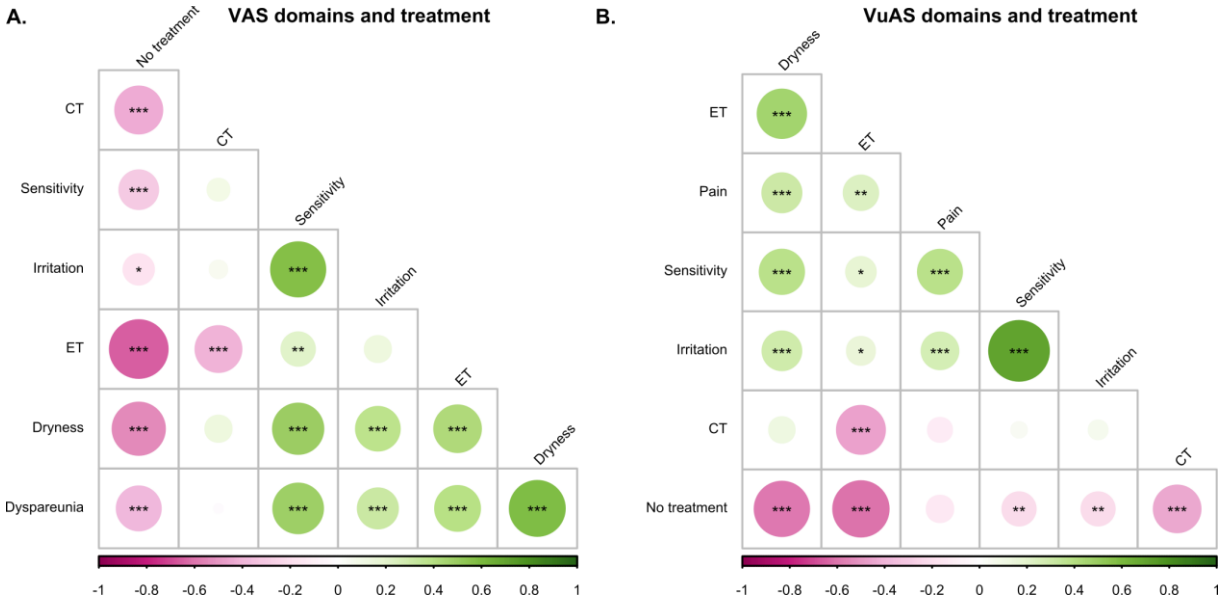
V1 = visit 1, V2 = visit 2, V3 = visit 3, V4 = visit 4, vas = vaginal assessment scale, vuas = vulvar assessment scale

A) *Longitudinal changes in the vaginal composite score from V1 (n=60) to V2 (n=55), V3 (n=52) and V4 (n=39). Box plots show results for vaginal composite score per study visit. Overall differences across visits were assessed using the Friedman test (Kendall's W = 0.455, P = 3.18 × 10 $^{-7}$). Pairwise comparisons were performed using two-sided paired Wilcoxon signed-rank tests; corresponding P- values are indicated in the figure. The VAS composite score increased significantly between consecutive study visits, except between V3 and V4, where no significant difference was observed.*

B) *Longitudinal changes in the vulvar composite score from V1, to V2, V3 and V4. Box plots show results for vulvar composite scores per study visit. Overall differences across visits were assessed using the Friedman test (Kendall's W = 0.378, P = 2.33 × 10 $^{-8}$). Pairwise comparisons were performed using two-sided paired Wilcoxon signed-rank tests; corresponding p-values are indicated in the figure. The vulvar composite score showed increased significantly between consecutive study visits compared to V1 and between V2-V4, there are no significant increases between V2-V3 and V3-V4.*

Figure 3

Figure 3| Pearson correlation for vaginal and vulvar symptoms and treatment modality



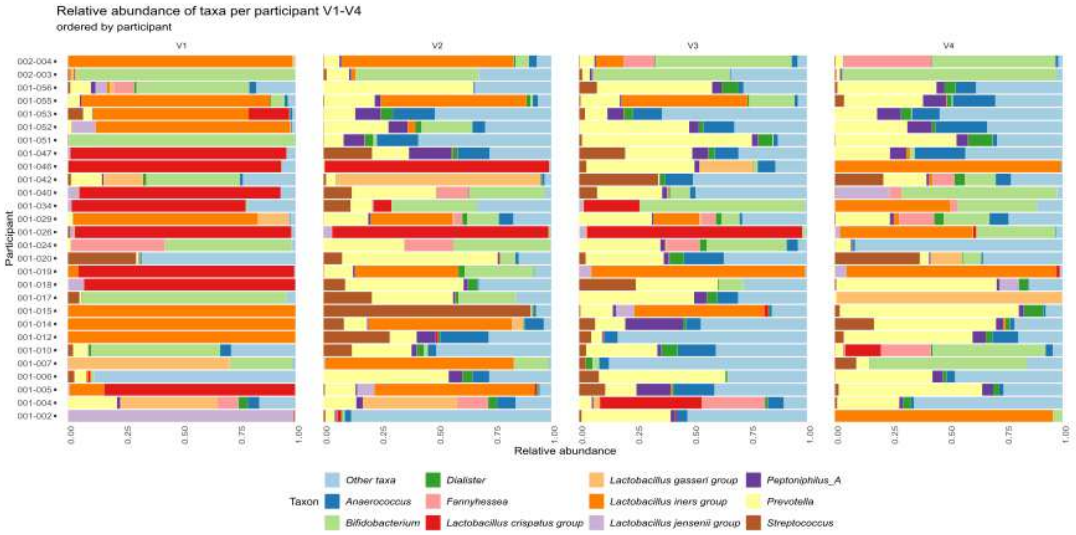
CT = chemotherapy, VAS = vaginal assessment scale, ET = endocrine therapy, VuAS = vulvar assessment scale

A) Pearson correlation for vaginal symptoms: *Pearson correlation matrix showing the pairwise associations between treatment timepoints, type of adjuvant treatment and VAS domains (sensitivity, irritation, dryness, and dyspareunia). The size and color of the circles represent the strength and direction of the correlation coefficients, with green indicating positive correlations and purple indicating negative correlations. Larger circles correspond to stronger correlations. Statistical significance is denoted by asterisks based on Benjamini-Hochberg adjusted P values (*P < 0.05, **P < 0.01, ***P < 0.001).*

B) Positive correlation for vulvar symptoms: *Pearson correlation matrix showing the pairwise associations between treatment timepoints, type of adjuvant treatment and VuAS domains (sensitivity, pain, irritation). The size and color of the circles represent the strength and direction of the correlation coefficients, with green indicating positive correlations and purple indicating negative correlations. Larger circles correspond to stronger correlations. Statistical significance is denoted by asterisks based on Benjamini-Hochberg adjusted P values (*P < 0.05, **P < 0.01, ***P < 0.001).*

Figure 4

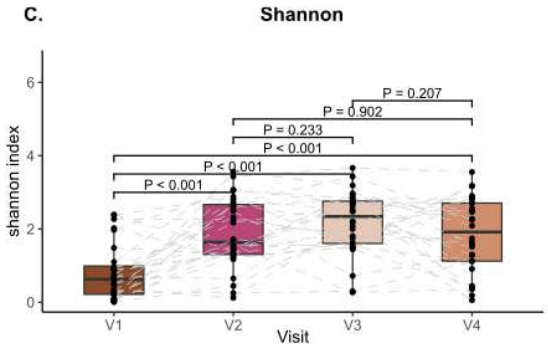
Figure 4| Relative abundance, dominant Lactobacillus taxa and alpha diversity per study visit



B.

Dominant Lactobacillus taxa per visit

visit	dominant_taxon	n	total	percentage	total_lacto_percentage
V1	*Lactobacillus crispatus group*	22	58	37.9	70.6
	Lactobacillus iners group	15	58	25.9	
	Lactobacillus gasseri group	2	58	3.4	
	Lactobacillus jensenii group	2	58	3.4	
V2	*Lactobacillus iners group*	15	54	27.8	40.8
	Lactobacillus crispatus group	4	54	7.4	
	Lactobacillus gasseri group	3	54	5.6	
V3	*Lactobacillus iners group*	4	45	8.9	22.2
	Lactobacillus crispatus group	3	45	6.7	
	Lactobacillus gasseri group	2	45	4.4	
	Lactobacillus jensenii group	1	45	2.2	
V4	*Lactobacillus iners group*	5	33	15.2	18.2
	Lactobacillus gasseri group	1	33	3.0	



V1 = visit 1, V2 = visit 2, V3 = visit 3, V4 = visit 4, n = number

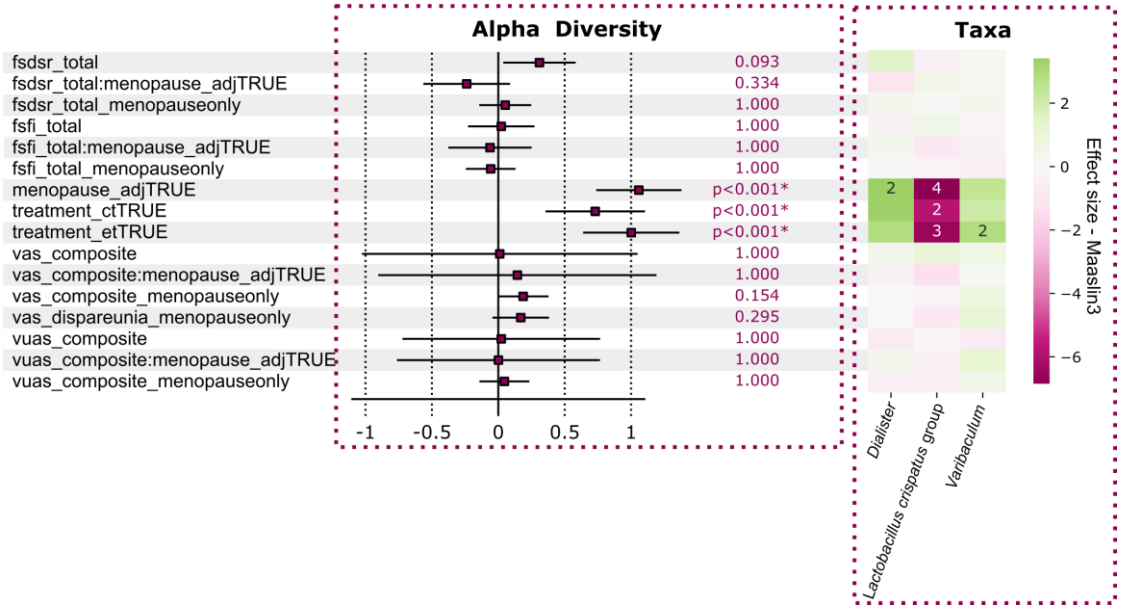
A. Relative abundance of taxa per participant (V1–V4): *Stacked bar plots showing the relative abundance of bacterial taxa at each study visit (V1–V4). This figure includes only participants with samples that passed quality control for each study visit. Visual inspection already suggests a progressive reduction in the relative abundance of Lactobacillus taxa (pink, red, and orange) over time, accompanied by an increase in the relative abundance of anaerobic taxa (blue, green, and yellow taxa).*

B. Dominant Lactobacillus taxa (V1-V4): *The table summarizes the dominant Lactobacillus subgroup at each study visit. The proportion of samples dominated by Lactobacillus decreases across visits, from 70.6% at V1 to 18.2% at V4, indicating a shift away from Lactobacillus-dominated vaginal microbiota over time.*

C. Alpha diversity plots (V1-V4): *Box plots show the differences in Shannon index for all study samples. Overall differences across visits were assessed using the Friedman test ($W = 0.296$, $P = 1.65 \times 10^{-5}$), showing a significant increase in diversity across visits. Pairwise comparisons were performed using two-sided paired Wilcoxon signed-rank tests; corresponding P-values are indicated in the figure. There is a significant increase in diversity comparing V1 to all other study visits, whereas no significant differences were observed between V2, V3, and V4.*

Figure 5

Figure 5| Association of differential abundance, clinical parameters, sexual function and the composition of the vaginal microbiome and alpha diversity



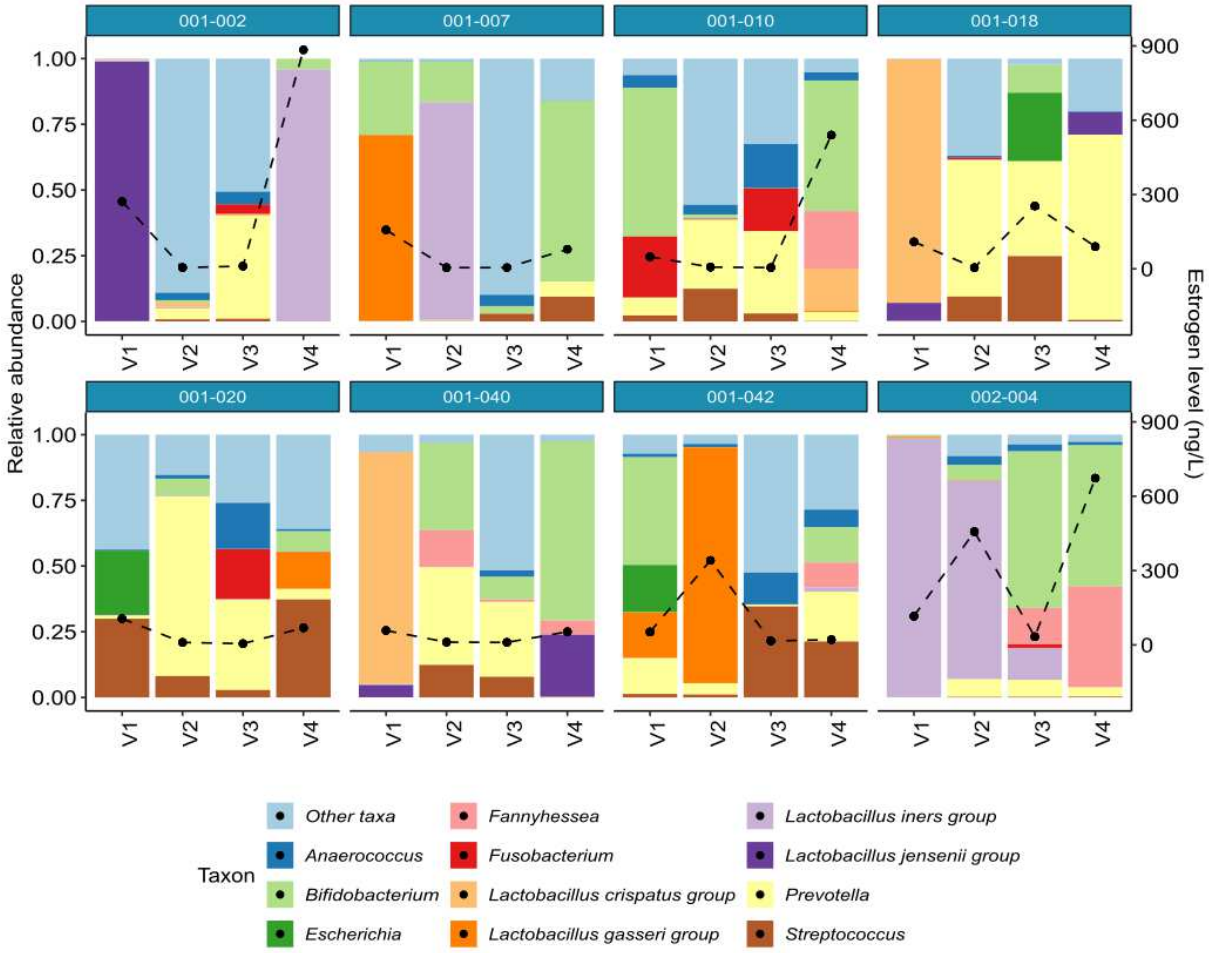
FSDSr_total = total scores on female sexual distress scale -revised, Menopause_adj_TRUE menopausal status (only applicable V2-V4), Menopauseonly refers to an adjusted menopause status, in which samples that are not in menopause following treatment (or reverted back to non-menopause after treatment) are excluded; FSFI = female sexual function index, ct = chemotherapy, et = endocrine therapy, VAS_composite = vaginal assessment scale – composite score, vuas_composite = vulvar assessment scale composite score

A. Differential abundance analysis: *Heatmap summarizing the differential abundance analysis across four statistical methods (MaAsLin3, ALDEx2, ANCOM-BC2, and a linear model of CLR-transformed taxa abundances). Cell colour represents the direction and magnitude of the effect size, with green indicating a positive association and pink/purple indicating a negative association. The number within each cell indicates how many methods identified a statistically significant association. Only associations supported by at least two methods were considered robust. Using this criterion, Lactobacillus crispatus was the only taxon consistently identified as differentially abundant across analyses.*

B. Alpha diversity analysis: *Forest plot showing the estimated effects of clinical variables on alpha diversity measures. Menopause and treatment were the only variable significantly associated with alpha diversity (both treatment groups, $P < 0.001$), whereas physical and psychosocial symptom scores, were not significantly associated with alpha diversity after multiple testing correction.*

Figure 6

Figure 6| Longitudinal follow-up in participants who regain ovarian function



Relative abundance of taxa (V1–V4 in participant who regain ovarian function: *Stacked bar plots showing the relative abundance of bacterial taxa at each study visit (V1–V4). The black dots in each stacked bar indicated serum estrogen levels. This figure includes only participants with samples that passed quality control for each study visit. Visual inspection already suggests an increase in relative abundance of anaerobic taxa (blue, green and yellow). Participants who returned to premenopausal status did not consistently revert to their premenopausal vaginal microbiome, suggesting that menopause-associated changes in the vaginal microbiome may persist beyond the menopausal transition.*

Methods

Study cohort and data collection:

VOLUPTA is a multicentric prospective longitudinal study conducted in Belgium, in the multidisciplinary breast units of University Hospitals Leuven (UZL) and AZ Sint Blasius Hospital Dendermonde (AZSB). The study was approved by the Regional Committees for Medical and Health Research Ethics (s66504) and conducted in accordance with the principles of the World Medical Association Declaration of Helsinki and its later amendments.

Participants were enrolled over a 22-month period, from October 26, 2022, to August 9, 2024. Of 120 eligible premenopausal women with stage I-IIIC breast cancer, 60 participants were included (56 at UZL and 4 at AZSB) (CONSORT scheme in Fig. 1A). Inclusion criteria were; being older than 18 years, reproductive stage or menopausal transition according to the Stages of Reproduction Aging Workshop +10 (STRAW+10)-criteria[46], expected iatrogenic menopause related to breast cancer treatment, fluent in Dutch, English or French, and any partner status. Exclusion criteria were hysterectomy in medical history, a history of abnormal cervical cytology, co-morbidities or co-medication that might influence sexual functioning, language barriers, or an asexual sexual identity. Full in- and exclusion criteria can be found in supplementary information, part 5. Written informed consent (ICF) was obtained from all participants before participation.

All eligible women were asked to participate at 4 time-points: Visit 1 was a baseline investigation where every participant was premenopausal (V1; *n*=60), visit 2 with 3 and visit 3 with 6 months interval after iatrogenic menopause (V2; *n*=55, V3; *n*=52). An amendment to conduct a fourth examination in the summer of 2025 (V4; *n*=39) was approved on 10-06-2025, this was 13-33 months after V1. At V4, participants were stratified into those who remained menopausal (*n*=31) and those who had exited the menopausal status (*n*=8), enabling evaluation of the longer-term effects of iatrogenic menopause and the potential for vaginal microbiome recovery following its cessation.

Study specific investigations

Hormonal measurements

Peripheral blood samples were collected in SST5 tubes (5 mL, clot activator with gel) and analyzed at the Department of Laboratory Medicine, UZL. Estradiol and FSH were measured using electrochemiluminescence immunoassays (ECLIA; cobas 8000 platform, Roche): a competitive assay for estradiol (postmenopausal reference <20 ng/L) and a sandwich assay for FSH (postmenopausal reference 25.8–134.8 IU/L). Menopausal status was defined by integrating biochemical results (estradiol <20 ng/mL) and clinical data (absence of vaginal bleeding).

Participants with absence of vaginal bleeding, but estradiol>20 ng/L were considered premenopausal. At visit 1, STRAW+10[46] criteria were applied; at subsequent visits, estradiol levels were primarily used for biochemical confirmation of menopause. Samples were discarded after analysis.

Psychosexual outcomes

Validated questionnaires were used to collect information regarding general mental well-being (World Health Organisation- 5 index (WHO5),[41] functional status (Eastern Cooperative Oncology Group score (ECOG)),[42] relational well-being (Dyadic adjustment Score (DAS)),[43] sexual function (Female Sexual Function Index (FSFI)), and sexual distress Female Sexual Distress Scale – revised (FSDS-r)).[44,49] Information regarding demographics and medical background were collected by a self-designed survey by the study group. Questionnaires were available for the participants on paper. We used RedCap to process the questionnaires.

Clinical assessment of GSM and sample collection

Participants were asked to abstain from sexual contact for 48 hours prior to clinical examination. Vulvovaginal signs of GSM were evaluated using the Vaginal Health Assessment (VHA), Vaginal Assessment Scale (VAS), and Vulvar Assessment Scale (VuAS); resulting in vaginal and vulvar composite scores.[45] During speculum examination, both a pH sample and an eNAT® (Copan) swab for microbiome sampling were collected. Standardized microbiome sample collection was taken at 3-5 cm beyond the introitus: rotating the swab 3-5 times 360°.

The pH was sampled at the mid vaginal wall, using a pH-indicator strip; Supelco®, (range 1-10, universal indicator); the indicator strip was discarded after the result was recorded. All gynecological examinations were performed by a dedicated group of gynecologists. Most examinations were performed by the first author. When clinical signs of GSM were noticed and the participant was symptomatic, treatment of GSM according to the current standard was proposed.[15,16]

Vaginal microbiome profiling

For sampling of the vaginal microbiome, eNAT swabs with 1 ml lysis buffer were used. Samples collected at UZ Leuven were stored at -20°C after collection, samples collected at AZSB were stored at 4° for a maximum of 3 days and later at -20°C for a maximum of 6 months before transport to the Lab of Applied Microbiology and Biotechnology, University Antwerp.[50] DNA was extracted using Dneasy PowerSoil Pro Kit (Qjagen, Hilden, Germany). The bacterial 16S rRNA gene was amplified using primers for the V4 hypervariable regions, libraries were created using barcoded primers, and amplicons were sequenced using the MiSeq (Illumina). Reads where

preprocessed and taxonomically classified using the nf-core ampliseq pipeline (v2.12.0) using default settings, truncLen = c(232, 215) and the SBDI Sativa curated 16S GTDB database (release S226) providing count tables of taxa present in the microbiome. *Lactobacillus* genus classifications were further subclassified into the subgenera as described in Lebeer et al.[29 51,52] Dominance was defined as the most abundant taxon that constituted at least 30% of the profile. Of note, of those samples that did not pass quality control, several samples had low reads (<600), this can be due to low biomass or technical reasons.

Bio-informatic and biostatistical analyses

Linear mixed-effects models were used to examine changes in general well-being (WHO-5), functional status (ECOG), relationship satisfaction (DAS), sexual function (FSFI), and sexual distress (FSDS-R) across the four study visits. Study visit was included as a categorical fixed effect of time, with a random intercept for each participant to account for within-participant correlations. For the binary outcome of sexual dysfunction, a generalized estimating equation (GEE) model was used to assess changes over time. Pairwise comparisons between follow-up visits and baseline (V1) were performed using Bonferroni correction for multiple comparisons. Statistical significance was defined as a two-sided p-value < .05. Wilcoxon rank sum tests were used to evaluate differences in the VAS and VUAS composite scores across time (visits), shown in figure 2. The correlations between vaginal and vulvar symptoms and treatment modality shown in figure 3 are Pearson correlations. Associated p-values were adjusted for multiple testing using the Benjamini-Hochberg procedure.

To evaluate changes in the microbial composition of the vagina and their relation to clinical and patient-reported outcomes related to sexuality and pain, different analyses were performed. First, the effects across time (visits) were analyzed at the alpha diversity level at the Shannon, Berger-parker, and observed diversity metrics. This was tested with a Wilcoxon rank sum test. Next, the effects of clinical measures were tested against microbial composition at both the bacterial alpha diversity (Shannon index) and the differential abundance of individual taxa, according to previously published approaches.[29] For each effect of interest, we tested the following models:

1. ~ e_i* menopause + moisturiser + age + plate + (1|participant)
2. ~ e_i(menopauseonly) + moisturiser + age + plate + (1|participant)
3. ~ menopause + moisturiser + age + plate + (1|participant) ")
4. ~ treatment_ct + treatment_et + moisturiser + age + plate + (1|participant)

e.i. refers to the variable of interest; FSDS-R_total: total scores on FSDS-R, FSFI_total: total scores on FSFI, VAS_composite, VuAS_composite, dyspareunia: reported scores for dyspareunia in

female sexual function index (indicating pain during intercourse), Menopause_adj_TRUE menopausal status (only applicable V2-V4).

Other variables in this model are: Menopauseonly refers to an adjusted menopause status, in which samples that are not in menopause following treatment (or reverted back to non-menopause after treatment) are excluded; this excluded 16 samples from the analysis whose effects we would be unable to model. Moisturizer indicates vaginal moisturizer use (0 if not, 1 if yes). Age is the age at inclusion. Plate refers to the sample batch effects. Treatment_et and treatment_ct refer to whether endocrine treatment (et) or chemotherapy (ct) was the most recent treatment received at that sampling point (0 if not, 1 if yes); Samples before treatment 0 for both variables.

A random intercept for participant (1|participant) was included to account for repeated measurements. To test whether variation in variable of interest was associated with the microbiome within only the menopausal samples, the fourth model with e_i(menopauseonly) refers to an analysis performed only in the subset of samples that were in menopause.

Alpha diversity was tested with a linear mixed effect model with Shannon diversity on the left-hand side of the formula. Differential abundance analyses were performed using multiple differential methods, including ALDeX3, ANCOM-BC2, MaAsLin3 and LMCLR, and consensus was examined. An effect was only reported if at least two methods reported a significant result. Multiple testing correction was done with the Benjamini-Hochberg procedure.

All data processing and visualization were performed in Python (3.13.0) and R (4.2.3) using the tidyverse suit of packages, the in-house developed tidytacos package and multidiffabundance package (https://github.com/thiesgehrmann/multidiffabundance).[53]

Data and code availability

Sequencing data are available at the European Nucleotide Archive (ENA) under accession code PRJEB121337. The Multiple Differential Abundance (MDA) tool can be found at: https://github.com/thiesgehrmann/multidiffabundance. All other scripts used for data processing and analysis are hosted on Github: https://github.com/thiesgehrmann/UA_volupta

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Author contributions

N.W., S.N.H., S.A., and S.L. designed the study, N.W and S.N.H worked on the conceptualization of the research project. P.E., and P.N. contributed to the conceptualization of the study. Clinical sampling was performed by N.W. and S.N.H. Data analysis was conducted by M.H., T.V.R., T.G., and M.P.B. Data were interpreted by N.W., S.A., M.H., T.V.R., T.G., S.L., S.N.H., and P.E., N.W., S.A., M.H., S.L., and S.N.H. drafted the original manuscript. All authors (N.W., S.A., M.H., T.V.R., T.G., I.D.B., M.P.B., J.D., P.E., P.N., S.L., and S.N.H.) reviewed and edited the manuscript and approved the final version.

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