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Colonization of the upper genital tract by vaginal bacterial species in non-pregnant women

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Abstract

Objective—Evaluate upper genital tract (UGT) presence of vaginal bacterial species using sensitive molecular methods capable of detecting fastidious bacterial vaginosis (BV)-associated bacteria.

Study Design—Vaginal swabs were collected prior to hysterectomy. The excised uterus was sterilely opened and swabs collected from endometrium and upper endocervix. DNA was tested in 11 quantitative PCR (qPCR) assays for 12 bacterial species: *Lactobacillus iners, L. crispatus, L. jensenii, Gardnerella vaginalis, Atopobium vaginae, Megasphaera spp., Prevotella spp., Leptotrichia/Sneathia*, BVAB1, BVAB2, BVAB3 and a broad-range16S rRNA gene assay. Endometrial fluid was tested with Luminex and ELISA for cytokines and defensins, and tissue for gene expression of defensins and cathelicidin.

Results—We enrolled 58 women: mean age 43 + 7 years, mostly white (n = 46; 79%) and BV-negative (n = 43; 74%). By species-specific qPCR, 55 (95%) had UGT colonization with at least one species (n = 52), or were positive by 16S PCR (n = 3). The most common species were *L. iners* (45% UGT, 61% vagina), *Prevotella* spp. (33% UGT, 76% vagina) and *L. crispatus* (33% UGT, 56% vagina). Median quantities of bacteria in the UGT were lower than vaginal levels by 2–4 $\log_{10}$ rRNA gene copies/swab. There were no differences in endometrial inflammatory

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This study was conducted in Seattle, WA.

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markers between women with no bacteria, *Lactobacillus* only or any BV-associated species in the UGT.

Conclusion—Our data suggest that the endometrial cavity is not sterile in most women undergoing hysterectomy, and that the presence of low levels of bacteria in the uterus is not associated with significant inflammation.

Keywords

Intrauterine bacteria; endometritis; upper genital tract infection; reproductive tract microbiota; uterine cavity; endometrium; sterile

Introduction

Bacterial colonization of the uterus is associated with adverse reproductive health outcomes, including preterm delivery and chorioamnionitis,[1] pelvic inflammatory disease and endometritis[2,3] and miscarriage.[4] Upper genital tract infection has been presumed to be due to pathologic ascent of vaginal bacteria in to the upper genital tract. The physical barrier of cervical mucous, its high concentrations of antimicrobial peptides and inflammatory cytokines,[5–9] and possibly immunoglobulins[10] or matrix degrading enzymes [11] in the mucous plug are thought to provide a defense against bacterial ascent and the uterine cavity of healthy women has long been considered sterile.

However, radioactively labeled albumin spheres placed in the vagina ascend into the uterus as early as 2 minutes after instillation,[12] suggesting that fluid and particles move between the vagina and uterus relatively freely. Studies of ostensibly healthy women report a variable rate of uterine bacterial colonization by culture, ranging from 0–82%.[13–22] This wide range is due in part to differences in sample collection: studies using hysterectomy or transfundal sampling had lower rates (0–24%) [13–16,22] compared to those using transcervical sampling (33–82%).[17,18,21]

Many studies using molecular characterization of the microbiota have demonstrated the ubiquitous presence of bacteria throughout the body, and their influence on health. [23,24] We hypothesized that bacterial colonization of the upper genital tract may be quite common and not pathologic in many cases. We undertook this study to assess the prevalence and concentrations of bacteria in the upper genital tract (UGT) using sensitive molecular methods in sterilely sampled hysterectomy specimens. Additionally, we measured the endometrial immune response to determine whether intrauterine bacterial colonization was associated with epithelial inflammation, which could suggest an adverse effect of the bacteria.

Materials & Methods

Study cohort and sample collection

Women undergoing hysterectomy for non-cancer indications were eligible. Exclusion criteria included presence of an IUD, use of antibiotics, endometrial biopsy, IUD removal or hysteroscopy in the past 30 days, or concern for cervical or endometrial neoplasia. Total

laparoscopic or laparoscopically-assisted vaginal hysterectomy specimens were only collected if the surgeon was able to complete the procedure using a non-invasive vaginal fornix delineator (Colpo-Probe, Cooper Surgical, Trumbull, CT) or a vaginal sponge stick rather than an intracervical manipulator. The University of Washington Human Subjects Division approved the study. All subjects signed informed consent. All patients received standard pre-operative antibiotic prophylaxis at least 30 minutes prior to surgery.

Prior to vaginal exams or prep, flocked swabs (Copan Diagnostics Inc., Murrieta, CA) were inserted 3–4cm into the vagina for 5 seconds. One was smeared on a glass slide for Gram stain and Nugent scoring.[25] The uterus was removed, wrapped in a sterile towel, taken to pathology without fixation and incised sagitally under sterile conditions, beginning at the fundus. Swabs were collected first from the endometrium and then from the upper endocervix by rolling the swab 2–3 times across the epithelium and frozen at −80ºC. In a subset of participants (n = 30, 52%) swabs were collected in the Port-A-Cul anaerobic system (Beckton, Dickinson and Company, Franklin Lakes, NJ), cultured in standard fashion, including selective broth to allow growth of mycoplasma species and isolates identified by routine biochemical methods. Tissue sections were collected from the endometrium contralateral to the swab collection, cut into 1 × 1 cm blocks, placed in RNALater at 4ºC for 24 hours, then placed at −80ºC.

Bacterial PCR assays

Frozen swabs were thawed and 400 uL of PBS added, mixed by vortex shaker for 1 minute, then the swab removed and the sample spun at 17,000 x g for ten minutes (all at 4 degrees). The pellet underwent DNA extraction with the MoBio Bacteremia DNA Isolation Kit (MoBio, Carlsbad, CA), while the supernatant was aliquoted and frozen for Luminex analysis. DNA underwent taxon-directed 16S rRNA gene TaqMan format qPCR assays for the following bacterial species: *Lactobacillus crispatus, L. jensenii, L. iners, Gardnerella vaginalis, Atopobium vaginae, Megasphaera* genus, *Prevotella* genus, Bacterial Vaginosis Associated Bacterium 1 (BVAB1), BVAB2, BVAB3 and an assay detecting two closely related bacteria (*Leptotrichia* and *Sneathia*).[26,27] For the *Prevotella* genus assay, the forward primer 384F (5′ - GC CTG AAC CAG CCA AGT A – 3′), reverse primer 513R (5′ - GGA ATT AGC CGG TCC TTA TT - 3′) and a taxon-specific probe (6FAM - GTG CAG GAI GAC GGC C – MGBNFQ) were used. The thermocycler (ABI 7500 Thermocycler, Applied Biosystems, Foster City, CA) program was 2 minutes 50°C, 10 minutes 95°C, and then 45 cycles of 15 seconds 95°C, 39 seconds 59°C and 30 seconds 72°C. UGT swabs were also tested using a broad-range 16S rRNA gene assay to assess for the presence of any bacteria. Limits of detection for the assays were as follows: *L. crispatus* 75 gene copies/swab, *L. jensenii* 125 gene copies/swab, all other species-specific assays 150 gene copies/swab, and broad-range 16S 6,400 gene copies/swab. [28] Negative assays were assigned a value of half the lower limit of detection for that assay.

Measurement of cytokines, chemokines and antimicrobial peptides

Supernatant from endometrial swabs was submitted for Luminex (Luminex Corporation, Austin, TX) analysis. Seven of the 14 analytes (IL4, IL10, IL17, IFN-γ, IFNα, TNFα, MIP1α) were undetectable in over 95% of samples and were not included in the final

analysis. ELISA for human beta defensin 2 (HBD2), HBD3 (Alpha Diagnostics International, San Antonio, TX) and human alpha defensins 1-3 (HNP 1-3; Hycult Biotech, Plymouth Meeting, PA) was performed. Homogenized endometrial tissue sections underwent RNA extraction using the RNEasy Fibrous Tissue Kit (Qiagen Inc., Valencia, CA). RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories, Waltham, MA) and amplified using primers and probes from Applied Biosystems (Foster City, California) for HBD2, HBD3, cathelicidin (CAMP) and IL1β, as well as the housekeeping gene β-actin.

Statistical analysis

All analysis was performed using Stata v.10. Prevalences were compared between groups using the chi square test. Quantities of bacteria and concentrations of cytokines were not normally distributed, so were compared across groups using Wilcoxon rank-sum or Kruskall Wallis tests.

Results

Cohort

We enrolled 58 women with mean age of 43 ± 7 years. Participants were primarily white (n = 46; 79%), with a small proportion of African American (n = 6; 10%) and Hispanic (n = 4; 7%) women (2 declined to answer the question). All underwent hysterectomy for benign disease: primarily bleeding (n = 20; 34%), fibroids (n = 15; 26%), pain (n = 17; 29%). Most had a normal Nugent score (n = 43; 74%), while 6 (10%) had bacterial vaginosis, 7 (12%) had an intermediate score and 2 (3%) could not be scored. Most (37; 64%) were on no hormonal medications, 5 (9%) were taking oral contraceptives, 13 (22%) were using Lupron, and 2 (3%) were using a different hormonal medication (testosterone, hormone replacement therapy). Eight women (14%) reported being menopausal. Only 37/50 (74%) pre-menopausal women provided information about last menstrual period (LMP). The median number of days since LMP was 28 (IQR 12, 64), and of the 24 women reporting < 40 days since their LMP only 8 (33%) were in the first 14 days of their cycle. Most women had never douched (n = 32; 55%) or douched more than 1 week prior to surgery (n = 10; 17%), with a minority who had douched within the past week (n = 2; 3%) and 14 (24%) who did not answer the question. Nineteen women (33%) reported sexual intercourse in the week prior to surgery.

UGT colonization

By species-specific qPCR, 55 (95%) of women had UGT colonization (i.e. in the endometrium or upper endocervix) with at least one of the assayed species (n = 52), or were positive by broad range 16S PCR (n = 3). The most commonly detected species in the vagina were *Prevotella* spp. (76%) *L. iners* (61%), and *L. crispatus* (56%). These were also the most commonly detected species in the UGT: *L. iners* (45%), *Prevotella* spp. (33%) and *L. crispatus* (33%)(Figure 1a). *G. vaginalis*, *A. vaginae* and *L. jensenii*, were detected in the vagina in over 40% of women, but were detected less frequently in the UGT (in 19%, 10% and 20%, of women). Mean quantities of bacteria detected in the UGT were lower than levels in the vagina by 2–4 $\log_{10}$ rRNA gene copies (Figure 1b). When detected in the

vagina, *A. vaginae* was the least likely species to also be detected in the UGT, while BVAB1 and *L. iners* were the most likely (Figure 1c). The mean vaginal quantity of *L. crispatus* and *G. vaginalis* was significantly higher in women who had UGT colonization with those species: 7.7 ± 1 vs. 5.5 ± 2.5 gene copies/swab for *L. crispatus* ($p = 0.006$) and 7.8 ± 1.2 vs. 4.9 ± 1.5 gene copies/swab for *G. vaginalis* ($p < 0.001$). There were no significant differences in vaginal quantity between women with and without UGT colonization with other bacteria (data not shown). The median number of species detected in the UGT was 2 (IQR 1,3; range 0–8), while the median number of species detected in the vagina was 3 (IQR 2,5; range 0–9). There was no correlation between number of species detected in the vagina and the UGT (correlation coefficient 0.21, $p = 0.12$). Of note, in several cases an organism present in the UGT was not present in the vagina (Figure 2).

As almost all women had at least one species detected in the UGT we were unable to evaluate risk factors for colonization in general. We divided women into those with no bacteria detected in the UGT (n = 3), *Lactobacillus* species only (n = 18; 31%), any non-*Lactobacillus* species (n = 34; 59%), 16S positive only (n = 3). The only demographic difference between these groups was race: UGT colonization with a non-*Lactobacillus* species was more common in African American women (5/6; 83%) and Hispanic women (3/4; 75%) than white women (25/46; 54%)($p = 0.01$). Rates of BV were slightly different between these groups: 17% for African American, 0% for Hispanic and 11% for white women. There was a trend to increasing UGT colonization by non-*Lactobacillus* species with increasing Nugent score: with Nugent score 0–3 the rate was 51% (22/43), score of 4–6 71% (5/7) and score 7–10 83% (5/6) ($p = 0.24$). However, the six women with the highest levels of non-*Lactobacillus* species detected in the UGT all had a Nugent score < 7. Age, menopausal status, treatment with GnRH agonist, gravidity, parity, douching or sex in the past week were not significantly different between the groups. (data not shown)

Of the subset of 30 women who also had cultures performed of upper genital tract swabs, 28 (93%) had bacteria detected by qPCR, and 26/30 (87%) had bacteria detected by culture. Both women with negative qPCR results were also negative by culture. The most commonly cultured organisms were *Diphtheroids* (n = 15; 50%), followed by anaerobic gram-positive cocci (12; 40%), *Proprionibacterium* spp. (n = 9; 30%) and *Lactobacillus* species (n = 8 species from 5 women; 17%) (Supplementary Table 1).

Immune response

Soluble markers of inflammation were measured from endometrial swabs by Luminex, antimicrobial peptides by ELISA, and gene expression for defensins, cathelicidin and IL1β from tissue RNA and results compared between women with no bacteria, only *Lactobacillus* species or any non-*Lactobacillus* species detected in the upper genital tract. (Figure 3) There were no significant differences in the median values of these markers between groups. However, the lowest quantities of beta-defensin proteins seemed to be samples from women with non-*Lactobacillus* species in the UGT. The one woman with high (> 100,000 gene copies/swab) of *L. iners* in the UGT had relatively high levels of several inflammatory markers – but the lowest levels of gene expression for the beta defensins, cathelicidin and IL1β. When compared between women who had surgery for fibroids, bleeding, pain or other

reasons, the only analyte that was significantly different between the groups was IL6: median 6 pg/mL (Interquartile Range (IQR) 1, 28) in women having surgery for fibroids, 21.9 pg/mL (IQR 9,154) in women having surgery for bleeding, 32 pg/mL (IQR 14, 323) in women having surgery for pain, and 1 (IQR 1, 7.8). There was no difference in the distribution of women with only *Lactobacillus spp.* in the UGT versus non-*Lactobacillus* species between the surgical indications. (data not shown)

Comment

We detected UGT bacteria by PCR in 95% of women undergoing hysterectomy for benign gynecologic conditions. These results confirm the growing consenus that the endometrial cavity is not sterile. However, the quantity of bacteria present in the uterus and high endocervix was significantly lower than that in the vagina, suggesting that either the cervix serves as a partial filter to ascent, or that the endometrial immune response clears bacteria that do ascend, or a combination of both. We found a much higher prevalence of UGT colonization, but less correlation between vaginal and UGT samples than we anticipated. In women with vaginal colonization by a given species, rates of UGT colonization varied widely, suggesting differences in microbial ability to evade cervical immunity, or greater permissiveness to some species. Many groups have shown that the vaginal microbial community is dynamic.[27,29] Our results suggest that microbes may remain in the UGT after they disappear from the vagina and/or have better growth in the UGT than the vagina.

Studies using a similar strategy of incising a hysterectomy specimen to collect samples, but using culture to identify bacterial colonization, report rates of intrauterine bacterial colonization ranging from 0% among 10 women from Finland[22] to 31% in a cohort of 100 women from England.[16] Studies using transcervical sampling report higher rates of intrauterine bacterial colonization, ranging from 33%[18] to 60%,[17] but the degree of cervical or vaginal contamination of the endometrial specimen is unknown.[30] Our qPCR results from surgically obtained samples suggest an even higher rate of low-level bacterial presence in the upper genital tract than culture-based studies using transcervical sampling. Many of the bacteria identified by qPCR in this study, such as BVAB1-3 and *Leptotrichia/Sneathia*, are fastidious and difficult to culture, which may account for the differences between our data and previous reports. The bacteria we identified by culture from a subset of women include several taxa that have been identified in vaginal communities but were not targeted by our PCR assays: *Corynebacteria (Diptheroids)*, *Proprionibacteria*, *Ureaplasma*, coagulase-negative *Staphylococcus*, as well as several anaerobic colonies that could represent any number of other common vaginal species. All women in this study received pre-operative antibiotics intravenously, which likely affected our culture results.

Surprisingly, we saw few differences in endometrial immune markers between women with and without upper genital tract colonization by BV-associated microbes. This could be due to trauma-induced cytokine release at the time of surgical removal of the tissue, but our median values are similar to reported median values from endometrial aspirates in women with intact uteri undergoing in-vitro fertilization procedures[31] suggesting this is not the case. Alternatively, cytokines may be impacted by hormonal status, the underlying pathology leading to hysterectomy, or by viral or fungal pathogens not measured in our study. Our data

suggest that a low quantity of upper genital tract bacterial colonization by common vaginal species does not induce a strong inflammatory stimulus in most cases.

This is an exploratory analysis with a small sample size, which limits our ability to detect small associations or to perform well-powered subgroup analyses to look at factors associated with UGT colonization for different species. However, it is the first study using molecular methods to assess upper genital tract colonization in non-pregnant women. Our analysis is cross-sectional, which limits our ability to make conclusions about causation or direction of associations. However, opportunities to sterilely collect endometrial samples with minimal risk of contamination from the lower genital tract are becoming scarce, and preclude longitudinal sample collection from the UGT. Changing patterns of surgery mean that fewer hysterectomies are being performed, and many are now performed use minimally invasive techniques where an intracervical manipulation device is used. Intracervical instrumentation could introduce an uncontrolled amount of endometrial contamination, so these cases were excluded. Another limitation of our study is the use of selective qPCRs, which do not capture the entire microbiota. However, we did not have sufficient concentrations of bacterial DNA to perform broad range bacterial PCR with pyrosequencing. We did obtain additional information using bacterial culture in some cases, but prophylactic antibiotics potentially impact the sensitivity of cultures, and culture is not able to identify fastidious bacterial species. Finally, since these samples were collected from surgical specimens, all participants had pathology and thus the study population may not reflect the conditions present in normal, healthy women.

Recent advances in our understanding of the human microbiome reveals the important role that microbes play in many facets of human health.[32] The microbiome plays an important role in the immunologic homeostasis of the gut, encouraging proper development of mucosal immunity and preventing excessive inflammation (reviewed in [33]). T-regulatory cells in the gut mucosa maintain a tolerogenic environment and appear to be selected by interactions with commensal gut microbiota.[34] In the uterus, T-regulatory cells are important for implantation of the embryo and early placental development.[35,36] In one study, the presence of hydrogen peroxide producing *Lactobacillus* species on the tip of the embryo transfer catheter for in-vitro fertilization increased the chance of live birth compared to women who did not have these bacteria detected by culture.[37] This finding raises the possibility that the presence of intrauterine commensal bacteria may have a similar role in the selection of uterine T-regulatory cells as commensal gut microbiota do for colonic T-regulatory cells. While many other factors contribute to a successful pregnancy, it is worth noting that germ-free mice raised in sterile conditions have lower rates of reproductive success after embryo transfer than conventional animals,[38] suggesting a potential role for intrauterine bacteria in successful pregnancy. This potentially critical issue is largely unexplored.

It is clear that intrauterine bacterial colonization is not always benign, nor positive. In patients undergoing in vitro fertilization cycles, the presence of *Streptococcus viridans* on the embryo transfer catheter tip was associated with decreased chance of live birth compared to women without *S. viridans* detected.[37] Women with preterm birth are more likely to have intra-uterine placental infection than women who deliver at term. (reviewed in [39]) Women

with significant inflammatory sequelae in the upper genital tract with pelvic inflammatory disease often have anaerobic Gram-negative rods and mixed communities of bacteria in the uterus and fallopian tubes.[3,40] Pathologic effects of intrauterine bacteria may occur only with particularly virulent strains or species, only with high concentrations of bacteria, or only in the presence of a mixed bacterial community at the endometrial surface.

In summary, these data indicate that the endometrial cavity is not sterile in most women undergoing hysterectomy for benign indications. Additionally, detection of bacteria in the upper genital tract is not associated with a significant inflammatory immune response. While bacterial concentrations in the endometrium are much lower than that in the vagina, a low-level bacterial presence in the uterus appears common and not pathologic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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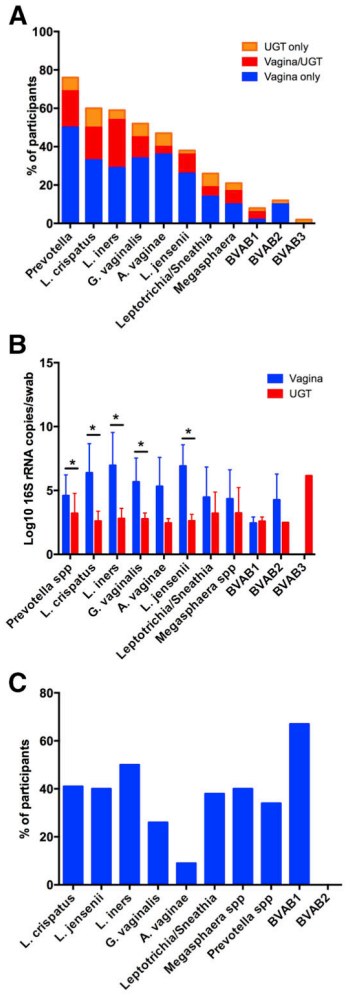

Figure 1. Distribution of upper genital tract bacterial colonization

A. Proportion of participants with detection of bacteria by species-specific qPCR in the vagina alone, both vagina and upper genital tract (UGT) and UGT alone. B. Comparison of mean quantity of bacteria in the vagina and UGT of women with either vaginal or upper genital tract detection of that species. * denotes comparisons that are significantly different ($p < 0.05$) by t-test. C. Proportion of women with vaginal detection of a given species by qPCR who also had UGT detection of that species.

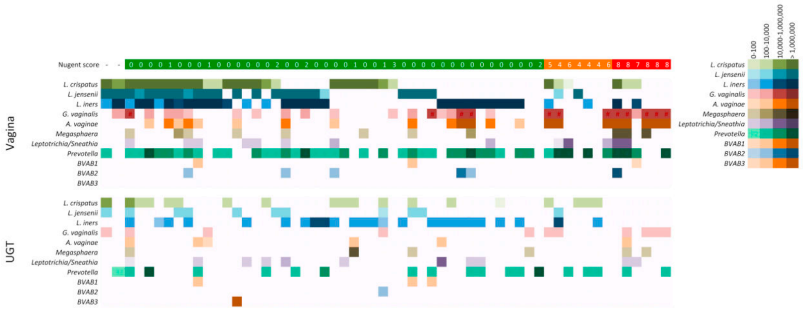

Figure 2. Comparison of vaginal and upper genital tract detection of bacteria

Detection and quantity of each species in the vagina and UGT for each participant, organized by Nugent score. Each bacterium is represented by a row, and the quantity is represented by a gradient of color, with darker colors representing higher quantities. The color gradients represent grouping of 1–100, 101–10,000, 10,000 – 1,000,000 and > 1,000,000 16S rRNA gene copies/swab. A white space means that the bacterium was not detected in that sample.

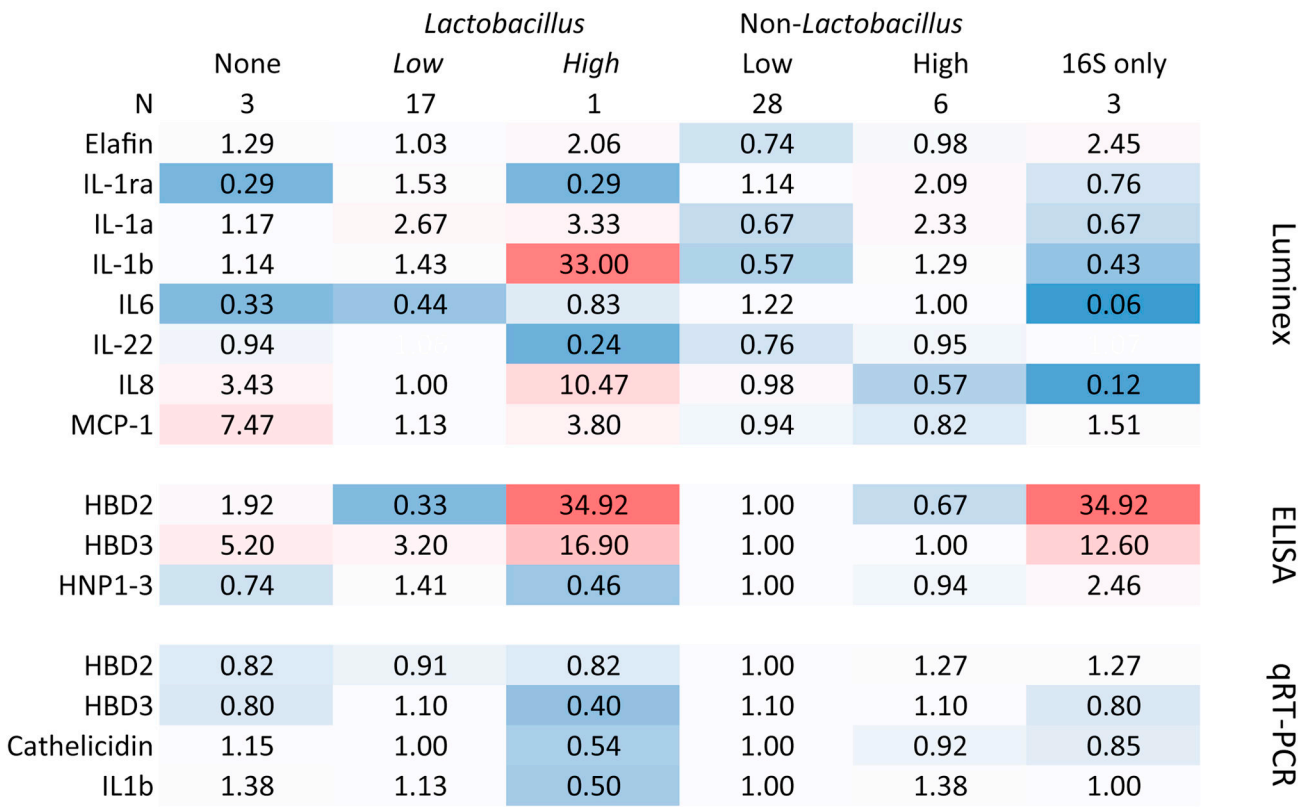

Figure 3. Endometrial immune markers

Comparison of markers of the immune response in the upper genital tract between women with no bacteria detected by PCR in the upper genital tract, only *Lactobacillus* species detected, or any non-*Lactobacillus* species detected. Numbers in boxes are multiple of the median, calculated by taking the median value for the whole cohort and dividing the individual group value by that number. There were no significant differences between these three groups. Values highlighted in red are higher than the group median, and those in blue are lower than the group median.