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Title: Relationship between vaginal microbial dysbiosis, inflammation, and pregnancy outcomes in cervical cerclage.

One Sentence Summary: Cervical cerclage using braided suture material disrupts vaginal microbial stability and increases inflammation.

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Abstract:

Preterm birth, the leading cause of death in children under five, may be caused by inflammation triggered by ascending vaginal infection. About two million cervical cerclages are performed annually to prevent preterm birth. The procedure is thought to provide structural support and maintain the endocervical mucus plug as a barrier to ascending infection. Two types of suture material are used for cerclage: monofilament or multifilament braided. Braided sutures are most frequently used, though no evidence exists to favor them over monofilament sutures. In this study we assessed birth outcomes in a retrospective cohort of 678 women receiving cervical cerclage in 5 UK university hospitals and showed that braided cerclage was associated with increased intrauterine death (15% v 5%, $P = 0.0001$) and preterm birth (28% v 17%, $P = 0.0006$) compared to monofilament suture. To understand the potential underlying mechanism, we performed a prospective, longitudinal study of the vaginal microbiome in women at risk of preterm birth because of short cervical length (≤ 25 mm) who received braided ($n=25$) or monofilament ($n=24$) cerclage under otherwise comparable circumstances. Braided suture induced a persistent shift towards vaginal microbiome dysbiosis characterized by reduced *Lactobacillus* spp. and enrichment of pathobionts. Vaginal dysbiosis was associated with inflammatory cytokine and interstitial collagenase excretion into cervicovaginal fluid and premature cervical remodeling. Monofilament suture had comparatively minimal impact upon the vaginal microbiome and its interactions with the host. These data provide in vivo evidence that a dynamic shift of the human vaginal microbiome toward dysbiosis correlates with preterm birth.

Main Text:

Introduction

Each year, preterm birth (PTB, defined as delivery before 37 weeks of gestation) causes over one million deaths worldwide (1). Although preterm birth has multiple etiologies, infection is thought to be a causal mechanism in up to 50% of cases (2). It has been postulated that microbiota may spread hematogenously (3), or ascend from the vagina along mucosal surfaces. During a healthy pregnancy, the uterus and fetus are protected from ascending infection from the vagina by the cervix, which acts as a functional and physical barrier to bacteria and pathogens (4). In the vagina, *Lactobacillus* spp. stability and dominance are central to reproductive health. Pregnancy induces a shift in the vaginal microbiome from a temporally dynamic community structure in non-pregnant women (5) towards stable, *Lactobacillus* spp. dominance that inhibits growth of pathobionts (6, 7). The vaginal microbiome at delivery also acts as an important source of pioneering microbiota for the neonatal gut microbiome, thus implicating it in long-term health outcomes (8, 9). An association between vaginal dysbiosis during pregnancy, characterized by reduced quantities of *Lactobacillus* spp., and preterm birth has long been recognized (2, 10); women diagnosed with bacterial vaginosis have a 2- and 6-fold increased risk of preterm birth and late miscarriage, respectively (10). Recent analyses of the vaginal microbiome in pregnancy using culture-independent methods lend further support to associate vaginal dysbiosis and preterm birth (11).

Cervical cerclage (12, 13) and progesterone supplementation (14) are the only widely used clinical strategies for the prevention of preterm birth, with an estimated two million cerclage procedures performed annually (15). Cervical cerclage reduces the risk of preterm birth by approximately 20% in women with a history of spontaneous preterm birth and/or a short cervical length (16, 17), and its use in these circumstances is recommended by both the

American and the UK Royal College of Obstetricians and Gynecologists (12, 13). Its mechanisms of action are uncertain, but it is thought to provide mechanical support to a weakened cervix (18) as well as to support the cervical mucosal plug as a barrier to ascending infection (4). However, cervical cerclage is associated with increased risk of infection (17, 19). The procedure involves placing a purse-string stitch around the cervix with or without a dissection of the bladder away from the cervix although there is no evidence for a benefit of bladder dissection (20). Two different suture materials, braided or monofilament, are used for the procedure, with braided preferred by 80% of surgeons without an evidence base (21, 22). Braided suture is composed of non-absorbable polyester ethylene terephthalate fibers braided together to form a 5 mm wide mesh tape. The tape is characteristically high in tensile strength and is thought to provide a secure structural support to a weakened cervix because of its high coefficient of friction (18). Monofilament sutures are made of a single strand of non-absorbable polyamide polymer and because of their simple structure, they provide less mechanical resistance when passed through tissue. As a result, they have a tendency to slip and therefore require a greater number of throws to secure the knot than a braided suture (18), which is why braided suture is usually preferred.

However, an association between braided suture use and increased risk of infection in other disciplines (23, 24) has informed the hypothesis that pregnancy outcome after cervical cerclage may be influenced by suture material (15). Aiming to assess the impact of cerclage suture on vaginal microbiota, we hypothesized that the braided suture material promotes pathobiont colonization of the vagina, resulting in activation of inflammatory parturition pathways and premature cervical ripening. In this study we have undertaken a retrospective analysis of pregnancy outcomes in women receiving a clinically indicated cervical cerclage over a ten-year period across five university hospitals in England (UK). After this, a

prospective cohort of women at risk of preterm birth were randomized to either braided or monofilament suture material. Longitudinal profiling of their vaginal microbiome in the context of cerclage insertion was undertaken using 16S rRNA gene sequencing. Cytokine expression profiling of matched cervico-vaginal fluid samples and cervical vascular assessment by 4-D ultrasound were concurrently performed as measures of physiological responses to cerclage insertion and changing microbial composition.

1 Results

Retrospective assessment of suture materials' effects on pregnancy outcomes

A total of 671 women receiving cervical cerclage during pregnancy were identified from five UK university hospitals within a 10-year period. Of these, 327 (49%) received a braided suture material and 344 (51%) received monofilament suture for their cervical cerclage. In women receiving a braided cerclage, higher rates of non-viable births (delivery <24 weeks or intrauterine death) were observed compared to those receiving the monofilament alternative (15% vs 5% respectively; $P < 0.0001$, Fig 1A). Increased rates of preterm birth (24-37 weeks gestation) were also observed in women receiving braided cerclage (28% braided vs 17% monofilament; $P < 0.0001$, Fisher's exact test, Fig 1A). Comparison of available demographics demonstrated that consistent with known clinical practice (21, 22), preference of suture material varied across hospital sites (Table S1). Linear mixed effects modeling excluding hospital location, which is linked to choice of suture material, demonstrated that although history of a previous preterm birth was a significant contributor to non-viable births, braided suture was the primary driver of the observed outcome independent of potential confounders including maternal age, ethnicity, and parity (Fig. S1) but not hospital location. The effect of previous preterm birth was lost when hospital location was included in the model (Table S2). In both analyses, suture material was the major variable influencing the risk of preterm birth independent of maternal age, ethnicity, parity, and history of a previous preterm birth.

Data on gestational age at cerclage insertion and corresponding cervical length were available for women receiving an ultrasound-indicated cerclage (for CL ≤ 25 mm). Distribution of cervical length was comparable among monofilament and braided groups ($P = 0.2$; Mann-Whitney, Table S1), and as would be expected, a shorter cervix significantly contributed to the risk of adverse outcome ($P = 0.019$). A sub-analysis using linear mixed effects regression

models demonstrated that suture material remained a significant contributing factor for both preterm birth ($P = 0.00002$) and non-viable pregnancy ($P = 0.006$) among ultrasound-indicated cerclages after adjusting for potential confounders including gestational age at insertion (Table S2).

Baseline characteristics of the prospective study subjects

Women who were attending prematurity surveillance clinic after a history of preterm birth and were sonographically identified as having a short cervix (≤ 25 mm) were prospectively recruited and randomized to receive a cerclage using either braided Mersilene ($n=25$) or monofilament Ethilon ($n=24$) suture material. Demographics among suture material groups, including gestation and cervical length at cerclage insertion were comparable (Table 1).

Suture material impact on the vaginal microbiome

The data set consisted of 2,792,842 high quality gene sequences, with a mean sequence read count of 13,825 per sample (range 689 to 1,396,421). Using bacterial genera sequence data, samples were classified according to their vaginal bacterial communities as normal ($>90\%$ *Lactobacillus* spp.), intermediate (30-90% *Lactobacillus* spp.), or dysbiotic ($<30\%$ *Lactobacillus* spp.) (Fig. 1B and Table S3). Before cerclage insertion, prevalence of intermediate and dysbiotic microbiomes in monofilament and braided patient groups was similar (16.7% v 17.3%, $P = 0.7$, Fig. 1C). These were higher than the rates of dysbiosis observed in the background low-risk pregnant population at the same gestational age not requiring intervention who had normal pregnancy outcomes (6%, Fisher's exact, $P = 0.03$) (6).

Insertion of the braided cerclage caused a dramatic shift towards dysbiosis at 4 weeks after the procedure, which persisted until the final follow up timepoint, at 16 weeks after cerclage

insertion ($P = 0.008$, ANOVA, Fig. 1C, Table S4). When assessed at the species level (Fig. S3, Fig. S4, and Tables S4 and S5), braided cerclage insertion was associated with an increasing proportion of women with community state type (CST) IV, characterized by reduced numbers of *Lactobacillus* spp. and increased diversity of anaerobic bacteria (6, 25). Before the insertion of braided cerclage, 13% were classified as CST IV, increasing to 45% at 4 weeks and 50% at 16 weeks after cerclage ($P = 0.02$, Fig. S4A, Table S4). In contrast, microbial disruption was not observed in women receiving monofilament cerclage, who instead demonstrated maintenance of high *Lactobacillus* spp. abundance (CSTs I, II, III, and V) and stability throughout longitudinal sampling ($P = 0.9$; Fig. S4A and Table S4). To identify degrees of dysbiosis that were not identified by CST classification, we also undertook alternate species level classification based upon dominance of vaginal bacterial communities by *Lactobacillus* species associated with stability and health, *L. iners* dominance associated more frequently with transition to dysbiosis, as well as intermediate and severe dysbiosis (26). Although *L. iners* has previously been observed as an intermediary towards dysbiosis, there was no significant change in *L. iners* abundance in association with insertion of a monofilament or braided suture material (Fig. S4B and Table S5).

An in vitro adhesion assay showed that braided suture cultured with the vaginal commensal *L. jensenii* or the pathobiont *E. coli* resulted in a 16- and 20-fold greater bacterial load adherence per unit length (cm), respectively, compared to monofilament suture ($P = 0.03$ and $P = 0.0003$, respectively, Student's t-test; Fig. S5).

To identify bacteria specifically associated with braided suture insertion, we performed linear discriminant analysis with effect size (LEfSe)(27) on the 16S rRNA sequence data collected before and 4 weeks after the procedure. Although no differences were identified between

patient groups before insertion, braided cerclage resulted in enriched numbers of Gram-negative bacteria at 4 weeks (Fig. 2A and B). This correlated with a 5-fold increase in the number of dysbiotic samples collected after braided cerclage insertion compared to monofilament cerclage ($P = 0.04$, ANOVA; Fig. 2C). Use of braided cerclage was characterized by increased numbers of bacteria associated with bacterial vaginosis, including species of *Prevotella* ($P = 0.02$), *Finegoldia* ($P = 0.02$), and *Dialister* ($P = 0.04$), and reduced *Lactobacillus* spp. (Fig. 2A, B and Fig. S6). Targeted qPCR suggested that insertion of braided suture was associated with an increase in mean copy numbers of both *A. vaginae* (594,326 before cerclage v 5,081,000 after cerclage; $P = 0.07$) and *G. vaginalis* (961,805 before cerclage v 10,170,000 after cerclage; $P = 0.05$; Fig. S7, Table S6). In contrast, no change in the amount of *G. vaginalis* or *A. vaginae* was detected after monofilament cerclage. Consistent with these observations, indices of bacterial community richness (Fig. 2D) and alpha-diversity (Fig. 2E) were increased in samples collected after braided suture compared to monofilament, with the greatest differences observed at 16 weeks after cerclage ($P = 0.02$; ANOVA, Bonferroni multiple comparison).

Inflammatory response to cerclage insertion

Insertion of braided, but not monofilament cerclage, increased the release of inflammatory cytokines into cervico-vaginal fluid, including IL-1 β , IL-6, IL-8, TNF α , and MMP-1 (Fig. 3A-F). No change was detected in anti-inflammatory cytokines IL-4 (Fig. 3G) IL-2, or IL-10 (Table S7). We observed a strong association between severe dysbiosis (<30% *Lactobacillus* spp.) and increased cervico-vaginal fluid concentrations of pro-inflammatory cytokines ICAM-1, IL-1 β , IL-6, MMP-1, MCP-1, TNF- α , GM-CSF, and IFN- γ , as well as the anti-inflammatory cytokine IL-10 when compared to women harboring *Lactobacillus* spp. dominated microbiomes (Fig. 3H).

Impact on cervical vasculature after cerclage

We constructed three-dimensional cervical vascular trees from ultrasound data and assessed the indices of cervical vasculature (Vascularity index, VI) (28) for morphological differences in the cervix according to suture material. The vascularity was not significantly different between the two groups before cerclage insertion (Fig. 4A). Braided cerclage was strongly associated with increased cervical vascularization 4 weeks after the procedure, and this relationship persisted until 16 weeks after insertion ($P = 0.0003$; ANOVA, Bonferroni multiple comparison, Fig. 4A). A positive correlation of cervical vascularity with both the number of bacterial species (Fig. 4B, $R^2 = 0.09$, $P = 0.002$) and alpha diversity (Fig. 4C, $R^2 = 0.14$, $P = 0.001$) was observed in women receiving a braided cerclage. No relationship between monofilament suture, cervical vascularization, and indices of microbial diversity or richness was observed.

Discussion

Braided suture material is commonly used in preference to monofilament for cervical cerclage (21), because it is assumed that braided suture provides a more secure cerclage that is less likely to slip or tear the cervix (18), however this is not evidence based. Here we show that use of braided suture material is associated with an increased risk of preterm birth and non-viable pregnancy, although this will need to be confirmed in a prospective randomized study. Braided cervical cerclage induces vaginal dysbiosis, increases excretion of inflammatory cytokines into the cervico-vaginal fluid, and induces premature cervical vascular remodeling. In contrast, monofilament suture has minimal impact upon the vaginal microbiome and inflammatory pathways associated with premature onset of parturition. These findings have clinical relevance for cerclage procedures in pregnancy and wider implications for braided suture use in other surgical procedures, particularly in potentially contaminated sites. Based upon an approximated two million cervical cerclages per annum (15), 80% of which are performed using braided suture (21), we estimate that a global shift to monofilament suture use for cervical cerclage would prevent 170,000 preterm births (number needed to treat NNT 9.4; 95% CI 5.9 to 22.6) and 172,000 fetal losses (NNT, 9.3; 95% CI 6.6 to 16.0) per annum world-wide.

Although cervical cerclage is effective in preventing preterm birth in singleton pregnancies with a previous preterm birth (17, 29), our data show that braided cervical cerclage increases vaginal dysbiosis and inflammation, and likely accounts for the doubled risk of puerperal sepsis (19) with no improvement in neonatal outcome (17) after cerclage insertion. Moreover, increasing evidence suggests that the braided cerclage is of no benefit, and may even be detrimental in other groups that are at high risk for preterm birth, such as multiple pregnancies (30) and women with a shortened cervix after excisional treatment for cervical intra-epithelial neoplasia (31-34). The role of suture material in cerclage efficacy has largely

been neglected, with studies rarely detailing the suture material used for the procedure (35). To date, only one randomized controlled trial has been conducted to examine the impact of suture material on pregnancy outcomes, but this study was limited to the comparison of two braided materials, and no differences in preterm birth rates were observed (22). Re-evaluation of existing literature on cervical cerclage use in pregnancy for the prevention of preterm birth is required in light of our findings.

Our study is limited by the retrospective nature of the comparisons of pregnancy outcome. However, the cohort size is large, and preterm delivery rates for each suture material were similar at each of the five centers. The size of the prospective study was limited by practicalities and cost of intensive multiple investigations; however, the experimental data provide supporting evidence for the mechanism of poorer outcomes in patients treated with braided suture cerclage. Analysis of the vaginal microbiome was undertaken using three alternate approaches. We primarily used a genera based classification of normal, intermediate and severe dysbiosis, which demonstrated that the principal effect of braided cerclage is to reduce lactobacillus numbers and induce vaginal dysbiosis. We next classified samples at species level into 5 previously described CSTs, however, this analysis was limited to the consideration of only one dysbiotic group. Therefore an alternate species level classification considering two levels of dysbiosis (intermediate and severe) was also undertaken.

The inherent capacity of braided suture material to facilitate bacterial growth has been previously described in other surgical arenas (36, 37); however, our in vivo and in vitro data provide evidence for preferential pathobiont colonization over commensal vaginal species that are important for reproductive health outcomes. Vaginal dysbiosis associated with braided cerclage insertion was characterized by reduced *Lactobacillus* species and increased diversity and enrichment of bacteria associated with poor pregnancy outcomes, including *Peptoniphilus harei* (38), species of *Bacteriodes* (38, 39), *Prevotella* (40), and *Clostridium*

(41). However, it is recognized that sequencing of specific hypervariable regions of the 16S rRNA gene can result in underrepresentation of key vaginal microbiota such as *G. vaginalis*, which together with *A. vaginae* is characteristic of bacterial vaginosis (BV) (42, 43), a condition associated with adverse reproductive health outcomes including pelvic inflammatory disease (44), HIV transmission (45), and preterm birth(46). Using targeted qPCR, we showed that dysbiosis associated with braided cerclage insertion may involve increased abundance of *G. vaginalis*. The virulence of *G. vaginalis* is thought to relate to its biofilm-producing capacity and adherence to vaginal epithelial cells (42, 47). It is possible that such characteristics may promote biofilm formation on the surface of cerclage suture material, and this should be examined in future studies. Other bacteria clinically associated with adverse pregnancy outcome include *S. agalactiae* (Group B streptococcus) and *E. coli*. However, we did not observe any changes in the amounts of *S. agalactiae*. *E. coli* was not detected in our data set, but this may reflect a limitation of the primer set used for 16S rRNA sequencing (48, 49).

Increased bacterial diversity in the vagina corresponded to the induction of a pro-inflammatory cytokine profile in cervico-vaginal fluid involving known mediators of cervical vascular remodeling, including IL-1 β , IL-6, IL-8, and TNF α (50-52). Concentrations of MMP-1, a matrix metalloproteinase central to collagenous remodeling preceding preterm birth (53, 54), were also increased after insertion of braided cerclage. Increased cervical vascularity occurs before term parturition (28, 55), and an association between increased cytokine excretion and cervical angiogenesis, vasodilation, and vascular permeability has been previously described (56). Consistent with a role in untimely cervical remodeling preceding preterm birth, increased cervical vascularity was observed as early as 4 weeks after insertion in women receiving a braided cerclage. Our study therefore provides a human model for understanding how pregnant host-vaginal microbial interactions may underpin poor

pregnancy outcomes. Cerclage-induced inflammation resulting in premature weakening of the cervix could also provide a mechanism for high rates of intrauterine death in women receiving a braided cerclage, because in-utero exposure of the fetus to elevated concentrations of circulating pro-inflammatory cytokines is known to associate with fetal brain injury (57, 58) and stillbirth (59-61).

In summary, our data provide evidence that cervical cerclage using braided suture associates with increased rates of preterm birth and non-viable pregnancy. Promotion of vaginal bacterial dysbiosis after insertion of braided suture material likely contributes to these adverse pregnancy outcomes through activation of local tissue inflammation and premature cervical remodeling. Because monofilament suture has minimal impact on the host microbiome or inflammation in pregnancy and associates with improved pregnancy outcome, we advocate its use for cervical cerclage. Further clinical trials addressing the impact of cerclage suture material, powered to assess outcomes of preterm birth, neonatal morbidity, and mortality, are therefore urgently required.

Materials and Methods

The study was approved by NHS National Research Ethics Service (NRES) Committee London - City & East (REC 12/LO/2003), and all participants provided written informed consent.

Study design

We initially performed a retrospective data collection to assess outcomes of cervical cerclages in singleton pregnancies considered at risk of preterm birth over a ten-year period between January 2003 and 2013 across five UK hospitals in London, Cambridge, and Birmingham. Cases were identified from operating theatre logs, and all case notes were reviewed where possible. Details regarding cerclage suture insertion, suture material used, outcomes of preterm birth (between 16⁺⁰ and 36⁺⁶ weeks' gestation), and non-viable birth (still birth or miscarriage >16⁺⁰ weeks' gestation) were collected. Other metadata collected included maternal age, parity, previous spontaneous preterm birth / midtrimester miscarriage, indication for cerclage (ultrasound indicated or elective), and cervical length at cerclage insertion.

After this, we prospectively recruited pregnant women at risk of preterm birth with sonographic indications for cervical cerclage at preterm surveillance clinics from January 2013 until August 2014 at a single London site (Queen Charlotte's and Chelsea Hospital). Inclusion criteria were pregnant women with history of spontaneous preterm birth (<37⁺⁰ weeks) and a cervical length (CL) measurement below the 10th centile (≤ 25 mm) on transvaginal scan at $\leq 23^{+6}$ weeks' gestation in the index pregnancy. A normally distributed cervical length range not associated with preterm birth risk is typically 35 mm \pm 8.3 mm (mean \pm SD) (62).

Exclusion criteria included multiple pregnancy, previous iatrogenic preterm births, HIV positive status, and sexual intercourse or vaginal bleeding in the preceding 48 hours. Eligible

women were randomized to either braided Mersilene (n=25) or monofilament Ethilon (n=24) cerclage suture material. The same obstetrician performed the procedure using the MacDonald technique (63). Participants were recruited before cerclage insertion and followed up longitudinally at 4, 8, 12, and 16 weeks after insertion. At each time point, cervico-vaginal fluid was sampled from the posterior fornix under direct visualization using 2 swabs for later 16S rRNA gene sequencing and cytokine analysis: a BBL CultureSwab MaxV Liquid Amies swab (Becton, Dickinson and Company) and a Transwab MW170 with rayon bud type (Medical Wire & Equipment), respectively. Both swabs were immediately placed on ice and snap frozen at -80°C. A transvaginal ultrasound scan was then immediately performed to assess cervical vascularization in the dorsal lithotomy position with an empty bladder, taking care to avoid undue pressure on the cervix.

DNA extraction and 16S rRNA sequencing

DNA extraction from the BBL CultureSwab was performed as previously described (6). Integrity of the extracted bacterial DNA was confirmed by PCR amplification using universal forward and reverse primers (6). The V1-V3 hypervariable regions of 16S rRNA genes were amplified for sequencing using a forward and reverse fusion primer. The forward primer was constructed with (5'-3') the Illumina i5 adapter (AATGATACGGCGACCACCGAGATCTACAC), an 8 bp barcode, a primer pad (forward: TATGGTAATT), and the 28F-GAGTTTGATCNTGGCTCAG primer (64). The reverse fusion primer consisted of (5'-3') the Illumina i7 adapter (CAAGCAGAAGACGGCATACGAGAT), an 8 bp barcode, a primer pad (reverse: AGTCAGTCAG), and the reverse primer (519R-GTNTTACNGCGGCKGCTG). Sequencing was performed on an Illumina MiSeq platform (Illumina, Inc.) at Research and Testing Laboratory (Lubbock, TX, USA). Resulting sequence reads were analyzed using the MiSeq SOP Pipeline of the Mothur package (65), which is designed to analyze a multiplexed set of

samples. Sequence alignment was performed using the Silva bacterial database (www.arb-silva.de/), and classification of sequences was undertaken using the RDP database reference sequence files and the Wang method (66). OTU taxonomies (phylum to genus) were determined using the RDP MultiClassifier script. Species level taxonomies were determined using USEARCH with 16S rRNA gene sequences from the cultured representatives from the RDP database (67). Sequence alignment data describing the capacity of the V1-V3 amplicons to discriminate *Lactobacillus* spp. are provided in Table S8 and Fig. S8. Data were re-sampled and normalized to the lowest read count in Mothur (n=689).

Quantitative PCR

Targeted quantitative PCR was carried out to detect 16S rRNA genes from *Atopobium vaginae* and *Gardnerella vaginalis*. The assays were SYBR green based and performed on Applied Biosystem's StepOnePlus. PCR reaction mixes are as follows: 1x SYBR Green Jumpstart *Taq* Ready Mix (Sigma-Aldrich), 5 µl of bacterial DNA isolated from the vaginal swabs, and 0.8 µM final concentration of forward and reverse primers. Oligonucleotide primers used for *A. vaginae* were: forward- 5'-TAGGCGGTTTGTAGGTCAGGA-3'; reverse- 5'-CCTACCAGACTCAAGCCTGC-3' (68) and for *G. vaginalis*; forward- 5'-GGAAACGGGTGGTAATGCTGG-3'; reverse- 5'-CGAAGCCTAGGTGGGCCATT-3') (69). Thermocycle profile was 95°C for 2 minutes, followed by 40 cycles at 95°C for 15 sec and 65 °C for 1 min. For both assays, vaginal samples and corresponding standards (*A. vaginae* and *G. vaginalis* DNA) were run in duplicates, and mean numbers were used to calculate 16S rRNA gene copies per 5 µl of vaginal DNA.

In vitro adhesion assay

Propensity of bacteria to adhere to braided and monofilament suture material was assessed using an in vitro adhesion assay (70). Briefly, 1 cm segments of sterile braided Mersilene or monofilament Ethilon were prepared with a sterile blade and tweezers. Suture fragments were

placed into a sterile screw-capped tube and incubated with 1 ml of 100% ethanol for 1 hour at room temperature. Fragments were washed 3x using 1 mL of sterile water before inoculation with *E. coli* (Nissle 1917) using LB broth and LB plates or *Lactobacillus jensenii* (Cultech Ltd.) using MRS broth and MRS plates.

For each bacterial isolate we tested the following groups: inoculated Mersilene thread fragments (n=3), uninoculated Mersilene thread fragment (sterility control, n=1), inoculated Ethilon thread fragments (n=3), uninoculated Ethilon thread fragment (sterility control, n=1). An overnight bacterial culture was centrifuged at 3,000 x g for 10 min, and the supernatant was discarded. Cell pellets were resuspended in fresh broth to a final concentration of 10^7 CFU/mL and 1 mL of this cell suspension was added to each of the suture fragments. For sterility controls, 1 mL of sterile broth was added to an uninoculated Mersilene and Ethilon thread fragment. Suture thread fragments were incubated at 37°C for 24 h before being washed 3x with sterile PBS and transferred into tubes containing 1 ml of sterile PBS. Each tube was vortexed 3 x for 30 seconds to detach bacterial cells. The cell suspension was vigorously passed through a 25G needle 10 times to break up cell clumps. Aliquots of 100 μ L were collected from the cell suspension and used for colony counts on LB or MRS agar plates. After incubation, we used plate colony counts to calculate the CFU/mL of cell suspension. Thread fragment length was accurately measured after cell suspension plating to calculate CFU/cm.

Cytokine Analysis

The transwab cervico-vaginal fluid samples were thawed on ice and resuspended in 350 μ L of phosphate-buffered saline solution with protease inhibitor (5 μ L/ml; Sigma Aldrich). The suspension was centrifuged at 3000 x g for 2 min and the supernatant collected into a new microcentrifuge tube before repeating the centrifugation step to ensure removal of any

cellular debris. Cell-free supernatants were analyzed by Human Magnetic Luminex Screen Assay (15-plex) (Luminex Corporation) with a Bioplex 200 system (Biorad Laboratories Ltd.). Analyte-specific Luminex Screening Assays were performed for 15 analytes: interleukin (IL)-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , regulated on activation normal T expressed and secreted/chemokine ligand 5 (RANTES/CCL5), vascular endothelial growth factor (VEGF), intercellular adhesion molecule 1 (ICAM-1), and matrix metalloproteinase 1 (MMP-1). Analytes were selected according to evidence of involvement in inflammatory change related to preterm birth, cervical ripening, and angiogenesis. Samples were analyzed on 96-well plates at two dilutions (1:1 and 1:50) optimized for detection of analytes within the range of the standards as specified by Luminex Human premixed analyte kit.

Cervical vascularization assessment

Voluson E ultrasound (GE Healthcare) in 3D/4D mode with medium persistence, high sensitivity, and normal line density was used for transvaginal cervical vascular assessment. A sagittal plane of volume acquisition, set at 90°, was analyzed using Virtual Organ Computer-aided AnaLysis software program (VOCAL, GE Medical Systems) (28). The “histogram facility” of the software was used calculated the vascularization index (VI) within the defined volume.

Statistical analysis

Assessment of differences in outcomes of viability and preterm birth between cerclage suture material groups (braided versus monofilament) was performed using the Fisher exact test for categorical variables and Mann-Whitney for continuous variables. We used a linear mixed-

effects model incorporating suture material group, maternal age, parity, previous preterm birth, and hospital location as fixed effects and ethnicity (Asian, Black, or Caucasian) as a random effect to compare braided versus monofilament suture material for the two primary outcomes (viability and preterm birth). The contributions of fixed-effects terms (p-value and F statistics) were calculated using the analysis of variance (ANOVA) with Satterthwaite approximation for degrees of freedom.

Examination of statistical differences between vaginal microbiota were performed at bacterial genera and species levels using the Statistical Analysis of Metagenomic Profiles (STAMP) software package (71). Ward linkage hierarchical clustering analysis (HCA) of bacterial genera was performed using a clustering density threshold of 0.75. Samples were classified according to the percentage of *Lactobacillus* spp. reads as a proportion of the total number of reads per sample into the following groups: normal (>90% *Lactobacillus* spp.), intermediate (30-90% *Lactobacillus* spp.), or dysbiotic (<30% *Lactobacillus* spp.). Bacterial species data were classified into community state types (CSTs) as described by Ravel et al (25): CST I (*L. crispatus*), CST II (*L. gasseri*), CST III (*L. iners*), CST IV (mixed bacterial species), and CST V (*L. jensenii*). To identify potential associations between suture material and differing degrees of dysbiosis, an alternative classification of the species data was performed, as described by Borgdorff et al (26) into communities characterized by healthy *Lactobacillus* spp. dominance, *L. iners*, or moderate or severe dysbiosis.

The effects of suture material and time from cerclage insertion on bacterial genera, number of species observed and alpha diversity were assessed using One-way ANOVA, Kruskal-Wallis, and Dunn's multiple comparisons where appropriate.

Linear discriminant analysis (LDA) effect size (LEfSe) method (27) characterized differentially abundant taxonomic features of the two suture materials before and 4 weeks after cerclage insertion. An alpha value of 0.01 was used for factorial Kruskal-Wallis test

between classes, and a threshold of 3.0 was used for logarithmic LDA score for discriminative features.

The Wilcoxon signed rank test compared cytokine analyte concentrations before and 4 weeks after cerclage insertion. The Mann-Whitney test was used to test for differences among suture material types. Analyte expression was classified according to the corresponding microenvironments (Fig. 1C), and the Mann-Whitney test compared cytokine expression in the presence of a normal or dysbiotic microbiome.

Cervical vascularization was compared according to suture material from the time of cerclage insertion and as a function of the corresponding bacterial classification, using Kruskal-Wallis and ANOVA multiple comparison analyses where appropriate. We used linear regression to assess for correlations between cervical vascularity, the number of observed species, and Shannon index of alpha diversity, according to suture material.

Supplementary information

Figure S1. Linear mixed effects modeling of retrospective outcome data.

Figure S2. Gestation at birth as a function of suture material used in the prospectively recruited cohort.

Figure S3. Ward hierarchical clustering analysis of species sequence data.

Figure S4. Longitudinal assessment of vaginal bacterial community structure following suture insertion.

Figure S5. In vitro adherence assay of suture material with *E. coli* or *L. jensenii*.

Figure S6. Longitudinal comparison of bacterial genera increased following braided suture insertion.

Figure S7. Quantitative PCR assessment of *A. vaginae* and *G. vaginalis* at 4 weeks after cerclage

Figure S8. V1-V3 hypervariable region sequence alignment against major vaginal *Lactobacillus* species

Table S1. Patient demographics for retrospective study cohort.

Table S2. Contributing confounder analysis for non-viable pregnancy and preterm birth <37 weeks.

Table S3. Bacterial genus classification according to time from cerclage

Table S4. Bacterial species classification into community state types according to time from cerclage.

Table S5. Species classification into normal, *L. iners* dominant, intermediate and severe dysbiosis according to time from cerclage.

Table S6. Mean bacterial counts of *Atopobium vaginae* and *Gardnerella vaginalis* before and 4 weeks after cerclage insertion, as assessed by quantitative PCR.

Table S7. Mean fold change in analyte expression detectable in cervico-vaginal fluid before and after cerclage insertion.

Table S8. DNA identity for the V1-V3 region used in the analysis for the 4 main lactobacilli in CST I, II, III, and V.

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Author contributions L.M.K., D.A.M., C.L., P.T-H., M.S., T.G.T. and P.R.B. conceived and designed the retrospective study. L.M.K., D.A.M., T.G.T and P.R.B. conceived and designed the prospective study. Retrospective data collection and collation was performed by L.M.K., V.T., J.R.C., C.L., F.I-B., Y.F., P.T-H., M.S., T.G.T., and P.R.B. Prospective patient enrollment and sample collection and transvaginal scans were undertaken by L.M.K. and J.R.C. Experiments were performed by L.M.K., Y.S.L. and J.A.K.M. Data analysis was performed by L.M.K., D.A.M., J.R.M., A.S., S.C., E.H., J.K.N and P.R.B. All figures and tables were generated by L.M.K., D.A.M., S.C., and J.R.M. The manuscript was written by L.M.K., D.A.M. and P.R.B, and critically reviewed by all authors.

Competing interests: The authors declare that they have no competing interests

Data and materials availability: Public access to sequence data and accompanying metadata can be obtained at the European Nucleotide Archive's (ENA) Sequence Read Archive (SRA) (PRJEB11895).

Figures:

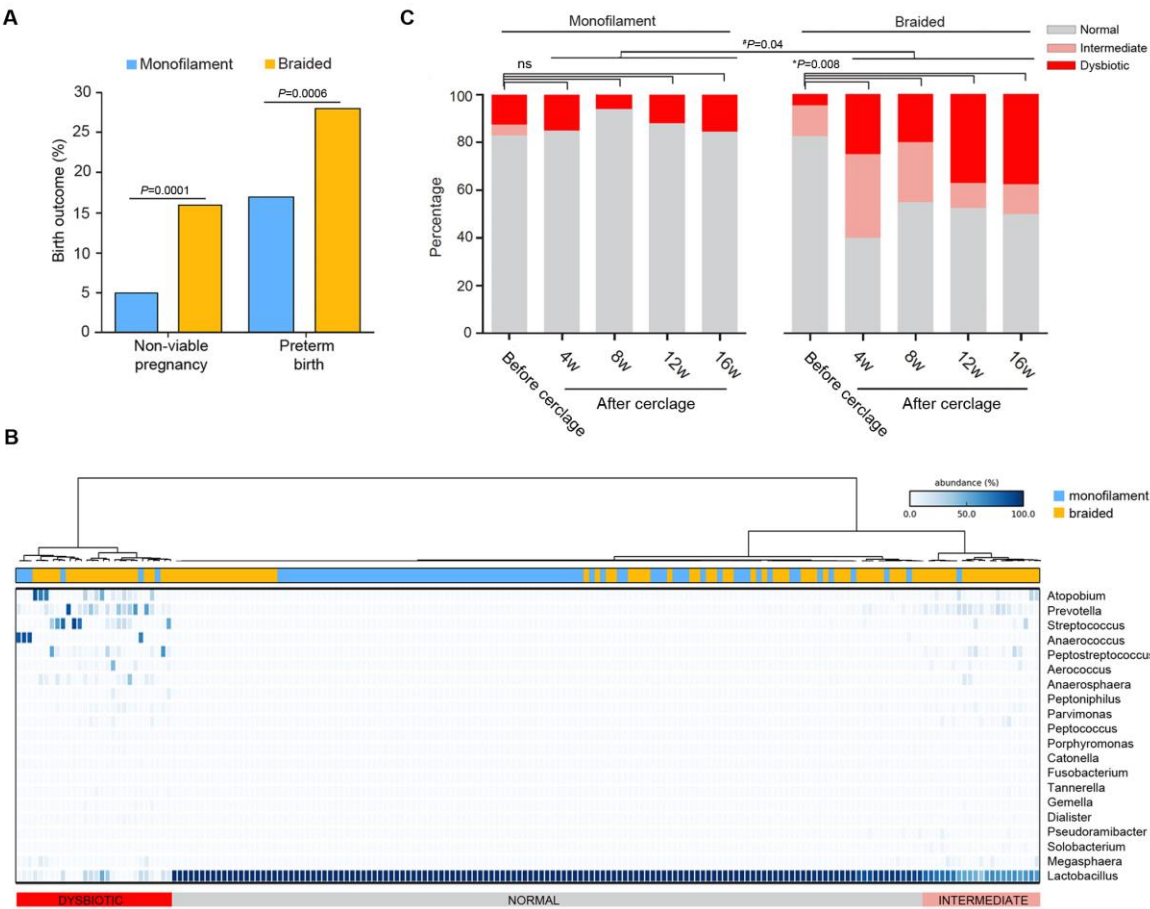


Figure 1. Braided suture material for cervical cerclage is associated with worse outcomes.

(A) Retrospective comparison of 10 years of birth outcomes for women receiving a cerclage based on suture material (monofilament, n=344 vs braided, n= 337) revealed higher rates of non-viable births (delivery <24 weeks or intrauterine death) in women receiving a braided cerclage compared to a monofilament alternative (15% vs 5% respectively; $P = 0.0001$, Fisher's exact test) and increased rates of preterm birth (24-37 weeks gestation) in women receiving braided cerclage (28% braided vs 17% monofilament; $P = 0.0006$, Fisher's exact test) (see Table S3 for details). (B) Ward-linkage analysis of vaginal bacterial genera of cervical vaginal fluid samples (n=197) collected longitudinally before and after insertion of a monofilament (n=24) or braided (n=25) cervical cerclage permitted classification of vaginal bacterial communities into three groups: normal (>90% *Lactobacillus* abundance), intermediate (30-90% *Lactobacillus* abundance), or dysbiotic (<30% *Lactobacillus* abundance). (C) Braided cerclage was associated with a 5-fold increase in microbial dysbiosis within 4 weeks of insertion, which persistent until at least 16 weeks, whereas no change was observed in women receiving a monofilament cerclage (P values = fishers exact before v after cerclage, and 2 way ANOVA: monofilament vs braided at comparable time points).

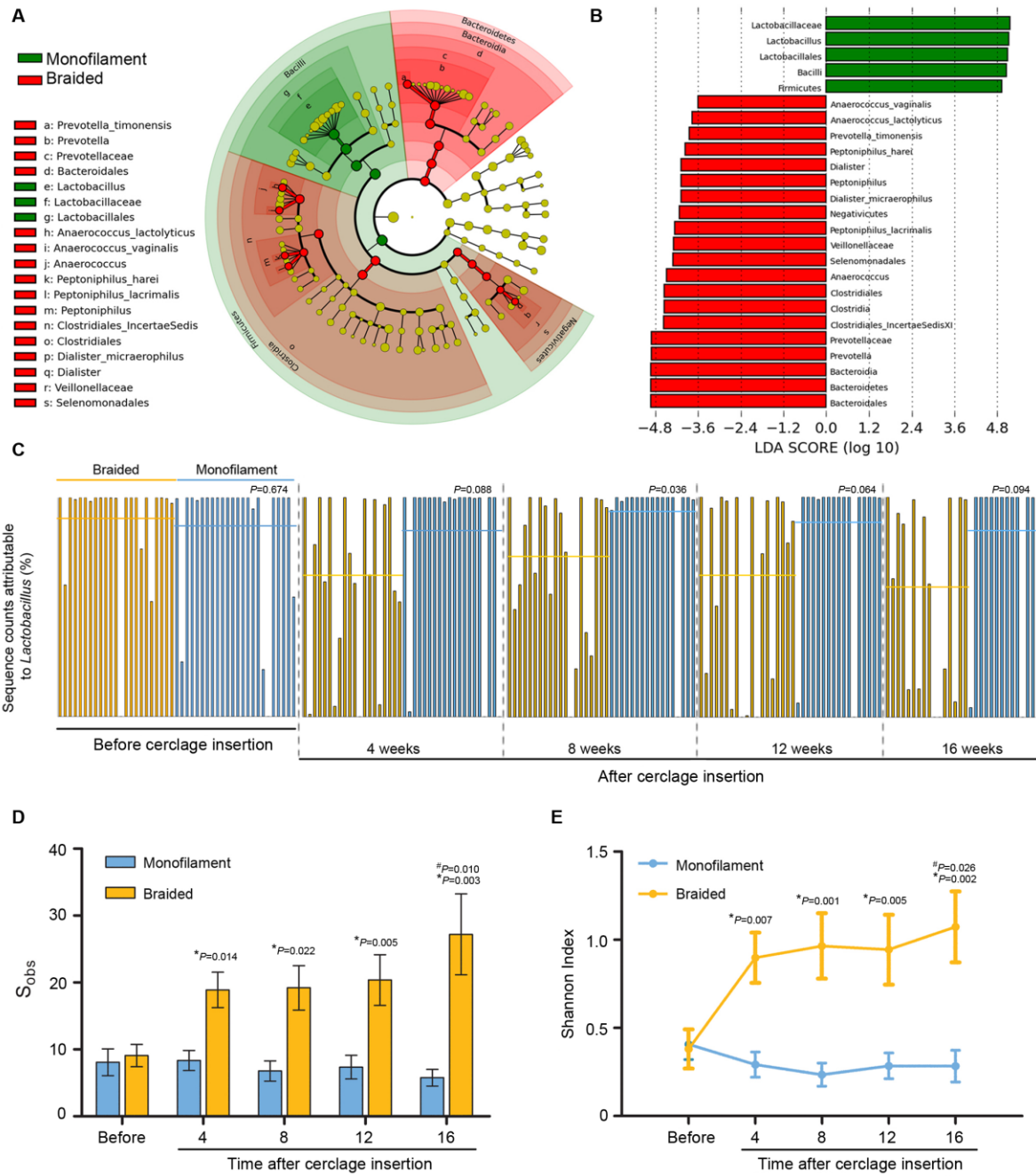
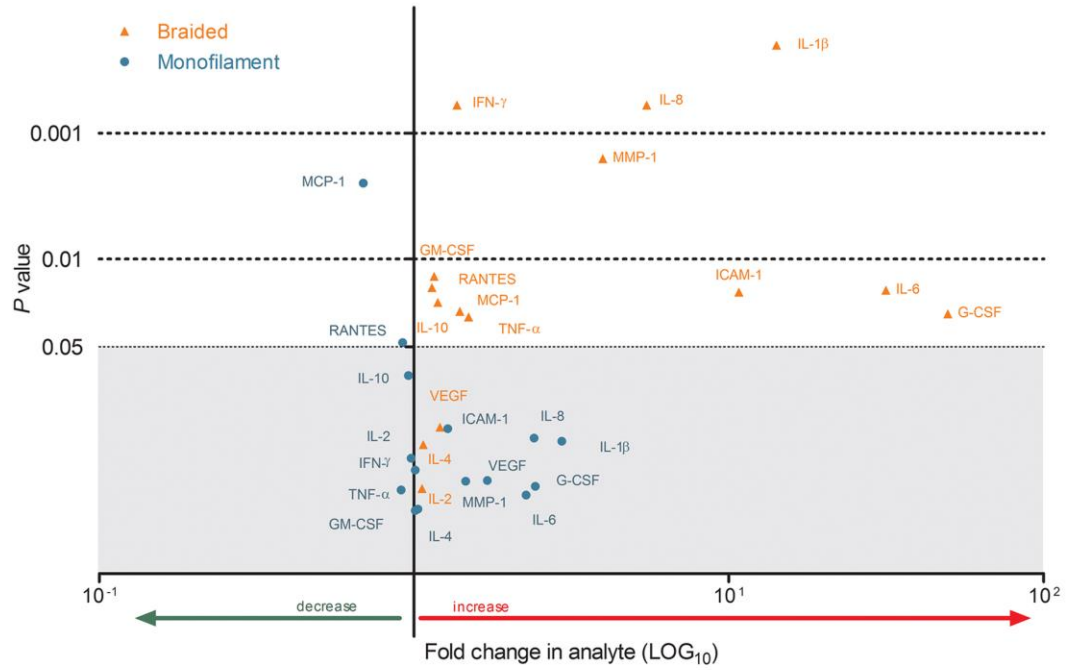
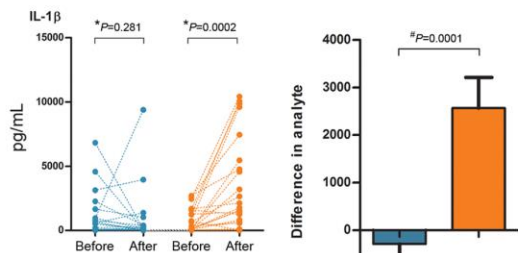


Figure 2. Bacterial taxonomic groups discriminate between monofilament and braided cerclage. (A) Differentially abundant microbial clades and nodes according to suture material four weeks after insertion were identified using LEfSe analysis and presented as a cladogram. (B) Linear Discriminant Analysis (LDA) was used to estimate the effect size for each differentially abundant species. The vaginal microbiome of patients receiving a monofilament cerclage was enriched with bacilli, whereas those receiving a braided cerclage were comparatively enriched in *Bacteroides* spp., and *Clostridia*. (C) Relative abundance bar charts for individual samples highlight maintenance of *Lactobacillus* genus stability after insertion of monofilament cerclage ($P = 0.9$; ANOVA), in contrast to the decreased numbers after braided cerclage ($P = 0.043$; ANOVA) (P values = corrected two-sided Welch's t-test for monofilament vs. braided). (D) Comparison of total number of bacterial species observed reveals an increase after braided cerclage compared to a monofilament cerclage. (E) Alpha diversity was increased at 16 weeks after braided cerclage insertion compared to monofilament. (* P values = corrected two-sided Welch's t-test for monofilament vs. braided, # P values = ANOVA Bonferroni multiple comparison to before-cerclage samples).

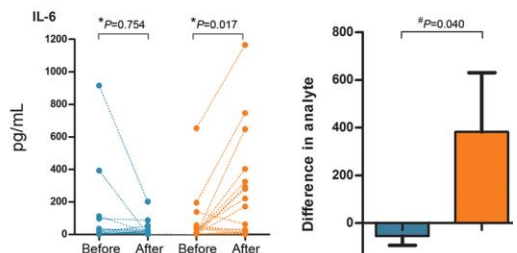
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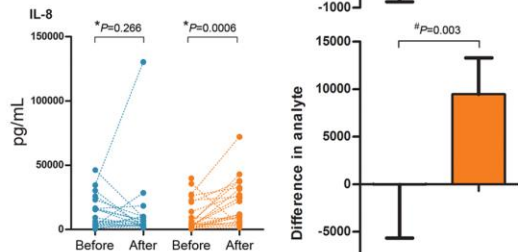
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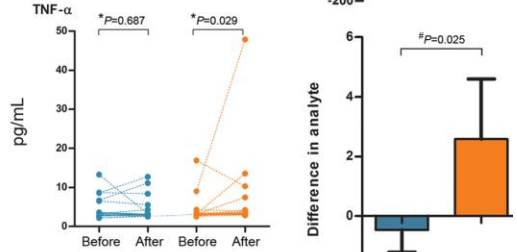
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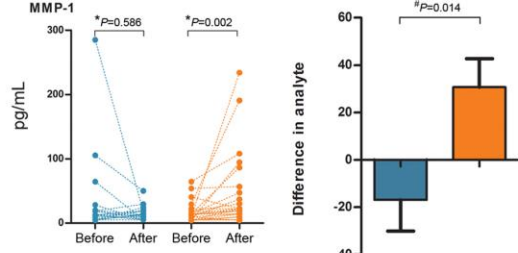
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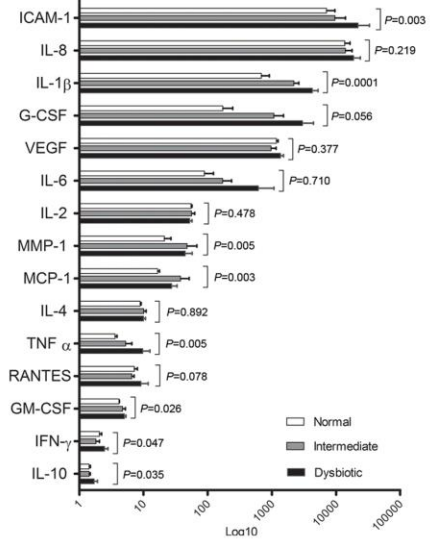
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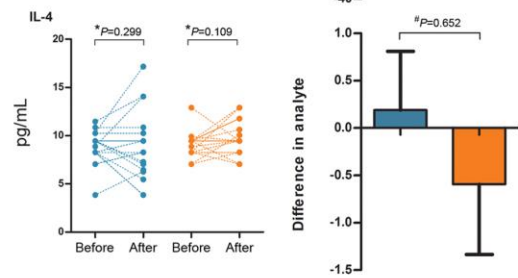


Figure 3. Braided suture induces cytokine release into the cervico-vaginal fluid. (A)

Cytokines were detected using a multiplex assay before and 4 weeks after cerclage insertion. An increase in pro-inflammatory cytokines was detected in the cervico-vaginal fluid after braided cerclage (see Table S7 for details). Relative to the concentrations before cerclage, braided suture was associated with an increase in pro-inflammatory cytokines **(B)** IL-1 β , **(C)** IL-6, **(D)** IL-8, **(E)** TNF- α , and **(F)** MMP-1 but not **(G)** IL-4 (**P* values = Wilcoxon signed rank test for cytokine concentration before and after cerclage). Similar changes were observed when the braided cerclage samples were compared for cytokine concentrations 4 weeks after monofilament cerclage ([#]*P* values = Mann-Whitney for fold change monofilament vs. braided). **(H)** Mean cytokine profiles grouped by the corresponding microbial classification (normal, intermediate, and dysbiotic) revealed that dysbiosis is associated with increased expression of pro-inflammatory cytokines ICAM-1, IL-1 β , IL-6, MMP-1, MCP-1, TNF- α , GM-CSF, and IFN- γ and anti-inflammatory IL-10, but not G-CSF, IL-8, VEGF, RANTES, IL-2 or IL-4. (*P* value = Mann-Whitney for normal vs dysbiotic).

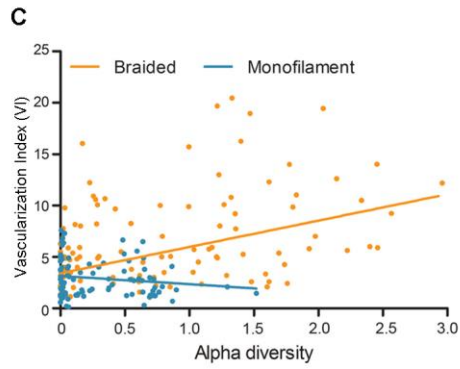
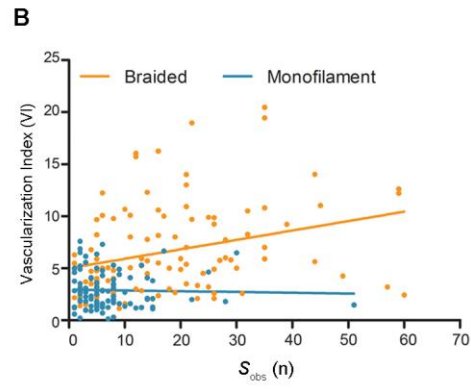
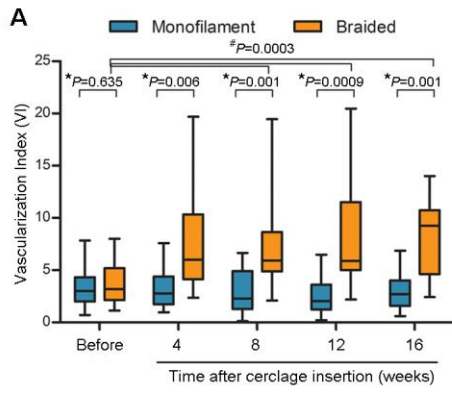


Figure 4. Braided cerclage induces premature cervical vascularization. (A) Cervical vascularization index (VI), as assessed by transvaginal ultrasound, was greater in patients receiving braided cerclage compared to monofilament cerclage at 4, 8, 12, and 16 weeks after insertion (* P value = Welches corrected t-test for monofilament vs braided, # P value = ANOVA, Bonferroni multiple comparison for before vs after cerclage). (B) Linear regression analyses demonstrated a positive correlation between VI and the number of species observed in braided ($R^2=0.09$, $P = 0.002$) but not monofilament ($R^2=0.001$, $P = 0.75$) cerclages. (C) A similar relationship was observed between VI and alpha diversity index (braided: $R^2 = 0.14$, $P = 0.001$ and monofilament: $R^2=0.02$, $P = 0.14$), indicating an interplay between braided suture, increased cervical vascularity, and vaginal microbial dysbiosis.

Tables

Table 1. Patient characteristics for women randomized to receive monofilament and braided cervical cerclages

	Monofilament suture	Braided suture	Total population
n (%)	24/49 (49%)	25/49 (51%)	49/49
Age, Mean \pmSD (range) years	32.8 \pm 3.0 (27-39)	33.9 \pm 3.8 (25-42)	33.5 \pm 3.5 (25-42)
BMI, Mean \pmSD (range)	24.1 \pm 4.2 (18-35)	26 \pm 3.6 (21-36)	25.1 \pm 4.5 (18-36)
Ethnicity, n (%)			
Caucasian	16 (67%)	11 (44%)	27 (55%)
Asian	2 (8%)	7 (28%)	9 (18%)
Black	6 (25%)	7 (28%)	13 (27%)
Parity, n (%)			
Para 0	12 (50%)	13 (52%)	25 (51%)
Smoking, n (%)	1 (4%)	2 (8%)	3 (6%)
<u>Cerclage insertion</u>			
GA at insertion, mean \pmSD w	17 ⁺⁶ \pm 2.8	18 ⁺¹ \pm 3	17 ⁺⁰ \pm 2.9
CL at insertion, mean \pmSD mm	18 \pm 5.1	19 \pm 4.5	19 \pm 5.3
<u>GA at delivery, n (%)</u>			
<34⁺⁰ w	4/24 (16%)	0/25 (0%)	4 (8%)
34⁺¹-36⁺⁶ w	2/24 (8%)	8/25 (32%)	10 (20%)
\geq37⁺⁰ w	18/24 (75%)	17/25 (68%)	35 (71%)

BMI= body mass index; CL = cervical length (mm); GA = gestational age; w= weeks; SD=standard deviation