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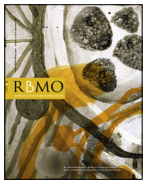
RBMO



ARTICLE

Female reproductive tract microbiota and recurrent pregnancy loss: a nested case-control study

BIOGRAPHY

Pirkko Peuranpää is conducting her PhD studies at the University of Helsinki. Her research focuses on understanding the aetiology of recurrent pregnancy loss. She is a general gynaecologist and reproductive medicine specialist who has cared for patients with infertility at Helsinki and Uusimaa District Hospital, Finland.

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KEY MESSAGE

Lactobacillus crispatus was less abundant, and *Gardnerella vaginalis* more abundant, in endometrial samples from 47 women with recurrent pregnancy loss compared with 39 healthy control women. Dysbiotic endometrial microbiota may be a novel risk factor for recurrent pregnancy loss, a condition that currently often remains unexplained after standard examinations.

ABSTRACT

Research question: Is the composition of the endometrial or vaginal microbiota associated with recurrent pregnancy loss (RPL)?

Design: Endometrial and vaginal samples were collected from 47 women with two or more consecutive pregnancy losses and 39 healthy control women without a history of pregnancy loss, between March 2018 and December 2020 at Helsinki University Hospital, Helsinki, Finland. The compositions of the endometrial and vaginal microbiota, analysed using 16S rRNA gene amplicon sequencing, were compared between the RPL and control women, and between individual vaginal and endometrial samples. The mycobiota composition was analysed using internal transcribed spacer 1 amplicon sequencing for a descriptive summary. The models were adjusted for body mass index, age and parity. False discovery rate-corrected *P*-values (*q*-values) were used to define nominal statistical significance at $q < 0.05$.

Results: *Lactobacillus crispatus* was less abundant in the endometrial samples of women with RPL compared with controls (mean relative abundance 17.2% versus 45.6%, $q = 0.04$). *Gardnerella vaginalis* was more abundant in the RPL group than in controls in both endometrial (12.4% versus 5.8%, $q < 0.001$) and vaginal (8.7% versus 5.7%, $q = 0.002$) samples. The individual vaginal and endometrial microbial compositions correlated strongly ($R = 0.85$, $P < 0.001$). Fungi were detected in 22% of the endometrial and 36% of the vaginal samples.

Conclusions: Dysbiosis of the reproductive tract microbiota is associated with RPL and may represent a novel risk factor for pregnancy losses.

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KEY WORDS

Endometrium
Microbiota
Recurrent miscarriage
Recurrent pregnancy loss
Vagina

INTRODUCTION

Recurrent pregnancy loss (RPL) is one of the most challenging issues in reproductive medicine because its causes are often unknown and effective treatment is rarely available. The European Society of Human Reproduction and Embryology defines RPL as the spontaneous loss of two or more pregnancies (*Bender Atik et al., 2018*), and it affects 1–3% of couples trying to have a child. Recognized causes of RPL are chromosomal abnormalities, uterine malformations, antiphospholipid syndrome and endocrinological disorders (*Rai and Regan, 2006*), but over half of RPL still remains unexplained.

Several studies have suggested an infectious aetiology behind miscarriages (*Giakoumelou et al., 2016*; *McQueen et al., 2015*). Chronic endometritis has been associated with RPL (*McQueen et al., 2021*) and bacterial vaginosis has been linked to the risk of miscarriage (*Haahr et al., 2019*; *Ralph et al., 1999*). Recent studies using high-throughput DNA sequencing techniques have shown that *Lactobacillus* spp. dominate the vaginal bacterial composition in healthy early pregnancy (*Freitas et al., 2017*; *MacIntyre et al., 2015*), while dysbiosis, the reduced prevalence of lactobacilli, especially *Lactobacillus crispatus*, has been associated with pregnancy loss (*Al-Memar et al., 2020*). The mycobiota, consisting of various species of fungi, is another important element of the vaginal ecosystem (*Bradford and Ravel, 2017*). However, there are no studies examining the mycobiota in relation to reproductive outcomes.

The uterine cavity has long been thought to be sterile, but recent studies have reported that the endometrium may have a distinct microbiome (*Chen et al., 2017*; *Mitchell et al., 2015*; *Moreno et al., 2016*). As in the vagina, the dominant species in the endometrium are usually lactobacilli (*Chen et al., 2017*, *Oberle et al., 2021*), and an alteration of this composition may affect reproductive outcomes. In patients undergoing IVF, a non-*Lactobacillus* dominated endometrial microbiota has been associated with lower implantation rates, clinical pregnancy rates and live birth rates compared with a *Lactobacillus*-dominated microbiota (*Moreno et al., 2016*). However, knowledge of the endometrial microbiota in RPL is scarce.

The goal of this study was to explore the composition of the microbiota and mycobiota in endometrial and vaginal samples in women with RPL and compare the results with those of healthy women without a history of miscarriages. The study also investigated whether the composition of the vaginal microbiota reflected the composition of the endometrial microbiota.

MATERIAL AND METHODS

Study population

TOIVE is a prospective cohort study of the immunological and microbiological causes of RPL, conducted at the University of Helsinki and Helsinki University Hospital (HUS), Finland. Between March 2018 and June 2020, the study recruited 51 women referred to the Reproductive Medicine Unit and the Department of Obstetrics and Gynaecology of Hyvinkää Hospital because of RPL (FIGURE 1). Women were eligible if they had a history of three or more consecutive clinical first-trimester pregnancy losses or two losses with at least one in the second trimester, and had no concomitant infertility. Clinical pregnancy loss was defined as a spontaneous loss of an intrauterine pregnancy confirmed by ultrasonography or a positive urine or serum human chorionic gonadotrophin test, and over 6 weeks of amenorrhoea.

As controls, 40 women investigated for male factor infertility between June 2018 and December 2020 were recruited. They represented healthy Finnish women as they had no history of pregnancy loss, endometriosis, anovulation or Fallopian tube defects. Participants were excluded if they were <18 or ≥40 years of age, had hepatitis or HIV infection, or had an irregular menstrual cycle (<21 or >42 days).

Power calculations were not applicable to this associative and novel setting as earlier studies on microbiota composition and RPL were lacking. The ethics committee of HUS approved the study (no. HUS/3635/2017; date of approval 17 January 2018).

Collection of clinical data

All participants gave their written informed consent. Upon enrolment, a detailed medical and reproductive history was taken, and participants received a questionnaire related to previous infections, the use of anti- or probiotics, sexual behaviour and educational background. The basic examinations for RPL included transvaginal 2D-ultrasonography, a complete blood count, phospholipid antibodies, karyotyping (of both partners), thyroid function tests, thyroid peroxidase antibodies, fasting glucose and glycated haemoglobin. Screening for thrombophilia, coeliac disease, hyperprolactinaemia and uterine anomalies with 3D-ultrasonography or hysteroscopy was performed when necessary. Women were not routinely tested for sexually transmitted diseases or chronic endometritis.

Endometrial and vaginal sampling

Vaginal and endometrial samples were collected in the mid-luteal phase, 6–8 days after a positive ovulation test (Clearblue Digital; Swiss Precision Diagnostics, Switzerland). Couples were advised to use a condom or abstain from sexual intercourse during the menstrual cycle in which the samples were taken, to ensure contraception and to avoid the effects of seminal fluid on the microbiota. Samples from the control women were collected during a natural menstrual cycle preceding the woman's first IVF/intracytoplasmic sperm injection stimulation; women with RPL were not undergoing active fertility treatment.

Vaginal samples were collected during a speculum examination from the right and left fornices with sterile flocked swabs (FLOQSwabs; Copan, Italy) and severed to 1.5 ml Eppendorf tubes. The researchers used non-sterile examination gloves and a white coat during sampling, but not a mask or hair cover. Lubricants were not used. Endometrial samples were collected in 1.5 ml Eppendorf tubes using an endometrial biopsy curette (Pipelle; Prodimed, France), inserted gently through the cervix into the uterine cavity, without contact with the vaginal walls but unprotected when passing through the cervical canal. In the uterine cavity, a vacuum was created by retracting the internal piston, and the sample was collected by rotating the device and gently moving it back and forth. The Eppendorf tubes were frozen at −20°C immediately after sampling, and moved to −80 °C within 2 weeks.

DNA extraction

Microbial DNA was extracted from the vaginal samples using a beat beating method as previously described

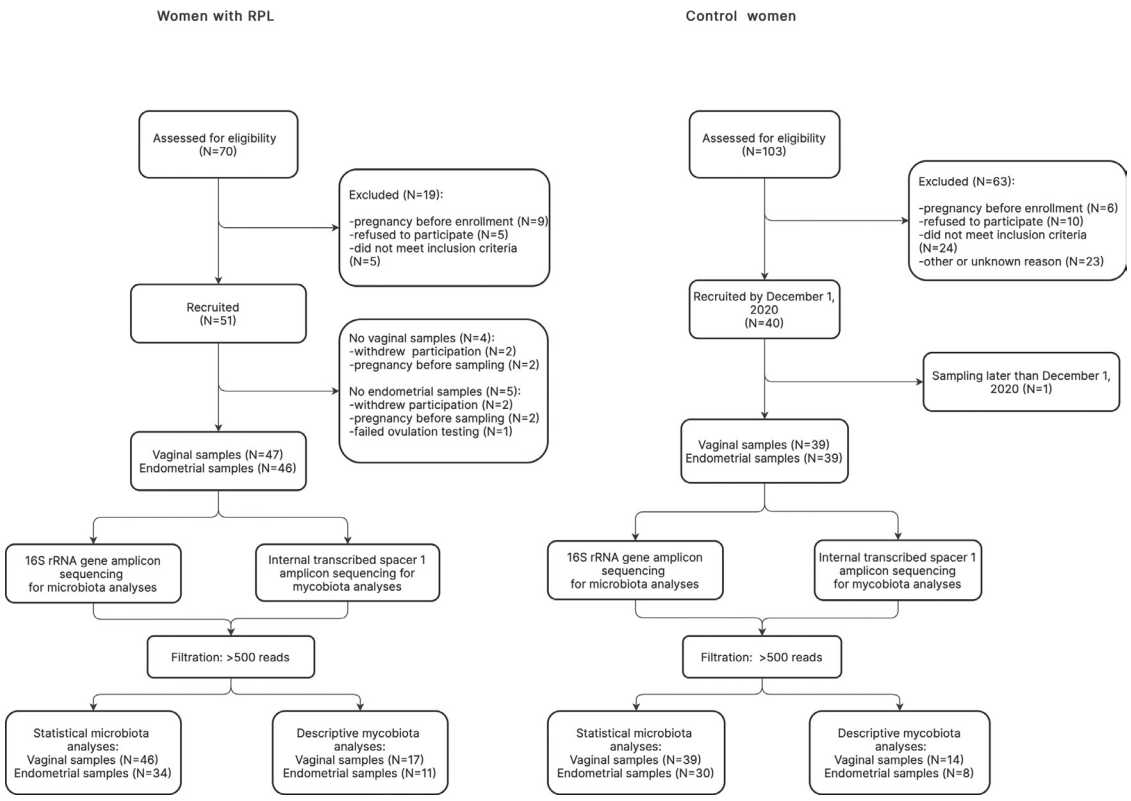

FIGURE 1 Flow chart describing the study design. RPL, recurrent pregnancy loss.

(*Virtanen et al., 2019*). One-sixth of the endometrial biopsies (average weight 33 mg; SD 22 mg) were used for DNA extraction, with negative controls using the same method as for the vaginal samples. DNA was quantified using a Quanti-iT PicoGreen dsDNA Assay (Invitrogen, USA). The DNA yields for both sample types were comparable, with a mean of 78.8 ng/μl (SD 60 ng/μl) for the vaginal and 43.8 ng/μl (SD 30 ng/μl) for the endometrial samples. The negative controls (no input sample for DNA extraction) did not contain detectable amounts of DNA. Nine samples from an earlier project were resequenced as positive controls.

16S rRNA gene and internal transcribed spacer (ITS) amplicon sequencing

MiSeq (Illumina , USA) paired-end sequencing of PCR amplicons from the hypervariable V3–V4 regions of the bacterial 16S rRNA gene (primers 341F/785R) and fungal ITS-1 region (primers ITS1F and ITS2) was prepared and performed as explained in detail elsewhere (*Virtanen et al., 2021*). Briefly, bacterial and fungal amplicons were prepared separately and combined for indexing in a 1:1 ratio, and an equimolar pool was sequenced using 2 × 300 bp reads and a MiSeq v3 reagent kit at the Biomedicum Functional Genomics Unit, Helsinki, Finland.

Sequence processing and analysing

The primary step of the analysis was to split the combined 16S rRNA gene and ITS sequence FASTQ files into separate datasets. This was done by first removing ambiguous (N) bases from the reads, followed by primer-based separation using cutadapt v3.5, removing the primers in the process (*Martin, 2011*). From this step onwards, all pre-processing was carried out individually on both datasets.

The bacterial 16S rRNA gene dataset was processed using dada2 v1.20, following the pipeline tutorial v1.16, while the ITS pipeline tutorial v1.8 was used for processing the ITS sequence dataset (*Callahan et al., 2016*). The dada2 tutorials were followed only until the stage of amplicon sequence variant (ASV) table construction and removal of chimaeras, after which the taxminer package was used to assign taxonomic annotations (*Saqib, 2021*). This is a Basic Local Alignment Search Tool (BLAST)-based annotation tool combined with text-mining-based filtration to assign the most likely annotations. A detailed description of this approach has been reported elsewhere (*Virtanen et al., 2021*).

Briefly, for sequence alignment, a stringent threshold of 98% was set for both percentage identity and query coverage, and was supplemented the authors' taxonomic filtration approach, which extracts the host and isolation source of each sequence alignment hit. The former eliminates low-quality alignments, and the latter filters the results based on user-defined parameters, in this case *Homo sapiens* (host) and female reproductive tract/clinical isolates/gut (isolation source), effectively minimizing the presence of potential contaminants and misannotations within the results.

The taxonomic profiles of vaginal bacterial communities can be sorted into categories called community state types (CST), a classification method based on the dominance, depletion or absence of prominent vaginal bacteria, mainly lactobacilli, within the bacterial profiles. CST were assigned in both vaginal and endometrial samples using the VALENCIA method (*France et al., 2020*).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics, version 25 (IBM Corporation, USA) and R (version 4.1.0; The R Foundation, Austria). A two-sample t-test and Mann–Whitney *U*-test were used to compare the continuous background variables, and Pearson chi-squared and Fisher's exact tests to compare the categorical variables between the RPL and control groups. Parity was categorized as nulliparous and parous (≥1 delivery).

The primary outcomes were the mean relative abundances of bacteria and fungi in the endometrial and vaginal samples. Associations between background variables and the microbiota were analysed using permutational analysis of variance (PERMANOVA) for the overall microbiota variation using the adonis2 function with 99,999 permutations from the vegan package (*Oksanen et al., 2020*). Potential confounders to adjust models for differential abundance analysis downstream were identified based on the following criteria: (i) factors previously identified to influence microbiota and RPL; and (ii) confounders with statistical significance, determined by PERMANOVA analysis, ordination plots and the GroupTest function from the package mare (*Korpela, 2016*). GroupTest performs differential abundance analysis for taxa within user-defined groups, in this case each background variable. The function finds the most suitable model for individual taxa and uses read counts as an offset within the model formula to account for sequencing depth. Finally, cases and controls were used as the grouping variable in GroupTest to determine the significantly different taxa in endometrial and vaginal samples between the RPL and control groups. Based on the criteria mentioned above, body mass index (BMI), parity and age were selected as confounders. The *P*-values obtained were adjusted for multiple testing by false discovery rate correction and reported as *q*-values to define nominal statistical significance at $q < 0.05$. The correlations between an individual's vaginal and endometrial microbial profiles were investigated using Pearson's correlation coefficient.

RESULTS

Samples were taken from 47 women with RPL and 39 control women to study the composition of the endometrial and vaginal microbiota and mycobiota. In one woman with RPL, only a vaginal sample was obtained. The participants with RPL had a history of three (range 2–5) consecutive miscarriages, of which 13 (15.3%) had occurred during the second trimester of pregnancy. Nine women had a likely explanation for their miscarriages: five had been diagnosed with congenital uterine malformation, three with acquired thrombophilia, and one with antiphospholipid syndrome and chromosomal translocation.

The women with RPL were older (mean 33.2 [range 22–39] versus 32.1 [26–38] years, $P = 0.04$) and had a higher BMI (mean 25.4 [19.5–39.4] versus 23.3 [19.4–33.1] kg/m^2, $P = 0.02$), and were more often parous (50.0% versus 15.4%, $P < 0.001$) than the controls (TABLE 1). The RPL group more often had self-reported recurrent (≥3) vaginal candidiasis, bacterial vaginosis and regular oral or vaginal probiotic use than the control group.

16S rRNA gene sequencing results

After taxonomic annotations and quality filtration (>500 reads), 74% (34/46) of the RPL and 77% (30/39) of control women's endometrial samples remained for bacterial analysis, with a mean read count of approximately 4500 (535–20,829), and approximately 4500 (569–11,540), respectively. Similarly, 98% of the vaginal RPL samples (46/47) and 100% of the controls (39/39) remained, with an average read count of approximately 23,600 (3728–47,923) and approximately 24,300 (1322–55,011).

PERMANOVA analysis showed a difference in the overall composition of the endometrial microbiota between the RPL and control women (R^2 (effect size) = 0.050, $P = 0.01$; see supplemental material Figure S1, Table S1), but the vaginal bacterial compositions were similar between the groups (Figure S2, Table S1). BMI was a strong clinical explanatory factor for the endometrial microbiota ($R^2 = 0.057$, $P = 0.005$), especially among the women with RPL ($R^2 = 0.09$, $P = 0.005$). Current vaginitis symptoms, use of vitamin D and folic acid, microbiota diversity and read count explained the variability of the vaginal microbiota in the whole study population, as did BMI, age and probiotics among the RPL group. Parity, gravidity or curettage had no impact on the endometrial or vaginal microbiota variation.

For the endometrial samples, 37 bacterial species were identified (FIGURE 2). *Lactobacillus crispatus* was significantly less abundant and *L. jensenii* more abundant in the RPL group compared with the controls (mean relative abundance of *L. crispatus* 17.2% versus 45.6%, $q = 0.04$; *L. jensenii* 5.6% versus 3.6%, $q = 0.004$) (FIGURE 3, Table S2). *Lactobacillus iners* was the dominant endometrial bacterium in the RPL group (mean relative abundance 32.2% in the RPL group, 20.0% in the controls). *Gardnerella vaginalis* was more abundant in the RPL group compared with the control group (12.4% versus 5.8%, $q < 0.001$). None of the intestinal bacteria, such as *Escherichia coli*, *Blautia* spp. and *Faecalibacterium* spp., or the uncultured bacteria (bacteria that lack culture-based genomic and physiological characterization) were significantly differentially abundant in the women with RPL compared with the control participants.

For the vaginal samples (FIGURE 2), the RPL group had more *G. vaginalis* (mean relative abundance 8.7% in the RPL group versus 5.7% in the control group, $q = 0.002$) and less *Garderella leopoldii* (1.0% versus 3.2%, respectively, $q < 0.001$), whereas *L. crispatus* was the most abundant bacterium (35.1% in the RPL group versus 47.5% in the controls) (FIGURE 3, Table S2). In the pooled endometrial and vaginal samples, *L. crispatus* was less abundant and *G. vaginalis* more abundant in the women with RPL than the controls (*L. crispatus* 27.5% versus 46.6%, $q = 0.04$; *G. vaginalis* 10.2% versus 5.7%, $q < 0.001$). When women with another explanation for RPL (congenital uterine malformation, acquired thrombophilia or chromosomal translocation) were excluded from the analyses, the differences in endometrial *L. crispatus* and endometrial and vaginal *G. vaginalis* abundances between the RPL and control women remained (Figure S3).

The results of the VALENCIA analyses (Figure S4, Table S3) were in line with the species-specific results presented above. In the endometrium, CST I, characterized by *L. crispatus* dominance, was most frequently assigned to controls (56.7%, 17/30), while its prevalence in women with RPL was only 23.6% (8/34) (Figure S4, Table S4). Meanwhile, assignments to CST III, characterized by *L. iners* dominance, were observed in 44.1% (15/34) of the RPL group and 26.7% (8/30) of the controls.

TABLE 1

Variable	RPL group (*n* = 46)	Control group (*n* = 39)	*P*-value
Age, years			0.04
Mean (SD [range])	33.2 (3.9 [22–39])	32.1 (3.0 [26–38])	
BMI, kg/m^2			0.02
Mean (SD [range])	25.4 (4.2 [19.5–39.4])	23.3 (3.1 [19.4–33.1])	
Parity, *n* (%)			<0.001
Nulliparous (*n* = 56)	23 (50.0)	33 (84.6)	
Parous (*n* = 29)	23 (50.0)	6 (15.4)	
Prior curettage, *n* (%)			<0.001
Yes (*n* = 18)	18 (39.1)	0	
No (*n* = 67)	28 (60.9)	39 (100.0)	
Folic acid use, *n* (%)[a]			0.02
Yes (*n* = 71)	34 (79.1)	37 (97.4)	
No (*n* = 10)	9 (20.9)	1 (2.6)	
Vitamin D use, *n* (%)[a]			0.21
Yes (*n* = 70)	35 (81.4)	35 (92.1)	
No (*n* = 11)	8 (18.6)	3 (7.9)	
Iron use, *n* (%)[a]			0.34
Yes (*n* = 30)	18 (41.9)	12 (31.6)	
No (*n* = 51)	25 (58.1)	26 (68.4)	
Smoking status, *n* (%)			0.19
Current or former smoker (*n* = 16)	11 (23.9)	5 (12.8)	
Non-smoker (*n* = 69)	35 (76.1)	34 (87.2)	
Alcohol use, *n* (%)[b]			0.11
Yes (*n* = 60)	29 (65.9)	31 (81.6)	
No (*n* = 22)	15 (34.1)	7 (18.4)	
Prior chlamydia, gonorrhoea or genital herpes, *n* (%)[c]			0.49
Yes (*n* = 9)	6 (14.3)	3 (7.9)	
No (*n* = 71)	36 (85.7)	35 (92.1)	
Prior vaginal candidiasis, *n* (%)[c]			0.45
Yes (*n* = 52)	29 (69.0)	23 (60.5)	
No (*n* = 28)	13 (31.0)	15 (39.5)	
Recurrent vaginal candidiasis (≥3), *n* (%)[c]			0.006
Yes (*n* = 8)	8 (19.0)	0	
No (*n* = 72)	34 (81.0)	38 (100.0)	
Prior bacterial vaginosis, *n* (%)[c]			0.15
Yes (*n* = 16)	11 (26.2)	5 (13.2)	
No (*n* = 64)	31 (73.8)	33 (86.8)	
Recurrent bacterial vaginosis (≥3), *n* (%)[c]			0.01
Yes (*n* = 7)	7 (16.7)	0	
No (*n* = 73)	35 (83.3)	38 (100.0)	
Antibiotic use during the past 3 months, *n* (%)[c]			0.05
Yes (*n* = 11)	9 (21.4)	2 (5.3)	
No (*n* = 69)	33 (78.6)	36 (94.7)	
Use of probiotics, *n* (%)[d]			0.008
Daily or weekly (*n* = 19)	15 (36.6)	4 (10.5)	
Less than weekly or never (*n* = 60)	26 (63.4)	34 (89.5)	
Level of education, *n* (%)[d]			0.75
Low (comprehensive or vocational secondary school) (*n* = 20)	11 (26.8)	9 (23.7)	
High (upper secondary school, university) (*n* = 59)	30 (73.2)	29 (76.3)	

Missing data were excluded from the analyses.
[a] Data missing in four cases.
[b] Data missing in three cases.
[c] Data missing in five cases.
[d] Data missing in six cases.
BMI, body mass index; RPL, recurrent pregnancy loss.

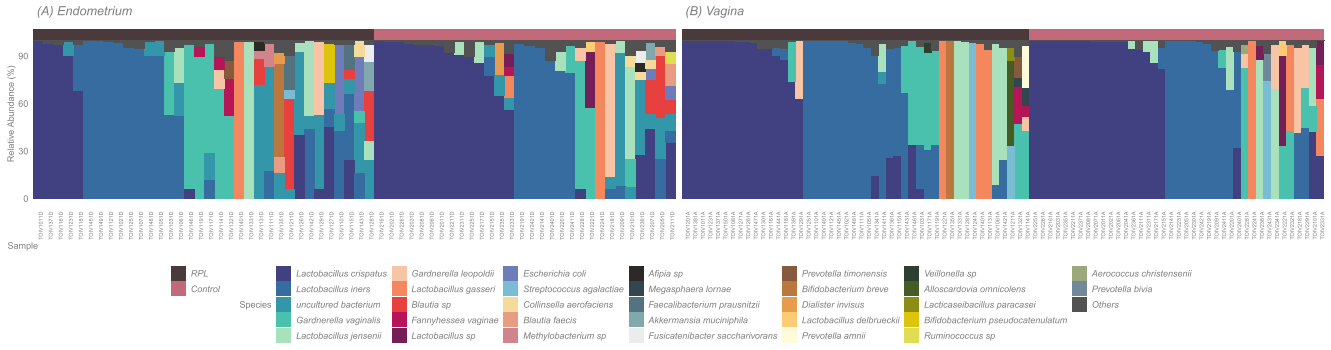

FIGURE 2 Stacked bar plots showing bacterial relative abundances in women with recurrent pregnancy loss (RPL) and control women, ordered based on the top three most prevalent and abundant taxa (*Lactobacillus crispatus*, *Lactobacillus iners* and *Gardnerella vaginalis*). (A) Endometrial samples, and (B) vaginal samples.

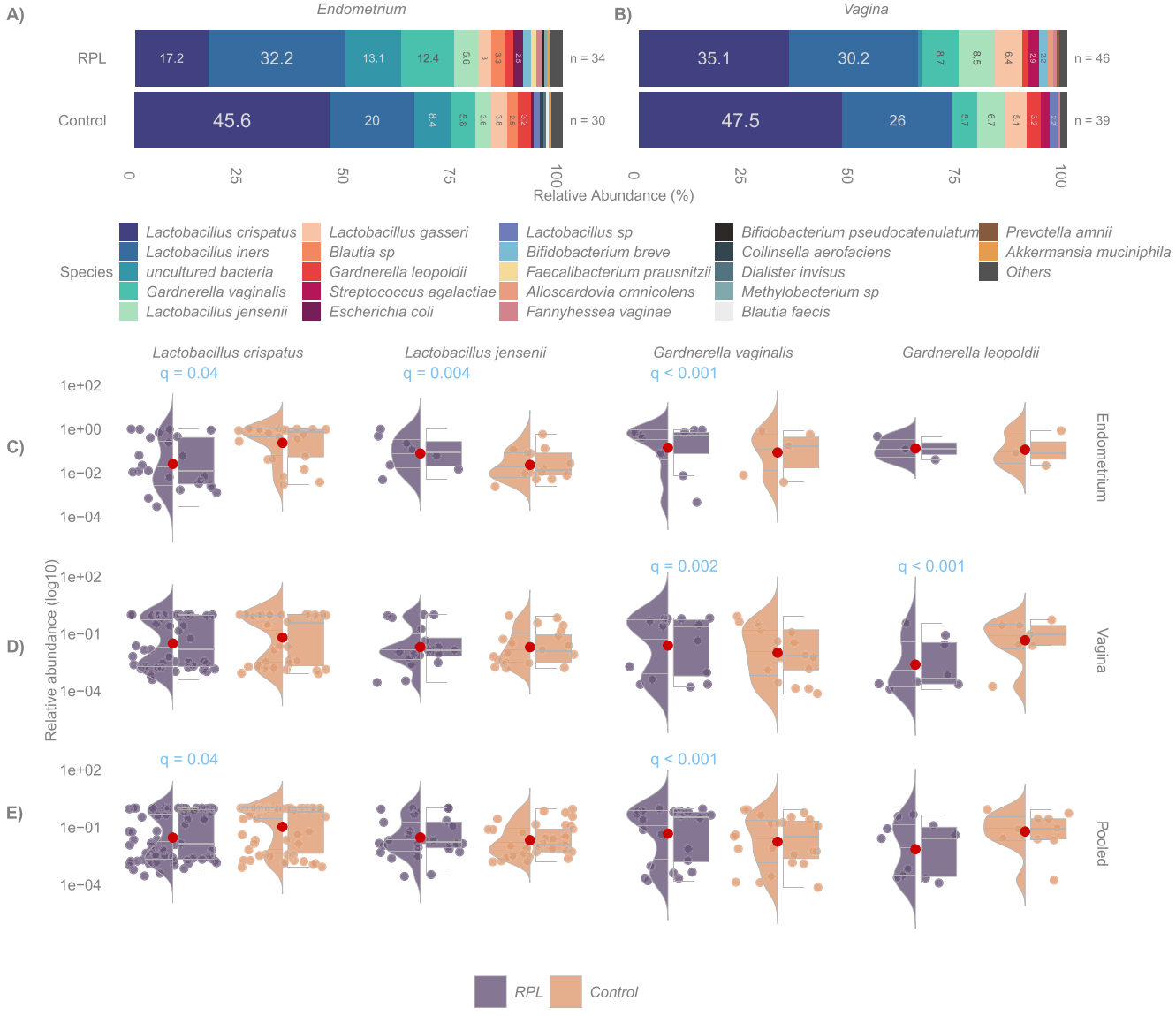

FIGURE 3 Illustration and summary of the main results and statistical analysis. (A, B) Bacterial mean relative abundances within the endometrial/vaginal and recurrent pregnancy loss (RPL)/control subgroups. (C–E) Violin-boxpots showing the distribution of data in each subgroup for taxa that were significantly differentially abundant after adjusting for age, parity and body mass index in the endometrial (C), vaginal (D) and pooled (E) samples. False discovery rate-corrected *P*-values (*q*-values) <0.05 indicate statistically significant differences between the RPL and control (reference) subgroups. 'Pooled' refers to the combined results for the endometrial and vaginal samples.

For non-*Lactobacillus*-dominated CST of interest, *G. vaginalis*-rich CST IV-B (14.7%, 5/34) and CST IV-C (5.9%, 2/34), characterized by heterogenous bacteria such as *Streptococcus, Bifidobacterium and Prevotella* spp., were detected in the RPL group and CST IV-B (6.7%, 2/30) in control participants. In the vagina, CST I was most frequently assigned to both the RPL (34.8%, 16/46) and control (48.7%, 19/39) groups, while assignment to CST III was nearly proportional (30.4%, 14/46; 30.8%, 12/39). CST IV-B and CST IV-C were marginally more frequently assigned to the RPL group (13.0%, 6/46; 6.5%, 3/46) than the control group (5.1%, 2/39; 2.6 1/39).

There were three blank samples within this pipeline that were analysed with the same pre-processing and filtration criteria. Only two out of these three samples had a considerable number of reads, which totalled to approximately 8500 (Figure S5A). These annotated to *Streptococcus oralis* (around 4000), *Fannyhessia vaginae* (around 600), *Prevotella amnii* (around 1700) and *Sneathia vaginalis* (around 1300). *Streptococcus oralis*, a potential oral contaminant, was not detected in any other samples. *Fannyhessia vaginae*, *P. amnii* and *S. vaginae* are known vaginal microbes, and their presence in blank samples is most likely due to cross-contamination from the primary study samples. Due to these reasons, these reads were not subtracted from the sample data. Furthermore, none of these bacteria were significant in the downstream analysis, and were therefore not crucial for the main study comparisons. Nine positive controls were used within this sequencing run (Figure S5B), which had been independently sequenced, processed and annotated for an earlier project. The microbial profiles obtained from the two runs were nearly identical (Pearson's correlation coefficient 0.996).

ITS sequencing results

After taxonomic annotations and quality filtration, 24% (11/46) of the RPL group's and 21% (8/39) of the control group's endometrial samples remained for the fungal analysis, with an average read count of approximately 14,500 (1031–81,809) and approximately 12,000 (1383–23,999) respectively. Similarly, 36% (17/47) of the RPL and 36% (14/39) of the control women's vaginal samples remained after filtration, with an average read count of around 6000 (742–22,500) and around 13,000 (542–30,685). As fungi could only be detected in a small fraction of the samples, they may not sufficiently represent the entire study cohort. The authors therefore refrain from statistical analysis and present a descriptive overview of the taxonomic profiles.

The most prevalent genus was *Candida*, which was detected in 19/39 samples (Figure S6). *Candida albicans* was not detected in the endometrium of women with RPL, although it was the most abundant taxon in their vaginal samples (mean relative abundance 26.9%) and in the endometrial and vaginal samples of the control group (mean relative abundance 49.9% and 42.9%, respectively). On the other hand, *Candida parapsilosis* was detected only in the RPL group, with an average relative abundance of 18.2% in endometrial and 11.8% in vaginal samples.

Comparison of microbiota in vaginal and endometrial samples

To study the relationship between the microbiota colonizing the endometrium and vagina, a comparison was made between 63 paired samples collected from these anatomical sites in the same woman. There was a strong within-individual correlation between the composition of the vaginal and endometrial microbiota (mean Pearson's correlation coefficient 0.85, $P < 0.001$; FIGURE 4, Figures S7 and S8), and 90.5% (57/63) of these sample pairs were assigned to the same CST, while 71.4% (45/63) were assigned to the same sub-CST, illustrating a high overlap in their bacterial profiles (Figure S4, Table S4). This overlap was the strongest for the *Lactobacillus*-dominated samples, while intestinal and uncultured bacteria were more abundant in the endometrium, especially in women with RPL.

DISCUSSION

An association was observed between RPL and reduced *L. crispatus* and increased *G. vaginalis* abundances in the endometrium, and increased *G. vaginalis* abundance in the vagina. The composition of vaginal microbiota was in concordance with that of the endometrial microbiota.

The finding of a reduced abundance of *L. crispatus* in RPL endometrial samples is in line with prior studies reporting that, in the vagina, *L. crispatus* dominance is associated with a healthy microbial environment (*Kindinger et al., 2017*; *Petrova et al., 2017*). Furthermore, *L. crispatus* has been shown to be less abundant in the endometrium of women with chronic endometritis (*Liu et al., 2019*), a condition associated with RPL (*McQueen et al., 2021*). In contrast, *L. iners*, which was the most dominant microbe in the endometrium of the RPL group, has been associated with dysbiosis (*Petrova et al., 2017*) and adverse reproductive outcomes, including subfertility (*Campisciano et al., 2021*), spontaneous miscarriage (*Nasioudis et al., 2017*) and preterm birth (*Kindinger et al., 2017*).

The results also showed an association between endometrial and vaginal *G. vaginalis* colonization and RPL. *G. vaginalis* is typically dominant in bacterial vaginosis, which has been associated with early (*Garcia-Grau et al., 2019*; *Haahr et al., 2019*; Moreno at al., 2016; *Ralph et al., 1999*) and especially with late (*Leitich and Kiss, 2007*) miscarriages. Miscarriage has also been linked with *Lactobacillus* depletion and high bacterial diversity in vaginal samples collected during early pregnancy (*Al-Memar et al., 2020*). Kuon and colleagues (*Kuon et al., 2017*) reported that women with RPL who had vaginal *G. vaginalis* colonization showed higher peripheral blood natural killer cell levels, suggesting a link between dysbiotic reproductive tract microbiota, inflammation and miscarriage. Furthermore, *Lactobacillus* depletion and the presence of pathogenic bacteria such as *Bifidobacterium*, *Gardnerella*, *Klebsiella* and *Neisseria* in endometrial biopsies has been associated with unsuccessful reproductive outcomes in IVF treatment (*Moreno et al., 2022*). In the current study, women with RPL reported a history of bacterial vaginosis, vaginal candidiasis and the use of probiotics more often than controls. Although bacterial vaginosis and vaginal candidiasis were not clinically or microscopically verified, symptoms and the use of probiotics may reflect these women's susceptibility to vaginal infection.

The genital tract microbiota is dynamic, influenced by several factors, including ethnicity (*Fettweis et al., 2014*), age (*Wang et al., 2021*), BMI (*Allen et al., 2022*), pregnancy (*Romero et al., 2014*) and childbirth (*Jie et al., 2021*). The

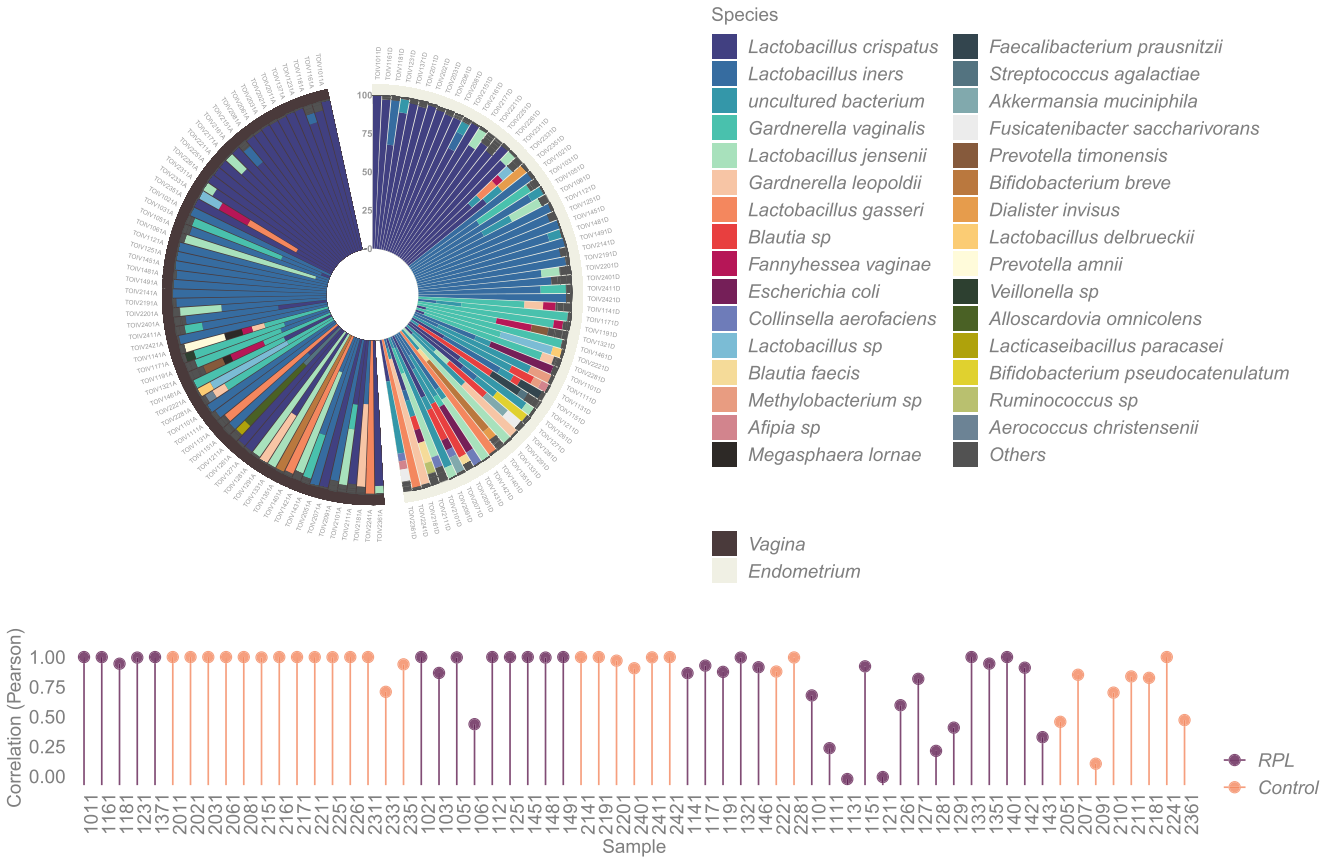

FIGURE 4 (A) Polar stacked bar plot illustrating the similarity of taxonomic profiles between the endometrial (right) and vagina (left) samples. The samples are arranged in the same order from top to bottom, creating a mirror image between the two semi-circles and allowing a direct comparison between the sample types. (B) Pearson's correlation between the paired endometrial and vaginal samples, arranged in the same order.

vaginal microbiota is significantly different in overweight women versus women with a normal weight, demonstrating higher diversity and lower *Lactobacillus* dominance (*Allen et al., 2022*). As women with RPL had a higher BMI than the control group in the current study population, the results were adjusted by BMI to eliminate its potential effect on the findings. Endometrial and vaginal microbiota alter as women age (*Wang et al., 2021*). The most significant changes occur after the age of 50 years, while the microbiota seems to be rather stable between ages 20 and 40 (*Wang et al., 2021*). Although age seems not to affect the reproductive tract microbiota for the ages of the current study population, it was selected as a confounder because age is strongly associated with RPL (*Lund et al. 2012*), it differed between the RPL and control groups, and it explained the variability in endometrial and vaginal microbiota in PERMANOVA. Because the control group had had significantly fewer childbirths than the RPL group, and the lack of previous childbirths has been associated with cervicovaginal *L. crispatus* colonization (*Jie et al., 2021*), the results were also adjusted for parity.

Lactobacillus-dominated endometrial microbiota may support early pregnancy, while more diverse microbiota can be detrimental, although the underlying mechanisms are still poorly understood (*Al-Nasiry et al., 2020*; *Bardos et al., 2020*; *Benner et al., 2018*). Liu and co-workers (*Liu et al., 2022*) observed higher bacterial richness and diversity with altered cytokine concentrations in the endometrial fluid samples of women with RPL compared with control women. *In vitro*, *L. crispatus* has been shown to attach to the decidualized endometrial cells and prevent pathogenetic microbes occupying the attachment sites (*Shiroda and Manning, 2020*). Conversely, dysbiotic endometrial microbiota may weaken the epithelial tight junctions, allowing pathogens to enter the endometrial stroma and induce a harmful immune reaction (*Al-Nasiry et al., 2020*). The activation of Toll-like receptors on the surface of endometrial cells by microbial molecules may elicit the secretion of cytokines that alter the local immune environment (*Benner et al., 2018*), leading to poor natural killer cell maturation. This may provoke disturbances in placentation (*Al-Nasiry et al., 2020*) as natural killer cells are essential in trophoblast invasion (*Moffett and Shreeve, 2015*) and remodelling of the spiral arteries (*Smith et al., 2009*). Abnormal endometrial microbiota may also favour endometrial T-helper 1 cell types (*Bardos et al., 2020*), which is thought to predispose to RPL (*Wang et al., 2020*).

The origin of endometrial microbes is still unclear, but the vagina and the gastrointestinal tract have been suggested (*Bardos et al., 2020*). The current findings and those of other researchers (*Walther-António et al., 2016*; *Wang et al., 2021*) of a concordance between vaginal and endometrial microbiota speak in favour of the vaginal route. Other possible mechanisms include haematogenous spread from the gastrointestinal tract

or retrograde ascension from the peritoneal cavity through the Fallopian tubes. Interestingly, oxygen-sensitive intestinal bacteria, including *Blautia* and *Faecalibacterium* spp., were detected in endometrial samples. In addition, Verstraelen and collaborators (*Verstraelen et al., 2016*) detected *Bacteroides* spp. in the endometrial samples of women with RPL or recurrent implantation failure, which supports the theory of a peritoneal route. However, although intestinal bacteria were detected solely in the endometrial samples in the current study, it cannot be excluded that these bacteria have ascended from the perineum through the vagina and remained under the detection level due to unfavourable conditions, such as a low pH. It is unlikely that the intestinal bacteria found in the endometrial samples were reagent-derived contaminants as those are typically water- and soil-associated bacterial genera (*Salter et al., 2014*) that were either not detected or were disregarded in the final sample read counts. In addition, intestinal bacteria were not found within the negative control samples. Finally, the role of intestinal versus vaginal microbes in the uterus and their potential effects on reproductive health remain to be elucidated.

This study has several strengths and limitations. The study population was ethnically and even genetically homogenous. Sampling was timed to the mid-secretory phase to analyse the microbiota during the receptive state of the endometrium, as the composition of the vaginal (*Lopes dos Santos Santiago et al., 2012*) and endometrial (*Kadogami et al., 2020*; *Khan et al., 2016*) microbiota may vary throughout the menstrual cycle. The recommended technique of transcervical biopsy was used for collecting endometrial tissue (*Molina et al., 2021*). In addition to the microbiota, the endometrial mycobiota were also analysed, which has not previously been explored. The control group in this study represent the general population as closely as possible as they were healthy and did not have any previous miscarriages or conditions known to be associated with alterations in reproductive tract microbiota, such as endometriosis (*Khan et al., 2016*; *Wei et al., 2020*), endometrial polyps (*Fang et al., 2016*), polycystic ovary syndrome (*Tu et al., 2020*) or Fallopian tube occlusion (*Haahr et al., 2019*). However, the applicability of these results to the general population remains to be explored.

As a limitation, low microbial abundance specimens, such as the endometrium, are susceptible to contamination (*O'Callaghan et al., 2020*). Although the authors avoided contacting the vaginal walls during sampling, cervicovaginal contamination cannot be ruled out as the back-and-forth movement of the biopsy device can inevitably push cervical mucus into the uterine cavity and contaminate the endometrial sample, and similarities existed between individual vaginal and endometrial microbial profiles. However, growing evidence supports the theory that the vaginal and endometrial ecosystems are not separate but can share microbes (*Chen et al., 2017*; *Walther-António et al., 2016*; *Wang et al., 2021*), and only a minority of studies have questioned the existence of a uterine microbiota (*Winters et al., 2019*). Although cervical mucus protects the uterine environment, spermatozoa pass from the vagina to the uterus, and vaginally administered radioactively labelled albumin macrospheres spread in the uterine cavity within minutes (*Kunz et al., 1997*). Therefore, it is likely that microbes ascending from the vagina may colonize the endometrium. In addition, the composition of the endometrial microbiota has been reported to be highly similar in laparoscopically and transcervically taken samples (*Chen et al., 2017*), and common vaginal microbes, including lactobacilli, have been found in the endometrium even after hysterectomy (*Chen et al., 2017*; *Miles et al., 2017*; *Mitchell et al., 2015*). Overall, even if there were cervicovaginal contamination of the endometrial samples, this would not explain the observed differences between the RPL and control groups.

Environmental, reagent and cross-contamination are a major concern in every low microbial biomass microbiome study (*Eisenhofer et al., 2019*; Salter, 2014). Several steps were taken at various stages of this project to identify and eliminate potential contaminants from the results. Environmental or reagent contamination was not likely as bacteria found in the negative controls, such as *S. oralis*, were not found in the women's samples, while reads for *Lactobacillus* and *Gardnerella* species were not substantial. The presence of vaginal microbes such as *F. vaginae*, *P. amnii* and *S. vaginae* could be explained as contamination, but their low abundance and prevalence in the study samples, as well as their lack of significance within the group comparisons, indicates that they did not have a significant effect on the overall results.

CONCLUSIONS

RPL was associated with a dysbiotic female reproductive tract microbiota, especially in the uterus. A divergent endometrial microbial environment may be a new background cause for RPL, possibly contributing to an adverse immunological response during implantation and placentation. Further research should examine the mechanisms of how altered microbiota may contribute to RPL, evaluate whether the prognosis of subsequent pregnancies could be assessed according to the microbiota profile, and investigate whether the endometrial microbiota could be modified to increase the success of future pregnancies in some couples affected by RPL.

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DATA AVAILABILITY

The sequencing data that support the findings of this study are available in the European Nucleotide Archive

(PRJEB48310). All code and the clinical metadata have been deposited to GitHub: https://github.com/SchahzadSaqib/TOIVE.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2022.06.008.

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