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Changes in the cervical microbiota of cervical cancer patients after primary radiochemotherapy

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Highlights

- Cervical microbiota composition differs between patients with histologically proven cervical cancer.
- There is a strong reduction in the cervical bacterial loads after chemoradiation.
- There is no change in terms of the composition of the cervical microbiota under chemoradiation.

ABSTRACT

Objective Several recent studies have identified a potential interaction between the vaginal microbiota and gynecological cancers, but little is known about the cervical microbiota and its changes during cancer treatment. Therefore, the aim of the study was to evaluate the quantitative and qualitative changes of cervical microbiota in patients undergoing concurrent chemotherapy and radiation treatment for locally advanced cervical cancer.

Methods Cervical cytobrush samples of 15 cervical patients undergoing chemoradiation treatment were collected 1 day before starting external beam radiation therapy and on the day of the last fraction of brachytherapy. After DNA extraction, 16S rRNA amplicon sequencing of the V3–V4 region was performed on the MiSeq platform, followed by data processing and statistical analyses concerning the alpha and beta diversity of 16 samples (7 samples were excluded because of incomplete sample sets).

Results The amount of amplicon yield after polymerase chain reaction analysis in post-radiation samples was significantly lower compared with the baseline samples (pre 31.49±24.07 ng/µl; post 1.33±1.94 ng/µl; p=0.007). A comparison of pre-treatment and post-treatment samples did not show significant differences regarding beta diversity (weighted UniFrac). There was no significant difference in alpha diversity, which is used to characterize species diversity within a particular community and takes into account both number and abundance (Shannon Diversity Index pre-treatment samples: 2.167±0.7504 (95% CI 1.54 to 2.79); post-treatment samples: 1.97±0.43 (95% CI 1.61 to 2.33); p=0.38). Interindividual differences in patients could partly explain some variation of the samples (permutational multivariate analysis of variance).

Conclusion There was a strong reduction in cervical bacterial loads after chemoradiation. Neither alpha nor beta diversity varied significantly when baseline samples were compared with post-treatment samples.

INTRODUCTION

The gastrointestinal tract harbors several hundred bacterial phylotypes, which not only play a significant role in aiding digestive processes, but also help maintain a healthy immune system. Chronic inflammation as a reaction to a disrupted microbiota appears to be a key mechanism by which specific bacterial species contribute to the development of cancer.[1] Several studies have emphasized the role of the gut microbiota in carcinogenesis, but data regarding the role of specific bacteria in certain cancers are sparse.

More recent analyses have identified potential interactions between the microbiota and gynecological cancers.[2–4] Although smaller in population, the cervicovaginal microbiota may affect local immune regulation and oncogenesis. It comprises 20–140 bacterial species with *Lactobacillus* species commonly being most abundant. *Lactobacillus* acidifies the vaginal pH by producing lactic acid. While this is well tolerated by *Lactobacillus*, a pH <4.5 inhibits the growth of several other bacterial species, including many pathogenic bacteria. Vaginal microbiota signatures identified in environments with a significantly higher pH are not dominated by *Lactobacillus,* but instead are characterized by high bacterial diversity and a higher prevalence of anaerobic species.[5 6]

In addition to their carcinogenic potential, recent findings in the area of onco-immunology suggest a significant role of the gut microbiota in priming cells of the immune system that improve the endogenous antitumor response, mainly regulatory and CD8+ T cells. This type of immune activation has been shown to enhance the efficacy of checkpoint inhibitors, a class of immunomodulatory drugs that also relies on activation of the T cell response.[7–10] The abscopal effect describes a systemic immune response to tumor metastases as a consequence of localized radiation therapy. It has equally been identified as a potential booster of checkpoint inhibition effects.[11] It could be hypothesized that there should be a synergistic effect between microbiota based and radiation based immune activation. Such synergism might in turn offer the chance of therapeutic options in the field of gynecological oncology.

Table 1 Patients characteristics

Patient No	Age at diagnosis (years)	FIGO stage	Pretreatment tumor size (cm)	Pelvic nodes (removed/infiltrated)	Para-aortic nodes (removed/infiltrated)	Histology	Grading
1	21	IB2	4.1×2.7	1/28	0/12	Adenosquamous	G3
2	36	IIB	4×5	1/16	0/15	Squamous cell	G2
3	44	IIIB	4.4×3.7	1/1	0/7	Squamous cell	G3
4	37	IIB	5×3.2	0/15	0/12	Squamous cell	G3
5	33	IB1	2.5×3	2/16	0/5	Squamous cell	G3
6	52	IIB	4.2×3.6	0/17	0/15	Squamous cell	G3
7	39	IIA1	3.7×2.5	6/36	0/5	Squamous cell	G1
8	30	IA2	0.4×0.7	SN positive; left pelvic SN: 2 mm metastasis; right pelvic SN: isolated tumor cells	0/12	Squamous cell	G2

FIGO, International Federation of Gynecology and Obstetrics; SN, sentinel node procedure.

Few data, however, are available on the cervical microbiota of affected patients over the course of their treatment. Hence the aim of this study was to evaluate the quantitative and qualitative changes in the cervical microbiota in patients undergoing concurrent chemotherapy and radiation treatment for cervical cancer.

METHODS

Fifteen patients with histologically proven cervical cancer and an indication for primary chemoradiation treatment were included. Because of incomplete sample sets, seven patients were excluded from the analysis. Patient characteristics are shown in Table 1. The analysis was approved by the local ethics committee (ISI Study, ethics committee No 08–160). After CT planning in the supine position with an emptied rectum and filled bladder with kneefix and footfix on a big bore TOSHIBA CT, radiation was performed. It included external beam radiation with 6/10 MV photons using volumetric arc techniques on a linear accelerator (TrueBeam, Varian) and daily cone beam CT with 5 weekly single doses of 1.8 Gy to the primary tumor, including the uterus, pelvic and, in case of histologically confirmed para-aortic lymph nodes including the para-aortic node, up to the renal vessels, to a total dose of 50.4 Gy in 28 fractions. A simultaneous boost was given with 5 weekly single doses of 2.12 Gy to both parametric regions, to a total dose of 59.36 Gy in 28 fractions. Brachytherapy started in the third–fourth week of external beam with MRI planned three dimensional generated plans and five fractions of 5 Gy single doses to the cervix and residual tumor volume. Concomitant chemotherapy was given to every patient once a week (cisplatin 40 mg/m^2 body surface area) for 5–6 applications. At the time of chemotherapy, comedication with dexamethasone 8 mg on day 1 of cisplatin and 4 mg on days 2 and 3 were administered. Proton pump inhibitor treatment was prescribed with oral pantoprazole 40 mg/day concomitantly with chemoradiation.

Cervical cytobrush samples of each patient were collected 1 day before starting external beam radiation (baseline) and on the day of the last fraction of brachytherapy. Each sample was transferred into a clean collection tube and stored at −80°C within 1 hour. For microbiome analysis, samples were thawed and directly subjected to DNA extraction using the ZymoBIOMICS DNA miniprep Kit (Zymo Research Corp, Irvine, USA) following the manufacturer's instructions. Afterwards, 16S rRNA amplicon sequencing of the V3–V4 region was performed as described in the Illumina 16S Sample Preparation Guide on the MiSeq platform (Illumina, San Diego, USA) in a 300 bp paired end run.[12] The sequencing data were processed using the DADA2 pipeline and analyzed using QIIME 2.[13 14] Quality profiles of the reads were analyzed. Reads were trimmed (trunc_len_f=290, trunc_len_r=220) and processed by the QIIME DADA2 plugin with the denoise paired option and standard parameters (trunc_q=2, max_ee=2, chimera_method=consensus). Taxonomic classification was performed by a Naïve Bayes classifier (sklearn),

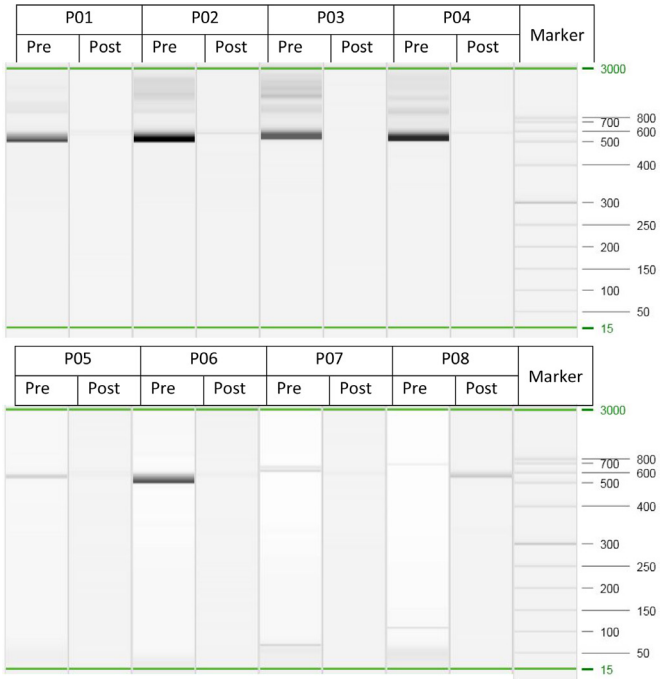

Figure 1 Polymerase chain reaction (PCR) product of the amplicon PCR. Capillary electrophoresis 'gel' showing the PCR product obtained after 25 cycles, targeting the 16S V3 and V4 region (Klindworth 2013) followed by clean-up using AMPure XP beads. The expected size of the PCR product is approximately 550 bp.

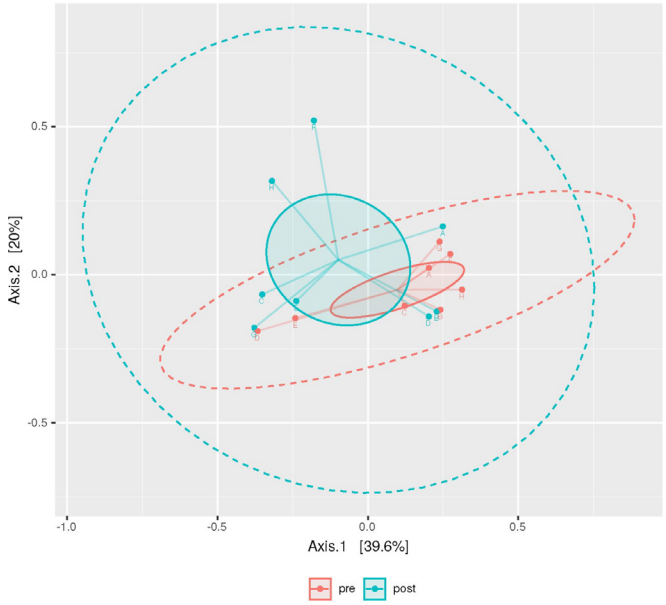

Figure 2 Principal coordinate analysis of bacterial community structures on the basis of weighted UniFrac distances of all samples; 95% confidence levels assuming normal (- - -) distribution and 95% confidence ellipses (---). Red dots represent the samples collected before chemoradiation and blue dots represent the samples collected after chemoradiation.

who was trained on the SILVA database release 128.[15 16] Rarefaction curves were determined based on the feature table, and analysis of the relative proportion of each bacterial taxon was made after the data were rarefied at a depth of 3500 sequences per sample. Statistical analyses were carried out using R for Statistical Computing (V.3.2.5, R Foundation for Statistical Computing, Vienna, Austria).[17] Alpha and beta diversity scores were calculated using the R package phyloseq.[18] Beta diversity, in this case the weighted UniFrac distances between the samples, was visualized using principal coordinate analysis. The effect of radiation status on beta diversity was tested by a permutational multivariate analysis of variance.

RESULTS

During the entire sample collection period, 23 cervical cytobrush samples were collected, 16 of which were included in further microbiome analyses. Seven samples were excluded from analysis because of incomplete sample sets, (ie, either the pre-treatment or post-treatment sample was missing because sampling had to be stopped due to pain). The mean concentration of the (total) extracted genomic DNA was 43.6±12.1 ng/µl (Qubit fluorometer). The product of the amplicon polymerase chain reaction—that is, of the 16S V3–V4 region—was checked by capillary electrophoresis. Although equal amounts of DNA template were used, the yield of the amplicon was much less in post-radiation samples (Figure 1). The QIAxcel Software was used to quantify the V3–V4 amplicons. DNA concentration of the pre-radiation amplicons was significantly higher compared with post-radiation amplicons (pre 31.49±24.07 ng/µl; post 1.33±1.94 ng/µl; p=0.007).

There was no significant difference in alpha diversity, which is used to characterize species diversity within a particular community and takes into account both number and abundance (Shannon Diversity Index pre-treatment samples 2.167±0.7504 (95% CI 1.54 to 2.79); post-treatment samples: 1.971±0.4296 (95% CI 1.61 to 2.33); p=0.38) when we compared baseline samples with samples after chemoradiation samples. The same was true for beta diversity, which refers to diversity between two communities by measuring variation between multiple samples. In terms of beta diversity, principal coordinate analysis of the weighted UniFrac distances did not show any shift in the cervical microbiota composition after radiation therapy (permutational multivariate analysis of variance F=1.42, p=0.197)—that is, the 95% confidence ellipses around the centeroids of both groups overlapped (Figure 2).

Another permutational multivariate analysis of variance revealed that there was some spread of the samples that (in part) could be explained by interindividual differences in patients (unweighted UniFrac F=2.15, p<0.001; weighted UniFrac F=1.42, p=0.106). The effect of the patient identification number (PID) on several dissimilarity indices is listed in Table 2.

The relative abundances of the bacterial families detected within the patients' cervical microbiota is shown in Figure 3.

DISCUSSION

There is a growing body of data which suggests that the microbiota plays an underestimated role in the development of chronic inflammation, human papillomavirus infection, and human papillomavirus clearance, progressing to invasive cancer, and may also play a role in prevention of cancer.[2–6 19–25] This pilot study is the first to analyze the effects of chemoradiation for cervical cancer on the cervical microbiota. Previous studies performed in this patient population have focused on analysis of the gut microbiota and its correlation with radiation related gastrointestinal toxicity. A systematic review[26] analyzed data from three cohort studies[27–29] on changes in the gut microbiome of 23 women with gynecological cancer. It has been shown that there is a change in microbiota quality in patients who developed gastrointestinal toxicity. An increase in unspecified bacterial species was seen in those with diarrhea, but patients without diarrhea maintained their initial bacterial profiles.[26] Supplementation with *Lactobacillus acidophilus* and *Bifidobacterium bifidum* before and during radiation decreased

Table 2 Effect of the patient identification number (PID) on the dissimilarity indices

Dissimilarity index	Bray–Curtis	Jaccard	Unweighted UniFrac	Weighted UniFrac
Test statistic	1.71	1.54	2.15	1.42
P value	0.004	0.003	<0.001	0.106

Test=pseudo-F; sample size=16; No of groups=8; No of permutations=999.

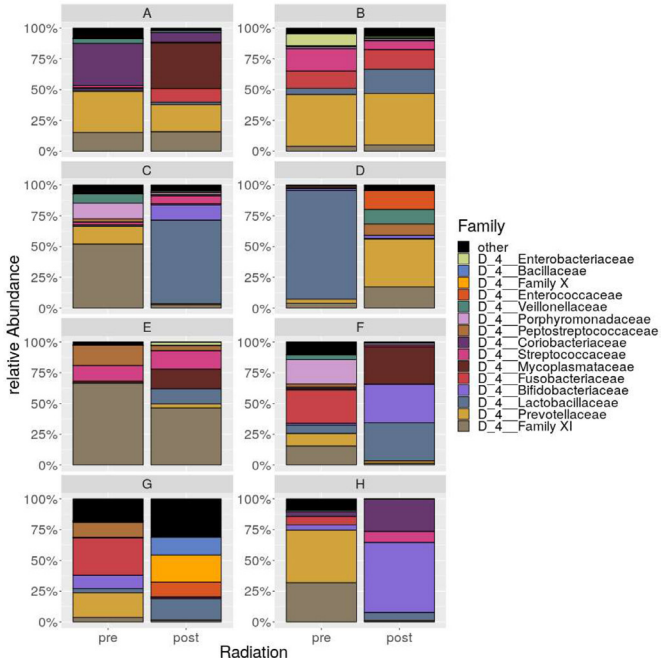

Figure 3 Relative abundance of different bacterial families in each sample. (A–H) Different patients, family XI=Clostridiales.

the rate of grade 2 and 3 diarrhea significantly (treatment group 9% vs no treatment group 45%).[30]

Our results suggest that there is an effect of chemoradiation therapy on the cervical microbiota. Although the DNA concentration measurement after DNA extraction from the swab samples showed high values, we have to consider that this could in part be due to high levels of simultaneously extracted host DNA. The results of electrophoresis nevertheless indicated a strong reduction in cervical bacterial loads after radiation therapy, as expected, as chemotherapy and radiation both lead to damage or destruction of cells.

Although the cervical microbiota of all patients considerably changed in terms of quantity, as revealed by electrophoresis, this conclusion cannot be drawn concerning quality—that is, the composition of the microbiota. Neither alpha nor beta diversity differed significantly when we compared pre-radiation with post-radiation samples. Clearly, this conclusion is limited by the small sample size of our study. What we could see, however, was inter-individual differences in the composition of the cervical microbiota. Some women showed a stronger dominance of certain families, such as *Clostridiales*, *Lactobacillaceae*, and *Prevotellacecae*, while others showed a more diverse composition.

To our knowledge, no data on changes to the cervical microbiota during chemoradiation treatment have been published previously. As a first step, we analyzed eight patients before and after treatment. We demonstrated that chemoradiation results in quantitative, but not qualitative, changes in the cervical microbiota. Quantification is an aspect that has been under appreciated in microbiome research to date, but may have relevant clinical implications. It remains to be determined in future studies whether there is any impact of local bacteria on the response to treatment of cervical tumor or whether such responses are mediated by the much more abundant microbiota colonizing the gut.

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Data availability statement All data relevant to the study are included in the article or available upon reasonable request.

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