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ARTICLE

The high-risk human papillomavirus continuum along the female reproductive tract and its relationship to infertility and endometriosis

BIOGRAPHY

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KEY MESSAGE

High-risk human papillomavirus (hrHPV) infection in the upper genital tract is associated with infertility and endometriosis. Additionally, there is a hrHPV infection continuum along the female genital tract.

ABSTRACT

Research question: Is there an association between the presence of sexually transmitted pathogens in the lower (LGT) and upper (UGT) female genital tract with endometriosis and infertility?

Design: Case–control study with 60 women submitted to gynaecological laparoscopic surgery. Samples from the UGT and LGT were collected and analysed by single polymerase chain reaction (PCR) for human papillomavirus (HPV) and by multiplex PCR for other sexually transmitted infections (STI). Patients were initially divided into two clinical groups: infertile patients (n = 25) with conjugal infertility and fertile control patients (n = 35). After the surgical findings patients were further divided for additional analysis: an endometriosis group (n = 29) and non-endometriosis control group (n = 31).

Results: Sixty per cent of patients were positive for DNA-HPV in some of the genital tract sites sampled. Infertile patients were associated with high-risk HPV (hrHPV) positivity in the UGT sites (P = 0.027). The endometriosis group was associated with hrHPV positivity in the LGT and UGT sites (P = 0.0002 and P = 0.03, respectively). Only hrHPV types were detected in the UGT in both groups. It may be that there is a hrHPV infection continuum, from LGT to UGT, in infertile and endometriosis patients. No association was observed among the other seven STI studied.

Conclusions: This study shows both an association between hrHPV infections in the UGT with infertility and endometriosis, and a possible hrHPV infection continuum, from LGT to UGT. Larger studies are needed to fully investigate the role of hrHPV as a cause of endometriosis and infertility.

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KEYWORDS

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Infertility
Upper female genital tract

INTRODUCTION

Infertility is a complex human health situation and a common public health concern worldwide (*Boivin et al., 2007; Chandra et al., 2013*). The inability to conceive creates not only a major psychological stressor for millions of couples but is also a considerable cost burden for patients and the healthcare system (*Cousineau et al., 2006*). Infertility results from multiple factors that are responsible for impairments in the reproductive function in men and/or women. It is considered to be 'idiopathic' in a large proportion of cases (*Quaas and Dokras, 2008*). Further insight into the causes of infertility is necessary to help alleviate this multifactorial burden on society.

Endometriosis is one of the leading causes of female infertility, as well as reduced quality of life and psychological problems. It is an oestrogen-dependent gynaecological disorder characterized by the implantation of endometrial tissue outside of the uterus (*Acién and Velasco, 2013; Burney and Giudice, 2012*). The symptoms vary widely, which contributes to delayed diagnosis (*Campos et al., 2018*). Although some patients are asymptomatic, most women present with dysmenorrhoea, dyspareunia, intestinal and urinary alterations, pelvic pain, chronic fatigue and infertility (*Burney and Giudice, 2012; Culley et al., 2013; Gao et al., 2006; Macer and Taylor, 2012; Missmer and Cramer, 2003*).

Nevertheless, the pathogenesis and pathophysiology of endometriosis remain unclear (*Heidarpour et al., 2017; Senapati et al., 2016*). Among the most plausible explanations, the theory proposed by Sampson in 1927 (*Vinatier et al., 2001*) is based on the flowback of endometrial tissue from the fallopian tubes into the peritoneal cavity with potential implantation and ectopic growth of the endometrium (*Augoulea et al., 2012*). Retrograde menstruation could explain most events associated with the disease (*Vinatier et al., 2001*). Other plausible hypotheses suggest that abnormalities of the eutopic endometrium confer intrinsic resistance to elimination by the immune system, and the disease could result from the inability of macrophages and natural killer cells to eliminate endometrial implants (*Acién and Velasco, 2013*). It is still difficult to identify a single mechanism that consistently explains all pathogenesis aspects of endometriosis (*Khan et al., 2014*).

The exposure of the lower genital tract (LGT) to microbes allows them to enter the upper genital tract (UGT) (*Larsen and Hwang, 2010*). The 'theory of contamination' or 'infectious theory' was proposed to explain these findings in the endometriosis context (*Campos et al., 2018; Khan et al., 2014, 2016; Kobayashi et al., 2014*). It has been suggested that during retrograde menstruation, endometrial tissue and microorganisms are carried to the ovary and peritoneal cavity (*Khan et al., 2014*). Most studies have focused on bacteria because of the broad bacterial composition of the vaginal microbiome. However, the involvement of other microorganisms such as viruses is plausible, as some pathogenic viruses are common in the LGT, such as the human papillomavirus (HPV). HPV is one of the most common viral sexually transmitted diseases worldwide in both males and females (*Tota et al., 2011*). Low-risk types of HPV (lrHPV) can cause genital warts, whereas persistent infection with high-risk types of HPV (hrHPV) is associated with anogenital and oropharyngeal cancers. HPV infections are often asymptomatic and people are usually infected without being aware (*Ho et al., 1998*). Even if the infection does not necessarily lead to cellular lesions or proliferation (*Ho et al., 1998; Tota et al., 2011*), it is unclear whether HPV infections can ascend from the LGT to the UGT and silently lead to damage that alters the reproductive function. Nevertheless, although some recent studies (*Heidarpour et al., 2017; Oppelt et al., 2010*) have reported a higher rate of hrHPV infection among patients with endometriosis, other authors have described the opposite (*Vestergaard et al., 2010*). Additionally, there are not thought to be any studies that have systematically sampled the LGT and UGT for HPV infection to evaluate the involvement of a possible continuum infection in women with infertility and/or endometriosis. Based on the above considerations, the present study aimed to evaluate the influence of a possible HPV infection continuum in the pathogenesis of infertility and endometriosis by systematically sampling six sites along the female reproductive tract in women of reproductive age. Seven other important sexually transmitted pathogens were also analysed for their possible involvement in infertility.

MATERIALS AND METHODS

Study population

This case–control study was performed at a single private medical centre (Instituto Da Vinci Obstetrics, Gynecology and Reproductive Medicine), Toledo, Paraná, Brazil. The subjects included women of reproductive age with a medical request for laparoscopic gynaecological surgeries. Vaginal and endocervical samples were collected before the laparoscopy, and peritoneal fluid and biopsy tissue samples were collected during the laparoscopy, from March 2014 to June 2017. Overall, the centre treated 2983 women during the period, 489 of whom underwent medical consultation due to conjugal infertility and 2494 for other causes but with no suspicion of infertility. Participants with any of the following criteria were excluded: younger than 18 years of age, positive HIV status or other immunosuppressive condition, pregnancy, evidence of active opportunistic infections or immune deficiencies leading to active opportunistic infections, documentation of pelvic inflammatory disease within the preceding 6 months, antibiotic treatment within 30 days, hormonal treatment within the preceding 3 months, previous or current history of cervical lesions or cancer, clinical or laboratorial evidence of hypogonadism, and diagnosed sexually transmitted infections (STI) in the preceding 6 months. Also excluded were women vaccinated for HPV, with infertility due to bilateral tubal factor, with altered serum FSH and thyroid-stimulating hormone levels on the third day of the menstrual cycle, and with severe male factor of infertility (altered semen analysis). Considering the inclusion and exclusion criteria described, a total of 60 Caucasian women were included in the study (FIGURE 1), with an age range of 28 to 47 years and a mean age and SD of 37.25 ± 5.49 years.

Clinical and sociodemographic data

Infertility is classically defined as the inability of couples to conceive after 12 or more consecutive months of regular unprotected sexual intercourse. Endometriosis was clinically suspected based on the clinical symptoms of chronic pelvic pain, severe or incapacitating dysmenorrhoea, deep dyspareunia, cyclic urinary abnormalities

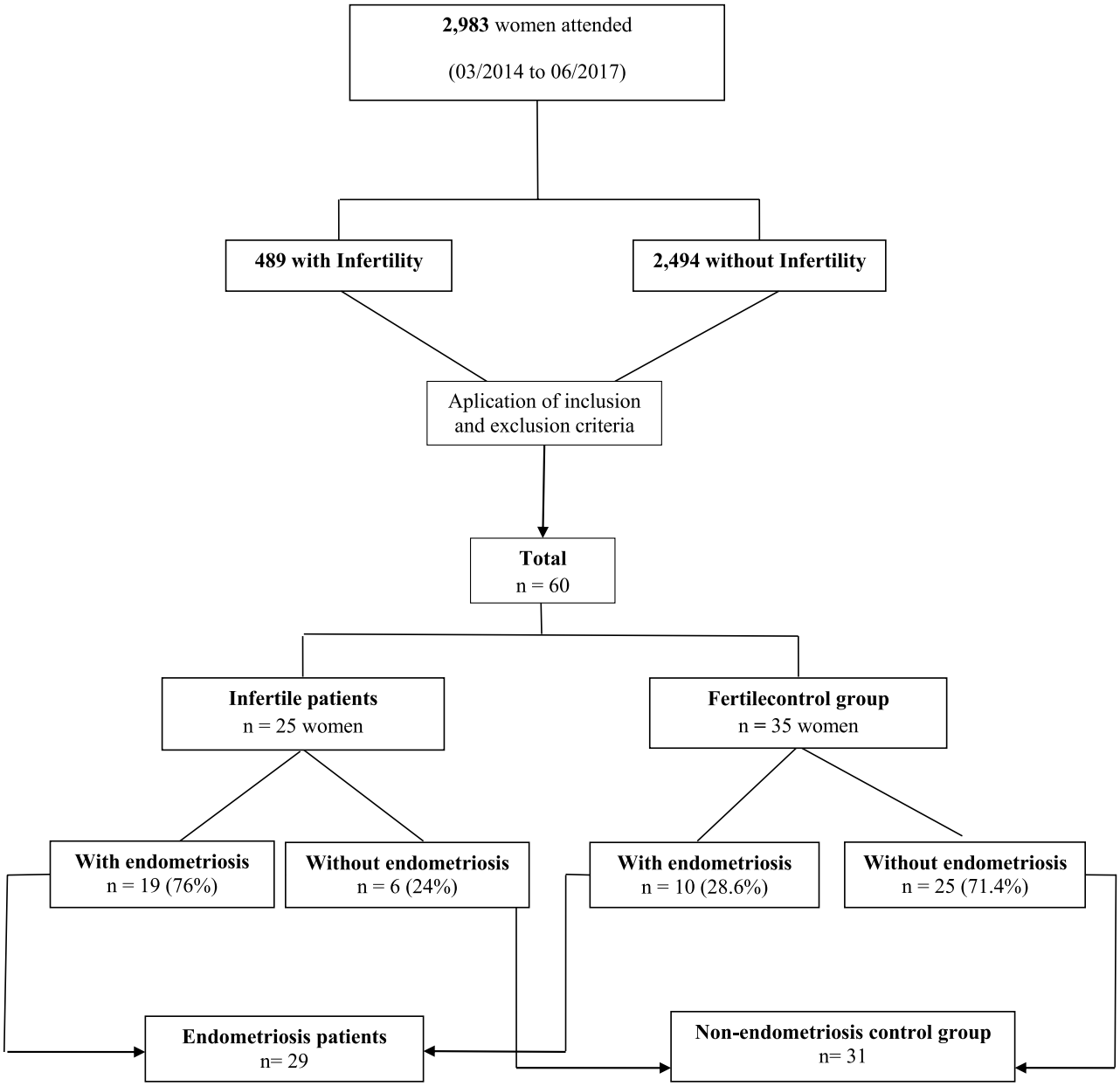

FIGURE 1 Flow chart of the study design.

(pain and/or bleeding) or cyclic bowel abnormalities (pain and/or bleeding) and infertility. Endometriosis was diagnosed when lesions were observed during surgery and subsequently confirmed by histology.

Before surgery, each woman included in the study self-completed validated questionnaires. Demographic data collected included age, ethnicity, level of education, marital status, deliveries, family income, job/profession and age of first sexual intercourse. All clinical data were collected from medical records. All procedures performed in the study were in accordance with the ethical standards of the Committee for Ethics in Research Involving Humans at the State University of Maringá (UEM), Paraná, Brazil (report no. 38254214.7.0000.0104; 12/15/2014) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Each participant signed an informed consent form, after which they were enrolled for laparoscopy and sample collection.

Gynaecological laparoscopic procedures and sample collection

The same surgical staff performed all surgical procedures. A gynaecological specialist (RM) recorded the laparoscopic surgical findings, such as the extent and location of endometriosis and the adhesion severity, using a standard checklist. Laparoscopy was performed during the first phase of the menstrual cycle in all patients. Endometriosis (stages I–IV) was scored according to the American Society for Reproductive Medicine/American Fertility Society (AFS) Revised Classification System (*'Special Contribution', 1997; Dunselman et al., 2014*).

Before laparoscopy, samples from the lower third of the vagina and cervical canal were obtained with the help of a sterile Ayre spatula and Cytobrush (Cralpast, Brazil), respectively.

Laparoscopy was performed with the use of an umbilical puncture and pneumoperitoneum, with no instillation of saline solution into the cavity. Endometrial samples were taken through a Pipelle curette. During laparoscopy, free fluid was collected from the pouch of Douglas. Uterine tube lavages were provided and collected by flushing the uterine tube with 10 ml of 0.9% saline from the infertile patients. Tubal tissues were obtained from the fertile control group. Peritoneal biopsies were performed on macroscopic sites of endometriosis or over the uterosacral ligaments if no lesions were visible. Ovary biopsies were taken from the lateral end portions. These materials were immediately packed in sterile Eppendorf tubes with 0.9% saline and kept refrigerated at –4°C until analysis. A similar approach had been used previously by other authors (*Pelzer et al., 2011*).

For analysis of the results, data from the samples from the lower third of the vagina and cervical canal were grouped and designated as the lower genital tract (LGT), and endometrial tissue, pouch of Douglas fluid, uterine tube lavage and ovarian biopsy samples were grouped as upper genital tract (UGT) samples.

Laparoscopy findings

Among a total of 25 infertile patients, 19 (76%) had endometriosis, 13 (52%) had endometriosis stage I, 3 (12%) had stage II, and 3 (12%) had stage III. According to the surgical findings, some women in the fertile control group also had endometriosis (n = 10/35; 28.6%), 7 (20%) had endometriosis stage I, and 3 (8.6%) had stage II. No women with grade 4 endometriosis were detected. Again, as expected, the infertile group had higher rates and more severe endometriosis than the control fertile group (P = 0.0001). Considering all patients with endometriosis (n = 29), 20 (69%) had stage I, 6 (20.7%) had stage II, and 3 (10.3%) had stage III.

Clinical groups

The 60 women included in the study were divided into the following clinical groups: infertile patients (n = 25) with conjugal infertility and fertile control patients (n = 35) without infertility (FIGURE 1). Initially, the infertile patients were thought to have unexplained infertility (n = 10), endometriosis (n = 7), unilateral tubal factors (n = 4), ovulatory factors (n = 3) and uterine factors (n = 1). The control group consisted of women with no evidence of infertility and at least one term pregnancy. In these patients, the causes that justified the laparoscopy were abnormal uterine bleeding (n = 27) and family planning (n = 8).

According to the surgical findings, some women of the fertile control group patients had endometriosis. Therefore, to allow for proper analysis of results, patients were divided into two additional groups: those with (n = 29) and without (n = 31) endometriosis (FIGURE 1). The endometriosis patients had confirmation of the initial diagnosis during laparoscopy, with histopathology. The non-endometriosis control group consisted of women without endometriosis, according to laparoscopy and histopathology, regardless of whether they had been classified as infertile in initial medical consultation.

Genomic DNA extraction

To remove any polymerase chain reaction (PCR) inhibitors from the samples, they were incubated for 15 min with proteinase K in phosphate-buffered saline and then centrifuged for 30 s/6.800*g*. DNA was extracted using an AxyPrep™ BodyFluid Miniprep Kit (Axygen, CA, USA), according to the manufacturer's instructions. The quality and quantity of purified DNA were determined by spectrophotometry on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, MA, USA).

HPV detection by single-target PCR (sPCR)

An independent validation cohort was used, comprised of additional cervical samples from reproductive age women included between November 2014 and June 2015. The conditions for cervical sampling were the same as those in the study cohort. Informed consent was also obtained from all participants. A total of 10 cervical samples (five positive and five negative for HPV) previously analysed by our molecular assay were selected for validation by the Cobas 4800 HPV testing platform (Roche Molecular Systems, Pleasanton, CA), a qualitative in-vitro assay for detection of 14 hrHPV types. This automated system simultaneously extracts cellular (including β-globin) and viral HPV-DNA. The target DNA is then amplified by PCR using primers specific to HPV and β-globin. Cobas 4800 HPV testing detects HPV in three separate channels that discriminate HPV16 individually, HPV18 individually, and a pool of 12 other hrHPV types (11 definite high-risk, cancer-associated HPV types [HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68] plus one possibly hrHPV type [HPV66]). A fourth channel measures β-globin for specimen adequacy. The Cobas HPV test is approved by the US Food and Drug Administration as a primary HPV test for cervical cancer screening (*Gosvig et al., 2013*). All samples tested showed consistent results between the two assays.

Next, HPV was detected using a sPCR method with the primers MY09 (5′-CGTCCMAARGGAWACTGATC-3′) and MY11 (5′-GCMCAGGGWCATAAYA ATGG-3′) as described previously (*Manos et al., 1994*). PCR was performed using a thermal cycler (Applied Biosystems, CA, USA) under the following conditions: 5 min denaturation at 94°C; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 60 s, elongation at 72°C for 60 s, and final extension at 72°C for 8 min. This reaction produced a final amplified product of 450 base pairs (bp). The quality of the DNA was tested by amplification of a 268 bp gene fragment of the human β-globin gene using the primers GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PC04 (5′-CAACTTCATCC ACGTTCACC-3′) under the same conditions as the HPV-PCR. Two types of controls were used in the reaction: a sample without DNA (negative control) and one HPV-positive cervical sample (positive control). The final amplified products were loaded onto a 1.0% agarose gel stained with 150 ng/μl ethidium bromide and subjected to electrophoresis in a horizontal tank at 110 V for 45 min in 0.5 × TBE buffer (45 mmol/l Tris-borate, 1 mmol/l EDTA, pH 8.0). A 100 bp marker (Invitrogen, Carlsbad, CA, USA) was used as a size standard. The amplified DNA fragments were visualized on a transilluminator with UV light and then photographed.

The HPV-positive samples were typed by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis, in which amplified DNA was cleaved with restriction enzymes to generate DNA fragments of different molecular sizes. Aliquots of each amplified product were subjected to digestion with the restriction enzyme HpyCH4V (New England Biolabs, Ipswich, MA, USA)

(*Santiago et al., 2006*). To better differentiate between the HPV types with similar RFLP patterns, such as HPV 11/30, 18/68, 44/55 and 61/83/84, a second enzyme was used (NlaIII, New England Biolabs) (*Chen et al., 2013*). The restriction digest fragments were subjected to electrophoretic analysis on 8% polyacrylamide gels. Both 100 and 25 bp ladders (Invitrogen) were used as molecular size standards. After electrophoresis, polyacrylamide gels were analysed using LabImage ID software (Loccus Biotechnology, Cotia, SP, Brazil), and the size of each fragment was determined. Typing was performed by comparing the molecular weights of the fragments for each HPV type, as described by *Santiago et al. (2006)*. A total of 40 individual HPV types can be determined using the PCR-RFLP method: 17 types are considered to be either high risk or potentially high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82); 22 low-risk types are not associated with carcinogenesis (6, 11, 30, 34, 40, 42, 43, 44, 54, 55, 61, 62, 64, 67, 69, 70, 72, 74, 81, 83, 84 and 91); and the carcinogenic risk for one type has not yet been determined (22) (*Bouvard et al., 2009; Chen et al., 2013; Monsonego et al., 2015; Santiago et al., 2006*).

Other STI detection by multiplex PCR (M-PCR)

A M-PCR assay was performed to simultaneously detect *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Trichomonas vaginalis*, herpes simplex virus (HSV)-1, HSV-2 and *Treponema pallidum*, as previously described by *Souza et al. (2013)*. M-PCR was performed using a thermal cycler (Applied Biosystems) using the following conditions: 10 min denaturation at 94°C; 35 cycles of denaturation at 94°C for 60 s, annealing at 62°C for 60 s, elongation at 72°C for 60 s; and final extension at 72°C for 10 min. The M-PCR products were electrophoresed in 8% polyacrylamide gel. Positive controls for all STI studied were derived from positive clinical samples that were detected using reference methods, including culture and/or sPCR. All clinical samples were also tested using human β-globin-specific primers GH20/PC04 as an internal control for amplification and DNA integrity under the same conditions as the M-PCR.

Statistical analysis

The means with SD are shown for continuous data and percentages for the categorical data. To allow for proper analysis of the results, patients were distributed into the following groups: the infertile patients (n = 25) and control non-infertile group (n = 35), and the endometriosis patients (n = 29) and control non-endometriosis group (n = 31). Among the groups, Student's t-test was used to calculate the differences between the patient characteristics related to the independent samples, with equal variances of the continuous variables. Chi-squared tests were performed for contingency tables of the specified groups (with infertility versus without infertility groups, and with endometriosis versus without endometriosis groups). Odds ratios (OR) with a confidence interval of 95% (95% CI) were calculated. Statistical significance was considered to be when $P < 0.05$. Statistical analysis was performed using R software (available at https://www.r-project.org).

RESULTS

Patient characteristics

Among the women included in the study, 100% were married, 76.7% had their first sexual intercourse at 16 years of age and older, 100% had a family income ≥$1500/month, and 79.7% had jobs outside the home. There were no significant differences in these characteristics among the infertile patients and endometriosis patients compared with the respective control groups. At the time of surgery, the infertile patients and endometriosis patients were significantly younger (34.1 and 35.3 years old, respectively) than women in the fertile and non-endometriosis control groups (39.5 and 39.1 years, respectively; P = 0.0001 for both) (TABLE 1). However, as expected, the number of deliveries was higher among women in the control non-endometriosis group compared with the endometriosis patients (P = 0.0001) (TABLE 1).

hrHPV types positivity in the UGT is associated with infertility and endometriosis

A total of 13 different HPV types were detected: 7 (53.8%) lrHPV and 6 (46.2%) hrHPV. Of the 60 women included in the study, 36 (60%) were positive for DNA-HPV in the LGT, UGT or in both. Of these DNA-HPV positive women, 25 (69.4%) had only hrHPV, 7 (19.4%) had only lrHPV, and 4 (11.1%) had multiple HPV infections, with lrHPV and/or hrHPV types. hrHPV 16 was the most prevalent (n = 17 women, 47.2%) among the patients and controls, followed by hrHPV 82 (n = 5/36, 13.9%), and lrHPV6 (n = 3/36, 8.3%). Other HPV types had a frequency ≤5.6% (TABLE 2).

Considering the infertile patients, 15 had HPV-DNA (60%), 13 of whom had hrHPV (87%). The infertile patients were statistically associated with hrHPV type positivity in the UGT sites (n = 11/25, 44.0%) compared with the fertile control group (n = 6/35; 17.1%), with an increased risk of over three times (OR 3.78, 95% CI 1.10–12.30; P = 0.027). Interestingly, only hrHPV types were detected in the UGT, both in the infertile patients and in the fertile control group (TABLE 3, FIGURE 2A).

The endometriosis patients were associated with DNA-HPV positivity (n = 24/29; 82.8%) compared with the non-endometriosis control group (n = 12/31; 38.7%), with an increased risk of infection greater than six times (OR 6.64, 95% CI 2.00–22.04; P = 0.001). Considering hrHPV specifically, the results were similar (OR 6.5, 95% CI 2.08–20.30; P = 0.0007). Nevertheless, endometriosis patients were associated with hrHPV in the LGT sites, with an increased risk of infection over eight times (OR 8.5, 95% CI 2.50–28.70; P = 0.0002), and in the UGT sites, with an increased risk (OR 3.6, 95% CI 1.09–12.30; P = 0.03). Again, only hrHPV types were detected in the UGT, both in endometriosis patients and in controls (TABLE 3).

hrHPV continuum along the female reproductive tract

In addition to the association between infertility and endometriosis patients with hrHPV in the UGT, it was possible to observe that hrHPV 16, 31, 66 and 82 had an infection continuum from the LGT to the UGT (TABLE 2). More specifically, in 13/36 (36.1%) of the HPV-positive cases, the same hrHPV type was detected in both the LGT and UGT. Additionally, it was found that only infertile (FIGURE 2A) and endometriosis (FIGURE 2B) patients had hrHPV in the ovaries, whereas their control groups did not. Nevertheless, the endometriosis patients had hrHPV in the uterine tubes, ovaries and peritoneum, but their controls did not (FIGURE 2B). FIGURE 3 schematically represents the

TABLE 1 SOCIODEMOGRAPHIC CHARACTERISTICS OF WOMEN (*N* = 60)

Patient characteristics	Infertile patients (*n* = 25)		Fertile control group (*n* = 35)			Endometriosis patients (*n* = 29)		Non-endometriosis control group (*n* = 31)		
Age ranges (years)	***n***	**%**	***n***	**%**	**[a]*P*-value**	***n***	**%**	***n***	**%**	**[a]*P*-value**
25 to 30	2	8	1	2.9		3	10.3	0	0	
31 to 40	22	88	16	45.7		23	79.3	15	48.4	
>40	1	4	18	51.4		3	10.3	16	51.6	
Median	34.1		39.5		0.0001	35.3		39.1		0.0001
Marital status										
Married/cohabiting	25	100.0	35	100.0		29	100.0	31	100.0	
Single/non-cohabiting	0	0	0	0		0	0	0	0	
Education (years)										
<8	0	0	0	0		0	0	0	0	
≥8	25	100.0	35	100.0		29	100.0	31	100.0	
Deliveries										
0	25	100.0	0	0	0.0001	20	69.0	5	16.1	
≥1	0	0	35	100.0		9	31.0	26	83.9	0.0001
Family income (per month)										
<$1500	0	0	0	0		0	0	0	0	
≥$1500	25	100.0	35	100.0		29	100.0	31	100.0	
Job/profession										
Housewife	3	12	10	28.6	0.22	7	24.1	6	19.4	NS
Outside home	22	88	25	71.4		22	75.9	25	80.6	
Age of first sexual intercourse (years)										
<16	5	20	9	25.7	0.84	7	24.1	7	22.6	NS
≥16	20	80	26	74.3		22	75.9	24	77.4	

NS = not statistically significant.
*Student's *t*-test.

hrHPV infection continuum along the female reproductive tract of endometriosis patients.

hrHPV is associated with the severity of endometriosis

In terms of the endometriosis staging, there was a significant association between endometriosis grade I–II and hrHPV in the UGT ($P = 0.017$), with the highest percentage of patients presenting with hrHPV in the UGT also presenting with grade I–II endometriosis ($n = 11$; 61.1%). In addition, the chance of a patient with grade I–II endometriosis presenting with hrHPV in the UGT was 3.71 (95% CI 1.29–15.91) times greater than that of the non-endometriosis control group. However, there was no significant association between grade III endometriosis and hrHPV in the UGT ($n = 2$; 11.1%). The chance of an individual with grade III endometriosis presenting with hrHPV in the UGT was 5.4 times greater than the chance of an individual in the non-endometriosis control group (95% CI 1.07–97.25).

Other STI

The seven STI agents studied were not statistically associated with infertility or endometriosis patients (TABLE 4). Among seven STI analysed, *C. trachomatis* was the most common and was always detected in the LGT sites, except for one infertile patient with endometriosis, in whom it was detected in the cervix and in the endometrium. Note that *T. pallidum*, *N. gonorrhoeae* and *M. genitalium* were detected in the endometrium; HSV-2, *C. trachomatis* (as described above) and *N. gonorrhoeae* in the ovaries; HSV-2 was detected in the tubes, and *M. genitalium* in the peritoneum (data not shown).

Furthermore, STI agents alone or in combination were not associated with hrHPV in the UGT. Finally, the positivity for the STI agents together was associated with HPV-DNA in the LGT ($P = 0.015$), with a chance of a patient with some STI presenting HPV-DNA in the LGT of 3.67 (95% CI 1.37 to 13.70) times greater than in women negative for STI.

DISCUSSION

The present study aimed to evaluate the influence of a possible HPV infection continuum in the pathogenesis of infertility and endometriosis by sampling systematically six sites along the female reproductive tract from women of reproductive age submitted for gynaecological laparoscopy. In addition, seven other important STI described for their possible involvement in infertility were analysed. For the first time, it was shown that there is a continuum of hrHPV infection, from the LGT to the UGT, and an association between hrHPV infection in the UGT with infertility, and particularly with endometriosis.

TABLE 2 FREQUENCY OF HPV INFECTION GROUPED BY CARCINOGENIC RISK AND GENITAL TRACT SITE OF DETECTION

HPV carcinogenic risk	HPV type	LGT (*n*)	UGT (*n*)	LGT and UGT (*n*)	Total women *n* (%)
Single high-risk (hr) infection					
	16	5	2	10	17 (47.2)
	82	2	1	0	3 (8.3)
	31	–	–	2	2 (5.6)
	56	1	–	–	1 (2.8)
	58	–	2	–	2 (5.6)
Total single hrHPV infection	–	8	5	12	25 (69.4)
Single low-risk (lr) infection					
	70	1	–	–	1 (2.8)
	62	1	–	–	1 (2.8)
	11	1	–	–	1 (2.8)
	72	1	–	–	1 (2.8)
	06	3	–	–	3 (8.3)
Total single lrHPV infection	–	7	–	0	7 (19.4)
Multiple HPV (lr and/or hr) infection					
	66+82 (hr+hr)	–	–	1	1 (2.8)
	82+62 (hr+lr)	1	–	–	1 (2.8)
	54+83 (lr+lr)	1	–	–	1 (2.8)
	11+72 (lr+lr)	1	–	–	1 (2.8)
Total multiple HPV infections	–	3	–	1	4 (11.1)
Total	–	18	5	13	36 (100.0)

The numbers do not represent a sum, due to multiple HPV type infections (*n* = 4).
HPV = human papillomavirus; LGT = lower genital tract; UGT = upper genital tract.

Nevertheless, no association was found between the other seven STI agents and infertility or endometriosis in the anatomical sites evaluated.

It is thought that only three previous studies have evaluated the possible association of HPV with endometriosis (*Heidarpour et al., 2017; Oppelt et al., 2010; Vestergaard et al., 2010*). The results of this study align with two of these other studies (*Heidarpour et al., 2017; Oppelt et al., 2010*) and highlight additional important information, particularly in relation to the hrHPV continuum infection from LGT to UGT sites. More specifically, *Oppelt et al. (2010)* have investigated the association between viral STI infections and endometriosis in 66 tissues, including peritoneum, ovary and endometrium, using PCR-based enzyme-linked immunosorbent assay. hrHPV was detected in 11.3% and 27.5% of lesions in

TABLE 3 DISTRIBUTION OF DNA-HPV TYPING ACCORDING TO GENITAL TRACT SITES IN INFERTILITY AND ENDOMETRIOSIS VERSUS CONTROL GROUPS

HPV oncogenic risk	Infertile patients (*n* = 25)	Fertile control group (*n* = 35)	OR	CI	[a]*P*-value	Endometriosis patients (*n* = 29)	Non-endometriosis control group (*n* = 31)	OR	CI	[a]*P*-value
	N	*n*				*n*	*n*			
DNA-HPV	15	21	1	–	1	24	12	6.64	2.00–22.04	0.001
hrHPV	13	12	1.8	0.64–5.19	NS	19	6	6.5	2.08–20.30	0.0007
hrHPV-LGT	12	11	2.014	0.69–5.80	NS	18	5	8.5	2.50–28.70	0.0002
hrHPV-UGT	11	6	3.78	1.10–12.30	0.027	12	5	3.6	1.09–12.30	0.03
lrHPV	2	9	0.2	0.05–1.20	–	5	6	0.86	0.20–3.20	NS
lrHPV-LGT	2	9	0.34	0.06–1.83	–	5	6	0.86	0.20–3.20	NS
lrHPV-UGT	0	0	–	–	–	0	0	–	–	–

The numbers do not represent a sum, due to multiple HPV type infections (*n* = 4).
[a] Chi-squared test.CI = confidence interval; HPV = human papillomavirus; hrHPV = high-risk HPV; LGT = lower genital tract; lrHPV = low-risk HPV; NS = not statistically significant; OR = odds ratio; UGT = upper genital tract.

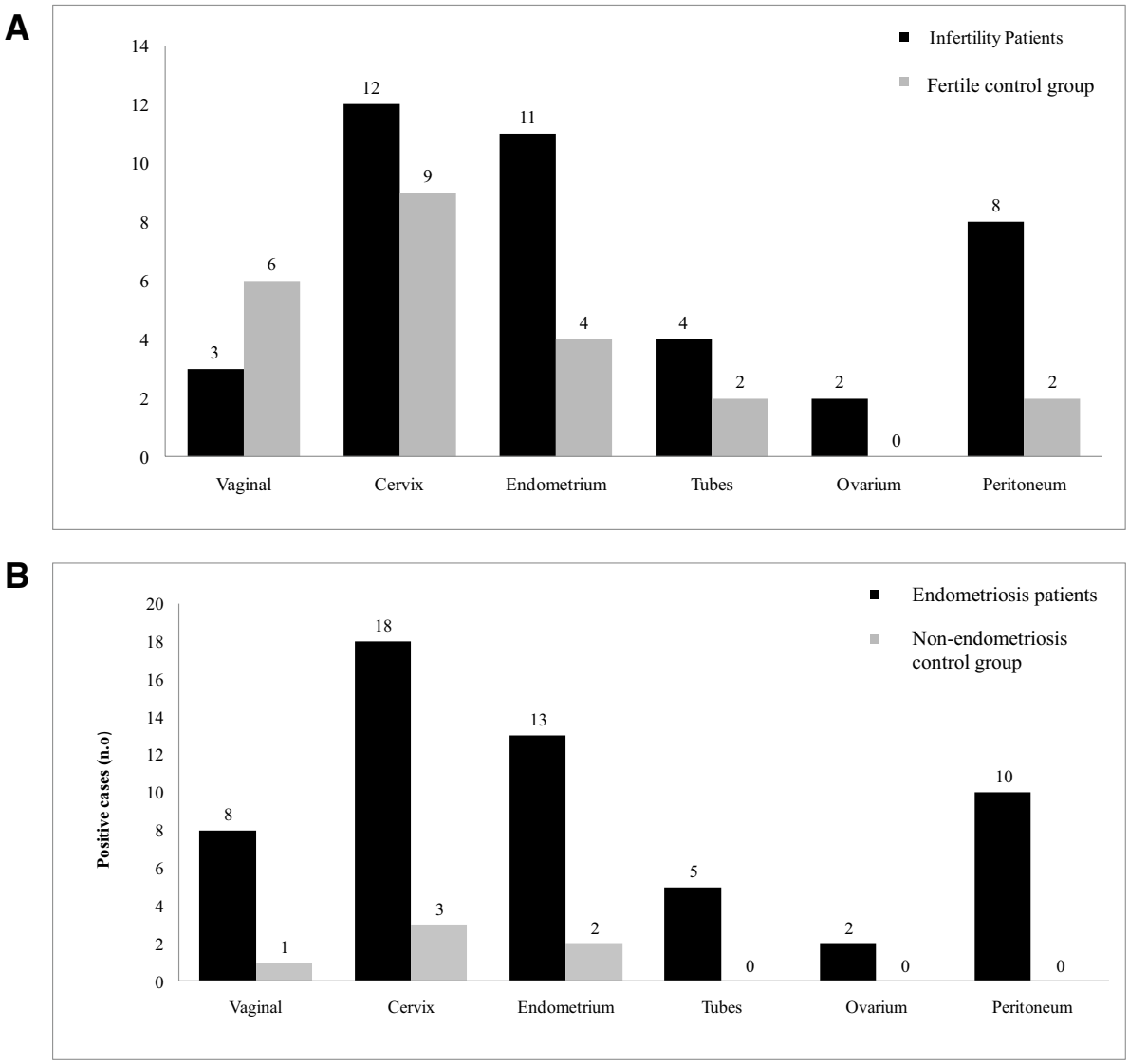

FIGURE 2 A comparison of HPV-DNA and hrHPV positivity between (A) infertility patients with the fertile control group, and (B) endometriosis patients with the non-endometriosis control group, systematically sampling six sites along the female reproductive tract. The total number of hrHPV infections is greater than the number of women with hrHPV positivity (n = 27) as some women presented the infection in both lower and upper genital tracts, as well as infections by multiple types of hrHPV. HPV = human papillomavirus; hrHPV = high-risk human papillomavirus.

the case and control groups, respectively. The authors supposed that the presence of HPV infection, in patients and in controls, supports the spreading of HPV-infected endometrial cells through retrograde menstruation. *Heidarpour et al. (2017)* have investigated the presence of hrHPV in ovarian endometriosis. The findings of this investigation indicated that hrHPV infection was significantly higher in women with endometriosis than in the control group. Based on the results of this study, which are in accordance with these other two studies, new and fundamental information has been added. First, a possible hrHPV infection continuum from the LGT to UGT was observed. Second, only hrHPV ascended to the UGT, and third, only women hrHPV positive in the UGT were associated with infertility, primarily with endometriosis. Regarding the third study that evaluated the presence of HPV in endometriotic tissues (*Vestergaard et al., 2010*), data from this study are quite different. These authors studied the possible involvement of pathogenic viruses, including HPV, in patients with and without endometriosis using sensitive PCR tests. The endometriosis tissues analysed were collected from endometrium and were of ectopic focus, such as the ovaries or peritoneum. They reported three HPV-positive cases from 77 patients with endometriosis, with both hrHPV and lrHPV types identified. They concluded that regarding the low prevalence of HPV in endometriotic lesions, HPV could not be the cause of endometriosis (*Vestergaard et al., 2010*).

Furthermore, in relation to the HPV frequency detected in this study compared with that described in these three previous studies, our rates were higher. DNA-HPV was detected in 60% of women studied, 69.4% of whom had only hrHPV. *Heidarpour et al. (2017)* have described hrHPV frequencies of 26% and 10.2% of samples with and without endometriosis, respectively. *Oppel et al. (2010)* detected hrHPV and medium-risk HPV in 11.3% of lesions (13.2% of patients) versus 27.5% of control tissues.

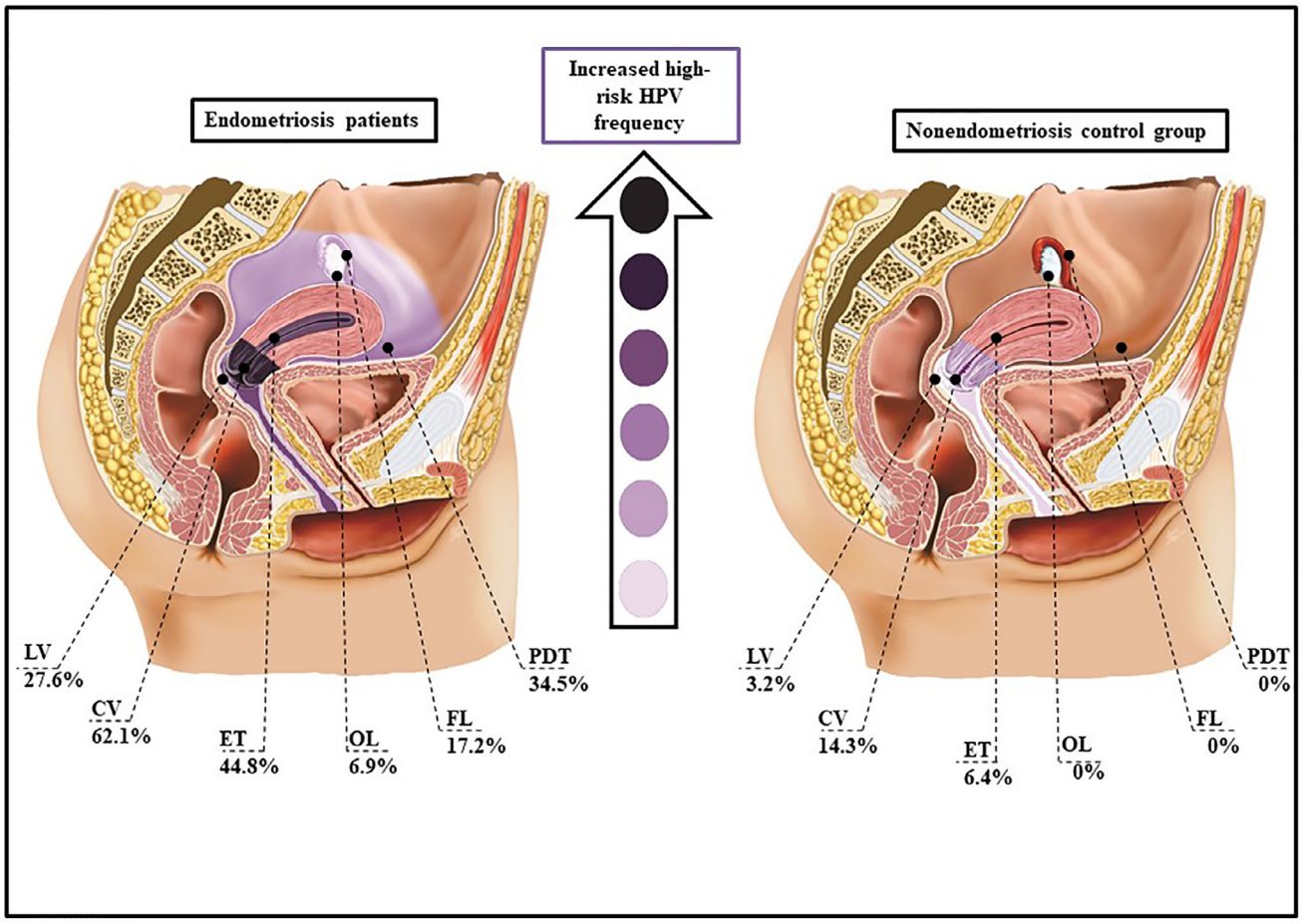

FIGURE 3 A schematic representation of continuous hrHPV infection along the female reproductive tract of patients with endometriosis. (A) Endometriosis patients with a high rate of hrHPV infection that is a continuum from the lower to the upper genital tract, including the uterine tubes, ovarium and peritoneum. (B) The non-endometriosis control group showed a low rate of hrHPV infection, most of them in the lower genital tract up to the endometrium. CV = cervical canal; ET = endometrium; FL = uterine tubes; LV = vagina; OL = ovary; PD = pouch of Douglas.

Finally, for *Vestergaard et al. (2010)*, only 3.9% of the endometriosis patients were HPV-positive for both hrHPV and lrHPV types.

An interesting aspect of this study was a possible continuous infection of the LGT to UGT only in women positive for hrHPV but not in those positive for lrHPV. According to *Castle et al. (2007)*, hrHPV types appeared to have a similar affinity for vaginal and cervical epithelium, but lrHPV types may have a tropism for vaginal epithelium. Considering this evidence, our results highlight the possibility of hrHPV having affinity with the UGT epithelia. However, there is a need for future studies that address and prove this hypothesis.

Together, our current findings are consistent with a recent theory of infection involvement in endometriosis. As previously mentioned, endometriosis is considered to be a common gynaecological problem that has a multifactorial pathogenesis. Considering that immunological events are possibly involved in endometriosis, the concept of a causal role of infectious agents has recently developed (*Burney and Giudice, 2012; Kobayashi et al., 2014*). It has been suggested that infections could initiate the endometriosis process by altering pro-inflammatory pathways and innate immunity. Moreover, clinical studies have indicated that endometriosis lesions can resemble 'punctuate blister-like lesions', which leads to the hypothesis of the association of viral infections with endometriosis lesions (*Oppelt et al., 2010*). In support of this hypothesis, a recent study showed an association between hrHPV and ovarian endometriosis, and the authors concluded that HPV could predispose the capacity of the invasiveness of the endometrial tissue, possibly through impairment of the immune response (*Heidarpour et al., 2017*). Our results align with these theories because we showed a possible ascending and hrHPV infection continuum that is associated with endometriosis and infertility. Nevertheless, we observed a significant association between endometriosis grade I–II and hrHPV in the UGT and no association between grade III endometriosis and hrHPV in the UGT. Considering these data and the fact that few women in this study had grade III endometriosis, and none had grade IV, we can postulate that the positivity for hrHPV in the UGT seems to be more associated with the initial pathogenesis of endometriosis than its worsening. However, we are aware, once again, that our results are the first to point in this direction and that future studies should be performed to clarify this hypothesis.

Two other aspects regarding our results for hrHPV in the UGT and its association with infertility and endometriosis are worth highlighting. First, considering

TABLE 4 DISTRIBUTION OF OTHER STI POSITIVITY ACCORDING TO INFERTILITY AND ENDOMETRIOSIS PATIENTS

Other STI	Infertile patients (*n* = 25)	Fertile control group (*n* = 35)	OR	CI
	n	***n***		
C. trachomatis	7	7	1.55	0.46–5.10
N. gonorrhoeae	1	2	0.68	0.06–7.30
T. pallidum	0	1	–	–
M. genitalium	1	1	0.04	0.09-21.30
T. vaginalis	0	1	–	–
HSV-1	0	1	–	-
HSV-2	1	1	0.04	0.09-21.30
Other STI	**Endometriosis patients (*n* = 29)**	**Non-endometriosis control group (*n* = 31)**	**OR**	**CI**
	n	***n***		
C. trachomatis	10	5	2.70	0.82–5.50
N. gonorrhoeae	2	1	0.48	0.04–5.20
T. pallidum	0	1		
M. genitalium	1	1	0.93	0.06–14.20
T. vaginalis	0	1	0.48	0.04–5.20
HSV-1	0	1	0.48	0.04–5.20
HSV-2	1	1	0.93	0.06–14.20

Chi-squared test; the seven STI agents studied were not statistically associated with infertility or endometriosis patients.
CI = confidence interval; OR = odds ratio; STI = sexually transmitted infections.

the oncogenic potential of hrHPV, our evidence could lead to the hypothesis that there is a higher risk of UGT cancer development in women with infertility, primarily with endometriosis. Consistent with this hypothesis, *Oppelt et al. (2010)* have proposed that persistent HPV infection of endometriosis lesions could contribute to malignant progression. Secondly, it has been shown that HPV infection was three times more prevalent in spontaneous abortion specimens compared with specimens from elective abortions (60% versus 20%, respectively). The authors suggested that HPV might be an aetiologic agent of some miscarriages and that these viruses may be closely linked to fetal pathology (*Hermonat et al., 1997*). Additionally, only a limited number of studies have investigated the influence of HPV specifically in female fertility, including assisted reproductive technology (*Zacharis et al., 2018*). In this sense, our results point to a possible negative effect of HPV on human reproduction. Considering the importance of the two themes discussed above, they should be investigated in detail in future studies in different populations.

In terms of the other seven STI analysed, a recent study by our group described a case of infertile women with ovarian endometriosis and concomitant infection by HSV-2, suggesting ascending infection and a relationship with endometriosis/infertility for this virus (*Rocha et al., 2017*). However, in this study no significant association between HSV-1/2 and infertility or endometriosis was observed. Similar results were found for the other STI agents studied. Furthermore, STI agents alone or in combination were not associated with hrHPV in the UGT, indicating that these agents are not associated with hrHPV in the pathogenesis of endometriosis and infertility. Nevertheless, some interesting results can be highlighted. *T. pallidum*, *N. gonorrhoeae* and *M. genitalium* were detected in the endometrium; HSV-2, *C. trachomatis* and *N. gonorrhoeae* were detected in the ovaries; HSV-2 was detected in the tubes, and *M. genitalium* in the peritoneum. These data point to a possible ascending infection, not only by hrHPV but also by other STI, similar to what has recently been described for vaginal microbiota (*Chen et al., 2017*). However, one aspect of our study to be considered is that all women studied (cases and controls) had high family incomes (higher than $1500/month). These data show that although this study was performed in Brazil, it was also performed in a private health network clinic that is frequented by women of high socioeconomic levels. Data on the prevalence of HPV and other STI, as well as their association with infertility and endometriosis, may be much higher in populations with a low socioeconomic status, known to have a high prevalence of STI (*Götz et al., 2019*).

This study has some limitations that deserve to be discussed. As is inherent in any case–control study, we cannot establish precise cause and effect mechanisms. A statistical advantage was the possibility of establishing similar groups, considering patients with and without infertility and with and without endometriosis. Despite this, the sample size is relatively small, making it necessary to increase this in future studies in order to confirm our findings. Also, the endometriosis group according to the grading of the disease is small, which compromises statistical analysis of the data.

It is thought that no other published studies have considered a hrHPV infection continuum in the context of infertility and endometriosis. We have demonstrated that there is a possible ascending and hrHPV infection continuum, from the LGT to the UGT. Additionally, an association between hrHPV infection and infertility and/or endometriosis was observed in the UGT. Although the exact mechanisms of this remain to be elucidated, the results of this study may contribute to understanding of the physiopathology of endometriosis and may be helpful to better understand the contribution of HPV to the development and/or maintenance of the disease. Overall, our findings should serve as the basis for future larger studies that more fully investigate the role that hrHPV may play in causing endometriosis and infertility.

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