Histopathological examination of the ectocervical biopsy in non-transplanted uteri: A study contributing to the provisional scoring system of subclinical graft rejection after uterus transplantation

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Abstract

Introduction: Uterus transplantation is a causal treatment for absolute uterine factor infertility. Assessing rejection signs using a histopathological examination of the ectocervical biopsy from the transplanted uterus is common practice in all human uterus transplants worldwide to date. A provisional scoring system was used for the histopathological assessment of subclinical rejection signs in uterus recipients. Here we hypothesized that histopathological and immunohistochemical findings in the normal uteri would differ from the borderline category of subclinical rejection in uterine transplants.

Material and methods: This prospective observational study included ectocervical biopsies of 54 women who underwent hysterectomy for benign reasons. All biopsy samples were assessed histopathologically and immunohistochemically.

Results: Most of the ectocervical biopsies showed clustering lymphocytic infiltrates affecting the stromal–epithelial interface with the epithelial influx of lymphocytes, primarily CD45RO-positive activated T-cells with CD8 T-lymphocyte predominance. CD4-positive T-lymphocytes and B-cells were rarely detected in the ectocervix. These morphological findings and immunoprofiles of lymphocytic populations overlapped with the so-called borderline changes defined in the provisional scoring system for rejection in the transplanted uteri. The immunoprofiles of ectocervical and endocervical lymphocytic populations differed, with strikingly prominent B-cell participation in the endocervix vs the rare detection of B-cells in the ectocervix.

Conclusions: The histopathological and immunohistochemical findings in the uteri of premenopausal women were similar to the borderline category of the currently used provisional scoring system of subclinical uterine rejection utilized in all uterine transplant studies. However, future similar studies are required to validate our findings.

Abbreviations: H&E, hematoxylin and eosin; UTx, uterine transplantation.
1 | INTRODUCTION

The first human uterus transplantation (UTx) trial for the treatment of absolute uterine factor infertility began with procurement and transplant procedures using the living donor concept. Since then, this experimental treatment method has undergone significant progress and expansion. In late 2014, the first-ever child after living donor UTx was born in Gothenburg, Sweden, followed by several others, including two uterine recipients having two offspring each. The first Swedish UTx study commenced in 2012–2013 and was the first-ever concluded human experimental trial worldwide. In late 2017, the first childbirth after deceased donor UTx was achieved in Sao Paolo, Brazil. The onset of the Czech mixed living and deceased donor UTx trial was preceded by a UTx study of interest in a group of women with congenital agenesis of the uterus in whom a neovagina was surgically created using laparoscopic Vecchietti’s vaginoplasty. The surgical results of the nine Czech UTx cases were published in 2019 and the preliminary assisted reproductive outcomes were recently reported.

Several aspects of UTx remain under investigation, including monitoring of the signs of uterine graft rejection after transplantation and during pregnancy. Given the lack of less invasive methods to control UTx rejection, the current practice is based on regular ectocervical biopsies and their histopathological evaluation according to the provisional scoring system of uterine rejection suggested by Mölne et al. Based on this classification, histopathological rejection of the uterine graft is divided into mild, moderate and severe grades, and a borderline change category. It is currently uncertain whether the above changes seen in the uterine ectocervix correspond to changes occurring in the entire transplanted uterus. However, based on the detailed histological assessment of the seven uterine explants, the Swedish pioneers in UTx research recently reported that the inflammatory changes in the uterine cervix represent those throughout the entire uterus; therefore, ectocervical biopsy seems suitable for allograft control. Here we report analyses of histopathological and immunohistochemical findings of ectocervical biopsies among a group of healthy premenopausal women who underwent hysterectomy. We further aimed to compare our findings with the scoring system for subclinical rejection in women with transplanted uterus.

2 | MATERIAL AND METHODS

A total of 54 premenopausal women with benign uterine conditions such as uterine leiomyoma (n = 25) and abnormal uterine bleeding (n = 29) confirmed by hysteroscopy who were scheduled for an abdominal or laparoscopic-assisted vaginal hysterectomy at our obstetrics and gynecology department were included in this prospective non-randomized study. We aimed to perform an ectocervical biopsy without colposcopy magnification at the start of surgery under general anesthesia to minimize the morbidity of the participants related to an otherwise painful cervical biopsy. A biopsy was performed close to the transformation zone and the cervical canal from the anterior portion of the uterine cervix.

The inclusion criteria for the study participants were as follows: premenopausal age, non-malignant indication for surgery, normal preoperative cervical Papanicolaou smear findings, no vaginal or cervical infection at the time of biopsy, no perioperative uterine bleeding, no immunological or autoimmune disease, no immunosuppressant use, and no use of corticosteroids or any medication altering the immunological status. Informed consent was obtained from each participant after we explained the principles and aims of the trial and the risks related to ectocervical biopsies. The participants’ main demographic characteristics were documented.

The study hypothesis was that histopathological and immunohistochemical findings of the normal uteri would differ from the borderline categories of rejection of uterine transplants suggested as an analogy to the rejection classifications for other organ transplants to avoid overtreatment by antirejection therapy. Borderline changes in transplanted uteri are histopathologically characterized as a few small and nonconfluent, at least two nested foci of inflammation, predominantly with lymphocytes in the epithelial–stromal interface, and frequently accompanied by intercellular edema. Minimal inflammation in the papillary stroma can also be detected. The morphological findings of the cervix in the proliferation and secretion phases of the menstrual cycle were also compared, particularly with respect to cellular infiltrates in the epithelial–stromal interface and the cervical stroma.

2.1 | Histopathological examination

Biopsy samples (3–4 × 6–8 mm) were fixed in neutral buffered 4% formaldehyde, transported to the histopathological laboratory, postfixed and embedded in paraffin. Subsequently, the paraffin blocks were sectioned into 4-µm-thick histological sections and
stained with hematoxylin and eosin (H&E) and Masson’s trichrome staining (to differentiate collagen fibers and fibrosis foci, including intimal sclerosis of the arteries). The histopathological examination focused on the epithelial–stromal interface and cellular infiltration into the cervical stroma. Cervical biopsies were assessed for the presence of inflammatory infiltrates (including interface and microvascular and perivascular stromal inflammation), arteriopathy (including endothelialitis and intimal sclerosis) and epithelial dysplasia. All samples were evaluated by two pathologists specializing in gynecological and lung and heart transplantations with experience in histological assessment of cervical biopsies of transplanted uteri.

2.2 Immunohistochemistry

The biopsies were immunostained for CD45RO, CD8, CD4, CD20 and C4d, particularly to characterize cellular infiltration. Thin histological sections (3 µm thick) were used, and each sample was stained using the following antibodies and protocols: anti-CD45RO antibody (clone UCHL1 [Agilent–Dako, Santa Clara, CA, USA], dilution 1:300, pretreatment by heating in a buffer solution of pH 6 in a water bath); anti-CD8 antibody (clone C8/144B-[Agilent], dilution 1:200, pretreatment by heating in a buffer solution of pH 9 in a water bath); anti-CD4 antibody (clone 4B12 [BioGenex Laboratories, Fremont, CA, USA], dilution 1:250, pretreatment by heating in a buffer solution of pH 9 in a water bath); anti-CD20 antibody (clone L26 [Agilent], dilution 1:300, pretreatment by heating in a buffer solution of pH 6 in a water bath); anti-C4d antibody (clone A24-T [Zytomed Systems GmbH, Berlin, Germany], dilution 1:150, pretreatment by heating in a buffer solution of pH 6 in a water bath). The detection was performed using a one-step micropolymeric non-biotin system (Bio SB– Bioscience for the World, Santa Barbara, CA, USA) with a peroxidase complex and 3,3’-diaminobenzidine tetrahydrochloride. The nuclei were counterstained with hematoxylin.

2.3 Semiquantitative scoring system

To characterize lymphocyte populations using H&E staining and immunohistochemistry, a semiquantitative scoring system was used instead
of absolute cell count to increase the reproducibility of the routine biopsy evaluations. The five-tier score was assessed (Figure 1) as follows.

Category 1: None or up to 20 isolated lymphocytes within the entire biopsy sample (number of lymphocytes corresponding to the normal cervical biopsy finding according to the provisional scoring system for UTx rejection).10

Category 2: More than 20 scattered cells containing sparse lymphocytes without clustering within the biopsy sample (corresponding to the normal finding according to the provisional scoring system for UTx rejection).10 However, these two categories were established for classification purposes, as healthy ectocervical tissues were examined assuming slight inflammatory changes only.

Category 3: Clustering cells containing lymphocytes tending to form small clusters at the epithelial-stromal interface (this can be seen in borderline changes according to the provisional scoring system for UTx rejection).10

Category 4: Focal infiltrates containing lymphocytes tending to form large nests or even lymphatic follicles with possible participation of other types of inflammatory cells (this can be seen in grade 1 and 2 rejections according to the provisional scoring system for UTx rejection). Both grades share the same degree of inflammation, and grade 2 was recognized if inflammatory cells were accompanied by stromal edema and reduced surface epithelial thickness.10

Category 5: Diffuse infiltrates within the tissue (this can be seen in grade 3 according to the provisional scoring system for UTx rejection). These changes might be accompanied by epithelial erosions/ulcerations and focal necrosis.10

The basic subsets of T-lymphocytes were evaluated using immunohistochemical markers CD4 and CD8 to identify the dominant types of inflammatory infiltrates and assess similarities to and differences from rejection in the transplanted organ. Both cytotoxic and helper T-cells are T-cell subsets; therefore, a semiquantitative system for the evaluation of the entire T-cell population (using CD45RO) was not used to evaluate these subsets. The subgroup of patients with more than 80% CD4+ or CD8+ lymphocytes was recorded. In the subgroups with lymphocytic infiltration without the prevalence of any subtype or with sparse lymphocytic infiltrate, an admixture of these cells was recorded. Cases with isolated or no T-cells were also recorded.

### 2.4 Ethical approval

The study protocol was approved by the Institutional Review Board and Ethics Committee of Motol University Hospital (EK-34/20) on 29 January 2020.

### 3 RESULTS

Among the patients in the study group, the mean age was 45.1 ± 3.97 years (range 36–54 years), mean body mass index was 26.6 ± 6.1 (range 17.1–40.8) and the mean parity was 1.9 ± 0.89 (range 0–4). Of the 54 total biopsies, 26 were taken in the proliferation phase vs 28 in the secretion phase of the menstrual cycle; the phases were confirmed by histopathological dating of the endometrium in the removed uteri. A subgroup comparison did not confirm the impact of menstrual cycle phase on histopathological findings in terms of cellular infiltrates in the epithelial–stromal interface and the cervical stroma.

### 3.1 Ectocervical results

Evaluation of the biopsies revealed that 52 of 54 samples were suitable for assessment and contained some degree of lymphocytic infiltrate within the ectocervical portion of the uterine cervix; however, none correlated to the first category of the proposed semiquantitative scoring system (Table 1). Similar to other study findings,10,11 immunohistochemical analysis of the ectocervical lymphocytic population proved that the vast majority of inflammatory cells were T-lymphocytes (positive for immunohistochemical marker CD45RO in all cases). In five (9.6%) samples, lymphocytes were scattered and corresponded to the second category (Figure 2). However, the majority of cases fulfilled the criteria for the third category: 43 (82.7%) showed clustered lymphocytes, and in every sample, inflammatory changes were identified at the epithelial–stromal interface with the intraepithelial influx of isolated lymphocytes (Figure 3). Another four cases (7.7%) contained focal lymphocytic infiltrates (fourth category) (Figure 4), whereas tissue sampling was inadequate in the remaining two participants, from whom only endocervical tissue was obtained. Additionally, no ectocervical samples showed diffuse lymphocytic infiltration correlating with the fifth category. The epithelial influx of lymphocytes within the ectocervix was recorded in 51 (98%) samples. Moreover, lymphocytes were found in the perivascular areas of patients with focal lymphocytic infiltrates (Figure 4). These signs

| Category | Samples (n) | % |
|----------|------------|---|
| Category 1 | 0 | 0 |
| Category 2 | 5 | 9.6 |
| Category 3 | 43 | 82.7 |
| Category 4 | 4 | 7.7 |
| Category 5 | 0 | 0 |

| Category | Samples (n) | % |
|----------|------------|---|
| Category 1 | 50 | 96 |
| Category 2 | 1 | 2 |
| Category 3 | 0 | 0 |
| Category 4 | 1 | 2 |
| Category 5 | 0 | 0 |
overlap with the borderline change category. None of the cervical biopsies revealed signs of human papilloma virus infection (epithelial dysplasia and/or koilocytosis), endothelialitis or intimal sclerosis of the vessels. These findings are consistent with the histopathological results of the removed uteri.

The T-cells were subdivided into subtypes using the immunohistochemical markers CD8 and CD4. In 24 (46%) cases, CD8^+ cytotoxic T-lymphocytes constituted the major cellular fractions. In six (11.5%) cases, T-lymphocytes were scattered and represented an admixture of the inflammatory infiltrate; in 17 (33%) cases, only isolated cells were observed. Five (9.5%) cases were completely negative for the immunohistochemical marker CD8. Two samples contained only endocervical tissue and were inadequate for assessment. There were no (in 49 cases) or only isolated (in three cases) CD4^+ helper T-lymphocytes among the lymphocytic population.

Compared with T-cells, B-lymphocytes represented a minority of the total immune cells within the ectocervix, being absent in 41 (79%) cases or isolated in nine (17%) cases (first category), scattered in one (2%) case (second category), and forming focal infiltrates in one (2%) case (fourth category). This finding was consistent with that of a previously suggested provisional scoring system for UTx rejection.10 The majority of the ectocervical biopsies showed clustered lymphocytic infiltrates affecting the stromal–epithelial interface with epithelial influx, consisting mainly of CD45RO-positive activated T-cells with CD8 T-lymphocyte predominance. CD4^+ T-lymphocytes and B-cells were rarely detected. Such immunoprofiles of the immune cell populations shared rejection infiltrates in other transplanted solid organs, such as the lungs.12,13

3.2 | Endocervical results

The aim of the biopsy was to take a sample of the ectocervical tissue only, but in 15 (27.8%) cases, a sample of the endocervix was also obtained; all showed some degree of inflammation (Table 2). Compared with the ectocervix, endocervical inflammatory infiltrates were usually larger and recognizable even at low magnification (Figure 5). They were composed of CD45RO-positive T-cells; in 11 of 15 cases, focal infiltrates were noted, representative of the fourth category according to the proposed semiquantitative scoring system. Another three cases contained diffused inflammatory infiltrations (fifth category), whereas the remaining sample showed clustered cells (third category).

The vast majority of the endocervical T-cells were CD8^+ T-lymphocytes, representing the main subpopulation of T-cells in 11 cases similar to the ectocervix, but three cases contained isolated Tc lymphocytes and one case was negative. No CD4^+ helper cells were identified in the endocervical tissues.

A significant difference was observed in the immunophenotype of the endocervical lymphocytic population vs the ectocervical
population: A large cohort of endocervical B-lymphocytes stained positive for immunohistochemical marker CD20. The endocervical B-cells were clustered in four of 15 cases, forming focal infiltrates in another 10 and being isolated in one case only. C4d staining was negative, showing weak non-specific positivity in all samples.

**FIGURE 3** The third category of the semiquantitative scoring system proposed by the study. (A) Light microscopy of the ectocervical biopsy (H&E staining). Clustered lymphocytes at the epithelial–stromal junction of the ectocervix appreciated even on low magnification. (B) Light microscopy of the ectocervical biopsy (H&E staining). Details of the intraepithelial influx of clustered lymphocytes found in the majority of cases even with routine H&E staining. (C) Light microscopy of the ectocervical biopsy (CD45RO staining). Immunohistochemical detection of activated T-lymphocytes using CD45RO staining. (D) Light microscopy of the ectocervical biopsy (CD45RO staining). Details of the intraepithelial T-cells. H&E: hematoxylin and eosin

**FIGURE 4** The fourth category of the semiquantitative scoring system proposed by the study. (A) Light microscopy of the ectocervical biopsy (H&E staining). Focal lymphocytic infiltrates within the subepithelial stroma. (B) Light microscopy of ectocervical biopsy (H&E staining). Perivascular focal lymphocytic infiltrate and the intraepithelial influx of inflammatory cells. (C) Light microscopy of the ectocervical biopsy (CD45RO staining). The same case is stained with immunohistochemical marker CD45RO. Note the striking intraepithelial influx of lymphocytes. (D) Light microscopy of ectocervical biopsy (CD45RO staining). Details of the previous section. H&E: hematoxylin and eosin

4 | DISCUSSION

Here we report the histopathological results of ectocervical biopsies in a group of premenopausal women in relation to rejection changes in the transplanted uterus according to Mölne et al.10 Findings similar to the borderline changes were noted in 90.4% of our ectocervical
biopsies. This study showed that the histopathological and immunohistochemical findings of ectocervical biopsies from the uteri of premenopausal women without immunity-altering therapy were similar to the borderline category of the provisional scoring system of subclinical graft rejection after UTx.

To our knowledge, no similar data on the histopathological and immunohistochemical assessments of ectocervical biopsies were published previously. However, in a recently reported study of seven uterine explants, in one of six women of a control group, an ectocervical “focal inflammation with borderline pattern” corresponding to our findings was recorded. Although our study of 54 women showed similar histopathological and immunohistochemical ectocervical findings to the borderline category of uterine rejection used in the ongoing UTx studies, further research in larger groups of non-transplanted premenopausal women are required to validate our findings.

To date, the control of post-transplant uterine rejection using a histopathological examination of the ectocervical biopsy is performed regularly before conception and several times during pregnancy. When borderline changes are histopathologically confirmed, an ectocervical re-biopsy is usually conducted. A repeated biopsy can be followed by prolongation of the interval to embryo transfer to allow healing of the post-biopsy epithelial–stromal defects (the cervical biopsy should be taken from the ectocervix close to the cervical canal as suggested by Mölne et al.).

| TABLE 2 | Endocervical biopsy results |
|----------|-----------------------------|
|          | n  | %         |
| Samples  | 15 | 100       |
|         |
| Semiquantitative scoring system of CD45RO-positive T-lymphocytes |
| Category 1 | 0  | 0         |
| Category 2 | 0  | 0         |
| Category 3 | 1  | 6.7       |
| Category 4 | 11 | 73.3      |
| Category 5 | 3  | 20        |
|          |
| Semiquantitative scoring system of CD20+ B-lymphocytes |
| Category 1 | 1  | 6.7       |
| Category 2 | 0  | 0         |
| Category 3 | 4  | 26.6      |
| Category 4 | 10 | 66.7      |
| Category 5 | 0  | 0         |

**FIGURE 5** Endocervical inflammatory infiltrates. (A) Light microscopy of the endocervical biopsy (H&E staining). Inadequate biopsy sample showing only endocervical tissue. Diffuse inflammatory changes are visible even at low magnification (corresponding to the fifth category of the semiquantitative scoring system). (B) Light microscopy of the endocervical biopsy (H&E staining). Details of the lymphocytic infiltrate within the endocervical stroma. (C) Light microscopy of the endocervical biopsy (CD45RO staining). T-cells are the main lymphocytic population within the endocervix and ectocervix, sometimes forming diffuse infiltrates. (D) Light microscopy of the endocervical biopsy (CD8 staining). CD8+ Tc cells in a population of T-lymphocytes. (E) Light microscopy of the endocervical biopsy (CD4 staining). CD4 staining was negative. (F) Light microscopy of the endocervical biopsy (CD20 staining). Unlike in the ectocervix, there are numerous endocervical B-cells. H&E: hematoxylin and eosin
Posttransplant cervical biopsies should also be performed strictly according to the study protocols regardless of embryo transfer timing. However, although the biopsy itself is not painful, because of the interrupted sensory innervation of the transplanted uterus, frequently performed biopsies may increase the morbidity and discomfort of uterine recipients, which could make its usefulness questionable, particularly in experimental trials testing the efficacy and safety of new methods.

The grade 0 category of the organ transplantation rejection scale was established to reveal an imminent rejection of an organ that is either vital (such as the kidney or lungs) or to improve quality of life (such as the hand or face). Based on current knowledge of UTx research, it is highly speculative to claim that borderline changes in the ectocervical biopsy after UTx are normal, although our study results suggest this. We agree with the recent proposal to keep the category of “inflammation of uncertain significance” (borderline category), although a similar inflammatory pattern has been observed in non-transplanted ectocervical tissue as well as in women with uterine transplants and rejection signs at other points.

We believe that using the optimal technique for cervical biopsy is crucial to ensuring an accurate assessment of cervical samples. Our data showed differences between lymphocytic infiltration within the ectocervical and endocervical tissues. The endocervical interstitium contained lymphocytes that correlated with higher scores on the proposed semiquantitative scoring system. The ectocervical biopsy can also contain areas of endocervical tissue; however, when only an endocervical stromal component is obtained, this may be confused with rejection. Therefore, a cervical biopsy after UTx should be performed by an experienced gynecologist using colposcopy magnification, which aids the identification of the optimal part of the ectocervix to biopsy to minimize the risk of incorrect sampling, for example, from a scar after a previous cervical biopsy. However, when only the endocervical stroma and no characteristic endocervical epithelial structures are detected, immunohistochemistry of the lymphocytes can identify the tissue’s origin because numerous CD20+ B-lymphocytes are detected in the endocervix but rarely in the ectocervix.

Until the discovery of less invasive methods of rejection control, ectocervical biopsy was the only safe method for the early detection of rejection signs after UTx. The pioneering experience of the first Swedish UTx trial was based solely on signs of cellular histopathological rejection; subsequent UTx studies confirmed these findings. The only different case report on uterine rejection after transplantation citing the first-ever severe mixed cell/humoral rejection, which was reversed by multiple thymoglobulin administrations, was recently published. However, only further data can confirm the above experience with the humoral component of rejection in uterine transplants.

This study had several strengths. First, it included a large number of participants. Second, it had a prospective design. Third, it suggested a semiquantitative scoring system to enhance its reproducibility. Finally, the ectocervical biopsies were taken from a group of women undergoing hysterectomy under general anesthesia and did not impact their morbidity.

This study also had some limitations. First, to the best of our knowledge, no previous reports are available for comparison. Second, this was only a single-center study. Third, our data lacked statistical support.

5 | CONCLUSION
To our knowledge, this prospective non-randomized study was the first to assess the histopathological and immunohistochemical findings of ectocervical biopsies in premenopausal women in relation to the provisional scoring system of rejection changes in transplanted uteri. Our study showed similar histopathological findings to the borderline category of the above scoring system of rejection used in previous UTx studies. However, similar studies are required to validate our findings.

CONFLICT OF INTEREST
None.

AUTHOR CONTRIBUTIONS
JB: data collection, data interpretation, manuscript writing. MN and RC: study design, data collection, data interpretation, manuscript writing. PS, ZP and RCJr: study design, data interpretation, manuscript writing. JZ: data interpretation, manuscript writing.

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