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The feasibility of a less invasive method to assess endometrial maturation—comparison of simultaneously obtained uterine secretion and tissue biopsy

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Objective To compare the assessment of endometrial maturation parameters in endometrial secretion samples obtained by a novel minimally invasive technique with those assessed in tissue biopsies.

Design Prospective study.

Setting University Hospital.

Population Healthy female volunteers attending a gynaecological outpatient clinic.

Methods Endometrial secretion fluid and tissue sampling 5 days after a spontaneous ovulation assessed with ultrasound.

Main outcome measures Progesterone (P) receptor, Ki-67 expression and the Noyes criteria were used to date endometrial biopsies. In the endometrial fluid samples, glycodelin A (GdA), leukaemia inhibitory factor (LIF) and P levels were analysed, and protein content and electrophoresis patterns were determined.

Results All data were correlated to estradiol (E_2) and P serum concentrations. The dating according to histology and immunohistochemical staining patterns correlated significantly with GdA levels ($r = 0.376$, $P = 0.048$) in endometrial fluid samples as well with serum levels of E_2 ($r = 0.568$, $P = 0.001$) and P ($r = 0.408$, $P = 0.023$). No correlation was observed between tissue dating and LIF levels and protein content in endometrial fluid samples.

Conclusions The measurement of GdA in endometrial secretion samples may provide a less invasive method for assessing endometrial maturation in potential conception cycles without disrupting implantation.

Keywords Biopsy, endometrial, glycodelin A, leukaemia inhibitory factor, maturation, uterine secretion.

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Introduction

Human implantation is a complex process that is dependent on multiple, successive interactions between the embryo and the endometrium. It is only successful when it occurs during a brief period of the secretory phase of the menstrual cycle,[1,2] usually referred to as the 'implantation window' or 'window of receptivity'.[3] The traditional 'gold standard' technique for assessing endometrial differentiation and maturation was described by Noyes *et al.*,[4] and these histological criteria are widely applied. However, the clinical value of these criteria in terms of predicting endometrial receptivity and consequently fertility are limited.[5–7] Moreover, the histological approach to monitor endometrial maturation requires an invasive biopsy that excludes its use during the luteal phase of cycles in which implantation is the end-point objective as in *in vitro* fertilization (IVF).[8]

In recent years, less invasive techniques have become available to study endometrial maturation. Aspiration and

flushing of the endometrial cavity in the peri-implantation period of menstrual cycles has been performed without detrimental effect on pregnancy rates.[8–10] Analysis of endometrial secretions showed proteins originating from transudate of serum, leakage products of apoptotic epithelial cells, and glandular secretion.[11] The composition of the secretions varies during the menstrual cycle as a result of changing ovarian steroid levels. Estradiol (E_2) regulates transudation by blood vessel dilatation and permeability, and progesterone (P) controls secretory activity of the endometrial glands. Protein composition analysis has been performed throughout the spontaneous menstrual cycle and revealed significant protein pattern changes for normal endometrium transition from proliferative to secretory.[12] Endometrial gene expression studies have shown glycodelin A (GdA) to be upregulated during the implantation window,[13] and both GdA and another putative marker of endometrial receptivity, leukaemia inhibitory factor (LIF), have been identified in endometrial secretions. These markers vary during the menstrual cycle[14–17] and appear to be differentially expressed between fertile and subfertile women.[16,18–20] However, the degree of correlation between the endometrial secretion levels of P, LIF, and GdA with the endometrial tissue markers of maturation PR and Ki-67, and Noyes criteria, is not known.

The principle aim of this study was to examine whether putative markers of endometrial maturation obtained in aspirated endometrial secretion fluids correlated with endometrial maturation monitored by the traditional histological Noyes criteria combined with immunohistochemical detection of endometrial PR and Ki-67 expression. In addition, markers of endometrial maturation were correlated with serum E_2 and P levels.

Methods

Subjects

This prospective study was approved by the local ethics committee of the Erasmus Medical Centre. Women attending the fertility clinic were asked to participate in the study. To limit the heterogeneity of the study population, inclusion criteria were: age between 18 and 40 years, normal regular menstrual cycles (ranging between 25 and 35 days), no uterus anomaly detectable by ultrasound, no hormonal contraception for at least 2 months before the study, and negative chlamydia screening by polymerase chain reaction analysis. Further exclusion criteria were evidence of endometriosis or a medical history of disease that may impair implantation or pregnancy. Thirty-four women meeting the inclusion and exclusion criteria consented to participate in the study.

Assessments

Ovarian follicle development was monitored by transvaginal ultrasound (TVS) by a single operator (M.H.v.d.G.) on cycle day 2 and 8, and then daily until the diameter of the leading follicle measured 18 mm. As changes in endometrial markers of maturation and receptivity were to be related to the day of ovulation, daily TVS was continued until spontaneous follicle rupture had been identified by the disappearance of the leading follicle and the presence of free fluid in the pouch of Douglas.[21] This validated method has been shown to be a reliable means of detecting ovulation for the purposes of endometrial dating.[22,23] On the fifth day after ovulation, the endometrial cavity and cervix length were measured by TVS, blood sampling was performed and transcervical aspiration of endometrial secretion fluid was carried out, as described previously.[8] In short, after removal of excessive cervical mucus, an insemination catheter (ASSA med GmbH, Bexbach, Germany) was cautiously inserted in the uterine cavity at a depth of the measured cervix length and 1 cm beyond. Suction was gently applied while simultaneously rotating the catheter. Immediately after the aspiration, the outside of the tip of the double outlet soft insemination catheter was cleaned with a sterile cloth. The tip containing the secretion fluid was cutoff into an Eppendorf cup and stored at –20°C until final assessment. Immediately following aspiration, an endometrial biopsy was obtained using a suction curette (Pipelle de Cornier®; C.C.D. Laboratoire, Paris, France).

Each specimen was labelled using a coding system for which the observers in the laboratory were blinded.

Serum hormone assay

Blood was centrifuged for 10 minutes at 1500 × *g* immediately after obtaining the samples, and the aliquots of serum were extracted and stored at –20°C until assessment. Serum E_2 and P were measured by an immunofluorometric assay (Immulite 2000; Diagnostic Products Corp., Los Angeles, CA, USA) performed in the same laboratory. Respective intra- and inter-assay coefficients of variation for P were less than 10% for both, and less than 5 and 7% for E_2.

Endometrial tissue analysis

After fixation in neutral buffered 3.7% formalin, the biopsied tissue samples were dehydrated with increasing ethanol concentration before they were embedded in paraffin. A portion of endometrial tissue from each specimen was routinely cut, mounted and stained with haematoxylin–eosin for histological dating according to the criteria of Noyes *et al.*[4]

For immunohistochemistry, 5-micrometer-thick paraffin sections were cut, deparaffinised and rehydrated in phosphate-buffered saline (PBS). The slides were heated in citrate buffer by microwave (4 × 5 min; 600 W) for epitope retrieval and the immunohistochemical staining was performed by a streptavidin–biotin–peroxidase method (Histostain-SP Kit; Zymed Laboratories Inc., Berlin, Germany).

The primary monoclonal antibody MIB-1 (M 7240; Dako-Cytomation, Hamburg, Germany) that was used to detect the

nuclear proliferation marker Ki-67 in endometrial tissue was diluted 1:100 in PBS/1.5% bovine serum albumin and applied overnight at 4°C. Visualisation of the Ki-67 antigen was performed by peroxidase catalysing the substrate and converting the chromogen aminoethylcarbazole (AEC) (Zymed Laboratories Inc., Berlin, Germany) to a red deposit.

For the detection of estrogen and P receptor (ER and PR), the same immunohistochemical procedure was used as described for Ki-67. The primary monoclonal anti-PR antibody (clone PgR 636, M 3569, DakoCytomation), which recognises the A and B isoform, was diluted 1:50. The anti-ER-α antibody (clone ER 6 F11; Novocastra, Newcastle upon Tyne, UK) was diluted 1:40.

As a negative control, the primary antibodies were replaced with nonimmune mouse immunoglobulin G at the same concentration. None of the negative controls revealed a positive staining. As a positive control, we used archival endometrial paraffin blocks with known positive reactivity for the studied antibodies.

All endometrial tissue assessments were performed by two observers (I.C-L., C.A.K.) who were blinded for the results of endometrial secretion fluid assessment and E and P serum levels. Since all samples were taken exactly on the fifth day after ovulation, we looked for the typical criteria according to Noyes *et al.*,[4] that is, the occurrence of glycogen vacuoles that first appear subnuclear and then shift from a subnuclear to a supranuclear location.[24] This has also been taken into account by a recent study by Tuckermann *et al.*[17] In addition to these data, we evaluated immunohistochemical staining of Ki-67 to obtain the proliferation status of epithelial versus stromal cells as well as the PR and ER expression in the glands. Endometrial epithelial expression of PR and ER was graded on a scale of 0–2+, based on the intensity of cells with positive nuclear staining: 0 if none stained, 1+ if weak staining, and 2+ if strong staining. The expression of Ki-67 in the glandular epithelium was graded on a scale of 0–3+ based on the percentage of cells with positive nuclear staining: 0 if none stained, 1+ if <10% stained, 2+ if 10–50% stained and 3+ if >50% stained. The correlation between the scorings of the two independent observers assessing Ki-67 staining was 95%.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and laser densitometry

Secretion fluids were removed from the tip of the catheter, incubated for 10 minutes in an ultrasound bath at 4°C and then centrifuged at 20 800 × g (10 minutes, 4°C). Protein concentration of the supernatant was determined[25] and the protein composition was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using 8.3–16.6% acrylamide separating gel with 5% acrylamide containing stacking gel.[26] Each lane was loaded with 50-microgram protein diluted with sample buffer. The gels were stained with Coomassie brilliant blue. Molecular weight was estimated by comparison with protein standards electrophoresed in adjacent lanes of the gel. Many protein bands appear among the lower molecular weight fractions (68-and 6.5-kDa area) in electrophoresed endometrial secretions.[12] The most pronounced and heavily staining are albumin at 68 kDa and the two of α- and β-chains of haemoglobin, close to the position of 12.5 kDa. Bands below 68 kDa that form three groups of similarly sized, partly faintly staining bands are the focus of our assessments. Group A is represented by bands between 45 and 34 kDa, group B from 29 to 25 kDa, and group C from 18 to 12 kDa. Group C awaits its completion at the time of about 2 days after ovulation. Particular attention is paid to the three intensely staining bands in the range of 15–18 kDa. The 12.5-kDa protein fraction decreases in width during the periovulatory period and remains less prominent during the luteal phase. Such pattern of the luteal phase is designated as 'optimal luteal phase pattern'. Suboptimal or impaired luteal phase patterns showed some or significant changes in groups B and C fractions.

The relative density of each band was measured using a scanning laser densitometer, He-Ne-Laser 633 nm (LKB Ultrascan XL; Pharmacia-LKB, Freiburg, Germany) and the GelScan XL software package. Protein pattern analyses were scored semiquantitatively as an optimal, suboptimal, or nonluteal protein pattern, according to previously published criteria.[27] All samples were analysed independently by two observers (K.B-H. and H.M.B.), who were blinded to the endometrial tissue assessment results.

LIF, GdA, and P assessment in endometrial secretions by enzyme-linked immunosorbent assay

The intra- and interassay variations of the LIF enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Wiesbaden-Nordenstadt, Germany) were 2.4 and 6.1%, respectively. The detection limit was 8 pg/ml. The intra- and interassay variation of the GdA ELISA (BIOSERV, Rostock, Germany) was 8.3 and 4.58%, respectively.

Statistical analysis

Because of the novel nature of this study, it was not possible to carry out an appropriate power calculation. However, it was considered that the study of 30 subjects would be sufficient to reveal important correlations.

Pearson's correlation coefficients were used for relation analysis of endometrial dating (histological Noyes criteria and cellular maturation markers), serum steroid levels, and endometrial secretion P, LIF, and GdA levels. In all statistical analyses, $P < 0.05$ was considered statistically significant. The analysis was performed with a commercially available software package (GraphPad Prism 3.00).

Results

Of the 34 women recruited to the study, three yielded insufficient endometrial tissue and these were excluded from further analysis. The median age was 32 years (range 21–38) and the median luteal cycle length was 14 days (range 11–16) (Table 1).

Although the endometrial biopsy and the endometrial secretion samples had been taken on the 5th day after ovulation as checked by TVS, the stage of endometrial maturation differed substantially between subjects with respect to both Noyes criteria combined with immunohistochemical markers. Glycogen vacuoles in the endometrial glandular epithelium were observed in all but one subject (patient 23 showed already a nonvacuolated secretory appearance). Since glycogen vacuoles are the hallmark of the early secretory phase (postovulatory days 2–5) and disappear after postovulatory day 5, all biopsies could be dated between postovulatory days 2 and 5. Analysis of tissue Ki-67 and PR expression enabled more precise assessment of maturation. When Ki-67 staining cells within the glandular epithelium were few or absent, the endometrium was dated as postovulatory days 4–5.5, reflecting 'adequate' endometrial maturation. When 10–50% of glands showed Ki-67-positive cells, the endometrium was dated as

Table 1. Characteristics of 31 women

No.	Age (years)	Parity	Cycle length (days)		Serum		Endometrial tissue, dating (POD)	Endometrial secretion				
			Follicular	Luteal	E_2 (pmol/ml)	P (nmol/ml)		SDS-PAGE scored protein profile	P (ng/ml)	Protein (mg/ml)	LIF (pg/ml)	GdA (ng/ml)
1	28	0	14	16	315	50.4	3.0	2	0.61	0.19	—	—
2	28	1	16	15	264	40.5	2.5	3	0.74	1.06	5.23	14.26
3	38	0	11	14	295	29.4	3.5	3	0.37	0.36	19.25	18.58
4	28	0	17	13	305	16.1	2.0	1	0.79	0.77	10.35	8.39
5	34	1	13	16	293	50.1	3.0	3	0.60	0.62	7.27	5.87
6	29	0	12	15	555	52.1	4.5	1	0.34	0.32	19.5	14.26
7	30	0	16	14	307	20.5	2.5	2	0.51	0.39	11.56	1.15
8	24	0	16	15	314	34.5	2.0	2	0.48	0.46	7.54	0.13
9	37	2	10	16	269	22.7	2.5	3	0.55	0.86	5.64	2.17
10	21	1	19	15	594	29.0	3.5	3	0.30	0.5	7.62	1.92
11	32	0	18	14	464	28.4	2.5	1	0.31	0.27	16.7	5.61
12	34	1	9	13	314	34.9	2.5	3	0.47	0.44	6.29	—
13	34	0	13	14	547	30.8	3.5	3	0.33	0.32	8.66	0.38
14	26	1	16	15	250	21.9	2.5	2	0.45	0.4	16.47	0.60
15	33	3	14	16	295	28.5	3.0	3	0.48	0.65	7.46	1.66
16	31	0	16	14	404	15.5	2.5	3	0.59	0.65	8.54	3.06
17	33	0	10	16	264	28.6	2.5	3	0.35	0.41	13.54	3.19
18	35	0	12	13	590	191	4.5	2	0.96	0.67	10.86	4.21
19	26	0	17	15	402	53.8	4.5	3	0.60	0.59	12.93	—
20	27	0	19	13	602	100	5.0	3	0.71	0.47	8.85	2.04
21	31	1	21	12	459	33.9	3.0	1	0.48	0.78	9.78	4.09
22	27	2	13	14	324	33.4	2.5	3	0.92	1.01	2.74	4.73
23	24	1	22	11	576	43.1	5.5	2	0.31	0.51	6.78	4.98
24	36	2	17	14	362	21.1	3.5	2	0.31	0.54	13.48	1.40
25	38	2	10	16	324	22.8	3.5	3	0.63	0.63	10.46	14.35
26	38	7	14	11	358	20.5	3.5	1	0.44	0.45	7.71	14.35
27	32	2	15	13	462	42.2	4.5	2	0.21	0.34	15.55	3.2
28	32	2	14	13	1177	53.0	5.0	2	0.40	0.64	6.50	25.76
29	38	1	16	13	256	20.9	5.0	3	0.33	1.09	8.25	4.13
30	32	2	13	13	402	27.6	4.5	2	0.61	0.75	—	28.76
31	32	2	15	11	392	36.5	4.5	3	0.30	0.52	—	40.18

POD, postovulatory days. Each serum, endometrial and secretion sample was obtained on the fifth day after spontaneous ovulation. Endometrial tissue dating (POD), assessed with histological Noyes criteria and immunohistochemical markers, is expressed in POD. SDS-PAGE scored protein profile of the endometrial secretions means: 1, impaired; 2, suboptimal; 3, optimal luteal phase pattern.

postovulatory days 3–4, characterising slightly retarded maturation since proliferation was continuing. When more than 50% of glands showed Ki-67-positive cells, the endometrium was dated as postovulatory days 2–3 referring to retarded maturation (Figure 1).

Strong staining of PR in the glands was dated as postovulatory days 2–4. Less intense staining of the PR in the glandular epithelial cells in contrast to the stromal cells, indicating the down-regulation of PR, was dated as postovulatory day 4–5.5 (Figure 1).

All results combined resulted in the final dating of the endometrial biopsies as given in Table 1.

Endometrial tissue dating relation with serum hormone levels

Serum E_2 and P levels showed a significant positive correlation with endometrial dating by Noyes criteria in combination with the immunohistochemical assessment of markers Ki-67 and PR ($r = 0.568$ $P = 0.006$ for serum E_2, and $r = 0.408$ $P = 0.023$ for serum P; Figure 2A, B).

Endometrial tissue dating relation with proteins and endometrial secretion fluid marker levels

Endometrial dating using the histological Noyes criteria combined with PR and Ki-67 immunostaining showed no correlation to the protein content in endometrial secretion fluids ($r = 0.013$, $P = 0.945$; Figure 3C). In the ten subjects who demonstrated adequate or advanced endometrial maturation at the tissue level, analysis of USE revealed an optimal pattern in four (40%), a suboptimal pattern in five (50%), and an impaired luteal phase pattern in only one subject (10%) (Figure 4).

Of the 21 subjects with slightly delayed endometrial maturation according to the dating of endometrial biopsies, 12 (57%) subjects showed an optimal luteal phase pattern, 5 subjects (24%) a suboptimal, and 4 subjects (19%) an impaired luteal phase pattern according to the assessments of the endometrial secretion samples (Figure 4).

Endometrial secretion levels of GdA revealed a positive correlation with endometrial dating ($r = 0.376$ $P = 0.048$; Figure 3B). In contrast to GdA, secretory LIF ($r = 0.105$, $P = 0.594$ in Figure 3A) was not correlated with endometrial dating.

Discussion

The principle aim of this study was to compare the assessment of endometrial maturation parameters that can be measured in endometrial secretion samples obtained by minimally invasive techniques with those assessed in tissue biopsies. The results of endometrial dating assessed by the combination of the Noyes criteria and the immunohistochemical staining patterns of endometrial PR and Ki-67 expression were compared with the overall protein content, the one-dimensional gel electrophoresis protein pattern as well as LIF and GdA content of endometrial secretion fluids. As far as we are aware,

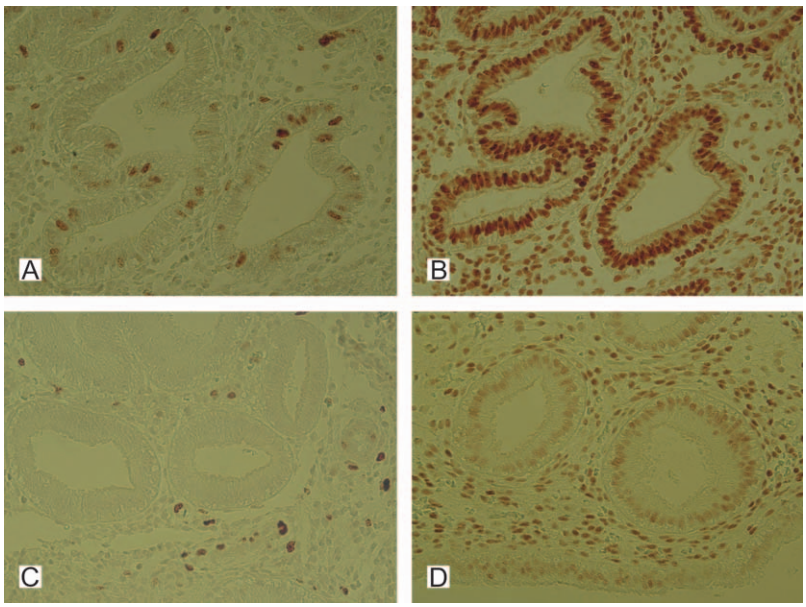

Figure 1. Immunostaining for the proliferation marker Ki-67 (MIB-1) (A, C) and the PR (B, D). Representative staining of two differently dated endometrial biopsies obtained on the fifth day after ovulation (postovulatory days). Sample A, B was dated as postovulatory days 2–3 (retarded maturation) according to strong staining of PR and Ki-67 in the glands. Samples C and D was dated as postovulatory days 4–5 (adequate maturation) according to weak staining of PR and no staining of Ki-67 in the glands. Magnification: 400×.

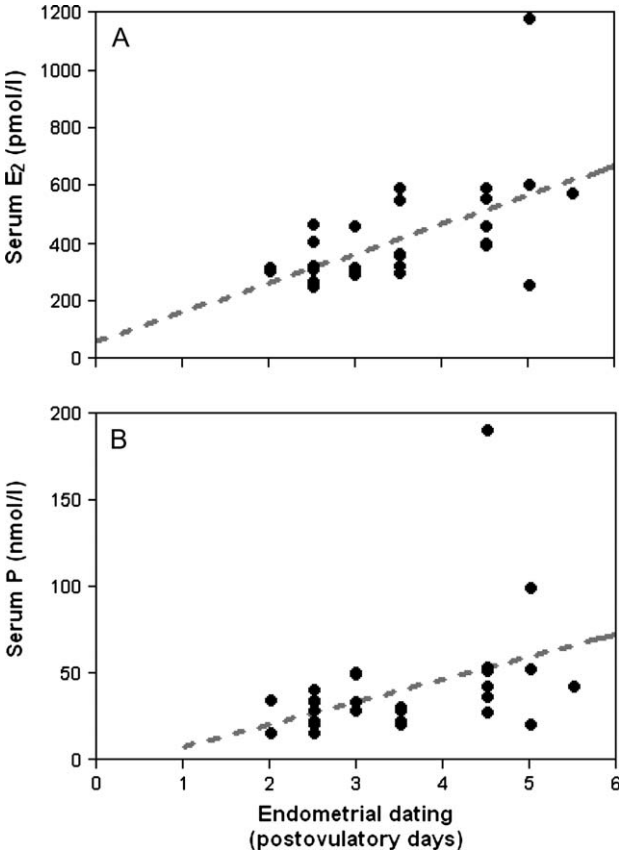

Figure 2. Correlations between the endometrial tissue dating (postovulatory days) with the serum concentration of estradiol (E_2) (A; $r = 0.568$, $P = 0{,}001$) and with the serum concentration of P (B; $r = 0.408$, $P = 0.023$).

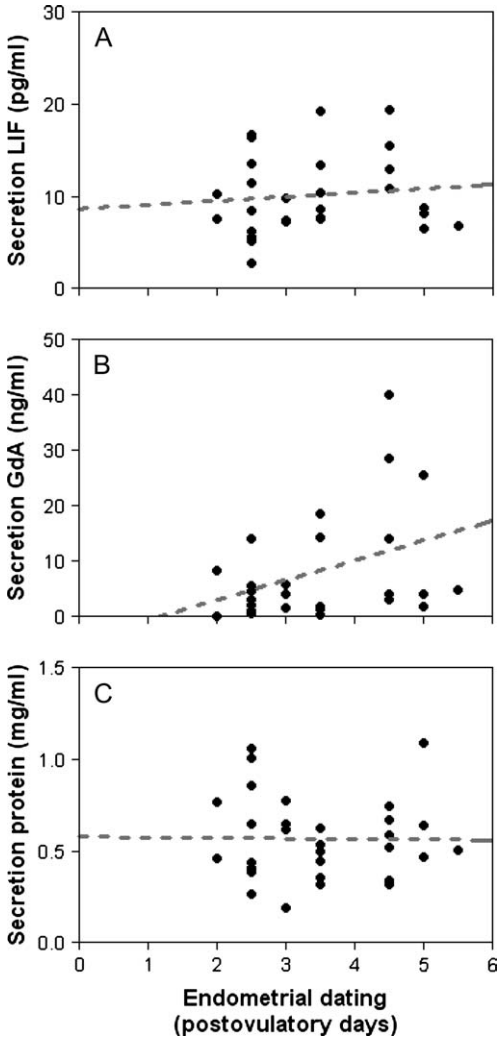

Figure 3. Correlations between the endometrial tissue dating (postovulatory days) and LIF in endometrial secretions (A; $r = 0.105$, $P = 0.594$), GdA (B; $r = 0.376$, $P = 0.048$) in endometrial secretions and protein content in endometrial secretions (C; $r = 0.013$, $P = 0.945$).

these factors have never been studied simultaneously in endometrial secretions (obtained by aspiration or flushing) and conventional endometrial maturation parameters in tissue from the same subject.

Endometrial biopsy before embryo transfer in IVF cycles negatively affects implantation rates.[8,10,28] In contrast, no decrease in pregnancy rates have been observed following transcervical aspiration of endometrial secretion fluids immediately before embryo transfer.[8,10,28] This technique can be applied during the window of implantation without disrupting the implantation process,[8,10,28] and is associated with less discomfort than endometrial suction microbiopsy.[9] Moreover, a single aspiration sample from 1 cm above the internal os and a single tissue biopsy was previously observed in hysterectomy specimens to be representative of other areas sampled within the uterine cavity (H. Beier, pers. comm.). No major complications following this approach have been reported.

Endometrial dating by assessment of the protein patterns of endometrial secretion fluids did not significantly correlate to that based on the combination of Noyes criteria and immunohistochemical PR and Ki-67 staining patterns. Previous studies have indicated that protein profile expression in endometrial secretions undergo cyclical changes, demonstrating significant differences between the proliferative, periovulatory, and late secretory phases.[27,29] However, the resolution of one-dimensional SDS-PAGE may be insufficient to demonstrate changes in protein profile expression within only few days between the early to midluteal phase.

E_2 and P are the key modulators of endometrial maturation. Consistent with this, a significant correlation was observed between endometrial maturation determined by the Noyes criteria combined with immunohistochemical assessments, and serum E and P levels.

The total protein content of endometrial secretion samples did not vary according to endometrial dating at the tissue

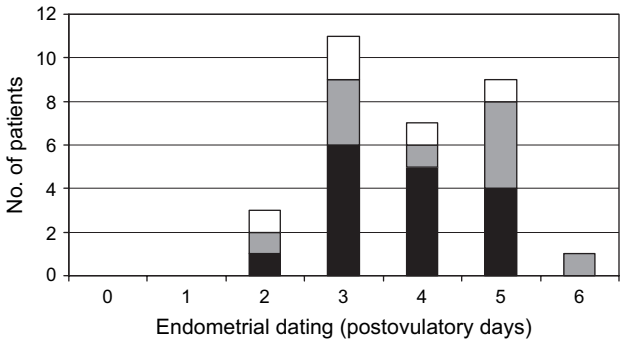

Figure 4. Number of women with optimal luteal phase pattern (black bars), suboptimal (grey bars), and impaired luteal phase pattern (white bars) according to scored protein profiles in endometrial secretions compared with endometrial dating (postovulatory days) determined in endometrial tissue obtained on the fifth day after ovulation. Only five women showed an impaired luteal phase pattern but independent of the endometrial dating.

level. This finding is consistent with several other studies that did not find a significant cycle-dependent variation of the total protein content of endometrial flushings.[30–32] In contrast, two studies reported a higher protein content in endometrial secretion samples retrieved from secretory phase.[33,34] This discrepancy may be because of differences in analysing techniques. Moreover, different proteins may subtly vary during the menstrual cycle without measurable variations in the total protein content. It is likely that changes are too small and the present method of analysis too crude to discriminate cyclic variation.

Two specific proteins reported to have key roles in implantation, GdA and LIF,[35] were analysed in the endometrial secretion samples. LIF has been shown to be essential for implantation in mice[36] and is upregulated in the human endometrium at the time of implantation.[37,38] LIF modulates the invasiveness of trophoblast cells[39] and affects immune tolerance by controlling human leucocyte antigen G expression of invasive cytotrophoblast cells[40] during implantation. It has been suggested that impaired LIF production may underlie some cases of recurrent miscarriage.[41,42] Furthermore, the amount of LIF in endometrial flushings was described to be highly predictive for pregnancy in the future menstrual cycles.[43] However, this study was based on returned voluntary questionnaires.

We detected LIF in aspirated endometrial secretions in all women with endometrial tissue dating of 2 days or later after ovulation. This is consistent with previous studies which have shown that LIF appears in endometrial flushings in the early luteal phase from postovulatory day 2 and onwards.[16,20] However, the present study showed no correlation between LIF levels in endometrial secretion samples with the P serum concentration in the early luteal phase. Furthermore, we found no significant correlation between LIF and endometrial maturation based on the combined assessments at the tissue level. This may be because of the large range of the LIF concentrations observed in endometrial secretions.[10,16,20,44] Furthermore, there is evidence that LIF expression is not primarily modulated by steroid hormones,[45] but by transforming growth factor-β, tumour necrosis factor-α and interleukin 1β.[46] Although LIF has been shown to be crucial for implantation in mice,[36] its importance and regulation pathways in human implantation remains unclear. In a recent study, LIF therapy failed to improve the outcome of IVF treatment in women with recurrent implantation failure.[47] In our study, we could not show a distinct correlation between endometrial maturation and LIF content of endometrial secretion fluids, probably because LIF is also strongly regulated by cytokines. In conclusion, the LIF content of endometrial secretion fluids does not appear to reflect endometrial maturity.

The second candidate marker for endometrial maturation was GdA. Global gene profiling studies revealed a significant increase of GdA expression during the window of implantation.[13] Furthermore, endometrial gene expression studies suggest that sex steroids play an important role in regulating endometrial GdA expression.[48] Like LIF, GdA may also suppress the maternal immune response possibly through suppression of the natural killer cells,[49] and an immunoprotective role for GdA during implantation and placentation has been proposed.[50]

In this study, GdA was detected in endometrial secretions when the endometrium was dated as postovulatory day 2 and onwards. Additionally, GdA expression increased together with the endometrial maturation detected at the tissue level. This is consistent with a previous study showing a positive correlation between GdA levels in endometrial flushings and endometrial maturation.[17] Other studies have identified GdA in uterine flushings 3–4 days after ovulation.[19,51] Moreover, fertile patients showed higher levels of GdA in uterine flushings compared with the subfertile controls,[52] suggesting that an increase of GdA might facilitate implantation. In the present study, GdA protein levels in the endometrial fluid clearly correlated with the combined endometrial dating using the Noyes criteria and immunohistochemical marker. These findings support a possible role for endometrial secretion GdA level as a marker of endometrial maturation. Further studies are required to ascertain whether endometrial secretion GdA concentrations provide a more specific and sensitive predictor of implantation.

Although the study subjects met certain inclusion and exclusion criteria and were similar in terms of cycle characteristics, they differed in cause of subfertility. However, since both endometrial secretion and biopsy material were compared within each subject and each subject was its own control, the effect of heterogeneous fertility on the presented analysis is likely to be limited.

Conclusions

In conclusion, we showed that putative secretory receptivity markers can be simultaneously analysed in endometrial secretions, which can be retrieved without disrupting endometrial receptivity. Only the GdA level was significantly correlated with endometrial dating. Therefore, the measurement of GdA in noninvasively obtained endometrial secretion samples may represent a novel diagnostic tool to monitor endometrial maturation in a way that does not affect implantation. This study also demonstrated that it is possible to assess a small array of putative receptivity markers simultaneously in a single endometrial secretion sample. Although further work will be required to confirm the findings of this preliminary study, this approach opens the possibility for studying the complex intrauterine regulatory networks before implantation and the identification of further important regulators of endometrial maturation and receptivity. In contrast to more invasive techniques, endometrial fluid aspiration may make it possible to correlate such markers directly with successful implantation. Clinical applications could include the assessment of endometrial maturation during an IVF treatment cycle, allowing embryos to be frozen rather than transferred to a suboptimal intrauterine milieu, and the evaluation of interventions designed to improve endometrial receptivity.

Disclosure of interest

None.

Contribution to authorship

M.H.v.d.G. recruitment and sampling of patient, writing of manuscript. N.S.M. concept, design and supervision of study, writing of manuscript. K.B-H. assessment of endometrial secretions. C.A.K. endometrial tissue analysis. B.C.J.M.F. concept of study, contributed to manuscript. H.M.B. design of study, assessment of endometrial secretions, contributed to manuscript. I.C-L. design of study, endometrial tissue analysis, writing of manuscript.

Details of ethics approval

Ethical approval was obtained from the Ethical Committee of the Erasmus Medical Centre (METC) to perform the study.

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References

1 Hertig AT, Rock J, Adams J. A description of 34 human ova within the first 17 days of development. *Am J Anat* 1956;98:435–94.
2 Wilcox AJ, Baird DD, Weinberg CR. Time of implantation of the conceptus and loss of pregnancy. *N Engl J Med* 1999;340:1796–9.
3 Psychoyos A. *The implantation window: basic and clinical aspects. Perspectives in assisted reproduction*. 1993;57–62.
4 Noyes N, Hertig AT, Rock J. Dating the endometrial biopsy. *Fertil Steril* 1950;1:3–25.
5 Myers ER, Silva S, Barnhart K, Groben PA, Richardson MS, Robboy SJ, *et al*. Interobserver and intraobserver variability in the histological dating of the endometrium in fertile and infertile women. *Fertil Steril* 2004;82:1278–82.
6 Coutifaris C, Myers ER, Guzick DS, Diamond MP, Carson SA, Legro RS, *et al*. Histological dating of timed endometrial biopsy tissue is not related to fertility status. *Fertil Steril* 2004;82:1264–72.
7 Murray MJ, Meyer WR, Zaino RJ, Lessey BA, Novotny DB, Ireland K, *et al*. A critical analysis of the accuracy, reproducibility, and clinical utility of histologic endometrial dating in fertile women. *Fertil Steril* 2004;81:1333–43.
8 van der Gaast MH, Beier-Hellwig K, Fauser BC, Beier HM, Macklon NS. Endometrial secretion aspiration prior to embryo transfer does not reduce implantation rates. *Reprod BioMed Online* 2003;7:105–9.
9 Li TC, MacKenna A, Roberts R. The techniques and complications of out-patient uterine washing in the assessment of endometrial function. *Hum Reprod* 1993;8:343–6.
10 Olivennes F, Ledee-Bataille N, Samama M, Kadoch J, Taupin JL, Dubanchet S, *et al*. Assessment of leukemia inhibitory factor levels by uterine flushing at the time of egg retrieval does not adversely affect pregnancy rates with in vitro fertilization. *Fertil Steril* 2003;79:900–4.
11 Beier HM. Oviducal and uterine fluids. *J Reprod Fertil* 1974;37:221–37.
12 Beier HM, Beier-Hellwig K. Molecular and cellular aspects of endometrial receptivity. *Hum Reprod Update* 1998;4:448–58.
13 Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, *et al*. Global gene profiling in human endometrium during the window of implantation. *Endocrinology* 2002;143:2119–38.
14 Bell SC, Dore-Green F. Detection and characterization of human secretory "pregnancy-associated endometrial alpha 2-globulin" in uterine luminal fluid. *J Reprod Immunol* 1987;11:13–29.
15 Li TC, Ling E, Dalton C, Bolton AE, Cooke ID. Concentration of endometrial protein PP14 in uterine flushings throughout the menstrual cycle in normal, fertile women. *Br J Obstet Gynaecol* 1993;100:460–4.
16 Laird SM, Tuckerman EM, Dalton CF, Dunphy BC, Li TC, Zhang X. The production of leukaemia inhibitory factor by human endometrium: presence in uterine flushings and production by cells in culture. *Hum Reprod* 1997;12:569–74.
17 Tuckerman E, Laird SM, Stewart R, Wells M, Li TC. Markers of endometrial function in women with unexplained recurrent pregnancy loss: a comparison between morphologically normal and retarded endometrium. *Hum Reprod* 2004;19:196–205.
18 MacKenna A, Li TC, Dalton C, Bolton A, Cooke I. Placental protein 14 levels in uterine flushing and plasma of women with unexplained infertility. *Fertil Steril* 1993;59:577–82.
19 Dalton CF, Laird SM, Serle E, Saravelos H, Warren MA, Li TC, *et al*. The measurement of CA 125 and placental protein 14 in uterine flushings in women with recurrent miscarriage; relation to endometrial morphology. *Hum Reprod* 1995;10:2680–4.
20 Mikolajczyk M, Skrzypczak J, Szymanowski K, Wirstlein P. The assessment of LIF in uterine flushing–a possible new diagnostic tool in states of impaired fertility. *Reprod Biol* 2003;3:259–70.

21 Ecochard R, Gougeon A. Side of ovulation and cycle characteristics in normally fertile women. *Hum Reprod* 2000;15:752–5.

22 Shoupe D, Mishell DR Jr, Lacarra M, Lobo RA, Horenstein J, d'Ablaing G, *et al*. Correlation of endometrial maturation with four methods of estimating day of ovulation. *Obstet Gynecol* 1989;73:88–92.

23 Guermandi E, Vegetti W, Bianchi MM, Uglietti A, Ragni G, Crosignani P. Reliability of ovulation tests in infertile women. *Obstet Gynecol* 2001; 97:92–6.

24 Hendrickson MR, Kempson RL. Histology of the uterus and fallopian tubes. In: Sternberg SS, editor. *Histology for Pathologists*. Philadelphia, PA: Lippincott-Raven, 1997. pp. 879–927.

25 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.

26 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.

27 Beier-Hellwig K, Sterzik K, Bonn B, Beier HM. Contribution to the physiology and pathology of endometrial receptivity: the determination of protein patterns in human uterine secretions. *Hum Reprod* 1989;4 (8 Suppl):115–20.

28 Ledee-Bataille N, Dubanchet S, Coulomb-L'hermine A, Durand-Gasselin I, Frydman R, Chaouat G. A new role for natural killer cells, interleukin (IL)-12, and IL-18 in repeated implantation failure after in vitro fertilization. *Fertil Steril* 2004;81:59–65.

29 Beier-Hellwig K, Sterzik K, Bonn B, Hilmes U, Bygdeman M, Gemzell-Danielsson K, *et al*. Hormone regulation and hormone antagonist effects on protein patterns of human endometrial secretion during receptivity. *Ann N Y Acad Sci* 1994;734:143–56.

30 Voss HJ, Beato M. Human uterine fluid proteins: gel electrophoretic pattern and progesterone-binding properties. *Fertil Steril* 1977;28:972–80.

31 Sylvan PE, MacLaughlin DT, Richardson GS, Scully RE, Nikrui N. Human uterine luminal fluid proteins associated with secretory phase endometrium: progesterone-induced products? *Biol Reprod* 1981;24:423–9.

32 MacLaughlin DT, Richardson GS. Analysis of human uterine luminal fluid proteins following radiolabeling by reductive methylation: comparison of proliferative and secretory phase samples. *Biol Reprod* 1983; 29:733–42.

33 Sullivan DA, Richardson GS, MacLaughlin DT, Wira CR. Variations in the levels of secretory component in human uterine fluid during the menstrual cycle. *J Steroid Biochem* 1984;20:509–13.

34 MacLaughlin DT, Santoro NF, Bauer HH, Lawrence D, Richardson GS. Two-dimensional gel electrophoresis of endometrial protein in human uterine fluids: qualitative and quantitative analysis. *Biol Reprod* 1986; 34:579–85.

35 Diedrich K, Fauser BC, Devroey P, Griesinger G. The role of the endometrium and embryo in human implantation. *Hum Reprod Update* 2007;13:365–77.

36 Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, *et al*. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* 1992;359:76–9.

37 Charnock-Jones DS, Sharkey AM, Fenwick P, Smith SK. Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *J Reprod Fertil* 1994;101:421–6.

38 Classen-Linke I, Alfer J, Hey S, Krusche CA, Kusche M, Beier HM. Marker molecules of human endometrial differentiation can be hormonally regulated under in-vitro conditions as in-vivo. *Hum Reprod Update* 1998;4:539–49.

39 Bischof P, Haenggeli L, Campana A. Effect of leukemia inhibitory factor on human cytotrophoblast differentiation along the invasive pathway. *Am J Reprod Immunol* 1995;34:225–30.

40 Bamberger AM, Jenatschke S, Schulte HM, Loning T, Bamberger MC. Leukemia inhibitory factor (LIF) stimulates the human HLA-G promoter in JEG3 choriocarcinoma cells. *J Clin Endocrinol Metab* 2000;85: 3932–6.

41 Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S. Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. *Nat Med* 1998;4:1020–4.

42 Steck T, Giess R, Suetterlin MW, Bolland M, Wiest S, Poehls UG, *et al*. Leukaemia inhibitory factor (LIF) gene mutations in women with unexplained infertility and recurrent failure of implantation after IVF and embryo transfer. *Eur J Obstet Gynecol Reprod Biol* 2004;112:69–73.

43 Mikolajczyk M, Wirstlein P, Skrzypczak J. The impact of leukemia inhibitory factor in uterine flushing on the reproductive potential of infertile women—a prospective study. *Am J Reprod Immunol* 2007;58:65–74.

44 Ledee-Bataille N, Lapree-Delage G, Taupin JL, Dubanchet S, Frydman R, Chaouat G. Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation. *Hum Reprod* 2002;17:213–18.

45 Ng EH, Laird SM, Li TC, Yeung WS, Ho PC. Concentrations of endometrial protein PP 14 and CA-125 in uterine flushings performed in natural and stimulated cycles. *Hum Reprod* 2004;19:905–10.

46 Arici A, Engin O, Attar E, Olive DL. Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. *J Clin Endocrinol Metab* 1995;80:1908–15.

47 Brinsden PR, Alam V, de Moustier B, Engrand P. Recombinant human leukaemia inhibitory factor does not improve implantation and pregnancy outcomes after assisted reproductive techniques in women with recurrent unexplained implantation failure. *Fertil Steril* 2008 (Epub ahead of print).

48 Horcajadas JA, Pellicer A, Simon C. Wide genomic analysis of human endometrial receptivity: new times, new opportunities. *Hum Reprod Update* 2007;13:77–86.

49 Okamoto N, Uchida A, Takakura K, Kariya Y, Kanzaki H, Riittinen L, *et al*. Suppression by human placental protein 14 of natural killer cell activity. *Am J Reprod Immunol* 1991;26:137–42.

50 Seppala M, Koistinen H, Koistinen R, Chiu PC, Yeung WS. Glycosylation related actions of glycodelin: gamete, cumulus cell, immune cell and clinical associations. *Hum Reprod Update* 2007;13:275–87.

51 Li TC, Dalton C, Hunjan KS, Warren MA, Bolton AE. The correlation of placental protein 14 concentrations in uterine flushing and endometrial morphology in the peri-implantation period. *Hum Reprod* 1993;8: 1923–7.

52 Dalton CF, Laird SM, Estdale SE, Saravelos HG, Li TC. Endometrial protein PP14 and CA-125 in recurrent miscarriage patients; correlation with pregnancy outcome. *Hum Reprod* 1998;13:197–202.

