Premature formation of nucleolar channel systems indicates advanced endometrial maturation following controlled ovarian hyperstimulation

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STUDY QUESTION: Is there a shift in the timing of nucleolar channel system (NCS) formation following controlled ovarian hyperstimulation (COH)?

SUMMARY ANSWER: NCSs appear prematurely following COH compared with natural cycles.

WHAT IS KNOWN ALREADY: During natural cycles, NCSs of endometrial epithelial cell (EEC) nuclei are exclusively present during the window of implantation and are uniformly distributed throughout the upper endometrial cavity.

STUDY DESIGN, SIZE, DURATION: Prospective two-cohort study. Cohorts I and II each consisted of seven volunteers for the duration of three menstrual study cycles that were separated by at least one wash-out or rest cycle, between December 2008 and May 2012.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Participants were recruited from a pool of healthy oocyte donors. Consecutive endometrial biopsies were obtained during the same luteal phase on cycle days (CD) 16, 20 and 26 for Cohort I, and on CD14, 22 and 24 for Cohort II, following random assignment to a natural cycle group, a COH cycle group (using a GnRH antagonist), or a COH cycle group receiving luteal phase hormonal supplementation (COH + S). The day of oocyte retrieval was designated CD14 in COH cycles and the day of the LH surge was designated CD13 in natural cycles. Prevalence of NCSs in the nuclei of EECs was quantified using indirect immunofluorescence with an anti-body directed against a subset of related nuclear pore complex proteins that are major constituents of NCSs. Progesterone and estradiol levels were measured on the day of each endometrial biopsy.

MAIN RESULTS AND THE ROLE OF CHANCE: The natural cycle group exhibited peak NCS prevalence on CD20 (53.3%; interquartile range (IQR) 28.5–55.8), which rapidly declined on CD22 (11.8%; IQR 6.3–17.6), CD24 (2.5%; IQR 0.0–9.2) and CD26 (0.3%; IQR 0.0–3.5), and no NCSs on CD14 and 16 defining a short NCS window around CD20. In contrast, in COH and COH + S cycles, NCS prevalence was high already on CD16 (40.4%; IQR 22.6–53.4 and 35.6%; IQR 26.4–44.5, respectively; \( P = 0.001 \) compared with CD16 of the natural cycle group, Mann–Whitney), whereas no significant difference in NCS prevalence was detected on any of the other five CDs between the three groups (\( P > 0.05 \)).

LIMITATIONS, REASONS FOR CAUTION: The cohort size was small (\( n = 7 \)) but was offset by the all-or-none presence of NCSs on CD16 in natural versus COH and COH + S cycles and the fact that each subject served as her own control.

WIDER IMPLICATIONS OF THE FINDINGS: Premature appearance of NCSs and hence maturation of the endometrium following COH is consistent with previous studies based on histological dating but contradicts studies based on mRNA expression profiling, which reported a lag in endometrial maturation. However, this is the first study of this kind that is based on consecutive endometrial biopsies within the same cycle and that reports such clear-cut differences: no versus robust NCS presence on CD16. Our observation of advanced endometrial maturation following COH may contribute to the reduced implantation rates seen in fresh compared with frozen and donor IVF-embryo transfer cycles. Therefore, the NCS window could serve as a sensitive guide for timing of embryo transfer in frozen and donor cycles.
**STUDY FUNDING/COMPETING INTEREST(S):** The study was supported by the March of Dimes Birth Defects foundation (1-FY09–363 to U.T.M.); Ferring Pharmaceuticals, Parsippany, NJ; East Coast Fertility, Plainview, NY and the CMBG Training Program (T32 GM007491 to M.J.S.). We report no competing interests.

**Key words:** nucleolar channel system / ovarian hyperstimulation / endometrial receptivity / window of implantation / immunodetection

**Introduction**

IVF protocols most often involve some form of controlled ovarian hyperstimulation (COH) prior to oocyte retrieval. Consequently, the normal progression of endometrial maturation is disturbed, as determined by histology and gene expression analysis (for review see Bourgain and Devroey, 2003; Horcajadas et al., 2007). Apparently, it is this aberrant maturation of the endometrium that leads to dysynchrony with the developing embryo causing reduced success rates after embryo transfer in the same cycle when compared with embryo transfer in frozen or donor oocyte recipient cycles (Richter et al., 2006; Sunkara et al., 2010; Shapiro et al., 2013). Despite these unwanted consequences, the exact nature of alteration of endometrial maturation by COH is not clear. Whereas histological approaches report advanced endometrial maturation, genomic approaches tend to show a delayed maturation (Kolb et al., 1997; Develegu et al., 1999; Nikas et al., 1999; Kolibianakis et al., 2002; Mirkin et al., 2004; Saadat et al., 2004; Horcajadas et al., 2005, 2008; Novin et al., 2007; Macklon et al., 2008; Humaidan et al., 2012; Roque et al., 2013). Obviously, it is important to have sensitive markers that inform on the status of the endometrium, specifically the timing of receptivity, i.e. the window of implantation. The gene expression profile identified by the endometrial receptivity array serves as one such marker (Diaz-Gimeno et al., 2011; Garrido-Gomez et al., 2013). Here we evaluate the behavior in COH cycles of a histological marker that sensitively and objectively marks the midluteal phase, the nucleolar channel system (NCS) of endometrial epithelial cells (EECs).

Discovered over half a century ago, NCSs are small (1 μm diameter) organelles in the nuclei of EECs appearing transiently, during an ~5-day window, in the midluteal phase (Dubrauszky and Pohlmann, 1960; Clyman, 1963; Terzakis, 1965; More and McSeveney, 1980; Guffanti et al., 2008). Together with giant mitochondria, subnuclear glycogen deposits and pinopodes, NCSs belong to the ultrastructural hallmarks of secretory transformation of EECs (Moricard and Moricard, 1964; Wynn and Woolley, 1967; Gordon, 1975; More and McSeveney, 1980; Cornillie et al., 1985; Dockery et al., 1988; Spornitz, 1992). By electron microscope, NCSs appear as three layers of membrane tubules embedded in an electron-dense matrix surrounding an amorphous core (Spornitz, 1992). Despite the partial characterization of the molecular composition of NCSs, their function remains as elusive as when originally identified (Kittur et al., 2007; Guffanti et al., 2008). Nevertheles, the identification of protein markers for NCSs now enables their immunodetection at the light microscopic level. Using indirect immunofluorescence, we showed that NCSs are indeed specific to healthy human EECs, that their transient midluteal appearance is robust and independent of fertility status, that they are evenly distributed throughout the endometrial cavity except for the lower uterine segment and that they reside preferentially in the functional luminal layers of the endometrium (Guffanti et al., 2008; Rybak et al., 2011; Szmyga et al., 2013). These findings are consistent with a role of NCSs in the endometrial preparation for attachment and implantation of the embryo.

In this study, we exploit NCSs as sensitive indicators for secretory transformation of the endometrium hypothesizing that NCS appearance is shifted in stimulated compared with natural cycles.

**Materials and Methods**

**Subjects**

The study was performed in two consecutive legs for each of which seven participants were recruited into two Cohorts, I and II, respectively (Fig. 1A). Participants were from a pool of healthy non-smoking oocyte donors. None had a history of infertility and one in each cohort was parous. Average weight, height and BMI did not differ between subjects of the two cohorts (Table I). Other requirements for inclusion were as follows: (i) age 21–32 years, (ii) CD3 FSH and estradiol (E2) within normal limits (Fig. 2A), (iii) regular 24–35 day cycles, (iv) normal baseline endometrial thickness on ultrasound and (v) willingness to participate in all three groups of the study to allow each subject to act as her own control. The maximum endometrial thickness did not vary significantly between treatments (Fig. 2B). The study was conducted at East Coast Fertility (Plainview, NY, USA) and was approved by the Western Institutional Review Board (Olympia, WA, USA). Informed consent was obtained from all participants.

**Cycle characteristics and assignment**

Consecutive endometrial biopsies were obtained during the same luteal phase on CD16, 20 and 26 for Cohort I and on CD14, 22 and 24 for Cohort II, following random assignment to a natural cycle group, a COH cycle group or a COH plus luteal hormone supplementation (COH + S) cycle group (Fig. 1). Following participation in the initial cycle, randomization took place between the remaining two groups to determine sequence completion. At least one wash-out cycle separated each study cycle (Fig. 1B). For Cohort I, CD20 was chosen to represent the peak day of NCS abundance in a natural cycle, whereas CD16 and 26 were chosen to fall just beyond the outer limits of known NCS abundance (Guffanti et al., 2008). The endometrial sampling days for Cohort II were selected based on the results from Cohort I. In particular, we probing CD14 to test whether in stimulated cycles NCSs might appear even more prematurely than on CD16 and we included CD22 and 24 to determine whether NCS prevalence in stimulated cycles declined more rapidly, i.e. whether the NCS window expanded or moved in its entirety to earlier luteal days.

The day of oocyte retrieval was designated CD 14 in COH cycles and the day of LH surge was designated CD 13 in natural cycles. Serial, follicular phase LH measurements and transvaginal ultrasound examinations were used to monitor natural cycles. CD 14 was confirmed in natural cycles by documenting follicular collapse on the day after the LH surge. Daily blood and ultrasound monitoring began when the dominant follicle reached about 16 mm in diameter (average of two perpendicular measurements).

**IVF protocols**

IVF protocols included the possible preliminary use of an oral contraceptive or E2, followed by ovarian hyperstimulation with a gonadotrophin: unfertilized (Bravelle, Ferring Pharmaceuticals, Parsippany, NJ, USA) or recombinant FSH (Gonal-f, EMD Serono, Inc., Rockland, MA, USA; or, Follistim, Schering-Plough Corporation, Kenilworth, NJ, USA); used in conjunction...
with hMG (Menopur, Ferring Pharmaceuticals). A GnRH antagonist (Ganirelix, Schering-Plough Corporation) was introduced when the lead follicle reached 14 mm in diameter or the serum E2 reached 400 pg/ml. A standard dose of hCG was administered to trigger final oocyte maturation prior to oocyte retrieval. The initial dose of gonadotrophins was determined based on subject BMI and basal antral follicle count. Dose adjustment during the stimulation cycle was based on the ovarian response as evidenced by E2 levels and by size and number of developing follicles. For the former, daily (during the first week of the cycle, every 1–3 days) hormone levels were monitored. The peak levels (Fig. 1C and D) usually occurred on the day of hCG administration but potentially earlier if a patient was at high risk of ovarian hyperstimulation syndrome. For those subjects randomized to receive luteal phase hormonal supplementation, administration of vaginal progesterone inserts (Endometrin, Ferring Pharmaceuticals) 100 mg three times daily was initiated on the day after oocyte retrieval (Doody et al., 2009). Additionally, oral generic E2, 2 mg twice daily, was initiated 6 days following oocyte retrieval (Fatemi et al., 2007).

FSH and LH levels were measured using the IMMULITE 2000 solid-phase, two-site chemiluminescent immunometric assay (Siemens Healthcare, Diagnostics Products Ltd., Llanberis, UK). E2 and progesterone levels were measured using IMMULITE 2000 solid-phase, competitive chemiluminescent enzyme immunoassay.

Endometrial biopsy protocols

Participants received 600 mg of ibuprofen, pre-procedure. All endometrial biopsies were performed using Uterine Explora Model I-MX 120 (Cooper Surgical, Trumbull, CT, USA). Prior to performing each endometrial biopsy, transvaginal ultrasonography was used to map the position of the cervical canal and the endometrial lining. This allowed for easy catheter placement without the need for cervical instrumentation, and for consistent,

Table I  Characteristics of patients in the study of NCSs and endometrial maturation with COH (mean ± SD).

<table>
<thead>
<tr>
<th>Cohort (n = 7)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>67.1 ± 13.6</td>
<td>165.7 ± 7.6</td>
<td>24.2 ± 3.6</td>
</tr>
<tr>
<td>II</td>
<td>63.5 ± 11.6</td>
<td>161.9 ± 7.0</td>
<td>24.1 ± 3.5</td>
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</tbody>
</table>
midline sampling within the endometrial cavity. Endometrial sampling occurred on the anterior surface in anteverted uteri, and on the posterior surface in retroverted uteri to minimize disruption of anatomy. As NCSs are uniformly distributed throughout the upper uterine cavity (Szmyga et al., 2013), the catheter was placed ≥5 cm depth within the uterus, suction was applied and the catheter was retracted from the uterus in a single motion, with the aim of sampling the fundal area and removing as little tissue as possible. The endometrial sample was immediately fixed with 4% paraformaldehyde for 4 h at room temperature and then kept at 4°C until paraffin embedding.

NCS detection and quantification

NCSs were detected on paraffin sections by indirect immunofluorescence using a monoclonal antibody (mAb414; Covance Research Products, Princeton, NJ, USA) against a subset of nuclear pore complex proteins that are enriched in NCSs (Guffanti et al., 2008; Szmyga et al., 2013). Briefly, nuclei were identified by double staining with the DNA stain 4′,6-diamidino-2-phenylindole (DAPI; Fig. 2E). All samples were imaged on a DeltaVision Core system (Applied Precision, Issaquah, WA, USA) with

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Figure 2 (A) Median with IQR of FSH (left Y-axis) and mean ± SD of E2 levels on CD3 (right Y-axis). (B) Medians with IQR of endometrial thickness of all subjects from both cohorts on the day of hCG administration show no significant difference between treatments. (C) Median with IQR of peak follicular progesterone levels for all subjects from both cohorts show no significant difference between treatments. (D) Mean ± SD of peak follicular E2 levels for all subjects from both cohorts show significant differences (indicated above) in natural versus COH and COH + S cycles. (E–G) Example of a DNA (E) and nuclear pore complex protein (F and G) stained paraffin section from an endometrial biopsy obtained on CD20 during a natural cycle (NCS prevalence = 53.2%). (E) The maximum projection of all optical planes (36) across the entire paraffin section (10.8 μm) used to identify glands and to count epithelial cell nuclei. Note this corresponds to 1/4th of 1 field of 10 that were analyzed for this (and every other) biopsy. (F) The maximum projection of the same field as in (F) but stained for nuclear pore complexes (punctate staining) and NCSs (arrows and arrowheads; arrows identify those NCSs that are also visible in the single optical plane in G). (G) Single 0.3-μm-thick optical plane used to unambiguously identify NCSs (arrows). Note the absence of NCSs from stromal cells. Some NCSs are barely visible in the maximum projection (F), hence, the scanning of individual sections, e.g. (G). The haze of the epithelium in the single section (G) is a result of autofluorescence that is artificially removed when only the maximum intensities are used for projection of all sections (F). CD, cycle day.
an Olympus IX71 stand using a 60X/1.42 NA planapo objective and a Cool-Snap HQ2 CCD camera (Photometrics, Tucson, AZ, USA). NCSs in EECs of each biopsy were quantified in 10 fields that were randomly selected regarding NCSs by using the DAPI channel. After selection of the 10 fields, they were imaged automatically and without human intervention across the entire paraffin section in 0.3 μm-step Z-series of optical planes and NCSs were analyzed using our continuous and absolute quantification method (Szmyga et al., 2013). NCSs were identified by visually scanning each optical plane assuring that they were globular rather than tubular structures, i.e. only visible in maximally four to five consecutive planes, allowing for some out of focus light radiating from the 1 μm-diameter organelles (Fig. 2G, arrows). DAPI stained nuclei were counted in maximum projections (Fig. 2E). In this manner, 15 513 NCSs in 64 120 EEC nuclei were counted in 84 endometrial biopsies, assessing between 440 and 1150 (720 ± 149, mean ± SD) EEC nuclei in each. A single observer quantified each sample because the method is observer independent (Szmyga et al., 2013). NCS prevalence was expressed as percentage of NCSs per EECs.

**Statistical analysis**

All statistical analysis was performed using Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). The distribution of our samples was assessed using D’Agostino and Pearson omnibus and Shapiro–Wilk normality tests. Due to the lack of normal distribution and the small sample size in most cases, the median and interquartile range (IQR) are reported and statistical significance between values was assessed by the Mann–Whitney test. Where mean and SD are reported, statistical significance was tested using an unpaired t-test. Statistical significance was defined as $P < 0.05$.

**Results**

**NCSs appear prematurely following COH**

NCSs were identified by their enrichment of a subset of related nuclear pore complex proteins (Fig. 2F and G, arrows and arrowheads) and epithelial cells were counted by their nuclei (Fig. 2E). In the natural cycle group, the window of NCS appearance was very narrow. Specifically, peak NCS prevalence was observed on CD20 (53.3%; IQR 28.5–55.8)—except in 1 patient (Fig. 3A, teal), which was linked to an infection indicated by massive lymphocyte infiltration of the stroma—and rapidly declined on CD22 (11.8%; IQR 6.3–17.6), CD24 (2.5%; IQR 0.0–9.2) and CD26 (0.3%; IQR 0.0–3.5) (Fig. 3A, natural cycle and Table II). No NCSs were detected on CD14 and CD16. In contrast, after COH and COH+S, NCS prevalence was high already on CD16 (40.4%; IQR 22.6–53.4 and 35.6%; IQR 26.4–44.5, respectively; $P ≤ 0.001$), whereas no significant difference in NCS prevalence was detected between the three groups on any of the other five CDs (Fig. 3A and Table II). Therefore, NCS formation was prominent already on CD16 (Fig. 3A, red arrows). This premature NCS appearance was induced by COH regardless of luteal hormone supplementation.

**Increased hormone levels after COH are insufficient to explain NCS induction**

Luteal hormone levels for all subjects are depicted in Fig. 3B and C. In natural cycles, median progesterone levels were ≤ 3.0 ng/ml on CD14 and CD16, and increased ≥ 3.0 ng/ml on CD20 (9.0 ng/ml; IQR 8.5–15.3) and CD22 (9.45 ng/ml; IQR 9.2–12.0) before declining on CD24 (6.3 ng/ml; IQR 4.3–11.0) and CD26 (4.1 ng/ml; IQR 1.2–6.6) (Fig. 3B). In COH cycles, median progesterone levels were
Hormone stimulation advances endometrial maturation

Table II: Median NCS prevalence [percentage (IQR)].

<table>
<thead>
<tr>
<th>CD</th>
<th>14</th>
<th>16</th>
<th>20</th>
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<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>None</td>
<td>0.0 (0.0–0.1)</td>
<td>0.0 (0.0–0.0)</td>
<td>53.3 (28.5–55.8)</td>
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<tr>
<td>COH</td>
<td>0.0 (0.0–0.2)</td>
<td>40.4 (22.6–53.4)</td>
<td>25.3 (15.8–39.6)</td>
</tr>
<tr>
<td>COH + S</td>
<td>0.0 (0.0–1.8)</td>
<td>35.6 (26.4–44.5)</td>
<td>52.8 (3.5–59.3)</td>
</tr>
<tr>
<td>CD 22</td>
<td>22.2 (0.0–25.0)</td>
<td>10.7 (0.0–17.9)</td>
<td>0.0 (0.0–8.7)</td>
</tr>
<tr>
<td>CD 24</td>
<td>10.4 (6.2–22.3)</td>
<td>3.6 (1.6–8.8)</td>
<td>0.0 (0.0–4.8)</td>
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<tr>
<td>CD 26</td>
<td></td>
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<tr>
<td>Treatment</td>
<td></td>
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<tr>
<td>None</td>
<td>11.8 (6.3–17.6)</td>
<td>2.5 (0.0–9.2)</td>
<td>0.3 (0.0–3.5)</td>
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<tr>
<td>COH</td>
<td>22.2 (0.0–25.0)</td>
<td>10.7 (0.0–17.9)</td>
<td>0.0 (0.0–8.7)</td>
</tr>
<tr>
<td>COH + S</td>
<td>10.4 (6.2–22.3)</td>
<td>3.6 (1.6–8.8)</td>
<td>0.0 (0.0–4.8)</td>
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COH: controlled ovarian hyperstimulation; COH + S, COH plus luteal hormone supplementation; CD, cycle day.

already significantly ($P \leq 0.002$) increased at CD14 (8.1 ng/ml; IQR 6.8–9.5) and CD16 (44.7 ng/ml; IQR 36.6–75.2) remaining supraphysiological on CD20 (59.3 ng/ml; IQR 44.1–71.8). Thereafter, on CD22 (3.6 ng/ml; IQR 2.4–11.1), CD24 (1.2 ng/ml; IQR 0.6–2.3) and CD26 (0.6 ng/ml; IQR 0.51.0), the levels dropped significantly below those in the natural cycle. In COH + S cycles, the only significant difference in NCS prevalence with that of COH cycles was detected on CD20 (3.6 ng/ml; IQR 1.3–7.3; $P = 0.02$) (Fig. 3B). No significant differences in peak follicular progesterone levels were noted between the different cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). 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Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C). Significantly, progesterone levels on CD14 in stimulated cycles were comparable to or higher than those observed in natural cycles (Fig. 2C).
Although the consecutive biopsies in the same luteal phase are a strength of our study, their mere performance and the resulting endometrial injury can influence endometrial histology, clinical pregnancy rates, endometrial gene-expression profiles and levels of midluteal estrogen receptors (Barash et al., 2003; Raziel et al., 2007; Zhou et al., 2008; Li and Hao, 2009; Kalm et al., 2009; Almog et al., 2010; Karimzade et al., 2010; El-Touhy et al., 2012). To minimize or avoid such effects on subsequent biopsy results, we required at least one wash-out or rest cycle between study cycles (Fig. 1B), and disturbed the endometrial lining as little as possible with the use of a single-pass biopsy technique and removing as small a sample of tissue as necessary. Indeed, the data from Cohort I suggest that NCS prevalence in the natural cycle was not influenced by the consecutive biopsies because they matched the previously published timing of NCSs (More and McSeveney, 1980; Spornitz, 1992; Guffanti et al., 2008; Rybak et al., 2011). Specifically, NCS prevalence was high on CD20 and low or absent on CD16 and CD26. Therefore, it seems unlikely that in Cohort II the biopsy on CD14 caused the rapid decline of NCSs on CD22 and CD24, which had been previously observed in at least one study (More and McSeveney, 1980). Importantly, our major finding, the high prevalence of NCSs already on CD16 in stimulated cycles, was not influenced by a preceding biopsy, as it was the first biopsy of the series in Cohort I.

To our knowledge, only two studies previously attempted to compare NCSs in natural and COH cycles (Dehou et al., 1987; Novotný et al., 1999). Both rely on electron microscopic detection of NCSs and report opposing results. Dehou et al. (1987) find NCSs ‘poorly represented’ in stimulated cycles, whereas Novotný et al. (1999) describe an increase in NCS number and size after hormonal stimulation. In addition to performing non-systematic endometrial sampling, the mean number of epithelial cells with NCSs on CD18–21 was only ~10% in natural and ~20% in stimulated cycles (Novotný et al., 1999). These numbers are below half of the actual number of NCSs observed when counting all NCSs in larger sections of the endometrium, which was possible by our light microscopic approach (Guffanti et al., 2008; Rybak et al., 2011; Smyga et al., 2013). Therefore, undersampling could account for the disparate outcomes in those two studies.

Overall, we report on an all-or-nothing change in the endometrial epithelium during the luteal phase caused by follicular estrogen regardless of luteal hormone supplementation. Therefore, assessment of NCS prevalence provides an objective and sensitive measure for the status of the luteal endometrium, i.e., secretory transformation and initiation of the window of implantation. In support of this statement, we are presently testing the performance of NCS prevalence against that of the gene expression profile identified by the endometrial receptivity array (Díaz-Gimeno et al., 2013).

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## Authors’ roles

G.Z. and U.T.M. designed the study, interpreted the results and wrote the manuscript. G.Z. performed all endometrial biopsies. M.J.S. quantified the NCSs in most samples and helped with the statistical analysis. E.A.R. performed initial semi-quantitative analysis of NCSs and contributed to the interpretation of the results. U.T.M. coordinated the study and all authors revised the manuscript and approved the final version.

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## Conflict of interest

None declared.

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