

ORIGINAL ARTICLE

Individual dynamics of uterine natural killer cells in natural and stimulated cycles monitored using a new endometrial dating method

Joachim Alfer^{1,2} | Amir Fattahi^{3,4} | Nathalie Bleisinger⁴ | Sophia Antoniadis⁴ |
Jürgen Krieg⁵ | Ralf Dittrich⁴ | Matthias W. Beckmann⁴ | Arndt Hartmann¹ |
Roxana M. Popovici⁶ | Kelton Tremellen⁷

¹Department of Pathology, Erlangen University Hospital, Friedrich-Alexander University of Erlangen-Nürnberg, Erlangen, Germany

²Kaufbeuren-Ravensburg Institute of Pathology, Ravensburg, Germany

³Department of Reproductive Biology, School of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

⁴Department of Obstetrics and Gynecology, Erlangen University Hospital, Friedrich-Alexander University of Erlangen-Nürnberg, Germany

⁵Kinderwunschzentrum Amberg, Amberg, Germany

⁶Kinderwunsch im Zentrum Kiz, Munich, Germany

⁷Department of Obstetrics, Gynaecology and Reproductive Medicine, Flinders University, South Australia, Australia

Correspondence

Joachim Alfer, Kaufbeuren-Ravensburg Institute of Pathology, Elisabethenstrasse 19, 88212 Ravensburg, Germany.

Email:

joachim.alfer@pathologie-ravensburg.de

Abstract

Problem: It is important to evaluate the dynamics of uterine natural killer (uNK) cells in hormone replacement therapy (HRT) cycles, given their potential role in implantation and the common usage of HRT cycles with in vitro fertilization (IVF).

Method of study: A total of 132 subfertile patients were evaluated during the secretory phase of either natural ovulation (OV) or HRT cycles, with two biopsies taken on approximately days 5 and 10 after ovulation/progesterone administration in a single menstrual cycle. Immunohistochemical Personal Endometrial Maturation Analysis (PEMA) was used to better quantify secretory-phase endometrial development, in combination with subsequent evaluation of uNK cell density.

Results: uNK cell density increased rapidly from the early to mid-secretory phase, with mean uNK densities of 113 and 117 per mm² in first biopsies and 315 and 387 per mm² in second biopsies for OV and HRT cycles, respectively. After reassessment of endometrial development with PEMA, the first and second biopsies in HRT and OV cycles were histologically dated to developmental ranges between days 15–20 (first biopsy) and days 19–25 (second biopsy).

Conclusion: Subfertile women showed variable endometrial development in PEMA assessment, with uNK cell density correlating with the dating results. Overall, comparable levels of uNK cell density were observed in OV and HRT cycles. Importantly, uNK cell density depends on the histological maturation stage, with similar low coefficients of determination. This observation suggests that aberrant uNK cell results more likely reflect displaced endometrial maturation, rather than an intrinsic anomaly in uNK cell trafficking.

KEYWORDS

CD56, dating, endometrium, reproduction, subfertile, uNK cells

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. *American Journal of Reproductive Immunology* published by John Wiley & Sons Ltd.

1 | INTRODUCTION

Embryo implantation is a complex event that requires precise synchronization of endometrial and embryo development. A better understanding of the mechanisms that regulate endometrial development may pave the way for improvements in assisted reproductive technology (ART) outcomes. Up to 15% of women undergoing in vitro fertilization (IVF) treatment experience recurrent implantation failure following the transfer of multiple high-quality embryos.¹ Although the cause of implantation failure is poorly understood, aberrant uterine natural killer (uNK) cell function² and a displaced window of implantation (WOI), leading to embryo–endometrial maturation disparity, have been suggested as explanations for implantation failure.³ Given the importance of endometrial development in implantation, Klentzeris et al. proposed that a precisely timed endometrial biopsy should be considered as part of the investigations for women with unexplained reproductive failure. They reported a strong increase in CD56-positive granulated lymphocytes around the time of implantation (LH +7), suggesting an important role for uNK cells in successful implantation. The morphometric findings indicated in situ proliferation of endometrial granulated lymphocytes in response to the ovulatory surge in progesterone, rather than migration from the peripheral circulation.^{4,5} Immune cells, such as T cells and macrophages, were found to be relatively constant in the endometrium throughout the menstrual cycle, whereas CD56-positive large granular lymphocytes (uNKs) are normally low between days 10 and 19 of the cycle, but strongly increase in the late luteal phase and again decrease after menstruation.^{6,7} A recent study investigated the influence of the immunosuppressive drug tacrolimus for patients with a higher Th1:Th2 ratio. The authors analyzed the quantity of uNK cells and the expression of cytokines. A lower proportion of uNK cells was detected in combination with up-regulation of leukemia-inhibiting factor (LIF), interleukin (IL)-10, and IL-17 in the cohort with tacrolimus treatment, resulting in an implantation rate of 40%. The quantity of uNK cells in the well-defined and dated endometrium in the present study may therefore provide evidence regarding subfertility in patients.⁸

King et al. reported that natural killer (NK) cells are closely associated with trophoblast invasion. The physical proximity of uNK cells to trophoblasts, their precise up-regulation in density around the time of implantation, and their ability to release cytokines known to stimulate trophoblast development^{2,9} all point to a key role of uNK cells in early pregnancy events.

Given the important role of uNK cells in pregnancy, previous studies have evaluated the density of uNK cells during natural ovulation cycles.^{10,11} These studies report that the density of uNK cells is low and relatively stable in the proliferative phase of the menstrual cycle. However, in response to the ovulatory surge in progesterone, the density of uNK cells starts to increase during the first half of the secretory phase and shows a strong 6–10-fold increase in the second half of the secretory phase.^{10,12} Since NK cell development appears to be significantly tied to progesterone exposure and resultant secretory changes in the endometrium, this poses a problem for clinical uNK cell assessment, as the dating of ovulation needs to be precisely determined by assess-

ing changes in the endocrine hormone profile (luteinizing hormone and progesterone) in natural ovulation cycles, or by using precisely timed artificial hormone replacement therapy (HRT) cycles. Further complicating the picture, many women with implantation failure exhibit delayed endometrial development/WOI,^{3,13–15} also potentially altering uNK cell dynamics. During IVF treatment, HRT cycles are regularly administered for transfer of frozen embryos. The dynamics of serum levels of progesterone (P4) will be more gradual in HRT cycles, due to constant ingestion of a defined amount of progesterone in comparison with natural cycles (OV), in which the individual release of P4 depends on corpus luteum activity. Moreover, the corpus luteum releases—in addition to P4—other hormones such as relaxin, which may potentially affect uNK cell activity. In primate pregnancy, circulating relaxin, solely a product of the corpus luteum, peaks in the first trimester of pregnancy and increases endometrial NK cells, macrophages, and neutrophils.¹⁶ It is believed that relaxin is involved in the process of decidualization, and that decidual cell production of cytokines, such as IL-15, alters the endometrial NK cell number.¹⁷ It is therefore of interest whether administration of P4 only in HRT cycles results in altered uNK cell density in comparison with natural cycles.

The aim of this study was to monitor dynamic changes in uNK cell density between and within individual subfertile women, in whom one biopsy was taken in the early phase and another in the mid-secretory phase of a single menstrual cycle. uNK cell density was also compared between natural cycles and—for the first time, to the best of our knowledge—HRT cycles, in combination with the enhanced immunohistochemical dating tool and endometrial monitoring test Personal Endometrial Maturation Analysis (PEMA).

2 | MATERIALS AND METHODS

2.1 | Study population

All participants were of reproductive age with a history of at least 12 months subfertility. They had experienced implantation failures after IVF a mean of four times. The study included 72 women (with a mean age of 37.66 ± 3.87 years) in well-monitored natural cycles and 60 women (with a mean age of 36.68 ± 4.66 years) in mock HRT cycles.

2.2 | Endometrial samples and dating method

Endometrial development was dated using two methods: firstly, based on the ovulation date, with assessment of serum hormones such as luteinizing hormone (LH) and progesterone (P4), or the starting date of progesterone therapy in an HRT cycle; and secondly, using the Personal Endometrial Maturation Analysis (PEMA) method. This method combines monitoring of one menstrual cycle by taking biopsy samples at OV/progesterone (P)+5 and OV/P+10 with a histomorphologically defined cycle day (Noyes criteria), supplemented by assessment of the immunohistochemical expression pattern of the estrogen receptor

(ER), progesterone receptor (PR), and proliferation marker Ki-67, with greater accuracy for the cycle day determined.^{18,19}

A total of 144 endometrial biopsies were taken at days OV+5 and OV+10 from individuals in the natural cycles, and 120 biopsies were collected on days P+5 and P+10 from patients in the HRT cycle, corresponding to days 19 and 24 of a 28-day menstrual cycle. A Pipelle endometrial suction curette (Gynemed GmbH, Lensahn, Germany) was used for sampling. The HRT was conducted on the basis of the standard Kaufmann protocol,²⁰ involving a minimum of 2 weeks of oral estrogen before the start of vaginal progesterone administration. Any patients with an abnormal uterine cavity on sonohysterography, unilateral or bilateral hydrosalpinx, and endometrium ≤ 6 mm after estrogen stimulation were excluded from the study. Couples with severe male-factor infertility (genetic translocation, possible poor sperm DNA integrity) were also excluded from the study, as the aim was to focus on cases with an endometrial factor leading to infertility.

Approval for the study was obtained from the ethics committee of the Medical Faculty of Friedrich-Alexander-University of Erlangen-Nürnberg. In accordance with the committee's findings, signed consent was not required, given the retrospective nature of the study.

2.3 | Immunohistochemistry

The endometrial biopsies were fixed and stained as described previously.¹⁸ Briefly, the slides were incubated with diluted primary monoclonal antibodies for 45 min at room temperature (estrogen receptor dilution 1:400, DCS, Hamburg, Germany; progesterone receptor dilution 1:300, DCS, Hamburg, Germany; Ki-67 dilution 1:500, Zytomed, Hamburg, Germany; CD56 dilution 1:800, DAKO, Glostrup, Denmark). After washing of the slides with washing buffer (Zytomed Systems GmbH, Berlin, Germany) and incubation with Post-Block reagent (POLAP-100 Kit, Zytomed), they were incubated with alkaline phosphatase (AP) polymer (POLAP-100 Kit, Zytomed) for 30 min at room temperature. To confirm the immunostaining method, positive control tissues were attached to each slide. For Ki-67 and CD56, an appendix cross-section served as the positive control and a cell control array was used for hormone receptors (Zytomed, cat. no. MB-CC REZ).

2.4 | Steroid hormone receptor and proliferation analyses

The analysis for estrogen and progesterone receptors focused on the glandular epithelium in the stratum functionale of the complete endometrium biopsy. The biopsies were analyzed and classified at interval steps of 5%. Counting was also done for results with less than 5% of stained nuclei. Ki-67 analysis was also done on the glands of the stratum functionale. Stromal cells were analyzed by counting 300 stromal cells in three representative high-powered fields (400 \times , field: .303 mm²).

2.5 | Analysis of CD56-positive cells

Positive cells close to the surface epithelium were counted. Representative areas without glandular spaces were assessed four times in each biopsy under a high-powered field (400 \times), together representing an area of approximately .3 mm² of endometrial tissue.

2.6 | Statistical analysis

The normal distribution of the data was confirmed by Kolmogorov–Smirnov testing. For comparison of the numbers of uNK cells between the two groups (OV and HRT), an independent-sample *t*-test was used. Since the number of cases was low on some days of the cycle after endometrial dating using PEMA, the nonparametric Mann–Whitney test (between two groups) and Kruskal–Wallis test (among cycle days) were used for the analysis. Pearson's correlation test was used to detect possible correlations in the number of uNK cells between early and late luteal phases. *P*-values < .05 were considered significant. The SPSS statistical package, version 16 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

3 | RESULTS

3.1 | Number of uNK cells based on hormonal dating

Analyses of uNK cells in the endometrial biopsies relative to the clinical timing details demonstrated very similar mean levels of uNK cell density on the first luteal-phase biopsy, at 117.5 ± 43.9 and 113.2 ± 49.3 cells per mm² in the HRT and OV cycles at P+5/OV+5, respectively ($p = .602$; Figure 1). Analysis of the late luteal-phase biopsy samples showed that the uNK cell density was significantly greater in the HRT cycles than in the OV cycles (387.3 ± 154.6 vs. 315 ± 108.5 uNK cells/mm²; $p = .002$).

3.2 | Endometrial development based on Personal Endometrial Maturation Analysis (PEMA)

After dating of the endometrial biopsies using the Personal Endometrial Maturation Analysis (PEMA) method, endometrial maturation in the biopsies from the day 5 secretory phase as anticipated by hormonally based dates showed a significant overall delay. The day 19 (day 5 secretory) biopsies in the OV cycles were histologically dated to between days 15 and 20 (a mean difference of 3.3 ± 1.3 days), while those in the HRT cycles were histologically dated to between days 15 and 20 (a mean difference of $3.1 \pm .9$ days). Although there were no significant differences in endometrial development between the OV and HRT cycles in the early secretory-phase biopsies, a large number of women in both groups thus had delayed endometrial development according to the PEMA analysis—equivalent to an average of 2 days'

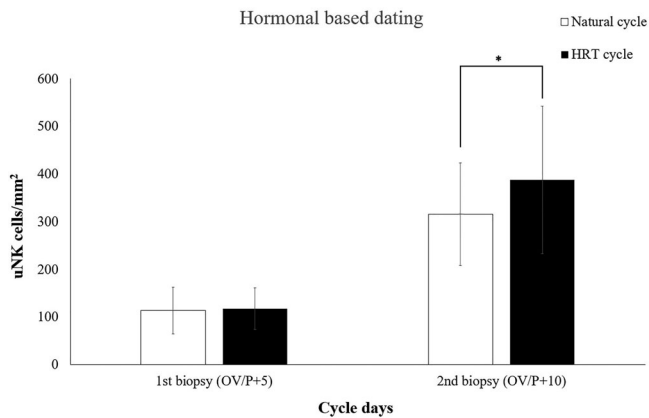


FIGURE 1 Numbers of uterine natural killer (uNK) cells in endometrial tissue obtained on days 5 and 10 of natural cycles (OV) and hormone replacement therapy (HRT) cycles. Each column indicates the mean uNK cell count for all cases relative to the first and second for each group, without dating. The x-axis corresponds to the clinical day of tissue sampling. The error bars show standard deviation (mean \pm SD). *Indicates significant differences between the groups ($p < .05$).

delay. The dating of the second biopsies (P+10/OV+10, or day 24 of the cycle) showed that endometrial development corresponded to a mean of 8.5 ± 1.0 days (range 20–25 days) in the OV cycles and 8.7 ± 1.3 days (range 19–25 days) in the HRT cycles.

3.3 | Number of uNK cells based on Personal Endometrial Maturation Analysis (PEMA) dating

After PEMA dating of the first biopsies, there were no significant differences between the two groups in relation to the numbers of uNK cells on different developmental days ($p > .05$). The numbers of uNK cells were significantly different between different days in the early luteal phase (first biopsy) in the OV group ($p = .005$); however, such differences were not observed in the HRT group ($p = .107$).

The numbers of uNK cells differed significantly among the endometrial development days in the second biopsies in both the OV and HRT cycles ($p = .003$ and $p < .001$). In the second biopsies, two more patients (6.12%) in the HRT cycles had reached day 10 in comparison with the OV cycles, and four more patients (6.95%) had reached day 11 in comparison with the OV cycles, with a mean amount of 414/480 uNK cells per mm^2 on day 10 and 554/632 uNK cells per mm^2 on day 11 in the OV and HRT cycles, respectively.

The density of uNK cells was slightly higher on the day 10 biopsy in the HRT group in comparison with the OV women, although the difference did not reach significance ($p = .129$). Continuous elevation of uNK cells was observed in both the OV and HRT cycles after progesterone exposure. Day-by-day comparison of increases in uNK cells showed significantly higher numbers in the HRT cycles in comparison with the OV cycles in the second biopsies, correlating after dating to histological day 8 ($p = .022$; Figure 2A,B).

3.4 | Individual maturation spread in OV cycles

The greatest increase in uNK cells in OV cycles (7.65-fold) was detected in a patient who had 3 days' delay at the first biopsy, corresponding to histological day 16 (OV+2), at 39.6 NK cells per mm^2 , and at the second biopsy with a delay of 1 day (OV+9), at 303 NK cells per mm^2 . The smallest increase (1.03-fold) in the OV cycles was detected in a patient who had 1 day of hypermaturation at the first biopsy, corresponding to histological day 20 (OV+6), with 244 NK cells per mm^2 and at the second biopsy with a 1-day delay (P+9) and 251 NK cells per mm^2 .

3.5 | Individual maturation spread in HRT cycles

The greatest increase in uNK cells in HRT cycles (12.32-fold) was detected in a patient who had 3 days' delay at the first biopsy, corresponding to histological day 16, at 39.2 NK cells per mm^2 and at the second biopsy with a delay of 1 day and 488 NK cells per mm^2 . The smallest increase (1.16-fold) was observed in a patient who had 4 days' delay at the first biopsy, corresponding to histological day 15, at 79 NK cells per mm^2 , and at the second biopsy with a delay of 3 days, corresponding to histological day 21 (92 NK cells per mm^2).

3.6 | Correlation in uNK cell numbers between early and late luteal phases

There were positive correlations in the numbers of uNK cells between the early and late luteal phases in both the OV and HRT cycles ($r = .344$, $p = .003$ and $r = .314$, $p = .015$, respectively; Figure 3A,B). There was a wide distribution of cases due to the individual maturation of the endometrium.

4 | DISCUSSION

Since the first report on uNK cells in the human endometrium,²¹ many researchers have investigated the functioning of these cells in fertile and subfertile patients. Their findings highlighted key role of uNK cells during the implantation process. Due to the abundance of uNK cells in endometrial tissue during the WOI, it has been postulated that these unique cells must play a key role in early pregnancy; there is therefore considerable interest in developing standardized diagnostic tests for uNK cell activity.^{10,12,22,23}

The present study has for the first time investigated uNK cell changes during the secretory phase in HRT cycles in comparison with natural cycles, on the basis of a standardized endometrial dating method using both endocrine and immunohistochemical assessments. As expected, a rapid increase in uNK cell density was observed during the secretory phase of both natural and HRT cycles dated either with endocrinological methods or with enhanced-sensitivity

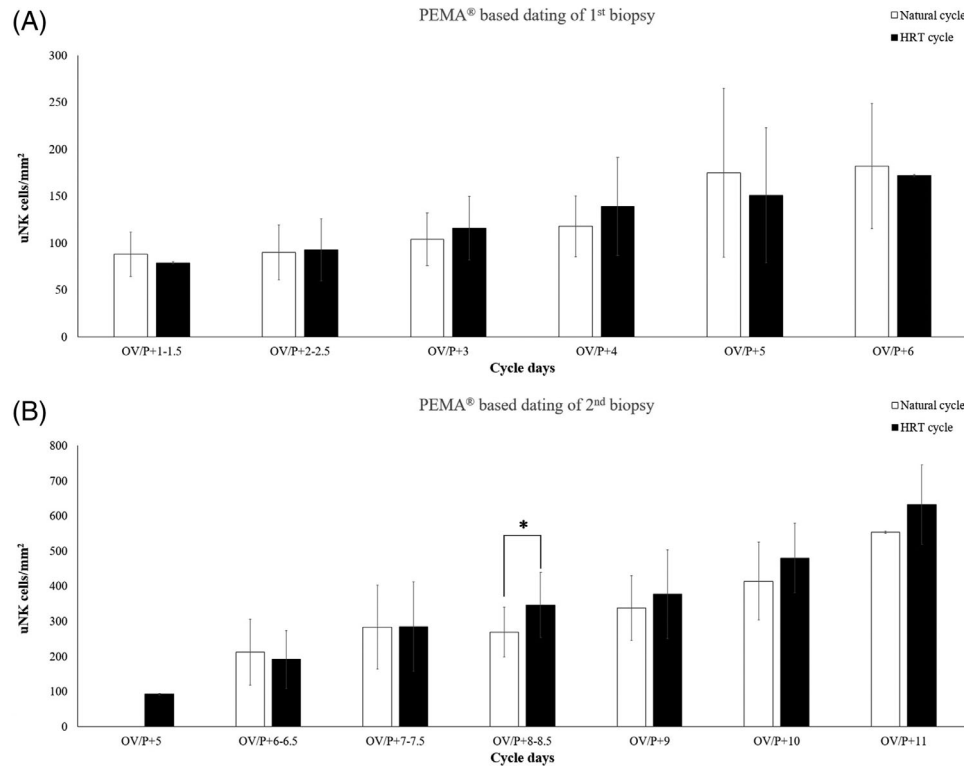


FIGURE 2 Numbers of uterine natural killer (uNK) cells in endometrial tissue according to the PEMA dating. Each column indicates the mean uNK cell count for all cases relative to a specified endometrial maturation day after dating. For each column, the x-axis indicates the day to which the endometrial maturation status corresponds. (A) Biopsies sampled on day 5 of natural cycles (OV) and hormone replacement therapy (HRT) cycles (OV/HRT +5) showed a range of different endometrial maturation rates according to PEMA dating, from OV/HRT +1 to +6. The numbers of uNK cells differed significantly between different days in the early luteal phase in the natural cycle group ($p = .005$). (B) Biopsies sampled on day 10 of natural cycles (OV) and HRT cycles (OV/HRT +10) showed a range of different endometrial maturation rates according to PEMA dating, from OV/HRT +5 to +11. The numbers of uNK cells differed significantly between endometrial development days at the second biopsy in both OV and HRT cycles ($p = .003$ and $p < .001$). The error bars show standard deviation (mean \pm SD). *Indicates significant differences between the groups ($p = .02$).

endometrial immunohistochemical methods (Personal Endometrial Maturation Analysis, PEMA).^{18,19} An interesting result was that the levels of uNK cells in HRT and natural cycles were comparable in the first biopsies of the endometrium (day 5), whereas the mean numbers of uNK cells in the second biopsies (day 10) were 22.8% larger in the HRT cycles than in the natural cycles. This might be because when PEMA dating was used, two more patients (6.12%) were found to have reached day 10 and four more patients (6.95%) had reached day 11 in the HRT cycles; it is already known that the number of uNK cells increases during the luteal phase. In addition, larger numbers of patients with accelerated maturation (day +11) were observed in the HRT group. Beyond the scope of this study, it may be speculated that the more accelerated pattern of endometrial development in HRT cycles possibly reflects the greater and possibly more rapid initial increase in levels of progesterone exposure in HRT in comparison with natural cycles. In a natural cycle, serum progesterone increases from a preovulatory baseline below 5 nmol to a mid-luteal peak of around 60 nmol. However, there is a very rapid increase in endometrial progesterone exposure in HRT cycles, equivalent to the mid-luteal ovular progesterone levels, right from the start of progesterone therapy. The

slower “take-off” in progesterone exposure in natural cycles compared with HRT cycles may explain why almost twice the number of endometrial samples showed large developmental delays (≥ 3 days) on day 5 in OV cycles in comparison with HRT, with a trend toward lower uNK cell density at the second biopsy. However, it should be noted that the number of samples was relatively small, so that the difference did not reach statistical significance.

Interestingly, PEMA-enhanced histomorphological dating of maturation resulted in a wide range of endometrial maturation dates, from days 1 to 6 (first biopsies) and 5 to 11 (second biopsies) after the start of progesterone administration. Endometrial development showed a mean delay of 2 days at the first biopsy in both the HRT and natural cycle groups. This finding is not unexpected, given that the study cohort consisted exclusively of a group of women experiencing recurrent implantation failure of likely uterine origin. Previous studies using sensitive molecular WOI tests, such as the endometrial receptivity array (ERA) test or ERPeakSM, have also confirmed that one-third of women who experience implantation failure have a displaced WOI, with mostly delayed endometrial development.³ The present results therefore highlight the clinical utility of PEMA for assessing

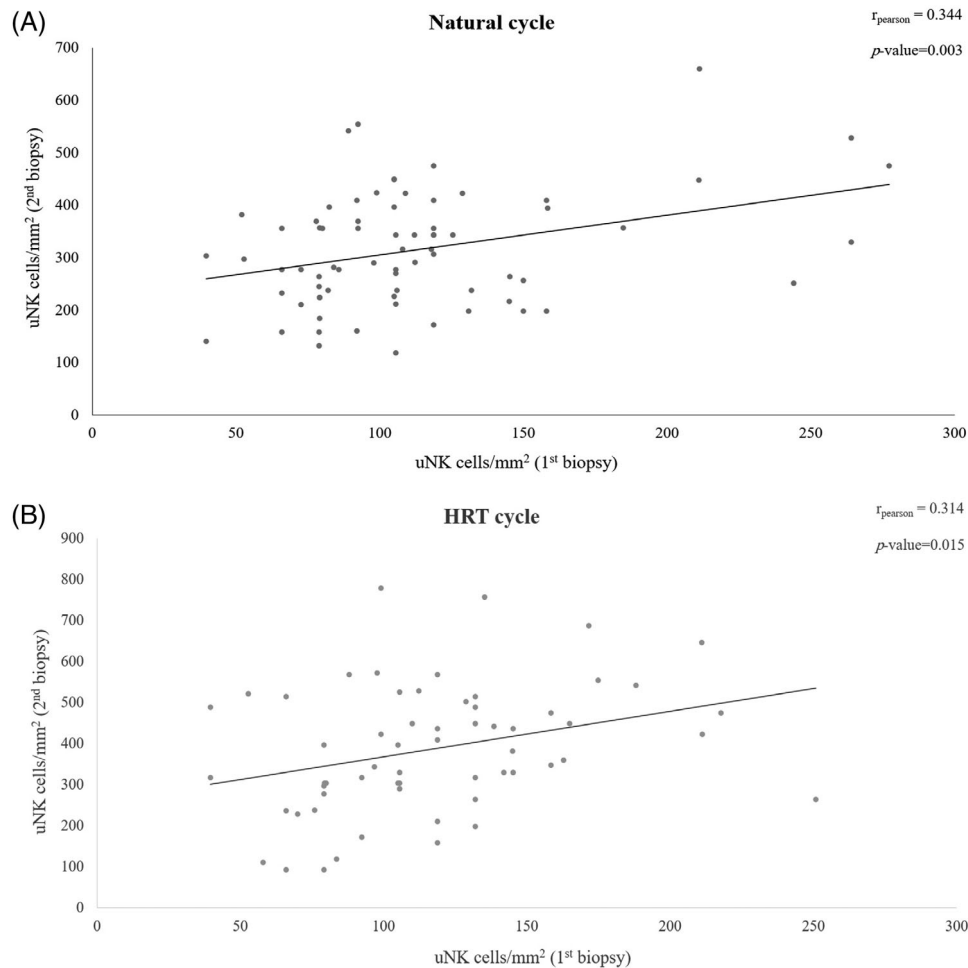


FIGURE 3 Correlation in uterine natural killer (uNK) cell numbers between the early and late luteal phases in (A) natural cycles (OV) and (B) hormone replacement therapy (HRT) cycles

displacement of the WOI, a common pathology in patients with recurrent implantation failure.

It has been documented that the density of uNK cells reflects the maturation status of the endometrium. This observation has been confirmed by a recent study that described a correlation between endometrial development and numbers of uNK cells.²⁴ With a focus on individual cases, the present results show that the quantity of uNKs was always higher in the second, late-secretory biopsy than in the first biopsy in the early mid-secretory phase. The largest increase in uNK cells observed in comparisons of the first and second biopsies was 12.32-fold during an HRT cycle, while the smallest increase was 1.03-fold during an ovulatory cycle. Assessing the quantity of uNK cells without dating the endometrium will therefore not be able to reflect the patient's personal situation, which depends on the individual rate of endometrial maturation. Primarily, the density of uNK cells correlated with histological maturation, with uNK cell density increasing with advancing secretory changes in the endometrium. This observation is consistent with the report by Liu et al., who also concluded that the prognostic value of the uNK cell count is significantly increased when the result is combined with histological dating.²⁵

Generally, it may be postulated that the density of uNK cells reflects the stage of endometrial development, since no obvious divergences in uNK cell numbers were found in relation to the status of endometrial maturation using PEMA dating. The numbers of uNK cells were within the expected range of 40–300 cells in the first biopsies, as reported by Kuon et al.²³ We were not able to find any reference data for numbers of uNK cells at the second biopsies; however, the large increase up to the end of the cycle was already described by Russell et al.¹² On the basis of these observations, it may be suggested that uNK cell density may not be a cause of implantation failure per se, but may rather merely represent a marker of endometrial development. Women with a low uNK cell density at a particular time in the cycle are most likely experiencing recurrent implantation failure due to delayed endometrial development (or a displaced WOI), rather than insufficient uNK cell activity. Similarly, women with elevated uNK cell numbers may have advanced/accelerated endometrial development. It should also be emphasized that immunohistochemistry can only quantify density, not individual uNK cell activity or cytokine production—a further impediment to the use of uNK cell density as an indicator for immunotherapy.

This shift from thinking of uNK cells as a direct cause of implantation failure to the view that they are only associated with implantation failure is of major clinical importance, for two reasons. Firstly, the current clinical practice of initiating immunosuppressive therapy for patients with elevated uNK cell numbers may be incorrect, as it does not directly treat the underlying cause, which may be accelerated endometrial maturation (advancement of the WOI). In addition, many of these mid-luteal biopsies are not precisely timed, so that assessment is difficult and the results are prone to false findings of immune dysregulation. Secondly, in patients with a low uNK cell density that is also associated with delayed endometrial development, the physician may consider personalizing the timing of embryo transfer a day or two later than is routinely done. This approach has been found to produce positive results with molecular techniques for endometrial WOI assessment such as ERA.³

Several weaknesses of this study need to be acknowledged. Firstly, we recognize that although the study is the largest of its kind describing uNK cell density changes throughout the secretory/luteal phase, the number of patients included for some days of development was still small, so the statistical assessment is underpowered. Furthermore, we did not correlate our results with the clinical outcome. Future papers correlating uNK cell density/PEMA assessment of endometrial development with subsequent IVF outcomes will be useful to prove a causal association between the extremes of uterine endometrial development/uNK cell density and pregnancy outcome. This would require a biopsy to be performed before commencing any IVF treatment, as it is possibly unethical to not inform a woman experiencing recurrent IVF implantation failure of her result, if that result may modify her subsequent treatment and increase the chances of pregnancy. Such a prospective study would also have the advantage of being able to generate a true recent fertile control population. We also acknowledge that the inclusion of a fertile control group would enhance the results, as it could prove that the altered endometrial development and associated uNK cell density are more commonly seen in infertile than fertile women. However, it is extremely difficult to enroll fertile patients in a study that involves an invasive endometrial biopsy. Finally, it is theoretically possible that the mere act of performing an endometrial biopsy may initiate a local inflammatory response that could possibly elevate uNK cell density at a second biopsy taken later in that same cycle, since an earlier study has shown an increase in proinflammatory cytokines in a second biopsy taken in the same menstrual cycle.²⁶ However, our own personal experience in comparing patients' uNK cell density on day OV/HRT +10 with or without an earlier biopsy does not show any major differences. Since the typical clinical practice is to perform only a single endometrial biopsy around the time of implantation, the possibility of a biopsy-initiated inflammatory reaction at a second biopsy is probably not a matter of material diagnostic concern.

5 | CONCLUSION

This is the first report of uNK cell analysis during monitored HRT cycles in well-dated endometrial samples (PEMA). The number of uNK

cells increased throughout the secretory phase and the coefficients of determination in the HRT and OV cycles were broadly comparable. uNK cell density significantly varied between individuals, but was always higher in the second biopsies (mid-luteal phase), and primarily reflected the endometrial maturation status. The uNK levels observed in this study were consistent with previous findings in fertile patients^{11,23} after extended dating and cycle monitoring using the PEMA method.^{18,19} It may therefore be concluded that the density of uNK cells appears to reflect the endometrial maturation status, and uNK cell density assessment should therefore not be used as a basis for initiating immunosuppressive therapies. Similarly, a low uNK cell density should not be used as an indication for induction of endometrial inflammation using a "scratching" biopsy, as has been suggested previously.²⁷ Instead, physicians treating the patients should consider the possibility of a displaced WOI as the underlying cause of implantation failure in patients with an aberrant uNK cell density relative to the cycle date.

ACKNOWLEDGEMENTS

The authors are grateful to all of the patients who participated in the study. They would also like to thank Dr. Michael Robertson for medical copy-editing.

CONFLICT OF INTEREST

The authors hereby declare that they had no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Coughlan C, Ledger W, Wang Q, et al. Recurrent implantation failure: definition and management. *Reprod Biomed Online*. 2014;28:14-38.
2. Lash GE, Bulmer JN. Do uterine natural killer (uNK) cells contribute to female reproductive disorders? *J Reprod Immunol*. 2011;88:156-164.
3. Ruiz-Alonso M, Valbuena D, Gomez C, Cuzzi J, Simon C. Endometrial Receptivity Analysis (ERA): data versus opinions. *Hum Reprod Open*. 2021;2021:hoab011.
4. Klentzeris LD, Li TC, Dockery P, Cooke ID. The endometrial biopsy as a predictive factor of pregnancy rate in women with unexplained infertility. *Eur J Obstet Gynecol Reprod Biol*. 1992;45:119-124.
5. Klentzeris LD, Bulmer JN, Warren A, Morrison L, Li TC, Cooke ID. Endometrial lymphoid tissue in the timed endometrial biopsy: morphometric and immunohistochemical aspects. *Am J Obstet Gynecol*. 1992;167:667-674.
6. Starkey PM, Clover LM, Rees MC. Variation during the menstrual cycle of immune cell populations in human endometrium. *Eur J Obstet Gynecol Reprod Biol*. 1991;39:203-207.
7. Zhao X, Jiang Y, Wang L, Li Z, Li Q, Feng X. Advances in understanding the immune imbalance between T-Lymphocyte subsets and NK cells in recurrent spontaneous abortion. *Geburtshilfe Frauenheilkd*. 2018;78:677-683.
8. Bahrami-Asl Z, Farzadi L, Fattahi A, et al. Tacrolimus improves the implantation rate in patients with elevated Th1/2 helper cell ratio and Repeated Implantation Failure (RIF). *Geburtshilfe Frauenheilkd*. 2020;80:851-862.

9. King A, Hiby SE, Gardner L, et al. Recognition of trophoblast HLA class I molecules by decidual NK cell receptors—a review. *Placenta*. 2000;21(Suppl A):S81-5.
10. Russell P, Sacks G, Tremellen K, Gee A. The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure. III: further observations and reference ranges. *Pathology*. 2013;45:393-401.
11. Chen X, Mariee N, Jiang L, et al. Measurement of uterine natural killer cell percentage in the periimplantation endometrium from fertile women and women with recurrent reproductive failure: establishment of a reference range. *Am J Obstet Gynecol*. 2017;217:680.e1-680.e6.
12. Russell P, Anderson L, Lieberman D, et al. The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure I: techniques. *J Reprod Immunol*. 2011;91:90-102.
13. Russell P, Hey-Cunningham A, Berbic M, et al. Asynchronous glands in the endometrium of women with recurrent reproductive failure. *Pathology*. 2014;46:325-332.
14. Enciso M, Aizpurua J, Rodriguez-Estrada B, et al. The precise determination of the window of implantation significantly improves ART outcomes. *Sci Rep*. 2021;11:13420.
15. Alfer J, Happel L, Dittrich R, et al. Insufficient angiogenesis: cause of abnormally thin endometrium in subfertile patients? *Geburtshilfe Frauenheilkd*. 2017;77:756-764.
16. Weiss G. Relaxin and the control of primate parturition. *Ital J Anat Embryol*. 2013;118(1 Suppl):17-18.
17. Dunn CL, Kelly RW, Critchley HO. Decidualization of the human endometrial stromal cell: an enigmatic transformation. *Reprod Biomed Online*. 2003;7:151-161.
18. Alfer J, Fattahi A, Bleisinger N, et al. Endometrial dating method detects individual maturation sequences during the secretory phase. *In Vivo*. 2020;34:1951-1963.
19. Alfer J, Popovici RM, Fattahi A, et al. Endometrial delay is found to be part of a normal individual dynamic transformation process. *Arch Gynecol Obstet*. 2021;304:1599-1609.
20. Kaufmann C. Therapeutics with hormones of the ovary: (section of therapeutics and pharmacology). *Proc R Soc Med*. 1934;27:849-863.
21. Hamperl H, Hellweg G. Granular endometrial stroma cells. *Obstet Gynecol*. 1958;11:379-387.
22. Diaz-Hernandez I, Alecsandru D, Garcia-Velasco JA, Dominguez F. Uterine natural killer cells: from foe to friend in reproduction. *Hum Reprod Update*. 2021;27:720-746.
23. Kuon RJ, Weber M, Heger J, et al. Uterine natural killer cells in patients with idiopathic recurrent miscarriage. *Am J Reprod Immunol*. 2017;78(4).
24. Hviid Saxtorph M, Persson G, Hallager T, et al. Are different markers of endometrial receptivity telling us different things about endometrial function? *Am J Reprod Immunol*. 2020;84:e13323.
25. Liu B, Mariee N, Laird S, Smith J, Li J, Li TC. The prognostic value of uNK cell count and histological dating in the mid-luteal phase of women with reproductive failure. *Eur J Obstet Gynecol Reprod Biol*. 2014;181:171-175.
26. Evans GE, Phillipson GTM, Sykes PH, McNoe LA, Print CG, Evans JJ. Does the endometrial gene expression of fertile women vary within and between cycles? *Hum Reprod*. 2018;33:452-463.
27. Gnainsky Y, Granot I, Aldo PB, et al. Local injury of the endometrium induces an inflammatory response that promotes successful implantation. *Fertil Steril*. 2010;94:2030-2036.

How to cite this article: Alfer J, Fattahi A, Bleisinger N, et al. Individual dynamics of uterine natural killer cells in natural and stimulated cycles monitored using a new endometrial dating method. *Am J Reprod Immunol*. 2022;88:e13620. <https://doi.org/10.1111/aji.13620>