

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

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1 **Title**

2 Measurement of uterine natural killer (uNK) cell percentage in the peri-implantation
3 endometrium from fertile women and women with recurrent reproductive failure:
4 establishment of a reference range

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25

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1 **Condensation**

2 A reference range for uNK cells percentage in ovulatory fertile women in natural cycles has
3 been established. Overall, the groups with recurrent reproductive failure had significantly
4 higher uNK cells percentage than the controls, but there was a subset of both groups that had
5 lower result.

6

7 **Short title**

8 Measurement of uNK cells in fertile women and women with reproductive failure

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1 **Abstract:**

2 **Background:** Uterine natural killer (uNK) cells are the major leucocytes present in the
3 peri-implantation endometrium. Previous studies have found controversial differences in uNK
4 cell percentage in women suffering from recurrent reproductive failure, compared with fertile
5 controls.

6 **Objective:** To compare the uNK cell percentage in women with recurrent reproductive failure
7 and fertile controls.

8 **Study Design:** It was a retrospective study carried out in university hospitals. A total of 215
9 women from 3 university centres participated in the study, including 97 women with recurrent
10 miscarriage (RM), 34 women with recurrent implantation failure (RIF) and 84 fertile controls.
11 Endometrial biopsy samples were obtained precisely 7 days after luteinization hormone surge
12 in a natural cycle. Endometrial sections were immunostained for CD56 and cell counting was
13 performed by a standardised protocol. Results were expressed as percentage of positive uNK
14 cell/ total stromal cells.

15 **Results:** The median uNK cells percentage in Chinese ovulatory fertile controls in natural
16 cycles was 2.5% (range 0.9-5.3%). Using 5th and 95th percentile to define the lower and upper
17 limits of uNK cells percentage, the reference range was 1.2% to 4.5%. Overall, the groups
18 with recurrent reproductive failure had significantly higher uNK cells percentage than the
19 controls (RM: median 3.2%, range 0.6-8.8%; RIF: median 3.1%, range 0.8-8.3%). However,
20 there was a subset of both groups (RM: 16/97; RIF: 6/34) that had lower uNK cells
21 percentage, comparing to fertile controls.

22 **Conclusions:** A reference range for uNK cells percentage in fertile women was established.
23 Women with recurrent reproductive failure had uNK cells percentages both above and below
24 the reference range.

25

26 **Key words:** uterine Natural Killer (uNK) cells; reference range; fertile women; recurrent
27 miscarriage, recurrent implantation failure

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1 **Introduction**

2 Uterine natural killer (uNK) cells are the major leucocytes present in the endometrium. The
3 phenotype of these uterine granulated lymphocytes in endometrium is CD56^{bright} CD16⁻
4 whereas in peripheral blood CD56^{dim}CD16⁺ NK cells constitute the major subpopulation (1).
5 The number of CD56⁺ NK cells varies throughout the menstrual cycle, with a dramatic
6 increase in the mid-secretory phase starting 6 to 7 days after the LH surge, the beginning of
7 the putative time of implantation. The number of CD56⁺ NK cells remains high during early
8 pregnancy and comprises around 70% of the lymphocytes at the interface between maternal
9 decidua and the invading trophoblast during the first trimester of pregnancy (2). Although the
10 increased number of uNK cells at the time of embryo implantation and their presence next to
11 the invading trophoblast suggests that they play a role in implantation, the exact function of
12 uNK cells is still unclear.

13 Studies of peri-implantation endometrium have found differences in uNK cells percentage
14 in women suffering from recurrent reproductive failure. Using immunohistochemistry,
15 increased percentage of CD56⁺ NK cells was detected in peri-implantation endometrium
16 from women with recurrent miscarriage (3-5) and women with recurrent implantation failure
17 after IVF (6,7). Nevertheless, the prognostic value of uNK cell measurement in women with
18 recurrent reproductive failure has not yet been confirmed (5,8). Furthermore, there is an
19 increasing demand from women with reproductive failure seeking an "uNK cell testing",
20 despite the fact that the normal reference range of uNK cells in a fertile population has not yet
21 been firmly established. One centre used the 75th percentile of a group of 18 fertile controls as
22 the cut off value of 5% (9), while another used 90th percentile of 13 fertile controls to define
23 the upper limit giving a value of 12.9% (5). One of the reasons for these differences is likely
24 to be differences in the counting methodology, which has recently been resolved (10).

25 Given the dramatic increase in uNK cell numbers during peri-implantation period, a
26 difference in endometrial sampling time as few as 1–2 days will make a big difference to the
27 number of uNK cells present. It is therefore important that the biopsy is precisely timed

1 according to the luteinising hormone (LH) surge and preferably taken 7 days following LH
2 surge (LH+7).

3 The aim of this study is to use the recently published methodology (10) to determine the
4 reference range of uNK cells percentage in precisely timed endometrial biopsies (LH+7) from
5 fertile women, and to compared these results to those in women with recurrent reproductive
6 failure.

7 **Materials and methods**

8 **Subjects**

9 Three groups of women were recruited for this study (Table 1). In the fertile control group,
10 72 non-smoking Chinese women with proven fertility were recruited from Prince of Wales of
11 Hospital (PWH), The Chinese University of Hong Kong and Shenzhen People's Hospital
12 (SZH) in China. Another 12 endometrial biopsies from fertile Caucasian women were
13 collected from Sheffield Teaching Hospitals (STH)-Jessop Wing, Hallamshire Hospital,
14 Sheffield, UK. All the women had regular menstrual cycles and had not used any hormonal
15 treatment for at least three months before the biopsy. Approvals from local ethical committees
16 in the participating centres were obtained for this study. All the endometrial biopsies were
17 collected with the informed consent of the participants.

18 In the recurrent miscarriage (RM) group (n=97), endometrial biopsies were obtained from
19 women with unexplained RM attending the Recurrent Miscarriage Clinic, Prince of Wales
20 Hospital, The Chinese University of Hong Kong. RM was defined as a history of three or
21 more consecutive miscarriages before gestational week 20, including biochemical losses. All
22 the subjects had normal karyotyping, normal 3D ultrasonography hysterosalpingogram, day 2
23 FSH<10IU/L, mid-luteal progesterone >30nmol/L, normal thyroid function and tested
24 negative for lupus anticoagulant and anti-cardiolipin IgG and IgM antibodies.

25 In the recurrent implantation failure (RIF) group (n=34), endometrial biopsies were
26 obtained from women attending the Assisted Reproductive Technology centre, Prince of

1 Wales Hospital, The Chinese University of Hong Kong. RIF was defined as the failure to
2 achieve a clinical pregnancy after transfer of at least four good-quality embryos in a minimum
3 of three fresh or frozen cycles in a woman under the age of 40 years (11).

4 **Endometrial biopsy**

5 All subjects in this study had daily urine dipstick test from day 9 of the menstrual cycle
6 onwards to identify the LH surge (ovulation), which was used to precisely time the
7 endometrial biopsies on day LH+7 of the peri-implantation period.

8 All biopsies were obtained using a Pipelle sampler (Prodimed, France) or Pipet Curet
9 (Cooper Surgical, USA). The specimens were immediately placed into 10% neutral buffered
10 formalin for over-night fixation at room temperature and then embedded into paraffin wax.

11 **Immunohistochemical staining of CD56+ cells**

12 In this study, CD56+ immunostaining was used to determine uNK cell. No attempt was made
13 to determine subsets of uNK cells which can be determined by differential expression of
14 CD16.

15 As described in the previous studies (10,12), paraffin-embedded human endometrial tissue
16 sections (3 µm) were dewaxed in xylene, rehydrated through descending ethanol to
17 Tris-buffered saline (TBS) and unmasking of antigen performed in citrate buffer. In all cases
18 the primary antibody used was a mouse anti-human monoclonal primary anti-CD56 antibody
19 (NCL-CD56-504; Novacastra Laboratories, Newcastle, UK) at a 1:100 dilution. The binding
20 of primary antibody was visualised using an avidin biotin peroxidase secondary antibody
21 complex (Elite ABC kit; Vector Laboratories, Peterborough, UK). The slides were then
22 incubated with peroxidase substrate DAB (3,3'-diaminobenzidine tetrahydrochloride) and
23 0.1% H₂O₂, counterstained with haematoxylin, dehydrated through alcohols, cleared in xylene
24 and mounted in DPX.

25 **Cell counting methodology**

1 The counting method was performed according to an agreed and published standardised
2 protocol by a multi-centre working group designed to reduce variance of results between
3 centres (10). The number of uNK cells (CD 56 positively stained) and stromal cells (CD56
4 negative stained) were counted manually using Image J and the result was expressed as a
5 percentage of total stromal cells. For cell counting, 10x40 fields (at least 3800 stromal cells)
6 were captured using a photomicroscope. The first field to be captured was selected at random,
7 ensuring that it contained the luminal epithelial border. Subsequent fields were obtained by
8 moving one field to the left or right of the original field, keeping the luminal epithelial border
9 in view. This was repeated until 10 fields had been captured. This methodology for
10 determining the location in the sample to be counted is important as it prevents inclusion of
11 CD56+ cell aggregates, which are present deeper in the tissue. Only membrane staining
12 associated with a cell nuclei was considered as specific. All stromal cells were counted,
13 including the cells surrounding the blood vessels. The number of CD56+ uNK cells as a
14 percentage of stromal cells for each image was calculated and the final cell count was
15 reported as an average of all 10 fields.

16 **Intra- and inter-observer variability**

17 To determine the intra-observer variability, the CD56+ cell counting was repeated by one of
18 the observers (X. C.) on 23 blinded slides on two separate occasions, without knowledge of
19 the results of the earlier measurement.

20 Inter-observer variability for uNK cells analysis was evaluated by two observers (X.C. and
21 N. M.) examining the same set of 23 randomly chosen sections using the agreed protocol (10),
22 independently of each other.

23 **Statistical analysis**

24 The data distribution was checked by the Shapiro-Wilk test. All results were presented as
25 mean \pm SD for normally distributed data or as median and range for skewed data. We used
26 the 5th and 95th percentile results of CD56+ cells percentage from fertile controls as the lower
27 limit and upper limit respectively, for the reference range. The non-parametric test

1 (Mann-Whitney U) test was used to compare (i) percentages of CD56+ cells in women with
2 RM and in controls, (ii) percentages of CD56+ cells in women with RIF and in controls. $P <$
3 0.05 was considered significant. All of the data were analysed with SPSS 18.0 software.

4 **Results**

5 **Demographics**

6 All participants had regular menstrual cycles. In the fertile group, the mean age of the Chinese
7 fertile women was 29 (range 22-38) years, whereas the mean age of the Caucasian women
8 was 36 (range 30- 42) years. None of the fertile controls had a history of spontaneous
9 miscarriage. In the RM group, the mean number of miscarriages was 3 (range 3-5) and the
10 mean age was 35 (range 26-40) years. In the RIF group, the mean number of failed IVF
11 cycles was 4 (range 3-6) and the mean age was 37 (range 32-40) years.

12 **Staining patterns**

13 Figure 1 shows the immunohistochemical staining for CD56+ cells, which were present as
14 individual or group cells throughout the stroma in selective LH+7 endometrium from fertile
15 women, women with RM and women with RIF. Visual examination suggested that increased
16 percentage of CD56+ cells were present in the endometrium from women with RM and
17 women with RIF, compared with controls. These observations were confirmed by further
18 statistical analysis.

19 **Intra- and inter-observer variability**

20 Intra- and inter-observer variability for uNK cells percentage is summarized in Table 2.
21 Regarding intra-observer variability, one observer measured the percentage of uNK cells in
22 23 biopsies on two separate occasions. There was significant correlation ($r = 0.96$, $p < 0.001$)
23 between the percentage of cells counted on each occasion. The median (range) of uNK cells
24 expressed as a percentage of the total stromal cells in the specimens was: measurement A,
25 2.70% (range 0.60-8.50%); measurement B, 2.67% (range 0.60-8.40%), respectively. The
26 coefficient of variance (CV) for intra-observer measurement was 9.9%.

1 For inter-observer variability, the same set of 23 specimens was measured by two observers
2 independently. There was significant correlation ($r=0.82$, $p<0.001$) between measurements
3 of observer A and observer B. The median (range) of uNK cells in the specimens was:
4 observer A, 2.73% (range 0.60-8.50%); observer B, 2.47% (range 0.20-13.10%), respectively.
5 The median (range) of the absolute difference in results between measurement of observer A
6 and B was 0.15% (range 0.03-4.60%). The CV for inter-observer measurement was 14.5%.

7 **uNK cells percentage in fertile women**

8 The median uNK cells percentage from 72 Chinese fertile controls was 2.5% (range
9 0.9-5.3%). We decided to use the 5th and 95th percentile results in peri-implantation
10 endometrium from these ovulatory fertile Chinese women in natural cycles as our reference
11 range, which gave a reference range of uNK cells percentage from 1.2% to 4.5%.

12 The median uNK cells percentage from 12 Caucasian normal fertile women in natural
13 cycles was 3.3% (range from 1.1-5.3%). There was no significant difference in uNK cells
14 percentage between fertile Chinese and Caucasian women.

15 **uNK cells percentage in women with recurrent reproductive failure**

16 The median (range) of uNK cells percentage in the women with RM was 3.2% (range
17 0.6-8.8%), and in the women with RIF was 3.1% (range 0.8-8.3%). Compared to the median
18 result in the fertile controls (2.5%), there was a significantly increased percentage of uNK
19 cells observed in women with RM ($p=0.042$), as well as women with RIF ($p=0.048$).

20 Using the 5th percentile (1.2%) as the lower limit and 95th percentile (4.5%) as the upper
21 limit to define the reference range of uNK cells percentage, 60 out of the 97 (around 62%)
22 women with RM were within the reference range, with 21 women above the range and 16
23 women below the range. In women with RIF, 18 out of the 34 (around 53%) women had uNK
24 cells percentage within the reference range, 10 women above the range and 6 women below
25 the range (Figure 2).

26

1 **Discussion**

2 In this study, we have established a reference range for uNK cells percentage in
3 peri-implantation endometrium using the 5th and 95th percentile results for a group of Chinese
4 fertile women. We have also observed a significantly increased uNK cells percentage in
5 women with recurrent reproductive failure. In addition, only around 62% of the women with
6 RM and around 53% of the women with RIF had uNK cells percentage within the established
7 reference range.

8 **Methods for uNK cells testing**

9 A lack of standardised method for the measurement of uNK cells percentage has been
10 considered a hindrance to the progress in understanding the role of these cells in pregnancy.
11 However, the recent publication of a working group, which addressed a standardised protocol
12 for the timing of sampling, processing of specimens, as well as the counting method including
13 the magnification and the number of fields/ cells to be counted (10) has started to resolve this
14 process. In this study, we adopted this standardised methodology and verified its use by
15 performing inter-observer variation analysis using people from two separate laboratories in
16 two different countries. The analysis showed acceptable levels of variation.

17 In the current study, the result for uNK cells measurement was expressed as a percentage
18 (number of CD56+ cells/ number of stromal cells) rather than the absolute number of uNK
19 cells per microscopic field, because the latter presenting method does not take into account
20 the relative size of the field, the presence of variable amounts of glandular components and
21 blood vessel and the amount of oedema in the stroma. The presentation of uNK cell
22 measurement as a percentage is in line with the recommendations on the standardization of
23 uNK cell measurement (10).

24 Given the very rapid changes in endometrial morphology and function around the time of
25 implantation and that the number of uNK cells increases rapid as the menstrual cycle
26 progresses (13,14), it is important that the biopsies are precisely timed. In this study, biopsies
27 from all women were precisely timed to take place seven days after LH surge. We accept that

1 it is not always possible to collect biopsy on a particularly chosen day, as it may not fit in the
2 schedule of the subject or the clinical team. However, we have chosen to include only
3 samples obtained on LH+7 to ensure the homogeneity of the collected specimens.

4 **Reference range for uNK cell**

5 Despite the fact there had been many publications on uNK cell numbers, there is a lack of
6 sufficiently robust data on the reference range derived from fertile subjects, with which
7 women with various reproductive failure can be compared. Our study has shown that the
8 reference range of uNK cells percentage in endometrium from ovulatory fertile Chinese
9 population in natural cycles can be defined as 1.2%-4.5%. However, the findings may not
10 apply to cycles employing controlled ovarian stimulation or in cycles with programmed
11 frozen embryo transfer.

12 As far as we are aware, the number of Chinese fertile controls in our study (n=72) presents
13 the largest series ever published and in particular is considerably larger than previous studies
14 from two different centres. These studies using small numbers of control women (n=18 and
15 n=13, respectively) have defined a high cell percentage as 5% and 12.9%. The study by
16 Quenby *et al.* defined “high” as the 75th percentile of the control population (9), while
17 Tuckerman *et al.* used a cut-off of 90th (5). In this study, we have chosen to use 5th and 95th
18 percentiles to define the lower and upper limits in accordance with general recommended
19 protocol. This gave an upper limit of 4.5% uNK cells, which is similar to that reported by
20 Quenby *et al.* (9).

21 In addition, all previous studies focused on the upper limit of the reference range without
22 attention to what may be the lower limit due to the assumption that high uNK cells percentage
23 is detrimental to the reproductive process. Limited attention had been made to “low uNK cell”
24 and the possible adverse impact of too few uNK cells on the endometrium. Based on our
25 proposed reference range, we defined a lower limit of 1.2% and found that around 16%
26 (16/97) of women with RM and around 18% (6/34) of women with RIF had uNK cells
27 percentage below this value. The clinical relevance of this observation requires further
28 investigations.

1 **uNK cells and recurrent reproductive failure**

2 Several studies have shown no significant difference in uNK cells percentage between women
3 with RM and fertile women (15,16), whereas the majority studies have found increased
4 percentage of uNK cells in the endometrium from women with RM (3-5). This study, which
5 is consistent with many of the earlier studies, has shown that median uNK cells percentage
6 was increased in women with RM. Around 22% (21/97) of women with RM had a high uNK
7 cells percentage as defined by our suggested upper limit.

8 An earlier study found no increase in uNK cells percentage in young women with RIF (17).
9 However, several other studies have reported an increase in uNK cells percentage in
10 endometrium from women with RIF after IVF (6,7). In this study, we also found a
11 significantly increased percentage of uNK cells in women with RIF, when compared to fertile
12 controls, with around 29% (10/34) of these women having a uNK cells percentage above the
13 upper limit.

14 **Comparison between ethnic groups**

15 An earlier study compared peripheral NK (pNK) cells between Chinese and Caucasian
16 populations and found that the number of pNK cell in Chinese women was higher than in
17 Caucasian women (18). However, to our knowledge, there has not been any study reporting
18 on the variation of uNK cells in various ethnic groups to date.

19 In this study, the median and range of uNK cells percentages in Chinese and Caucasian
20 fertile women were similar and the reference range established could be applied to both ethnic
21 groups. However, the number of Caucasian women was very small in this study and a larger
22 sample size is needed to confirm this observation.

23 **Strengths of this study**

24 There are several strengths to our study. Firstly, we have included only precisely timed
25 endometrial biopsies on day LH+7 in natural cycles. Secondly, we had used a standardised
26 protocol of immunostaining and cell counting method to obtain numbers of uNK cells.

1 Thirdly, we have recruited a large group of 72 fertile Chinese women to establish the
2 reference range, which is considerably larger than that of previous studies.

3 **Role of uNK cells in the endometrium**

4 Although a precise role for uNK cells in endometrial function and embryo implantation is not
5 clear, a recent review has highlighted their possible role in effective implantation by
6 controlling the depth and pattern of trophoblast invasion (19). There is also evidence for a
7 role of uNK cells in other important events in the endometrium and decidua, such as the
8 control of vascular remodelling in early pregnancy (20). *In vitro* models have shown that uNK
9 cell supernatants can alter markers of vascular remodeling (20). Moreover, uNK cells were
10 observed to be frequently aggregated around the spiral arteries in early pregnancy and this
11 distribution may reflect their role in mediating vascular changes in normal pregnancy (21).

12 **Conclusion**

13 In this study, we have established a reference range for uNK cells percentage in
14 peri-implantation endometrium on day LH+7 in fertile women and using this reference range
15 found that a significant proportion of women with recurrent reproductive failure had uNK
16 cells percentages both above and below the reference range. We found that around 38% of
17 women with RM and around 47% of women with RIF had uNK cell count outside the
18 reference range, with a majority above the range and a smaller proportion below the range.

19 **Funding**

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9

10 **Figure legend**

11 Figure 1. Photomicrograph of immunostaining for CD56 in LH+7 endometrium from (A) a
12 fertile woman, (B) a woman with recurrent miscarriage, and (C) a woman with recurrent
13 implantation failure. Positive staining cells indicated by arrow. Scale bar= 50µm. LE=luminal
14 epithelium.

15 Figure 2. uNK cells percentage in fertile women and women with recurrent reproductive
16 failure. Using the 5th percentile (1.2%) as the lower limit and 95th percentile (4.5%) as the
17 upper limit to define the reference range of uNK cells percentage, 21 out of the 97 women
18 with RM above the range and 16 women below the range. In women with RIF, 10 out of 34
19 women above the range and 6 women below the range. Compared to the fertile controls, a
20 overall significantly increased uNK cells percentage was observed in women with RM and
21 women with RIF, compared to controls.