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The effect of prednisolone on endometrial uterine NK cell concentrations and pregnancy outcome in women with reproductive failure. A retrospective cohort study

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Abstract

This retrospective study of prospectively collected data examines the effect of prednisolone therapy on raised uterine Natural Killer cell (uNK) concentrations and pregnancy outcomes in women with recurrent miscarriage (RM) and recurrent implantation failure (RIF) after IVF/ICSI treatment. 136 women diagnosed with RRF who had a timed midluteal endometrial biopsy taken for uNK cell analysis were included. Women with high uNK cell concentrations (n=45) were treated with prednisolone (10mg/day) for one month, after which a second biopsy was taken for repeat uNK cell analysis. Women for whom prednisolone caused a decrease in uNK cell concentrations continued on prednisolone until 12 weeks of pregnancy. Pregnancy outcomes (live birth, miscarriage and implantation rates) and pregnancy complications were compared for women who received prednisolone and those who did not. Results showed that the prevalence of high uNK cells was 33.1%. Prednisolone significantly decreased the uNK cell concentration ($P<0.001$), however reduction to normal limits was achieved in only 48.3% of patients. There was no difference in any of the pregnancy outcomes or complications between women who had received prednisolone and those who had not. In conclusion, this study showed a relatively high prevalence of raised uNK cells in women with recurrent reproductive failure and confirmed the effect of prednisolone on reducing uNK cell concentrations. We found however no evidence for a significant beneficial effect for prednisolone therapy on pregnancy outcomes. Until the results of an adequately powered RCT become available however, these findings should be considered preliminary

Key Words Endometrium; uNK cells; Prednisolone; Recurrent Miscarriage; Recurrent Implantation Failure

Introduction

The endometrium consists of a population of natural killer cells distinct from peripheral blood cells. The majority (90%) of peripheral blood natural killer cells (pNK) are CD56^{dim}CD16⁺ and a minority (10%) are CD56^{bright}CD16⁻, whilst in the endometrium the majority (90%) of cells are CD56^{bright}CD16⁻ and a minority (10%) are CD56^{dim}CD16⁺ (Bulmer *et al.* 1991). These cell populations also differ in function with the endometrial CD56^{bright}CD16⁻ population showing increased cytokine production and decreased cytotoxicity, compared to the CD56^{dim}CD16⁺ peripheral blood cells (Saito *et al.* 1993, Deniz *et al.* 1994, Koopman *et al.* 2003).

The numbers of endometrial NK (uNK) cells increase rapidly during the mid-secretory phase and remain high during the first trimester of pregnancy, suggesting that they play a role in embryo implantation. Their presence in close proximity to uterine blood vessels and the invading trophoblast suggest that they may play a role in spiral artery remodeling or trophoblast cell growth or function (Smith *et al.* 2009, Lash *et al.* 2010).

High uNK cell concentrations have been found in patients with reproductive failure including recurrent miscarriage (Quenby *et al.* 2005) and repeated implantation failure (RIF) after in vitro fertilization treatment (IVF) (Tuckerman *et al.* 2010), although other studies in women with RIF have suggested no change in NK cell concentrations in the endometrium of women with RIF (Matteo *et al.* 2007). Differences in findings may be because the definition of a "high" uNK cell count differs between research groups; even within the United Kingdom two groups have initially defined high as either greater than 5% (Quenby *et al.* 2005) or greater than 13% (Tuckerman *et al.* 2010). These differences in findings may be due to differences in the methods used to count uNK cell numbers and illustrate the

importance of adopting a standardized approach to cell counting in future studies (Lash *et al.* 2016).

RRF: Recurrent reproductive failure
 RM: Recurrent miscarriage
 RIF: Recurrent implantation failure
 NK cells: Natural Killer cells
 IVF: In vitro fertilization

Despite no clear understanding of the role that uNK cells may play in pregnancy outcomes, there are increasing requests from women with reproductive failure to undergo an endometrial NK cell count test and many centers are indeed offering this test. Furthermore there is a difference of opinion regarding a suitable treatment for women with "high" uNK cells, but many centers use prednisolone treatment (Laird *et al.* 2016). Prednisolone treatment is based solely on two small sample sized reports (Quenby *et al.* 2003, Quenby *et al.* 2005). In these studies, a repeat biopsy was not taken after prednisolone therapy, and therefore it is not known whether the prednisolone therapy resulted in a decrease in uNK cells or not. Despite this clear deficiency of the evidence, testing and treatment for raised uNK concentrations seems in many cases to have been rushed into clinical practice.

The main aim of the current study is to effect of prednisolone on raised uNK cells concentrations in a well selected group of women with recurrent early reproductive failure and to investigate the effect of prednisolone treatment on uNK cell concentrations and pregnancy outcomes. As far as we are aware it is the first to examine both the effect of prednisolone on NK cell concentrations as well as pregnancy outcomes.

Materials and Methods

Patients

This was a retrospective analysis of prospectively collected data from a consecutive series of 164 women who had an endometrial biopsy between the 22nd June 2010 and the 22nd May 2017, to test for uNK cells at the Jessop Wing, Royal Hallamshire Hospital, Sheffield, United Kingdom. The study examines data obtained from women undergoing investigations and treatment for raised uNK cells based on our Unit's established protocol (Figure 1).

Patients were included in the analysis if they had a diagnosis of recurrent miscarriage (RM) defined as having three or more consecutive pregnancy losses before 24 weeks gestation (Stirrat 1990) or RIF as defined by the failure to achieve a clinical pregnancy after the transfer of at least two embryos in a minimum of two fresh or frozen cycles. All patients underwent investigations including: thrombophilia screening, karyotyping, thyroid function tests and antibodies, androgen profile, gonadotrophins, ultrasonography and hysterosalpingography (Metwally *et al.* 2014). The study was approved by the University of Sheffield Ethics Application System.

Tissue sampling and uNK cell analysis

An endometrial biopsy sample was obtained using a Pipelle sampler on days LH + 6-9 of the cycle according to the LH surge, which was measured by a urine ovulation test. The technique for sample preparation has been previously described by our group (Tuckerman *et al.* 2007).

Cells were counted by one operator who assessed 10 x400 fields. The technique of counting uNK cells changed in July 2015. Prior to that date, fields of view were chosen at random throughout the tissue section. After July 2015, images were only chosen for analysis if adjacent to the luminal edge and a minimum of 3800 total stromal cells were counted. Counting the number of nuclei determined the number of total stromal cells. Counting the positively stained cells determined the number of

uNK cells. Data was expressed as the percentage of CD56+ uNK cells as a total of stromal cells.

Definition of high uNK cell. Prior to July 2015 (n=106) raised uNK was defined as a concentration of >13.9% (Tuckerman *et al.* 2007). The definition of raised uNK cells was redefined as more than 5% in July 2015 (n=30) with the change in our technique of measurement after comparison of methodology across a number of centers (Lash *et al.* 2016).

Prednisolone Administration

Patients with high uNK cells were offered 10 mg of prednisolone daily starting from the first day of the menstrual cycle. Nine patients with high uNK cells did not receive treatment due to the fact that even though, their uNK cells had been measured with the new technique, the new cut off limit for uNK had not yet been introduced and hence under the old classification system, they were considered to have normal uNK cells and hence not treated. Even though this group was not the result of a randomization process, they did provide an opportunity for comparison with those who had been treated. Prednisolone was continued until the biopsy was repeated again at LH +7-9 and was then gradually stopped while results were awaited.

If the uNK count had dropped to within normal range, patients were prescribed prednisolone 10 mg when pregnant until 12 weeks gestation. Those where the repeat biopsy did not show normalization of uNK cell concentrations, were prescribed a higher dose of 20 mg when pregnant, again until 12 weeks gestation. On discontinuation, the dose of prednisolone was always tapered gradually.

Outcome measures

- a. **Primary:** The effect of prednisolone on the uNK concentrations in women with raised uNK cells concentrations.

b. Secondary:

- i. 1- Prevalence of high uNK cell concentrations in women with recurrent reproductive failure
- ii. 2- The effect of prednisolone on pregnancy outcomes in women with raised uNK cells concentrations..

Pregnancy outcomes are defined as follows:

- a. Live birth rate (LBR) - calculated as the ratio between the number of patients achieving a live birth and the total number of patients.
- b. Miscarriage rate (MR) - calculated as the ratio between the number of miscarriages (confirmed pregnancies lost <24 weeks of gestation) and the total number of patients.
- c. Implantation failure rate (IFR) - calculated as the ratio between the number of cycles that ended in implantation failure and the number of women who underwent an embryo transfer (ET).
- d. Pregnancy complications
 - I. 1- Antepartum hemorrhage (APH): vaginal bleeding from 24 weeks gestation prior to birth of the baby
 - II. 2- Gestational Diabetes: hyperglycemia first diagnosed in pregnancy according to the WHO criteria; fasting venous plasma glucose level >9mmol/L or a two hours level of >11 mmol/L after ingestion of 75 g of oral glucose.
 - III. 3- Pregnancy induced hypertension (PIH): diastolic blood pressure of >90mmHg on two consecutive occasions taken at least four hours apart diagnosed for the first time after 20 weeks gestation

IV. 4- Emergency caesarean-section rate

V. 5- Preterm labor (PTL), the number of babies born before 37 weeks

VI. 6- Intrapartum fetal distress: a pathological heart trace pattern seen during continuous fetal monitoring

VII. 7- Small for gestational age (SGA): fetal growth less than two standard deviations below the mean for gestational age according to growth and development record charts

VIII. admission to special care baby unit (SCBU)

IX. 8- Stillbirth rate (number of babies born after 24 completed weeks of gestation without signs of life)

X. 9- Neonatal death rate (death of a baby within 28 days following birth)

Statistical Analysis

The normality in the distribution of data was established using a Kolmogorov-Smirnov test. Data were expressed as mean (\pm SD), median (range) and percentages as appropriate. For comparison between two independent groups an independent t test or a Mann Whitney test was performed as appropriate. A chi square test was used to compare proportions. A Pearson/Spearman correlation was used as appropriate to investigate the relationship between uNK cell concentrations and variables. For comparison between uNK cell concentrations before and after treatment a Wilcoxon test was performed. Logistic regression analysis was used to examine the relationship between uNK classification (normal or treated) and other factors, which could influence the chance of having a live birth. These included: maternal age, gravidity, parity, infertility duration, time to pregnancy/cycle, early

cleavage/blastocyst transfer and single/multiple embryo transfer. Statistical significance was defined as $P < 0.05$, analysis was performed using the Statistical Package for Social Sciences version 22 (SPSS, Inc., Chicago, IL).

Results

Demographics

164 patients underwent a biopsy. Tissue processing was not possible for three biopsies. 16 patients were lost to follow-up. Data from one patient could not be included as she was participating in a separate interventional study. Eight patients were undergoing other forms of assisted conception treatment and were excluded in order to reduce heterogeneity of the population. Therefore, 136 patients were included in the analysis (108 with RIF and 28 with RM).

The demographics for patients with high and normal uNK cell concentrations are shown in Table 1.

A total of 45 women (33.1%) had high uNK cells [9/28 (32%) In the RM group and 39/108 (36%) in the RIF group. For biopsies taken before July 2015 the median (range) uNK concentration was 9.0% (0.4%-48.2%) for the RIF group and 7.6% (1.5%-31.5%) for the RM group.

After our change of methodology in July 2015 as described above, the median (range) uNK cell concentration was 5.5% (0.5%-17.7%) for the RIF group and 2.3% (2%-3.4%) for the RM group.

There was no significant correlation between baseline uNK concentration and age, body mass index (BMI), duration of infertility, parity, gravidity, number of miscarriages or number of failed cycles. There was also no difference in any of the described demographics between the normal and high group.

There was also no significant association between a high uNK % and the type of miscarriage (primary or secondary), type of infertility or diagnosis (RM or RIF).

The cause of the infertility in each group is shown in Table 2. There were significantly fewer patients with tubal factor infertility in women with normal uNK cells compared to those with high uNK cells ($P=0.016$). There were significantly more patients with endometriosis in women with normal uNK cell numbers compared to those with high uNK cell numbers ($P=0.021$) and there were no patients with both high uNK cells and endometriosis.

The effect of Prednisolone treatment on uNK cell concentrations

Of the patients with high uNK cells ($n=45$), 36 (80%) were prescribed prednisolone. A repeat biopsy for uNK cell concentration was obtained on day LH+7-9 in a subsequent cycle for 29 (80.4%) of these patients. The repeat biopsy showed that uNK cell concentrations significantly decreased after treatment with prednisolone ($P<0.001$) (Figure 2). In 14 (48.3%) patients uNK cell concentrations returned to the normal range and were classified as 'responders'. In 15 (51.7%) patients uNK cell numbers did not return to the normal range and were classified as 'non-responders'. There were significantly more RM patients ($P=0.011$) in the responder group (Table 3). There was no significant difference in the etiology of the infertility in women who responded to prednisolone and those who did not.

The effect of prednisolone on pregnancy outcomes

There was no significant difference in any of the pregnancy outcomes in women with low and high uNK cells regardless of prednisolone treatment. In the subgroup of women with high uNK cells there was also no difference on any of the outcomes whether prednisolone was used or not. Similar results were obtained when considering the RIF and the RM groups of women separately.

The relationship between uNK cell concentration before and after treatment with 10 mg of prednisolone and the subsequent achievement of a live birth whilst remaining on treatment with prednisolone is shown in Figure 3. The uNK cell concentration before or after treatment was not predictive of a live birth nor was the drop in uNK cell concentration as a result of treatment. When comparing those who responded to one month of 10mg of prednisolone treatment and those that did not, there were also no significant difference in LBR, MR and IFR.

The effect of prednisolone pregnancy complications

There was no significant difference in the incidence of pregnancy complications between women who received prednisolone and those who had not (Table 4). Of the women in the treated group on 20 mg of prednisolone (n=2), only one baby was SGA.

Discussion

Despite the lack of sufficient studies regarding the effect of raised uNK cells on pregnancy outcomes uNK cell testing and treatment with prednisolone has become common practice. The first and foremost finding of this study was a relatively high prevalence of high uNK cell concentrations of 33%. Although a control group was not included in this study we used reports from previous studies to define a "high cell concentration". Prior to 2015 this was based on the analysis of Tuckerman et al, 2007 where 13.9% was shown to be the highest value in a small cohort of fertile control patients. After 2015 we reduced this to 5% as we had aligned our counting method to that of others including Quenby et al, 2005 who had previously shown that 5% represented the 75th percentile in fertile control women. This has been confirmed more recently by a further study which showed that the 95th percentile for uNK cells in a cohort of 84 control fertile women was 4.5% (Chen et al. 2017). The

incidence of 33% is higher than the incidence of other established causes for recurrent early pregnancy loss that are routinely tested for. For example, antiphospholipid syndrome has a prevalence of around 15% and uterine malformations have a prevalence of 1.8%-37.6% (RCOG 2011). If the clinical significance of uNK cells testing is confirmed, then recommendations for routine testing will need to be considered. However, at present, the evidence is insufficient to make such a recommendation.

Regarding possible confounding factors, we found a significant association between the presence of tubal factor infertility and high uNK cells. CD56(+) NK cells have been found to be present in small numbers within the normal fallopian tube (Ardighieri *et al.* 2014). Although we do not have details regarding specific tubal pathology a proportion could be due to hydrosalpinges (Al Subhi *et al.* 2013) which are known to be associated with poor reproductive outcomes. We hypothesize that this increase in uNK may be related to an increase in tubal NK cells or as a response to an endometrial inflammatory reaction induced by hydrosalpinges or other inflammatory tubal conditions.

Interestingly endometriosis, another condition which may also be associated with an inflammatory response, was conversely not found to be associated with an increased uNK cell concentration. Although this may be a chance finding, it is known that defective cytotoxicity of NK cells in the peritoneal space can lead to endometriosis, through poor tissue clearance of ectopic endometrial tissue (Thiruchelvam *et al.* 2015). Although rather speculative, it may be that raised uNK cells may be associated with raised peritoneal NK cells that could promote successful clearance of ectopic endometrial tissue preventing endometriosis.

Secondly, we found a significant drop in uNK after prednisolone treatment albeit with a lower dose than that used previously (i.e. 10mg) (Quenby *et al.* 2003). However, this drop only reached normal range in 48.3% of patients. A recent paper, which investigated the effect of prednisolone treatment on a variety of immunological markers also showed that only 54% of their cohort responded to treatment (Ledee *et al.* 2018). There is limited information available about the reproducibility of endometrial markers between different cycles in the same women. The reproducibility of uNK cell concentration in paired endometrial samples from two different cycles in a small group of women suggested that the diagnosis of high cell counts would have changed in 30% of women (Mariee *et al.* 2012) and this variability may have affected the observed reduction in uNK cell concentrations in the second biopsy. The mechanism by which prednisolone reduces uNK cell numbers is not clearly understood. However, uNK cells have been shown to express glucocorticoid receptors (Henderson *et al.* 2003) and therefore a direct interaction between the steroid and the cells is possible. The longevity of the effect of prednisolone treatment on uNK cell concentrations is unknown and further work is needed to determine this. Despite the drop in uNK concentrations we found no evidence for improvement in pregnancy outcomes with prednisolone treatment.

Other studies have explored the potential benefits of prednisolone treatment in women with high peripheral blood NK activity and found improvements in pregnancy outcomes (Ogasawara and Aoki 2000, Ubaldi *et al.* 2002). However, the clinical relevance of these findings is uncertain as the relationship between uNK0 and pNK cells is not clear (Laird *et al.* 2011). It remains to be seen if the use of a 20-mg dose would have resulted in improved outcomes. A large well powered RCT is therefore needed.

Until further evidence is available however it is reassuring that we found no evidence for an adverse effect on pregnancy. Prednisolone is assumed to have effect on the fetus as 90% is metabolized by the placenta(Addison *et al.* 1993). However other studies have suggested that administration may increase the likelihood of miscarriage and pre-term birth or growth restriction (Quenby *et al.* 2003, Gur *et al.* 2004, Robertson *et al.* 2016). In our study, there was only one case (9.1%) of a baby born that was with IUGR.

Limitations of the study

This is a cohort study that lacks the strengths of an RCT however so far evidence from RCT's is lacking. There has been one RCT addressing the use of prednisolone but was mainly a feasibility study and therefore underpowered to give an answer(Tang *et al.* 2013). Our study also included a number of patients registered in other hospitals (9.8%) where we could not examine their pregnancy outcomes and a number of women had unknown pregnancy outcome (22.2%). Furthermore as this is a retrospective study where no power calculation had been performed it is possible that the study was underpowered to show a clear effect. The data generated from this study however could form the basis for a sample size calculation in future prospective studies.

Secondly, a potential confounding variable is that of the possible therapeutic effect of the endometrial biopsy which has been reported to improve pregnancy rates in women with recurrent implantation failure after IVF (Zhou *et al.* 2008, Karimzade *et al.* 2010). It is possible that the procedure improved implantation rates in both treated and non-treated groups, which diluted the effect of prednisolone treatment and made it more difficult to detect a significant effect.

The non-treatment group also included some women who had received a course of prednisolone that was later discontinued after demonstrating a lack of significant drop in uNK cell concentrations. It is possible that this short course of steroids did to an extent affect uNK cell activity thus influencing pregnancy outcomes in the non-treatment group and diluting any significant difference between the groups. A pure placebo group with no intervention is therefore necessary, again this is best achieved through well designed RCT.

We also included women who were over 40. This is not ideal when analyzing pregnancy outcomes and maternal and fetal complications, as women over 40 have worse fertility and obstetric outcomes (Ziadeh and Yahaya 2001, Malizia *et al.* 2009). However, our analysis did show that in this particular study age was not an important predictor of pregnancy outcome and our previous study showed no relationship between number of CD56+ cells and age (Tuckerman *et al.* 2007). Due to the above factors and particularly the absence of a control group, the findings of this study should be considered preliminary.

Conclusions

This study demonstrates a relatively high prevalence of high uNK concentrations in women with RRF. The study did not demonstrate any beneficial effect for prednisolone at on pregnancy outcomes. Therefore, at present there is no evidence to support treating women with raised uNK cell concentrations. Until the findings of a larger randomized controlled study become available the results of this study should be considered preliminary.

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Table 1. Demographics of women with normal and high uNK cell numbers.

| Demographic | Normal (n=91) | High (n=45) | P value |
|--|----------------------|--------------------|----------------|
| Age (SD) | 35.9 (4.5) | 36.0 (4.2) | 0.881 |
| BMI (kg/m²) | 23.8 (3.3) | 24.1 (4.4) | 0.621 |
| Gravidity median (range) | 1 (0-8) | 1 (0-9) | 0.306 |
| Parity median (range) | 0 (0-1) | 0 (0-2) | 0.802 |
| Duration of Infertility median (range) | 96.0 (31.0-104.0) | 50.0 (33.0-60.0) | 0.440 |
| RM n (%) | 16 (17.6) | 6 (13.3) | 0.510 |
| RIF n (%) | 72 (79.1) | 36 (80) | 0.905 |
| Women with both RIF and RM n (%) | 3 (3.3) | 3 (6.7) | 0.368 |
| Consecutive miscarriages median (range) | 4 (3-6) | 4 (3-7) | 1.0 |
| Failed cycles median (range) | 3 (2-7) | 3 (2-6) | 0.970 |
| Embryos transferred median (range) | 5 (2-13) | 5 (2-10) | 0.667 |

Table 2. Etiology of infertility in women with normal and high uNK cell concentrations

| Etiology | Normal (n=91) | High (n=45) | P value |
|----------------------------|----------------------|--------------------|----------------|
| Unexplained n (%) | 35 (38.5) | 13 (28.9) | 0.272 |
| Uterine n (%) | 1 (1.1) | 1 (2.2) | 0.609 |
| Tubal n (%) | 7 (7.7) | 10 (22.2) | 0.016* |
| Mixed n (%) | 15 (16.5) | 9 (20) | 0.612 |
| Anovulation n (%) | 7 (7.7) | 5 (11.1) | 0.825 |
| Male factor n (%) | 14 (15.4) | 7 (15.6) | 0.975 |
| Endometriosis n (%) | 10 (11.0) | 0 (0.0) | 0.021* |

Table 3. Demographics of women who responded to prednisolone treatment and those who did not.

| Demographic | Non-Responders (n=15) | Responders* (n=14) | P value |
|--|----------------------------------|-------------------------------|----------------|
| Age (y) | 35.2 (3.3) | 35.9 (5.0) | 0.646 |
| BMI (kg/m²) | 23.7 (3.8) | 25.1 (5.9) | 0.448 |
| Time between initial and second biopsy (days) | 57 (29-270) | 56 (36-105) | 0.959 |
| Duration of infertility (months) | 60.0 (22.0-180.0) | 50.0 (15-204) | 0.356 |
| RM n (%) | 0 (0.0) | 5 (35.7) | 0.011* |
| RIF n (%) | 13 (86.7) | 8 (57.1) | 0.075 |
| Both RM and RIF n (%) | 2 (13.3) | 1 (7.1) | 0.585 |
| Parity n (range) | 0 (0-1) | 0 (0-2) | 0.949 |
| Gravidity n (range) | 1 (0-5) | 2.5 (0-7) | 0.146 |
| Failed cycles n (range) | 3 (2-6) | 3.5 (2-6) | 0.821 |

- Responders as defined by a drop in uNK concentrations to within normal range

Table 4 Fetal complications in the babies born to mothers who had received prednisolone and those who had not. The no prednisolone group consisted of women with both high and normal uNK cells.

| | No prednisolone (n=26) | Prednisolone(n=11) | P value |
|--|---------------------------|---------------------------|------------|
| Birth weight (gm) | 3110.0 (703-5441) | 3424.0 (1895.0-4224.0) | 0.603 |
| Gestation (weeks) | 39.0 (26.9-41.7) | 39.7 (32.9-41.9) | 0.603 |
| Neonatal complication* rate n (%) | 8 (30.8) | 4 (36.4) | 0.740 |
| Admission to SCBU/ITU n | 8 | 3 | |
| SGA n | 1 | 1 | |

* includes SGA, admission to SCBU/ITU, neonatal death

Note: there were 3 multiple births in the no prednisolone group, thus n=26 rather than n=23

Legends to Figures

Figure 1. Flow chart to show the treatment protocol for women suffering with repeated reproductive failure and high uNK cells.

Figure 2. uNK cell number before and after treatment with prednisolone. Women with 'high' uNK cells were prescribed daily 10 mg of prednisolone for one month before a repeat biopsy was taken to measure the resulting uNK cell number *P<0.05. uNK cells were counted according to our original methodology in all samples.

Figure 3. The relationship between uNK% before and after treatment and the subsequent pregnancy outcome. Figure shows the initial uNK cell number before treatment and the uNK cell number after one month of 10 mg of prednisolone treatment for each patient. Patients were then prescribed 10mg or 20 mg of prednisolone for their subsequent ART treatment or whilst conceiving naturally. uNK cells were counted according to our original methodology in all samples