OBSTETRICS

Decrease in peripheral natural killer cell level during early pregnancy predicts live birth among women with unexplained recurrent pregnancy loss: a prospective cohort study

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BACKGROUND: Previous studies have suggested that trophoblast cells inhibit the proliferation of peripheral natural killer cells and that the level of peripheral natural killer cells decrease in the middle and late pregnancy stage among healthy women. The change in peripheral natural killer cell level during early pregnancy and the relationship between the change in peripheral natural killer cell level and pregnancy outcomes among women with unexplained recurrent pregnancy loss have not been sufficiently explored.

OBJECTIVE: This study aimed to characterize the level of prepregnancy peripheral natural killer cells in comparison with those in early pregnancy among women with unexplained recurrent pregnancy loss and to determine if the change in the level of peripheral natural killer cells from prepregnancy to early pregnancy can predict pregnancy outcomes.

STUDY DESIGN: In this prospective cohort study, 1758 women with recurrent pregnancy loss were recruited between January 2017 and December 2021 among whom 252 women with unexplained recurrent pregnancy loss had prepregnancy and early pregnancy (4—6 weeks gestation) peripheral natural killer cell measurements. These 252 women were divided into 2 groups, namely those with a lower gestational peripheral natural killer cell level (group 1) when compared with prepregnancy levels and those who did not (group 2). The respective outcomes of these groups in terms of live birth and pregnancy loss were comparatively analyzed using chi-square and Student's *t* tests. Candidate factors that could influence live birth were selected using the Akaike information criterion. The participates were then randomly divided into training and testing groups. A multivariable logistic regression analysis was performed and a nomogram was created to assess the possibility of live birth. The predictive accuracy was determined by the area under the receiver operating characteristic curve and validated by plotting the predicted probabilities and the observed probabilities. A Hosmer-Lemeshow test was used to assess the goodness of fit.

RESULTS: When early gestational peripheral natural killer cell levels were compared with prepregnancy peripheral natural killer cell levels, 61.5% (154) of women had a comparatively lower early-gestational peripheral natural killer cell level and 38.9% (98) of women had an increase or no change in the peripheral natural killer cell level. The live birth rate in group 1 was 89.0% (137/154), which was significantly higher than the rate of 49.0% (48/98) in group 2 ($P<.001$). A decrease in the peripheral natural killer cell level (odds ratio, 1.36; 95% confidence interval, 1.22—1.55; $P<.001$) and the anti-Muellerian hormone level (odds ratio, 1.41; 95% confidence interval, 1.14—1.81; $P=.003$) were important predicting factors for a higher live birth rate. Female body mass index (odds ratio, 0.97; 95% confidence interval, 0.82—1.15; $P=.763$) and parity (odds ratio, 1.61; 95% confidence interval, 0.71—4.12; $P=.287$) also were predicting factors. Furthermore, the area under the receiver operating characteristic curve of the model to diagnose of live birth was 0.853 with a sensitivity of 81.6% and a specificity of 78.0% using the training data set. And the Hosmer-Lemeshow test showed that the model was a good fit ($p=6.068$).

CONCLUSION: We report a comparative decrease in the peripheral natural killer cell levels in early gestation when compared with prepregnancy cell levels in more than 60% of women with unexplained recurrent pregnancy loss at 4 to 6 weeks of gestation. When compared with prepregnancy peripheral natural killer cell levels, a decrease in the peripheral natural killer cell level during early pregnancy might be a useful predictor of the live birth rate among women with unexplained recurrent pregnancy loss.

Key words: early pregnancy, live birth, peripheral NK, unexplained recurrent pregnancy loss

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Introduction

Recurrent pregnancy loss (RPL) is generally defined as the loss of 2 or more fetuses before 24 completed gestational weeks and affects 1% to 5% of couples during their reproductive life.[1,2] Without medical management or intervention, the live birth rate decreases with an increasing of number of lost pregnancies.[3,4] RPL is a risk factor for obstetrical complications and long-term sequelae, including cardiovascular disease and venous thromboembolism.[5—7] Approximately 50% of RPL cases can be attributed to genetic, anatomic, endocrine, autoimmune, and environmental factors and thrombophilia and infectious diseases.[8] The remaining 50% of cases are classified as unexplained RPL (URPL), which is thought to be correlated with an undiagnosed immunologic abnormality, including a disequilibrium in the natural killer (NK) cell level.[9,10]

AJOG at a Glance

Why was this study conducted?

Peripheral natural killer (pNK) cell levels decrease in the middle and late pregnancy stage among healthy women, but the change in pNK cell level during early pregnancy and the relationship between the change in pNK cells level and pregnancy outcomes among women with unexplained recurrent pregnancy loss (URPL) have not been explored.

Key findings

A lower early gestational pNK cell level has been observed in 61.5% of women with URPL. Women with a lower gestational pNK cell level when compared with prepregnancy pNK cell level have a significantly higher live birth rate than those without (89% vs 49%; $P<.001$).

What does this add to what is known?

The decrease in pNK cell level in early pregnancy may be fundamentally important in the physiology of the establishment of a normal pregnancy.

Activated NK cells are essential innate immune cells that eliminate target cells by antibody-dependent, cell-mediated cytotoxicity, perforin-granzyme pathways, and death receptor-mediated pathways, lead to apoptosis of the target cells. Aberrant distributions of decidual NK (dNK) cells with low cytotoxicity and strong cytokine-producing capacity is known to suppress extravillous trophoblast invasion and embryo growth in women with URPL[11−15]; hence, endometrial sampling is proposed as an investigative and diagnostic tool,[16,17] but endometrial sampling can only be performed preconception. Thus, many studies look to peripheral blood NK (pNK) cells as an option for monitoring the immunologic process during pregnancy. pNK cells, which make up approximately 10% to 15% of the circulating lymphocytes, play a significant role in regulating the immune tolerance and establishing a successful maternal-fetal interface.[18,19] pNK cells could traverse the intervillous space and then display their function by contact with villi directly. In addition, pNK cells are recruited to the endometrium and participate in the process of decidualization. NK cells with natural cell adhesion molecule (CD56) and FcγRIII (CD16), which account for 90% to 95% of pNK cells, produce cytokines with strong cytotoxicity, such as perforin and granzyme B, to mediate cell killing, whereas CD56+CD16−NK cells have weak cytotoxicity.[20]

Early studies have observed elevated prepregnancy pNK cell levels with higher cytotoxicity among women with URPL.[21−23] Previous studies also indicated that trophoblast cells and serum β-human chorionic gonadotropin (β-hCG) might inhibit pNK cell proliferation and thus the pNK cell number decreases in the middle and late pregnancy stages from the nonpregnancy state levels among healthy women.[24−26] Hence, it is plausible that a comparison between the prepregnancy and gestational pNK cell levels may be predictive of pregnancy outcomes.[27,28]

In this study, we aimed to investigate the relative change in the prepregnancy pNK cell levels during early pregnancy among women with a history of URPL and to explored if the change is predictive of pregnancy outcomes.

Materials and Methods

Study design

In this prospective cohort study, women with RPL were enrolled between January 2017 and December 2021 at the Reproductive Medicine Center of the First Affiliated Hospital of Sun Yat-sen University. RPL was defined as 2 or more consecutive pregnancy losses before 24 weeks' gestation.[2] URPL was diagnosed among women with RPL without known causes based on the European Society of Human Reproduction and Embryology guidelines.[2] Known causes of RPL were (1) parental chromosomal translocations; (2) clinical endocrine disease, including polycystic ovarian syndrome, thyroid diseases such as hyperthyroidism, hypothyroidism, and thyroiditis, and diabetes mellitus; (3) structural uterine abnormalities observed using transvaginal ultrasonography; (4) primary and acquired thrombophilia, including antiphospholipid syndrome (including anticardiolipin antibody, lupus anticoagulant antibody, anti-b2 glycoprotein I antibody); (5) infectious diseases, including endometritis diagnosed on endometrial biopsy; and (6) a clinical autoimmune disease, including systemic lupus erythematosus and Sjogren syndrome.

All women with RPL provided consent, and blood samples were taken at their routine basal sex hormone test on day 2 to 5 of menstruation to determine the pNK cell level.

The exclusion criteria were (1) a previous pregnancy loss after 12 gestational weeks (because the causative factors for second vs first trimester pregnancy loss are different); (2) lack of consent; (3) positive for antinuclear antibodies or antithyroid antibody; (4) no conception within a year after initial blood tests or until study end, whichever was sooner; (5) unwilling to have a blood sample taken for pNK cell level measurement during pregnancy; and (6) the use of immunologic medication before or after 3 months of pregnancy. The pNK cell levels were also measured at 4 to 6 weeks of gestation.

An ultrasound was conducted at around 6 to 8 gestational weeks to confirm the fetal heartbeat. At around 12 weeks of pregnancy, a nuchal translucency test was carried out. If the women miscarried, a chromosome analysis of the chorionic tissue was recommended. The pregnancy outcomes were determine telephonically after 11 months of pregnancy.

The trial was approved by the ethics committee of the institutional review

board of the First Affiliated Hospital of Sun Yat-sen University (2016-116).

Relevant laboratory analysis

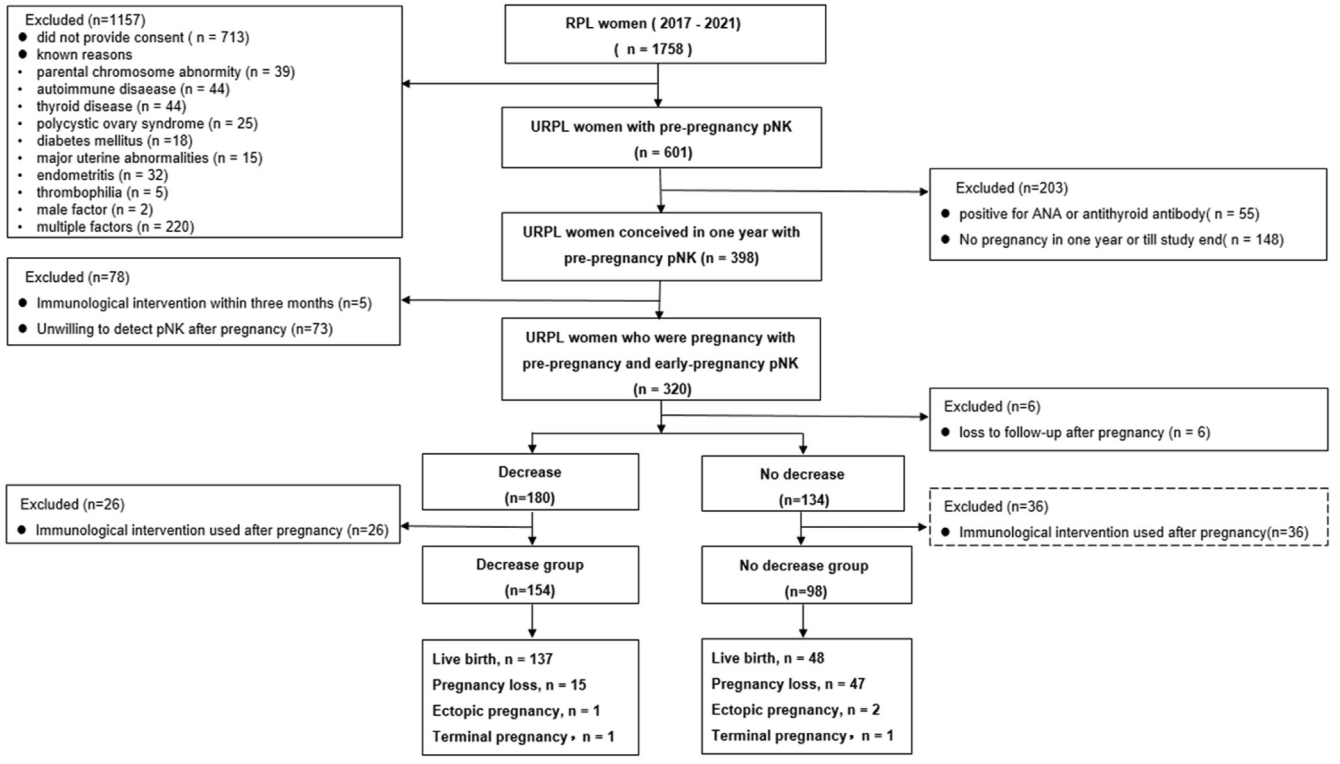
All the serological indexes were measured before 10 AM on day 2 to 5 of the menstrual cycle together with the routine basal sex hormone levels (follicle-stimulating hormone [FSH], leutinizing hormone [LH], and estradiol [E_2]). A total of 3 mL peripheral blood for pNK cell level determination was obtained aseptically by venipuncture in ethylenediaminetetraacetic acid–coated vacutainer tubes (BD, Franklin Lakes, NJ). The whole blood was stored at room temperature, transported to the laboratory, and processed within 2 hours. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus density gradient centrifugation (GE Healthcare, Uppsala, Sweden) and assessed using flow cytometric analysis within 1 hour.

Freshly thawed PBMCs were used for surface staining and analysis for NK and NKT-like cell markers. PBMCs were suspended in staining buffer and plated at 1×10^5 to 10^6 cells according to the manufacturer's instructions. After staining for 30 minutes with anti-human fliorescein isothiocyanate (FITC)-CD3, allophycocyanin (APC)-CD56, and phycoerythrin (PE)-CD16 (all from BD, San Jose, CA) in the dark, multicolor flow cytometry was performed using a BD FACS Canto Plus (BD Biosciences, San Diego, CA) instrument. The percentages of $CD3^-CD56^+$, $CD3^-CD56^+CD16^+$, $CD3^-CD56^+CD16^-$, $CD3^+$, and $CD3^+CD56^+$ PBMCs were calculated. NK cells were identified as the $CD3^-CD56^+$ cell population and NKT-like cells were identified as the $CD3^+CD56^+$ cell population within the lymphocyte population. $CD3^-CD56^+CD16^+$ cells and $CD3^-CD56^+CD16^-$ cells were subsets of NK cells. The pNK cell level was identified as the percentage of $CD3^-CD56+$ cells population within the lymphocyte population.

Assessment of outcomes

Based on the relative change in the level of prepregnancy pNK cells during early pregnancy, the outcomes were analyzed in 2 groups, namely those who had a lower early pregnancy pNK cell level (group 1, prepregnancy pNK − gestational pNK >0) and those who did not (group 2, prepregnancy pNK − gestational pNK <0). The primary pregnancy outcomes were live birth and early pregnancy loss. A live birth was defined as the delivery of at least 1 live baby after 24 weeks' gestation. Early pregnancy loss was defined as the spontaneous loss of pregnancy before 12 weeks of gestation, which included biochemical pregnancies and early miscarriages. A biochemical pregnancy was

FIGURE 1
Flowchart of the study



ANA, antinuclear antibody; *pNK*, peripheral natural killer cell; *RPL*, recurrent pregnancy loss; *URPL*, unexplained RPL.

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defined as the detection of hCG in maternal urine or blood, but no clinical pregnancy was achieved. Early miscarriage was defined as spontaneous intrauterine pregnancy demise confirmed by transvaginal ultrasound before 12 weeks' gestation. Pregnancy loss in the second trimester was defined as the spontaneous loss of pregnancy between 12 to 24 weeks of gestation. Termination of pregnancy was defined as an iatrogenic abortion when fatal fetal dysplasia or a chromosome abnormality was present between 12 to 24 weeks' gestation. An ectopic pregnancy was defined as embryo implantation outside the uterine cavity.

The secondary outcomes included preterm birth, preeclampsia or gestational hypertension, gestational diabetes, and small for gestational age (SGA). Preeclampsia or gestational hypertension was defined as a rise in the systolic blood pressure to ≥140 mm Hg or in the diastolic pressure to ≥90 mm Hg on 2 separate occasions in a patient before 20 weeks of gestation and new-onset proteinuria or, in the absence of proteinuria, new-onset hypertension with new onset of any of the following: thrombocytopenia, impaired liver function, renal insufficiency, pulmonary edema, or cerebral or visual disturbances.[29] Gestational diabetes was

TABLE 1
Baseline characteristics of women who had a decrease in the pNK cell levels measured in early gestation when compared with the prepregnancy levels (group 1) and those with no decrease in the pNK cell levels (group 2)

	Both (n=252)	Group 1 (n=154)	Group 2 (n=98)	
Characteristic	Mean (SD) or n (%)	Mean (SD) or n (%)	Mean (SD) or n (%)	*P* value
Female age (y)	32.55 (4.57)	32.47 (4.36)	32.67 (4.89)	.736
Male age (y)	34.36 (5.06)	34.16 (4.73)	34.67 (5.58)	.453
Female BMI (kg/m^2)	21.36 (2.34)	21.16 (2.27)	21.68 (2.43)	.087
Gravidity	2.66 (1.29)	2.66 (1.29)	2.74 (1.34)	.599
Parity	0.25 (0.51)	0.25 (0.52)	0.26 (0.50)	.899
Number of previous pregnancy losses				
2	153 (60.7)	94 (61.43)	59 (38.56)	.895
3	66 (26.2)	38 (57.58)	28 (42.42)	.493
≥4	33 (13.1)	22 (66.67)	11 (33.33)	.483
Mean gestational age at previous pregnancy loss (wk)	7.09 (1.80)	7.08 (1.81)	7.10 (1.80)	.944
Mode of conception (%)				
Natural	165 (65.5)	99 (60.0).	66 (40.0)	.899
IUI	4 (1.6)	3 (75.0)	1 (25.0)	.566
IVF-ET (fresh cycles)	12 (4.8)	8 (66.67)	4 (33.33)	.686
IVF-ET (frozen cycles)	71 (28.2)	44 (28.6)	27 (27.6)	.864
AMH (ng/mL)	3.71 (2.71)	3.85 (2.04)	3.50 (2.38)	.211
TPOAB (mU/L)	1.08 (1.54)	1.11 (1.64)	1.02 (1.38)	.652
TGAB (mU/L)	0.24 (0.61)	0.27 (0.69)	0.20 (0.46)	.413
ANA (U/mL)	4.42 (3.48)	4.71 (3.39)	3.94 (3.27)	.086
Prepregnancy pNK cell level (%)	17.60 (7.71)	19.57 (7.72)	14.50 (6.61)	<.001
Prepregnancy CD56$^+$CD16$^+$ cell level (%)	14.85 (7.39)	16.13 (7.53)	12.78 (6.70)	<.001
Prepregnancy CD56$^+$CD16$^-$ cell level (%)	6.52 (6.15)	6.58 (7.11)	6.44 (4.27)	.862
Prepregnancy CD3$^+$ cell level (%)	69.65 (9.72)	68.87 (8.62)	70.94 (11.19)	.100
Prepregnancy CD3$^+$CD56$^+$ cell level (%)	5.58 (4.32)	5.48 (3.86)	6.66 (5.14)	.303
Gestational pNK cell level (%)	15.88 (6.68)	14.78 (5.99)	17.59 (7.36)	.001
Months from the last miscarriage to testing the prepregnancy pNK cell level	14.65 (16.45)	15.35 (16.25)	13.57 (16.79)	.403

AMH, anti-Muellerian hormone; *ANA*, antinuclear antibody; *BMI*, body mass index; *IUI*, intrauterine insemination; *IVF-ET*, in vitro fertilization-embryo transfer; *pNK*, peripheral natural killer; *SD*, standard deviation; *TGAB*, antithyroglobulin antibody; *TPOAB*, antithyroid peroxidase antibody.

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defined as any degree of glucose intolerance with onset or first recognition during pregnancy and was diagnosed when ≥1 value exceeded the International Association of the Diabetes and Pregnancy Study Group criteria among women who underwent a 75 g oral glucose tolerance test (OGTT) after fasting for ≥8 hours.[30] SGA was defined as an estimated fetal weight or abdominal circumference below the 10th percentile of given reference ranges.[31] Spontaneous premature labor was defined as birth between 20 and 37 weeks of gestation following the spontaneous onset of labor, preterm prelabor rupture of membranes, or premature dilation of the cervix (cervical insufficiency).[32]

Statistical analysis

Statistical analyses were performed using SPSS, version 27.0 (IBM, Armonk, NY), GraphPad Prism version 8.0 (GraphPad Software, Inc., La Jolla, CA), and R, version 4.2.3 (R Core Team, Vienna, Austria). The normality of continuous variables was assessed using the Shapiro-Wilk normality test. Normally distributed continuous variables, expressed as mean±standard deviation (SD), were analyzed using Student t tests, whereas nonnormally distributed variables were compared using the Kruskal-Wallis test as medians and interquartile ranges (IQRs). Categorical variables, presented as number of cases (n) with occurrence percentage (%), were compared among the groups using z tests and chi-square analysis. Statistical significance was set at $P<.05$.

A total of 16 candidate factors that may influence live birth were included, including maternal age, male age, female body mass index (BMI), male BMI (Supplemental Table), parity, number of previous pregnancy losses, months from the last miscarriage to pregnancy, mode of conception, anti-Muellerian hormone (AMH) level, prepregnancy pNK cell level, prepregnancy $CD56^+CD16^+$ cell level, prepregnancy $CD56^+CD16^-$ cell level, prepregnancy $CD3^+$ cell level, prepregnancy CD3+CD56+ cell level, gestational pNK cell level, and change in the pNK cell level. The candidate influencing factors were calculated using pairwise correlations, and those with correlations larger than 0.7 were removed to reduce the effects of collinearity. The Akaike information criterion (AIC) was further used to select the remaining candidate influencing factors and the final candidate predictor variables were subsequently included in the multivariable logistic regression models.

Of the cohort of study participants, 70% were randomly selected to form the training data set for model construction, whereas the remaining 30% of participants were used for model testing to assess the final model performance. For the nomogram, logistic regression modeling was performed to examine the association between the decrease in pNK cell level and live birth. The predictive model performance was assessed using the discrimination by area under the receiver operating characteristic (ROC) curve (AUC) and calibration by plotting the predicted probabilities and the observed probabilities. The Youden index was used to find out the balanced sensitivity and specificity, and the Nagelkerke pseudo-R^2 and the Hosmer-Lemeshow tests were used to explain the model and the goodness of the fit.

Results

Characteristics of the subjects

A total of 1758 women with a history of RPL were enrolled from January 2017 to December 2021 (Figure 1). Among these, 713 women were excluded because consent was not provided. A further 444 women were excluded for having known causes for RPL, including 39 cases with a chromosome abnormity in either partner, 44 women with autoimmune diseases, 44 with thyroid disease, 25 with

TABLE 2
Pregnancy outcomes in group 1 and group 2

Pregnancy outcomes	Group 1	Group 2	*P* value
Biochemical	1 (9.09)	10 (90.91)	<.001
Early miscarriage	14 (28.57)	35 (71.43)	<.001
<6 wk	1 (50.0)	1 (50.0)	.746
<8 wk	3 (18.75)	13 (81.25)	<.001
<10 wk	6 (28.57)	15 (71.43)	.001
<12 wk	4 (40.0)	6 (60.0)	.162
Ectopic pregnancy	1 (33.33)	2 (66.67)	.321
Second-trimester miscarriage	2 (100.00)	0 (0)	.257
Terminated pregnancy	1 (50.0)	1 (50.0)	.746
Live births	137 (74.05)	48 (25.95)	<.001
Preterm birth	24 (92.31)	2 (7.69)	<.001
Term delivery	113 (71.07)	46 (28.93)	<.001
Preeclampsia or gestational hypertension	4 (80.0)	1 (20.0)	.758
Gestational diabetes	22 (84.62)	4 (15.38)	.232
SGA	6 (85.71)	1 (7.36)	.679
Spontaneous premature labor	16 (88.89)	2 (11.11)	.164
Chromosome karyotype of abortus			
Normal embryonic chromosome karyotypes	3 (20)	12 (80)	.378
Abnormal embryonic chromosome karyotypes	6 (42.9)	8 (57.1)	.162
Unknown	5 (25.0)	15 (75.0)	.646

SGA, small for gestational age.

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polycystic ovary syndrome, 18 with diabetes mellitus, 15 with uterine abnormality, 32 with endometritis, 5 with thrombophilia, and 220 with multiple factors. A total of 601 women with URPL with data on the prepregnancy pNK cell levels were enrolled. A total of 55 women were positive for antinuclear antibodies or antithyroid antibody, 148 women did not have another pregnancy in 1 year or until the study ended, 75 women were unwilling to have pNK cell levels measured after pregnancy, and 67 women received immunosuppressive medications 3 months before or after pregnancy and were excluded according to the exclusion criterion. Six women were lossed to follow-up after pregnancy. Thus, 252 women with UPRL were included for analysis.

Over a 5-year period, 185 women had a live birth, 62 women experienced pregnancy losses, 3 women had ectopic pregnancies, and 2 women terminated the pregnancy for fetal malformation.

When comparing prepregnancy pNK cell levels with gestational levels, 154 women had lower gestational pNK cell levels (group 1), whereas 98 women did not (group 2). Table 1 provides the comparative demographic characteristics of the 2 groups that showed no statistical differences in age, BMI, gravidity, parity, number of previous pregnancy losses, mode of conception including natural, intrauterine insemination, in vitro fertilization (IVF) frozen embryo transfer (fresh cycles), and IVF embryo transfer (frozen cycles), and blood tests, including AMH, antithyroid peroxidase antibody (TGAB), antithyroid peroxidase antibody (TPOAB), level of prepregnancy $CD56^{+}CD16^{-}$ cells, prepregnancy $CD3^{+}CD56^{+}$cells, and prepregnancy $CD3^{+}$cells between the 2 groups ($P>.05$). The total prepregnancy pNK and $CD56^{+}CD16^{+}$ pNK subset cell levels were higher in group 1 than in group 2 ($P<.001$). The gestational pNK cell levels were lower in group 1 than in group 2 ($P=.001$). Among 62 women who suffered pregnancy loss, 51 had an early miscarriage, and 11 had a biochemical pregnancy. Among those women with early miscarriage, 15 had an abnormal embryonic chromosome karyotype, 16 had a normal embryonic chromosome karyotype, and 20 did not have any karyotype results because no tissue was collected or because of the presence of maternal blood contamination. We did not find any difference in the decrease in pNK cell levels in the prepregnancy and gestational samples between those with normal or those with abnormal embryonic chromosome karyotypes (Table 2).

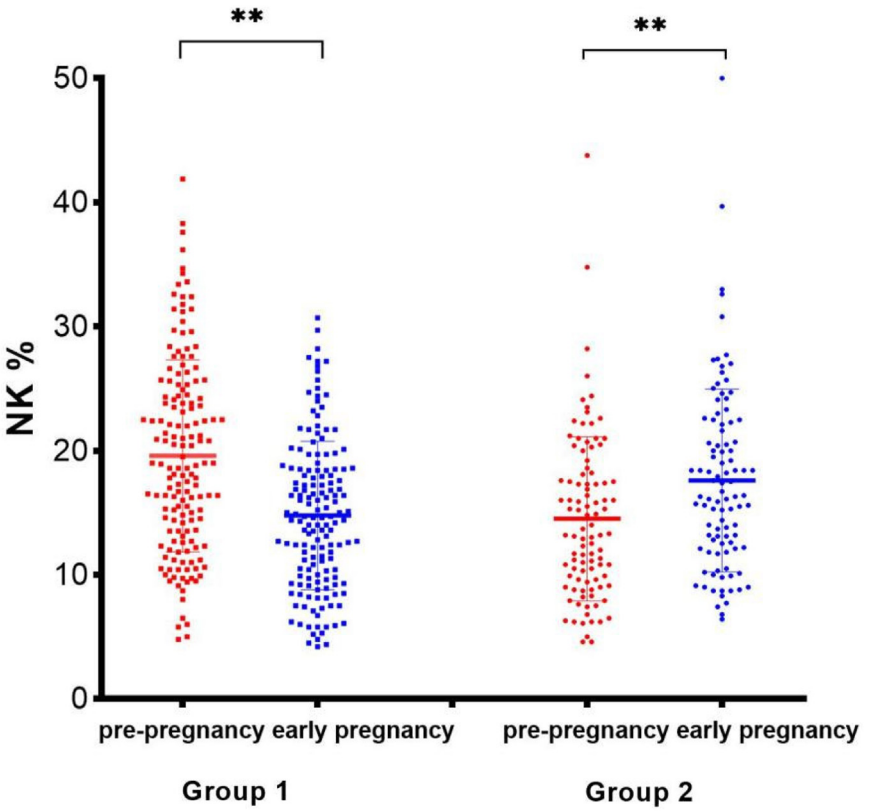
Prepregnancy and early gestational peripheral natural killer cell levels

Overall, when compared with the prepregnancy levels, the gestational pNK cell level was decreased in 61.1% (154) of women with URPL (group 1). In 38.9 % (98) of women, the pNK cell levels increased or remained unchanged at 4 to 6 gestational weeks (group 2). Women in group 1 had a significant decreased in the pNK cell levels (prepregnancy, 19.57%±7.73%; early gestation, 14.78%±5.99%; $P<.001$); group 2 had significantly increased pNK cell levels (prepregnancy, 14.50%±6.71%; early gestation, 17.59%±7.36%; $P<.001$) (Figure 2).

A decrease in gestational peripheral natural killer cell level when compared with the prepregnancy level is associated with a higher live birth rate among women with unexplained recurrent pregnancy loss

The mean live birth rate among 252 women was 73.4% and the mean rate of pregnancy loss among all women was 24.6%. The pregnancy outcomes for group 1 and group 2 are shown in Table 2. The percentage of women with a live birth and pregnancy loss in group 1 was 89% (137) and 9.7% (15),

FIGURE 2
pNK level of before and after pregnancy in group 1 and group 2



Double asterisks denote $P<.001$.

pNK, peripheral natural killer cell.

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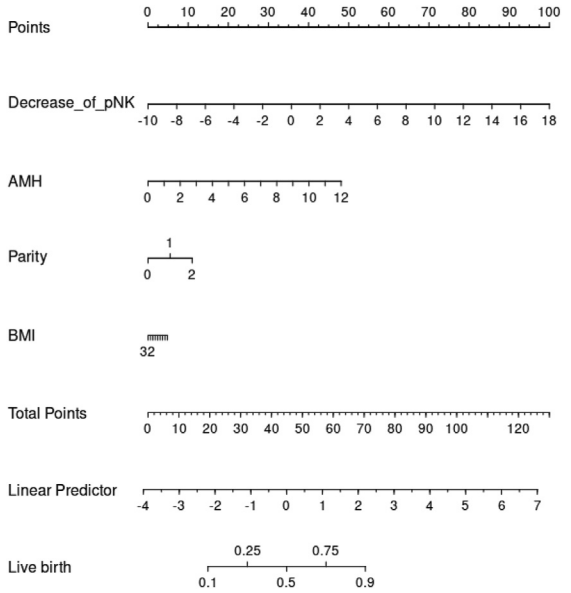
respectively; in group 2, 49% (48) of women had a live birth and 48% (47) had a pregnancy loss ($P<.001$) (Table 2). Among those in group 1, 9.09% (1) of women experienced a biochemical pregnancy, whereas 90.91% (10) of women in group 2 experienced the same ($P<.001$). In group 1, 30% (14) of women had an early miscarriage, whereas 70% (35) of women in group 2 had an early miscarriage ($P<.001$). Women in group 1 had a significant higher live birth rate (74.05%; n=137) than women in group 2 (25.95%, n=48) ($P<.001$). However, for the other pregnancy outcomes there were no differences between the groups (Table 2).

A decrease in the peripheral natural killer cell level during early pregnancy is a crucial factor that impact live birth among women with unexplained recurrent pregnancy loss

Of the 16 candidate variables for live birth, prepregnancy pNK cell level, prepregnancy $CD56^{+}CD16^{+}$ cell level, prepregnancy $CD3^{+}$ cell level, and gestational pNK cell level were found to be strong correlated when using pairwise correlations and then the last 3 factors were excluded. Of the remaining candidate variables, 4 variables were retained after selection using AIC, all of which exhibited markedly associations with live birth in the multivariable logistic regression model (Figure 3). Specifically, the decrease in pNK cell level (odds ratio [OR], 1.36; 95% confidence interval [CI], 1.22−1.55; $P<.001$) was positively associated with a higher live birth rate, whereas the AMH level (OR, 1.41; 95% CI, 1.14−1.81; $P=.003$) was also associated with live birth. However, female BMI (OR, 0.97; 95% CI, 0.82−1.15; $P=.763$) and parity (OR, 1.61; 95% CI, 0.71−4.12; $P=.287$) were risk factors for live birth but with no significance (Table 3).

FIGURE 3
Nomogram for the prediction of live birth in women with URPL



AMH, anti-Muellerian hormone; *BMI*, body mass index; *pNK*, peripheral natural killer cell.

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A decrease in the peripheral natural killer cell level from prepregnancy to early gestation was predictive of live birth

The nomogram demonstrated the possibility of each factor to influence live birth among women with live birth (Figure 3). For each woman, higher total points indicated a higher possibility of live birth. The multivariable logistic regression model based on these 4 factors yielded an AUC of 0.853 for the training data set (95% CI, 0.796−0.911) (Figure 4). The Youden index was 1.596, which yielded a specificity of 81.6% and a sensitivity of 78% for the training data set. Furthermore, the positive predictive value (PPV) of the training data set was calculated at 91.7% and the negative predictive value (NPV) was 58.8%. The model had good calibration with an average discrepancy (difference between

TABLE 3
Multivariate logistic regression for predictive variables comprising the final model for association with live birth

Variable	Coefficient (95% CI)	OR (95% CI)	Importance	*P* value
Female BMI	−0.03 (−0.19 to 0.15)	0.97 (0.82−1.15)	0.301	.763
Parity	0.47 (−0.35 to 1.42)	1.61 (0.71−4.12)	1.064	.287
AMH	0.35 (0.13−0.59)	1.41 (1.14−1.81)	2.934	.003
Decrease in pNK cell level	0.31 (0.20−0.44)	1.36 (1.22−1.55)	5.139	<.001

AMH, anti-Muellerian hormone; *BMI*, body mass index; *CI*, confidence interval; *OR*, odds ratio; *pNK*, peripheral natural killer.

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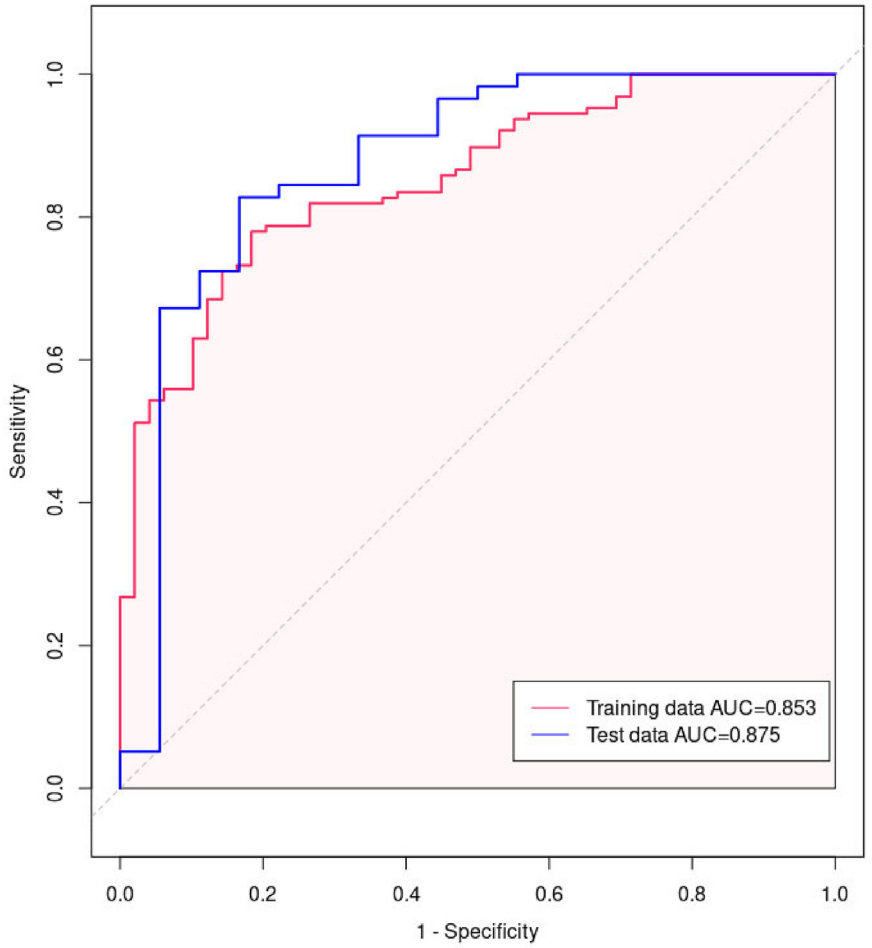
FIGURE 4
ROC curve for the predicting model in training and testing groups



AUC, area under the ROC curve; *ROC*, receiver operating characteristic.

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predicted and observed frequency) of 1.90% and maximum discrepancy of 7.1% (Figure 5, A). The calibration intercept was 0.00 and the calibration slope was 1.00 (Figure 5, A). The Hosmer-Lemeshow test and Nagelkerke pseudo-R^2 also demonstrated the goodness of the fit in the training test (p=6.068, R^2=0.442). For the test data set, the model yielded an AUC of 0.875 (95% CI, 0.763−0.986) (Figure 4). The Youden index was 1.661, which yielded a specificity of 83.3% and a sensitivity of 82.7% for the test data set. The PPV of the test data set was 94.1% and the NPV was 60%. There was no statistical difference between the ROC curve for the training data set and the test data set (P=.741). The calibration of the test data set had average and maximal discrepancies of 3.3% and 7.5%, respectively, whereas the intercept was 0.00 and the slope was 1.00 (Figure 5, B).

Comments

Principal findings

Women with a lower gestational pNK cell level when compared with the prepregnancy pNK cell level had a significantly higher live birth rate (89.0%) than those who did not (49.0%). Both a decrease in pNK cell level and a high AMH level were important predicting factors for live birth, and a decrease in pNK cell level showed the strongest influence. A lower female BMI and higher parity were also predicting factors for live birth. A logistic regression model that incorporated these factors demonstrated good predictive performance in the training data set and was validated by the test data set.

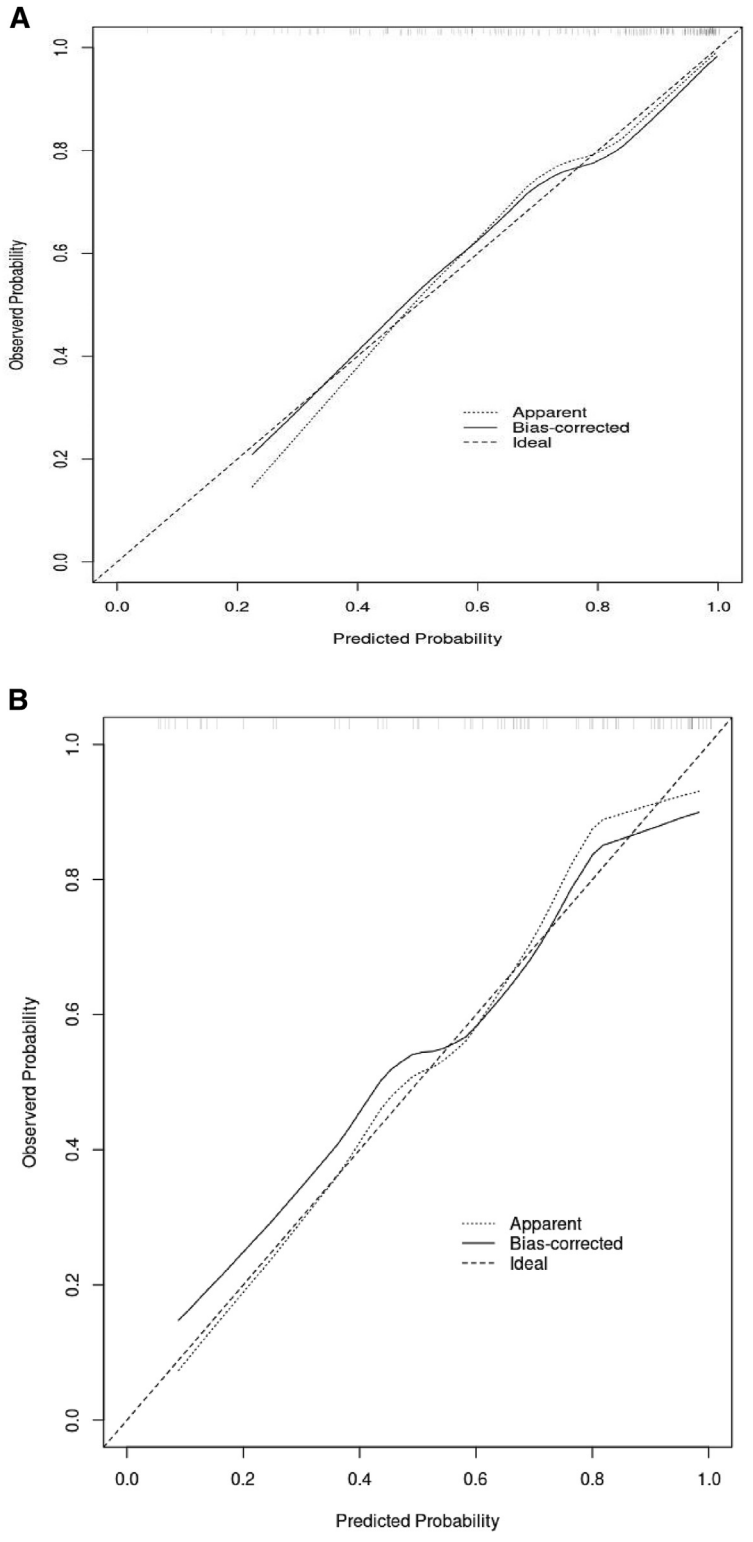
Results in the context of what is known

Our study found that the prepregnancy pNK cell level measured in the menstrual phase tends to decrease in early pregnancy among women with URPL with a subsequent live birth. A large cohort study have shown that a higher prepregnancy pNK cells level was not a risk factor for subsequent pregnancy outcomes,[33] which was in line with our previous study.[34] However, pNK cell levels have not been investigated during the transitional period from prepregnancy to early pregnancy, a critical time during trophoblast invasion and spiral artery remodeling for the establishment of a balanced maternal-fetal interface.[35] Lower pNK cell levels during the second and third trimester when compared with the levels in the nonpregnant state were observed previously,[25,36] which may indicate a modulation of inflammatory and immunity milieu among women with a good prognosis.[26] Indeed, immunomodulatory treatments were proposed to lower the immune status, including pNK cell levels, and benefits on live birth have been reported[37-41].

We observed a natural decrease in the pNK cell level from 19.57%±7.72% in the prepregnancy period to 14.78%± 5.99% in early pregnancy without any treatments among women with a subsequent live birth. None of the previous studies on decreased pNK cell levels after immunologic intervention with higher live birth rates have measured the pNK cell level in an untreated group,[42,43] and thus, the change in pNK cellslevel after pregnancy without treatment was unclear until this observation.

Several studies showed that higher prepregnancy pNK cell levels and pNK activity were found among women with RPL when compared with controls, but neither the prepregnancy pNK cell level nor the pNK activity could predict a subsequent live birth or

FIGURE 5
Calibration plots for the data sets



A, The plot for the training data set. **B,** The plot for the test data set.

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miscarriage.[10,33] In this study, a lower prepregnancy pNK cell level was found in the no decrease or increasing pNK group (group 2), and women in group 2 were more likely to experience a subsequent pregnancy loss. Nevertheless, we do not think that a lower prepregnancy pNK cell level could be a predictor for live birth because this factor was not included in the logistic predictive model with other factors. Furthermore, ROC curves of pre-pregnancy pNK, CD3+, CD56+CD16+, CD3+CD56+, and gestational pNK cell levels were also calculated for live birth, and all of the AUC were near to 0.5 (Supplemental Figure).

Clinical implications

Our study showed that it is the decrease in pNK cell level from prepregnancy to early pregnancy that may predict live birth rate among women with URPL, and this adds an additional layer of complexity when evaluating the impact of immunosuppressive treatment on this group of women. Specifically, according to our prediction model, a decrease in pNK cell level after pregnancy when compared with the level during the menstruation phase could forecast a live birth. Consistant with previous studies, we found that not only the pregnancy pNK cell level but also the prepregnancy total pNK cell level and the $CD3^+$, $CD3^+CD56^+$, and $CD56^+CD16^+$ pNK cell level subsets alone were not reliable predictive of live birth.[33] The fluctuation in pNK cell level during the menstrual cycle had been explored and the level of pNK has been reported to be lower in the menstrual state than in the luteal phase.[43] In this study, the prepregnancy pNK cell measurement was made on menstruation day 2 to 5.

Research implications

In the endometrium and decidua of healthy women, studies reported a relatively lower uterine NK cell level after menstruation with gradually increasing levels during the secretory phase, reaching a peak and transitioning to decidual NK (dNK) cells in the early pregnancy.[44,45] It is plausible that pNK cells could migrate to

decidua and be converted to dNK[46]; with the decreased gestational pNK population reflecting a higher recruitment and transformation of pNK in the decidua.[47,48] Hence, the decrease in pNK cells in early pregnancy may be fundamentally important in the physiology of the establishment of a normal pregnancy. The biologic role of the dynamic transition of pNK cells in prepregnancy to early pregnancy and its relations with placentation and embryo development requires further exploration.

Strengths and limitations

We report on a large, comparative, observational study in which pNK cell levels were assessed prepregnancy and during early gestation as a guide to determine the likelihood of live birth. We were limited by the nature of observational studies, but we have adjusted for known confounders. It would need to be further confirmed by comparative studies with normal controls. Twenty women did not have results for the embryonic chromosome testing because of detection failure or maternal blood contamination ,which could have confounded our results. Another limitation relates to the variable time rame in which the menstrual pNK cell levels were determined prepregnancy (within 1 year), which may confound the results, although the immune cell profile in the menstrual phase between different menstrual cycles has been shown to be similar.[26] The pNK cell levels do not necessarily reflect the NK cell population within the endometrial tissue, however, it is known that pNK cells could traverse the intervillous space and then display their function by contact with the villus directly.[40] Nevertheless, further research is required on the interrelationship of peripheral and uterine NK subpopulations.

Conclusion

The results of this study showed that the pNK cell level could decrease in early pregnancy among women with URPL, and this observed decrease in the NK cell level in early gestational may be a useful predictor of a higher live birth rate. ■

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The data sets generated and analyzed during this study are available from the corresponding author on reasonable request.

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University (2016-116).

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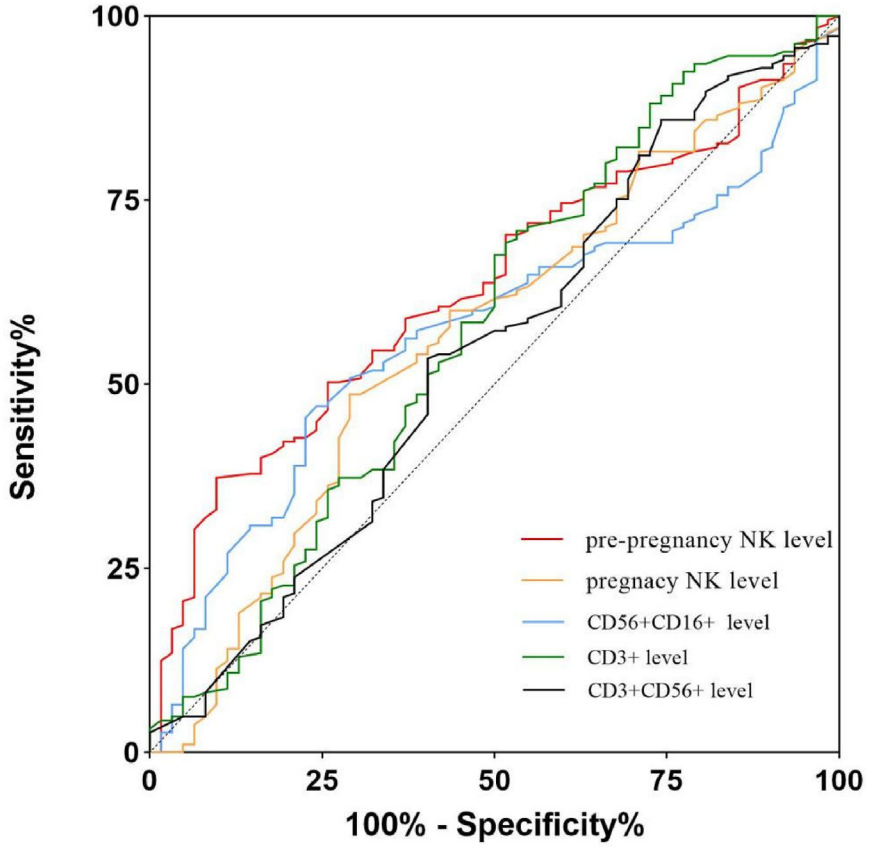
SUPPLEMENTAL FIGURE

ROC curves for the lymphocyte and live birth



pNK, peripheral natural killer; *ROC*, receiver operating characteristic.

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SUPPLEMENTAL TABLE

Baseline characteristics of live birth and pregnancy loss groups

Characteristic	Live birth women (n=185) Mean (SD) or n (%)	Pregnancy loss women (n=62) Mean (SD) or n (%)	*P* value
Female age (y)	32.22 (4.30)	33.48 (5.19)	.086
Male age (y)	33.98 (4.84)	35.53 (5.62)	.055
Female BMI (kg/m^2)	21.08 (2.29)	22.04 (2.34)	.006
Male BMI (kg/m^2)	24.48 (3.64)	24.47 (3.84)	.982
Gravidity	2.68 (1.26)	2.77 (1.44)	.628
Parity	0.27 (0.53)	0.19 (0.44)	.262
Number of previous pregnancy losses			
2	118 (78.67)	32 (21.33)	.089
3	43 (67.18)	21 (32.81)	.098
≥4	24 (72.7)	9 (27.3)	.757
Mean gestational age at previous pregnancy loss (wk)	7.19 (1.91)	6.84 (1.47)	.188
Pregnancy type (%)			
Natural	128 (79.01)	34 (21.99)	.040
IUI	4 (100.0)	0 (0.0)	.575
IVF-ET (fresh cycles)	7 (58.33)	5 (41.67)	.183
IVF-ET (frozen cycles)	46 (66.67)	23 (33.33)	.063
AMH (ng/mL)	3.94 (2.27)	3.13 (1.77)	.005
Basal FSH (IU/L)	6.16 (3.42)	5.63 (1.71)	.246
TPOAB (mU/L)	1.04 (1.55)	1.24 (1.55)	.371
TGAB (mU/L)	0.25 (0.64)	0.23 (0.52)	.816
ANA (U/mL)	4.60 (3.51)	4.04 (3.49)	.275
Prepregnancy pNK cell level (%)	18.38 (7.81)	15.01 (6.38)	<.001
Prepregnancy CD56$^+$CD16$^+$ cell level (%)	15.22 (7.53)	13.50 (6.44)	.083
Prepregnancy CD56$^+$CD16$^-$ cell level (%)	6.65 (6.72)	6.15 (432)	.584
Prepregnancy CD3$^+$ cell level (%)	70.11 (7.81)	69.43 (12.81)	.694
Prepregnancy CD3$^+$CD56$^+$ cell level (%)	5.75 (4.38)	6.30 (4.25)	.390
Gestational pNK cell level (%)	15.24 (5.92)	17.44 (7.87)	.046
Months from the last miscarriage to the test of pre-pregnancy pNK cells level	14.56 (15.61)	14.30 (18.38)	.912

AMH, anti-Muellerian hormone; *ANA*, antinuclear antibody; *BMI*, body mass index; *FSH*, follicle-stimulating hormone; *IUI*, intrauterine insemination; *IVF-ET*, in vitro fertilization-embryo transfer; *pNK*, peripheral natural killer; *SD*, standard deviation; *TGAB*, antithyroglobulin antibody; *TPOAB*, antithyroid peroxidase antibody.

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