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ORIGINAL ARTICLE

Association between Peripheral CD19$^+$ B Cells and Reproductive Outcome in Women with Recurrent Implantation Failure

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SUMMARY

Background: **To investigate the roles of T, B, and natural killer (NK) cells in pregnancy outcome of women with recurrent implantation failure (RIF).**
Methods: **This retrospective cohort study enrolled 196 patients with RIF. Peripheral lymphocyte subsets were measured before and during pregnancy. The relationship between pregnancy outcome and level of lymphocytes was analyzed.**
Results: **Peripheral CD19$^+$ B cells in women who experienced miscarriage were significantly lower than those who subsequently had live birth. After adjusting for potential confounders in the multiple logistic regression models, each 1% increment in the peripheral CD19$^+$ B cells before pregnancy [odds ratio (OR): 0.93] and during early pregnancy (OR: 0.83) was associated with a significantly decreased risk of miscarriage ($p < 0.05$). The risk of miscarriage in patients with ≥ 15% CD19$^+$ B cells before and during pregnancy was 39% and 21% lower, respectively, than in their counterparts with < 15% CD19$^+$ B cells. The association between CD19$^+$ B cells and the risk of miscarriage was nonlinear.**
Conclusions: **Measurement of peripheral CD19$^+$ subsets may help predict the pregnancy outcome in women with RIF.**
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recurrent implantation failure, CD19$^+$ B cells, miscarriage, cohort study

INTRODUCTION

Recurrent implantation failure (RIF) is often defined as failure of three *in vitro* fertilization (IVF) cycles in which viable human embryos were transferred [1]. An estimated 10% of women who undergo IVF are affected by RIF. RIF has been attributed to multiple etiological factors such as embryonal factors, maternal age, parental chromosomal anomalies, uterine factors, and immunological factors [2]; therefore, there are no specific preventive or therapeutic strategies for women affected by RIF. Presently, some IVF physicians acknowledge

the importance of immunological alterations in the management of RIF patients with reproductive outcomes [3]. Although the maintenance of pregnancy depends on the maternal-fetal interface, the evaluation of local immunological milieu is typically challenging. It is interesting to investigate whether peripheral immunological variations have an effect on implantation outcomes. Peripheral lymphocytes play an important role in successful pregnancy. Domination of the Th2 type immune response has been shown to be responsible for allograft tolerance [4]. Additionally, patients with higher levels of circulating $CD56^+CD16^+$ natural killer-like T cells were shown to achieve better outcomes of IVF [5]. Compared with non-pregnant fertile women, the number of interleukin-10 producing $CD3^+/CD8^+$ T-cells was significantly lower in women who experienced implantation failure [6]. Certain women with IVF failure were shown to exhibit deranged systemic immunity parameters. A strong predominance of Th1 immune response was detected in women with multiple implantation failure after IVF [7]. Women with elevated counts of peripheral $CD56^{dim}CD16^+CD69^+$ NK cells showed poorer outcomes of IVF [8]. Beer et al. first demonstrated an increase in circulating natural killer (NK) and $CD19^+/CD5^+$ B cells in women with documented prior IVF failure [9].

Therefore, we speculated some correlation between the peripheral lymphocytes and the pregnancy outcomes in women with RIF. Previous studies that focused on the number or percentage of various peripheral lymphocyte subsets did not demonstrate any robust correlation between lymphocytes and the pregnancy outcomes after RIF. The objective of the present study was to elucidate the relationship between peripheral lymphocytes and pregnancy outcomes in women who achieve pregnancy by IVF after a history of RIF.

MATERIALS AND METHODS

Ethical approval, study participants, and data collection

This study was approved by the institutional review board of the Shanghai First Maternity and Infant Hospital. Written informed consent was obtained from all subjects prior to the commencement of our research. Furthermore, the study protocol and methods complied with the guidelines of the Helsinki Declaration. A total of 196 women with 3 or more RIF were registered at the Infertility and Reproductive Immunology outpatient clinic at the Shanghai First Maternity and Infant Hospital between January 2014 and December 2016. Pregnancy outcomes were recorded and one-year follow-up was conducted until December 2017. Inclusion criteria for this study were: (i) infertile women undergoing IVF treatment cycles for tubal factors; (ii) women who had ≥ 3 embryo transfer failures including the transfer of two fresh or frozen embryos for each IVF cycle; (iii) those who became clinically pregnant through a standardized IVF method and were administered low molecular weight heparin (LMWH) for improving uterine blood flow according to our reproductive immunology outpatient clinic criteria (LMWH has been shown to improve placental function by increasing blood flow at the implantation site [10]); (iv) those whose pregnant outcome was recorded; (v) absence of known immunological disease, previous history of miscarriage, chromosomal, endocrine, infectious factors, or anatomic defects; (vi) patients who were not on any anti-inflammatory and immune drug; (vii) availability of data pertaining to blood tests prior to any treatment including IVF cycles.

Age ≥ 35 years was defined as advanced maternal age. Pre-gravid body mass index (BMI) was calculated using the equation weight (kg)/height $(m)^2$. Based on the BMI cutoff value for Asian populations, patients were classified into non-overweight (pre-gravid BMI < 23 kg/m^2) and overweight (pre-gravid BMI ≥ 23 kg/m^2) groups [11]. Fifteen percent peripheral $CD19^+$ B cell fraction among the lymphocytes was defined as the cutoff level according to the criteria from our own clinical laboratory (normal range of peripheral $CD19^+$ B cells for fertile woman in our lab was 8% - 15%).

Peripheral blood samples and flow cytometry

Blood samples were collected from patients during the mid-luteal phase prior to a new IVF procedure and at gestational week 6 - 8 after identification of RIF and anticoagulated in EDTA vacuum tubes. Gestational age was calculated based on the last menstrual period and confirmed by ultrasound examination.

Lymphocyte subsets were determined using a multicolor bench top flow cytometry system (Tru-Count®; FAC Scalibur BD Biosciences, San Jose, CA, USA) after staining with the following monoclonal antibodies (BD Multitest™, San Diego, CA, USA): anti-CD3-FITC, anti-CD4-APC, anti-CD8-PE (LOT: 82998), anti-CD19-APC, anti-CD16/CD56-PE and anti-CD45-PerCP (LOT: 25936). T cells were identified as $CD3^+$ cells, NK cells as $CD56^+CD16^+$ cells, Ts cells as $CD3^+CD8^+$ cells, Th cells as $CD3^+CD4^+$ cells, and B cells as $CD19^+$ B cells.

Statistical analyses

The characteristics of subjects before pregnancy are summarized in Table 1. Data are presented as mean ± standard deviation (SD). Between-group differences with respect to continuous variables were assessed using the two-sample *t*-test; those with respect to categorical variables were assessed using the Chi-squared test (Figure 1). We then explored the association between pregnancy outcome and peripheral blood $CD19^+$ B cells (%) at both the baseline and during pregnancy using a smooth spline (Figure 2). Univariate and multivariate logistic regression analyses were performed to assess the relationship of multiple prognostic factors with pregnancy outcome. Both crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) were

calculated (Tables 3 and 4). Statistical analyses were performed using R (http://www.R-project.org) and Empower Stats software (www. empowerstats.com, X&Y solutions, Boston, MA, USA). p-values < 0.05 were considered indicative of statistical significance.

RESULTS

Characteristics of patients

Out of the 196 women, the pregnancy outcome of 90 (45.92%) and 106 (54.08%) women were live birth and miscarriage, respectively. The mean age of subjects was 37.49 ± 6.02 years; 135 (68.88%) subjects were aged > 35 years. The mean BMI was 22.36 ± 3.50 kg/m^2; 77 women (39.29%) had BMI ≥ 23 kg/m^2. The average number of previous RIF was 6.80 ± 1.85; 86 (43.88%) women had less than 5 times RIF while 110 (56.12%) women had more than 5 miscarriages.

Comparison of the percentage of lymphocyte subsets

Figure 1 illustrates the differences in the percentage of lymphocyte subsets, NK, T, Ts, Th, and B cell in the two groups before and during early pregnancy. The mean percentage of peripheral CD19$^+$ B cells in the live birth group was 13.90 ± 4.37% before pregnancy (Figure 1A) and 13.83 ± 4.56% during early pregnancy (Figure 1B); these percentages were significantly higher than those in the miscarriage group (11.60 ± 3.81% and 11.45% ± 2.64%, respectively; p = 0.03 for both). However, no significant differences were observed with respect to the percentages of T, Th, Ts, and NK cells between the two groups. Furthermore, we compared the changes in the T-lymphocyte subsets at gestation. The difference value (DV) refers to the change in T lymphocyte subsets before and during pregnancy (Figure 1C) (DV = level of T lymphocyte subsets $_{\text{(during pregnancy)}}$ - level of T lymphocyte subsets $_{\text{(before pregnancy)}}$). The rate of change (RC) was calculated as: RC% = 100 * (level of T lymphocyte subsets $_{\text{during pregnancy}}$ - level of T lymphocyte subsets $_{\text{before pregnancy}}$)/level of T lymphocyte subsets $_{\text{before pregnancy}}$); comparisons were made between the two groups (Figure 1D). There was no significant between-group difference with respect to DV or RC in lymphocyte subsets.

Univariate and multiple regression analysis

Results of univariate analysis showing the effects of risk factors on pregnancy are shown in Table 2. Increased maternal age was associated with an increased risk of miscarriage (OR 1.07, 95% CI 1.02 - 1.12; p = 0.006). BMI and number of previous RIF were not associated with pregnancy outcome. Increase in CD19$^+$ B cell percentages before pregnancy (OR 0.92, 95% CI 0.85 - 0.99; p = 0.04) as well as during early pregnancy (OR 0.83, 95% CI 0.70 - 0.99; p = 0.04) was associated with decreased miscarriage rate. However, NK, T, Ts, and Th before and in early pregnancy showed no association with pregnancy outcome.

In the multiple regression analysis model (Table 3), age and CD19$^+$ B cells before and during early pregnancy showed an association with pregnancy outcome. After adjustment for BMI and the number of previous RIF, the odds ratio (OR) for miscarriage was significantly increased with increase in maternal age. OR for age ≥ 35 years was significantly higher than the OR for age < 35 years (OR 2.62, 95% CI 1.04 - 7.31; p = 0.04). Additional adjustment for age, BMI, and number of previous RIF did not reduce the ORs for the association between CD19$^+$ and the miscarriage. Each increase of 1% in the peripheral CD19$^+$ B cell percentage was related with a decrease in the risk of miscarriage with an OR value of 0.91 (95% CI 0.45 - 0.99; p = 0.04) before pregnancy and 0.78 (95% CI 0.40 - 0.99; p = 0.03) during early pregnancy. We also conducted analyses using CD19$^+$ as a categorical variable. In patients with ≥ 15% CD19$^+$ B cells, the risk of miscarriage was 56% lower (OR 0.44, 95% CI 0.20 - 0.96; p = 0.04) before pregnancy and 79% lower (OR 0.21, 95% CI 0.04 - 0.98; p = 0.04) during early pregnancy than those with < 15% CD19$^+$ B cells. After adjustment for age, BMI, and number of previous RIF, a similar conclusion was obtained. The risk of miscarriage in women who had > 15% CD19$^+$ B cells at the pre-pregnancy and early pregnancy stages was 39% and 21% lower, respectively, compared to that in women who had < 15% CD19$^+$ B cells.

Two-piecewise linear regression model

The two-piecewise linear regression model was applied to examine the threshold effect of the CD19$^+$ B cells on pregnancy outcome using a smoothing function. The turning point was accessed employing trial and error, including the selection of turning points along a pre-defined interval and then choosing the turning point that yielded the maximum model likelihood. A nonlinear relationship was observed between the CD19$^+$ B cells before pregnancy and the risk of miscarriage (Figure 2A). A one-percent increment in CD19$^+$ B cells within the range of 6.99% - 20.66% was associated with a 15% decrement in the risk of miscarriage (OR 0.85, 95% CI 0.76 - 0.96; p = 0.02) (Table 4). No significant relationship was observed between CD19$^+$ B cells and the risk of miscarriage, when the level of CD19$^+$ B cells was < 6.99% or > 20.66%. The adjusted smoothed plots showed a linear relationship between CD19$^+$ B cells during pregnancy and the risk of miscarriage (Figure 2B).

DISCUSSION

In this retrospective cohort study, the level of CD19$^+$ B cells showed an association with pregnancy outcome in women affected by RIF, both when used as a categorical variable or continuous variable. The relationship held true even after controlling for various clinical and demographic variables. Furthermore, we found a non-linear association between the CD19$^+$ B cells before

Table 1. Baseline characteristics of the entire cohort of recurrent implantation failure women.

Variable [*]	All participants (n = 196)
Age, years	37.49 ± 6.02
< 35	61 (31.12%)
≥ 35	135 (68.88%)
BMI [a], kg/m^2	22.36 ± 3.50
< 23	119 (60.71%)
≥ 23	77 (39.29%)
Number of previous RIF	6.80 ± 1.85
< 5	86 (43.88%)
≥ 5	110 (56.12%)

[*] - Values are presented as mean ± SD or n (%), [a] - BMI body mass index.

Table 2. Effect of risk factors on pregnancy outcome after univariate analysis.

Variable	Pregnancy outcome OR (95% CI)	p-value
Age	1.07 (1.02 - 1.12)	0.006
BMI	0.98 (0.87 - 1.11)	0.80
Num. of previous RIF	1.09 (0.96 - 1.24)	0.19
Level T lymphocyte subsets before pregnancy		
$CD3^+$	1.01 (0.97 - 1.05)	0.53
$CD3^+CD4^+$	1.03 (0.99 - 1.08)	0.15
$CD3^+CD8^+$	0.99 (0.94 - 1.03)	0.54
$CD16^+CD56^+$	1.01 (0.97 - 1.05)	0.56
$CD19^+$	0.92 (0.85 - 0.99)	0.04
Level T lymphocyte subsets during pregnancy		
$CD3^+$	0.98 (0.93 - 1.03)	0.46
$CD3^+CD4^+$	1.10 (0.94 - 1.08)	0.87
$CD3^+CD8^+$	0.99 (0.90 - 1.07)	0.73
$CD16^+CD56^+$	1.03 (0.97 - 1.10)	0.32
$CD19^+$	0.83 (0.70 - 0.99)	0.04

pregnancy and the risk of miscarriage, with two turning points of 6.99% and 20.66%. The percentage of peripheral $CD19^+$ B cells may serve as a clinically useful marker to predict pregnancy outcome for women with RIF. We would recommend that clinicians should understand the importance of peripheral $CD19^+$ B cell analysis in women with RIF.

In previous studies, B cell levels in blood or endometrium did not differ in patients affected by recurrent spontaneous abortion (RSA) and infertility [12-14]. Jablonowska et al. found that circulating B cells did not affect pregnancy outcome in RSA patients [15]. Lachapelle et al. reported that RSA patients with normal endometrial B cell numbers subsequently underwent successful pregnancies, while repetitive aborters with a strikingly increased proportion of B lymphocytes ($CD20^+$) suffered continuing abortions [16]. However, none of the above studies included women suffering from RIF and little attention was paid to live births. Both B cells as well as antibodies produced by B cells were shown to play a role in pregnancy associated pathophysiology [17]. During normal pregnancies, antibodies secreted by B cells against paternal antigens were shown to exhibit asymmetry, as a protective mechanism [18]. B cells

Table 3. Adjusted ORs for associations between CD19+ and pregnancy outcomes.

Variable	Non-adjusted [a] (OR 95% CI)	p-value	Adjusted I [b] (OR 95% CI)	p-value
Age	1.07 (1.02 - 1.12)	0.006	1.12 (1.03 - 1.22)	0.01
< 35	1.0		1.0	
≥ 35	2.16 (1.17 - 4.00)	0.01	2.62 (1.04 - 7.31)	0.04
CD19+before pregnancy	0.92 (0.47 - 1.00)	0.04	0.91 (0.45 - 0.99)	0.04
< 15%	1.0		1.0	
≥ 15%	0.44 (0.20 - 0.96)	0.04	0.39 (0.17 - 0.89)	0.02
CD19+ during pregnancy	0.83 (0.50 - 0.99)	0.04	0.78 (0.40 - 0.99)	0.03
<15%	1.0		1.0	
≥ 15%	0.21 (0.09 - 0.98)	0.04	0.21 (0.09 - 0.99)	0.04

[a] - Non-adjusted model adjusted for - None, [b] - Adjusted model adjusted for - (Age), BMI - Number of previous RIF.

Table 4. ORs for associations between pregnancy outcomes and CD19+, stratified by threshold levels.

Variable	OR (95% CI)	p-value
CD19+ before pregnancy		
< 6.99%	1.86 (0.66 - 5.250	0.24
≥ 6.99% and < 20.66%	0.85 (0.76 - 0.96)	0.02
≥ 20.66%	1.24 (0.78 - 1.97)	0.37

were shown to exhibit the ability to directly interact with Th2 T cells, which are known to play a role in allograft tolerance and successful pregnancy [19]. According to our study, a higher percentage of $CD19^+$ B cells before pregnancy or during early pregnancy seemed to be a clinical marker to predict miscarriage and live birth for women with RIF.

We found no significant correlation of circulating T ($CD3^+$) cells, Ts ($CD3^+CD8^+$) cells, Th ($CD3^+$ $CD4^+$) cells, and NK ($CD56^+CD16^+$) cells with pregnancy outcome in pregnant RIF women. These findings are consistent with those of previous studies. Thum et al. found no relationship between the absolute counts of circulating T cells and NK cells on the implantation and miscarriage rate post-IVF treatment [12]. Baczkowski et al. found no value of peripheral $CD56^+CD16^+$ NK and $CD3^+$ T cell levels in predicting the outcomes of intracytoplasmic sperm injection (ICSI). However, the total counts of $CD56^-CD16^+$ cells in the pregnant group were higher than those in the non-pregnant group, while the $CD56^{bright}CD16^-$ cells in the fertile group were more abundant compared to women with unsuccessful ICSI [14]. In the study by Chernyshov et al., higher expressions of CD8, CD158a, and HLA-DR in NK cells, and lower levels of $CCR4^+$ and $IL\text{-}4^+$ T lymphocyte subsets were associated with implantation failure [20]. These results and our data revealed that the variation in the percentage of peripheral NK cells, T cells, Th cells, and Ts cells had no prognostic relevance with respect to pregnancy outcome; however, lymphocytic activation markers and the other fractions of lymphocyte subpopulations were found to have an influence on pregnancy success.

Consistent with previous reports [21-23], age was identified as a potential risk factor for miscarriage in women with RIF. The mean age in the miscarriage group (38.65 ± 6.25 years) was higher than that in the live birth group (36.20 ± 5.49 years). Jansen investigated the effects of a woman's age on the possibility of a live birth after a single IVF treatment. They observed an age-dependent increase in the frequency of miscarriages, from 10.5% for women < 35 years, to 16.1% for those aged 35 - 39 years, and 42.9% for those aged > 40 years [23]. In the current study, the probability of miscarriage was further increased for women who were aged > 35 years compared with women aged < 35 years (OR: 2.62; 95% CI 1.04 - 7.31; p = 0.04).

Maternal obesity and the number of previously unsuccessful IVF treatments have been identified as risk factors for multiple adverse pregnancy outcomes [24,25].

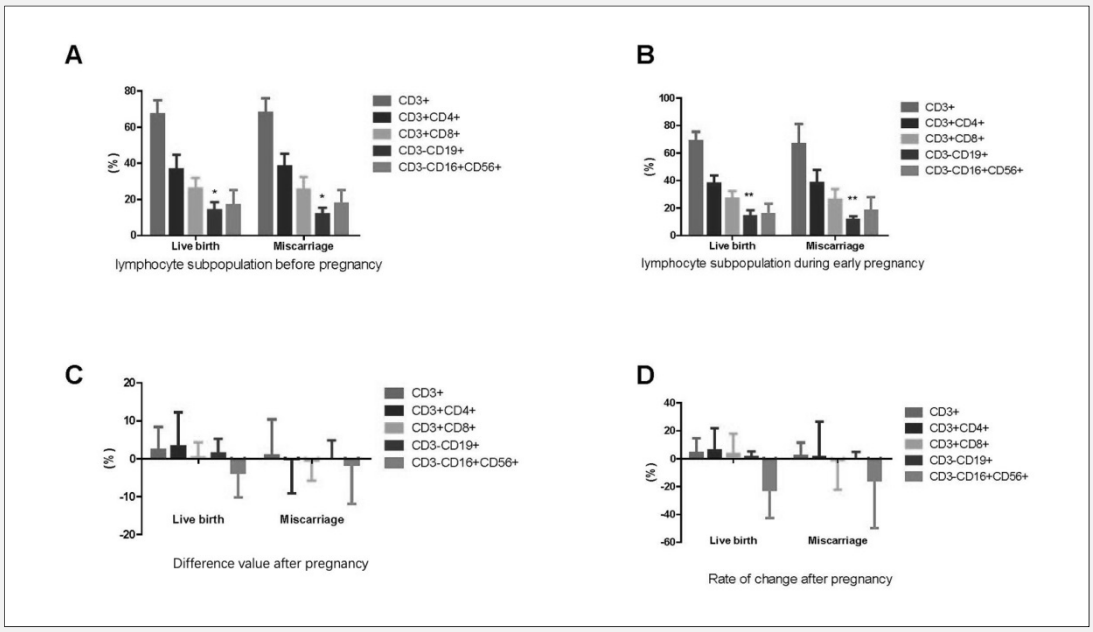

Figure 1. The change in lymphocyte subpopulations in peripheral blood of women with RIF before and during early pregnancy.

A: Percentage of peripheral lymphocyte subsets in RIF women before pregnancy in the two groups (* p = 0.03), B: percentage of peripheral lymphocyte subsets in RIF women during early pregnancy in the two groups (p = 0.03), C: The difference in the percentage of lymphocyte subpopulations after pregnancy in the two groups. Different value after pregnancy (DV), DV = level of T lymphocyte subsets $_{\text{during pregnancy}}$ - level of T lymphocyte subsets $_{\text{before pregnancy}}$, D: Rate of change in percentage of lymphocyte subsets after pregnancy in the two groups. Rate change after pregnancy (RC), RC (%) = 100 * (level of T lymphocyte subsets $_{\text{during pregnancy}}$ - level of T lymphocyte subsets $_{\text{before pregnancy}}$)/level of T lymphocyte subsets $_{\text{before pregnancy}}$).**

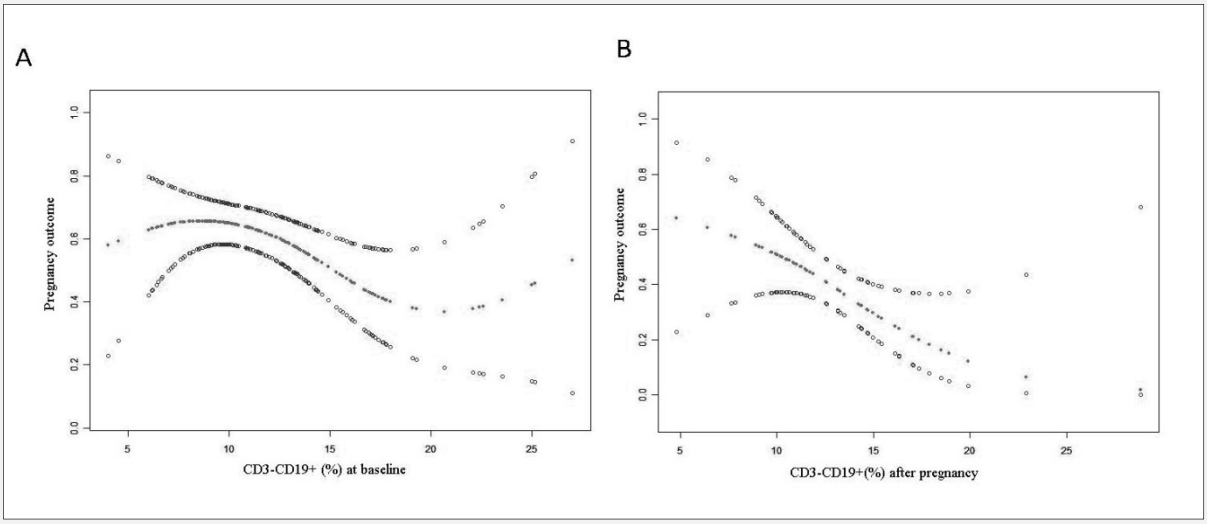

Figure 2. The smooth curve showing the estimated relationship of peripheral percentage of $CD19^+$ B cells before and during early pregnancy with the pregnancy outcome. The dash line in the middle represents the relative risk. The other two dash lines represent the 95% confidence intervals.

According to our results, no remarkable difference was found between our two groups with respect to BMI and the number of RIF.

There were several limitations in our study. For example, we did not analyze the different subtypes of B cells or various cytokines produced by B cells in our patients, which may have helped clarify the critical protective mechanism. In addition, the lack of cytogenetic analysis on miscarriage specimens and etiological analysis for RIF affected the accuracy and homogeneity of our studies. Further studies are required to better understand the role of B cells in RIF.

CONCLUSION

We demonstrated that peripheral $CD19^+$ B cell levels before and during pregnancy in women who had a miscarriage were significantly lower than those in the live birth group. A nonlinear association was observed between the $CD19^+$ B cells and the risk of miscarriage. Each one-percent increment in the peripheral $CD19^+$ B cells percentage was associated with a decreased risk of miscarriage. The risk of miscarriage in patients with $\geq$ 15% $CD19^+$ B cells before and during pregnancy was 39% and 21% lower, respectively, than that in their counterparts with < 15% $CD19^+$ B cells. Measurement of peripheral $CD19^+$ subsets may help predict the pregnancy outcome in women with RIF.

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Declaration of Interest:

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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