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Increase in FoxP3, CD56 immune cells and decrease in glands PGRMC1 expression in the endometrium are associated with recurrent miscarriages

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An increase in FoxP3, CD56 immune cells and decrease in glands PGRMC1 expression in the endometrium are associated with recurrent miscarriages

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Abstract

Objective: Recurrent miscarriage (RM) is a multifactorial condition that involves frequent uterine anatomical abnormalities, parental karyotype abnormalities, and clotting disorders. We investigate the potential roles of endometrium FoxP3⁺ Tregs and CD56⁺ cells (uNK cells) and endometrial expression of PGRMC1 in the development of recurrent miscarriage.

Study design: This prospective study included 102 out of 286 cases of SA patients. The cases were divided into groups with RM (+RM) and without RM (-RM). Immunohistochemistry staining was made using primary antibodies to FoxP3, CD56, and PGRMC1 in both groups. Morphometry analyses were carried out in 10 non-overlapping high power fields. Mann-Whitney U test, Fisher two-tail test, correlation analysis and relative risk (RR) were evaluated. A p<0.05 was considered statistically significant.

Results: An increased presence of CD56-positive (p<0.001) and FoxP3⁺ Treg (p= 0.0005) cells was found in the endometrium, with a reduction in PGRMC1 expression compared with -RM group (p= 0.004). A positive correlation was shown between the number of CD56-positive cells and FoxP3⁺ cells (r= 0.55), and an inverse correlation with PGRMC1 (r= -0.35) in the +RM group. A similar observation was found in the -RM group, with a positive correlation of uNK cell number with the number of pregnancies (p<0.001; r = 0.34). Endometrial infiltration of CD56-positive (p<0.0001) and FoxP3⁺ (p<0.0001) cells revealed an increased relative risk of RM. This increased risk was also revealed in SA with a loss of PGRMC1 expression (p<0.0001).

Conclusion: Our prospective study suggests, for the first time, that increased endometrial infiltration of uNK, FoxP3⁺ Treg cells and a decreased PGRMC1 expression may play potential roles in the development of RM.

Keywords: Recurrent miscarriage, uterine NK cells, CD56, FoxP3, PGRMC1

Introduction

Recurrent miscarriage (RM), is a multifactorial condition that is characterized by frequent uterine anatomical abnormalities, parental karyotype abnormalities, and clotting disorders such as protein C deficiency, factor V Leiden mutation, antiphospholipid syndrome and other thrombophilias. However, according to Tremellen and Russell (2011), the true diagnoses for miscarriage in over 50% of couples remain “unexplained”, after such investigation has been categorized as “unlucky” due to repeated abnormal embryo production and/or attachment (1). A number of factors may play a role in RM such as endometrium hormone receptivity, immunological factors, growth factors, trophoblast features, and local leukocyte populations (2).

Uterine natural killer (uNK) cells are the major leukocytes present in the endometrium. A precise role for uNK cells in endometrial function and embryo implantation is not fully clear, although it was recently highlighted that these cells may have a role in effective implantation by controlling the depth and pattern of trophoblast invasion (3). Evidence also suggests a role for uNK cells in other biological events in the endometrium and the decidua, such as the control of vascular remodeling in early pregnancy (4). These CD56-positive cells secrete cytokines and angiogenic factors, which play key roles in placental development and pregnancy establishment (5).

Endometrium regulatory T cells (Tregs) are essential for a successful pregnancy, protecting the semi-allogenic fetus from immune rejection (6). Foxp3⁺ Treg cells are a unique subset of suppressive CD4⁺ T helper cells involved in immune tolerance to self and foreign antigens in humans and mice. The periodic elevation of Treg cells on the day of embryo transfer was reported to be associated with higher embryo implantation rate (7). Only a few human studies of FoxP3 expression in peri-implantation period endometrium have been reported using different methods assessing the FoxP3 expression (6), thus the outcome remains largely inconclusive.

Progesterone receptor membrane component 1 (PGRMC1) expression in the endometrium may also play a role in successful implantation. Its expression in the uterus derives from a

microarray study in which PGRMC1 mRNA was found to be down-regulated from the proliferative to secretory phase of the human menstrual cycle. Beyond this, very little is known about PGRMC1 expression in the human endometrium (8), which raises a possibility that uNK, FoxP3 and PGRMC1 may be involved in the development of recurrent miscarriages. Thus, we hypothesize that dysregulation of uNK, FoxP3 cells, PGRMC1 expression and crosstalk between these factors play a pro-RM role in the development of recurrent miscarriages.

Materials and Methods

Patients

This is a prospective study that commenced on 1 January 2016 and completed on 31 January 2019 in Gomel region, Republic of Belarus. Informed consent was taken from all patients included in this study. Ethical approval was obtained and reviewed by the Institutional Review Board, Gomel, Belarus. Criteria of inclusion in this study were 1) the presence/absence of RM; 2) age range between 18 and 45 years; and 3) an absence of the following: malformations of the reproductive system, acute infections in the pelvis, tumors of female genital tract, hyperplastic lesions of the endometrium, hematological and autoimmune diseases and previous or current treatment by hormonal remedies. Criteria of exclusion from study were as follows: age less than 18 and more than 45 years old; malformations of reproductive system, acute infections of the pelvis; tumors of female genital tract; hyperplastic lesions of the endometrium; and use of hormonal remedies. During this period, we observed 286 patients from which we selected 102 cases according to our criteria of inclusion. Groups with (+RM) and without (-RM) recurrent miscarriage consisted of 39 and 63 patients respectively. The characteristics of the patients in the groups are presented in **Table 1**.

Endometrial Biopsy and Immunohistochemistry

Endometrial biopsy from patients was performed using an aspiration curette ProfiCombi (Simurg, Republic of Belarus) on days 7–9 after ovulation, depending on the duration of the menstrual cycle. **The ovulation was determined using transvaginal ultrasound examination.**

Biopsies were fixed in 10% buffered neutral formalin solution for 48 hours. Endometrial tissues were processed with isopropanol and paraffin in Thermo Scientific STP-120 spin tissue processor (Thermo Scientific, Germany). 3-4µm thick sections were made from paraffin blocks using a Microm HM 304E rotor microtome (Thermo Scientific, Germany). Next, the sections were mounted on Thermo Super-Frost poly-L-lysine coated slides (Thermo Scientific, Germany). Antigen retrieval was performed using a microwave. The sections were then allowed to cool down and endogenous peroxidase blocking was done in 5% hydrogen peroxide. Blocking of nonspecific antibody binding was carried out in 5% casein. Sections were washed and incubated in a moist chamber at room temperature with corresponding primary antibodies to CD56 (**ready-to-use**; Diagnostic Biosystems, URM), FoxP3 (**1:100**; Abcam, UK) and PGRMC1 (**1:150**; Abcam, UK), followed by incubation with secondary anti-mouse HRP antibodies. The visualization of product reaction was made using 3,3-diaminobenzidine (DAB) staining for 5 minutes, followed by Mayer's hematoxylin counterstaining (9-11).

Morphometry

The morphometrical analysis was carried out using NIS-Elements (Nikon, Japan) and a Nikon Eclipse 50i microscope (Nikon, Japan) in 10 non-overlapping high-power fields (HPF, ×400 magnification). CD56-positive cells were counted in the functional layer of the endometrium excluding areas containing infiltrated uNK cells such as glands and vessels as these are normal immune cells infiltration (9). FoxP3-positive cells were evaluated in the functional layer of the endometrium, specifically in areas of maximum concentration of these cells. The number of uNK and Tregs cells was presented as number of cells/mm². PGRMC1 expression has been shown to be weak to moderate at secretory phase of the menstrual cycle (8). Accordingly, we defined all

cases as PGRMC1+ (glands express this marker) or PGRMC1- (absence of marker expression or presence of few glands with expression).

Statistical analysis

Data for CD56⁺ and FoxP3⁺ cells/mm² was presented by the median, lower and upper quartiles. Fisher two-tail exact test was used to compare the groups according to their PGRMC1 expression. Mann-Whitney test was utilized to compare the study groups based on the number of uNK and Treg cells/mm². A threshold criterion of uNK and Treg cells number was evaluated by ROC-analysis. A relative risk (RR) analysis was performed using Fisher's two-tail test. Correlation between different groups was assessed using a Spearman correlation test. A $p < 0.05$ was considered statistically significant. GraphPad Prism v.8.1 (GraphPad Software Inc., San Diego, CA, USA) and R Software v 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria) were used for analysis.

Results

uNK cells

CD56-positive cells diffusely infiltrate the endometrium stroma epithelium in the +RM group (median: 151.3 (105.1-193.4) cells/mm²; **(Figure 1a)**) compared to the -RM group, with fewer CD56-positive cells (median: 42.0 (16.8-92.5) cell/mm²) in the endometrial stroma (**Figure 1b**). Statistically significant differences in CD56-positive cells ($p < 0.001$) were found between the two groups (**Figure 2a**). A ROC-analysis of uNK cells revealed the area under the curve is 0.77 ($p < 0.0001$). The sensitivity, specificity and threshold criteria were 76.9%, 68.3%, and 100.8 cells/mm², respectively.

FoxP3⁺ Treg cells

In the group of patients with RM, FoxP3-positive cells were observed in small clusters located near immune cells infiltrates (**Figure 1c**) (median: 58.0 (16.8-92.5) cells/mm²). In the cases of group without RM, 2-5 FoxP3⁺ cells were revealed in the stroma of the endometrium (**Figure**

1d) (median: 25.2 (0.0-42.0) cells/mm²). A Mann-Whitney test showed a statistically significant difference ($p = 0.0005$) between the groups in the expression of FoxP3 (Fig. 2b). On the basis of the ROC-analysis, a total Treg cells threshold of 56.3 was adopted as the best differentiating value between patients with or without RM, with 51.2% specificity and 90.8% sensitivity (area under the curve was 0.70; $p = 0.0007$).

PGRMC1 expression

Microscopically the intensity of PGRMC1 expression was shown to be weak but uniformed in the -RM group (**Figure 3a**), whereas in the +RM group this expression was absent (**Figure 3b**) or very weak. In the +RM group, we observed a loss of PGRMC1 expression in 25 (64.1%) cases, compared with the -RM group where the loss of expression was found in 21 (35.9%) cases. A fisher two-tail exact test showed statistically significant difference ($p = 0.004$) in PGRMC1 expression between these groups (Fig. 2c).

Correlation analysis

We differentially analyzed correlations in these two groups of patients. In the +RM group (**Figure 4a**), the number of uNK cells demonstrated a positive correlation with the number of FoxP3 Tregs ($p < 0.001$; $r = 0.55$) and an inverse correlation with PGRMC1 expression ($p < 0.001$; $r = -0.35$).

In the group without RM (**Figure 4b**), the number of uNK cells correlated positively with the number of FoxP3 Tregs ($p < 0.001$; $r = 0.35$) and inversely with the PGRMC1 expression ($p < 0.001$; $r = -0.48$), and interestingly, correlated positively with the number of pregnancies ($p < 0.001$; $r = 0.34$). PGRMC1 expression displayed an inverse correlation with the duration of the menstrual cycle ($p < 0.001$; $r = -0.34$).

Relative risk

The CD56-positive cells infiltration of the endometrium revealed an increased risk of RM (RR=3.46 95%CI 1.91 to 6.61; $p < 0.0001$) that was higher than the threshold criteria. The presence

of FoxP3⁺ cells also demonstrated a similar increase (RR=3.04 95%CI 1.92 to 4.78; p<0.0001). A loss of PGRMC1 expression showed an increase in the risk of recurrent miscarriage (RR=2.09 95%CI 1.25 to 3.56; p<0.0001). The reciprocal relative risk values are shown in **Figure 5**.

Discussion

The most abundant immune cells in the uterine decidua around the time of implantation and early placental development are the uNK cells. Altered numbers of uNK cells have been associated with several human reproductive disorders such as repeated miscarriage, implantation failure, fetal growth restriction, and preeclampsia (2). In this study, we evaluated a high number of uNK cell/mm² in cases of RM that may result in an increased risk of this condition. Similar results have been reported in cases of RM in previous studies (12, 13). **A correlation between the number of uNK cells and the number of childbirths in the group of patients with RM could be a manifestation of chronic endometritis which may be associated with medical manipulations** (14). Reports about low diagnostic value of uNK cells count due to their reaction to stress is indeed surprising (15). A few studies reported that autoimmune diseases and physical exercises could increase the number of peripheral NK cells in circulation (16). Although previous research utilized the count of uNK, they did not investigate that the CD56-positive cells infiltration of glands and microvessels are a variant of the norm (17, 18, 19). As Sehardi et al. (2013) reported in their work that the immune system is complex and one variable, such as NK cell levels, which cannot predict an outcome. Thus, it is important to understand that NK indices do not reflect specific immune responses in pregnancy and NK cell numbers and activity can fluctuate according to different variables, such as hormonal effect, exercise, time of day and sympathetic response to stressors (2)

Tregs are important regulators of almost all immune responses, for example, modulating host responses to tumors and infections, inhibiting the development of autoimmunity or preventing rejection of transplantation. Tregs mediate suppressive functions both through the production of cytokines and direct cell-cell contact (20, 21). The regulatory FoxP3⁺ Treg cells play a crucial role in controlling and suppressing immune responses, especially to sustain successful implantation

which is rare at all normal menstrual cycle stages (22). We report an increase in the number of FoxP3-positive Tregs in patients with RM. In addition, we revealed a correlation between the number of Treg cells and uNK cells. It could be associated with the fact that FoxP3 cells regulate a potent inflammation, which may favor a hostile uterine microenvironment that would hinder nidation of the embryo (23). Relative risk analysis showed that an increase in FoxP3⁺ immune cell infiltration elevates the risk of RM. We suggest that the elevation of these cells is a compensatory reaction to the intensification of uNK endometrial infiltration in the case of RM ($r=0.35$; $p<0.001$).

PGRMC1 is a member of a multi-protein progesterone-binding complex (also named Hpr6.6 (human membrane progesterone receptor)), albeit, not associated with progesterone receptor (24, 25). Predominant expression of PGRMC1 in the endometrial glands could be associated with their role in the implantation process. Possibly, PGRMC1 is a secreted factor of the endometrial glands that regulate uterine luminal fluids homeostasis and interact with other cell types in the uterus to influence the uterine receptivity and blastocysts implantation for the establishment of pregnancy (26). Our study showed that in the cases of RM, PGRMC1 expression decreased and had an inverse correlation with the number of infiltrated immune cells in the endometrium in both groups. It may be associated, in our opinion, with the role of PGRMC1 in the regulation of immune response of the uterus microenvironment. A correlation between PGRMC1 expression and the duration of the menstrual cycle could be associated with its regulatory role. Gerhardt (2016) reported a strong connection between PGRMC1 and fertility regulation, as well as the uterine health. Another study suggested that ablation of this progesterone receptor resulted in subfertility and that endometrial cysts are potentially related to the premature aging of the endometrial tissue. Nevertheless, the mechanism of this disruption remains largely unknown (25, 27).

Potential limitations of this study are a relatively small number of cases, due to the rare incidences of RM not associated with endometriosis, fibroids and hyperplastic lesions of the female reproductive tract which are the most common causes of miscarriages. Despite these

limitations, the characteristics in the study population, availability of good follow-up data, uniform immunohistochemical and histopathological analyses, and uniform standardized protocols of patients' examination and endometrial biopsy by the same gynecologists mitigated the weaknesses and increased the validity of our results.

In conclusion, this study presents an elevation of uNK cells, FoxP3-positive Tregs and a decrease in PGRMC1 expression in the endometrial glands that could be used as a diagnostic factor or target for specific therapy for RM. Correlations between immune cells and PGRMC1 were described in our study for the first time that could reveal a new role for this protein in the development of RM. A larger cohort study should be carried out to confirm our conclusions.

Statement of contribution

YAL contributed to the conception and design of the study, recruitment of participants, analysis and interpreted of data as well as wrote and revised this original article. ZDA and MZI contributed to the interpretation of data, drafting and revising of the article. YAL and MZI contributed to the conception of the study, interpretation of data as well as the drafting and revising of this article.

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Declaration of interests

None of the authors have any conflicts of interest to declare.

Patient consent

Written informed consent was obtained.

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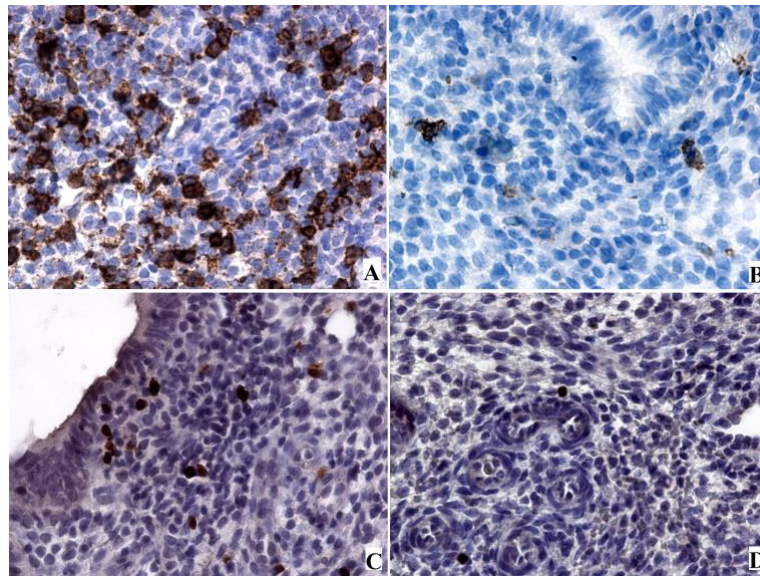


Figure 1. Infiltration of the endometrial stroma by lymphocytes: Dense infiltration by CD56-positive uterine NK lymphocytes in recurrent miscarriages group (**a**); A few uterine NK lymphocytes expressing CD56 situated near gland in comparison group (**b**); A small group of FoxP3-positive Tregs in the endometrium stroma near the surface epithelium in the recurrent miscarriages group (**c**); Two FoxP3 positive lymphocytes near the vessels in the group without recurrent miscarriages (**d**). Magnification: $\times 400$.

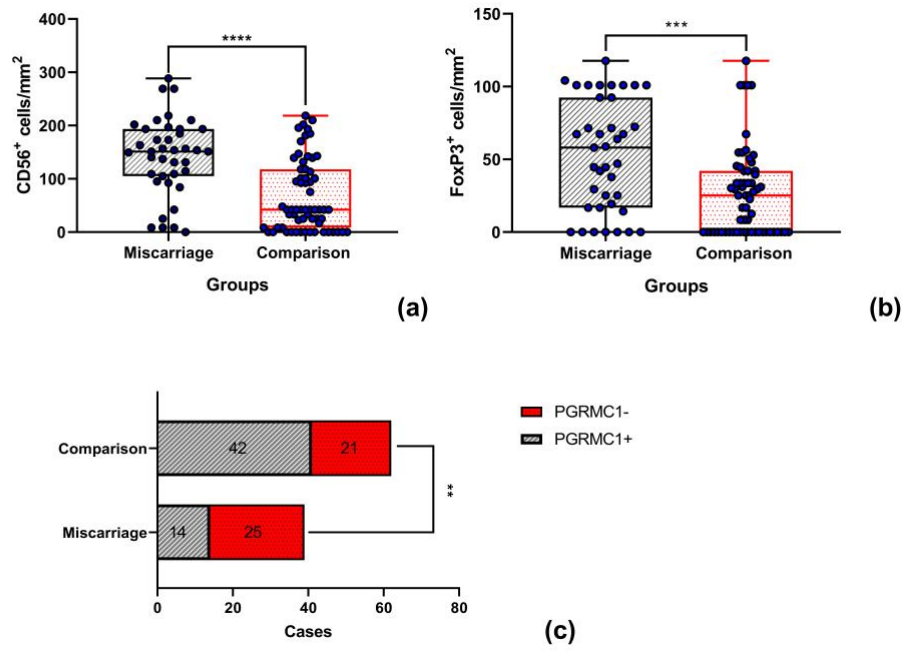


Figure 2. Statistical characteristics of groups: CD56-positive uterine NK lymphocytes (a); FoxP3 positive Tregs (b); Expression of PGRMC1 (c).

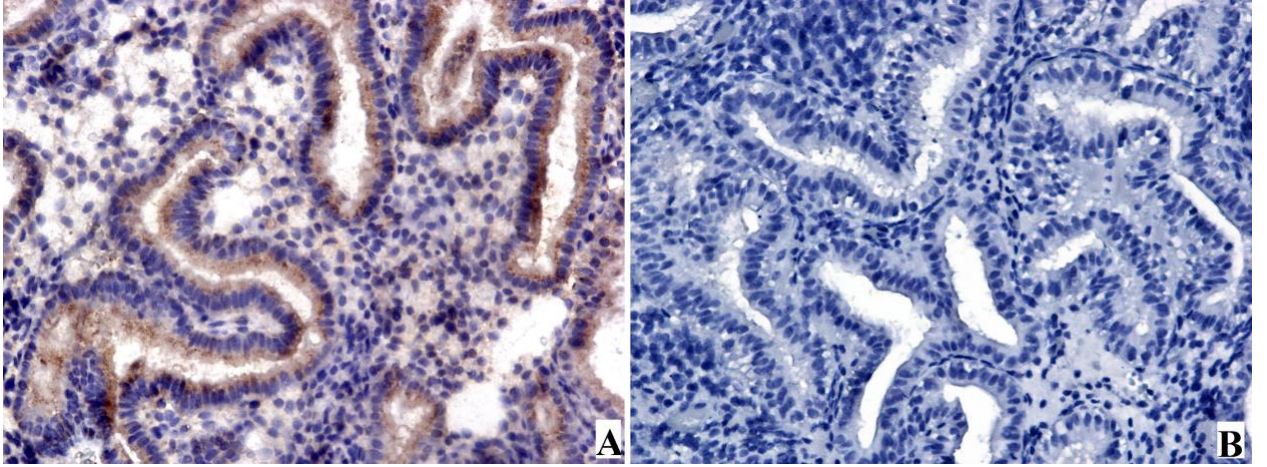
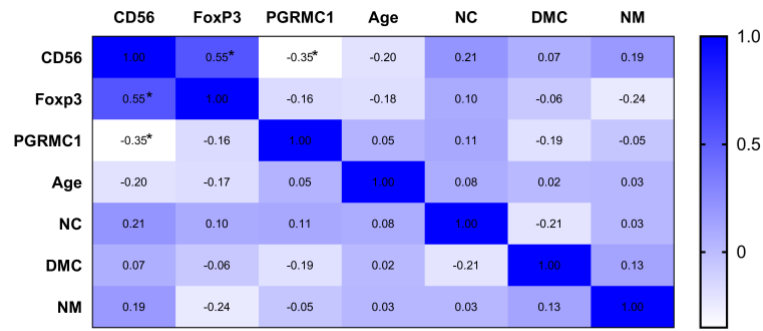
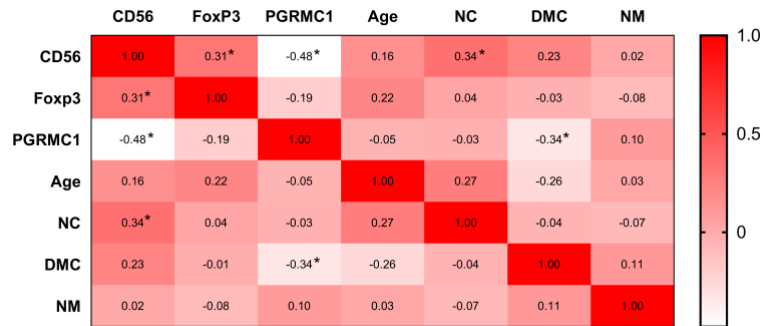


Figure 3. The Expression of PGRMC1: The endometrium of the group without recurrent miscarriages (**a**) and with recurrent miscarriages (**b**). Magnification: $\times 400$.



A



B

Figure 4. Correlation plots: +Recurrent miscarriage group (a); -Recurrent miscarriage group (b); Abbreviations: * $p < 0.001$; CD56 – uNK lymphocytes; FoxP3 – Treg lymphocytes; PGRMC1 – expression of PGRMC1; Age – age of the patients; NC – number of childbirths; DMC – duration of menstrual cycle, NM – number of miscarriages.

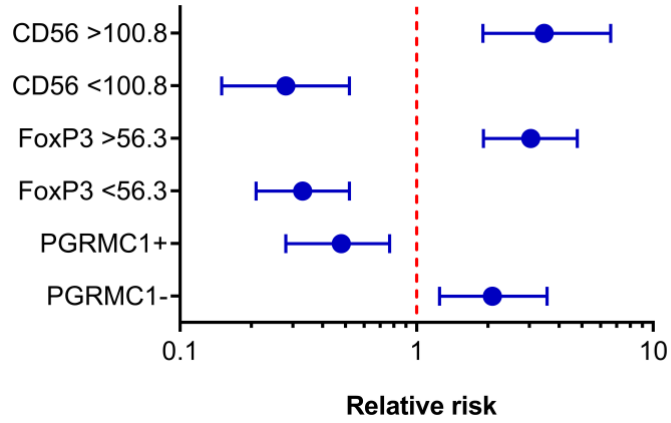


Figure 5. The forest plot represents a relative risk and 95% confidence interval of the association between threshold criteria of CD56+, FoxP3+ cells infiltration, PGRMC1 expression and recurrent miscarriages. The result of analyses demonstrated a statistically significant association between an increase of CD56+, FoxP3 cells, a decrease of PGRMC1 expression and recurrent miscarriages.

Table 1. Clinical characteristics of the patients

Parameters	Recurrent miscarriages group	Comparison group
Age	30.8±4.5	29.9±5.4
Number of childbirths	1.9±1.0	0.93±0.93
Duration of menstrual cycle	29.0±5.7	30.8±5.1
Number of miscarriages	3.9±0.4	0.9±0.29