

# Significance to investigate the TH1 /TH2 cytokines and their proportions in peripheral blood of women with unexplained recurrent spontaneous abortion and normal women based on pregnancy status

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## Research article

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# Abstract

**Background:** To investigate the significance of th1/th2 cytokines in the pathogenesis of unexplained recurrent spontaneous abortion(URSA) patients.

**Methods:** Flow cytometry was used to determine the level of th1/th2 cytokines in peripheral blood of URSA patients, comparison between two groups was analysed by Mann-Whitney U test, and Kruskal-Wallis H test was used to test three or more groups.

**Results :** The content of il-6 in peripheral blood of adverse pregnancy group (ad-pregnancy group) was significantly higher than that of URSA group and pregnancy group. The ratio of il-2 / il-4 and IFN- $\gamma$ /il-4 in peripheral blood of URSA group was significantly higher than that of pregnancy group, the ratio of IFN- $\gamma$ /il-10 and IFN- $\gamma$ /il-4 in peripheral blood of ad-pregnancy group was significantly higher than that of pregnancy group. The AUC of IFN- $\gamma$ /il-4 was 0.821, with high diagnostic efficiency, sensitivity as high as 84.09% and specificity as high as 69.05%, which were higher than other indicators.

**Conclusion :** The Variable of IFN- $\gamma$ /il-4 can be used for the initial diagnosis of Ursa to reduce the rate of missed diagnosis, the cytokine ratio in the Th1/Th2 immune response was more important than the expression of single cytokine.

## 1. Background

Abortion is a common complication in early pregnancy, with about 15% to 20% of pregnancies ending in spontaneous abortion, and two or more consecutive pregnancy losses before the 20th week of gestation are defined as recurrent abortion (RSA). It occurs in 1-2% of women of normal gestational age<sup>[1, 2]</sup>. The causes of recurrent abortion are diverse<sup>[3]</sup>, such as chromosomal abnormalities, intrauterine infections, autoimmune diseases, endocrine/metabolic diseases, including insufficient progesterone secretion from the corpus luteum, anatomical abnormalities, abnormal coagulation proteins and uterine abnormalities. However, the etiology of about 50% of recurrent abortion cases is still not clear<sup>[4]</sup>, it's called URSA. Due to the unclear specific immune mechanism, URSA, as a refractory pathological pregnancy, has brought great troubles to clinical treatment. Due to the lack of targeted treatment, the therapeutic effect has been unsatisfactory. URSA still lacks consensus on diagnosis and treatment options, and despite the lack of conclusive evidence, patients still have a strong desire to undergo diagnostic testing and begin treatment. Couples with URSA experience emotional stress, such as sadness, low self-esteem, guilt, anger, depression and anxiety, which continues for a long time during abortion<sup>[5]</sup>, which not only seriously affects female reproductive health, but also brings great negative effects on both couples<sup>[6]</sup>.

In recent years, with the development of molecular biology and flow cytometry, great progress has been made in the research on the mechanism of reproductive immune regulation, URSA has been reported to be associated with immune imbalance at the maternal-fetal interface<sup>[7-10]</sup>.

The maternal-fetal interface is composed of placental trophoblast cells and decidual tissue, which is the place where the maternal tissue comes into direct contact with the embryo. Since the embryo carries half of the allogenic antigen from father, it is the equivalent of an allograft. Therefore, establishing immune tolerance embryos may be associated with maternal immune system is the key to a successful pregnancy.

Raghupathy<sup>[11]</sup> studied the relative bias of Th1 and Th2 cytokines in 23 patients with URSA and 24 women with successful pregnancy history who volunteered to terminate pregnancy, the results showed that the concentration of Th1 cytokines in the URSA group was higher than that in the control group, while the concentration of Th2 cytokines in the control group was higher than that in the URSA group. M. akhseed<sup>[12]</sup> further studied the outcome of repregnancy in patients with URSA, the results of the study showed that the women who had abortion again with URSA had Th1 bias compared with the women who had successful pregnancy with URSA, and the women who had successful pregnancy with easy abortion had Th2 bias compared with the women miscarried again who had easy abortion. during the normal pregnancy, there existed Th2 type bias and pregnancy failure is there Th1 type bias [13-17].

Tom Wegmann<sup>[18]</sup> first proposed that the relationship between maternal and fetal immunity should be bi-directional, and that immune stimulation may be more important than immunosuppression, Th2-dominance has been found in URSA patients in some studies<sup>[19]</sup>. However, the Th1/Th2 paradigm is no longer relevant when the complexity of cytokine networks at the mater-fetal interface is taken into account.

In our study, the serum concentrations of Th1/Th2 cytokines including il-2, il-4, il-6, il-10, TNF- $\alpha$  and IFN- $\gamma$  in URSA and normal pregnancy women were measured to investigate the relationship between cytokines and the occurrence of URSA and whether Th1/Th2 cytokines in URSA patients conform to the Th1/Th2 balance paradigm.

## 2. Materials And Methods

### **Ethics Statement:**

The study was approved by the Ethics Committee of central south university of the second xiangya hospital.

### **2.1 Study population**

#### **2.1.1 Study participants and grouping**

A total of 44 USRA patients admitted to the second xiangya hospital from July 2018 to January 2019 were collected. At the same time, 42 cases of normal pregnancy without adverse pregnancy history and 51 cases of patients with adverse pregnancy history were collected. Flow cytometry was used to detect

URSA patients and each control group, and the concentration status of Th1 / Th2 cytokines was analyzed.

### **2.1.1.1 URSA group**

44 patients were collected with two or more consecutive early abortion in July 2018 to May 2019 in the second xiangya hospital of as the experimental group.

### **2.1.1.2 Control group**

At the same time, 51 cases with adverse pregnancy history were collected as the control group during the same period called adverse pregnancy group (ad-pregnancy group), 42 cases of normal pregnancy in the same period were collected as normal pregnancy control group (Pregnancy group). Recruitment criteria and grouping details are shown in figure 1.

## **2.1.2 Inclusion and exclusion criteria of cases**

**URSA group:** Recruitment of patients with pregnancy status. Excluded patients with diabetes and autoimmune thyroid diseases; B ultrasound, hysterosalpingography and hysteroscopy excluded the malformation of reproductive organs; the results of karyotype analysis of both couples were normal. Anti-sperm, anti-cardiolipin and anti-nuclear antibody were negative. The immune tests of cytomegalovirus and herpes simplex virus were negative. Those with infections such as ureaplasma and ureaplasma ureaplasma were excluded; the determination of reproductive endocrine hormones progesterone and estradiol and the diagnosis of curettage excluded endocrine factors. Routine examination of the semen of the male partner was normal. No history of exposure to toxic mercury or other lead-containing chemicals and radiation; No unhealthy habits such as smoking, drinking alcohol or using heroin, no excessive fatigue or strenuous exercise.

**ad-pregnancy group:** There is a history of spontaneous abortion, stillbirth or stillbirth, but chromosomal abnormalities, intrauterine infections, autoimmune diseases, endocrine/metabolic diseases, abnormal blood clotting and hemostatic proteins and uterine abnormalities are excluded, B ultrasound indicated pregnancy.

**Pregnancy group:** The group with regular menstrual cycle was included, and there was no history of spontaneous abortion, stillbirth or stillbirth, no history of chromosomal malformation, endocrine disorder, reproductive tract infection or autoimmune disease. There was no abdominal pain or vaginal bleeding during pregnancy, and B ultrasound indicated normal embryo development. No history of exposure to toxic mercury or other lead-containing chemicals and radiation, no unhealthy habits such as smoking, drinking or using heroin, no excessive fatigue or strenuous exercise.

## **2.2 Preparation of peripheral blood and detection of cytokine Levels**

10ml of the patient's blood was collected by vacuum blood collection vessel with heparin sodium anticoagulant, and the blood was tested within 8 hours. First, cell surface stain was added and incubated in dark for 15 minutes at room temperature. Then, hemolysin was added and incubated in dark for 10 minutes at room temperature. The supernatant was centrifuged and discarded. Then add fixative, incubate in the dark at room temperature for 15 minutes, centrifuge and discard the supernatant; Then, intraprep Permeabilization reagent was added and incubated in dark for 10 minutes at room temperature. Then add pbs 2-3ml lotion and centrifuge to discard supernatant. Finally, fluorescent labeled cytokine antibodies were added, mixed and incubated in dark for 30 minutes at room temperature. Concentrations of cytokine were determined using flow cytometry (Saiji.China) according to the manufacturer's protocol.

## **2.3 Statistical analysis**

SPSS version 21.0 Software (SPSS, Inc., Chicago, IL, USA) was used to analyse the results. Comparison between two groups was analysed by Mann-Whitney U test, and Kruskal-Wallis H test was used to test three or more groups.  $P < 0.05$  was considered statistically significant.

# **3. Statistical Analysis**

## **3.1 Comparison of background information between both groups**

A total of 47 patients were recruited in the URSA group, whose age ranged from 23 to 41 years, with an average age of  $30.84 \pm 4.182$  years. Ad-pregnancy group recruited a total of 54 patients, with the age ranging from 26 to 41 years, and the average age is  $31.86 \pm 4.252$  years; A total of 45 women were recruited as Pregnancy group. Their ages ranged from 22 to 44 years old, with an average age of  $31.65 \pm 4.672$  years. There was no significant difference in age between the groups (table1),The kruskal-wallis H test was used for comparison between groups,the general situation of all the data of the test indicators included in each group was described by median (quartile) [M (P25, P75)] (table 2).

## **3.2 comparison of cytokine and their proportions results in each group**

### **3.2.1 comparison of cytokines in peripheral blood of each group**

With the exception of il-6, the results of testing of all cytokines in the URSA group were not statistically significant compared with the ad-pregnancy group, the detection results of all cytokines in URSA group were not statistically significant compared with pregnancy group. With the exception of il-6, the results of testing of all cytokines in the ad-pregnancy group were not statistically significant compared with the pregnancy group(Table 3).The comparison of the contents of various cytokines was shown in figure 2.

Fig2 :(a)IL-2 content in peripheral blood of the four groups.(b)the IL-4 content in peripheral blood in the four groups. (c)the IL-6 content in peripheral blood in the four groups. (d)the IL-10 content in peripheral blood of four groups of patients. (e) the contents of TNF- $\alpha$  in peripheral blood in the four groups. (f)the IFN- $\gamma$  content in peripheral blood of patients in the four groups. The data were averaged and tested by Mann-Whitney U test. \* indicated that the difference between the two groups was statistically significant ( $P < 0.05$ ).

### 3.2.2 Comparison of Th1/Th2 ratio in each group

In order to compare the cytokine ratios in the Th1/Th2 immune response, the ratios of Th1/Th2 were calculated using a combination of IFN- $\gamma$ /il-4, IFN- $\gamma$ /il-10, TNF- $\alpha$ /il-4, TNF- $\alpha$ /il-10,il-2/il-4, il-2 / il-6, IFN- $\gamma$  / il-6, and TNF- $\alpha$  / il-6.The results indicated that the results of all cytokine ratios in the URSA group were not statistically significant compared with those in the ad-pregnancy group, which was consistent with the anticipated results of the experiment. Except for il-2/il-4 and IFN- $\gamma$ /IL-4, the ratio of all cytokines in URSA group was not statistically significant compared with that in the Pregnancy group. Except for IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10, the ratio of all cytokines in ad-pregnancy group was statistically significant compared with that in pregnancy group. (table 4).The ratio of cytokine content in each group was compared in figure 3.

### 3.3 Curve drawing and calculation of diagnostic efficiency of each Variable

ROC curves were plotted for all data from URSA group and Pregnancy group.ROC curves were drawn for the results of IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , IFN- $\gamma$ , IL-2/IL-4,IL-2/IL-10, TNF- $\alpha$ /IL-4, TNF- $\alpha$ /IL-10, IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10, as shown in figure 4.

The Area Under Curve (AUC) of peripheral blood for il-2, il-4, il-6, il-10, TNF- $\alpha$ , IFN- $\gamma$  was 0.523, 0.448, 0.515, 0.548, 0.473 and 0.482, respectively. The AUC of il-2 / il-4, il-2 / il-10, TNF- $\alpha$ /IL-4, TNF- $\alpha$ /IL-10, IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-10 were calculated to be 0.648, 0.521, 0.571, 0.457, 0.821 and 0.432, respectively. The AUC of il-4, TNF- $\alpha$ , IFN- $\gamma$ , TNF- $\alpha$ /IL-10 and IFN- $\gamma$ /IL-10  $< 0.5$ , indicating weak diagnostic efficacy, as shown in table 5.

According to the Youden's index , the best Cut-Off values were determined, and the best cut-off values of 7 indicators, including il-2, il-6, il-10, il-2 / il-4, il-2 / il-10, TNF- $\alpha$ /IL-4, and IFN- $\gamma$ / IL-4,were respectively as follows:Cut-Off<sub>IL-2</sub>=0.45pg/ml,Cut-Off<sub>IL-6</sub>=2.795pg/ml, Cut-Off<sub>IL-10</sub>=1.165pg/ml,Cut-Off<sub>IL-2/IL-4</sub>=0.4962,Cut-Off<sub>IL-2/IL-10</sub>=0.6896,Cut-Off<sub>TNF- $\alpha$ /IL-4</sub>=0.4487 and Cut-Off<sub>IFN- $\gamma$ /IL-4</sub>=0.6835.The diagnostic results of clinicians are taken as the "gold standard", and the corresponding sensitivity and specificity of each indicator are further calculated, as shown in table 6.

## 4. Discussion

Pregnancy is a complex physiological process, all the subjects included in this study were pregnant. Previous studies mostly compared the pregnant women with URSA history and normal gestational age, while the pregnant women with URSA and normal gestational age were compared in this study. The two groups of people had a common immune basis and were all pregnant. An identical immune basis between each group is a necessary condition for effective experimental results, and observation of the expression status of Th1/Th2 during pregnancy can more truly and effectively reflect the immune status and relative number of Th cells in patients with unexplained recurrent abortion.

Studies<sup>[20]</sup> have shown that the first three months of pregnancy is closely related to inflammation and may produce corresponding immune rejection. In the second trimester, anti-inflammatory and Th2 immune microenvironment are the main factors, which are necessary for fetal growth. There is a transition between Th1 and Th2 immune states between the first and second trimester of pregnancy, which is a necessary transition process for successful delivery. However, patients with URSA may show a continuous inflammatory immune response in the second trimester of pregnancy, which cannot be adjusted by increasing inhibitory immune mechanism, eventually leading to the intrauterine microenvironment with persistent inflammation, which is harmful to the fetus and leads to abortion.

various types of immune cells may be involved in promoting immune tolerance during pregnancy, the immune cells in the decidual tissues of the mother produce th2-related cytokines, including il-3, il-4, il-5, il-6, il-9, il-10 and il-13<sup>[21]</sup>, which can promote the growth of trophoblast cells and may be conducive to maintaining the success of pregnancy<sup>[22-25]</sup>, and some studies suggest that Th1-type inflammatory response may lead to embryo loss<sup>[26, 27]</sup>, Th1 cells are activated and release cytokines, including IFN- $\gamma$ , TNF- $\alpha$  and IL-2, and these cytokines mainly mediated immune cells and local inflammatory response<sup>[28]</sup>.

In addition to setting the Pregnancy group as the control group, the ad-pregnancy group was added as the other control group in this study. The recruiters in the ad-pregnancy group had a history of spontaneous abortion, stillbirth, or stillbirth, and B-mode ultrasound indicated gestational status, but excluded chromosomal abnormalities, intrauterine infections, autoimmune diseases, endocrine/metabolic disorders, thrombo-hemostatic protein abnormalities, and uterine abnormalities. With insufficient number of embryo losses to meet the criteria for diagnosis of URSA, we might consider the ad-pregnancy group to be the advance-ursa group. Further validation of the ad-pregnancy group with the URSA group, the pregnancy group. Our experimental research results showed that the ratio of il-2 / il-4 and IFN- $\gamma$ /il-4 in peripheral blood of URSA group was significantly higher than that of Pregnancy group ( $P < 0.05$ ), the ratio of IFN- $\gamma$ /il-10 and IFN- $\gamma$ /il-4 in peripheral blood of ad-pregnancy group was significantly higher than that of pregnancy group, it is consistent with the results of other people's research<sup>[11, 21, 29-31]</sup>, these results suggested that th1 cytokines increased and th2 cytokines decreased in the URSA patients.

The results of this paper indicated that, with the exception of il-6, there was no statistically significant difference between the URSA group and the ab-pregnancy group, the results of our study showed that,

when compared with the URSA group, the peripheral blood of the ad-pregnancy group had a higher il-6 content and had a statistically significant difference.

When we looked at the ab-pregnancy group as the advance-ursa group, this result was not in line with expectations, that was probably because il-6 is a nonspecific infection markers, normal serum il - 6 in the body content is relatively low, when some diseases occur, the content of il - 6 will rise sharply in the body, especially when the body infection, and at the time of the recruiting research object of this study, not to conduct a comprehensive physical examination, there may be some potential infection or other diseases affect the content of il - 6 in the body , perhaps the increasing of il-6 in ad-pregnancy group may be due to individual inconsistencies in the exclusivity response to foreign antigens.

The results of this study showed that when compared with the pregnancy group, the peripheral blood of the ab-pregnancy group had a higher il-6 content and had a statistically significant difference and from the level of il-6 in peripheral blood of the three groups (Fig. 2-c), it can be seen that the content of URSA group and ab-pregnancy group is higher than that of pregnancy group, the results of Lim KJ's study<sup>[32]</sup> showed that the level of IL-6 in the decidual tissue of URSA patients was low, which was inconsistent with the results of this study, possibly because IL-6 is a proinflammatory factor, which can promote the maturation and differentiation of T and B lymphocytes, and promote the immune activity of NK cells and CTL cells.IL-6 further promotes the production of other cytokines and inflammatory mediators, and interacts with each other, so that the balance between inflammatory factors and anti-inflammatory factors is out of control and the inflammatory reaction is excessive, and the accumulation of neutrophils in the inflammatory site leads to more inflammatory mediators, which further damages trophoblast cells and is not conducive to embryonic development.

But in some experiments<sup>[33]</sup> , it was found that the content of IL-6 and mRNA in the decidual tissues of URSA patients increased, the results are consistent with the results of our study. However, il-6 is a Th2 type cytokine, these results contradicted the th1/th2 equilibrium theory of maternal-fetal interface.

Previously, it was thought that the etiology of multiple miscarriages in URSA patients is that immune response from the Th2 immune response as the main guide to the Th1 immune response as the leading metastasis<sup>[34]</sup>.However, the results of our study showed that the reason of embryo loss was not only the transfer of immune response, but also the inflammation of maternal-fetal interface.

The results of our study showed that there was no significant difference between the groups of il-10 and TNF- $\alpha$ , however, there were studies<sup>[35-38]</sup> that showed that the expression level of TNF- $\alpha$  in URSA patients increased as the number of miscarriages increased, indicating that the increase in cytokines was positively correlated with the increase in the number of miscarriages and TNF- $\alpha$  might be an important reference for the results of pregnancy, and the data of Marzi M study suggested that the production of il-10 in peripheral blood mononuclear cells decreased during spontaneous abortion, manifesting that il-10 may be a protective factor in pregnancy<sup>[27]</sup>. In this paper, we can't get similar results in our data, this may be because the number of samples included in this study is slightly small, or it may be because there are

differences in environmental genes between different countries, ethnic groups or regions. Studies have<sup>[39-41]</sup> proved that unexplained recurrent abortion may be related to gene polymorphism.

ROC curve was plotted from data of URSA group and Pregnancy group in this study. The AUC of IFN- $\gamma$ /il-4 was 0.821, with high diagnostic efficiency, sensitivity as high as 84.09% and specificity as high as 69.05%, which were higher than other indicators, the initial diagnosis of URSA with this index could reduce the rate of missed diagnosis.

However, it was found in the study that there was no statistical difference in the content of il-4, il-2 and IFN- $\gamma$  in peripheral blood between each group, but the ratio of IFN- $\gamma$ /il-4 and il-2/il-4 in peripheral blood of URSA group was significantly higher than that of Pregnancy group. This may be because the decreased or increased amount of il-2, il-4 and IFN- $\gamma$  in patients is not statistically significant compared with Pregnancy group, but the ratio of the two variables is equal to the combination of the differences between patients and normal pregnant women, so that the new variable has obvious differences between the two; cytokines may be because of the complexity of the interaction of the formation of the complex network of cytokines to adjust a single factor can be the rest is influenced by many kinds of cell factors or other factors, when the two effects opposite factors taken together, can eliminate some indirect interactions between cytokines, amplifying the differences between different groups; it may also be because the sample size included in this study is slightly smaller.

## Conclusion

To sum up, Th1/Th2 balance paradigm in URSA patient was not an absolute increase in Th1 cytokine level and decrease in Th2 cytokine level, moreover, it is closely related to the effect of various cytokines at the maternal-fetal interface. The diagnostic efficacy of the variable il-2 / il-10 and IFN- $\gamma$ /il-4 was good, which can be used not only for the initial diagnosis of Ursa, but also for the monitoring of various clinical new treatments, the prediction of pregnancy outcome, and the evaluation of whether the new therapy is effective. The ratio of Th1/Th2 cytokines was more effective in URSA diagnosis than single cytokines, which may indicated that the cytokine ratio in the Th1/Th2 immune response was more important than the expression of single cytokine.

## Abbreviations

unexplained recurrent spontaneous abortion: URSA

recurrent spontaneous abortion: RSA

adverse pregnancy group: ad-pregnancy group

the Area Under Curve: AUC

## Declarations

## **Ethics approval and consent to participate**

In the ethics approval and consent for participate statement, the consent you obtained from study participants was verbal, and this was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University.

## **Consent for publication**

Any form of data (including personal details, test results and medical history) contained in the manuscript has been approved by the parties.

## **Availability of data and materials**

Data supporting the results of this study can be obtained from the Second Xiangya Hospital of Central South University, but the availability of such data is limited and the data has been used under the license of this study, so it is not publicly available. However, at a reasonable request and with the permission of the Second Xiangya Hospital of Central South University, I can provide the data.

## **Competing interests**

The authors declare that they have no competitive interest

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## **Authors' contributions**

YZ Peng collected specimens, analyzed and interpreted patient data, and was a major contributor to the manuscript;

M Wang provided the ideas for the whole work and supervised the collection and processing of the data, as the corresponding author.

All authors have read and approved the manuscript

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## Tables

Table 1 Basic information about subjects in case and control groups (median, p25-p75)

	URSA group	Ab-pregnancy group	Pregnancy group	P value
the number of cases	44	51	42	P=0.650
Age (median, range)	30.5(28-33)	31(28-34)	30(29-35)	

Table 2 General information of the participants (median, p25-p75)

	URSA group	Ad-pregnancy group	Pregnancy group	P value
IL-2(pg/ml)	0.6(0.17-1.05)	0.56(0.15-1.14)	0.43(0.16-1.11)	<0.001
IL-4(pg/ml)	0.97(0.21-1.95)	1.06(0.18-2.04)	1.26(0.55-2.24)	<0.001
IL-6(pg/ml)	2.2(1.49-3.62)	2.8(1.89-65.27)	2.38(1.43-3.30)	<0.001
IL-10(pg/ml)	1.67(0.88-2.33)	1.79(1.11-2.5)	1.37(0.85-2.19)	0.001
TNF- $\alpha$ (pg/ml)	0.77(0.25-1.48)	0.97(0.37-1.62)	0.71(0.29-2.14)	0.015
IFN- $\gamma$ (pg/ml)	1.60(0.38-2.73)	1.35(0.4-2.49)	1.54(0.53-3.33)	<0.001
IL-2/IL-4	0.56(0.38-1.01)	0.48(0.32-0.91)	0.42(0.22-0.64)	0.107
IL-2/IL-10	0.39(0.11-0.69)	0.25(0.10-0.51)	0.30(0.12-0.61)	<0.001
TNF- $\alpha$ /IL-4	0.74(0.45-1.36)	0.77(0.47-1.13)	0.67(0.27-1.14)	<0.001
TNF- $\alpha$ /IL-10	0.54(0.24-0.73)	0.48(0.22-0.73)	0.60(0.26-1.02)	0.444
IFN- $\gamma$ /IL-4	1.20(0.69-3.02)	1.17(0.69-3.02)	0.32(0.12-0.92)	0.178
IFN- $\gamma$ /IL-10	0.79(0.29-1.51)	0.73(0.22-1.32)	1.21(0.47-2.48)	<0.001

Table 3 comparison of cytokines in peripheral blood of each group

	IL-2	IL-4	IL-6	IL-10	TNF- $\alpha$	IFN- $\gamma$
URSA VS Ad-pregnancy group	0.679	0.771	0.031	0.263	0.395	0.785
URSA VS Pregnancy group	0.717	0.402	0.812	0.444	0.663	0.772
Ad-pregnancy group VS Pregnancy group	0.935	0.336	0.019	0.052	0.832	0.524

Table 4 Comparison of Th1/Th2 ratio results of each group

	IL-2/IL-4	IL-2/IL-10	TNF- $\alpha$ /IL-4	TNF- $\alpha$ /IL-10	IFN- $\gamma$ /IL-4	IFN- $\gamma$ /IL-10
URSA group VS Ad-pregnancy group	0.204	0.115	0.794	0.571	0.723	0.485
URSA group VS Pregnancy group	0.018	0.739	0.256	0.489	<0.001	0.280
Ad-pregnancy group VS Pregnancy group	0.294	0.270	0.438	0.228	<0.001	0.041

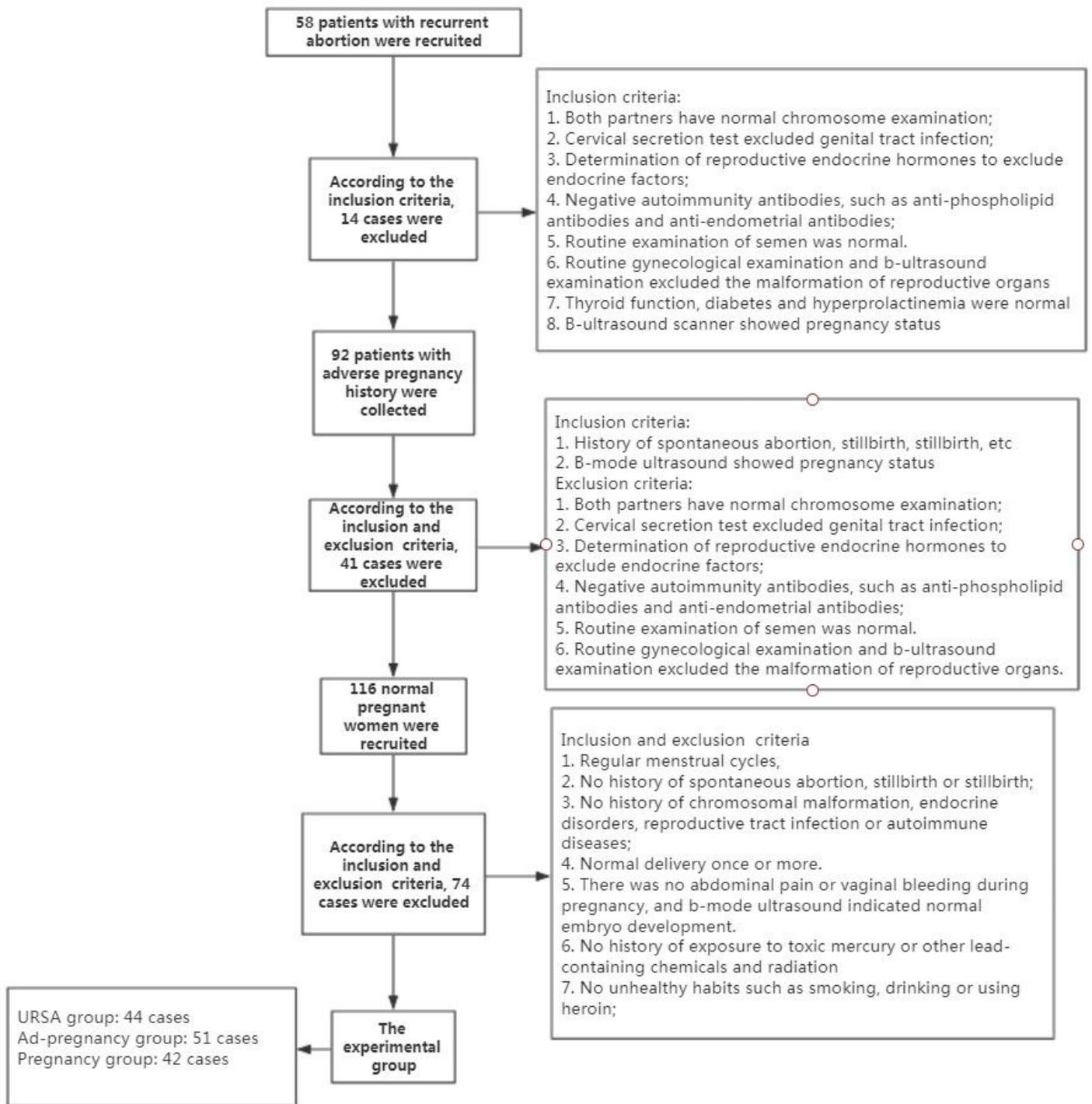
Table5 Area under the ROC curve for cytokine measurements

Variable(s)	the area under the curve [AUC]	Standard Error [SE]	95% confidence interval	
			Low limit	upper limit
IL-2	0.523	0.063	0.399	0.647
IL-4	0.448	0.062	0.325	0.570
IL-6	0.515	.063	0.392	0.638
IL-10	0.548	0.063	0.425	0.671
TNF- $\alpha$	0.473	0.064	0.348	0.598
IFN- $\gamma$	0.482	0.063	0.358	0.605
IL-2/IL-4	0.648	0.059	0.532	0.765
IL-2/IL-10	0.521	0.063	0.398	0.644
TNF- $\alpha$ /IL-4	0.571	0.062	0.449	0.693
TNF- $\alpha$ /IL-10	0.457	0.063	0.332	0.581
IFN- $\gamma$ /IL-4	0.821	0.045	0.733	0.910
IFN- $\gamma$ /IL-10	0.432	0.063	0.310	0.555

Table 6 Comparison of the cut-off value, sensitivity and specificity of URSA diagnosed by each Variable

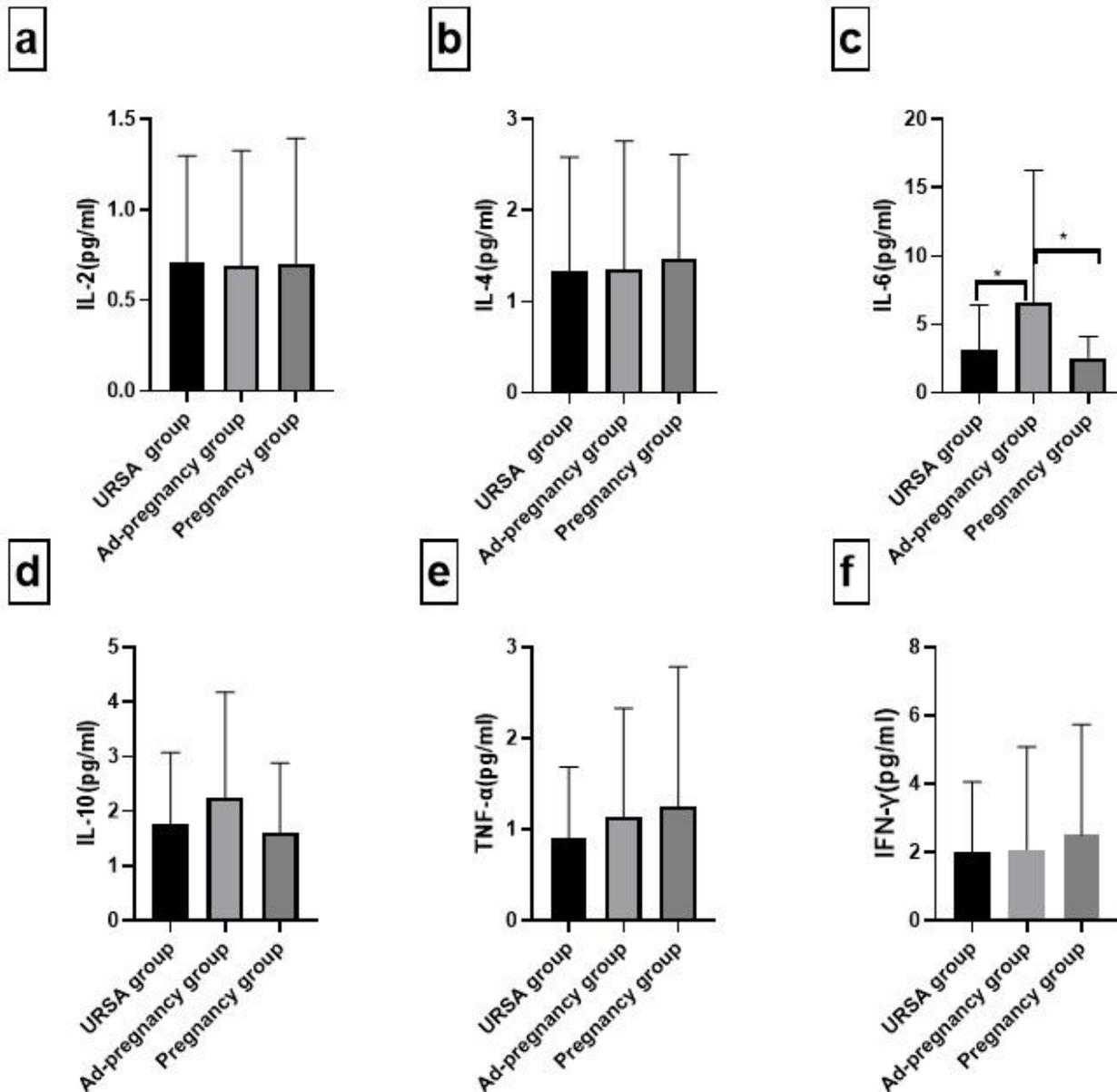
Variable(s)	Cut-off value	sensitivity(%)	specificity(%)
IL-2(pg/ml)	0.45	63.64	50
IL-6(pg/ml)	2.795	40.91	69.05
IL-10(pg/ml)	1.165	50	61.90
IL-2/IL-4	0.4962	63.64	64.29
IL-2/IL-10	0.6896	25	85.71
TNF- $\alpha$ /IL-4	0.4487	81.82	38.10
IFN- $\gamma$ /IL-4	0.6835	84.09	69.05

## Figures



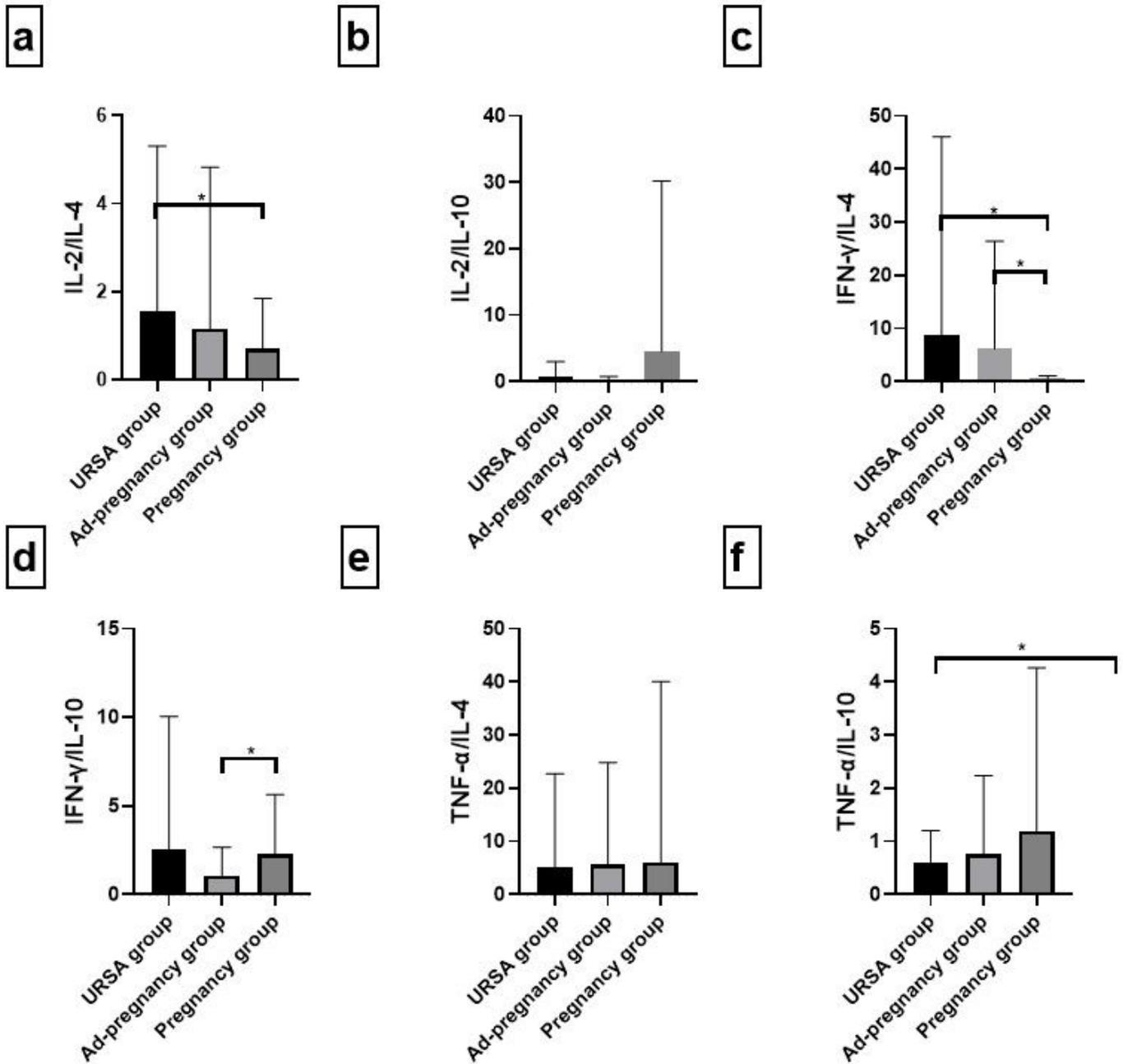
**Figure 1**

Recruitment criteria and grouping details



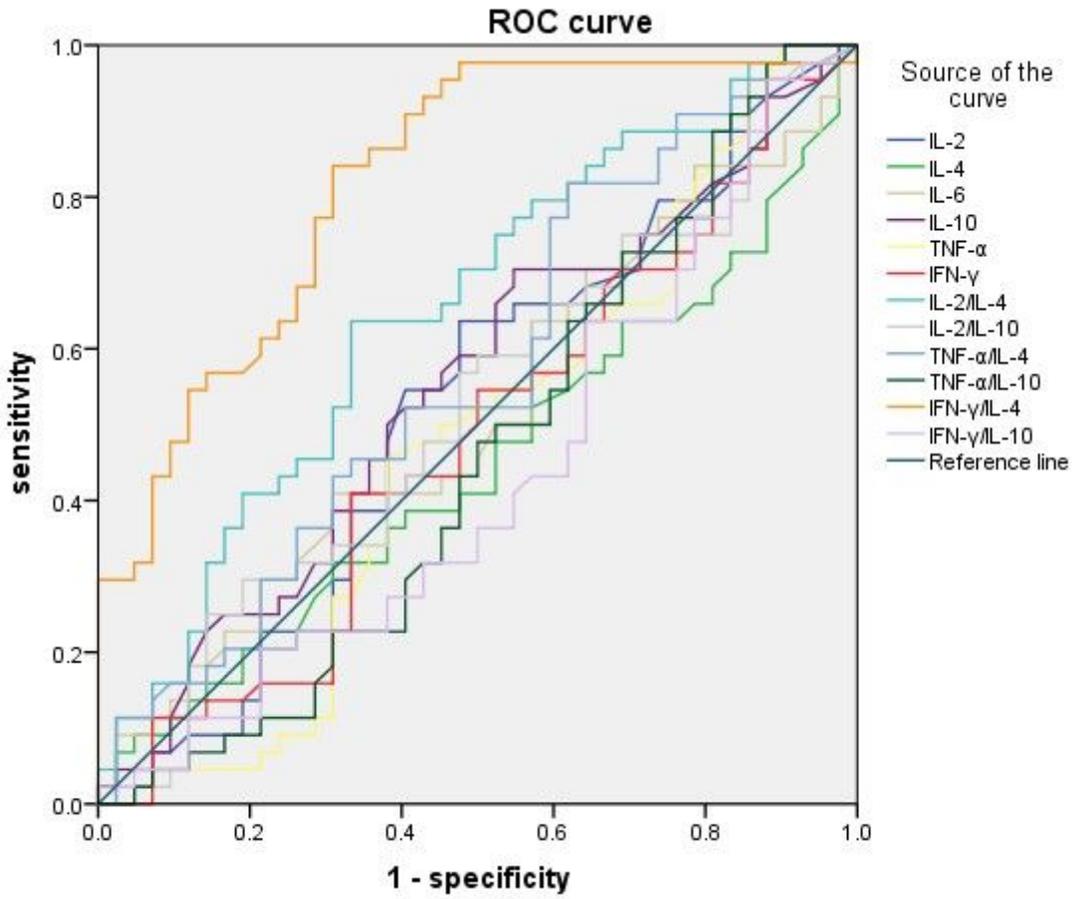
**Figure 2**

(a) IL-2 content in peripheral blood of the four groups. (b) the IL-4 content in peripheral blood in the four groups. (c) the IL-6 content in peripheral blood in the four groups. (d) the IL-10 content in peripheral blood of four groups of patients. (e) the contents of TNF- $\alpha$  in peripheral blood in the four groups. (f) the IFN- $\gamma$  content in peripheral blood of patients in the four groups. The data were averaged and tested by Mann-Whitney U test. \* indicated that the difference between the two groups was statistically significant ( $P < 0.05$ ).



**Figure 3**

(a) the ratio of il-2/il-4 in the peripheral blood of the four groups. (b) the ratio of il-2/il-10 in the peripheral blood of the four groups. (c)the peripheral blood IFN-γ / il-4 ratio in the four groups. (d)the peripheral blood IFN-γ/ il-10 ratio in the four groups. (e)the ratio of TNF-α / il-4 in peripheral blood of the four groups. (f)the ratio of TNF-α / il-10 in peripheral blood of the four groups. The data are represented by the mean of their ratios and are tested by Mann-Whitney U test. \* indicated that the difference between the two groups was statistically significant (P < 0.05)



Diagonal segment are produced by ties.

Figure 4

ROC curve of URSA diagnosis by each Variable