

## BASIC SCIENCE: OBSTETRICS

## Pro- and antiinflammatory cytokines in threatened miscarriages

Jean Calleja-Agius, MD; Shanthi Muttukrishna, PhD, DSc; Arnold R. Pizzey; Eric Jauniaux, MD, PhD

**OBJECTIVE:** The purpose of this study was to evaluate circulating and intracellular levels of Th1 and Th2 cytokines in women with threatened miscarriage (TM) and subsequent outcome.

**STUDY DESIGN:** Plasma levels of tumor necrosis factor (TNF)-receptors 1 and 2, TNF $\alpha$ , interferon gamma (IFN $\gamma$ ), and interleukins (IL) -6 and -10 were measured by flow cytometric bead assays in 80 women with TM: 53 women with normal outcome and 27 women who miscarried. Fluorescent antibody labeling was also performed on whole blood in a subgroup of 27 women of TM: 16 women with normal outcome and 11 women who miscarried.

**RESULTS:** Monocyte expression of TNF $\alpha$  and circulating levels of TNF $\alpha$ , IFN $\gamma$ , IL-10, IL-6, and TNF-R1 were significantly lower, whereas circulating levels of TNF $\alpha$ /IL-10, IFN $\gamma$ /IL-10, and TNF $\alpha$ /IL-6 ratios were significantly higher, in women with TM who subsequently miscarried, compared with the women with normal outcome.

**CONCLUSION:** An increased Th1 type of immune response, which was similar to that observed in preterm delivery, was found in TM cases that were complicated by a subsequent miscarriage.

**Key words:** cytokine, interleukin-10, threatened miscarriage, TNF receptor, tumor necrosis factor

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**T**hreatened miscarriage (TM) is defined as a history of vaginal bleeding in early pregnancy in the presence of a closed cervix and with ultrasonic evidence of an intrauterine gestational sac and a fetal heartbeat.<sup>1</sup> TM is the most common complication of ongoing preg-

nancy, which occurs in approximately 30% of pregnant women, and is associated with a 10-14% risk of full miscarriage.<sup>2,3</sup> Various management protocols have been developed that included conservative management and hormonal therapy that were based on progesterone<sup>4</sup> and human chorionic gonadotropin (hCG).<sup>5</sup> Overall, TM can generate great anxiety in patients, especially because bleeding in early pregnancy can lead to pregnancy loss and other obstetric complications.<sup>6</sup>

The pathophysiology of TM is linked to bleeding from uteroplacental vessels at the margin of the placenta with blood accumulating between the chorionic membrane and uterine wall.<sup>7</sup> The formation of a subchorionic hematoma may disrupt the placental bed. If the hematoma expands to the rest of the placental mass, it will induce complete miscarriage within a week of the first symptoms.<sup>8</sup> If bleeding is limited or happens close to the internal cervical os and is evacuated, the pregnancy may continue; however, this can lead to a chronic inflammatory reaction within the decidua and membranes.<sup>7,8</sup> Within this context, TM is associated with a higher incidence of preterm labor, prelabor rupture of membranes,

placental abruption, fetal growth restriction, and low birthweight.<sup>2,9-18</sup>

A strong association exists between maternal T helper-1 (Th1)-type immunity and pregnancy loss, whereas a shift towards Th2-type cytokine response has been observed in successful pregnancy.<sup>19</sup> Cytokines are known to play an important role in implantation. An imbalance in cytokine production occurs in early pregnancy loss.<sup>20-22</sup> Cytokine receptors such as tumor necrosis factor-receptor 1 (TNF-R1) have also been associated with miscarriage in their role as apoptosis-mediators through proinflammatory cytokines such as tumor necrosis factor alpha (TNF $\alpha$ ).<sup>23</sup>

Several factors in the maternal history (such as age and smoking, biomarkers that include serum hormones levels, and the presence of a retroplacental hematoma on ultrasound scanning) have been investigated as possible predictors of pregnancy outcome in women with TM.<sup>24-27</sup> It has been shown that after TM, maternal serum inhibin A in combination with other serum markers, such as hCG, are altered in women who subsequently have a first-trimester miscarriage.<sup>28,29</sup> The biomarkers that give the best predictive values are a combination of se-

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**TABLE 1**  
The limit of detection, intra- and interassay coefficients of variation for each cytometric bead assay

Cytokine/receptor	Assay limit of detection, pg/mL	Intraassay coefficient of variation, %	Interassay coefficient of variation, %
Tumor necrosis factor- $\alpha$	0.7	10.2	5.0
Interferon $\gamma$	1.8	10.8	5.0
Interleukin-10	0.13	6.4	11.0
Interleukin-6	1.6	9.4	8.0
Tumor necrosis factor-receptor 1	5.2	2.6	10.1
Tumor necrosis factor-receptor 2	1.4	7.1	5.6

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rum progesterone and  $\beta$ hCG, with a sensitivity of 88.1% and specificity of 84.3%.<sup>30</sup> However, no biomarker of inflammation in the peripheral maternal blood has been validated sufficiently to be of any clinical use in counseling women with TM regarding their risks of subsequent miscarriage and other complications or for providing advice about the benefit of specific treatment to reduce these risks.<sup>31</sup> Recent data have indicated that treatment that corrects Th1/Th2 imbalance, such as dydrogesterone, could help prevent TM from resulting in a full miscarriage.<sup>32-35</sup>

The objective of this study was to evaluate the changes in circulating levels and intracellular expression of Th1 and Th2 cytokines in patients with TM and to investigate whether these cytokines are altered in patients who subsequently miscarry. TNF $\alpha$  and its receptors TNF-R1 and TNF-R2 and interferon gamma (IFN $\gamma$ ) were measured to evaluate the Th1 cytokine response, whereas interleukins (IL)-6 and -10 were measured to evaluate Th2 cytokine response. IL-6 was classified as a Th2 cytokine because of its role in early pregnancy.<sup>36</sup> The role of these cy-

tokines in predicting miscarriage was also investigated.

## MATERIALS AND METHODS

### Subjects and samples

A group of 80 women with first-trimester TM were recruited prospectively from the Early Pregnancy Unit at University College London Hospital after clinical diagnosis of TM (ie,  $\leq 12$  weeks 6 days of gestation as calculated from the first day of their last menstrual period) with vaginal bleeding and after an ultrasound confirmation of a singleton viable pregnancy. Exclusion criteria included patients with a history of recurrent miscarriage, presence of twin pregnancy, hydatiform mole or a congenital uterine anomaly, presence of large leiomyomata that was distorting the uterine cavity, known history of cervical incompetence, thrombophilia, or any medical condition that needed chronic drug therapy.

After written informed consent, 10 mL of venous blood were collected by sterile venipuncture. One milliliter of blood was analyzed as part of a prospective study that involved flow cytometric analysis of fluorescent antibody-labeled whole blood. This analysis had to be carried out on fresh whole blood, without prior knowledge of the outcome of the index pregnancy. The remaining 9 mL of blood were centrifuged within 2 hours of collection, and the plasma supernatant was stored at  $-20^{\circ}\text{C}$  until it was assayed. Measurement of cytokines/receptors in plasma was done with knowledge of the outcome of the index pregnancy (ie, as a retrospective nested case control study). A normal pregnancy outcome was defined as a singleton live birth at term and of normal weight ( $\geq 3$  kg). A miscarriage was defined as pregnancy ending spontaneously at  $< 24$  weeks of gestation. The study was approved by University College London Hospital Committee on the Ethics of Human Research.

### Bioassays

**Multiplex bead assays.** TNF-receptors 1 and 2, TNF $\alpha$ , interferon gamma (IFN $\gamma$ ), IL-6, and IL-10 were assayed in maternal plasma using Cytometric Bead Array Human Soluble Protein Flex Sets (BD

**TABLE 2**  
Fluorochromes used for dual antibody labeling of whole blood

Patient code	Monocyte label	Cytokine/receptor label	Lipopolysaccharide, ng/mL
a	CD-14: APC	TNF $\alpha$ receptor I: PE	—
b	CD-14: APC	TNF $\alpha$ receptor II: PE	—
c	CD-14: APC	IgG1: PE	—
d	CD-14: APC	IgG2: PE	—
e	CD-14: APC	TNF $\alpha$ : FITC	40
f	CD-14: APC	TNF $\alpha$ : FITC	—
g	CD-14: PE	IFN $\gamma$ : APC	40
h	CD-14: PE	IFN $\gamma$ : APC	—
i	CD-14: PE	IL-10: PE	40
j	CD-14: PE	IL-10: PE	—
k	CD-14: PE	IL-6: biotin + streptavidin PE	40
l	CD-14: PE	IL-6: biotin + streptavidin PE	—

APC, allophycocyanin; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; IL, interleukin; PE, phycoerythrin; TNF, tumor necrosis factor.

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Biosciences, San Jose, CA). The scope of this cytometric bead array was to allow for multiplexed analysis of different cytokines and receptors on smaller quantities of plasma, when compared with the use of conventional enzyme-linked immunosorbent assay. The experiment was carried out according to the manufacturer's instructions and as previously published.<sup>37</sup> Acquisition of the sample data was performed with a bioanalyzer flow cytometer (BD FACSArray; BD Biosciences). The data were presented in graphic and tabular formats with the FCAP Array software (BD Biosciences). The limit of detection for each assay and the intraassay and interassay coefficients of variation are shown in Table 1.

**Flow cytometric analysis of fluorescent antibody-labeled whole blood.** Initial whole blood validation experiments showed that 40 ng/mL of lipopolysaccharide (40 LPS) and an incubation period of 12 hours gave the highest increment above basal level (0 LPS) in terms of cytokine expression by the activated monocytes, while retaining a high monocyte population. Dual antibody labeling was carried out with specific antibodies that were conjugated to spectrally distinct fluorochromes to enable easy discrimination. All samples were labeled with mouse anti-human antibody (AbD Serotec, Oxford, UK) that was specific for monocytes (CD 14). Different aliquots were also labeled with antihuman antibodies that were specific to the cytokine/receptor of interest (Table 2).

With the use of aseptic techniques, 1.5 mL of whole blood from each individual patient was mixed with 1.5 mL of Dulbecco phosphate-buffered saline solution without  $Ca^{++}$ ,  $Mg^{++}$ . One milliliter of this whole blood/Dulbecco phosphate-buffered saline solution was placed into 4 200- $\mu$ L aliquots (Table 2). The other 2 mL aliquots were separated into 2 separate 1-mL aliquots. Ten microliters of monensin sodium solution (Sigma-Aldrich, St. Louis, MO) and 10  $\mu$ L of 40 LPS solution were added to 1 aliquot (stimulated); only 10  $\mu$ L of monensin sodium solution (Sigma-Aldrich) without LPS was added to the other aliquot. Both

**TABLE 3**  
**Patient demographics**

Parameter	Outcome	Mean	SEM	P value <sup>a</sup>
Body mass index, kg/m <sup>2</sup>	Normal outcome	23.63	.47	.63
	Miscarriage	24.18	1.05	
Gestation, wk	Normal outcome	8.10	.40	.24
	Miscarriage	7.45	.37	
Parity, births	Normal outcome	.43	.10	.61
	Miscarriage	.52	.14	
Age, y	Normal outcome	30.57	.74	.07
	Miscarriage	32.41	.68	
White ethnicity, %	Normal outcome	77		.59
	Miscarriage	70		

Patient demographics of all the patients with threatened miscarriage (TM) who were recruited in this study (TM normal pregnancy outcome, 53 women; TM with subsequent miscarriage, 27 women) showed no significant difference between the 2 groups in terms of body mass index, gestation, parity, age, and ethnicity.

<sup>a</sup> Considered to be not significant.

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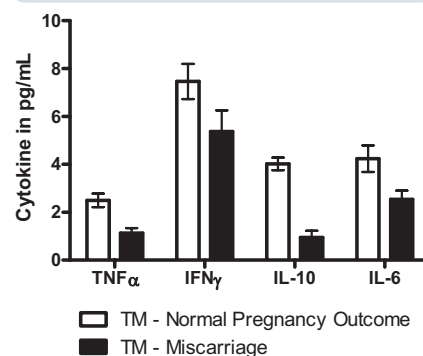
aliquots were placed in a water bath at 37°C and shaken at 20 rev/min. Normal atmospheric composition was used instead of a CO<sub>2</sub> system to ensure that the samples were kept as close to the physiologic state as possible. After 12 hours incubation, the samples in each tube were further divided into 4 200- $\mu$ L aliquots, to a total of 8 aliquots, each of which was labeled with the patient's code and lettered e-1, depending on the antibody combination to be added and whether LPS was added (Table 2). Ten microliters of CD14 antibody were added to the sample and incubated in the dark for 15 minutes at room temperature. Fix and Perm Cell Permeabilization Reagents (Caltag; Invitrogen, Paisley, Scotland, UK) were added according to the manufacturer's instructions, followed by 5  $\mu$ L of the second antibody (TNF $\alpha$ , IL-10, TNF $\alpha$  receptor, or negative control). The sample was again incubated in the dark for 20 minutes, washed, and centrifuged. The pellet was resuspended in 200  $\mu$ L of wash buffer. Each cryovial was kept at 4°C in the dark until the sample was read by the flow cytometer (Dako CyAn ADP; Advanced Digital Processing, Glostrup, Denmark), which was operated through the Summit software (version 4.31; Summit Software, Little Rock, AR).

The results were then saved in file format for post-acquisition spectral compensation and data analysis (FCS 2.0; Dako Colorado Inc, Fort Collins, CO).

### Statistical analysis

The results generated by the FCAP Array software and Summit software were entered in Microsoft Excel (version 2003; Microsoft Corporation,

**FIGURE 1**  
**Comparison of cytokine levels in plasma**

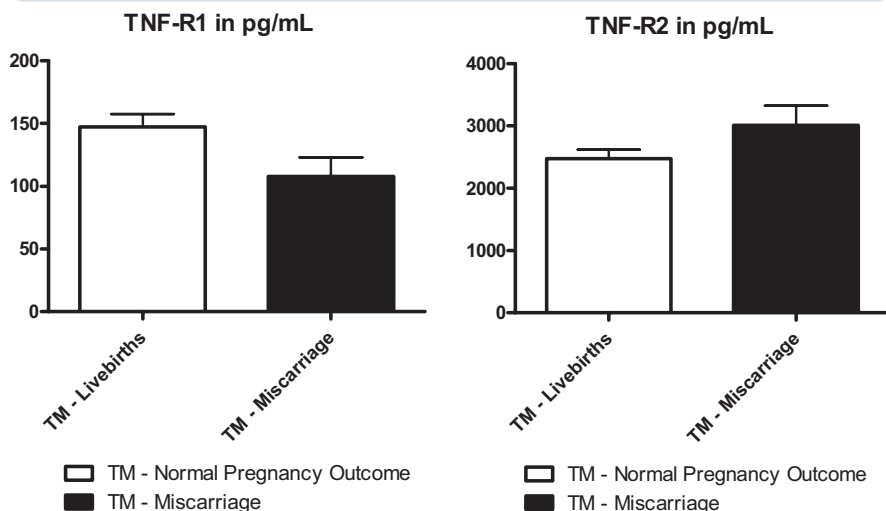


Graph shows significantly higher cytokine levels in patients with threatened miscarriage (TM) with normal outcome. See Table 4 for probability values.

IL, interleukin; IFN, interferon; pg/mL, picograms per milliliter; TNF, tumor necrosis factor.

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**FIGURE 2**  
Comparison of tumor necrosis factor (TNF) receptors in plasma

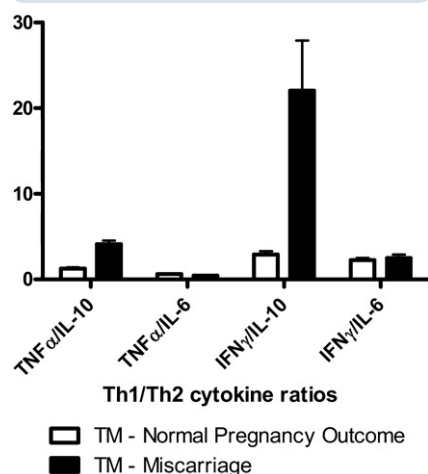


There is a significantly higher level of TNF-R1 in patients with threatened miscarriage (TM) with normal pregnancy outcome ( $P < .05$ ).

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Redmond, WA). For the purpose of analysis, patients were matched for ethnicity, gestation, body mass index, age, and parity. Data were normalized by log

**FIGURE 3**  
Comparison of Th1/Th2 cytokine ratios in plasma



There are significantly higher tumor necrosis factor (TNF)- $\alpha$ /IL-10, TNF- $\alpha$ /interleukin (IL)-6, and interferon (IFN) $\gamma$ /IL-10 ratios in patients with threatened miscarriage (TM) who subsequently miscarried than in women with a normal pregnancy outcome ( $P < .001$ ,  $P = .049$ ,  $P < .001$ , respectively).

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transformation, and unpaired  $t$  test was carried out for the plasma and intracellular cytokine and receptor levels. The mean and SEM of the actual values were entered in GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA) to generate graphs. Analysis was carried out with SPSS software (version 12 for Windows; SPSS Inc, Chicago, IL) for significance testing. A probability value of  $< .05$  was considered statistically significant.

Intercooled STATA 9.1 (StataCorp, College Station, TX) was used to generate nonparametric receiver operator characteristics curves for each cytokine/receptor. Each biomarker was ranked according to the area under the graph, and confidence intervals were calculated. To identify the best biomarker combination for the prediction of miscarriage, stepwise logistic regression was carried out, followed by discriminant analysis on the resulting combination to quantify the specificity, sensitivity, and positive predictive value.<sup>38</sup>

## RESULTS

Of the 80 patients with TM who were recruited in the retrospective nested case control study, 53 patients had normal pregnancy outcome that resulted in a singleton live birth at term and of normal

weight (10-90th percentile); 27 patients subsequently miscarried at  $< 24$  weeks of gestation. There was no statistical difference in body mass index, parity, age, and ethnicity between patients with TM with normal outcome and those who miscarried (Table 3). The mean gestational age for patients with TM who had normal pregnancy outcome and those who subsequently miscarried was not significantly different (9 weeks 6 days and 8 weeks 3 days, respectively;  $P = .73$ ). Flow cytometric analysis of fluorescent antibody-labeled whole blood was carried out on a subgroup of 27 patients: 16 patients had normal outcome, and 11 patients subsequently miscarried.

## Circulating cytokine and receptor levels in the plasma

Circulating levels of TNF $\alpha$ , IFN $\gamma$ , IL-10, IL-6, and TNF-R1 in plasma were significantly higher in patients with TM who had normal pregnancy outcome than in patients who subsequently miscarried ( $P < .001$ ,  $< .05$ ,  $< .001$ ,  $< .005$ , and  $< .05$ , respectively). TNF $\alpha$ /IL-10, TNF $\alpha$ /IL-6, and IFN $\gamma$ /IL-10 ratios were significantly higher in patients with TM who subsequently miscarried than in those with normal pregnancy outcome ( $P < .001$ ,  $< .05$ , and  $< .001$ , respectively; Figures 1-3; Table 4).

The biomarker that was the best predictor of miscarriage was TNF-R2, with the highest area under the curve (0.65; 95% confidence interval, 0.47-0.75; Table 5; Figure 4). At a cutoff value of 2202 pg/mL, TNF-R2 gave a positive predictive value of 44%, a sensitivity of 63%, and a specificity of 62%. Discriminant analysis showed that the best combination of biomarkers was TNF-R2 and TNF $\alpha$ , which gave a positive predictive value of 66%, a sensitivity of 54%, and a specificity of 92%.

## Intracellular cytokine and receptor levels in whole blood

In whole blood, the number of CD-14 cells that were positive for each individual cytokine was measured both at unstimulated basal level (0 LPS) and after LPS stimulation (40 LPS; Figure 4). In monocytes, there was a significantly higher level of stimulated TNF $\alpha$

**TABLE 4**  
**Comparison of circulating cytokine and receptor levels in the plasma in threatened miscarriage with normal pregnancy outcome and with subsequent miscarriage**

Cytokine/receptor	P value
TNF $\alpha$	< .001 <sup>a</sup>
IFN $\gamma$	< .05 <sup>a</sup>
IL-10	< .001 <sup>a</sup>
IL-6	< .005 <sup>a</sup>
TNF-R1	< .05 <sup>a</sup>
TNF-R2	Not significant
TNF $\alpha$ /IL-10	< .001 <sup>a</sup>
TNF $\alpha$ /IL-6	< .05 <sup>a</sup>
IFN $\gamma$ /IL-10	< .001 <sup>a</sup>
IFN $\gamma$ /IL-6	Not significant

*t* test showed that there were statistically significant elevated levels of TNF $\alpha$ , IFN $\gamma$ , IL-10, IL-6, and TNF-R1 in threatened miscarriage in women who proceeded to live birth; the women with threatened miscarriage who miscarried had statistically higher ratios of TNF $\alpha$ /IL-10, TNF $\alpha$ /IL-6, and IFN $\gamma$ /IL-10.

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

<sup>a</sup> Considered to be statistically significant.

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levels (TNF 40 LPS/TNF 0 LPS;  $P < .05$ ) in women with TM who had normal pregnancy outcome compared with those who miscarried. The median number of CD-14 cells that were positive for TNF-Rs were measured at unstimulated basal levels only, and there was no significant difference between women with TM who had normal pregnancy outcome and those who miscarried (Figure 5).

The ratio of Th1/Th2 cytokines was calculated both at unstimulated basal level and as a ratio of percentage fold increase in cytokine expression. At basal level, Th1/Th2 ratio was measured in whole blood without LPS stimulation as TNF $\alpha$ /IL-10, TNF $\alpha$ /IL-6, IFN $\gamma$ /IL-10, and IFN $\gamma$ /IL-6. Also, the percentage fold increase with LPS stimulation (40/0 LPS) of each individual Th1 cytokine over percentage fold increase of each individual Th2 cytokine was calculated. There was no significant difference in any of the ratios between patients with TM with

normal pregnancy outcome and those who miscarried (Figure 6).

### COMMENT

The results of this study indicated that women with TM during the first trimester who subsequently miscarried had an imbalance in the circulatory Th1/Th2 cytokine levels, with a shift towards a Th1 type of immune response. Similar findings have been found previously in women with recurrent or complete miscarriage,<sup>21,22</sup> whereas women with a normal pregnancy outcome do not have an imbalance in their plasma Th1/Th2 cytokine ratio.<sup>19,39,40</sup> These findings suggest that changes in levels of cytokines could play a role in the determination of the risk of miscarriage in women with first trimester TM.

The mechanism by which intrauterine bleeding alters maternal circulating hormone and cytokine levels is not well-defined. Up to week 9-10 of gestation, placental villi cover the entire surface of the chorionic sac. As the gestational sac grows during fetal life, the villi that are associated with the decidua capsularis surrounding the amniotic sac degenerate and form the chorion leave, whereas villi that are associated with the decidua basalis proliferate and form the definitive placenta.<sup>7</sup> We previously have shown that the underlying uteroplacental circulation in the center of the primitive placenta is plugged, whereas in periphery the mouth of the spiral arteries are never plugged by the trophoblastic shell, which allows limited maternal blood flow to enter the marginal zone of placenta from 8-9 weeks of gestation.<sup>8</sup> This leads to higher local O<sub>2</sub> concentration at a stage of pregnancy when the trophoblast possesses low concentrations of the main antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase.<sup>7</sup> Focal trophoblastic oxidative damage and progressive villous degeneration trigger the formation of fetal membranes, which remodels the uteroplacental interface. First-trimester vaginal bleeding has been associated with increased maternal levels of free serum  $\beta$ -hCG,<sup>41</sup> possibly because of a rise in hCG transfer into the maternal circula-

**TABLE 5**  
**Values of AUC of the ROC curves and CIs for each cytokine and receptor**

Biomarker	Area under the curve	95% CI
TNF-R2	0.65	0.47-0.75
TNF-R1	0.36	0.22-0.50
IFN $\gamma$	0.36	0.22-0.49
IL-6	0.31	0.18-0.43
TNF $\alpha$	0.29	0.19-0.39
IL-10	0.12	0.01-0.23

TNF-R2 is the best single predictor of miscarriage, with an area under the curve of 0.65.

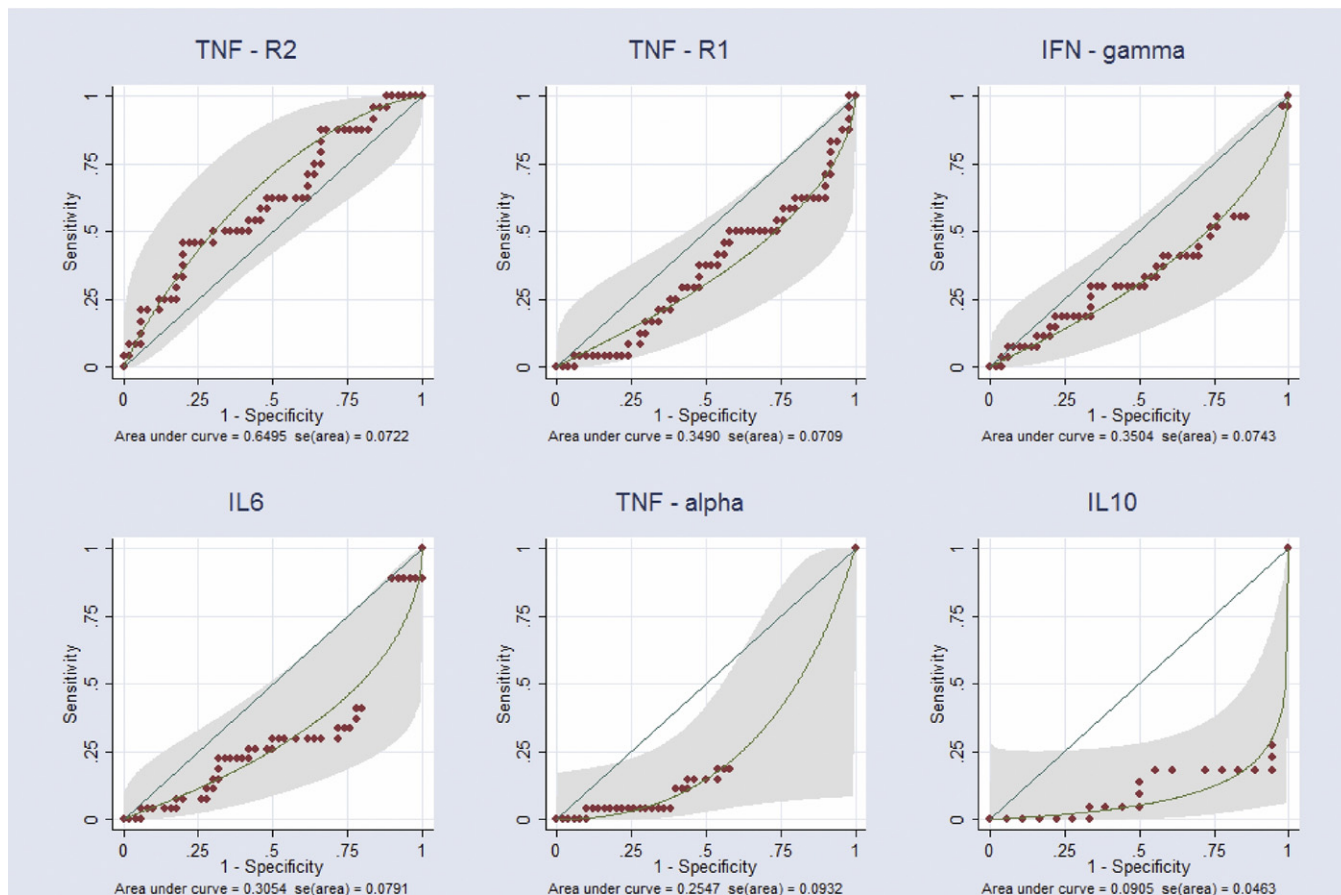
AUC, area under the curve; CI, confidence interval; IFN, interferon; IL, interleukin; ROC, receiver operator characteristic; TNF, tumor necrosis factor.

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tion because of the disruption of the maternofetal interface. Bleeding in the subchorionic space can result in elevated bioavailability of free iron, catalyzing the generation of hydroxyl radicals and subsequent free-radical damage to the surrounding tissues. The oxidative stress and focal rise in oxygenation may also stimulate the synthesis of various trophoblastic proteins such as hCG and estrogens.

We found that all cytokines that were investigated in our study, both proinflammatory (TNF $\alpha$  and IFN $\gamma$ ) and anti-inflammatory (IL-10 and IL-6) and TNF-R1, were significantly higher in the circulation of women with TM who had a normal pregnancy outcome than in those women who subsequently miscarried. Intracellular TNF $\alpha$  levels after LPS stimulation were also higher in TM with a normal pregnancy outcome. This is comparable with the findings of another study that shows that TNF $\alpha$  production in women with a history of recurrent miscarriage who subsequently miscarry is significantly lower than in normal pregnant women with successful outcome.<sup>42</sup> It has been proposed that TNF $\alpha$  is essential in preventing the birth of offspring with structural anomalies<sup>43</sup> and protects embryos who are exposed to developmental toxins.<sup>44</sup> Therefore, the rise in TNF $\alpha$  and other cytokines could be secondary to the insult caused by the pre-

**FIGURE 4**  
Receiver operator characteristics (ROC) curves for each cytokine and receptor



Tumor necrosis factor (*TNF*)-R2 was shown to be the biomarker that best predicts miscarriage, with an area under the curve of 0.65 (95% confidence interval, 0.47-0.75). A cutoff level of 2202 pg/mL of *TNF*-R2 gave a sensitivity of 63%, a specificity of 62%, and a positive predictive value of 44% for the prediction of miscarriage.

*IFN*, interferon; *IL*, interleukin.

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mature entry of oxygenated maternal blood in the developing placenta in TM. If the offspring is otherwise normal, elevated  $TNF\alpha$  levels, in fact, might have a protective effect to dampen the harm that is caused by oxidative stress, thus allowing the TM to proceed to a normal outcome.  $IFN\gamma$  production by peripheral lymphocytes in very early pregnancy is higher in normal women compared with women with recurrent miscarriage.<sup>42</sup> By contrast, IL-10 is known to be an antiinflammatory cytokine,<sup>45</sup> and as shown in our study and others,<sup>46,47</sup> high levels of IL-10 are associated with successful outcome.

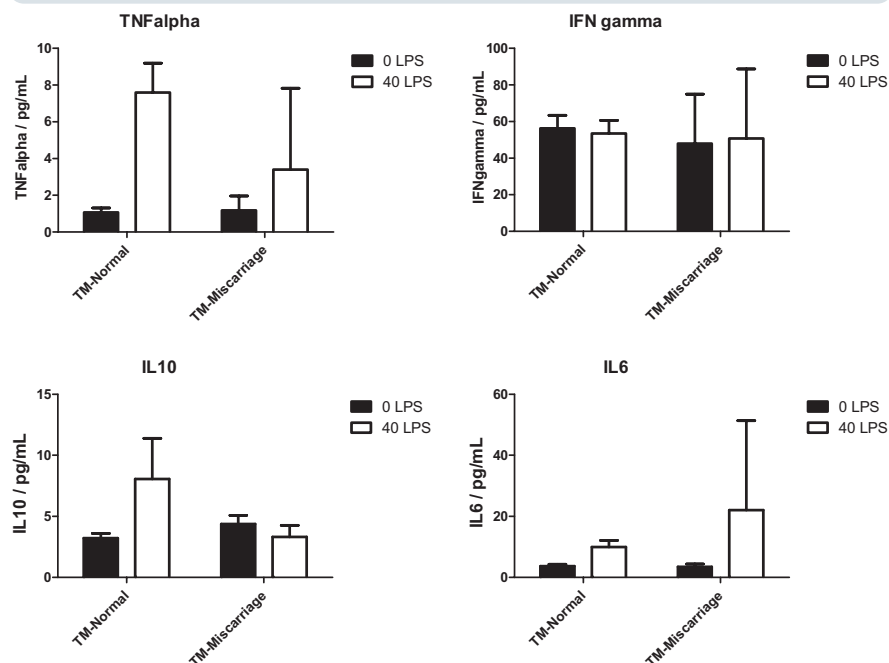
We also found that, when the ratios of Th1/Th2 cytokines were calculated,

there was a shift towards Th1 cytokines with elevated  $TNF\alpha/IL-10$  and  $IFN\gamma/IL-10$  ratios in patients with TM who subsequently miscarried. This suggests that, if there is a rise in proinflammatory cytokines in women with TM, there is an even bigger rise in the antiinflammatory cytokines to counteract the harmful effect of proinflammatory cytokines such as  $TNF\alpha$  and  $IFN\gamma$ .  $TNF\alpha/IL-10$  and  $IFN\gamma/IL-10$  ratios are more representative of the Th1/Th2 balance, rather than the absolute individual cytokine levels. If there is an overall imbalance with a predominance of Th1 cytokines, then TM ends up in miscarriage. This pattern is comparable with that found in women with recurrent miscarriages; the Th1/

Th2 cytokine balance of women with TM who proceed to normal pregnancy outcome is similar to women with successful pregnancy outcome.<sup>48-55</sup>

Our data also show that the  $TNF\alpha/IL-6$  ratio, but not the  $IFN\gamma/IL-6$  ratio, was also significantly higher in women with subsequent miscarriage. This could be explained by the fact that IL-6 has both pro- and antiinflammatory properties. The classification of IL-6 as a Th1-type cytokine in studies on preterm labor<sup>56,57</sup> or as a Th2 type cytokine in early pregnancy<sup>58-61</sup> remains controversial. Recently, it has been demonstrated that IL-6 is involved in the promotion of Th2 differentiation and the inhibition of Th1 polarization.<sup>62</sup> During the first trimester-

**FIGURE 5**  
Cytokines in monocytes in whole blood



There is a significantly higher tumor necrosis factor (TNF)- $\alpha$  elevation on lipopolysaccharide (LPS) stimulation in patients with threatened miscarriage (TM) with normal pregnancy outcome ( $P < .05$ ).

IFN, interferon; IL, interleukin; pg/mL, picograms per milliliter.

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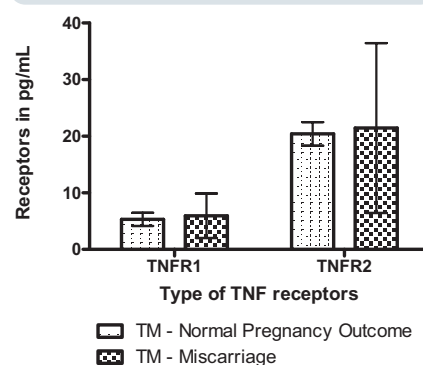
ter, IL-6 contributes to tissue remodeling that is associated with placentation, the hematopoiesis function of the secondary yolk sac, and the generation of new vessels in placental villi.<sup>36</sup> One possible mechanism of IL-6 in preventing miscarriage is modulation of the quality of the Th2 response by increasing the proportion of blocking asymmetric antibodies during implantation and placental vascularization.<sup>62</sup>

Although the sensitivity and specificity of the cytokines/receptors in our study are not accurate enough for treatment decisions, a combination of cytokines together with other biomarkers in the future may prove to be useful clinically. Previous studies have shown that TNF-receptors are useful in first-trimester screening of women who are at subsequent risk of preeclampsia.<sup>63,64</sup> By contrast, elevated levels of TNF-R1 and TNF-R2 have been described as markers of normal human pregnancy, although a lack of soluble TNF-receptors have been associated with spontaneous miscarriage in women who undergo recurrent mis-

carriage.<sup>65</sup> In our study, there was significantly higher TNF-R1 levels in the plasma of patients with TM who had a normal pregnancy. Circulating TNF-receptor levels reflect TNF $\alpha$  activity; thus, elevated plasma levels of TNF $\alpha$  can be explained by increased levels of TNF-receptors.

The novelty of this study is that blood sampling has been carried out in patients with TM at the time of presentation with a viable fetus who then were followed prospectively. Most other studies include women who had already miscarried.<sup>66-68</sup> In contrast to what previous investigators have carried out, where experiments were done on cultured peripheral blood mononuclear cells,<sup>69,70</sup> fluorescent antibody-labeled whole blood was analyzed by flow cytometry in this case. In view of the patient groups that were recruited (pregnant women who were experiencing vaginal bleeding in the first trimester), we aimed to sample smaller volumes of blood (1 mL) for the entire experiment (100  $\mu$ L/cytokine or receptor), compared with a minimum of

**FIGURE 6**  
Median levels of TNF receptors in monocytes at basal (unstimulated) levels



There were no significant differences between threatened miscarriage (TM) with normal pregnancy outcome and miscarriage.

pg/mL, picograms per milliliter; TNF, tumor necrosis factor.

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50 mL in the peripheral blood mononuclear cell culture experiments. Also, whole blood analysis with incubation, as carried out in our study, with the use of a water bath at 37°C in atmospheric conditions, rather than in a CO<sub>2</sub> incubator, mimics physiologic conditions and reflects the in vivo scenario, compared with experiments on isolated peripheral blood mononuclear cells. This method has been used successfully by other groups as well.<sup>71-73</sup>

The limitation of this study was that inevitably there were fewer women who were recruited whose threatened pregnancy ended up in miscarriage, especially in the flow cytometry subgroup. Epidemiologic studies have shown that the miscarriage rate in women with TM is up to 14%.<sup>74</sup> The other drawback of this study was that we did not have data on the karyotype of the products of conception of the patients who eventually miscarried. The reason for this is that most patients either had a complete miscarriage or were treated expectantly; therefore, it was not possible to carry out karyotyping. The reason that karyotyping would have been useful is that it is known that up to 50% of miscarriages occur because of a chromosomal abnormality.<sup>75,76</sup> Also, it has been shown that

patients with a history of miscarriage and decreased circulating levels of TNF $\alpha$  had a significantly higher chance of having a subsequent miscarriage with normal karyotype.<sup>77</sup>

The immune response towards elevated Th1 cytokines that has been demonstrated in our study in patients with TM who subsequently miscarry is similar to that described in preterm delivery. The onset of preterm labor is associated with increased proinflammatory cytokines such as TNF $\alpha$ .<sup>78</sup> Progesterone treatment reduces the rates of preterm delivery in singleton pregnancies with short cervical length<sup>79</sup> and in women with a history of preterm birth<sup>80</sup> by preventing TNF $\alpha$ -induced apoptosis of fetal membranes.<sup>81,82</sup> Future clinical trials and in vitro studies should be carried out to confirm the effect of progesterone in reversing the imbalance of Th1/Th2 in women with TM. ■

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