

## Increased Plasma Levels of Platelet Factor 4 and $\beta$ -thromboglobulin in Women with Recurrent Pregnancy Loss

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Thrombosis in decidual vessels is one of the mechanisms of pregnancy loss. However, few studies have assessed the relation between platelet activation, which is known to cause of thrombosis, and recurrent pregnancy loss (RPL). We investigated platelet activation in women with RPL compared to controls by measuring plasma levels of platelet factor 4 (PF4) and  $\beta$ -thromboglobulin ( $\beta$ TG), and assessed correlations between PF4/ $\beta$ TG and coagulative risk factors associated with RPL. The study group included 135 women who had experienced two or more consecutive pregnancy losses. The control group included 28 age-matched healthy women who had never experienced pregnancy loss. PF4 and  $\beta$ TG plasma levels were significantly higher in the women with RPL than controls (PF4: 14.0 [8.0-20.0] vs. 9.0 [6.0-12.0] ng/ml,  $p=0.043$ ;  $\beta$ TG: 42.0 [24.3-59.8] vs. 31.5 [26.6-36.4] ng/ml,  $p=0.002$ ). There was a significant association between  $\beta$ TG and anti-phosphatidylethanolamine antibody immunoglobulin M (aPE IgM) ( $p=0.048$ ). Among the women with RPL, 18 of those who were positive for PF4 (45%) and 18 of those who were positive for  $\beta$ TG (37%) were negative for all known coagulative risk factors associated with RPL. Measurements of PF4 and  $\beta$ TG may be important because they help identify women who are at risk of RPL.

**Key words:** recurrent pregnancy loss, platelet factor 4,  $\beta$ -thromboglobulin, platelet activation

Spontaneous pregnancy loss occurs in approximately 15-25% of pregnancies [1]. Recurrent pregnancy loss (RPL) is a disorder defined by two or more failed clinical pregnancies [1]. Fewer than 5% of women experience 2 consecutive miscarriages, and only 1% experience 3 or more [1, 2]. The Practice Committee of the American Society for Reproductive Medicine recommends that evaluation of RPL should proceed after 2 consecutive clinical pregnancy losses [1]. Potential causes of RPL include parental chromosomal abnormalities, untreated hypothyroidism, uncontrolled

diabetes mellitus, certain uterine anatomic abnormalities, antiphospholipid antibody syndrome (APS) [3], heritable and/or acquired thrombophilias, immunologic abnormalities, infections, and environmental factors; however, in 50% to 75% of women with RPL, no causative factors are identified [1, 3].

The association between acquired/inherited thrombophilias and pregnancy loss has been well established [1, 3, 4]. APS is known to be the most frequent risk factor associated with RPL in women with acquired thrombophilias [3]. Although the underlying pathophysiological mechanisms are not well known, throm-

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bophilias have been considered to be related to thrombosis in decidual vessels or impairment of placentation through hypercoagulability and inflammation [4]. Indeed, it has long been hypothesized that women with RPL are already in a prothrombotic state before pregnancy begins [5]. Moreover, an exaggerated hemostatic response is known to lead to thrombosis in decidual vessels and placentation defects in some cases of RPL [5,6].

Two of the known thrombosis-specific proteins, platelet factor 4 (PF4) and  $\beta$ -thromboglobulin ( $\beta$ TG) are chemokines released from  $\alpha$ -granules in platelets when the platelets are activated during the release reaction induced by ADP, epinephrine, arachidonic acid, collagen, and thrombin [7]. PF4 and  $\beta$ TG are elevated in the plasma of prethrombotic and thrombotic patients, and are considered to reflect in vivo platelet activation in these patients [7]. Elevated plasma levels of PF4 and  $\beta$ TG have been reported in patients with cardiovascular and thrombotic diseases such as atherosclerosis, ischemic heart diseases and diabetes mellitus with vasculopathy [8-12], and inflammatory diseases such as rheumatic diseases, atopic dermatitis, asthma and inflammatory bowel diseases [13-17]. However, to our knowledge there have been no studies investigating PF4 and  $\beta$ TG in the setting of RPL.

The purpose of this study was to determine whether or not women with RPL were in a stage of platelet activation. We measured the plasma levels of PF4 and  $\beta$ TG in women with RPL and compared them to those in healthy women who had never experienced pregnancy losses. We also assessed whether there was any correlation between PF4/ $\beta$ TG levels and the known coagulative risk factors associated with RPL.

## Materials and Methods

This study was carried out at the Department of Obstetrics and Gynecology of Okayama University Hospital over a period of 5 years (January 2010-December 2014). The study was approved by the ethics committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital (approval no.963). Explicit or opt-out consents were collected from each participant in conformity with the decisions of the ethics committee. The study group included 135 women who had experienced two or more consecutive preg-

nancy losses (pregnancy at <12 weeks, not including biochemical pregnancy). The control group consisted of 28 age-matched multiparous healthy women who had not had a previous pregnancy loss. All subjects in both groups showed no abnormal findings in physical exams and pelvic ultrasound tests. Women were excluded from both groups if they declined enrollment, were younger than 20 years old or older than 43 years old, and/or had chronic diseases such as hypertension, diabetes mellitus, renal diseases, infectious digestive diseases, other gynecologic problems (endometriosis, evident uterine anatomic abnormality, uterine fibroids, adenomyosis, uterine polyps and cervical incompetence). Women or women whose partners had been examined for and found to have chromosomal abnormalities causative of miscarriage were also excluded. Finally, we excluded women who had a history of thrombotic diseases, smoking, or use of medications affecting platelet functions, such as hormonal treatments, aspirin, other nonsteroidal anti-inflammatory drugs and anticoagulant drugs. All subjects were examined at least 3 months after previous pregnancies.

**Measurement of PF4 and  $\beta$ TG levels.** Venous blood samples were collected by single puncture and aspiration at a regular speed using a 20-gauge needle without a tourniquet. Samples were placed immediately on ice for 15 to 30 min. Blood samples were centrifuged at 2,000 g for 30 min at 4°C. Plasma samples were obtained from directly under the supernatant by a micropipette, then stored at -80°C in a refrigerator until analysis. Plasma levels of PF4 and  $\beta$ TG were measured by enzyme immune assay (EIA) (SRL, Tokyo). The reference values of PF4 and  $\beta$ TG were defined as <20 ng/ml and <50 ng/ml, respectively, which are the normal ranges provided by the manufacturer.

**Measurement of coagulative risk factors in RPL.** We performed blood tests in the RPL group as follows. The dilute Russell viper venom test was performed to measure lupus anticoagulant (LAC) (BML, Tokyo) in RPL. The levels of anti-phospholipid antibodies (aPA), including anti-cardiolipin antibody immunoglobulin G (aCL IgG), anti-cardiolipin antibody immunoglobulin M (aCL IgM), anti-phosphatidylethanolamine antibody immunoglobulin G (aPE IgG) and anti-phosphatidylethanolamine antibody immunoglobulin M (aPE IgM), were measured in the women with RPL by enzyme-linked immunosorbent assay (ELISA) (SRL). To diagnose protein S deficiency, we measured protein S activ-

ity by enzyme immunoassay (EIA) (BML) and/or protein S antigen by EIA (SRL). We defined protein S deficiency as protein S activity and/or protein S antigen of less than 60%. Protein C activity was measured by the synthetic substrate method (LSI, Tokyo). Protein C deficiency was defined as protein C activity of less than 60%. Coagulation factors XII (FXII) were measured by the coagulation time method (SRL) to evaluate FXII deficiency. We defined FXII deficiency as a plasma FXII level of less than 60%.

**Statistical analysis.** Continuous variables are expressed as the median and interquartile range if not noted otherwise. Student's *t*-test and Mann-Whitney *U* test were used to compare differences between the groups. A chi-square test was used to indicate correlations between variables. All analyses were performed using the software package IBM SPSS Statistics, ver. 20. Significance was established at the  $p < 0.05$  level.

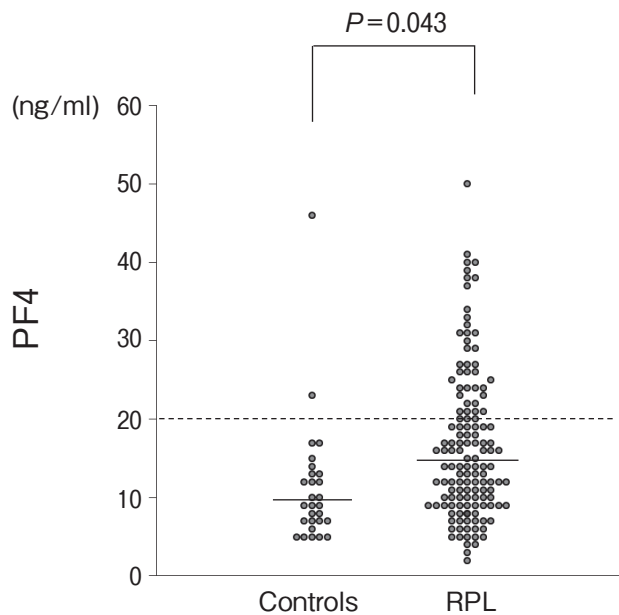
### Results

**PF4 and  $\beta$ TG in the RPL and control groups.** Characteristics and clinical findings of participants are

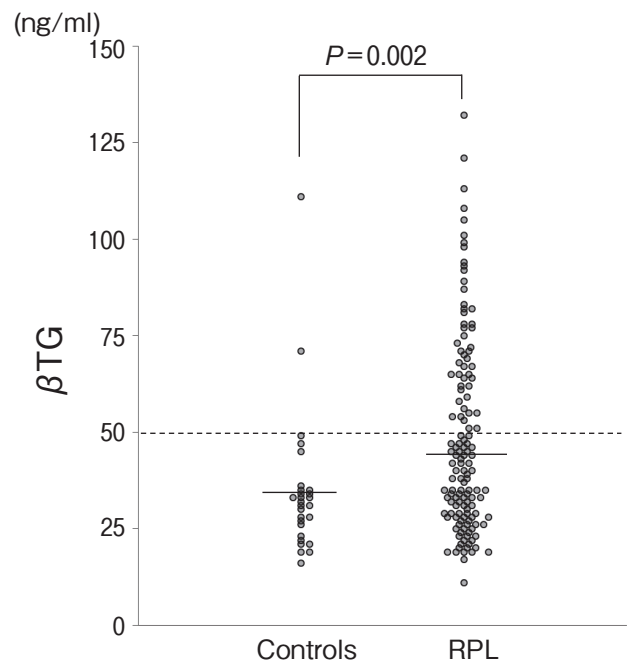
shown in Table 1. There were no statistically significant differences between subjects of the control and RPL groups in regard to age and body mass index (BMI).

Plasma levels of PF4 in the women with RPL were significantly higher than in the controls (14.0 [8.0-20.0] vs. 9.0 [6.0-12.0] ng/ml,  $p = 0.043$ ) (Fig. 1). In addition, plasma levels of  $\beta$ TG in the RPL group were significantly higher than in the controls (42.0 [24.3-59.8] vs. 31.5 [26.6-36.4] ng/ml,  $p = 0.002$ ) (Fig. 2).

**Coagulative risk factors in RPL.** The normal value of each coagulative risk factor of RPL and the frequency of positivity for each coagulative risk factor in the RPL group are shown in Table 2. Correlations between positivity for each coagulative risk factor and positivity for PF4/ $\beta$ TG are shown in Table 3. The chi-square test showed no significant correlations between positivity for PF4 and positivity for any of the risk factors. However, there was a significant association between positivity for  $\beta$ TG and positivity for aPE IgM ( $p = 0.048$ ). Eighteen of the women in the RPL group who were positive for PF4 (18/40; 45%) were negative for all coagulative risk factors. Similarly, 18 of the women in the RPL group who were positive for  $\beta$ TG



**Fig. 1** Comparison of plasma levels of PF4 between the controls and women with RPL. Plasma levels of PF4 in the RPL group were significantly higher than in controls (14.0 [8.0-20.0] vs. 9.0 [6.0-12.0] ng/ml,  $p = 0.043$ ). RPL, recurrent pregnancy loss; PF4, platelet factor 4.



**Fig. 2** Comparison of plasma levels of  $\beta$ TG between the controls and women with RPL. Plasma levels of  $\beta$ TG in the RPL group were significantly higher than in controls (42.0 [24.3-59.8] vs. 31.5 [26.6-36.4] ng/ml,  $p = 0.002$ ). RPL, recurrent pregnancy loss;  $\beta$ TG,  $\beta$ -thromboglobulin.

**Table 1** Characteristics and clinical findings of the women with RPL and controls

	Controls (n = 28)	RPL (n = 135)	P value
Age (year)	34.3 ± 5.3	34.3 ± 5.0	0.91
BMI	20.6 ± 2.5	21.0 ± 2.7	0.79
Parity (number of pregnancy losses < at 12 wks)	0	2.5 ± 0.8	

Data are presented as means ± 1 standard deviation. Student's *t*-test was used to compare differences between the groups. A *p* value of < 0.05 was accepted as statistically significant. There were no statistically significant differences between controls and the RPL group in regard to age and BMI (body mass index).

RPL, recurrent pregnancy loss; BMI, body mass index.

**Table 2** Frequency of positivity for coagulative risk factors in RPL

	Normal value	Positivity in RPL, n = 135 (%)
LAC	< 1.2	0 (0.0)
aCL IgG	< 10 U/ml	14 (10)
aCL IgM	< 8 U/ml	4 (3.0)
aPE IgG	< 0.3	22 (16)
aPE IgM	< 0.45	24 (18)
Protein S activity or antigen	> 60%	7 (5.2)
Protein C activity	> 60%	1 (0.7)
FXII	> 60%	26 (19)

The normal value of each coagulative risk factor for RPL and the frequency of positivity for each coagulative risk factor in the women with RPL are shown. RPL, recurrent pregnancy loss; LAC, lupus anticoagulant; aCL IgG, anti-cardiolipin antibody immunoglobulin G; aCL IgM, anti-cardiolipin antibody immunoglobulin M; aPE IgG, anti-phosphatidylethanolamine antibody immunoglobulin G; aPE IgM, anti-phosphatidylethanolamine antibody immunoglobulin M; FXII, coagulation factors XII.

**Table 3** Correlations between coagulative risk factors and PF4/ $\beta$ TG in RPL

Risk factors	PF4 positivity, n (%)	P value	$\beta$ TG positivity, n (%)	P value
LAC	0 (0.0)		0 (0.0)	
aCL IgG	3 (7.5)	0.50	4 (8.2)	0.49
aCL IgM	2 (5.0)	0.38	2 (4.1)	0.58
aPE IgG	4 (10)	0.20	4 (8.2)	0.053
aPE IgM	11 (28)	0.059	13 (27)	0.048**
Protein S deficiency	2 (5.0)	0.93	2 (4.1)	0.74
Protein C deficiency	0 (0.0)	0.54	0 (0.0)	0.47
FXII deficiency	7 (18)	0.79	12 (25)	0.22
All negatives	18 (45)		18 (37)	
Total	40		49	

Correlations between positivity for coagulative risk factors and positivity for PF4/ $\beta$ TG are shown.

A chi-square test was used to indicate correlations between variables. There were no significant correlations between positivity for PF4 and positivity for all risk factors. There were no significant correlations between positivity for  $\beta$ TG and positivity for each factor; LAC, aCL IgG, aCL IgM, aPE IgG, Protein S deficiency, Protein C deficiency and FXII deficiency. There was a significant association between positivity for  $\beta$ TG and positivity for aPE IgM ( $p = 0.048$ ). Eighteen of the PF4-positive women with RPL (45%) were negative all risk factors. Eighteen of the  $\beta$ TG-positive women with RPL (37%) were negative for all risk factors.

PF4, platelet factor 4;  $\beta$ TG,  $\beta$ -thromboglobulin; RPL, recurrent pregnancy loss; LAC, lupus anticoagulant; aCL IgG, anti-cardiolipin antibody immunoglobulin G; aCL IgM, anti-cardiolipin antibody immunoglobulin M; aPE IgG, anti-phosphatidylethanolamine antibody immunoglobulin G; aPE IgM, anti-phosphatidylethanolamine antibody immunoglobulin M; FXII, coagulation factors XII.

PF4 positivity: PF4  $\geq$  20 ng/ml;  $\beta$ TG positivity:  $\beta$ TG  $\geq$  50 ng/ml.

(18/49, 37%) were also negative for all coagulative risk factors (Table 3).

## Discussion

The present study demonstrated that plasma levels of PF4 and  $\beta$ TG were higher in women with RPL than in women who had never experienced pregnancy losses. Another interesting finding is that there were some women with RPL who were positive for plasma PF4 and/or  $\beta$ TG but did not show any increase in the levels of known coagulating risk factors for RPL.

Increased levels of PF4 and  $\beta$ TG reflect increased platelet activation *in vivo*. Platelets are activated through several receptor-mediated pathways [8]. In the denuded area of the vascular endothelium, collagen stimulates platelet activation and adhesion to the vessel wall [8]. Activated platelets extrude granule contents, including PF4,  $\beta$ TG, adenosine diphosphate, calcium, serotonin and so on, and induce irreversible platelet-platelet aggregation and thrombus formation [8]. Many studies have shown that increased plasma levels of PF4 and  $\beta$ TG in patients with diseases related to vascular endothelial dysfunction such as atherosclerosis, coronary artery, cerebrovascular and peripheral vascular diseases, and diabetic vascular disease [7-12]. Elevated PF4 and  $\beta$ TG have also been reported in patients with inflammatory diseases such as rheumatic arthritis, atopic dermatitis, asthma and inflammatory bowel diseases [13-17]. Platelets are now recognized as key players in inflammatory and innate immune responses [18]. Chemokines such as PF4 and  $\beta$ TG derived from platelets may play an important role in inflammation by attracting and stimulating leukocytes [19]. These chemokines regulate leukocyte movement, migration, and other proinflammatory functions such as phagocytosis and generation of reactive oxygen species [18].

With respect to obstetrics, PF4 and  $\beta$ TG levels were reported to be significantly higher in women with pre-eclampsia than women with uncomplicated pregnancy [20,21]. However, there has been no prior report of increases in PF4 and  $\beta$ TG in women with RPL, and our present results thus suggest the importance of measurement of PF4 and  $\beta$ TG even in the absence of coagulative risk factors. It is known that the hemostatic balance changes in the direction of hypercoagulability during pregnancy [22,23]. Thrombosis in the decidual vessels is believed to be one possible cause of recurrent preg-

nancy loss [24]. A previous study showed that patients with RPL exhibited greater platelet aggregation in response to arachidonic acid than controls [5]. Other studies have demonstrated increased levels of thrombin-antithrombin complexes [6], and increased endothelial microparticles in women with a history of RPL, suggesting that endothelial damage or activation might be associated with the pathogenesis of pregnancy loss, and the prothrombotic state of thromboelastography in RPL [24,25]. These studies support our hypothesis that some women with RPL might be in a chronic subclinical hypercoagulable status before pregnancy and platelet activation may pose a risk for thrombosis in decidual vessels.

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is related to platelet activation [26], induces secretion of PF4 [27,28], and plays an important role for maintenance of pregnancy [28]. Eser *et al.* reported that PAF levels were elevated in patients with RPL [29]. In their study, levels of serum PAF were significantly higher in women with recurrent miscarriage, although they did not evaluate the direct association between PAF and the platelet activation [29]. Some of the women with RPL with an increased level of PF4/ $\beta$ TG had positive coagulative risk factors, indicating that platelet activation plays an important role in the process of miscarriage, leading to RPL.

Among the coagulative risk factors analyzed in our study, only the aPE IgM level was elevated in women with RPL who had an increased level of  $\beta$ TG, suggesting that aPE IgM might be one of the factors causing platelet activation in RPL. Some studies have reported that aPE is significantly associated with fetal loss and/or thrombosis, and that aPE may be responsible for clinical features that are highly suggestive of APS [30,31]. aPE IgM is found more frequently in women with unexplained RPL compared to women who have experienced only normal pregnancy or those with explained RPL [30-32]. Some studies supported the role of aPE is to maintain placental homeostasis [33]. However, well-controlled prospective studies will be needed to elucidate the relationship between aPE and thrombotic diseases [31]. A previous study in which platelet aggregation induced by  $\gamma$ -thrombin was measured with a laser light scattering aggregometer reported that platelet aggregation was caused by aPE in women with RPL [34]. Together with the findings of the present study, these facts suggest that aPE IgM could be one of the risk

factors for RPL through platelet activation.

Our study did not show any significant correlations between PF4/ $\beta$ TG and any of the coagulative risk factors of RPL except aPE IgM. LAC, aCL IgG and aCL IgM are well known to be diagnostic of APS and to be risk factors for RPL [35]. The pathogenic mechanisms of aCL on negatively charged phospholipids located on the outer leaflet of the plasma membrane include blood coagulation or thrombosis and direct effects against trophoblasts [36].

Protein C deficiency and protein S deficiency result in loss of inactivation against activated factor V (aFV) [35]. None of the coagulative risk factors except aPE was correlated with platelet activation in this study, and it may be possible that pregnancy losses are caused by pathways other than platelet activation.

FXII plays a key role in coagulation and fibrinolytic processes [36]. FXII deficiency results in suppression of the fibrinolytic system and thereby causes thrombosis [37]. In previous studies, an association of FXII deficiency with RPL has been reported [36,38,39]. Our study showed that subjects with lower levels of FXII tended to have higher rates of positivity for PF4/ $\beta$ TG. However, this association did not reach the level of statistical significance. Studies employing larger numbers of subjects may be necessary in order to clarify the potential association between FXII and PF4/ $\beta$ TG.

Our study showed that 18 subjects who were positive for either PF4 or  $\beta$ TG were negative for coagulative risk factors of RPL. This finding indicates that there might be other unknown factors that cause platelet activation in RPL.

In RPL, an important question is whether women with RPL are in the platelet activation stage before and/or during pregnancy. If RPL patients are in a platelet-activated stage before pregnancy, they may be indicated for prophylactic anticoagulant therapy to avoid spontaneous abortions at next pregnancy. Aspirin inhibits platelet aggregation, and improves the levels of PF4 and  $\beta$ TG in many occlusive vascular diseases [7,40]. In obstetrics, aspirin is considered to reduce placental coagulation as a platelet inhibitor and to contribute to successful placental development [4]. Moreover, aspirin is known to help improve uterine perfusion [41]. Two randomized controlled studies showed that there were no differences in live birth rates and pregnancy complications among anticoagulant treatments (*i.e.*, enoxaparin, aspirin, or both) [42,43]. These studies

found that both the aspirin and enoxaparin treatments were associated with a high live birth rate and few late pregnancy complications respectively. However, the effectiveness of prophylactic antithrombotic therapy for unexplained RPL has been a subject of controversy [44]. A recent trial failed to support any role of aspirin in unexplained RPL [45]. However, in regard to the above findings, it should be noted that the unexplained RPL groups could have included both patients with unknown coagulative factors and patients who do not have any coagulative risk factors. If women with RPL are in the platelet-activated stage, prophylactic antithrombotic therapies could be one of the options to avoid pregnancy losses. If we could identify increased platelet activation, it might guide therapy in individual patients. Thus, measurements of PF4 and  $\beta$ TG would be useful to determine whether antithrombotic therapy is necessary in cases of unexplained RPL. Also, measurements of PF4/ $\beta$ TG are more convenient and less expensive than measurements of antiphospholipid antibodies and other coagulative risk factors of RPL. Since there were a considerable number of women with RPL who showed negative coagulative risk factors of RPL in our study, it may be important to assess platelet activation in RPL.

Elevated PF4 and  $\beta$ TG were observed in several women of the control group. We consider that healthy women also could show platelet activation, because PF4 and  $\beta$ TG are elevated by subclinical damage of the vascular endothelium [8]. In our study, PF4 and  $\beta$ TG were elevated when blood samples were collected in appropriately. Thus PF4 and  $\beta$ TG may be sensitive markers of prethrombotic states and thrombosis.

Several limitations must be considered when interpreting our findings. First, our study may not have had a sufficient sample size to detect significant correlations between PF4/ $\beta$ TG and coagulative risk factors of RPL. Second, we could not investigate coagulative risk factors in the controls, and therefore our study could not demonstrate the clinical meanings and risks of the coagulative risk factors in this study. Third, we did not measure the levels of PF4 and  $\beta$ TG after the antithrombotic therapy, observe the obstetric prognosis of subjects or verify the effectiveness of antithrombotic therapy in this study; further investigation of these issues in randomized controlled trials will be needed.

In conclusion, this is the first study to demonstrate higher plasma levels of PF4 and  $\beta$ TG in women with

RPL compared to those without. In addition, our study found that elevated levels of PF4 and  $\beta$ TG were significantly correlated with the presence of aPE IgM. Also, there were some women with RPL who were positive only for plasma PF4 and/or  $\beta$ TG, and not for any of the other known coagulating risk factors for RPL. Thus there may be other factors related to platelet activation and leading to miscarriage. Therefore, measurements of PF4 and  $\beta$ TG be important because they help identify women who are at risk of RPL. Further studies are necessary to investigate unknown factors that contribute to platelet activation and thereby to RPL.

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