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## **A Role for Platelets in Normal Pregnancy**

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### **1. Introduction**

#### **1.1. Haemostasis in pregnancy**

Normal haemostasis is a complex equilibrium involving a balance between blood-borne pro-coagulant proteins, the natural anticoagulation system and the fibrinolytic system. Normal human pregnancy is associated with profound alterations to the haemostatic balance wherein the pro-coagulant effects become dominant. A marked progressive increase in the concentration of pro-coagulant proteins (Factors VII, VIII, X, Von Willebrand factor, Fibrinogen) is observed in blood plasma at all stages of pregnancy. These changes coincide with decreases in natural anticoagulants and lower levels of natural fibrinolytic agents and are more pronounced in the uteroplacental circulation than in the systemic circulation during pregnancy [7]. In parallel, markers of platelet activation are markedly elevated throughout normal pregnancy suggestive of a functional role for platelet activation during normal gestation.

It is often argued that this altered haemostatic status is required as the maternal coagulation system prepares for the challenges of parturition, and aims to minimize intrapartum blood loss. However, the alterations in the haemostatic system begin as early as the first trimester, suggesting a requirement for such changes in the proper progression of the early stages of pregnancy, in addition to their role in regulation of post-partum bleeding. For example, alterations in haemostasis enable the necessary changes in the uteroplacental vasculature to support the establishment of the trophoblast invasion of the spiral arteries of the uterus early in gestation [12].

The altered haemostatic status during normal pregnancy presents a number of physiological challenges in the vasculature and results in an increased risk of excessive thrombosis, especially within the uteroplacental circulation. This enhanced pregnancy-associated thrombotic risk may provide the mechanistic basis for many of the major pregnancy complications, such as pre-eclampsia, HELLP syndrome (Hemolysis, Elevated Liver enzymes and Low Platelet count) and intrauterine growth retardation (IUGR) [13].

Pre-eclampsia (PE) is a multifaceted disorder that complicates between 2 and 8% of all pregnancies. It is the most common causes of morbidity and mortality in mothers and babies in the Western world [14]. Clinical signs of disease, such as an elevated maternal blood pressure and proteinuria, become apparent as early as gestation week-20 [9]. Such symptoms can dictate urgent delivery of a preterm baby (<40 weeks). The underlying pathology appears to be a complex interaction of the placental and maternal tissues [15] that leads to generalized endothelial dysfunction. This heightens the normal shift of haemostatic equilibrium toward hypercoagulability. As part of this generalized hypercoagulability status in pregnancy, evidence for enhanced platelet activation, observed in normotensive pregnancies [18], are further increased in women with preeclampsia giving rise to the formation of platelet-derived microthrombi in smaller vessels [20] and an associated disseminated intravascular coagulation [21]. Similar to changes in the coagulation parameters, changes in platelet activation status are recorded before gestational week 13 in PE. Intrauterine growth retardation is often associated with PE, and is correlated with reduced uteroplacental blood flow caused by platelet-rich microthrombi.

## 2. Changes in platelet parameters during pregnancy

The normal blood platelet count in healthy non-pregnant individuals varies between  $150\text{--}400 \times 10^9/\text{L}$ . Early studies into platelet parameters in normotensive pregnant women reported a progressive drop in the normal platelet count [22]. Indeed mild, non clinical, thrombocytopenia (platelets  $100\text{--}150 \times 10^9/\text{L}$ ), is observed in up to 10% of all pregnancies [6]. It is likely that this is largely a haemodilution effect that results from the maternal blood volume expansion [23]. The decrease in platelet count is accompanied by an increase in mean platelet volume [24] and a notable change in the granule content [25].

Changes in platelet function, and platelet activation status, have been widely reported during pregnancy. However, measurement of platelet activation is complex. There is no one accepted index of platelet activation, although a number of tests and assays are used as surrogate markers of platelet activation in studies on gestational platelet activation. Usually a blood sample is collected from the patient and mixed with an appropriate anticoagulant before being transported to a laboratory for analysis. Platelets are assayed either in whole blood samples or are processed to remove red blood cells and plasma to yield platelet-rich plasma (PRP) or washed platelets (WP), respectively. Thereafter standard or high throughput assays are utilized as appropriate. Platelet aggregation assays are used frequently (Light Transmission Aggregometry; LTA) and can be performed in PRP or WPs [27]. Alternatively high throughput assays of platelet function can be used to assess multiple samples, or multiple agonists, simultaneously. The Moran group have recently developed a high throughput assay of platelet dense-granule secretion to permit extensive assessment of a dose-range of agonists on a relatively small blood sample. In addition, mobile diagnostic analysers of platelet function, such as the PFA-100, have been developed to assess the acute capacity of platelets to form thrombotic aggregates and can be performed in whole blood [31].

In addition to standard assays of platelet function, platelet activation may be inferred, from the presence of activated cell adhesion molecules on the surface of isolated platelets. Such

markers include the activated platelet integrin  $\alpha\text{IIb}\beta 3$  (assessed by the monoclonal antibody PAC-1 or fibrinogen-binding) or the enhanced surface expression of the  $\alpha$ -granule protein P-Selectin (CD62P) or dense-granule derived protein, CD63 on the surface of circulating platelets.

Platelet granules are rich sources of bioactive agents that are selectively released in response to diverse platelet activating stimuli. Thus, evidence of the presence of secreted platelet-derived bioactive agents in plasma or urine can be used to determine if platelet activation has occurred *in vivo*. Typically, elevated levels of plasma  $\beta$ -thromboglobulin ( $\beta$ TG) and Platelet Factor-4 (PF4) are an indication that platelet release of the contents of platelet  $\alpha$ -granules has occurred [36]. Similarly, plasma adenosine or ATP levels reflect release from dense granules. Finally, elevated concentrations of plasma or urinary 11-dehydro Thromboxane  $B_2$ , a stable metabolite of platelet-generated Thromboxane  $A_2$ , reflect recent platelet activation. One concern with the use of secreted proteins as markers of platelet activation is the fact that, once released from platelets, biomolecules will have variable half-lives and sensitivity to storage conditions [39], making it difficult to determine how recently the sampled platelets were activated. The use of thromboxane as a marker is also complicated, as this eicosanoid, previously presumed to be only synthesized in platelets, can also be produced by fetal and maternal macrophages in the uteroplacental unit [40].

Proteins that are shed from the surface of activated platelets are also used as surrogate markers of platelet activation. These include soluble P-Selectin (CD62-P) and soluble CD40 Ligand (CD154). Activated platelets also release microparticles (MPs) [43]. However, MPs found in blood may originate from a number of different sources in addition to platelet  $\alpha$ -granules. Hence they have not been widely used in assessments of platelet function in pregnancy.

Basal levels of P-Selectin and  $\alpha\text{IIb}\beta 3$  are progressively elevated on the surface of platelets during gestation, suggesting that an inherent activation of platelets occurs during normal pregnancy. In addition, the capacity of platelets to aggregate [31] and adhere [32] in response to various stimuli is enhanced in normotensive pregnant women but is somewhat reduced in platelets from women with preeclampsia [45]. Enhanced fibrinogen binding to circulating platelets, indicative of platelet activation, is also observed in pregnant women compared to non-pregnant subjects [34]. Plasma levels of  $\beta$ TG and PF4, secreted from platelet  $\alpha$ -granules, and ADP/ adenosine, secreted from platelet dense granules are elevated during pregnancy.

Many indices of platelet activation have been shown to correlate with gestational age [32], though some, such as platelet responsiveness to agonist stimulation, peak at weeks 30-36 and decline thereafter (Table 1). Only a few studies have assessed platelet function at multiple time points throughout pregnancy and so, information is limited regarding the absolute indices of platelet activation and their temporal relationship to gestational events. However, in general it can be concluded that platelets are hyperactivated from as early as gestational week 10, in the first trimester of pregnancy.

### 3. Changes in platelet parameters in preeclampsia

The gestational changes in haemostatic and platelet responses are altered in women with PE. Accordingly,  $\beta$ -TG and PF4 are elevated in PE above levels observed in normotensive preg-

nancy. CD63 and P-Selectin levels are increased on the surface of platelets from patients with PE, indicative of recent secretion of dense and  $\alpha$ -granules, respectively. However, the lack of elevated plasma levels of adenosine or ATP suggest that the secretory events were not acute [26]. The half-life of adenosine is less than 1 minute in plasma in contrast to  $\beta$ -TG, which has a half-life > 120 minutes. Thus, the data suggests that a slow sustained secretion from platelet granules occurs. This is distinct from the pattern that is expected following a thrombotic event where markers of dense granules and  $\alpha$ -granule release occur acutely and simultaneously. Furthermore, the capacity of platelets from PE patients to acutely aggregate *ex vivo* appears to be attenuated compared to normotensive subjects. This is indeed consistent with the evidence, reported by Janes & Goodall [34] that although activated, degranulated platelets are observed in the circulation in PE, and no evidence for platelet thrombi or platelet aggregates are observed. Together this data shows strong evidence for widespread and sustained platelet secretion in PE. Although platelet thrombi are not observed, activated circulating platelets are observed that contain bound fibrinogen[20]; a strong indication of a thrombotic tendency.

Marker of platelet activation	Normotensive pregnancy	Preeclampsia
Platelet Aggregation (LTA; PFA-100)	Responsiveness is greater in normotensive pregnant women than in non pregnant . Platelet hyperaggragation peaks at weeks 20 & 30; declines at week 36	Platelet aggregation is decreased in both mildly and severely preeclamptic women in comparison with non-pregnant women . Increased numbers of platelets binding to fibrinogen are observed [20].
Surface expression of CD62P on Platelets	Increases with gestational age	Evidence of activated, degranulated platelets in the circulation [34]. CD62P expression is accentuated in PIH<PE<severe PE
Surface expression of CD63 on Platelets	elevated in normotensive women	Further elevated in women with non-proteinuric and proteinuric pre-eclampsia [34]. Increased first-trimester CD63 expression is an independent risk factor for development of preeclampsia
ATP Secretion	Increases with gestational age [26]. Adenosine in plasma is elevated [46]	Significantly lower in PE than in normotensive pregnant subjects [26]
Thromboxane metabolites in plasma or Urine	Increased in normotensive pregnancies . Elevated at 20 weeks and continues to increase thereafter	An imbalance between the production of two metabolites of arachidonic acid, thromboxane and prostacyclin, that favors thromboxane. [53] Greater enhancement of TxA <sub>2</sub> production in PIH than in normotensive pregnancies
Plaasma levels of $\beta$ -Tg/ PF4 (secreted from platelet $\alpha$ -granules)	Elevated in normotensive pregnancy; Peaks at gestational weeks 30, & 36	Greater than in normotensive pregnancy
Soluble CD62P in plasma	Elevated by 24 weeks	No difference vs normotensive pregnancy

**Table 1.** Please add caption



Other haemostatic changes that occur in systemic and uteroplacental circulations during normotensive pregnancies appear to be accentuated in patients with preeclampsia [7]. For example, thromboxane A<sub>2</sub> biosynthesis is increased in PE above that observed in normotensive pregnancy and appears to correlate with disease severity. It is preceded by a decreased endogenous production of the endothelial-derived eicosanoid, Prostaglandin I<sub>2</sub> (PGI<sub>2</sub>) [55]. The altered ratio of these thrombo-regulatory eicosanoids appears to play a critical pathological role in preeclampsia. In addition, blood-borne microparticles (MPs) are elevated in preeclampsia and have demonstrable adverse effects on endothelial function. Isolated MPs from preeclamptic women, though not those from normotensive pregnant women, have been shown to downregulate endothelial NO production. NO is a critical regulator of platelet activation, and a regulator of the sensitivity of vascular endothelial cells to trophic agents. By altering the endothelial response and reducing the endogenous production of NO in preeclamptic vascular beds, MPs in preeclamptic women can markedly affect platelet activation and vascular remodeling. Thus in preeclamptic women, many elements of the delicate balance governing platelet activation appear to be dysregulated, predisposing towards premature and excessive platelet secretion. It is tempting to suggest that this dysregulation underlies the pathophysiological mechanisms in PE.

It is postulated therefore that platelets are hyper-reactive in normal pregnancy, and are primed to undergo granule-release at appropriate times and vascular locations. Thus, platelet passage through the placental vasculature can cause acute platelet activation and secretory events, which result in the release of bioactive mediators from platelet  $\alpha$ -granules. The nature of these bioactive mediators and their functional roles in normal, and preeclamptic, pregnancies remain to be elucidated. Women at risk of developing preeclampsia may have an abnormality in their platelet activation or a temporal dysregulation of these events resulting in atypical vascular events. In fact, increased first-trimester CD63 expression on platelets is an independent risk factor for development of preeclampsia, confirming that early subclinical platelet defects occur in this population.

#### **4. Mechanisms of platelet activation during pregnancy**

It has been strongly suggested that platelets contribute to protective mechanisms against excessive bleeding during childbirth. Whilst this is indeed relevant, it seems premature to start such thrombotic preparations as early as gestational week 10-12. Thus, platelets must play a role into other gestational requirements. Any roles for platelets are likely to result from their activation in the circulation. However, the mechanisms underlying platelet activation in pregnancy remain largely unknown.

A number of studies have indicated that platelets are hyperresponsive during pregnancy. This may be explained by the increased gestational production of the platelet-activating prostaglandin, thromboxane A<sub>2</sub> (TXA<sub>2</sub>) by platelets. TXA<sub>2</sub> can enhance platelet responsiveness to low levels of physiological activation. Thus increases in ambient TXA<sub>2</sub> may prime platelets for activation by other agonists. Indeed platelets generate more intracellular calcium in response

to standard pro-thrombotic stimuli as pregnancy progresses. In parallel, a reduced synthesis of inhibitory cyclic AMP is observed in platelets from pregnant donors. This reduced production of platelet cAMP may be secondary to altered endothelial function during pregnancy whereby endothelial cells release less of the inhibitory regulators of platelet function (Nitric Oxide and PGI<sub>2</sub>). Overall, the sensitivity of platelets to activation is enhanced during pregnancy by a parallel increase in ambient pro-thrombotic agents, TxA<sub>2</sub> and calcium, and a decrease in anti-thrombotic influences such as intraplatelet cAMP. It is possible that such changes may be initiated by the elevated progesterone level extant during pregnancy, as it has previously been noted that cyclical changes in progesterone during the luteal phase of the ovarian cycle similarly affects platelet function and activation status. Platelets are therefore 'primed' to response to stimulation by various gestational adaptations.

The molecular mechanisms underlying the direct activators of platelets in the placental beds remain unclear, but two independent activators have been identified. Firstly, circulating primed, platelets adhere to the extracellular matrix in the uteroplacental vascular beds and are activated to release various soluble factors to regulate trophoblastic vascular infiltration and differentiation. Secondly, local generation of thrombin, and its interaction with platelet thrombin receptors (PARs) is critical for efficient gestation [62]. However, although generated locally from the plasma coagulation cascade, this thrombin does not participate in fibrin cross-linking, the usual culmination step in blood coagulation. Instead, it directly activates platelet PAR receptors and induces platelets to release their stored contents.

The capacity and sensitivity of platelets to secrete their granular contents therefore is enhanced as pregnancy progresses. Indeed, substantial evidence that this occurs is presented in Table 1. Moreover, data from the Moran laboratory confirms that alterations in platelet function are evident as early as 10-12 gestational weeks (unpublished data; MAO, NM).

## 5. Functional roles for platelet activation during normotensive pregnancy

As discussed above, the gestational role of platelets in pregnancy is likely to be mediated via the secretion of the cargo from platelet  $\alpha$ -granules. This cargo is comprised of cytokines and other bioactive agents, stored in the abundant platelet granules and includes coagulation proteins such as Fibrinogen and Factors V and XIII. Up to 800 different proteins have been identified in the platelet  $\alpha$ -granule proteome. Moreover, the contents of platelet  $\alpha$ -granules can be selectively released in response to discrete activation signals.

In normotensive pregnancy, maternal platelets adhere to the extracellular matrix in the uteroplacental vasculature and are activated in the spiral arteries. As a consequence of this activation of maternal platelets, elevated levels of platelet derived cytokines and bioactive agents are released, that assist and enable trophoblastic arterial infiltration. In addition, platelet derived biomolecules drive morphological changes of trophoblasts and enable angiogenesis in the placental beds. This enables localized physiological vascular remodeling to ensure the appropriate development of embryonic and maternal placental circulations. The precise nature of the platelet releasate in these circumstances has not been fully explored to date. However,

there is some evidence that the nature of the releasate differs between normotensive and pre-eclamptic subjects,

Thus, the platelet activation detected in pregnancy (Table 1) is an indicative record of  $\alpha$ -granule secretion events in the placental vasculature. Importantly, no overt thrombosis is observed in normotensive pregnancies, despite the localized platelet activation and secretion. The requirement for localized platelet regulation of vascular remodeling probably underlies the need for platelets to be primed at early gestational stages in normal pregnancy. The consequent elevation in markers of platelet activation, as shown in Table 1, is simply a confirmation that platelet secretion events have occurred, rather than being suggestive of a pro-thrombotic role for platelets during normal gestation.

## 6. Non-thrombotic roles of platelets in pregnancy

Platelets serve as mobile stores of active biomolecules that can be transported around the body via the vasculature. This function of platelets is well defined in cancer, where platelet stored biomolecules can be released in a bespoke manner by circulating tumour cells to enable the development of a novel, bespoke network of new blood vessels to supply a growing metastatic tumour with suitable nutrients and waste removal functions (See Chapter 6: Mitrugno et al). It is tempting to speculate therefore, that platelets store a relevant and bespoke collection of bioactive compounds during pregnancy. Thus platelet  $\alpha$ -granules serve as vectors of biological messages for vascular homeostasis during pregnancy. Similarly, platelet-derived MPs may serve to deliver bespoke cocktails of bioactive molecules to uteroplacental vascular beds to facilitate the required vascular changes to support gestation. In support of this, MPs from women with preeclampsia, compared to those from normotensive pregnant women, showed greater pro-inflammatory effects on the vascular wall, inducing vascular hyporeactivity in small blood vessels [65].

However, the key question then becomes one of how the body balances the dual needs of requiring primed platelets to release their cargo at relevant vascular beds in the dynamic, fast-flowing environment of the uteroplacental vasculature, whilst preventing inappropriate thrombosis from occurring; yet maintaining the potential to respond to thrombotic needs should they arise elsewhere in the body.

## 7. Regulation of prothrombotic responses in platelets during normotensive pregnancy

Pregnancy-specific glycoproteins (PSGs) are a family of soluble cell adhesion proteins found in the plasma at various stages of pregnancy. They are largely derived from trophoblastic cells during pregnancy and are abundantly expressed in maternal blood [66]. There are ten human PSG genes (PSG1 - PSG9, PSG11). Several recent studies have indicated that individual PSGs



have immunoregulatory, pro-angiogenic, and anti-platelet functions, though their precise functions during pregnancy remain largely speculative.

Recently Shanley et al demonstrated that PSG1 had a high affinity for binding to the major platelet integrin  $\alpha\text{IIb}\beta 3$ . It competes with fibrinogen for binding to this integrin, thereby inhibiting local platelet aggregation [27]. However, the capacity of platelets to secrete the contents of their  $\alpha$ - and dense granules is unaffected by PSG1. Thus PSG1 enables the secretory responses of circulating platelets, whilst attenuating their thrombotic tendencies.

In parallel, a role for PSG1 in the activation of the anti-inflammatory cytokine, transforming growth factor-beta (TGF) has recently been established. TGF- $\beta$  regulates many biological events essential for the successful completion of pregnancy including trophoblast invasion and proliferation, angiogenesis, and tolerance to the fetus to the fetal allograft during pregnancy. Of note, platelets serve as a major storage site for TGF- $\beta$  and release it in response to platelet-activating stimuli. Plasma concentrations of active TGF- $\beta$  are significantly higher in preeclamptic women than in normotensive pregnant women [70]. In the presence of a high concentration of PSG1, TGF- $\beta$ , released from primed platelets in the maternal uteroplacental circulation, is activated and enabled to exert its vascular remodeling effects. Yet due to the high locally-produced concentrations of PSG1, aggregation of platelets does not occur.

Thus PSG1 can simultaneously inhibit platelet aggregation and enable the release of platelet granular-contents including TGF- $\beta$ , to promote vascular remodeling. This strongly supports the contention that the role of platelet activation during pregnancy is to permit the local delivery of cytokines, via the secretion of  $\alpha$ -granule contents, rather than the more widely accepted role of inducing thrombotic events. It is likely that complications of pregnancy such as preeclampsia arise when the balance between the thrombotic function of platelets and their secretory functions is disturbed.

## 8. The 'priming' of platelets may contribute to complications of pregnancy

As discussed previously, platelets are primed for activation at early gestational stages. One of the mechanisms of platelet priming is their enhanced ability to synthesize the platelet-specific prothrombotic eicosanoid  $\text{TxA}_2$ , in an environment where opposing antithrombotic influences, namely  $\text{PGI}_2$  and NO, are downregulated. Measuring  $\text{TxA}_2$  levels during pregnancy can therefore yield important information on platelet status during gestation.

The concentrations of  $\text{TxA}_2$  observed in normotensive pregnancy are significantly raised above levels seen in normal healthy non-pregnant donors. In fact, gestational  $\text{TxA}_2$  levels equate to pathological concentrations identified in cardiovascular patients (Table 2). Yet, despite these pathological levels of circulating prothrombotic eicosanoids, there is little evidence for enhanced platelet-rich thrombotic events during normotensive pregnancy, confirming therefore, that the intended physiological purpose of the elevated platelet activation is not thrombotic in nature. Together, these studies suggest an underlying physiological balance during pregnancy to prime platelets for activation whilst regulating thrombosis.

The need to understand the physiological and molecular mechanisms underlying the enhanced gestational platelet activation is underscored by observations that platelet activation is further accentuated in patients with PE and that pathological thrombotic events can occur if the balance is disturbed.

Population	[urinary 11-dehydro TXB <sub>2</sub> ]	Reference
Non-pregnant, healthy subjects	Basal levels approx. 275 pg/mg creatinine	
Normotensive, pregnant subjects	3 fold ↑ over basal levels	
Patients with Preeclampsia	↑ ↑ 1.3 fold increase relative to normotensive pregnant subjects	
Patients with Ischemic disease	↑ donors; similar to normotensive pregnant women	

**Table 2.** Please add caption

Thus, bioactive agents are released from platelets in a controlled, systematic way during pregnancy to enable required hemostatic changes in the uteroplacental vasculature. However, platelet aggregation and thrombus formation are not required, or could be contraindicated during pregnancy, and are therefore suppressed by PSG1 [27] or similar soluble proteins.

Our limited knowledge of how platelets might contribute to complications of pregnancy evolves from our understanding of the nature of the delicate balance between platelet-activating forces during normotensive pregnancy and the need to prevent thrombosis in the uteroplacental vascular beds. Thus, under the influence of gestational progesterone, endothelial cells produce less NO and PGI<sub>2</sub> and platelets produce more TXA<sub>2</sub>. This tilts the haemostatic balance towards platelet activation and thrombosis. Exposed extracellular matrix proteins in the placental vascular beds bind and activate the primed platelets inducing secretion of their α-granule contents. Any tendency for platelets to aggregate is however balanced by the local production of high concentrations of PSG1 [27], which prevents integrin mediated platelet aggregation. In parallel, local generation of Thrombin, the procoagulant, proteolytic enzyme of the coagulation cascade, occurs in normotensive pregnancy [7]. Yet its function is not to generate fibrin, as would be expected in the coagulation cascade. Instead, thrombin in the placental beds acts directly on platelet thrombin receptors to induce platelet release from α-granules. The fibrinogenic actions of thrombin are moderated by Thrombomodulin (TM) and are essential for successful development of the placenta [62]. Interestingly, TM is also stored in platelet α-granules, comprising up to 10% of total granule stores, and is released in response to platelet activation [74]. Absence of TM causes fatal arrest of placental morphogenesis in mice, but this action is not related to its ability to affect fibrin formation, leading the authors to conclude that TM regulates the capacity of thrombin to activate platelets [62]. Moreover, the experiments of Sood and colleagues demonstrate that the delicate balance between the required functions of platelets in pregnancy and their regulation, to prevent unwanted thrombosis, can all-too-easily be shifted to cause placental failure and complications of pregnancy.

## 9. Summary

Therefore we suggest that platelets are not minor participants in the gestational events of pregnancy. Instead, they are active mediators of the complex regulatory system which has several, as yet uncharacterized mechanisms. They serve as vectors for vascular homeostasis during pregnancy, that co-ordinate a delicate balance between delivering relevant and potent biological messages through their granule-delivery system, whilst avoiding platelet-related thrombotic events.

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