



Regular Article

Platelet activation and endogenous thrombin potential in pre-eclampsia

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ABSTRACT

Introduction: Platelets and the coagulation system may be involved in the pathogenesis of pre-eclampsia. We investigated whether platelet and coagulation activation markers, are elevated in pre-eclampsia.

Materials/methods: Case-control study in which activated platelets, platelet-monocyte/ neutrophil aggregates, platelet microparticles (measured by flow cytometry) and four markers of thrombin generation capacity (endogenous thrombin potential (ETP), peak height, lag time and time to peak) using the Calibrated Automated Thrombogram system were assessed in pregnant women of similar gestational age with (n = 46) and without (n = 46) pre-eclampsia, and in healthy non-pregnant women (n = 42).

Results: The percentage of, CD62P+ platelets (p = 0.013), CD62P+ platelet microparticles (p = 0.029) and platelet-monocyte aggregates (p = 0.019) were significantly higher in women with pre-eclampsia than the pregnant controls. Both groups of pregnant women had significantly higher ETP and peak height (p < 0.001) than the healthy non pregnant group and the women with pre-eclampsia had significantly higher ETP and peak height (p < 0.001) than the normotensive pregnant controls.

Conclusion: In the most comprehensive laboratory analysis to date, we found evidence of both platelet and coagulation activation in women with pre-eclampsia.

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Introduction

Pre-eclampsia is a multisystem disorder of unknown cause that occurs during the second half of pregnancy and is characterised by an abnormal vascular response to placentation associated with increased systemic vascular resistance, enhanced platelet aggregation, activation of coagulation and endothelial cell dysfunction [1]. It is a major obstetric complication affecting 2–7% of pregnancies and causing substantial maternal and perinatal morbidity and mortality worldwide, particularly in developing countries [1]. Within the United Kingdom pre-eclampsia and eclampsia remain the second most common direct cause of maternal death [2]. The diagnostic features of pre-eclampsia include the new onset of hypertension and proteinuria from the 20th week of pregnancy onwards. Pre-eclampsia cannot be prevented so management involves screening asymptomatic women and inducing delivery when necessary. There is a need to improve the ability of clinicians to identify women at risk and also to better understand the pathogenesis of pre-eclampsia to promote the development of specific therapies for prevention and treatment [1].

In this case-control study of pregnant women with and without pre-eclampsia we focus on the haemostatic component of this condition. During normal pregnancy the haemostatic balance shifts to favour clotting over bleeding, probably as an evolutionary advantage in view of the substantial risk of the latter at delivery. Haemostasis does not fully normalise for over 4 weeks *post partum* [3].

In pre-eclampsia the haemostatic system is further activated compared to normal pregnancy with increased thrombin generation [4,5]. However, the most appropriate method to measure thrombin generation is uncertain. The endogenous thrombin potential (ETP) has been developed as one method with the advantage that it measures the composite effect of the multiple factors that determine coagulation capacity [6]. In this assay coagulation is initiated *ex vivo* with the addition of tissue factor to patient plasma and thrombin generation is continuously registered by measuring cleavage of a fluorogenic substrate. A thrombin generation curve is produced from which parameters such as peak thrombin generation and the area under the thrombin generation curve (the ETP) are derived. Such measures have been used to predict, for example, risk of venous thromboembolism [7], but there has been little study to date of this method in pre-eclampsia.

Platelets are thought to play a crucial role in the pathophysiology of pre-eclampsia by promoting vascular damage and obstruction,

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leading to tissue ischaemia and further damage [4]. The circulating platelet count is reduced reflecting a decreased platelet lifespan [8,9]. Features of platelet activation in pre-eclampsia include an increased expression of platelet surface P-selectin (CD62P) [10–13]. Activated platelets have the potential to bind to neutrophils and monocytes initially via engagement of platelet surface P-selectin with leucocyte P-selectin ligand-1 (PSGL-1). The resultant intracellular signalling causes leucocyte surface expression of tissue factor and integrin α M β 2 (CD11b/CD18) [14,15]. The activation-dependent conformational change in monocyte CD11/CD18 results in the binding of coagulation factor Xa and/or fibrinogen increasing procoagulant activities [14]. This represents one mechanism whereby platelet activation and coagulation are linked. One *in vitro* study has found increased numbers of platelet leucocyte aggregates in women with pre-eclampsia compared to normotensive pregnant women [16].

The interaction of activated platelets with activated monocytes also induces blebbing of the cell surface membrane to generate microparticles bearing tissue factor [17]. Recently several studies have shown the presence of increased levels of microparticles in pre-eclampsia [18–20].

A major problem in assessing platelet and neutrophil activation is that activation can occur rapidly during venesection, or afterwards *ex vivo* before the blood is analysed. Artefactual activation may be minimised by adequate blood collection procedures but an appropriate anticoagulant and storage temperature must also be used, so that the degrees of activation that existed *in vivo* can be preserved. In previous studies, a mixture of K₃EDTA and CTAD (E/C) has been shown to have distinct advantages for flow cytometric studies of platelet and neutrophil activation *ex vivo* in whole blood [15,21].

The aim of this study, one of the largest to compare findings in pre-eclampsia with normal pregnancy, was to provide a more comprehensive view of the prothrombotic changes in pre-eclampsia. We therefore developed a routine assay to measure thrombin generation potential (ETP) and, using flow cytometry, platelet activation, platelet–leucocyte aggregates and platelet microparticles in whole blood. We also assessed how discriminatory the observed changes were between normal pregnancy and pre-eclampsia to see whether evaluation of haemostasis in this manner might have any potential role in prediction of pre-eclampsia.

Methods

Patients and sample collection

The study was undertaken in a single obstetric unit (Royal London Hospital) in East London that provides secondary care for an inner city population with a high proportion of individuals from ethnic minorities, particularly from South Asia (predominantly Bengali). All mothers presenting during weekdays with pre-eclampsia were invited to participate. Pre-eclampsia was defined as new hypertension and proteinuria at or after 20 weeks gestation. New hypertension was defined as a blood pressure of at least 140 mm Hg (systolic) or at least 90 mm Hg (diastolic) on at least two occasions and at least 4–6 hours apart at or after the 20th week of pregnancy in women known to be normotensive beforehand. New proteinuria was defined as protein excretion ≥ 300 mg per 24 h or $\geq 1+$ on dipstick in at least 2 random urine samples taken at least 4–6 hours apart. Blood samples were taken from mothers when they presented with pre-eclampsia. Mothers of the same gestational age and from the same wards and clinics but without pre-eclampsia were invited to act as controls. Controls were selected as normal pregnancies without complications. Women with medical or obstetric complications were not included. All tests on the blood samples from the mothers were undertaken blinded. Healthy women (23–53 years) who had not taken any medication including aspirin or aspirin containing products in the previous 72 h were also

recruited from the Dental School at the Royal London Hospital as non-pregnant controls. The study was approved by the Local Research Ethics Committee and all participants gave informed consent.

Blood samples for thrombin generation were collected into 0.109M tri-sodium citrate vacutainers. Samples were centrifuged at 1500 g for 10 minutes. Following this time the top two thirds of the plasma were removed and aliquoted into LP4 tubes. These aliquots were then centrifuged for a further 10 minutes at 1500 g. The top two thirds of the double centrifuged plasma were then removed, placed in a corning tube and frozen at -70°C .

For flow cytometric analysis, whole blood samples were taken into Vacutainers (BD) containing a mixture of K₃EDTA and CTAD (E/C). For this, blood was collected first into K₃EDTA and then immediately transferred to a Vacutainer containing CTAD. Anticoagulated blood was held at 4°C until flow cytometric analysis which was always within 6 h post venesection.

Thrombin generation assay

Thrombin generation was performed on a Fluoroscan Ascent FL (Thermo Electron Corporation) using the Calibrated Automated Thrombogram® system (Thrombinoscope bv) and reagents produced by Diagnostica Stago. Thrombin generation was initiated by the addition of $20\ \mu\text{l}$ of both PPP reagent and FluCa reagent to $80\ \mu\text{l}$ patients plasma, giving final concentrations of 5pM tissue factor and $4\ \mu\text{M}$ phospholipid. Thrombin calibrator wells contained $20\ \mu\text{l}$ of thrombin calibrator and FluCa reagents and $80\ \mu\text{l}$ of patient plasma. Both calibrator and thrombin generation wells were measured in triplicate. Fluorescence was measured at 390/460 nm every 30 seconds for 40 minutes.

Immunolabelling platelet leucocyte aggregates

The percentage of leucocytes that had platelets attached (platelet–leucocyte aggregates) was determined by labelling anticoagulated blood ($10\ \mu\text{l}$) for 5 min at 4°C with either FITC–isotype control ($5\ \mu\text{l}$) or FITC–CD42a ($5\ \mu\text{l}$), PE–CD14 ($5\ \mu\text{l}$) and 0.3% (v/v) of a saturated methanolic solution of LDS-751 (Molecular Probes, UK), in $90\ \mu\text{l}$ of Tyrodes Salt Solution (TS Sigma). All antibodies were from Beckman Coulter, UK. Samples were then diluted and analysed immediately as described before [15]. In brief, leucocytes in whole blood were identified based upon their side scatter and log red fluorescence associated with the vital nuclear dye LDS-751. Leucocytes were then analysed in a plot of forward scatter and side scatter. Neutrophils were identified based upon their characteristic light scatter properties. Monocytes were identified in a plot of side scatter and PE–CD14. Aggregates were identified as neutrophils or monocytes that expressed FITC–CD42a.

Platelet and microparticle CD62P analysis

Possible changes in the numbers of platelets and platelet microparticles and their expression of CD62P, was investigated. Anticoagulated blood ($5\ \mu\text{l}$) was incubated for 5 min at 4°C with either FITC–isotype control ($2\ \mu\text{l}$) and PE–isotype control ($2\ \mu\text{l}$), or with FITC–CD42a ($2\ \mu\text{l}$) and PE–CD62P ($2\ \mu\text{l}$). Samples were diluted to 1 ml with filtered ($0.2\ \mu\text{m}$) TS. To each tube was added $1\ \mu\text{l}$ of $1.09\ \mu\text{m}$ diameter unlabelled polystyrene spheres (at 1×10^{-6} from the stock solution supplied, (Sigma, UK), then analysed immediately by flow cytometry as previously described [15]. In brief platelets were identified in a plot of side scatter logarithmic scale and forward scatter logarithmic scale. The platelet component was confirmed by the expression of CD42a. CD42a positive events that were smaller than $1.09\ \mu\text{m}$ were identified based upon the position of the polystyrene spheres and were analysed as microparticles.

The Percentage of microparticles that expressed CD62P was then determined.

Statistical analysis

Data was assessed using the Mann-Whitney U test to compare the results of the pregnant women with and without pre-eclampsia and with normal female controls. The median and 25th and 75th quartile ranges are given for the different test groups. Significance was considered at $p < 0.05$. The inter and intra assay CVs for all tests were less than 10.

Results

Patient and control characteristics

Pregnant women with and without pre-eclampsia were of similar age, parity and gestational age at blood sampling although a slightly greater proportion of cases were from Asian ethnic groups (Table 1). Blood samples from pregnant controls and women with pre-eclampsia were drawn at the same time of gestation with a median in both groups of 38 weeks. No women were in labour. The median number of weeks to delivery of the pre-eclampsia women was 1 week less than for the normal pregnant women ($p = 0.046$) as would be expected because women with pre-eclampsia are more likely to be induced. Babies born to women with pre-eclampsia had significantly lower birth weights ($p = 0.0125$).

Endogenous thrombin potential

Four parameters associated with thrombin generation were measured, the ETP, the peak height, the lag time and the time to peak. Of these parameters the ETP and the peak height were significantly raised ($p < 0.001$) in the pregnant women compared to the non pregnant controls. These two parameters were also significantly raised ($p < 0.001$) in pre-eclamptic pregnant women compared to normotensive pregnant women (Table 2). The lag time and time to peak were significantly lower ($p < 0.01$) in the pregnant women compared to the normal controls.

Table 1
Details of the controls and cases.

	Normal Controls N = 42	Pregnant Controls N = 46	Pregnant Cases N = 46
Age (Years)			
Median	34	27	29
Quartile Range*	29–42	25–31	25–35
Parity			
Median	NA	1	0
Quartile Range		0–2	0–2
Gestational Age Blood sample (Weeks)			
Median	NA	38	38
Quartile Range		34–40	35–39
Gestational Age Delivery (Weeks)			
Median	NA	39	38
Quartile Range		38–40	35–39
Birth weight (g)			
Median		3348	2910
Quartile Range	NA	2844–3536	2490–3200
Ethnicity (%)			
Black	25	13	17
Asian	25	52	57
Caucasian	50	35	26

*25th – 75th Quartiles.

Shows the age, parity, gestational age at sampling, gestational age at birth, birth weight and ethnicity for the normal controls, pregnant controls and pre-eclampsia cases. The median and 25th–75th quartiles are given for each parameter.

Table 2
Endogenous Thrombin Potential Results.

	Normal Controls N = 42	Pregnant Controls N = 46	Pregnant Cases N = 46
ETP (nM Ila)			
Median	2149	2515	2814
Quartile Range*	1873 – 2307	2289 – 2726	2512 – 3034
Peak Height (nM Ila)			
Median	359	452	500
Quartile Range	338 – 383	433 – 498	453 – 534
Lag Time (Min)			
Median	3.0	2.8	2.6
Quartile Range	2.7 – 3.3	2.5 – 2.9	2.5 – 2.9
Time to Peak (Min)			
Median	6.0	4.9	4.6
Quartile Range	5.3 – 6.7	4.6 – 5.1	4.81 – 5.55

* 25th – 75th Quartiles.

Shows the ETP, peak height, lag time and time to peak for the normal controls, pregnant controls and pre-eclampsia cases. The median and 25th–75th quartiles are given for each parameter.

Expression of CD62P on platelets

A small percentage of platelets expressed CD62P in both the pregnant women and healthy controls (HC). The median and interquartile range for the HC ($n = 42$) was 0.5 (0.3 – 1.0)% CD62P expressing platelets and that for the pregnant women ($n = 92$) was 0.4 (0.1 – 1.25)%. There was no significant statistical difference in percentage of CD62P expressing platelets in blood from HC and pregnant women. However blood from women with pre-eclampsia ($n = 46$) had a significantly higher ($p = 0.013$) percentage of CD62P expressing platelets (0.8, 0.1 – 2.7) than platelets in blood from normotensive pregnant women ($n = 46$) (0.3, 0.1 – 0.6). There was no significant difference between the HC and normotensive pregnant women (Table 3).

Platelet microparticle CD62P expression

Low percentages of CD62P expressing microparticles could be detected in the blood of all women. The percentage of CD62P expressing microparticles for the pre-eclampsia women ($n = 46$) was significantly higher at 1.0 (0.2 – 2.5)% than that for the normotensive women ($n = 46$) which was 0.4 (0.2 – 1.0)% ($p = 0.029$) (Table 3). That for HC was 0.7 (0.5 – 1.3)% and was not significantly different from the normotensive or pre-eclamptic women.

Table 3
Flow cytometry results.

	Normal Controls N = 42	Pregnant Controls N = 46	Pregnant Cases N = 46
Platelet CD62P (%)			
Median	0.5	0.3	0.8
Quartile Range*	0.3 – 1.0	0.1 – 0.6	0.1 – 2.7
CD62P Platelet Microparticles (%)			
Median	0.7	0.4	1.0
Quartile Range	0.4 – 1.3	0.2 – 1.0	0.2 – 2.5
Platelet Monocyte Aggregates (%)			
Median	2.1	2.8	4.8
Quartile Range	0.4 – 4.9	0.5 – 5.6	2.0 – 8.0
Platelet Neutrophil Aggregates (%)			
Median	1.6	1.9	1.7
Quartile Range	0.6 – 2.1	0.7 – 3.4	1.1 – 2.9

* 25th – 75th Quartiles.

Shows the % of CD62P positive platelets, CD62P positive platelet microparticles, platelet monocyte aggregates and platelet neutrophil aggregates. The median and 25th–75th quartiles are given for each parameter.

Platelet monocyte aggregate formation

Only low percentages of monocytes associated with platelets were detected in blood from controls and pregnant women. The percentage platelet monocyte aggregates (median, interquartile range) for the HC ($n = 42$) was 2.2 (0.4 – 5.0)%, for the pre-eclampsia women ($n = 46$) it was 4.8 (2.0 – 8.0)% and this was significantly higher ($p = 0.019$) than for the normotensive women which was 2.8 (0.5 – 5.6)%. There was no significant difference in the percentage of platelet monocyte aggregates found in the HC and the pregnant women bloods.

Platelet neutrophil aggregate formation

A low percentage of neutrophils associated with platelets were found to be present in blood taken from controls and patients. The percentage of platelet neutrophil aggregates for HC ($n = 42$) was 1.6 (0.6 – 2.1)% and for the pre-eclampsia women ($n = 46$) it was 1.7 (1.1 – 2.9)% and for the normotensive pregnant women it was 1.9 (0.7 – 3.4)%. There were no significant differences between the groups.

Predictive value of tests

All laboratory tests on mothers were performed blinded as to case or control status. We then analysed whether values higher than normal (mean + 2SD for the normal non-pregnant controls) for ETP (mean + 2SD; 2920), platelet activation (mean + 2SD; 2.52%), platelet monocyte aggregates (mean + 2SD; 8.82%) and level of microparticles (mean + 2SD; 12.6%) could be used to distinguish mothers with and without pre-eclampsia. If one or more of these parameters was higher than normal (mean + 2SD) in the women at time of sampling then 75/92 (82%) of tests were predictive for outcome (38 true positive, 37 true negative) and 17/92 (18%) were not (8 false negative, 9 false positive). This gave a sensitivity of 83% and a specificity of 80% and a positive predictive value of 81% and negative predictive value of 82%.

Discussion

A prothrombotic state has long been recognised in pre-eclampsia but details of its causation, critical features and contribution to the pathogenesis of pre-eclampsia remain to be clarified. Although thrombin generation, platelet activation and formation of platelet leucocyte aggregates and microparticles have been investigated individually in pregnant women in mostly small studies, none of these studies has investigated all four parameters together in pregnant women with and without pre-eclampsia. We have shown that pregnant women with pre-eclampsia have higher levels of circulating activated platelets, platelet monocyte aggregates and CD62P positive microparticles compared to normotensive pregnant women. Moreover, these changes in pre-eclampsia are associated with increased thrombin generation in plasma. Interestingly, in the pregnant women without pre-eclampsia, thrombin generation was increased but there was no evidence of increased platelet activation or formation of platelet leucocyte aggregates or microparticles compared to non-pregnant controls. This suggests that normal pregnancy is characterised by increased activity of the plasma coagulation system without significant platelet activation, but in pre-eclampsia platelet activation does occur leading to platelet monocyte aggregate and microparticle formation and a further increase in thrombin generation. These pathological disturbances, and platelet activation and its consequences in particular, may contribute to some of the clinically adverse effects of pre-eclampsia on the mother and fetus. This may also partly explain the modest benefit of aspirin in pre-eclampsia [22]. Analysis of ETP showed that pregnant women had a significantly higher ETP and peak height compared to non pregnant healthy controls ($p = <0.001$) and that pre-eclamptic women also have significantly

higher values for these parameters than normotensive pregnant women ($p < 0.001$). This is consistent with the greater thrombotic risk in pregnancy and with pre-eclampsia as an additional risk factor for thrombosis.

Our finding of increased numbers of CD62P positive platelets in pre-eclampsia is in agreement with other studies [23–26]. Like us these studies used whole blood immunofluorescent flow cytometry. Konijnenberg et al. [26] found elevated CD62P positive platelets in 10/10 of patients with pre-eclampsia and 4/10 women with normal pregnancy. Here we found that 31/46 patients with pre-eclampsia and 7/46 normotensive patients had evidence of platelet activation above normal control levels. In the past 10 years there have been a number of studies using a variety of methods to confirming increased platelet activation in pre-eclampsia compared to normotensive pregnancy. These have included measuring platelet β -thromboglobulin secretion [10], platelet thromboxane A_2 production [11], changes in ATP secretion and aggregation [27], levels of soluble CD40 ligand, RANTES and GRO-alpha [28], platelet aggregation and plasma levels of TGF-beta [29].

Our study investigated the number of platelet microparticles expressing CD62P and found them to be increased in pre-eclampsia, confirming the findings of Lok et al. [30]. However, other studies on microparticles have generated a variety of results and probably reflect the methods used. Redman & Sargent [31] reported that microparticles are increased during normal pregnancy and that they are further increased with pre-eclampsia. VanWijk et al. [18] found that the number of microparticles was unaltered in pre-eclampsia and normotensive pregnancy and Freeman et al. [32] showed that there was no difference in phosphatidylserine-exposing microparticles in these two groups. Other studies have found reduced numbers of microparticles in women with pre-eclampsia compared to women with normal pregnancies [16,19,33]. Recently, increased numbers of monocyte-derived [33] endothelial-derived [34], lymphocyte-derived [35] and FLT-1-positive [36] microparticles have been reported in women with pre-eclampsia. All these studies were performed on microparticles that had either been isolated or were in plasma and so not comparable with the whole blood flow cytometric assay used in our study.

This is the first study to show that circulating platelet-monocyte aggregates are increased in women with pre-eclampsia compared to normotensive pregnant women. One previous study showed that *in vitro* stimulation of blood with adenosine diphosphate (ADP) resulted in increased numbers of platelet-monocyte and platelet-neutrophil aggregates in women with pre-eclampsia and normotensive pregnant women compared to non-pregnant normal controls [37]. However, clearly the two studies are not directly comparable.

VanWijk et al. [38] reported that thrombin generation by isolated microparticles was similar in samples from women with pre-eclampsia, normal pregnancy and normal controls. They also enumerated the number of tissue factor-expressing, annexin V-positive microparticles in the three groups of women and found them to be similar. However the method used for enumeration could have been improved by the inclusion of standardised beads and they did not enumerate microparticles based upon size. In our study we found that the proportion of CD62P + microparticles less than 1 μ m was increased in women with pre-eclampsia compared to that found in normotensive women. We found only a weak correlation between the expression of CD62P on microparticles and ETP (data not shown). In contrast VanWijk et al. [38] did find a significant correlation between the number of circulating microparticles and thrombin generation but concluded that microparticles were not directly involved in the increased coagulation activation in pre-eclampsia. Hamad et al. [39] recently investigated women with a history of pre-eclampsia and found them to have signs of hypercoagulability as indicated by higher thrombin generation and higher platelet derived microparticles.

Much research in pre-eclampsia has centred around the identification of laboratory tests for prediction and prevention [40]. Clinical assessment alone based on maternal risk factors for the development of pre-eclampsia, for example, using the pre-eclampsia community guideline [41], does not provide an effective screening test based on our findings (manuscript in preparation). Biochemical screening also has been found unsatisfactory because the sensitivity is too low or the false positive rate is too high [40]. Furthermore most of the available assays are relatively expensive, which precludes their routine use in clinical practice. A number of studies have assessed the impedance to blood flow in the uterine arteries by Doppler to identify pregnancies at risk of complications due to impaired placentation. Most of these studies suggested that a Doppler screening programme identifies most women who subsequently develop severe pre-eclampsia and or fetal growth restriction [42,43]. However such a routine screening programme would be costly.

In this series, ETP had a sensitivity of 64% and a specificity of 75%. Including the platelet parameters in the assessment of risk gave a sensitivity of 83% and a specificity of 80% and a positive predictive value of 81% and negative predictive value of 82%. The measurement of ETP may be performed on batched frozen plasma samples and is therefore suitable for routine diagnostic analysis. Here we show that it is possible to perform routine ETP analysis. The measurement of platelet activation, platelet leucocyte aggregates and platelet microparticles is measured in whole blood held at 4 °C and needs to be assessed within 6 hours of venesection. This does not lend itself to large batch analysis but it is feasible to keep a smaller number (eg 10) of samples and analyse them together. Although the flow cytometry equipment is expensive the cost of each test is low. Only small volumes of blood are required (5–10 µl) so that the volumes of antisera used are also small and the set up and analysis is rapid (15 min per sample). Thus the test could be accommodated in most routine diagnostic flow cytometry laboratories. Importantly we have used a pre-analytical procedure which maintains the *ex vivo* state of platelets, platelet monocytes aggregates [15,21] and microparticles [44].

In conclusion our data confirm firstly that normal pregnancy is associated with a hypercoagulable state resulting in an increased thrombin generation potential and secondly that pre-eclampsia is associated with evidence of platelet activation and formation of platelet monocyte aggregates and microparticles, together with a further increase in thrombin generation capacity.

Conflict of interest statement

None of the above authors have a conflict of interest and no sponsors were involved with any part of the study.

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