

## ORIGINAL ARTICLE

## Deposition of whole blood platelets on extracellular matrix under flow conditions in preterm infants

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**Background:** A previous study showed greater adhesion by platelets of healthy full term infants to sub-endothelial extracellular matrix (ECM) under flow conditions compared with healthy adult platelets.

**Aim:** To investigate the adhesion and aggregation of platelets from preterm infants on ECM under defined shear conditions.

**Methods:** In vitro platelet function was investigated in 106 preterm infants, 74 full term infants, and 26 healthy adults. Blood samples were obtained from all infants within 24 hours of birth, and weekly until discharge from preterm infants only. Citrated whole blood was placed in ECM precoated tissue culture plates and subjected to shear stress ( $1300\text{ s}^{-1}$ ) for two minutes using a rotating Teflon cone. Platelet adhesion (surface coverage) and aggregation (average size) to ECM were assayed using an image analyser. Assays for von Willebrand factor (vWF) antigen, ristocetin cofactor, and vWF collagen-binding activity were performed on samples from an additional 70 preterm infants, 23 healthy full term infants, and 24 healthy adults. Preterm infants with hyaline membrane disease (HMD) were analysed separately in both cohorts.

**Results:** Platelets from preterm infants displayed significantly less platelet adhesion than those from full term infants but similar aggregation and levels of vWF antigen, ristocetin cofactor, and collagen binding activity. Mean surface coverage was 22.0 (8.4)% for preterm infants with HMD, 28.7 (8.0)% for healthy preterm infants, and 35.7 (7.9)% for full term infants. Surface coverage in the preterm infants correlated with gestational age during the first 24 hours only, and did not reach full term levels during 10 weeks of follow up.

**Conclusion:** Platelet adhesion to ECM is significantly poorer in preterm than in full term infants, and poorer in preterm infants with HMD than in healthy preterm infants. Intrinsic platelet properties rather than the concentration or activity of vWF may be responsible for this difference.

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Preterm infants have a higher rate of bleeding diathesis than full term infants and significantly lower levels of most coagulation and anticoagulation factors.<sup>1,2</sup> A reduced haemostatic functional reserve capacity could contribute to the occurrence of bleeding, especially in the presence of additional risk factors.

Adequate platelet function is crucial to the maintenance of normal haemostasis. However, the conventional physiological tests used until recently were limited by the relatively large amounts of blood required and their performance without shear or without the presence of solid phase subendothelial components. Although several sophisticated methods for studying platelet interaction with subendothelial protein ligands under shear conditions have been described,<sup>3–5</sup> none had the potential for wide clinical use, and they possessed a limited ability to differentiate between the various pathological conditions. The method that we used which requires only small volumes of blood is based on the study of Varon *et al.*,<sup>6</sup> who found that subjection of citrate whole blood to flow on bovine endothelium extracellular matrix (ECM) results in platelet adhesion and aggregation. In our previous study,<sup>7</sup> we used the cone and plate(let) analyser (CPA) to examine platelet adhesion and aggregation on ECM under shear conditions, mimicking most of the complex series of events occurring in vivo during thrombogenesis. We showed that full term infants have increased adhesion and normal aggregation compared with adult controls. This shift in platelet function may provide neonates with a balanced primary haemostasis, compensating for neonatal platelet hyporeactivity, which has been shown in vitro without the application of shear stress.<sup>8,9</sup>

This study was designed to extend the investigation of platelet adhesion and aggregation under flow conditions to preterm infants. The findings may enable us to reach a better understanding of primary haemostasis in this patient population.

## MATERIALS AND METHODS

### Patients and blood sampling

The study was approved by the local and national ethics committees, and informed consent was obtained for each participant. Two cohorts were defined. The first, in which platelet adhesion and aggregation were measured under defined shear stress, included 106 preterm infants ( $\leq 35$  weeks gestation), born consecutively between March and August 1997, and 74 healthy full term infants ( $\geq 37$  weeks gestation, one and five minute Apgar scores 9 and 10 respectively; birth weight  $> 2500$  g) born during April 1997. All infants were born at the Rabin Medical Center, Petah Tiqva, Israel. Twenty six healthy adult volunteers who had not taken medication known to affect platelet function for at least 10 days before blood sampling served as controls for this cohort. The second cohort, in which von Willebrand factor (vWF) antigen assay, ristocetin cofactor activity test, and collagen binding activity assay were performed, included 70 preterm infants born consecutively

**Abbreviations:** CPA, cone and plate(let) analyser; ECM, extracellular matrix; GP, glycoprotein; HMD, hyaline membrane disease; PBS, phosphate buffered saline; vWF, von Willebrand factor

**Table 1** First cohort: clinical characteristics and platelet function in infants and adult controls

Characteristics	Preterm		Full term (n=74)	Adult (n=26)
	Healthy (n=52)	HMD (n=23)		
Gestational age (weeks)	32.1 (2.2)	28.6 (3.0)	39.6 (1.5)	–
Birth weight (g)	1611.0 (429.9)	1190.5 (457.1)	3184.5 (540.7)	–
Surface coverage (adhesion) (%)	28.7 (8.0)	22.0 (8.4)	35.7 (7.9)	19.4 (4.6)
Average size (aggregation) ( $\mu\text{m}^2$ )	44.0 (12.7)	43.9 (16.8)	44.7 (4.9)	47.52 (15.2)
Platelet count (cells $\times 10^6/\text{ml}$ )	268.7 (92.5)	217.1 (115.8)	242.0 (61.0)	208.0 (48.0)

Note: all values are mean (SD).

$p < 0.01$  for preterm v full term and healthy preterm v HMD preterm;  $p < 0.001$  for full term v HMD preterm.

between April and December 1999, and 23 full term infants born during April 1999. Twenty four healthy adult volunteers served as controls for the second cohort.

### Sample collection

Venous blood samples were obtained during the first 4 hours of life from all infants. In the first cohort, additional samples were collected weekly from the preterm infants until discharge. All blood samples were drawn into plastic syringes and immediately transferred to plastic tubes containing trisodium citrate (0.129 M).

### Procedure for the cone and plate(let) analyser (CPA)

The method has been described in detail elsewhere.<sup>6</sup> Briefly, 0.2 ml samples of citrated blood were placed in tissue culture wells precoated with subendothelial ECM derived from bovine corneal endothelium. Blood was subjected to flow ( $1300 \text{ s}^{-1}$ ) for two minutes using a rotating Teflon cone. The wells were washed with phosphate buffered saline (PBS) stained with May-Grünwald stain and analysed with an image analysis system (Galai, Migdal Haemek Israel).

Two parameters of platelet function were evaluated: platelet adhesion to ECM, defined as the percentage of total area covered by platelets (surface coverage), and platelet aggregation on the ECM, defined as the average size of the ECM bound objects.

### Immunoturbidimetric assay of vWF antigen

vWF antigen was tested with the immunoturbidimetric assay, which measures the change in light transmission by a suspension of microlatex particles precoated with antibodies against vWF, in the presence of tested plasma. The STA-Liatest vWF kit (Stago, France)<sup>10</sup> was used.

### Assay of ristocetin cofactor

Ristocetin makes conformational changes in the vWF structure, allowing it to bind to glycoprotein Ib (GPIb) on the platelet membrane. The rate of ristocetin induced platelet agglutination is related to vWF activity. The ristocetin cofactor assay kit<sup>11</sup> (Helena Laboratories, Beaumont, Texas, USA) measures the agglutination of formalin fixed normal platelets by ristocetin in the presence of test plasma. The percentage of vWF activity is obtained from aggregometer tracings.<sup>12</sup>

### vWF collagen binding activity

This assay was performed according to a previously described method<sup>10</sup> with minor modifications. Plasma samples diluted 1:100 with PBS containing 0.2% bovine serum albumin were placed on multiwell plates precoated with collagen type III (5  $\mu\text{g}/\text{ml}$ ) and blocked with 1% bovine serum albumin in PBS for 15 minutes. After incubation for two hours, the bound vWF was incubated for one hour with a solution of peroxidase conjugated antibody to vWF (1:4000). After each step the microtitre wells were washed three times with PBS. The chromogenic reaction was performed by addition of phenylenediamide (0.5 mg/ml) containing 0.5  $\mu\text{l}/\text{ml}$  30%  $\text{H}_2\text{O}_2$ . The reaction was

terminated by the addition of 1.6%  $\text{H}_2\text{SO}_4$ , and the absorbance was read at 2932 nm on an ELISA reader.

### Statistical analysis

Statistical analysis was performed with BMDP Statistical Software, 1990. One way and two way analysis of variance and covariance with repeated measures, Fisher's exact test, Pearson's  $\chi^2$  test, paired  $t$  test, and logistic regression analysis were used, as appropriate. Correlations were derived by Student's linear regression analysis.

## RESULTS

### First cohort

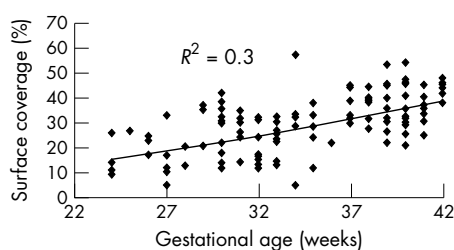
The mean (SD) birth weights of the 106 preterm infants and 74 full term infants were 1412.2 (482.8) g and 3184.5 (540.7) g respectively; mean (SD) gestational ages were 30.5 (3.2) weeks and 39.6 (1.5) weeks respectively. A healthy preterm group of 52 infants was formed after the exclusion of 54 infants with one or more major pathologies including: hyaline membrane disease (HMD;  $n = 26$ ), hyperbilirubinaemia (serum bilirubin  $> 150 \text{ mg}/\text{l}$ ;  $n = 9$ ), sepsis (either positive blood or cerebrospinal fluid culture any time during their stay in the neonatal unit;  $n = 16$ ), intraventricular haemorrhage ( $n = 4$ ), thrombocytopenia, or coagulation disorder ( $n = 8$ ).

Twenty three preterm infants who were found to have HMD, without sepsis, serum bilirubin of  $> 150 \text{ mg}/\text{l}$ , or coagulation disorders were separated into a distinct subgroup for comparison. All were diagnosed by a qualified radiologist, and all required supplementary oxygen with either mechanical ventilation or continuous positive airway pressure support. Table 1 gives the characteristics of the healthy preterm infants, the preterm infants with HMD, the healthy full term infants, and healthy adult controls of the first cohort.

The healthy preterm infants were older ( $p < 0.001$ ), had higher birth weights ( $p < 0.001$ ), and higher Apgar scores at one and five minutes ( $p < 0.001$ ) than the preterm infants with HMD. Significantly more preterm (whole group) than full term infants were born by caesarean section (76/106, 72% v 6/74, 8%,  $p < 0.01$ ). However, within the healthy preterm subgroup, there were no significant differences in gestational age or birth weight between infants delivered by caesarean section and those delivered vaginally.

Table 1 presents platelet adhesion (surface coverage) and aggregation (average size) during the first 24 hours of life for all the infant groups studied. Surface coverage was lower in the total preterm than the full term population (24.2 (9.3)% v 35.7 (7.9)%;  $p < 0.001$ ), and lowest in the preterm infants with HMD (22.0 (8.4)%) compared with the healthy preterm infants (28.7 (8.0)%;  $p < 0.01$ ) and the full term infants (35.7 (7.9)%;  $p < 0.0001$ ). On multivariate analysis, HMD was a significant factor for low surface coverage ( $p < 0.05$ ), independent of weight, gestational age, or Apgar score. Aggregation was similar in the healthy preterm, HMD preterm, and full term infants.

During the first 24 hours of life, surface coverage correlated with gestational age in all infants (fig 1) ( $R^2 = 0.30$ ,  $p < 0.05$ ).



**Figure 1** Surface coverage during the first 48 hours of life correlated with gestational age.

However, during follow up (weeks 1–10 of life), the preterm infants showed no increase in surface coverage or correlation with corrected postconceptional age.

Platelet count correlated with both surface coverage and average size, but packed cell volume, white blood cell count, and absolute neutrophil count did not. No correlations were found between mode of delivery and surface coverage or average size.

### Second cohort

The mean birth weights of the 70 preterm and 23 full term infants were 1417.3 (642.1) g and 3250.4 (530.7) g respectively; mean gestational ages were 29.9 (3.3) weeks and 39.1 (1.3) weeks respectively. Using the same criteria as for the first cohort, six preterm infants with intraventricular haemorrhage, sepsis, hyperbilirubinaemia, and coagulation disorders were excluded. Two preterm subgroups were defined: healthy preterm infants ( $n = 38$ ) and preterm infants with HMD ( $n = 26$ ). Table 2 shows the results for the preterm infant subgroups, full term infants, and adults. There were no significant differences between the whole preterm group and full term infants with respect to vWF antigen (165.3 (33.5)%  $\nu$  166.5 (33.5)%), ristocetin cofactor 215.9 (73.3)%  $\nu$  253.3 (88.2)%), and collagen binding activity (0.88 (0.27)  $\nu$  1.11 (0.12) units). Adult values were significantly lower than all infant values ( $p < 0.01$ ). No correlation was found between the levels in any of the assays and gestational age.

### DISCUSSION

The CPA test has been found suitable for diagnosis and monitoring of treatment in various platelet disorders. Blood samples from patients with severe von Willebrand disease show very low adhesion (surface coverage) and aggregation (average size) compared with samples from healthy volunteers, mainly under a relatively high shear rate (1300  $s^{-1}$ ). In contrast, blood samples from patients with Glanzmann's thrombasthenia had a surface coverage similar to that of normal blood, but with a smaller average size than that of a single platelet. The CPA method has also been used to assess platelet

function in thrombocytopenic patients when conventional platelet aggregometry is not available<sup>14</sup> and to monitor GPIIb-IIIa antagonist treatment in patients undergoing carotid stenting.<sup>15</sup> One recent study reported increased platelet adhesion to ECM under flow conditions in healthy full term neonates compared with normal adults.<sup>3</sup> This finding contradicts the previously described impaired response of neonatal platelets to various agonists as assessed by conventional platelet aggregometry and flow cytometry,<sup>1, 2, 5</sup> and it explains why full term neonates have no tendency to bleed.

This study shows that healthy preterm infants have significantly lower surface coverage than healthy full term infants, and preterm infants with HMD have even lower surface coverage than healthy preterm infants. In the preterm infants, gestational age correlated with surface coverage at delivery, but surface coverage did not rise with increasing postconceptional age during the first 10 weeks of life, possibly because these infants had not been exposed to a maturational signal to instigate an increase in surface coverage close to term.

The surface coverage of both the healthy preterm infants and those with HMD was still higher than that in adults. This may be due to the presence of more active high molecular mass multimers of vWF in neonates.<sup>24</sup> However, the latter may not be enough to compensate for the primary haemostasis disorder in preterm infants, which is manifested by a more defective platelet response, compared with full term infant platelets, to various agonists, as assessed by platelet aggregometry and flow cytometry.<sup>1, 2, 5</sup> Thus, surface coverage and average size alone, as assessed by the CPA test, cannot serve as absolute parameters of platelet function.

Although it has been suggested that the platelets of full term infants are activated during the birth process,<sup>17</sup> other studies indicate that this is not the case.<sup>2, 4, 5, 18</sup> In our study, no difference in parameters of platelet function was observed between infants delivered by caesarean section or vaginally.

Under shear conditions, erythrocytes enhance platelet transport, leading to the formation of platelet receptor regions near to solid surfaces<sup>19</sup> and facilitating platelet deposition on ECM.<sup>9, 20, 21</sup> The large packed cell volume observed in neonates could be responsible for the increased adhesion of neonatal platelets to ECM. However, in the study, no correlation was found between packed cell volume and surface coverage or average size in either preterm or full term infants, suggesting that the influence of packed cell volume on platelet function may be insignificant when it is maintained above 25%.

Adhesion of platelets to the exposed subendothelium under shear stress is mediated principally by platelet binding to vWF through the GPIb component of the surface membrane glycoprotein complex GPIb-V-IX, and to collagen through specific receptors on the platelet surface.<sup>22</sup> In our previous study,<sup>3</sup> we showed that the presence of higher vWF multimers in full term neonatal plasma allowed increased adhesion of platelets to subendothelial ECM under shear conditions. Thus, the

**Table 2** Second cohort: clinical characteristics of infants and von Willebrand factor (vWF) function

Characteristics	Preterm		Full term (n=23)	Adult (n=24)
	Healthy (n=38)	HMD (n=26)		
Gestational age (weeks)	32.2 (3.0)	28.5 (2.7)	39.1 (1.3)	–
Birth weight (g)	1613.1 (693.3)*	1192.3 (479.9)*	3250.4 (530.7)	–
vWF antigen (%)	165.1 (27.4) (n=20)	162.8 (39.7) (n=23)	166.5 (33.5) (n=17)	93.4 (22.6) (n=24)
vWF collagen binding (arbitrary units)	0.89 (0.26) (n=25)	0.77 (0.30) (n=10)	1.11 (0.12) (n=6)	0.55 (0.09) (n=24)
Ristocetin cofactor (%)	193.8 (73.3) (n=20)	228.6 (74.7) (n=23)	253.3 (88.2) (n=17)	89.6 (16.8) (n=24)

All values are mean (SD).

Adult values significantly lower than all infant values for each characteristic,  $p < 0.01$ .

\* $p < 0.01$  compared with full term.



short bleeding time shown in full term newborns may result from raised levels of circulating vWF and/or increases in high molecular mass vWF multimers.<sup>23, 24</sup> On the basis of these data, the second part of the study (cohort 2) was designed to investigate the levels and activity of circulating vWF in preterm and full term infants. Unexpectedly, neither the concentration of vWF nor its activity, as determined by the level of vWF antigen in plasma, ristocetin cofactor, and assay of collagen binding activity, differed significantly between the two groups.

The reason for the lower adhesion of preterm platelets may be an intrinsic platelet hypoactivity. Platelets from preterm infants have been shown to be less reactive than those from full term infants and adults to physiological agonists.<sup>5</sup> Furthermore, preterm platelets have reduced thromboxane A2 productivity. Decreased platelet function has not been related to altered binding characteristics of the thromboxane A2 receptor, but may lie in the transduction pathway after the receptor.<sup>25</sup>

As described in our previous study,<sup>3</sup> unlike surface coverage, average size did not differ between full term neonates and adults. This was also true in the present study for preterm infants. This finding suggests that vWF functions under high shear conditions to maintain platelet adhesion to the ECM surface.<sup>21, 26</sup> Average size is an important variable in solid phase platelet aggregation, depending mainly on the GPIIb-IIIa receptor,<sup>21, 26, 27</sup> which is present at similar levels in adults and neonates.<sup>25</sup> The decrease in average size to that of a single platelet in patients with Glanzmann's thrombasthenia<sup>6, 28</sup> is confirmed in patients receiving treatment with GPIIb-IIIa antagonist.<sup>15</sup>

In conclusion, platelet deposition on ECM under flow conditions may be an accurate and specific representation of the state of platelet function in preterm infants. Platelet adhesion to ECM is significantly poorer in preterm than full term infants. Intrinsic platelet properties, rather than concentration or activity of vWF, may be responsible for this difference.

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