Platelet function alterations in dengue are associated with plasma leakage

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Summary
Severe dengue is characterised by thrombocytopenia, plasma leakage and bleeding. Platelets are important for preservation of endothelial integrity. We hypothesised that platelet activation with secondary platelet dysfunction contribute to plasma leakage. In adult Indonesian patients with acute dengue, we measured platelet activation status and the response to the platelet agonist TRAP using flow cytometer-based assays. Patients were monitored daily for plasma leakage by ultrasonography. Acute dengue was associated with platelet activation with an increased expression of the activated fibrinogen receptor (αIIbβ3), the lysosomal marker CD63 and the alpha-granule marker CD62P (P-selectin). Upon maximal platelet activation by TRAP, platelet function defects were observed with a significantly reduced maximal activated αIIbβ3 and CD63 expression and reduced platelet-monocyte and platelet-neutrophil complexes. Patients in the lowest tertile of activated αIIbβ3 and CD63 expression had an odds ratio for plasma leakage of 5.2 (95% confidence interval [CI] 1.3–22.7) and 3.9 (95% CI 1.1–13.7), respectively, compared to the highest tertile. Platelet-derived serotonin has previously been related to plasma leakage and we found increased intra-platelet serotonin concentrations in our patients. In conclusion, platelet activation with platelet function alterations can be found in patients with acute dengue and this may contribute to dengue-associated plasma leakage.

Keywords
Dengue virus infection, platelet function, flow cytometry, plasma leakage, serotonin

Introduction
Dengue has become the most important arthropod-borne viral infection in the world (1). Severe dengue is characterised by thrombocytopenia, vascular leakage and haemorrhage. The pathogenic mechanisms underlying these complications, which typically occur during or shortly after defervescence and are not associated with morphological endothelial damage, are still incompletely understood (2–4). In recent years, the role of platelets in regulating endothelial integrity and dengue-associated plasma leakage has gained increased interest (5–9).

Sufficient numbers of functional platelets are required for preservation of endothelial integrity during inflammation (10). The observation that petechiae and bleeding complications in dengue patients frequently occur with platelet counts well above the threshold for spontaneous bleeding, suggests that dengue is not only associated with thrombocytopenia, but also with functional platelet defects. Studies using platelet aggregometry indeed showed reduced aggregation in dengue patients (11–13), but because the reliability of aggregometry is compromised in conditions with thrombocytopenia, these findings should be interpreted with caution. We have developed a novel flow cytometer-based platelet function test using anti-coagulated unprocessed blood that is well suited for use in thrombocytopenia (14). In this test, expression of the alpha-granule marker CD62 (P-selectin), the lysosomal marker CD63 and binding of the monoclonal antibody PAC-1 to the activated αIIbβ3 (the GPIIb/IIIa complex, CD41/ CD61) receptors are used as markers for platelet activation (15–17). Reduced expression of these markers upon addition of a platelet agonist suggests the presence of thrombocytopenia (18). Activated platelets form complexes with monocytes and neutrophils by binding of platelet CD62P to its counter-ligand on leukocytes, P-selectin glycoprotein ligand-1 (PSGL-1) (19). These complexes can also be...
quantified using flow cytometry and it has frequently been used as a sensitive marker of platelet activation.

The hypothesis which formed the basis of this study was that dengue is not only associated with thrombocytopenia, but also with functional platelet alterations due to excessive platelet activation and that these functional alterations are associated with plasma leakage. We therefore determined platelet activation status, platelet-monocyte (PMC) and platelet-neutrophil complexes (PNC) and the functional response to platelet stimulation by a platelet agonist in adult Indonesian patients with dengue. In addition, we determined soluble platelet activation markers as a measure of recent platelet degranulation and intra-platelet serotonin concentrations. Plasma leakage in dengue occurs at predilection locations and can be easily detected by ultrasonography as gallbladder wall oedema, ascites and/ or pleural effusion. We compared results of the platelets assays with daily ultrasonography as previously reported (20).

Material and methods

Patients and study design

This study was performed from March 2011 – March 2012 in Hasan Sadikin General Hospital, an academic referral hospital in Bandung, Indonesia. Febrile patients clinically suspected for dengue infection were eligible for inclusion in the study. Patients with chronic diseases, use of platelet function inhibitors and pregnant patients were excluded. Patients were systematically followed by daily history, physical examination, laboratory tests and by daily ultrasonography for detection of plasma leakage. Special attention was paid to bleeding manifestations. Demographic, clinical, laboratory and ultrasonography data were collected using a standardised data collection form. Blood was drawn at admission and in each clinical phase of dengue infection: the febrile phase (temperature of 37.5°C or higher), the critical phase (period within 48 hours (h) after defervescence when complications usually occur and before platelet counts start to recover), early recovery phase (recovering platelet counts together with clinical improvement) and convalescence phase (> 2 weeks after discharge). Plasma leakage was defined as an increase in haematocrit of ≥ 20%, a single high haematocrit value (≥ 50% for men and > 44% for women), and/or plasma leakage in the form of ascites and/or pleural effusion detected by ultrasonography, according to WHO guidelines (21). A thickened gall bladder wall was not used as a criterion for plasma leakage. Handheld ultrasonography was performed by three study physicians who had received a four-week training to examine patients for presence of ascites, pleural effusion and measurement of gallbladder wall thickness. All images were daily reviewed by the coordinating physician. Results of the handheld ultrasonography were also compared with results obtained by conventional ultrasonography performed by a trained radiologist. Details on the methods and findings of the ultrasonography in our cohort have previously been reported. Patients were retrospectively classified as non-severe or severe dengue according to the 2009 WHO guidelines (21). A group of healthy volunteers was recruited among hospital staff to serve as controls. None of the controls had had fever or other complaints in the past two weeks and all had a normal complete blood count.

The study was approved by the local Medical Ethical Committee of the Medical Faculty of Padjadjaran University, Hasan Sadikin General Hospital and written informed consent was obtained before enrolment from all patients and healthy controls.

Laboratory diagnosis

Samples of clinically dengue suspected cases were tested for the presence of viral RNA by reverse-transcriptase PCR and for dengue specific IgM and IgG (Panbio, Windsor, Australia). A proven dengue infection was defined as a positive RT-PCR result, a ≥ 4-fold increase in dengue IgG titre and/or IgM or IgG seroconversion. Patients with a positive IgM titre in at least one sample and/or IgG levels comparable to a HI titre of at least 1:2,560 (the IgG cut off point set by the manufacturer to detect secondary infection) were diagnosed with acute dengue infection. The remaining patients with clinically suspected dengue who failed to fulfil the diagnostic laboratory criteria outlined above were excluded.

Flow cytometer-based platelet activation and platelet function assays

Venous blood was collected using a 21-gauge needle in 3.2% citrate anti-coagulated vacutainers (BD Biosciences, Franklin Lakes, NJ, USA) after a light tourniquet application, which was released as soon as a good blood flow was established. The first 2 ml of blood were used for other purposes. The blood was kept at room temperature and processed immediately.

For the platelet activation assay, 5 µl whole blood was added to a mixture of monoclonal antibodies (MoAb), fixed after 20 minutes (min) incubation with 0.2% formyl saline (0.2% formaldehyde in 0.9% NaCl) and analysed within 3 h on a Guava® EasyCyte™ 6–2L flow cytometer (Merck KGaA, Darmstadt, Germany). The following MoAb were used: fluorescein isothiocyanate (FITC)-labelled or phycoerythrin (PE)-labelled anti-CD42b (anti-GP1b, a platelet identification marker abundantly present on platelets), PE-labelled anti-CD62P (anti-P-selectin, an alpha granule marker), allophycocyanin (APC)-labelled anti-CD63 (dense-body/ lysosomal marker; all from Biolegend, San Diego, CA, USA), and FITC-labelled PAC-1 MoAb which recognise the activated fibrinogen binding conformation of αIIbβ3 (BD Biosciences). Platelets were gated based on their forward- and sideward-scatter (FSC/SSC) properties and positivity for CD42b. CD42b positivity was defined as a mean fluorescence intensity (MFI) exceeding the MFI of the matched isotype control. Next, all CD42b positive events were gated for the platelet activation markers CD62P, CD63 and activated αIIbβ3.

Platelet-monocyte complexes (PMC) and platelet-neutrophil complexes (PNC) were measured by incubating 50 µl of citrate anti-coagulated whole blood with anti-CD14 and with anti-CD42b MoAb (both Biolegend) for 15 min. Erythrocytes were lysed using lysing solution (OptiLyse B, Beckman Coulter, Ville...
pinte, France). Monocytes and neutrophils were identified based on their FSC/SSC and CD14 staining as has been described before (22). PMC and PNC were determined by using the proportion of monocytes and neutrophils, respectively, which were positive for the platelet identification marker CD42b.

Platelet responsiveness to stimulation by the PAR-1 agonist thrombin receptor activating peptide (TRAP) (Bachem, Bubendorf, Switzerland) and the formation of PMC and PNC were measured after 20 min of incubation with 625 µM of TRAP before fixation. In addition, a dose response curve using eight serial dilutions of TRAP, ranging from 0.038 to 625 µM, was made using CD62P as readout for platelet activation.

### Soluble platelet activation markers

Concentrations of the soluble platelet activation markers platelet factor 4 (PF4), P-selectin (sCD62P) and RANTES were determined in plasma derived from venous blood collected in CTAD tubes (BD Biosciences). These tubes contain citrate and the platelet stabilising agents theophylline, adenosine and dipyridamole, preventing in vitro platelet activation. Plasma was obtained by centrifugation the tubes at 1700 g for 15 min followed by immediate storage at −80°C until further analysis. Concentrations of the platelet activation markers were measured using a semi-automated ELISA on a TECAN Freedom Evo robot in the Department of

<table>
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<th>Parameter</th>
<th>Dengue (n=77)</th>
<th>Healthy controls (n=30)</th>
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<tr>
<td>Age; years</td>
<td>23 (19–32)</td>
<td>25 (24–31)</td>
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<tr>
<td>Male sex; n (%)</td>
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<td>Duration of illness; days</td>
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<td>Ascites; n (%)</td>
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<tr>
<td>Pleural effusion; n (%)</td>
<td>6 (8)</td>
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<td>Plasma leakage during study; n (%)</td>
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<tr>
<td>Ascites or pleural effusion; n (%)</td>
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<td>Albumin; g/dl</td>
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### Table 1: Characteristics of dengue patients and healthy controls.

The first day of fever was defined as the first day of illness. Data are presented as medians (interquartile range) or numbers (n) with percentage (%). Mann-Whitney U tests or Chi-square tests were performed whenever appropriate. A p-value < 0.05 was considered significant and was indicated as *** (p<0.001); ** (p<0.01); or * (p<0.05).
Clinical Chemistry and Hematology of the University Medical Center Utrecht as described in detail earlier (23) and had a lower detection limit of 80 pg/ml for PF4, 5.0 ng/ml for sCD62P and 70 ng/ml for RANTES. All intra-assay CVs were below 10% and inter-assay CVs were below 12%.

Intra-platelet and plasma serotonin levels

Blood was drawn into CTAD containing vacutainers and centrifuged at 150g for 10 min without brake to obtain platelet-rich plasma (PRP) which was pipetted off and further processed by centrifuging at 1350g for 15 min to a platelet pellet with a known number of platelets. This platelet pellet was subsequently resuspended and lysed by perchloric acid. Platelet-poor plasma (PPP) was obtained from the sample that remained after pipetting off the PRP, by centrifugating at 1700g for 15 min. Lysed platelet pellets and PPP were stored at –80°C until analysis. Plasma serotonin levels were determined by commercially available ELISA (Genway Biotech, San Diego, CA, USA), which had a lower detection limit of 0.3 ng/ml, according the manufacturer's instructions. Intra-platelet serotonin levels were determined in the supernatant of the platelet pellets, which was obtained after centrifuging the thawed samples for 10 min at 2000g. The supernatant was aspirated and hydrochloric acid was added before reading the fluorescence of serotonin after exciting at 310 nm and registering at 510 nm by a Fluorescence Spectrophotometer (F-7000, Hitachi High Technologies, Schaumburg, IL, USA). This assay had a lower detection limit of 170 nmol/1011 platelets and an intra-assay CV below 10%.

Data presentation and statistical analysis

Data are expressed as medians with interquartile ranges (IQR) or numbers with percentages, unless otherwise specified. Differences in non-continuous data of two groups were analysed by Pearson’s Chi-square test or by Fisher’s exact test in case of expected counts less than five. Continuous variables between two groups were analysed by unpaired t-test in case of normally distributed data and by Mann-Whitney U test in case of non-parametric data. Continuous variables with repeated measurements in the different phases of dengue infection were analysed by a linear mixed model with repeated measurements after log transformation in case of non-parametric data. Relationships between continuous data were examined by Spearman’s correlation for non-parametric data. TRAP-CD62P expression dose-response graphs were produced with Prism 5.02 software (Graphpad Software, La Jolla, CA, USA). A p-value < 0.05 was considered significant. All analysis were performed using SPSS (version 18.0). Binding of PAC-1 was considered the primary outcome. Assuming that an increase in PAC-1 positive platelets from 25% in the convalescent phase to 50% in the critical phase of dengue would be relevant, a total number of 74 patients had to be included.

Results

Clinical characteristics and baseline data

A total number of 77 patients with a dengue infection and 30 healthy controls were included. Demographics and clinical and laboratory characteristics at baseline are summarised in Table 1. Twenty-nine (38%) of the dengue patients had a positive RT-PCR with DENV-2 being the most common serotype (n=13), followed by DENV-3 (n=7), DENV-1 (n=5) and DENV-4 (n=4). The 48/77 (62%) PCR negative patients in this study were included based on a positive dengue-specific serology. Most patients were admitted in the critical phase around defervescence (n=62; 81%), the others in the febrile (n=11; 14%) or the early recovery phase (n=4; 5%). Based on serology results, 58 (75%) had a secondary infection (n=58; 75%), two (3%) a primary infection and the remainder was inconclusive. Thrombocytopenia (platelet count < 150 * 109/l) was present in all dengue patients at enrolment with a median platelet count of 44 * 109/l. Seventy-five percent of patients had some form of bleeding manifestation, of which skin and gingival bleeding and epistaxis were the most common. Plasma leakage was confirmed in 39 (51%) of patients during hospitalisation. Ascites and/or pleural effusion were demonstrated by ultrasonography in 18/74 (24%) patients at enrolment and developed in an additional 15/77 (19%) patients. In the remaining six patients, the diagnosis of plasma leakage was based on their haematocrit values, which is defined by WHO criteria (21) as a significant haematocrit change (minimal 20%) and/or single high haematocrit values (> 50 and > 44% for men and women, respectively). Thickening of the gallbladder wall is a common event in dengue, which is also thought to result from

![Image of a graph showing platelet count during dengue](image-url)
plasma leakage (24). Thickening of the gallbladder wall to more than 0.5 cm was found in 15 patients (20%) during hospitalisation. All patients were discharged from hospital in good health.

**Dengue is associated with platelet function alterations**

The course of platelet number and platelet activation markers is shown in Figure 1 and Figure 2A, respectively. The most striking finding was a strong increase in the binding of PAC-1 antibodies, indicating that the integrin \( \alpha_{IIb}\beta_3 \) had undergone a conformational change to a fibrinogen binding state. The median expression of CD63 and CD62P, signifying degranulation of lysosomes and \( \alpha \)-granules, respectively, were also significantly higher in the dengue patients.

Subsequently, the capacity of platelets to be maximally activated was tested by adding a high concentration of the platelet agonist TRAP. Platelets of patients in the febrile and critical phase of dengue had a reduced expression of activated \( \alpha_{IIb}\beta_3 \) and CD63, implying a reduced haemostatic function (Figure 2B). In some patients, CD63 expression to high dose TRAP remained low up to the convalescent phase, while expression of CD62P and activated \( \alpha_{IIb}\beta_3 \) had recovered. The difference in CD62P expression between groups after high dose TRAP was only modest, but a clear differ-

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**Figure 2: Dengue is associated with baseline platelet activation and a decreased expression of platelet activation markers after TRAP-stimulation.** The binding of the platelet activation markers was determined in citrate anti-coagulated whole blood by flow cytometry before and after addition of the PAR-1 agonist thrombin receptor activating peptide (TRAP) in patients with dengue infection (n=77) and in healthy controls (n=30). The proportion of platelets positive for the platelet activation markers PAC-1, CD63 and P-selectin (CD62P) was increased during dengue (A), while expression of these markers was decreased upon incubation with a high concentration of TRAP (B). Expression of PAC-1, CD63P and CD62P indicate platelet activation with a conformational change of activated \( \alpha_{IIb}\beta_3 \) to a fibrinogen binding state, degranulation of \( \alpha \)-granules and degranulation of lysosomes and dense granules, respectively. The boxes indicate the median and interquartile ranges and the whiskers indicate 10th to 90th percentile. Differences between each phase of dengue were compared to healthy controls values with the Mann-Whitney U test. A p-value < 0.05 was considered significant; *** denotes p < 0.001; ** p < 0.01 and * p < 0.05.
ence in the dose-response curve with a downward and vertical shift of the curve, suggesting impaired CD62P secretion from α-granules, was seen in the dengue patients when eight concentrations of TRAP were used (▶Figure 3).

**Functional platelet defects are associated with plasma leakage**

Patients who developed plasma leakage had a significantly lower expression of activated αIIBβ3 and CD63 in TRAP-stimulated samples at enrolment compared to patients without plasma leakage (▶Figure 4). When these results were stratified in tertiles, the patients in the lowest tertile of activated αIIBβ3 and CD63 expression had an odds ratio for plasma leakage of 5.2 (95% confidence interval [CI] 1.3–22.7) and 3.9 (95% CI 1.1–13.7) compared to the highest tertile, respectively. Thickening of the gallbladder wall was also associated with a reduced activated αIIBβ3 expression upon TRAP stimulation (▶Figure 4). There was no association between either plasma leakage or gallbladder wall thickening and CD62P expression in TRAP-stimulated samples, nor with the expression of activated αIIBβ3 and CD63.

**Figure 3: P-selectin expression is decreased at sub-maximal TRAP stimulation during dengue.** The dose-response curve of platelets expressing the platelet activation markers P-selectin (CD62P) after stimulation with eight serial dilutions ranging from 0.038 to 625 µM of the PAR-1 agonist thrombin receptor activating peptide (TRAP) was assessed in dengue patients (n=77) and in healthy controls (n=30) in citrate anti-coagulated whole blood by flow cytometry. Data are presented as medians of the TRAP dose-response curves for each group. Differences in CD62P expression at each dilution between each phase of dengue were compared to healthy controls values with the Mann-Whitney U test. A p-value < 0.05 was considered significant; *** denotes p < 0.001; ** p < 0.01 and * p < 0.05.

**Figure 4: Platelet dysfunction is associated with plasma leakage and gallbladder wall thickening during dengue.** The percentage of platelets showing PAC-1 binding and CD63P expression after stimulation with 625 µM of the PAR-1 agonist thrombin receptor activating peptide (TRAP) is lower in dengue patients with (n=39) plasma leakage than in dengue patients without (n=38) plasma leakage. The PAC-1 expression was also lower in patients with a maximal gallbladder wall thickness (GBWT) of more than 0.50 cm (n=15) compared to a maximal GBWT of less than 0.50 cm (n=39) during the study. Activation markers were determined in citrate anti-coagulated whole blood by flow cytometry. The boxes indicate the median and interquartile ranges and the whiskers indicate 10th to 90th percentile. Differences between patients with and without plasma leakage or patients with and without a gall bladder wall thickness of 0.50 cm or more were compared with the Mann-Whitney U test. A p-value < 0.05 was considered significant; *** p < 0.001; ** p < 0.01 and * p < 0.05.
Figure 5: Decreased platelet monocyte and platelet neutrophil complexes during dengue are associated with plasma leakage. Platelet-monocyte (PMC) and platelet-neutrophil complexes (PNC) were determined in baseline (A) and TRAP stimulated (B) citrate anti-coagulated whole blood of dengue patients (n=77) and healthy controls (n=30) as the % of CD42b positive cells within the monocyte and neutrophil population, respectively. Enrolment PMC and PNC values were lower for patients with plasma leakage (n=39) than for patients without plasma leakage (n=38). The boxes indicate the median and interquartile ranges and the whiskers indicate 10th to 90th percentile. Differences between each phase of dengue were compared to healthy controls values and differences between patients with and without plasma leakage were determined by the Mann-Whitney U test. A p-value < 0.05 was considered significant; *** denotes p < 0.001; ** p < 0.01 and * p < 0.05.
pression of activated αIIBβ3, CD63 and CD62P in baseline samples. Bleeding manifestations were also not associated with expression of these platelet markers in non-stimulated and TRAP-stimulated samples (data not shown). Finally, patients with plasma leakage had significantly lower platelet counts with median value of 28 *10^9/l (16–51 *10^9/l) vs 63 *10^9/l (40–83 *10^9/l).

Reduced PMC and PNC in plasma leakage

In dengue patients, the proportion PMC and PNC were decreased at baseline (Figure 5A) and after stimulation with TRAP (Figure 5B). This decrease was especially outspoken in those with plasma leakage, in whom PMC and PNC at enrolment were significantly lower at baseline and in TRAP-stimulated samples compared to those without plasma leakage.

Concentrations of soluble platelet markers

We found that serotonin concentrations within platelets were higher in platelets from dengue patients (Figure 6). This was not associated with a detectable increase in plasma serotonin concentrations, as 70% (n=39/56) of the tested dengue samples and 100% (n=15/15) of the control plasma samples had levels below the detection limit of the assay (data not shown). Intra-platelet serotonin concentrations correlated inversely with platelet number in the critical phase of dengue (R_s = – 0.75; p<0.0001). Intra-platelet serotonin levels were not significantly different between patients with...
and without plasma leakage with median values of 5824 nmol/10¹¹ platelets (4373–9850) vs 4795 nmol/10¹¹ platelets, (3903–5484 nmol/10¹¹ platelets), respectively (p = 0.07).

The soluble platelet activation markers soluble CD62P (sCD62P), PF4 and RANTES were subsequently measured to determine recent platelet degranulation (Figure 7). Plasma levels of sCD62P were lowest in the febrile and critical phases of dengue and recovered in the convalescent phase. In contrast, levels of PF4 and RANTES were high in the febrile phase of dengue but relatively stable in the further course of the infection. None of these soluble markers were different significantly between patients with and patients without plasma leakage (data not shown).

Discussion

Our study shows that dengue is associated with platelet activation and multiple platelet function alterations, of which the reduced expression of the activated fibrinogen receptor (αIIβ₃) and the lysosomal marker CD63 following TRAP stimulation were the most striking. These platelet function defects correlated with plasma leakage, which is a cardinal feature of severe dengue. The pathogenesis of plasma leakage in dengue is multifactorial, but inflammatory cytokines are thought to play an important role. Increasing evidence supports the notion that sufficient numbers of functioning platelets are required to maintain vascular integrity during inflammation (5, 6, 10). Platelets have a dual role in endothelial integrity and can have both vascular protective and permeability-enhancing effects. Different mechanisms may therefore have contributed to the correlation between platelet function alterations and plasma leakage in our study. First, platelet granules contain multiple vasculoprotective proteins, including angiopoietin-1 and sphingosine-1 phosphate. The reduced expression of CD63 and CD62P following TRAP stimulation and the lower plasma concentrations of soluble platelet granule proteins suggests that platelets in our patients were degranulated and in a sort of ‘exhausted’ state. This may result in a local shortage of such vascular protective proteins as we have previously shown for angiopoietin-1 (8). Second, release of CD62P from platelet granules and the change in the conformation in αIIβ₃ allowing fibrinogen to bind are critical steps in thrombus formation (25–28). Platelet function defects may impair platelet adhesion and aggregation on inflamed endothelium, promoting inflammatory bleeding. Infusion of thrombin–degranulated platelets indeed did not prevent intra-tumour bleeding in thrombocytopenic mice in contrast to normal resting platelets (29). Moreover, mice lacking integrin β₃ were prone to severe bleeding during infection with lymphocytic choriomeningitis virus (30). Third, platelet activation itself may promote vascular leakage by local release of pro-permeability mediators, such as serotonin and VEGF. Platelets are the most important vehicle for serotonin in the blood and uptake of serotonin by platelets through their serotonin transporter (SERT) (31) was a prerequisite for the enhancement of microvascular permeability in a murine experimental inflammatory arthritis model, while either pharmacologic blockage of SERT or the use of SERT-deficient mice reduced leakage (32). Platelet serotonin concentrations were increased in our study patients, which may have been due to the effects of thrombocytopenia itself and the upregulation of SERT, as has been described previously in association with activation of the αIIβ₃ integrin (33). Fourth, DENV-induced platelet activation may promote leakage by activation of the NLRP3 inflammasome inside platelets leading to shedding of interleukin-β-rich microparticles, as recently shown by Hottz et al. (9). Finally, platelets interact with inflammatory cells and endothelial cells and as such promote immune responses and leukocyte infiltration in inflamed tissues (34, 35). Multiple platelet receptors are involved in platelet leukocyte interaction and leukocyte infiltration, including CD62P, GPIb and αIIβ₃ (36, 37). Platelet activation is likely to enhance infiltration of inflammatory cells, while platelet dysfunction may eventually impair clearing of DENV.

Bleeding in dengue usually manifests as skin bleeding, epistaxis and mucosal bleeding (38, 39). The majority of dengue patients in our study had some manifestation of haemorrhagic tendency, but clinically important bleeding was rare. This may explain the absence of an association between the degree of platelet hypo-responsiveness and bleeding complications in our study. In other studies, similar platelet function defects such as reduced expression of activated αIIβ₃ and CD62P were associated with a higher bleeding risk in immune-mediated thrombocytopenia (40) and in acute myeloid leukaemia (41). Pharmacological inhibition of αIIβ₃ by drugs such as abciximab are also associated with an increased risk for bleeding and CD62P-deficient mice have a prolonged bleeding time (42).

Our findings are in line with the few studies which have reported data on platelet activation and function in dengue (11–13). These studies used platelet aggregometry which is a less reliable method in thrombocytopenia. Reduced aggregation was found in
these studies, which is explained by the finding of reduced expression of activated αIIbβ3 in our study since this receptor is critical for platelet aggregation. Hottz et al. recently reported increased CD62P expression in isolated platelets of dengue patients (43) supporting our observations of platelet activation in dengue. None of these studies, however, related platelet dysfunction to plasma leakage.

The finding that PMC and PNC were lower in dengue was unexpected and is in contrast to findings by Tsai et al. (44) and Onlamoon et al. (45) who found an increase in platelet-leukocyte aggregates in humans and macaques infected with dengue, respectively. One possible explanation is that Tsai et al. used the platelet activation marker CD62P to identify PMC and PNC, whereas we used the platelet identification marker CD42b which is the abundantly present on platelets. In our study, platelet function defects and thrombocytopenia may have resulted in decreased complex formation. Another explanation may be the shedding of PSGL-1 from monocytes and neutrophils, which is known to occur when these cells are activated (46). Platelet-leukocyte complexes are generally considered a sensitive marker for platelet activation, since a small increase in CD62P expression already promotes complex formation via PSGL-1. Our present data, however, suggest that quantification of platelet-leukocyte complexes may not always be a reliable marker of platelet activation, especially in conditions with thrombocytopenia. Moreover, platelet-leukocyte interaction is important in enhancement of innate immunity (47) and removal of micro-organisms form the circulation by formation of neutrophil extracellular traps (NETs) (48).

Our study has several limitations. First, our study is observational and the association between platelet function abnormalities and plasma leakage does not does not necessarily imply causation. Second, our patients presented relatively late in our referral hospital, which limited the number of patients that could be included early in dengue infection. Third, we used only TRAP as platelet agonist. Additional platelet agonists, such as ADP and collagen-related peptide would have given a broader insight in dengue-associated platelet function abnormalities. Moreover, inclusion of samples using other anticoagulants not chelating calcium, such as direct thrombin inhibitors, and estimation of the numbers of molecules per platelet may be considered for future studies (49, 50). A particular strength of our study is the use of daily ultrasonography to reliably diagnose plasma leakage.

In conclusion, platelet activation with secondary platelet function alterations were detected in patients with dengue and these alterations correlated with plasma leakage. In case our findings are confirmed in other studies, interventions aimed at reversal of platelet activation and platelet dysfunction may be explored as new means of preventing the severe complications of dengue.

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Conflicts of interest
None declared.

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