

**Association of microvascular function and endothelial biomarkers with clinical outcome in dengue:
an observational study**

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Abstract

Background: The hallmark of severe dengue is increased microvascular permeability however alterations in the microcirculation and their evolution over the course of dengue are unknown.

Methods: We conducted a prospective observational study to evaluate the sublingual microcirculation using sidestream dark field imaging in patients presenting early (<72 hours fever) and patients hospitalized with warning signs or severe dengue in Vietnam. Clinical, microvascular function, global haemodynamics assessed by echocardiograms and serological markers of endothelial activation were performed at 4 time points.

Results: 165 patients were enrolled. No difference was found between the microcirculatory parameters comparing dengue with other febrile illnesses. Proportion of perfused vessels (PPV) and Mean Flow Index (MFI) were lower in dengue patients with plasma leakage (PPV: 88.1% vs. 90.6%, $P=0.010$ and MFI: 2.1 vs. 2.4, $P=0.007$) most marked during the critical phase. PPV and MFI correlated with the endothelial activation markers, VCAM-1 ($P<0.001$ for both) and Angiopoietin-2 ($P<0.001$ and $P=0.001$ respectively) with a negative association.

Conclusion: Modest microcirculatory alterations occur in dengue, are associated with plasma leakage and correlate with molecules of endothelial activation, Angiopoietin-2 and VCAM-1.

Introduction

Dengue is the most prevalent vector-borne viral illness globally, with an estimated annual incidence of ~96 million clinically apparent infections [1]. The majority of patients experience a self-limited febrile illness lasting 4-8 days. However, a small proportion develop potentially life-threatening complications including bleeding, organ impairment and, most importantly, plasma leakage that can result in hypovolaemic shock [2]. Plasma leakage manifests relatively late in the disease course with haemoconcentration, clinical fluid accumulation and haemodynamic compromise. However although the 2009 WHO classification highlights a set of warning signs intended to identify patients likely to progress to severe dengue, particularly shock [3], early detection of patients who develop significant vascular leakage remains challenging and more sensitive methods are needed [4].

The mechanisms underlying the vascular leak syndrome remain poorly understood, but alterations to microvascular function through modulation of the endothelial glycocalyx layer by viral and NS1 proteinbinding [5-7], and endothelial activation [8], have been implicated. Imbalance of vasoactive mediators such as TH1 cytokines, angiopoietin (Ang) 1 and 2 [9, 10], vascular endothelial growth factor (VEGF) and its receptors [11], and mast cell products [12], may also play a role. A number of novel methods to assess microvascular function have become available in recent years, including sidestream dark field imaging (SDF), a technique which assesses microcirculatory flow in real-time using videomicroscopy [13]. SDF imaging provides semi-quantitative measurement of various parameters that describe vessel density, blood flow, the proportion of perfused vessels, and heterogeneity of flow between vessels. Recent studies using this technique in sepsis and malaria have demonstrated that alterations in the microcirculation are associated with organ failure and worse patient outcomes, and have prognostic value independent of global haemodynamics [14, 15]. Mechanisms that may underlie these microcirculatory alterations include reduced perfusion pressure, endothelial dysfunction, reduced red cell deformability, and obstruction by sequestered red cells in the case of severe malaria [16].

We hypothesized that clinical assessment of microvascular function in dengue could lead to identification of early markers of vascular leakage that are associated with disease progression. To date, visualization of the microcirculation using SDF imaging has only been described in 2 patients, both with dengue shock syndrome (DSS), and showed severe microcirculatory abnormalities with reduced flow and perfusion [17]. However neither the evolution of microcirculatory alterations over the disease course, nor potential associations with clinical outcomes or endothelial activation markers have been evaluated.

Therefore we set out to investigate changes in the sub-lingual microcirculation in patients with dengue, and in a comparison group with other febrile illnesses (OFI), using SDF imaging performed at serial time-points throughout the disease course, aiming to explore associations with plasma leakage and patient outcomes. We also evaluated relationships between these microcirculatory parameters and global hemodynamics, as well as with serological markers of endothelial activation.

Methods

Clinical methods and patient recruitment

We performed a STROBE [18] compliant prospective observational study at the National Hospital for Tropical Diseases (NHTD), Hanoi, Vietnam between June 2013 and February 2014. Ethical approvals were obtained from the Oxford Tropical Research Ethics Committee and the Ethics Review Committee at NHTD, and written informed consent was obtained from all participants or the parent/guardian of children.

Adults and children above 5 years of age with a clinical diagnosis of possible dengue were eligible for enrolment into either of two study arms. In the outpatient arm, participants presenting within 72 hours of fever onset could be enrolled, if no alternative cause for the fever was identified [19]. For the inpatient arm, any patient admitted to NHTD with suspected dengue with warning signs or severe dengue was eligible [3]. All patients were reviewed daily until fully recovered and afebrile, or for up to 6 days from enrolment. Standardized clinical information was recorded daily, including detailed

clinical examination and haemodynamic assessment. A full blood count was performed daily, with additional samples obtained for a biochemical profile and dengue diagnostics at enrolment, defervescence, and at a follow-up visit 10-14 days after the illness onset. Any outpatient requiring admission continued to be followed daily in hospital, with the indication for admission documented, and all management interventions recorded. Additional investigations including ultrasound examinations and/or chest radiology were performed if clinically indicated.

Laboratory investigations

Dengue diagnostics: An NS1 test (Platelia ELISA, Bio-Rad) and commercial IgM and IgG serology assays (Capture ELISA, Panbio, Australia) were used to confirm the diagnosis on acute and convalescent plasma. In addition RT-PCR was performed on enrolment samples to identify the viral serotype and measure plasma viremia [20]. Patients were defined as having laboratory confirmed dengue if the RT-PCR, NS1 or IgM assays were positive at enrolment, or if there was IgM seroconversion between paired specimens. A diagnosis of OFI was assigned to participants with no laboratory evidence of acute or recent dengue – i.e. if they were negative for RT-PCR, NS1, and IgM/IgG on paired serology. Patients with negative tests at enrolment, but for whom convalescent plasma was not available, were considered unclassifiable.

Endothelial biomarkers: Plasma concentrations of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), VEGF, E selectin, and Ang 1 and 2 were measured at four time-points; enrolment, 24 hours later, defervescence or hospital discharge, and follow-up 10-14 days after illness onset. These tests were performed using a magnetic bead-based assay on a Luminex 200 analyzer, according to the manufacturer's specifications (R&D Systems, Abingdon, UK).

Sublingual videomicroscopy

SDF imaging was performed using a handheld videomicroscope with a X5 objective lens (Microvision Medical, Amsterdam, Netherlands) and disposable lens caps, at the same four time-points noted above. Image acquisition and subsequent analysis of the videos was performed using AVA 3.2 software following consensus guidelines described previously [21]. Briefly, video clips were obtained

by three of the investigators (SY, LL, TTT) from 3 different sublingual sites for 60 seconds, with attention to minimize pressure artefacts and reduce secretions. Videos were analysed later by SY, VM and LL who were blind to the time-point and the disease category. The software separates vessels according to their size, with small vessels defined as those < 20µm in diameter, and the total vessel density (TVD) is calculated as the number of vessels identified in the area analysed. One of the investigators then graded the average flow per quadrant using standardized techniques; (0=no, 1=intermittent, 2=sluggish and 3=normal flow), allowing for calculation of the microvascular flow index (MFI). The flow in all vessels was then categorized as normal, intermittent, or absent, and the proportion of perfused vessels (PPV) was calculated. The heterogeneity of flow was determined using the heterogeneity index (HI), (highest minus lowest MFI/mean MFI across all the sublingual sites.) Extravasated red cells (eRBC) were graded according to an internally agreed classification, as these have not been described using SDF imaging before; the presence of eRBC was first noted in each quadrant, and then scored from 0-4 with 0 indicating no eRBC seen, and 4 being eRBC noted in all quadrants. Intra and inter-user variability of the video analysis was checked at regular intervals, and was consistently <10%.

Portable echocardiography

Echocardiograms were performed at the bedside by one of the investigators (SY) on patients enrolled after September 2013, using an M-turbo Sonosite with cardiac settings. The echocardiograms were performed at the same time-points as SDF, and included two-dimensional, M-mode and Doppler studies. The following measurements were made as per standardized techniques: ejection fraction, stroke volume index and cardiac index (see Appendix 1).

Clinical endpoint definitions

The primary clinical endpoint was presence/absence of plasma leakage. A minimum dataset was required to fulfill this definition, encompassing all the following in addition to daily clinical examination; at least 3 haematocrit recordings during the acute illness with one or more value obtained during the critical period (illness day 4-6); a baseline haematocrit, being the lowest value

(in the following order) of the follow-up sample, a sample obtained within 72 hours of fever onset, or, for hospitalized patients only, the discharge sample, provided no parenteral fluid therapy was administered within the preceding 12 hours; a radiological assessment for vascular leakage within the critical period.

Participants were classified as having no evidence of leakage if the percentage haemoconcentration $((\text{peak minus baseline haematocrit/baseline hematocrit}) \times 100)$ was less than 15% and there were no clinical or radiological signs of leakage. Conversely, if the percentage haemoconcentration was 15% or more, or any radiological or clinical signs of fluid accumulation were identified, the individual was defined as having significant vascular leakage. We also carried out a sensitivity analysis omitting the requirement for a radiological examination, assuming that where such a test was not deemed necessary by the treating clinician it would be likely to be negative.

Other outcomes assessed included presence/absence of mucosal bleeding and overall dengue severity using the WHO 2009 classification; detailed information on the criteria for this is included in the supplementary Appendix.

Statistical analysis

Data are presented as frequency (percentage) for categorical variables and median (interquartile range) for continuous parameters. All analyses were defined *a priori* in a written analysis plan. First, comparisons of microcirculatory parameters were performed between confirmed-dengue and OFI patients in the outpatient arm. Among confirmed-dengue patients in both study arms, associations with plasma leakage and bleeding were then explored. All analyses were based on logistic regression models with dengue diagnosis, plasma leakage, or bleeding as the outcome of interest, and the microcirculatory/vascular parameters as covariates. For each covariate, an initial comparison with the outcome of interest used all measurements from the various time-points assessed apart from the follow-up values; subsequently separate analyses were performed for each disease phase: early, day of illness 1-3; critical, day of illness, 4-6; recovery, day of illness 7-13; and follow-up, after 14 days or more.

As most patients had multiple microcirculatory measurements during the study, potentially with more than one measurement within each disease phase, robust sandwich standard errors based on working independence covariance structure were used throughout. Comparisons were adjusted for day of illness, age, sex, and hospitalization, as appropriate.

Associations between microcirculation parameters, serological endothelial biomarkers and haemodynamics measurements in patients with dengue were assessed by partial correlations controlling for the following potential confounding variables: age, sex and day of illness of measurement. Significance of partial correlations was assessed based on their Fisher transformation and corresponding bootstrap standard errors. The cluster bootstrap which resamples patients rather than individual parameter values accounted for multiple measurements per patient.

To informally adjust for multiplicity, a significance level of 0.01 was used for all comparisons. All analyses were performed with the statistical software R version 3.2.2 and the companion package geepack version 1.2-0.

Results

A total of 165 patients were enrolled, 91 in the outpatient arm and 74 in the inpatient arm (Figure 1, Table 1). After excluding 16 patients who withdrew or had an inconclusive diagnosis, 149 patients (79 and 70 in the outpatients and the inpatient arm, respectively) were included in the analysis. Among the outpatients, 63 participants had laboratory confirmed dengue while 16 were assigned a diagnosis of OFI; in the inpatient arm there were 69 confirmed dengue cases, and 1 OFI. Twenty-six outpatients were subsequently hospitalized (24 with confirmed-dengue and 2 OFI), but all participants recovered fully in the end. Considering all study participants, 50% were female and the median age was 26 (IQR 21-35) years. Inpatients were generally recruited slightly later in the illness course than outpatients, which likely explains the greater derangements observed in the laboratory parameters measured at enrolment. Using the 2009 WHO classification, among the 132 confirmed dengue cases there were 57 (43%) with dengue, 68 (52%) dengue with warning signs and 7 (5%)

severe cases (Table 1). Of the 106 patients that were PCR positive, the serotypes identified were as follows; 21 DENV-1 (20%), 18 DENV-2 (17%), 35 DENV-3 (33%) and 32 DENV-4 (30%).

Associations between microcirculatory parameters and dengue diagnosis

This analysis was limited to participants enrolled in the outpatient arm of the study. No differences were identified between the microcirculatory variables (small vessel TVD, PPV, MFI and HI) between the confirmed-dengue (59 participants) and OFI (15 participants) patient groups when considered overall, or during any of the individual time-periods (Table 2). However, there was a trend towards lower MFI in the OFI compared to the dengue patients during the critical phase ($P=0.045$). Time trends for perfusion, and mean flow are shown in Figure 2A. Considering all the time-points, a higher proportion (33/141, 23%) of dengue patients had eRBC seen in one or more of the video quadrants, but this was not significantly different to the findings in the OFI group (3/20, 15%). Collectively, these data indicate similar patterns of microcirculatory disturbance in the dengue and OFI patient groups.

Associations between microcirculatory parameters and clinical endpoints in confirmed-dengue patients.

With respect to plasma leakage, the primary analysis included all confirmed-dengue patients in both arms who had full clinical and radiological assessments for leakage (Table 3). There were no differences identified in total small vessel density (TVD) between the patients with and without plasma leakage at any of the time-points. However, the proportion of perfused small vessels (PPV) over all time-points was lower in patients with plasma leakage (median PPV: 88.1% vs. 90.6%, $P=0.010$), most markedly during the critical phase (Table 3, Figure 2B). Mean flow index (MFI) was also lower in the patients with plasma leakage compared to those without when considered overall (2.1 vs. 2.4, $P=0.007$). The heterogeneity index (HI) was greater for patients with plasma leakage compared to those without leakage (Table 3). The associations between plasma leakage and PPV, but not between plasma leakage and MFI or HI, were confirmed in the sensitivity analysis using the larger cohort classified for plasma leakage using clinical and haematocrit information only

(Supplementary data, Table 5). In terms of prediction, the MFI on day 3 of illness was associated with subsequent plasma leakage (OR = 0.39, 95% CI 0.08-0.94 per unit increase in the flow index), although the association did not reach significance at the predefined 1% significance level ($P=0.034$) (Supplementary data Table 6). The other microcirculatory parameters of TVD, PPV, HI and eRBC on days 1-3 were not predictive.

With respect to bleeding, we found no associations between any of the microcirculatory variables and mucosal bleeding, except a trend for more patients with mucosal bleeding to have eRBC seen during the early phase (9/24, 38% vs. 8/42, 19% among patients with and without mucosal bleeding respectively) (Supplementary data, Table 7). Collectively, these results indicate that although microcirculatory indices of perfusion and flow were worse in patients with plasma leakage than in those without evidence of leakage, the differences observed were relatively minor.

Associations between microcirculation parameters, serological endothelial biomarkers and global haemodynamics.

Although plasma levels of VCAM-1 were raised, and the ratio of Ang-2/Ang-1 was increased during the acute dengue illness, there were no statistically significant differences between patients with and without plasma leakage (Figure 3). Nor were there differences between VEGF and E-selectin levels between patients with and without plasma leakage (data not shown).

Amongst patients with dengue, there was a negative correlation of PPV and MFI with molecules associated with endothelial activation; VCAM-1 (partial correlations: -0.45, $P<0.001$ and -0.46 $P<0.001$ respectively) and Ang-2 (partial correlations: -0.33, $P<0.001$ and -0.29 $P=0.001$). No associations were identified between the microcirculatory parameters assessed and the other endothelial biomarkers including ICAM-1, E-selectin, VEGF, and Ang-1 (Table 4).

We found no correlations between the microcirculatory variables and global haemodynamics assessed contemporaneously, such as pulse, mean arterial blood pressure, stroke volume or cardiac

index. Collectively, these results indicate microcirculatory parameters of flow and perfusion correlate negatively with endothelial activation markers, but not with global haemodynamics.

Discussion

We have shown that modest microcirculatory disturbances occur in dengue, with a reduction in blood flow and perfusion indices that was most marked in patients with plasma leakage during the critical phase of the disease. MFI and PPV were lower in patients with plasma leakage than those without when assessed overall during the acute illness, and the reduction in PPV was borderline significant during the critical phase (illness day 4-6), but not earlier in the febrile phase. These findings are in agreement with the only other published report assessing the microcirculation in dengue, which showed altered perfusion and flow during the critical phase in 2 severe dengue cases [17]. In addition to flow disturbances, we have shown that extravasated RBCs were visible in a quarter of the dengue patients assessed, a finding not previously reported in SDF studies. However, unlike flow disturbances the presence of eRBC was not associated with the clinical outcomes of interest.

Interestingly, these microcirculatory changes do not appear to be exclusive to dengue, as similar disturbances were seen in the OFI group. Specific diagnoses were not established for the entire OFI group, but it included a mixture of other viral illnesses such as influenza, measles and acute hepatitis, as well as some bacterial infections including scrub typhus. Microcirculatory dysfunction has been demonstrated in numerous studies of severe infections including sepsis [14], and severe influenza [22], but this study demonstrates that some degree of microcirculatory change likely also occurs in less severe infections. The majority of microcirculatory studies have been performed in bacterial sepsis and, similar to our findings, PPV was found to be the most robust parameter associated with outcome [23], although higher HI indices were also associated with more severe disease [24].

Our results suggest that microcirculatory parameters assessed in the early febrile phase of dengue have limited prognostic potential, as the flow abnormalities generally occurred later in the disease course and lower MFIs on day 3 were only weakly associated with subsequently developing plasma leakage in the critical phase. This is also in agreement with one study performed in early sepsis, where microcirculatory abnormalities were not observed in the early phase of low acuity sepsis [25]. The mechanisms underlying the microcirculatory abnormalities observed in dengue may relate to increased blood viscosity, causing capillary flow to become more sluggish as plasma leakage progresses and haemoconcentration occurs. In addition endothelial activation may contribute to the flow disturbances; of the endothelial activation markers we investigated, VCAM-1 and Ang-2 were negatively associated with small vessel MFI and PPV. A correlation between VCAM-1 and perfusion/flow parameters has previously been described in paediatric meningococcal sepsis [26]. The causality of the association between VCAM-1 and flow disturbances may be bidirectional, as the expression of VCAM-1 is known to be up-regulated in low blood flow and shear stress states [27], but also VCAM-1 mediated increases in endothelial cell-leukocyte adhesion may interfere with capillary flow. Ang-2 is known to be up-regulated in vessels with low flow states [28], and has been implicated in the haemodynamic and microvascular alterations seen in animal models of sepsis [29], and in pulmonary capillary leak in human sepsis [30].

Evidence from human studies and animal models of sepsis also suggests the microcirculation functions independently of systemic haemodynamics, with derangements demonstrated despite normal mean arterial pressures (MAP) and cardiac index [31, 32]. Our data confirms this independence since we found no associations between the microcirculatory parameters and MAP, cardiac output or stroke volume. This uncoupling of micro and macro-circulations, together with the observation that microcirculatory parameters correlate more closely with markers of tissue perfusion like lactate, has encouraged critical illness researchers to consider using the microcirculation as a target for goal directed therapy [33]. Fluid resuscitation has been shown to increase the proportion of perfused vessels and flow in early but not late septic shock, independent

of MAP, CI and type of fluid used [34]. Other attempts to improve the microcirculation in sepsis have included use of therapeutics such as activated protein C [35], and corticosteroids [36]. However larger randomised controlled trials are needed to link these microcirculatory improvements with better organ function and improved patient outcomes in severe infections.

There were several limitations of this study. First, in the inpatient arm the patients were often enrolled several hours after fluid therapy had been commenced, potentially resulting in underestimation of the microcirculatory changes. Second, prediction modelling was limited by the small number of patients experiencing a severe outcome in the outpatient arm. As patients were generally admitted to hospital later in the disease course, early assessments (within 72 hours of fever onset) were rarely available for these participants, making comparisons with the outpatients difficult. Lastly, due to a certain level of cooperation required by patients undergoing sublingual videomicroscopy, we were unable to study younger children; this group are at greatest risk for severe plasma leakage, shock and poor outcomes [37], and may have more marked microcirculatory abnormalities.

In summary, this study has identified moderate abnormalities in microcirculatory flow and perfusion in dengue patients, with the most severe disturbances seen in patients with plasma leakage during the critical phase of the disease. These disturbances were poorly specific, however, since similar changes occurred in patients with OFI. Perfusion indices correlated negatively with VCAM-1 and Ang 2, suggesting endothelial activation may underlie the flow disturbances observed in dengue.

Although microcirculatory assessment in early dengue is unlikely to be useful for risk prediction, future studies should be considered in established severe dengue to evaluate the utility of including microcirculatory perfusion outcomes alongside other haemodynamic endpoints in clinical trials assessing novel therapeutic strategies.

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Conflict of Interest

We declare no conflict of interest

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Figure 1. Study flow chart

Figure 2A. Scatter plot showing the proportion of perfused vessels (top panel), and mean flow index (bottom panel), by day of illness in patients with confirmed-dengue and other febrile illness.

Figure 2B. Scatter plot showing the proportion of perfused vessels (top panel) and mean flow index (bottom panel) by day of illness in patients with confirmed dengue with and without plasma leakage (primary endpoint definition)

The short grey line represents the median value of the parameter during each illness phase. The numbers at the bottom of the figures represent the number of individuals that contributed to that group (each individual could have more than 1 measurement during the illness phase). Black dots represent values from patients who developed dengue shock syndrome.

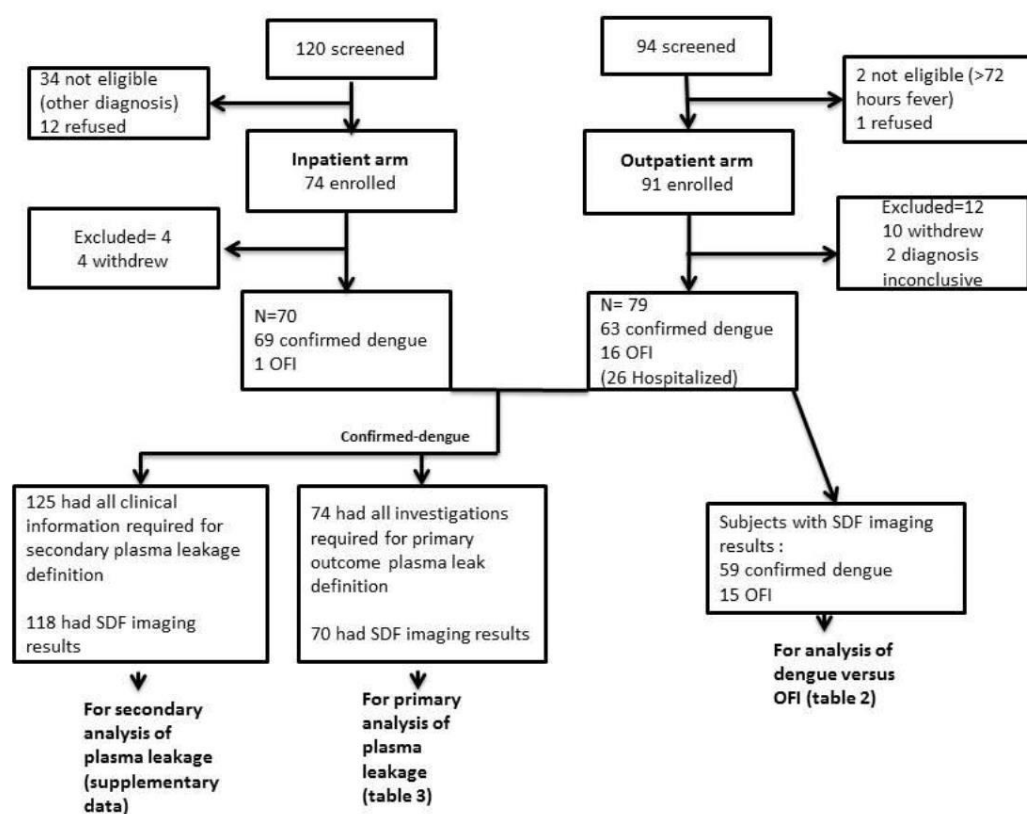
2A. There were no significant differences between the dengue and OFI groups for either PPV or MFI.

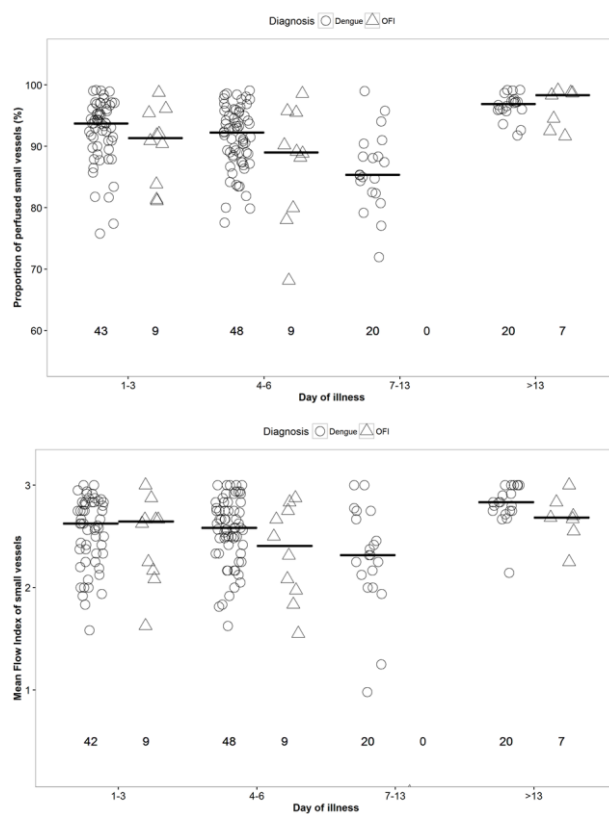
2B. There was a significant difference between patients with and without plasma leakage during the acute illness (days 1-13) for both PPV ($P=0.010$) and MFI ($P=0.007$).

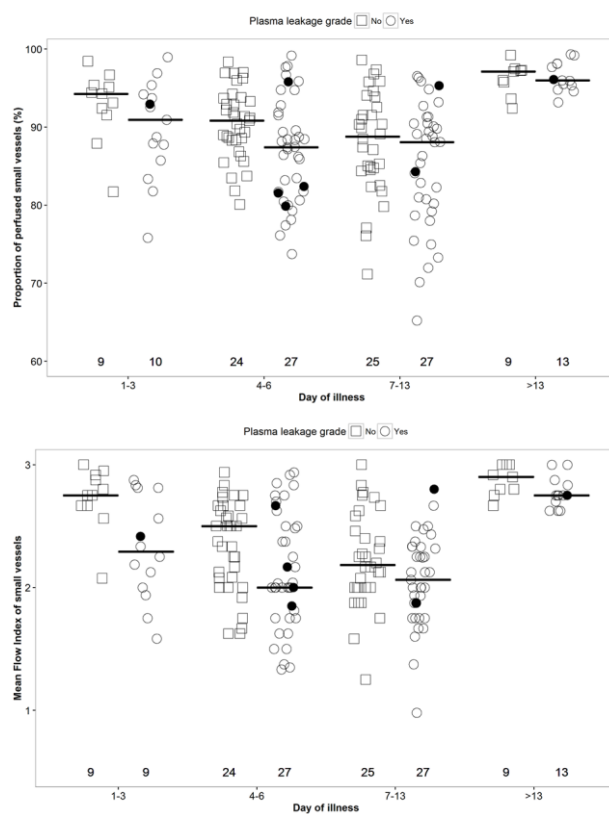
Figure 3. Scatter plot showing VCAM-1 (top panel) and angiopoietin2/1 ratio (bottom panel) by day of illness in patients with confirmed dengue with and without plasma leakage (primary endpoint definition)

The short grey lines represent the median value of the parameter of interest during each illness phase. Black dots represent values from patients who developed dengue shock syndrome. The numbers at the bottom of the figures represent the numbers of individuals that contributed to the measurements in that illness phase.

There were no significant differences between VCAM-1 and Ang2/1 for patients with and without plasma leakage.







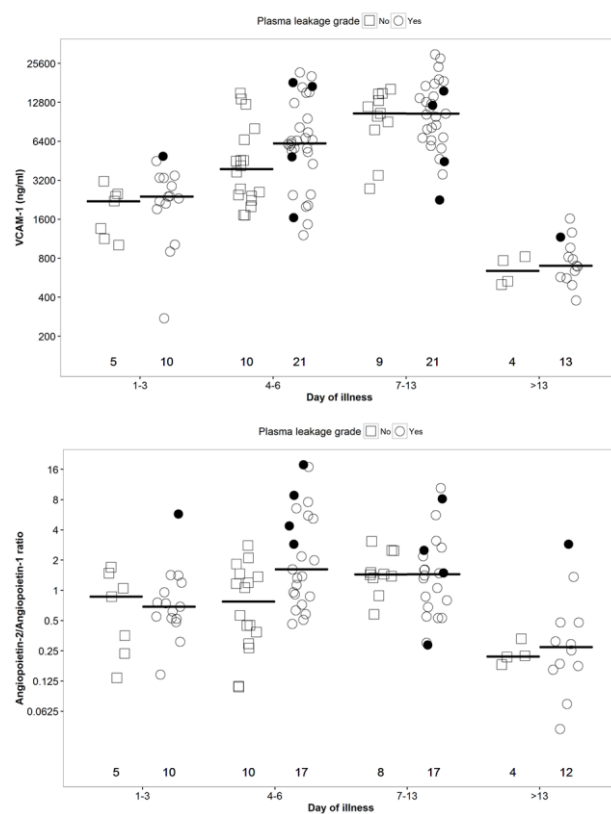


Table 1. Baseline characteristics and clinical outcomes of participants

	All patients (n=149)		Inpatient (n=70)		Outpatient (n=79)	
	n		n		n	
Baseline characteristics						
Age (years)	149	26 (21-35)	70	28.5 (21-36)	79	26 (21-33)
Male sex	149	75 (50)	70	36 (51)	79	39 (49)
Illness day at enrolment	149	3 (3-5)	70	5 (4-6)	79	3 (2-3)
Confirmed-dengue	149	132 (89)	70	69 (99)	79	63 (80)
Other febrile illness	149	17 (11)	70	1 (1)	79	16 (20)
Platelet count (10 ⁹ /L)	144	121 (61-161)	67	58 (31-108)	77	151 (126-183)
White blood cells(10 ⁹ /L)	144	3.9 (2.7-5.7)	67	3.4 (2.5-4.6)	77	4.5 (3.0-6.5)
Albumin (g/L)	96	44 (40-48)	24	39 (36-45)	72	45 (42-48)
AST (U/L)	116	34 (25 -57)	40	62 (33-117)	76	32 (23-41)
Clinical outcomes						
Primary endpoint plasma leakage-yes ¹	74	38 (51)	47	27 (57)	27	11 (41)
Clinical definition plasma leakage-yes ²	125	30 (24)	63	21 (33)	62	9 (15)
Mucosal bleeding	132	59 (45)	69	39 (56)	63	20 (32)
Dengue severity ³	132		69		63	
- Dengue		57 (43)		19 (28)		38 (60)
- With warning signs		68 (52)		45 (65)		23 (37)
- Severe		7 (5)		5 (7)		2 (3)

Summary statistic is absolute count (%) for categorical variables and median (IQR) for continuous data, AST: aspartate transaminase. n refers to number of subjects with complete data for the respective characteristic

¹Plasma leakage, primary endpoint definition based on full clinical and radiological information, ² Plasma leakage based on clinical and routine haematology data only. ³ Dengue severity according to the WHO 2009 classification.

Table 2. Small-vessel microvascular variables in study participants with confirmed-dengue versus other febrile illness, by disease phase

	Other Febrile Illness			Dengue			OR	(95% CI)	p value
	n	N		n	N				
Total vessel density (mm/mm³)	15	20	14.2 (12.6-15.5)	59	143	14.1 (12.6-15.4)	0.92	(0.74-1.16)	0.493
Day 1-3	9	10	14.0 (13.1-15.3)	43	58	13.4 (12.2-15.3)	0.92	(0.69-1.22)	0.554
Day 4-6	9	10	14.3 (12.6-15.6)	48	65	14.1 (12.0-15.3)	0.96	(0.68-1.36)	0.810
Day 7-13	0	0	-	20	20	15.3 (14.3-16.0)	-	-	-
Day >13	7	7	14.6 (13.0-16.4)	20	20	15.8 (14.5-17.4)	1.22	(0.70-2.12)	0.485
Proportion of perfused vessels (%)	15	20	90.3 (83.2-95.5)	59	143	92.4 (87.9-95.5)	1.08	(0.99-1.17)	0.085
Day 1-3	9	10	91.3 (85.5-94.6)	43	58	93.7 (90.2-96.1)	1.07	(0.94-1.22)	0.301
Day 4-6	9	10	89.0 (82.0-94.2)	48	65	92.2 (88.5-95.5)	1.11	(0.98-1.27)	0.105
Day 7-13	0	0	-	20	20	85.3 (82.5-88.9)	-	-	-
Day >13	7	7	98.3 (93.5, 98.7)	20	20	96.9 (96.0-97.5)	1.12	(0.74-1.70)	0.601
Mean flow Index	15	20	2.6 (2.1, 2.7)	59	142	2.6 (2.3-2.8)	1.27	(0.98-1.64)	0.071
Day 1-3	9	10	2.6 (2.2, 2.7)	42	57	2.6 (2.3-2.8)	1.16	(0.70-1.92)	0.574
Day 4-6	9	10	2.4 (2.0, 2.7)	48	65	2.6 (2.4-2.8)	1.69	(1.01-2.84)	0.045
Day 7-13	0	0	-	20	20	2.3 (2.1-2.7)	-	-	-
Day >13	7	7	2.7 (2.6, 2.8)	20	20	2.8 (2.8-3.0)	2.45	(0.54-11.07)	0.246
Heterogeneity Index	14	19	0.1 (0.1, 0.2)	59	138	0.2 (0.1-0.3)	1.04	(0.80- 1.36)	0.768
Day 1-3	8	9	0.2 (0.1, 0.3)	41	56	0.2 (0.1-0.3)	1.00	(0.68- 1.46)	0.983
Day 4-6	9	10	0.1 (0.1, 0.2)	48	65	0.2 (0.1- 0.3)	1.01	(0.65- 1.58)	0.959
Day 7-13	0	0	-	17	17	0.2 (0.1-0.4)	-	-	-
Day >13	6	6	0.1 (0.0, 0.2)	19	19	0.1 (0.0-0.1)	0.35	(0.10, 1.22)	0.099
eRBC score >0	14	20	3 (15)	59	141	33 (23)	1.60	(0.31, 8.28)	0.576
Day 1-3	9	10	2 (20)	42	56	13 (23)	1.24	(0.22, 7.18)	0.807
Day 4-6	8	9	1 (11)	48	65	13 (20)	1.95	(0.24, 16.06)	0.536
Day 7-13	1	1	0 (0)	20	20	7 (35)	-	-	-
Day >13	6	6	1 (17)	20	20	0 (0)	-	-	-

Data are presented as absolute count (%) for categorical variables and median (IQR) for continuous data. n corresponds to number of participants, N corresponds to number of measurements. eRBC: extravasated red blood cells, OR: odds ratio, CI: confidence interval.

For each variable, the highlighted rows (bolded) corresponds to the overall comparison which included all values except for values on day of illness > 14, and were adjusted for age, sex and day of illness. Other rows correspond to comparisons for each day of illness category, and were adjusted for age, sex. All comparisons were based on generalized estimating equations with independence covariance structure to take into account multiple measurements per patient. The OR describes the predicted change in the odds of a dengue diagnosis corresponding to an increase of 1mm/mm³ in TVDs, 1% in PPVs, 0.25 unit in MFIs (or increase by 1 unit in the total flow index from all 4 quadrants), 0.1 unit in HI, having positive eRBC score.

Table 3. Small-vessel microvascular variables in dengue patients with and without plasma leakage (primary endpoint definition)

Characteristic	No leakage			Plasma leakage			OR	(95% CI)	p value
	n	N	Summary statistic	n	N	Summary statistic			
Total vessel density (mm/mm³)	33	78	14.5 (13.3-5.3)	37	91	13.3 (11.8-15.0)	0.82	(0.68-0.98)	0.029
Day 1-3	9	11	14.7 (13.3-16.2)	10	15	13.4 (11.6-14.7)	0.74	(0.51-1.07)	0.108
Day 4-6	24	35	14.7 (13.5-15.4)	27	39	13.1 (11.6-14.9)	0.84	(0.65-1.10)	0.207
Day 7-13	25	32	14.4 (13.4-15.2)	27	37	13.6 (12.2-15.1)	0.85	(0.67-1.08)	0.193
Day >13	9	9	17.2 (15.7-17.5)	13	13	14.9 (14.2-15.8)	0.46	(0.18-1.19)	0.111
Proportion of perfused vessels (%)	33	78	90.6 (85.8-94.0)	37	91	88.1 (81.6-92.2)	0.94	(0.89-0.98)	0.010
Day 1-3	9	11	94.3 (92.0-95.3)	10	15	90.9 (86.7-94.0)	0.93	(0.77-1.13)	0.489
Day 4-6	24	35	90.8 (88.3-93.5)	27	39	87.4 (81.6-91.7)	0.89	(0.81-0.98)	0.018
Day 7-13	25	32	88.8 (84.5-92.9)	27	37	88.1 (80.2-90.5)	0.96	(0.91-1.02)	0.162
Day >13	9	9	97.1 (95.7-97.3)	13	13	96.0 (95.3-98.1)	1.53	(0.90-2.62)	0.119
Mean Flow Index	33	78	2.4 (2.1-2.7)	37	90	2.1 (1.9-2.4)	0.75	(0.61-0.92)	0.007
Day 1-3	9	11	2.8 (2.7-2.9)	9	14	2.3 (2.0-2.8)	0.24	(0.05-1.25)	0.090
Day 4-6	24	35	2.5 (2.1-2.6)	27	39	2.0 (1.8-2.5)	0.72	(0.51-1.02)	0.063
Day 7-13	25	32	2.2 (2.0-2.5)	27	37	2.1 (1.9-2.3)	0.81	(0.58-1.11)	0.189
Day >13	9	9	2.9 (2.8-3.0)	13	13	2.8 (2.7-2.8)	0.12	(0.01-1.33)	0.084
Heterogeneity Index	33	69	0.2 (0.1-0.2)	37	81	0.2 (0.1-0.3)	1.33	(1.07-1.66)	0.010
Day 1-3	8	10	0.2 (0.1-0.2)	9	14	0.3 (0.2-0.4)	2.02	(0.95-4.31)	0.069
Day 4-6	22	31	0.2 (0.1-0.3)	27	35	0.2 (0.1-0.3)	1.02	(0.74-1.39)	0.910
Day 7-13	21	28	0.2 (0.1-0.2)	25	32	0.3 (0.1-0.4)	1.39	(0.97-1.99)	0.073
Day >13	7	7	0.1 (0.0-0.1)	10	10	0.1 (0.0-0.1)	0.18	(0.0-11.65)	0.422

Summary statistic is absolute count (%) for categorical variables and median (IQR) for continuous data. n corresponds to number of participants, N corresponds to number of measurements. Day: day of illness. For each variable, the highlighted rows (bolded) correspond to the overall comparison which included all values except for values on day of illness > 14, and was adjusted for age, sex, hospitalization and day of illness. Other rows correspond to comparisons for each day of illness group, which included all values obtained during that time-period. All comparisons were based on generalized estimating equations with independence covariance structure to take into account multiple measurements per patient. The OR describes the predicted change in the odds of plasma leakage corresponding to an increase of 1mm/mm³ in TVDs, 1% in PPVs, 0.25 unit in MFIs (or increase by 1 unit in the total flow index from all 4 quadrants), 0.1 unit in HI

Table 4. Partial correlation between small-vessel microcirculatory variables, endothelial biomarkers and global haemodynamics in patients with dengue

	TVD		PPV		MFI	
	rho	p value	rho	p value	rho	p value
Serological endothelial biomarkers						
Log2 VEGF	-0.254	0.061	-0.156	0.248	-0.030	0.838
Log2 ICAM-1	-0.093	0.297	-0.136	0.178	-0.083	0.455
Log2 VCAM-1	-0.097	0.275	-0.448	<0.001	-0.464	<0.001
Log2 E-selectin	0.165	0.149	0.069	0.565	-0.002	0.985
Log2 Angiopoietin 1	0.160	0.119	0.114	0.248	0.123	0.208
Log2 Angiopoietin 2	0.042	0.611	-0.332	<0.001	-0.290	0.001
Global haemodynamics parameters						
Pulse rate	-0.019	0.797	-0.005	0.935	0.093	0.145
Mean arterial blood pressure	-0.036	0.620	0.074	0.354	-0.015	0.848
Stroke volume index	-0.161	0.163	0.096	0.443	0.122	0.444
Cardiac Index	-0.009	0.939	0.167	0.146	0.256	0.024

VEGF: vascular endothelial growth factor, ICAM-1: Intercellular adhesion molecule 1, VCAM-1: Vascular cell adhesion molecule 1. Rho is the partial correlation coefficient between the respective two parameters of interest controlling for age, sex and day of illness of measurement. Significance of partial correlations was assessed based on their Fisher transformation and corresponding bootstrap standard errors.