Association Between Increased Vascular Nitric Oxide Bioavailability and Progression to Dengue Hemorrhagic Fever in Adults

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In a prospective longitudinal adult study, vascular nitric oxide bioavailability measured as reactive hyperemia index was significantly higher at enrollment in patients who developed dengue hemorrhagic fever (DHF) (n = 11), compared with the non-DHF group (n = 63) and those with other febrile illnesses (n = 25) (P = .01). After adjustment for age, fever day, and body mass index, enrollment reactive hyperemia index was associated with a 4-fold increased risk for DHF, and predicted DHF with an area under the receiver operating curve of 0.86. Increased vascular nitric oxide in dengue is associated with increased vascular permeability and impaired homeostasis and may have utility as a predictor of DHF.

Keywords. adult; dengue hemorrhagic fever; endothelial function; nitric oxide; noninvasive; peripheral arterial tone; reactive hyperemia index; vascular.

Dengue fever (DF) results from infection with dengue viruses from the family Flaviviridae, transmitted by the Aedes mosquito [1]. Urbanization, global warming, and lack of effective vaccines or antiviral drugs have caused dengue to be a major global public health threat [1]. It is estimated that almost 2.5 billion persons or half the world’s population are at risk, and annually there are approximately 390 million infections, resulting in 500,000 severe cases with a mortality rate of about 2.5% [1].

The diagnosis of dengue has improved significantly with new rapid diagnostic tools. However, despite numerous studies, prediction of severe disease remains challenging [1]. In adult and pediatric studies, abdominal pain, vomiting, fever day, hepatomegaly, hematocrit >50%, leukopenia, platelet counts <75,000/mm³ and proteinuria were predictive of severe disease [2, 3]. Other studies found increased viral load and inappropriate activation of the host immune response were predictors [4]. However, these require specialized laboratory assays and further clinical validation, with none performed at the bedside or in the clinic.

Vietnamese children with dengue hemorrhagic fever (DHF) and dengue shock syndrome had short-lived periods of increased microvascular permeability [5]. However, the pathogenesis of vascular leak remains to be delineated, with increased proinflammatory cytokines and angiogenic factors postulated to play a role [1]. In vitro and in vivo animal studies have shown that nitric oxide (NO) produced by endothelial NO synthase (eNOS) modulates vascular permeability during acute inflammation [6]. Clinical studies in dengue that measured NO have shown conflicting results; South American study showed increased NO in patients with DF compared with patients with DHF and controls [7], whereas Thai children with DF had decreased NO compared with healthy controls [8]. However, these studies measured total NO, with the major contribution from immune cells compared with endothelium. The half-life of NO is short, most of the physiological effects are from local sources, and the above-mentioned studies did not assess vascular NO bioavailability and the effect on endothelial homeostasis and permeability in dengue. Endothelial dysfunction is observed in systemic infections such as malaria and bacterial sepsis [9]. However, to our knowledge, vascular NO bioavailability and the association with DF and DHF has not been evaluated.

Using a portable device, we undertook a prospective longitudinal study and measured endothelial function, a measure of vascular NO bioavailability, at the bedside or clinic in patients with dengue and those with other febrile illness (OFI). We hypothesize that endothelial function (1) will be decreased during acute infection and will increase during recovery and (2) may be a predictor of disease severity.

METHODS

Acutely febrile patients were recruited prospectively from August 2011 to September 2012 at the Communicable Disease
Centre, Tan Tock Seng Hospital, Singapore. Details of the study have been described elsewhere [10]. Adult patients (aged ≥18 years) who presented with acute undifferentiated fever were recruited, and dengue infection was confirmed by reverse-transcription polymerase chain reaction (PCR) or non-structural protein 1 (NS1) antigen positivity. Dengue-negative patients (OFI group) had neither confirmed dengue nor probable dengue. Patients with a history of potential confounders, such as cardiovascular or peripheral vascular disorders, were excluded. Demographic data was collected at enrollment, with clinical and laboratory details recorded daily until clinical recovery and discharge and at a convalescent visit (21–35 days after enrollment). The decision for admission was made by nonstudy physicians based on hospital guidelines.

Patients with DHF had all 4 of the required World Health Organization (WHO) 1997 criteria: fever (≥37.5°C), bleeding manifestations (petechiae, purpura, mucosal or gastrointestinal bleeding), plasma leakage (rise in hematocrit ≥20% above the mean for age, sex, and population), and low platelet counts (<100 000/µL). Viral load (the inverse of the cycle threshold) was measured by means of PCR at the Environmental Health Institute, Singapore, WHO collaborating Center for Reference and Research on Arboviruses [10]. This study was approved by the Domain Specific Review Board, National Healthcare Group, Singapore (DSRB/E/09/575). Written informed consent was obtained from every patient.

**RESULTS**

We enrolled 110 patients, 74 (67%) with laboratory-confirmed dengue and 25 (23%) classified as having OFI. Eleven patients (10%) classified as having probable dengue clinically but negative for dengue by either reverse-transcription PCR or NS1 tests were excluded in subsequent analyses. The proportions with ≥1 follow-up visit were 90.9% in the DHF group, 84.1% for non-DHF, and 72% for OFI.

Among the confirmed dengue cohort (n = 74), almost all (99%) were male, 67% were of Chinese ethnicity, and 24% were hospitalized (Table 1). Only 1 had an existing comorbid condition (ie, diabetes mellitus). The median age in this group was 35 years (5th–95th pctl, 22–47 years) and the median duration of fever at enrollment was 6 days (5th–95th pctl, 3–10 days). Patients with OFI (n = 25) had characteristics similar to those of patients with dengue, but the proportion of Chinese ethnicity was smaller (Table 1). Of 11 patients (15%) who fulfilled DHF criteria during the study, 3 had DHF diagnosed at the enrollment visit. The DHF group (n = 11) included older patients, more Chinese patients, and more inpatients than the non-DHF group (n = 63) (Table 1).

At enrollment, RHIs of patients who had or subsequently developed DHF were significantly higher than those in the OFI and non-DHF groups (medians, 2.7, 2.19, and 2.03, respectively; \( P = .01 \)). There was no difference in RHI between the patients with DHF at enrollment, and those whose DHF was diagnosed later. Paired comparison showed that RHI at enrollment differed significantly between DHF and non-DHF groups (\( P < .01 \)) but not between all patients with dengue and those with OFI. At the convalescent visit, the RHI trend remained highest in DHF group, followed by the OFI and non-DHF groups, although this difference was not significant in group-wide (median, 2.38, 2.31, and 1.97, respectively; \( P = .22 \)) or pairwise comparisons (Table 1).

Longitudinal mixed effects modeling did not show any significant difference in RHI between the enrollment, follow-up, and convalescent visits for patients with DHF (\( P = .49 \)) or those in the non-DHF group (\( P = .33 \)). However, there was a significant increase in the OFI group with clinical recovery (\( P = .01 \)). There was no difference in viremia (inverse of the cycle threshold) at enrollment between patients with DHF (n = 7; median, 3.62 \times 10^{-2}; 5th–95th pctl, 3.17–5.60) and those with non-DHF (n = 25; median, 3.64 \times 10^{-2}; 5th–95th pctl, 3.2–5.6) (\( P = .84 \)), and there regression was used to determine whether RHI is predictive of developing DHF, with adjustment for age, body mass index, and fever day. Longitudinal trends were estimated using a linear mixed effects model or paired \( t \) tests. All statistical tests were carried out in R version 15.3 (R Foundation for statistical computing, Vienna, Austria) and Stata version 13 (Stata Corp., College Station, Texas) with 5% level of significance.

**Statistical Analysis**

For descriptive analyses, numbers and percentage were used for categorical variables, and medians (5th–95th percentile [pctl]) for continuous variables. The Kruskal–Wallis test was used to assess the differences in RHI across the 3 groups (ie, DHF, non-DHF and OFI groups). Fisher exact or Mann–Whitney \( U \) tests were used to assess the differences between paired groups (ie, DHF and non-DHF, all dengue and OFI) (Table 1). Cox
was no association with RHI. There was also no association either cross-sectionally or longitudinally between RHI and systolic/diastolic blood pressure or mean arterial pressure.

A multivariate Cox regression model using RHI at the enrollment visit was found to be predictive of DHF after adjustment for age, body mass index, and fever day (adjusted odds ratio, 4.15; 95% CI, 1.32–13.03), with an area under receiver operating curve of 0.86 (Table 2 and Supplementary Figure 1B). The sensitivity and specificity for the development of DHF were 0.56 and 0.79, respectively, at an RHI of 2.5, 0.56 and 0.84 at 2.6, and 0.56 and 0.85 at 2.7.

**DISCUSSION**

In contrast to our hypothesis, patients who developed DHF during follow-up had increased baseline vascular NO bioavailability compared with patients with uncomplicated DF or OFI.

Longitudinally, vascular NO bioavailability showed no significant change in either the patients with DHF or those without DHF, but it was increased in the OFI group. With RHI used as a surrogate for NO bioavailability, increased RHI was associated with a 4-fold greater odds of developing DHF. These results suggest that vascular NO may contribute to impaired vascular homeostasis or leakage and DHF in adults with dengue. Endothelial function measured noninvasively as RHI may also be a prognostic marker for the development of DHF with an area under the receiver operating curve of 86%.

The results are in contrast to findings in other acute febrile infections, such as malaria and bacterial sepsis, where impaired vascular NO bioavailability was demonstrated [9]. In mice infected with dengue, those with inducible NOS deficiency or treated with NG-monomethyl-L-arginine, a NOS inhibitor, had decreased endothelial cell death and hemorrhage [11]. This is consistent with findings from Brazil, where patients with DHF or DF had increased intraplatelet NO production with impaired platelet aggregation, but no increase in protein levels of eNOS or inducible NOS compared with controls [12]. Platelet NO production was not reduced with use of NOS inhibitors in patients with DHF but was decreased in controls [12]. This suggests that the mechanisms affecting NOS activity in dengue may be related to mechanisms involving increased phosphatidylinositol 3 kinase activity with increased eNOS phosphorylation and activity [13].

In vitro studies have shown binding of vascular endothelial growth factor (VEGF) to VEGF receptor 2 (VEGFR2) increased eNOS activity via a phosphatidylinositol 3 kinase–mediated mechanism [13]. In an adult study from Taiwan, VEGF levels

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**Table 1. Patient Characteristics and RHI at Enrollment and Convalescent Visits**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients With Dengue</th>
<th>Patients With OFI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHF (n = 11)</td>
<td>Non-DHF (n = 63)</td>
</tr>
<tr>
<td></td>
<td>Total (n = 74)</td>
<td></td>
</tr>
<tr>
<td>Patient characteristic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, median (5th–95th percentile), y</td>
<td>39 (23–48)</td>
<td>33 (22–47)</td>
</tr>
<tr>
<td>Male sex, No. (%)</td>
<td>11 (100)</td>
<td>62 (98)</td>
</tr>
<tr>
<td>Chinese ethnicity, No. (%)</td>
<td>11(100)</td>
<td>39 (62)</td>
</tr>
<tr>
<td>Comorbid condition, No. (%)</td>
<td>1 (9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Inpatient status, No. (%)</td>
<td>6 (54)</td>
<td>12 (19)</td>
</tr>
<tr>
<td>Fever day at enrollment, median (5th–95th percentile)</td>
<td>5 (3–7)</td>
<td>6 (4–9)</td>
</tr>
<tr>
<td>RHI, median (5th–95th percentile)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrollment visit</td>
<td>2.70 (1.74–3.94)</td>
<td>2.03 (0.86–3.14)</td>
</tr>
<tr>
<td>Convalescent visitd</td>
<td>2.38 (1.8–3.06)</td>
<td>1.97 (1.34–3.61)</td>
</tr>
</tbody>
</table>

Abbreviations: DHF, dengue hemorrhagic fever; OFI, other febrile illness, RHI, reactive hyperemia index.

a Comparison between DHF and non-DHF groups (Fisher’s exact or Mann–Whitney U test).

b Comparison between dengue and OFI groups (Fisher’s exact or Mann–Whitney U test).

c Diabetes mellitus.

d Nine patients in the DHF group, 52 in the non-DHF group, and 18 in the OFI group had a convalescent visit.

**Table 2. Multivariate Cox Regression Model Used to Predict Dengue Hemorrhagic Fever**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Adjusted OR (95% CI)</th>
<th>P Value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHI at enrollment</td>
<td>4.15 (1.32–13.03)</td>
<td>.02</td>
</tr>
<tr>
<td>Fever day at enrollment</td>
<td>0.73 (.48–1.09)</td>
<td>.12</td>
</tr>
<tr>
<td>Age</td>
<td>1.03 (.94–1.14)</td>
<td>.52</td>
</tr>
<tr>
<td>BMI</td>
<td>1.03 (.68–1.56)</td>
<td>.90</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; CI, confidence interval; OR, odds ratio; RHI, reactive hyperemia index.

a P values calculated using Cox regression.
were increased in patients with DHF relative to those with DF and controls but did not differ between patients with DF and controls [14]. In other studies, decreased soluble VEGFR2 has been associated with plasma leakage [15]. Acting as a decoy by binding VEGF to decrease its availability to vascular VEGFR2, decreased soluble VEGFR2 may result in increased binding and eNOS activity.

RHI may be useful as a biomarker for the progression of DF to DHF, with an area under the receiver operating curve of 0.86. However, the sensitivity was low, although the specificity was >80%, similar to findings of other studies that have looked at clinical features and laboratory biomarkers to predict the severity of dengue. Our study has several limitations, including relatively small sample size, a disproportionate number of male subjects, and enrollment later in the febrile phase. However, we did perform detailed follow-up and longitudinal physiological measurements in a large proportion of patients.

In summary, increased vascular NO bioavailability is associated with the development of DHF, and mechanisms underlying the increase in endothelial function need to be further delineated. This finding may be important for identifying potential targets to reduce the occurrence of DHF. Peripheral arterial tonometry may have potential use as a noninvasive instrument for predicting the development DHF, but larger studies are required to further validate our results.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No potential conflicts of interest.

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