Discovery and Characterization of Potential Prognostic Biomarkers for Dengue Hemorrhagic Fever

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Abstract. Half a million patients are hospitalized with severe dengue every year, many of whom would die without timely, appropriate clinical intervention. The majority of dengue cases are uncomplicated; however, 2–5% progress to severe dengue. Severe dengue cases have been reported with increasing frequency over the last 30 years. To discover biomarkers for severe dengue, we used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry to analyze dengue virus positive serum samples from the acute phase of infection. Using this method, 16 proteins were identified as candidate biomarkers for severe dengue. From these 16 biomarkers, three candidates were selected for confirmation by enzyme-linked immunosorbent assay and Western blot: vitronectin (Vtn, 55.1 kDa), hemopexin (Hx, 52.4 kDa), and serotransferrin (TF, 79.2 kDa). Vitronectin, Hx, and TF best differentiated between dengue and severe dengue.

INTRODUCTION

Dengue is caused by infection with any one of the four mosquito-borne dengue viruses (family Flaviviridae, genus Flavivirus; DENV1–4). Identifying patients with dengue early in the course of illness can be challenging: initial symptoms are often non-specific (fever, headache, retro-orbital pain, and malaise) and may mimic other febrile illnesses such as malaria, leptospirosis, and influenza. Dengue patients have a 2–5% risk of advancing to severe dengue, which manifests at 3–5 days post onset (DPO) of fever as a rapid drop in blood platelets, and fluid accumulation or hemorrhaging. Currently, there are no commercially available vaccines for prevention of dengue. There are no anti-viral drugs for dengue but severe dengue may be successfully treated with aggressive intravenous rehydration. It is critical that patients with severe dengue receive prompt treatment to manage shock, hemorrhage, and organ impairment. Although methods for dengue diagnosis are well established, there are no prognostic tests to help the clinician evaluate the risk of progressing to severe dengue. This is especially important because often the onset of severe disease occurs as the patient’s fever is resolving.

Previously identified markers of dengue severity include cytokines (i.e., tumor necrosis factor alpha [TNFα] and Interleukin 6 [IL-6]), vascular permeability proteins, clotting cascade regulators, and gene expression profiles. These severity markers are not, however, currently being used to guide patient management. It is unclear why these severity markers are not being used, possibly because detection methods are expensive, slow, or require sophisticated equipment. This study focused on stable functional biomarkers, specifically proteins, for dengue severity, as they could potentially be incorporated into point-of-care (POC) tests, thus ensuring their use. We used a classical proteomics approach for biomarker discovery. We focused on high-throughput fractionation and proteomic analysis using the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) platform to identify specific biomarkers that can differentiate between dengue and severe dengue. Biomarker discovery is an iterative process, which combines several rounds of SELDI-TOF MS discovery with confirmation of protein biomarkers by enzyme-linked immunosorbent assay (ELISA) or Western blot. Early users of this technology focused primarily on the identification of biomarkers for cancer; more recently, researchers have begun using SELDI-TOF MS for biomarkers of infectious diseases such as human immunodeficiency virus (HIV). Using this approach, we have identified three unique biomarkers for severe dengue.

MATERIALS AND METHODS

Ethics statement. Protocol no. 6048.0 entitled “The development of a host biomarker diagnostic assay for dengue fever and the differentiation of dengue hemorrhagic fever” was reviewed by the Centers for Disease Control and Prevention (CDC) Human Research Protection Office and determined to be exempt.

Study design. We used an iterative biomarker discovery design of three groups of serum samples for three rounds of experiments: discovery panel (Panel 1), serotype exclusion panel (Panel 2), and confirmation panel (Panel 3). Figure 1 illustrates the methods for discovery, serotype exclusion, and confirmation; Table 1 contains the patient characteristics for each panel. Results from each round of experiments were used to improve methods for the next round.

Samples. Serum samples were obtained from suspected dengue cases submitted for testing during 2007–2010 to the Passive Dengue Surveillance System (PDSS) of the CDC Dengue Branch, at San Juan, Puerto Rico. Samples were confirmed as DENV laboratory positive cases by reverse transcriptase polymerase chain reaction or anti-DENV immunoglobulin M (IgM) assay and clinically identified as dengue hemorrhagic fever (DHF) or dengue fever (DF) based on a dengue case investigation form (DCIF) containing clinical data submitted with the specimen. Clinical classification of DF and DHF samples was based on the 1997 World Health
Figure 1. Iterative biomarker study design. (A) Panel 1 discovery methods and samples, (B) Panel 2 DENV serotype exclusion methods and samples, and (C) Panel 3 confirmation methods and samples. All samples were obtained in the acute phase (0–5 days post onset of fever), and met the 1997 World Health Organization (WHO) DF/DHF criteria. DF = dengue fever; DHF = dengue hemorrhagic fever; Fatal = confirmed fatal dengue cases; HC = laboratory-negative healthy controls; OFI = other febrile illnesses.
Dengue disease state
Causative dengue virus and controls

Included DHF (DENV = dengue virus; DF = dengue hemorrhagic fever; Fatal = confirmed fatal dengue; OFI = other febrile illness; HC = healthy control; DV UNKN = dengue positive, serotype unknown. Percentages are rounded up to a whole number.

Panel 1: discovery. The purpose of this panel was to discover host biomarkers that distinguish DHF from DF by SELDI-TOF MS. A panel of 115 serum samples, which included DHF (N = 68), DF (N = 11), dengue fatal cases (N = 6), and OFI (N = 30) was used for biomarker discovery. Classification of DF and DHF was based on single specimens. Samples were analyzed by SELDI-TOF MS to generate protein spectra. Protein spectra were analyzed by ProteinChip, Integrated Biomarker Wizard (Bio-Rad Laboratories, Hercules, CA), and Biomarker pattern software (BPS; Bio-Rad Laboratories) to detect proteins, candidate biomarkers, which can differentiate between DF and DHF. Candidate biomarkers were separated by gel electrophoresis and identified by tandem mass spectrometry (MS-MS).

Panel 2: serotype exclusion. The purpose of this panel was to include only pan-dengue host biomarkers and exclude any DENV serotype specific biomarkers by SELDI-TOF MS. A panel of 133 serum samples including OFI (N = 30), HC (N = 30), and 73 DF (DENV-1 [N = 25], DENV-2 [N = 27], DENV-3 [N = 13], DENV-4 [N = 8]), was used to exclude serotype-specific candidate biomarkers. After completing the discovery study with Panel 1, an analysis revealed that 20% of DF cases in PDSS were misclassified as DHF cases (CDC, unpublished data). Therefore, to ensure that classification as DF was correct, sample selection for Panel 2 used paired specimens. The use of paired specimens, acute phase specimens (DPO = 0–5), and convalescent phase specimens (DPO > 5), verified that the specimens were correctly clinically classified. Panel 2 was analyzed by SELDI-TOF MS to generate protein spectra, which were analyzed by ProteinChip, Integrated Biomarker Wizard, and Biomarker pattern software. Panel 2 results were used to exclude any serotype-specific candidate biomarkers discovered from Panel 1. Non-serotype-specific candidate biomarkers were further refined by literature mining.

Panel 3: confirmation. The purpose of this panel was to confirm the host biomarkers discovered by SELDI-TOF MS distinguish between DHF and DF in ELISA format. A panel of 153 serum samples consisting of laboratory-confirmed DF (N = 105), DHF (N = 23), and HC (N = 25) samples were selected from the most recent samples submitted to PDSS. Sample selection for Panel 3 was improved by using the most recent paired samples available, from the 2010 epidemic, to ensure specimen integrity by minimizing any potential effects of storage.24 Samples were analyzed by quantitative anti-biomarker ELISAs to determine biomarker concentrations in serum samples. In addition, Western blot analysis was used to further evaluate the Vtn biomarker.

Discovery: SELDI-TOF MS data acquisition and analysis. Serum samples were fractionated using the ProteinChip Serum Fractionation kit (Bio-Rad Laboratories) by anion-exchange chromatography and pH gradient as previously described by Ndao. The purpose of this panel was to discover host biomarkers that distinguish DHF from DF by SELDI-TOF MS. A panel of 115 serum samples, which included DHF (DPO = 0–5), overall, 401 samples were used, which included the following: DF, DHF, confirmed fatal dengue cases (Fatal), laboratory-negative healthy controls (HC), and other febrile illnesses (OFI).

Figure 1. Continued.
to cationic (CM10), metal affinity (IMAC), and hydrophobic (H50) ProteinChip arrays (Bio-Rad Laboratories). The fractions were then washed to remove non-specific binding, and the energy absorbing molecule was applied. Arrays were analyzed in a ProteinChip biology system reader series (PCS 4000) equipped with an autoloader using ProteinChip software, version 3.5 (Bio-Rad Laboratories). Ionization energy was optimized on pooled human serum samples. Each spot of the arrays was then read at low-energy intensity for low molecular weight (LMW) and high-energy intensity for high molecular weight (HMW).

Spectral analyses. Spectra were analyzed using ProteinChip software and Ciphergen Express Data Manager (Bio-Rad Laboratories). Spectra were externally calibrated using an equation generated from a spectrum of protein standards with molecular weights ranging from 5733.6 Da (bovine insulin) to 147,300 Da (bovine IgG), which were collected at the same SELDI-TOF MS settings. Spectra were baseline subtracted, and normalized to total ion current within a mass/charge (m/z) range corresponding to optimized LMW or HMW ranges, and with an external normalization coefficient of 0.2 for both conditions. As a quality control measure for the comparison of spectra processed on different days, the average normalization factor was first calculated for all spectra. Any spectra that did not fall within the overall average normalization factor twice were discarded from the analysis.

Two-step spectral analysis was performed with Integrated Biomarker Wizard software (version 5, Bio-Rad Laboratories). Initially automatic peak detection was used to determine qualified mass peaks (signal/noise [S/N] > 3; cluster mass window at 0.3%) and (S/N > 3; cluster mass window at 2%) for LMW and HMW ranges, respectively. A peak cluster was determined as a peak that was found in at least 10% of the spectra for 1 condition (i.e., fraction 1 bound on CM10 chip read at LMW; F1CSL). The P values for these peaks were calculated for differences between different groups (DF, DHF, and OFI), and cluster peaks with a P value ≤ 0.05 (Mann-Whitney U test) were visually inspected and manually relabeled. Second-pass peak detection was performed on the user-defined peaks only, with the same settings as the first-pass, but the cluster mass window was increased to 2%. The P values for differences in average peak intensity between groups (DF versus OFI, DHF versus OFI, DF versus DHF) (Wilcoxon exact test) were determined; OFI comparison was used to exclude biomarkers representative of other febrile illnesses. A candidate biomarker was defined as a peak with receiver operator curve (ROC) ranging from 0.30 or lower for downregulated proteins to 0.70 or greater for upregulated proteins with a P value ≤ 0.05 and an intensity ratio between the two compared groups being at least two. Cluster data were analyzed with BPS (Bio-Rad Laboratories) as previously described by Ndao 2010.25 The BPS used the classification and regression tree (CART) method, to identify peaks that best discriminated between groups.

Protein identification. Candidate biomarkers were initially identified from spectra as proteins of a particular molecular weight. To identify these proteins, samples from Panel 1 minus OFI were pooled according to disease state: 11 DF, 68 DHF, and 30 OFI. Samples were then fractionated using the ZOOM Isoelectric Focusing Fractionator (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 5 ZOOM disks were used to obtain pH 3.0–10.0 range (pH 3.0, pH 4.6, pH 5.4, pH 7.0, pH 9.1, and pH 10.0) fractions that were stored at −20°C for further analysis by gel electrophoresis. Before analysis, each fraction was precipitated using 3 volumes of methanol, 1 volume of chloroform, and 4 volumes of distilled water. Precipitated protein samples were dissolved in sodium dodecyl sulfate (SDS) sample buffer, and separated at 200 V for 45 min using a 4–12% Bis-Tris NuPAGE gel under denaturing conditions with Mark12 unstained protein standards (Invitrogen). Gels were stained using Coomassie blue, and candidate biomarker bands were identified based on molecular weight, excised, and stored in 2% acetic acid for MS-MS. The proteins of interest were sequenced by MS-MS. The resulting spectra were submitted to the database-mining tool MASCOT (Matrix Sciences, Boston, MA) for identification.

Exclusion: SELDI-TOF MS. To exclude any serotype-specific proteins identified in Panel 1, Panel 2 samples (representative of the four dengue serotypes) were analyzed as described in the SELDI-TOF MS Acquisition and Analysis methods and Spectral Analyses methods. Candidate biomarkers classified as serotype specific were excluded from further analyses.

Literature mining. After refining the pool of candidate biomarkers by exclusion of serotype-specific markers, we conducted a literature review of the remaining candidate biomarkers. We consulted the Protein Data Bank for basic protein characteristics, PubMed to understand the role of candidate biomarkers in healthy and diseased individuals, and Linscott’s Directory to determine availability of reagents for confirmation experiments. From the literature data, we selected candidate biomarkers that had not yet been evaluated for dengue severity, and there were antibodies and/or ELISA reagents available for confirmatory experiments.

Confirmation. Enzyme-linked immunosorbent assays. The ELISAs were used to determine biomarker concentration in serum samples from Panel 3 (Table 1). Serum samples were tested for human vitronectin (Innovative Research, Novi, MI), hemopexin, and transferrin, (MyBioSource, San Diego, CA) according to the manufacturer’s instructions. Vitronectin (Vtn) was further analyzed by Western blot because the Vtn isoform (55 kDa) detected by SELDI-TOF MS was not detected by the commercially available ELISA.

Gel electrophoresis and Western blot analysis. Each individual sample from Panel 3, 105 DF, 23 DHF, and 25 HC was separated using Novex NuPAGE 4–12% Bis-Tris gels (Invitrogen/Life Technologies Grand Island, NY) under denaturing conditions with MagicMark XP Protein standards (20–220 kDa, Invitrogen) and 0.125 μg Vtn control (Sigma, St. Louis, MO). Proteins were transferred to nitrocellulose membranes (Invitrogen, 0.22μm), and blocked at room temperature overnight in 5% nonfat dry milk in phosphate-buffered saline with 0.05% Tween20. Membranes were incubated with 1:1,000 anti-human Vtn antibody (Cedarlane, Hornby, Ontario), followed by 1:10,000 anti-sheep peroxidase (KPL, Gaithersburg, MD). SuperSignal West Pico solution (Pierce/Thermo Scientific, Rockford, IL) was used to detect proteins.

Anti-Vtn western was used to compare Vtn isoforms of DF, DHF, and HC samples from Panel 3 (Table 1). The Vtn concentration for all samples was determined using quantitative ELISA as described in the ELISA analysis section (Figure 2C, Table 2). Samples were divided by disease state (DF, DHF, and HC), ranked according to total Vtn concentration, and gels loaded based on the optimized detection of Vtn isoforms.
Serum samples were diluted in Tris-buffered saline, and loaded at the following total Vtn concentrations: 1) DF 500 ng (Vtn = 2,400–10,000 μg); 2) DHF 125 ng; 3) HC 125 ng (Vtn = 550–4,500 μg); and 4) 31.5 ng (Vtn < 550 μg).

**Volume analysis of Vitronectin isoforms.** Western blots were imaged at 3-min exposures using a charge-coupled device camera (Gel Doc Bio-Rad Laboratories), and analyzed using version 4.6.9 Quantity One software (Bio-Rad Laboratories) as previously described. Briefly, lanes were auto-framed and bands auto-detected without background correction. All Lanes Report function was used to determine the percentage of total Vtn for each band per lane. Bands within molecular weights 125–140 (oligomer), 80, 75, 65, 55, 52, 45, and 10 kDa were selected for analysis, and the mean percentage of total Vtn protein was determined (Table 2).

**Statistical analysis.** Integrated Biomarker Wizard software (version 5, Bio-Rad Laboratories) used the Mann-Whitney U test and Wilcoxon exact test for spectral analysis. The ELISA concentration values for Hx, Tf, and Vtn from DF and DHF samples were compared using Prism 5 software and P values were calculated using a Kruskal-Wallis test with Dunn’s post hoc test (* = 0.01 to 0.05, ** = 0.001 to 0.01, *** < 0.001). DF = dengue fever; DHF = dengue hemorrhagic fever; HC = healthy control.

**Figure 2.** Enzyme-linked immunosorbent assay (ELISA) confirmation of biomarkers identified by surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS). Mean micrograms per milliliter (μg/mL) were determined by quantitative ELISAs: (A) hemopexin (Hx), (B) serotransferrin (Tf), (C) vitronectin (Vtn), and (D) Vtn by age. DF, DHF, and HC were compared for each biomarker by the Kruskal-Wallis test with Dunn’s post hoc test (* = 0.01 to 0.05, ** = 0.001 to 0.01, *** < 0.001). DF = dengue fever; DHF = dengue hemorrhagic fever; HC = healthy control.

**Table 2**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession no.</th>
<th>Predicted mass (Da)</th>
<th>Actual mass (Da)</th>
<th>Mean biomarker concentration (μg/mL)</th>
<th>Ratio of mean concentration</th>
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<td></td>
<td>NCBI UniProt</td>
<td>DF</td>
<td>DHF</td>
<td>HC</td>
<td>(DF/DHF)</td>
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<td>Hx</td>
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<td>51,600</td>
<td>3,593</td>
<td>[3,346, 3,841]</td>
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<td></td>
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<td>1,571</td>
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<td>76,500</td>
<td>11,163</td>
<td>[11,163, 9,674]</td>
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<tr>
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<td>55,165‡</td>
<td>10,000 +</td>
<td>3,011</td>
<td>[2,720, 3,302]</td>
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<tr>
<td></td>
<td></td>
<td>75,000</td>
<td>2,089</td>
<td></td>
<td>[1,561, 2,616]</td>
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</tbody>
</table>

| Accession nos., predicted mass by SELDI-TOF MS, actual mass in Daltons (Da), and biomarkers identified as hemopexin (Hx), serotransferrin (Tf), and vitronectin (Vtn). Mean micrograms per milliliter (μg/mL) and 95% confidence interval were determined for each biomarker by disease state (DF = dengue fever, DHF = dengue hemorrhagic fever, HC = healthy control).‡Deglycosylated vitronectin ~55,000 Da.

†Overall P values calculated using Kruskal-Wallis test.
RESULTS

Discovery of 16 candidate biomarkers discriminating DF from DHF. The SELDI-TOF MS analysis of 115 samples from Panel 1 yielded 2,070 spectra. Using the ProteinChip software analysis, we identified 251 unique biomarkers that discriminated between DF and DHF. These unique biomarkers were selected based on differences in average mass spectrometry peak intensity between DF and DHF samples (P values < 0.05 and ROC analysis ≥ 0.9). Further analysis was performed using BPS, which allowed selection of the most significant biomarkers by generating decision trees thus identifying 16 candidate biomarkers.

Serotype exclusion: biomarkers were consistent across all four DENV serotypes. The SELDI-TOF MS analysis of 133 samples from Panel 2 yielded 2,394 spectra, from which ProteinChip software analysis determined no candidate biomarkers that differed by DENV serotypes 1–4. From these 16 DENV biomarkers that were non-serotype-specific candidate biomarkers, Hx, Tf, and Vtn were selected for confirmation analysis because their role in dengue severity had never been evaluated. For Vtn, the predicted size based on the NCBI accession number differed from the actual size observed in SELDI-TOF MS (Table 2).

Confirmation. The ELISA analysis of 153 serum samples (Table 1, Panel 3) was used to determine the average concentration of the three candidate biomarkers based on disease state (DF versus DHF). On the basis of a Kruskal-Wallis test performed using BPS, which allowed selection of the most significant biomarkers by generating decision trees thus identifying 16 candidate biomarkers.

DISCUSSION

Analysis of SELDI-TOF MS results identified 16 unique proteins that discriminated between DF and DHF across all DENV serotypes. We selected three of these, Vtn, Hx, and Tf, which operate to maintain the hemostatic balance between coagulation and fibrinogenesis, for further analysis. By ELISA, three of these (Vtn, Hx, Tf) were elevated in DF relative to DHF cases; Hx and Vtn were elevated in dengue cases relative to healthy controls. Currently the molecular mechanisms of dengue coagulopathy, which occur during DHF are attributed to increased viral replication, apoptosis, complement activation, and proinflammatory cytokines. Although many molecular mechanisms of coagulation malfunction have been proposed, exact roles of effector proteins, such as Vtn, Hx, and Tf in pathology are not yet known.

Based on ELISA confirmation of SELDI-TOF MS results, Vtn is our single most statistically promising biomarker for distinguishing between DHF and DF. The Hx and Tf require further analysis to determine why our data differed from other researchers. After further characterization of Vtn, Hx, and Tf, we may find that a combination of biomarkers has the greatest utility as a diagnostic test for severe dengue as noted by other researchers.

Vitronectin is a multifunctional glycoprotein that circulates in the blood, usually as an inactive monomer, until it is recruited to regulate coagulation and platelet aggregation. In our study, the Vtn levels of DF patients and DHF patients were much higher compared with HCs. There was an overall increase in Vtn after dengue compared with HCs, with overall Vtn concentrations decreasing in DHF cases compared with DF cases. The recruitment of Vtn monomers to oligomers

![Figure 3](image-url)
occurs after the initiation of the clotting cascade.32 In addition to its role in the clotting cascade, Vtn also assists in the regeneration of the vascular extracellular matrix. The decline of Vtn levels in DHF cases might be caused by Vtn recruitment to damaged vascular tissues.33,34 A decline in Vtn levels has been observed in other hemorrhagic viral infections, such as hemorrhagic fever renal syndrome (HFRS), which is caused by Hantaan virus, showing that Vtn is essential for vascular integrity. The Vtn levels were lower in HFRS patients during all phases of the disease, except the convalescent phase, and may have reduced our ability to identify biomarkers during any patient in the study developed symptoms of severe dengue later in the course of their illness. An estimated 20% of DF cases in PDSS are misclassified; these cases are actually HF patients, indicating that this isoform could be used as a biomarker for severe dengue.36 The Vtn oligomer has two distinct functions that could have implications for DENV infection and pathogenesis. First, the oligomer binds to heparin, a known receptor for DENV.37 Second, it regulates coagulation by forming a bridge between integrin and fibrin to induce platelet aggregation to inhibit plasma leakage.38,39 Western blot analysis did not identify any single isoform as having a greater association with dengue severity compared with the overall Vtn concentration measured by ELISA. To determine the role of Vtn, fine discriminatory analysis of Vtn isoforms after infection may require sequential sampling throughout the clinical course of severe dengue.

Hemopexin is a glycoprotein that binds free heme, preventing oxidative tissue damage.40–42 Elevated Hx levels were observed in DF and DHF patients relative to healthy controls. Our findings differ from some earlier studies. Our healthy controls had 2-fold higher Hx (1,571 µg/mL) compared with a previous report (770 µg/mL).40 Ray and colleagues28 observed higher Hx levels in healthy controls compared with DF. This discrepancy could be caused by a difference in methods (Western blot versus ELISA), or to sample size (HC = 6, DF = 6). Kumar and colleagues37 observed elevated levels of Hx in DHF cases relative to DF cases over time, in contrast to our observation of lower Hx levels in DHF relative to DF (DPO = 0–5). This discrepancy may be caused by age differences between the samples analyzed or quantitation methodology (SOLDI-TOF MS and ELISA versus isobaric tagging). The potential of Hx as a biomarker will depend on resolution of these differences through further studies.

Serotransferrin, an iron-binding and transport protein that maintains hemostasis by transferring iron from sites of heme degradation, thus preventing tissue damage; it also stimulates cell proliferation.43,44 We observed approximately equivalent Tf levels in DF (11,163 µg/mL) and HC (11,536 µg/mL); however, Tf levels were lower in DHF cases (9,674 µg/mL). Inadequate levels of Tf in DHF cases may lead to vascular tissue damage and plasma leakage. The Tf levels were higher in classical swine fever compared with uninfected swine, contrary to our observation with DENV in humans. Clinical manifestations of classical swine fever, which is also a flavivirus, pathologically resemble DHF, including thrombocytopenia and the hemorrhage of skin, mucosa, and organs. This difference in Tf levels after a flavivirus infection may be explained by virus or host-specific protein differences. A protein basic local alignment search tool (BLAST) search of human and swine Tf predicted 98% homology; therefore functional differences between human and swine Tf may exist.

The fact that our samples had been collected as part of PDSS somewhat limits our knowledge of the course of disease in the donors. Biomarker expression may vary during the clinical course of dengue; hence, serially prospectively collected patient serum samples would be preferred to the single samples obtained from PDSS. It is not possible retrospectively to re-examine patients or charts to determine whether any patient in the study developed symptoms of severe dengue later in the course of their illness. An estimated 20% of DF cases in PDSS are misclassified; these cases are actually DHF cases (CDC, unpublished data). This misclassification may have reduced our ability to identify biomarkers during the initial discovery study. Another limitation of our retrospective analysis is that the samples had been classified as DF and DHF using the 1997 WHO case classifications rather than those published in 2009, which categorize all infections as either dengue, dengue with warning signs, or severe dengue. Finally, although age-related differences for one of our biomarkers (Vtn) have been reported in healthy individuals; we did not have age-matched DF and DHF specimens in the serum panels for analysis by age.45–48

Further validation is required before any of these biomarkers could be formulated as prognostic tests. Future studies should verify the prognostic utility of the biomarkers by analyzing biomarker levels over time and using the 2009 WHO severity classifications. Diagnostic cut-off values should then be developed for each biomarker. After diagnostic cut-off values have

### Table 3

**Analysis of vitronectin (Vtn) isoforms by dengue disease state***

<table>
<thead>
<tr>
<th>Isoform (kDa)</th>
<th>Mean Vtn/lane</th>
<th>Range</th>
<th>Isotform absent (%)</th>
<th>Mean Vtn/lane</th>
<th>Range</th>
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<th>Mean Vtn/lane</th>
<th>Range</th>
<th>Isotform absent (%)</th>
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<tbody>
<tr>
<td>Oligo</td>
<td>4</td>
<td>3–1–2</td>
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<td>3</td>
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<td>12 (52)</td>
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<tr>
<td>80</td>
<td>9</td>
<td>40–1–72</td>
<td>67 (67)</td>
<td>5</td>
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<td>3</td>
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<td>9 (9)</td>
<td>3</td>
<td>7–0–0</td>
<td>0 (0)</td>
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*Western blots were analyzed using Bio-Rad Laboratories Quantity One software. Isoform bands were auto-detected and reported as the percentage of total vitronectin (Vtn) protein/lane for each isoform. The mean and range of the percentage of total Vtn protein/lane from each molecular weight isoform (oligomer 125 or 140, 80, 75, 65, 55, 52, 45, 10 kDa) and the percentage of absent bands was calculated by disease state. Percentages are rounded up to a whole number.**


