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MOLECULAR CLASSIFICATION OF OUTCOMES FROM DENGUE VIRUS -3 INFECTIONS

Allan R. Brasier^{a,b,c,†,*}, Yingxin Zhao^{a,b,c,†}, John E. Wiktorowicz^{b,c,d,†}, Heidi M. Spratt^{b,c,e,†}, Eduardo J. M. Nascimento^h, Marli T. Cordeiro^f, Kizhake V. Soman^{b,d}, Hyunsu Ju^e, Adrian Recinos III^a, Susan Stafford^g, Zheng Wu^g, Ernesto T.A. Marques Jr^{f,h}, and Nikos Vasilakis^{i,j,k}

^a Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, United States of America

^b Sealy Center for Molecular Medicine, UTMB

^c Institute for Translational Sciences, UTMB

^d Department of Biochemistry and Molecular Biology, UTMB

^e Department Preventive Medicine and Community Health, UTMB

^f Laboratório de Virologia e Terapê Experimental do Centro de Pesquisas Aggeu Magalhães-CPqAM, Fiocruz, Recife, Pernambuco, Brazil

^g Biomolecular Resource Facility, UTMB

^h Department of Infectious Diseases and Microbiology and Immunology, University of Pittsburgh, United States of America

ⁱ Department of Pathology and Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch, Galveston, TX

^j Center for Tropical Diseases, University of Texas Medical Branch, Galveston, TX

^k Institute for Human Infection and Immunity, University of Texas Medical Branch, Galveston, TX

Abstract

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* Corresponding author: arbrasie@utmb.edu.

† Equal contributors

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Competing interests

All authors have no conflicts of interest to declare.

Ethical approval

This study was conducted under approval by the ethics committee of the Brazilian Ministry of Health, number, CONEP: 4909; Process 25000.119007/2002-2003; CEP: 68/02 and the Johns Hopkins Institutional Review Board (IRB) as protocol JHM-IRB-3:03-08-27-01. Research at the UTMB was conducted under IRB Nos. 10-804 and 08-258. This study was compliant with all applicable federal regulations governing the protection of human subjects. All adult subjects provided written, informed consent; and parents or guardians provided informed consent on behalf of any children participating in the study.

Objectives—Dengue virus (DENV) infection is a significant risk to over a third of the human population that causes a wide spectrum of illness, ranging from sub-clinical disease to intermediate syndrome of vascular complications called Dengue Fever Complicated (DFC) and severe, dengue hemorrhagic fever (DHF). Methods for discriminating outcomes will impact clinical trials and understanding disease pathophysiology.

Study Design—We integrated a proteomics discovery pipeline with a heuristics to develop a molecular classifier to identify an intermediate phenotype of DENV-3 infectious outcome.

Results—121 differentially expressed proteins were identified in plasma from DHF vs dengue fever (DF), and informative candidates were selected using nonparametric statistics. These were combined with markers that measure complement activation, acute phase response, cellular leak, granulocyte differentiation and viral load. From this, we applied quantitative proteomics to select a 15 member panel of proteins that accurately predicted DF, DHF, and DFC using a Random Forest Classifier. The classifier primarily relied on acute phase (A2M), complement (CFD), platelet counts and cellular leak (TPM4) to produce an 86% accuracy of prediction with an area under the receiver operating curve of >0.9 for DHF and DFC vs DF.

Conclusions—Integrating discovery and heuristic approaches to sample distinct pathophysiological processes is a powerful approach in infectious disease. Early detection of intermediate outcomes of DENV-3 will speed clinical trials evaluating vaccines or drug interventions.

Keywords

Dengue; biomarker pipeline; selected reaction monitoring; acute phase reaction

1. Background and objectives

DENV is an arthropod-borne RNA virus that causes significant health and/or economic impact to over 100 dengue-endemic countries in which approximately half of the world's population resides (1). As a result, dengue fever (DF) is the most common vector-borne viral infection in humans (2). Symptomatic DENV infections manifest as a flu-like disease characterized by a sudden onset of fever, maculopapular rash, and leucopenia (3). A small group develop severe dengue disease (SDD), a later-onset syndrome characterized by vascular leakage and/or hemorrhage, leading to 500,000 annual hospitalizations with a case fatality rate of about 5% (4).

Risk factors for complicated DENV infections are multifactorial; these include host genetic factors (5-10), viral strains (11-13), anamnestic immune response from previous heterologous exposure (14-16), age (17, 18) and/or gender effects (12). Not surprisingly, then, the clinical manifestations of DENV infection is highly variable in terms of times of onset, involvement of thrombocytopenia (19, 20), manifestations of endothelial injury/vascular leak (21), myositis, arthralgias and hepatic involvement (22, 23). As a result, the World Health Organization (WHO) refined dengue case classification schemes in 2009 to include the identification of non-severe dengue with early warning signs (24). Accordingly, approaches seeking to discriminate acute, self-limited infections vs those that will progress to SDD have yielded inconsistent results (12, 22, 23, 25).

Recent advances in global proteomic technologies enable more unbiased methods for disease discrimination than previously possible. Here we report, for the first time, a multi-level strategy for discovery and biomarker qualification of severity of DENV infections in a cohort of DENV-3 infections. This unique cohort represented a valid sampling of the entire infected population, including a balanced mixture of primary and secondary infections (26). We tested the performance of robust, clinical grade quantitative proteomics measurements that sampled multiple pathophysiological processes, including complement activation, acute phase response, cellular leak, granulocyte differentiation and viral load, on clinical outcome. This study will be of value to produce objective case definition to better evaluate diagnostics and clinical interventions to reduce the impact of DENV-3 infections.

2. Study design

2.1 Study population and case definitions

Patients >5 years of age with suspected DF who were admitted to one of three hospitals in the city of Recife, Pernambuco, Brazil. After written informed consent (or assent) was obtained from each subject (or guardian) clinical and laboratory evaluations were performed on enrollment (33). Dengue cases were classified according to the 1997 WHO Guidelines. DF was assigned by a high fever accompanied by at least two associated symptoms: headache, retro-orbital pain, myalgia, arthralgia and rash. DHF was defined by the same clinical manifestations as for DF, but with hemorrhagic manifestations, including a positive tourniquet test, thrombocytopenia ($<100,000/\text{mm}^3$), hemoconcentration or other sign of plasma leakage (24). DFC was designated when subjects met the criteria for DF and who presented with hemorrhagic manifestations and thrombocytopenia ($<100,000/\text{mm}^3$), but the clinical/laboratory parameters did not fulfill the WHO DHF criteria as described by us earlier (26). Infectious outcomes were 29.4% DF, 44% complicated DF (DFC), those with hemorrhagic or thrombocytopenia that did not meet the classical WHO criteria, and 8.2 % DHF (26).

2.2 Biofluid Analysis Platform (BAP) Fractionation and 2DE

The BAP pre-separation fractionation system is a semi-automated device to fractionate plasma into protein and peptide pools (12). The protein pools were depleted of the most highly abundant proteins and labeled with BODIPY FL-maleimide (BD) (27, 28). BD-labeled proteins were separated by 2DE, imaged and analyzed using Progenesis/SameSpots software (Nonlinear Dynamics, Ltd. Newcastle Upon Tyne, UK).

2.3 Stable Isotope Dilution (SID)-Selected Reaction Monitoring (SRM)-MS

Two technical replicates of analysis for each sample were analyzed by SID-SRM-MS. All of the SRM measurements had within-technical replicates coefficient of variation (CV) of 20% or less. To detect CFD, we developed an immuno-precipitation (IP)- SID-SRM-MS assay. An aliquot of 20 μL of plasma was immunoprecipitated with 4 μL of biotin conjugated anti-CFD Ab (Thermo Scientific, Waltham, MA). The samples were captured, washed and CFD abundance quantified with SID-SRM-MS.

2.4 Statistical analysis

Statistical comparisons were performed using SPSSv18 (SPSS, Inc., Chicago, IL). 2-way ANOVA, post-hoc testing using Tukey's t-test, and Kruskal Wallis tests were applied as indicated. Student's t-tests were performed on the 2DE spots. The significance level in all instances was 0.05.

3. Results

The clinical parameters of the one hundred and ten (110) study volunteers are shown in Table I. The three study groups were matched for age, gender [male/female (M/F)] distribution and days of fever. At the time of presentation, hematocrit (HCT) was significantly different between the DF and DHF diagnoses ($p = 0.046$, Tukey's t-test). The platelet concentration was also significantly different in all groups ($p < 0.001$, Tukey's t-test). These data suggested that the DFC was a distinct category of disease.

3.1 Discovery of candidate biomarkers related to DENV severity

We first applied discovery proteomics to identify any unique proteins using an unbiased protein profiling strategy (BAP; Methods). In this platform, plasma from a matched set of extremes (29 cases of DF and 22 cases of DHF) were subjected to analysis (Figure 1). We detected 1,311 protein spots after complete analysis; Student's t-tests were performed on the $\log_2$ -transformed intensities for each. Of these 1,311 spots, 121 were significantly different between the two classes ($p < 0.05$). The top 10 canonical biological pathways dysregulated by DHF were identified by pathway enrichment analysis. The top 3 pathways included acute phase response signaling, LXR/RXR activation and coagulation system (Figure 2). Pathways analysis of the dysregulated network showed an interconnected network enriched in acute phase response proteins, coagulation pathways, and cytoskeletal elements (Figure 3).

3.2 Candidate panel for disease severity

We subjected the proteins identified in our discovery analysis to statistical feature reduction using a non-parametric method that identifies informative proteins that correlate with clinical phenotype (12, 29, 30). Three proteins were identified as being most informative: (1) a 250 kDa high molecular weight albumin (HMWAlb) complex; (2) a native alpha 2 macroglobulin (A2M); and (3) a 34 kDa fragment of desmoplakin (DESP); shown in Figure 4.

Discriminant markers were combined with a heuristic approach selecting informative protein markers that monitored distinct biological functions known to be dysregulated in DHF. These candidates included complement factors -D, -H, and -4A (31); acute-phase reactants alpha-1 anti-trypsin (A1AT), fibrinogen α/β (FIBA/FIBB), ferritin (FRIL) and haptoglobin (HPT); and the granulocyte differentiation marker, leucine rich alpha 2 glycoprotein (AG2L). The immunoglobulins, IgG-1, IgG κ and IgJ are differentially expressed in DHF, consistent with previous work identifying increased expression of plasmablasts in DHF (31-33). The presence of cytoskeletal leak proteins, keratin-1 (KRT1), tropomyosin (TPMH), desmoplakin (DESP), and vimentin (VIME) have been observed in

acute DENV infection. Additionally we measured DENV NS1 protein as a surrogate of viral load (Table II).

3.4 Targeted proteomic assays

Selected reaction monitoring (SRM) was applied as a 'targeted' MS approach for the accurate quantification of predetermined proteins in plasma (34). For highly informative candidates, such as A2M, the SID-SRM-MS assay shows a LLOQ of 0.064 fmol, and a dynamic range of 10,000 (Supplementary Table 1). Complement Factor D (CFD) was measured using an IP-SRM assay. To measure the 34 kDa DESP and 250 kDa HMWAlb fragment, both proteins representing small fractions of the total circulating DESP and Alb respectively, a capillary electrophoresis (CE) pre-fractionation method was developed. Technical replicates indicated that these methods were highly reproducible with <20% CoV.

3.5 Panel measurement

The circulating levels for the 19 candidate biomarkers were measured in the plasma of 110 study volunteers (59 DF, 29 DFC, 22 DHF) obtained at the time of initial presentation of DENV infection (Figure 5). Consistent with our previous studies (12), the biomarker measurements were not normally distributed and non-parametric statistical methods were used to identify biomarkers that were significantly different between the groups. Of the initial 19 candidates measured, 15 were significantly different ($p < 0.05$, Kruskal-Wallis test; Supplementary Table 2). We noted that 8 of these, including Dengue NS1, CFD, A1AT, A2M, CO4A, TPM4, FRIL and DESP were significantly different between all three groups. Interestingly, 5 markers, AG2L, KRT1, IGJ, HPT, and VIME were distinct between DFC vs DHF and DF vs DFC, but not between DF vs DHF. These 15 verified biomarkers were then combined with discriminant clinical variables (platelet counts, HCT, and gender) and subjected to nonparametric classification.

3.6 Ensemble classification of DENV disease severity

To develop a classifier of DENV severity, we used a random forests classifier, an ensemble classifier that uses many decision tree models to predict outcome (30, 35). The best model produced 79.7% accuracy for DF (47 of 59 were correctly predicted), 100% accuracy for DFC, and 90.9% accuracy for DHF (20 of 22 were correctly predicted) in 10-fold cross-validation evaluation of model performance (Table IV).

3.7 Post-hoc classifier evaluation

Post-hoc analysis of variable importance was used to identify the features that were most important in the random forest classifier (Figure 6). The four most significant variables were A2M, CFD, platelet and TPMH. The ROC of the classifier shows overall model performance in disease classification. From this analysis, the area under the ROC curve (AUC) for DHF prediction is 0.901 (Figure 7A). Similarly, the ROC for DFC prediction is 0.945, indicating a high model performance for prediction of intermediate severities of DENV infection (Figure 7B).

4. Discussion

The rapid re-emergence of dengue infections worldwide merits its consideration as an international public health emergency by the WHO (24). Recent cartographic estimates indicate that the number of acute infections annually may be as high as 400 million (1). During the acute febrile illness, it is not possible to clinically distinguish self-limited DF from those that will develop complicated DENV infections. Because the early detection of those at risk for SDD will aid in management of disease outbreaks, the identification of those at risk for SDD is of high impact. Here we apply a multivariate strategy in a representative cohort of primary and secondary infections associated with endemic DENV-3 spread (26) to produce more a rigorous, molecular case definition.

Although consensus groups have agreed that dengue represents a single disease entity with different clinical presentations, it is currently controversial as to which criteria can be used to describe the severity of the disease. DENV infection produces a wide spectrum of clinical presentations, with currently an unpredictable disease progression. Following a period of incubation, most patients present with an acute, self-limited, febrile syndrome (1, 3). A small subgroup will progress to severe disease; currently using clinical case definitions it is not possible to reliably distinguish the two. In response to this difficulty the WHO criteria was modified in 2009 to include the separation of non-severe dengue into those with or without warning signs. However, even patients who present with non-severe dengue can develop DHF or shock, and up to 29% of patients could not be classified suggesting that the current categories will need further refinement (36).

In our previous characterization of the Recife cohort, we designed a group associated with hemorrhagic complications and thrombocytopenia that did not fulfill the 1997 WHO Guidelines for DHF (24), yet these manifestations were clearly separate from those with uncomplicated DF. Our studies identify objective criteria that indicate DFC is an intermediate state distinct from DF and DHF. Not only are the platelet counts significantly different between these three groups (Table I), but the NS1, CO4A, TPMH, FRIL and DESP biomarkers are as well (Figure 4). Together, these findings indicate that DFC is not just an intermediate stage between DF and DHF, but rather may have distinct pathophysiology. More work will be needed to identify subgroups of DENV infection; we contend that addition of objective, molecular criteria may be useful in disease classification.

Plasma is a complex mixture of proteins over a high dynamic concentration range, circulating in protein complexes (37), and containing alternatively processed protein fragments. The BAP platform deals with many of these difficulties not addressable by conventional “bottom-up” profiling. Denaturation of the plasma prior to its fractionation enables the detection of low abundance biomarkers associated with carrier proteins (38). Application of saturation fluorescence labeling provides enhanced quantitation over conventional fluorescent dyes in 2DE (27). Finally, the use of 2DE enables detection of post-translationally modified proteins that would be missed by “bottom-up” approaches. Our discovery of post-translationally modified 250kDa high molecular weight albumin and the detection of the 34kDa DESP fragment was possible only by 2DE.

Our BAP discovery proteomics indicate that severe DENV infection dysregulates pathophysiological processes, including the acute phase response, coagulation cascade, and cytoskeletal proteins generated from cytotoxicity; many have been identified previously (12, 39-44). The four most informative protein biomarkers identified in this study indicate dysregulation of hepatic acute phase (A2M), the complement alternative pathway (CFD), and cytoskeletal leakage proteins (TPMH), followed by the viral load (NS1). The acute-phase represents a non-specific systemic switch in the hepatic production of plasma proteins to facilitate macrophage opsonization, coagulation and wound repair (45). A2M is the most informative marker in the DENV classifier, previously identified by 2DE analysis (39). Our SID-SRM-MS results indicate that A2M is increased in DFC, and is decreased in DHF cases, relative to DF patients (Figure 3). This finding suggest that consumption of plasma anti-proteases may play a role in the evolution to DHF. Our finding that CFD is an informative marker is consistent with earlier studies from our group (31). The expression of CFD in DFC was not examined in this previous study. Additionally, we showed that levels of complement factor B and C3 are reduced on DHF patients (31, 33). Altogether, our data confirms the role of the alternative pathway of complement system on dengue disease immune-pathogenesis. TPMH is an actin-binding, contractile protein expressed in striated and smooth muscle. Muscle involvement in DENV is documented (46).

Interestingly, DENV NS1 was the fourth most informative biomarker of DENV severity that we identified. NS1 is a highly conserved nonstructural glycoprotein of all flaviruses that is secreted into plasma as a soluble hexameric complex in DENV-infected patients (47). Previous work has shown that NS1 is responsible for the complement fixing activity in DENV infection (47) forming active SC5b membrane attack complexes on DENV-infected cells in the presence of anti-DENV antibodies (21), perhaps playing a role in vascular leak. Currently clinical assays rely on enzyme-linked immunosorbent assays for detection of NS1, a detection method that may be unreliable in the presence of anti-NS1 antibodies produced during infection. The SRM assay we have developed would not be sensitive to the interference. Our studies here indicate that NS1 is lower in DHF relative to DF or DFC, perhaps suggesting that DFC may be primarily driven by complement activation, whereas DHF may involve the host inflammatory response.

Our results illustrate the adoption of the targeted SID-SRM-MS assays to provide independent, clinical quality assays for informative protein measurements. SID-SRM-MS is increasingly being accepted for applications in clinical monitoring due to its portability and reproducibility in of assay performance across laboratories and detection platforms (48). We demonstrate how the SID-SRM-MS assay can be coupled with CE pre-fractionation to detect and quantify informative protein fragments produced from tissue leak (DESP). Moreover, coupling IP enrichment to the SID-SRM-MS assay enables the detection of complement proteins (CFD) at levels comparable to ELISA.

In summary, we report the identification and verification of a nonparametric classifier of severity of DENV infection in an epidemic of DENV-3 infection. Our studies verify 15 markers statistically associated with disease severity, that when combined with platelet counts in the clinical laboratory produce a highly accurate classifier of DENV outcomes.

Early detection of intermediate outcomes of DENV will speed clinical trials evaluating clinical vaccine or drug interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We conduct a biomarker study associated with complications of Dengue infection
- We apply a novel liquid separations-proteomics profiling to detect Dengue markers
- Differences in plasma proteins are measured in subjects with 3 infectious outcomes
- A machine learning classifier (random forest) accurately distinguishes disease outcomes
- Our results suggest that distinct pathophysiologic processes underly dengue fever outcomes

Figure 1. Schematic of classification strategy

Schematic view of strategy for identification of disease intermediates associated with DENV disease severity. Extremes of phenotypes were compared for differential expression using BAP pipeline in 51 subjects. Candidate biomarkers were then assembled based on proteins identified in the discovery phase and those that had been identified by previous studies (see Table III for further details). For each candidate, targeted proteomics assays using stable isotope dilution (SID)-selected reaction monitoring (SRM) assays were developed, standardized, and used to quantitate the abundance of the candidate biomarker in the entire population (110 patients). Nonparametric random forests classification was then applied to identify informative features of DENV severity.

Figure 2. The top 10 canonical pathways dysregulated by DHF

Shown are canonical pathways dysregulated by DHF (Table II). The horizontal bar for each pathway is its score, calculated as $-\log_{10}(p)$, where p is the probability that the specific combination of the implicated proteins occur in the pathway by pure chance. The yellow dots on the bars mark the ratios of the number of pathway proteins in the uploaded data set to the total number of proteins that make up the pathway.

Figure 3. Network of interactions

Shown is the top network dysregulated by DHF. The fold changes of protein abundance are marked below the nodes, with a positive value representing up-regulation and a negative value, down-regulation (DHF vs. DF). Molecules shown in the network: A2M: alpha-2 macroglobulin; ALB: albumin; APOE: apolipoprotein E; C3: complement component 3; C4A/C4B: complement component 4B; CFB: complement factor B; Chymotrypsin; CLU: clusterin; collagen(s); Elastase; ERK1/2: map kinase; FG: fibrinogen gamma chain; Fibrinogen; GOT: aspartate aminotransferase; HDL: high-density lipoprotein; Hemoglobin; HP: haptoglobin; IgG: immunoglobulin G; IgG1: immunoglobulin G1; IGHG1: immunoglobulin heavy constant gamma 1 ; IGKC: immunoglobulin kappa constant; Kallikrein; KRT9: keratin 9; KRT10: keratin 10; LDL: low-density lipoprotein; Pro-inflammatory cytokine; SAA4: serum amyloid A4; Serine protease; SERPINA1: alpha-1-antitrypsin; SERPINA3: alpha-1-antichymotrypsin; SERPINA5: plasma serine protease inhibitor; SERPINC1: antithrombin-III; Stat3: signal transducer and activator of transcription 3; TCF: T cell factor; Trypsin.

Figure 4. Reference gel of plasma proteins dysregulated by DHF

Shown is a reference gel of 2DE of SEC fractionated and IgY depleted plasma proteins from the study subjects. The location of spots 179, 76 and 646, identified as discriminant proteins in the MARS model are shown. Insets are the 3D view of the spots for DF and DHF generated by Totalabs SameSpots software. The image shown represents the pI range of 3-10 and the molecular size range of 25-200+ kDa. Gel-derived protein size and pIs are: high molecular size ALB (spot 76) - MSize of >250 kDa and pI of 6.23; A2M (spot 179) exhibited a MSize of 128 kDa and pI of 5.27; and DESP (spot 646) exhibited an MSize of 34 kDa and pI 9.84.

Figure 5. SID-SRM-MS measurements in DENV infections

For each candidate biomarker, the SID-SRM-MS measurement by disease category is shown in a box plot format. Box plots show the 25% and 75% interquartile range, and the mean value indicated by horizontal dark line. Outliers are indicated as circles and asterisks. Note that the mean horizontal line is not symmetrically located within the box plot, indicating that the data is not normally distributed.

Figure 6. Variable importance measures in RF classifier
Shown is a rank ordered list of the relative importance for each feature predictive of the outcome for DENV infection.

Figure 7. Receiver Operating Characteristic (ROC) curve for the predictive model for severity DENV infections

A, ROC for DHF prediction. Y axis, Sensitivity; X axis, 1-Specificity. The area under the ROC curve (AUC) = 0.901. **B**, ROC for DFC prediction. The AUC=0.945.

Table I

Clinical demographics.

Disease phenotype	Characteristic	Mean $\pm$ SE
DF (n=59)	M/F	25/34
	Age (y)	22.7 $\pm$ 2.1
	Days of symptoms at Sample 1	4.7 $\pm$ 0.26
	qPCR viral copies (per mL)	5.3E+07 $\pm$ 2.0E+07
	Platelet count (per mL)	195,945 $\pm$ 6280.31 †
	HCT (%)	39.3 $\pm$ 0.4 †
DFC (n=29)	M/F	17/12
	Age (y)	36 $\pm$ 3.16
	Days of symptoms at Sample 1	5.1 $\pm$ 0.58
	qPCR viral copies (per mL)	4.5E+10 $\pm$ 0.43E+11
	Platelet count (per mL)	125,536 $\pm$ 6513.64 †
	HCT (%)	41 $\pm$ 0.7 †
DHF (n=22)	M/F	7/15
	Age (y)	38.5 $\pm$ 4.43
	Days of symptoms at Sample 1	5.4 $\pm$ 0.55
	qPCR viral copies (per mL)	1.70E+08 $\pm$ 1.14E+08
	Platelet count (per mL)	86,750 $\pm$ 5354.75
	HCT (%)	41.7 $\pm$ 1.0 †

 † p<0.05 ANOVA.

Table II

Proteins identified from 2DE gel spots

Spot #	Spot pI ¹	Spot MW (kDa) ¹	Protein Name	UniProt Accession	Pep-tide count ²	Seq. coverage ³	Fold change	p (t-test)
identified by MALDI TOF/TOF								
73	7.20	>250	Complement C3	P01024	21	18.3	-1.81	0.03335
80	6.18	>250	Serum albumin; Flags: Precursor	P02768	14	29.2	-1.33	0.06969
83	7.30	200	Complement C3	P01024	26	22.7	-1.72	0.01575
201	5.74	119	Alpha-2-macroglobulin	P01023	21	20.6	-1.43	0.01962
204	5.80	117	Alpha-2-macroglobulin	P01023	22	21.2	-1.46	0.01788
224	3.55	100	Keratin, type I cytoskeletal 10	P13645	16	32.4	-2.80	0.06689
306	7.30	80	Complement C3	P01024	31	27.2	-2.18	0.04624
330	9.19	71	Complement C4-B	P0C0L5	18	13.8	-1.74	0.05309
335	9.09	70	Complement C4-B	P0C0L5	20	15.9	-1.79	0.05494
434	9.20	49	Ig gamma-1 chain C region	P01857	6	23.6	-2.41	0.08858
444	9.09	48	Ig gamma-1 chain C region	P01857	6	23.6	-2.71	0.06011
486	5.27	44	Antithrombin-III	P01008	13	34.9	-1.64	0.04141
719	9.92	31	Keratin, type I cytoskeletal 10	P13645	16	33.9	1.38	0.01334
784	6.89	28	Complement C3	P01024	15	8.9	-1.52	0.06435
1434	4.11	13	Keratin, type II cytoskeletal 1	P04264	16	30.4	2.03	0.10884
1483	4.13	12	Keratin, type I cytoskeletal 9	P35527	19	49.4	1.91	0.01366
1516	7.96	11	Keratin, type I cytoskeletal 9	P35527	20	50.08	1.43	0.05599
Identified by LC-MS								
6	4.76	>250	Alpha-2-macroglobulin	P01023	5	3.7	-1.54	0.06468
76	6.15	>250	Serum albumin	P02768	11	20.1	-1.21	0.16639
81	6.68	>250	Complement C3	P01024	11	9.0	-1.69	0.05986
85	3.62	191	Desmoplakin	P15924	7	2.8	-2.00	0.02492
90	3.49	184	Alpha-2-macroglobulin	P01023	15	12.1	-2.01	0.01044
94	6.40	184	Alpha-2-macroglobulin	P01023	12	10.4	-1.15	0.07054
108	6.55	181	Alpha-2-macroglobulin	P01023	10	7.6	-1.56	0.04054
127	4.86	169	Alpha-2-macroglobulin	P01023	6	4.3	-1.52	0.01788
179	5.18	129	Alpha-2-macroglobulin	P01023	14	13.0	-1.11	0.16626
217	3.62	106	Alpha-1 acid glycoprotein 2	P19652	2	6.0	1.68	0.40325
221	6.53	102	Complement factor B	P00751	3	2.2	-1.43	0.03297
303	7.69	81	Isoform 2 of Complement C4-A	P0C0L4	7	4.1	-1.93	0.03246
325	9.39	73	Isoform 2 of Complement C4-A	P0C0L4	10	6.2	-1.72	0.05882
346	7.86	70	Complement C3	P01024	13	11.1	-1.23	0.14246

Spot #	Spot pl ¹	Spot MW (kDa) ¹	Protein Name	UniProt Accession	Pep- tide count ²	Seq. cover- age ³	Fold chang e	p (t-test)
350	9.78	69	Isoform 2 of Complement C4-A	P0C0L4	7	4.2	1.38	0.00536
373	3.36	63	Alpha-1-antichymotrypsin	P01011	2	5.7	-2.04	0.00162
385	8.08	61	Complement C3	P01024	2	1.4	-1.52	0.03671
394	3.36	60	Alpha-1-antichymotrypsin	P01011	4	9.9	-2.48	0.02543
411	3.40	56	Ig gamma-1 chain C region	P01857	5	19.4	-3.23	0.00698
421	3.47	52	Ig gamma-1 chain C region	P01857	3	12.1	-2.69	0.01273
441	4.73	48	Alpha-1-antichymotrypsin	P01011	2	5.7	-1.23	0.03502
450	8.66	48	Ig gamma-1 chain C region	P01857	2	9.4	-1.62	0.04675
451	8.91	48	Ig gamma-1 chain C region	P01857	5	19.4	-2.11	0.04242
457	5.73	47	Fibrinogen gamma chain	P02679	3	6.5	-1.16	0.03002
458	8.06	47	Ig gamma-1 chain C region	P01857	3	13.0	-1.60	0.05222
465	4.49	46	Leucine-rich alpha-2-glycoprotein	P02750	2	5.5	-1.43	0.02089
493	5.07	44	Alpha-1-antitrypsin	P01009	2	4.8	-2.61	0.00261
506	9.47	43	Isoform 2 of Complement C4-A	P0C0L4	2	1.1	1.48	0.02166
539	6.51	39	Plasma serine protease inhibitor	P05154	4	10.6	-1.75	0.00800
546	4.79	39	Desmoplakin	P15924	24	10.9	-1.42	0.02830
556	5.38	38	Complement C3	P01024	5	3.7	-1.41	0.05469
563	5.07	38	Zinc-alpha-2-glycoprotein	P25311	3	10.4	-1.43	0.02424
564	5.24	38	Complement C3	P01024	3	2.8	-1.41	0.01681
565	5.29	38	Haptoglobin	P00738	2	6.4	-1.54	0.02545
566	5.52	38	Haptoglobin	P00738	1	3.0	-1.74	0.02893
567	5.67	38	Complement C3	P01024	5	4.4	-1.85	0.05336
584	5.68	36	Apolipoprotein E	P02649	3	12.0	-1.69	0.06733
604	7.78	36	Complement C3	P01024	11	9.3	-1.62	0.02817
646	9.97	34	Desmoplakin	P15924	3	1.6	1.38	0.01476
721	4.83	31	Isoform 2 of Clusterin	P10909	55	2.0	1.60	0.01231
776	6.55	29	Complement C3	P01024	4	3.3	-1.44	0.03974
891	3.45	23	60 kDa heat shock protein, mitochondrial	P10809	2	9.1	-2.08	0.07175
964	5.77	20	Junction plakoglobin	F5GWP8	6	19.6	1.65	0.00919
1013	9.48	19	Dermicidin	P81605	1	10.0	-1.00	0.82643
1031	9.82	19	Ig kappa chain C region	P01834	2	35.8	1.98	0.00745
1138	6.38	18	Haptoglobin	P00738	2	6.2	-1.99	0.02162
1159	5.35	18	Haptoglobin	P00738	2	6.2	-1.93	0.05555
1232	8.03	17	60 kDa heat shock protein, mitochondrial	P10809	1	5.2	1.41	0.02449
1256	7.06	16	Desmoplakin	P15924	11	4.6	1.57	0.01826
1318	7.54	15	Isoform 2 of Dermicidin	P81605	3	21.5	2.42	0.00207
1416	9.06	13	Serum amyloid A-4 protein	P35542	2	14.6	1.47	0.01291

Spot #	Spot pI ¹	Spot MW (kDa) ¹	Protein Name	UniProt Accession	Pep-tide count ²	Seq. cover-age ³	Fold change	p (t-test)
1459	9.32	12	Alpha-1-antitrypsin	P01009	2	4.8	1.42	0.00300
1490	8.44	12	Isoform 2 of Dermcidin	P81605	2	11.6	1.67	0.03968

¹The pI and MW are from 2DE spots by gel calibration

²“Peptide count” for MALDI and “Exclusive unique peptide count” for LC-MS identifications

³Percent of the protein sequence covered by the mapped peptides

Table III

Candidate biomarkers.

Biological Function	Biomarker candidate	Short name	Swiss Prot #	Source of identification	Fractionation
Dengue					
	Dengue NS1	NS1	Q67431	(42)	–
Complement					
	Complement factor 4A	CO4A	P0C0L4	This study; (12, 40, 44)	-
	Complement Factor H	CFH	P08603	This study; (31, 40)	-
	Complement Factor D	CFD	P00746	(31, 33)	IP
Acute Phase					
Reactant					
	A2-macroglobin	A2M	P01023	This study; (39)	-
	Alpha 1 anti-trypsin	A1AT	P00760	This study; (39, 40, 42, 44)	-
	Fibrinogen, alpha	FIBA	P02671	(12)	-
	Fibrinogen, beta	FIBB	P002675	(40)	-
	Ferritin, light chain	FRIL	P02792	(40)	-
	Haptoglobin	HPT	P00738	(40)	-
Plasma protein					
	Leucine rich alpha 2 glycoprotein	AG2L	P02750	This study; (40, 44)	-
	High MW albumin	HMWAlb	P02761	(12)	CE
Immunoglobulin					
	Immunoglobulin J	IGJ	P01591	(12)	-
	Immunoglobulin kappa, C region	IGKC	P01834	(12)	-
	IgG-gamma-1, C region	IGHG1	P01857	This study;(32)	-
Cytoskeletal					
	Keratin 1	KRT1	P04264	This study; (12)	-
	Tropomyosin 4	TMPH	P67936	(12)	-
	Low MW desmoplakin	DESP	P15924	This study	CE
	Vimentin	VIME	P08670	This study	-

IP, immunoprecipitation; CE, capillary electrophoresis

Table IV

Confusion matrix for RF classifier of disease severity.

Class	Total	Prediction		
		DF (n=59)	DFC (n=29)	DHF (n=22)
DF	59	47	5	7
DFC	29	0	29	0
DHF	22	2	0	20
Total	110	correct =79.66%	correct =100%	Correct=90.91%

For each disease (class), the prediction success of the RF classifier is shown.