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## Biomarkers in differentiating clinical dengue cases: A prospective cohort study

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## ABSTRACT

**Objective:** To evaluate five biomarkers (neopterin, vascular endothelial growth factor-A, thrombomodulin, soluble vascular cell adhesion molecule 1 and pentraxin 3) in differentiating clinical dengue cases.

**Methods:** A prospective cohort study was conducted whereby the blood samples were obtained at day of presentation and the final diagnosis were obtained at the end of patients' follow-up. All patients included in the study were 15 years old or older, not pregnant, not infected by dengue previously and did not have cancer, autoimmune or haematological disorder. Median test was performed to compare the biomarker levels. A subgroup Mann-Whitney *U* test was analysed between severe dengue and non-severe dengue cases. Monte Carlo method was used to estimate the 2-tailed probability (*P*) value for independent variables with unequal number of patients.

**Results:** All biomarkers except thrombomodulin has *P* value < 0.001 in differentiating among the healthy subjects, non-dengue fever, dengue without warning signs and dengue with warning signs/severe dengue. Subgroup analysis for all the biomarkers between severe dengue and non-severe dengue cases was not statistically significant except vascular endothelial growth factor-A (*P* < 0.05).

**Conclusions:** Certain biomarkers were able to differentiate the clinical dengue cases. This could be potentially useful in classifying and determining the severity of dengue infected patients in the hospital.

## 1. Introduction

Only nine countries had experienced severe dengue epidemics before the year of 1970. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-east Asia and the Western Pacific. More than 3.9 billion people, who constitute 40% of the world population, are at risk to the exposure of dengue infection[1]. The incidence rate is approximately four times higher in 15 year-old and above as compared to less than 15 year-old Malaysians[2]. This has an impact on the economy of the country's health care system.

Dengue clinical manifestation is divided into three phases: febrile, critical and recovery phase. Critical phase is the most important phase where the patient can either recover or die from the disease[3-5].

Unfortunately, critical phase is difficult to predict even with the new World Health Organization classification which hope to serve better clinical management of the patient[6,7]. This led to unnecessary admission and in turn increased the health care burden due to the lack of specificity of warning signs[8]. The criteria of severe dengue in the revised classification indicate that the pathological changes have already occurred and thus only a short window period for treatment and intervention. The list of warning signs were based on symptoms and signs one day prior to the cases that requiring major intervention[7]. This also does not allow clinician to timely institute the treatment within the short time frame. Furthermore, a significant 14% of patients with severe dengue do not develop warning signs at all[9].

In order to reduce the burden of the disease and for safer management of dengue infected patients, a prediction method is useful to help guide the clinicians. Numerous biomarkers for dengue infection were identified for the prediction of dengue infection and its progress[10,11]. Unfortunately, these markers have not been substantiated so that it could be applied in the prediction of the infection and its outcome. The selection of biomarkers was based on future use at primary care setting with test kit that is cheap and easily to be performed in a large population. Therefore, biomarkers which tested using ELISA technique

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along with literature evidence to indicate its use in dengue infection were selected[12-19]. Hence, this study identified five most potential biomarkers [neopterin, vascular endothelial growth factor-A (VEGF), thrombomodulin, soluble vascular cell adhesion molecule 1 (VCAM-1) and pentraxin 3 (PTX-3)] to be evaluated in differentiating clinical dengue cases. The secondary objective was to compare the different clinical dengue cases with healthy subjects and non-dengue fever.

## 2. Material and methods

### 2.1. Sample collection

This was a prospective cohort study conducted in Ampang Hospital from January to September 2014 where patients were recruited consecutively at day of presentation and upon discharged from the hospital. Informed consent and patient information leaflet were provided to all patients. This study was approved by the Medical Research and Ethics Committee (National Institute of Health Secretariat, Malaysia) and Scientific and Ethical Review Committee of Universiti Tunku Abdul Rahman.

The blood samples were obtained at the day of presentation. The demography and final diagnosis of the patients were obtained from the medical records at the end of patient follow-up with the hospital. The levels of the biomarkers were compared among healthy subjects, non-dengue fever, dengue without warning signs and dengue with warning signs/severe dengue. The clinical classification of dengue infection was based on clinical practice guidelines published by World Health Organization[6].

All patients included in the study were 15 years old or older, not pregnant, has not been infected by dengue before (secondary dengue) and did not have autoimmune disorder, haematological disorder or cancer. All healthy subjects which derived mainly from university students and some were staffs working in the hospital, did not have fever at the time when blood was taken. Non-dengue fever was defined as patients having fever but did not have the final diagnosis of dengue such as upper respiratory tract infection, acute gastroenteritis and pneumonia. The sample size of 95 per group with a power of 80% were based on the highest calculated sample size using previous available studies of various biomarkers[12,16,18].

### 2.2. Blood sampling and biomarkers assay

The blood samples were obtained either by the investigator or by the attending practitioners. Plain tubes and ethylene diamine tetraacetic acid tubes were used for the collection of blood samples. The serum of the blood samples was stored below -20 °C. The duration of storage to the analysis of blood samples were around 8 to 12 months. Ideally the laboratory analysis should be performed immediately to avoid degradation of biomarkers. The laboratory analysis was unable to be performed much earlier because the different test kits from different company did not arrived to our laboratory at the same time. The five test kits were chosen to be performed together rather than individually to avoid repeated freeze-thaw cycle which could lead to degradation of biomarkers as well. The results of this study produced significant finding even though

the test were performed at 8 to 12 months' time. The biomarkers were expected to be degraded equally in all samples since they were tested around the same time which allows better comparison.

The levels of the biomarkers were measured using commercially available sandwich ELISA test kits: neopterin (IBL International), VEGF (eBioscience and R&D Systems), VCAM-1 (eBioscience and R&D Systems), thrombomodulin (R&D Systems) and PTX-3 (R&D Systems).

### 2.3. Statistical analysis

The demography (age, sex and race), day of fever when the blood was taken, dependent variables (healthy subjects, non-dengue fever, dengue without warning signs and dengue with warning signs/severe dengue) and independent variables (levels of the biomarker) were described.

Median test was used when the distribution of the biomarker levels among the dependent variables were not similar as indicated by the non-parametric Levene test. A subgroup Mann-Whitney *U* test was analysed by splitting the severe dengue cases from dengue with warning signs to compare severe dengue with non-severe dengue cases (combined dengue with and without warning signs). Monte Carlo method was used to estimate the 2-tailed probability (*p*) value for independent variables with unequal number of patients. A *p*-value of less than 0.05 is considered statistically significant. SPSS (Statistical Package for Social Science) version 20 was used in the analysis.

## 3. Results

The median age was 25 years old with interquartile range (IQR) of 12. Number of patients included in the study was 195 (56.7%) male and 149 (43.3%) female. There were 230 (66.9%) Malays, 85 (24.7%) Chinese, 23 (6.7%) Indians and 6 (1.7%) Malaysians of other ethnicity. The median day of fever when the blood was taken was Day 4 of fever (IQR: 2 days). Missing biomarker levels were found one each in thrombomodulin and VCAM-1, and three in PTX-3. The median, IQR and number of patients of the five biomarkers stratified by the groups of dependent variable were shown in Table 1.

Comparison of neopterin, VEGF, thrombomodulin, VCAM-1 and PTX-3 levels among the dependent variables along with the *post-hoc* analysis was tabulated in Table 2. Subgroup analysis for all the biomarkers between severe dengue and non-severe dengue cases was not statistically significant except VEGF ( $P < 0.05$ ) as in Table 3.

**Table 3**

Subgroup analysis between severe dengue and non-severe dengue cases.

	Mean rank		Mann-Whitney <i>U</i>	<i>P</i> value*
	Non-severe dengue cases	Severe dengue		
Neopterin (nmol/L)	91.56	118.20	623.00	0.13
VEGF (pg/mL)	91.23	124.00	565.00	< 0.05
Thrombomodulin (pg/mL)	93.38	77.15	716.50	0.35
VCAM-1 (ng/mL)	94.32	60.75	552.50	0.05
PTX-3 (ng/mL)	89.72	122.10	554.00	0.06

\* Monte Carlo method.

**Table 1**

The median, IQR and number of patients of the five biomarkers stratified by the groups of dependent variable.

	Biomarkers [median (IQR)]					Number of patients [n (%)]
	Neopterin (nmol/L)	VEGF (pg/mL)	Thrombomodulin (pg/mL)	VCAM-1 (ng/mL)	PTX-3 (ng/mL)	
Healthy subjects	23.30 (41.36)	0.00 (571.00)	4376.61 (12645.17)	73.20 (106.21)	0.00 (5.70)	62 (18.00%)
Non-dengue fever	53.04 (125.73)	89.95 (822.50)	4188.54 (4448.81)	71.49 (385.34)	1.24 (12.19)	97 (28.20%)
Dengue without warning signs	67.94 (210.56)	0.00 (117.00)	4461.82 (2548.27)	273.13 (1223.84)	5.66 (16.46)	93 (27.00%)
Dengue with warning signs	81.43 (219.18)	0.00 (350.00)	5244.17 (11649.14)	241.85 (1627.39)	10.13 (25.14)	82 (23.80%)
Severe dengue	141.84 (245.96)	362.35 (1019.06)	4293.01 (1954.89)	88.32 (453.95)	26.48 (53.96)	10 (2.90%)

**Table 2**

Comparison of the biomarker levels among the dependent variables: healthy subjects, non-dengue fever, dengue without warning signs and dengue with warning signs/severe dengue.

	Comparison among the four dependent variables <sup>a</sup>			Post-hoc analysis of two dependent variables [ <i>Chi-square (df)</i> ]		
	Median	<i>Chi-square (df)</i>	<i>P</i> -value	Healthy subjects, non-dengue fever	Non-dengue fever, dengue without warning signs	Dengue without warning signs, dengue with warning signs
Neopterin (nmol/L)	55.61	38.75 (3)	< 0.001	12.02 (1)**	1.36 (1)	4.26 (1)*
VEGF (pg/mL)	0.00	17.73 (3)	< 0.001	1.33 (1)	17.16 (1) <sup>#</sup>	4.23 (1)*
Thrombomodulin (pg/mL)	4432.33	2.66 (3)	0.450	0.03 (1)	0.41 (1)	0.79 (1)
VCAM-1 (ng/mL)	123.33	57.19 (3)	< 0.001	1.64 (1)	29.91 (1)**	0.91 (1)
PTX-3 (ng/mL)	3.88	30.13 (3)	< 0.001	4.46 (1)*	3.57 (1)	2.82 (1)

<sup>a</sup>: Median test; \*:  $P < 0.05$  with non-dengue fever has higher biomarker level than healthy subjects; dengue with warning signs has higher biomarker level than dengue without warning sign; \*\*:  $P < 0.001$  with non-dengue fever has higher biomarker level than healthy subjects, dengue without warning signs has higher biomarker level than non-dengue fever; <sup>#</sup>:  $P < 0.001$  with non-dengue fever has higher biomarker level than dengue without warning signs.

#### 4. Discussion

Neopterin and PTX-3 levels have a clear increasing median trend from healthy subjects to severe dengue. All except thrombomodulin have statistically significant difference among the four groups of dependent variable. The *post-hoc* analysis indicated that these biomarkers were able to discriminate certain groups of dependent variables. Subgroup analysis between severe dengue and non-severe dengue cases indicated that only VEGF was able to discriminate the two categories. Though VCAM-1 and PTX-3 were not statistically significant, the  $P$  values were at the margin of the pre-determined  $P$  value of less than 0.05. However, VCAM-1 has a lower level in severe dengue compared to non-severe dengue cases which was not theoretical coherent.

Analysis of neopterin appeared to be consistent with previous studies where the serum neopterin could differentiate dengue fever from dengue haemorrhagic fever/ dengue shock syndrome and dengue infection from healthy individuals[12,13]. Other studies have shown that neopterin is a non-specific biomarker which its level also elevated in other conditions such as cardiovascular disease, pneumonia and colorectal cancer[20-22]. Hence, this could possibly explain the failure to differentiate between non-dengue fever and dengue without warning signs in our study. Another possible explanation is that neopterin is only elevated during the plasma leakage which is seen in dengue with warning signs[23].

Similar to some studies, VEGF levels could not differentiate between the healthy subjects, non-dengue fever and dengue without warning signs[24,25]. However, VEGF is elevated in dengue with warning signs/severe dengue[26], but care must be taken when comparing with studies conducted in children[27-29].

Our study on thrombomodulin level in dengue infection is in agreement with some studies where thrombomodulin could not differentiate the clinical dengue cases[24,30], but not in many other studies[14,18,19,30-33]. However, those studies conducted were in children which itself is a higher risk of severe complication and thus reflected in the elevated thrombomodulin levels[14,18,19,31].

VCAM-1 was only able to differentiate dengue without warnings signs from non-dengue fever. As opposed to previous study, VCAM-1 could not differentiate among the different severity of dengue cases in our study[34]. Other studies were conducted in children indicated VCAM-1 could differentiate the different severity of dengue cases[17,35,36].

To our knowledge, our study was the first to evaluate PTX-3 in dengue infection of adult population. There was a study conducted in children with similar results whereby the levels of PTX-3 were higher in dengue shock syndrome compared to dengue fever/dengue haemorrhagic fever in the first five days of admission[37].

Several limitations identified in this study: (i) the inclusion criteria was based on the clinical diagnoses made by the attending practitioners, not by the laboratory diagnosis. There could be misclassification among the non-dengue fever and dengue fever cases. Ideally, it should be supplemented with a confirmatory dengue diagnosis. (ii) the day of diagnosis made especially to those who developed severe dengue

should be noted down. There is a possibility that the blood withdrawn from the patients coincided with the same day as the diagnosis being made. Furthermore, the blood was taken at around day four of fever in our study coincides during the critical phase of dengue infection. Hence, this study's result might not be able to predict severe dengue. (iii) the sample size for severe dengue in the subgroup analysis was low as this was not the primary objective of this study. (iv) there could be variation among the commercial test kit used. However, few of the same samples were tested in each subsequent test kit to ensure the results were reliable. For those test kits were not consistent, all the samples were repeated in subsequent test kits. (v) this study was conducted in a hospital setting and therefore, it is expected to have much severe cases compared to primary care clinics. The result is perhaps applicable to the hospital population.

In our study, only 10 cases of all dengue cases (5.7%) progressed to severe dengue which was also seen in previous study[7]. Hence, the study should have focused on group of patients developing complication instead of warning signs because many of the patients with warning signs do not progress to severe dengue. Perhaps, the recovery of these patients who do not progress to severe dengue could also be due to the supportive treatment provided after admitting to the hospital. The subgroup analysis indicated that VEGF and PTX-3 could be a potential biomarker in differentiating severe dengue cases from non-severe dengue cases. The results though favourable, the results should be interpreted cautiously as mentioned in the limitation of the study: blood could have been taken during the same day as the diagnosis being made whereby the pathogenesis of complication has already begun. This will not be helpful in predicting the severity of dengue infection. Since these biomarkers were also non-specific to dengue infection, the use of biomarkers in suspected dengue patient should be accompanied by a diagnostic test. Otherwise, non-dengue fever cases could be misdiagnosed as dengue which will in turn affect the treatment and the disease outcome. Thus, further appropriate study to address the limitations is to be conducted before concluding that the results were favourable in clinical management of dengue infection.

In conclusion, some of the biomarkers were able to differentiate the clinical dengue cases. This could be potentially useful in classifying and determining the severity of dengue infected patients in the hospital.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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