

## Cut-off Value of HSPG for Early Marker of Plasma Leakage in Adult Dengue Patient

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### ABSTRACT

Plasma leakage is a state of increased vascular permeability due to loss of interendothelial junction and focal adhesion. Endothelium glycocalyx plays a major role in the pathogenesis of plasma leakage. Proteoglycans glycocalyx consists of heparan sulfate (HSPG) around 50-90%. Plasma leakage in dengue infection can be fatal and early detection is essential. This study aimed to determine the optimal cut-off value of HSPG levels for early marker of plasma leakage in dengue infection. The study was a retrospective cohort study as a part of the Community Based Dengue Study of the Faculty of Medicine, University of Indonesia, which was conducted between February 2010 and January 2011. Subjects were recruited by consecutive sampling. Dengue infection was confirmed by conventional PCR serotyping. Subjects were categorized into 2 groups, i.e. those with and without plasma leakage. Plasma leakage was confirmed by hemoconcentration or hypoalbuminemia or USG results. The level of HSPG was measured using the ELISA method from the first until the third day of the hospital admission. A total of 40 subjects were involved in this study, consisting of 21 with leakage and 19 without leakage. The optimal cut-off level of HSPG as a marker of early plasma leakage in dengue infection patients on the 1<sup>st</sup> day, 2<sup>nd</sup> day, and 3<sup>rd</sup> day was 2179.73 pg/mL, 2538.66 pg/mL, and 1294.06 pg/mL, respectively. HSPG could as an early marker of plasma leakage in dengue infection with an optimal cut-off value for each of the first 3 days of the patient's fever. A pediatric study was recommended to obtain the optimal cut-off value for HSPG.

**Keywords:** Dengue, plasma leakage marker, HSPG

### INTRODUCTION

Dengue is an acute infectious disease that has spread to almost all over the world, especially in tropical countries. The Ministry of Health Republic of Indonesia in 2021 reported that the incidence rate was 27 per 100,000 people and the case fatality rate was 0.96%.<sup>1</sup> European Centre for Disease Prevention and Control in 2022 announced that there were 4,110,465 cases of dengue and 4,099 deaths worldwide; whereas there have been 125,888 cases reported in Indonesia with the highest death rate of 1,082 deaths.<sup>2</sup>

Dengue infection is caused by one of five serotypes of dengue viruses, Denv-1, 2, 3, 4, and 5. The fifth variant, Denv-5, was isolated in October

2013 in India.<sup>3,4</sup> Denv-2 is the most dominant serotype in Jakarta which is followed by Denv-3, Denv-1, and Denv-4.<sup>5</sup> In Surabaya, Denv-3 is dominant and the incidence of plasma leakage was 56.67%.<sup>6,7</sup> A person can be infected by different serotypes of dengue virus (heterologous infection), which may lead to Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS). They can be fatal when prompt treatment is not available.<sup>8</sup>

The main complication of dengue infection is plasma leakage, which may result in shock and death. Plasma leakage is a state of increased vascular permeability due to loss of interendothelial junction and focal adhesion caused by the host's response to infectious pathogens. Plasma leakage and the severity of the leakage can only be identified through

its secondary effects on plasma volume and body fluid distribution, such as hemoconcentration, pleural effusion, and ascites.<sup>9,10</sup> Nowadays, the common method for monitoring plasma leakage in DHF/DSS patients is through serial measurement on hematocrit or albumin level, which can identify plasma leakage at a critical phase. However, these methods are considered less sensitive since the baseline data of hematocrit or albumin levels of the patients are usually unknown.<sup>9,11,12</sup> Other methods used for this purpose are abdominal and thoracic ultrasonography (USG). Serial USG examination can identify plasma leakage at the defervescence phase.<sup>13,14</sup> Therefore, a new parameter is needed to be used as an early marker of plasma leakage.

Previous studies have shown that vascular endothelial cells, especially endothelium glycocalyx, play a major role in the pathogenesis of plasma leakage. Glycocalyx is a thin layer of endothelial endocapillary lining the entire surface of the luminal endothelium.<sup>15</sup> Glycocalyx serves as a primary hindrance to water and molecule movement. It also serves as a selective molecular sieve towards molecular restriction.<sup>10</sup>

Another study found that damage to the glycocalyx endothelial layer precipitously disrupts microvascular flow.<sup>16</sup> Proteoglycan is considered as the most important structure since it is the largest component of glycocalyx. It consists of a long glycosaminoglycan chain (GAG-chain), which is negatively charged, and embedded in the plasma membrane. On vascular endothelial cells, 50-90% proteoglycans glycocalyx consists of heparan sulfate known as heparan sulfate proteoglycans (HSPG).<sup>17</sup> A biomolecular study shows that HSPG plays a role in detecting and amplifying shear stress of vascular flow that contributes to inflammatory responses. In general, it is suggested that glycocalyx may serve as the protector of blood vessels.<sup>18</sup>

This study aimed to determine the cut-off value of vascular endothelial HSPG levels for an early marker of plasma leakage in adult dengue patients. An optimal cut-off value for each day of the first 3 days of the patient's fever was analyzed.

## METHODS

This study was a part of the Community-based Dengue Study (Commbads) at the Faculty of Medicine, University of Indonesia. It had been approved by The Joint Committee on Medical Research and Ethics, Faculty of Medicine, University of Indonesia (No 63/PT02.FK/ETIK/2010). A retrospective cohort design was used in this study.

Subjects were recruited by consecutive sampling method and the study was carried out from March 2010 to December 2011 in Jakarta.

Blood samples were collected when participants came to public health centers or research centers in the community. To confirm dengue infection among the subjects, the following procedure was carried out, i.e. on the first day at the community health center, blood samples were collected and NS1 dengue rapid test (Standard Diagnostic, South Korea) was used to detect the presence of NS1 dengue. A conventional PCR serotyping was used for the sample, which gave a positive result. Participants with positive test results were immediately transferred to Cipto Mangunkusumo Hospital, Jakarta for further observation and treatment. Blood samples were taken in the hospital every day at 6 A.M. for the first 3 days of admission. Samples were collected in a K3EDTA anticoagulant tube for hematocrit and a plain tube for measurement of albumin and HSPG levels.

In the community setting, the inclusion criteria were patients with a fever of  $\geq 38^{\circ}\text{C}$  for  $\geq 48$  hours, age of  $\geq 14$  years, and patients who provided informed consent to participate in the study. Patients with current pregnancy, menstruation, or comorbidities such as diabetes mellitus, or chronic kidney disease were excluded.

Since there had been no studies on HSPG levels in the Indonesian population, a pilot study was used to determine the study sample size. A minimum of 30 subjects consisting of 10 subjects without dengue infection, 10 subjects who had dengue infection with plasma leakage, and 10 subjects who had dengue infection without plasma leakage were required.

Pilot studies showed that the optimal cut-off value of HSPG levels was 4741.23 pg/mL. The cut-off value was used to determine proportion size. The proportion of increased HSPG levels ( $>4741.23$  pg/mL) in the subject group with no plasma leakage (P1) was 0.4. Delta P was set at 0.3, which resulted in the proportion of increased HSPG levels in the leakage group (P2) of 0.7. After P1 and P2 were obtained, the sample size was calculated using the hypothesis test formula for two proportions, which resulted in 21 subjects as the minimum sample size.<sup>19,20</sup> Dengue status in this study was determined according to the WHO 1997 definition.<sup>21</sup>

The criteria of plasma leakage in this study were determined by the presence of pleural effusion or ascites using USG examination and/or hemoconcentration (hematocrit level  $>20\%$  than normal value for Indonesian people) and/or hypoalbuminemia.

Hematocrit level was determined using the Sysmex XE-2000 automatic hematology analyzer (Sysmex, Japan). The normal range for the Indonesian population used in this study was 40-52% (male) and 35-47% (female).<sup>22</sup>

The albumin level was measured using an automated clinical chemical analyzer [Cobas 501 (Roche, Germany)]. The normal value range for the Indonesian population used in this study was 3.4 - 4.8 g/dL (both for males and females).<sup>22</sup>

The heparan sulfate levels were quantitatively measured with the ELISA method by using an HSPG ELISA kit (Clone-Cloud Corp., USA) with a detection range of 62.5 - 4,000 pg/mL.

A two-step RT-PCR test was used as the gold standard to confirm the presence of dengue virus infection and determine the dengue virus serotype. RNA was extracted using Qiagen (Amsterdam, Netherlands). The presence of dengue viral genome serotypes was indicated by clear DNA bands. A DNA band at 482 bp, 119 bp, 290 bp, and 392 bp showed the serotypes of Denv-1, Denv-2, Denv-3, and Denv-4, respectively. PCR was repeated on specimens with results showing mixed serotypes of dengue by using primers for each serotype.

Abdominal and thoracic USG examinations were performed every day. USG was performed using the Aloka SSD 3500 series with a convex probe at 2.5-5 MHz. The examinations were carried out daily at the same time during admission by an experienced hepatologist to avoid interobserver bias.

Demographic characteristics of the subjects, dengue virus serotypes, plasma leakage, hypoalbuminemia, and USG results were presented in a tabular and percentage. Hematocrit was presented in mean and standard deviation. The normality of the HSPG level was tested using the Shapiro-Wilk test. Bivariate analysis of HSPG levels between subjects in the leakage and no leakage group was performed using a T-test for data with normal distribution and a Mann-Whitney test for data with abnormal distribution.

ROC was used to determine the optimal cut-off value of HSPG levels as an early marker of plasma leakage in dengue infection with optimal sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) for each of the first 3 days of the patient's fever.

## RESULTS AND DISCUSSIONS

A total of 40 subjects were recruited for this study. Characteristics of the subjects are shown in Table 1. The subjects were categorized into two groups, such as a group with plasma leakage and without plasma leakage.

Graph 1 shows that the HSPG levels were significantly different between subjects in the leakage group and the no-leakage group. These results showed that HSPG were potential to be a marker for early detection of plasma leakage in dengue infection, particularly ranging between the

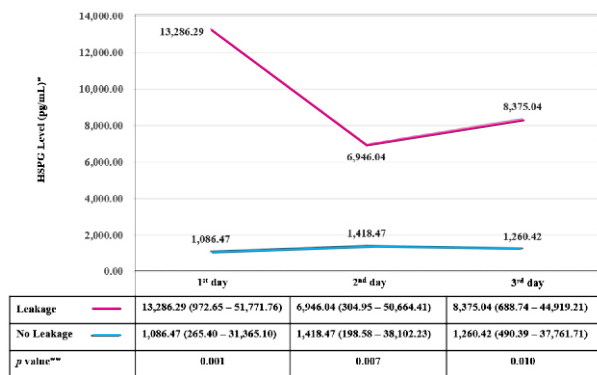
**Table 1.** Demographic characteristics of the study population

		Leakage n=21	No Leakage n=19
Age (year)*		24±10	25±11
Gender**	Male	10 (47.6)	10 (52.6)
	Female	11 (52.4)	9 (47.4)
Diagnosis**	DF	0 (0.0)	19 (100.0)
	DHF	21 (100.0)	0 (0.0)
Grade**	DF	0 (0.0)	19 (100.0)
	DHF grade I	9 (42.9)	0 (0.0)
	DHF grade II	12 (57.1)	0 (0.0)
Serotypes**	DEN-1	6 (28.5)	5 (23.8)
	DEN-2	6 (28.5)	9 (47.3)
	DEN-3	5 (23.8)	3 (17.4)
	DEN-4	0 (0.0)	1 (4.5)
	Mixed	4 (19.2)	1 (4.5)
Hypoalbumin**	Negative	13 (62.0)	19 (100.0)
	Positive	8 (38.0)	0 (0.0)
USG**	Negative	6 (28.5)	19 (100.0)
	Positive	15 (71.5)	0 (0.0)
Delta hematocrit*		36.2±3.1	3.7±1.4

\*Age, delta hematocrit shown in mean and SD

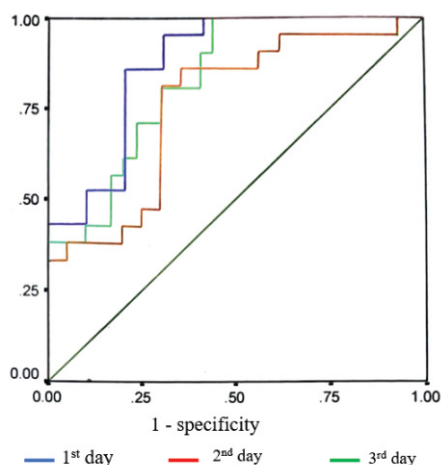
\*\*Gender, diagnosis, grade, dengue serotypes, hypoalbumin, and USG shown in percentage

first day and the third day of the febrile phase. ROC was used to obtain the optimal cut-off value of HSPG levels for each day (Graph 2).



**Graph 1.** Kinetics of median HSPG Level from 1<sup>st</sup> day until 3<sup>rd</sup> day

\*Shapiro-Wilk test, data with abnormal distribution were shown in the median and range; \*\*Mann-Whitney test



**Graph 2.** ROC of a cut-off value of HSPG levels from 1<sup>st</sup> day until 3<sup>rd</sup> day

Table 2 shows that the optimal cut-off value of HSPG levels on the first day was 2179.73 pg/mL with 89.4% sensitivity, 79.3% specificity, 73.9% PPV, and 92.0% NPV. On the second day, the value was 2538.66 pg/mL with 89.5% sensitivity, 68.4% specificity, 73.9% PPV, and 86.7% NPV. On the third day, the value was 1294.06 pg/mL, with 80.9% sensitivity, 68.9% specificity, 65.3% PPV and 83.3% NPV.

**Table 2.** Optimal cut-off value of HSPG levels on the first, second, and third day for early markers of plasma leakage in dengue infection

Day	HSPG (pg/mL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
1	2179.73	89.4	79.3	73.9	92.0
2	2538.66	89.5	68.4	73.9	86.7
3	1294.06	80.9	68.9	65.3	83.3

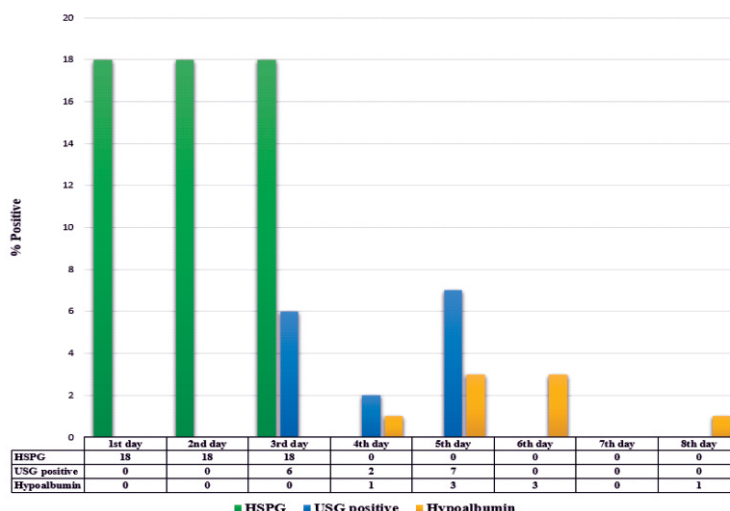
PPV: Positive Predictive Value, NPV: Negative Predictive Value

Cell-surface heparan sulfate proteoglycan plays key roles in regulating cell behavior, cell signaling, and interactions between cell and matrix in both physiological and pathological conditions. Their soluble forms of glycocalyx shedding are not merely waste products and are detectable in serum, which may be useful as diagnostic and prognostic markers.<sup>23-25</sup>

Reitsma *et al.* found that glycocalyx damage can be observed by the hematocrit differences.<sup>15</sup> These data were similar to the study by Desjardins and Duling in 1990, which reported an increase of up to two-fold of hematocrit when glycocalyx damage occurred.<sup>26</sup> This found that the group with plasma leakage obtained higher mean delta hematocrit (36.2±3.1%) and HSPG level (13,286.29 pg/mL) on the first day. On the contrary, a lower mean delta hematocrit (3.7±1.4 %) and HSPG level (1,086.47 pg/mL) were found on the first day in the group without plasma leakage. These findings clarified the role of glycocalyx as the protector of blood vessels.<sup>15</sup>

Hypoalbuminemia is another marker of plasma leakage. Hypoalbuminemia in dengue infection occurs at a critical phase, specifically after the third day of fever onset.<sup>10</sup> This study has demonstrated that all incidences of hypoalbuminemia occurred for more than 3 days after admission. There were only 8 out of 21 subjects (38%) in the leakage group who experienced hypoalbuminemia. In comparison, HSPG levels increased at 18 subjects (86%) since the first day. This comparison data indicated that the HSPG level was more sensitive and able to detect earlier plasma leakage compared to hypoalbuminemia (Graph 3).

Detection of plasma leakage using USG examination has been investigated. Serial bedside ultrasound can detect plasma leakage earlier than hemoconcentration.<sup>27</sup> However, some studies have shown that USG assessments are only able to identify plasma leakage at the defervescence phase.<sup>14</sup> Similar data have also been found in our study, which has demonstrated that 15 out of 21 subjects (71.5%) in the plasma leakage group had positive USG results after the third day. In contrast, HSPG levels can



**Graph 3.** Comparison of the sensitivity of HSPG, hypoalbuminemia and USG examination to detect plasma leakage in dengue infection.

detect 18 subjects (86%) who were likely to experience plasma leakage from the first day. It demonstrated that HSPG was more sensitive and earlier compared to the USG examination (Graph 3).

This study demonstrated that HSPG level was associated with dengue infection and plasma leakage. Those who were infected and experienced plasma leakage had higher HSPG levels than those without plasma leakage and the HSPG levels had already been high since the first day of admission.

The kinetics of the median of HSPG levels was slightly decreased on the second day and third day. Greater HSPG destruction compared to its production and fluid therapy received by the subjects with plasma leakage in this study resulting to the dilution of HSPG levels might be the main cause for this finding.

Cut-off values with the highest sensitivity and NPV were selected for the early detection of plasma leakage. Lower sensitivity and NPV value would increase the risk of false negative results. False interpretation of such results would be very harmful since it would lower the vigilance against the danger of dengue shock.<sup>19</sup>

Although 90% of dengue infections occur in children, this study was unable to provide an overview of the role of HSPG levels in this age group. Therefore, further research was needed in the age group of children.

**CONCLUSIONS AND SUGGESTIONS**

This study has successfully established an optimal cut-off level of HSPG as an early marker to detect plasma leakage in patients with dengue infection in

Indonesia, for each day from the first day until the third day. It is necessary to conduct further research on pediatric patients because many children suffer from dengue infection.

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