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Expression of inducible nitric oxide synthase, nitric oxide and salivary oxidized LDL as early markers of severe dengue

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Abstract

Objectives: To identify suitable biomarkers during early stages of dengue to predict which patients would develop severe forms of dengue before the warning signs appear.

Methods: Expression of inducible nitric oxide synthase (iNOS) and resultant changes in nitric oxide (NO) and oxidized low density lipoprotein (oxLDL) levels in plasma and saliva were analyzed.

Results: Expression of iNOS in patients who later developed dengue hoemorrhagic fever (DHF) showed significant ($P<0.05$) down regulation compared to dengue fever (DF) patients while those who later developed DHF showed a corresponding significant ($P<0.05$) decrease of plasma NO levels (18.1±3.1 µM) compared to DF patients (23.6±4.4 µM) within 4 days from fever onset. OxLDL levels in plasma showed a decrease in patients who later developed DHF compared to DF patients although this value was significantly different only within 3 days from fever onset. The salivary NO levels did not show a significant difference. However, salivary oxLDL levels were significantly ($P<0.05$) low in patients who later developed DHF (0.6±0.2 ng/mL) compared to DF patients (1±0.4 ng/mL) collected within 4 days from fever onset.

Conclusions: The expression level of iNOS, plasma NO and salivary oxLDL levels may serve as early markers of severity of dengue infection.

Keywords: Dengue, Dengue Hemorrhagic Fever, inducible nitric oxide synthase, nitric oxide, oxidized LDL, salivary biomarkers

Highlights

- Severity of dengue infection correlates with early differential expression of iNOS
- Plasma NO and salivary oxLDL levels may also serve as early markers of severe dengue
- Saliva may serve as a non-invasive source of early biological markers for severity of dengue infection

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Introduction

Dengue is an extremely prevalent mosquito-borne viral disease in many tropical countries including Sri Lanka. It is the second most important tropical disease (after malaria) with 284 - 528 million dengue infections resulting in 67 – 136 million clinically manifested dengue cases with half the global population at-risk posing a significant public health threat worldwide.[1,2] In 2017, 1,86,101 suspected dengue cases were reported to the Epidemiology Unit of Sri Lanka from all over the island.[3] Most people infected with dengue viruses are asymptomatic while others may suffer a wide range of clinical manifestations from mild fever to severe dengue. Although a serious, debilitating condition, dengue fever (DF) is not fatal while severe manifestations of the disease such as dengue hoemorrhagic fever (DHF) and dengue shock syndrome (DSS) are major causes of hospitalization and death, globally.[1,4] Currently, the number of DHF cases in Sri Lanka has dramatically increased.[5] Unlike DF, severe dengue is characterized by severe possibly lethal vasculopathy marked with plasma leakage, intrinsic coagulopathy and massive internal bleeding.[6,7]

Despite the social and clinical impact, there are no antiviral therapies available for treatment of dengue.[8] The vaccine that is licensed in 18 countries has several limitations because it is only recommended for individuals aged 9-45 years who have had previous exposure to dengue.[9] As such, prevention is mostly limited to vector control measures. While several efficient and relatively reliable diagnostic tests based on PCR or serological testing are available for detection of dengue virus infections, these diagnostic tests do not distinguish between DF and severe dengue.[10] Limited progress has been made in finding markers that can indicate the evolution of dengue infection to the severe form of the illness at an early stage of infection. Infact, a diagnosis of disease severity is usually made after the patient is presented with severe dengue symptoms. Several studies have compared the transcriptomes of patients that developed DF with those who developed DHF to identify molecular markers such as cytokines associated with disease severity. [7,11-17]. In a genome-wide association study, genetic varients in MICB and PLCE1 has been found to be associated with severe dengue.[18] Allelic forms of MICA and MICB, on the other hand, has been found to strongly associate with susceptibility to illness but not severe cases.[19] Recent studies have also reported differential expression in microRNA in dengue patients and infected cultured cells.[20-24] However, most of the patient studies do not limit the sample pool to acute phase of infection at which differentail diagnosis is not possible. Thus, despite these efforts, endeavors to discover a prognostic test for severe dengue is yet to become part of the dengue clinical tests, leaving much to be done in finding a solution to this public health crisis.

Inducible nitric oxide synthase (iNOS) has been implicated in host response to dengue virus infection.[25] Expression of iNOS results in nitric oxide (NO) biosynthesis resulting in generation of a highly reactive nitrogen oxide species, peroxynitrite, via a radical coupling reaction of NO with superoxide which in turn causes potent oxidation and nitration reactions of various biomolecules including lipids. NO which plays a complex and diverse physiological and

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pathophysiological role may serve as an early prognostic marker in dengue.[26] iNOS activity and plasma NO has been implicated in inflammatory responses and plasma leakage.[25] Severe dengue is characterized by thrombocytopenia (low platelet count) and plasma leakage. A recent study evaluating the levels of NO in patient samples from DF and DHF patients indicate the potential of these molecular markers to serve as early markers of disease prognosis in serum.[27] However, the levels of these reactive oxygen species (ROS) and reactive nitrogen species (RNS) were not evaluated for their potential as early markers of the disease in other biological fluids. Therefore, in this study, we evaluated whether the severity of dengue infection is correlated with early differential expression of iNOS and resultant changes in NO levels and oxidized low density lipoprotein (oxLDL) levels in plasma from patients who tested positive for dengue within 4 days from fever onset before severe symptoms are presented. Levels of biomarkers such as LDL in saliva has been reported to correlate with serum and plasma LDL levels.[28] Since saliva is a safe and easy to handle biological fluid that can be collected using non-invasive measures, we also evaluated the salivary NO and oxLDL levels in samples from patients presented with symptoms of dengue fever during the early stages of infection with those who later developed DHF.

The development and the severity of DHF can be mitigated with proper disease management. If diagnosed early before severe symptoms are presented, effective disease management of severe dengue only involves hospital care and hydration. Identifying early molecular markers of severe dengue may help distinguish dengue patients who would benefit from early intensive therapy and hospitalization before severe symptoms appear and increase the availability of public health resources and also mitigate the cost of public health and the impact on the national economy.

Materials and Methods

Sample collection and processing: Patients presented with clinical symptoms of dengue viral infection according to WHO Dengue case classification (fever, with two of the following criteria: headache, retro-orbital pain , myalgia, arthralgia, rash, hemorrhagic manifestations with no plasma leakage, and following laboratory findings leucopenia, thrombocytopenia, rising hematocrit with no evidence of plasma loss) within 4 days from fever onset who tested positive for onsite NS1 rapid test (SD Bio) were recruited for the study from the North Colombo Teaching Hospital, Ragama, Sri Lanka with informed consent. Patients who later develop DHF were determined according to the WHO guidelines; Fever and Hemorrhagic manifestation (positive tourniquet test) with evidence of plasma leakage (pleural effusions and ascites detected using a portable bedside ultrasonogram), spontaneous bleeding, circulatory failure, profound shock with undetectable BP and pulse, thrombocytopenia < 100 000 cells / mm^3, and HCT rise > 20%.[29] A questionnaire was used to collect information pertaining to alcohol consumption, smoking habits and dietary intake. 2.5 mL blood was collected in to EDTA tubes and approximately 500 µL saliva was collected by spit method. The samples were collected from patients within 4 days from fever onset and transported and processed at 4 °C within an hour

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from sample collection. Isolated peripheral blood cells (PBC) and saliva samples (after adding 30 mM NaOH) were stored at – 80 °C until sample analysis. Ethical clearance for patient sample collection was obtained from the ethics review committee of the Faculty of Medicine, University of Kelaniya, Kelaniya, Sri Lanka (Reference number-P/119/07/2015).

Quantitative Real-time PCR: Gene specific human primers against the reference gene GAPDH and iNOS were mined from previously published literature.[30] Total RNA was isolated from peripheral blood cells using miRNeasy serum/plasma kit (Qiagen) with 700 µL QIAzol Lysis buffer and 140 µL chloroform according to the manufacturer's instructions. cDNA was synthesized using the miScript II RT Kit with Hiflex buffer from 12 µL extracted total RNA according to product manual (Qiagen). Expression of mRNA was quantified using QuantiTect SYBR Green PCR kit (Qiagen) according to product manual. Each reaction was carried out in triplicates in 20 µL reaction volume using StepOne real-time PCR Thermal Cycler (Applied Bio). The efficiency of amplification for iNOS was 106 % and GAPDH was 110 % based on the standard curve analysis. No-template reactions and melting curve analysis was used to confirm specificity of target amplification.

Quantification of plasma and salivary nitric oxide by Greiss reaction: Nitrite content in plasma was measured according to a previously reported method using Griess reaction against a standard series of $NaNO_2$.[31] Plasma sample (60 µL) was deproteinized with 7.5 µL of 200 mM $ZnSO_4$ prior to assay for nitrites.

Salivary nitrite levels were detected by Griess colorimetric reaction as total nitrates and nitrites as described by Miranda, Espey and Wink, (2000) with some modifications[32]. Saliva sample (120 µL) was deproteinized with 15 µL of 200 mM $ZnSO_4$ and mixture was centrifuged for 3 min at 10,000 g at room temperature. 50 µL of supernatant, 50 µL of 2 % (w/v) sulfanilamide in 5 % HCl (v/v) and 50 µL of 1 % (w/v) N-(1-Naphthyl) ethylenediamine dihydrochloride in water were mixed to a final volume of 200 µL. Mixture was incubated at room temperature for 20 mins and absorbance at 540 nm was measured using Multiskan Go spectrophotometer (Thermo Scientific). Conversion of nitrates to nitrites by VCl_3 followed by colorimetric analysis for nitrites using Greiss reaction did not give higher nitrite reading indicating that there are no nitrates in the samples.

Quantification of plasma and salivary oxLDL by ELISA: oxLDL content in plasma and saliva was measured using human oxLDL ELISA kit (Elabscience) according to the manufactures protocol with minor modifications. 5 µL of plasma was diluted 1:1000 in phosphate buffered saline (PBS, pH 7) and 25 µL of diluted sample was assayed. 10 µL saliva was assayed in antibody pre coated well of micro ELISA plate after dilution with 15 µL of PBS (pH 7). The oxLDL concentration was calculated based on the concentration series of reference standards of oxLDL provided with the assay kit as follows. Diluted saliva sample was removed from the well and 100 µL of biotinylated detection Ab was added into the well and incubated for 60 mins at 37 °C. Liquid was aspirated and 100 µL of horse radish peroxidase conjugate was added into the well after 3

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washes with wash buffer and incubated for 30 mins at 37 °C. Liquid was aspirated and 90 µL of substrate reagent was added following 5 washes with wash buffer and incubated for 15 mins at 37 °C. 50 µL of stop solution was added and the absorbance was read at 450 nm by using Multiskan Go spectrophotometer (Thermo Scientific).

Statistical Analysis: q-q plots and Shapiro-Wilk test were used to determine normality at a 95% confidence interval. For the Shapiro-Wilk test $P > 0.05$ was determined as normal distribution. The fold change of expression was calculated using the ΔΔCq method. A difference in expression based on fold change of log base 2, less than 0.5 between DF and DHF cases was considered as downregulation and above 1.5 was considered as upregulation. Statistical significance for differentially expressed targets were determined based on the SEM of ΔCq using independent t-test (SEM: Standard error of mean). Statistically significant differences among the mean ± SD and mean ± SD of $5^{th} – 95^{th}$ percentile was determined using independent t-test. Statistically significant differences among the median ± MAD and median ± MAD of $5^{th} – 95^{th}$ percentile was determined using Mann-Whitney U test for non-parametric independent samples (MAD: median absolute deviation). $P < 0.05$ was considered statistically significant. Logistic regression analysis for odds ratio, receiver operator characteristics, area under curve, specificity and sensitivity was determined using IBM SPSS Statistics, 2013 version at a 95 % confidence interval. Sample sizes were calculated using G*Power 3.1.9.2 software at 95 % confidence interval with a power of 80 % for normally distributed samples using parametric test and skewed distributions using non-parametric test. Pearson correlation analysis at 95 % confidence interval was used to determine correlation between the different parameters against the platelet counts.

Results and Discussion

iNOS expression in Dengue patients within four days from fever onset

Thirty-nine patients suspected of having dengue based on clinical classification adopted by WHO (2012) with positive diagnosis for dengue infection by viral NS1 rapid test, hospitalized at the North Colombo Teaching Hospital, Sri Lanka were recruited for the gene expression analysis. Among them, 19 (48.7 %) patients were classified as dengue fever (DF) and 20 (51.2 %) patients who later showed evidence of plasma leakage (pleural effusions and ascites) as detected using a portable ultrasonogram were classified as DHF according to the clinical classification adopted by WHO.[29] Most of the subjects were male (77 %), with a median age of 30 (18-60) while the female subjects had a median age of 24 (19-60) years. At enrollment, there were no statistically significant differences in mean, median, and $5^{th} – 95^{th}$ percentile of mean and median laboratory clinical parameters such as thrombocytopenia, leukopenia, hematocrit count and AST and ALT levels in patients who later developed DHF compared with DF (**Table 1, Table S1**). Circulating AST and ALT levels were not significantly different between these two groups throughout the course of infection (**Table S2**).

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Table 1

Clinical characteristics of dengue patients subjected to iNOS expression analysis at the time of admission (Mean ± SD).

	DF patients (n=19)	DHF patients (n=20)	P value CI, 95 %
Gender (Male % / Female %)	68/32	85/15	
Age	34 (18-60)	29 (19-60)	
Hematocrit (%)	39.9 ± 4.7	39.4 ± 3.9	0.74
Hemoglobin (g/dL)	13.6 ± 1.5	12.4 ± 3.1	0.14
White blood cells (x1000 cells/mm^3)	15.1 ± 26.8	20.6 ± 67.7	0.74
Neutrophils (%)	58.2 ± 23.1	68.7 ± 20.8	0.16
Lymphocytes (%)	28.2 ± 15.7	19.2 ± 13.6	0.08
Eosinophils (%)	3.9 ± 10.1	1.2 ± 1.2	0.28
AST (U/L)	43.9 ± 17.9	54.4 ± 24.2	0.26
ALT (U/L)	39.3 ± 17.5	45.8 ± 23.1	0.47

Since iNOS has been implicated in host response to dengue virus infection, iNOS expression in PBC harvested from EDTA blood collected during the acute phase (within four days from fever onset) was evaluated by qRT-PCR. DHF patients showed significant ($P < 0.05$) down regulation of iNOS expression within 4 days from fever onset. Furthermore, iNOS expression in DHF patients significantly decreased on day three from fever onset and continued to decrease till day four from fever onset (**Fig. 1a**, **Table 2**). Further analysis of iNOS expression among the male patients (DF; n = 13 and DHF; n = 17) and female patients (DF; n = 6 and DHF; n = 3) showed similar downregulation of iNOS expression (**Fig. 1b**). Expression analysis of iNOS among the patients who showed signs of thrombocytopenia (< 100,000/mm^3 platelets) during illness against those who did not develop thrombocytopenia, and a platelet count of < 60000/mm^3 as an indicator of severe dengue during illness against the patients of whom the platelet count did not drop below 60000/mm^3 during the course of illness did not show differential iNOS expression indicating that the expression of iNOS is not correlated with the drop in platelet count ($r = 0.05$) (**Fig. 1c**).

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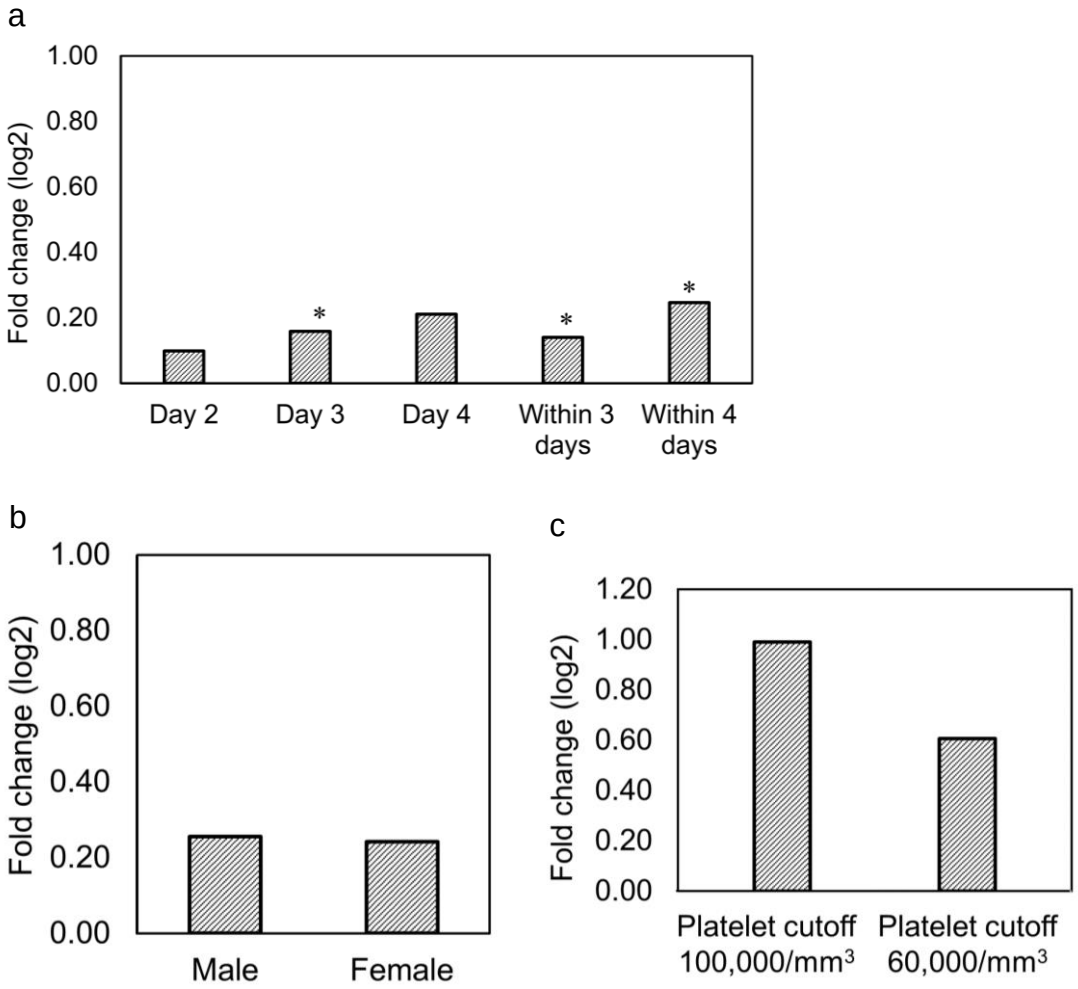

Fig. 1. Expression of iNOS in PBC from DF patients and patients who later developed DHF collected within 4 days from fever onset (**a**) Fold change of iNOS between DF and DHF patient samples collected from patients recruited on, day 2 (nDF=2, nDHF =3), day 3 (nDF=6, nDHF=12), day 4 (nDF=11, nDHF=5), within 3 days (nDF=8, nDHF=15) and within 4 days (nDF=19, nDHF=20) from fever onset. (**b**) Fold change of iNOS expression among the male patients (nDF=13 and nDHF=17) and female patients (nDF=6 and nDHF=3) within 4 days from fever onset (**c**) iNOS expression in dengue patients with thrombocytopenia. Fold change of iNOS expression between PBC collected within 4 days from fever onset from patients with platelet count above 100,000 cells/mm^3 (n=8) compared to those below 100,000 cells/mm^3 (n=31) and platelet count above 60,000 cells/mm^3 (n=12) compared to those below 60,000 cells/mm^3 (n=27) during the course of infection. Fold change of expression based on ΔΔCt values against GAPDH presented as log values to the base 2, where a fold change >1.5 was considered as up regulation and < 0.5 considered as down regulation. *P < 0.05 based on ΔCt ± SEM using independent t - test.

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Table 2

Relative expression of iNOS (log value to the base 2 of ΔΔCq)

	Relative expression [CI, 95%]	P values
Day 2	0.10 [0.00 – 3565.78]	0.53
Day 3	0.16 [0.03 – 0.95]*	0.04*
Day 4	0.21 [0.03 – 2.39]	0.22
Within 3 days	0.14 [0.03 – 0.98]*	0.05*
Within 4 days	0.24 [0.08 – 0.76]*	0.02*
Male, within 4 days	0.24 [0.06 – 1.13]	0.07
Female, within 4 days	0.26 [0.06 – 1.04]	0.06

Level of nitric oxide in plasma and saliva from acute dengue patients

Expression of iNOS results in NO biosynthesis. We quantified the levels of NO in plasma using the Greiss reaction. Preliminary measurement of NO by Greiss reaction after conversion of nitrate to nitrite revealed that there was no detectable level of nitrate in the plasma samples. Therefore, the nitrite levels as measured by the Greiss reaction was taken as the total NO in the plasma and saliva samples. Among 59 patients with positive diagnosis for dengue infection, 51(%) patients were classified as DF and 49 (%) patients who later showed evidence of plasma leakage (pleural effusions and ascites) were classified as DHF. Among the patients recruited for the study, 60 % of the DF subjects were male, with a median age of 25 (18-63) years and 40 % of the subjects were female with a median age of 36 (18-57) years while 83 % of the DHF subjects were male with a median age of 21 (18-46) years and 17 % of the DHF subjects were female with a median age of 30 (18-56). At enrollment, there were no statistically significant differences in mean, median, and 5^{th} – 95^{th} percentile of mean and median laboratory clinical parameters such as thrombocytopenia, leukopenia, hematocrit count and AST and ALT levels in patients who later developed DHF compared with DF (**Table S3**). Mean plasma NO concentration within four days from fever onset in DF group (23.6 ± 4.4 µM; n = 30) was significantly ($P < 0.05$) higher than that of those who later developed DHF (18.1 ± 3.1 µM; n = 29). A significant decrease in mean NO levels ($P < 0.05$) in the DHF patients was observed in samples collected on day 2, day 3, day 4 and within 3 days from fever onset (**Fig. 2a**). The data are not normally distributed. Therefore, Mann-Whitney U test was used to determine significant differences. However, this decrease of plasma NO in DHF patients within four days from fever onset was observed among male patients ($P < 0.05$) but not in female patients (**Fig. 2b**). This may be due to fewer number of samples collected from females who later developed DHF. Patients presenting with thrombocytopenia (20.6 ± 4.1 µM; n = 48) and patients presented with a platelet count <60000/mm^3 showed a statistically significant ($p < 0.05$) decrease in plasma NO levels (19.7 ± 3.5 µM; n = 34) compared to those with platelet count >100000/mm^3 (23.4 ± 7.1 µM; n = 11) and >60,000/mm^3 (23.4 ± 5.7 µM; n = 25) (**Fig. 2c**).

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287

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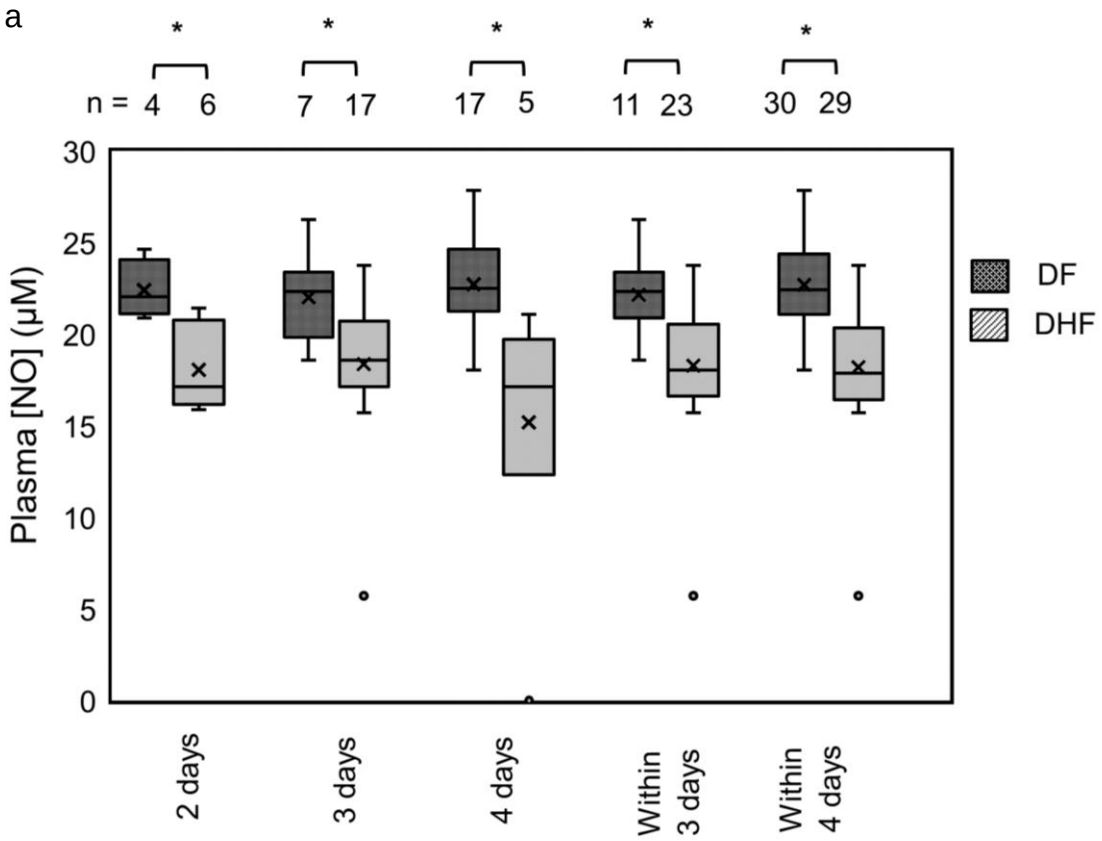

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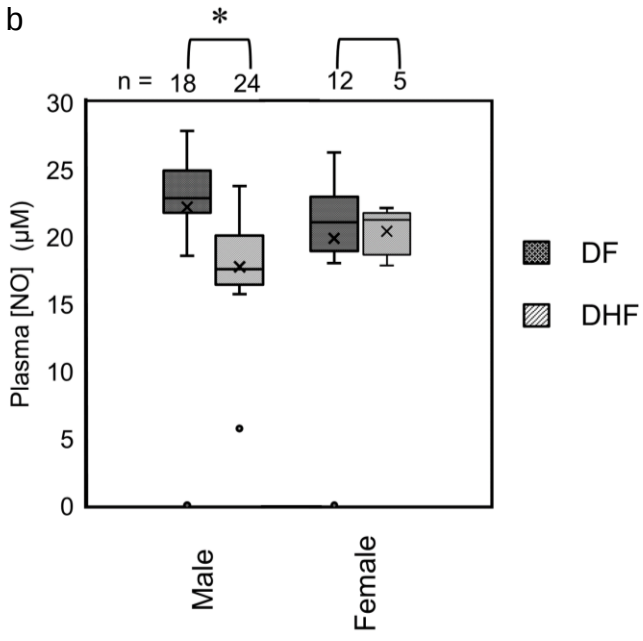

289

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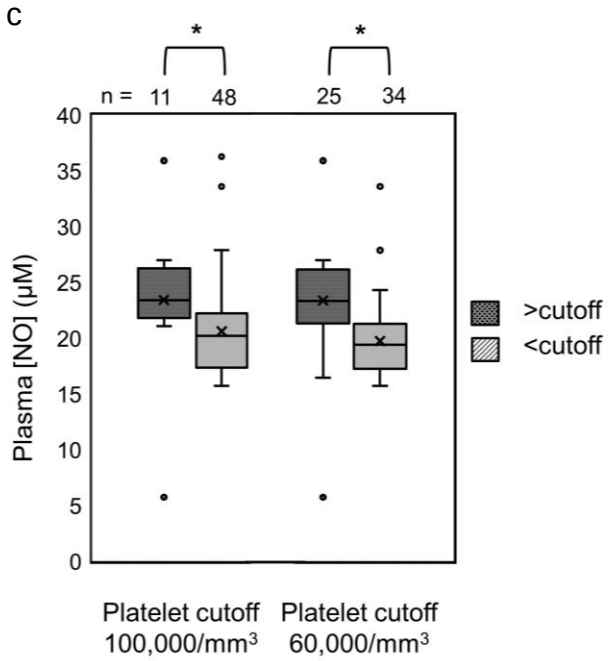

Fig. 2. NO in plasma collected from DF patients and patients who later developed DHF within 4 days from fever onset**.** (**a**) Plasma nitric oxide levels in patient samples collected on day 2 (nDF=4, nDHF=6), day 3 (nDF=7, nDHF=17) day 4 (nDF=17, nDHF=5), within 3 days (nDF=11), nDHF=23) and within 4 days (nDF=30, nDHF=29) from fever onset. (**b**) Plasma NO levels in males (nDF=18, nDHF=24) and females (nDF=12, nDHF=5) within 4 days from fever onset. (**c**) Plasma NO level in patients presented with no thrombocytopenia (n=11) against those with thrombocytopenia (n=48) and patients presented with platelet count >60,000/mm^3 (n=25) against <60,000/mm^3 (n=34) during illness. * $P < 0.05$

Since saliva is a non-invasive source of biological markers, we also quantified the salivary NO levels in 38 patients with positive diagnosis for dengue using the Greiss reaction to evaluate the potential as an early biomarker for severe dengue. Among them, 19 (50 %) patients were classified as DF and 19 (50 %) patients who later showed evidence of plasma leakage (pleural effusions and ascites) were classified as DHF. Approximately 95 % of the DF subjects were male and 5 % were female with DF patients having a median age of 23 (18-63) years while 79% of the DHF patients were male and 21 % were female with DHF patients having a median age of 22 (18-43). At enrollment, there was a statistically significant difference in mean (DF; 14.4 ± 1.2, DHF; 12.8 ± 2.5 g/dL), median, 5th – 95th percentile of mean and median hemoglobin levels ($P<0.05$) and median (DF; 42.1 ± 1.3, DHF; 39.7 ± 2.3 %), 5th – 95th percentile of mean and median hematocrit levels among DF and DHF patients while there was no significant difference in other laboratory clinical parameters such as thrombocytopenia, leukopenia, AST and ALT levels in patients who later developed DHF compared with DF (Table S4**)**. This statistical difference in hemoglobin levels during the acute phase of infection was not observed in the larger cohort of patient samples used for the plasma NO analysis. q-q plots and Shapiro-

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Wilk test revealed that the salivary NO levels were not normally distributed ($P<0.05$). Therefore, Mann-Whitney U test was used to determine significant differences among the salivary NO levels. Salivary NO concentration in groups did not show a statistically significant difference in patient saliva collected within 4 days from fever onset (**Fig. 3a**). The salivary NO levels in dengue patients also did not correlate with the platelet count or the plasma NO concentration ($r = -0.02$; $r = 0.21$) (**Fig. 3b**).

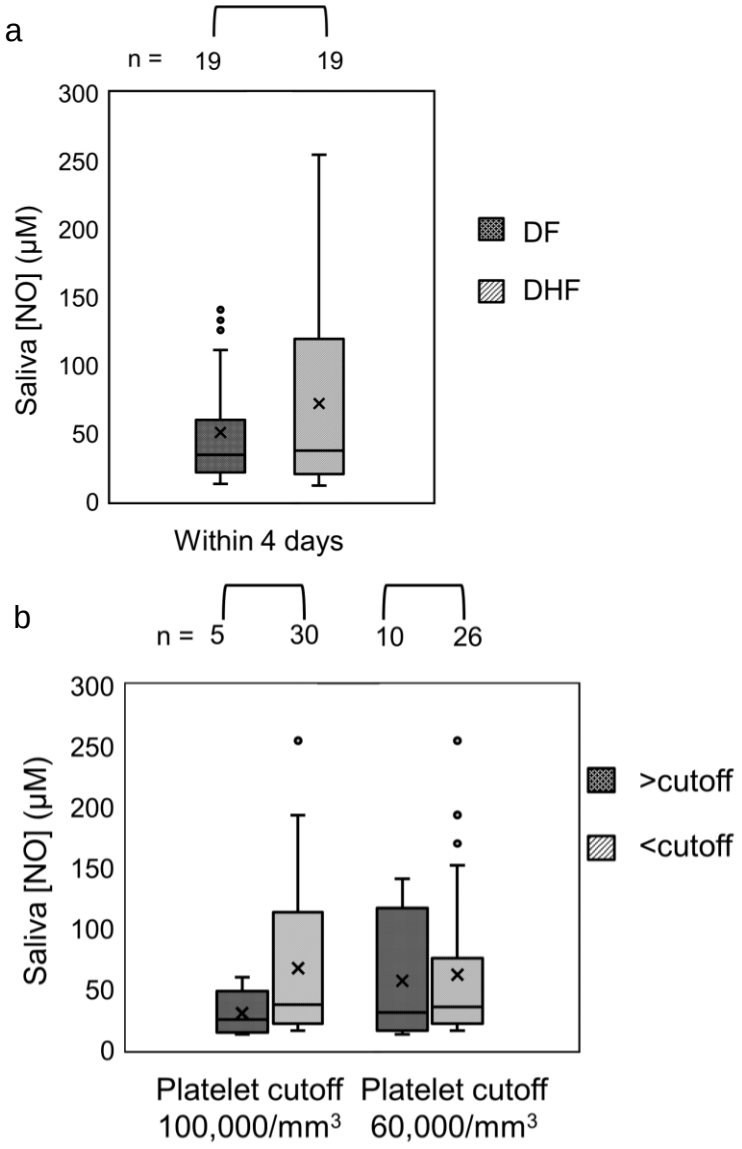

Fig. 3. NO levels in Saliva from DF patients and patients who later developed DHF collected within 4 days from fever onset**.** (**a**) Salivary NO in DF patients (n=19) and patients who later developed DHF (n=19). (**b**) Saliva nitric oxide levels in patients with the platelet count >100,000/mm^3(n=6) against those presented with <100,000/mm^3 (n=30) and patients with platelet count >60,000/mm^3 (n=10) against those presented with <60,000/mm^3 (n=26) during the course of illness. *p < 0.05

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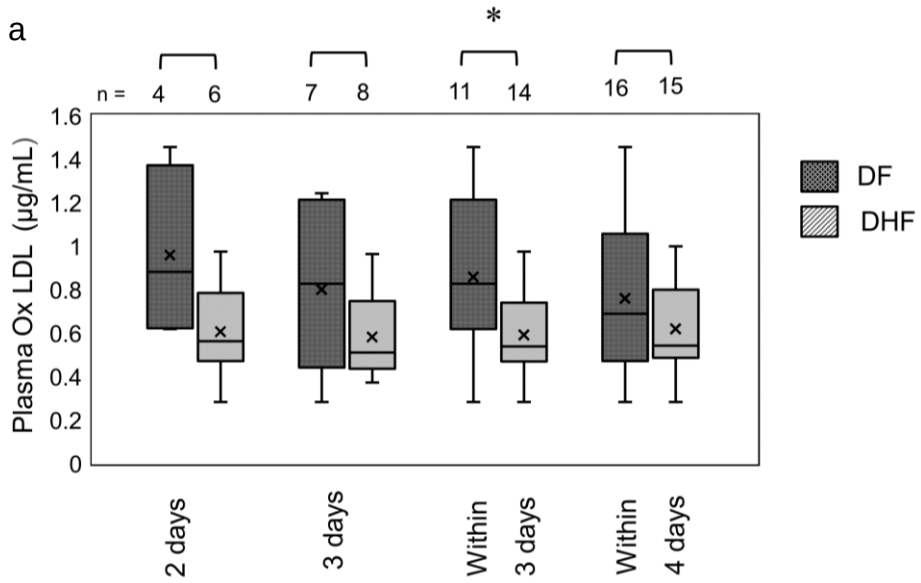
Oxidized LDL levels in plasma and saliva

L-arginine-NO pathway is involved in the effects of ox-LDL on platelet function (Chen et al., 1996). Therefore, plasma oxLDL levels were analyzed in 31 dengue positive patients within four days from fever onset using ELISA. The clinical characteristics of the patients are given in **Table S5**. Mean plasma oxLDL concentration in DF (757.5 ± 343.9 ng/mL; n = 16) was higher than that of DHF group (618.4 ± 224.2 ng/mL; n = 15). The data are normally distributed ($P > 0.05$). Although the sample numbers in each group were low, the oxLDL levels in the DHF group also showed a decrease in samples collected on day 2, day 3 and within 3 days from fever onset with a statistically significant decrease in oxLDL levels in plasma collected within 3 days from fever onset from patients who later developed DHF ($P < 0.05$) (**Fig. 4a**).

oxLDL in sixteen saliva samples from patients with positive diagnosis for dengue within four days from fever onset were analyzed using ELISA to evaluate whether the differences observed in the plasma samples were detectable in saliva samples as well. Clinical characteristics of the patients are given in Table S6. Mean salivary oxLDL in DF patients (1.0 ± 0.4 ng/mL; n = 8) was significantly higher ($p < 0.05$) than that of DHF patients (0.6 ± 0.2 ng/mL; n=8) within four days from fever onset suggesting that salivary oxLDL may serve as an early marker for DHF (**Fig. 4b**). The data are normally distributed.



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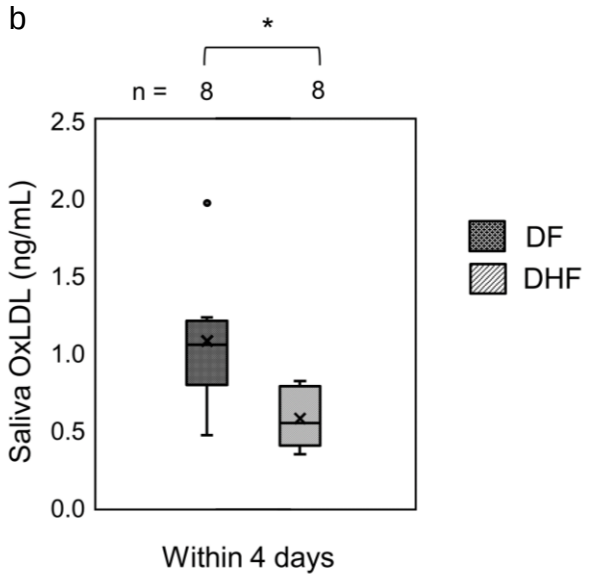

Fig. 4. oxLDL in plasma and saliva from DF patients and patients who later developed DHF collected within 4 days from fever onset**.** (**a**) oxLDL levels in plasma collected on day 2 (nDF=4), nDHF=6), day 3 (nDF=7, nDHF=8), within 3 days (nDF=11, nDHF=14) and within 4 days (nDF=16, nDHF=15) from fever onset. (**b**) oxLDL levels in saliva from DF patients (n=8) and patients who later developed DHF (n=8) within four days from fever onset. *$p < 0.05$

Discussion

iNOS expression, plasma NO, plasma oxLDL and salivary Ox LDL levels showed significant differences between the DF patients and patients who later developed severe dengue during the acute phase of infection. iNOS is implicated in host response to dengue virus infection and plasma leakage symptomatic of severe dengue (Fialho, 2017)[25]. Logistic regression analysis of iNOS expression within 4 days from fever onset was found to be predictive of DHF (odds ratio, 1.39; 95 % CI 1.04-1.85; $P < 0.05$) with an area under the receiver operating curve of 0.75. The sensitivity and specificity for the development of DHF were 0.80 and 0.63 respectively at ΔCq for iNOS expression of -0.41. The data were determined to be normally distributed and the calculated sample size to assess the potential of iNOS as an early prognostic marker for severe dengue based on the iNOS expression levels among DF patients and patients who later developed DHF within 4 days from fever onset is 50 (n_{DF} = 24; n_{DHF} = 26). While iNOS has been reported to overexpress in response to dengue infection compared to healthy subjects with a corresponding increase in the NO levels, the opposite was observed between the DF patients and DHF patients during the early phase of infection[25] (Fialho, 2017). This may be due to the role of iNOS and NO as part of the host defense mechanism which appears to be compromised even during the early phase of infection in the individuals who later developed DHF.

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Due to the role of iNOS in NO biosynthesis, corresponding decrease in NO levels were expected as a result of observed downregulation of iNOS expression during the acute phase of infection, which was significant within 2 days from fever onset and remained significantly low in plasma during the acute phase of infection. Logistic regression analysis of plasma NO level within 4 days from fever onset was found to be predictive of DHF (odds ratio, 0.54; 95% CI 0.40-0.72; $P < 0.01$) with an area under the receiver operating curve of 0.89. The sensitivity and specificity for the development of DHF were 0.90 and 0.70 respectively at plasma NO level of 21.3 µM ($P < 0.01$). The calculated sample size to assess the potential of plasma NO to serve as an early prognostic marker for severe dengue within 4 days from fever onset is 20 ($n_{DF} = 10$; $n_{DHF} = 10$) indicating that the sample size used for the analysis is within the calculated limit. These findings are consistent with the reported differences of serum NO and nitrite levels suggesting a role as early marker of disease severity for dengue[27] (Mapalagamage 2018). However, the plasma NO levels are at least four fold higher than serum NO levels with a clear distinction of the levels between DF patients and patients who later developed DHF with an odds ratio of approximately 0.5 for samples collected on day 3, day 4, within 3 days and 4 days from fever onset, indicating that plasma NO may serve as a more robust marker.

Oxidation of lipids by NO can result in increased levels of plasma oxLDL which promotes vasoconstriction and platelet activation with alterations in platelet function which has been connected to dengue-associated plasma leakage[33,34] (Michels et al., 2014). A decrease of plasma oxLDL levels corresponding to the decrease in plasma NO were observed in dengue patients who later developed DHF. Therefore, the plasma oxLDL levels do not appear to participate in development of symptoms of severe dengue such as plasma leakage during the early phase of infection. Logistic regression analysis of plasma oxLDL level within 3 days from fever onset was not predictive of DHF after adjustment for number of days from fever onset (adjusted odds ratio, 1.00; 95% CI 1.00-1.00, $P < 0.05$) with an area under the receiver operating curve of 0.62. The sensitivity and specificity for the development of DHF were 0.73 and 0.50 respectively at plasma oxLDL level of 729.7 ng/mL ($P = 0.27$). The calculated sample size to assess the potential of plasma oxLDL to serve as a biomarker of severity of dengue infection within 4 days from fever onset based on the normally distributed data (Shapiro-Wilk test P value > 0.05) is 392 and within 3 days from fever onset is 166.

Similarly, salivary oxLDL levels showed a significant decrease in the patients who later developed DHF compared to the DF patients within 4 days of infection proving to be an excellent non-invasive biological source for predictive markers for dengue. Salivary oxLDL levels have been shown to correlate with the serum oxLDL levels[28] (De Giuseppe et al., 2015). Logistic regression analysis of salivary oxLDL levels within 4 days from fever onset was found to be predictive of DHF with an area under the receiver operating curve of 0.91. The sensitivity and specificity for the development of DHF were 0.88 and 0.75 respectively at salivary oxLDL level of 0.8 ng/mL ($P < 0.01$). The calculated sample size to assess the potential of salivary oxLDL to serve as an early marker of severe dengue is 18. However, oxLDL has also been associated with cardiovascular risks[28] (De Giuseppe et al., 2015). Therefore, oxLDL levels in a

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larger cohort of dengue patients during acute phase of infection may be necessary to validate the role of oxLDL in saliva as a non-invasive early prognostic biomarker of DHF and evaluate the effect of the confounding factors. Although saliva may serve as a non-invasive source for NO levels, saliva was proven to be an unreliable biological source due to high standard deviation of NO concentration that may be resulting from the influence of oral health and diet[35] (Mobarak). Our study is limited by the relatively small sample sizes for 2, 3 and 4 days from fever onset and relatively few samples from female patients to assess the potential of these markers to predict the outcome within these parameters. Therefore, further analysis in a larger cohort is needed to assess the full potential of these biomarkers to distinguish DF patients from those who progress to DHF during the early stages of infection. Analysis of iNOS expression, plasma NO, plasma oxLDL and saliva oxLDL in larger cohorts of dengue patients within each day from fever onset during the acute phase and analysis of each of the above variables in a larger cohort of samples from female patients is needed to evaluate the full potential of these markers to serve as early markers of severity of infection. We were also unable to determine whether dietary habits, social habits such as smoking and non-communicable conditions such as high cholesterol and diabetes influence the iNOS expression, plasma NO and plasma and salivary oxLDL among the DF and DHF patients within four days from fever onset, due to unreliable response rate from the subjects. However, differential expression of iNOS, plasma NO and salivary oxLDL levels may serve as reliable early biomarkers to predict the development of severe dengue within 4 days from fever onset. Our findings also suggest saliva as a potential new non-invasive biological source for early prognosis of disease outcome for dengue.

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Conflicts of interest

The authors declare to have no conflicts of interest

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