Plasma levels of Ceramides and their association with Hematocrit and Thrombocytopenia in Severe and Non-severe dengue

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Abbreviation:

SD: Severe dengue, DWOW: Dengue without warning sign, DWW: Dengue with warning sign, AD: All dengue, OFI: Other febrile illnesses, HPLC: High-performance liquid chromatography, DOA: Day of Admission, DOD: Day of Defervescence, HUVECs: Human umbilical vein endothelial cells, ECs: Endothelial cells, FBS: Foetal bovine serum, HCT: Haematocrit, WNV: West Nile virus DENV: Dengue virus, HCV: Hepatitis C virus, HIV: Human Immunodeficiency virus, JEV: Japanese encephalitis virus, ZIKV: Zika virus

Abstract

Background: Plasma leakage due to endothelial permeability is regarded as a hallmark process in the pathophysiology of severe dengue. In recent years, the importance of ceramide in regulating vascular tone during viral infection and metabolic diseases has gained attention. The present study aimed to evaluate the plasma levels of ceramide and its association with plasma leakage in dengue patients.

Methods: The study involved 30 dengue samples involving severe dengue (SD-10), Dengue with Warning sign (DWW-10), Dengue without Warning Sign (DWOW-10), along with other febrile illness (OFI-10) controls. Samples were collected on the day of admission (DOA), day of defervescence (DOD), and day of convalescence (DOC). Total plasma ceramides (d18:1/22:0) were quantified using RP-HPLC. The correlation between ceramides and hematocrit/platelet count was evaluated using Spearman Rho Correlation. All the statistical analyses were performed using SPSS software.

Results: During the febrile phase, a significant ($P \leq 0.05$) decrease in the levels of ceramides was observed in dengue compared to OFI (control). Towards the defervescences, the ceramides levels were substantially ($P \leq 0.001$) elevated in dengue groups compared to baseline. Most importantly, the ceramide levels were found to be higher in SD patients compared to non-severe dengue (DWW & DWOW) and OFI, particularly at the critical phase of infection. We observed a significant negative correlation ($r = -0.867$, $P \leq 0.001$) between the platelet count and ceramide levels in SD subjects. Notably, a negative correlation was observed between ceramide and hematocrit during the defervescence phase ($R = -0.355$, $P \leq 0.05$) in the AD group.

Conclusion: Elevated levels of circulating plasma ceramides during the defervescence phase of severe dengue show an essential role of ceramides in disease pathogenesis, however further studies are required to ascertain it.

Keywords: Dengue, vascular leak, ceramide, HPLC, endothelial cells

Introduction:

Dengue fever is a resurgent arbovirus disease that causes 100-400 million infections each year (1). Dengue disease is characterized by fever, headache, muscle, and joint pains, rash, nausea, and vomiting. Some infections result in dengue hemorrhagic fever (DHF), a syndrome that in its most severe form can threaten the patient's life, primarily through increased vascular permeability and shock (2). Nevertheless, on the 3rd to 7th day of the illness, as the fever subsides, some patients manifest severe signs like endothelial dysfunction, which induces vascular permeability. Thrombocytopenia and vascular dysfunction are the cardinal features of dengue pathogenesis. Currently, there is no commercially available vaccine for dengue, but treatment is supportive. Early prediction of disease outcomes could provide prompt medical attention and reduce the risk of fatality (2)

The exact mechanism that regulates vascular dysfunction and platelet destruction in the pathogenesis of dengue remains obscure. Recently, we have reported the role of platelet lipids in the pathogenesis of dengue(3). Lipids play a role in structural, energy storage, and cellular barriers. The role of lipids in the pathogenesis of single-stranded RNA virus disease is not well established. We have reported an alteration in the platelet fatty acids in severe dengue cases(4). A lipidomic study on dengue plasma revealed a differential expression of phospholipids and sphingolipids in the severe form of dengue compared to the non-severe form. For example, DENV has been shown to increase the expression of sphingolipids (ceramide and sphingomyelin) in mosquito cells(5).Sphingolipids have recently been identified as an important contributor to vascular integrity and preventing endothelial cell hyper-permeability(5). The intracellular biosynthesis of ceramide takes place through two pathways namely, the *de novo* synthesis and salvage pathways (6) Several studies highlighted the role of two pathways in altering the life cycle and replication of some viruses (7–9). Thus, validating the role of selected lipids in disease progression during virus infection could shed light on understanding disease pathogenesis and develop novel therapeutics.Other studies have also shown that lipid changes could alter lipid microenvironments in the dengue disease(10).However, the association between lipid alterations and platelet count as well as haematocrit has not been studied. Therefore, this study aimed to determine the ceramide concentration in plasma from dengue patients using RP-HPLC and to assess their associations with the dengue severity symptoms.

Methods:

Patients and study design:

The study was approved by the Institutional Human Ethical Review Committee (ECR/451/Inst/PO/2013/RR-16), and all patients gave written informed consent. The patient presented to General Medicine at Mahatma Gandhi Medical College and Research Institute, Pondicherry with symptoms suggestive of dengue and confirmed by antigen (NS1) and an antibody test (IgM, IgG) were included in the study. Patients will be classified according to WHO 2009 criteria into two groups, namely, Group A, which includes severe dengue (SD-10) and non-severe dengue, which is further sub-categorised into dengue with warning signs (DWW-10) and dengue without warning signs (DWOW-10). Group B subjects (controls) are patients with other febrile illnesses (OFI-10). They would be defined as those patients who are negative for dengue NS1 antigen and anti-IgM/IgG dengue antibodies. About 3 mL of blood was collected in a tube containing 10% EDTA as an anticoagulant. Plasma was separated from the whole blood by centrifuging at 3000 g for 10 minutes and stored at -80°C.The ceramide (d18:1/22:0) levels from the plasma samples were thereafter measured.

Lipid Extraction Protocol:

Reagents and Chemicals:

HPLC grade methanol (Thermo Fisher Scientific (Sydney, Australia), 1-butanol (AR grade, Asia Pacific Specialty Chemicals (APS), Acetonitrile (Honeywell, Korea), Ammonium formate (Honeywell, Germany), Milli-Q water, were used for lipid extraction and quantification. Lipid standard - Ceramide (d18:1/22:0) (Avanti Polar Alabaster, Alabama, USA) was added to all test samples before lipid extraction (10 μL of a 1:10 dilution of Avanti polar lipids to 10 μL of neat plasma) for normalisation of raw peak areas and to correct for differences in extraction(**11**).

Quantification of ceramide in plasma:

Lipids were extracted by the Alshehry Extraction Method (Single Phase 1-Butanol/Methanol 1:1 (v/v)) as previously described(12). The mobile phase was prepared by dissolving 0.0038 M of K_2HPO_4 in water and methanol, then sonicating. The organic phases were evaporated and the dried lipids were dissolved in a mixture (50 μl) of methanol and 0.07 M K_2HPO_4 (9:1 v/v). A derivatization mixture of 10 mg of o-phthaldialdehyde, 200 μl of ethanol, 10 μl of 2-mercaptoethanol, and 10 ml of boric acid (3% v/w) was prepared (pH 10.5). 5μl of the derivatization mixture, 45 μl of 9:1 ratio methanol, and 0.07 M K_2HPO_4 were added. The derivatives were analysed with a Shimadzu (Shlm-pack HPLC column system using an RP 18 Shimadzu column (4.6 mm x 250 mm) maintained at 40°C. Followed by, 20 μl of sample

mixture being injected into the column. Then, set the flow rate at 1.000 mL/min for 20 minutes. The retention time of the selected peak was observed with the emission wavelength at 274 nm.

**In vitro study growth and maintenance using the HUVEC cell model:**

Human Umbilical Vein Endothelial Cells (HUVEC) purchased from Lonza (Walkersville, USA) were maintained in the laboratory as prescribed (13). In brief, HUVECs were seeded $(1.5 \times 10^4/\text{well})$ in a 6-well plate. Cells were starved in EBM2s basal medium for 24 hours at 37° C and 5% CO_2 before incubation in media containing 10% human serum collected from SD, DWW, and OFI plasma samples(14). After 24 h of treatment, the HUVEC cells were harvested and resuspended in PBS solution. The levels of ceramide in the cells were estimated by HPLC methods.

Statistical analysis:

Results were expressed as median (interquartile range) or n (%). *P*-values of 0.05 or less were considered statistically significant. The normality test (Shapiro-Wilk Test) was performed to analyse the data. A comparison within the groups was performed using a two-tailed related sample t-test (Wilcoxon Signed Rank Test). Similarly, a comparison between the groups was performed using a two-tailed Independent Sample t-test (Mann-Whitney U test). The correlation was assessed using Spearman's Rho correlation. A correlation analysis was directed to study the association between haematocrit and ceramide. All statistical tests were performed using SPSS software version 23 (SPSS, Chicago, IL, USA).

Results:

During the defervescence phase of dengue illness, platelet numbers fall as ceramide levels rise. In our current study, total plasma ceramide levels at the time of admission were elevated significantly (*P≤ 0.05) during the defervescence phase of infection, releasing an excess of ceramides into circulation.
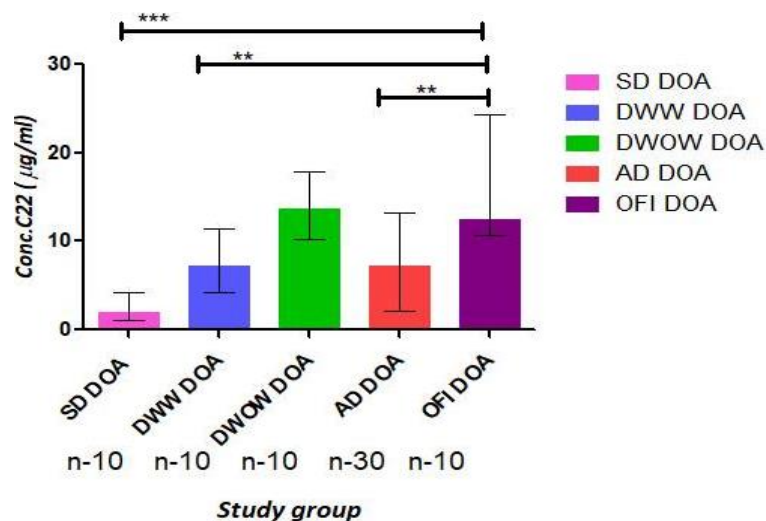
Figure 1 Decreased levels of ceramide (C22) at the time of admission. Statistical analysis was done using two-tailed Mann–Whitney tests to compare the data between the groups since the data was found to be non-parametric. P ≤ 0.05 was considered statistically significant. **AD** – All Dengue; **DWOW** - Dengue without Warning Sign; **DWW**- Dengue with Warning Sign, **SD** – Severe Dengue; **OFI** - Other Febrile Illness.

Plasma ceramide concentration:

Table 1 shows the levels of plasma ceramide in the study groups. Except for DWOW; there was a significant reduction in ceramide levels in SD, DWW, and AD cases as compared to OFI at the time of admission (Fig 1). Within the dengue groups, the severe group noted a significant (P≤ 0.01) drop in lipid levels as compared to the non-severe group.

Group	DOA	DOD
AD (n-30)	7.239(2.136-13.16)	7.591(4.826-11.04)
SD (n-10)	2.026(1.075-4.277)	3.592(1.959-8.798) *
DWW(n-10)	7.239(4.185-11.47)	12.49(5.736-20.97)
DWOW (n-10)	13.63(10.26-17.92)	8.165(6.221-9.200) **
OFI (n-10) (Control)	12.46(10.65-24.40)	8.577(7.269-18.26)

The concentration of plasma ceramide (μg/ml) in the study groups

**Plasma levels of ceramide within the DOA and DOD:**

Furthermore, in dengue groups such as SD and DWOW patients, plasma levels of ceramides increased drastically towards defervescence (Fig.2). However, there was an enhanced amount of ceramide in DWW that had no significance (Fig 2). Ceramide levels in the dengue and OFI groups did not change significantly from the time of admission. Ceramide levels were found to be higher in the DWOW and AD groups during the defervescence phase of dengue infection.
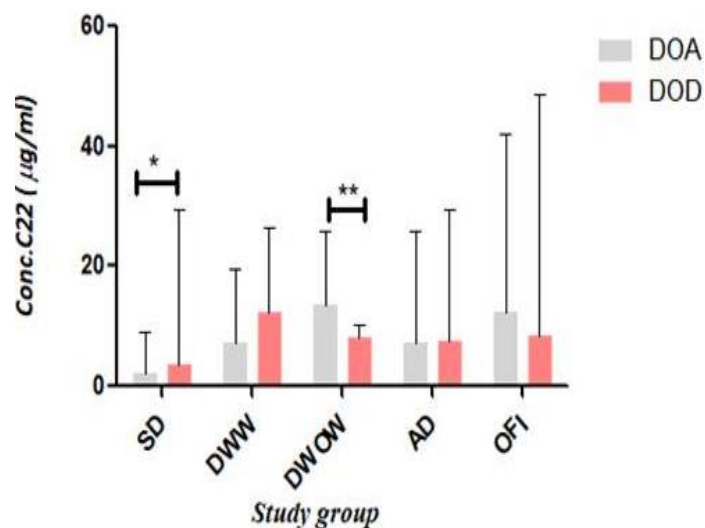
Figure 2: Ceramide levels are elevated in dengue patients. In comparison to OFI, our findings show that there is no significant difference across dengue groups in the febrile phase. Statistical analysis was done using two-tailed Wilcoxon signed-rank tests to compare data within groups since the data was found to be non-parametric. P≤0.05 was considered statistically significant. **DOA**- Day of admission and **DOD**-Day of defervescence

Comparison between different groups at the time of admission and defervescence

When compared to control, there was a significant ($P \leq 0.05$) downregulation of ceramides in the SD, DWW, and DWOW during the febrile phase (**Fig 3A**). In contrast, towards the critical phase, we noted a significant (P≤0.05) decrease in the levels of ceramide 22 in SD compared to DWW and OFI (**Fig 3B**).
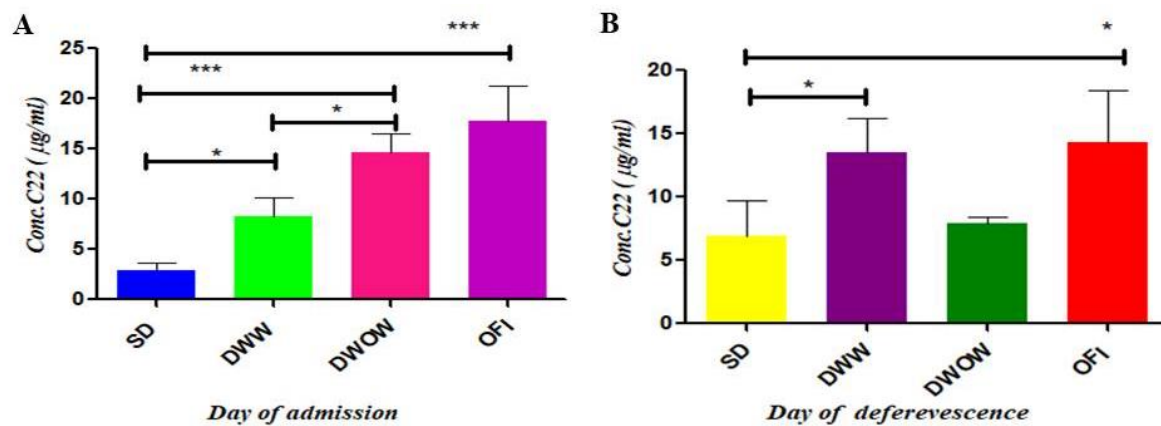

Figure 3: Ceramide concentrations in plasma (µg/ml) differed between the Dengue and OFI groups. In the Fig 3A and 3B, we depict the day of admission and Day of defervescence between the dengue groups and control. Statistical analysis was done using two-tailed Mann–Whitney tests to compare the data between the groups since the data was found to be non-parametric. P≤0.05 was considered statistically significant.

Plasma levels of ceramides in HUVECs— *In vitro* study:

The vascular endothelium serves as a barrier between the blood and the body's surrounding tissue, which is a vital function. Ceramides may play a protective effect in improving vascular barrier function by reducing vascular leakage by maintaining tight junctions between endothelial cells. We examined the total plasma ceramide levels by RP-HPLC using the HUVECs model to look for changes in the endothelial cells.
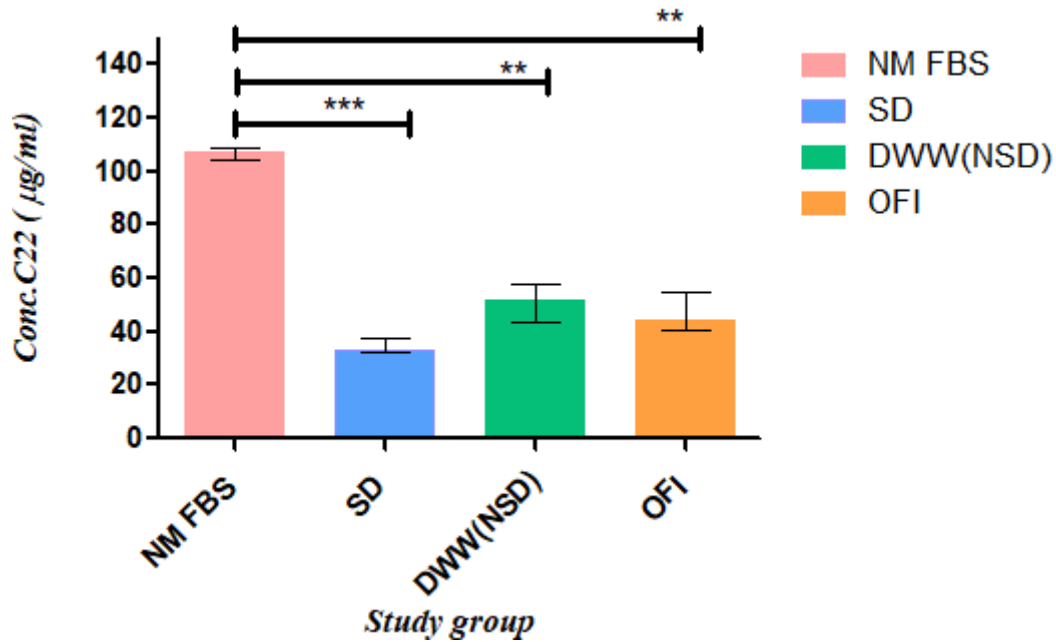
Figure 4: Comparison of Normal Foetal Bovine Serum (FBS) to other study groups. HUVEC cells treated with serum obtained from a severe form of dengue showed a significant decrease in the levels of ceramide compared to the cells treated with normal FBS. There were no significant differences observed when comparing the study groups and OFI. Median and interquartile ranges are shown. $P \leq 0.05$ was considered statistically significant.

Association between haematocrit and ceramide levels:

A negative correlation was observed between the plasma concentration of ceramide and haematocrit during the admission (R = -0.443, $P \leq 0.05$) and defervescence phase (R = -0.355, $P \leq 0.05$) in the AD group (Fig. 5A & B). However, no such correlation was observed in other dengue groups (SD, DWW, and DWOW) during infection. This result suggests that the lipid may be associated with vascular integrity, yet the role of this ceramide in endothelial leakage needs further investigation.
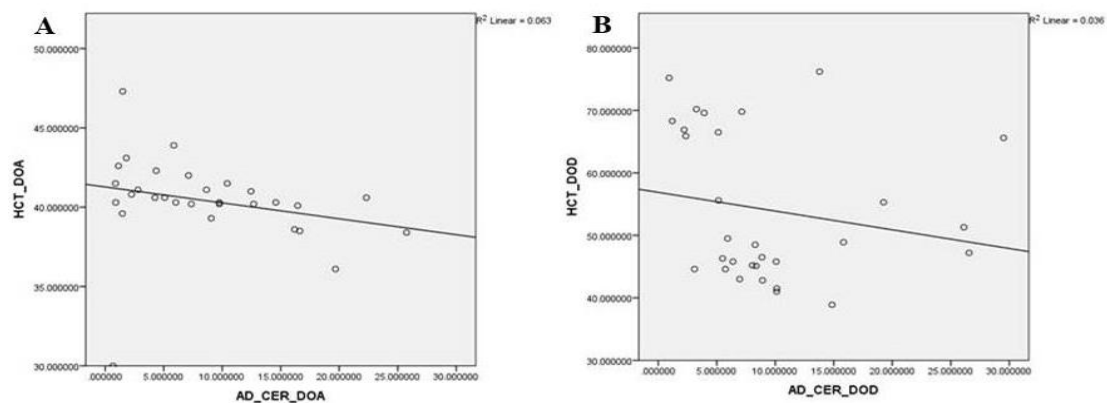
Figure 5: A negative correlation between ceramide and haematocrit during dengue. In Fig. 5 A and B, there is a correlation between ceramide and haematocrit during febrile phase (R = -0.355, P≤0.05) and the defervescence phase (R = -0.443, P≤0.05) of the AD group. Since the data was nonparametric, Spearman's Rho correlation was used to compare the data. A P-value ≤ 0.05 is considered significant.

Relationship between Ceramide levels and Platelet count:

We performed a correlation analysis to assess the relationship between platelet count and ceramide. A significant negative correlation was observed during the defervescence phase of the SD group (R =-0.867, P≤0.001) (Fig 6 A & B). But no such correlation was noted in other dengue groups (DWW, DWOW, and AD) throughout the infection.
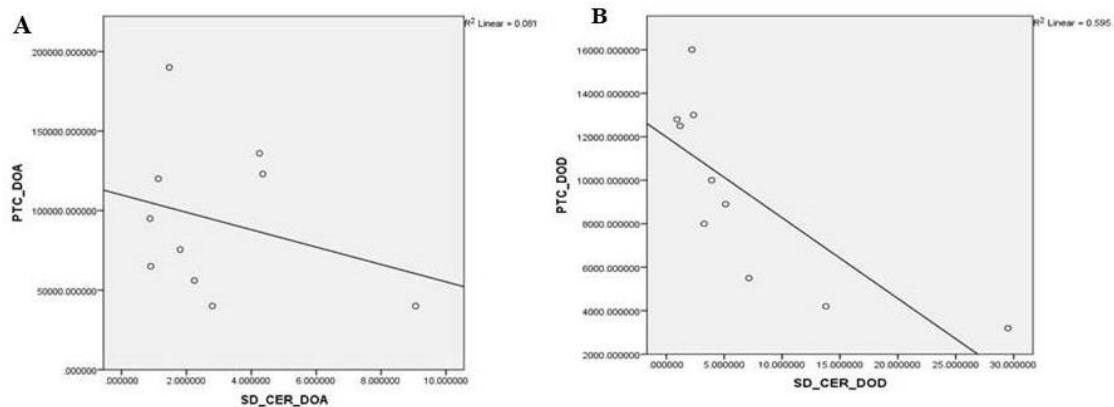
Figure 6: The negative correlation between ceramide and platelet counts during dengue. There was a correlation between ceramide and platelet count during the defervescence phase of the SD group (R = -0.867, P≤0.001). Since the data was nonparametric, Spearman's Rho correlation was used to compare the data. A P-value ≤ 0.05 is considered significant.

Discussion:

A lipid in the pathogenesis of dengue is not explored much. We have reported a set of 23 differentially expressed platelet lipids in dengue patients(4).The present study assessed plasma levels of ceramide (d18:1/22:0) in dengue cases compared to other febrile controls. Previous studies suggested that ceramide and downstream products would interfere with the various stages of the life cycle of influenza A virus including replication, protein transport, and assembly(15-16). Ceramide is reported to have a role in the virus propagation like WNV, dengue(17).Tafesse et al. (2019) demonstrated that ceramide is a key signalling mediator in ZIKV replication and pathogenesis(18).Another study showed that treatment of cells with SMase markedly enhanced Japanese encephalitis virus infection and also indicates that

ceramide plays a crucial role in the entry and exit of JEV into host cells[19].A significant decrease in the levels of ceramides was observed in SD, DWW, and AD cases compared to OFI at the time of admission, except for DWOW. The plasma level of ceramides was found to be decreasing towards DOD in DWOW cases, whereas the study observed a significant increase in ceramide levels towards DOD in SD and DWW cases. This enhanced ceramide found only in severe cases suggests the role of ceramides in the disease pathogenesis which typically lasts 24–48 hours during the defervescence phase. Most patients clinically improve during this phase, but those with substantial plasma leakage could develop severe dengue as a result of a sudden increase in vascular permeability. As a result, platelet count decreases, and ceramide levels eventually rise during infection. Together, these results show that ceramide is differentially expressed in severe dengue and patients characterised by severe symptoms during a critical phase. But it is still unclear whether the accumulation of ceramide in circulation could be attributed to vascular damage and platelet dysfunction, which could provide some light on the disease's pathogenesis. However, an elevated level of ceramides in circulation may be a counter response by the host against virus infection.

The molecular basis by which ceramides impact endothelial cells remains poorly understood [20].The hyper-host responsive factors may be involved in the down regulation of ceramides during dengue infected patients. In support of this notion, the HUVEC cells treated with positive serum obtained from the critical phase of severe dengue showed a decrease in the levels of ceramide compared to the cells treated with normal FBS and non-severe dengue groups (DWW serum), serum from SD cases during febrile phase as well as OFI. This shows that the host inflammatory response can alter the ceramides in ECs during the course of dengue infection. In in vitro study, experiments indicated that Zika infection of microglial cells indicates up-regulation of ceramide and sphingosine in 48 hpi infection[21].

Plasma leakage is caused by increased capillary permeability and may manifest as haemoconcentration, as well as pleural effusion, hypoproteinaemia, and ascites[14].The 20% increase in haemoglobin levels(haematocrit) in severe dengue is a widely used sign of plasma leakage[22].In our study, a significant negative correlation ($P \leq 0.05$) was observed between the plasma concentration of ceramide and haematocrit during the time of admission and the defervescence phase of the AD group.

A study reported that sphingosine-1-phosphate enhances endothelial integrity while ceramide tends to promote vascular leak[23]. Indeed, there is more evidence to support the elevated

levels sphingolipids, including ceramides, exhibit an inhibitory effect during the entry of viral infection. Besides viral infections, plasma ceramide levels are elevated in inflammatory conditions such as hypertension, diabetes, and cardiovascular diseases(24-25).The differentially expressed plasma lipids levels increase in SD reveals its importance in disease pathogenesis. Though, plasma levels of ceramides showed a negative association with HCT as well platelet count, the exact role of ceramides in endothelial dysregulation is not known. Perhaps, further investigations are required to ascertain the ceramides as an effective prognostic marker in a large clinical setup.

Conclusion:

A negative association between ceramide and platelet count could be used as early indicator for disease progression. An increased levels of ceramide (d18:1/22:0) towards the defervescence phase during dengue infection may serve has a robust biomarker for the early prediction of disease severity.

Acknowledgment:

Conflict of interest:

The authors declare no competing interests.

Funding source:

Ethics:

The study protocol has been approved by the Institutional Human Ethics committee (ECR/451/Inst/PO/2013/RR-16)

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