SMJ Singapore Medical Journal

Association of systemic vitamin D on the course of dengue virus infection in adults: a single-centre dengue cohort study at a large institution in Singapore

Sapna P Sadarangani[1,2,3], MBBS, Htet Lin Htun[4], MBBS, MPH, Weiping Ling[1], MPH, Robert Hawkins[5], MBBS, Tsin Wen Yeo[1,2,3], MBBS, PhD, Laura Rivino[6], PhD, Paul A MacAry[7], PhD, Yee-Sin Leo[1,2,3], MBBS, FRCP

[1]Department of Infectious Diseases, Tan Tock Seng Hospital, [2]National Centre for Infectious Diseases, [3]Lee Kong Chian School of Medicine, Nanyang Technological University, [4]Department of Preventive and Population Medicine, Office of Clinical Epidemiology, Analytics and Knowledge (OCEAN), [5]Department of Laboratory Medicine, Tan Tock Seng Hospital, [6]Emerging Infectious Diseases Programme, Duke-NUS Medical School, [7]Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Correspondence: Dr Sapna P Sadarangani, Consultant, National Centre for Infectious Diseases, 16 Jalan Tan Tock Seng, Singapore 308442. sapna_sadarangani@ttsh.com.sg

---

Singapore Med J 2022, 1–22
https://doi.org/10.11622/smedj.2022064
Published ahead of print: 2 June 2022

ABSTRACT

Introduction: Host immune responses may impact dengue severity in adults. Vitamin D has multiple immunomodulatory effects on innate and adaptive immunity.

Methods: We evaluated the association between systemic 25-hydroxyvitamin D [25-(OH) D] and dengue disease severity in adults. We measured plasma for total 25-(OH) D levels with an electrochemiluminescence immunoassay using stored samples from participants with laboratory confirmed dengue who were prospectively enrolled in 2012–2016 at our institution.

Results: 80 participants (median age 43 years) were enrolled. Six participants had severe dengue based on the World Health Organisation (WHO) 1997 criteria (i.e. dengue haemorrhagic fever/dengue shock syndrome) and another six had severe dengue based on the WHO 2009 criteria. Median 25-(OH) D at acute phase of dengue was 6.175 µg/L (interquartile range 3.82–8.21; range 3.00–15.29) in all participants. 25-(OH) D showed inverse linear trend with severe dengue manifestations based on the WHO 2009 criteria (aRR 0.72; 95% confidence interval 0.57–0.91; $p < 0.01$) after adjustment for age, gender and ethnicity.

Conclusion: Limited studies have evaluated the role of systemic 25-(OH) D on dengue severity. Our study found low systemic 25-(OH) D was associated with increased dengue disease severity, particularly for severe bleeding that was not explained by thrombocytopenia. Further studies investigating the underlying immune mechanisms and effects on the vascular endothelium are needed.

Keywords: dengue, severe dengue, vitamin D, 25-hydroxyvitamin D

INTRODUCTION

Dengue remains a globally important vector-borne infection with World Health Organisation (WHO) estimates of 50 million annual dengue infections and approximately 2.5 billion individuals at risk in dengue-endemic areas.[1] A more recent estimate using cartographic approaches revealed an annual burden of 390 million (95 percent credible interval 284-528) dengue infections, of which 96 million (67-136) are symptomatic.[2] The risk of severe dengue in adults is associated with host co-morbidities such as diabetes mellitus and other components of the host immune response.[3,4] The critical phase during dengue infection occurs during viral clearance, suggesting host immune responses may play an important role and could be targeted in approaches to mitigate severe dengue infection. Various immunopathogenesis and virus-host interaction factors have been studied, including role of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-10), innate immunity, cell-mediated immunity, antibody-mediated enhancement (ADE) and endothelial activation.[3,5,6]

There is now a licensed live-attenuated dengue vaccine CYD TDV (Dengvaxia ®) which may be used in certain patient sub-populations and there are other candidate dengue vaccines in development.[7,8] However, at the time of writing there is no licensed vaccine for use in older adults, and CYD TDV may not be appropriate for widespread implementation in all populations of risk. There is a need for further research to delineate the mechanisms of dengue pathogenesis in the context of rational development of therapeutic and immuno-modulatory interventions to prevent dengue-related complications in adults.[9,10]

Recently, there has been escalating interest in vitamin D's immunomodulatory actions, and its association with susceptibility to certain infections.[11,12] Vitamin D has robust actions on the innate immune response, acting as a chemo-attractant for monocytes, T cells and

neutrophils. It triggers a shift to a Th2 type cytokine response (characterized by increased levels of IL-4, IL-5, IL-10, and reduced levels of IL-2, IFN-γ, and TNF-α, i.e. pro-inflammatory cytokines). 1,25-$(OH)_2$ D3, the active metabolite produced endogenously from 25-(OH) D, inhibits IL-17 and IL-22 producing Th17 cells and increases CD4+/CD25+ Treg cells.[13,14] Vitamin D also has an influence on peripheral homing and the migration of T cells to the skin.[14]

Few studies have evaluated the association between vitamin D and dengue disease severity.[15-22] Several recent studies suggest a dose-response relationship between exposure to vitamin D and dengue pathogenesis and severity.[15,18,20] In contrast, other studies have shown contrasting results, i.e. higher 25-(OH) D associated with more severe dengue (DHF/DSS).[17,19] Importantly, the threshold of systemic 25-(OH) D for its immune-active actions is not yet known and may not be directly congruent to the levels relevant to skeletal health.[23,24] It is unknown if 1,25-$(OH)_2$-D3 has any specific actions on the vascular endothelium. Dengue disease course is dynamic and the timing of 25-(OH) D assessment, extent of plasma leakage, patient's prior 25-(OH) D status, co-morbidities all would play a role in studying this association.

We measured systemic 25-(OH) D in adult dengue patients with uncomplicated and severe disease prospectively enrolled at our institution, the largest tertiary teaching hospital in Singapore for dengue management. We hypothesized low systemic 25-(OH) D would be associated with more severe dengue clinical outcomes.

METHODS

We conducted a cohort study among adult (≥21 years) patients presenting with acute dengue infection to the Department of Infectious Diseases, Tan Tock Seng Hospital (TTSH), a 1700-bed adult tertiary-care public hospital in Singapore, measuring 25-(OH) D on stored samples.

The source population was identified from an ongoing prospective adult dengue cohort study active since 2009, henceforth referred to as "Study A". Study A included individuals with acute dengue confirmed by either positive dengue polymerase chain reaction (PCR),[25] or non-structural protein 1 (NS1) antigen or serology (IgM and IgG) tests based on a single acute sample.[26,27] Study A included 3 study visits – first visit on hospital presentation (acute illness), second visit at day 14-28 of illness (early convalescence) and third visit at day 45-120 of illness (late convalescence). Each study visit involved clinical assessment and venepuncture. For the present study, we performed convenience sampling to obtain our study population from the source population with the following eligibility criteria: (a) individuals who had completed study visit during acute illness phase, (b) sufficient residual sample available at acute time point for testing of plasma 25-(OH) D. Age-groups of individuals was also considered to ensure an adequate representation of various age-groups in final cohort. We excluded patients who did not give consent to be included in this study. This study was designed as a pilot exploratory study hence formal sample size calculation *a priori* was not performed.

25-hydroxyvitamin D assessment

We utilized residual cryopreserved plasma samples following informed consent. The plasma was frozen in aliquots at -80 °C immediately after processing of blood following collection and thawed only prior to the 25-(OH) D assay. Plasma total 25-(OH) vitamin D was measured on a Roche e601 immunoassay analyser (electrochemiluminescence immunoassay) using the Elecsys Vitamin D total II assay with manufacturer-supplied reagents and calibrators (Roche Diagnostics, Mannheim, Germany). The assay uses a vitamin D binding protein to bind 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2. The mean cross-reactivity of 25-hydroxyvitamin D_3 in the assay is 100% while the cross reactivity of 25-hydroxyvitamin D_2 is 93.7%. Cross-reactivity to 24,25–dihydroxyvitamin D is blocked by a specific monoclonal

antibody. The method has been standardised using internal standards which are traceable to an isotope dilution – liquid chromatography – tandem mass spectrometry (ID-LC-MS/MS) method, which is in turn traceable to the National Institute of Standards and Technology Standard Reference Material 2972.[28] The limit of blank was 2 µg/L.

Severe dengue manifestations

Severe clinical presentation of dengue was classified as dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) according to the WHO 1997 criteria, and severe dengue (SD) according to WHO 2009 criteria. The hospital and outpatient course for each dengue infected patient was documented using a standardized dengue care path that records relevant clinical, laboratory and radiological data in a standardized manner. Clinical data was extracted from the first day of hospital presentation until discharge date for inpatients, or until follow-up for outpatients by the trained study team. We retrospectively classified the severity of patients' illness based on the WHO dengue criteria.

DHF cases (WHO, 1997) met all the following criteria: fever and all three of (i) haemorrhagic manifestations, (ii) thrombocytopenia <100 x 10^9/L; and (iii) plasma leakage evidenced by pleural effusion or ascites or change in haematocrit ≥20% or hypoproteinaemia. DSS (WHO, 1997) was defined as presence of tachycardia with narrow pulse pressure lower than 20 mmHg or hypotension (systolic blood pressure <90 mmHg) in addition to DHF.[28] SD cases (WHO, 2009) met the following criteria (i) severe plasma leakage with respiratory distress or shock, (ii) severe bleeding defined as a minimum of WHO grade 2 bleeding scale or any bleeding required whole blood or packed red cell transfusion, (iii) severe organ involvement – acute liver injury with aspartate transaminase (AST) and alanine transaminase (ALT) ≥1000 IU/L or acute kidney injury or myocarditis or encephalopathy.[1]

Data collection and statistical analysis

Data collection was performed independently by trained research assistants following standardized procedures. Systemic 25-(OH) D was analysed as a continuous variable, using median value and interquartile ranges (IQR) for descriptive statistics. Chi-square test was implemented for bivariate inference method. We used univariable and multivariable Poisson regression with robust error variance[29] to estimate crude and adjusted risk ratio (cRR and aRR) respectively with 95% confidence interval (CI) assessing the association between serum 25-(OH) D concentration and severe dengue manifestations, as well as each sub-category signifying severity, namely plasma leakage leading to shock, bleeding, organ involvement. In view of small sample size, we adjusted only demographic variables in the adjusted model. Statistical significance threshold was set at $P < 0.05$. All analyses were carried out with Stata 13.1 (College Station, TX, USA: StataCorp LP).

Ethics approval

The study was approved by the Domain Specific Review Board of the National Healthcare Group, Singapore (DSRB - 2016/01167). Informed consent was obtained via completed returned reply slips posted to invited participants. Study team followed up with phone call if no response was received at two weeks following mailing of a letter, and informed consent was obtained verbally and documented in patient's medical record.

RESULTS

199 participants who had been admitted for dengue infection between 2012 and 2016 were screened for eligibility for enrolment (Fig. 1). 119 participants were not eligible either due to lack of informed consent or insufficient residual samples. 80 participants were enrolled, aged 21 to 69 years with male predominance. Six participants had severe dengue based on WHO

1997 (i.e. DHF/DSS) criteria and another six participants had severe dengue based on WHO 2009 criteria. Two participants had severe dengue fulfilling both WHO 1997 and 2009 classifications. 70 participants had uncomplicated dengue. Median day of illness at time of acute visit for all participants was 5 days (IQR 4-6). Table I presents the demographic, 25-(OH) D levels, and clinical characteristics of the study population. Median 25-(OH) D was lower in younger age group (4.50 µg/L in 21-40 years *vs.* 6.59 µg/L in 41-60 years *vs.* 6.87 µg/L in 61-69 years, P=0.042), and in non-Chinese patients (5.83 µg/L amongst Malays and Indians *vs.* 6.76 µg/L in Chinese, P=0.009). Median 25-(OH) D was 4.42 ug/L (IQR: 3.00 - 6.74) in those with dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS) based on WHO 1997 criteria compared to 6.39 ug/L (IQR: 3.93- 8.36) in those without DHF/DSS (p=0.115), and 5.41 ug/L (IQR: 3.00-5.84) in those with severe dengue (WHO 2009) compared to 6.64 ug/L (IQR:3.82- 8.36) in those without severe dengue (p=0.101).

Multivariable analysis

(1) WHO 1997 dengue classification

No statistically significant association was found between serum 25-(OH) D and (i) DHF/DSS (aRR 0.82, 95% CI 0.64-1.05, P=0.113), or its severity indicators including (ii) haemorrhagic manifestations (aRR 0.98, 95% CI 0.86-1.12, P=0.801) and (iii) plasma leakage (aRR 0.98, 95% CI 0.84-1.13, P=0.749) based on the WHO 1997 dengue criteria (Table II).

(2) WHO 2009 dengue classification

A significant inverse linear trend of association between serum 25-(OH) D and SD (aRR 0.72, 95% CI 0.57-0.91, P=0.005) was observed after adjusting for age, gender and ethnicity, based on the WHO 2009 dengue criteria as shown in Table II. Similarly, serum 25-(OH) D had statistically significant association with severe bleeding (aRR 0.71, 95% CI 0.53-0.96,

P=0.024). However, there was no significant association for severe plasma leakage leading to shock (aRR 0.73, 95% CI 0.48-1.114, P=0.142). The association of low 25-(OH) D with severe bleeding does not appear to be mediated by thrombocytopenia as median 25-(OH) D levels were higher in patients with thrombocytopenia as defined in Table I. Table III shows a more detailed clinical course of these patients who had severe dengue.

DISCUSSION

We report an association of low systemic 25-(OH) D with higher dengue severity (WHO, 2009) particularly for bleeding manifestations which is not explained by thrombocytopenia in our adult cohort study. The bleeding manifestations were mainly mucosal bleeding and none of the patients required blood transfusions or ICU care (table III). A small number received platelet transfusions in the setting of bleeding. The strength of our study is it is one of few clinical studies to investigate association between systemic 25-(OH) D on dengue disease severity outcomes based on WHO 1997 and 2009 criteria in adults in a cohort that includes older adults. The use of standardized dengue clinical care path that contains clinical and laboratory data for the course of dengue illness ensures systematic method of collection and minimises bias.

Examining the potential role of immunomodulators and modifiable factors, such as systemic 25-(OH) D is an approach that may have translational potential to attenuate disease severity. Importantly, the 25-(OH) D threshold defining 'deficiency' is based on its role in bone health, and thresholds defining actions relevant to immune relevant actions is not known. 25-(OH) D is the main systemically available form of vitamin D with a half-life of 2-3 weeks and is reflective of an individual's vitamin D stores.[23,24] Of significance, biologically active form of vitamin D, i.e. calcitriol or 1, 25-dihydroxy vitamin D_3 [1,25-$(OH)_2$ D_3], is also locally produced (CYP27B1, 1 alpha hydroxylase) in various immune cells from systemic 25-hydroxy vitamin D [25-(OH) D]. The Vitamin D receptor (VDR) is expressed in many human tissues

including cells from the innate and adaptive immune system, and VDR binds systemically available and locally produced 1,25-$(OH)_2$ D, leading to downstream tissue-specific intracrine and paracrine actions.[11,12]

As shown by other studies, vitamin D deficiency is not uncommon in Singapore and other tropical dengue-endemic areas despite higher year-round UV exposure.[30,31] Our study participants had overall low 25-(OH) D levels at acute time-point, and lower levels were observed in those of Malay and Indian ethnicity compared to Chinese as has been reported in other studies.[30] The comparatively higher 25-(OH) D levels in older participants may have been from supplementation (non-prescription), however this data was not available to the study team.

The immune mechanisms for observations of 25-(OH) D association with dengue disease course and severity are not entirely elucidated. Few authors have evaluated this in more detail. Of interest, an *in vitro* study involving human myelomonocytic and hepatic cell lines exposed to various concentrations of 1,25-$(OH)_2$ D3 which were subsequently infected with DENV-4 found significantly reduced percentage of infected cells, and reduced production of TNF α, IL-1B, IL-6, IL-12p70 with a dose-response relationship observed with 1,25-$(OH)_2$ D3.[15] The underlying immune mechanisms are not yet clear. Arboleda Alzate *et al* exposed monocyte-derived macrophages (from healthy volunteers) *in vitro* to varying concentrations of 1,25-$(OH)_2$D3 with subsequent infection with DENV-2. The macrophages differentiated in the presence of higher 1,25-$(OH)_2$D3 concentrations had decreased DENV-2 infectivity, potentially due to reduced expression of receptors required for DENV entry into macrophages and also had reduced pro-inflammatory cytokine levels (specifically TNF α, IL-1β, IL-10) in response to DENV infection.[20] Another *in vitro* study challenged monocyte-derived macrophages from participants enrolled in a vitamin D supplementation study with DENV-2. Macrophages from participants exposed to higher-dose (4000 IU/day) supplementation were not as susceptible to

DENV-2 infection compared to those who received lower dose supplementation, thereby having a protective effect.[18] TNF-α levels were lower while IL-10 and IL-8 were higher in the higher dose supplementation group. However, serum 25-(OH) D levels were not quantified in this study. Interestingly, a recent in-vitro study examining seven VDR agonists found five of the compounds significantly inhibited DENV-2 infection of HEK293T/17 cells with reduced virus production of up to $3Log_{10}$.[32] There are many immunological postulations as to how Vitamin D may be influencing the susceptibility to infection and inflammatory response, however this still needs further study.[18]

There are a few limitations in our study which could be addressed in future studies. Since we invited previously enrolled participants to participate in this study, there is possibility of bias in recruitment due to participants who were not contactable for informed consent. The number of severe dengue patients in this cohort was limited. We also did not have control groups of non-dengue febrile patients or well patients without any febrile illness. We did not perform a sample size calculation *a priori* as this was designed as a pilot study, hence our study was not sufficiently powered to examine the effects of 25-(OH) D might exert on different subgroups of patients and severity indicators of dengue. Although multivariable models were used to control for the main confounding variables, residual confounding might persist.

A commonly used immunoassay bench method was used for total 25-(OH) vitamin D measurement rather than an ID-LC-MS/MS reference method. Such methods generally show poorer precision than reference methods and do not allow differentiation of vitamin D_2 from vitamin D_3. However, the assay was traceable to the reference method, and the lesser accuracy and precision should not have affected the conclusions of this study.

In conclusion, further studies are needed in cohorts with a higher number of severe dengue patients to validate our findings, and preferably include control groups. Underlying immune and other mechanisms should also be studied, such as effects on vascular endothelium,

certain markers of innate and adaptive immunity as well as cytokine responses where appropriate. We note that few other clinical studies have shown higher 25-(OH) D associated with higher probability of DHF/DSS[17, 19] which is contrary to findings from human monocyte studies.[18, 20] Whether this is related to the timing of venepuncture, phase of dengue illness, population variability, performance of assay or other factors remains unclear.

An emerging concept in the understanding of 25-(OH) D's non-skeletal actions is the "personal vitamin D response index", which is thought to arise from a set of molecular and epigenetic variations in the vitamin D-signalling pathway.[34] This may in turn explain variable 'threshold' of 'sufficiency' or vitamin D-responsiveness for certain individuals and population groups, and in turn potentially explain the conflicting results of vitamin D observational and supplementation studies as also mentioned here. Ideally, well-designed human intervention studies with vitamin D-supplementation or VDR agonists should include baseline 25-(OH) D, evaluate various dosing regimens, while also stratifying based on the "vitamin D-response index" of the study population once this is better defined.

In summary, our study found low systemic 25-(OH) D was associated with increased dengue disease severity as based on WHO 2009 criteria, particularly for severe bleeding, which was not explained by thrombocytopenia. Further studies are needed in cohorts with larger numbers of severe dengue patients. 25-(OH) D's impact on the course of dengue infection in terms of underlying immune mechanisms and effects on the vascular endothelium are needed.

ACKNOWLEDGMENTS

We thank all the study participants. We acknowledge funding support from National Medical Research Council (NMRC) Centre Grant (Ref. NMRC/CG/003/2013). Residual sera are received from NMRC Translational and Clinical Research (TCR) STOP Dengue Programme (ref. NMRC/TCR/005/2008). The authors have no conflict of interests to declare.

REFERENCES

1. World Health Organization. Dengue: guidelines for diagnosis, treatment, prevention and control 2009 [cited 2019 October 1]. Available from: https://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf.
2. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013;496(7446):504-7.
3. Ben-Shachar R, Koelle K. Minimal within-host dengue models highlight the specific roles of the immune response in primary and secondary dengue infections. Journal of The Royal Society Interface. 2015;12(103):20140886.
4. Htun NSN, Odermatt P, Eze IC, Boillat-Blanco N, D'Acremont V, Probst-Hensch N. Is diabetes a risk factor for a severe clinical presentation of dengue?--review and meta-analysis. PLoS Negl Trop Dis. 2015;9(4):e0003741-e.
5. Lee I-K, Hsieh C-J, Chen R-F, Yang Z-S, Wang L, Chen C-M, et al. Increased Production of Interleukin-4, Interleukin-10, and Granulocyte-Macrophage Colony-Stimulating Factor by Type 2 Diabetes' Mononuclear Cells Infected with Dengue Virus, but Not Increased Intracellular Viral Multiplication. BioMed research international. 2013;2013:965853.
6. Rivino L, Lim MQ. CD4+ and CD8+ T-cell immunity to Dengue – lessons for the study of Zika virus. Immunology. 2017;150(2):146-54.
7. World Health Organization. Dengue vaccine: WHO position paper. 93:457-476 2018 [cited 2019 October 1]. Available from: https://www.who.int/wer/2018/wer9336/en/.
8. Biswal S, Reynales H, Saez-Llorens X, Lopez P, Borja-Tabora C, Kosalaraksa P, et al. Efficacy of a Tetravalent Dengue Vaccine in Healthy Children and Adolescents. New England Journal of Medicine. 2019;381(21):2009-19.
9. Liu Y, Liu J, Cheng G. Vaccines and immunization strategies for dengue prevention. Emerging Microbes & Infections. 2016;5(1):1-6.

10. Low JGH, Ooi EE, Vasudevan SG. Current Status of Dengue Therapeutics Research and Development. The Journal of Infectious Diseases. 2017;215(suppl_2):S96-S102.

11. Lang PO, Aspinall R. Can We Translate Vitamin D Immunomodulating Effect on Innate and Adaptive Immunity to Vaccine Response? Nutrients. 2015;7(3).

12. Haussler MR, Whitfield GK, Kaneko I, Haussler CA, Hsieh D, Hsieh J-C, et al. Molecular Mechanisms of Vitamin D Action. Calcified Tissue International. 2013;92(2):77-98.

13. Sadarangani SP, Whitaker JA, Poland GA. "Let there be light": the role of vitamin D in the immune response to vaccines. Expert Review of Vaccines. 2015;14(11):1427-40.

14. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nature Reviews Immunology. 2008;8(9):685-98.

15. Puerta-Guardo H, De la Cruz Hernández SI, Rosales VH, Ludert JE, del Angel RM. The 1α,25-dihydroxy-vitamin D3 reduces dengue virus infection in human myelomonocyte (U937) and hepatic (Huh-7) cell lines and cytokine production in the infected monocytes. Antiviral Research. 2012;94(1):57-61.

16. Loke H, Bethell D, Phuong CXT, Day N, White N, Farrar J, et al. Susceptibility to dengue hemorrhagic fever in vietnam: evidence of an association with variation in the vitamin d receptor and Fc gamma receptor IIa genes. The American journal of tropical medicine and hygiene Am J Trop Med Hyg Am J Trop Med Hyg. 2002;67(1):102-6.

17. Alagarasu K, Bachal RV, Bhagat AB, Shah PS, Dayaraj C. Elevated levels of vitamin D and deficiency of mannose binding lectin in dengue hemorrhagic fever. Virology Journal. 2012;9(1):86.

18. Giraldo DM, Cardona A, Urcuqui-Inchima S. High-dose of vitamin D supplement is associated with reduced susceptibility of monocyte-derived macrophages to dengue virus infection and pro-inflammatory cytokine production: An exploratory study. Clinica Chimica Acta. 2018;478:140-51.

19. Villamor E, Villar LA, Lozano A, Herrera VM, HerrÁN OF. Vitamin D serostatus and dengue fever progression to dengue hemorrhagic fever/dengue shock syndrome. Epidemiology and Infection. 2017;145(14):2961-70.

20. Arboleda Alzate JF, Rodenhuis-Zybert IA, Hernández JC, Smit JM, Urcuqui-Inchima S. Human macrophages differentiated in the presence of vitamin D3 restrict dengue virus infection and innate responses by downregulating mannose receptor expression. PLoS Negl Trop Dis. 2017;11(10):e0005904.

21. Voge NV, Perera R, Mahapatra S, Gresh L, Balmaseda A, Loroño-Pino MA, et al. Metabolomics-Based Discovery of Small Molecule Biomarkers in Serum Associated with Dengue Virus Infections and Disease Outcomes. PLoS Negl Trop Dis. 2016;10(2):e0004449.

22. Langerman SD, Ververs M. Micronutrient Supplementation and Clinical Outcomes in Patients with Dengue Fever. The American Journal of Tropical Medicine and Hygiene. 2021;104(1):45-51.

23. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, Treatment, and Prevention of Vitamin D Deficiency: an Endocrine Society Clinical Practice Guideline. The Journal of Clinical Endocrinology & Metabolism. 2011;96(7):1911-30.

24. Medicine Io. Dietary Reference Intakes for Calcium and Vitamin D. Ross AC, Taylor CL, Yaktine AL, Del Valle HB, editors. Washington, DC: The National Academies Press; 2011. 1132 p.

25. Barkham TM, Chung YK, Tang KF, Ooi EE. The performance of RT-PCR compared with a rapid serological assay for acute dengue fever in a diagnostic laboratory. Transactions of The Royal Society of Tropical Medicine and Hygiene. 2006;100(2):142-8.

26. Gan VC, Tan L-K, Lye DC, Pok K-Y, Mok S-Q, Chua RC-R, et al. Diagnosing Dengue at the Point-of-Care: Utility of a Rapid Combined Diagnostic Kit in Singapore. PLOS ONE. 2014;9(3):e90037.

27. Blacksell SD, Newton PN, Bell D, Kelley J, Mammen MP, Jr., Vaughn DW, et al. The Comparative Accuracy of 8 Commercial Rapid Immunochromatographic Assays for the Diagnosis of Acute Dengue Virus Infection. Clinical Infectious Diseases. 2006;42(8):1127-34.

28. Carter GD. 25-Hydroxyvitamin D: A Difficult Analyte. Clinical Chemistry. 2012;58(3):486-8.

29. World Health Organization. Dengue haemorrhagic fever : diagnosis, treatment, prevention and control. 2nd ed ed. Geneva: World Health Organization; 1997.

30. Zou G. A Modified Poisson Regression Approach to Prospective Studies with Binary Data. American Journal of Epidemiology. 2004;159(7):702-6.

31. Man REK, Li L-J, Cheng C-Y, Wong TY, Lamoureux E, Sabanayagam C. Prevalence and Determinants of Suboptimal Vitamin D Levels in a Multiethnic Asian Population. Nutrients. 2017;9(3):313.

32. Bi X, Tey SL, Leong C, Quek R, Henry CJ. Prevalence of Vitamin D Deficiency in Singapore: Its Implications to Cardiovascular Risk Factors. PLOS ONE. 2016;11(1):e0147616.

33. Jaratsittisin J, Xu B, Sornjai W, Weng Z, Kuadkitkan A, Li F, et al. Activity of vitamin D receptor agonists against dengue virus. Scientific Reports. 2020;10(1):10835.

34. Carlberg C, Haq A. The concept of the personal vitamin D response index. The Journal of Steroid Biochemistry and Molecular Biology. 2018;175:12-7.

Table I. Demographic, clinical characteristics of enrolled patients and serum 25-(OH) D at acute time-point (n = 80)

Characteristics	**No.**	**%**	**Serum 25-hydroxyvitamin D (µg/L) median (IQR)**	**P**
Age (years)				0.042
21 - 40	36	45.0	4.50 (3.06 - 7.75)	
41 – 60	32	40.0	6.59 (4.74 - 8.76)	
61 – 69	12	15.0	6.87 (5.95 - 10.17)	
Gender				NS
Male	56	70.0	5.90 (3.96 - 8.15)	
Female	24	30.0	6.52 (3.08 - 8.44)	
Ethnicity				0.009
Chinese	63	78.8	6.76 (4.46 - 8.68)	
Non-Chinese	17	21.2	5.83 (3.93 - 7.89)	
Charlson's comorbidity index (CCI)				NS
CCI = 0	75	93.7	5.96 (3.66 - 8.21)	
CCI ≥ 1	5	6.3	6.98 (6.54 - 9.30)	
Hypertension				0.049
No	66	82.5	5.83 (3.25 - 8.09)	
Yes	14	17.5	6.94 (6.07 - 9.20)	
Hyperlipidaemia				NS
No	68	85.0	6.12 (3.58 - 8.21)	
Yes	12	15.0	6.31 (5.29 - 7.85)	
Past dengue infection				NS
No	74	92.5	5.90 (3.81 - 8.04)	
Yes	6	7.5	8.38 (6.07 - 9.68)	
Antihypertensive drugs				NS
No	70	87.5	5.84 (3.49 - 8.09)	
Yes	10	12.5	6.94 (6.54 - 9.2)	
Antihyperlipidemic drugs				NS
No	74	92.5	6.12 (3.66 - 8.21)	
Yes	6	7.5	6.31 (5.53 - 6.98)	
Antidiabetic drugs				NS
No	78	97.5	6.02 (3.81 - 8.21)	
Yes	2	2.5	6.76 (6.54 - 6.98)	
Aspirin				NS
No	77	96.3	6.07 (3.81 - 8.21)	
Yes	3	3.7	6.54 (5.11 - 9.30)	
Thrombocytopenia *				NS
No	28	35.0	5.22 (3.20 - 7.98)	
Yes	52	65.0	6.75 (4.23 - 8.70)	
WHO 1997 dengue criteria				
DHF / DSS				NS
No	74	92.5	6.39 (3.93 - 8.36)	
Yes	6	7.5	4.42 (3.00 - 6.74)	
Plasma leakage				NS
No	67	83.8	6.28 (3.82 - 8.21)	

Yes	13	16.2	6.07 (3.81 - 7 .08)	
Haemorrhagic manifestations				NS
No	57	71.3	6.54 (3.82 - 8.67)	
Yes	23	28.7	5.84 (3.49 - 7.08)	
WHO 2009 dengue criteria				
Severe dengue				NS
No	74	92.5	6.64 (3.82 - 8.36)	
Yes	6	7.5	5.41 (3.00 - 5.84)	
Severe plasma leakage leading to shock				NS
No	78	97.7	6.39 (3.82-8.21)	
Yes	2	2.3	4.54 (3.00-6.07)	
Severe bleeding				NS
No	74	95.0	6.52 (3.88 - 8.29)	
Yes	6	5.0	4.35 (3.00 - 5.77)	
Epidemic year †				NS
2013, 2014 (DENV1)	9	11.3	6.93 (5.11 - 9.48)	
2012, 2015, 2016 (DENV2)	71	88.7	5.96 (3.81 - 8.21)	

**Thrombocytopenia was defined as lowest platelet count during hospital stay <100 x 10^9/L.*
†Epidemic year was used as a surrogate index to estimate the circulating dengue serotype
NS; p > 0.05. DHF: dengue haemorrhagic fever; DSS: dengue shock syndrome; WHO: World Health Organization

Table II. Risk ratio for association between plasma 25-(OH) D level and severe dengue manifestations based on the WHO 1997 and 2009 criteria

Severe dengue manifestations	No. (%) of outcomes	Crude			Adjusted *		
		RR	95% CI	P	RR	95% CI	P
WHO 1997 dengue classification							
DHF/ DSS	6 (7.5)	0.76	0.55 - 1.05	NS	0.82	0.64 - 1.05	NS
• Hemorrhagic manifestations	23 (28.8)	0.95	0.84 - 1.08	NS	0.98	0.86 - 1.12	NS
• Plasma leakage	13 (16.3)	0.94	0.79 - 1.12	NS	0.98	0.84 - 1.13	NS
WHO 2009 dengue classification							
Severe dengue	6 (7.5)	0.77	0.61 - 0.97	0.025	0.72	0.57 - 0.91	0.005
• Severe bleeding	4 (5.0)	0.69	0.46 - 1.02	NS	0.71	0.53 - 0.96	0.024
• Severe plasma leakage leading to shock	2 (2.3)	0.72	0.41 - 1.26	NS	0.73	0.48 - 1.114	NS

**Adjusted for age, gender and ethnicity. NS; p > 0.05.,CI: confidence interval; DHF: dengue haemorrhagic fever; DSS: dengue shock syndrome; RR: risk ratio*

Table III

Table III a: Clinical characteristics of participants with severe dengue based on WHO 1997 Dengue Classification

Subject ID	Subject 041	Subject 044	Subject 047	Subject 050†	Subject 001†	Subject 061
Age	36	44	21	46	31	34
Gender	Female	Male	Female	Female	Female	Male
Ethnicity	Chinese	Chinese	Chinese	Chinese	Chinese	Others
Comorbidities	Nil	Nil	Nil	Hyperlipidaemia, Hypothyroidism	Nil	Nil
Year of presentation	2012	2012	2012	2012	2015	2016

Day of fever at hospital presentation	1	5	2	5	4	4
WHO dengue 1997 classification	DHF, DSS	DHF, DSS	DHF	DHF	DHF	DHF
Hemorrhagic manifestations/ mucosal bleeding	Yes; petechiae	Yes; gum bleeding, petechiae	Yes; petechiae	Yes; menorrhagia, petechiae	Yes; hematemesis	Yes; gum bleeding
Severe plasma leakage	Yes; hypoproteinemia	Yes; hypo-proteinemia	Yes; hemo-concentration	No	Yes; hemo-concentration	Yes; hemoconcentration
Key Physical exam findings	Hypotension SBP < 90 mmHg. No hepatosplenomegaly	Hypotension SBP < 90 mmHg. No hepato-splenomegaly	Not hypotensive, no hepato-splenomegaly	Hypotensive SBP < 90 mmHg. Hepato-splenomegaly	Tachycardic > 100. No hypotension or hepatospleno megaly	Tachycardic > 100. No hypotension or hepatosplenomegaly
Transaminitis *	Moderate	No	Moderate	Mild	Mild	Unknown
Lowest platelet count (L X 10^9/L)	70	73	49	16	56	33
Platelet transfusion	No	No	No	Yes	No	No
Length of Inpatient stay (days)	6	5	3	4	5	5
Serum 25-hydroxyvitamin D (ug/L)	7.08	5.83	6.74	3.00	3.00	3.00

**Transaminitis definition: "mild" defined as transaminase elevation up to 2 times the upper limit of normal laboratory reference range; "moderate" between two to five times upper limit of normal, and "severe" more than 5 times upper limit of normal. (Reference range for AST: 7-55 units/L, ALT: 8-48 units/L). †Subjects 001 and 050 had severe dengue both based on WHO 1997 and 2009 definitions.*

Table III b: Clinical characteristics of participants with severe dengue based on WHO 2009 Dengue Classification

Subject ID	Subject 050[†]	Subject 023	Subject 001[†]	Subject 054	Subject 008	Subject 032
Age	46	63	31	50	63	31
Gender	Female	Male	Female	Female	Female	Male
Ethnicity	Chinese	Chinese	Chinese	Chinese	Chinese	Chinese
Comorbidities	Hyperlipidaemia, hypothyroidism	Hypertension, hyperlipidaemia	Hyperlipidaemia	Chronic Hepatitis B (normal transaminases at baseline)	Hyperlipidaemia, hyperthyroidism, psoriasis, osteoarthritis	Nil
Year of presentation	2012	2013	2015	2015	2015	2016
Day of fever at hospital presentation	5	5	4	2	3	2
WHO dengue 2009 classification	Severe dengue	Severe dengue	Severe dengue	Severe dengue	Severe dengue	Severe dengue
Hemorrhagic manifestations/ mucosal bleeding	Yes; menorrhagia	No	Yes; hematemesis	Yes; rectal bleeding, gum bleeding	No	Yes; rectal bleeding
Severe plasma leakage	No	No	Yes; hemo-concentration	No	Yes; pleural effusion, radiologically diagnosed	No
Key Physical exam findings	Hypotension SBP < 90 mmHg and hepato-splenomegaly	Hypotension SBP < 90 mmHg. No hepato-splenomegaly	Tachycardia, HR > 100. No hepato-splenomegaly or hypotension	Tachycardia, HR > 100. No hepato-splenomegaly or hypotension	Hypotension SBP < 90 mmHg. No hepato-splenomegaly.	None.
Transaminitis [*]	Mild	Mild	Mild	Moderate	Moderate	No

Lowest platelet count (L X 10^9/L)	16	40	56	12	12	140
Platelet transfusion	Yes	No	No	Yes	No	No
Length of Inpatient stay (days)	4	5	5	3	3	2
Serum 25-hydroxyvitamin D (ug/L)	3.00	5.11	3.00	5.84	6.07	5.70

**Transaminitis definition: "mild" defined as transaminase elevation up to 2 times the upper limit of normal laboratory reference range; "moderate" between two to five times upper limit of normal, and "severe" more than 5 times upper limit of normal. Reference range for AST: 7-55 units/L, ALT: 8-48 units/L). †Subjects 001 and 050 had severe dengue both based on WHO 1997 and 2009 definitions.*

FIGURE

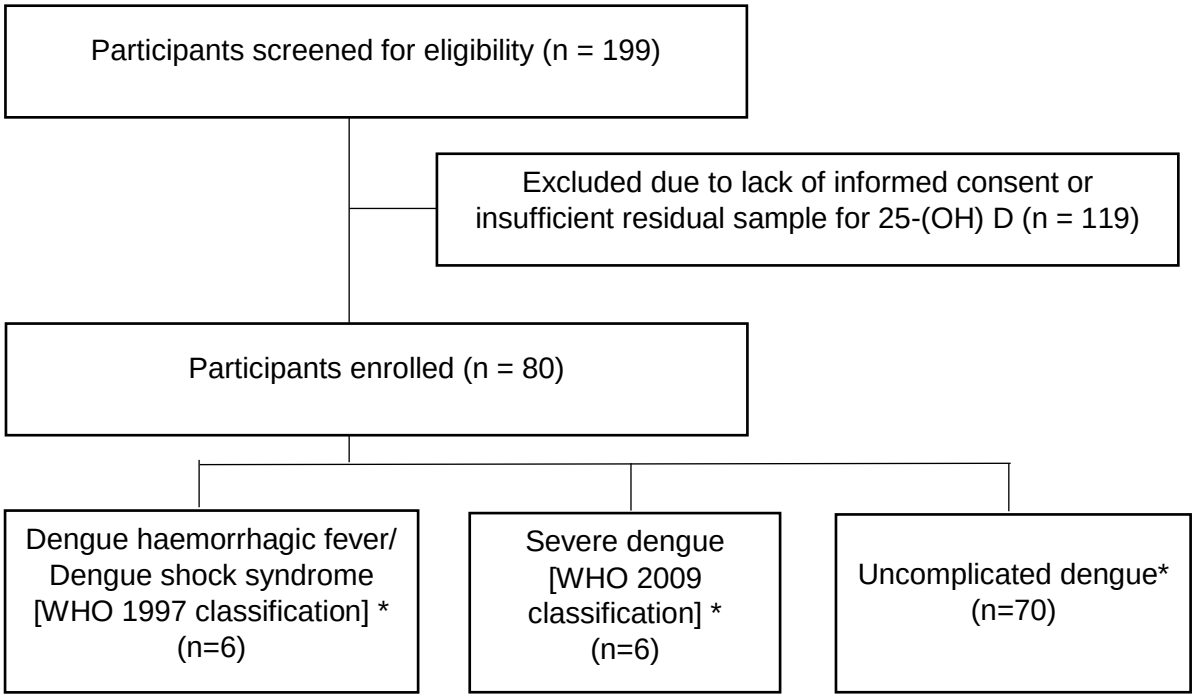

Figure 1. Study flow diagram. *There were two patients who were classified as severe dengue on both WHO 1997 and 2009 classifications