CLINICAL, GENETIC AND MOLECULAR RISK FACTORS OF DENGUE SEVERITY

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NATIONAL UNIVERSITY OF SINGAPORE

2014

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B.Sc. (Hons.), M.Sc., NUS

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

SAW SWEE HOCK SCHOOL OF PUBLIC HEALTH

NATIONAL UNIVERSITY OF SINGAPORE

2014

DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety except on the following Chapter which are jointly worked on with the collaborator for publication purpose:

- Chapter 3 was performed with collaborator Assistant Professor Khor Chiea Chuen, Principal Investigator & Senior Research Scientist, Division of Human Genetics, Genome Institute of Singapore, Singapore. I was involved in performing the quality control of the DNA samples, and the genotyping of the replication cohort, and subsequently, the statistical analysis of the genotypes of the replication cohort. I was also involved in editing the manuscript draft.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Vincent Pang

Mr. Pang Junxiong, Vincent

29 July 2014

ACKNOWLEDGEMENTS

My PhD journey has been full of twists and turns, just like running a triathlon; a new sport that I enjoy doing and which I have only picked up during my PhD candidature. This new sport has reemphasized the importance of self-discipline, perseverance, ample preparation and to remain optimistic at all time in my life. I would like to attribute the success of my PhD journey to these core values that I firmly believe in. With these values, it has been the most fruitful and memorable learning journey that I have ever experience in my life. Furthermore, I am very grateful and thankful to the following supervisors and mentors, who have generously shared their precious time, expertise, resources, and guidance during this rigorous learning journey:

I. Professor Chia Kee Seng, Dean, Saw Swee Hock School of Public Health (SSHSPH) of the National University of Singapore (NUS). Prof. Chia had accepted my PhD application at SSHSPH four years ago, and had passionately guided me on epidemiological concepts for about two years before taking up the Dean position of our school on a full time basis.

II. Professor Martin Lloyd Hibberd, Senior Group Leader and Associate Director of Infectious Diseases Group in Genome Institute of Singapore (GIS). Prof. Hibberd had kindly provided laboratory space, resources, expertise and encouragement for my laboratory experiments and pursuits over the last four years.

III. Professor Leo Yee Sin, Head of Institute of Infectious Diseases and Epidemiology (IIDE) of Tan Tock Seng Hospital (TTSH) and Clinical Director of Communicable Disease Centre (CDC), Singapore. Prof. Leo had kindly provided clinical datasets, samples, expertise, and support for the clinical epidemiological studies over the last four years. She had also generously provided conference

opportunities for me to share my research findings and to stay current in dengue research.

IV. Assistant Professor Mark I-Cheng Chen, SSHSPH, NUS and Head, Research of IIDE, TTSH. Asst. Prof. Chen had kindly took over the role of Prof. Chia to support and guide me in the last two years of my PhD journey. Asst. Prof. Chen had critically provided his guidance and advice on my manuscript and thesis writing. I am also grateful to him for his guidance on career planning and research inspirations.

V. Assistant Professor Khor Chiea Chuen, Principal Investigator & Senior Research Scientist, Division of Human Genetics, GIS. Asst. Prof. Khor was an important pillar of my early PhD training. His passion and knowledge in genetics had provided strong motivation and inspiration in our dengue genetic studies. I am honoured to have him as one of my mentors to overcome my academic limits and to seek excellence in research.

VI. Assistant Professor David Lye Chien Boon, Senior Consultant, Department of Infectious Diseases, TTSH, and CDC, Singapore. Dr. Lye, as my clinical mentor, had provided tremendous amount of encouragement and guidance when I needed most. I am truly grateful for his strong support in my academic endeavours, and my first PhD first-authored publication.

Besides my honorary supervisors and mentors, I am also very grateful to have received continuous strong support, encouragement and assistance from the following individuals and groups during my PhD journey:

I. Associate Professor Teo Yik Ying, Vice-Dean (Research), SSHSPH, NUS.

II. Assistant Professor Hsu Li Yang, Consultant, Department of Medicine, Yong Loo Lin School of Medicine, NUS.

III. Dr. Agus Salim, Senior Lecturer, Faculty of Science, Technology and Engineering, La Trobe University, Australia.

IV. Dr. Thein Tun-Linn, Research Fellow, CDC, IIDE, TTSH, Singapore.

V. Dr. Anna Lindblom, Postdoctoral Fellow, Center for Molecular Medicine (CMM), Karolinska Institutet (KI), Karolinska University Hospital, Stockholm, Sweden

VI. Dr. Thomas Tolfvenstam, Clinician-Scientist, CMM, KI, Karolinska University Hospital, Stockholm, Sweden

VII. Professor Cameron Simmons, Oxford University Tropical Research Unit, Vietnam

VIII. Dr. Hoang Truong Long, Postdoctoral Fellow, Infectious Diseases Group, GIS.

IX. Dr. Eileen Png, Postdoctoral Fellow, Infectious Diseases Group, GIS.

X. Dr. Rajkumar Dorajoo, Postdoctoral Fellow, Human Genetics Group, GIS.

XI. STOP Dengue Team of CDC, TTSH. Special thanks to Ms. Linda Kay Lee and Ms. Soh Siew Hwa.

XII. Genotyping Team of GIS, Singapore. Special thanks to Mr. Meah Wee Yang and Mr. Wong Chang Hua.

XIII. Infectious Diseases Team of GIS, Singapore. Special thanks to Dr. Ling Ling, Ms. Pauline Aw, Mr. Ahmad Nazri Mohamed Naim and Mr. Louie Low.

XIV. To all dengue patients who have sincerely consented to our dengue research in Singapore and Vietnam.

XV. My previous supervisors Professor Wong Sek Man and Professor Ng Mah Lee and their team members for the continuous encouragement and guidance.

In addition, I am very thankful to have the financial support from NUS PhD scholarship, research funding from the STOP Dengue Translational and Clinical Research (TCR) Flagship Program, Singapore, the Wellcome Trust, United Kingdom and The Centre for Infectious Diseases Epidemiology and Research, SSHSPH.

Finally but most importantly, I am indebted to my beloved wife Ms. Ang Xiaoman, my newborn Vera Pang, my parents and in-laws for being extremely supportive, caring and understanding throughout these busy four years. Thank you so much for the support!

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SUMMARY

Dengue, the most rapidly spreading mosquito-borne viral infection, is prevalent in tropical and subtropical regions of the world and results in diverse clinical outcomes. It causes a significant disease burden globally as there is currently neither vaccine nor antiviral therapy. Treatment is largely supportive. In order to provide prompt treatment management, accurate early prognosis of dengue patients according to risk profiles and disease phase is critical. This would also enable more cost-effective resource management and future pharmaceutical interventions. Currently, the capability to identify adult dengue patients who are at higher risk of progressing into severe disease is still lacking, especially during early infection of less than 72-hour post fever. Even though the World Health Organization (WHO) have recently recommended using clinical warning signs during triage as guidance for strict observation and potential clinical interventions in hospital, many challenges remain in the clinical setting.

In the development of early prognostic tools, this thesis explored risk factors from three different perspectives of dengue; the genetic, molecular and the clinical profiles. In Chapter 3, genetics risk variants of MICB and PLCE1 genes were observed to affect the baseline risk of an individual towards developing dengue shock syndrome (DSS). The top common variant of MICB gene [rs3132468: meta-odds ratio (OR) =1.34; p-value=4.41×10^{-11}] was observed to be positively associated with DSS, while the top common variant of PLCE1 gene (rs3765524; meta-OR=0.80; p-value=3.08×10^{-10}) was likely to have a protective effect against DSS. In Chapter 4, dengue patients with diabetes and hypertension were observed to be twice more likely to develop dengue haemorrhagic fever (DHF) [adjusted odd ratios (AOR) =2.16; 95% confidence interval (CI) =1.18-3.96] as compared to patients without these pre-existing comorbidities. In

Chapter 5, RNA biomarkers CCL8, VPS13C with protein biomarkers uPAR or platelets count were proposed and independently validated as biomarkers for early stratification of patients who are at risk of progressing into severe disease, with sensitivity and specificity up to 96% and 54.6% respectively. In Chapter 6, neutrophil proportion, alanine aminotransferase and serum urea were proposed as a risk model for ICU triage at first presentation with sensitivity and specificity of about 88%. In addition, monocyte and lymphocyte proportions, blood pressure, and pulse rate were proposed as a risk model for ICU triage 24 hours prior to ICU admission with sensitivity and specificity of 88.9% and 78%, respectively.

The application of these early risk models will be beneficial in guiding dengue prevention policies, early dengue diagnosis and prognosis in the primary healthcare setting, as well as guiding early clinical and therapeutic management in tertiary healthcare setting, with the overall objective to reduce disease burden and cost in dengue endemic regions.

(437 words)

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LIST OF ABBREVIATIONS

AST- Aspartate aminotransferase

ALT- Alanine aminotransferase

DALYs- Disability-adjusted life years

DENV- Dengue virus

DENCO- Dengue and control study

DF- Dengue fever

DHF- Dengue haemorrhagic fever

DSS- Dengue shock syndrome

HLA- Human leucocyte antigen

ICU- Intensive care unit

IL- Interleukin

LTA- Lymphotoxin-alpha

MBL- Mannose-binding lectins

NPV- Negative predictive value

PPV- Positive predictive value

SD- Severe dengue

SNPs- single nucleotide polymorphisms

TGF- Transforming growth factor

TNF- Tumor necrosis factor

VDR- Vitamin D receptor

WHO- World Health Organization

WS- Warning signs

CHAPTER 1: BACKGROUND

1.1 OVERALL SUMMARY OF CURRENT CHALLENGES IN DENGUE

Dengue incidence has been increasing over the past decades, which is likely due to the increasing trend of globalization, urbanization, and global warming. Although extensive studies have been conducted in the past two decades focusing on host-virus interactions, the pathogenesis of dengue disease remains unclear. This is likely due to the complex interplay between the viral and host factors. Furthermore, the absence of a robust animal model of disease made the investigation of dengue pathogenesis even more challenging. Currently, there is no licensed vaccine or specific antiviral therapy available for the prevention or treatment of dengue. Vector control programmes have been inefficient and unsustainable over time. The current strategies applied to reduce burden are mainly close observation of patients for signs of bleeding and circulatory compromise, and providing early and appropriate supportive fluid management. Therefore, identification of patients who are susceptible to developing severe dengue disease or who warrant strict medical attention in hospital at the early phases of the disease is critical for proper clinical management (WHO, 2009, 2012). However, there were limitations in using the WHO 1997 and 2009 classifications to triage the increasing proportion of adults who are at risk of dengue severity. This is mainly due to the fact that the classifications were derived largely from pediatric cases, and have also been shown not be useful in the early phase of infection (less than 72 hours). Furthermore, clinical signs and symptoms among adults are likely to present late around one day before progression to severe disease, which makes any form of intervention very challenging.

Even though there have been reported risk factors that may be effective for identifying high risk individuals, these are often not generalizable to all countries of interest due to differences in host genetic background, predominant circulating viral strains, and geographical areas and public health priorities as well as standard of medical services available. Moreover, there have been proposals to look at dengue severity in a multi-factorial manner as each risk factor is unlikely to account for all the cases observed (Guzman & Kouri, 2008; Guzman et al., 1990).

The objectives of this thesis are to explore several complementary approaches to identifying susceptibility to severe dengue, namely the use of genetic, molecular, epidemiological, clinical risk stratification. We will start with a review of existing literature on the topic in Chapter 1, followed by a description of methods used in Chapter 2. We will then detail individual studies we conducted using the genetic (Chapter 3), epidemiological (Chapter 4), molecular (Chapter 5) and clinical approaches (Chapter 6) to risk stratification, followed by Chapter 7 which will discuss the applicability of the various approaches to different contexts for improving clinical management of dengue disease. We will also touch on the implications of our findings for understanding the causes of severe dengue, which could be the subject for future research.

1.2 EPIDEMIOLOGY AND BURDEN OF DENGUE INFECTION

Dengue, the fastest spreading vector-borne disease, is endemic in the tropical and subtropical regions of the world due to the high suitability of its environment for dengue transmission (Figure 1) by Aedes mosquitoes, with *Aedes aegypti* being the more efficient vector as compared to *Aedes albopictus*. Dengue infection is caused by dengue virus (DENV), which belongs to the family *Flaviviridae* and genus *flavivirus*. There are four antigenically distinct serotypes of dengue virus (DENV-1, DENV-2, DENV-3, DENV-4), which are usually co-circulating in these endemic regions. Rapid urbanization over the past decades has facilitated endemicity as dengue is predominantly found in semi-urban and urban areas (Simmons, Farrar, Nguyen v, & Wills, 2012).

1.2.1 Burden of dengue infection

The population at risk of dengue infection residing in these endemic regions is approximately 2.5 to 3.6 billion people (Murray, Quam, & Wilder-Smith, 2013), with about 50 million reported symptomatic dengue infections per year as estimated by the World Health Organization (WHO) (WHO, 2009). However, a recent work estimated 390 million infections per year, of which only 25% was estimated to be symptomatic infections (Bhatt et al., 2013). This estimate of symptomatic infections is twice as high as the estimate of the WHO, which highlights the increasing trend and the scale of underreporting of global burden due to dengue over the past decades. Nevertheless, it was also highlighted in the study that the determination of an accurate dengue burden will always be a challenge due to inadequate surveillance and reporting systems, as well as dengue misdiagnosis. In addition, the rapid expansion of dengue can be due to factors such as the dynamic changes of climate, globalization, travel, trade, socioeconomics, settlement and also viral evolution (Murray et al., 2013).

Approximately, 75% of the global burden (Figure 2) due to dengue was derived from the WHO South-East Asia and Western Pacific regions, where more than 70% of the population at risk are residing (Bhatt et al., 2013; WHO, 2009). The recent annual economic burden due to dengue in South-East Asia, as an example, was estimated to be US$950m (cost per capita of US$1.65) with 372 disability-adjusted life years (DALYs) per million inhabitants, which is higher than the burden due to other diseases of public health importance such as Japanese encephalitis, upper respiratory infections, and hepatitis B (Shepard, Undurraga, & Halasa, 2013). Furthermore, developed countries such as Singapore are more affected economically compared to developing countries such as Vietnam. Singapore had the highest cost per capita GDP (2010 US$) of US$14.99 (Shepard et al., 2013) due to dengue with a mean total cost of US$1,500 per dengue patient (Carrasco et al., 2011) compared to Vietnam, with the lowest cost per capital GDP of US$0.28 (Shepard et al., 2013) with a mean total cost of US$147.77 per dengue patient (P. T. Tam et al., 2012).

1.2.2 Epidemiology of dengue infections

Dengue infection is predominantly a pediatric disease in most parts of Southeast Asia and Western Pacific regions (WHO, 1997), where children less than 15 years of age are at higher risk of dengue infection with high DHF/DF ratio compared to adults (Guzman et al., 2002). However, over the years, there has been a gradual increase in the reported number of dengue infections among older children and young adults age 15 and above in Singapore (Goh, 1995; Ooi, Goh, & Chee Wang, 2003; Ooi, Goh, & Gubler, 2006), Vietnam (Anders et al., 2011), Thailand (Chareonsook, Foy, Teeraratkul, & Silarug, 1999; Pongsumpun, Yoksan, & Tan, 2002; Tantawichien, 2012; Wichmann et al., 2004), South China (Guo et al., 2014), Taiwan (M. S. Lee, Hwang, Chen, Lu, & Chen, 2006; C. C. Lin

et al., 2010) and Sri Lanka (Kularatne, Gawarammana, & Kumarasiri, 2005). Furthermore, this phenomena was also observed outside Southeast Asia in Hawaii (Effler et al., 2005), Cuba (Guzman et al., 2000; Pongsumpun et al., 2002) and Brazil (Guilarde et al., 2008; Souza et al., 2013). It was hypothesized that the increasing average age of dengue illness is due to a shift in the age structure of the population, and its impact on the force of infection (Cummings et al., 2009). While most dengue infections, particularly primary infections in young children, are mild or silent (Burke, Nisalak, Johnson, & Scott, 1988; Endy et al., 2002), infections in adults are more likely to be clinically symptomatic, resulting in a higher proportion of DF than DHF/DSS (Egger & Coleman, 2007; Ooi et al., 2003; Thai et al., 2010).

1.2.3 Clinical manifestations of dengue infection.

In addition, there were significant differences in the clinical manifestations between young children and adults (Tantawichien, 2012). In comparing adults and children at mean duration post fever prior to admission of 4.2 days, headache, nausea, myalgia/arthralgia, petechiae, clinical bleeding and severe liver involvement were significantly more common in adults, while cough and hepatomegaly were significantly more frequent in children (Thai et al., 2010; Wichmann et al., 2004). Moreover, haemoconcentration, thrombocytopenia, increased alanine aminotransferase, and longer prothrombin time were found to be significantly higher in adults than in children (Kittigul, Pitakarnjanakul, Sujirarat, & Siripanichgon, 2007; Souza et al., 2013; C. C. Wang et al., 2009)

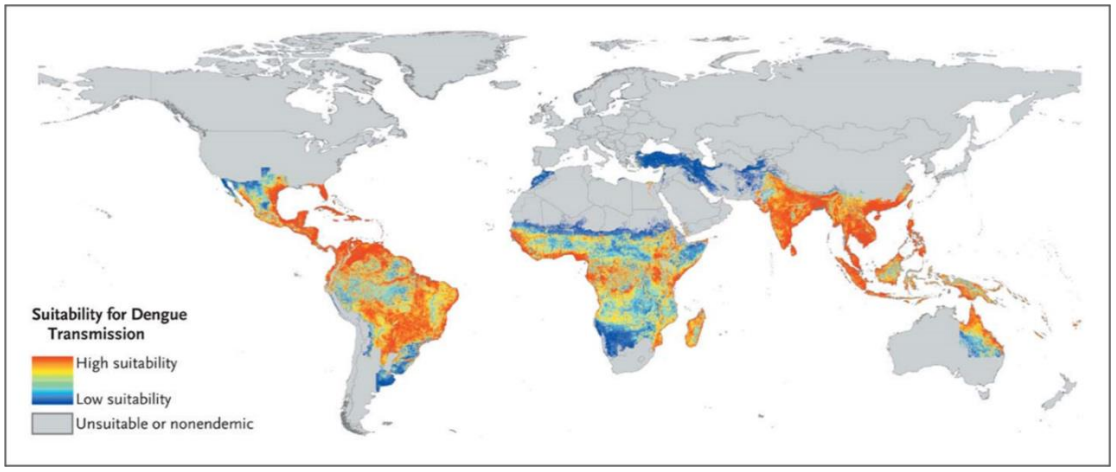

Figure1. Global Dengue Risk.
(Reproduced with permission from Simmons, Farrar et al. *NEJM* 2012, Copyright Massachusetts Medical Society)

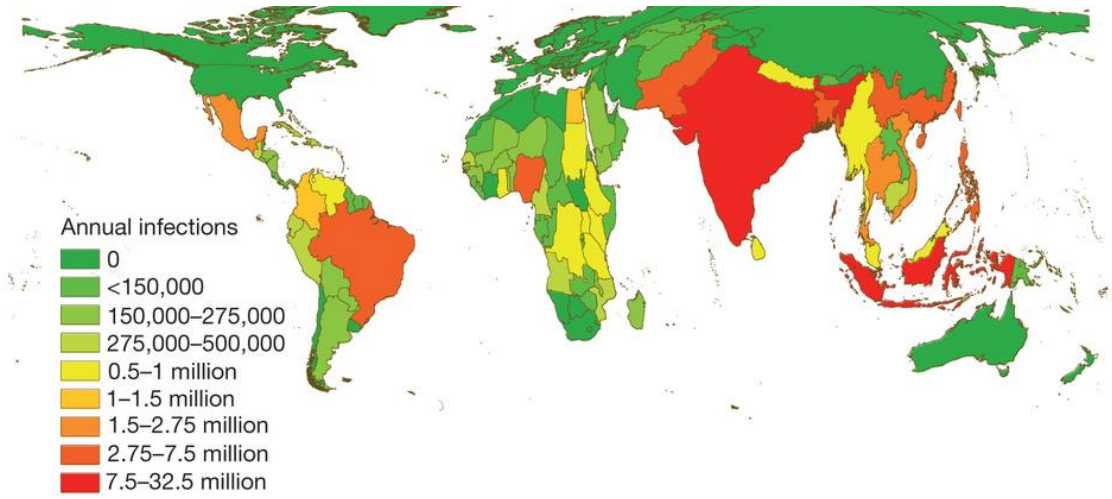

Figure 2. Global Dengue Burden.
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1.3 DENGUE PREVENTION, TREATMENTS AND THEIR CHALLENGES

1.3.1 Challenges of vector control.

Prevention is always better than cure. Currently, vector control is the main area of emphasis for prevention. Vector control measures have been effective in reducing populations of the main transmission vector, the mosquito *Aedes. aegypti* (Kay & Vu, 2005), but these have largely failed to slow the current dengue pandemic as a cost-effective and sustainable strategy in an urban endemic setting (Luz, Vanni, Medlock, Paltiel, & Galvani, 2011).

Control of dengue vectors relies on the removal of larval breeding containers, such as old tyres or flower vases or on insecticide spraying in homes. This approach has been used successfully in some locations, but is not sustainable (Gubler, 1989, 2002; Gubler & Clark, 1996; Hemingway, Beaty, Rowland, Scott, & Sharp, 2006; Rigau-Perez et al., 2002). In addition, continuous insecticide-based larval control with high efficacy has been shown to be counterproductive. This is because as resistance against insecticides increases while the population herd immunity decreases over time, it is likely to result in an increased magnitude of future epidemics (Luz et al., 2011).

One other potential of vector control is the use of wMel Wolbachia infection, introduced into *Aedes aegypti* from Drosophila melanogaster, which has been recently shown to have successfully invaded two natural *Aedes aegypti* populations in Australia and blocked transmission of DENV-2 in caged mosquitoes (Hoffmann et al., 2011; Walker et al., 2011). However, data on whether the population replacement has reduced the incidence of human dengue cases will take many years to assess and more studies would still be required to understand the potential impact on the natural ecology of releasing this

Wolbachia-infected mosquitoes on the long term basis as the mechanism behind this supercharged host protection remains elusive (LePage & Bordenstein, 2013).

1.3.2 Challenges of vaccine development

Currently, there is still no licensed vaccine yet as there are many clinical development challenges to develop an effective vaccine. Safe vaccination should generate durable immune responses to all four dengue serotypes due to the potential implication of antibody-dependent enhancement theory such as the phenomena of high immune reactogenicity, resulting in severe disease. Other challenges also include the lack of a suitable animal model and immunological correlates, the lack of a robust methodology to assess efficacy and the necessity to evaluate the vaccine in different populations at risk of disease, and to have sufficient dengue cases in the control group to statistically highlight efficacy against, ideally, all 4 serotypes (Wallace, Canouet, Garbes, & Wartel, 2013).

Nevertheless, there were many clinical trials ongoing (Webster, Farrar, & Rowland-Jones, 2009). However, concerns still remain on the potential reversion of attenuated strain to wild type, induction of antibody-dependent enhancement in individuals with pre-existing immunity, as well as the reduction of serotype-specific response due to over proportion of the fusion-loop-specific antibodies compared to the serotype-specific antibodies (K. Fink & Shi, 2014).

Currently, the most promising vaccine candidate is from Sanofi Pasteur, a recombinant, live, attenuated, tetravalent dengue vaccine (CYD-TDV),which is made up of recombinant infectious cDNA clones of yellow fever 17D vaccine strain as a backbone, substituting membrane precursor protein and envelope protein genes with those of dengue viruses (Guy et al., 2011). It has been shown to be immunogenic and safe in population of the South-east Asia regions (R. Z. Capeding et al., 2011; Lanata et al., 2012; Leo et al.,

2012) with efficacy estimates against DENV-1, 3, and 4, but not DENV-2, in a range consistent with the assumed overall efficacy of 70% after three injections, and these estimates were also significant after at least one vaccination (Sabchareon et al., 2012).

Similarly, the full evaluation of the phase 3 clinical trial showed consistently high efficacy against serotype 1, 3 and 4, but not for serotype 2 with efficacy of about 34.7% (M. R. Capeding et al., 2014; Hss et al., 2013), even though serotype 2 is one of the more predominant serotype in Asia such as in Singapore. Therefore, further studies would still be required to refine the potential vaccine for dengue prevention. As such, there is currently a stronger need to focus on the treatment strategies to minimize the burden of infection.

1.3.3 Challenges of anti-viral therapy.

Thus far, there are also no licensed antiviral drugs available against DENV infection as a specific treatment for dengue patients. Over the last decades, there were a number of antiviral therapies proposed and evaluated in clinical trials on humans. They were balapiravir (N. M. Nguyen et al., 2013), chloroquine (Tricou et al., 2010), corticosteroid (T. H. Nguyen et al., 2013; D. T. Tam et al., 2012), steroids (Sumarmo, Talogo, Asrin, Isnuhandojo, & Sahudi, 1982; Tassniyom, Vasanawathana, Chirawatkul, & Rojanasuphot, 1993), statins (Whitehorn, Van Vinh Chau, et al., 2012) and Celgosivir (Rathore et al., 2011).

1.3.4 Challenges of current clinical interventions.

Unfortunately, none turned out to be significantly effective in reducing viral load to prevent disease at the early phase of infection. Current treatment strategy remains largely supportive, which includes careful fluid management, bed rest, antipyretics, and analgesics

(Harris et al., 2003; Simmons et al., 2012; WHO, 2009). In addition, constant clinical monitoring of patients suspected of severe dengue, together with anticipatory and supportive care can help to reduce mortality rate (WHO, 2009). Intensive care is required for severely ill patients, including prompt intravenous fluids resuscitation, blood or plasma transfusion and medicines.

Prompt fluid resuscitation, either with isotonic crystalloid solutions or colloids, remains the mainstay of treatment to counteract massive plasma leakage (Dung et al., 1999; Ngo et al., 2001; Wills et al., 2005). Platelet transfusion has been routinely performed as part of management of dengue (L. Thomas et al., 2009), but it has been observed not to be effective in prevention of severe bleeding or shorten the bleeding period recently (Khan Assir et al., 2013). In addition, prophylactic platelet transfusion, which is thought to reduce the risk of hemorrhage, has been shown to be unsuccessful so far to prevent or to treat severe thrombocytopenia significantly, and to minimize the chance of severe disease progression (Kurukularatne, Dimatatac, Teo, Lye, & Leo, 2011; Lum, Abdel-Latif Mel, Goh, Chan, & Lam, 2003; Lye, Lee, Sun, & Leo, 2009).

However, there is still an existing large variation in clinical practice and a lack of stronger evidence for prophylactic platelets transfusion from large prospective controlled trials (Whitehorn, Rodriguez Roche, et al., 2012).

1.4 DENGUE CLASSIFICATIONS AND ITS LIMITATIONS

1.4.1 Dengue classifications

Dengue infection results in a wide spectrum of clinical manifestations from asymptomatic infection, undifferentiated fever, dengue fever (DF) to dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). Recommendations for the classification and management of DHF were developed based on key findings of pediatric dengue studies in Bangkok in the 1960s (Cohen & Halstead, 1966) and were further used as WHO guidelines in 1974, updated in 1986, 1994, and 1997 (WHO, 1997). Dengue fever is clinically defined as an acute febrile illness with two or more manifestations (headache, retro-orbital pain, myalgia, arthralgia, rash, haemorrhagic manifestations, or leucopenia) and occurrence at the same location and time as other confirmed cases of dengue fever (Figure 3).

The hallmark of DHF is the presence of hemorrhagic manifestations and evidence of plasma leakage (besides having fever lasting two to seven days and thrombocytopenia), which can lead to the loss of intravascular volume and circulatory insufficiency, known as hypovolemic shock or DSS and even death, if not treated timely. Early detection and management of severe disease are essential to prevent morbidity and mortality (WHO, 2009).

Therefore, an ideal dengue classification system should be one which facilitates early triage, and prognosis of patients, through early recognition of key warning signs/markers, guides clinical management, and is useful as an endpoint evaluation of potential

interventions, epidemiological surveillance, reporting and for comparisons among other dengue endemic countries.

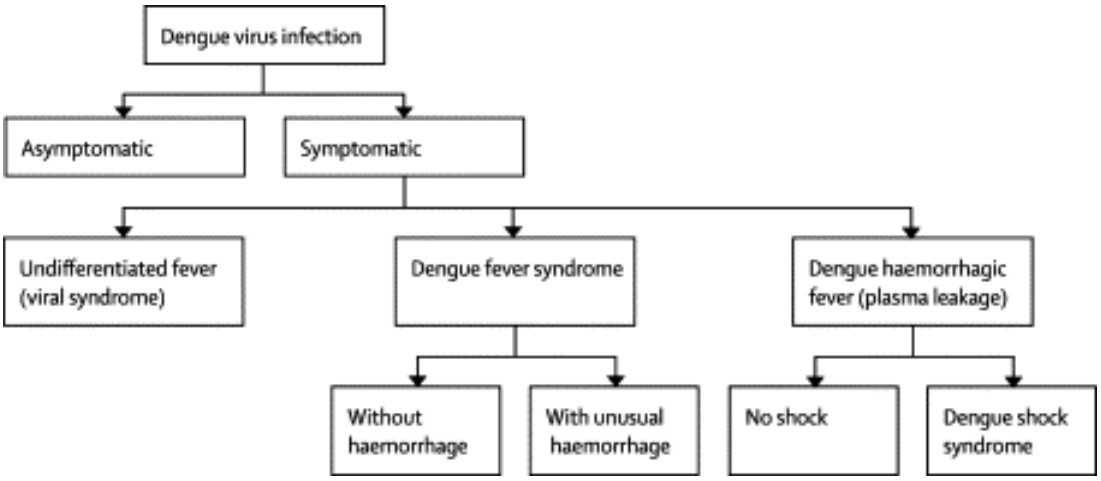


Figure 3.The WHO 1997 classification system.
(Reprinted from The Lancet, 368/9530, Deen JL, Harris E, Wills B et al., The WHO dengue classification and case definitions: time for a reassessment, 170-173, Copyright 2006, with permission from Elsevier.)

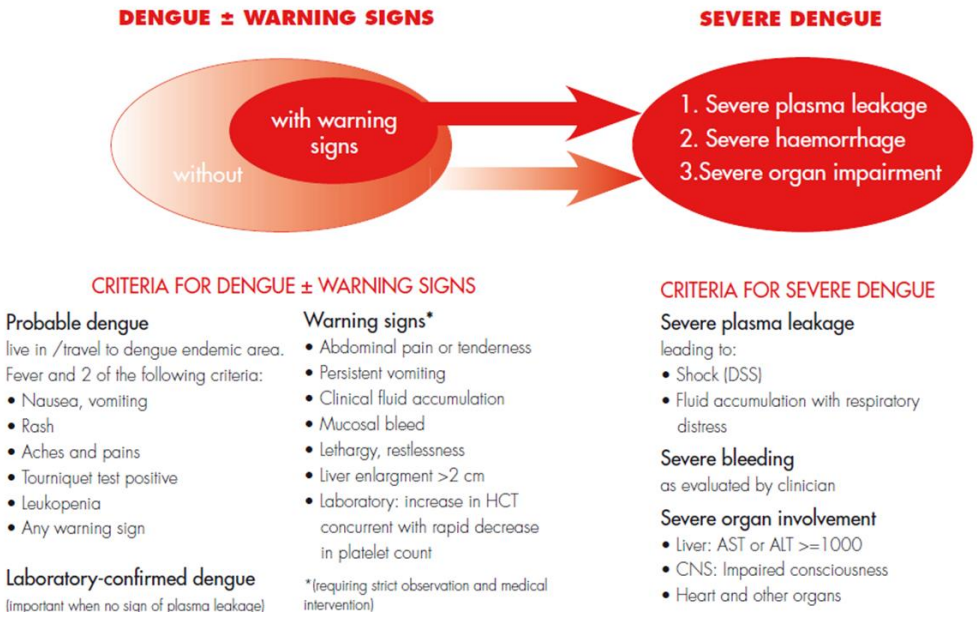


Figure 4. The WHO 2009 classification of severity.
(Reprinted with permission from the WHO Dengue Diagnosis, Treatment, Prevention and Control 2009)

1.4.2 Challenges of current classifications

Over the years, there were increasing reports of the challenges and difficulties in applying the WHO 1997 classification guidelines (Balmaseda et al., 2005; Bandyopadhyay, Lum, & Kroeger, 2006; Deen et al., 2006; Hadinegoro, 2012; Phuong et al., 2004; Rigau-Perez, 2006; Setiati et al., 2007; Srikiatkhachorn et al., 2010) and proposal was made to review and develop a more evidence-based clinical guideline (Horstick et al., 2012; Santamaria et al., 2009). Briefly, this was mainly due to the fact that it was developed based on substantial clinical observations of pediatric dengue cases in Thailand only, which may not be generalizable or applicable in other countries as dengue increasingly spread to new geographical areas and started affecting more adults (aged more than 15 years old) in the past decade (Horstick et al., 2012; Rahman et al., 2002).

In addition, the requirement for repetition of clinical tests impose substantial burden to endemic countries with limited resources, and the recommended usage of the tourniquet test had limited effectiveness in stratifying DF and DHF (Horstick et al., 2012). Furthermore, the emphasis on hemorrhage instead on plasma leakage, and the lack of emphasis on organ failure in the WHO 1997 classification had greatly hindered the classification of patients with severe dengue, which is also a significant cause of dengue-related morbidity and fatality (Deen et al., 2006; Horstick et al., 2012; Phuong et al., 2004; Rigau-Perez, 2006). These limitations had led to a clinical dilemma of clinicians not being able to classify substantial number of severe patients as DHF, particularly in adults as they failed to satisfy the four criteria (Balmaseda et al., 2005; Horstick et al., 2012). In view of these limitations, the WHO Dengue Scientific Working Group recommended additional research into dengue diagnostics and triaging of patients into non-severe and severe groups to optimize clinical management in 2006 (Barniol et al., 2011; Horstick et al., 2012). Subsequently, WHO introduced a new classification system, with guidance from

the results of DENCO (Alexander et al., 2011), to guide clinicians in triaging dengue severity into two groups to facilitate early and appropriate clinical management. The two groups were dengue with/without warning signs and severe dengue. These warning signs were proposed as indicators for disease progression towards severe dengue. They were mucosal bleeding, abdominal pain, persistent vomiting, lethargy, clinical fluid accumulation and rapid decrease in platelet count (WHO, 2009). The WHO recommended dengue patients who presented with warning signs should be hospitalized for observation so as to provide prompt appropriate clinical management to minimize progression into severe disease (WHO, 2009). Evaluation of the revised WHO 2009 dengue classification has shown high acceptance and applicability (Barniol et al., 2011), higher sensitivity and specificity of classifying pediatric severe dengue cases (Basuki et al., 2010; Narvaez et al., 2011; Prasad, Kumar, Jain, & Kumar, 2013) and higher sensitivity of classifying adult dengue cases with higher proportion of cases requiring hospitalization as compared to that using the WHO 1997 classification (Gan et al., 2013; T. L. Thein, Gan, Lye, Yung, & Leo, 2013; Tsai, Lee, Lee, Yang, & Liu, 2013).

However, there is a need for more precise definition of warning signs to enable optimal triaging for more accurate identification of patients who require hospitalization as opposed to those who can be treated as outpatients. It was observed that adult dengue patients without any warning signs can be treated as outpatients, and no single warning sign can independently predict disease progression (Leo et al., 2013; T. L. Thein, Gan, et al., 2013; Tsai et al., 2013). Furthermore, most of these warning signs appeared at median 4th day of illness and only up to 50% of the severe patients had warning signs preceding occurrence of DHF or severe dengue (T. L. Thein, Gan, et al., 2013), or one day prior to the development of severe illness/ requirement of intervention (Alexander et al., 2011), and this narrow window makes any form of intervention challenging, particularly when

appropriate healthcare facilities are not accessible or available near their place of residences. Alternative means of stratifying high risk patients during triage into the various dengue severity levels for prevention and prompt clinical management would be to explore epidemiological, genetic, molecular and clinical risk factors as potential early markers for severe dengue disease.

1.5.RISK FACTORS OF DENGUE SEVERITY

1.5.1. Epidemiological risk factors of dengue severity among children

Unravelling of risk factors is challenging but important for screening and prevention as well as for implementation of vaccines and therapeutics when they are available. General epidemiological risk factors of DHF/DSS among children were dengue-serotype 2 (Guzman et al., 1990; S. Thein et al., 1997; Vaughn et al., 2000), dengue-serotype 3 (Guilarde et al., 2008; Harris et al., 2000), Asian genotype (Watts et al., 1999), prior dengue infections (Bravo, Guzman, & Kouri, 1987; Burke et al., 1988; Guzman et al., 1984; Vaughn et al., 2000), age less than 15 years old (Anders et al., 2011; Guzman et al., 2002; Ooi et al., 2003), white females (Anders et al., 2011; Guzman et al., 1984), and obesity (S. Kalayanarooj & Nimmannitya, 2005; Pichainarong, Mongkalangoon, Kalayanarooj, & Chaveepojnkamjorn, 2006). Epidemiological risk factors of mortality among children were age under five (Anders et al., 2011), female (Anders et al., 2011), rural residents (Anders et al., 2011).

1.5.2 Epidemiological risk factors of dengue severity among adults.

Even though there have been efforts in unravelling the risk factors among adults, usually defined as age greater than 15 years old, the findings were still limited for use in early prevention, triage and clinical management. The risk factors among adults were age greater than 65 years (M. S. Lee et al., 2006), low income (Blanton et al., 2008) and having co-morbidities. Co-morbidities were reported as risk factors for DHF in a number of studies from dengue endemic countries. These co-morbidities included sickle cell anaemia(Bravo et al., 1987), asthma (Bravo et al., 1987; Cunha et al., 1999; D. Gonzalez

et al., 2005), hypertension (Cunha et al., 1999; Figueiredo et al., 2010; M. S. Lee et al., 2006), uremia (M. S. Lee et al., 2006), allergies treated with corticosteroid (Figueiredo et al., 2010) and diabetes mellitus (Bravo et al., 1987; Cunha et al., 1999; Figueiredo et al., 2010; M. S. Lee et al., 2006).

However, these co-morbidities may not be generalized to all populations and epidemics of all dengue serotypes. Furthermore, most of these risk factors were identified from univariate analysis (Bravo et al., 1987; Cunha et al., 1999; D. Gonzalez et al., 2005) instead of multivariate analysis whichtake into account of potential confounders (Figueiredo et al., 2010; M. S. Lee et al., 2006). Increasingly, there were more evidence of substantial risk of death among adults whose age is greater than 55 years old (Leo et al., 2011; C. H. Lin et al., 2012; Ong, Sandar, Chen, & Sin, 2007). These may be due to the fact that elderly dengue patients were at higher risk of DHF, SD and hospital-acquired infections (Low et al., 2011; Rowe et al., 2014).

Furthermore, there are several challenges in reducing the dengue burden in the elderly. First, there is a lack of evidence-based clinical management protocols for managing elderly patients. It is especially important if fluid replacement therapy, which was mainly used on paediatric patients to prevent shock, is not monitored carefully, it could result in fluid overload and death. The situation would be more complicated particularly for those who have pre-existing or undiagnosed cardiovascular disease (Low & Ooi, 2013). Second, it is clinically challenging to diagnose dengue-infected elderly as they may present atypically as compared to younger dengue infected adults, with the elderly being more likely to have hepatomegaly and malaise/lethargy (C. C. Lee, Hsu, Chang, Hong, & Ko, 2013; Low et al., 2011; Rowe et al., 2014). Nevertheless, it has been proposed that elderly with fever and leukopenia be tested for dengue, even in the absence of other symptoms as

this combination can achieve a sensitivity of 82% and specificity of 87% (Low et al., 2011).

1.5.3 Genetic risk factors of dengue severity

Genetic risk factors include single nucleotide polymorphisms (SNPs), gene copy number variants and genetic deletions. These subtle changes might have important consequences for susceptibility to disease (Casanova & Abel, 2007). Indirect evidence implicating host genetic background in predisposing to DHF/DSS has been reported in Cuban dengue epidemics whereby a reduced risk for DHF/DSS was observed in those with an African ancestry compared to those with European ancestry (Blanton et al., 2008; Bravo et al., 1987; D. Gonzalez et al., 2005; Guzman, 2005; Guzman et al., 1999; Guzman & Kouri, 2008; Guzman et al., 2002; Sierra et al., 2007). These Cuban observations are of significant epidemiological interest, as the differences in susceptibility to DHF among racial groups in Cuba coincide with the low susceptibility reported in African and Black Caribbean populations (Halstead et al., 2001; Saluzzo, Cornet, Adam, Eyraud, & Digoutte, 1986).

Genetic studies in Thailand, Cuba, Mexico, Sri Lanka, Malaysia and Vietnam have also reported associations between certain Class I HLA alleles and DHF (Appanna, Ponnampalavanar, Lum Chai See, & Sekaran, 2010; Chiewsilp, Scott, & Bhamarapravati, 1981; LaFleur et al., 2002; Loke et al., 2001; Malavige et al., 2011; Nguyen et al., 2008; Stephens et al., 2002). Cuban studies showed that HLA-B14 and A-29 are negatively correlated with DHF risk and HLA-A1 and B-blank were positively associated with severe disease. Mexican studies showed that HLA-DRB1*04 is an important resistance allele to DHF and its high frequency in Mexican population could explain at least partly the atypical course of DHF in that country (LaFleur et al., 2002). In Vietnam, HLA-

DRB1*0901 (HLA Class II) was generally found to be a protective allele against the severe form of dengue, while HLA-A*24 (HLA Class I) was found to be an enhancing allele (Nguyen et al., 2008). Furthermore, a recent study suggested that HLA-DRB1*07/*15 genotype in combination with TNF polymorphisms influence progression to DHF (Alagarasu et al., 2013).

Studies carried out in different populations have shown the association of genetic variations in the CD209 (Sakuntabhai et al., 2005), vitamin D receptor (VDR) (Alagarasu et al., 2012; Loke et al., 2002), tumor necrosis factor (TNF) (Fernandez-Mestre, Gendzekhadze, Rivas-Vetencourt, & Layrisse, 2004; Perez et al., 2010; Vejbaesya et al., 2009), transforming growth factor-beta1 (TGF-β1) (Perez et al., 2010), interleukin-10 (IL10) (Perez et al., 2010), lymphotoxin-alpha (LTA) (Vejbaesya et al., 2009) and FCGR2A (Loke et al., 2002) genes with DHF. Interestingly, wild type AA mannose-binding lectin 2 (MBL2) genotype was observed to augment susceptibility for the risk of manifesting dengue thrombocytopenia (Acioli-Santos et al., 2008).

Though there may be a fair amount of research on the implication of host genetics on dengue severity, it is still very limited as most of these studies were based on candidate-gene approach with small numbers of subjects (less than a thousand of patients) involved, and mostly it is hard to replicate the findings. Furthermore, this approach is strategically-biased as it does not explore other genetic regions of the whole human genome due to their very limited efficiency and statistical power. An assessment of the whole human genome for these susceptibility or resistance polymorphisms in different populations at risk (Chaturvedi, Nagar, & Shrivastava, 2006; Coffey et al., 2009; Stephens, 2010)using whole genome chip would minimize selection biases and is highly established and favorable for a more comprehensive understanding of the role of host genetics on dengue severity of a specific population at risk(Khor & Hibberd, 2012).

1.5.4 Clinical risk factors of dengue severity

Clinical risk factors for death in children were severe bleeding (hemoptysis), epistaxis and persistent vomiting during hospitalization (Branco et al., 2014). Whereas, in adults, clinical risk factors observed were hematemesis and melena during the acute phase in Mexico (Navarrete-Espinosa, Gomez-Dantes, Celis-Quintal, & Vazquez-Martinez, 2005), pre-fatal (48 hour before fatality) profound thrombocytopenia and leukocytosis in Taiwan (I. K. Lee, Liu, & Yang, 2012), absence of myalgia, leukocytosis (T. L. Thein, Leo, et al., 2013) and tachycardia on admission in Singapore (Ong et al., 2007). In addition, cases of gastrointestinal bleeding, ascites, pericardial and/or pleural effusion, hepatomegaly, hypotension and shock were proposed to have higher risk of progression to death in Brazil (Cavalcanti et al., 2010), and Acute Physiology and Chronic Health Evaluation (APACHE) II may predict higher risk for death among dengue patients admitted to intensive care unit (ICU) in India (Juneja et al., 2011).

Clinical risk factors of DHF/DSS of children were thrombocytopenia and bleeding (Srikiatkhachorn et al., 2010), spontaneous bleeding, hepatomegaly, signs of capillary leakage like ascites and pleural effusion (Gupta et al., 2011), and hemoconcentration of more than 22% from baseline hematocrit (Tantracheewathorn & Tantracheewathorn, 2007), whereas for adults the factors were bleeding, particularly mucosal bleeding (M. S. Lee et al., 2006; V. J. Lee et al., 2008; Rowe et al., 2014; Vasanwala et al., 2014), abdominal pain and persistent vomiting (T. L. Thein, Gan, et al., 2013).

1.5.5 Laboratory and molecular risk factors of dengue severity.

Laboratory and molecular risk factors for DHF/DSS of children were higher viral load than DF children (Endy et al., 2004; Vaughn et al., 2000), lower platelet count and higher AST level than DF patients (S. Kalayanarooj et al., 1997), lower WBC count, percent

monocytes, platelet count, and hematocrit level, but higher neutrophil and AST level than non-DSS children (Potts et al., 2010), sTNRr80, IFN-γ, sCD8, and sIL2R (Green, Vaughn, Kalayanarooj, Nimmannitya, Suntayakorn, Nisalak, Lew, et al., 1999), sTNRr75 (Bethell et al., 1998), free sNS1 (Libraty, Young, et al., 2002), sVCAM-1 (Murgue, Cassar, & Deparis, 2001) and IL-10 (Green, Vaughn, Kalayanarooj, Nimmannitya, Suntayakorn, Nisalak, Rothman, et al., 1999).

Whereas, laboratory and molecular risk factors for DHF/DSS relevant to dengue-infected adults were higher urine protein creatinine ratio (UPCR), platelet count, serum hematocrit, serum protein (Vasanwala et al., 2011; Vasanwala et al., 2014), interferon-gamma, interleukin-17, fibroblast growth factor, RANTES, IP-10, serum amyloid A2 (SAA) and haptoglobin (HPT) (Y. Kumar et al., 2012), lower lymphocyte proportion and total protein and higher urea (V. J. Lee et al., 2008), CD3, 4, 5, 8 (Fadilah et al., 1999), AST and ALT (Guilarde et al., 2008; L. K. Lee et al., 2012; W. K. Wang et al., 2006), platelet-associated IgM (Saito et al., 2004), C5a, sIL-2R and viral load (W. K. Wang et al., 2006), soluble thrombomodulin and VEGF (Butthep et al., 2006; Del Moral-Hernandez et al., 2014; Seet, Chow, Quek, Chan, & Lim, 2009; Wills et al., 2002). Furthermore, a study which defined severity as death and intensive care admission proposed using four laboratory criteria [creatinine (>140 mmol/L), free bilirubin (>18 umol/L), amylase (>220IU/L) and platelets (<45,000/mm3)] to triage adult dengue patients for closer monitoring and hospitalization requirement (Bouldouyre, Baumann, Berlioz-Arthaud, Chungue, & Lacassin, 2006).

In addition, laboratory and molecular risk factors of hospitalization due to thrombocytopenia at early febrile phase of dengue infection of less than 72hr were higher viral load, lower platelet counts, white blood cells, lymphocyte count and neutrophil counts (Low et al., 2011; Tanner et al., 2008), presence of IgG (Tanner et al., 2008),

higher proportion of chemokines CCL2 (MCP-1), CCL8 (MCP-2), CXCL10 (IP-10) and CCL3 (MIP-1α), antimicrobial peptide β-defensin 1 (DEFB1), desmosome/intermediate junction component plakoglobin (JUP) and a microRNA 147 (NMES1) (Tolfvenstam et al., 2011).

Lastly, it is important to note that some of these laboratory and molecular factors were measured at the point of severity, and hence, were not prognostic biomarkers. Laboratory and molecular factors which were assessed at the point of admission or before the development of severe dengue conditions were evaluated for their prognostic performance as triage biomarker as listed in Table 1.

Table 1. Significant risk factors of dengue severity with potential clinical prognostic impact.

Variables	Severity	Subjects Type	Disease Phase	Country	Sens (%)	Spec (%)	PPV (%)	NPV (%)	Reference
sTNFR80b cut-off 1.6 ng/mL	DHF	Children	2 days before fever abates	Thailand	67	80	66	69	(Green, Vaughn, Kalayanarooj, Nimmannitya, Suntayakorn, Nisalak, Lew, et al., 1999)
sTNF75 > 55pg/ml	DSS	Children: Median 6 yrs old	4 days post symptom onset	Vietnam	93	34	27	95	(Bethell et al., 1998)
sNS1 level ≥600 ng/mL	DHF	Children: Median 9.5 yrs old	1-3 days post symptom onset	Thailand	72	79	-	-	(Libraty, Young, et al., 2002)
WBC count (≤8500), percent monocytes (≤9%), platelet count (≤160,200), and hematocrit achieved (≤40)	DSS	Children: Mean age 8.5 yrs old	Median 2 days post illness onset	Thailand	97	48			(Potts et al., 2010)

WBC count (<13700), percent neutrophils (>68%), AST (>50), platelet count (≤282,000), and age (>6.75)	DSS/ pleural effusion index >15				99	44			
sTM (8.8-21.1ng/ml)	DHF	Adult: Median 25 yrs old	4 days post illness	Mexico	94.1	98.6	-	-	(Del Moral-Hernandez et al., 2014)
VEGF (281.4-1047pg/mL)					55.8	80	-	-	
Dengue viral load ≥7.5 log RNA copies/ml	DHF	Adult: Median 51 yrs old	1 day before fever abates	Taiwan	-	-	71	94	(W. K. Wang et al., 2006)
PAIgM > 20ng/10^7 platelets	DHF	Young Adults: Median 18.2 yrs old	Median 5.9 days post illness onset	Philippines	48.6	92.1	-	-	(Saito et al., 2004)
UPCR cutoff 29mg/mmol	DHF	Adults: Median 34 yrs old	Median 6 days post fever onset	Singapore	76	77	-	-	(Vasanwala et al., 2014)
UPCR, WBC & platelet count, serum hematocrit and protein, bleeding					92	80	-	-	
Bleeding, lymphopenia, serum protein and urea (Cutoff P=-2.9)	DHF	Adults: Median 32 yrs old	Median 5 days post illness	Singapore	83	83.5	18	99	(V. J. Lee et al., 2008)
Cutoff P=-5.1					97.6	60.2	10	>99	
Independent Validation	DHF	Adults:	Median 6	Singapore	94	17	16	94	(T. L. Thein, Leo,

Bleeding, lymphopenia, serum protein and urea (Cutoff P=-5.1)		Median 35 yrs old	days post illness						Lee, Sun, & Lye, 2011)
Bleeding, serum protein and urea (decision tree)	DHF	Adults: Median 34 yrs old	Median 5 days post illness	Singapore	100	46	7.5	100	(V. J. Lee, Lye, Sun, & Leo, 2009)
Independent Validation Bleeding, serum protein and urea (decision tree)	DHF	Adults: Median 35 yrs old	Median 6 days post illness	Singapore	99	12	16	99	(T. L. Thein et al., 2011)
AST/ ALT	DHF	Adults: Median 34 yrs old	Median 4 days post illness	Singapore	50%	40%	-	-	(L. K. Lee et al., 2012)
Platelet count, viral Ct, Den IgG (decision tree)	platelet count 50,000 /mm^3 or less	Adults: Median 40 yrs old	<72hr post fever	Singapore	78.2	80.2			(Tanner et al., 2008)
Any number of seven warning signs	DHF	Adults: Median 34 yrs old	Median 4 days post illness	Singapore	87	18	30	77	(T. L. Thein, Gan, et al., 2013) Validation in (Leo et al., 2013)
Abdominal pain/ tenderness					29	73	17	85	
Persistent vomiting					6	93	16	82	
Hepatomegaly					1	99	20	81	

Hematocrit rise and rapid platelet count drop					9	92	17	83	
Clinical fluid accumulation					2	98	18	83	
Mucosal bleeding					42	88	31	93	
Lethargy					33	55	28	61	
Any number of seven warning signs	Severe Dengue				96	18	15	96	
Abdominal pain/ tenderness					21	72	9	87	
Persistent vomiting					8	93	18	85	
Hepatomegaly					0	99	6	84	
Hematocrit rise and rapid platelet count drop					5	94	9	89	
Clinical fluid accumulation					2	98	16	87	
Mucosal bleeding					17	82	10	89	
Lethargy					34	56	17	76	
Creatinine (>140 mmol/L), free bilirubin (>18 umol/L), amylase (>220IU/L) and platelets (<45,000/mm^3)	Death/ ICU	Adults: Mean 47 yrs old	Mean 7.9 days	New Caledonia (South Pacific)	84	94	29.8	96.1	(Bouldouyre et al., 2006)

1.6. SIGNIFICANCE OF THESIS

Based on the paradigm of disease progression as illustrated in Figure 5, there is a significant increase in disease burden and cost as the disease progresses from pre-clinical to the late clinical phase. This reinforces the importance of prevention, screening and early triage as the way forward to reduce disease burden due to dengue severity. Dengue severity can be defined as a spectrum of clinical manifestations from non-severe (i.e. DF, Dengue with or without warning signs) to severe classifications (i.e. DHF/DSS/Severe Dengue). Dengue severity can also be defined based on clinical interventions such as the requirement for strict observation in hospital based on the WHO 2009 recommendation and the requirement for intensive care unit admission. Much focus has been on looking at the risk factors of dengue severity among children in the past two decades as dengue was primarily a pediatric disease at the time.

As the epidemiology of dengue changes, there is an increasing trend of adults being affected by dengue, and there have been limited studies on the risk factors of dengue severity in adults, hence, the lack of simple but effective early predictors that can reliably predict dengue severity in adults at early phase of infection. In addition, these current risk factors may not be generalizable to all unique populations, and epidemics. Furthermore, most of the current studies were likely to be case series studies or univariate analyses, which provide less convincing evidence for causal inference. As a result, these may either lead to over-hospitalization, or under-prognosis, particularly in resource-limited settings. Both scenarios increase the public health burden and cost, which would be detrimental to the country's economy overtime. Therefore, the discovery of risk factors may provide meaningful policies and guidance on dengue prevention, early screening and triaging of high risk patients who require appropriate clinical interventions. These findings may also

further provide insights into the development and management of clinical trial protocols, as well as distribution and usage strategy and recommendations of vaccine and anti-viral therapy, when made available in future.

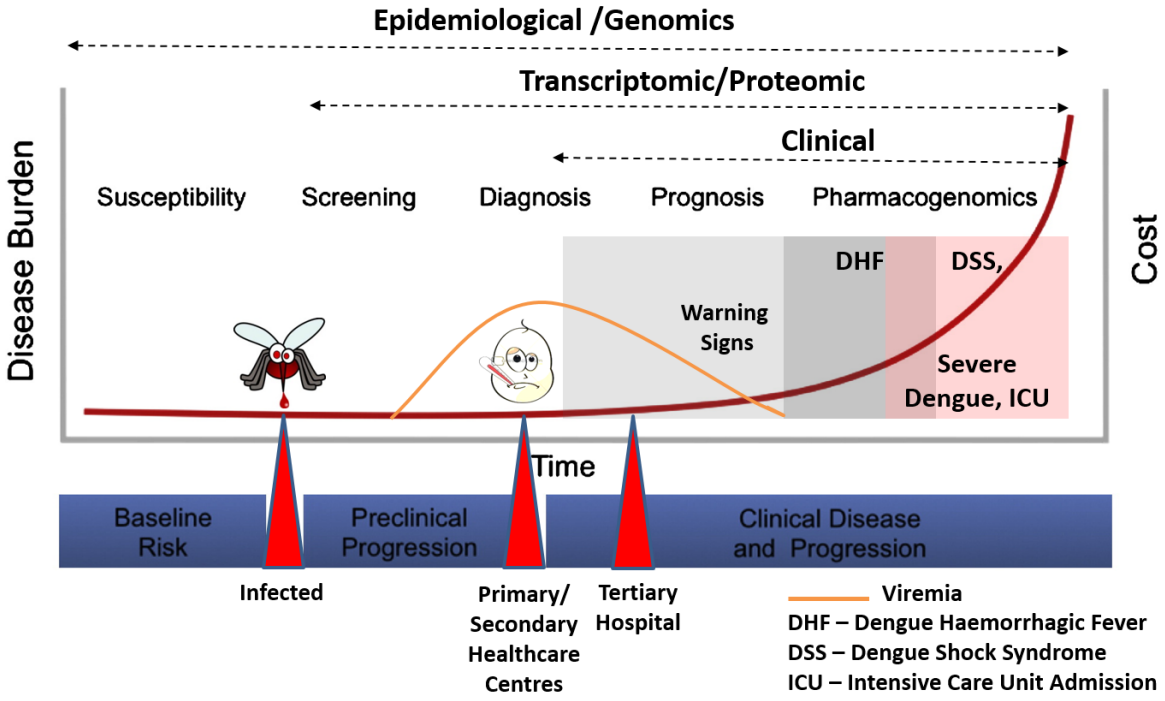


Figure 5. Diagram of the course of dengue disease over time and the application of epidemiological, genetic, molecular and clinical risk factors to refine prediction risk of developing disease severity, for screening, diagnosis, prognosis and therapeutic selection.
(Adapted from Translational Research 154/6, Ginsburg GS and Willard, Genomic and personalized medicine: foundations and applications, 277-286, Copyright 2009, with permission from Elsevier.)

1.7. AIMS OF THESIS

With the recent changing epidemiology of dengue disease, with increasingly more adults infected by dengue compared to a decade ago, it would be critical to understand the risk factors involved in the progression of dengue severity to guide prevention and control strategies, and to have effective triage tools to stratify these potentially, high risk patients at the early phase of infection. Triaging of dengue disease severity early and effectively allows the appropriate level of medical care and interventions such as resuscitative measures, fluid management, anti-viral (when available) and vaccine (when available, in the pre-infection phase to individuals susceptible to severe dengue) to be administered at an appropriate time point so as to reduce dengue progression. As such, the aims of the thesis are to **explore clinical, genetic and molecular risk factors/biomarkers of dengue disease severity** and to **propose early prognostic models of adult dengue disease severity,** ideally, simultaneously with dengue diagnosis for early and appropriate clinical management of adult patients.

A total of four studies were performed to address the aims of the thesis, covering relevant risk factors from pre-infection, post-infection to pre-ICU admission (Figure 6). **Study I** is focused on unraveling the **human genetic risk factors** of dengue disease severity that predispose dengue patients to severe dengue disease. **Study II** is focused on unraveling the **epidemiological risk factors** that are associated with higher risk of severe dengue disease. As such, **Study I and II** aim to explore the baseline risk factors before dengue infection as well as at the primary care level to guide prevention and control measures against progression to severe disease (Figure 6). **Study III** is focused on unraveling **molecular risk factors** at early post-infection as potential early warning signs of severe disease progression. **Study IV** is focused on unraveling **clinical and laboratory risk factors** of dengue disease severity at earliest presentation to the hospital. Both **Study**

III and IV hence aim to explore early prognostic tools to guide clinicians in primary care (study III) and tertiary care (study IV) on stratifying patients who are likely to develop severe dengue disease for closer monitoring in hospital and appropriate clinical management to reduce burden.

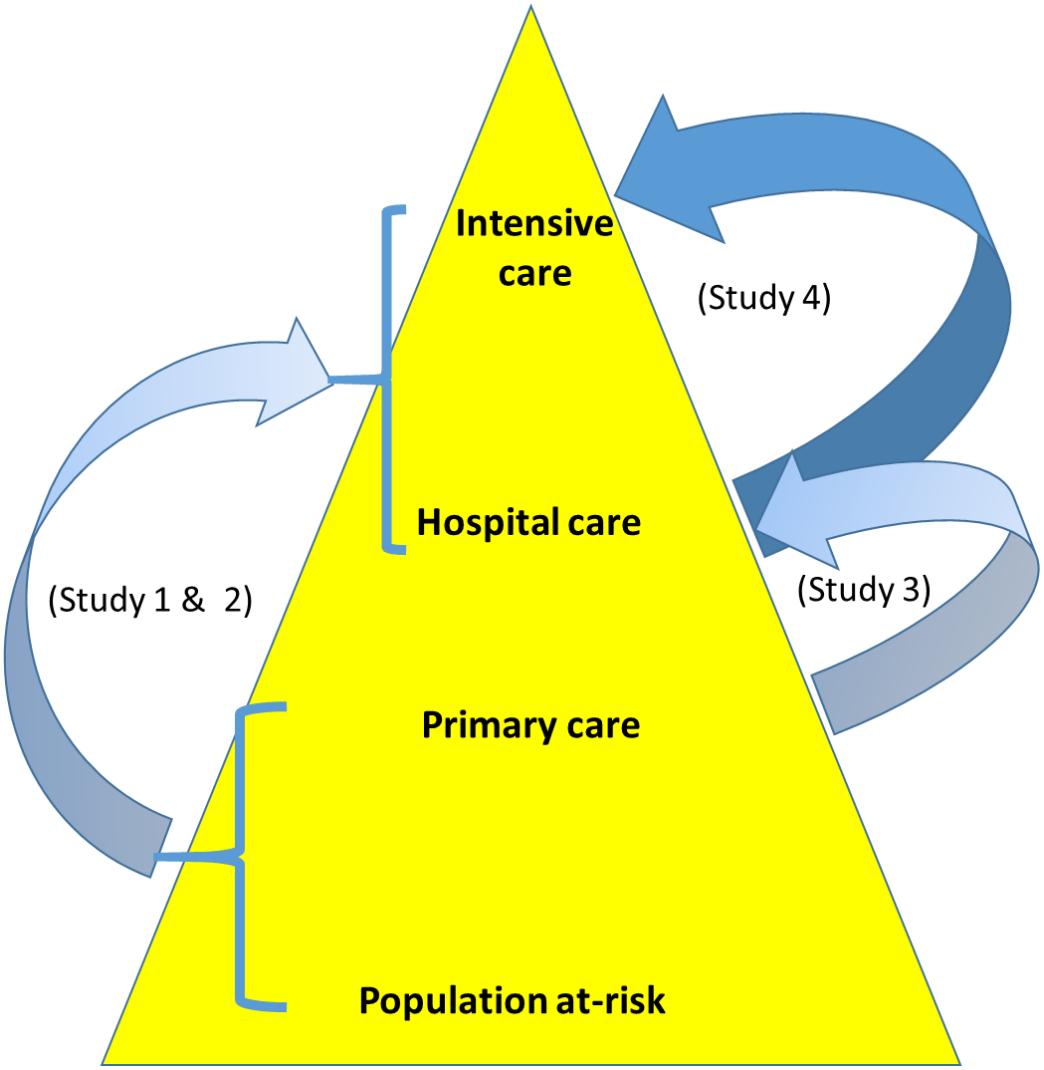


Figure 6. Diagram of the pyramid of dengue severity.

CHAPTER 2: METHODOLOGY

2.1. THE INTEGRATIVE APPROACH WITH GENOMIC MEDICINE

Disease progression and severity are mainly outcomes of the dysfunctional regulation and expression of genomic molecules, via complex and dynamic interactions, resulting in disruption of the steady-state of a host system. (Ginsburg & Willard, 2009). Genomic medicine, which is the use of information from genomes and their derivatives (RNA and proteins) to guide medical decision making—is a key component of stratified medicine, which is a rapidly advancing field of health care that is informed by each person's unique family history, clinical, genetic, molecular, and environmental information. The fundamentals of genomic and stratified medicine will require the development, standardization, and integration of this important information into health systems and clinical workflows. With the advent of the next generation technologies, more novel genomic information could be derived, to enable a paradigm shift to a comprehensive approach that will redefine individual risks and guide clinical management and decision making. All of these could form the basis for a more informed and effective approach to patient care. Therefore, current research studies involving the integration of genetic, epidemiological, clinical, and molecular perspectives of dengue disease, could serve as the way forward to improve the public health system in the near future.

2.2.DENGUE DIAGNOSIS

Dengue diagnosis was established by expert clinicians, following the World Health Organization (WHO) 1997 (WHO, 1997), 2009 (WHO, 2009) criteria or both, depending on the hypothesis of each study, unless stated otherwise in the specific study. Based on the WHO 1997 criteria, the diagnosis for dengue fever includes fever, laboratory diagnosis and any two of the following signs and symptoms: headache, retro-orbital pain, myalgia (muscle pain), arthralgia (bone or joint pain), rash, leukopenia, and haemorrhagic manifestations. Based on the WHO 2009 criteria, the diagnosis for dengue fever includes living in or travelling to dengue endemic areas, having fever and two of the following signs and symptoms: nausea or vomiting, rash, aches and pains, and leukopenia with laboratory- confirmed dengue test. The tourniquet test was not performed. Laboratory diagnosis of dengue virus infection was defined as either polymerase chain reaction (PCR) assay and/ or Dengue Duo IgM & IgG Rapid Strip Test (Panbio Diagnostic), unless specified otherwise in the respective studies. Different genetic backgrounds and exposures such as demographics, pre-existing clinical conditions and different dominant circulating virus serotypes may have different implications on disease severity of the host. As such, these are addressed as critically as possible in the design and analysis phase of the studies.

2.3. THE WHO 1997 AND 2009 CLASSIFICATIONS OF DENGUE INFECTION

Based on the WHO 1997 classification, diagnosis of dengue haemorrhagic fever (DHF) was made on the basis of the four following characteristics: (i) high continuous fever lasting two to seven days, (ii) haemorrhagic manifestations, (iii) thrombocytopenia ($<100\times10^9$/L) and (iv) evidence of plasma leakage (haematocrit change ≥20%, hypoproteinemia [serum protein <63 g/dL] or if signs of clinical fluid accumulation were present). For dengue shock syndrome (DSS), DHF cases required either (i) tachycardia (pulse >100/minute) with narrow pulse pressure (<20 mmHg) or (ii) hypotension for age (systolic blood pressure <90 mmHg).

Based on the WHO 2009 classification, patients classified as dengue with warning signs had to have laboratory-confirmed dengue infection with one of the following: (i) abdominal pain/tenderness, (ii) persistent vomiting (≥2 consecutive days), (iii) clinical fluid accumulation (pleural effusion or ascites on examination or radiography), (iv) mucosal bleed, (v) liver enlargement, and (vi) increase in haematocrit concurrent with rapid decrease in platelet count (interpreted as any haematocrit ≥20% over baseline with platelet <50000/mm^3). For severe dengue, the criteria were: for severe plasma leakage, either clinical fluid accumulation or evidence of plasma leakage (haematocrit change of ≥20%) with at least one of tachycardia (pulse >100/minute), hypotension (systolic blood pressure <90 mmHg), or narrow pulse pressure (<20 mmHg);severe bleeding was defined as WHO Grade 2 or above: hematemesis, melena, menorrhagia or clinical drop in haemoglobin requiring whole blood or packed red cell transfusion; severe organ involvement comprised hepatic injury (aspartate [AST] or alanine transaminase [ALT] levels >1000 unit/L), renal impairment (Stage 2 Acute Kidney Injury (Mehta et al., 2007)

defined as serum creatinine increase of 100% over baseline or calculated norm for age/gender/race), or impaired consciousness.

2.4.METHODS FOR HUMAN GENETIC RISK FACTORS STUDY (CHAPTER 3)

Patient enrollment and diagnosis. Blood samples for genotyping were collected from patients enrolled into either of two research studies of children with dengue. In both studies, children were eligible if ≤15 years of age and had clinical signs, symptoms and hematological findings that led to a clinical diagnosis of incipient or established DSS, as defined by WHO criteria. All patients were resuscitated with bolus intravenous fluid therapy (≥15 ml per kg body weight in the first hour). Summary laboratory and clinical findings were recorded into case record forms during the inpatient period until the patient was discharged from hospital, died or was transferred to another hospital. Blood samples for research and diagnostic tests were collected at the time of enrollment and again before patient discharge from hospital. The first study enrolled patients in the pediatric intensive care unit of the Hospital for Tropical Diseases (Ho Chi Minh City, Vietnam) between 2001 and 2009. The second study enrolled patients in high dependency rooms or the intensive care departments of Children's Hospital No. 1 and Children's Hospital No. 2 (Ho Chi Minh City, Vietnam), Tien Giang Provincial Hospital (My Tho City, Vietnam), Dong Thap Provincial Hospital (Cao Lanh City, Vietnam) and Sa Dec Hospital (Sa Dec Town, Vietnam) between 2008 and 2010.

The GWAS was performed on DNA samples (n = 1,039) from patients enrolled between 2001 and 2009 at the Hospital for Tropical Diseases and from patients (n = 969) enrolled at the other five participating hospitals during 2008 only. The replication study was performed in patients (n = 1,737) enrolled between 2009 and 2010 at Children's Hospital No. 1, Children's Hospital No. 2, Tien Giang Provincial Hospital, Dong Thap Provincial Hospital and Sa Dec Hospital. All patients represented in the GWAS and

replication phases had laboratory evidence of dengue, as shown by reverse transcription PCR detection of viral RNA in plasma collected at the time of enrollment and/or by serological detection of dengue-virus–reactive IgM or IgG in single or paired plasma specimens.

Cord blood DNA samples. Blood from the cord of newborn infants was collected in one of two prospective studies. The first study was conducted at Hung Vuong Hospital (Ho Chi Minh City, Vietnam) between 2004 and 2006. The second study was conducted at Hung Vuong Hospital and Dong Thap Hospital between 2009 and 2010. All participants gave written informed consent to participate. The Scientific and Ethical Committees of each study site approved the study protocols, as did the Oxford University Tropical Research Ethical Committee. DNA was extracted from cord blood using Nucleon BACC Genomic DNA Extraction Kits (GE Healthcare, USA).

The use of population controls. The number of potentially misclassified cord blood controls in the GWAS and replication stages was estimated to be 11 (out of 2,018) in the GWAS stage, and 15 (out of 2,394) in the replication stage, based on the following three assumptions that: a) all individuals in a given birth cohort will experience two sequential infections by different serotypes during their lifetime(Chau et al., 2009) b) that only up to 25% of these infections are clinically apparent (Balmaseda et al., 2010; Endy et al., 2011; Endy et al., 2002; Porter et al., 2005; Tien et al., 2010), c) 2% of clinically apparent secondary infections develop DSS. These assumptions estimate a life-time population risk of DSS to be 0.5%. This is consistent with estimates of the prevalence of DSS cases expected over the first 15 years in a given birth cohort under the assumption that the incidence of DSS is constant (DSS incidence in Southern Viet Nam in 2009 was

26.59/100,000; based on statistics obtained from the Dengue Control program, Ministry of Health Viet Nam, 2010). Under this assumption we would expect 0.4% of a birth cohort to experience DSS before the age of 15yrs.

Genotyping. Cases and controls were randomized on plates and were genotyped with Illumina Human 660W Quad BeadChips following manufacturer instructions. For the replication stage, 72 of the selected SNPs that were not on the broad MHC region were genotyped with the Sequenom MassArray primer extension iPLEX system. *MICB* rs3132468 and rs3134899 were genotyped using the Applied Biosystems TaqMan platform.

Statistical analysis. Stringent quality control filters were applied to remove poorly performing SNPs and samples using tools implemented in PLINK (version 1.7) (Purcell et al., 2007). The quality control criteria were as follows: SNPs that had genotypes with more than 5% missing, showed gross departure from Hardy–Weinberg equilibrium (a departure of $P < 10-7$) or had a minor allele frequency below 1% were excluded from downstream analysis. For sample quality control, samples with an overall genotyping call rate of <95% were excluded from analysis. The remaining samples were then subjected to biological relationship verification by using the principle of variability in allele sharing according to the degree of relationship. Identity-by-state information was derived using PLINK (Purcell et al., 2007). For those pairs of individuals who showed evidence of cryptic relatedness (possibly owing to duplicated or biologically related samples), we removed the sample with the lower call rate before performing principal component analysis. Principal component analysis was undertaken to account for spurious associations resulting from

ancestral differences of individual SNPs (Price et al., 2006), and principal component plots were performed using the R statistical program package.

For both the GWAS and replication stages, analysis of association with DSS was carried out using a 1 degree of freedom score-based test. This test models for a trend per copy of the minor allele on disease risk and has been extensively described (Purdue et al., 2011; G. Thomas et al., 2009). It has the best statistical power to detect association for complex traits across a wide range of alternative hypotheses, with the exception of those involving rare recessive variants (Lettre, Lange, & Hirschhorn, 2007). The threshold for significant independent replication was set at $P < 0.05$ in the combined replication data sets.

Meta-analysis was conducted using inverse variance weights for each cohort, which calculates an overall Z statistic, corresponding *P* value and accompanying ORs for each SNP analyzed (Asano et al., 2009; McGovern et al., 2010).Genotyping clusters were directly visualized for the 85 SNPs exceeding $P < 10^{-4}$ and confirmed to be of good quality before inclusion for statistical analysis. Analysis of linkage disequilibrium was performed using Haploview (Barrett, Fry, Maller, & Daly, 2005).

Statistical findings after routine GWAS analysis. Analysis of genetic ancestry using principal components revealed no significant population substructure between the DSS cases and controls. As the individual principal components were non-significant (Bonferroni corrected $P > 0.05$) when tested as continuous covariates using logistic regression, we did not adjust for them in subsequent association analysis. Single SNP analysis was performed using logistic regression assuming additive genetic effects relating

genotype dosage (scoring for 0, 1 or 2 copies of the minor allele) to DSS. A quantile–quantile plot of the single SNP analysis showed a clear excess of extreme *P* values compared to the null distribution. As this excess was observed against a background of minimal genome-wide inflation of test statistics (λgc = 1.024), it excludes the possibility of substantial population substructure and differential genotyping call rate between cases and controls as a reason for the excess. Instead, this suggests that at least some of these extreme *P* values ($P < 1 \times 10^{-4}$) may represent true genetic associations with DSS.

Overall, we genotyped 2,118 DNA samples from Vietnamese children with established or incipient DSS and 2,089 cord blood controls in a genome-wide association study (GWAS). After exclusion of samples for discrepancies between clinical and genetically inferred gender, relatedness or for per-sample call rates of less than 95 percent, there were 2,008 DSS cases and 2,018 controls available for analysis. The clinical and virological characteristics of the case population were also described. A total of 657,366 SNPs were initially included within the Illumina 660W Beadchip used for genome-wide genotyping. After various stringent QC exclusions, which include removal of CNV SNPs, SNPs with minor allele frequency (MAF) less than 1%, call rate less than 95%, non-autosomal SNPs, SNPs with intensity only, a total of 481,342 SNPs were retained for downstream association analysis. We were able to design assays for 72 out of these 85 SNPs using the Sequenom Mass-Array platform. The remaining 13 SNPs in the broad MHC region were refractory to assay design, thus necessitating ABI Taqman assays to be designed for the sentinel SNP at MICB (rs3132468) and rs3134899 (also within MICB; GWAS P = 1.03 x 10-4, OR = 1.31). We then genotyped these 74 SNPs (72 non-MHC SNPs and two SNPs within MICB) in a replication sample of 1,824 DSS cases and 3,019 controls. We applied the same GWAS QC filters for the replication set: five SNPs had

poor genotyping clusters and were excluded from analysis, and 132 samples (87 cases and 85 controls) had per-sample call-rates of less than 95 percent; these were excluded from further analysis. This left 69 SNPs to be analyzed in 1,737 cases and 2,934 controls for the replication stage.

2.5.METHODS FOR EPIDEMIOLOGICAL RISK FACTORS STUDY (CHAPTER 4)

Patient source and diagnosis. A retrospective case-control study was conducted using data collected from all adult dengue patients admitted from 1 January 2006 to 31 December 2008 to the Department of Infectious Diseases at Tan Tock Seng Hospital (TTSH). In this hospital, which treats the most dengue patients in Singapore, dengue patients are managed using a standardized dengue care path. The extracted data was de-identified in analysis. Probable dengue patients had positive acute dengue serology, as measured by Dengue Duo IgM & IgG Rapid Strip Test (Panbio Diagnostic, Queensland, Australia) (Blacksell et al., 2006; Cuzzubbo et al., 2001), and clinical diagnostic criteria of dengue fever by WHO 1997. The IgG test line for this Rapid Strip Test is set to detect the high levels of IgG characteristic of secondary virus infection, and has been validated to detect acute secondary infection (Cuzzubbo et al., 2001). Confirmed dengue patients had positive dengue polymerase chain reaction (PCR) assay (Barkham, Chung, Tang, & Ooi, 2006) and clinical diagnostic criteria according to WHO 1997.

Case-control groups and data extracted. Patients with outcome diagnosed as DHF and DF were classified as the case group and control group respectively. In order to compare the demographic and co-morbidity profiles between DHF and DF patients, data were extracted from chart review. Demographic data extracted were age, gender and ethnicity. Co-morbidities data extracted were diabetes mellitus, hypertension, asthma, hyperlipidaemia, stroke, chronic obstructive pulmonary disease, corticosteroid use and HIV/AIDS. However, stroke, chronic obstructive pulmonary disease, corticosteroid use and HIV/AIDS were not included in the analysis because these co-morbidities were either

not reported or reported by very few cases (DHF) or controls (DF). Patients with diabetes mellitus tend to have other co-morbidities, and the risk effect of diabetes mellitus with an additional co-morbidity on DHF outcome was also investigated.

Statistical analysis. For descriptive analysis, Pearson's chi-square and Fisher's exact tests were used to compare categorical variables, and Mann-Whitney U test was used to compare continuous variables with non-normal distribution. Univariate and multivariate logistic regression were used to calculate crude and adjusted odds ratios (COR, AOR), respectively, and their 95% confidence intervals (CI) were used to assess the association of the variables with DHF. Confounding effects were minimized by performing multivariate logistic regression adjusting for potential confounders identified in the descriptive analysis. These potential confounders are exposures that were found to be statistically different ($p<0.05$) between DHF and DF patients and the model that fits best in the multivariate regression was then identified using the likelihood-ratio test. In Singapore, dengue infections were predominantly due to dengue serotype 1 (detected in 75% to 100% of dengue samples collected each month) during the epidemic in the year 2006, and dengue serotype 2 (detected in up to 91% of dengue samples) during the epidemic in the year 2007 and 2008 (K. S. Lee et al., 2010). Given that different dengue serotypes may cause different disease severity (Fried et al., 2010), the data from year 2006 and the data from year 2007 to 2008 were analyzed separately to minimize any possible confounding effect due to different predominant dengue serotypes, since information on the dengue serotype was not available for individual infections. Stratified analyses using logistic regression were performed to evaluate the presence of effect modification between diabetes mellitus and other co-morbidities on the risk of DHF outcome. There is at least 70% power of detecting a significant ($P<0.05$) odds ratio of 1.3 with a sample size of 400

DHF and 400 DF. Correction for multiple comparisons was not performed as each of the variables of interest was tested independently, instead of simultaneously. There was no a priori analytical plan to adjust for risk factors associated with DHF in previous studies, unless it was also shown to be significantly different in the univariate analysis in this study. All statistical analyses were performed using Stata 10.0 (Stata Corp., College Station, TX, 2005). All tests were conducted at the 5% level of significance, with OR, P-value and corresponding 95% CI reported where applicable.

2.6.METHODS FOR MOLECULAR RISK FACTORS STUDY (CHAPTER 5)

Patient enrolment and sample collection. The early dengue infection and control study (EDEN) was launched in April 2005 and has been described in previously published articles (Low et al., 2006; Tanner et al., 2008; Tolfvenstam et al., 2011). Patients fulfilling the inclusion criteria (18 years of age or older; within 72 hrs from acute onset of fever of 38^{o}C or above and; no clinically obvious alternative diagnosis to fever) presenting at any of the participating primary care polyclinics were eligible for enrolment. Enrolment of study participants was conditional on appropriate informed consent administered by a study research nurse. A standardized case report form (CRF) was used for collecting clinical data. Additionally, venous blood for hematological and molecular analyses was collected. Clinical data and samples were obtained at time of inclusion (within 72 hrs post fever onset), at time of defervescence (4-7 days post fever onset) and finally at time of convalescence (3-4 weeks post fever onset). The clinical data for hospitalized patients were obtained from the electronic medical records. No study criteria for hospitalization were specified with the decision to admit left to the discretion of the attending physician. The training cohort consists of samples collected from patients enrolled from year 2005 to 2008 while the validation cohort consists of samples collected from patients enrolled from year 2009 to 2012.

Patient classification criteria. In 2009, WHO published new guidelines for the management of patients with acute dengue infection. These are based on clinical signs and symptoms, and divides patients into “probably dengue”, “dengue with warning signs” and “severe dengue”. The warning signs stated in the guidelines are; abdominal pain or

tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleed, lethargy, restlessness, persistent vomiting, liver enlargement >2cm and increase in HCT concurrent with rapid decrease in platelet account. Severe dengue was defined by severe plasma leakage, internal bleeding and/or severe organ impairment (WHO, 2009). In the EDEN study, only three warning signs were recorded during the study period and hence, can only be evaluated in our study setting. They are abdominal pain, mucosal bleeding and persistent vomiting. Patients were classified as having "persistent vomiting" either when they report vomiting at both the first and second study clinic visit, or at the second visit and at time of hospitalization. Patients who had at least one of these warning signs were classified with a more marked dengue disease and referred to as "patients with warning signs", whereas, patients who did not present any of these warning signs in all three study clinic visits are referred to as "patients without warning signs".

Hospital admission criteria. The decision to hospitalize a patient was left to the discretion of the treating physician. However, national guidelines on dengue management are available and are adopted by the healthcare institutions in Singapore. Hospitalization criteria in these guidelines include: significant bleeding, fall in blood pressure, dehydration and postural hypotension, rise in haematocrit of 20% or greater compared to the baseline, platelet count <80,000 cells/mm3, severe vomiting or diarrhoea, severe abdominal pain, and elderly patients with co-morbidities who are unwell.

Hematology. A full blood count was performed on anticoagulated whole blood collected at all three time points. A bench-top, FDA-approved hematocytometer was used for this application (iPoch-100, Sysmex, Japan). Calibration by internal and external QC controls was also performed on a regular basis.

RNA extraction. RNA was extracted using QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Viral detection and quantification. Dengue virus RNA detection was carried out by PCR using a set of generic pan-dengue primers that targeted the 3' non-coding region of dengue viruses as previously described (Lai et al., 2007). Results were analyzed with the LightCycler software version 3.5. Reactions with high crossover threshold (Ct) value or ambiguous melting curve results were further analyzed by electrophoresis on a 2% agarose gel, to confirm the presence of the correctly sized amplicon. Quantification of viremia was performed by a Taqman based PCR using earlier published primers and probes detecting DENV 1-4 (Ito et al., 2004). Standard ABI conditions were used, incorporating primers at 900nM and probes at 50nM.

Serology. IgM and IgG antibodies against dengue virus were detected using commercially available ELISAs (PanBio, Brisbane, Australia) according to manufacturer's instructions.

Microarray. Total RNA (500ng) was amplified in a single-round of IVT amplification that allowed incorporation of Biotin-labeled nucleotides using the Illumina® TotalPrep™ RNA Amplification Kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. cRNA (850ng) of each sample was hybridized to an Illumina HumanRef-8 V3.0 BeadChip (containing probes to approximately 24,500 transcripts) at 55 °C for 18 hrs following the manufacturer's instructions (Illumina, Inc., San Diego, CA). This was followed by washing, blocking, and streptavadin-Cy3 staining steps and finally by

scanning with a high resolution Illumina Bead Array Reader confocal scanner, all carried out following manufacturer's instructions (Illumina, Inc., San Diego, CA). Data extraction was performed using Illumina Bead Studio software.

Microarray normalization and gene selection. The detection *p*-value was calculated by Beadstudio software (Illumina). Standard normalization procedures (GenespringGX software; version10.0; Silicon Genetics) for one color array data were used. In brief, array (mean) normalization accounted for chip variability was performed by dividing all of the measurements on each chip by a 75^{th} percentile value. After normalization, the data was filtered according to flags present there at least 75% of the samples in any 1 of the 2 conditions had flags present leaving 6844 genes for further analyzes. Significance Analysis of Microarray (SAM) was used to detect transcripts that were relatively more or less abundant in one group of samples. SAM also corrected significance values for multiple testing using a false discovery rate threshold of 5%. False discovery rate of less than 5 percent and fold difference of at least 1.5 fold were used to identify the significant genes. Pathway analysis was done using Ingenuity Pathway Analysis software (version 7.5; Ingenuity Systems).

Measurement of RNA expression using Fluidigm technology. In order to develop a potential point-of-care device, a simple PCR based technology should be applied instead of using a microarray based technology to reduce cost and processing time. Fluidigm platform was used. The required amount of RNA is 500ng/10ul per reaction. The protocols were according to the manufacturer's recommended instructions. Briefly, cDNA is synthesized through reverse transcription using MultiScribe reverse transcriptase with the following program: 25 ˚C for 10 min, 37 ˚C for 120 min, 85˚C for 5 min and 4˚C for

30min. This is followed by pooling of Taqman assays and preamplification reaction with the following program: 95 for 10min, and 14 ˚C cycles of 95˚C for 15sec and 60˚C for 4 min. Lastly, this is followed on with final amplification using the BioMark and the 48.48 dynamic array as instructed in the manufacturer's instructions. The signal of the gene expression was normalised by 18S rRNA expression. The expression level and quality control checks were determined using the BioMark Real-Time PCR Analysis Software.

Statistical analyses of demographics and haematological parameters. Group comparisons were performed by a Mann Whitney *U* test, for viremia, lymphocyte counts, platelet levels, age and a Chi-square test for gender. Two tailed tests were used and a *P*-value of < 0.05 was considered as statistically significant. All statistical analyses were performed using Stata 10.0 (Stata Corp., College Station, TX, 2005).

Protein measurements. Serum samples collected at three time points (within 72 hours post fever onset, day 4-7 and three to four weeks post fever onset) were assayed for 22 cytokines and chemokines related to inflammation and immune response based on published and unpublished data using a luminex bead array approach (Bioplex) (BioRad Carlsbad, CA) according to the manufacturer's protocol. Quantitative sandwich enzyme-linked immunosorbent assays (ELISA) were used to measure fibrinogen (Immunology Consultants Laboratory Inc, Newberg, OR), urokinase plasminogen activator receptor (uPAR) as well as IP-10 (R&D Systems, Minneapolis, MN). The assay from R&D Systems was needed as a significant number of serum samples from dengue patients had concentration of IP-10 above the detection range of the Bioplex system. All assays were carried out according to the manufacturers' instructions.

Statistical analysis of protein expression. Two-sample t test for independent samples were used to compare the means of protein levels in the serum between the patient groups. For proteins expression with unequal variance, two-sample t test for independent samples with unequal variance was used. Two-tailed P value of <0.05 were considered statistically significant.

Biomarkers Selection and Prognostic Model Development. RNA and proteins that were statistically significant ($P<0.05$) between the two groups within 72 hrs (Day1-3) were chosen for the model development. Each RNA and protein was assessed as a potential single biomarker model based on the area under the curve (AUC) from the receiver operating characteristic curve (ROC) analysis. In addition, Hosmer-Lemeshow Goodness-Of-Fit (GOF) test was used to assess whether the model provides a good fit to the training data. These single biomarker models were then ranked according to their stratification performance based on the AUC analysis and the GOF test. Forward stepwise and backward elimination estimation using multiple logistic regressions, AUC analyses and GOF test were performed using the top 10 RNA and protein single biomarker models to determine the optimal multi-biomarkers (RNA only, proteins only and combination of RNA and proteins) model for stratification of patients with warning signs that require hospitalization from patients without warning signs and do not require hospitalization. Likelihood ratio test was performed to evaluate if a model with N variables is as good fit as a model with N+1 variables to achieve a more parsimonious model, and to compare the AUC of RNA-Protein model against that of the viral load AUC, lymphocyte AUC and platelet AUC All analyses were carried out using STATA 10 (StataCorp LP Texas, USA).

2.7.METHODS FOR CLINICAL AND LABORATORY RISK FACTORS STUDY (CHAPTER 6)

Patient source and study design. A retrospective matched case-control study was conducted using anonymized data collected from all adult dengue patients admitted into intensive care unit (ICU) from 1 January 2004 to 31 December 2008 to the Department of Infectious Diseases at Tan Tock Seng Hospital (TTSH), where dengue patients were managed using a standardized dengue care path. In Singapore, dengue infections were predominantly due to dengue serotype 1 (detected in 75% to 100% of dengue samples collected each month) during the epidemics in the year 2004 to 2006, and dengue serotype 2 (detected in up to 91% of dengue samples) during the epidemic in the year 2007 and 2008 (K. S. Lee et al., 2010). Each case who was admitted into ICU was randomly matched to four patients who did not require intensive care by the year of dengue presentation as controls. Dengue-confirmed patients with positive dengue polymerase chain reaction (PCR) assay, and probable dengue patients with positive dengue immunoglobulin-M (IgM) or immunoglobulin-G (IgG) (Dengue Duo IgM & IgG Rapid Strip, Panbio Diagnostic, Queensland, Australia) and fulfilling either the WHO 1997 or 2009 probable dengue criteria, were included in this study.

Data extraction. The following data at first presentation in hospital and 24 hours prior to ICU admission were obtained from medical records: demographic, epidemiological, co-morbidity, clinical, laboratory, treatment and outcome data. The severity of the patients as evaluated by the WHO 1997 and 2009 classifications was determined at first presentation and during hospitalisation. The duration of progression to ‘DHF’ or ‘severe dengue’ was determined only for patients who were either classified as

‘DF’ or ‘probable dengue with/without warning signs’ at first presentation. The number of days post presentation (DPP) was used to define the period since the first presentation in hospital. The number of days post fever (DPF) was used to define the period since the day of fever onset.

Statistical analysis. Univariate and multivariate conditional logistic regression were performed to assess the association between the variables and dengue severity as defined by ICU admission. Conditional logistic regression was used to account for the matching factor selected in the analyses (Sedgwick, 2012). Matching was performed based on the year of presentation to control for potential confounding by the predominant serotype in each year. Any potential confounding effects were further minimized by performing multivariate conditional logistic regression adjusting for significant demographic & co-morbidity factors associated with ICU admission only. This parsimonious approach would minimize overloading the multivariable model to achieve higher statistical power to detect true association as the sample size available was small (Sedgwick, 2010, 2013). The laboratory variables were analyzed in the continuous format to maximize all the data available, and to minimize reporting bias when the variables were categorized into the expected normal or hypothetical range. For analysis of risk factors 24 hour prior to ICU admission, four ICU patients were excluded as they were admitted into ICU less than 24 hour post presentation to hospital. The most optimal predictive model was derived using the stepwise forward and backward elimination method, with the top 10 most significantly associated variables based on the *P*-value of the univariate conditional regression analyses. Only variables with *P*-value <0.05 would remain in the optimal model during the model development. Hosmer–Lemeshow Goodness of fit test was used to check if the model fit the observed dataset with $P > 0.05$. Likelihood ratio test was used to assess

if a model is more efficient than the other similar model with more significant variables. Fisher's exact test and Wilcoxon rank-sum test were used for categorical and continuous variables, respectively, for the subgroup analysis of death cases in ICU group. All statistical analyses were performed using Stata 10.0 (Stata Corp., College Station, TX, 2005). All tests were conducted at the 5% level of significance, with conditional odds ratio (COR) and/or adjusted COR (ACOR), *P*-value and corresponding 95% CI reported where applicable.

2.8.METHODS FOR THE POTENTIAL PUBLIC HEALTH IMPACT OF RISK FACTORS ASSOCIATED WITH ADULT DENGUE SEVERITY (CHAPTER 7)

Epidemiological risk factors. Using the epidemiological risk factors identified in Chapter 4, which includes age range from 30-49, Chinese, female and having diabetes as individual risk factors of DHF, a discrete and weighted screening/triage risk score was computed for about 2600 laboratory confirmed dengue patients upon first presentation to the clinicians in Singapore. A discrete score was computed based on whether each of the four risk factors is present for each dengue patient upon presentation, up to a total score of four. The sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) was calculated for each risk score of one, two and three of predicting whether the patient is at high risk of developing DHF. The weighted risk score was computed using the logistic regression coefficients of each these epidemiological risk factors, and the score can range from 0 to 21.The score cut-offs used for assessing the sensitivity, specificity, NPV and PPV for predicting DHF outcome were greater than zero, greater than three, greater than six, greater than nine and greater than 12, with dengue patients having scores greater than the respective cut-offs were predicted at higher risk of DHF outcome.

$$DHF\ Outcome = 0.6(Diabetes) + 0.45(Female) + 0.34(Age30 - 39) + 0.3(Age40 - 49) - 0.09(Age50 - 59) + 0.16(Age > 60) + 0.51(Chinese) + 0.27(Malay) - 0.64(Indian) - 1.2$$

Genetic risk factors. The top genetic locus at MICB (rs3132468) that is associated with DSS (Khor et al., 2011) and non-DSS cases (Whitehorn et al., 2013) in Vietnam were used to assess its sensitivity, specificity, PPV and NPV using a Singapore cohort of about 1017 Chinese dengue adult cases to evaluate the prognostic performance of the genetic loci rs3132468 to predict DHF outcome. The genetic marker of rs3132468 was genotyped as per described in method for Chapter 3.

2.9. ETHICAL APPROVAL

Studies in Chapter 3 was approved by the Scientific and Ethical Committees of each study site as well as the Oxford University Tropical Research Ethical Committee. The parent or guardian of each participant gave written informed consent to participate.

Studies in Chapter 4 and 6 werea pproved by Domain Specific Review Board, National Healthcare Group, Singapore (DSRB-E/08/567) with waiver of informed consent as this was a retrospective study and the data were analysed anonymously.

Studies in Chapter 5 was approved by Domain Specific Review Board, National Healthcare Group, Singapore (DSRB B/05/013) as well as the Institutional Review Boards of DSO National Laboratories with waiver of informed consent as this was a retrospective study and data were analyzed anonymously.

CHAPTER 3: HUMAN GENETIC RISK FACTORS OF DENGUE DISEASE SEVERITY

3.1.INTRODUCTION.

As highlighted in Chapter 1, dengue disease results in a wide spectrum of disease manifestations ranging from subclinical infection to severe and fatal disease. Severe dengue, most commonly found in children, is characterized by an increase in vascular permeability that leads to life-threatening hypovolemic shock (dengue shock syndrome-DSS). This is often accompanied by thrombocytopenia and haemostatic dysfunction, which may result in severe bleeding. Although children are at greatest risk of developing DSS, with careful supportive care the case fatality rate is less than 1% (Anders et al., 2011). In southern Vietnam, serological studies have estimated the population based exposure to dengue virus infection to reach 85% by the end of childhood (15 years old) (Thai et al., 2005), while the incidence of DSS is estimated to occur at less than 1% of exposed individuals (Anders et al., 2011)(see "The use of population controls" in the Methods section). A host genetic basis to susceptibility to severe dengue has been alluded to in epidemiological studies, and various candidate gene studies of modest sample sizes have only been performed(Loke et al., 2002; Loke et al., 2001; Sakuntabhai et al., 2005; Stephens et al., 2002; Vejbaesya et al., 2009). Therefore, there is a critical lack of a well-powered assessment of the whole genome that aims to determine the genetic risk factors that may predispose individuals with a higher risk of severe dengue disease such as DSS.

3.2.RESULTS

(Publication 1: Khor CC, Chau TN, **Pang J**, Davila S. et. al. **Genome-wide association study identifies susceptibility loci for dengue shock syndrome at MICB and PLCE1.***Nat. Genet.* 2011. 43(11):1139-1141.)

(Publication 2: Whitehorn J, Chau TNB, Nguyet NM, Kien DTH, Quyen NTH, Trung DT, **Pang J** et al. **Genetic variants of MICB and PLCE1 and association with non-severe dengue.***PLoS ONE* 2013 8(3):e59067.)

Susceptibility loci for dengue shock syndrome at MICB and PLCE1

Upon conducting the routine GWAS statistical tests (see Statistical findings in the Methods section), detailed examination of the overall scan results revealed strong evidence of disease association at two distinct loci; (Figure 7) MICB on Chromosome 6 and PLCE1 on Chromosome 10, both represented by SNPs which were close to the formal threshold for genome-wide significance (P_{GWAS} = 5.38 x 10^{-8} for MICB rs3132468 and P_{GWAS} = 5.84 x 10^{-8} for PLCE1 rs3740360) (Table 2). Together with the SNPs at MICB and PLCE1, a total of 85 SNPs exceeded $P_{GWAS} < 10^{-4}$ on single SNP analysis and were extended for replication phase with an independent cohort. In keeping with the GWAS observations, the strongest evidence of association in the replication phase was observed with SNPs at MICB (rs3132468, P_{repl} = 9.32 x 10^{-5} and rs3134899, P_{repl}= 0.0082) and PLCE1 (3 SNPs with P_{repl} ranging from 5.23 x 10^{-4} to 1.6 x 10^{-4}, Table 1). Using inverse-variance weights, data from both the GWAS and replication cohorts (N = 3,745 DSS cases and N = 4,952 controls) were combined in formal meta-analysis, and this revealed strong evidence of association with rs3132468 at MICB (P_{meta} = 4.41 x 10^{-11}; per-allele odds ratio (OR) = 1.34, [1.23 - 1.46]) and 7 SNPs at PLCE1 (4.18 x $10^{-9} \leq P_{meta} \leq$ 3.08 x 10^{-10}; 0.75 ≤

OR ≤ 0.87, Table 2). To aid in refining the original signal of association, we performed imputation analysis at regions flanking both loci (Chr. 6: 30 - 32 Mb, and Chr. 10: 95.5 - 96.5 Mb). This did not reveal signals of association over and above that of the directly genotyped SNPs. The associations observed at MICB and PLCE1 were not specific to any Dengue virus serotype on subgroup analysis of viral serotype, nor were they associated with the degree of thrombocytopenia or the degree of clinical shock.

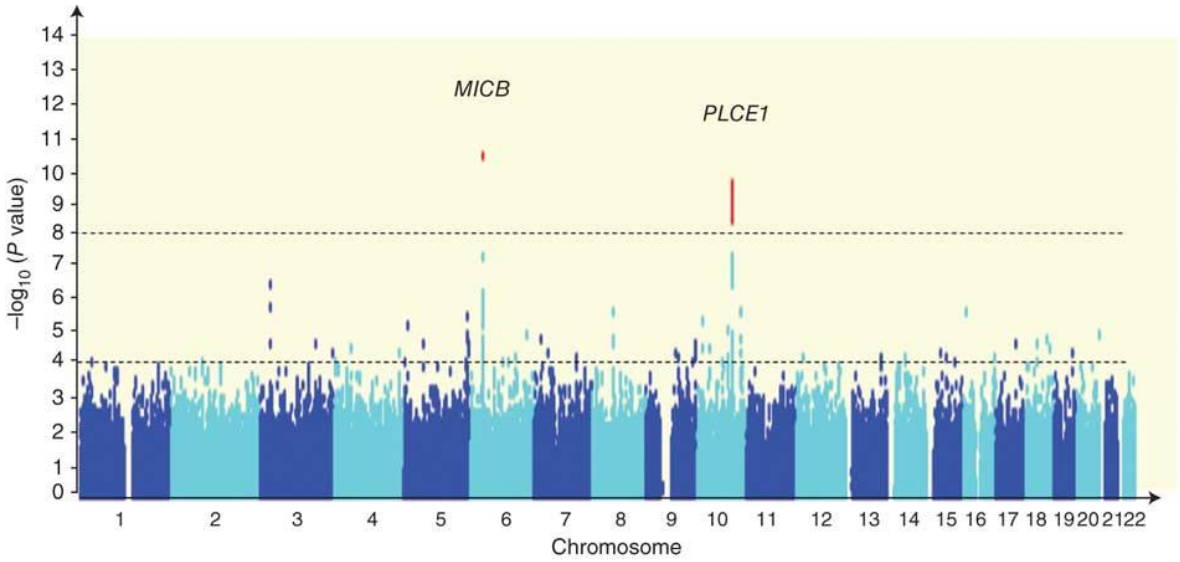


Figure 7. Manhattan plot showing directly genotyped SNPs plotted according to chromosomal location (x axis), with –log10 P values (y axis) derived from the 1-degree-of- freedom score test.

SNPs colored in red achieved genome-wide significance of $P < 10^{-8}$ on meta-analysis.

Table 2. Association analysis between dengue shock syndrome and SNP genotypes at MICB and PLCE1

Gene/Marker (Alleles)	Chromosome (Position)	Stage	MAF Cases	MAF Controls	OR	*P*	OR_{meta} (95% CI)	P_{meta}
MICB/rs3132468	6	GWAS	0.176	0.132	1.41	5.39×10^{-8}		
(C/T)	31583465	Replication	0.163	0.134	1.27	9.32×10^{-5}	1.34 (1.23 - 1.46)	4.41×10^{-11}
MICB/rs3134899	6	GWAS	0.130	0.102	1.31	1.09×10^{-4}		
(G/A)	31581265	Replication	0.114	0.096	1.20	0.0082	1.26 (1.14 - 1.38)	4.08×10^{-6}
PLCE1/rs3765524	10	GWAS	0.249	0.300	0.77	2.68×10^{-7}		
(T/C)	96048288	Replication	0.265	0.302	0.83	1.60×10^{-4}	0.80 (0.75 - 0.86)	3.08×10^{-10}
PLCE1/rs2274223	10	GWAS	0.250	0.303	0.77	1.19×10^{-7}		
(G/A)	96056331	Replication	0.267	0.300	0.85	5.23×10^{-4}	0.81 (0.75 - 0.86)	6.89×10^{-10}
PLCE1/rs3740360	10	GWAS	0.219	0.271	0.75	5.84×10^{-8}		
(C/A)	96015481	Replication	0.242	0.273	0.85	0.0012	0.80 (0.75 - 0.86)	1.15×10^{-9}
PLCE1/rs12263737	10	GWAS	0.250	0.301	0.77	3.73×10^{-7}		
(A/G)	96034903	Replication	0.266	0.300	0.84	3.95×10^{-4}	0.81 (0.75 - 0.87)	1.22×10^{-9}
PLCE1/rs11187842	10	GWAS	0.219	0.269	0.76	1.19×10^{-7}		
(T/C)	96042501	Replication	0.240	0.271	0.85	0.0011	0.80 (0.75 - 0.86)	1.78×10^{-9}
PLCE1/rs753724	10	GWAS	0.219	0.269	0.76	1.28×10^{-7}		
(T/G)	96041407	Replication	0.242	0.272	0.85	0.0012	0.81 (0.75 - 0.86)	2.27×10^{-9}
PLCE1/rs3781264	10	GWAS	0.229	0.278	0.77	3.43×10^{-7}		
(G/A)	96060365	Replication	0.250	0.280	0.85	0.0011	0.81 (0.76 - 0.87)	4.18×10^{-9}

Found within the broad Major Histocompatibility (MHC) locus, MICB lies just outside both the type I and type II HLA regions, ~140,000 base-pairs centromeric to the nearest Class I gene (HLA-B) and more than 1 million base-pairs away from the nearest Class II gene (HLA-DR). Apart from the peak signal at rs3132468 which was observed directly within MICB, twelve other SNPs in this region also showed association signals exceeding $P < 10^{-4}$ on single-SNP analysis. We thus performed conditional analysis to assess the independence of the association observed at MICB rs3132468 from that of the nearby genes.

Although the most significant SNP from the GWAS (rs3132468) could account for the majority of the association signal across the locus, we observed residual signals of association ($0.0003 < P < 0.05$) with SNPs near the vicinity of HLA-B and HLA-C as well as other neighboring genes. These residual associations indicate that definitive identification of MICB as a gene associated with DSS could be complicated by its location within the broad MHC region, which is known for its extensive linkage disequilibrium (LD) spanning multiple genes. This precludes definitive identification of the causative gene without extensive further fine-mapping and re-sequencing.

With regards to PLCE1 on Chromosome 10, association analysis conditioning for the lead SNP (rs3743060, directly genotyped) did not reveal any secondary signals of association, which suggests that the lead SNP -or any of its close correlates in complete LD with it and confined within their distinct genomic region- best explains the association signal at the locus. We did not observe any evidence of epistasis between SNPs at MICB and PLCE1 ($P = 0.11$).

Genetic variants of MICB and PLCE1 and associations with non-severe dengue.

Pooled analysis of genotype data from the pediatric patient cohorts revealed that non-DSS dengue cases were significantly more likely to carry the MICB risk allele rs3132468 than controls (per-allele odds ratio (OR) = 1.16; 95%CI: 1.07 – 1.25). Pooled analysis of cohorts of adult non-DSS dengue cases revealed a similar pattern of effect with the MICB risk allele, but this did not reach statistical significance (OR = 1.10, P = 0.11; Figure 8). However, a pooled analysis of all pediatric and adult patient cohorts indicated a significant association (OR = 1.15, P = 0.0014; Figure 8) compared to controls, with no heterogeneity between children and adults (Phet = 0.19). In the relatively small number of adult and pediatric DSS cases (N = 297), we were able to confirm the association and effect size reported in our previous GWAS (OR = 1.42, P = 0.0014; Figure 8). We observed significant association between PLCE1 risk allele rs3740360 with non-DSS cases upon pooled analysis of all adults and children (OR = 0.92, P = 0.018; Figure 8). Amongst DSS cases, the association at PLCE1 revealed by the previous GWAS was also confirmed (OR = 0.77: P = 0.0094).

Since each of the infant cohorts was relatively small in their own right, a meta-analysis was performed. Consistent with the findings in older children, this pooled analysis revealed a significant association between dengue in infants and MICB rs3132468 (OR = 1.48; P = 0.0075), as well as PLCE1 rs3740360. (OR = 0.75, P = 0.041) (Figures 8 and 9). Although the infant cohorts included 16 cases of DSS, removal of these samples did not affect the associations demonstrated.

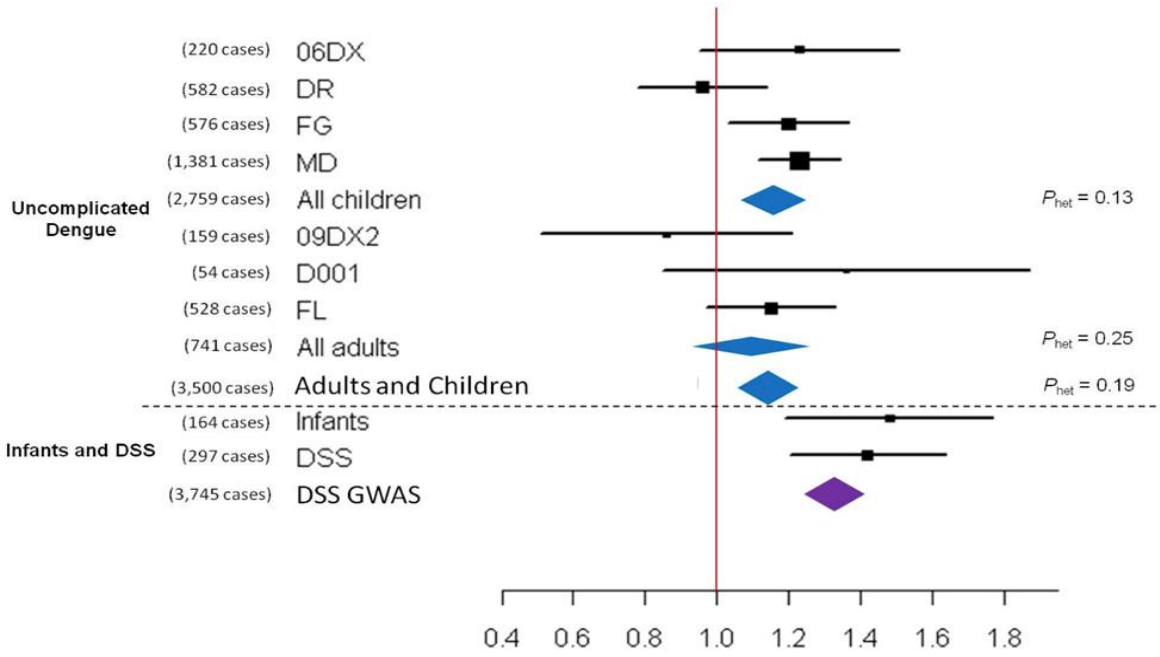


Figure 8. Forest plot illustrating the association between MICB rs3132468 and susceptibility to dengue.

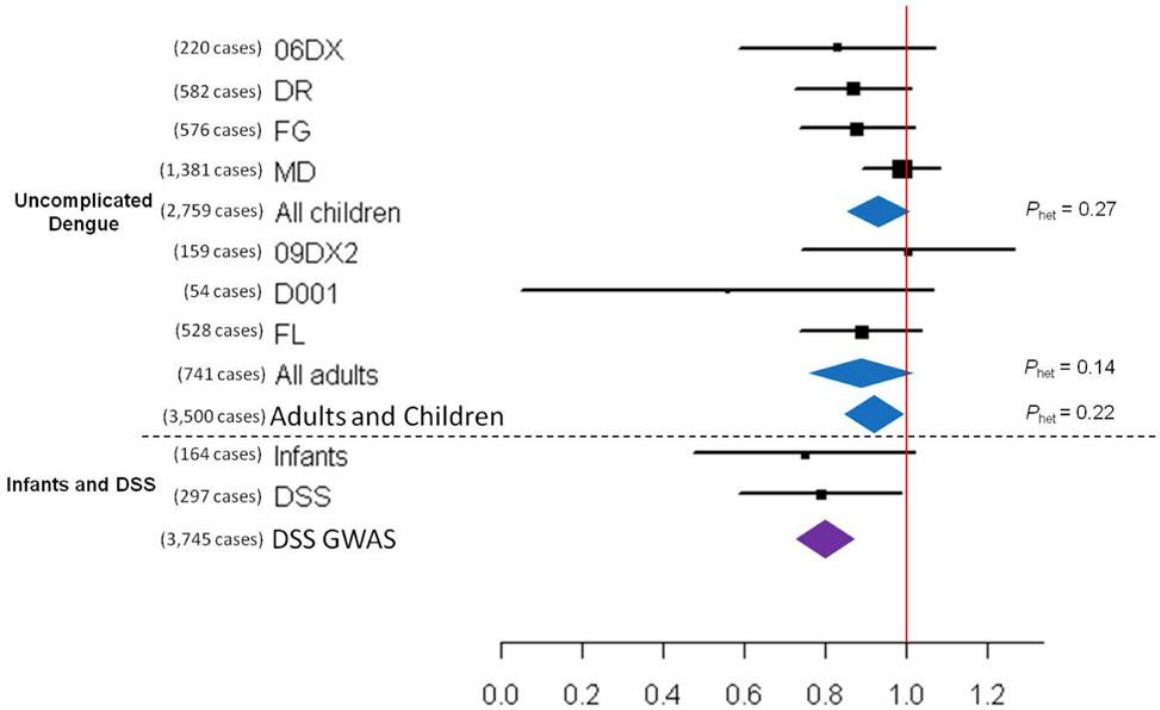


Figure 9. Forest plot illustrating the association between PLCE1 rs3740360 and susceptibility to dengue.

3.3.DISCUSSION AND CONCLUSION

MICB appears to be a promising candidate based on the present strength of the statistical associations observed in the Chromosome 6 hit region. MICB encodes for MHC class I polypeptide-related sequence B, an inducible activating ligand for the NKG2D type II receptor on natural killer (NK) and CD8+ T cells (S. Gonzalez, Lopez-Soto, Suarez-Alvarez, Lopez-Vazquez, & Lopez-Larrea, 2008; Steinle et al., 2001). Ligation of NKG2D by MICB stimulates anti-viral effector functions in NK cells including cytokine expression and the cytolytic response (Garrity, Call, Feng, & Wucherpfennig, 2005). We have previously reported that MICB, together with other genes associated with NK cell activation, are highly expressed in the leukocytes of acute dengue patients (Hoang et al., 2010). We therefore propose the association between the MICB rs3132468 genotype and susceptibility to severe dengue might reflect altered or dysfunctional NK and/or CD8+ T cell activation early in infection that results in a higher viral burden in vivo, a recognized factor in clinical outcome (Libraty, Endy, et al., 2002; Vaughn et al., 2000). The recent finding that a SNP near the closely related MICA gene (rs2596542) is associated with Hepatitis C virus induced hepatocellular carcinoma is suggestive of a pivotal role for MIC proteins in the pathogenesis of these Flaviviridae infections (V. Kumar et al., 2011).

Mutations within PLCE1 are associated with nephrotic syndrome(Hinkes et al., 2006). Nephrotic syndrome is a kidney disorder in which dysfunction of the glomeruli basement membrane results in proteinuria and hypoproteinemia that when severe leads to reduced vascular oncotic pressure and edema. These elements of nephrotic syndrome have striking similarities with severe dengue and suggest an important role for PLCE1 in maintaining normal vascular endothelial cell barrier function. In addition, mutations within PLCE1 are

associated with cancer such as colorectal (Duan et al., 2014), esophageal (Cui et al., 2013), and gastrointestinal cancer (Hao et al., 2013), which suggested that the progression of DSS may involve similar mechanism as the progression of cancers. However, future studies would be required to determine the association between DSS and cancer.

Significant associations were observed between *MICB* rs3132468 and *PLCE1* rs3740360 and both clinical phenotypes of non-DSS and DSS. In addition, we observed association between both SNP genotypes and infants with non-DSS dengue at effect sizes comparable to that seen with children DSS in the GWAS study. Finally, amongst children and adults with DSS in a separate cohort, we were able to confirm association of *MICB* rs3132468 and *PLCE1* rs3740360 that was first observed in the previous GWAS. The association of the MICB rs3132468 genotype with DSS and non-DSS indicates a role for this variant in susceptibility to overall clinically apparent dengue and not just severe disease. The effect size between the PLCE1 rs3740360 risk genotype and non-DSS was less pronounced than that observed for DSS cases. In light of the observed association with PLCE1 and nephrotic syndrome it is interesting that the degree of proteinuria has been proposed as a potential predictor in determining which dengue patients are at risk of developing more severe disease (Vasanwala et al., 2011; Vasanwala et al., 2014). Recently, the association of these SNPs was also replicated in children dengue patients in Thailand (Dang et al., 2014) .

The association between MICB rs3132468 and dengue in infants, with effect sizes in keeping with that observed in DSS patients reflects a prominent role for innate immunity and particularly NK cells in controlling early viral infection in infants; impaired control of viral replication could be a risk factor for clinically apparent dengue in this age group.

This is further supported by the elevation of soluble MICB during acute primary DENV infections in infants, as a potential immune evasion strategy that may have contributed to the severity of the acute illness (Libraty, Zhang, Obcena, Brion, & Capeding, 2014). The effect size observed at PLCE1 rs3740360 in infants was also similar to that observed in DSS patients. It has been noted that hospitalized infants with dengue represent a group with the highest risk of death, and it is thought that this is partly related to an intrinsically more permeable vascular endothelium in this age group (Anders et al., 2011). In infants with dengue, carriage of either risk alleles thus represent an additional risk variable alongside the presence of maternally-derived non-neutralizing antibodies and poor compensatory reserve (Chau et al., 2010; Kliks, Nimmanitya, Nisalak, & Burke, 1988).

Our study has several limitations. Misclassified control samples will be more common in non-severe dengue study than in the original GWAS of DSS cases because dengue without shock is a more common clinical outcome for a given cohort of children in an endemic area. Reassuringly, the fact that consistent associations were observed despite this limitation lends credence to our observations. In addition, as the functional basis of these mutations is yet to be clearly defined, our conclusions are to an extent speculative. As dengue without shock includes a diverse range of clinical manifestations, our ability to determine this functional basis is more limited.

We have shown that the MICB rs3132468 and PLCE1 rs3740360 genotypes are associated with clinically apparent dengue in both adults and children, including infants, which is a significant extension from the earlier GWAS on children DSS cases alone. As expected, the effect sizes of these variants is small and underscores that susceptibility to symptomatic dengue is multifactorial and includes epidemiological risk factors (e.g. age).

However, we have not performed multivariate analysis in this study as the majority of risk factors for symptomatic (non-severe) dengue are not clearly defined. The challenge now is also to define the functional basis for these observed genetic associations at MICB and PLCE1 and thus increase our understanding of disease pathogenesis. Future studies would also include targeted resequencing of these two genes (MICB and PLCE1) to determine the potential causal variant of dengue severity.

CHAPTER 4: EPIDEMIOLOGICAL RISK FACTORS OF DENGUE DISEASE SEVERITY

4.1.INTRODUCTION

In several Asian countries, dengue is one of the leading causes of hospitalization and death among children (WHO, 1997, 2009, 2010a). However, in Singapore, there has beena decreasing trend of children (aged <15 years) and an increasing trend of adults (aged ≥25 years) being infected with dengue since 1982 (Ooi et al., 2006). Molecular determinants of DHF such as virus variation, viral load, antibody-dependent enhancement (ADE), 'original antigenic sin', 'cytokine storm' and plasma factors were proposed in the pathophysiology of DHF (Rothman, 2003, 2011; Srikiatkhachorn & Green, 2010). However, predicting or preventing the occurrence of DHF remains a challenge. Identifying epidemiological risk factors for DHF can facilitate prevention, clinical, and healthcare resource management. Epidemiological risk factors for DHF that have been identified include dengue-serotype 2 (Guzman et al., 1990; S. Thein et al., 1997), Asian genotype (Watts et al., 1999), prior dengue infections (Burke et al., 1988; Guzman et al., 1984), children (Guzman et al., 2002; Ooi et al., 2003), age >65 years (M. S. Lee et al., 2006), and white females (Figueiredo et al., 2010; Guzman et al., 1984)

Co-morbidities were reported as risk factors for DHF in a number of studies from dengue endemic countries. These co-morbidities included sickle cell anemia (Bravo et al., 1987), asthma (Bravo et al., 1987; Cunha et al., 1999; D. Gonzalez et al., 2005), hypertension (Cunha et al., 1999; Figueiredo et al., 2010; M. S. Lee et al., 2006), uremia

(M. S. Lee et al., 2006), allergies treated with corticosteroid (Figueiredo et al., 2010) and diabetes mellitus (Bravo et al., 1987; Cunha et al., 1999; Figueiredo et al., 2010; M. S. Lee et al., 2006). However, these co-morbidities may not be generalized to all populations and epidemics of all dengue serotypes. Furthermore, most of these risk factors were identified from univariate analysis (Bravo et al., 1987; Cunha et al., 1999; D. Gonzalez et al., 2005; M. S. Lee et al., 2006) instead of multivariate analysis to adjust for potential confounders (Figueiredo et al., 2010). In this study, we explored demographic and co-morbidity risk factors for DHF in Singapore in the year 2006 (where dengue serotype 1 predominated) and in the year 2007 and 2008 (where dengue serotype 2 predominated) (K. S. Lee et al., 2010).

4.2.RESULTS

(Publication 3: **Pang J,** Salim A, Lee VJ, et al. **Diabetes with hypertension as risk factors for adult dengue hemorrhagic fever in a predominantly Dengue serotype 2 epidemic: A case control study.** *PLoS NTD*. 6(5): e1641.)

Demographic and co-morbidity profiles of DHF & DF patients during the two epidemics

In year 2006 epidemic, there were 149 DHF and 326 DF patients. Among these patients, there were 131 (27.6%) patients who were PCR positive and 344 (72.4%) patients who were serology positive but PCR negative. The mean age was 37.3 (±12.8) years and 34.0 (±11.0) years for DHF patients and DF patients respectively. Among DHF patients, there were 67.8% male and 77.2% Chinese. Of the 326 DF patients, 71.5% were male and 62.9% were Chinese (Table 3). In year 2007 and 2008 epidemic, there were 669 DHF and 1,141 DF patients. Among these patients, there were 590 (32.6%) patients who were PCR positive and 1220 (67.4%) patients who were serology positive but PCR negative. The mean age was 38.4 (±13.4) years and 36.2 (±12.9) years for DHF patients and DF patients respectively. Among the DHF patients, there were 58.7% male and 77.1% Chinese. Of the 1,141 DF patients, 70.9% were male and 62.8% were Chinese (Table 3).

Of the demographic variables, statistically significant differences ($P<0.05$) were found between DHF and DF with respect to mean age ($P=0.008$), age groups ($P=0.017$) and ethnicity ($P=0.021$) in year 2006, and mean age ($P<0.001$), age groups ($P=0.002$), gender ($P<0.001$) and ethnicity ($P<0.001$) in year 2007 and 2008 (Table 3). Using the number of fever days before hospital presentation as a surrogate index of health-seeking behavior between DHF and DF patients, no significant difference was observed in both

year 2006 (P=0.941) as well as year 2007-2008 (P=0.308) (Table 3). Notably, statistically significant differences were found between DHF and DF with respect to hypertension (P=0.036) and diabetes mellitus (P=0.004) in year 2007 and 2008 but not year 2006 (Table 3).

Table 3. Distribution of demographic characteristics and co-morbidities by cases (DHF) and controls (DF) in year 2006 and year 2007-2008 epidemics.

Year	2006 (N=475)						2007-2008 (N=1810)					
Exposure	Cases (N=149)		Controls (N=326)		Case : Control		Cases (N=669)		Controls (N=1141)		Case : Control	
	N	%	N	%		P-value$^{\Delta}$	N	%	N	%		P-value$^{\Delta}$
Age (Years)												
Mean (SD)	37.3	(12.8)	34.0	(11.0)		**0.008^**	38.4	(13.4)	36.2	(12.9)		**<0.001^**
<30	43	28.9	115	35.3	1 : 2.67		180	26.9	405	35.5	1 : 2.25	
30-39	46	30.9	127	39.0	1 : 2.76		209	31.2	328	28.8	1 : 1.57	
40-49	39	26.2	58	17.8	1 : 1.49		152	22.7	213	18.7	1 : 1.40	
50-59	12	8.1	19	5.8	1 : 1.58		76	11.4	132	11.6	1 : 1.74	
≥ 60	9	6.0	7	2.2	1 : 0.78	**0.017**	52	7.8	63	5.5	1 : 1.21	**0.002**
Gender												
Male	101	67.8	233	71.5	1 : 2.31		393	58.7	809	70.9	1 : 2.06	
Female	48	32.2	93	28.5	1 : 1.94	0.414	276	41.3	332	29.1	1 : 1.20	**<0.001**
Ethnicity												
Others	15	10.1	60	18.4	1 : 4.00		79	11.8	188	16.5	1 : 2.38	
Chinese	115	77.2	205	62.9	1 : 1.78		516	77.1	717	62.8	1 : 1.39	
Malay	4	2.7	12	3.7	1 : 3.00		38	5.7	62	5.4	1 : 1.63	
Indian	15	10.1	49	15.0	1 : 3.27	**0.021**	36	5.4	174	15.3	1 : 4.83	**<0.001**
Fever DBP												
Mean (SD)	4.9	(1.5)	4.9	(1.6)		0.941^	4.9	(1.7)	5.0	(1.8)		0.308^
Hypertension												
No	140	94.0	315	96.6	1 : 2.25		594	88.8	1047	91.8	1 : 1.76	
Yes	9	6.0	11	3.4	1 : 1.22	0.179	75	11.2	94	8.2	1 : 1.25	**0.036**
Diabetes												
No	147	98.7	319	97.9	1 : 2.17		626	93.6	1101	96.5	1 : 1.76	
Yes	2	1.3	7	2.2	1 : 3.50	0.726$^{\#}$	43	6.4	40	3.5	1 : 0.93	**0.004**
Hyperlipidemia												
No	145	97.3	317	97.2	1 : 2.19		612	91.5	1061	93.0	1 : 1.73	
Yes	4	2.7	9	2.8	1 : 2.25	1.000$^{\#}$	57	8.5	80	7.0	1 : 1.40	0.241
Asthma												
No	145	97.3	311	95.4	1 : 2.15		637	95.2	1082	94.8	1 : 1.70	
Yes	4	2.7	15	4.6	1 : 3.75	0.323	32	4.8	59	5.2	1 : 1.84	0.740

$^{\Delta}$Person's Chi-square, unless otherwise annotated; ^ Mann-Whitney U test; $^{\#}$ Fisher's Exact test;
DHF-Dengue hemorrhagic fever; DF- Dengue fever; DBP- Days before presentation in hospital

Independent risk factors for DHF

Chinese ethnicity was the only significant risk factor independently associated with DHF in year 2006, after adjustment for statistically significant univariate risk factors (Table 4). Although marginally significant, the likelihood (AOR) of a Chinese patient developing DHF was 1.90 (95% CI: 1.01-3.56) times higher than that of other ethnicity (not Chinese, Malay or Indian). In year 2007 and 2008, age groups, gender and ethnicity were observed to be independently associated with DHF, following adjustment for statistically significant univariate risk factors (Table 4). The likelihood (AOR) of an individual who were 30 to 39 years of age and 40 to 49 years of age developing DHF was 1.41 (95% CI:1.09-1.81) and 1.34 (95% CI:1.09-1.81) times higher than that of an individual below 30 years old respectively. Females had 1.57 (95% CI: 1.28-1.94) times higher risk developing DHF than males. In addition, the likelihood (AOR) of a Chinese patient developing DHF was 3.15 (95% CI: 2.34-4.23) and 1.67 (95% CI: 1.24-2.24) times higher than that of Indian and other ethnicity respectively (Table 4).

For co-morbidities, after adjustment for statistically significant univariate risk factors, only diabetes mellitus remained an independent risk factor for DHF outcome (AOR=1.78; 95% CI:1.06-2.97) in year 2007 and 2008 (Table 5). Diabetic patients tend to have other co-morbidities. We investigated the risk effect of DHF outcome on patients having diabetes mellitus with hypertension, hyperlipidemia or asthma. Diabetes mellitus with hypertension (COR=2.43; 95% CI: 1.42-4.15), diabetes mellitus with hyperlipidemia (COR=1.82; 95% CI: 1.06-3.12) and diabetes mellitus with no asthma (COR=1.74; 95% CI: 1.10-2.76) were observed to be significantly associated with DHF outcome (Table 5). However, only diabetes mellitus with hypertension (AOR=2.16; 95% CI: 1.18-3.96) and diabetes with no asthma (AOR=1.68; 95% CI: 1.02-2.76) were observed to be

independently associated with DHF outcome after adjustment for statistically significant univariate risk factors (Table 5). Interestingly, the likelihood (AOR) of an individual having diabetes mellitus with asthma developing DHF was 4.38 (95% CI: 0.80-23.85) times higher than that of an individual having no diabetes with no asthma. However, there is a lack of statistical significance and it is most likely due to the small sample size with only 7 subjects having diabetes mellitus with asthma (Table 5). In order to confirm this observed phenomenon, further studies with larger sample size are required. In addition, among patients with hypertension, the likelihood (AOR) of developing DHF due to diabetes mellitus was higher (AOR=2.39; 95% CI: 1.21-4.71) compared to that of patients without hypertension (AOR=1.28; 95% CI: 0.56-2.93; Table 6). This provided preliminary evidence of effect modification between diabetes mellitus and hypertension on the risk of DHF outcome. Moreover, it was observed that the mean hospitalization days was longer for diabetic patients (4.99±3.34 days) compared to non-diabetic patients (4.04±1.62 days; P=0.001). A significant difference was also observed in the mean hospitalization days between diabetic DHF patients and non-diabetic DHF patients (diabetic DHF: 5.21±3.12 days; non-diabetic DHF: 4.33±1.75 days; P=0.046) (data not shown).

Subgroup analyses of patients with dengue IgG data and of Chinese patients were carried out. In the subgroup analysis of 1,220 (67.4%) patients hospitalized during the year 2007-2008 that had dengue IgG data, we further showed that diabetes (AOR: 1.92; 95% CI: 1.02-3.61) as well as diabetes with hypertension (AOR: 4.41; 95% CI: 1.16-16.82) remained as a risk factor of DHF. Furthermore, in a subgroup analysis of cases (DHF) and controls (DF) identified as Chinese in year 2007 and 2008, diabetes mellitus (AOR=2.23; 95% CI:1.21-4.11), diabetes mellitus with hypertension (AOR=2.1; 95% CI:1.07-4.12), diabetes mellitus with no hyperlipidemia (AOR=3.75; 95% CI:1.27-11.02) and diabetes

mellitus with no asthma (AOR=1.96; 95% CI:1.09-3.52) were independently associated with DHF outcome, after adjustment for age groups, gender, and hypertension (data not shown).

Table 4. Crude and adjusted odds ratios of the association of DHF with age groups, gender and ethnicity in year 2006 and year 2007-2008 epidemics.

Year	2006				2007-2008			
Exposure	**COR**	**95% CI**	**AOR***	**95% CI**	**COR**	**95% CI**	**AOR****	**95% CI**
Age (Years)								
<30	1		1		1		1	
30-39	0.97	0.60 - 1.58	0.92	0.56 - 1.50	**1.43**	**1.12 - 1.84**	**1.41**	**1.09 - 1.81**
40-49	**1.80**	**1.05 - 3.07**	1.53	0.88 - 2.67	**1.61**	**1.22 - 2.11**	**1.34**	**1.09 - 1.81**
50-59	1.69	0.76 - 3.77	1.47	0.65 - 3.31	1.30	0.93 - 1.81	0.91	0.63 - 1.30
≥ 60	**3.44**	**1.20 - 9.81**	2.71	0.94 - 7.88	**1.86**	**1.24 - 2.79**	1.14	0.70 - 1.85
Gender								
Male	1		1		1		1	
Female	1.19	0.78 – 1.81	1.14	0.74 – 1.78	**1.71**	**1.40 – 2.09**	**1.57**	**1.28 – 1.94**
Ethnicity								
Others	1		1		1		1	
Chinese	**2.24**	**1.22 – 4.13**	**1.90**	**1.01 – 3.56**	**1.71**	**1.29 – 2.28**	**1.67**	**1.24 – 2.24**
Malay	1.33	0.38 – 4.72	1.15	0.32 – 4.13	1.46	0.90 – 2.36	1.30	0.79 – 2.13
Indian	1.22	0.55 – 2.75	1.19	0.53 – 2.68	**0.49**	**0.32 – 0.77**	**0.53**	**0.34 – 0.83**

* Adjusted odds ratio was obtained from a multivariate logistic regression being adjusted by age groups and ethnicity.
** Adjusted odds ratio was obtained from a multivariate logistic regression being adjusted by age groups, gender, ethnicity, diabetes mellitus and hypertension.
DHF- Dengue Hemorrhagic Fever
COR- Crude odds ratio
AOR- Adjusted odds ratio
CI- Confidence interval

Table 5. Crude and adjusted odds ratios of the association of DHF with co-morbidities in year 2006 and year 2007-2008 epidemics.

Year	2006				2007-2008			
Exposure	**COR**	**95% CI**	**AOR***	**95% CI**	**COR**	**95% CI**	**AOR****	**95% CI**
Hypertension								
No	1		1		1		1	
Yes	1.84	0.74 - 4.54	0.97	0.31 - 3.00	**1.41**	**1.02 - 1.94**	1.06	0.70 - 1.60
Diabetes								
No	1		1		1		1	
Yes	0.62	0.13 - 3.02	0.34	0.06 - 1.89	**1.89**	**1.21 - 2.94**	**1.78**	**1.06 - 2.97**
Hyperlipidemia								
No	1		1		1		1	
Yes	0.97	0.29 - 3.20	0.54	0.15 - 1.96	1.24	0.87 - 1.76	0.79	0.50 - 1.26
Asthma								
No	1		1		1		1	
Yes	0.57	0.19 - 1.75	0.51	0.16 - 1.62	0.92	0.59 - 1.43	0.86	0.55 - 1.35

*Adjusted Odds Ratio was obtained from a multivariate logistic regression being adjusted by age groups and ethnicity.
** Adjusted odds ratio was obtained from a multivariate logistic regression being adjusted by age groups, gender, ethnicity, diabetes mellitus and hypertension.
DHF- Dengue Hemorrhagic Fever
COR- Crude odds ratio
AOR- Adjusted odds ratio
CI- Confidence interval

Table 6. Crude and adjusted odds ratios of the association of DHF with multiple co-morbidities in year 2007-2008 epidemic.

Exposures	Cases N	Controls N	COR	95% CI	AOR*	95% CI
Diabetes						
No	626	1101	1		1	
Yes	43	40	**1.89**	**1.21 - 2.94**	**1.78**	**1.06 - 2.97**
Diabetes, Hypertension						
No diabetes with no hypertension	584	1031	1		1	
No diabetes with hypertension	42	70	1.06	0.71 - 1.57	0.97	0.62 - 1.52
Diabetes with no hypertension	10	16	1.1	0.50 - 2.45	1.26	0.55 - 2.87
Diabetes with hypertension	33	24	**2.43**	**1.42 - 4.15**	**2.16**	**1.18 - 3.96**
Diabetes, Hyperlipidemia						
No diabetes with no hyperlipidemia	597	1048	1		1	
No diabetes with hyperlipidemia	29	53	0.96	0.60 - 1.53	0.82	0.50 - 1.37
Diabetes with no hyperlipidemia	15	13	2.03	0.96 - 4.29	2.03	0.93 - 4.47
Diabetes with hyperlipidemia	28	27	**1.82**	**1.06 - 3.12**	1.62	0.90 - 2.92
Diabetes, Asthma						
No diabetes with no asthma	599	1044	1		1	
No diabetes with asthma	27	57	0.83	0.52 - 1.32	0.79	0.49 - 1.27
Diabetes with no asthma	38	38	**1.74**	**1.10 - 2.76**	**1.68**	**1.02 - 2.76**
Diabetes with asthma	5	2	4.36	0.84 - 22.53	4.38	0.80 - 23.85

*Adjusted odds ratio was obtained from a multivariate logistic regression being adjusted by age groups, gender, ethnicity, diabetes mellitus and hypertension; DHF- Dengue Hemorrhagic Fever
COR- Crude odds ratio; AOR- Adjusted odds ratio; CI- Confidence interval

Table 7. Stratified analysis between diabetes mellitus and other comorbidities in year 2007-2008 epidemic.

Exposures	Cases N	Controls N	COR	95% CI	AOR*	95% CI
Diabetes						
No	626	1101	1		1	
Yes	43	40	**1.89**	**1.21 - 2.94**	**1.78**	**1.06 - 2.97**
No hypertension						
No diabetes	584	1031	1		1	
Diabetes	10	16	1.10	0.50 – 2.45	1.28	0.56 – 2.93
Hypertension						
No diabetes	42	70	1		1	
Diabetes	33	24	**2.29**	**1.20 – 4.39**	**2.39**	**1.21 – 4.71**
No hyperlipidemia						
No diabetes	597	1048	1		1	
Diabetes	15	13	2.03	0.96 – 4.29	1.95	0.88 – 4.36
Hyperlipidemia						
No diabetes	29	53	1		1	
Diabetes	28	27	1.90	0.94 – 3.80	2.03	0.93 – 4.41
No asthma						
No diabetes	599	1044	1		1	
Diabetes	38	38	**1.74**	**1.10 – 2.76**	**1.77**	**1.03 – 3.00**
Asthma						
No diabetes	27	57	1		1	
Diabetes	5	2	5.28	0.96 – 29.0	1.01	0.04 – 25.15

*Adjusted odds ratio was obtained from a multivariate logistic regression being adjusted by age groups, gender, ethnicity, diabetes mellitus and hypertension; DHF- Dengue Hemorrhagic Fever
COR- Crude odds ratio; AOR- Adjusted odds ratio; CI- Confidence interval.

4.3.DISCUSSION AND CONCLUSION

The results of this study showed that female, Chinese, age group between 30 to 49 years, pre-existing diabetes mellitus or diabetes mellitus with hypertension were risk factors of developing DHF during the year 2007 and 2008 epidemic when dengue serotype 2 was predominant. In contrast, Chinese ethnicity was the only risk factor observed during the year 2006 epidemic when dengue serotype 1 was predominant. This might be due to the different predominant circulating dengue serotypes during the two epidemics (K. S. Lee et al., 2010). Notably, dengue serotype 2 was known to be associated with more severe dengue disease than serotype 1 (Fried et al., 2010; Guzman et al., 1990; S. Thein et al., 1997). In a combined analysis of year 2006, 2007 and 2008 epidemic, all the risk factors identified in the year 2007-2008 epidemic remained as independent risk factors except for diabetes mellitus. This may suggest potential confounding effect of different serotypes. Furthermore, it was observed that age, gender and co-morbidities were not independently associated with DHF outcome in a previous study of 1,973 adult dengue patients in the year 2004 epidemic when dengue serotype 1 was also predominant (V. J. Lee et al., 2008). However, it is not possible to conclusively demonstrate serotype difference during epidemics as the main factor that accounted for the differences in risk factors in this study. This was because we do not routinely serotype all individual infections and this was hence not available for analyses. Instead, the differences in risk factors may be due to the small sample size of patients admitted in year 2006, and the significant differences in mean age, number of patients with co-morbidities and DHF outcome admitted during the two epidemics. It is beyond the scope of this study to highlight other potential factors, such as climate change, viral genotype change as well as change in health-seeking behaviors that may have also resulted in the differences.

It is not surprising that female and Chinese ethnicity were independent risk factors of DHF as gender (Anders et al., 2011; Guzman et al., 1984) and ethnicity (Figueiredo et al., 2010; Guzman et al., 1984) were shown to be risk factors for DHF in Cuba and Brazil studies as well as in Vietnam for dengue shock syndrome (DSS). Age groups between 30 and 39 and between 40 and 49 were independent risk factors of DHF in our adult dengue cohort. This observation is different from previous studies in Cuba (Guzman et al., 2002) and in Singapore (Ooi et al., 2003) where children <14 years of age had higher risk of developing DHF compared to young adults aged 15 years or greater. The rationale behind this difference could be due to lowered herd immunity and change in transmission patterns (Ooi et al., 2003; Ooi, Hart, Tan, & Chan, 2001). The elderly (>65 years of age) in Taiwan (M. S. Lee et al., 2006) had a higher risk of developing DHF. However, the age group ≥60 year was not an independent risk factor of DHF outcome (Table 3) in our current study which is also consistent with our previous study on dengue in older adults (Lye, Lee, Sun, & Leo, 2010). It is still not well understood how these risk factors contribute to the pathophysiology of DHF, and understanding the underlying mechanism may facilitate clinical management.

Co-morbidities such as hypertension, diabetes mellitus, hyperlipidemia and asthma are among the few leading causes of mortality and morbidity in Asia (WHO, 2005) and globally (Geneau et al., 2010; WHO, 2010b). Co-morbidities were shown to be associated with severe clinical manifestations of several infectious disease such as SARS (Booth et al., 2003; Lau et al., 2010), pandemic influenza H1N1 (Jain et al., 2009), tuberculosis (Dooley & Chaisson, 2009; Dooley, Tang, Golub, Dorman, & Cronin, 2009), hepatitis C (Charlton, Pockros, & Harrison, 2006) and community-acquired infections (Jackson, 2005; Muller et al., 2005). Many studies found association between various co-morbidities and

DHF outcome (Bravo et al., 1987; Cunha et al., 1999; Figueiredo et al., 2010; D. Gonzalez et al., 2005; M. S. Lee et al., 2006) but only one study was carried out with multivariate analysis to adjust for potential confounders (Figueiredo et al., 2010). Furthermore, none has evaluated the risk effect of two existing co-morbidities and the effect modification between two co-morbidities on DHF outcome. In this study, we showed that diabetes mellitus was associated with DHF outcome as observed by other studies (Bravo et al., 1987; Cunha et al., 1999; Figueiredo et al., 2010). In addition, we observed that individuals reported as having diabetes mellitus with hypertension had higher risk of developing DHF compared with individuals with no diabetes mellitus and no hypertension. Our study may be the first that provide the preliminary evidence of synergy of risk effects between diabetes mellitus and hypertension on DHF outcome (Tables 5 & 6). Our study showed concomitant diabetes mellitus with hypertension as an independent risk factor for DHF in a large number of adult DHF cases in Singapore, and supported the initial evidence of association between hospitalization with a diagnosis of DHF and diabetes mellitus in Brazil(Figueiredo et al., 2010). However, the pathophysiology behind diabetes leading to DHF outcome is not well understood yet, even though numerous studies had suggested that diabetes mellitus can result in immune and endothelial dysfunction (Dandona, Aljada, Chaudhuri, & Mohanty, 2004; Geerlings & Hoepelman, 1999; Gonzalez-Curiel et al., 2011; Hsueh, Lyon, & Quinones, 2004; Kaye et al., 1986).

Identifying risk factors for DHF can guide clinicians to triage dengue patients for the right site of care for closer monitoring and early intervention with fluid resuscitation. In an epidemic where healthcare resources may be stretched, risk factors for DHF can be used to prioritize hospitalization of dengue patients. In our study, we observed that diabetic patients with DHF outcome required longer stay and, presumably, required more medical

attention in the hospital compared to non-diabetic patients with DHF. Additionally, policy makers can prioritize population groups at high risk of developing DHF such as female patients, patients in age group 30-49, and patients having diabetes or diabetes with hypertension for vaccination when dengue vaccines are available. Demographic and co-morbidity risk factors may help public health clinicians raise awareness among high-risk individuals to take preventive measures against dengue infections.

As this is a retrospective study, the quality of the study was dependent on the quality of the data available and collected. Information bias was minimized by the use of the standardized dengue care path for consistent clinical documentation. Reporting bias was minimized by the fact that patients with comorbidities tend to know their existing condition and are likely to be on constant medication. However, it is challenging to exclude the fact that there are no undiagnosed existing comorbidities among some of these patients as this study is performed retrospectively. In addition, there may be selection bias because the subjects were all hospitalized and hence were likely to have active health care-seeking behaviour, and the controls were hospitalized DF patients who may not truly represent the general population. In the general population, less active health care-seeking, asymptomatic or mild DF patients may not visit a doctor or hospital and may also have diabetes mellitus or other co-morbidities assessed in this study. However, it is technically challenging to identify these less active health care-seeking, asymptomatic or mild DF patients for inclusion in the study. We also did not have patient-specific dengue serotype data and could only extrapolate our observations from a previous population level study in Singapore (K. S. Lee et al., 2010). Lastly, we acknowledge the importance of accounting for prior infection as it is a main risk factor for DHF. The result of IgG test carried out within seven days of fever onset can be used to classify patients with or without prior

infection (Cuzzubbo et al., 2001; Thomas et al., 2008). However, we only had IgG results of 67.4% of all patients during the year 2007-2008. In the subgroup analysis, we showed that prior infection was not significantly associated with DHF in adult patients. It has been shown that prior infection was strongly associated with DHF, but this was in children under the age of 15 years (Fried et al., 2010; Gubler, 1998). Our study instead suggests that diabetes as well as diabetes with hypertension may be one of the key risk factors of DHF in adults, regardless of prior dengue infections. Further studies involving larger number of patients with acute secondary infections are required to confirm this hypothesis. Lastly, there is a lack of Body Mass Index (BMI) or obesity data which could be a potential confounder for the relationship between diabetes, hypertension and dengue severity.

In conclusion, we found age between 30 and 49 years, female gender, Chinese ethnicity, diabetes mellitus and diabetes mellitus with hypertension to be independent risk factors for DHF in an adult dengue epidemic with predominantly dengue serotype 2. The two co-morbidities appeared to have effect modification on the risk of DHF outcome. More studies, particularly prospective studies are required to confirm these findings. Our finding raised the likely association between the pathophysiology of diabetes mellitus, hypertension and dengue severity. An ongoing genome-wide association study in Singapore may help elucidate genetic predisposition to severe dengue disease including the role of diabetes mellitus.

CHAPTER 5: MOLECULAR RISK FACTORS OF DENGUE DISEASE SEVERITY

5.1.INTRODUCTION

Based on the WHO 2009 new classification of dengue disease, patients with symptomatic infection may be classified as having dengue without warning signs, dengue with warning signs or severe dengue as possible outcomes of infection, in order of increasing severity. Severe dengue infection is characterized by severe plasma leakage, severe bleeding, and severe organ involvement which can proceed to shock if left untreated, that usually occurs at time of defervescence, four to seven days after fever onset. Specific antiviral therapy is currently not available and case management is entirely supportive. The standard-of-care for dengue disease is not specific, but rather directed towards constantly monitoring patients for early warning signs, with the aim of providing appropriate and timely fluid support, to prevent the development of hypovolemic shock (S Kalayanarooj, 1999). As such, the WHO 2009 classification advocates clinicians to look out for specific presentation of warning signs, as indicators of possible severe dengue manifestations (WHO, 2009).

However, there are a number of challenges encountered when applying the warning signs-guided revised WHO dengue classification. Firstly, it has been found to be too highly sensitive and less specific in identifying severe illness, potentially resulting in significant increase in hospitalization, workload to medical personnel and burden to resource-limited endemic regions (Barniol et al., 2011; S. Kalayanarooj, 2011; Narvaez et al., 2011). Secondly, warning signs generally occurred one day prior to the development of

severe illness/ requirement of intervention or 4-7 days post illness, and this narrow window makes any form of intervention challenging, particularly when appropriate healthcare facilities are remote from their place of residence. On the other hand, it was observed that patients with no warning signs, which typically occurred on median 4-5 days post fever were generally less likely to develop severe dengue and these patients, and it has been proposed that these can be monitored and managed as outpatients(T. L. Thein, Gan, et al., 2013). As such, further refinement of the triage process of patients with warning signs and required hospitalization in the early phase of infection would be particularly useful in the healthcare setting to reduce over-hospitalization(Barniol et al., 2011; Srikiatkhachorn et al., 2011).

As the expression of RNA and proteins are dynamic and sensitive to stimulus from environmental, diet and metabolic changes, as well as pathogen infection, these molecular features when assessed systematically at early infection may provide potential signals of the emerging warning signs, and more importantly, severe clinical outcomes. In 2005, a prospective study was set up in Singapore with the major aim to characterize adults with acute dengue infection at the early stage of illness when a specific intervention could be introduced effectively (Low et al., 2006). In this study, we aim to investigate the molecular features associated with warning signs and the requirement for hospitalization at early infection that could be applied as a point-of-care tool to complement the triage process with the WHO 2009 classification within 72 hr post fever onset for adult patients.

5.2.RESULTS

(Publication 4: **Junxiong Pang**[*], Anna Lindblom[*],Thomas Tolfvenstam,Tun-Linn Thein, Ahmad Nazri Mohamed Naim, Ling Ling, Angelia Chow, Mark Chen-I-Cheng, Eng Eong Ooi, Yee-Sin Leo, Martin L. Hibberd. **Discovery and Validation of Early Biomarkers to Guide Clinical Management of Dengue in Adults with Warning Signs**- Manuscript under review)

Between May 2005 and December 2008, a total of 1,315 dengue patients were enrolled. Among the 212 (16%) patients who had RT-PCR confirmed dengue infection, 47 patients with WS were subsequently hospitalized (**WS+Hosp. Group**; Figure 10). These patients were representative of those that should be prioritized for strict monitoring and interventions at early infection. In addition, 14 (30%) of these patients were classified as severe dengue as the disease progressed (Table 8). Furthermore, 45 patients with no WS and were not hospitalized (**Non-WS+Non-Hosp. Group**; Figure 10) represented a group of mild dengue patients who were safely managed as outpatients and did not develop severe dengue.

Clinical and Laboratory Characteristics of Discovery Cohort.

Among the WS+Hosp. group, 44 (94%), 10 (21%) and 5 (11%) reported mucosal bleeding, persistent vomiting and abdominal pain respectively. Six (14%) patients had signs of mucosal bleeding at Day 1-3 post fever onset (p.f.), 14 (32%) at Day 4-7 p.f., 27 (61%) at time of hospitalization. Moreover, 32 (73%) showed signs of bleeding during hospitalization. Gum bleeding was the most common (n=18) followed by skin (n=12), menstrual bleeding (n=8), nose bleeding (n=5), hematuria (n=3) and per rectal bleeding (n=2). Six of the 10 patients reported persistent vomiting at Day 1-3 p.f. and Day 4-7 p.f.,

and nine of 10 reported it at Day 4-7 p.f. and during hospitalization. Two patients had abdominal pain at Day 1-3 p.f. while 3 patients had abdominal pain at Day 4-7 p.f. The patients in the WS+Hosp. group were admitted in a median of 4 days (range 1-7) p.f. and hospitalized for a median of 3 days (range 1-7) (Table 8). Among patients with severe dengue, 6 had severe plasma leakage, 5 had severe bleeding (2 hematuria, 3 rectal bleeding) and 3 had severe organ involvement (transaminase >1000 U/L). During hospitalization, 44 out of 47 (94%) patients received intravenous fluid replacement.

There were significant differences ($P<0.05$) in viral, platelet and lymphocyte levels between the WS+Hosp. and Non-WS+Non-Hosp. groups (Figure 10). Within the WS+Hosp. group, there was no significant differences in viral, platelet and lymphocyte levels at Day 1-3 p.f. between the 14 patients who progressed to severe dengue and the remaining 33 patients with no progression to severe dengue (Figure 12). However, severe dengue patients had significantly higher viral, lower platelet and lymphocyte levels than Non-WS+Non-Hosp. group (Figure 13).

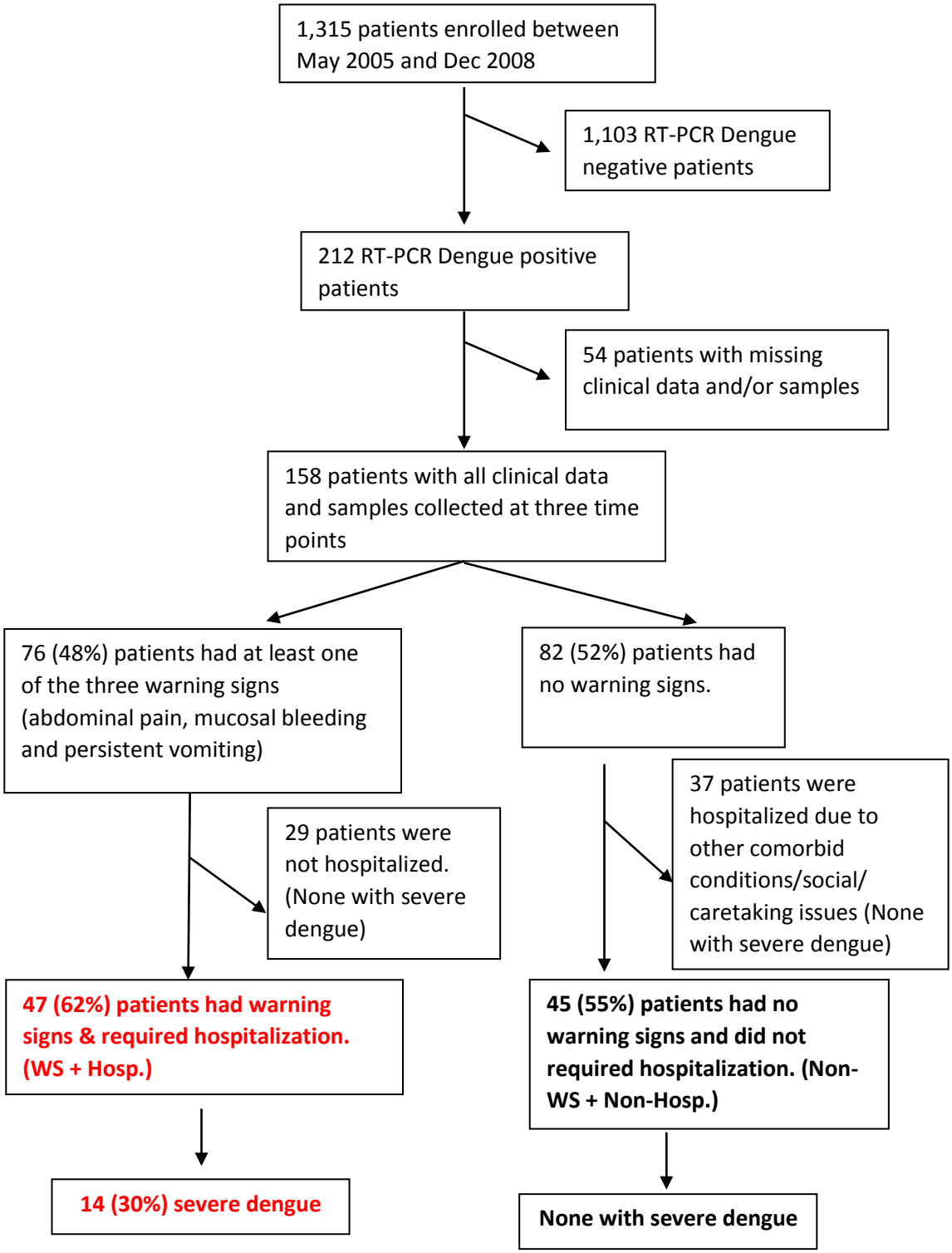


Figure 10. Selection workflow of dengue patients with warning signs that required hospitalization and patients with no warning signs and no hospitalization required.

Table 8. Demographic descriptions of dengue RT-PCR positive patients classified according to designated clinical outcomes.

	EDEN 2005-2008 (Discovery Cohort)					EDEN 2009-2012 (Validation Cohort)				
	Non-WS + Non-Hosp		WS + Hosp			Non-WS + Non-Hosp		WS + Hosp		
	(N=45)	%	(N=47)	%	p-value	(N=55)	%	(N=25)	%	p-value
Age										
Median (Range)	41 (21-63)		37 (19-77)		0.229[#]	33 (25.5-42.5)		41 (25-52)		0.211[#]
Gender										
Female	22	48.9	23	48.9	0.996	7	12.7	10	40	0.006
Ethnicity										
Chinese	32	71.1	39	83.0		31	56.4	16	64.0	
Malay	1	2.2	4	8.5		5	9.1	6	24.0	
Indian	7	15.6	3	6.4		8	14.5	1	4.0	
Others	5	11.1	1	2.1	0.082	11	20.0	2	8.0	0.122
Pre-Existing Comorbid										
Yes	7	15.6	6	12.8	0.701	3	5.5	4	16	0.196^
Serotype										
1	16	35.6	25	53.2		3	5.5	3	12	
2	13	28.9	14	29.8		35	63.6	19	76	
3	16	35.6	8	17.0		0	0	2	8	
4	0	0	0	0	0.098	6	10.9	1	4	0.011
Unknown	0	0	0	0		11	20	0	0	
IgG Status at Presentation										
Positive	20	44.4	25	53.2	0.401	20	36.4	17	68	0.009
Hospitalization										
Median days p.f. on admission (Range)	n.a.		4 (1-7)			n.a.		5 (1-8)		
Length of stay (Range)	n.a.		3 (1-7)			n.a.		4 (2-9)		
Severe Disease										
Yes	0	0	14	30.0	**<0.001^**	0	0	1	4	0.312^

[#] Mann-Whitney Test
^ Fisher's Exact Test
p.f. - post fever onset
n.a. – not applicable

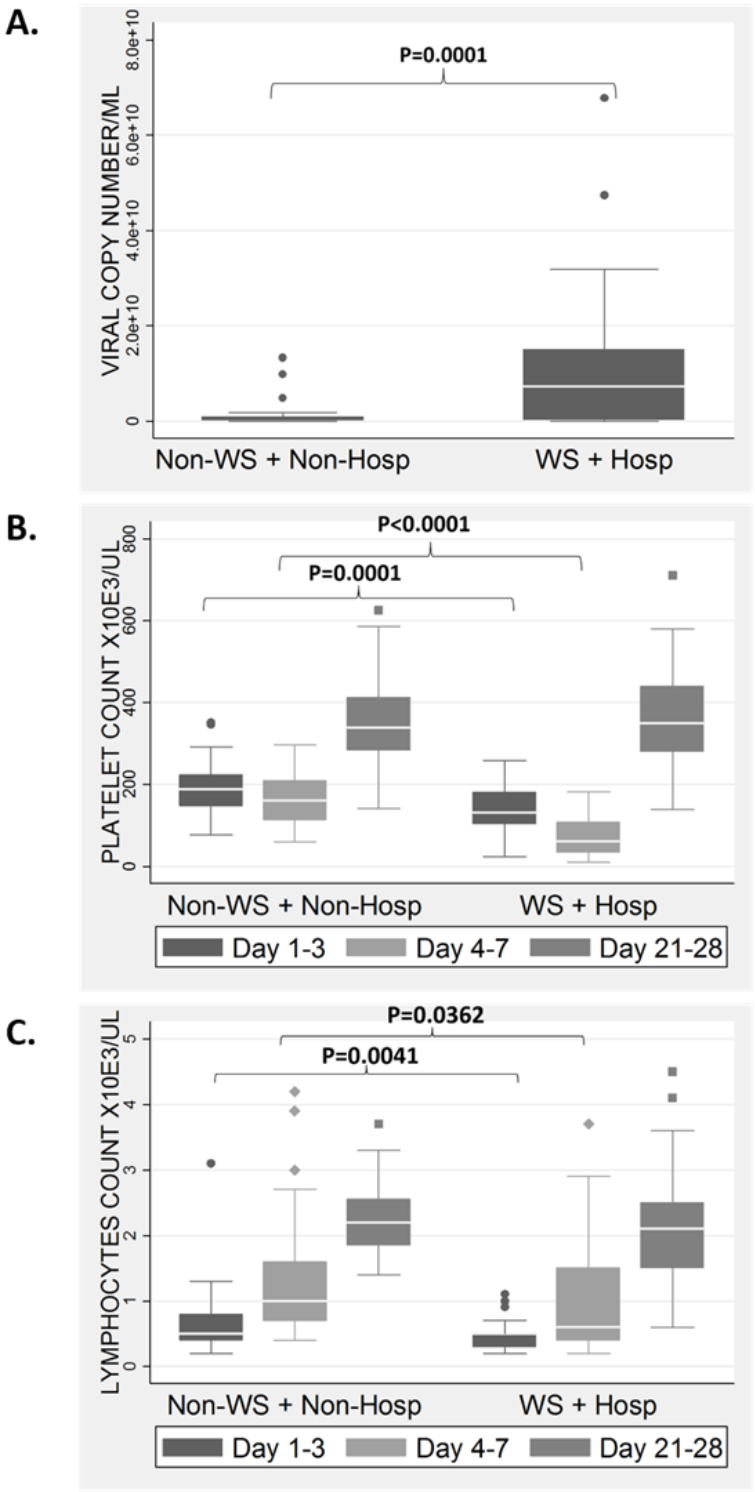


Figure 11. Laboratory characteristics (A-Viral copy number at Day 1-3; B-Platelet count; C-Lymphocytes count) of hospitalized dengue patients with warning signs (WS + Hosp. Group) compared to non-hospitalized patients with no warning signs (Non-WS + Non-Hosp. Group).

P-value (P) is shown only for statistically significant comparisons on Day 1-3 and Day 4-7.

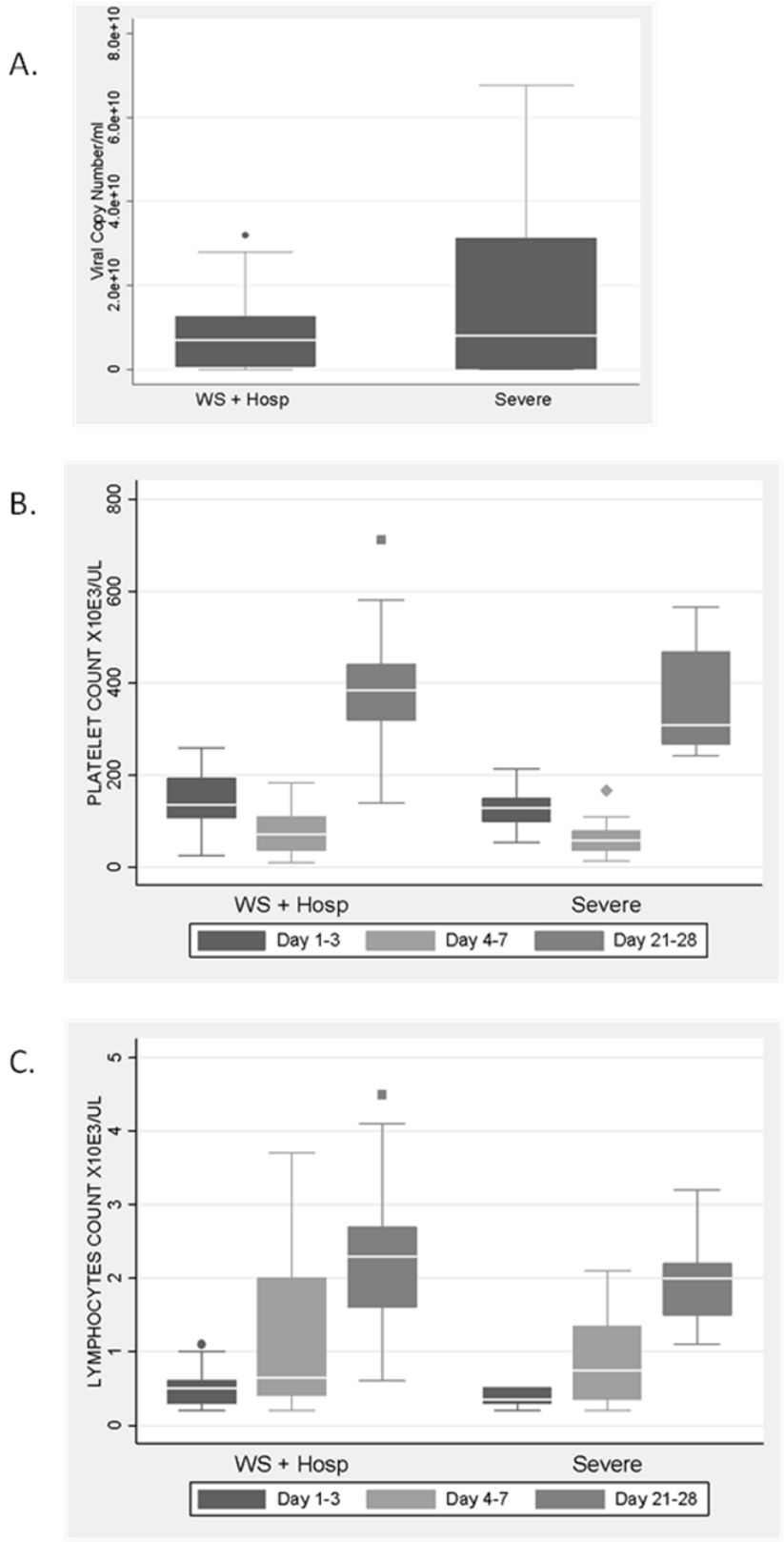


Figure 12. Laboratory characteristics of patients with severe dengue among the hospitalized dengue patients with warning signs (WS + Hosp. Group).
P-value (P) is shown only for statistically significant comparisons on Day 1-3 and Day 4-7.

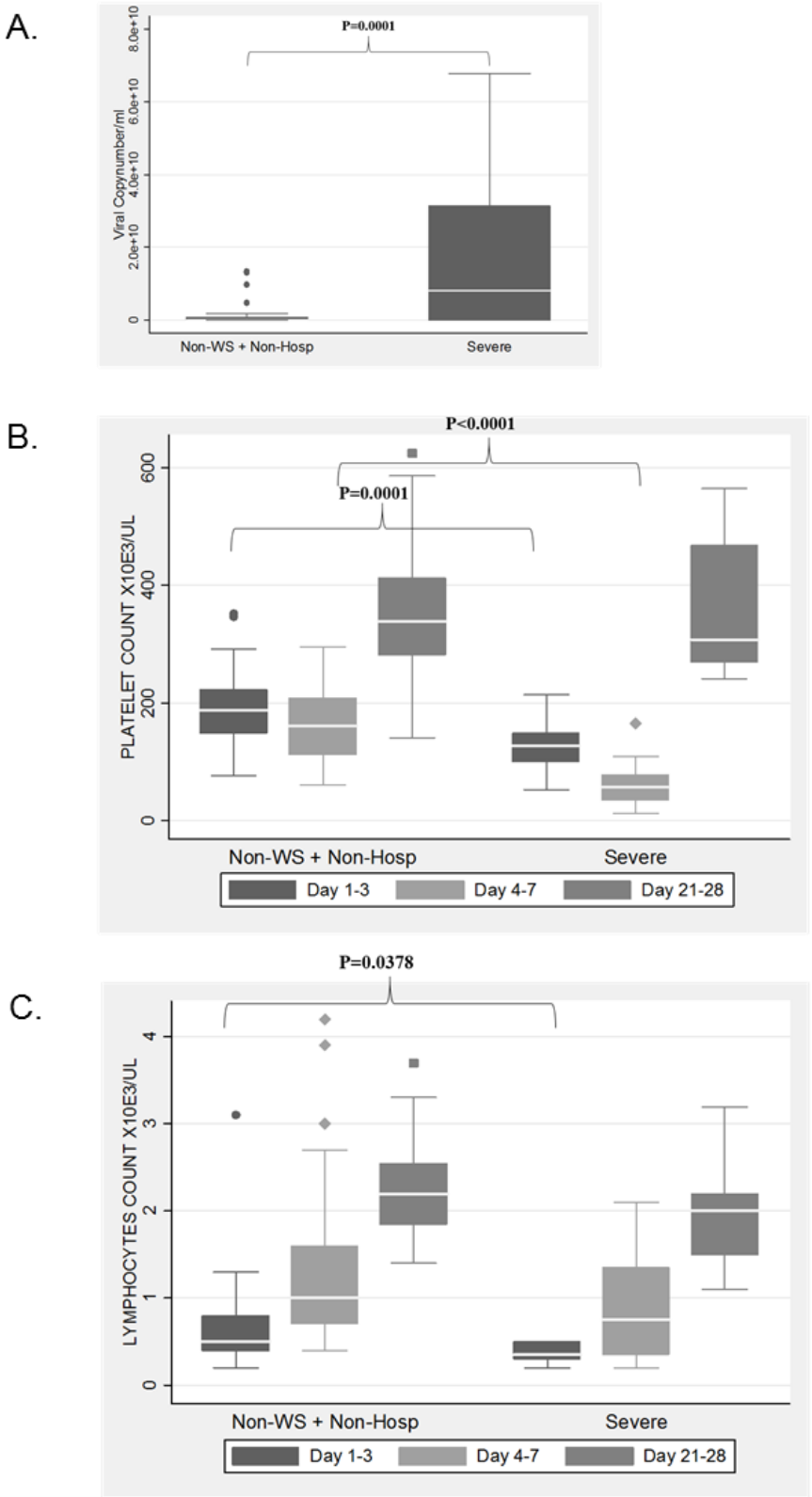


Figure 13. Laboratory characteristics of patients with severe dengue compared to non-hospitalized dengue patients with no warning signs (Non-WS + Non-Hosp. Group).

P-value (P) is shown only for statistically significant comparisons on Day 1-3 and Day 4-7.

Differential transcriptome between WS + Hosp. group and Non-WS + Non-Hosp. group.at less than 72hrs post fever.

To identify the genomic background behind the clinical and laboratory characteristics of the patients with and without warning signs, we compared gene transcript abundance in whole blood from the first sampling point between two groups of patients. After analysis by SAM, 23 genes were found to be significantly differentially expressed. 17 genes were over expressed and six were under expressed in the group of patients with warning signs when compared to the group without warning signs (**Table 9**). Eight of these genes are known to be related to innate immune activation and in particular CCL2 and CCL8, which have previously been described as key responses to dengue infection (Tolfvenstam et al., 2011). Interestingly, when comparing the 14 patients with severe dengue against the non-hospitalized patients without warning signs, IL-8 was also significantly differentially expressed, besides CCL2 and CCL8 (**Table 9**). The expression level of these RNA responses may be informative of the strength of the innate response during the early dengue infection, which may in turn be related to the clinical outcomes observed.

Table 9. Differential genes between hospitalized patients with warning signs (WS + Hosp.), including patients with severe dengue (SD) and non-hospitalized patients without warning signs (Non-WS + Non-Hosp.) at less than 72hr post fever.

Symbol	WS + Hosp. VS Non-WS + Non-Hosp. ≥1.5 Fold change	SD VS Non-WS + Non-Hosp. ≥2 Fold change	Acute vs convalecent[a]*	Dengue vs non dengue[b]
IL-8	-	3.2	N.S.	N.S.
PKD2L1	2.9	2.4	Up reg.	Up reg.
CCL8	2.7	2.5	Up reg.	Up reg.
HESX1	2.6	-	Up reg.	Up reg.
BRDG1	2.5	-	Up reg.	Up reg.
OLFM4	2.4	-	N.S.	N.S.
CCL2	2.3	2.3	Up reg.	Up reg.
SERPINE2	-	2.3	N.S.	N.S.
CD69	2.2	-	N.S.	N.S.
C15orf48	2.0	2.4	N.S.	N.S.
ARL5B	2.0	-	N.S.	N.S.
RIN2	1.8	-	Up reg.	Up reg.
HIST1H4E	1.8	-	N.S.	N.S.
NCOA7	1.6	-	Up reg.	Up reg.
LOC440093	1.6	-	N.S.	N.S.
KCTD14	1.6	-	Up reg.	Up reg.
CCL3	1.6	2.2	Up reg.	Up reg.
RGL1	1.6	-	Up reg.	Up reg.
NFIL3	1.5	-	Up reg.	N.S.
ESPN	-1.5	-	Down reg.	N.S.
VPS13C	-1.6	-	N.S.	N.S.
CYP27A1	-1.6	-	Down reg.	Down reg.
CDKN1C	-1.6	-	Up reg.	Up reg.
SH3KBP1	-1.8	-	N.S.	N.S.
FECH	-	-2.2	N.S.	N.S.
PI3	-2.5	-	Down reg.	Down reg.

[a]Data from 32 patients generated by comparing samples from day 1-3 after fever onset with convalescent samples from day 21-28 after fever onset (Tolfvenstam et al., 2011).

[b]Data generated by comparing samples from dengue positive patients sampled day 1-3 after fever onset with samples from dengue negative febrile patients sampled day 1-3 after fever onset (Tolfvenstam et al., 2011).

Reg.- regulated; N.S.-not significant

Differential targeted proteomic between WS + Hosp. group and Non-WS + Non-Hosp. group.

Targeted differential protein expressions between hospitalized patients with warning signs and non-hospitalized patients without warning signs were analyzed. Out of the 22 proteins analyzed, four proteins (IP-10, IL-1ra, FGA, uPAR) were significantly higher in abundance at Day 1-3in hospitalized patients with warning signs compared to non-hospitalized patients without warning signs. The four proteins were IP-10 (p-value=0.0001), IL-1ra (p-value=0.0094), FGA (p-value=0.0423) and uPAR (p-value=0.0047) (**Table 10**). RANTES was the only protein observed to be significantly lower (p-value=0.0207) in abundance in hospitalized patients with warning signs compared to patients without warning signs at Day 1-3. Interestingly, CCL-4, IP-10 and uPAR protein expression were differentially expressed when comparing patient who developed severe dengue with patients with no warning signs and non-hospitalized at Day 1-3 post infection (**Table 11**).

Table 10. Targeted proteomic expression between Non-WS + Non-Hosp. group and WS + Hosp. group.

	DAY 1- 3						
Proteins	**Non-WS + Non-Hosp**			**WS + Hosp**			
	N	**Mean**	**SD**	**N**	**Mean**	**SD**	**p-value**
Chemokines							
MCP-1 (CCL2)	45	351.29	382.65	47	537.16	701.50	0.117
MCP-2 (CCL8)	45	693.78	354.13	47	896.23	821.28	0.127
MIP1a (CCL3)	45	6.63	8.74	47	10.15	14.75	0.1661
MIP1b (CCL4)	45	252.48	151.46	47	325.81	225.47	0.0697
RANTES (CCL5)	26	16877	9103.60	27	11593	6900.10	**0.0207**
IP-10 (CXCL10)	45	10167	19784	46	23010	18217	**0.0001**
Interleukins							
IL-1b	45	1.49	2.99	39	1.70	1.08	0.6662
IL-1ra (IL1RN)	45	1362.40	1524.60	47	3707.60	5757.80	**0.0094**
IL-2	45	6.32	17.60	47	7.73	12.83	0.6617
IL-4	45	0.68	2.28	47	0.62	0.40	0.8731
IL-8	45	18.55	9.38	47	26.94	34.81	0.1171
IL-10	45	44.04	85.91	47	53.63	94.40	0.6118
IL-12	45	9.48	11.50	47	7.41	5.98	0.286
IL-18	35	228.41	113.43	43	281.46	157.41	0.0988
Interferons							
IFN-a2	45	112.91	74.97	47	121.91	61.27	0.529
IFN-g	45	60.49	95.61	47	76.70	89.56	0.4034
Tumor Necrosis Factor							
TNF-a (TNF)	45	24.83	18.48	47	32.32	26.03	0.114
TRAIL(TNFSF10)	45	994.08	525.45	47	1060.30	538.45	0.5525
Others							
Fibrinogen (FGA)	35	4820.70	3476.70	35	7672.30	7304.20	**0.0423**
ICAM-1	26	231917	64163	27	260042	97853	0.2207
VCAM-1	26	409475	227158	27	528265	252640	0.0781
uPAR (PLAUR)	35	5870.50	1990.30	35	7786.40	3298.20	**0.0047**

Table 11. Targeted proteomic expression between Non-WS + Non-Hosp. groupand severe dengue patients among the WS + Hosp. group.

	DAY 1- 3						
Proteins	**Non-WS + Non-Hosp**			**Severe Dengue**			
	N	**Mean**	**SD**	**N**	**Mean**	**SD**	**p-value**
Chemokines							
MCP-1 (CCL2)	45	351.29	382.65	14	822.98	1119.20	0.159
MCP-2 (CCL8)	45	693.78	354.13	14	1015.52	761.18	0.162
MIP1a (CCL3)	45	6.63	8.74	14	12.23	16.49	0.258
MIP1b (CCL4)	45	252.48	151.46	14	433.63	289.68	**0.048**
RANTES (CCL5)	26	16876.54	9103.57	7	11405.57	7966.89	0.147
IP-10 (CXCL10)	45	10166.67	10784.03	14	32301.95	22130.61	**0.004**
Interleukins							
IL-1b	45	1.49	2.99	10	1.92	1.44	0.507
IL-1ra (IL1RN)	45	1362.43	1524.61	14	5787.56	8647.18	0.091
IL-2	45	6.32	17.60	14	6.17	8.56	0.968
IL-4	45	0.68	2.28	14	0.74	0.55	0.858
IL-8	45	18.55	9.38	14	41.55	63.10	0.116
IL-10	45	44.04	85.91	14	38.92	30.23	0.739
IL-12	45	9.48	11.50	14	7.48	3.11	0.301
IL-18	35	228.41	113.43	14	338.87	227.58	0.116
Interferons							
IFN-a2	45	112.91	74.97	14	133.62	57.89	0.300
IFN-g	45	60.49	95.61	14	71.24	43.59	0.568
Tumor Necrosis Factor							
TNF-a (TNF)	45	24.83	18.477	14	31.05	16.86	0.265
TRAIL(TNFSF10)	45	994.08	525.96	14	1233.53	587.51	0.202
Others							
Fibrinogen (FGA)	35	4820.73	3476.66	10	10825.44	10865.57	0.117
ICAM-1	26	231916.5	64162.6	7	257704.26	104522.33	0.553
VCAM-1	26	409475	227158.3	7	510908.98	264411.17	0.379
uPAR (PLAUR)	35	5870.54	1990.26	10	8522.53	3022.12	**0.023**

Prognostic Performance of RNA and Protein Biomarker Models to discriminate patients between WS+Hosp. group and Non-WS+Non-Hosp. group at Day 1-3 p.f.

We next investigated if these molecular markers were significantly associated with hospitalization and warning signs, and whether they could be utilized as early prognostic biomarkers models. The top single RNA biomarker model, is *CCL8* with an AUC of 0.73 (Hosmer-Lemeshow GOF p-value=0.14) for differentiating the patients into either hospitalized with warning signs group, or without warning signs and no hospitalization group. Whereas, the top single protein biomarker is IP-10 with an AUC of 0.74 (Hosmer-Lemeshow GOF p-value: 0.57) (**Table 12**). Combining the RNA and protein measurements together, the top RNA-protein biomarker model, includes *CCL8*, *VPS13C* and uPAR with an AUC of 0.90 (Hosmer-Lemeshow GOF p-value=0.24; **Table 12**). Furthermore, these biomarkers were shown to be significantly associated with hospitalized patients with warning signs (**Table 13**). Using the default cut-off of $P = 0.5$, the model 13 had sensitivity of 82.9% sensitivity, 80.0% specificity, positive predictive value (PPV) of 80.6% and negative predictive value (NPV) of 82.4%. Using Fluidigm technology to replace microarray technology for assessment of RNA expression level of *CCL8* and *VPS13C* provides comparable AUC of 0.84 (GOF p-value=0.34). Hence, the predicted probability of warning signs and hospitalization (p_i) within 72 hour post fever onset was calculated as follows:

$$P_{model13} = \frac{\ln\ pi}{1 - pi} = -0.4418\ \ x_{1i} + 0.9915\ \ x_{2i} + 0.0004\ \ x_{3i} - 5.4221$$

For each individual i, x_1 to x_3 are indicator variables taking on respective values, where x_1 represents *CCL8*, x_2 represents *VPS13C* and x_3 represents uPAR (pg/ml). x_1 and x_2 are obtained from Fluidigm delta Ct, while x_3 is obtained from ELISA. Optimizing the sensitivity and specificity may be achieved through the varying probability cut-offs as shown in Figure 14A.

We next investigated if these models are more efficient than just using laboratory indicators. Viral Ct, lymphocytes as well as platelets level were used in the modeling development to assess the overall performance of stratification between the two groups of patients. The results suggested that the RNA-protein biomarker model has better predictive power with AUC of 0.90, compared to that of viral load (AUC=0.71; GOF p-value=0.56), lymphocytes (AUC=0.68; GOF p-value=0.0.1) and platelets level (AUC=0.74; GOF p-value=0.84) to differentiate hospitalized patients with warning signs from non-hospitalized patients without warning signs (**Table 12**). Furthermore, combining the biomarkers with the laboratory features, the top model which comprised of *CCL8* and *VPS13C,* together with platelets level produced an AUC of 0.88 (**Table 12**). Furthermore, these biomarkers were shown to be significantly associated with hospitalized patients with warning signs (**Table 13**). Using the default cut-off of *P* = 0.5, the model 14 had sensitivity of 80.9% sensitivity, 84.4% specificity, positive predictive value of 80.6% and negative predictive value of 82.4%. Using Fluidigm technology to replace microarray technology for assessment of RNA expression level of *CCL8* and*VPS13C* also provides comparable AUC of 0.87 (GOF p-value=0.74). Hence, the predicted probability of warning signs and hospitalization (p_i) within 72 hour post fever onset was calculated as follows:

$$P_{model14} = \frac{\ln pi}{1 - pi} = -0.3606\ x_{1i} + 0.9421\ x_{2i} - 0.0197\ x_{3i} + 0.2971$$

For each individual i, x_1 to x_3 are indicator variables taking on respective values, where x_1 represents *CCL8,* x_2 represents *VPS13C* and x_3 represents platelet (x10 ^{3}/ul). x_1 and x_2 are obtained from Fluidigm delta Ct, while x_3 is obtained from ELISA. Optimizing the sensitivity and specificity may be achieved by varying the probability cut-off as shown in **Figure 14B.**

Table 12. Combination of RNA, proteins and laboratory variables as potential biomarkers of warning signs and hospitalization.

Model	Variables (*RNA*/Proteins/Lab)	AUC	Sen (%)	Spe (%)	PPV (%)	NPV (%)	GOF test (p-value)
	Models without Laboratory Features						
1	**IP-10**	**0.7353**	**54.35**	**77.78**	**71.43**	**62.50**	**0.57**
2	IL-1ra	0.7348	55.32	84.44	78.79	64.41	0.29
3	***CCL8***	**0.7277**	**72.34**	**66.67**	**69.39**	**69.77**	**0.14**
4	*HIST1H4E*	0.7229	76.60	55.56	64.29	69.44	0.06
5	*PKD2L1*	0.7047	72.34	53.33	61.82	64.86	0.68
6	*CCL3*	0.6979	63.83	64.44	65.22	63.04	0.57
7	uPAR	0.6955	60.00	74.29	70.00	65.00	0.77
8	*VPS13C*	0.6950	61.70	53.33	58.00	57.14	0.05
9	*RGL1*	0.6946	68.09	53.33	60.38	61.54	0.70
10	*NCOA7*	0.6927	70.21	60.00	64.71	65.85	0.13
11	IP-10, *CCL8*	0.7942	71.74	73.33	73.33	71.74	0.48
12	*HIST14HE*, *VPS13C*, IL-1RAuPAR*	0.9045	82.86	77.14	78.38	81.82	0.62
13	***CCL8, VPS13C,* uPAR^§**	**0.8988**	**82.86**	**80.00**	**80.56**	**82.35**	**0.24**
	Models with Laboratory Features						
14	***CCL8, VPS13C,* Platelets Level^***	**0.8757**	**80.85**	**84.44**	**84.44**	**80.85**	**0.32**
15	Platelets Level	0.7390	72.34	68.89	70.83	70.45	0.84
16	Viral Ct Level	0.7058	73.91	56.82	64.15	67.57	0.56
17	Lymphocytes Level	0.6792	76.60	48.89	61.02	66.67	0.01#
18	Platelets and Viral Ct Level	0.8370	73.91	79.55	79.07	74.47	0.20
19	Platelets ,Viral Ct Level, IP10	0.8520	77.78	81.82	81.40	78.26	0.08
20	Platelets Level, IP10	0.8097	76.09	77.78	76.09	77.78	0.33
21	Platelets ,Viral Ct Level, *CCL8*	0.8696	84.78	79.55	81.25	83.33	0.02#
	Models with Warning Signs						
22	Abdominal Pain	0.5102	100.0	0	51.09	-	N.A
23	Persistent Vomiting	0.5615	23.40	88.89	68.75	52.63	N.A
24	Mucosal Bleeding	0.5000	0	100	-	52.33	N.A
	Internal Validation using Fluidigm for *CCL8, VPS13C*						
13	***CCL8, VPS13C,* uPAR**	**0.8420**	**77.27**	**61.90**	**68.00**	**72.22**	**0.34**
14	***CCL8, VPS13C,* PLT**	**0.8682**	**69.57**	**83.87**	**76.19**	**78.79**	**0.74**
11	IP10, *CCL8*	0.7451	41.18	88.89	70	70.59	0.83

Continued from Table 12

^ Forward Stepwise Estimation from top 10 single RNA and protein molecules based on AUC;

* Backward Elimination Estimation from top 10 single RNA and protein molecules based on AUC;

§ Likelihood-Ratio test shows model 12 provides the same fit as model 11 (p-value=0.1688);

#Model has significant lack of fit for the data;

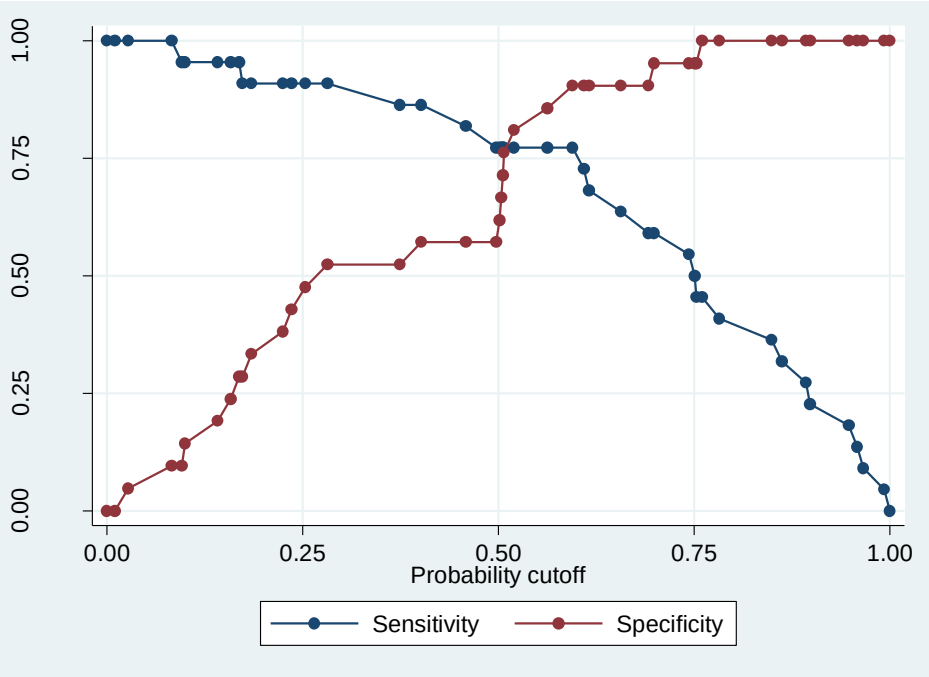
GOF- Goodness-of-fit test showed significant “lack-of-fit” when $p<0.05$

Table 13. Risk association analyses of RNA and protein biomarker in selected models.

A. Biomarkers in Model	Data Sources	Variable	Coefficient	OR	p-value	95% CI
***CCL8, VPS13C,* uPAR**	RNA from **Microarray** (Normalised Signal); Protein from ELISA	***CCL8***	**0.7383**	**2.09**	**0.002**	**1.32-3.32**
		VPS13C	**-2.4363**	**0.09**	**0.002**	**0.02-0.40**
		uPAR	**0.0004**	**1.01**	**0.003**	**1.00-1.02**
		cons	-4.8011			
***CCL8, VPS13C,* Platelets (PLT)**		***CCL8***	**0.6855**	**1.98**	**<0.001**	**1.41-2.79**
		VPS13C	**-1.3869**	**0.25**	**0.02**	**0.08-0.80**
		PLT	**-0.0200**	**0.98**	**<0.001**	**0.97-0.99**
		cons	0.9761			
CCL8, Viral Ct, IP-10, uPAR		***CCL8***	**0.6501**	**1.00**	**0.014**	**1.14-3.21**
		Viral Ct	-0.0065	0.99	0.944	0.83-1.19
		IP-10	0.000003	1.00	0.890	0.99-1.01
		uPAR	**0.0003**	**1.00**	**0.010**	**1.00-1.01**
		cons	-4.9307			
CCL8, IP-10		**CCL8**	**0.37449**	**1.45**	**0.013**	**1.08-1.95**
		IP-10	**0.00005**	**1.00**	**0.006**	**1.00-1.01**
		cons	-2.3953			
IP-10		**IP-10**	**0.00064**	**1.00**	**0.001**	**1.00-1.01**
		cons	-0.9495			
CCL8		***CCL8***	**0.47861**	**1.61**	**0.001**	**1.22-2.14**
		cons				

B. Biomarkers in Model	Data Sources	Variable	Coefficient	OR	p-value	95% CI
***CCL8, VPS13C,* uPAR**	RNA from **Fluidigm** (Delta Ct value); Protein from ELISA	***CCL8***	**-0.4418**	**0.64**	**0.034**	**0.43-0.97**
		VPS13C	**0.9915**	**2.70**	**0.041**	**1.00-7.59**
		uPAR	**0.0004**	**1.00**	**0.019**	**1.00-1.01**
		cons	-5.4221			
***CCL8, VPS13C,* Platelets (PLT)**		***CCL8***	**-0.3606**	**0.70**	**0.035**	**0.50-0.98**
		VPS13C	**0.9421**	**2.57**	**0.014**	**1.21-5.43**
		PLT	**-0.0197**	**0.98**	**0.013**	**0.967-0.99**
		cons	0.2971			
CCL8, Viral Ct, IP-10, uPAR		*CCL8*	-0.20534	0.81	0.461	0.47-1.41
		Viral Ct	-0.20766	0.81	0.136	0.62-1.07
		IP-10	0.00001	1.00	0.742	0.99-1.01
		uPAR	0.00033	1.00	0.075	0.99-1.01
		cons	2.0693			
CCL8, IP-10		*CCL8*	-0.1513	0.86	0.394	0.61-1.22
		IP-10	**0.00007**	**1.00**	**0.029**	**1.00-101**
		cons	-1.0832			
CCL8		***CCL8***	**-0.30604**	**0.74**	**0.026**	**0.56-0.96**
		cons	0.39050			

A.



B.

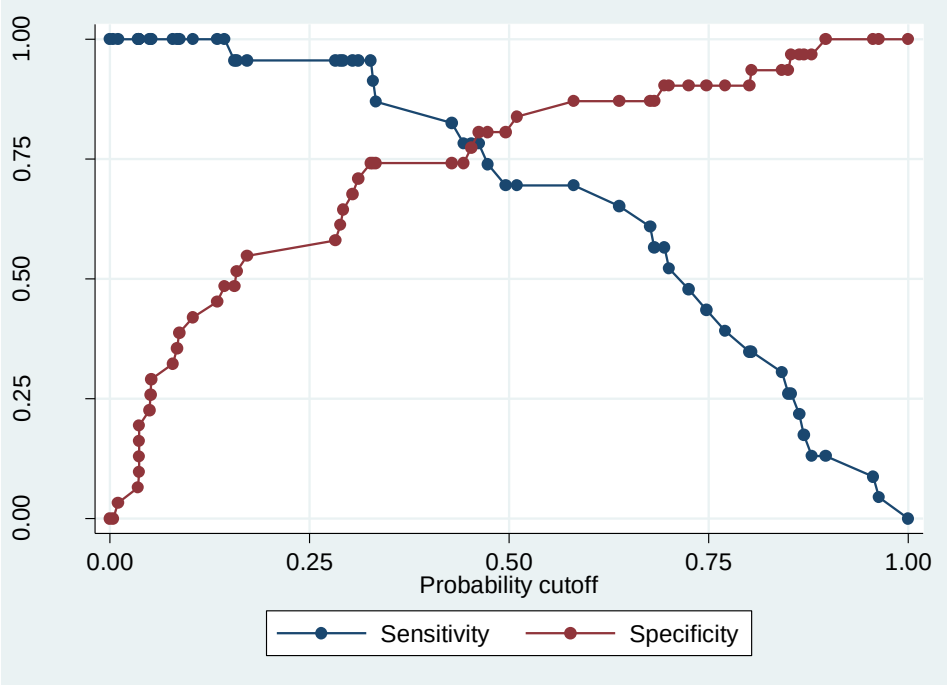


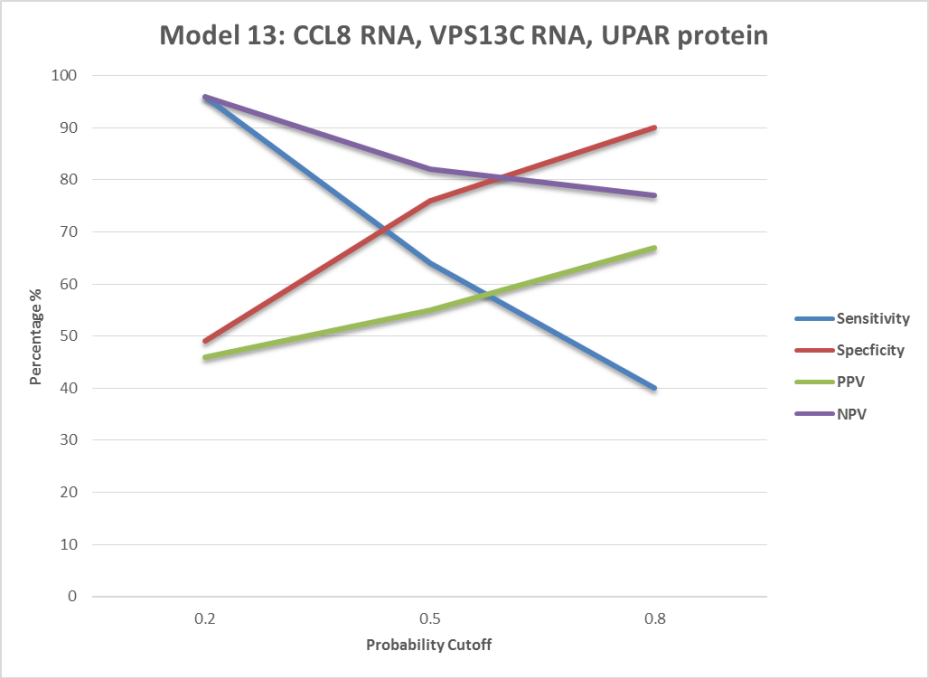
Figure 14. Sensitivity and specificity plots of Model 13 (A.) and Model 14 (B.) with the varying probability cut-offs.

Validation of top two prognostic models

For validation of our two optimal models, we utilized samples collected from patients enrolled between January 2009 and December 2012. There were 1,895 patients enrolled and only 117 patients were dengue RT-PCR positive. Of these, 2 were excluded due to lack of data and the remaining were 115 patients. Using the same classification criteria as described in Methods, there were 55 patients who had no warning signs and do not need hospitalization, and 25 patients who had warning signs and hospitalization. Patient characteristics are outlined in **Table 8**. Model 13 consisting of *CCL8, VPS13C* and uPAR were used to stratify the patients at the first visit (≤72H post fever onset) and achieved 64% sensitivity, 76% specificity, positive predictive value of 55% and negative predictive value of 82% with the default cutoff of 0.5(**Figure 15andTable 14**). Model 14 consisting of *CCL8, VPS13C* and platelets were also used to stratify the patients at the first visit (≤72H post fever onset) and achieved 60% sensitivity, 78% specificity, positive predictive value of 56% and negative predictive value of 81%with the default cutoff of 0.5 (**Figure 15 and Table 14**). Only one patient was defined as having severe dengue in this validation cohort, and both models classified this patient into the hospitalized with warning signs group. Viral levels were significantly higher in the predicted warning signs and hospitalization group within 72 hour post fever onset compared to the predicted no warning signs and non-hospitalization group using both optimal models (). Platelet level was only significantly different at time point Day 4-7 using model 13 between the two predicted groups, but it was significantly lower at both time points Day 1-3 and Day 4-7 using Model 14 (**Figure 16**). The lymphocyte level was only significantly lower in the predicted warning signs and hospitalization group at time point Day 1-3 compared to the predicted no warning signs and non-hospitalization group using both models (**Figure 16**). The optimal model 14, which consisted of platelet as one of variables also act as a positive

control, where it is expected for the model to be more sensitive to pick up patients with thrombocytopenia even at Day 1-3.

A.



B.

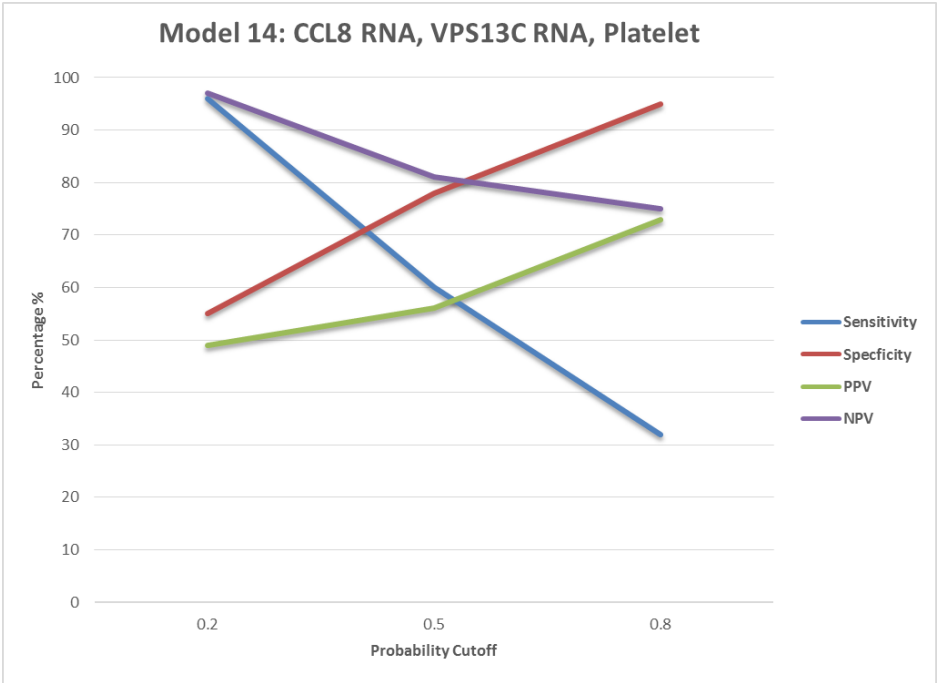


Figure 15. Performance of the optimal model 13 (A.) and model 14 (B.) on the validation cohort with different probability cut-off.

Table 14. Prognostic performance of the top selected models with different probability cutoff as positive for falling into WS+Hosp. group in the validation cohort.

A.

Model 13: *CCL8, VPS13C*, uPAR

Probability Cutoff	Sensitivity	Specificity	PPV	NPV
0.2	96.0	49.1	46.2	96.4
0.5	64.0	76.4	55.2	82.4
0.8	40.0	90.9	66.7	76.9

B.

Model 14: *CCL8, VPS13C*, Platelets

Probability Cutoff	Sensitivity	Specificity	PPV	NPV
0.2	96.0	54.6	49.0	96.8
0.5	60.0	78.2	55.6	81.1
0.8	32.0	94.6	72.7	75.4

C.

Model 1: IP-10

Probability Cutoff	Sensitivity	Specificity	PPV	NPV
0.2	100.0	0	31.3	-
0.5	80.0	50.9	42.6	84.9
0.8	32.0	96.4	80.0	75.7

D.

Model 3: *CCL8*

Probability Cutoff	Sensitivity	Specificity	PPV	NPV
0.2	96.0	16.4	34.3	90.0
0.5	48.0	67.3	40.0	74.0
0.8	8.0	98.2	66.7	70.1

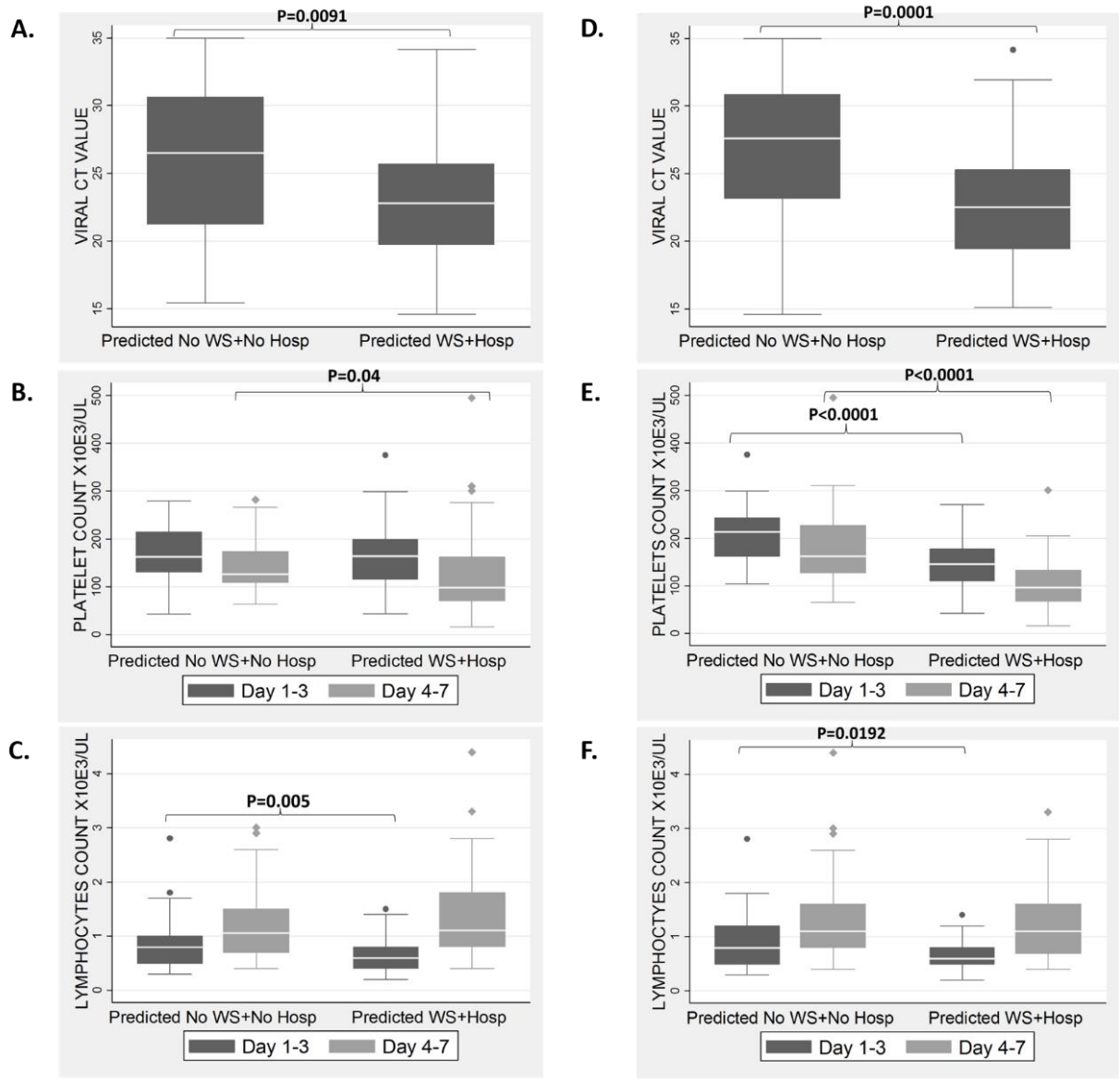


Figure 16. Laboratory characteristics of patients who are likely to require hospitalization and develop warning signs.

Viral (A, D), platelet (B, E) and lymphocyte (C, F) levels of the patients predicted into either the "Non-WS + Non-Hosp" group or "WS + Hosp" group. The predicted groups of A, B and C are based on Model 13, while D, E and F are based on Model 14. WS-Warning Signs; Hosp-Hospitalisation. P-value is shown only for statistically significant comparisons.

5.3.DISCUSSION AND CONCLUSION

The diverse clinical spectrum of dengue disease is still a challenge for health care workers in dengue endemic regions, especially to identify patients early that will later require clinical interventions to prevent progression of severe illness, particularly in adults. WS were found to be associated with severe illness, but they typically occurred only one day prior to the development of severe illness (Alexander et al., 2011; Leo et al., 2013; T. L. Thein, Gan, et al., 2013; Tsai et al., 2013), which would be a challenging window for any effective intervention. In this study, we aimed to identify and validate distinct pathophysiological and molecular features associated with adult dengue patients who presented WS and required hospitalization within 72 hours of fever.

The WHO 2009 WS guidelines and clinical judgment were applied to identify patients who did and did not require clinical observation, dividing the patients into those hospitalized with WS of abdominal pain, bleeding and/or persistent vomiting (WS+Hosp. group); and those without WS and did not require hospitalization (Non-WS+Non-Hosp. group). We found that the WS+Hosp. group had significantly higher viral, lower platelet and lymphocyte levels compared with Non-WS+Non-Hosp. group at early infection (Day 1-3). These novel observations clearly illustrated that viral (AUC=0.71), platelet (AUC=0.74) and lymphocyte (AUC=0.68) levels as potential biomarkers to triage patients into the two groups at early infection, probably at an earlier time point than the presentation of WS, typically on Day 4-7 p.f. (Alexander et al., 2011; Leo et al., 2013). At Day 4-7, when hospitalization typically occurred, the platelet level was lower in the WS+Hosp. group suggesting hospitalization due to low platelet levels was common in our cohort. The lack of statistical differences in viral, platelet or lymphocyte levels between the severe dengue group and the remaining patients from the WS+Hosp. group highlighted

the difficulties in recognizing these severe dengue patients at early infection as they are likely to be indistinguishable from other hospitalized patients with WS, even on Day 4-7 p.f. This reflects the similar observations in children with severe illness and hospitalized non-severe illness published previously (Hoang et al., 2010). However, in spite of the small number of severe dengue patients, they remained significantly different in pathophysiology from the Non-WS+Non-Hosp. group at early infection. High viremia was associated with severe dengue outcomes (Low et al., 2011; Tanner et al., 2008; Vaughn et al., 2000). Similarly, our findings showed that viremia was significantly associated with WS and close monitoring at early infection; this is similar for platelet and lymphocyte level, suggesting the important role of these parameters in dengue disease severity.

Biomarkers associated with WS+Hosp. group were involved in innate immunity (CCL2, CCL3, CCL8, CD69, RANTES, IL1RA), type II interferon (IP-10) and coagulation (uPAR, FGA) pathways which were associated with dengue severity (Becquart et al., 2010; J. Fink et al., 2007; Green, Pichyangkul, et al., 1999; Hsieh et al., 2006; Y. R. Lee et al., 2006; Tolfvenstam et al., 2011). The high expression level of these biomarkers except RANTES at Day 1-3 may be informative of the strength of the innate response during early infection, which may be related to the progression of disease severity after Day 1-3. CCL8 (MCP-2), the top RNA biomarker is a chemokine that had been previously associated as a biomarker for tuberculosis diagnosis (Ruhwald et al., 2008) and outcome of hepatitis C virus infection (Hellier et al., 2003). IP-10 (CXCL-10), the top protein biomarker is a pro-inflammatory chemokine (Luster, 1998), which had been highly associated as a biomarker to predict severity of inflammatory diseases including infectious diseases, immune dysfunction and tumor development (Liu et al., 2011). Soluble uPAR is a versatile signaling proteinase receptor (Blasi & Carmeliet,

2002)that was associated as a biomarker to predict survival of HIV-1 infection (Sidenius et al., 2000) and to discriminate primary focal segmental glomerulosclerosis (Huang et al., 2014), which may be related to the protective PLCE1 loci associated with DSS (Khor et al., 2011). Furthermore, VPS13C is a vascular protein was previously associated with the pathophysiology of type-2 diabetes (Strawbridge et al., 2011), which may support the association of diabetes with dengue severity (Pang et al., 2012). These biomarkers showed high biological relevance to dengue pathophysiology but they may not fully explain the development of severe disease, as it may also be influenced by other mechanisms such as the host genetic susceptibility (Khor et al., 2011).

Many of the WS stated by the WHO 2009 classification are seen typically Day 4-7 p.f. in the clinical course (Leo et al., 2013; T. L. Thein, Gan, et al., 2013; Tsai et al., 2013). Mucosal bleeding was a common WS in our cohort and in others (Leo et al., 2013; T. L. Thein, Gan, et al., 2013), and a majority of the patients showed WS mainly during admission into hospital at Day 4-7 p.f. and during hospitalization, which was also observed in other studies (Binh, Matheus, Huong, Deparis, & Marechal, 2009; Leo et al., 2013). Similarly, in other studies (Leo et al., 2013; T. L. Thein, Gan, et al., 2013), WS namely abdominal pain (AUC=0.51), persistent vomiting (AUC=0.56) and mucosal bleeding (AUC=0.50) had less optimal prognostic performance in this cohort, reemphasizing the importance to assess the molecular biomarker as a potential prognostic tool.

Ideally, in primary healthcare facilities, the clinician should have a reliable test that can diagnose and predict at Day 1-3 p.f. if a patient may progress to severe disease which requires close monitoring and hospitalization. Our findings showed that by combining

RNA and protein biomarkers, a model (CCL8, VPS13C, and uPAR) was established with 82.9% sensitivity, 80.0% specificity using the discovery cohort. Furthermore, by adding platelet counts to the biomarkers, a model (CCL8, VPS13C and Platelets) with 81% sensitivity and 84% specificity could be established. When validating in an independent cohort, the top two models achieved high sensitivity of up to 96% with a corresponding specificity of 49.1% (CCL8, VPS13C, and uPAR), and 56% (CCL8, VPS13C and Platelets), where sensitivity is more critical during triage as compared to specificity. Moreover, these models may be tested simultaneously with the dengue virus PCR assay as diagnosis, particularly in dengue endemic regions with high prevalence, to reduce the turn-around time required to guide prompt clinical management. Furthermore, we showed that the predicted WS+Hosp. group using the top two models displayed significant differences in pathophysiology compared with the predicted Non-WS+Non-Hosp. group, illustrating the optimistic performance of the optimal models. In addition, we showed that both models were able to pick up patients who do not present with thrombocytopenia at Day 1-3 p.f., but only present at Day 4-7 p.f.

The generalizability of these optimal models may be limited until validation is performed in larger cohorts of adult dengue patients. The different predominant serotype in the discovery and validation cohorts may have also affected the results to some extent. However, the results suggested that the optimal models may still be applicable during epidemics of both serotype 1 and 2, which are the two most common serotypes that cause significant disease burden in Singapore and South-East Asia. Nevertheless, it is also important to emphasize that this study had presented other potential models such as IP-10 protein and CCL8 RNA for prognosis, even though they may not be most optimal in this population (Table 2 and Supplementary Table 5). They may be more useful in other

population when validated with large number of patients. The small number of severe dengue patients is due to the low prevalence of severe dengue in adults in Singapore (Leo et al., 2013). Therefore, it was statistically challenging to develop optimal models in stratifying patients of high risk of severe dengue. Nevertheless, with the low prevalence of severe dengue patients, it highlighted the need to focus more on patients with warning signs to prevent them from severe disease progression. Furthermore, the clinical management may vary in the two different periods of the discovery and validation cohorts, which may account for the variation in the performance of the models. Lastly, innovation will be needed to reduce the cost and complexity of the current methods used to detect multiple RNA transcripts and protein simultaneously with a blood test based application, particularly for application in a developing countries.

In summary, this is the first study, to our best knowledge, that showed hospitalized adult dengue patients with WS at Day1-3 p.f. have differential pathophysiological features from non-hospitalized patients with no WS. Selected laboratory and molecular features were potential biomarkers for triage at early infection. With future independent larger cohorts for validation, these optimal models may be refined for stratification of adult patients at early infection who are more likely to progress in disease severity that require close monitoring and potential interventions. These biomarkers can be integrated with viral detection assays as a potential point-of care tool for diagnosis and prognosis, simultaneously, to guide clinical management and potentially treatment, when antivirals becomes available, during early dengue infection.

CHAPTER 6: CLINICAL AND LABORTORY RISK FACTORS OF DENGUE DISEASE SEVERITY

6.1.INTRODUCTION

Dengue results in a wide spectrum of non-specific clinical manifestations with unpredictable clinical course and outcome. Patients can be clinically classified as ‘dengue fever’ (DF), ‘dengue hemorrhagic fever’ (DHF) or ‘dengue shock syndrome’ (DSS) according to the 1997 WHO dengue classification system (WHO, 1997). More recently, based on the WHO classification system (WHO, 2009), patients can be clinically classified as ‘probable dengue’, ‘dengue with warning signs’ or ‘severe dengue’ according to the 2009. Although a large proportion of patients recover after a mild self-limiting disease, a small proportion may progress to develop severe dengue clinical manifestations, some of which require interventions in the intensive care unit (ICU). The progression to severe clinical manifestations is usually unpredictable. Without prompt and appropriate therapy, case fatality rate may exceed 20%(WHO, 2009).

There are currently no systematic studies, but only case series reported (Bouldouyre et al., 2006; Juneja et al., 2011) focusing on epidemiological, clinical and laboratory risk factors at first presentation in hospital that are predictive of clinical severity, as defined by the requirement for ICU admission, instead of the WHO classification criteria. These studies and classifications may not be generalizable to all populations at risk as well as in different clinical settings and healthcare systems. Furthermore, with the recommendation of close monitoring of patients with warning signs in hospital (WHO, 2009), there is a

greater need to identify these high risk dengue patients 24 hours prior to ICU admission, and information on this is also currently lacking. Therefore, it is important to identify and evaluate the clinical and laboratory risk factors of ICU admission at first presentation in hospital as well as 24 hours prior to ICU admission, particularly in the predominantly dengue serotype 1 and 2 epidemics which are most commonly found and responsible for the greatest disease burden in Singapore and South-east Asia.

6.2.RESULTS

(**Junxiong Pang**, Tun-Linn Thein, Yee-Sin Leo, David C Lye. **Clinical and Laboratory Risk Factors of Dengue Patients Requiring Admission into Intensive Care Unit during 2004-2008 Dengue Epidemics: A Matched Case-Control Study-*BMC Infect Dis*. 2014 Dec 5;14(1):649.**)

A total of 8,123 dengue-infected patients were admitted into Tan Tock Seng Hospital during the dengue epidemics between January 2004 and December 2008. Among these admissions, there were a total of 27 dengue-infected cases that required intensive care interventions in the intensive care unit (ICU). Out of these 27 ICU cases, 22 cases had shock, four cases had severe organ involvement, two cases had severe bleeding, two cases had acute respiratory disease syndrome (ARDS), one case had encephalopathy and one case had intracranial hemorrhage. Each ICU case was matched to four non-ICU dengue cases by year of presentation, with a total of 108 dengue-infected matched controls that did not require intensive care interventions randomly selected. The median age of the cases was 44 years (25th-75th percentiles [pctl]: 36-53 years), with 30% female and 78% Chinese. The median age of the controls was 34 years (25th-75th pctl: 25-44 years), with 36% female and 71% Chinese (**Table 15**). Among the cases, 48% were positive for dengue polymerase chain reaction assay (PCR) and 52% were positive by serology. Among the controls, 29% were positive for dengue PCR, and 71% were positive by serology. The median DPF at presentation was 3 (25th-75th pctl: 3-5 days) and 5 days (25th-75th pctl: 4-5 days) for cases and controls, respectively. This may suggest that the ICU patients were sicker, requiring earlier admission to hospital. However, there was no significant association observed in gender, ethnic groups, IgG status and median days post fever (DPF) at first presentation with ICU admissions.

Demographic and Co-morbidity Risk Factors of ICU

Age groups 50-59 years old [conditional odds ratio (COR) =8.51; 95% confidence interval (CI) =1.38-52.6] and equal to or greater than 60 years old (COR=21.98; 95% CI=1.97-245) had significantly higher risk of ICU admission compared with age group 14 to 30 years old. However, after adjusting for diabetes mellitus, only age group 50-59 years old was observed to be a significant risk factor (adjusted COR (ACOR)=6.9; 95% CI=1.25-38.23) for ICU admission (**Table 15**). Among the co-morbidities analyzed, patients with diabetes mellitus were found to have significantly higher risk (COR=11.57; 95% CI=1.25-1077) of ICU admission compared with patients with no diabetes. After adjusting for age, the risk remained high with ACOR of 5.53 (95% CI=0.56-54.15), but it was statistically insignificant, probably due to the small sample size of cases (**Table 15**). In addition, patients with a cardiac disorder (dysfunction) were observed to be at higher risk (ACOR=3.18; 95% CI=0.21-47.52) compared with patients without. This may also be a potential risk factor that requires further investigation, even though it was statistically insignificant in this study (**Table 15**).

Table 15. Demographic and co-morbidities of dengue-infected non-ICU (controls) and ICU (cases) patients

Variables	Den-infected Non-ICU Controls (n=108)	%	Den-infected ICU Cases (n=27)	%	COR	p-value	95% CI	ACOR*	p-value	95% CI
Median age (25% -75%)	34 (25 - 44)		44 (36-53)		**1.07**	**0.003**	**1.02-1.11**	**1.05**	**0.014**	**1.01-1.10**
Age groups										
14-30	36	33	4	15	1					
30-39	32	30	7	26	3.64	0.138	0.66-20.0	2.48	0.244	0.54-11.41
40-49	24	22	7	26	5.9	0.061	0.92-37.9	4.1	0.126	0.67-25.04
50-59	13	12	6	22	**8.51**	**0.021**	**1.38-52.6**	**6.9**	**0.027**	**1.25-38.23**
≥60	3	2.8	3	11	**21.98**	**0.012**	**1.97-245.2**	5.49	0.165	0.50-60.54
Gender										
Female	39	36	8	30	0.74	0.52	0.29-1.87	0.59	0.318	0.21-1.65
Ethnic groups										
Chinese	77	71	21	78	1					
Malay	5	4.6	2	7.4	1.44	0.667	0.28-7.49	2.47	0.36	0.36-17.15
Indian	15	14	2	7.4	0.45	0.344	0.09-2.37	0.39	0.315	0.06-2.47
Others	11	10	2	7.4	0.65	0.606	0.13-3.36	0.95	0.952	0.17-5.29
IgG at presentation										
IgG+	42	39	12	44	1.26	0.596	0.53-2.98	1.19	0.708	0.47-3.03
Median DPF at presentation (25%-75%)	5 (4-5)		3 (3-5)		0.75	0.059	0.56-1.01	0.78	0.112	0.58-1.06
Diabetes mellitus										
Yes	3	2.8	4	15	**11.57**	**0.031**	**1.25-1077**	5.53	0.142	0.56-54.15
Hypertension										
Yes	10	9.3	5	19	2.22	0.184	0.69-7.17	0.42	0.296	0.08-2.14
Hyperlipidemia										
Yes	7	6.5	4	15	2.57	0.168	0.67-9.83	1	0.998	0.20-4.87
Cardiac disorder										
Yes	2	1.9	3	11	9.83	0.051	0.99-97.23	3.18	0.402	0.21-47.52

DPF- Days Post Fever; COR- Conditional Odds Ratio

*Adjusted Conditional Odds Ratio (ACOR) was obtained from a multivariate conditional logistic regression with test variables being adjusted by age and diabetes mellitus.

Dengue Severity Classification and Clinical Outcomes

The DHF/DSS classification (WHO 1997) at presentation was not significantly associated with ICU admission (ACOR=1.8; 95% CI=0.54-6.08), with sensitivity of about 19% and specificity of 87%. However, Severe Dengue classification (WHO 2009) at presentation was significantly associated with ICU admission (ACOR=8.79; 95% CI=2.65-29.16) (**Table 16**), with sensitivity of 52% and specificity of 94%. By the end of the hospitalization, both DHF/DSS and Severe Dengue classifications were observed to be significantly associated with ICU admission (WHO 1997: ACOR=2.99; 95% CI=1.15-7.78) (WHO 2009: ACOR=116.4; 95% CI=8.79-1541) (**Table 16**). By the end of the hospitalization, DHF/DSS classification achieved sensitivity and specificity of 67% and 78%, respectively, while Severe Dengue classification achieved sensitivity and specificity of 89% and 90%, respectively.

The median days for progression to DHF/DSS and severe dengue for cases was 4 (25th-75th pctl: 3-5 days) and 3 DPP (25th-75th pctl: 3-5 days), respectively. For the controls, the median days for progression to DHF/DSS and severe dengue was 3 (25th-75th pctl: 2.3-3.8 days) and 2.5 DPP (25th-75th pctl: 1.8-3.3 days). The median length of stay (LOS) in hospital was 8 (25th-75th pctl: 6-13.5 days) and 4 days (25th-75th pctl: 3-5 days) for cases and controls, respectively. The median DPF and DPP to ICU admission was 6 (25th-75th pctl: 4-7.5 days) and 3 (25th-75th pctl: 2-4.5 days) days respectively. The median LOS in ICU for the cases was 3 days (25th-75th pctl: 2-4 days). Among the cases, there were seven deaths (case fatality rate=26%), with median DPF of 7 days (25th-75th pctl: 7-15.5 days) and median DPP of 3 days to death (25th-75th pctl: 2.5-7.5 days) (**Table 16**).

Table 16. Severity classification and duration of illness of dengue-infected non-ICU (controls) and ICU (cases) patients

Variables	Den-infected Non-ICU Controls (n=108)	%	Den-infected ICU Cases (n=27)	%	COR	p-value	95% CI	ACOR*	p-value	95% CI
WHO 1997 (Presentation)										
DHF/DSS	14	13	5	19	1.5	0.469	0.50-4.4	1.8	0.34	0.54-6.08
WHO 2009 (Presentation)										
Severe dengue	7	6.5	14	52	**10.3**	**<0.001**	**3.70-28.84**	**8.79**	**<0.001**	**2.65-29.16**
WHO 1997 (Outcome)										
DHF/DSS	24	22	18	67	**7.65**	**<0.001**	**2.74-21.35**	**2.99**	**0.025**	**1.15-7.78**
WHO 2009 (Outcome)										
Severe dengue	11	10	24	89	**73.1**	**<0.001**	**9.82-543.96**	**116.4**	**<0.001**	**8.79-1541**
Median DPP to DHF/DSS (25%-75%)	3 (2.3-3.8)		4 (3-5)		1.79	0.206	0.73-4.43	3.08	0.677	0.02-608
Median DPP to Severe dengue (25%-75%)	2.5 (1.8-3.3)		3 (3-5)							
Median LOS in hospital (25%-75%)	4 (3-5)		8 (6-13.5)		**1.73**	**<0.001**	**1.30-2.32**	**1.76**	**0.001**	**1.28-2.43**
Median DPF on ICU admission (25%-75%)	N.A		6 (4-7.5)							
Median DPP on ICU admission (25%-75%)	N.A		3 (2-4.5)							
Median LOS in ICU (25%-75%)	N.A		3 (2-4)							
Death	0	0	7	26						
Median DPF to death (25%-75%)	N.A		7 (7-15.5)							
Median DPP to death (25%-75%)	N.A		3 (2.5-7.5)							

DPF- Days Post Fever; DPP- Days Post Presentation; LOS- Length of Stay; COR- Conditional Odds Ratio

*Adjusted Conditional Odds Ratio (ACOR) was obtained from a multivariate conditional logistic regression with test variables being adjusted by age and diabetes mellitus.

ICU Risk Factors at First Presentation in Hospital

At first presentation, warning signs from the WHO 2009 classification were not observed to be significantly associated with ICU admission. In addition, other signs and symptoms such as haemorrhagic manifestation, rash, leucopenia, nausea/vomiting, ache and pains, thrombocytopenia and tardycardia were not observed to be significantly associated with ICU admission. Hypoproteinemia (ACOR=4.65; 95% CI=1.09-19.74), hypotension (ACOR=7.63; 95% CI=1.83-31.79), severe organ involvement (ACOR=33.04; 95% CI=3.30-331.3) and hematocrit rise with rapid platelet drop (ACOR=8.66; 95% CI=2.46-30.53) were observed to be significantly associated with ICU admission (**Table 17**).

Pulse rate (ACOR=1.05; 95% CI=1.01-1.09), neutrophil proportion(ACOR=1.11; 95% CI=1.04-1.17), serum urea (ACOR=1.26; 95% CI=1.02-1.56), creatinine (ACOR=1.02; 95% CI=1.01-1.04), aspartate (ACOR=1.01; 95% CI=1.001-1.06) (AST) and alanine aminotransferases (ALT),(ACOR=1.01; 95% CI=1.001-1.06) at first presentation were positively correlated with ICU admission (Table 17). On the contrary, lymphocyte (ACOR=0.90; 95% CI=0.85-0.96) monocyte proportions (ACOR=0.86; 95% CI=0.77-0.95), platelet count (ACOR=0.98; 95% CI=0.96-0.99), serum protein (ACOR=0.88; 95% CI=0.79-0.98) and albumin (ACOR=0.81; 95% CI=0.70-0.94) at first presentation were significantly and negatively correlated with ICU admission (**Table 17**).

These risk factors of ICU admission at first presentation in hospital were used to create a predictive model. The most optimal model derived was a combination of neutrophil proportion, ALT and serum urea level with an area under the curve of 0.92 (Hosmer–Lemeshow Goodness of fit test *P*=0.52), indicating that the model had good

discrimination between non-ICU and ICU patients at first presentation in hospital. A prognostic index (*P*) was created using this model. Using the default cut-off of *P* = 0.5, the model had sensitivity of 58.8%, specificity of 96.3%, positive predictive value of 76.9% and negative predictive value of 91.8% (**Figure 17**). Using a cut-off of *P* = 0.25 (maximizing sensitivity and specificity; **Figure 17A**.), the model had sensitivity of 88.2%, specificity of 88.9%, positive predictive value (PPV) of 62.5% and negative predictive value (NPV) of 97.3%. The predicted probability of ICU admission at presentation was calculated as follows:

$$P_{presentation} = \ln\left(\frac{pi}{1 - pi}\right) = 0.106\ x_{1i} + 0.004\ x_{2i} + 0.326\ x_{3i} - 10.601$$

For each individual i, x_1 to x_3 are indicator variables taking on respective values, where x_1 represents neutrophil proportion (%), x_2 represents ALT (IU/L) and x_3 represents serum urea (mmol/L).

Table 17. Clinical and laboratory variables at first presentation of dengue-infected non-ICU (controls) and ICU (cases) patients

Variables	Den-infected Non-ICU Controls (n=108)	%	Den-infected ICU Cases (n=27)	%	COR	p-value	95% CI	ACOR*	p-value	95% CI
Hypoproteinemia										
Yes	23	21	10	37	2.28	0.085	0.89-5.85	**4.65**	**0.037**	**1.09-19.74**
Hypotension										
Yes	4	3.7	7	26	**8.62**	**0.002**	**2.21-33.63**	**7.63**	**0.005**	**1.83-31.79**
Severe organ involvement										
Yes	1	0.9	10	37	**40**	**<0.001**	**5.12-312.5**	**33.04**	**0.003**	**3.30-331.3**
Hematocrit rise with rapid platelet drop										
Yes	7	6.5	11	41	**9.4**	**<0.001**	**2.95-29.83**	**8.66**	**0.001**	**2.46-30.53**
Systolic blood pressure (25%-75%)	105 (100-114)		110 (91.5-118.5)		0.98	0.216	0.96-1.01	**0.97**	**0.046**	**0.94-0.99**
Pulse rate (25%-75%)	90 (80-96)		95.5 (88.5-102)		**1.03**	**0.049**	**1.00-1.07**	**1.05**	**0.012**	**1.01-1.09**
Proportion neutrophils (25%-75%)	60.3 (46.2-70.4)		76.8 (70.7-81.5)		**1.11**	**<0.001**	**1.05-1.18**	**1.11**	**0.001**	**1.04-1.17**
Proportion lymphocytes (25%-75%)	30.3 (22-39)		16.5 (9.8-19.9)		**0.90**	**<0.001**	**0.85-0.95**	**0.90**	**0.001**	**0.85-0.96**
Proportion monocytes (25%-75%)	12.5 (8.9-17.3)		7.9 (5.1-9.9)		**0.84**	**0.002**	**0.76-0.94**	**0.86**	**0.004**	**0.77-0.95**
Platelet (25%-75%)	68 (44-84)		42.5 (20.3-74.8)		**0.98**	**0.01**	**0.96-0.99**	**0.98**	**0.017**	**0.96-0.99**
Serum urea (25%-75%)	3.5 (2.6-4.6)		5.1 (3.7-6.8)		**1.33**	**0.002**	**1.11-1.58**	**1.26**	**0.030**	**1.02-1.56**
Serum creatinine (25%-75%)	77 (65-88.3)		102 (82.3-130)		**1.03**	**0.001**	**1.01-1.04**	**1.02**	**0.006**	**1.01-1.04**
Serum AST (25%-75%)	114 (70-195)		156.5 (96.5-516)		**1.01**	**0.017**	**1.00-1.04**	**1.01**	**0.019**	**1.00-1.04**
Serum ALT (25%-75%)	74 (38-121)		92 (55.8-293.3)		**1.01**	**0.012**	**1.001-1.06**	**1.01**	**0.017**	**1.001-1.06**
Serum protein (25%-75%)	67 (63-72)		66 (60.5-68.5)		**0.89**	**0.016**	**0.79-0.98**	**0.88**	**0.022**	**0.79-0.98**
Serum albumin (25%-75%)	37 (35-40)		37 (31-39)		**0.8**	**0.004**	**0.69-0.93**	**0.81**	**0.006**	**0.70-0.94**

DPF- Days Post Fever; DPP- Days Post Presentation; LOS- Length of Stay; COR- Conditional Odds Ratio
*Adjusted Conditional Odds Ratio (ACOR) was obtained from a multivariate conditional logistic regression with test variables being adjusted by age and diabetes mellitus.

ICU Risk Factors at 24 Hours Prior to ICU Admission

Close monitoring of high risk patients in hospital for ICU admission may be challenging as clinical conditions can evolve unpredictably. We further explored potential risk factors that can provide stratification at 24 hours prior to ICU admission after their first presentation in hospital. Pulse rate (COR=1.07; 95% CI=1.002-1.14), white cell count (COR=1.59; 95% CI=1.04-2.44), lymphocyte (COR=1.19; 95% CI=1.03-1.38) and monocyte proportions (COR=1.35; 95% CI=1.02-1.78) were observed to be significantly higher in ICU dengue patients 24 hour before ICU admission compared with these at first presentation (**Table 18**). On the contrary, blood pressure (COR=0.96; 95% CI=0.92-0.996) and hematocrit (COR=0.81; 95% CI=0.66-0.99) were observed to be significantly lower in ICU dengue patients 24 hour prior to ICU admission compared with that at the first presentation (**Table 18**). The most optimal model derived was a combination of the monocyte and lymphocyte proportion, blood pressure and pulse rate, which had an area under the curve of 0.94 (Hosmer–Lemeshow Goodness of fit test *P*=0.96), indicating that the model had good predictive potential of whether a high risk dengue-infected patient is likely to require ICU admission in the next 24 hour. In addition, this model is more accurate than the model comprising of only pulse rate and blood pressure, which are known critical factors for ICU admission, and has an AUC of only 0.74. A prognostic index (*P*) was thus created using the model with monocyte and lymphocyte proportion, blood pressure and pulse rate. Using the default cut-off of $P = 0.5$, the model had sensitivity of 74.1%, specificity of 88%, positive predictive value of 87% and negative predictive value of 75.9% (**Figure 17B**). Using a cut-off of $P = 0.40$ (maximizing sensitivity and specificity; **Figure 17B**), the model had sensitivity of 88.9%, specificity of 86%, PPV of 80% and NPV of 86.4%. The predicted probability of ICU admission in the next 24 hour was calculated as follows:

$$P_{24\ HpriorICU} = \ln\left(\frac{pi}{1-pi}\right) = 0.333\ x_{1i} - 0.105\ x_{2i} + 0.079\ x_{3i} + 0.188\ x_{4i} - 5.284$$

For each individual i, x_1 to x_3 are indicator variables taking on respective values, where represents x_1 monocyte proportion (%), x_2 blood pressure (mHg), x_3 pulse rate (bpm) and x_4 lymphocyte proportion (%).

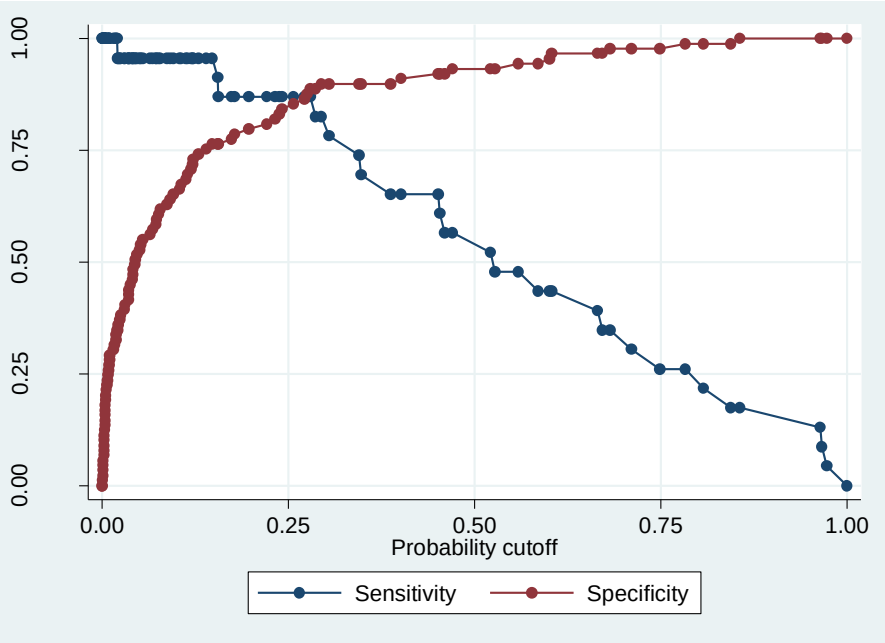
Table 18. Significantly associated laboratory variables at 24h prior to ICU admission compared to first presentation of dengue-infected ICU (cases) patients

Variables	Den-infected ICU at Presentation (n=23)^	Den-infected ICU at 24H prior ICU (n=23)^	COR	p-value	95% CI
Systolic blood pressure (25%-75%)	**110 (91.5- 118.5)**	**90 (80- 101.5)**	**0.96**	**0.03**	**0.92-0.996**
Pulse rate (25%-75%)	**95.5 (88.5- 102)**	**101 (90.5- 117.5)**	**1.07**	**0.044**	**1.002-1.14**
White cell count (25%-75%)	**3.3 (2.4- 4.2)**	**4.9 (3- 7.4)**	**1.59**	**0.033**	**1.04-2.44**
Proportion lymphocytes (25%-75%)	**16.5 (9.8- 19.9)**	**23.5 (18.1- 28.6)**	**1.19**	**0.020**	**1.03-1.38**
Proportion monocytes (25%-75%)	**7.9 (5.1- 9.9)**	**12.1 (9.1- 17.9)**	**1.35**	**0.033**	**1.02-1.78**
Serum haematocrit(25%-75%)	**44.9 (42.8- 47.2)**	**40.2 (33.6- 43.6)**	**0.81**	**0.037**	**0.66-0.99**

COR- Conditional Odds Ratio

^ - Four cases were excluded as they were admitted into ICU less than 24 hours after first presentation at hospital.

A.



B.

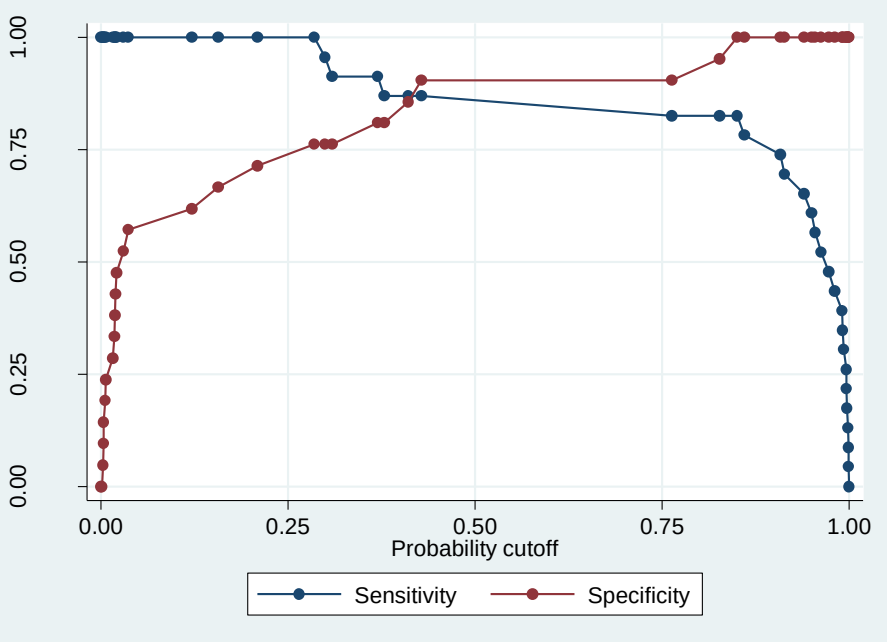


Figure 17. Sensitivity and specificity plots of Model at presentation (A.) and Model for 24hr Pre-ICU (B.) with the varying probability cut-off.

Differential Clinical and Laboratory Features of Death Cases at First Presentation among the ICU Cases

A subgroup analysis was performed among the ICU cases to explore the significantly different clinical and laboratory features of death cases at first presentation in hospital (**Table 19**). The median age for death ICU cases (n=7) and non-death ICU (n=20) subgroups was 48 (25th-75th pctl: 27-50.5) and 43 (25th-75th pctl: 36.8-54.3) years old, respectively. There were about 30% female in both the death and non-death subgroups (*P*=1). Based on the 2009 WHO classification, 100% and 85% had severe dengue in the death and non-death subgroups, respectively (*P*=0.545). The median length of stay in ICU was 3 and 3.5 days for the death and non-death subgroups, respectively (*P*=0.383). The median length of stay in hospital was 4 and 9 days for death and non-death subgroups (P=0.029). Importantly, the following characteristics at first presentation were observed to be significantly different between the death and non-death ICU subgroups: having any warning signs (*P*=0.022), monocyte (*P*=0.035) and eosinophils proportions (*P*=0.035), serum AST (*P*=0.049) and ALT (*P*=0.010), and protein (*P*=0.039) (**Table 19**).

Table 19. Significantly different characteristics between deaths and non-deaths at first presentation in the subgroup analysis of ICU Dengue-infected patients.

Variables	Den-infected ICU Non-Death (n=20)	%	Den-infected ICU Death (n=7)	%	p-value*
Median LOS in hospital (25%-75%)	9 (7-17.25)		4 (3.5-8.5)		**0.0285**
Median DPF to death (25%-75%)	0		3 (2.5-7.5)		
Median DPP to death (25%-75%)	0		7 (7-15.5)		
Any warning sign					
Yes	9	45	7	100	**0.022^**
Proportion monocytes (25%-75%)	9.1 (6.55-12.55)		5.4 (3.8-7.15)		**0.0348**
Proportion eosinophils (25%-75%)	0.1 (0-0.4)		0.5 (0.4-0.9)		**0.0351**
Serum AST (25%-75%)	11 (10-17)		19 (16-29)		**0.0487**
Serum ALT (25%-75%)	126 (70.25-263.25)		939 (442.75-1577.75)		**0.0103**
Serum protein (25%-75%)	85.5 (39.25-146.25)		491 (159-653.5)		**0.0328**

^- Fisher's exact test

*- Wilcoxon rank-sum test

6.3.DISCUSSION AND CONCLUSION

Dengue infection results in a wide spectrum of clinical severity, from self-limiting dengue fever to severe dengue. Timely and appropriate monitoring and clinical management, mainly with fluid interventions of dengue patients is critical to reduce morbidity and mortality (WHO, 1997, 2009). Some of these patients may also require critical clinical management in the intensive care unit (ICU). Healthcare resources such as ICU beds and close monitoring may be limited during a large epidemic, making effective triage of patients who may require ICU admission more crucial. However, there is currently a lack of systematic studies looking at potential early risk factors for ICU admission. Simple and vital clinical and laboratory indicators during first presentation at hospital or even 24 hours prior to ICU admission may assist clinicians in triaging these patients.

From this matched case-control study, dengue patients who were either between 50 to 59 years old or with pre-existing diabetes had about five times the risk of ICU admission, compared with dengue patients who are less than 30 years old or without diabetes, respectively (Table 15). Age group 50 to 59 years old was also implicated in ICU admission in a case-control study of dengue fatality (Ong et al., 2007) and the recent multi-center mortality cohort study in Singapore (Leo et al., 2011). However, the age group at risk of dengue hemorrhagic fever (DHF)observed in a dengue 2 epidemic in our other study was those from 30-49 years old (Pang et al., 2012). This suggests that different severity outcome measures, either defined as DHF, fatality or the requirement for ICU admission, may affect different age groups. Furthermore, age group of 60 years old and above was not observed to be a risk factor of ICU admission previously (I. K. Lee et al., 2012; Lye et al., 2010) which supports the observations in this study. On the contrary, it

was observed that the mean age of ICU admission in New Delhi case series was younger (39.6 years) (Juneja et al., 2011), which may be due to the lower proportion of dengue cases aged greater than 55 years in that study. In addition, in southern Taiwan, dengue patients 65 years old or above were reported to have a higher fatality risk compared with patients less than 65 years of age (I. K. Lee, Liu, & Yang, 2008). Interestingly, there was no significant difference in age between severe cases requiring ICU admission and benign casesin a dengue serotype 1 epidemic in New-Caledonia (South Pacific), with mean age of 47 years in both groups (Bouldouyre et al., 2006). These findings highlighted the potential differences in epidemiology of ICU dengue patients in different geographical regions, which would be interesting for further evaluation. In general, the risk of ICU admission was observed to increase from age 40 years old and above (**Table 15**). Diabetes was not an independent risk factor of ICU admission in this study (even after adjusting for DHF), which has also been similarly reported in dengue mortality studies (I. K. Lee et al., 2012; Leo et al., 2011; Ong et al., 2007). However, diabetes was shown to be an independent risk factor of DHF as an outcome (Figueiredo et al., 2010; Pang et al., 2012), and DHF as an outcome was significantly associated with ICU admission in this study. This may suggest having diabetes may not predispose a patient to ICU admission or death directly, but it may increase the risk of DHF, resulting in ICU admission. However, caution in interpretation remains as the sample size of cases is small in this study.

Based on the WHO classification at presentation, patients who fulfilled the WHO 2009 severe dengue classification were at a higher risk of ICU admission, eight times more than patients classified as non-severe, but not for the WHO 1997 DHF/DSS criteria at first presentation(Table 16). This suggests that the WHO 2009 classification for severe dengue is more sensitive in identifying clinically more severe patients than the WHO 1997

classification for DHF/DSS at first presentation; similar observations have been reported in other studies (Gan et al., 2013; Leo et al., 2011; Narvaez et al., 2011). However, the application of both WHO 1997 and 2009 classification systems for severe dengue conditions only achieved sensitivity of about 19% and 52%, respectively, in differentiating patients who required ICU admission at first presentation, although both classification systems for classifying severe conditions achieved high specificity of 87% and 94%, respectively. In contrast, at the time of final clinical outcome, the WHO 1997 classification system achieved sensitivity and specificity of about 67% and 78% respectively, and the WHO 2009 classification system achieved sensitivity and specificity of about 89% and 90% respectively. Our results are comparable to the sensitivity and specificity of identifying patients requiring category III care (includes ICU admission) for DHF/DSS (39.0% and 75.5%, respectively) and for severe dengue (92.1% and 78.5%, respectively) as described by Narvaez F et al. (Narvaez et al., 2011).

Most warning signs and symptoms at first presentation in this study were not observed as risk factors of ICU admission in this study, except for hypoproteinemia, hypotension, and hematocrit rise with rapid platelet drop (**Table 17**). Other studies also reported that most warning signs were not able to identify patients progressing into DHF, severe dengue, category III care or fatality efficiently (I. K. Lee et al., 2012; Narvaez et al., 2011; T. L. Thein, Gan, et al., 2013). Gastrointestinal symptoms were proposed as an important warning sign for severe dengue and fatality (Figueiredo et al., 2010; I. K. Lee et al., 2012), but it was not associated with ICU admission in this study. Hypoproteinemia was observed in most (>80%) of the fatal cases in other studies (Leo et al., 2011), and was observed at first presentation of severe patients who required ICU admission (Sam, Omar, Teoh, Abd-Jamil, & AbuBakar, 2013) . Similarly, hypotension was observed in 71% of

fatal cases (Ong et al., 2007), and was observed to be significantly more in severe cases who required ICU admission (Sam et al., 2013). Interestingly, having any warning signs at first presentation may be a risk factor for death among ICU cases (**Table 19**).

The following laboratory parameters at presentation may be potential indicators of ICU admission at first presentation in hospital, and were reported in ICU admission case series and/or fatality case-control studies: pulse rate (Ong et al., 2007), neutrophil (Bouldouyre et al., 2006), lymphocytes and monocytes proportions, platelet count (Chandralekha, Gupta, & Trikha, 2008; I. K. Lee et al., 2012; Ong et al., 2007; Sam et al., 2013; Schmitz et al., 2011), serum urea, creatinine (Bandyopadhyay et al., 2006; Ong et al., 2007; Schmitz et al., 2011), AST, ALT (Bandyopadhyay et al., 2006; Chandralekha et al., 2008; Ong et al., 2007; Sam et al., 2013; Schmitz et al., 2011)[5, 10, 15, 20, 22], protein (Bouldouyre et al., 2006; Schmitz et al., 2011)[5, 22] and albumin (Bouldouyre et al., 2006; Ong et al., 2007; Sam et al., 2013; Schmitz et al., 2011). In our study, a prognostic model using a combination of neutrophil proportion, ALT and serum urea level provided a sensitivity of 88.2%, specificity of 88.9%, PPV of 62.5% and NPV of 97.3%. This is comparable to the models for predicting severity, defined as death using a model comprised of creatinine, free bilirubin, amylase and platelets with PPV 66.7% and NPV 75.5% (Bouldouyre et al., 2006), and the prediction of DHF in adult patients at presentation with median DPF of 5 days using a model comprised of clinical bleeding, serum urea, serum protein, and lymphocyte proportion with sensitivity 83%, specificity 84%, PPV 18%, NPV 99% (V. J. Lee et al., 2008; V. J. Lee et al., 2009; T. L. Thein et al., 2011). Serum urea level was a common variable in these models, which suggests urea level in serum may be an important risk factor for severity. Monocyte proportion, AST, ALT and serum proteins were also observed to be significantly different between the death

and non-death among ICU cases. This suggests that these could be important factors involved in the progression of fatality after ICU admission, and should be considered for close monitoring during hospitalization. On the contrary, only bandemia (excess of immature white blood cells) was highlighted as a significant risk factor at first presentation between non-fatal and fatal patients (I. K. Lee et al., 2012)

Dengue severity is unpredictable during hospitalization. Pulse rate, lymphocyte and monocyte proportions, blood pressure, white cell count, and hematocrit were observed as risk factors at 24 hours prior to ICU admission, and were reported in ICU admission case series and/or fatality case-control studies (Juneja et al., 2011; I. K. Lee et al., 2012; Ong et al., 2007). In the fatality study, leukocytosis and platelet count were also shown to be significantly different between the first presentation and pre-fatal data (I. K. Lee et al., 2012). Interestingly, lymphocyte and monocyte proportions of ICU dengue patients at 24 hours prior to ICU admission was significantly greater compared with that at first presentation, but lower compared with non-ICU dengue patients at first presentation. This suggests the dynamic differences in immune response between dengue patients who required ICU admission and dengue patients who did not require ICU admission were already evident even at first presentation, which is about three to five days post fever. In addition, this highlighted the fact the immune response changes rapidly over time as a patient progresses from first presentation at hospital to 24 hours before ICU admission (Hoang et al., 2010). A prognostic model using a combination of monocytes and lymphocytes proportion, blood pressure and pulse rate provided a sensitivity of 88.9%, specificity of 86%, PPV of 80% and NPV of 86.4%.Some of these significant variables were also part of the model used for predicting pediatric DHF which comprise white cell count, monocytes proportion, platelet count, and hematocrit with sensitivity of 97% and

specificity of 48% (Potts et al., 2010). Furthermore, this model is clinically relevant as pulse rate and blood pressure were known critical factors for ICU admission, and the predictors were selected in an unbiased manner based on its statistical significance by regression techniques. Lastly, this model was observed to be more effective in stratifying potential ICU cases requiring ICU intervention in about 24 hours' time before ICU as compared to the predictive model with only pulse rate and blood pressure as significant variables.

There are some limitations in our study that should be taken into consideration when interpreting the results. First, the study had a relatively small number of patients with ICU admission that may result in low statistical power to detect true association, even though Tan Tock Seng Hospital is a major infectious disease center in Singapore, which provided care for about 40% of all reported dengue patients in Singapore. As such, the power was maximized by using four matched controls to each case. Second, no dengue serotype data and BMI/obesity data were available for individual study subjects. In order to account for the serotype variable, we used the year of dengue presentation as a surrogate for serotype (as describe in methods) as well as a matching factor for our selection of controls to minimize confounding effect due to the different predominant serotype each year. Third, this model is derived from adult dengue patients presenting to hospital at a median of 5 DPF (25^{th}-75^{th}pctl=3-5 days). Hence, the model may be less accurate when used for triage of patients outside this period range, or when used at community healthcare facilities, where patients may present to clinicians at lesser days post fever than in our study. Further validation studies are still required to confirm the validity and reliability of this model. Lastly, three parameters in our probability equation were laboratory test results, which may not be easily available in resource-poor countries.

However, it is hoped that as the awareness of dengue prevention and clinical management is raised with the guidance of the WHO, there will be an increase in laboratory capabilities that can help to resolve this challenge gradually.

Studies to assess risk factors for severity as defined by DHF/DSS and severe dengue for effective triage are important. However, information on dengue severity as defined by the requirement of ICU admission is also critical, but lacking. This is the first systematic study, to our best knowledge, that highlighted the differential pathophysiological background of dengue patients at presentation and at 24 hours prior to ICU admission. Based on this study, the key demographic and clinical risk factors for triage at first presentation that we propose are age group 50 to 59 years old, pre-existing diabetes, WHO 2009 classification at first presentation, hypoproteinemia, hypotension and hematocrit rise with rapid platelet drop. For laboratory risk factors, two optimal models with significantly associated variables were derived and proposed to complement the WHO classification system. An optimal model at first presentation comprises neutrophil proportion, ALT and serum urea level, and another optimal model for assessing severity 24 hours prior to ICU admission comprises monocytes and lymphocytes proportions, blood pressure and pulse rate. This would be useful to identify dengue patients who may require ICU admission early at presentation as well as during hospitalization for closer monitoring and clinical management to reduce morbidity and mortality, when validated in independent larger studies.

CHAPTER 7: THE POTENTIAL PUBLIC HEALTH IMPACT OF RISK FACTORS ASSOCIATED WITH ADULT DENGUE SEVERITY- THE CLOSING CHAPTER.

7.1.INTRODUCTION

Dengue caused substantial morbidity and mortality globally, resulting in an alarming public health burden (Bhatt et al., 2013; Carrasco et al., 2011; Shepard et al., 2013; P. T. Tam et al., 2012; WHO, 2009, 2010a, 2012). Substantial improvements are possible. Dengue burden can be reduced through an integrative top-down government and bottom-up community approach. This strategy involves execution of sustainable prevention measures and programmes, and strong recommendation for early diagnosis and prognosis to guide interventions and clinical management (WHO, 2009, 2012) at different stages of dengue disease: the pre-infection, pre-clinical (post-infection), and clinical stages (Ginsburg & Willard, 2009).

There is no one single risk factor assessed so far that can account for all severe dengue disease outcomes, which was also highlighted by Guzman M.G. and Kouri G.P. as a "multifactorial and unifying view of the problem" (Guzman & Kouri, 2008; Kouri, Guzman, & Bravo, 1987). Therefore, the integrated approach also involves unravelling of risk factors associated with dengue severity from different aspects of dengue diseases, and to apply these risk factors in an integrative manner to achieve maximum cost-effectiveness in reducing dengue burden. Moreover, it is of paramount importance for the whole community (both residents and government), particularly in dengue endemic countries to

support prevention measures/programmes, and for the clinicians to recognise the risk factors early for early diagnosis, prognosis and interventions to reduce dengue severity. Severity is usually defined according to the WHO classifications(WHO, 1997, 2009), but this could also be defined by clear and objective clinical criteria such as the requirement of intensive care unit admission. Even though it has its own limitations and advantages as a measurement of severity, as described in Chapter 1, it remains critically relevant to have an early prognosis capability of dengue severity.

7.2. APPLICATION OF RISK FACTORS, ITS POTENTIAL PUBLIC HEALTH IMPACT, AND IMPLICATIONS FOR PATHOGENESIS

Epidemiological and genetic risk factors as baseline risk

Susceptible individuals in dengue endemic countries who possessed the epidemiological and genetic risk factors identified in Chapter 3 and 4 may be classified as the "potentially susceptible group" who are at an elevated risk of complications should they get infected with dengue; the genetic factors identified in Chapter 3 would be of particular relevance to both children and adult as baseline risk, while the epidemiological risk factors identified in Chapter 4 would be of relevance to only adults. This "potentially susceptible group" could be educated and guided by clinicians and public health professionals about the importance of primary prevention and protective measures such as vector control, the use of insect repellent and mosquito nets, and to avoid countries where dengue outbreaks are occurring as much as possible. In addition, this "potentially susceptible group" may be recommended to have the dengue vaccine when it is made available. However, evidence on the effectiveness of personal protective measures for dengue prevention is currently lacking, and further evaluation on vaccine safety and efficacy for this "potentially susceptible group" is critically required prior to the recommendation (**Figure 18**).

This "potentially susceptible group" in the endemic countries should also be highly encouraged to visit the primary/secondary healthcare for both screening (for epidemiological and genetic risk factors) and early diagnosis at less than 72 hour post fever, where rapid diagnostic tests such as the viral RNA detection and NS1/IgM/IgG-based assays would be available and diagnosis can be made usually within 24-hour.

However, there is currently an urgent need to raise the awareness and importance of early dengue diagnosis in the public primary healthcare settings to maximise these diagnostic and prognostic tools. Even in country such as Singapore which has great accessibility and availability to medical care, a large proportion of primary care physicians were not as active as expected in ordering dengue diagnostic test for patients who fulfilled clinical criteria(L. K. Lee et al., 2011). The situation may be worse in the developing countries, where a diagnostic test may not be readily available. Fortunately, in a global effort, much guidance and research has been gradually put in place on developing and sharing assays and raising awareness on early diagnosis of dengue, particularly in dengue endemic developing countries (Peeling et al., 2010). With increasingly more active health-seeking behaviour of the population towards screening of risk factors and early diagnosis of dengue at the primary/secondary healthcare centres, prompt and appropriate interventions/clinical management such as antiviral therapy (when made available) and hospitalisation for closer monitoring can be applied to ensure the progression to severe dengue is prevented. Furthermore, early prescription of antivirals would only be effective during early infection to inhibit viral replication, hence, the importance of early diagnosis (**Figure 18**).

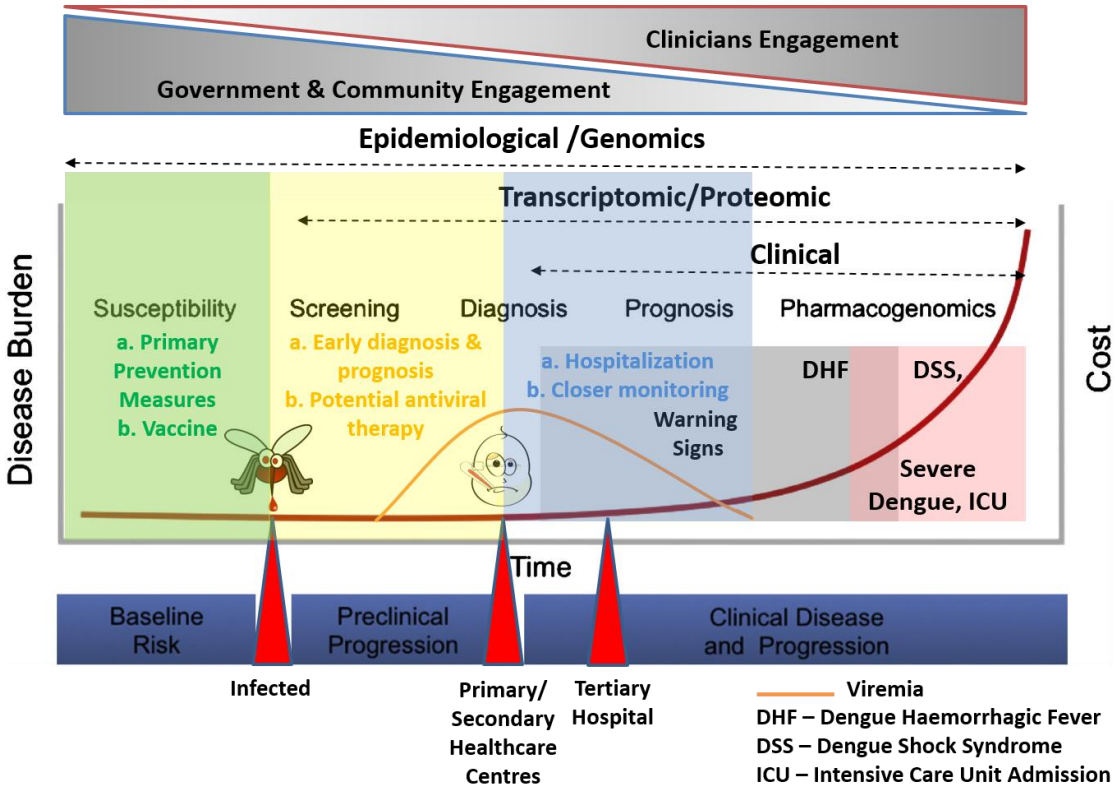
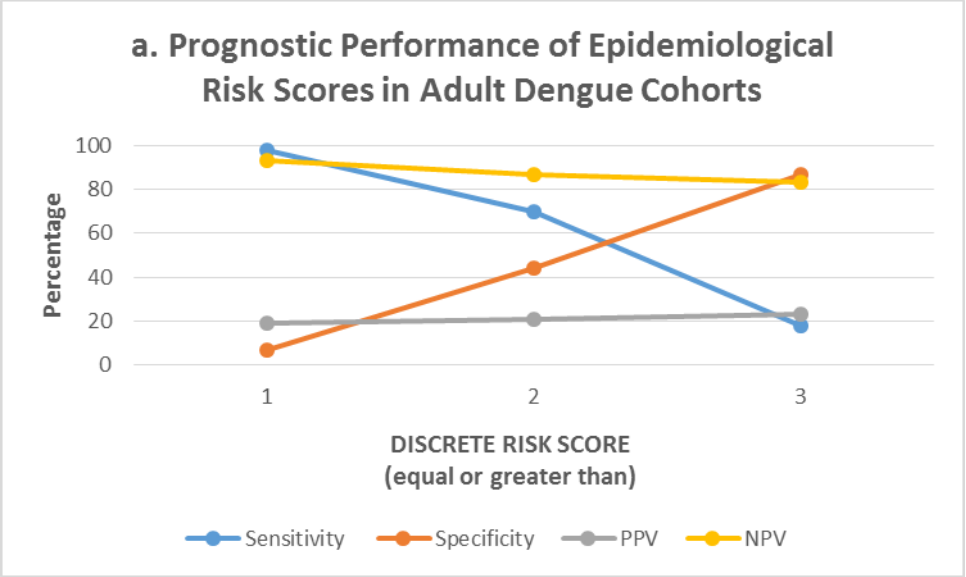


Figure 18. Potential interventions to reduce the public health burden due to dengue at different stage of the disease, from pre-infection, pre-clinical (post-infection) to clinical stage of the disease.

(Adapted from Translational Research 154/6, Ginsburg GS and Willard, Genomic and personalized medicine: foundations and applications, 277-286, Copyright 2009, with permission from Elsevier)

Using age range from 30-49, Chinese, female and having diabetes as individual risk factors of DHF (Chapter 4) to compute a screening/triage risk score upon first presentation to the clinicians of about 2600 laboratory confirmed dengue patients in Singapore, sensitivity drops but specificity increases to about 85% as the risk score increases. Negative predictive value (NPV) remains relatively high at about 80% as the risk score increases (**Figure 19a**). Similarly, using logistic regression algorithm of these epidemiological risk factors listed above to compute the weighted risk score provided comparable performance as the discrete risk scoring method (**Figure 19b**). This suggests that using a discrete risk score of 3 or a weighed risk score of 9 and above as a cut-off for DHF in a setting with DHF prevalence of about 3% is more reliable for identifying patients who are less likely to develop DHF as dengue outcome.

On applying the top genetic locus at MICB rs3132468 that was associated with DSS (Khor et al., 2011) and non-DSS cases (Whitehorn et al., 2013) in Vietnam on a cohort of about 1000 Singapore Chinese dengue adult cases to evaluate the prognostic performance for DHF, it can only achieve a sensitivity of 17% and specificity of 82%, PPV of 16% and NPV of 84%. The low PPV is likely due to the low DHF prevalence rate in Singapore. The high specificity but low sensitivity suggests that the MICB genetic locus is also only effective to rule out individuals not likely to develop severe dengue, rather than to identify those who are at higher risk of severe dengue.



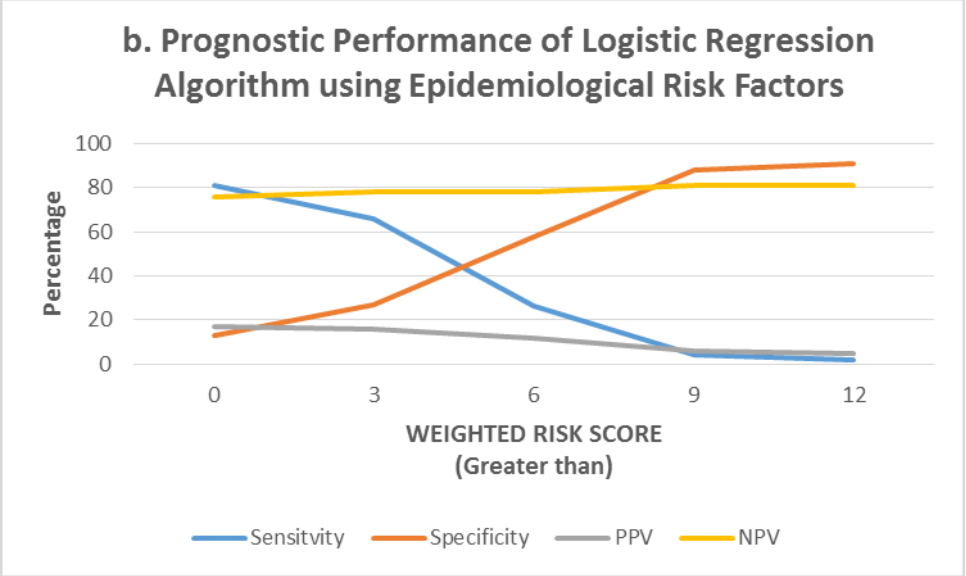


Figure 19. Prognostic performance of using discrete (a) and weighted (b) risk scoring method to triage patients at first presentation.

RNA and protein biomarkers to complement clinical and laboratory risk factors as dengue disease progresses from pre-clinical to clinical stage.

Taking advantage of the dynamic and sensitive transcriptome and proteome changes at the pre-clinical stage (post-infection), there would be opportunity at less than 72 hour post infection for both antiviral therapy and triage of patients who are at high risk of developing severe clinical manifestation, and the need for closer monitoring in the tertiary hospital, as described in Chapter 5. Similarly, this molecular biomarkers could be applied during the early clinical phase of the disease at the tertiary hospital (**Figure 18**), to complement the application of the clinical risk factors to triage adult dengue patients who are likely to develop severe outcomes such as the requirement of intensive care unit admission, as described in Chapter 6.

Due to the challenges of utilising warning signs as an early prognostic tool on adult dengue patients at primary healthcare setting, particularly at less than 72 hour post fever as described in Chapter 1 and 5, two molecular prognostic models were built. The model with RNA *CCL8, VPS13C* and protein uPAR has a sensitivity of 82.9%, specificity of 80.0%, PPV of 80.6% and NPV of 82.4%. The other model with RNA *CCL8, VPS13C* and platelet counts with sensitivity of 80.9% sensitivity, 84.4% specificity, positive predictive value of 80.6% and negative predictive value of 82.4% (Chapter 5). These molecular prognostic tools would be useful for clinicians to make well-informed decisions early at less than 72 hour post fever to guide clinical management, particularly in the primary/secondary healthcare centres, after validation with a larger cohort.

As these high risk dengue adult patients are recommended to be hospitalized from primary healthcare setting, it would be optimal to have another prognostic capabilities

upon presentation at the hospital for dengue patients who are more likely to progress in dengue severity and who would require intensive care unit admission (ICU) during hospitalization (Chapter 6). The requirement for ICU may be considered as an important clinical outcome of severity, and is usually neglected due to the popular usage of the WHO classification (WHO, 1997, 2009). Epidemiological risk factors for requiring ICU care were age group 50-59 years old and probably patients with diabetes and cardiac disorders. One prognostic model was built for ICU triage at first presentation with risk factors of significant discriminatory power. The model with neutrophil proportion, ALT and serum urea had sensitivity of 88.2%, specificity of 88.9%, PPV of 62.5% and NPV of 97.3%. Another model was also built for ICU triage 24hr before ICU admission with risk factors of significant discrimatory power. This model with monocyte proportion, blood pressure, pulse rate (bpm) and lymphocyte had sensitivity of 88.9%, specificity of 76%, PPV of 80% and NPV of 86.4%. These models would also be useful to complement the use of warning signs for recommendation of closer monitoring during triage at first presentation as well as during hospitalisation to guide clinical management and to reduce progression of dengue severity of adult patients. Table 20 summaries the results of this thesis.

Potential reduction in healthcare cost and burden due to dengue

In Singapore, about 80% of the notified adult dengue cases were hospitalized from 2000 to 2005 despite low rates of DHF (1.8–2.8%) (Lye, Chan et al. 2008). Furthermore, the usage of the new WHO 2009 classification, particularly with the warning signs may be too sensitive and resulted in significant increase in admission and clinical workload, which may not be clinically necessary (S. Kalayanarooj, 2011; Leo et al., 2013). As warning signs are recommended as criteria for hospitalization, both sensitivity and specificity are

important. High specificity is important to optimize the use of scarce hospital resources to manage only patients who are at high risk of progressing to severe dengue (Srikiatkhachorn et al., 2011). At the same time sensitivity is critical to ensure that dengue patients are not being sent home with subsequent progression to DHF or SD. These epidemiological, genetic, and molecular risk factors found in this thesis would provide additional guidance for clinicians to triage adult dengue patients who are not likely to develop severe dengue disease (Chapter 3 and 4), and those who are more likely to require close monitoring (Chapter 5 and 6) at early infection (less than 72 hour post fever). These risk factors may also augment the previously derived clinical and laboratory risk factors which are mostly looking at dengue patients who presented more than 72hour post fever (V. J. Lee et al., 2008; V. J. Lee et al., 2009; Leo et al., 2013; T. L. Thein, Gan, et al., 2013) to ensure that there were tools available to cover a reasonable time period of first presentation of these dengue patients for triage. With these, it is hopeful that unnecessary hospitalisation can be reduced in tertiary hospitals, but also at the same time, increase the chance of early diagnosis and prognosis for appropriate clinical management of those who are at higher risk of severe dengue. As a result, there will be much reduced overall healthcare cost incurred (Carrasco et al., 2011; L. K. Lee et al., 2013) and dengue burden in Singapore if we could accurately triage adult patients who do require close monitoring and are likely to develop severe dengue from those patients that can be safely managed as outpatients without progression to severe clinical conditions, as reflected hypothetically in Figure 18 (Ginsburg & Willard, 2009) .

Table 20.Summary of risk factors as potential early clinical prognosis tools and proposed actions.

Variables	Severity	Subjects Type	Recommended Disease Phase of Application	Sens (%)	Spec (%)	PPV (%)	NPV (%)	Proposed Actions
30-49, Chinese, female and having diabetes	DHF	Adult	Pre-infection and pre-clinical	20	85	25	80	Active prevention measures; Vaccination; Early dengue diagnosis & antiviral therapy
MICB variant			Pre-infection and pre-clinical	17	82	16	84	
***RNA VPS13C,* *CCL8* and protein uPAR**	Warning signs & hospitalization		Less than 72 hrs post fever	82.9	80.0	80.6	82.4	Early dengue diagnosis & antiviral therapy; Hospitalization
***RNA VPS13C,* *CCL8* and platelet**				80.9	84.4	80.6	82.4	
Neutrophil proportion, ALT and serum urea	ICU admission		Median 6 days post fever	88.2	88.9	62.5	97.3	Hospitalization; closer monitoring and more active clinical management
Monocyte proportion, blood pressure, pulse rate (bpm) and lymphocyte	24hrs to ICU admission			88.9	76	80	86.4	

Implications from studies in thesis for dengue pathogenesis

Risk factors such as MICB, diabetes and older age have been associated with impaired immunity. These high risk individuals may have difficulties in mounting an early immune response to inhibit viral entry, replication and infection, which manifests as elevated viral load and altered blood monocyte and lymphocyte counts, including increased in CCL8 (MCP-2), a chemokine that had been previously associated as a biomarker for tuberculosis diagnosis (Ruhwald et al., 2008) and outcome of hepatitis C virus infection (Hellier et al., 2003), IP-10 (CXCL-10), a pro-inflammatory chemokine (Luster, 1998), highly associated as a biomarker to predict severity of inflammatory diseases including infectious diseases, immune dysfunction and tumour development (Liu et al., 2011). These may then result in adverse clinical outcomes via more complex pathways, which remains to be elucidated.

However, many of the factors identified in the thesis do emphasize the role of dysfunctional vascular permeability. Plasma leakage has long been recognised as a key part of the pathogenesis pathway to DSS, and this thesis adds to the evidence. Firstly, we identified the importance of the PLCE1 gene in DSS, a gene thus far implicated in nephrotic syndrome; while this may seem to be an unrelated non-communicable disease, we note that the key commonality here is leakage of proteins across the vascular endothelium. Likewise, diabetes, and to some extent older age also affects vascular endothelial function, and provides an alternative non-genetic basis for increased plasma leakage. The molecular markers uPAR is an indicator of diabetes, systemic inflammation, and active vascular endothelial dysfunction such as segmental glomerulosclerosis (Huang et al., 2014). In addition, VPS13C is a vascular protein was previously associated with the

pathophysiology of type-2 diabetes (Strawbridge et al., 2011). Therefore, these biomarkers potentially reflect a downstream manifestation on the pathway of plasma leakage. As a consequence of plasma leakage, altered serum urea levels and subsequent hemodynamic changes like low blood pressure and elevated pulse rate necessitate ICU resuscitation, as identified in Chapter 6.

In addition, blood coagulation may be hindered by means of fibrinolysis, where a fibrin clot, the product of coagulation, is broken. This process is catalysed by the enzyme plasmin, which is regulated by tissue plasminogen activator and urokinase (regulated by uPAR) that convert plasminogen to the active plasmin, thus allowing fibrinolysis to occur. The fibrinolytic system is also closely linked to control of inflammation, and plays a role in disease states associated with inflammation. Plasmin, in addition to lysing fibrin clots, also cleaves the complement system component C3, and fibrin degradation products have some vascular permeability inducing effects. As such, it is hypothesized that the increased uPAR level in the blood may be highly associated with the risk of severe bleeding, by increasing fibrinolysis, with decreasing platelet count as dengue infection progresses, and at the same time, affects the vascular permeability of the endothelium, resulting in increased risk of plasma leakage. Future research to prove this hypothesis would be critical as the cause of severe dengue may arise from this pathway.

Overall limitations of thesis

Even though a number of potential risk factors and prognostic models had been identified and derived in this thesis, further validation with more well-described prospective cohorts would be important prior to their application in the clinical setting. There are a few limitations that may limit the generalizability of the findings. There is low prevalence of severe dengue of adult patients in Singapore, therefore, the PPV and NPV may not be applicable in endemic countries with high prevalence of severe dengue among adult patients. However, severe dengue is usually more common among the paediatric as compared to adult patients. Furthermore, the prevalence of diabetes may vary in different endemic countries, and this is mostly likely to affect the PPV and NPV of the risk scoring algorithm. Intensive care unit (ICU) admission reflects a more realistic outcome of dengue severity compared to the WHO classification of DHF/DSS/Severe Dengue. However, the threshold for ICU admission may vary in different hospitals in different countries, and hence, an independent study for risk factors of ICU is highly recommended at different hospitals. Furthermore, most of these studies were performed in the hospital setting, and so to some extent, there may be bias towards selection of patients who had more active healthcare-seeking behaviour, or those who had more severe clinical manifestations. There may also potentially be non-differential misclassification bias of outcomes and exposures due to recall bias or interviewer bias. However, the extent of this bias should be minimal as patients were reviewed based on a standardised dengue care path or standardised review method of medical records. Last but not least, these prognostic models would only be useful if both the medical community and the public understand the importance of early dengue diagnosis, which would require the long-term commitment and support from the governments as well as the non-profit organisations to drive awareness on these risk factors of dengue severity (**Figure 18**).

Conclusion

In conclusion, this thesis has explored the risk factors of dengue severity, which comprised of DSS, DHF (WHO, 1997), the requirement of closer monitoring in hospital (WHO, 2009), and ICU admission. In addition, this thesis has taken up the challenge of exploring the risk factors from various aspects of adult dengue disease, looking into the epidemiological, genetic, molecular and clinical risk factors. Risk factors and models derived from each of these aspects for early triage of adult dengue patients would be potentially beneficial at every phase of dengue disease, from pre-clinical to clinical, to increase the intervention window of reducing the development of severe disease and mortality, and ultimately to reduce public health burden due to dengue. They also have important implications for prioritising utilisation of dengue vaccines and therapeutics should these become available in the near future. Potential future work would include prospective intervention studies of these risk factors and models for application in the primary and tertiary setting in endemic countries, as well as further basic science and clinical research to elucidate the mechanisms behind the risk factors for dengue severity that we have identified.

Manuscripts Published

Chapter 3

1. Whitehorn J, Chau TNB, Nguyet NM, Kien DTH, Quyen NTH, Trung DT, **Pang J** et al. Genetic variants of MICB and PLCE1 and association with non-severe dengue. ***PLoS NTD* 8(3):e59067**.

2. Khor CC, Chau TN, **Pang J**, Davila S.et al. Genome-wide association study identifies susceptibility loci for dengue shock syndrome at MICB and PLCE1**.** ***Nat. Genet.* 2011. 43(11):1139-1141**.

Chapter 4

3. **Pang J**, Salim A, Lee VJ, et al. Diabetes with hypertension as risk factors for adult dengue haemorrhagic fever in a predominantly Dengue serotype 2 epidemic: A case control study. ***PLoS NTD*. 6(5): e1641.2011**

Chapter 6

4. **Junxiong Pang**, Tun-Linn Thein, Yee-Sin Leo, David C Lye. Clinical and Laboratory Risk Factors of Dengue Patients Requiring Admission into Intensive Care Unit during 2004-2008 Dengue Epidemics: A Matched Case-Control Study. ***BMC Infect Dis. 2014 Dec 5;14(1):649.***

Manuscripts under Review

Chapter 5

5. **Junxiong Pang*,** Anna Lindblom*, Thomas Tolfvenstam, Tun-Linn Thein, Ahmad Nazri Mohamed Naim, Ling Ling, Angelia Chow, Mark Chen-I-Cheng, Eng Eong Ooi, Yee-Sin Leo, Martin L. Hibberd. Discovery and Validation of Early Biomarkers to Guide Clinical Management of Dengue in Adults with Warning Signs- Manuscript under review in ***Genome Medicine***

Oral/Poster Presented

1. American Society of Tropical Medicine and Hygiene 61th Annual Meeting 13-17 Dec 2013, Washington, DC, USA."Clinical and Laboratory Characteristics of Dengue and HIV: A Matched Case-Control Study"**(Poster)**
2. American Society of Tropical Medicine and Hygiene 61th Annual Meeting 13-17 Dec 2013, Washington, DC, USA."Clinical and Laboratory Risk Factors of Dengue Patients Requiring Admission into Intensive Care Unit during 2004-2008 Dengue Epidemics: A Matched Case-Control Study"**(Poster)**
3. Yong Loo Lin School of Medicine Graduate Scientific Congress 2013 "Diabetes with hypertension as risk factors for dengue haemorrhagic fever in a predominantly dengue serotype 2 epidemic: A case-control study" **(Poster)**
4. Singapore International Conference on Dengue and Emerging Infections 21-23 Nov 2012 "Early laboratory and molecular indicators of hospitalized adult dengue patients with warning signs" **(Poster)**
5. American Society of Tropical Medicine and Hygiene 60th Annual Meeting 4-8 Dec 2011, Philadelphia, PA, USA "Risk factors of dengue haemorrhagic fever in a predominantly dengue serotype 2 epidemic" **(Oral & Poster)**

Awards Received

1. Best Poster Award- Yong Loo Lin School of Medicine Graduate Scientific Congress 2013, National University of Singapore
2. 2nd Prize for the Elsevier-ASTMH Clinical Research Award 2014, America Society of Tropical Medicine and Hygiene Annual Meeting 2014, USA

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