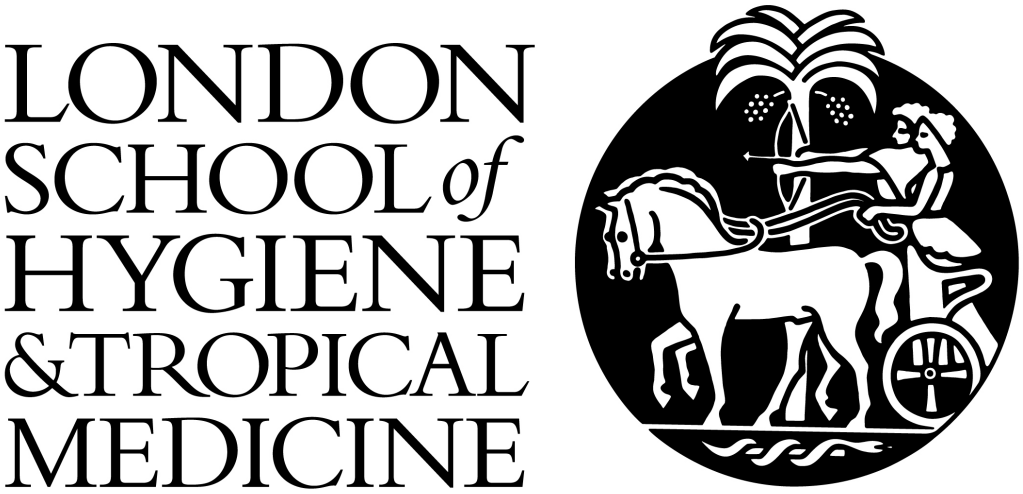
THE PATHOGENESIS AND CLINICAL MANAGEMENT OF DENGUE

JAMES WHITEHORN



Department of Clinical Research

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DECLARATION BY CANDIDATE

I have read and understood the School's definition of plagiarism and cheating given in the Research Degrees Handbook. I declare that this thesis is my own work. Where appropriate, I have acknowledged the contributions and work of other people.

Signed:

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ABSTRACT

Dengue is an arboviral disease that exerts a significant public health burden on the tropical world. Currently there is no available vaccine or specific therapeutic. My reviews show that our understanding of dengue pathogenesis, transmission dynamics and optimal case management is incomplete. The work presented in this thesis is a compilation of expert reviews/perspectives and primary research that addresses some of these knowledge gaps.

Understanding dengue pathogenesis and in particular, risk factors for progression to severe dengue, is an important priority to reduce morbidity and mortality, especially in young children. Genetic variants of the *MICB* and *PLCE* genes have been shown to be associated with severe dengue. I tested the hypothesis that these variants are also associated with less severe dengue infection and with higher early viraemia levels in two studies involving the genotyping of 3961 and 2742 dengue cases respectively. My studies showed that these genetic variants are associated with less severe but clinically apparent dengue infection but showed no evidence of an association with higher viraemia levels. The functional basis of these susceptibility mutations remains unclear.

Dengue transmission dynamics are shaped by the prevalence of the permissive vectors, *Aedes aegypti* and *Aedes albopictus.* My research hypothesis was that susceptibility and transmission of dengue might differ between the two species. I conducted a clinical study that compared the susceptibility of *Ae. aegypti* and *Ae. albopictus* to both initial and disseminated dengue after direct blood feeding experiments on viraemic patients. This work showed that both mosquito types were equally susceptible to initial infection with dengue but that *Ae. albopictus* was less likely to develop salivary infection, and, thus, an infectious phenotype. These results have important implications for the development of dengue transmission models, especially in areas of dengue emergence where the influence of *Ae. albopictus* is thought to be greatest. In addition, the results confirm the central importance of patient plasma viraemia in causing successful DENV transmission, suggesting that reducing this through the use of antivirals could potentially reduce transmission.

Clinical management of dengue patients remains an enormous challenge. Statins are rational candidate drugs for dengue because of their previously identified positive influence on vascular endothelial function. I conducted a clinical trial of lovastatin therapy in adult dengue patients. The trial showed that lovastatin was safe and well tolerated in dengue patients but it did not show any positive effects on the kinetics of viraemia or on any of the pre-specified clinical or laboratory features. I conducted a survey of platelet management in 20 countries and found a wide variety of approaches to the use of platelets in dengue underscoring the need for prospective clinical trials to inform evidence in this area. To reduce the large sample size normally required for the development of dengue therapeutics, I considered the use of a

human dengue infection model in dengue drug development. This model has the potential to a game-changer in drug development and in the design of future trials.

PREFACE

In accordance with LSHTM Research Degree Regulations much of this thesis is presented as a series of published or accepted review and research manuscripts. The papers are supplemented by additional material that explains how the research forms a coherent body of work. There is therefore some repetition of background information in the manuscripts. In addition, the differing editorial conventions used by various journals may have resulted in some inconsistencies with terminology and formatting.

Research conducted in Vietnam by foreigners must be conducted in partnership with a local "competent authority" under the overall jurisdiction of the Vietnam Ministry of Health. All the work presented in this thesis was conducted under the umbrella of a project agreement between the Hospital of Tropical Diseases in Ho Chi Minh City and the Oxford University Clinical Research Unit.

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ABBREVIATIONS

ADE	Antibody-dependent enhancement
APTT	Activated partial thromboplastin time
ARDS	Acute respiratory distress syndrome
COPD	Chronic obstructive pulmonary disease
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DHIM	Dengue human infection model
DIC	Disseminated intravascular coagulation
DSS	Dengue shock syndrome
FDP	Fibrinogen degradation product
GWAS	Genome-wide association studies
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme-A
HCMC	Ho Chi Minh City
IMCI	Integrated Management of Childhood Illness
LSHTM	London School of Hygiene and Tropical Medicine
MHC	Major histocompatibility complex
MICB	MHC Class I polypeptide-related sequence B
OFI	Other febrile illnesses
OUCRU-VN	Oxford University Clinical Research Unit Vietnam
PLCE1	Phospholipase C, epsilon 1
PT	Prothrombin time
RT-PCR	Reverse transcriptase polymerase chain reaction

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Above all I would like to thank my wife Saras and my children Theresa, Sophie and Emily for their love and support and everything else.

ADMG

1. INTRODUCTION AND AIMS OF THESIS

Background

Dengue represents a major global health challenge. It is the most important human arboviral infection with an estimated 390 million infections annually.[1] Dengue causes considerable suffering to those affected but also exerts a significant economic cost to the communities and countries in which they live.[2] Treatment is limited to supportive care and we currently have no way of predicting which patients are at most risk of progressing to severe disease with the result that health facilities are often overwhelmed by patients with dengue in endemic countries. Despite decades of research our understanding of disease pathogenesis is limited hindering both vaccine and therapeutic development.[3] It was hoped that the Sanofi recombinant live attenuated vaccine candidate would be the answer to dengue control. However the vaccine trials showed mixed results with poor responses in those without pre-existing immunity and against DENV-2.[4,5] The phase 3 results suggest that the vaccine may reduce hospitalisation suggesting a possible disease modifying effect.[5,6] These results imply that a vaccine might prove to have a role as part of a multifaceted approach to dengue control but is unlikely to be the sole answer that had been hoped for. Addressing the global problem of dengue requires a multidisciplinary approach encompassing therapeutics, vaccine research and vector control. The successful development of these areas requires clinically relevant basic science research as a foundation.

Aims of thesis

The work described in this thesis aims to improve our understanding of dengue with the hope of improving the clinical and public health management of the disease. To these ends the thesis has four major aims.

Aim 1: To review the current state of understanding of dengue pathogenesis, transmission dynamics and clinical management of dengue patients

Specific objectives:

1. To conduct an overall review of dengue considering virology, epidemiology, pathogenesis, clinical features, and clinical management
2. To conduct a in-depth review of dengue pathogenesis

Aim 2: To understand host factors associated with the outcome of dengue infection

Specific objectives:

1. To conduct a review of host factors in dengue pathogenesis
2. To determine if genetic variants of the *MICB* and *PLCE* genes are associated with less severe dengue

3. To determine if genetic variants of the *MICB* and *PLCE* are associated with higher early DENV viraemia levels

Aim 3: To improve our understanding of dengue transmission dynamics

Specific objectives:

1. To determine if *Ae aegypti* and *Ae albopictus* are equally susceptible to dengue infection
2. To determine if the 2 species are equally capable of transmission to humans

Aim 4: To improve the management of dengue patients

Specific objectives:

1. To conduct a review of the current dengue therapeutics landscape
2. To determine the safety and efficacy of lovastatin therapy in adult patients with dengue
3. To survey the approach to platelet management in dengue infection in 20 countries
4. To consider the potential role of a human dengue infection model in drug development

Outline of peer-reviewed, published review papers in this thesis

Dengue overview

This manuscript provides an introduction to dengue. The article considers dengue virology, epidemiology, transmission, clinical features, pathogenesis, diagnosis, prevention and control.

Dengue pathogenesis

This monograph provides a detailed overview of dengue pathogenesis and considers the relevance of aspects of disease pathogenesis for vaccine and therapeutic development.

LITERATURE REVIEW 1: DENGUE FEVER VIRUSES

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SECTION A – Student Details

Student	James Whitehorn
Principal Supervisor	Rosanna Peeling and Cameron Simmons
Thesis Title	The pathogenesis and clinical management of dengue

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

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Dengue Fever Viruses

James Whitehorn, *London School of Hygiene and Tropical Medicine, London, UK and Oxford University Clinical Research Unit – Vietnam, Ho Chi Minh City, Vietnam*

Based in part on the previous version of this eLS article 'Dengue Fever Viruses' (2003) by Duane J Gubler.

Advanced article

Article Contents

- Classification
- Structure
- Viral Replication
- Vectors
- Transmission Cycles
- Epidemiology
- Clinical Features of Infection and Pathogenesis
- Pathogenesis
- Diagnosis
- Prevention and Control
- Future

Online posting date: 16th July 2012

Dengue viruses are the most important arboviruses causing disease in humans. Approximately 3 billion people live in areas of risk, and there are an estimated 50–100 million cases occurring each year. These estimates reflect an enormous burden of morbidity, predominantly affecting urban centres of the developing world. There is currently no specific treatment and disease control is limited to tackling the vector. Despite considerable scientific attention disease pathogenesis remains incompletely understood. A better understanding of dengue virus biology and disease pathogenesis has the potential to pave the way to successful therapeutic interventions. This article aims to review current understanding of virus and vector biology, disease pathogenesis and clinical manifestations and management. Finally the article will consider novel approaches to clinical management and disease control.

Classification

Dengue viruses belong to the family Flaviviridae, genus *Flavivirus*. There are four serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. They belong to a larger, heterogeneous group of viruses called arboviruses. This is an ecological classification, which implies that transmission between vertebrate hosts including humans is dependent on haematophagous (blood-sucking) arthropod vectors. **See also**: Human Pathogenic Viruses

There are over 70 antigenically related viruses in the genus *Flavivirus*, including the type species, *Yellow fever virus*. The genus includes several antigenic complexes, including the dengue complex, the Japanese encephalitis complex and the tick-borne encephalitis complex. The Japanese encephalitis complex includes several well-known disease pathogens of humans, including *Japanese encephalitis*, *Murray Valley encephalitis*, *St Louis encephalitis*, *West Nile*, *Kunjin*, *Zika* and other viruses, all of which are transmitted by mosquitoes. The tick-borne flaviviruses include *Tick-borne encephalitis*, *Omsk haemorrhagic fever* and *Kyasanur Forest disease viruses*. Some *flaviviruses* have no arthropod vector, probably having lost the need for this type of transmission during the process of developing evolutionary relationships with their vertebrate hosts. **See also**: Flaviviruses; Flavivirus Infections in Humans; *Yellow Fever Virus*

The four *dengue viruses* make up a unique complex within the genus *Flavivirus* (Holmes and Twiddy, 2003). Although the four serotypes are antigenically distinct, there is evidence that serological subcomplexes may exist within the group. For example, a close genetic relationship has been demonstrated between DENV-1 and DENV-3 by using sequence homology and complementary deoxyribonucleic acid (cDNA) hybridisation probes. In addition, DENV-2 shows a high-sequence homology (71%) with *Edge Hill virus*, an ecologically distinct flavivirus from Australia. **See also**: Flaviviruses; Flavivirus Infections in Humans

Structure

The dengue viruses have a structure similar to other flaviviruses; they are spherical, about 40–50 nm in diameter (**Figure 1**), with a lipid envelope, which is apparently derived from the host cell membrane from which the viruses bud (Perera and Kuhn, 2008). The envelope encloses an isometric nucleocapsid core of 30–35 nm in diameter, which consists of a capsid protein and single-stranded, positive-sense ribonucleic acid (RNA) genome. The virion envelope is fringed with fine surface projections, the envelope and

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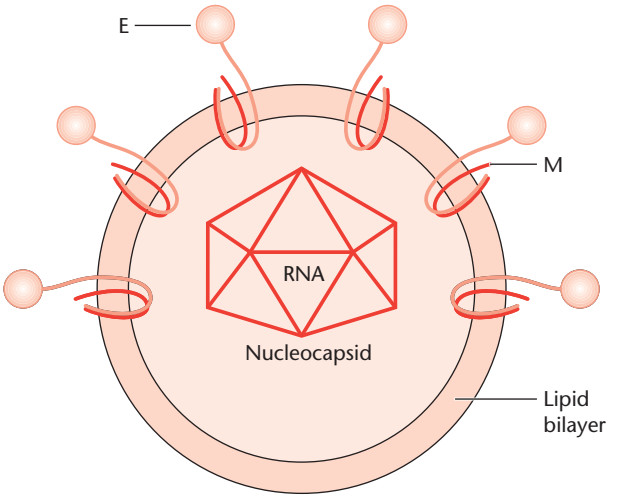

Figure 1 The *Flavivirus* virion structure. Structural proteins: E, envelope glycoprotein and M, membrane glycoprotein.

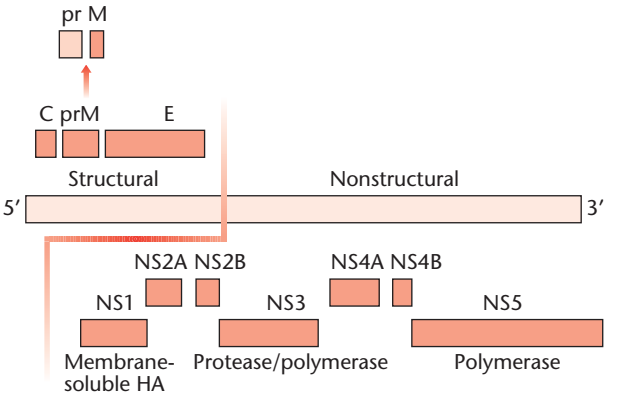

Figure 2 Genome of the *Flavivirus* genus within the family Flaviviridae, showing gene order. Structural proteins: C, capsid; E, envelope and prM, premembrane. NS, nonstructural proteins and HA, haemagglutinin.

membrane structural proteins. The envelope protein facilitates virus maturation and membrane fusion. Non-structural protein 1 (NS1) is found on the outer membrane of infected cells, however its function remains unclear. NS5 acts as an RNA-dependent RNA-polymerase. NS3 is a helicase and aids successful viral replication (Paranjape and Harris, 2010). Both NS3 and NS5 have potential as therapeutic drug targets. **See also**: RNA Virus Genomes

The dengue (*Flavivirus*) genome is approximately 11 000 bases in length, and consists of short untranslated regions at the 3′ and 5′ ends with an uninterrupted open reading frame in between (**Figure 2**; Kuhn *et al*., 2002). The genome has a cap at the 5′ end but no poly A tail at the 3′ end. The 3′-untranslated region appears to regulate RNA replication in place of the poly A tail (Alvarez *et al*., 2005). The complete genome sequences are known for several strains of all four dengue virus serotypes. There are three structural proteins (capsid, premembrane and envelope), encoded by sequences near the 5′ end of the genome, and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) encoded by the remainder of the genome (**Figure 2**). **See also**: Viral Capsids and Envelopes: Structure and Function

Viral Replication

It is thought that dengue and other flaviviruses infect cells by attaching to cellular receptors through the envelope protein, although specific receptor proteins have not been identified (Kuhn *et al*., 2002; Bartenschlager and Miller, 2008). The viruses are internalised by endocytosis, after which the nucleocapsid is released into the cytoplasm of the cell by membrane fusion, a process that is initiated by a low-pH-dependent conformational change in the envelope protein. **See also:** Virus Host Cell Receptors

The dengue gene order (**Figure 2**) is encoded in open reading frames as follows 5′-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′ (Chiu *et al*., 2005). Translation of the viral messenger RNA (mRNA) is initiated at the 5′ end, and the resulting polyprotein goes through extensive cotranslational and post-translational proteolytic processing and cleavage to form at least 10 mature viral proteins (Kuhn *et al*., 2002; Chiu *et al*., 2005). Both viral and host proteases appear to be involved in processing the dengue polyprotein. A *C*-terminal hydrophobic membrane anchor domain in the capsid, premembrane and envelope proteins function both as internal signal sequences involved in the transfer of the polypeptide into the lumen of the rough endoplasmic reticulum, and as membrane anchor domains (Markoff *et al*., 1994). Charged amino acid sequences that follow this anchor act to stop the peptide transfer through the endoplasmic reticulum membrane as well as a signalase cleavage recognition site of the host cell signalase in the lumen of the endoplasmic reticulum. The initial processing events that separate the structural protein precursors and the *N*-terminus of the NS1 are mediated by this signalase. Cell-associated virions are constructed from the structural proteins, and mature virions are released from infected cells through a process involving cleavage of the pre-M protein in the Golgi vesicles (Barth, 1999). **See also:** RNA Plant and Animal Virus Replication

Dengue viruses have three natural hosts: *Aedes* mosquitoes, lower primates and humans (Weaver, 2005). Dengue viruses are known to cause clinical illness and disease only in humans. After introduction by a mosquito bite the initial site of DENV replication is the cutaneous Langerhans dendritic cell (Limon-Flores *et al*., 2005). The migration of these infected cells around the lymphatic system triggers the production of cytokines and the recruitment of other immune cells. These include monocytes and macrophages, which are the primary target of infection and the main site of DENV replication (Durbin *et al*., 2008). Other tissues from which the viruses have been isolated include the liver, lungs, kidneys, lymph nodes,

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stomach, intestine and brain, but it is not known to what extent virus replicates in these tissues (Jessie *et al*., 2004). There is some evidence that the viruses also replicate in endothelial cells and possibly in bone marrow cells. Although encephalopathy and encephalitis has been documented in dengue infection, there is limited evidence to support viral replication within the CNS (Soares *et al*., 2006).

Viraemia in humans may last 2–12 days (average, 4–5 days) with titres ranging from undetectable to over 10^8 mosquito infectious doses 50 (MID_{50}) mL (Halstead, 2008). Experimental evidence shows that several species of lower primates (chimpanzees, gibbons and macaques) become infected and develop viraemia titres high enough to infect mosquitoes, but do not develop illness (Vasilakis *et al*., 2011). Viraemia levels in lower primates are more transient, often lasting only 1–2 days if detectable, with titres seldom reaching 10^6 MID_{50} mL.

Low-passage or unpassaged dengue viruses can only be propagated with consistent results in laboratory-reared mosquitoes and in mosquito cell lines (Tesh, 1979; Gubler *et al*., 1984). Mosquito species most commonly used for *in vivo* propagation include *Aedes aegypti*, *Aedes albopictus* and *Toxorhynchites* spp., all of which can be reared with ease in the laboratory. In the mosquito vectors, dengue viruses infect and replicate to high titre in nearly all tissues, including midgut epithelial cells, ovaries, fat body, brain and central nerve cord ganglia and salivary glands. Only three mosquito cell lines show high susceptibility to dengue viruses: C6/36 from *A. albopictus*, AP-61 from *Aedes pseudoscutellaris* and TRA-284 from *T. amboinensis*.

Dengue viruses can also be propagated in baby mice and in several vertebrate cell lines (Vorndam and Kuno, 1997). These all have lower susceptibility to infection than mosquito cells however, and dengue viruses must be adapted to each system by serial passage before consistent results can be obtained. Baby mice, which are used for the isolation and assay of many other arboviruses, generally show no signs of illness after intracerebral inoculation with most unpassaged strains of dengue viruses. Mammalian cell lines commonly used include LLC-MK2 and Vero (monkey kidney), BHK-21 (baby hamster kidney), FRhL (fetal rhesus lung) and PDK (primary dog kidney). **See also**: Viral Culture Methodologies

Vectors

Only species of the genus *Aedes* appear to be natural mosquito hosts for dengue viruses (Halstead, 2008). Species of the subgenus *Stegomyia* are the most important vectors in terms of human transmission, and include *A. aegypti*, the principal urban vector worldwide, *A. albopictus* (Asia–Americas), *Aedes scutellaris* spp. (Pacific), *Aedes africanus* and *Aedes luteocephalus* (Africa). Species of the subgenus *Finlaya* (Asia) and *Diceromyia* (Africa) appear to be important mosquito hosts involved in dengue forest maintenance cycles. Two other species, *Aedes (Gymnometopa) mediovittatus* (Caribbean) and *Aedes (Protomacleaya) triseriatus* (North America) have been shown to be excellent experimental hosts for dengue viruses (Fuentes *et al*., 1992).

Transmission Cycles

Dengue viruses exist in nature in three basic maintenance cycles (**Figure 3**; Vasilakis *et al*., 2011). The primitive forest cycle involves canopy-dwelling mosquitoes and lower primates. A rural cycle, primarily in Asia and the Pacific, involves peridomestic mosquitoes (*A. albopictus* and *A. scutellaris* spp.) and humans (Vasilakis *et al*., 2010). The urban cycle, which is the most important epidemiologically and in terms of public health impact, involves the highly domesticated *A. aegypti* mosquito and humans (Halstead, 2008). Multiple virus serotypes are maintained in an endemic cycle in most large urban centres of the tropics, with epidemics occurring at periodic intervals.

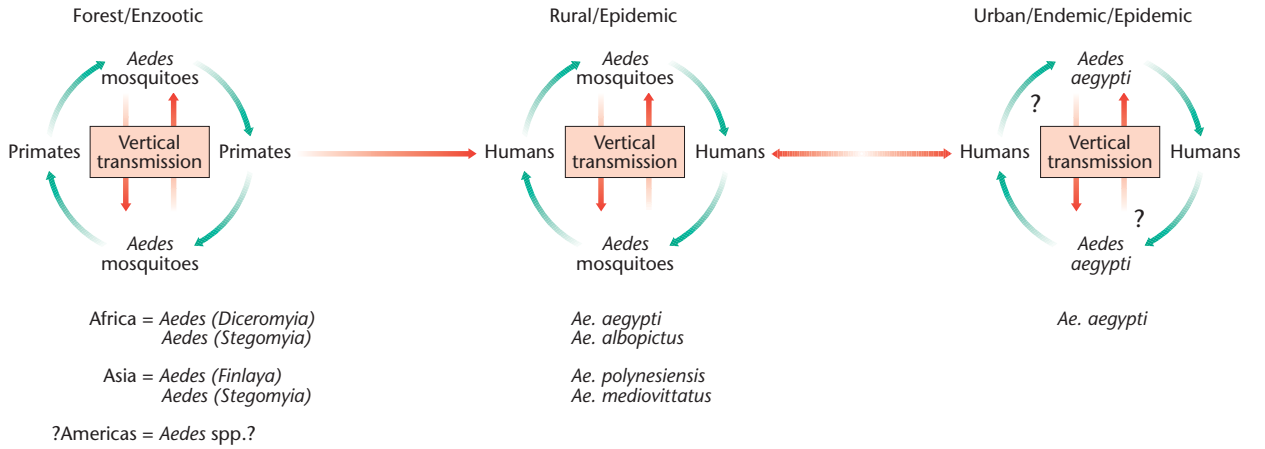

Figure 3 Transmission cycles of dengue viruses.

Dengue viruses are only transmitted to humans and lower primates by the bite of an infective mosquito vector (Halstead, 2008). When a competent mosquito vector takes a blood meal from a person during the viraemic phase (see earlier discussion), virus is ingested with the blood and infects the cells of the mosquito mesenteron. After 8–12 days, depending on ambient temperatures, the mosquito vector and the virus strain, the virus will disseminate and infect other tissues, including the mosquito salivary glands (Halstead, 2008). When the mosquito takes a subsequent blood meal, virus is injected into the person with the salivary fluids. The mosquito is infected for life.

A. aegypti is a highly competent epidemic vector of dengue viruses. It lives in close association with humans because of its preference for laying eggs in artificial water-holding containers in the domestic environment resting inside houses and feeding on humans rather than other animals. It has a nearly undetectable bite and is very restless, in the sense that the slightest movement will make it interrupt feeding and fly away. It is not uncommon, therefore, for a single mosquito to bite several persons in the same room or general vicinity over a short period of time.

In addition to transmitting the virus to humans or lower primates, the female mosquito may also transmit the virus vertically through the eggs to her offspring (Arunachalam *et al*., 2008). Although the implications of vertical transmission are not fully understood, it is thought to be an important mechanism in the natural maintenance cycles of dengue viruses, especially in rural and forest settings (Vasilakis *et al*., 2011).

Epidemiology

Geographic and seasonal distribution

Dengue viruses have a worldwide distribution in the tropics (**Figure 4**). The viruses are endemic in most urban centres of the tropics, with transmission occurring throughout the year (Scott and Morrison, 2010). Epidemic transmission occurs periodically in most virus-endemic areas, usually at 3–5 year intervals (Halstead, 2008). As surveillance in most endemic countries is poor, cases are not usually reported during interepidemic years, thus potentially downplaying the risk of infection. It is well documented, however, that dengue viruses are maintained during interepidemic periods in most tropical areas and, although risk of infection is lower than during epidemic periods, it is still substantial to unsuspecting visitors.

Peak transmission of dengue viruses is usually associated with periods of higher rainfall in most dengue-endemic countries (Halstead, 2008). Factors influencing seasonal transmission patterns of dengue viruses are not well understood, but obviously include mosquito density, which may increase during the rainy season, especially in those areas where the water level in larval habitats is dependent on rainfall. In areas where water storage containers are not influenced by rainfall, however, other factors such as higher humidity and moderate ambient temperatures associated with the rainy season increase survival of infected mosquitoes, thus increasing the chances of secondary transmission to other persons (Halstead, 2008).

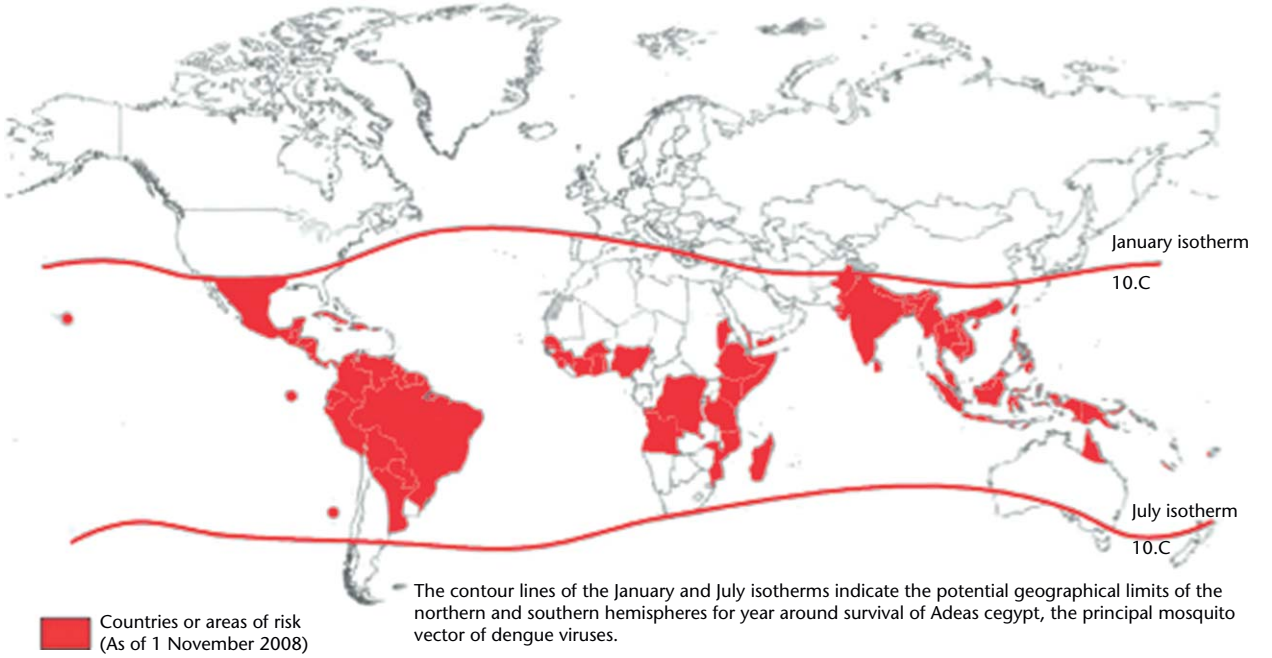

Figure 4 Countries and areas at risk of dengue transmission. The contour lines represent the potential geographic limits of *A. aegypti* (WHO, 2009).

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Changing epidemiology of dengue

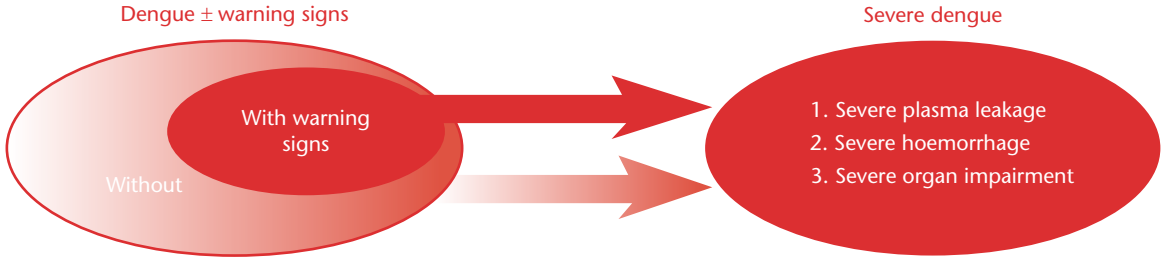
A combination of increased and unplanned urbanisation, changing life styles and lack of effective mosquito control has made most tropical cities highly permissive for efficient dengue transmission by *A. aegypti*. Increased air travel by humans provides the ideal mechanism for the transport of dengue viruses between population centres. Consequently, in the past 20 years there has been a dramatic increase in the movement of dengue viruses within and between regions, resulting in increased epidemic activity, development of hyperendemicity (the co-circulation of multiple virus serotypes) and the increased incidence of severe dengue, including dengue shock syndrome. Once principally observed in Southeast Asia, severe dengue has spread in epidemic form to west Asia, the People's Republic of China, the Pacific Islands and the Americas in the past two decades.

The factors responsible for the emergence and spread of severe dengue as an epidemic phenomenon are not fully understood. The changing disease pattern described above provides support for both principal hypotheses on the pathogenesis of severe dengue, secondary infection and virus virulence (see later discussion). Increased transmission of multiple dengue serotypes (hyperendemicity) increases incidence and thus the probability that severe disease will occur, regardless of whether the underlying cause is due to increased virulence, immune enhancement or, more likely, a combination of both.

Dengue in humans is primarily an urban disease. Most major epidemics of severe dengue occur in tropical urban centres, where large and crowded human populations live in intimate association with the principal mosquito vector, *A. aegypti*.

Clinical Features of Infection and Pathogenesis

Dengue infection causes a spectrum of illness in humans, ranging from inapparent to severe disease characterised by plasma leak resulting in shock and haemorrhage (WHO, 2009). The most severe disease is observed in infants and young children. The previously used WHO disease classification categorised dengue into either 'dengue fever' or 'dengue haemorrhagic fever' (WHO, 1999). Dengue haemorrhagic fever (DHF) was further subdivided into four severity grades, with the two most severe grades referred to as 'dengue shock syndrome' (DSS). There were problems with this classification system, and it had a low sensitivity for diagnosing severe disease (Srikiatkhachorn *et al.*, 2010). The updated WHO guidelines recognise that clinical dengue is a dynamic entity and categorise disease into either 'dengue' or 'severe dengue'. The new guidelines place emphasis on the warning signs that may indicate a patient is progressing to severe disease (WHO, 2009; **Figure 5**). The differential of dengue is broad and varies depending on the local epidemiology of other infectious diseases. It includes other haemorrhagic fevers, hepatitis, leptospirosis, typhoid, malaria, measles and influenza,



Criteria for dengue ± warning signs

Probable dengue

Live in /travel to dengue endemic area.

Fever and 2 of the following criteria:

- Nausea, vomiting
- Rash
- Aches and pains
- Toumiquet test positive
- Leukopenia
- Any warning sign

Laboratory-confirmed dengue
(Important when no sign of plasma leakage)

Warning signs•

- Abdominal pain or tenderness
- Persistent vomiting
- Clinical fluid accumulation
- Mucosal bleed
- Lethargy, restlessness
- Liver enlargment >2 cm
- Laboratory: increase in HCT concurrent with rapid decrease in platelet count

- (Requiring strict observation and medical intervention)

Criteria for severe dengue

Severe plasma leakage
leading to:

- Shock (DSS)
- Fluid accumulation with respiratory distress

Severe bleeding
as evaluated by clinician

Severe organ involvement

- Liver: AST or ALT >=1000
- CNS: Impaired consciousness
- Heart and other organs

Figure 5 Dengue classification and levels of severity (WHO, 2009).

among others. **See also**: Haemorrhagic Fever Viruses; Hepatitis Viruses; Leptospirosis; Malaria; Measles Virus

In 'classical' dengue a sudden onset of fever follows a 4–7 days incubation period. This is frequently accompanied by headache, myalgia and severe retro-orbital pain (WHO, 2009). A range of other clinical features may be observed, and include sore throat, diarrhoea, vomiting, anorexia and conjunctival injection. Rashes are commonly seen in dengue: early in the course of illness the skin often appears flushed with petechiae developing in the 'critical' phase and a macular rash occurring in the convalescent period. Severe arthralgia can be a feature of the illness and explains the use of 'break-bone fever' as a descriptive term for dengue (McBride, 2010). The clinical features differ between age groups with clinical features such as cough, vomiting and abdominal pain being more frequently observed in children. The early febrile phase lasts for 2–7 days. This is followed by defervescence and the start of the 24–48 h 'critical' phase when an increase in capillary permeability with an associated rise in haematocrit can be observed. The degree of plasma leakage is variable. If a critical volume of plasma is lost patients will develop clinical shock. Thrombocytopenia is almost universally seen in dengue infection and minor mucosal bleeding can be a feature of uncomplicated infection. Severe haemorrhage can occur – gastrointestinal bleeding is well described in those with a history of peptic ulcer disease (Tsai *et al*., 1991). Intracerebral and pulmonary haemorrhage can occur (Kumar *et al*., 2009). Other organs can be affected in dengue, and it is possible that these atypical presentations are under appreciated. These include encephalitis, myocarditis, hepatitis, pancreatitis, retinitis and the acute respiratory distress syndrome (Gulati and Maheshwari, 2007).

The management of dengue remains limited to supportive treatment. Uncomplicated infections can usually be managed in the outpatient setting. It is important that patients managed in the outpatient setting are encouraged to maintain a good oral intake. Paracetamol is the antipyretic of choice as non-steroidal anti-inflammatory drugs are associated with an increased risk of haemorrhage, and aspirin has an association with the development of Reye's syndrome (WHO, 2009).

Patients who develop warning signs suggestive of severe disease should be admitted. Patients who have warning signs should have a baseline haematocrit measured as this could be used as a guide to treatment response or disease progression. Patients should be started on parenteral isotonic fluids and the infusion rate should be adjusted according to the clinical response. Parenteral fluids are generally needed for only 24–48 h – the 'critical' phase (WHO, 2009). Continuing fluids for longer than this carries a risk of fluid overloading the patient. In severe cases the administration of parenteral fluid is life saving. Patients with shock require more intensive management. The use of isotonic crystalloid fluids in the resuscitation of children with shock is supported by clinical evidence, and starch-based solutions are advocated for more severe cases (Wills *et al*., 2005). In cases of haemorrhage the transfusion of blood products is often indicated.

There are currently no licensed antivirals for use in dengue. Owing to the immune system's key role in disease pathogenesis it is possible that drugs that modulate the immune response may be future therapeutic options.

Pathology and histopathology

The pathology of dengue virus infection is not well understood because systematic postmortem studies have not been done on patients representing all types of clinical expression. The major pathophysiological abnormality in classic DHF/DSS is an increase in vascular permeability, which leads to leakage of plasma. Twenty-four Patients may have serous effusion in the pleural and abdominal cavities and a variable amount of haemorrhaging in most major organs. Studies have not revealed destructive inflammatory vascular lesions, but some swelling and occasional necrosis have been observed in endothelial cells, as well as some perivascular oedema (Bhamarapravati *et al*., 1967).

Limited studies on patients with a fatal outcome have demonstrated focal central necrosis of the hepatic cells, presence of Councilman bodies and hyaline necrosis of Kupffer cells in the liver (Huerre *et al*., 2001). Changes in the kidney are suggestive of an immune-complex type of glomerulonephritis. There is depression of bone marrow elements, which improves when the patient becomes afebrile. Biopsy studies of the skin rash have demonstrated perivascular oedema with infiltration of lymphocytes and monocytes (de Andino *et al*., 1985).

Pathogenesis

The pathogenesis of dengue involves a complex interaction between virus and host factors, and remains incompletely understood. The severe manifestations of DENV infection are observed at the point the virus is being cleared from the host by the immune response as opposed to when the viral load is highest, suggesting that the immune system plays a key role in disease pathogenesis (Green and Rothman, 2006).

Prospective cohort studies in Asia and Latin America have demonstrated that secondary infections are associated with more severe disease (Kyle and Harris, 2008). The accepted explanation for this observation is that non-neutralising cross-reactive antibodies elicited in a primary infection bind virus in a secondary infection and then have a greater ability to infect Fc-receptor bearing cells. This is called antibody-dependent enhancement (ADE), and potentially leads to an increased viral biomass, and therefore more chance of developing severe disease (Halstead, 2003). ADE was supported by epidemiological observations in Cuba where DHF was seen more frequently in patients with secondary DENV infections. In addition, there is evidence that ADE immunologically modulates

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infected cells in such a way that the microenvironment becomes more supportive of DENV replication. A recent study has provided further support for the concept of ADE (Dejnirattisai *et al*., 2010). This work has shown how antibodies to dengue structural precursor membrane protein (prM), a component of the humoral immune response to DENV, are highly cross-reactive between serotypes, are non-neutralising and appear to mediate ADE in Fc-receptor bearing cells. Despite epidemiological and laboratory support for ADE, severe disease can occur in primary infections and most secondary infections do not result in severe disease. This implies that other factors contribute to the dengue pathogenesis.

The cellular immune response is also involved with the clearance of dengue virus from the host, and is thought to play a role in the development of severe disease (Rothman, 1999). The proliferation of activated memory T cells and the production of pro-inflammatory cytokines are thought to contribute to the development of plasma leak observed in dengue. In addition, the level of T cell activation is proposed to correlate with disease severity. It has been demonstrated that the T cells produced in severe disease have a low affinity for the current infecting serotype, but a high affinity for a different serotype responsible for a past infection (Mongkolsapaya *et al*., 2003). This observation is consistent with the concept known as 'original antigenic sin'.

There is increased evidence that the observed clinical severity varies depending on the infecting serotype. For example, higher viraemia and NS1 antigenaemia were observed in Vietnamese infants infected with DENV-2 as compared to DENV-1 (Chau *et al*., 2008). In addition, a prospective cohort study in Thai children suggested infection with DENV-2 and DENV-3 was twice as likely to result in DHF than infection with DENV-4 (Fried *et al*., 2010). Within each serotype there are distinct phylogenetic genotypes that vary in their geographical ranges, and appear to have differences in fitness. There is evidence that the Asian/American genotype of DENV-2 is associated with more severe disease and has a fitness advantage in mosquitoes as compared with the American genotype of DENV-2, perhaps reflecting the higher viraemia observed with this phenotype (Rico-Hesse *et al*., 1997).

Many host factors appear to contribute to the development of severe disease. A retrospective study in Vietnam demonstrated that children admitted to hospital with dengue aged between 1 and 5 years were four times more likely to die than children aged between 11 and 15 years (Anders *et al*., 2011). Various single nucleotide polymorphisms (SNPs) appear to have an association with both protection and vulnerability to severe disease. For example, a polymorphism in the vitamin D receptor appears to protect against severe disease, perhaps reflecting the role of the vitamin D receptor in immune modulation (Loke *et al*., 2002). There have been conflicting results from studies looking at polymorphisms in the tumour necrosis factor α (TNF-α) gene with some studies showing an association and others not. Genome-wide association studies (GWAS) allow a broad approach in understanding genetic susceptibility to diseases, including dengue. SNPs at two loci with a significant association with DSS have been identified from a GWAS conducted on samples from Vietnamese children (Khor *et al*., 2011). These SNPs were in the major histocompatibility complex class I polypeptide-related sequence B gene (MICB) on chromosome 6, and in the phospholipase C epsilon 1 (PLCE 1) gene on chromosome 10. The MICB gene encodes a surface protein that contributes to natural killer (NK) and CD8 T cell activation. The observed association with an SNP in the MICB gene and DSS may reflect dysfunctional NK and CD8 cell activity in severe disease, suggesting a key role for these cells in disease control and pathogenesis. GWAS technology has the potential to advance understanding of pathogenesis, and may have a future clinical application within the context of dengue pharmacogenomics.

Diagnosis

In many areas dengue remains a clinical diagnosis. Whereas certain clinical and laboratory features are suggestive of the diagnosis, the non-specific nature of early infection means relying on this strategy is unreliable (WHO, 2009). Three major laboratory methods have been developed for dengue diagnosis. A patient is said to have laboratory-confirmed DENV infection if they have symptoms suggestive of dengue in the presence of one of the following: (a) DENV IgM seroconversion or high and rising levels of DENV-reactive IgM/IgG in paired plasma samples, (b) DENV viraemia, (c) NS1 antigenaemia or (d) DENV isolated in cell culture (WHO, 2009).

Prevention and Control

The development of a safe and effective dengue vaccine would be a major advance in the global control of the infection. There have been barriers to the successful development of a vaccine. For example a candidate vaccine would need to offer protection against all four serotypes and there are concerns that incomplete protection may actually result in more severe disease, reflecting the immune system's role in pathogenesis (Webster *et al*., 2009). In addition, there are no animal models of dengue that may assist pre-clinical development. Despite these challenges there are currently several vaccine candidates in clinical trials. On the bases of the success in developing vaccines against yellow fever and Japanese encephalitis, initial focus was on developing a live attenuated vaccine. Whereas initial results with monovalent candidates were encouraging, the development of a tetravalent formulation proved difficult and development efforts were suspended. The most promising candidate, a chimeric vaccine based on a yellow fever backbone, is now in phase 3 trials (Chimerivax, Sanofi Pasteur) (Coller and Clements, 2011). The on-going safety concerns will need to be addressed by long-term follow-up

of vaccine recipients. The economic justification for a dengue vaccine and the identification of its place in the current schedule of immunisation remain important areas to address. **See also**: Vaccination of Humans

Vector control has previously been used to control dengue with some success, most recently in Singapore and Cuba, and previously in the Panama canal region (Ooi *et al*., 2006). Unfortunately these successes have generally been short-lived, and dengue is increasing in many parts of the world. Improving our understanding of the virus–vector interface would aid our understanding of transmission of DENV in the community, help construct transmission models and potentially allow us to assess the impact of new disease control interventions.

Environmental management aims to modify the environment in such a way that viable larval habitats are minimised and human contact with the vector is reduced. This would include strategies such as regular emptying of water containers, proper disposal of used tires and other waste, and construction of mosquito screens in homes. This approach has the potential to reduce the incidence of dengue infection, however the success of such strategies is dependent on active engagement with the local community (WHO, 2009).

Insecticides are often effective, but are expensive as they require repeated application and may have been unwanted environmental side effects. In addition mosquitoes are able to develop resistance to insecticides. However, due to the domesticated habits of *A. aegypti*, larviciding has traditionally been seen as an adjunct to environmental management.

Imaginative new approaches to vector control are needed. Infection of mosquitoes with fruitfly strains of symbiont *Wolbachia* bacteria appears to reduce adult lifespan, affect reproduction and interfere with pathogen replication, including dengue (Walker *et al*., 2011). This exciting new approach to vector control has been commenced by releasing *Wolbachia*-infected *A. aegypti* in parts of Australia with a view to expanding the programme to endemic countries in Asia.

Future

Dengue represents a major challenge to global health. Its global expansion, reflecting huge demographic changes, has resulted in approximately 40% of the world's population being at risk of infection. Despite this, diagnosis remains largely clinical, case management is supportive, and disease control is limited to tackling the vector. The pathogenesis of dengue, despite its enormous interest to scientists, remains incompletely understood.

Understanding disease pathogenesis has been hampered by the lack of a suitable animal model of dengue infection. It is possible that future insights into pathogenesis may come from detailed observations within intervention studies, such as vaccine trials.

Improving the clinical management of dengue remains an important area of research focus. The new dengue case classification needs to be validated. Appropriate fluid management is critical in the management of dengue. However, the fluid management of those with co-morbidities or pregnancy is particularly challenging and is an area that warrants further research. There remains a need for an effective antiviral agent. It is possible that developments in the treatment of hepatitis C, another flavivirus, may be transferable to dengue. However, the nature of dengue pathogenesis suggests that immunomodulatory agents such as steroids or statins may have more potential in the treatment of dengue than an antiviral. Dengue can cause outbreaks that can overwhelm health facilities. In the early stages of illness it is difficult to distinguish dengue from other febrile conditions. Distinguishing dengue from other febrile illnesses, and predicting which patients are at risk of disease progression would be major advances in dengue management.

The development of a vaccine would be a major advance in the control of disease. As discussed above, the most promising candidate is in phase 3 trials. If the candidate vaccine is successful there will still be hurdles to overcome before it becomes integrated into immunisation programmes in endemic countries. Understanding community transmission dynamics by researching the virus–vector interface may inform mathematical models of disease transmission. These could potentially aid prediction of the impact of various interventions, for example the introduction of *Wolbachia*-infected *A. aegypti* into endemic areas. **See also**: Disease Eradication by Vaccination: Prospects

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LITERATURE REVIEW 2: THE PATHOGENESIS OF DENGUE

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Review

The pathogenesis of dengue

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ABSTRACT

Dengue is an important cause of childhood and adult morbidity in Asian and Latin American countries and its geographic footprint is growing. The clinical manifestations of dengue are the expression of a constellation of host and viral factors, some acquired, others intrinsic to the individual. The virulence of the virus plus the flavivirus infection history, age, gender and genotype of the host all appear to help shape the severity of infection. Similarly, the characteristics of the innate and acquired host immune response subsequent to infection are also likely determinants of outcome. This review summarises recent developments in the understanding of dengue pathogenesis and their relevance to dengue vaccine development.

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1. Introduction

Dengue is a globally important arboviral infection transmitted by *Aedes* mosquitoes that endangers an estimated 2.5 billion people and represents a rapidly growing public health problem [1]. There are between 50 and 100 million infections each year, with approximately 500,000 cases admitted to hospital with severe and potentially life-threatening disease [2–4]. Dengue is an icosahedral,

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enveloped virus with a single-stranded positive sense genome; it is a member of the *flaviviridae* family and has 4 antigenically distinct serotypes (DENV1-4) [3,5,6]. After infection of a susceptible host, an acute, self-limiting febrile systemic syndrome ensues. Resolution of infection occurs within 4–7 days and is associated with a robust innate and adaptive immune response. At present diagnosis is largely clinical, treatment is supportive and disease control is limited to tackling the vector [1]. Development of a vaccine would be a major advance in disease control but efforts have been hampered by the lack of an animal model of the disease and concerns about the role of the immune system in disease pathogenesis [6]. The lack of an animal model further limits our understanding of immunopathogenesis.

Dengue is a syndrome and its pathogenesis an interplay between virus and host factors that remains incompletely understood [1,7]. Explaining the heterogeneity between clinical presentations within a similar cross-section of the population remains a vital area of research. A better understanding of disease pathogenesis would greatly aid vaccine development by addressing specific concerns about vaccine safety and efficacy in light of the immune system's role in the development of clinical disease [8]. This review will examine the different components of our current understanding of dengue pathogenesis and then consider their implications for dengue vaccine development.

1.1. Clinical signs, symptoms and management

Clinically apparent dengue (DEN) virus infection is associated with a range of syndromes. Classical dengue fever is observed more frequently in adults and occurs after an incubation of 4–7 days [9,10]. The clinical manifestations of children with DEN can differ from adults – cough, vomiting and abdominal pain appear to be more common [11]. The mortality rate in young children with DEN is significantly higher than in older children or adults [12]. The incidence of severe disease is perhaps highest in infants, which may reflect increased capillary fragility and lower compensatory reserve in this age group [13]. In classical DEN fever there is an abrupt onset of fever often associated with myalgia, headache and sometimes severe retro-orbital pain. Early in the illness the skin is flushed with petechiae appearing in the "critical" phase; a macular rash is observed in convalescence. The critical phase occurs around the time of defervescence, typically on days 3–7, and is associated with an increased propensity for capillary leakage and haemorrhage. In some individuals, capillary permeability manifests as a rise in haematocrit, pleural effusions or ascites [14]. This is the stage when life-threatening clinical complications such as circulatory shock are typically observed [14]. Disabling fatigue and depression may complicate recovery [15]. Thrombocytopenia is almost universal and minor bleeding may occur in mild infections – this can be severe in those with peptic ulcer disease [16]. Atypical clinical features of DEN are being more frequently reported but are probably still underappreciated [17]. These include encephalitis, myocarditis, hepatitis, pancreatitis, retinitis and the acute respiratory distress syndrome (ARDS) [8,10,18,19]. These atypical presentations likely reflect pathology at different endothelial surfaces [17]. Recognizing the warning signs that may indicate progression to severe disease is essential for successful case management – these signs include abdominal pain, hepatomegaly, evidence of fluid accumulation and a rising haematocrit (or conversely a falling haematocrit suggestive of haemorrhage) [3]. The current WHO guidelines recognize dengue as a clinical continuum from dengue to severe dengue [3]. Careful fluid resuscitation is life-saving in DEN [3]. Ringer's lactate has been shown to be efficacious in moderately severe DEN, and starch or dextran have been suggested for more severe cases [20]. Platelets are often given as prophylaxis to prevent haemorrhage; however this is a controversial area without a clear evidence base [21,22]. In many settings DEN is diagnosed clinically; however the features of early infection are non-specific and mimic those of other febrile illnesses [23–25]. An early diagnosis would assist in patient triage and will have an increasingly important role as therapeutic drugs, e.g. anti-virals, become available for DEN [26].

2. Immunopathogenesis of dengue

The severe phenotypes of dengue are observed not at the time when the viral burden is at its highest in vivo, but paradoxically when the virus is being rapidly cleared from host tissues by the innate and adaptive immune response. This has led to the suggestion that the pathogenesis of clinically important complications is closely linked to the host immune reponse [27].

2.1. The humoral immune response and antibody-dependent enhancement

Although no correlate of immunity to dengue has been defined, it's hypothesized that the humoral immune response is vital for controlling DEN virus infection and for the expression of acquired immunity. In part, the belief that immunity to dengue is antibody-mediated stems from observations in other medically important flavivirus infections, e.g. Japanese encephalitis virus and Yellow Fever, where the accepted vaccine-elicited correlate of immunity is virus-neutralizing antibody [28–31]. Confirmation that virus neutralizing antibody is a correlate of dengue immunity is most likely to come from expanded phase II and phase III trials of the Sanofi Pasteur developed ChimeriVax vaccine that are currently underway in Asia and Latin America [32]. (See Guy et al. article in this Special Issue).

Sabin demonstrated in the post-war era how infection with one serotype gave long-lasting protection to that specific serotype (homotypic immunity) and short-lived protection against the other serotypes (heterotypic immunity) [33]. The basis for homotypic immunity is believed to be virus-neutralizing antibodies. The transient nature of heterotypic immunity is likely due to cross-reactive E-protein specific antibodies that when above a certain concentration threshold are protective. However, over time, these antibody concentrations decline and the individual is then susceptible to infection with other DEN virus serotypes.

Multiple prospective cohort studies in Asia and Latin America have identified secondary infection as an epidemiological risk factor for severe dengue [34–38]. Third, or even fourth infections also probably occur in endemic settings, but hospital data and the age-related burden of dengue (children) suggests the vast majority of these tertiary or quaternary infections are clinically silent or very mild [39]. The leading explanation for increased risk of disease in secondary infection is that non-neutralising, cross-reactive antibodies elicited by a primary infection bind the virus which then have greater potential to infect Fc-receptor bearing cells. This phenomenon, called antibody-dependent enhancement (ADE), potentially increases the risks of developing severe disease by virtue of increasing the number of virus infected cells and therefore the viral biomass in vivo [40,41]. Some evidence also suggests cells infected via an ADE process are immunologically modulated such that the local environment becomes more permissive for virus replication [42]. Cells of the monocyte–macrophage lineage in particular are believed to be a major site of dengue replication under conditions of ADE [27,43]. The concept of ADE was initially proposed for dengue in 1977 and was supported by epidemiological observations in Cuba [44,45]. Data from Cuba demonstrated that DHF was more frequently observed in those patients who had evidence of previous infection with a different serotype. Recent work has provided further support for the concept of ADE. This research

has demonstrated that antibodies to dengue structural precursor-membrane protein (prM), a component of the humoral response to infection, are highly cross-reactive between DEN virus serotypes [46]. In vitro studies indicate that even when anti-prM antibodies are at high concentrations, they are non-neutralising but potently mediate ADE in Fc receptor bearing cells [46,47]. The proposed basis for prM-mediated ADE is that on a proportion of virus particles prM is only partially cleaved from the virus surface during the virus maturation process. In this scenario, such "immature" virus particles that would otherwise be non- or less-infectious, are rendered infectious in an environment where anti-prM antibodies can mediate ADE [46]. These in vitro observations have led to the suggestion that a vaccine candidate should be designed in a way that would minimise the anti-prM response [46].

A second epidemiological setting that implicates a role for ADE is primary infection of infants born to dengue-immune mothers. Primary infections in infants aged between 4 and 12 months of age can result in severe dengue, an outcome that is epidemiologically less common in younger infants and children aged between 1 and 2 yrs of age. At the age of 3–4 months, maternally derived virus neutralizing antibodies have generally declined below measurable levels in infants, however non-neutralising antibodies, which represent a much greater fraction of the virion-binding antibody population, remain present [48–50]. These virion-binding, non-neutralising antibodies are thought to enhance the risk of clinically apparent and severe dengue through a process of ADE [51–54]. In support of this hypothesis, neat plasma from 6-month old healthy Vietnamese infants enhances the infectivity of DENV-2 in Fc receptor bearing cells significantly more than plasma collected at the time of birth or at 1-yr of age [52].

Concerns about ADE have led to fears that a vaccine could contribute to increased disease severity due to re-infection in the presence of incomplete protection to one or more serotypes. This hypothetical concern needs to addressed by careful, long-term follow-up of vaccine recipients. It is difficult however to envisage a scenario whereby vaccination actually increases the risk of dengue or severe dengue to a level where it exceeds the "natural history" of dengue epidemiology in endemic regions.

2.2. The cellular immune response

Cellular immune responses are also suggested to play a role in clearing virus infection and potentially triggering the development of severe disease. Activated memory T cells recognizing both conserved and altered peptide ligand epitopes, are suggested to be involved in the development of plasma leakage [55]. It is proposed that the expression of viral epitopes on the surface of infected cells trigger the proliferation of memory T cells and the production of pro-inflammatory cytokines that have an indirect effect on vascular endothelial cells resulting in plasma leak. The level of T cell response is thought to correlate to disease severity [56,57]. Mongkolsapaya and colleagues in Thailand showed that T cells in severe infection have a relatively low affinity for the current infecting serotype but a high affinity for a past infection with a different serotype, i.e. they display characteristics of original antigenic sin [57]. Moreover, T cell responses in severe patients are mainly mono-functional in that they produce IFN-γ and/or TNF-α only and rarely CD107a, a marker of cytotoxic degranulation. Conversely, in patients with uncomplicated dengue, relatively more $CD8^+$ T cells expressed CD107a and only a few expressed only IFN-γ and/or TNF-α [58]. This is suggested to delay viral clearance, and via cytokine-mediated effects, potentially increase the risk of severe manifestations of disease. However, it remains to be shown, in even a temporal way, that T cells contribute to capillary leakage in vivo. Recent data in Vietnamese children suggests the emergence of activated T cells in blood of children with dengue is not synchronous with commencement of capillary permeability [62]. One possibility is that activated T cells are sequestered in tissues during acute dengue and are therefore difficult to detect at the time capillary permeability becomes apparent. The role of T regulatory cells is not clear in dengue. Their role in chronic infectious diseases has been studied but their role in acute viral infections is not well established [63]. However Luhn and colleagues demonstrated that they are functional and expand in acute dengue infection [64]. They may have a role in suppression of the production of vasoactive cytokines – perhaps the regulatory response is inadequate in severe disease?

The absence of good animal models of disease (as opposed to infection) is a hurdle to understanding the role of memory T cells in immunity and immunopathogenesis. For this reason, insights into the role of T cells in immunity will derive from prospective cohort studies, or vaccine trials [65]. A detailed commentary on the challenges of using cellular immune parameters as correlates of immunity to dengue has recently been published [66].

2.3. Cytokines

It is thought that in some individuals with secondary infection, high viral burdens trigger expression of a wave of cytokine and other inflammatory molecules from innate and activated, cross-reactive T cells. This inflammatory mileau is hypothesized to mediate permeability in the vascular endothelium, allowing water and small molecules to leak from the intravascular space and in some cases leading to the severe manifestations of dengue [67]. Increased levels of many different cytokines have been observed in dengue infection [68]. In particular, higher concentrations of cytokines such as IFN-γ, TNF-α and IL-10 have been observed in the sera of patients with severe dengue in Vietnam, Cuba and India [69–71]. Reduced levels of nitric oxide (NO) associated with increased levels of IL-10 have been described in patients with severe dengue [72]. NO contributes to immune regulation – lower levels of NO may result in the increased expression of pro-inflammatory cytokines [73]. In addition it is known that increased IL-10 levels correlate to reduced levels of platelets and reduced platelet function [74]. This phenomenon could, in part, contribute to the development of the bleeding complications observed in severe disease. Elevated levels of IL-6 have been observed in children with ascites [75]. TNF-α promotes increased endothelial permeability and it is plausible that increased levels of this cytokine could result in a more severe disease course [76]. It is likely that cytokines play a mutually synergistic role at the endothelial surface contributing to the development of the transient plasma leak, however the exact dynamic of this interaction has yet to be elucidated. It is worth noting that other infectious diseases and inflammatory disorders result in elevated cytokines without the attendant increased vascular permeability seen in severe dengue. Indeed, one of the challenges in dengue is to dissect those elements of the host immune response that are causally linked to capillary permeability from those that simply reflect the normal host immune response to a pathogen. This challenge is made more difficult by the absence of good animal models of disease.

2.4. Complement

Reduction in the levels of complement components have been described in patients with severe dengue, suggesting that complement activation may have a role in the pathogenesis of severe disease [77,78]. In particular it has been suggested that excessive complement activation at endothelial surfaces contributes to the vascular leak observed in severe disease [79]. NS1, a non-structural viral protein that is secreted from infected cells and present in blood in concentrations exceeding 1 μg/ml in some patients, could be an important modulator of the complement pathway. DEN virus

NS1 attenuates classical and lectin pathway activation of complement by directly interacting with C4 [80]. NS1 promotes efficient degradation of C4 to C4b and by this mechanism, NS1 is suggested to protect DENV from complement-dependent neutralization in solution [80]. The significance of these observations with the independent observation that early NS1 concentrations in blood are positively associated with disease severity are not yet clear, but are consistent with a role for NS1 and complement in pathogenesis [81,82]. Finally, it is plausible that the low levels of complement observed in severe dengue are merely a marker of a severe systemic disease rather than an indicator of their role in capillary permeability.

2.5. Moving beyond descriptive studies in immunopathogenesis

Much of the literature describing the immunopathogenesis of dengue has been correlative in nature, e.g. temporal associations between elevated cytokine concentrations in the febrile phase of dengue is often interpreted as being causally linked to the important clinical events of plasma leakage or bleeding manifestations. The challenge in this area of research is to move beyond descriptive studies and begin to identify causal immunopathogenic mechanisms. Therapeutic randomized controlled intervention trials, with either anti-viral drugs or immunomodulatory agents, e.g. corticosteroids, perhaps will offer insights into pathogenesis in a more direct fashion. Such trials are underway (ClinicalTrials.gov identifier NCT01096576 and ISRCTN39575233). Alternatively, improvements in animal models of dengue have occurred such that a productive, sometimes fulminant, acute virus infection can be established [83]. As yet however, no small animal model mimics the virological and pathophysiological events that occur in a child with severe dengue, in particular the relatively slowly evolving capillary permeability that clinically manifests between day 3 and 6 of illness coupled with a fast declining viral burden. Clearly then there is scope for further research into animal models and strong justification for studying the host response during randomized controlled treatment trials in dengue patients.

3. Host determinants of disease severity

Only a small proportion of individuals with secondary DEN virus infections (and an even smaller proportion with primary infection) develop severe disease [1]. Therefore it follows that other variables, besides pre-existing immunity, shape the outcome of infection. This section will consider the role of age, gender, genetic susceptibility determinants and pre-existing medical conditions in determining the clinical phenotype.

3.1. Age and gender

Age is a risk factor for severe dengue and death. For example, the odds of a Vietnamese child 1–5 yrs of age dying as an inpatient in a Ho Chi Minh City Hospital between 2001 and 2009 was four-fold higher than a child 11–15 yrs of age. The greater relative prevalence of DSS in children relative to adults is likely tied to their having an intrinsically more permeable vascular endothelium, as demonstrated previously by Gamble et al. in healthy Vietnamese children and adults [84]. The risk for more severe outcomes in young children argues for vaccination implementation strategies to include this most vulnerable population.

Gender is also a risk factor for severe dengue and death-females are over-represented in several case series of children with DSS [85–87]. Girls were also over-represented amongst severe (odd ratio 1.19) and fatal cases (odd ratio 1.57) in the cohort of dengue inpatients at three large hospitals in Ho Chi Minh City between 2001 and 2009 [85]. The basis for an over-representation of females amongst severe and fatal cases has been suggested to be due to differences in health-care seeking behavior on behalf of girls and boys in Asian countries, i.e. girls may be presenting later in the course of their illness, leading to a higher likelihood of severe outcomes [85]. An alternative hypothesis is that physiological or immunological differences exist between males and females that explain these gender differences. Clearly, more research is required to understand the role of age and gender as determinants of outcome.

3.2. Genetic determinants of dengue susceptibility

Epidemiological studies suggest that people with African ancestry are less susceptible to the severe manifestations of dengue infection – the evidence for this is strongest in studies from Cuba and Haiti [88,89]. Other evidence for a host genetic basis to susceptibility stems from case-control association studies. In the immune response to DEN virus infection, viral antigens are presented to T cells in association with the major histocompatability complex (HLA) class I and II system. Some HLA alleles are suggested to be associated with different clinical phenotypes of dengue [90]. In addition, there is a suggestion of an association between HLA allele and susceptibility to specific dengue serotypes, for example DENV-1 and HLA *0207 and DENV-2 and HLA *B52 [91]. In addition some associations appear to be protective, for example HLA DR alleles in Mexican and Vietnamese populations [92,93]. However, most studies of genetic association with dengue are undermined by issues of sample size, multiple testing, unknown population stratification and variable case definitions. Indeed, with the exception of DC-SIGN, no association in an HLA allele or candidate gene has been replicated in an independent study.

Despite these limitations in study design, various single nucleotide polymorphisms (SNPs) have been associated with both protection and vulnerability to dengue infection [90]. The vitamin D receptor modulates the immune response by stimulating cell-mediated immunity and inhibiting lymphocyte proliferation; a polymorphism in the receptor appears to protect against severe dengue [94]. The Fcγ receptor mediates entry of antibody-coated DENV into target cells; the same study demonstrated that homozygotes for the arginine variant at position 131 of the Fcγ receptor gene may also be protected from severe disease [94]. This is perhaps because these homozygotes have less ability to opsonise IgG2 antibodies. There have been conflicting results from studies looking at polymorphisms in the TNF-α gene – data from Vietnam did not show an association, whereas investigators in Venezuela reported a significantly increased prevalence of the TNF-308A allele in patients with severe dengue [94,95]. Dengue infection of dendritic cells is mediated by the attachment factor, dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN1, encoded by CD209). The G allele of the variant of the promoter region (DC-SIGN-336) was associated with protection against dengue fever, but not dengue haemorrhagic fever [96]. This finding suggests that different disease phenotypes may be partly related to the activity of CD209 and the degree of DC-SIGN expression. Another area of focus in determining susceptibility to dengue has been polymorphisms in human platelet antigens (HPA). HPAs mediate the interaction between platelets and endothelium. Data from an Indian cohort showed that HPA-1a and HPA-2b alleles were expressed more frequently in patients with severe disease [97]. In addition the study demonstrated that clinical shock was seen more frequently in HPA-1 heterozygotes. It is possible that cross-reactive antibodies formed in the context of dengue infection may bind to HPA leading to platelet reduction.

In the era of genome-wide association studies (GWAS), a more expansive approach to understanding genetic susceptibility to severe dengue is now possible and desirable. Equally, this approach could be used in a pharmacogenomics fashion to understand the

genetic basis for adverse events, immunogenicity and durability of immune responses in participants of large dengue vaccine trials. This approach typically requires thousands of cases that fit a well-defined clinical outcome and population-matched controls. The strength of the approach is that it has power to detect relatively small contributions to genetic risk whilst also discounting any population stratification in cases or controls. The next major advances in our understanding of host genetic susceptibility to severe dengue, or the immune response to vaccination, are likely to come from GWAS methods.

3.3. Pre-existing comorbidities and predisposition to severe dengue

There have been few studies that have explored associations between underlying chronic diseases and predisposition to severe dengue. It was observed during the Cuban epidemics that chronic conditions, for example bronchial asthma, diabetes mellitus and sickle cell disease, were over-represented in patients with severe disease [98–101]. In addition it has been observed that a chronic medical condition is present in up to 70% of fatal cases in some case series of adults [102]. Data from Singapore suggests that co-morbidities such as hypertension are common in older patients with DEN, but infections in this age group are relatively benign [103]. Case control studies are necessary to further determine the role that chronic illness plays in predisposing the host to the severe manifestations of disease. Chronic hepatitis B virus infection is a common health problem in DEN-prevalent areas; co-infection does not appear to affect the clinical course but is associated with higher liver enzyme elevations [104]. DEN-HIV co-infection is an area of interest. There has been a recent intriguing observation that DEN NS5 protein inhibits HIV replication [105]. However a small case series in Singapore does not suggest the clinical course of dengue is any different in patients with HIV [106]. Pre-existing morbidities, more common in adults, could also be a determinant in the safety profile of live attenuated dengue vaccines. For example, it is unknown how HIV co-infection impacts the safety profile of live attenuated dengue vaccines currently in development. Similarly, it is unknown if secondary immunodeficiency (e.g. through long-term corticosteroid use) is a factor in the safety and reactogenicity of live attenuated dengue vaccines. Insights in these areas will accumulate as clinical development of candidate vaccines progresses, or alternatively, from post-marketing surveillance.

4. Thrombocytopenia and coagulopathy in dengue pathogenesis

4.1. Thrombocytopenia

As discussed above thrombocytopenia is an almost universal finding in dengue. This occurs as a result of both reduced production and increased destruction of platelets [107–109]. It has been suggested that the degree of thrombocytopenia correlates with clinical severity and complement activation [110]. As a result of reduced platelet counts and increased complement activation there is increased vascular fragility and thus an increased risk of haemorrhage.

4.2. Coagulopathy

Disordered coagulation, together with plasma leak and thrombocytopenia is likely to contribute to the haemorrhage observed in some cases of severe dengue [111]. This coagulopathy is likely to be mediated by cytokines. In a mouse model of the disease elevated levels of TNF-α correlated with endothelial cell dysfunction and haemorrhage [112]. Elevations of IL-6 and IL-8 have been associated with disordered coagulation and fibrinolysis in dengue [109,111]. Data from children in Vietnam has shown that increased APTT and reduced fibrinogen are consistently observed in patients with dengue [113]. The same study showed minor elevations in prothrombin time (PT). Infections normally result in increased fibrinogen, unless these conditions are complicated by disseminated intravascular coagulation (DIC). However the minor changes in PT together with the infrequent observation of fibrinogen degradation products (FDPs) do not support DIC being the cause of dengue-associated coagulopathy [113]. There are conflicting opinions about whether fibrinolysis or impairment of the fibrinolytic pathway occurs in dengue pathogenesis [114]. It is clear that the exact mechanisms contributing to dengue coagulopathy are incompletely understoodand that a better understanding is needed to allow for improvements in supportive care.

5. Viral factors in pathogenesis

5.1. Virus epidemiology and virulence

There are 4 antigenically distinct serotypes of dengue virus – DENV-1, DENV-2, DENV-3 and DENV-4. Each of the DEN virus serotypes is capable of causing severe dengue and early viral burdens in the course of illness are associated with severe disease [74,82,115]. Oscillations in the prevalence of each serotype are common in endemic settings. Typically, one serotype is dominant for a period of 2–4 yrs, after which it declines in prevalence as a different serotype(s) emerges to replace it [116,117]. The basis for the decline in prevalence of a serotype is presumably the accumulation of herd immunity such that the number of susceptible humans available is diminished. This cycling may also be due to immune enhancement when a new serotype is encountered. Each DENV serotype isas phylogenetically distinct from one other as the DENV groupis from Japanese encephalitis virus, suggesting that each serotype could be considered a separate virus with distinct in vivo characteristics. This concept is now gaining wider recognition. For example, the kinetics of viremia and NS1 antigenemia in DENV-1 infections is distinctly different (higher) from DENV-2 infections in Vietnamese children [52,60]. Similarly, Vietnamese infants with primary DENV-1 infections have significantly lower plasma NS1 concentrations than DENV-2 infected infants at the time of hospital presentation. Furthermore, a prospective study of hospitalized Thai children suggestedDENV-2 and -3 were twice as likely to result in DHF as DENV-4 [118]. Collectively, these data are examples that suggest each DEN virus serotype has its own constellation of virological characteristics in human hosts. Further research is needed to understand the breadth of these differences in humans, but also to understand differences with the mosquito host.

Adding to the complexity of DEN virus biology, within each DEN virus serotype there are distinct phylogenetic genotypes that appear to have differing geographical ranges. Increasingly, it is becoming clear that fitness differences exist between genotypes of the same serotype [119]. For example, there is evidence that the Asian/American subtype of DENV-2 is associated with more severe disease and has a fitness advantage in infected mosquitoes compared to the American genotype of DENV-2 [120]. Conversely, in Viet Nam, the introduction of the Asian 1 genotype of DENV-2 led to the complete replacement of the resident Asian/American genotype of DENV-2 [117]. The transmission fitness advantage of Asian 1 viruses was attributed to this virus attaining higher viremia levels in humans [117]. Other examples of genotype replacement events have occurred in Sri Lanka and Thailand. In Sri Lanka, the introduction of a new genotype DENV-3 virus was associated with an increased incidence of severe dengue [121]. Recently, evidence of antigenic differences between genotypes of the same serotype

has emerged [122,123]. To date these differences have been elucidated using mAbs specific to the E protein and it remains to be seen whether such differences are present in the face of polyclonal immune sera. Prospective cohort studies that can follow the introduction of different DEN virus genotypes into the same population are needed to understand the clinical and epidemiological significance of antigenic variation between DEN virus genotypes. It is worth remembering however that clinically apparent recurrent infections caused by the same serotype of DENV virus have not been documented, suggesting antigenic variation between genotypes might not be clinically relevant. Nevertheless, this is an important question since any evidence of "immune escape" within a serotype would have implications for vaccine development.

5.2. Cellular and tissue targets of DEN virus infection

The very early events in DEN virus infection of the human host are poorly understood. After the bite of an infected mosquito it is thought that initially immature Langerhan's cells in the dermis are infected first [124]. These infected cells migrate to the lymph nodes, resulting in infection of cells of the macrophage-monocyte lineage. This amplifies the infection, which is then disseminated via the lymphatic and vascular system. After this primary viremia circulating monocytes within the blood and macrophages within the liver, spleen and bone marrow are infected [125,126]. The range of tissues and cell types infected with DEN viruses suggests that the host receptor(s) are broadly distributed. To date, there is evidence for several candidate host receptors, for example mannose binding protein, heparan sulphate, chondroitin sulphate and DC-SIGN [127–129]. The factors that determine the number of cells infected at specific sites may also influence the level of immune activation, and thus have a role in determining the disease phenotype [111,125]. The time between the bite of an infected mosquito and the onset of clinical symptoms is believed to be 3–5 days [130,131]. Whether infected individuals in this pre-syndromic phase are capable of transmitting virus to a biting mosquito is unknown.

The study of viral tropism and in vivo pathology in dengue has been hampered by the lack of an animal model of disease and limited autopsy data from fatal cases. Nevertheless, post-mortem studies have detected suggested DEN viral antigen in a range of host cells and tissues [132]. There was also selective apoptosis of endothelial cells in the pulmonary and intestinal vasculature in one study, although this needs to qualified since differentiating true disease pathology from post-mortem tissue changes can be difficult [133].

6. Conclusions

Dengue continues to be a growing public health threat in many parts of the world. It is clear that vector control is an inadequate public health tool in endemic areas. Whilst careful clinical management can reduce the case-fatality rate amongst hospitalized patients to less then 1%, the disease burden places enormous pressure on health services and has a substantial social and economic impact [134]. The virulence of the virus plus the flavivirus infection history, age, gender and genotype of the host all appear to be determinants of outcome. The host immune response appears to play a central role in pathogenesis and much new information on this topic has been derived in the last 10 yrs. Nonetheless, much work is still needed to identify the precise causal mechanisms of the dengue capillary permeability syndrome, and in turn, how this can be modulated to improve patient management. A better understanding of dengue immunopathogenesis will assist not only development of therapeutic interventions but also the understanding of dengue vaccine efficacy or vaccine adverse events.

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2. HOST SUSCEPTIBILITY

Background

A key question in dengue is understanding why some patients infected with DENV get severe disease whereas others do not. Understanding the factors that determine the outcome of DENV infection is important and has the potential to expand our understanding of pathogenesis and, in the long-term, improve the clinical management of the disease.

Outline of papers

Dengue pathogenesis: host factors

This monograph considers the role of various host factors in dengue pathogenesis including host genetics. It provides an introduction to this section of the thesis and considers the relevance of these factors to clinical practice.

MICB and *PLCE1* and host susceptibility

SNPs at two genomic loci are associated with DSS in Vietnamese children.[7] These SNPs were in the major histocompatibility complex class I polypeptide-related sequence B gene (*MICB*) on chromosome 6, and in the phospholipase C epsilon 1 (*PLCE 1*) gene on chromosome 10. The *MICB* gene encodes a surface protein that contributes to natural killer (NK) and CD8 T cell activation. The observed association with a SNP in the *MICB* gene and DSS may reflect dysfunctional NK and CD8 cell activity in severe disease, suggesting a key role for these cells in disease control and pathogenesis. Mutations in *PLCE1* are associated with nephrotic syndrome, a condition characterised by proteinuria, reduced vascular oncotic pressure and subsequent oedema.[8] Severe dengue is characterised by plasma leak suggesting the possibility of some similarities with the pathophysiology of nephrotic syndrome. It is possible that mutations in *PLCE1* result in a reduced ability to maintain normal endothelial integrity, and therefore an increased risk of a more severe disease phenotype.[7] However these identified associations were in the context of paediatric patients with DSS, leaving unanswered the question whether they are also associated with less severe clinical phenotypes of dengue. To this end, the aim of this component of the thesis was to define the extent to which these alleles are associated with milder clinical phenotypes of paediatric and adult dengue. This work represents a natural extension of the original GWAS and has the potential to improve our understanding of disease susceptibility and pathogenesis and, in the longer term, pave the way to improved clinical management.

LITERATURE REVIEW 3: DENGUE PATHOGENESIS: HOST FACTORS

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SECTION A – Student Details

Student	James Whitehorn
Principal Supervisor	Rosanna Peeling and Cameron Simmons
Thesis Title	The pathogenesis and clinical management of dengue

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Dengue and Dengue Hemorrhagic Fever 2nd Edition, CAB International		
When was the work published?	2014		
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Student Signature: _____ **Date:** 4/6/2015

Supervisor Signature: _____ **Date:** 4/6/2015

11 Dengue Pathogenesis: Host Factors

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Introduction

Fortunately, severe dengue is an uncommon outcome of both primary and secondary DENV infections. The syndromic nature of severe dengue (see Chapter 8, this volume) is a reminder that no single host or viral factor should be considered a sole mechanistic cause of this outcome. Instead, a constellation of virological and host variables contribute to the expression of the clinical phenotype. These host and virus variables need to be thought of as risk factors that are measurable on a population-wide scale, but are not necessarily risk factors in individual patients. Here we will review some of the host risk factors associated with severe dengue and identify knowledge gaps where future research could be directed.

Age and Gender as Risk Factors for Severe Dengue

Age is a risk factor for the development of severe disease and death from DENV infection. A retrospective study in Vietnam showed that while the incidence of dengue shock syndrome (DSS) was highest in children aged between 6 and 10 years, younger children had a significantly higher chance of dying from their infection (Anders *et al.*, 2011). This study, of over 100,000 clinically diagnosed dengue cases, showed the odds of a child aged between 1 and 5 years dying of dengue as a hospital inpatient in Ho Chi Minh City was four times higher than for a child aged between 11 and 15 years. These findings are consistent with physiological observations that suggest that younger children have an intrinsically more permeable vascular endothelium and are therefore more prone to significant plasma leak and are thus more vulnerable to the development of shock and a poor clinical outcome (Gamble *et al.*, 2000).

A number of case series and cohort studies have suggested that gender is an additional risk factor for both the development of severe dengue and death (Halstead *et al.*, 1970; Kabra *et al.*, 1999; Anders *et al.*, 2011). In the retrospective cohort study in Vietnam, Anders and colleagues demonstrated that females were over-represented in both severe (odds ratio 1.19) and fatal cases (odds ratio 1.57) (Anders *et al.*, 2011). In this study the authors suggest that these observed differences between males and females might reflect different health-seeking behavior on behalf of different genders in Asian countries. This explanation seems plausible given the striking overall bias towards males among dengue cases in this same cohort, suggesting that the over-representation of

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females among the severe and fatal cases may reflect later presentation of girls to health services. It has been suggested that the over-representation of females in other case series may reflect a stronger, and thus pathological, cellular immune response to DENV, or a capillary endothelium more vulnerable to leak in females (Halstead *et al.*, 1970; Halstead, 1997; Kabra *et al.*, 1999). Further research is needed to assess the contribution gender makes on the eventual disease phenotype.

Dengue in the first year of life

Severe dengue is strongly associated with secondary DENV infections (Sangkawibha *et al.*, 1984; Burke *et al.*, 1988; Thein *et al.*, 1997; Graham *et al.*, 1999). Severe dengue also occurs during primary DENV infection of infants born to DENV-immune mothers (Halstead *et al.*, 1970; Hongsiriwon, 2002; Nguyen *et al.*, 2004; Simmons *et al.*, 2007). Clinically, severe dengue in infants is associated with all of the clinical features seen in older children, with the distinguishing feature being that infants with dengue have primary DENV infections and are born to dengue immune-mothers. Halstead has proposed that infants born to dengue-immune mothers and previously infected children or adults have in common a single immune risk factor – DENV-reactive IgG antibodies (Halstead, 1988). The capacity of sub-neutralizing concentrations of DENV-reactive IgG antibodies to enhance DENV infections in Fc-receptor bearing cells (Halstead, 1988; Morens and Halstead, 1990; Gagnon *et al.*, 2002), the process called antibody-dependent enhancement (ADE), provides a unifying basis to explain severe dengue in secondary infections and in infants with primary infections. Support for this hypothesis stems from the observation that infants with severe dengue are typically between 4 and 11 months of age, a time when maternally derived neutralizing antibody has usually waned but when non-neutralizing, virion-binding antibodies remain and experimentally can cause ADE (Kliks and Halstead, 1983; Kliks *et al.*, 1988; Chau *et al.*, 2008). Refinement of this hypothesis as it relates to infants has implicated maternal IgG as a risk factor for symptomatic dengue as well as severe dengue (Libraty *et al.*, 2009). Further, maternally derived anti-prM IgG antibodies are a logical candidate for supporting ADE in infants (Chau *et al.*, 2008; Dejnirattisai *et al.*, 2010). Primary dengue in infants is not associated with an anamnestic cellular immune response and therefore this aspect of the host immune response cannot explain the clinical complications of capillary leakage, bleeding and coagulopathy that are often observed in infants with severe dengue. It is possible that severe dengue in infants reflects their intrinsically poor compensatory reserve in the face of a severe, systemic viral infection that has been exacerbated by ADE. Further research is needed to understand the similarities and differences in the pathogenesis of dengue in infants compared with older children. Future studies should utilize prospective cohort studies, as described previously (Libraty *et al.*, 2009), to gain insights into the role of maternal antibody and the clinical, virological and immunological phenotype of dengue in infants.

Host Genetics and Susceptibility to Severe Dengue

A genetic component to susceptibility to severe DENV infection was indirectly suggested in 1906, when Agramonte observed, 'Black people seem to have remarkable degree of resistance to dengue disease' (Agramonte, 1906). Agramonte's statement has been supported by epidemiological observations in Cuba and Haiti that suggest that people with an African ancestry have a reduced risk of developing severe disease as compared to those with European ancestry (Guzman *et al.*, 1990; Halstead *et al.*, 2001; Guzman and Kouri, 2002; Guzman, 2005). Whether this reflects intrinsic genetic or physiological differences, or reduced exposure because of *A. aegypti* biting preferences, requires further study.

Most studies exploring genetic association in dengue have been case-control association studies. Unfortunately many of these studies have been compromised by poor design and sometimes vague reporting. Inadequate sample sizes, unclear case definitions, multiple

testing and undetermined population stratification are prominent potential confounders to the results of these studies. The vast majority of previous studies examined polymorphisms in candidate genes where there was pre-analysis justification for an association. However, following the completion of sequencing for the human genome project and the advent of genome-wide association studies (GWAS) there have been considerable advances in our understanding of the genetic susceptibility to diseases, including infectious diseases, as a more comprehensive and rigorous approach has become possible (Hill, 2006; Casanova and Abel, 2007; Manolio, 2010). Despite the limitations of many earlier case-control genetic association studies, a panel of single nucleotide polymorphisms (SNPs) has been nominated as being associated with both protection and vulnerability to dengue. The validity of many of these associations has yet to be tested in independent cohorts from either the same population or a different population.

HLA genotype and susceptibility to dengue

As the immune system is strongly implicated in DENV pathogenesis, it is natural that the genes involved in regulation of the immune response have been among the most closely studied. In the course of DENV infection, viral antigens are presented to T cells in association with the major histocompatibility complex (HLA) class I and II systems. Some of the highly polymorphic HLA alleles have been proposed to have an association with either disease susceptibility or protection (Coffey *et al.*, 2009). Studies have been conducted in different geographic and ethnic settings that have shown an association between certain HLA class I alleles and vulnerability to severe DENV infection (Chiewsilp *et al.*, 1981; Paradoa Perez *et al.*, 1987; Loke *et al.*, 2001). A case-control study conducted in Thailand showed a serotype-specific association between various HLA class I alleles and the clinical outcome of infection (Stephens *et al.*, 2002). For example, HLA-A*0203 was associated with less severe disease irrespective of the infecting serotype, whereas HLA-A*0207 was associated with more severe disease only in patients with secondary DENV-1 and DENV-2. In contrast, HLA-A*B51 was associated with more severe disease irrespective of serotype, and HLA-A*B52 was associated with a less severe disease phenotype in secondary infections with DENV-1 and DENV-2. In addition, various HLA-B alleles (B44, B62, B76 and B77) appeared to protect against the development of clinical disease from secondary DENV infection (Stephens *et al.*, 2002). Work in Mexico and Vietnam has suggested that certain HLA DR alleles protect against severe infection (LaFleur *et al.*, 2002; Nguyen *et al.*, 2008). For example, in the Mexican case-control study there appeared to be a protective association between HLA-DR4 and the risk of developing severe disease (LaFleur *et al.*, 2002). The Vietnamese case-control study showed HLA-A*A24 was over-represented in patients with severe disease, whereas HLA-DRB1*0901 was under-represented, suggesting an association with susceptibility and protection respectively (Nguyen *et al.*, 2008). There have been additional studies assessing the association between polymorphisms in the TNF-α gene and susceptibility to DENV infection (Fernandez-Mestre *et al.*, 2004; Perez *et al.*, 2010; Garcia-Trejo *et al.*, 2011). These studies suggested an association between the TNF-308A allele and increased susceptibility to severe manifestations of DENV infection (Fernandez-Mestre *et al.*, 2004; Perez *et al.*, 2010), and a possible protective association between the TNF-238A allele and severe DENV infection (Garcia-Trejo *et al.*, 2011). Another case-control study showed an increased frequency of the transforming growth factor β-1 (TGFβ-1)-509 CC genotype in cases of dengue hemorrhagic fever, as compared to the milder dengue fever (Chen *et al.*, 2009). This same study showed that the presence of the TGFβ-1-509 CC genotype together with the cytotoxic T-lymphocyte antigen 4 (CTLA-4) +49G allele was associated with a higher viral load in DENV-2 infections and a more severe disease phenotype (Chen *et al.*, 2009). These findings support disordered regulation of T cell responses playing a key role in the development of severe disease.

The vitamin D receptor (VDR) appears to play an integral role in the modulation of the immune response (Baeke *et al.*, 2010).

The VDR is present on virtually all cells of the immune system and activation of the receptor modulates the immune response by triggering cell-mediated immunity and inhibiting the proliferation of lymphocytes (Baeke *et al.*, 2010). The tt genotype of a SNP at position 352 of the VDR gene was associated with protection from severe dengue (Loke *et al.*, 2002). Interesting this same SNP has been observed more frequently in other infectious disease phenotypes. For example, it is over-represented in tuberculoid as opposed to lepromatous leprosy (Roy *et al.*, 1999). As the tuberculoid phenotype of leprosy is associated with a robust cell-mediated immune response, these observations support the role of enhanced cell-mediated immunity in controlling DENV infection. Loke and colleagues (2002) also showed that homozygotes for the arginine variant at position 131 of the Fcγ receptor II (FcγRII) gene appeared to be protected from the development of severe DENV infection. FcγRII is a widely distributed receptor for all IgG classes, and has been shown to mediate antibody-dependent enhancement of DENV in *in vitro* experiments (Littaua *et al.*, 1990). The arginine variant of the FcγRII receptor appears to be associated with reduced opsonization of IgG_2 antibodies (Clark *et al.*, 1991). It is plausible that the association between the arginine variant of the FcγRII gene and a less severe clinical phenotype may reflect reduced antibody-mediated enhancement at this receptor. Loke and colleagues showed no association between polymorphisms in mannose-binding lectin (MBL), interleukin-1 and interleukin-4 receptor antagonist genes and severe dengue (Loke *et al.*, 2002). However a different study has shown a potential association between polymorphisms in the MBL gene and protection from severe thrombocytopenia associated with DENV infection (Acioli-Santos *et al.*, 2008).

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a common inherited enzyme deficiency with a particularly high prevalence in Asian countries. An *in vitro* study with DENV-2 showed a higher infection in monocytes from patients with G6PD deficiency as compared to those with normal G6PD levels (Chao *et al.*, 2008). The authors suggest that this observation might, in part, explain the high DENV burdens seen in Asian countries, although these findings have not been validated by larger case-control studies. Dengue virus–antibody complexes have been detected on platelets in patients with severe DENV infection and it is thought that viral binding to platelets via human platelet antigens (HPA) may result in the thrombocytopenia observed in severe infection. A case-control study in India showed that HPA-1a and HPA-2b alleles were observed more frequently in patients with more severe disease (Soundravally and Hoti, 2007). The study also demonstrated that DSS was observed more frequently in HPA-1 heterozygotes. Interestingly, HPA-1 and HPA-2 homozygotes appeared to be relatively protected from severe primary DENV infection, whereas heterozygotes were vulnerable to severe primary infection (Soundravally and Hoti, 2008).

Dendritic cell infection by DENV is effected by the attachment factor, dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN1), encoded by CD209. The G allele of DC-SIGN1-336 appears to protect against milder DENV infection, dengue fever, but not against more serious disease (Sakuntabhai *et al.*, 2005). These findings suggest that the severity of disease may be related to CD209 activity and the extent of DC-SIGN expression.

The limited strength of case-control study design for demonstrating a genetic association is illustrated by the fact that, with the exception of DC-SIGN, no associations have been replicated in an independent cohort or study. As the technology of GWAS becomes more accessible as a result of reduced costs of genotyping and the development of high-throughput sequencing, a more comprehensive approach to understanding genetic susceptibility to severe dengue is possible. GWAS typically requires thousands of cases with a well-defined clinical outcome and population-matched controls. The advantage of GWAS is that it can robustly detect relatively small contributions to genetic risk as seen in previous studies in, for example, type 2 diabetes mellitus, obesity and systemic lupus erythematosus (Frayling *et al.*, 2007; Scott *et al.*, 2007; Hom *et al.*, 2008). A recent GWAS demonstrated susceptibility loci for severe dengue at MHC class I polypeptide-related sequence

B (*MICB*) (rs3132468) on chromosome 6 and phospholipase C, epsilon 1 (*PLCE1*) (rs3765524) on chromosome 10 (Khor *et al.*, 2011). The cases used in this study were children with DSS. The controls were from cord-blood samples. *MICB* encodes an activating ligand for the NKG2D type II receptor present on natural killer (NK) cells and CD8+ T cells. Ligation of NKG2D by *MICB* results in a range of antiviral effector functions including cytokine expression and the cytolytic response (Raulet, 2003). *MICB* and other genes associated with NK cell activation are highly expressed in acute dengue infection – the association described between a mutation in *MICB* and severe dengue may reflect altered or dysfunctional NK or CD8+ T cell activation perhaps resulting in higher viral load (Hoang *et al.*, 2010; Khor *et al.*, 2011). Mutations in *PLCE1* are associated with nephrotic syndrome, a condition characterized by proteinuria, reduced vascular oncotic pressure and subsequent edema (Hinkes *et al.*, 2006). Severe dengue is characterized by plasma leak, suggesting some similarities with the pathophysiology of nephrotic syndrome. It is possible that mutations in *PLCE1* result in a reduced ability to maintain normal endothelial integrity, and therefore an increased risk of a more severe disease phenotype (Khor *et al.*, 2011).

The 'Cytokine Storm' in Immunopathogenesis

The host immune response is widely considered to contribute to many of the clinical complications associated with severe dengue. Support for a role of the host immune response in mediating capillary permeability is based on the temporal mismatch between the highest virus burdens *in vivo* and the observed clinical manifestations of capillary permeability (the first 48 hours of illness, followed by rapid clearance, compared with day 3–6 of illness, respectively). Thus, capillary permeability develops at a time when the viral burden is in sharp decline, arguing against a direct virus-mediated effect on the vascular endothelium. Further, the most severe complication of capillary permeability, DSS, manifests when many patients have low or undetectable viremia levels and are already or very nearly afebrile (Simmons *et al.*, 2012). Finally, the characteristic capillary permeability of severe dengue manifests relatively slowly and in many patients it is sufficiently well compensated for by homeostatic mechanisms that intravenous fluid therapy is not required (see Chapter 10, this volume). This is quite unlike the direct cytopathic insult on the vascular endothelium that occurs in fulminant Ebola virus infection and that leads to hemorrhage, rapid hypovolemic shock and a high mortality, despite supportive care (Zampieri *et al.*, 2007).

A second line of evidence in support of the immune pathogenesis model of severe dengue lies in the observation that the vast majority of severe dengue complications occur in patients with immunological evidence of a previous history of DENV exposure, or in the case of infants, those with maternally acquired anti-DENV antibody. These observations have provided support to the hypothesis that ADE is an important risk factor for severe dengue. In this model, ADE supports virus infection of Fc receptor bearing cells such as tissue macrophages that in turn secrete vasodilatory cytokines. An alternative, but not mutually exclusive model, is that cross-reactive memory T cells secreting vasoactive cytokines have a role in the pathogenesis of severe dengue during secondary infections (discussed in depth in Chapter 14, this volume), although memory T cells cannot explain severe dengue in infants with primary dengue. In all of these models of pathogenesis, the production of pro-inflammatory cytokines by immune cells during the course of infection is nominated as being central to the mechanistic process of capillary permeability. In short, it is believed that the vasodilatory potential of some cytokines that occur in elevated concentrations during acute dengue are causally linked to endothelial cell dysfunction and capillary permeability. This hypothesis of a 'cytokine storm' mediating capillary permeability is attractive since it helps unify the ADE hypothesis (greater virus burden) with the concept of immune activation of memory, cross-reactive T cells in secondary infection.

There are however important knowledge gaps that undermine the strength of the 'cytokine storm' hypothesis. In Table 11.1, a non-exhaustive summary of publications since 1995 that describe cytokine or chemokine concentrations in clinical specimens from dengue cases is shown. The number of these studies is large (n = 38). Of note, there is significant heterogeneity in study designs, patient sample size, quality of clinical descriptions and data reporting. Collectively, some of the obstacles in this body of publications that limit the development of a well-evidenced viewpoint on the role of a 'cytokine storm' in immunopathogenesis include:

- Study designs: many studies were hospital-based and therefore lacked useful control groups, such as uncomplicated, ambulatory dengue patients.
- Study reporting: inadequate descriptions of the inclusion/exclusion criteria and the enrolment process were common in many of the publications. Thus, in most studies it was unclear how many participants were screened for eligibility but were not enrolled, how many patients were screened and identified as eligible for enrolment but were not consented and for those enrolled how many completed the study investigations and were followed-up.
- Variable sampling strategies: many studies were cross-sectional in nature, whereas others performed serial measurements. In general, few studies investigated very early time points in disease (e.g. within the first 48 hours of illness).
- Variable clinical descriptions and phenotypes: frequently, data from dengue hemorrhagic fever patients have been compared to dengue fever patients. In some studies, it is unclear if some dengue fever patients would have been re-classified if more investigations had been performed.
- Control groups: some studies recruited patients with other febrile illnesses (not dengue), used healthy controls or dengue convalescent blood samples.
- Pediatric vs. adult dengue: well-recognized differences in the clinical phenotype of dengue between children and adults (more DSS in children; more bleeding and comorbidities in adults) make it challenging to compare between studies of different patient age-groups.
- Multiple testing: none of the published studies stated that they followed an *a priori* analysis plan. Instead, there is a hint of *post-hoc* testing, possible bias towards reporting of 'favorable comparisons', and all without correction for multiple testing. This is a major point of weakness in the literature.

In spite of these challenges in interpreting the existing literature, there are some important themes relevant to understanding pathogenesis that can be graded according to the strength of evidence supporting them. These themes, and their level of evidence, are summarized in Table 11.2.

Table 11.2 identifies important knowledge gaps in our understanding of the natural history of changes in soluble pro-inflammatory mediators and their association with capillary permeability during dengue. Addressing these knowledge gaps is important because it helps provide a rational basis to move towards specific therapeutic approaches, e.g. anti-cytokine therapy with mAbs or other inhibitors. In the future, observational studies of dengue could learn from the rigorous approach used in clinical trials, with detailed attention to data collection, sample handling, analysis and most of all, transparent reporting of patient enrolment and an *a priori* analysis and reporting plan. Observational studies would also be enhanced by early enrolment of patients, inclusion of ambulatory and hospitalized patients coupled with serial observations during the acute phase and again in convalescence. Inclusion of patients with other febrile illnesses (not dengue) provides a valuable reference population.

Conclusions and Relevance to Clinical Practice

Multiple host risk factors for severe dengue have been identified. Age, gender and host genotype all contribute to the outcome of

Table 11.1. Publications that describe cytokine and chemokine levels in dengue.[a]

Article	Country	Sample year	Inclusion criteria	Inpatients/outpatients	Molecules measured
Agarwal *et al.* (1999)	India	1996	Patients 8 months–55 years presenting with dengue as per the 1993 WHO guidelines	Inpatients	TGF-β1
Assuncao-Miranda *et al.* (2010)	Brazil	2002	Severe dengue cases	Inpatients	MIF, TNF-α, IL-6, IFN-γ
Avila-Aguero *et al.* (2004)	Costa Rica	2000–2001	Patients 13–70 years presenting with dengue as per the 1996 WHO guidelines	Inpatients	IL-6, IL-1β, IL-8, IL-10, TNF-α
Azeredo *et al.* (2006)	Brazil	2005	Patients 18–50 years presenting with dengue as per the 1997 WHO guidelines	Outpatients	IL-15
Azeredo *et al.* (2006)	Brazil	2001–2002	Patients 15–73 years presenting with dengue as per the WHO guidelines, 2002 (with modifications)	Both	TGF-β, IL-18, sICAM-1
Bethell *et al.* (1998)	Vietnam	1993	Patients 1.2–8 years with dengue as confirmed by elevated dengue virus specific IgM titers, and exclusion of other diagnoses	Inpatients	TNF, IL-6, IL-8, sTNFR-55, sTNFR-75, sICAM-1, CRP
Bozza *et al.* (2008)	Brazil	Unspecified	Patients 15–73 years presenting with dengue as per the WHO guidelines, 1997 (with modifications)	Both	IL-1β, TNF-α, IFN-γ, IL-12, IL-4, IL-5, IL-6, IL-10, IL-13, IL-2, IL-7, IL-17, GM-CSF, IL-8, MCP-1, MIP-1β
Braga *et al.* (2001)	Brazil	1997, 1999	Patients presenting with dengue as per the 1994 WHO guidelines	Outpatients	TNF-α, IFN-γ, TNF-Rp75, IL-1β, IL-6, IL-12
Chakravarti and Kumaria (2006)	India	2003	Patients 8–52 years presenting with dengue as per the 1999 WHO guidelines	Outpatients	TNF-α, IFN-γ
Chaturvedi *et al.* (1999)	India	1996	Patients 7 months–55 years presenting with dengue as per the 1993 WHO guidelines	Inpatients	TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10
Lee *et al.* (2006)	Taiwan	2002	Patients 7–79 years presenting with dengue as per the 1997 WHO guidelines	Both	MIF, IL-6, TNF-α, IFN-γ, IL-10

De Kruif *et al.* (2008)	Indonesia	2002–2003	Patients 3–14 years presenting with dengue as per the 1997 WHO guidelines	Inpatients	IFN-γ, IL-12, IL-1b, IL-8, MIF, NFR1, MCP1, MIP1α, MIP1β, IL-1A, IL-2, IL-6, IL-10, IL-18, MCP2
De Oliveira-Pinto *et al.* (2012)	Brazil	2001–2008	Patients 1–77 years presenting with acute exanthematic manifestation	Outpatients	IFN-γ, IP-10 (CXCL10/IP-10), MCP-1, MIP-1β, IL-1Ra
Djamiatun *et al.* (2011)	Indonesia	2005–2006	Patients <15 years presenting with dengue as per the 1997 WHO guidelines	Inpatients	TGF-β1
Gagnon *et al.* (2002)	Thailand	1994	Patients 6 months–14 years presenting with dengue as per the 1997 WHO guidelines	Inpatients	TNF-α, TNF-β, IFN-γ, IL-2, IL-4
Green *et al.* (1999)	Thailand	1994–1998	Children presenting with dengue fever of <72 hours	Inpatients	IL-10, IL-12p70, IL-12 (p40+p70)
Hober *et al.* (1998)	Vietnam	Unspecified	Patients 3–15 years presenting with dengue grades 2–4 as per the 1994 WHO guidelines	Inpatients	TNF-α
Houghton-Trivino *et al.* (2010)	Colombia	2005–2006	Patients 3 months–55 years presenting with dengue as per the 2005 WHO guidelines	Unspecified	IL-1β, TNF-α, IL-8, IL-4, IL-5, IL-10, IL-12, IFN-γ, IL-2
Iyngkaran *et al.* (1995)	Malaysia	1995	10-year-old Chinese male with dengue fever <2 days	Inpatient	TNF, IL-6, IL-1
Juffrie *et al.* (2001)	Indonesia	1995–1996	Children presenting with dengue as per the 1997 WHO guidelines	Inpatients	IL-6, CRP
Juffrie *et al.* (2000)	Indonesia	1995–1996	Children presenting with dengue as per the 1997 WHO guidelines	Inpatients	IL-8
Kadhiravan *et al.* (2010)	India	2006	Patients >12 years presenting with dengue as per the 1997 WHO guidelines with no HIV or other illness	Inpatients	IFN-γ, IL-4, TNF-α
Kittigul *et al.* (2000)	Thailand	1997	Patients presenting with dengue as per the 1997 WHO guidelines	Inpatients	TNF-α
Kubelka *et al.* (1995)	Brazil	1995	Adults with dengue	Inpatients	TNF-α
Laur *et al.* (1998)	French Polynesia	1996–1997	Patients 1 month–15 years presenting with dengue as per the 1986 WHO guidelines	Inpatients	TNF-α, TGF-β1
Lee *et al.* (2006)	Taiwan	Unspecified	Vietnamese patients with dengue as per WHO guidelines	Unspecified	MCP-1

Continued

Table 11.1. Continued.

Article	Country	Sample year	Inclusion criteria	Inpatients/outpatients	Molecules measured
Levy *et al.* (2010)	Venezuela	2005–2006	Patients 3–53 years presenting with dengue as per the 1986 WHO guidelines	Outpatients	IL-6, IL-1β, TBF-α
Mangada and Rothman (2005)	India	2001	Patients presenting with dengue as per the 1993 WHO guidelines	Inpatients	IL-13, IL-18
Masaki *et al.* (2002)	Japan	1997	A 37-year-old male with fever <1 day	Inpatient	TFN-α, IFN-γ, IL-12
Nguyen *et al.* (2004)	Vietnam	1998–2002	Patients <1 year presenting with dengue as per the 1997 WHO guidelines	Inpatients	IFN-γ, TNF-α, IL-10, IL-6, IL-4, IL-12
Pacsa *et al.* (2000)	Northern India	1996	Patients presenting with dengue as per the 1993 WHO guidelines	Inpatients	IL-12
Perez *et al.* (2004)	Cuba	1997	Patients 16–59 years presenting with dengue as per the 1997 WHO guidelines	Inpatients	IL-10, IL-12 (p70 + p40), RANTES
Pinto *et al.* (1999)	Brazil	1995–1996	Patients diagnosed with dengue	Inpatients	TNF-α, IL-6, IL-1β, sTNFR-55 and sTNFR-75, IL-1Ra
Priyadarshini *et al.* (2010)	India	2005	Patients 1–64 years presenting with dengue as per the WHO guidelines	Both	IL-6, IL-8, TNF-α, IFN-γ
Raghupathy *et al.* (1998)	India	1996	Patients 8 months–55 years with dengue as per the 1993 WHO guidelines	Inpatients	IL-8
Restrepo *et al.* (2008a)	Colombia	2005–2007	Patients presenting with dengue as per the 1995 Pan-American Health Organization guidelines	Outpatients	IL-6, TNF-α, IFN-γ
Restrepo *et al.* (2008b)	Colombia	2000–2002	Patients <1 year presenting with dengue as per the 1995 Pan-American Health Organization guidelines	Both	IL-6, TNF-α, IFN-γ
Tolfvenstam *et al.* (2011)	Singapore	Unspecified	Patients 23–66 years presenting with dengue as per the 2009 WHO guidelines	Both	MCP-1, MCP-2, IP-10, MIP-1α

[a]The search terms used to capture these publications were: dengue, cytokine, IFN, interferon, IL, interleukin, CSF, TNF, LT, lymphotoxin, CD40 ligand, CD40L, Fas ligand, FasL, CD27L, CD27 ligand, CD30 ligand, CD30L, 4-1BBL, Trail, AP0-2L, OPG-L, RANK-L, APRIL, LIGHT, TWEAK, BAFF, CD257, BlyS, TGF, MIF, CXCL, CCL, MIP, chemokine, HCC, TARC, DC-CK1, PARC, ELC, ELR, LARC, SLC, MDC, MPIF, CK, Eotaxin, CTACK, MEC, SCM.

Table 11.2. The evidence base for associations between pro-inflammatory cytokines and dengue pathogenesis.

Observation on plasma/serum cytokine levels	Strength of evidence	Example references
Elevations in pro- and anti-inflammatory cytokines occur during the febrile phase of dengue (both primary and secondary)	Strong	De Oliveira-Pinto *et al.* (2012); Green *et al.* (1999); Juffrie *et al.* (2001); Kitigul *et al.* (2000); Laur *et al.* (1998); Nguyen *et al.* (2004); Perez *et al.* (2004); Raghupathy *et al.* (1998); Restrepo *et al.* (2008a,b); Tolfvenstam *et al.* (2011)
Cytokines with functional relevance to inhibition of virus infection, e.g. IFN-α/β, IFN-γ, TNF-α, are elevated during the acute, viremic phase	Strong	Bozza *et al.* (2008); Braga *et al.* (2001); Chen *et al.* (2006); De Kruif *et al.* (2008); Houghton-Trivino *et al.* (2010); Masaki *et al.* (2002)
During the critical phase, cytokine concentrations are significantly elevated in severe dengue cases compared to non-severe dengue cases	Variable	Bethell *et al.* (1998); Bozza *et al.* (2008); Green *et al.* (1999); Juffrie *et al.* (2001); Kittigul *et al.* (2000); Levy *et al.* (2010); Mangada *et al.* (2005); Perez *et al.* (2004)
Elevations in cytokine concentrations occur in parallel to hemoconcentration or other signs of capillary permeability	Weak	Bethell *et al.* (1998); Djamiatun *et al.* (2011); Houghton-Trivino *et al.* (2010); Juffrie *et al.* (2001)
Elevations in cytokine concentrations during dengue are qualitatively or quantitatively different from those observed in other acute systemic virus infections that *do not* involve a capillary permeability syndrome	Weak	De Oliveira-Pinto *et al.* (2012); Kubelka *et al.* (1995); Gagnon *et al.* (2002); Green *et al.* (1999); Lee *et al.* (2006); Houghton-Trivino *et al.* (2010) respiratory infection; Juffrie *et al.* (2000, 2001) bacterial infections
Evidence for a causal association between one or more soluble factors and capillary permeability during dengue	None	None

infection. Passively acquired antibody might be a unique risk factor in infants. However, each of these risk factors is not at the level of impact where they can be integrated into routine clinical care. Pro-inflammatory mediators probably contribute to the capillary permeability syndrome but there are gaps in the quality of indirect evidence around this link. More fundamentally, it will be extremely difficult to identify *causal* mediators of capillary permeability in observational studies. 'Drug probe' randomized controlled clinical trials are one way to interrogate this process, with a recent trial of early corticosteroid therapy being one example of how this can be done (Tam *et al.*, 2012). That early therapy with oral corticosteroids did not improve clinical or laboratory features of dengue should not necessarily be interpreted as indicating immunological drivers are not important in pathogenesis, but possibly that earlier, more potent or better targeted immunomodulatory therapy is required. The field should be encouraged to explore a range of options for similar drug probe studies that encompass careful evaluation of the host immune response and clinical outcome.

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RESEARCH PAPER 1: GENETIC VARIANTS OF *MICB* AND *PLCE1* AND ASSOCIATIONS WITH NON-SEVERE DENGUE

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Student	James Whitehorn
Principal Supervisor	Rosanna Peeling and Cameron Simmons
Thesis Title	The pathogenesis and clinical management of dengue

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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Genetic Variants of *MICB* and *PLCE1* and Associations with Non-Severe Dengue

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Abstract

Background: A recent genome-wide association study (GWAS) identified susceptibility loci for dengue shock syndrome (DSS) at *MICB* rs3132468 and *PLCE1* rs3740360. The aim of this study was to define the extent to which *MICB* (rs3132468) and *PLCE1* (rs3740360) were associated with less severe clinical phenotypes of pediatric and adult dengue.

Methods: 3961 laboratory-confirmed dengue cases and 5968 controls were genotyped at *MICB* rs3132468 and *PLCE1* rs3740360. Per-allele odds ratios (OR) with 95% confidence intervals (CI) were calculated for each patient cohort. Pooled analyses were performed for adults and paediatrics respectively using a fixed effects model.

Results: Pooled analysis of the paediatric and adult cohorts indicated a significant association between *MICB* rs3132468 and dengue cases without shock (OR = 1.15; 95%CI: 1.07 – 1.24; $P = 0.0012$). Similarly, pooled analysis of pediatric and adult cohorts indicated a significant association between dengue cases without shock and *PLCE1* rs3740360 (OR = 0.92; 95%CI: 0.85 – 0.99; $P = 0.018$). We also note significant association between both SNPs (OR = 1.48; P = 0.0075 for *MICB* rs3132468 and OR = 0.75, $P = 0.041$ for *PLCE1* rs3740360) and dengue in infants.

Discussion: This study confirms that the *MICB* rs3132468 and *PLCE1* rs3740360 risk genotypes are not only associated with DSS, but are also associated with less severe clinical phenotypes of dengue, as well as with dengue in infants. These findings have implications for our understanding of dengue pathogenesis.

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Introduction

Dengue is the most important arboviral disease of humans.[1] Dengue viruses (DENV) cause a spectrum of clinical manifestations ranging from asymptomatic infection through to life-threatening shock and haemorrhage.[1,2] The clinical outcome of an individual infection is influenced by a variety of virus and host-related factors. The host factors that influence the clinical course of an individual infection include flavivirus infection history, host genotype, sex, age, and the presence of underlying medical conditions.[3–5] The first GWAS in dengue identified susceptibility loci for dengue shock syndrome (DSS) at MHC class I polypeptide-related sequence B (*MICB*) (C/T, rs3132468) on chromosome 6 and phospholipase C, epsilon 1 (*PLCE1*) (C/A, rs3740360) on chromosome 10.[6] The *MICB* gene encodes an activating ligand of natural killer (NK) cells (and possibly CD8+ T cells). We have previously speculated that mutations in *MICB* might result in impaired induction of anti-viral effector functions in NK cells with the consequence being a greater DENV-infected cell mass in vivo [6], a recognised risk factor for severe dengue.[7]

The identification of variants of *PLCE1* as being associated with severe dengue is intriguing.[6] Rare mutations of high penetrance within *PLCE1* are associated with nephrotic syndrome, a condition characterised by oedema secondary to proteinuria and reduced vascular oncotic pressure.[8] Since plasma leak, proteinuria and hypovolemia are also characteristic features of severe dengue, it's plausible that nephrotic syndrome and severe dengue share some common underlying pathophysiological processes. Furthermore, there are data implicating *PLCE1* in the homeostatic regulation of blood pressure.[9] These findings have the potential to help us define more clearly the functional basis of *PLCE1* variants in severe dengue.

The SNP associations identified at *MICB* (rs3132468) and *PLCE1* (rs3740360) by the GWAS study were in the context of

pediatric patients with DSS, leaving unanswered the question whether they are also associated with less severe clinical phenotypes of dengue. To this end, the aim of this study was to define the extent to which these alleles were associated with milder clinical phenotypes of pediatric and adult dengue. We analyzed a total of 3961 laboratory-confirmed dengue cases, independent from the initial GWAS study, and 5968 cord blood controls.[6]

Materials and Methods

Ethics statement

All participants gave written informed consent to participate in the prospective studies summarised in **Table 1** (details of the inclusion and exclusion criteria are available in **Supplementary Table 1**). Parents or guardians provided written informed consent on behalf of the children involved in the studies. The protocols for these studies were approved by the Institutional Review Boards of each study site (Hospital for Tropical Diseases HCMC, Children's Hospital 1 and 2 HCMC, Hung Vuong Hospital HCMC, Dong Thap Hospital, Sa Dec Hospital and Tien Giang Hospital) and by the Oxford University Tropical Research Ethics Committee. Each ethical committee approved of the consent procedure detailed above.

Enrolment and diagnosis

Blood samples for genotyping were collected in one of several prospective studies of dengue in Vietnamese patients detailed in **Table 1**. Dengue cases were laboratory-confirmed by one of three methods: IgM-seroconversion by ELISA assay on paired samples, detection of DENV RNA by RT-PCR, or detection of non-structural protein 1 (NS1) by ELISA (BioRad Platelia). The control samples used in this study were from blood samples collected from the umbilical cord of newborn infants enrolled into the birth cohort study detailed in Table 1. Within each cohort, dengue cases were classified in a binary fashion as being "DSS" or "not-DSS".[6] Consistent with the original GWAS study, DSS cases were defined as laboratory-confirmed dengue cases with cardiovascular decompensation secondary to plasma leakage and requiring fluid resuscitation.[2]

Table 1. Summary of the cohort studies used in the analysis.

Group	Number (% male)	Median age (range 5th – 95th)
Control	1068 (52)	At birth
Infants	165 (55)	7 months (3 – 12 months)
DC	59 (52)	6 (3 – 11)
DT	88 (57)	7 (4 – 11)
DZ	8 (50)	8 (7 – 11)
FB	9 (55)	8 (7 – 15)
Children/Young adults	2759 (56)	12 years (6 – 16 years)
FG	576 (56)	11 (4 – 22)
06DX	220 (71)	13 (11 – 14)
DR	582 (56)	11 (6 – 15)
MD	1381 (60)	12 (7 – 14)
Adults	741 (46)	22 (15 – 35)
09DX	159 (41)	23 (19 – 27)
D001	54 (43)	20 (14 – 25)
FL	528 (48)	22 (15 – 35)

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Genotyping

DNA extractions were performed using a MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, Germany) according to the manufacturer's instructions. Candidate SNPs were genotyped using a TaqMan® genotyping assay to amplify and detect the specific alleles in the DNA samples as per manufacturer instructions.

Statistical analysis

The data were analysed using PLINK version 1.07 and the R statistical software package version 2.12.0 (2010 The R Foundation for Statistical Computing). For each cohort study per-allele odds ratios with 95% confidence intervals were calculated to assess the relationship between SNP genotypes (rs3132468 at *MICB* and rs3740360 at *PLCE1*) and susceptibility to dengue. For the analysis we considered non-DSS cases in children separately from adults and considered infants with dengue as a distinct group. Infants were defined as less than 1 year in age, children were defined by ages between 1 and 15 years, and adults were defined by being 16 years and older. Other variables known to be associated with disease severity were not included in this analysis, consistent with the GWAS primary analysis for DSS as well as other infectious diseases.[6,10–12] Pooled odds ratios across the different sample collections were calculated using the inverse-variance, fixed effects model, as previously described.[13] This model used the weighted average of each study's odds ratio. The weights used were the inverse of the variance of the study's estimated odds ratio, ensuring

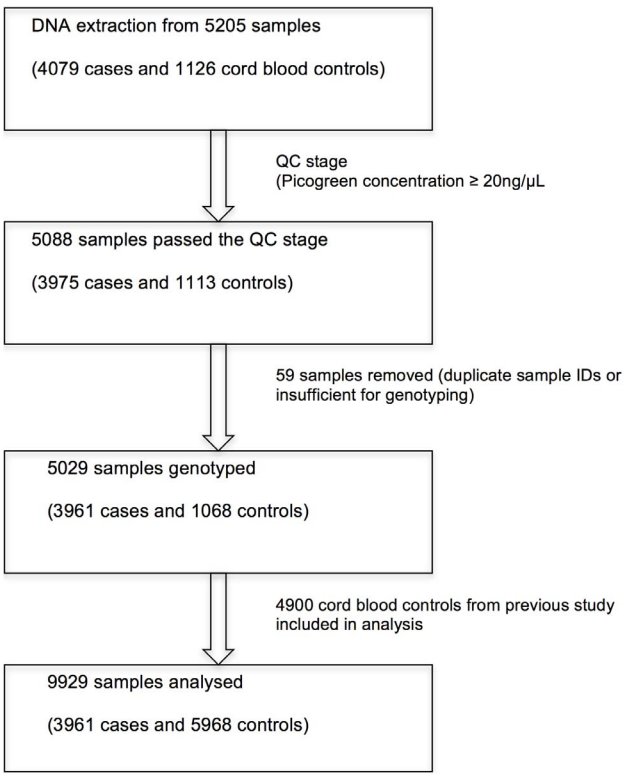

Figure 1. Genotyping and sample quality control process flowchart.
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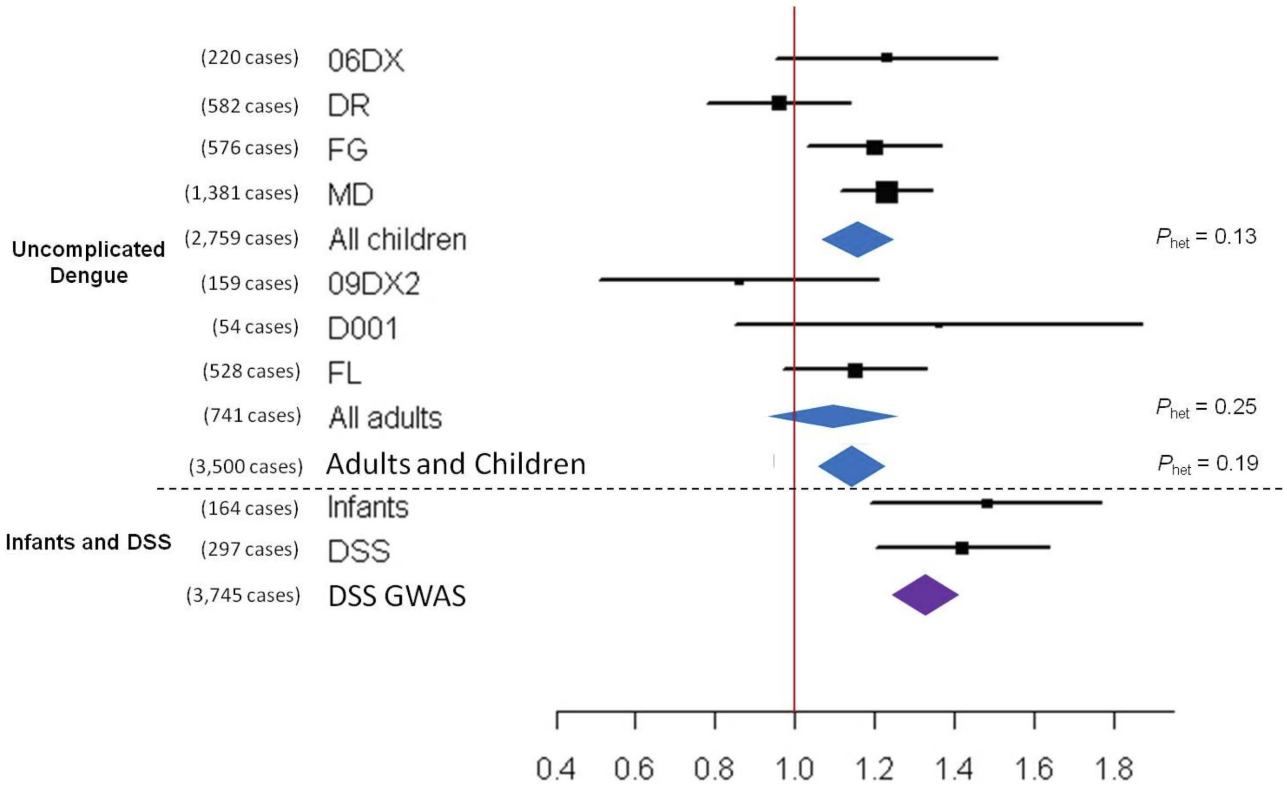

Figure 2. Forest plot illustrating the association between *MICB* rs3132468 and susceptibility to dengue. 06DX, DR, FG and MD were cohort studies of children, and 09DX, D001 and FL were cohort studies of adults. The oblongs represent point estimates (referring to the per-allele odds ratio, expressed on the horizontal axis), with the height of the oblongs inversely proportional to the standard error of the point estimates. Horizontal lines indicate the 95% confidence interval for each point estimate. Meta-analysis of children, adults, as well as children and adults with uncomplicated dengue are reflected by blue diamonds. Data from our previously reported GWAS on DSS is reflected by the purple diamond.[6] The width of the diamonds indicates their 95% confidence intervals. Each meta-analysis is accompanied by a test of heterogeneity between the sample collections summarized by it (expressed as P_{het}).
doi:10.1371/journal.pone.0059067.g002

that larger studies command greater weight. Forest plots demonstrating these results were created in R.

Results

Case and control cohorts

The patient cohorts that provided the laboratory-confirmed dengue cases that were genotyped in this study are summarised in **Table 1**. Across the 7 cohorts (4 pediatric and 3 adult) there were 297 DSS cases (161 pediatric and 136 adult), 3500 non-DSS dengue cases (2759 pediatric and 741 adult cases), and 164 cases of infants with dengue that were successfully genotyped at *MICB* rs3132468 and *PLCE1* rs3740360. Umbilical cord blood DNA samples (n = 1068) were similarly genotyped at *MICB* rs3132468 and *PLCE1* rs3740360 and served as population controls. Data from this was complemented with genotype data from 4900 controls studied previously.[6] In *a priori* analyses we considered non-DSS cases in children separately from adults. Infants with dengue (n = 164) were also treated as a distinct cohort because of the unique context of dengue in this age group. The sample quality control process is illustrated in **Figure 1**.

Association between MICB rs3132468 and dengue in children and adults

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 2**, and **Table 2**). However, a pooled analysis of all pediatric and adult patient cohorts indicated a significant association (OR = 1.15, P = 0.0014; **Figure 2**, and **Table 2**) compared to controls, with no heterogeneity between children and adults (P_{het} = 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 2** and **Table 2**).

Association between PLCE1 rs3740360 and dengue in children and adults

We observed significant association with non-DSS cases upon pooled analysis of all adults and children (OR = 0.92, P = 0.018; **Figure 3** and **Table 3**). Amongst DSS cases, the association at *PLCE1* revealed by the previous GWAS was confirmed (OR = 0.77: P = 0.0094).

Table 2. Per-collection analysis for *MICB* rs3132468.

Children							
Collection	**Number**	**MAF cases**	**MAF controls**	**OR**	**95% CI**	**Weight**	***P***
06DX	220	0.160	0.134	1.23	0.95 – 1.51	49.45393	0.14
DR	582	0.129	0.134	0.96	0.78 – 1.14	119.4422	0.66
FG	576	0.156	0.134	1.20	1.03 – 1.37	134.8943	0.035
MD	1381	0.159	0.134	1.23	1.12 – 1.35	289.2313	0.00047
All children	2759	0.152	0.134	1.16	1.07 – 1.25	-	0.0011
Adults							
Collection	**Number**	**MAF cases**	**MAF controls**	**OR**	**95% CI**	**Weight**	***P***
09DX2	159	0.118	0.134	0.86	0.51 – 1.21	31.03638	0.42
D001	54	0.173	0.134	1.36	0.85 – 1.87	14.72486	0.24
FL	528	0.151	0.134	1.15	0.97 – 1.33	120.2293	0.12
All adults	741	0.145	0.134	1.10	0.94 – 1.26	–	0.22
All mild cases (Adults and children)	3500	0.151	0.134	1.15	1.07 – 1.24	–	0.0014
Collection		**MAF cases**	**MAF controls**	**OR**	**95% CI**	**Weight**	***P***
All infants	164	0.186	0.134	1.48	1.19 – 1.77	–	0.0075
Collection		**MAF cases**	**MAF controls**	**OR**	**95% CI**	**Weight**	***P***
All DSS	297	0.180	0.134	1.42	1.20 – 1.64	–	0.0014

Number: Number of cases
MAF cases: minor allele frequency in dengue cases
MAF controls: minor allele frequency in controls
OR: per-allele odds ratio.
SE: Standard error of the OR
Weight: study-specific weight when meta-analyzed, not calculated for infants and DSS collections, which were not meta-analyzed.
P: *P*-value for association with disease (unadjusted).
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Association between MICB rs3132468 and dengue in infants

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 2** and **3; Tables 2** and **3**). Although the infant cohorts included 16 cases of DSS, removal of these samples did not affect the associations demonstrated.

Discussion

MICB rs3132468 and *PLCE1* rs3740360 genotypes are associated with DSS in Vietnamese children.[6] Here, we have extended this finding by follow-up genotyping in a large number of Vietnamese adult and paediatric dengue cases, together with a new cohort of population control samples. The data revealed significant association between *MICB* rs3132468 and *PLCE1* rs3740360 and clinical phenotypes of dengue less severe than DSS, albeit with smaller effect sizes than observed between these alleles and DSS.[6] In addition, we observed association between both SNP genotypes and infants with dengue at effect sizes comparable to that seen with DSS. Finally, amongst children and adults with DSS in these cohorts, we were able to confirm association of *MICB* rs3132468 and *PLCE1* rs3740360 that was first observed in the previous GWAS.[6] Collectively, these findings provide further validation of the importance of *MICB* rs3132468 and *PLCE1* rs3740360 risk genotypes to shaping the clinical phenotype of dengue and raises intriguing questions about their roles in disease pathogenesis.

The association of the *MICB* rs3132468 genotype with clinical phenotypes of dengue less severe than DSS indicates a role for this variant in susceptibility to overall clinically apparent dengue and not just severe disease. Given the role of *MICB* in activation of NK and $CD8^+$ cells these findings support a central role for these cell types in shaping the outcome of DENV infection. For example, it is plausible that the *MICB* rs3132468 genotype is associated with an impaired NK cell response, potentially resulting in a higher *in vivo* virus titre and an increased risk of developing both symptomatic and severe dengue. Furthermore, inefficient induction of regulatory NK cells might result in dysregulated T cell responses that may also shape the clinical phenotype.[14]

The effect size between the *PLCE1* rs3740360 risk genotype and milder clinical phenotypes of dengue was less pronounced than that observed for DSS cases.[6] Whilst a degree of endothelial leak is likely to occur in most clinically evident DENV infections, patients with DSS experience the most severe vascular permeability.[15,16] Our current data, together with the recently demonstrated association between *PLCE1* rs3740360 and DSS, may indicate a central role for *PLCE1* in the context of vascular leakage.[6] Further genetic fine mapping studies will be required to pinpoint functional mutations that could mechanistically explain the association between *PLCE1* and DSS. In doing so,

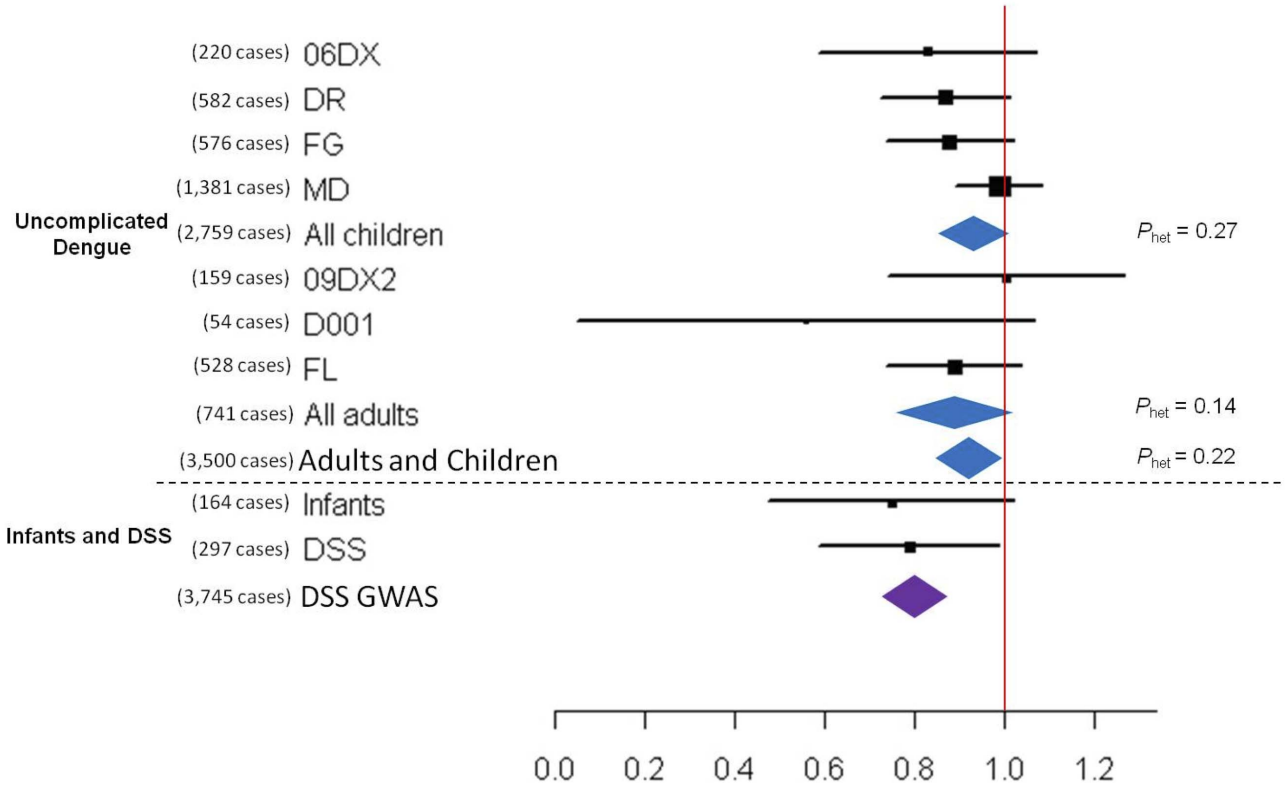

Figure 3. Forest plot illustrating the association between *PLCE1* rs3740360 and susceptibility to dengue. 06DX, DR, FG and MD were cohort studies of children. and 09DX, D001 and FL were cohort studies of adults. The oblongs represent point estimates (referring to the per-allele odds ratio, expressed on the horizontal axis), with the height of the oblongs inversely proportional to the standard error of the point estimates. Horizontal lines indicate the 95% confidence interval for each point estimate. Meta-analysis of children, adults, as well as children and adults with uncomplicated dengue are reflected by blue diamonds. Data from our previously reported GWAS on DSS is reflected by the purple diamond.[6] The width of the diamonds indicates their 95% confidence intervals. Each meta-analysis is accompanied by a test of heterogeneity between the sample collections summarized by it (expressed as P_{het}).
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we expect to contribute to the wider understanding of the role of *PLCE1* in health and disease, particularly in light of its association with nephrotic syndrome, regulation of blood pressure and esophageal cancer.[8,9,17] 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.[18]

Infants with dengue were analysed independently of other patient populations. Primary infection in the context of waning maternal antibody levels, immunological immaturity and vulnerable physiology make infants with dengue a distinct group.[19] Our data shows association between *MICB* rs3132468 and dengue in infants, with effect sizes in keeping with that observed in DSS patients.[6] We speculate that this 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. The effect size observed at *PLCE1* rs3740360 was also similar to that observed in DSS patients.[6] It has been noted that hospitalised 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.[4] In infants with dengue, carriage of either risk alleles thus represent an additional risk variable alongside the presence of maternally-derived non-neutralising antibodies and poor compensatory reserve.[19–20]

Our study has limitations. Misclassified control samples will be more common in this 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 as 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, which is a significant extension from the earlier GWAS on DSS cases alone. As expected, the effect sizes of these variants is small and underscores that susceptibility to symptomatic dengue is multifactorial and includes demographic risk factors (e.g. age).[21] 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 to define the functional basis for these observed genetic associations at *MICB* and *PLCE1* and thus increase our understanding of disease pathogenesis.

Table 3. Per-collection analysis for *PLCE1* rs3740360.

Children							
Collection	**Number**	**MAF cases**	**MAF controls**	**OR**	**95% CI**	**Weight**	***P***
06DX	220	0.235	0.271	0.83	0.59 – 1.07	66.09822	0.12
DR	582	0.244	0.271	0.87	0.73 – 1.01	190.7756	0.046
FG	576	0.248	0.271	0.88	0.74 – 1.02	189.726	0.089
MD	1381	0.270	0.271	0.99	0.90 – 1.08	426.8834	0.90
All children	2759	0.257	0.271	0.93	0.86 – 1.00		0.054
Adults							
Collection	**Number**	**MAF cases**	**MAF controls**	**OR**	**95% CI**	**Weight**	***P***
09DX2	159	0.272	0.271	1.01	0.75 – 1.27	56.10964	0.97
D001	54	0.173	0.271	0.56	0.05 – 1.07	14.7929	0.025
FL	528	0.249	0.271	0.89	0.74 – 1.04	171.3221	0.13
All adults	741	0.248	0.271	0.89	0.76 – 1.02		0.065
All mild cases (Adults and children)	3500	0.255	0.271	0.92	0.85 – 0.99		0.018
Collection		**MAF cases**	**MAF controls**	**OR**	**95% CI**	**Weight**	***P***
All infants	164	0.219	0.271	0.75	0.48 – 1.02		0.041
Collection		**MAF cases**	**MAF controls**	**OR**	**95% CI**	**Weight**	***P***
All DSS	297	0.223	0.271	0.77	0.59 – 0.99		0.0094

Number: Number of cases
MAF cases: minor allele frequency in dengue cases
MAF controls: minor allele frequency in controls
OR: per-allele odds ratio.
SE: Standard error of the OR
Weight: study-specific weight when meta-analyzed, not calculated for infants and DSS collections, which were not meta-analyzed.
P: *P*-value for association with disease (unadjusted).
doi:10.1371/journal.pone.0059067.t003

Supporting Information

Table S1 Details of the cohort studies used in the analysis.
(DOCX)

Author Contributions

Critically reviewed and approved the manuscript: JW TNBC NMN DTHK NTHQ DTT JP BW NVVC JF MLH CCK CPS. Conceived and designed the experiments: JW TNBC DTT JP BW NVVC JF MLH CCK CPS. Performed the experiments: JW TNBC NMN DTHK NTHQ DTT JP CCK. Analyzed the data: JW TNBC JP MLH CCK CPS. Wrote the paper: JW TNBC CCK CPS.

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Supplementary Table 1: Details of the cohort studies used in the analysis

Patient cohort name (sample size)	Inclusion criteria	Exclusion criteria	Study site and study period	Age Median (IQR)	Serotypes present
BC (n=1068)	Babies born to mothers living in areas served by recruiting district hospitals	+HIV Mothers < 15yrs	Hung Vuong Hospital, HCMC Dong Thap Hospital, DT Province	At birth	Controls
FG (n=622)	Inpatients Febrile and clinical suspicion of dengue. Fever less than 72hrs Age >18 months	N/A	Dong Thap Hospital, DT province Sa Dec Hospital, DT province Tien Giang Hospital, TG province 2007-2009	11 (7-14)	DENV-1 (63.7%) DENV-2 (15%) DENV-3 (10.8%) DENV-4 (0.5%) Unknown (10.1%)
06DX (n=220)	Inpatients Febrile and clinical suspicion of dengue. Fever less than 72hrs Age < 20 years	Pregnant woman	Hospital for Tropical Diseases, HCMC 2009-2010	13 (11-14)	DENV-1 (60.9%) DENV-2 (25%) DENV-3 (10.5%) DENV-4 (3.2%) Unknown (0.5%)
09DX (n=159)	Inpatients Febrile and clinical suspicion of dengue.	Pregnant woman	Hospital for Tropical Diseases, HCMC 2011-2012	23 (19-27)	DENV-1 (37.1%) DENV-2 (45.3%) DENV-3 (9.4%)

	Fever less than 72hrs Age > 15 years				DENV-4 (7.5%) Unknown (0.6%)
DR (n=597)	Outpatients Febrile and clinical suspicion of dengue. Fever ≤ 72 hours (flexible) Age 5 - 15 years	N/A	District 8 Hospital Outpatients, HCMC 2005-2009	11 (9-13)	DENV-1 (62%) DENV-2 (15.2%) DENV-3 (17.8%) DENV-4 (1.2%) Unknown (3.9%)
MD (n=1464)	Inpatients Febrile and clinical suspicion of dengue. Fever ≤ 72 hours (flexible) Age 5 - 15 years	N/A	Hospital for Tropical Diseases, HCMC 2001-2009	12 (10-13)	DENV-1 (65%) DENV-2 (14.5%) DENV-3 (11.7%) DENV-4 (0.7%) Mixed DENV (0.1%) Unknown (8%)
D001 (n=76)	Clinical suspicion of dengue ≤ 72 hours illness: infection wards; ≤ 6 days illness ICUs Age 12 – 25 years	N/A	Hospital for Tropical Diseases, HCMC 2010-2011	18 (16-23)	DENV-1 (27.6%) DENV-2 (30.2%) DENV-3 (5.3%) DENV-4 (5.3%) DENV mixed (5.3%) Unknown (26.3%)

FL (n=627)	Clinical suspicion of dengue Any day of illness Infection wards and ICUs Age ≥ 15 years	N/A	Hospital for Tropical Diseases, HCMC 2006-2008	21 (18-26)	DENV-1 (16.6%) DENV-2 (10.0%) DENV-3 (4.8%) DENV-4 (0.3%) Not done (68.3%)
DC (n=68)	Inpatients Febrile and clinical suspicion of dengue Any day of illness Age <18 months	N/A	Children's Hospital 1, HCMC 2005-2007	6 (4-8) months	DENV-1 (23.5%) DENV-2 (42.6%) DENV-3 (11.8%) DENV-4 (1.5%) Unknown (20.6%)
DT (n=93)	Inpatients Febrile and clinical suspicion of dengue Any day of illness Age <18 months	N/A	Children's Hospital 2, HCMC 2005-2007	7 (6-9) months	DENV-1 (34.4%) DENV-2 (33.3%) DENV-3 (12.9%) Unknown (19.4%)
FB (n=11)	Inpatients Febrile and clinical suspicion of dengue Any day of illness Age <18 months	N/A	Dong Thap Hospital, DT province 2005-2007	8 (7-11) months	DENV-1 (63.6%) DENV-2 (18.2%) Unknown (18.2%)
DZ (n=8)	Outpatients Febrile and clinical suspicion of dengue Any day of illness Ae <18 months	N/A	Children's Hospital 1, HCMC 2005-2007	8 (7-11) months	DENV-1 (12.5%) DENV-2 (37.5%) Unknown (50.0%)

Associations between MICB and PLCE1 and laboratory features of dengue

The work described above suggests that mutations in the *MICB* and *PLCE1* genes have important associations with dengue of milder severity than DSS. However the functional basis of these observed associations remains unclear. The aim of the analysis described in this section was to define the association between these specific mutations and various laboratory features of dengue that have been shown to have correlation with the clinical outcome of infection. For example, the level of viraemia in the first days of illness has also been shown to have an association with the outcome of DENV infection with higher viraemia being associated with a more severe clinical phenotype.[9] In addition, the degrees of lymphopenia and thrombocytopenia have been shown to have an association with subsequent development of DSS.[10,11] This analysis has the potential to expand our understanding of these associations and further clarify host susceptibility and dengue pathogenesis.

Aims

The specific aims of this analysis were to measure the association between individuals with different *MICB* rs3132468 and *PLCE1* rs3740360 genotypes (wild-type, heterozygous carriers, and homozygous variant) and various laboratory features. Specifically the features that were compared between the groups were:

- Early viraemia level
- Platelet nadir
- White cell count nadir
- Maximum haematocrit
- Proportions of patients that develop “severe dengue” as per WHO criteria[12]

Methods

All participants gave written informed consent to take part in the prospective studies detailed in **Table 1**. All these studies took place in southern Vietnam. The parents or guardians of the children involved in the studies gave written informed consent on their behalf. The protocols of these studies were reviewed and approved by the Institutional Review Boards of each study site (Hospital for Tropical Diseases HCMC, Children’s Hospital 1 and 2 HCMC, and Tien Giang Hospital) and by the Oxford University Tropical Research Ethics Committee.

This was a case-only analysis of laboratory-confirmed dengue patients obtained from 2 prospective cohort studies and 1 randomised clinical trial in Vietnam. The cohort studies were conducted in both adults and children and aimed to create diagnostic and prognostic algorithms to improve the clinical management of dengue in this setting. The randomised clinical trial was an investigation of lovastatin therapy in adult patients with dengue and is described elsewhere in this thesis. The studies are summarised in **Table 1**. The inclusion

criteria for 13DX were (1) fever or history of fever for less than 72 hours, (2) clinical suspicion of dengue, (3) 1 – 15 years of age, (4) written informed consent, and (5) accompanying family member has a mobile phone. The exclusion criteria were (1) deemed unlikely to attend follow-up, and (2) in whom an alternative diagnosis was thought more likely. The inclusion criteria for 22DX were (1) fever or history of fever for less than 72 hours, (2) clinical suspicion of dengue, (3) aged 5 years or over, (4) written informed consent. The exclusion criteria were (1) localising features suggesting an alternative diagnosis, and (2) inability to attend daily follow-up. The inclusion and exclusion criteria for 26DX (lovastatin in dengue trial) are detailed in the therapeutics section of this thesis but, in brief, patients aged 18 years or over with less than 72 hours fever and a positive NS1 rapid test were eligible for recruitment

The study methodology varied – 13DX only obtained laboratory samples on the enrolment day, whereas 22DX and 26DX had multiple laboratory sampling points. Therefore all the studies were included for the analysis of the viraemia and clinical outcome data, however to ensure appropriate comparisons 13DX data were excluded from the analysis of other laboratory variables.

Study	Setting	Number (% male)	Median age (range 5^{th}-95^{th} centile)
13DX	Outpatient	1951 (57)	9 (3 – 14)
22DX	Outpatient	749 (60)	18 (6 – 39)
26DX	Inpatient	42 (57)	24 (18 – 51)

Table 1: Summary of the studies used in the analysis

DNA extractions were performed using a MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, Germany) according to the manufacturer's instructions. Candidate SNPs were genotyped using a TaqMan genotyping assay to amplify and detect the specific alleles in the DNA samples as per the manufacturer instructions. Samples failing picogreen QC (<5ng/uL in concentration) were excluded from further analysis. Similarly, during the genotyping process, samples which fail to show adequate allelic discrimination on the Taqman RT-PCR melt-curve were also be excluded from analysis. Each SNP has been shown previously to show good call rate exceeding >95%.

Statistical analysis was performed in Stata 13 (Stata Statistical Software: Release 13. College Station, TX. StataCorp LP). Laboratory variables were compared between genotypes and stratified by DENV serotype using the binary chi-squared test, the Kruskal-Wallis non-parametric test and the analysis of variance test.

Results

2742 dengue cases were successfully genotyped at *MICB* rs3132468 and *PLCE1* rs3740360. 573 cases were in adults (age $\geq$ 15) and 2180 were in children. There were 109 cases of severe dengue across the cohorts (6 in adults and 103 in children).

The baseline characteristics of the study participants are summarised in **Table 2**.

Association between MICB rs3132468 and enrolment viraemia

The mean viraemia was compared between the *MICB* genotypes. The mean viraemia in patients carrying the C/C allele was 7.16 (95%CI: 6.84-7.47) log_{10}-copies/mL. In those with the C/T allele it was 7.05 (95%CI: 6.92-7.18) log_{10}-copies/mL, and in those with the T/T allele it was 7.08 (95%CI: 7.01-7.15) log_{10}-copies/mL. The difference between these values was not statistically significant. The comparison in viraemia levels between *MICB* genotypes was repeated with data stratified by DENV serotype. Again, no significant differences were demonstrated. These data are summarised in **Table 3**. Collectively, these data indicate some variation in the viraemia between the DENV serotypes but no significant difference between the *MICB* genotypes.

Association between PLCE rs3740360 and enrolment viraemia

The mean viraemia was compared between the *PLCE* genotypes. The mean viraemia is patients carrying the A/A allele was 7.08 (95%CI: 7.00-7.15) log_{10}-copies/mL. In those carrying the A/C allele it was 7.09 (95%CI: 6.99-7.19) log_{10}-copies/mL, and in those with the C/C allele it was 7.02 (95%CI: 6.78-7.26) log_{10}-copies/mL. The difference between these values was not statistically significant.

The comparison in viraemia levels between *PLCE* genotypes was repeated with data stratified by DENV serotype. Again, no significant differences were demonstrated. These data are summarised in **Table 3**. As above, these data indicate some variation in the viraemia level between the DENV serotypes but no significant difference between the *PLCE* genotypes.

Association between MICB rs3132468 and PLCE rs3740360 and severe dengue

The proportions of patients developing severe dengue were compared between the genotypes in an overall analysis and after stratification by serotype.

When analysing *MICB,* 6 patients (7.7%) with the C/C allele developed severe dengue, 24 (4.1%) with the C/T allele developed severe dengue, and 79 (3.8%) with the T/T allele developed severe dengue. These differences were not statistically significant in both an overall analysis and when stratified by serotype.

When analysing *PLCE,* 68 patients (4.4%) with the A/A allele developed severe dengue, 32 (3.2%) with the A/C allele developed severe dengue, and 9 (4.9%) with the C/C allele

developed severe dengue. These differences were not statistically significant in both an overall analysis and when stratified by serotype.

Collectively, these data indicate that there is no clear association between *MICB* and *PLCE* genotype and the development of severe dengue.

Association between MICB rs3132468 and other laboratory variables

After excluding data from 13DX, which only included enrolment results, the platelet nadir, the maximum haematocrit and the minimum white cell count were compared between the *MICB* genotypes in both an overall analysis and then stratified by serotype. The median platelet nadir in patients with the C/C allele was 82.5 (IQR: 47-101) x 10^9/L, in those with the C/T allele was 80 (IQR: 48-115) x 10^9/L, and in those with the T/T allele was 70 (41-113) x 10^9/L. These differences were not statistically significant. The minimum white cell count in those with the C/C allele was 2.6 (IQR: 2.3-3.1) x 10^9/L, in those with the C/T allele was 2.9 (IQR: 2.3-3.8) x 10^9/L and in those with the T/T allele was 2.7 (IQR: 2.1-3.4) x 10^9/L. These differences were not statistically significant. The median maximum haematocrit in patients with the C/C allele was 45.4% (IQR: 44-49.2%), in those with the C/T allele was 45% (IQR: 42-48.3%), and in those with the T/T allele was 44.7% (IQR: 41.8-48.4%). These differences were not statistically significant. When these data were stratified by DENV serotype again no significant differences were demonstrated. These data suggest no measurable association between *MICB* genotype and the laboratory variables explored in this analysis.

These data are summarised in **Tables 4, 5, 6, 7** and **8**.

Association between PLCE rs3740360 and other laboratory variables

The platelet nadir, the maximum haematocrit and the minimum white cell count were compared between the *PLCE* genotypes in both an overall analysis and then stratified by serotype. The median platelet nadir in patients with the A/A allele was 71 (IQR: 41-109) x 10^9/L, in those with the A/C allele was 76.5 (IQR: 45-118) x 10^9/L, and in those with the C/C allele was 66 (37-114) x 10^9/L. These differences were not statistically significant. The minimum white cell count in those with the A/A allele was 2.7 (IQR: 2.1-3.4) x 10^9/L, in those with the A/C allele was 2.8 (IQR: 2.2-3.6) x 10^9/L and in those with the C/C allele was 2.9 (IQR: 2.2-3.6) x 10^9/L. These differences were not statistically significant. The median maximum haematocrit in patients with the A/A allele was 44.6% (IQR: 41.9-48.1%), in those with the A/C allele was 45.1% (IQR: 42-48.3%), and in those with the C/C allele was 45% (IQR: 42.4-50.3%). These differences were not statistically significant. When these data were stratified by DENV serotype again no significant differences were demonstrated. These data suggest no measurable association between *PLCE* genotype and the laboratory variables explored in this analysis.

These data are summarised in **Tables 4, 5, 6, 7** and **8**.

Discussion

This analysis showed no association between *MICB* and *PLCE* genotype and early viraemia level, platelet nadir, white cell count nadir, maximum haematocrit or the development of severe disease in both overall analysis and in analysis stratified by serotype.

Given the possibility that *MICB* may have an important role in the establishment of antiviral effector function we proposed that mutations in this gene might be associated with higher dengue viraemia. Our analysis in 2742 adults and children with dengue showed no difference in the enrolment viraemia levels between the different *MICB* and *PLCE1* genotypes in both an overall analysis and analysis stratified by DENV serotype. The enrolment viraemia was taken in the vast majority of patients in the first 72 hours of clinical illness (**Table 2**). While our understanding of DENV kinetics suggests that this is likely to correspond near to the peak viraemia level it only gives a snapshot of the *in vivo* viral dynamics. It is possible that the viral clearance patterns varied across genotypes. In addition, measuring plasma viraemia only provides a surrogate of what is happening in other tissues – obviously, however, invasive assessments of this would not be appropriate in large prospective studies. However, an interesting finding was the range of early viraemia levels across the DENV serotypes and between the different *MICB* and *PLCE* genotypes. While the observed variation was not statistically significant, it does suggest that there are factors that influence the early viraemia level. This may be a combination of intrinsic genetic susceptibility, host immune status and various viral factors. Determining the factors that influence the DENV "set point" would be intriguing and would advance our understanding of dengue pathogenesis.

As no associations between viraemia and genotype were shown, it is perhaps not surprising that no associations were demonstrated between genotype and the pre-selected routine haematological laboratory variables both in overall analysis and in analysis stratified by DENV serotype. These variables were chosen because they have been previously shown to have some correlation with dengue severity.[10,11] However, differences in the study designs, particularly the frequency of laboratory investigations, across the cohorts described meant that the largest study in patient numbers (13DX) was excluded from this component of the analysis.

While previous work has demonstrated an association between genetic variants of *MICB* and *PLCE1* and both severe and non-severe dengue, the functional basis of these associations is not clear.[7,13] *MICB* encodes an activating ligand for natural killer cells, and possibly CD8+ T cells, raising the possibility that mutations in this gene may result in altered antiviral effector functions and an associated increased viral burden, a recognised risk factor for the development of severe dengue.[9] It is possible that the associations reflect an aspect of dengue pathogenesis unrelated to control of early viral replication. The association of *PLCE1* and dengue is harder to explain. It is interesting to note that mutations in this gene have been associated with nephrotic syndrome, regulation of blood pressure and oesophageal

malignancy.[8,14,15] While it is plausible that the relationship between mutations in *PLCE1* and both severe dengue and nephrotic syndrome suggests some role in the maintenance of endothelial integrity, it is more difficult to relate this potential function to oesophageal malignancy. The role of *PLCE* in control of blood pressure warrants further exploration as it raises the possibility of some overlap in function in dengue. This relationship with diverse conditions suggests an important, but as yet unclear, role for *PLCE1* in human health and disease.

Our study has limitations. The number of patients with the "risk" alleles for *MICB* and *PLCE* was relatively small. This raises the possibility that the lack of a clear association demonstrated in this analysis is not a true finding. In addition, the study methodology varied between the studies included in the analysis meaning that it was only possible to explore the relationship between genotype and laboratory variable in a smaller group of patients. The functional basis of these observed associations remains unclear but it is possible that pooled data from prospective intervention studies may provide clearer insights. The potential offered by this study approach reflects the more frequent sampling that typically occurs in this type of study. Greater insights into dengue host susceptibility have the potential to expand our knowledge of disease pathogenesis and, in the longer term, assist the development of vaccines and therapeutics.

Table 2. Collated study population characteristics (n=2730)

	DENV1 (n=1042)	DENV2 (n=593)	DENV3 (n=263)	DENV4 (n=832)	All (n=2730)
Age (median, IQR), years	10(7-13)	10(7-14)	9(6-13)	11(8-15)	10(7-14)
Sex (n,%)					
Male	596(57.2)	357(60.2)	147(55.9)	474(57.0)	1574(57.7)
Female	446(42.8)	236(39.8)	116(44.1)	358(43.0)	1156(42.3)
Days of illness at enrolment (n,%)					
0	9(0.86)	1(0.17)	0(0)	3(0.36)	13(0.48)
1	323(31.0)	174(29.3)	85(32.3)	195(23.4)	777(28.5)
2	437(41.9)	271(45.7)	113(43.0)	354(42.5)	1175(43.0)
3	269(25.8)	146(24.6)	64(24.3)	279(33.5)	758(27.8)
4	2(0.19)	1(0.17)	1(0.4)	0(0)	4(0.15)
Viremia (mean, 95%CI), log10 copies/mL	7.39(7.29-7.50)	6.89(6.77-7.01)	7.14(6.94-7.33)	6.80(6.71-6.88)	7.08(7.02-7.14)
Clinical classification (n,%)					
Dengue	950(91.2)	553(93.3)	257(97.7)	780(93.8)	2540(93.0)
Severe dengue	39(3.74)	32(5.4)	6(2.3)	32(3.85)	109(3.99)

Table 3: Viremia by *MICB* and *PLCE* genotype by dengue serotype and overall (mean (IQR) (N))

MICB				
Serotype	C/C	C/T	T/T	P-value
DENV1	7.32 (6.7-7.95) (n=24)	7.28 (7.03-7.53) (n=211)	7.43 (7.31-7.55) (n=761)	0.238
DENV2	7.04 (6.32-7.76) (n=18)	7.03 (6.77-7.29) (n=125)	6.84 (6.7-6.98) (n=450)	0.833
DENV3	6.99 (5.24-8.75) (n=7)	7.15 (6.7-7.6) (n=50)	7.14 (6.91-7.36) (n=206)	0.836
DENV4	7.13 (6.67-7.59) (n=28)	6.75 (6.56-6.93) (n=184)	6.8 (6.7-6.9) (n=620)	0.86
Overall	7.16 (6.84-7.47) (n=78)	7.05 (6.92-7.18) (n=583)	7.08 (7.01-7.15) (n=2081)	0.315
PLCE				
Serotype	A/A	A/C	C/C	P-value
DENV1	7.38 (7.24-7.53) (n=578)	7.39 (7.22-7.56) (n=397)	7.47 (7.03-7.91) (n=67)	0.972
DENV2	6.83 (6.67-7) (n=334)	6.99 (6.79-7.19) (n=215)	6.83 (6.38-7.29) (n=44)	0.938
DENV3	7.08 (6.82-7.34) (n=157)	7.19 (6.87-7.5) (n=87)	7.32 (6.35-8.29) (n=19)	0.198
DENV4	6.87 (6.75-6.98) (n=472)	6.74 (6.99-7.19) (n=306)	6.51 (6.18-6.83) (n=54)	0.867
Overall	7.08 (7-7.15) (n=1546)	7.09 (6.99-7.19) (n=1012)	7.02 (6.78-7.26) (n=184)	0.7

Table 4: Laboratory features by *MICB* and *PLCE* genotype for DENV1

MICB				
Laboratory/clinical feature	C/C (n=4)	C/T (n=46)	T/T (n=171)	P-value
Platelet nadir (median(IQR))	60.5 (39.5-85)	80.5 (44-128)	80 (48-127)	0.608
Minimum white cell count (median(IQR))	2.25 (1.75-2.65)	2.65 (2-3.8)	2.6 (2.1-3.5)	0.442
Maximum haematocrit (median(IQR))	44.8 (43-49.5)	45.3 (42.1-48.8)	44.2 (41.5-48.1)	0.421
PLCE				
Laboratory/clinical feature	A/A (n=119)	A/C (n=89)	C/C (n=13)	P-value
Platelet nadir (median(IQR))	72 (49-124)	83 (41-128)	61 (46-172)	0.961
Minimum white cell count (median(IQR))	2.6 (2.1-3.3)	2.7 (2-3.6)	3 (2.2-4.1)	0.802
Maximum haematocrit (median(IQR))	44 (41.7-47.2)	44.5 (41.9-48.8)	49 (42.7-51)	0.0719

Table 5: Laboratory features by *MICB* and *PLCE* genotype for DENV2

MICB				
Laboratory/clinical feature	C/C (n=4)	C/T (n=31)	T/T (n=121)	P-value
Platelet nadir (median(IQR))	85 (39-110.5)	70 (33-107)	57 (29-90)	0.497
Minimum white cell count (median(IQR))	2.7 (2.6-2.95)	3.3 (2.5-3.9)	2.7 (2.2-3.4)	0.115
Maximum haematocrit (median(IQR))	47.2 (44.3-49.3)	45 (43.3-47.6)	45.2 (42.5-48.4)	0.719
PLCE				
Laboratory/clinical feature	A/A (n=88)	A/C (n=55)	C/C (n=13)	P-value
Platelet nadir (median(IQR))	62.5 (30-101.5)	58 (31-91)	66 (30-116)	0.912
Minimum white cell count (median(IQR))	2.8 (2.2-3.6)	2.6 (2.3-3.5)	2.8 (2.3-3.4	0.987
Maximum haematocrit (median(IQR))	45.6 (43.4-48.4)	44.9 (41.1-47.9)	44.7 (42-50)	0.44

Table 6: Laboratory features by *MICB* and *PLCE* genotype for DENV3

MICB				
Laboratory/clinical feature	C/C (n=3)	C/T (n=13)	T/T (n=57)	P-value
Platelet nadir (median(IQR))	92 (82-102)	67 (51-95)	77 (48-111)	0.803
Minimum white cell count (median(IQR))	2.7 (1.9-3.7)	2.7 (1.9-3.9)	2.6 (2.1-3.2)	0.987
Maximum haematocrit (median(IQR))	48.6 (45.1-50.6)	43.6 (40-47)	44.1 (41.9-47.9)	0.229
PLCE				
Laboratory/clinical feature	A/A (n=44)	A/C (n=24)	C/C (n=5)	P-value
Platelet nadir (median(IQR))	83 (50-117.5)	70.5 (49-105)	83 (67-96)	0.876
Minimum white cell count (median(IQR))	2.4 (2-3.1)	2.85 (2.1-3.8)	2.6 (2.4-3.2)	0.349
Maximum haematocrit (median(IQR))	43.4 (41.1-46.7)	45.1 (42.2-48.5)	45.7 (45-50.4)	0.12

Table 7: Laboratory features by *MICB* and *PLCE* genotype for DENV4

MICB				
Laboratory/clinical feature	C/C (n=7)	C/T (n=59)	T/T (n=194)	P-value
Platelet nadir (median(IQR))	83 (19-107)	86 (51-113)	72 (40-109)	0.698
Minimum white cell count (median(IQR))	2.5 (2.3-4)	2.9 (2.2-3.5)	2.8 (2.1-3.5)	0.747
Maximum haematocrit (median(IQR))	44.7 (43-49.2)	45 (41.9-48.6)	45.1 (41.9-48.6)	0.88
PLCE				
Laboratory/clinical feature	A/A (n=153)	A/C (n=91)	C/C (n=16)	P-value
Platelet nadir (median(IQR))	72 (36-101)	84 (54-121)	61 (44.5-111.5)	0.132
Minimum white cell count (median(IQR))	2.7 (2.1-3.4)	3 (2.4-3.8)	2.9 (2.15-3.5)	0.342
Maximum haematocrit (median(IQR))	44.4 (41.8-48.6)	45.7 (42.3-48.6)	43.5 (42-46.1)	0.342

Table 8: Laboratory features by *MICB* and *PLCE* genotype for all serotypes

MICB				
Laboratory/clinical feature	C/C (n=18)	C/T (n=150)	T/T (n=554)	P-value
Platelet nadir (median(IQR))	82.5 (47-101)	80 (48-115)	70 (41-113)	0.494
Minimum white cell count (median(IQR))	2.6 (2.3-3.1)	2.9 (2.3-3.8)	2.7 (2.1-3.4)	0.214
Maximum haematocrit (median(IQR))	45.4 (44-49.2)	45 (42-48.3)	44.7 (41.8-48.4)	0.244
PLCE				
Laboratory/clinical feature	A/A (n=409)	A/C (n=266)	C/C (n=47)	P-value
Platelet nadir (median(IQR))	71 (41-109)	76.5 (45-118)	66 (37-114)	0.675
Minimum white cell count (median(IQR))	2.7 (2.1-3.4)	2.8 (2.2-3.6)	2.9 (2.2-3.6)	0.268
Maximum haematocrit (median(IQR))	44.6 (41.9-48.1)	45.1 (42-48.3)	45 (42.4-50.3)	0.336

3. PATHOGENESIS OF DENGUE TRANSMISSION

Background

The principle vectors of DENV are the mosquitoes *Aedes aegypti* and *Aedes albopictus* of the subgenus *Stegomyia*.[16] Both have adapted to live in urban and peri-urban areas, and their larvae can be found in water containers around and inside houses.[16] *Ae. aegypti* is the more domesticated of the two, and thus more closely implicated in DENV transmission. The global spread of dengue is, in part, linked to changes in human behaviour, notably the expansion of large urban centres that support the breeding of *Ae. aegypti*.[17] However *Ae. albopictus* also has an important role in dengue transmission and has been implicated in various outbreaks. For example, outbreaks of dengue in the Seychelles in 1976-77, Ningbo, China in 2004, Gabon in 2007, Mauritius in 2009, Dongguan, China in 2010, Guandong, China and Japan in 2014 were all associated with *Ae. albopictus*.[18-25] Numerous features have supported the expansion of the geographic footprint of *Ae. albopictus,* including the ability of the eggs to undergo diapause thus aiding their survival through unfavourable conditions, relative refractoriness of adults to cold, and speculation around their ability to out-compete established species of mosquitoes.[26-28] This global expansion and the potential competitive displacement of *Ae. aegypti* may prove to have an important role in the changing epidemiology of dengue.[29,30]

Recent work has defined the infectious dose of DENV to *Ae. aegypti* mosquitoes by conducting biting experiments on viraemic dengue patients.[31] This component of the thesis used similar methodology to directly compare the vector competence of *Ae. aegypti* and *Ae. albopictus* by assessing the susceptibility to both initial and disseminated DENV infection. This research has the potential to improve our understanding of transmission dynamics particularly in areas of dengue emergence where the contribution of *Ae. albopictus* is thought to be greatest. In addition, understanding the levels of virus that are infectious to mosquitoes have the potential to inform the design of therapeutic and chemoprophylactic candidates thus potentially improving the clinical management of the disease.

Outline of paper

Comparative susceptibility of *Ae. aegypti* and *Ae. albopictus* to dengue

This manuscript describes the results of experiments that directly compared the susceptibility of *Ae. aegypti* and *Ae. albopictus* to both initial and disseminated DENV infection after blood feeding on viraemic dengue patients. *Ae. albopictus* is generally regarded as a secondary dengue vector – the aim of this work was to establish whether there are intrinsic differences in DENV susceptibility between the two mosquito species. We demonstrated that both mosquito

types were equally susceptible to acquiring an initial abdominal infection but that *Ae. albopictus* was less likely to acquire an infectious phenotype as defined by the presence of DENV in the mosquito saliva (OR=0.70; 95%CI:0.52-0.93).

Previous vector competence studies have been based on laboratory experiments using artificial blood meals and laboratory-passaged viruses. This is the first comparative vector competence study using “field” conditions and the results have the potential to inform accurate transmission models which may be of particular use where the contribution of *Ae. albopictus* to dengue transmission is the greatest.

Interestingly, our study showed that once infected *Ae. albopictus* had higher DENV RNA concentrations in their abdomens as compared to *Ae. aegypti*. We explored the possibility that this difference could result in greater virus sequence diversity by determining the consensus nucleotide sequence of the DENV-1 Env gene in the plasma of 10 patients and the corresponding abdominal tissue of 20 *Ae. aegypti* and 20 *Ae. albopictus*. We showed that the median number of nucleotide differences from the consensus sequence in plasma to the consensus sequence in the abdominal tissue of *Ae. aegypti* was 9 (IQR: 4-15), and for *Ae. albopictus* was 17 (IQR: 11-18) (P=0.02, Wilcoxon test (stratified by patient)). This difference raises the possibility that *Ae. albopictus* disproportionately contributes to the genetic diversity that exists within DENV populations.

RESEARCH PAPER 2: COMPARATIVE SUSCEPTIBILITY OF *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* TO DENGUE VIRUS INFECTION AFTER FEEDING ON BLOOD OF VIREMIC HUMANS: IMPLICATIONS FOR PUBLIC HEALTH

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Student	James Whitehorn
Principal Supervisor	Rosanna Peeling and Cameron Simmons
Thesis Title	The pathogenesis and clinical management of dengue

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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MAJOR ARTICLE

Comparative Susceptibility of *Aedes albopictus* and *Aedes aegypti* to Dengue Virus Infection After Feeding on Blood of Viremic Humans: Implications for Public Health

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***Aedes albopictus* is secondary to *Aedes aegypti* as a vector of dengue viruses (DENVs) in settings of endemicity, but it plays an important role in areas of dengue emergence. This study compared the susceptibility of these 2 species to DENV infection by performing 232 direct blood-feeding experiments on 118 viremic patients with dengue in Vietnam. Field-derived *A. albopictus* acquired DENV infections as readily as *A. aegypti* after blood feeding. Once infected, *A. albopictus* permitted higher concentrations of DENV RNA to accumulate in abdominal tissues, compared with *A. aegypti*. However, the odds of *A. albopictus* having infectious saliva were lower than the odds observed for *A. aegypti* (odds ratio, 0.70; 95% confidence interval, .52–.93). These results quantitate the susceptibility of *A. albopictus* to DENV infection and will assist parameterization of models for predicting disease risk in settings where *A. albopictus* is present.**

Keywords. Dengue; *Aedes aegypti*; *Aedes albopictus*; susceptibility; transmission.

Dengue is the most important arboviral infection of humans, with an estimated 00 million clinically apparent infections annually [1, 2]. In dengue-endemic countries, *Aedes aegypti* (Linnaeus) is widely accepted to be the primary vectors of dengue viruses (DENVs), with *Aedes albopictus* (Skuse) regarded as a secondary vector. Yet *A. albopictus* can clearly transmit DENV at scales that are important to public health. For example, outbreaks of dengue during 1976–1977 (in the Seychelles), 2004 (in Ningbo, China), 2007 (in Gabon), 2009 (in Mauritius), 2010 (in Dongguan, China), and 2014 (in Guangdong, China, and Japan) were associated with *A. albopictus* [3–10]. These outbreaks demonstrate that, although *A. albopictus* can be a competent vector of DENV, there must be biological features that render *A. albopictus* generally less well equipped to transmit DENV, compared with *A. aegypti*.

Aedes albopictus originated in Asia but has geographically spread through global trade, particularly via used car tires infested with *A. albopictus* eggs and larvae [11]. It is now distributed throughout the United States, Central America, and South America and in temperate African and European countries [12–14]. Numerous features have supported the expansion of *A. albopictus*, including the ability of the eggs to undergo diapause [15]. There is also speculation around their ability to outcompete established species of mosquitoes [16, 17].

The expansion of *A. albopictus* has led to concern of an associated increase in the range of dengue transmission. Lambrechts et al reviewed vector competence

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literature and reported that, although *A. albopictus* were more susceptible to DENV midgut infection, rates of virus dissemination to other tissues were significantly lower in *A. albopictus* than in *A. aegypti* [18]. Additionally, laboratory-reared *A. albopictus* became increasingly susceptibility to DENV, which may have been a confounding variable in the literature. Furthermore, the comparative vector competence literature has been derived entirely from laboratory experiments using artificial blood meals and laboratory-passaged viruses. How well these laboratory conditions replicate the pathogenesis of DENV transmission from viremic humans to mosquitoes in the field is uncertain.

The benefits of understanding why *A. albopictus* is largely a secondary vector of DENV in settings of endemicity are numerous. First, existing risk-analysis models of the likelihood of dengue outbreaks in southern Europe and the United States could be improved with quantitative estimates of *A. albopictus* vector competence [19, 20]. Second, probability maps of dengue occurrence could be refined with better estimates of the relative vector competence of *A. albopictus* versus *A. aegypti.* Third, if the historical literature is correct and *A. albopictus* is more resistant than *A. aegypti* to disseminated DENV infection, then this provides an opportunity to identify species-specific antiviral defense mechanisms. Last, novel dengue control efforts using *Wolbachia*-infected *A. aegypti* will be better informed with an understanding of the vector competence of *A. albopictus* in candidate intervention settings [21]. To these ends, the current study compared the susceptibility of *A. aegypti* and *A. albopictus* to initial and disseminated DENV infection after direct blood-feeding episodes on viremic patients with dengue.

METHODS

Ethics Statement

All participants provided written informed consent to participate in the study. The study protocols were reviewed and approved by the Scientific and Ethical Committee of the Hospital for Tropical Diseases (CS/ND/12/15) and the Oxford Tropical Research Ethical Committee (OxTREC 29–12). All investigations were conducted in accordance with the principles expressed in the Declaration of Helsinki.

Patient Cohorts

The study was performed at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam, between September 2012 and November 2013. The inclusion criteria were age of ≥15 years, fever duration of ≤96 hours, and clinical suspicion of dengue; positive result of an NS1 rapid test; and provision of written informed consent. The exclusion criteria were current pregnancy, determined by clinical examination or a urine dipstick test for β-human chorionic gonadotropin; current intensive care unit stay; intellectual disability; a history of severe reactions to mosquito bites; and severe dermatological conditions. Demographic and clinical information were recorded prospectively in a standard case report form.

Mosquitoes for Blood-Feeding Experiments

All of the mosquitoes (*A. aegypti* and *A. albopictus*) that fed on patients with dengue were F3 generation and derived from 2 independent pooled larval collections, each sampled from 3 locations within 5 km of each other in Ho Chi Minh City. The *A. aegypti* used were distinct from those in our previous study [22]. Briefly, field-caught larvae were pooled and fed commercial dry fish and dog food. Mosquitoes were housed as described previously [22]. Briefly, adults (F1 generation) were kept in cages containing males and females in an environmental chamber with 12-hour cycles of light and dark at 27°C and 70% relative humidity. F1 females were provided blood meals by direct feeding on afebrile healthy human volunteers for multiple gonotrophic cycles over 45 days, with 15% sucrose provided freely in addition. Eggs from F1 females were hatched and reared and the subsequent F2 females provided with human blood meals as described above. When female F2 mosquitoes were 12 days old, they were killed and pooled into groups of 10 mosquitoes. Each pool was homogenized and tested, along with appropriate controls, by reverse transcription–polymerase chain reaction (RT-PCR) to confirm the absence of DENV, Japanese encephalitis virus, and chikungunya virus. Eggs collected and stored from F2 females were the source of F3 females that were used for direct feeding on patients with dengue.

Experimental Exposure of Patients to Mosquitoes

Each patient was assigned a schedule of 2 exposures to mosquitoes on 2 different study days during the first 4 days after enrollment. The patient's forearm was exposed to 30–40 *A. aegypti* and *A. albopictus* aged 3–7 days (using a 2:3 ratio of *A. aegypti* to *A. albopictus* because preliminary experiments indicated that a higher fraction of *A. aegypti* took a blood meal) contained in a mesh-covered plastic cup that was held against the patient's forearm for 5 minutes. After 5 minutes, mosquitoes were returned to the insectary and subjected to cold anesthesia at 4° C for 45 seconds. Engorged mosquitoes were transferred to 500-mL plastic cups, separated by species, and maintained in an environmental chamber with 12-hour cycles of light and dark at 27°C and 70% relative humidity for 14 days. The number of dead mosquitoes was recorded daily.

Clinical Adverse Events

Postexposure severe adverse events were defined as any event that was clinically significant (ie, requiring clinical intervention, prolonged hospitalization, or admission to an intensive care unit) and possibly, probably, or definitely related to experimental exposure to mosquitoes.

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Detection of DENV in Mosquito Tissues and Saliva

Technicians blinded to clinical and virological details of the participants performed laboratory assays of the mosquitoes. Mosquitoes were killed by cold exposure. The abdomen was dissected from the rest of the mosquito body and suspended in 0.5 mL of mosquito diluent (2% [v/v] heat-inactivated fetal calf serum [FCS] in Roswell Park Memorial Institute 1640 medium, antibiotics, and antimycotics). Individual mosquito abdomens were homogenized with 1-mm zirconia/silica beads for 15 minutes at 30 Hz by using a TissueLyser II system (Qiagen), as described previously [22]. Mosquitoes were scored as being infected with DENV, using a previously described quantitative RT-PCR analysis of homogenized tissue, with the results expressed as copies per abdomen [23]. Infectious virus in the saliva of individual mosquitoes was detected as described previously [22]. Briefly, the proboscis of dewinged and delegged mosquitoes was inserted into the end of a micropipette tip containing 6 μL of filtered saliva medium (a 1:1 solution of 15% [v/v] sucrose in normal saline and inactivated FCS). After 30 minutes, the 6 μL of saliva medium was ejected and then drawn into a pointed glass capillary tube (tip diameter, <0.3 μm), after which the volume of saliva medium derived from 1 mosquito was injected into the thorax of 4–6 *A. aegypti* (age, 4–7 days; volume injected, approximately 1 μL per mosquito). These injected mosquitoes were maintained for 7 days in an environmental chamber as described above. After 7 days, the mosquitoes were killed and the bodies pooled, homogenized, and tested by quantitative RT-PCR for DENV infection, with saliva samples scored as positive or negative depending on this result.

Sequence Amplification and Sequencing of the Gene Encoding DENV Envelope Protein

Viral RNA was extracted as previously described from plasma and mosquito abdomen tissues [22]. Complementary DNA (cDNA) was synthesized from 8 μL of viral RNA by using the Superscript III First-Strand Synthesis System for RT-PCR according to manufacturers' instructions. The genomic region spanning the genes encoding premembrane and envelope proteins was amplified from the cDNA in 16 amplimers. Subsequent steps were performed according to the manufacturers instructions for 454 GS-Junior Next Generation Sequencing method (Roche). Briefly, the primers in this first PCR round contained universal tails at the 5′ end to allow the addition of 454 sequencing-specific nucleotides and isolate-specific multiplex identifiers (also known as "barcodes") in a second PCR round. The first-round and second-round PCR analyses used FastStart High Fidelity polymerase (Roche). The long-read sequencing performance of the 454 GS-Junior (between 400 and 500 bases), in combination with a sample pooling strategy that uses barcoded amplicons, was used for parallel analysis of pooled samples. GS Mapping software was used for primer trimming and alignment of reads against a reference sequence DENV-1/VN/BID-V2732/2007 (GenBank accession number GQ199773.1). Sequence quality was measured using Phred (Q) scores with a minimum acceptable threshold of 95% of sequencing reads having Q scores of >20 (1/100 errors per base).

Dengue Diagnostic Tests

Serological responses were detected using immunoglobulin M (IgM) and immunoglobulin G (IgG) antibody-capture enzyme-linked immunosorbent assays in accordance with the manufacturer's instructions (Panbio, Australia). In accordance with the manufacturer's instructions, we classified serological profiles as "probable secondary" when >22 U of IgG were detected in either acute or early convalescent samples. If acute or early convalescent samples were IgM positive but IgG negative, serological profiles were classified as "probable primary." When IgM or IgG tests result were equivocal, we classified the serological profile as "indeterminate."

DENV plasma viremia levels were measured by a validated quantitative RT-PCR assay that has been described previously [23].

Statistical Analysis

The probability of successful human-to-mosquito transmission was compared between *A. aegypti* and *A. albopictus*, using marginal logistic regression models. Models for assessment of abdomen samples were adjusted for the patient's $\log_{10}$-transformed viremia level, and models for assessment of saliva samples were further adjusted for the abdominal tissue viremia level. Analyses were performed for all patients and stratified by serotype. To account for potential within-patient correlation, model-robust sandwich standard error estimates were used throughout to construct confidence intervals (CIs) and *P* values. We derived 50% mosquito infectious doses (MID_{50} values; defined as plasma viremia levels corresponding to a 50% probability of infection) for abdomen infection on the basis of marginal logistic regression coefficients for each mosquito type, and corresponding 95% CIs were calculated using the delta rule. Since the proportion of mosquitoes with infectious saliva was <50%, MID_{50} values were not estimated. In the initial analysis of the transmission of DENV to saliva, mosquitoes with uninfected abdomens were assigned a log value of 0. This analysis was also run with exclusion of mosquitoes with uninfected abdomen's. The relationship between various covariates and the probability of successful human-to-mosquito transmission was assessed for each mosquito type, using a similar multivariable marginal regression model. The covariates assessed were day of illness, plasma viremia level, serotype, serological result, and abdominal viral burden. The number of mutations in the DENV-1 consensus sequence was compared between mosquito types, using a stratified version of the Wilcoxon test with stratification by patient [24]. All the analyses were performed using the R statistical software package, version 2.13.2 (R Foundation for Statistical Computing; Vienna, Austria).

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RESULTS

Study Population Characteristics

Between September 2012 and November 2013, 120 patients with dengue were enrolled and experimentally exposed to field-derived *A. aegypti* and *A. albopictus* on 2 randomly allocated days within the first 4 study days. The patient enrollment flowchart is shown in Supplementary Figure 1. The final cohort for analysis comprised 118 DENV viremic patients with 232 independent mosquito exposure events. The baseline characteristics of the patients enrolled are shown in Table 1. Experimental exposure to mosquitoes was well tolerated, and no patient experienced a severe adverse event or required withdrawal from the study. DENV serotype 1 (DENV-1) and DENV-4 were responsible for 26% and 44% of cases, respectively, with DENV-2 (13%) and DENV-3 (16%) also represented.

Susceptibility of *A. aegypti* and *A. albopictus* to Acquisition of DENV Infection After Direct Feeding

There was a dose-response relationship between plasma viremia levels at the time of mosquito feeding and the proportion of mosquitoes of either species with DENV-infected abdomens 14 days later (Figure 1). The overall DENV MID_{50} for *A. aegypti* was lower than that observed for *A. albopictus*, but this difference was not statistically significant (7.0 $\log_{10}$ copies/mL [95% CI, 6.77–7.23] vs 7.1 $\log_{10}$ copies/mL [95% CI, 6.9–7.3]; Table 2). Viral RNA concentrations of all 4 DENV serotypes were significantly higher in abdomen tissues from infected *A. albopictus* than in those from *A. aegypti* (Supplementary Table 1). Given that *A. albopictus* accommodated higher DENV RNA concentrations once infected, we examined whether this could result in greater virus sequence diversity. As a test case, the consensus nucleotide sequence of the gene encoding DENV-1 envelope protein was determined directly by analysis of plasma specimens from 10 patients and abdominal tissues from 20 *A. aegypti* and 20 *A. albopictus* that had taken blood meals from these cases (ie, 2 *A. aegypti* and 2 *A. albopictus* per patient). The median number of nucleotide differences between the consensus sequences of the gene recovered from plasma specimens and the gene recovered from abdominal tissue was 9 (interquartile range [IQR], 4–15) for *A. aegypti* and 17 (IQR, 11–18) for *A. albopictus* ($P = .02$, by the Wilcoxon test [stratified by patient]; Supplementary Figure 2).

Table 1. Characteristics of 118 Patients With Dengue Virus (DENV) Infection, by DENV Serotype

Characteristic	DENV-1 (n = 31)	DENV-2 (n = 15)	DENV-3 (n = 19)	DENV-4 (n = 52)	Overall (n = 118)[a]
Age, y	23 (19–31)	23 (21–32)	25 (20–27)	27.5 (22–34)	26 (20–32)
Sex					
Male	12 (38.7)	7 (46.7)	5 (26.3)	17 (32.7)	41 (34.8)
Female	19 (61.3)	8 (53.3)	14 (73.7)	35 (67.3)	77 (65.3)
Illness duration at enrollment, d[b]					
1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	7 (22.3)	5 (33.3)	0 (0)	7 (13.4)	19 (16.2)
3	11 (36.7)	3 (20.0)	7 (36.8)	20 (38.4)	42 (35.9)
4	12 (40.0)	7 (46.7)	11 (57.9)	24 (46.2)	54 (46.2)
5	0 (0)	0 (0)	1 (5.3)	1 (2.0)	2 (1.7)
Viremia level, $\log_{10}$ copies/mL	8.1 (6.9–8.7)	7.6 (7.1–8.9)	6.6 (5.5–7.1)	7.2 (6.5–7.8)	7.2 (6.6–8.1)
Serological profile[c]					
Primary	10 (32.3)	0 (0)	1 (5.3)	1 (2.0)	12 (10.3)
Secondary	17 (54.8)	12 (80.0)	16 (84.2)	48 (94.1)	94 (80.3)
Indeterminate	4 (12.9)	3 (20.0)	2 (10.5)	2 (3.9)	11 (9.4)
Clinical classification					
Dengue	26 (83.9)	14 (93.3)	16 (84.2)	47 (90.4)	104 (88.1)
Dengue with warning signs	5 (16.1)	1 (6.7)	2 (10.5)	4 (7.7)	12 (10.2)
Severe dengue	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Others	0 (0)	0 (0)	1 (5.3)[d]	1 (1.9)[e]	2 (1.7)
Transferred to ICU	1 (3.2)	0 (0)	0 (0)	0 (0)	1 (0.9)

Data are no. (%) of patients or median value (interquartile range).

Abbreviation: ICU, intensive care unit.

[a] The viremia level for 1 patient was below the limit of detection.

[b] Data for 1 patient were missing.

[c] Data for 1 patient with DENV-4 infection were missing.

[d] One patient was categorized as having a "viral infection" but was actually infected with DENV-3.

[e] One patient was categorized as having a "viral infection" but was actually infected with DENV-4.

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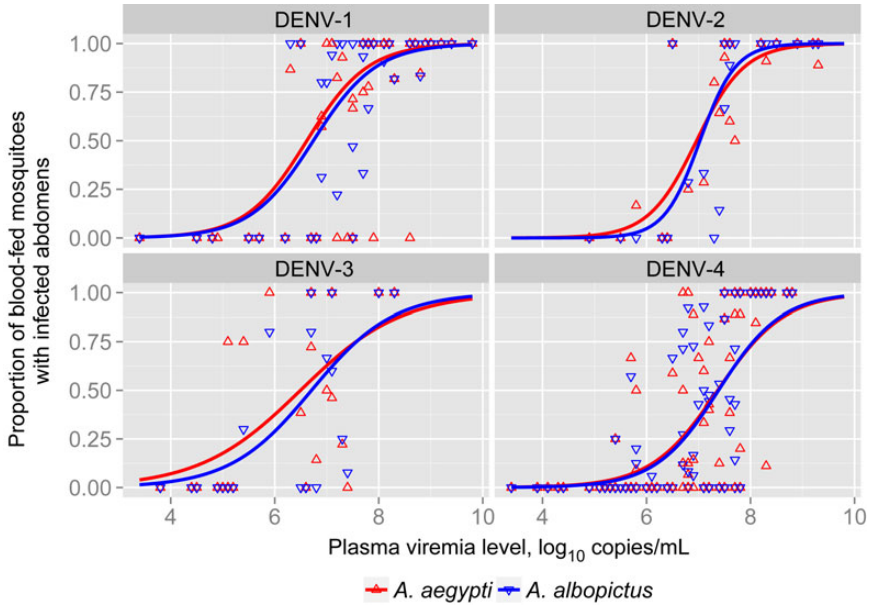

Figure 1. Dose-response scatterplot and curve (derived from logistic regression models) of plasma viremia versus the proportion of mosquitoes with dengue virus (DENV)–infected abdomens after feeding on 118 DENV-infected patients, showing curves for *Aedes aegypti* and *Aedes albopictus*. Each dot represents the proportion of mosquitoes that took a blood meal during an exposure event and had DENV-infected abdomens 14 days later and the corresponding plasma viremia level in the patient at the time of mosquito exposure, stratified by DENV serotype.

Acquisition of Infectiousness Among *A. aegypti* and *A. albopictus*

As was observed in abdomen tissues, there was a dose-response relationship between the plasma viremia level and the proportion of mosquitoes with infectious saliva 14 days after blood feeding (Figure 2). However, the likelihood of detecting infectious saliva differed by mosquito species and DENV serotype. Table 3 summarizes the odds of abdomen and saliva infection and demonstrates that the detection of infectious saliva was less likely in blood-fed *A. albopictus*, compared with blood-fed *A. aegypti*, in both unadjusted analysis and analysis that adjusted for plasma viremia level (adjusted odds ratio [OR], 0.70; 95% CI, .52–.93). By serotype, the odds of *A. albopictus* having infectious saliva were significantly lower for blood meals involving uptake of DENV-2 and DENV-4, compared with those involving uptake of DENV-1 or DENV-3 (Table 3). These data identified the odds of *A. albopictus* becoming infectious as lower than the odds of *A. aegypti* becoming infectious after feeding on the blood of viremic patients. This analysis used all engorged mosquitoes as the denominator. Supplementary Table 2 shows the odds of having infectious saliva, using mosquitoes with DENV-positive abdomens as the denominator. This analysis confirms that the odds of *A. albopictus* becoming infectious were lower than those for *A. aegypti* after viremic blood feeding (OR, 0.69; 95% CI, .49–.96).

Covariates and Their Association With Successful Human-to-Mosquito Transmission

In multivariable regression analysis, a higher plasma viremia level at the time of exposure was independently associated with a greater likelihood of DENV transmission to abdominal tissue for both mosquito types. Each 1-log increase in the

Table 2. 50% Mosquito Infectious Doses (MID_{50} Values) for *Aedes albopictus* and *Aedes aegypti* Abdomen Infection, by Dengue Virus (DENV) Serotype

Serotype	*A. aegypti*	*A. albopictus*	Absolute Difference	*P* Value
DENV-1	6.62 (6.10–7.13)	6.74 (6.27–7.21)	0.12 (−.11 to .35)	.29
DENV-2	6.96 (6.54–7.38)	7.03 (6.62–7.43)	0.07 (−.17 to .30)	.59
DENV-3	6.49 (5.69–7.30)	6.70 (5.86–7.56)	0.21 (−.39 to .82)	.49
DENV-4	7.37 (7.04–7.70)	7.38 (7.07–7.69)	0.01 (−.21 to .24)	.89
Overall	7.00 (6.77–7.23)	7.10 (6.90–7.30)	0.10 (−.04 to .24)	.15

MID_{50} values (defined as plasma viremia levels corresponding to a 50% probability of infection) were derived on the basis of marginal logistic regression coefficients for each mosquito type, and corresponding 95% confidence intervals were calculated using the delta rule.

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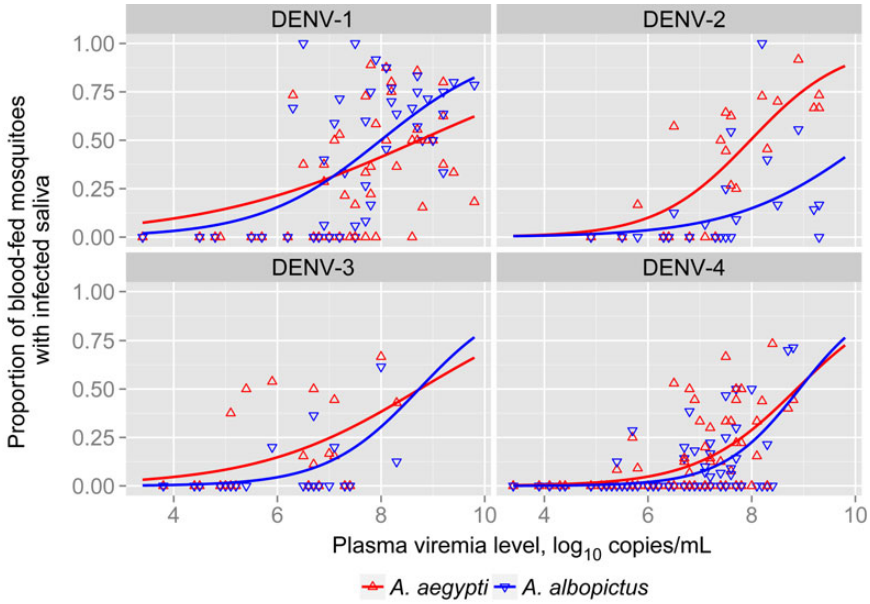

Figure 2. Dose response scatterplot and curve (derived from logistic regression models) of plasma viremia versus the proportion of mosquitoes with infectious saliva after feeding on 118 dengue virus (DENV)–infected patients, showing curves for *Aedes aegypti* and *Aedes albopictus*. Each dot point represents the proportion of mosquitoes that took a blood meal during an exposure event and had saliva containing infectious DENV 14 days later and the corresponding plasma viremia level in the patient at the time of mosquito exposure, stratified by DENV serotype.

$\log_{10}$ plasma viremia level was associated with a 4.65-fold increase in the odds of abdominal tissue infection in *A. aegypti* (95% CI, 3.34–6.48) and a 5.3-fold increase in the odds of abdominal tissue infection in *A. albopictus* (95% CI, 3.80–7.43; Table 4). Each 1-log increase in the $\log_{10}$ plasma viremia level was associated with 2.16-fold increase in the odds of saliva infection in *A. aegypti* (95% CI, 1.68–2.78) and a 2.79-fold increase in the odds of saliva infection in *A. albopictus* (95% CI, 2.17–3.60; Table 4). These data highlight plasma viremia level as a risk factor for infectiousness among *A. aegypti* and *A. albopictus*. Supplementary Table 3 shows the association between these covariates and successful transmission, with mosquitoes with DENV-negative abdomens excluded from the model.

Table 3. Comparison of the Odds of Abdomen and Saliva Infection Among *Aedes albopictus* Versus the Odds Among *Aedes aegypti*, by Dengue Virus (DENV) Serotype

Specimen, Serotype	Patients, No.	*A. albopictus* Infected/Tested, No. (%)	*A. aegypti* Infected/Tested, No. (%)	Unadjusted OR (95% CI)	*P* Value	Adjusted OR (95% CI)[a]	*P* Value
Abdomen							
DENV-1	27	291/411 (70.8)	278/379 (73.4)	0.88 (.62–1.25)	.48	0.79 (.52–1.20)	.28
DENV-2	13	108/185 (58.4)	143/220 (65.0)	0.76 (.52–1.09)	.13	0.97 (.52–1.82)	.93
DENV-3	16	67/173 (38.7)	83/208 (39.9)	0.95 (.55–1.66)	.86	0.76 (.39–1.48)	.42
DENV-4	49	229/777 (29.5)	232/751 (30.9)	0.93 (.70–1.24)	.64	0.95 (.69–1.31)	.77
Overall	105	695/1546 (45.0)	736/1558 (47.2)	0.91 (.78–1.07)	.27	0.85 (.69–1.06)	.16
Saliva							
DENV-1	27	177/411 (43.1)	142/379 (37.5)	1.26 (.89–1.80)	.20	1.31 (.90–1.93)	.16
DENV-2	13	22/185 (11.9)	91/220 (41.4)	0.19 (.11–.33)	<.001	0.17 (.09–.31)	<.001
DENV-3	16	17/173 (9.8)	35/208 (16.8)	0.54 (.21–1.37)	.19	0.44 (.19–1.00)	.051
DENV-4	49	60/777 (7.7)	92/751 (12.3)	0.60 (.36–.99)	.044	0.60 (.37–.98)	.041
Overall	105	276/1546 (17.9)	360/1558 (23.1)	0.72 (.55–.95)	.022	0.70 (.52–.93)	.014

Abbreviations: CI, confidence interval; OR, odds ratio.

[a] Marginal logistic regression models adjusted for plasma viremia.

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Table 4. Covariates and Their Association With Successful Dengue Virus (DENV) Transmission Among *Aedes albopictus* and *Aedes aegypti*

	Abdomen				Saliva (Among All Mosquitoes)			
	A. aegypti		*A. albopictus*		*A. aegypti*		*A. albopictus*	
Variable	Adjusted OR (95% CI)	*P* Value	Adjusted OR (95% CI)	*P* Value	Adjusted OR (95% CI)	*P* Value	Adjusted OR (95% CI)	*P* Value
Illness duration at enrollment (per 1-d increase)	0.58 (.36–.93)	.025	0.60 (.39–.92)	.018	0.87 (.61–1.24)	.45	0.91 (.62–1.34)	.63
Viremia level (per 1-$\log_{10}$ copies/mL increase)	4.65 (3.34–6.48)	<.001	5.32 (3.80–7.43)	<.001	2.16 (1.68–2.78)	<.001	2.79 (2.17–3.60)	<.001
DENV serotype								
DENV-1	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
DENV-2	0.57 (.18–1.78)	.34	0.74 (.24–2.34)	.59	1.43 (.69–2.95)	.34	0.18 (.08–.39)	<.001
DENV-3	1.29 (.30–5.49)	.73	1.31 (.31–5.47)	.71	1.08 (.41–2.86)	.88	0.40 (.12–1.38)	.15
DENV-4	0.27 (.10–.71)	.008	0.36 (.15–.88)	.026	0.48 (.24–.99)	.046	0.23 (.12–.44)	<.001
Serological profile								
Primary	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
Secondary	0.84 (.18–3.86)	.83	0.73 (.18–2.96)	.66	0.76 (.29–1.99)	.58	1.01 (.45.2.26)	.99
Indeterminate	0.27 (.06–1.16)	.08	0.51 (.11–2.43)	.40	0.37 (.13–1.09)	.07	0.31 (.09–1.02)	.054

Data are from marginal models.

Abbreviations: CI, confidence interval; OR, odds ratio.

DISCUSSION

There is speculation on the arboviral disease risks posed by the invasion of *A. albopictus* into southern Europe and the United States [25]. Central to calibrating these risk assessments is an understanding of *A. albopictus* vector competence. There is conflicting literature on whether *A. albopictus* is less susceptible than *A. aegypti* to disseminated DENV infection [26–32]. Furthermore, all of the previous work in this area has used laboratory methods that do not mimic natural infection. Here, in experiments involving direct feeding on viremic patients with dengue, we provide evidence that *A. albopictus* had a susceptibility to acquiring DENV infection that was similar to that of *A. aegypti*. However, blood-fed *A. albopictus* was significantly less likely to become infectious with DENV-2 and DENV-4, suggesting an interspecies difference of epidemiological importance.

A first step in the establishment of DENV infection in mosquitoes is attachment of virus particles to luminal receptors on the midgut epithelium, followed by virus entry via endocytic pathways. There are few insights into the nature of these receptors for DENV, although it has been proposed that the R67 and R80 proteins may act as midgut receptors leading to subsequent systemic infection [33]. Our finding that the plasma viremia MID_{50} values for *A. aegypti* and *A. albopictus* were broadly similar suggests that DENV uses common mechanisms to attach and initially infect the midgut epithelium in these specimens. The MID_{50} values for DENV-1 and DENV-4 measured in this study were concordant with those observed previously, whereas the MID_{50} values differed for DENV-2 and DENV-3 [22]. We speculate that this reflects the smaller numbers of patients with DENV-2 and DENV-3 in this current study and hence decreased accuracy.

Our results identified that *A. albopictus* and *A. aegypti* were equally likely to develop infectious saliva containing DENV-1 and DENV-3. However, DENV-2 and DENV-4 were detected in saliva from *A. albopictus* less frequently than in saliva from *A. aegypti*. This might simply reflect intrinsic differences between the virus types in their virulence for *A. albopictus*, with DENV-1 and DENV-3 being fitter in this instance. However, it is interesting to note that, while DENV-1 was the dominant serotype in the Key West outbreak of dengue with *A. aegypti* as the vector, DENV-1 has not become further established in the southern United States, where *A. albopictus* is prevalent [34].

We explored the relationship between various covariates and the likelihood of successful human-to-mosquito transmission. We confirmed the importance of the plasma viremia level in successful human-mosquito transmission: each 1-log increase in plasma viremia level was associated with an approximately 5-fold increase in the odds of successful human-to-mosquito transmission for both *A. aegypti* and *A. albopictus* [22]. This finding again suggests that interventions that reduce DENV viremia during natural infection could have both an individual benefit and a public health benefit through reducing human infectiousness and thus reducing the risk of further transmission [19].

DENV RNA concentrations were significantly higher in abdominal tissues of *A. albopictus* than *A. aegypti* for all serotypes.

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When examined for DENV-1, this was also associated with higher levels of sequence drift away from the consensus sequence of the gene encoding envelope protein in the patient's plasma sample. Plausibly, by being more permissive to DENV replication in its abdominal tissues, *A. albopictus* might contribute to the genetic diversity that exists within DENV populations. Despite *A. albopictus* harboring higher viral burdens in abdominal tissues, significantly fewer *A. albopictus* had infectious saliva resulting from DENV-2 or DENV-4 infections. These data are broadly consistent with previous observations demonstrating that *A. albopictus* was more susceptible to fulminant DENV infection of the mosquito body but that viral dissemination to saliva was less than in *A. aegypti* [18]. That such nuanced interspecies differences can exist is perhaps not surprising, given the literature reporting variation in the susceptibility of *A. aegypti* populations to DENV infection, albeit under laboratory conditions [35–39]. Future studies to understand the basis for these phenotypes, particularly when using field-derived mosquitoes and direct feeding on human viremic hosts, will require large sample sizes to overcome the intrinsic variance in this system.

While our findings have identified interspecies differences in susceptibility to DENV infection, the absolute differences between the mosquito types are nonetheless small. This points to factors other than outright susceptibility to DENV infection as important reasons why *A. albopictus* is a marginal contributor to dengue transmission in urban and peri-urban settings in dengue-endemic countries. Behavioral attributes are also important for vector competence, and thus we speculate that the tendency of *A. aegypti* to live proximate to humans is the major reason why it is the primary DENV vector.

Our study has limitations [18]. First, for practical reasons we measured the phenotype of blood-fed, field-derived mosquitoes at 14 days after feeding; data acquisition at other time points might have led to additional insights. Second, the results were limited in scope to the virus serotypes and genotypes in circulation during the study period, and consequently we acquired sparse data on DENV-2 and DENV-3. Additionally, there may be specific interactions between DENV types and mosquito genotypes that influence infection outcome [40]. Studies in other settings should be encouraged to understand the generalizability of this work.

In summary, we are the first to demonstrate that *A. albopictus* are less likely than *A. aegypti* to develop an infectious phenotype 14 days after direct blood feeding on viremic patients with dengue due to DENV-2 or DENV-4 infection. These results will enable more-accurate parameterization of DENV transmission models in regions where dengue is endemic and those that are at risk for endemicity. In addition, we have confirmed the central importance of plasma viremia in determining the likelihood of mosquito tissue infection, suggesting that interventions that attenuate viremia will have both individual and community benefits.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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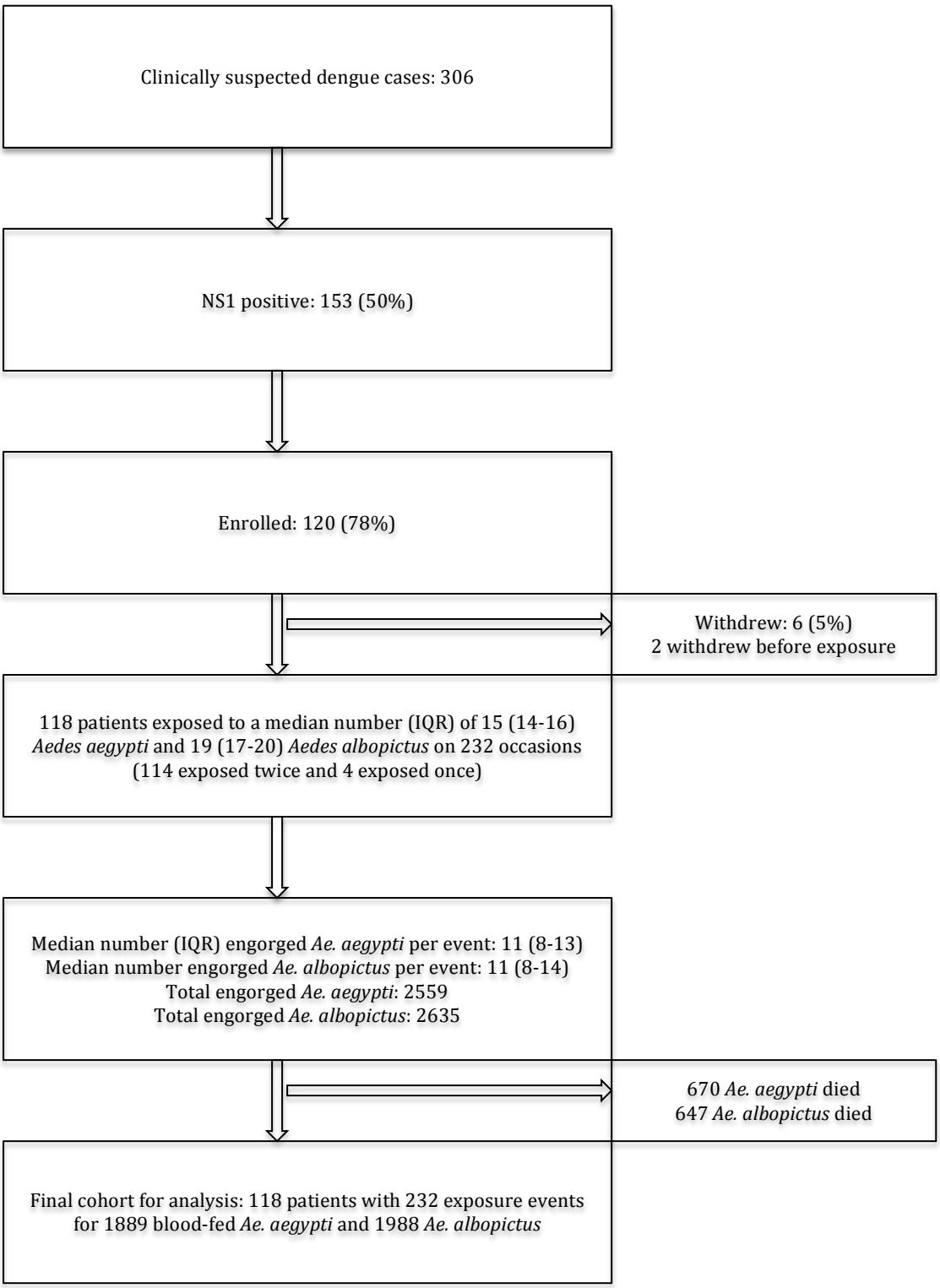
Figure S1: Flow chart of patient enrolment and exposure to mosquitoes. IQR, interquartile range



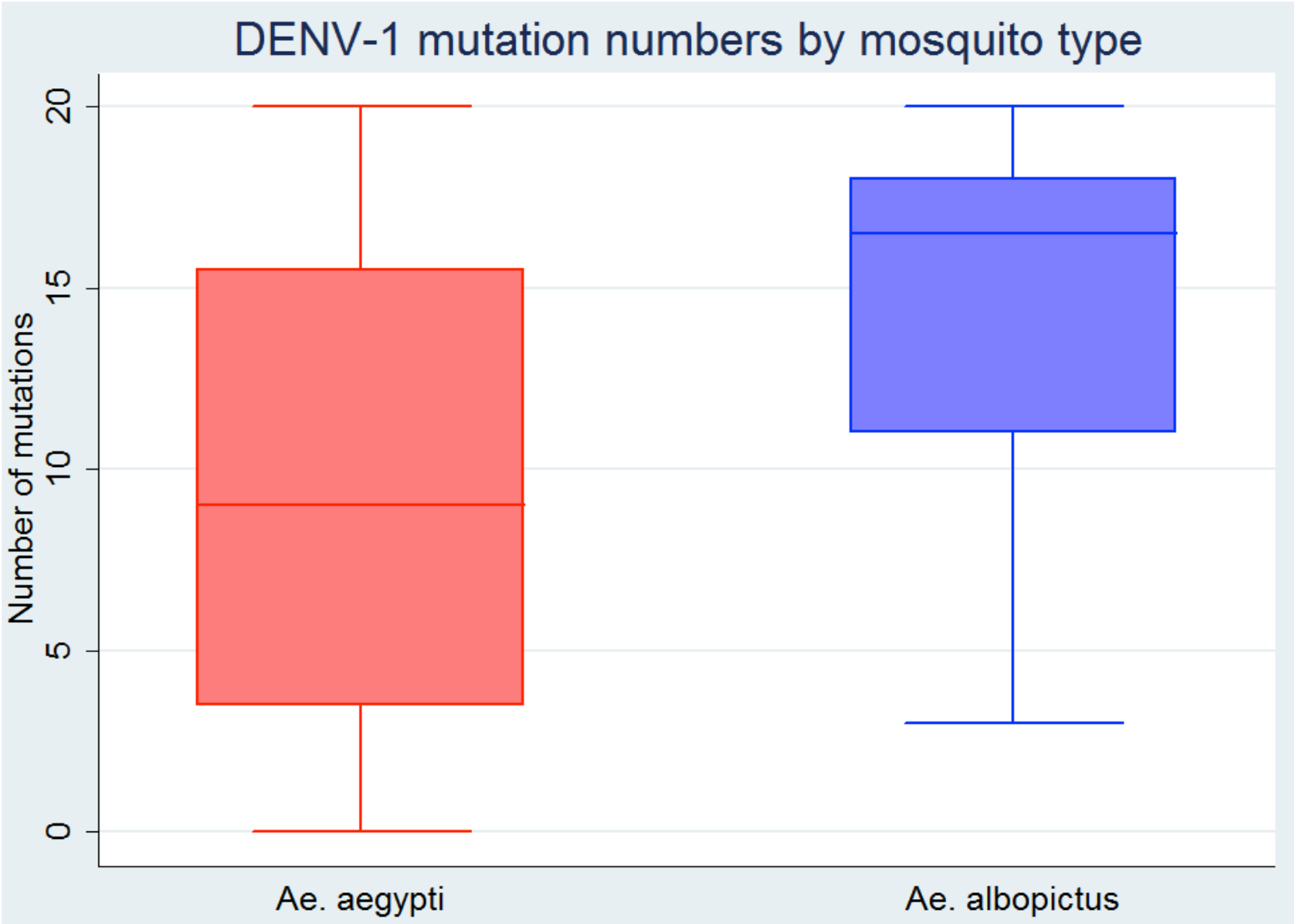


Figure S2: Box plot showing the number of mutations by mosquito type stratified by patient

Supplementary Table 1: Abdomen viral burden (log10 RNA copies per abdomen) according to mosquito type *

Serotype	Number of Ae. aegypti	Ae. aegypti (median, IQR)	Number of Ae. albopictus	Ae. albopictus (median, IQR)	p value
All	1472	7.3(6.9-7.6)	1390	7.7(7.4-8.0)	<0.0001
DENV 1	556	7.0(6.7-7.3)	582	7.6(7.4-7.9)	<0.0001
DENV 2	286	7.5(7.2-7.8)	216	7.8(7.4-7.9)	<0.0001
DENV 3	166	7.1(6.6-7.5)	134	7.4(7.1-7.7)	<0.0001
DENV 4	464	7.4(7.2-7.7)	458	7.8(7.6-8.1)	<0.0001

*Among infected abdomen tissues only.

IQR: Interquartile range; p value from Wilcoxon rank-sum tests

Supplementary Table 2. Saliva infection among positive abdomen

	No of patients	Ae. albopictus		Ae. aegypti		Unadjusted OR (95CIs)	p-values	Adjusted OR (95CIs)*	p-values
		# infected/ N abdomen positive	%	# infected/ N abdomen positive	%				
DENV1	27	177/291	60.8	142/278	51.1	**1.49(1.01-2.19)**	**0.045**	**1.49(1.01-2.19)**	**0.046**
DENV2	13	22/108	20.4	91/143	63.6	**0.15(0.08-0.27)**	**<0.001**	**0.15(0.08-0.28)**	**<0.001**
DENV3	16	17/67	25.4	35/83	42.2	0.49(0.23-1.06)	0.07	**0.45(0.24-0.85)**	**0.014**
DENV4	49	60/229	26.2	92/232	39.7	**0.54(0.30-0.96)**	**0.037**	**0.55(0.31-0.97)**	**0.040**
All	105	276/695	39.7	360/736	48.8	**0.69(0.49-0.98)**	**0.035**	**0.69(0.49-0.96)**	**0.030**

*Marginal logistic regression models for adjusted for plasma viremia

Supplementary Table 3. Covariates and their association with successful DENV transmission among positive abdomen

	Saliva (among positive abdomen)			
	Aegypti		Albopictus	
Variable	Adjusted OR(95%CI)	p value	Adjusted OR(95%CI)	p value
Day of illness at enrolment (+1 day)	0.99(0.73-1.34)	0.95	1.07(0.78-1.46)	0.70
Viremia (+1 log -copies/ml)	1.01(0.78-1.31)	0.95	**1.57(1.20-2.05)**	**<0.001**
Serotype				
DENV1	1.00(reference)		1.00(reference)	
DENV2	1.68(0.93-3.01)	0.08	**0.17(0.08-0.35)**	**<0.001**
DENV3	0.65(0.31-1.36)	0.26	**0.31(0.11-0.91)**	**0.032**
DENV4	0.62(0.34-1.11)	0.10	**0.29(0.16-0.51)**	**<0.001**
Serology				
Primary	1.00(reference)		1.00(reference)	
Secondary	0.91(0.49-1.71)	0.77	1.11(0.63-1.95)	0.72
Indeterminate	0.74(0.32-1.73)	0.49	**0.42(0.17-0.99)**	**0.049**

4. CLINICAL MANAGEMENT

Background

There is an unmet need for an effective dengue therapeutic. An intervention that shortens the duration of illness and prevents or attenuates severe manifestations of disease would represent a major public health advance. In addition, there remain many questions about the optimal way to manage those patients with severe disease in terms of fluid management and the use of blood products. Dengue drug development is hampered by the lack of a suitable animal model of disease – it is possible that novel approaches such as the use of a dengue human infection model (DHIM) may assist this effort.

Outline of papers

Dengue therapeutics review

This monograph aims to explain the rationale for a dengue therapeutic and to consider the current state of dengue therapeutics research.

Lovastatin for dengue trial protocol

HMG-CoA reductase inhibitors, or statins, were originally developed for lipid lowering and mortality reduction in cardiovascular disease.[32] More recent research has demonstrated that statins have effects beyond their lipid-lowering properties, and it is thought that part of the mortality benefit seen with statins in cardiovascular disease is mediated through these anti-inflammatory pleiotropic effects.[33] These pleiotropic effects include the restoration or improvement of endothelial cell function, increasing production of nitric oxide, reducing the release of cytokines and acute phase proteins and reducing inflammatory responses within the vessel wall.[34,35] As protein isoprenylation is involved in various intracellular signalling processes, it is thought that these beneficial effects are, in part, mediated through statin inhibition of the mevalonate pathway and thus inhibition of isoprenoid formation.[33] These isoprenoid intermediates serve as attachment sites for proteins such as Rho, which is involved in the expression of pro-inflammatory cytokines. Furthermore, statins result in increased nitric oxide bioavailability and thus improved endothelial function as a result of their effect on the production of reactive oxygen species and on the activity of the enzyme nitric oxygen synthase.[36] Statins have been shown to reduce the expression of pro-inflammatory cytokines in patients with hypercholesterolaemia, therefore controlling the migration of leucocytes to areas of endothelial inflammation.[37] There is thought to be some overlap between the inflammatory processes seen in atherosclerosis and sepsis, and it has been proposed that chronic exposure to lipopolysaccharide is important in the pathogenesis of atherosclerosis.[38] Various observational studies have suggested that a prior history of statin therapy is associated with improved outcomes for a diverse range of conditions including sepsis and pneumonia.[39-47] A study in

healthy volunteers showed that the inflammatory response to endotoxin was suppressed in those receiving a statin at a high dose, perhaps explaining its beneficial effect observed in sepsis.[35,48] One of the major features of both dengue and sepsis is endothelial dysfunction resulting, in part, from exposure to inflammatory mediators.[49-51] This suggests that in view of their pleiotropic effects, it is plausible that statins may favourably augment the pathophysiological mechanisms of these two conditions. However prospective clinical trials investigating their role as an adjunctive therapy in severe conditions have recently reported and the results have been disappointing. For example, a trial investigating the role of simvastatin as an adjunctive therapeutic for ventilator-associated pneumonia was stopped early for futility.[52] A trial investigating the role of atorvastatin as an adjunctive therapy in patients with severe sepsis showed no benefit of commencing *de novo* statin therapy in this group but suggested that prior statin use was associated with more favourable cytokine concentrations and better survival results.[53] Furthermore, trials investigating the potential role of statins as adjuncts in acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD) showed no benefit.[54,55] All of these trials reported after the date of commencement of patient recruitment into the trial of lovastatin in adult dengue cases reported in this thesis.

Flavivirus replication is lipid-dependent and *in vitro* work has demonstrated that lovastatin may have some antiviral properties by reducing DENV virion assembly.[56-58] This is postulated to occur through the blocking of viral transport from the endoplasmic reticulum to the Golgi apparatus in infected cells. It is therefore possible that statins may have a useful adjunctive role in dengue in view of both their endothelial stabilising effects and their potential antiviral activity. In addition, the low cost and safety profile of statins make them attractive therapeutic candidates. However the rare but recognised adverse events associated with statin therapy such as hepatitis and muscle inflammation, overlap with the clinical features of dengue making the safety of such a therapeutic approach a key question to address. To this end we designed and conducted a randomised, double-blind, placebo-controlled trial investigating the effects of short course lovastatin in adult patients with dengue. The primary endpoint of this trial was safety but it was also sufficiently large to address *a priori* defined secondary endpoints related to clinical and virological features of dengue.

Lovastatin for dengue trial results

The results of a randomised double-blind, placebo-controlled trial of five days lovastatin are presented. The trial was conducted in two phases. The first phase investigated 40mg lovastatin versus placebo in 30 patients, and the second phase investigated 80mg of lovastatin versus placebo in 300 patients. There was no difference in adverse event rates between treatment arms and no evidence of a beneficial effect on the *a priori* clinical and virological endpoints. These results showed that lovastatin was safe and well tolerated in dengue but that there was no evidence of a beneficial therapeutic effect.

Platelets in dengue survey

As discussed above thrombocytopenia is a very common feature of dengue. In some settings the concern about bleeding leads clinicians to administer platelet transfusions as prophylaxis against haemorrhage. This approach lacks an evidence base and is potentially dangerous as it exposes patients to blood products and may represent an unnecessary fluid challenge at a time when fluid management is critical. To establish a clearer idea about clinical practice related to this area we designed a survey with different clinical scenarios and distributed it to clinicians involved with dengue management in 20 countries. The survey responses showed a wide variety in approaches to the use of platelets in dengue. The survey findings underscore the need to conduct clinical trials to better establish an evidence base to guide management in this area. It is possible that the on-going ADEPT trial currently recruiting in Singapore will better clarify the evidence in this area (ClinicalTrials.gov identifier NCT01030211).

Dengue human infection model

This monograph considers how a DHIM has the potential to be a game changer in the development of dengue therapeutics. It was written in light of a recently performed DENV challenge given to human recipients of a live dengue vaccine candidate.[59] We consider how the controlled approach inherent to a DHIM would further dengue therapeutic research by allowing the study of pre-selected susceptible volunteers thus potentially reducing the sample size required to conduct an efficacy study. In addition, the approach would facilitate the study of pharmacokinetics and aid the choice of the optimal dosing strategy and potentially answer the question as to whether chemoprophylaxis is feasible in dengue.

LITERATURE REVIEW 4: DENGUE THERAPEUTICS, CHEMOPROPHYLAXIS, AND ALLIED TOOLS: STATE OF THE ART AND FUTURE DIRECTIONS

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SECTION A – Student Details

Student	James Whitehorn
Principal Supervisor	Rosanna Peeling and Cameron Simmons
Thesis Title	The pathogenesis and clinical management of dengue

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	PLOS Neglected Tropical Diseases		
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Review

Dengue Therapeutics, Chemoprophylaxis, and Allied Tools: State of the Art and Future Directions

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Abstract: Dengue is the most common arboviral disease of humans. There is an unmet need for a therapeutic intervention that reduces the duration and severity of dengue symptoms and diminishes the likelihood of severe complications. To this end, there are active discovery efforts in industry and academia to develop interventions, with a focus on small molecule inhibitors of dengue virus replication that are suitable for therapy or chemoprophylaxis. Advancements in animal models of dengue virus infection together with the possibility of a dengue human infection model have further enhanced the platform for dengue drug discovery. Whilst drug discovery efforts gestate, there are ongoing clinical research designed to benefit today's patients, including trials of supportive care interventions, and descriptive studies that should improve the ability of clinicians to make an accurate diagnosis early in the illness course and to identify patients most at risk of progression to severe disease. This review provides a state of the art summary of dengue drug discovery, clinical trials, and supportive allied research and reflects discussions at the 2nd International Dengue Therapeutics Workshop held in Ho Chi Minh City, Vietnam, in December 2013.

Introduction

The global dengue pandemic represents a major 21st-century public health challenge, with approximately 3,000,000,000 people living in areas at risk of transmission [1,2]. Dengue causes individual suffering to those affected but also significant economic costs to endemic countries, as hospitals are frequently overwhelmed by dengue patients and those affected are unable to attend school or go to work [1].

Dengue is a systemic viral infection caused by any of the dengue viruses (DENV), of which there are four types, DENV-1–4. DENV are members of the Flaviviridae family and possess a single-stranded positive-sense RNA genome that encodes three structural proteins and seven nonstructural proteins. Infection of susceptible human hosts occurs after the bite of an infectious mosquito, usually *Aedes aegypti*. In susceptible human hosts, DENV replication likely occurs predominantly in cells of the reticuloendothelial system [3,4]. Virus produced from infected tissues results in a viremia that can allow for onward transmission of DENV to naïve mosquitoes that take a blood meal [5]. Dengue is a self-limiting febrile illness, which, although often debilitating, typically resolves after 4–7 days of symptoms without major complications. Common symptoms include lethargy, headache, myalgia, nausea, vomiting, and rash. Viremia is apparent 1–2 days prior to the onset of symptoms, peaks during the first 1–2 days of fever, and then declines over the next 3–5 days until resolution.

A small proportion of people who develop dengue do experience severe complications during the illness. The most common complication identified is a significant increase in vascular permeability secondary to a transient vasculopathy; typically, this becomes apparent between the fourth and sixth day of illness, at a time when viremia is in steep decline or has resolved and the patient is afebrile [6]. In some patients, predominantly but not exclusively older children and young adults, significant vascular leakage occurs and can result in life-threatening hypovolaemic shock, with or without haemorrhage [1]. Various host and viral factors influence the eventual clinical phenotype of a DENV infection [7]. Current treatment is limited to fluid resuscitation and supportive care [8]. To prevent the complications that are typically observed between the fourth and sixth day of illness would require the administration of a therapeutic earlier in the course of illness. This raises a number of challenges—patients may not present to healthcare settings early in their illness, differentiating dengue from other febrile illnesses (OFI) is not easy, rapid diagnostic tests are relatively costly and not always available, and identifying patients at risk of severe disease and thus most likely to benefit from a therapeutic remains difficult. In addition, despite considerable research efforts, the pathogenesis of dengue remains incompletely understood [7].

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Competing Interests: I have read the journal's policy and have the following conflicts: PYS is an employee of Novartis Institute for Tropical Diseases, a public-private entity dedicated to developing drugs for tropical infectious diseases. CPS is an occasional paid consultant to GSK, Tibotec and Unither Virology on the development of antiviral drugs for dengue. This does not alter our adherence to all PLOS policies on sharing data and materials.

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These factors are challenges to the programmatic use of a dengue therapeutic in routine clinical practice.

To date, few trials of new specific therapeutic interventions have been conducted. However, this landscape is rapidly evolving, driven by a growing awareness of the scale of the disease burden [2], economic changes in endemic countries that are creating viable markets, and strong basic science that is supporting rational drug discovery and testing in small animal models. This article reflects discussions held at the 2nd International Dengue Therapeutics Workshop held in Ho Chi Minh City in December 2013 and attended by delegates from academia, industry, and funding agencies. It aims to illustrate the "state of the art" in dengue therapeutics discovery, clinical intervention trials, and enabling allied research.

Target product profile for a dengue therapeutic

There remains an unmet need for an effective dengue therapeutic that can shorten the duration of illness, reduce the severity of common symptoms, and prevent the development of severe complications such as dengue shock syndrome (DSS). An example of a target product profile for an antiviral dengue drug is shown in Table 1. Desirable properties of a therapeutic candidate include low cost, ease of administration, and an excellent risk-benefit profile. While preliminary studies will typically be conducted in adults, it is important that children also be taken into consideration when developing the target product profile, as the disease burden of dengue falls most heavily on this age group.

State of the Art in Small Molecule Therapeutic Trials in Dengue

Between 2007 and 2013, ten therapeutic trials in dengue patients were reported [9–18]. These have varied in size, quality, and design. Studies that have adopted a conventional randomised controlled approach have included trials of chloroquine, prednisolone, lovastatin, celgosivir, and balapiravir [16–20]. Chloroquine was investigated for both its antiviral properties and its potential ability to modulate the immune response to infection; however, the trial (n = 307 adult patients) did not demonstrate any clear antiviral or clinical benefits [16]. Prednisolone was investigated for its immunomodulatory properties with the hope that early initiation of therapy would prevent or attenuate severe manifestations of disease. The trial (n = 225 paediatric patients) was powered for safety but yielded no evidence of therapeutic benefit [18] and very limited evidence of attenuation of the host immune response [21]. Balapiravir, a prodrug of a nucleoside analogue, was clinically investigated as a candidate dengue antiviral on the basis of in vitro findings. However, the absence of a strong antiviral signal in 69 adult dengue patients led to cessation of the trial [17]. Subsequent work has indicated balapiravir is poorly metabolised to its active moiety in immune-activated cells, suggesting a possible explanation for the clinical trial outcome [22]. Another antiviral candidate, celgosivir, is a cellular glucosidase inhibitor. In vitro studies suggested celgosivir had antiviral activity against all four serotypes of DENV, and further in vivo work using a lethal mouse model showed celgosivir had antiviral activity against DENV-2 [23]. A clinical trial of celgosivir in 50 adult dengue cases suggested celgosivir was safe and well tolerated, but there was no evidence of an antiviral effect at the doses used [20,24]. Inhibitors of HMG-CoA reductase, known as statins, were originally developed as lipid-lowering agents and have an established role in cardiovascular risk modification [25]. More recent research has shown that they have anti-inflammatory and endothelial-stabilising properties, and this has prompted the investigation of these drugs as adjunctive therapeutics for a range of conditions such as sepsis, pneumonia, and acute lung injury [26–28]. Based on the potential benefit of the properties of statins on the endothelium and as an immunomodulatory agent, it is plausible that they may have a beneficial effect in dengue. A trial of early lovastatin therapy in adult dengue cases is ongoing [19].

State of the Art in Supportive Care Trials

Judicious fluid resuscitation is critical to the successful management of patients with severe dengue [8]. Isotonic crystalloid fluids (e.g., 0.9% normal saline and Ringer's lactate) are recommended for initial resuscitation of those with shock [1,29,30]. Colloid solutions (e.g., hydroxyethyl starch and Gelofusine) are suggested for patients with profound shock or for those who do not respond to initial resuscitation with crystalloids [8,29]. There remain questions surrounding the optimal fluid management of the critically ill dengue patient, particularly for the estimated 30% of DSS cases who suffer recurrent episodes of shock [31,32]. The safety of starch solutions, currently integral to resuscitation of severe dengue patients in many endemic countries, has also been called into question [33,34]. While these safety concerns have generally arisen in elderly patients, often with comorbidities, and thus may not be relevant to the management of previously healthy children and young adults, they make the establishment of a clear evidence base for the optimal fluid resuscitation of patients with

Table 1. Target product profile for a dengue therapeutic.

Profile	Ideal
Target population	Adults and children (including infants and pregnant women)
Dosing frequency	Once daily
Formulation	Water-soluble tablet that can be dissolved in a small amount of liquid
Pill burden	One tablet daily
Pharmacokinetic	Half-life that enables once daily dosing
Bioavailability	Fast acting, high volume of distribution
Interactions	Minimal interactions with commonly used supportive care drugs
Safety	Well tolerated; no need for lab monitoring
Stability	No need for cold chain; 1–2-year shelf life at room temperature
Cost	To be investigated; needs to be affordable in dengue-endemic countries

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single or recurrent episodes of shock ever more pressing. In addition, the previous fluid intervention studies in dengue were all conducted in children; given the diverse epidemiology of dengue, with adults representing the majority of cases in some countries, it is important that future fluid trials include adult patients [35,36].

Thrombocytopenia is almost universally observed in patients with dengue [1]. Despite a lack of evidence, prophylactic platelets are commonly administered in the belief that they can prevent haemorrhage [37]. This practice is both costly and potentially dangerous, as it constitutes administration of a blood product and a fluid challenge at the point of infection when fluid balance is critical [38–41]. A recently completed trial (n = 87 patients) suggested that administration of prophylactic platelets has no therapeutic benefit and is associated with an increased risk of harm—three patients who received platelets developed severe transfusion reactions [42]. The Adult Dengue Platelet Study (ADEPT) trial aims to further clarify the evidence for the use of prophylactic platelets in dengue (ClinicalTrials.gov identifier NCT01030211).

State of the Art in Discovery of Antiviral Therapies

Encouragingly, a number of institutions, both academic and pharmaceutical, are actively engaged in dengue therapeutic discovery and development.

Novartis Institute of Tropical Diseases (NITD)

NITD has made a concerted effort to search for dengue antiviral candidates. NITD has investigated host and virus protein targets using both a cell-based infection approach and a target-based rational approach [43]. Although several interesting inhibitors with various modes of action have been identified, some of which have demonstrated in vivo efficacy in mouse models, as yet none have advanced to the point of a clinical trial [44,45]. However, the knowledge gained from these efforts has provided a better rationale for ongoing dengue drug discovery.

Unither Virology

Unither Virology is attempting to develop a broad-spectrum, host-enzyme-targeted antiviral drug using an iminosugar platform. Iminosugars inhibit host alpha-glucosidases that are required for viral glycoprotein modifications. Inhibition of cellular glucosidase suppresses viral replication by disruption of productive folding pathways of the envelope glycoproteins prM (the intracellular glycosylated precursor of M [membrane protein]) and E (envelope protein). Encouraging results were seen with the leading candidate, UV-4, in a mouse model of DENV infection [46].

Monoclonal antibodies for dengue

Therapeutic antibodies are also being explored to block dengue virus infection. Several potent monoclonal antibodies have been developed, including one that selectively neutralises DENV-1 [47]. Two challenges were perceived for the antibody approach: (1) it is likely that a panel of antibodies will be needed to inhibit all four serotypes of virus, and (2) the relatively high cost and requirement for parenteral administration may limit deployment in many endemic countries. It remains to be determined whether a single antibody that can potently neutralize all four serotypes can be developed.

University of Leuven

Drug discovery at the University of Leuven has focused primarily on viral proteins using a cell-based screening approach and a wealth of experience gained from their research on hepatitis C and HIV therapeutics [48]. Currently, the lead dengue antiviral candidate is an inhibitor of nonstructural protein 4B (NS4B), which shows antiviral activity across all four serotypes and in a variety of cell lines.

University of Marseilles

Work at the University of Marseilles is mainly focused on the screening of potential inhibitors of viral RNA-dependent RNA polymerase activity. A panel of polymerase inhibitors with both enzymatic and cellular activities has been identified. Efforts are ongoing to improve the potency of these inhibitors, as well as to identify their binding sites through biophysical and structural studies.

University of Queensland

Investigators at the University of Queensland have been using cell electrical impedance measurements as a correlate of cell fusion mediated by enveloped viruses. This novel approach provides a high-throughput screening platform for the investigation of potential inhibitors of viral fusion. Besides flaviviruses, the approach could be applied to screen for fusion inhibitors of other enveloped viruses.

Monash University

Researchers at Monash University are exploring the possibility of using inhibitors of nuclear transport as anti-DENV compounds [49]. In DENV infection, nonstructural protein 5 (NS5) localises in the nucleus through an interaction with importin-α1/β1 (IMPα1/β1). Intriguingly, the antiparasitic agent, ivermectin, has been shown to inhibit this interaction and reduce viral production [50]. Interestingly, ivermectin was also reported to inhibit flavivirus helicase activity [51]. Furthermore, the well-established safety profile of ivermectin demonstrated through its use in mass drug administration programmes makes this an attractive therapeutic candidate to investigate further.

State of the Art in Research Tools Supporting Dengue Drug Development

Funding resources for developing dengue therapeutics

Several government funding agencies and research charities around the world are currently supporting research on dengue, including the United States National Institutes of Health (NIH), the European Framework Programme, the Wellcome Trust (United Kingdom), and the Agency for Science, Technology, and Research (Singapore). The NIH, mostly through the National Institute of Allergy and Infectious Diseases (NIAID), is currently supporting several research projects to elucidate the basic biology of dengue virus and the mechanisms of disease development and to develop and evaluate therapeutics, diagnostics, and vaccines. A list of NIH-supported projects on dengue can be found on NIH Research Portfolio Online Reporting Tools (RePORT) (http://report.nih.gov/index.aspx). There are different funding mechanisms at the NIH to obtain support for dengue research. These include the following: (1) grant mechanisms, some of which focus on international research or product development partnerships (http://www.niaid.nih.gov/researchfunding/ann/pages/opps.aspx), and (2) preclinical and clinical research resources. These research resources include dengue reagents, bioinformatic databases, in vitro antiviral screening, evaluation of therapeutics in animal models, therapeutic preclinical development services, and clinical evaluation. Information about NIAID research services and contact information can be found at http://www.niaid.nih.gov/labsandresources/resources/Pages/default.aspx.

A list of additional funding sources can be found at http://www.niaid.nih.gov/researchfunding/ann/pages/found.aspx.

Mouse models of DENV infection

Mice deficient in interferon-α/β and interferon-γ receptor (AG129) are susceptible to DENV infection and can experience a fulminant and fatal infection under certain experimental conditions using DENV2 strains [52,53]. Ongoing work aims to develop mouse models of disseminated disease with other DENV serotypes and to replicate the severe disease that occurs in primary infections in some infants born to immune mothers [54]. As such, mouse models like the AG129 system provide a valuable mechanism to evaluate inhibitors of DENV infection and replication under in vivo conditions. One limitation of mouse models, however, is that they do not replicate the temporal sequence of the virological and common clinical events seen in humans with severe dengue, in particular the occurrence of hypovolemic shock relatively late in the illness course at a time when DENV infection of tissues has very nearly or already resolved. It may be that the vasculopathy seen in some DENV-infected patients is a phenomenon unique to humans. Thus, dengue mouse models may have limited utility in assessing interventions that target the cascade of host-mediated responses that are believed to partly underlie the syndrome of severe dengue in humans.

Nonhuman primates and dengue

Nonhuman primates (NHPs), such as rhesus macaques (*Macaca mulatta*), are naturally susceptible to DENV infection and develop a viremia of similar duration to humans, yet they rarely manifest clinical signs or symptoms [55]. A trial of nonpegylated interferon in rhesus monkeys yielded a delay to peak viremia but with no change in the overall area under the curve. In another trial of recombinant pegylated interferon therapy versus placebo, a log decrease in viremia was observed over several days with a trend towards improved viral clearance, but the magnitude of the response was not deemed to be clinically useful [56]. NHPs have rarely been used in dengue drug development, perhaps because of the cost and the scarcity of laboratories capable of performing such studies. However, several NHP models have been developed that address different dengue manifestations [56–58]. These models could be utilized as part of a rational drug development plan, particularly for the advancement of novel drug entities, including direct-acting antiviral agents. In these scenarios, NHPs could provide the opportunity to conduct prophylactic and therapeutic trials in an animal model in which the virological and immunological features of disease are likely more "human-like" than in mice.

Dengue human infection models (DHIM)

US Army researchers are developing a DHIM, whereby flavivirus-naïve adults are experimentally challenged with a DENV-1 virus strain that previously proved insufficiently attenuated to be used as a vaccine candidate for the purposes of vaccine development [59]. This is not a novel concept. Over 700 subjects have participated in such trials spanning from 1902 until the present time [60]. Rederivation of the challenge strain is in process, and an initial small-scale human trial to demonstrate the safety of the strain is currently being planned.

The administration of a dengue human infection will be conducted with all the current safeguards to protect human volunteers. The study would be done under a Food and Drug Administration (FDA) Investigational New Drug (IND) application, with independent review by both scientists and ethicists. The human volunteers would be informed of the study design, why the study was being done, and of the risks and the benefits of being in the trial. One challenge for the DHIM is that it should replicate the features of naturally acquired DENV infection, and early studies suggest this is possible [60]. A DHIM would also support the fast-track development of potential therapeutics, as it would allow for experimentally controlled trials of chemoprophylaxis and therapy and for detailed pharmacokinetic and basic research studies in preselected individuals.

Physiological endpoints in therapeutic studies

As early phase research of novel therapeutic candidates is often exploratory and focused on safety, it is necessary to investigate surrogate markers of clinical impact rather than the major clinical complications themselves, e.g., DSS. Sensitively measuring the endothelial response to specific interventions in early-phase clinical trials would be ideal. Various noninvasive techniques have been developed that assess functional properties of the endothelium [61]. Peripheral artery tonometry is a user-independent method of measuring endothelium-dependent microvascular reactivity. Changes in microvascular reactivity have been shown to correlate with disease severity and outcome in sepsis and malaria [62,63]. Alternatively, techniques such as videomicroscopy can be used to directly evaluate microcirculatory networks and perfusion status; studies using this method have demonstrated altered microcirculation in severe sepsis, again with correlations with the severity of organ dysfunction and outcome [64,65]. These techniques have been used to monitor responses to particular therapies in other infectious diseases and may have a role as proxy endpoints for clinical trials in dengue [66–68]. It is possible that using noninvasive techniques that assess endothelial function and microcirculation may provide useful correlates of the capillary leak that is characteristic of severe dengue—ongoing observational research aims to evaluate this potential role [63,69].

Clinical descriptive studies and diagnostic/prognostic signs and symptoms

A number of important issues complicate the management of potential dengue cases in endemic areas. First, given the nonspecific nature of the symptoms and signs during the early febrile phase, establishing a firm clinical diagnosis without reliance on expensive diagnostics is difficult. Second, prediction of risk for the development of complications such as shock due to systemic vascular leakage is currently poor. A prospective multicentre observational study, aiming to enrol 10,000–12,000 outpatients presenting with a febrile illness consistent with possible dengue, is currently underway in seven countries across Southeast Asia and Latin America and is expected to report in 2016 (www.idams.eu). The availability of improved strategies for early diagnosis and risk prediction, ideally using a simple laboratory or a laboratory and clinical algorithm, would not only greatly facilitate patient triage but likely also enhance the productivity of clinical trials of early therapeutic interventions by focusing enrolment towards patients at greatest risk of complications. In high-risk patients, it may be easier to differentiate the treatment effect between the existing standard of care versus the standard of care plus the clinical intervention. From a practical perspective, a clinical trial enrolling patients at the highest risk of developing complications could reduce the size, duration, and cost of dengue randomised controlled trials, since the patient population would be enriched for the main endpoints of clinical relevance and interest.

State of the Art in Prospects for Dengue Chemoprophylaxis

The concept of chemoprophylaxis for dengue has not been widely discussed as yet. Nonetheless, there are plausible scenarios and population groups that might benefit from an orally available chemoprophylactic agent. For example, aid workers, missionaries, and military travellers to dengue epidemic settings or "at risk" individuals living in areas of focal transmission in an endemic setting might benefit from chemoprophylaxis. Prophylactic delivery of a dengue antiviral compound has the theoretical advantage of interrupting the course of infection earlier than a therapeutic drug, i.e., prior to the development of peak viremia. However, a chemoprophylactic agent would need to be orally available, have an extremely good safety profile, and possess pharmacokinetic characteristics that allow for relatively infrequent dosing. Additional issues requiring further consideration, particularly for endemic settings, concern the identification of suitable target groups, the duration of dosing, and the risk-benefit ratio.

Conclusions

There remains an unmet need for an effective dengue therapeutic or prophylactic, particularly in light of the continuing geographic expansion of dengue and the lack of an effective vaccine [2,70]. This article has summarised the state of the art in the field of dengue therapeutics and the potential future landscape of therapeutic research. Increased collaboration between academia and industry, perhaps through public-private partnerships, is likely to be necessary for the successful development of an effective dengue therapeutic [43]. There remain many questions, foremost of which is whether an antiviral intervention can have a positive clinical impact when peak viremia typically occurs in the first 48 hours of illness and before most patients seek medical care. The dengue drug development field will benefit from a consensus on methodological approach in the conduct of early phase trials, common case report forms, and data sharing; efforts to provide these to the research community are underway [71]. In the long run, it is likely that a dengue therapeutic (or prophylactic) small molecule would fit into a "package" of dengue control measures comprising immunisation, vector control, and individual therapy.

Box 1. Key Learning Points

- There is an unmet need for specific interventions to improve the clinical management of dengue.
- Ongoing clinical research aims to improve diagnosis and prognosis and to test the efficacy of repurposed drugs.
- Discovery and development of specific therapeutics is directed at inhibiting virus replication or modifying host physiology.
- Therapeutic development is supported by animal models, and a human dengue virus challenge model is under development.

Box 2. Top Five Papers in the Field

- Thomas SJ (2013) Dengue human infection model: Re-establishing a tool for understanding dengue immunology and advancing vaccine development. Hum Vaccin Immunother 9: 1587–1590.
- Schul W, Yip A, Shi PY (2013) Testing antiviral compounds in a dengue mouse model. Methods Mol Biol 1030: 269–281.
- Simmons CP, Farrar JJ, Nguyen vV, Wills B (2012) Dengue. N Engl J Med 366: 1423–1432.
- Low J, Sung C, Wijaya L, Wei Y, Rathore A, et al. (2014) Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): A phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial. Lancet Infect Dis 14: 706–715.
- Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, et al. (2013) The global distribution and burden of dengue. Nature 496: 504–507.

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RESEARCH PAPER 3: LOVASTATIN FOR ADULT PATIENTS WITH DENGUE: PROTOCOL FOR A RANDOMISED CONTROLLED TRIAL

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Student	James Whitehorn
Principal Supervisor	Rosanna Peeling and Cameron Simmons
Thesis Title	The pathogenesis and clinical management of dengue

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TRIALS

STUDY PROTOCOL Open Access

Lovastatin for adult patients with dengue: protocol for a randomised controlled trial

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Abstract

Background: Dengue is the most important vector-borne viral infection of man, with approximately 2 billion people living in areas at risk. Infection results in a range of manifestations from asymptomatic infection through to life-threatening shock and haemorrhage. One of the hallmarks of severe dengue is vascular endothelial disruption. There is currently no specific therapy and clinical management is limited to supportive care. Statins are a class of drug initially developed for lipid lowering. There has been considerable recent interest in their effects beyond lipid lowering. These include anti-inflammatory effects at the endothelium. In addition, it is possible that lovastatin may have an anti-viral effect against dengue. Observational data suggest that the use of statins may improve outcomes for such conditions as sepsis and pneumonia. This paper describes the protocol for a randomised controlled trial investigating a short course of lovastatin therapy in adult patients with dengue.

Methods/design: A randomised, double-blind, placebo-controlled trial will investigate the effects of lovastatin therapy in the treatment of dengue. The trial will be conducted in two phases with an escalation of dose between phases if an interim safety review is satisfactory. This is an exploratory study focusing on safety and there are no data on which to base a sample size calculation. A target sample size of 300 patients in the second phase, enrolled over two dengue seasons, was chosen based on clinical judgement and feasibility considerations. In a previous randomised trial in dengue, about 10% and 30% of patients experienced at least one serious adverse event or adverse event, respectively. With 300 patients, we will have 80% power to detect an increase of 12% (from 10% to 22%) or 16% (from 30% to 46%) in the frequency of adverse events. Furthermore, this sample size ensures some power to explore the efficacy of statins.

Discussion: The development of a dengue therapeutic that can attenuate disease would be an enormous advance in global health. The favourable effects of statins on the endothelium, their good safety profile and their low cost make lovastatin an attractive therapeutic candidate.

Trial registration: International Standard Randomised Controlled Trial Number ISRCTN03147572

Keywords: Clinical trial, Dengue, Lovastatin, Statins

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Background

Dengue is the most common and important vector-borne viral infection of man, with at least 2 billion people living in areas of risk [1]. Clinical dengue varies from asymptomatic infection to severe disease characterised by shock and haemorrhage. There are currently no specific therapeutic agents and disease management is limited to careful fluid management [2,3].

Statins

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. They were first used clinically in the late 1980s and quickly became established as an effective drug for both lipid lowering and mortality reduction in cardiovascular disease [4]. They are currently one of the most prescribed classes of drugs globally. Statins have an excellent safety profile [5]. The most common adverse effects are rises in the level of liver transaminases and myopathy. These effects are rare and appear to be dose-related [5,6]. More recent research has demonstrated that statins have additional beneficial effects. These pleiotropic effects include the restoration or improvement of endothelial cell function, an increased production of nitric oxide, and a reduction in the release of cytokines and acute phase proteins. These effects lead to a reduction of inflammation within the vessel wall [7,8]. Observational studies suggest that statin therapy may result in improved outcomes for a diverse range of conditions, including sepsis and pneumonia [9-13]. In addition, there are a number of on-going randomised clinical trials to assess the therapeutic efficacy of statins in sepsis and acute lung injury (NCT00528580, NCT00676897, NCT00979121).

Statins and dengue

One of the major features of both dengue and sepsis is widespread vascular endothelial disruption resulting, in part, from exposure to inflammatory mediators [14-16]. This suggests that in view of their pleiotropic effects, it is plausible that statins may favourably augment the pathophysiological mechanisms of these two conditions. A study in healthy volunteers showed that the endotoxin-induced inflammatory response was suppressed in those receiving a statin at a high dose (simvastatin 80 mg), perhaps explaining the beneficial effect observed in sepsis [8,17]. Furthermore, *in vitro* work has demonstrated that lovastatin may have an anti-viral effect in dengue by reducing virion assembly [18,19].

Choice of study drug

We have chosen lovastatin as the study drug for both scientific and pragmatic reasons. In light of the US Food and Drug Administration's safety recommendations about the risks of muscle toxicity with simvastatin, there were concerns from the regulatory authorities in Vietnam about the safety of using this agent in dengue. An additional reason for our choice was the dengue anti-viral effect observed with lovastatin in *in vitro* experiments [18]. As this effect may also be observed *in vitro*, we believe it is rational to test the drug used in the original work. Furthermore, lovastatin is commonly prescribed and available in Vietnam as a generic and will therefore be immediately available to patients if there is a positive result from the trial.

Aims of the trial

Typically, severe vascular leakage and shock occur around the fifth day of illness in patients with dengue [20]. It is possible that initiation of statin therapy early in the course of the illness may prevent or favourably modulate these effects. As the proportion of patients who develop shock or other complications after presentation to hospital is low (approximately 5%), a large trial would be required to demonstrate a clinical benefit [2,21]. However, this study presents an opportunity to assess formally the safety of using statins in the treatment of this disease. In addition, such a study will also provide an opportunity to investigate the effect of statin usage on the immune response during dengue infection and to generate preliminary data for planning a Phase III trial in the future.

Although there is extensive experience of using statins as a lipid-lowering agent, and an increase in the amount of observational data from their use in critically ill patients, this will be the first study looking at the use of statins in dengue. We propose to investigate the effect of lovastatin for 5 days in adult dengue patients presenting in the first 72 hours of illness. The rational of a 5-day treatment course is to cover the transient 'critical' phase of infection when complications arise, which typically occurs around the fifth day of illness [3]. Our proposed treatment course will start on the second or third day of illness and continue to the seventh or eighth day of illness, which is into the recovery phase. As this is the first study investigating statin therapy in dengue with a particular focus on safety, we propose a dose-escalation study, investigating 40 mg lovastatin versus placebo with a safety review after recruitment of the first 30 patients. If this review is satisfactory, we will increase the lovastatin dose to 80 mg and conduct a further safety review after the next 30 patients.

Methods/design

Design

This study is a randomised, placebo-controlled, double-blind trial investigating lovastatin therapy in Vietnamese adults with dengue infection. The trial will be conducted in two phases, with an escalation of dose between phases

if the results of an interim data review show no safety concerns within the first cohort of patients treated with the lower dose.

Patients will be followed for clinical and laboratory endpoints in hospital until study day 6 (or daily as outpatients from discharge to day 6) and reviewed at an outpatient visit on day 28.

Inclusion and exclusion criteria

All patients aged 18 or more presenting to the Hospital of Tropical Diseases, Ho Chi Minh City with a clinical suspicion of dengue, less than 72 hours of fever and a positive rapid test for dengue non-structural protein 1 (NS1) will be eligible for recruitment into the study. Exclusion criteria are: signs or symptoms suggestive of another acute infectious disease, alanine transaminase levels greater than 150 U/l, creatine kinase levels greater than 1000 U/l, liver cirrhosis, myopathy, current or use within past week of statins, pregnancy and lactation. In addition, patients taking medications contraindicated for use with statins, for example, isoniazid for treatment of tuberculosis, will be excluded.

Primary endpoint

The primary endpoint of this study is an evaluation of the safety and tolerability of lovastatin therapy in adult patients with dengue. Comparing the rates of adverse events between randomised treatment arms will assess this.

Secondary endpoints

The secondary endpoints of this study are fever clearance time (see definition), plasma viraemia (area under the log-transformed viraemia curve from enrolment until study day 6), platelet nadir between day 3 and 8 of illness, maximum haematocrit between day 3 and 8 of illness, percentage increase in haematocrit between day 3 and 8 of illness from baseline, maximum alanine transaminase (ALT) and creatine kinase (CK) recorded between day 3 and 8 of illness, lowest oxygen saturation recorded between day 3 and 8 of illness, number of patients in each group requiring colloid, and disease progression as defined by one or more of the following: (a) admission to the ICU, (b) diagnosis of shock (see definition), (c) diagnosis of severe bleeding (see definition), (d) development of encephalitis, (e) death. In addition, quality of life scores obtained using a visual analogue scale will be compared between treatment groups. The times from enrolment to the first sample with viraemia less than 1000 copies/ml and the first negative NS1 antigenaemia sample will be compared between treatment groups.

Definitions

1. Fever clearance time is the time from enrolment to the first time the temperature falls to <37.5°C and remains below this level for 48 hours.
2. Shock: cardiovascular decompensation requiring fluid resuscitation and considered to be due to plasma leakage.
3. Severe bleeding is clinically severe if it results in haemodynamic instability or requires fluid resuscitation or a blood transfusion. Any bleeding resulting in death and any intracranial bleed are considered severe.
4. Baseline haematocrit: the haematocrit value obtained at the day 28 follow-up visit, or (if the day 28 value is missing) the expected age- and sex-matched population value.

Randomization procedure

Randomization to either treatment arm will be in a 1:1 ratio. Randomization will be stratified according to the ward of recruitment. A randomization list will be prepared and maintained confidentially from study staff by the clinical trials pharmacist. Block randomization using variable block sizes will be used.

A chronological log of all enrolled patients will be maintained. Each enrolled patient will be assigned the next available sequential study code. The assigned number will correspond to a coded, sealed, pre-packaged bottle containing six doses of either lovastatin or visually matched placebo. Blinding will be maintained amongst the attending physicians and nurses by ensuring that the study drug and the placebo have an identical appearance. In addition the administration schedule will be identical.

Enrolment

Patients presenting to the out-patients department or in-patient wards with a clinical suspicion of dengue and less than 72 hours of fever will be identified to study staff. Study staff will approach these patients, check eligibility criteria and confirm dengue by NS1 rapid test. Eligible and consenting patients will have screening blood tests sent and provided these are satisfactory will be allotted the next consecutive study number and enrolled to the study.

Safety reviews

This trial will be conducted in two phases with an escalation of dose if the results of an interim data review show no safety concerns within the first cohort treated with the lower dose. The dose will begin at 40 mg per day in cohort 1 and may continue at 80 mg per day in cohort 2. A DSMB (data and safety monitoring board) review will take place when day 6 study data are

available from the 30th patient enrolled in cohort 1. If this review is satisfactory, the dose will be increased to 80 mg per day and recruitment into cohort 2 will commence. Further DSMB reviews will take place when day 6 study data are available from the 30th and 100th patients enrolled in cohort 2.

Treatment and drug dispensation

Patients will be assigned to one of two treatment arms:

- Active medicinal product: 40 mg (stage 1) or 80 mg (stage 2) lovastatin once daily for 5 days.
- Placebo: visually matched placebo once daily for 5 days.

The first dose will be given as soon as practically possible after enrolment.

An unblinded study pharmacist will prepare study drug bottles centrally and will distribute the bottles as required. Drugs will be stored in accordance with the manufacturers' recommendations in a secure area. Lovastatin and the placebo must be maintained below 25°C. All movements of study medication will be recorded. Both individual subject and overall drug accountability records will be kept up to date by the study staff.

Data collection

Clinical evaluation

Patients will be followed by a study physician daily until discharge, and all signs and symptoms recorded in the case report form. An ultrasound scan will be performed on day 6 of illness to detect signs of plasma leakage. Clinical management decisions will remain in the hands of the attending ward doctors. In the event that shock or any other serious complication develops, the patient will be transferred to the appropriate ICU. Details of all adverse events will be recorded on specific forms, together with an assessment as to whether the event is likely to be related to any treatment received, and all serious events will be reported promptly to the DSMB.

Quality of life will be measured by questionnaire and visual analogue scale daily.

Patients who are fit to discharge on or after study day 3 may be followed as an outpatient until study day 6. All patients will be asked for attend a follow-up visit for review after 4 weeks.

Laboratory evaluation

Haematocrit, platelet and total cholesterol measurements will be carried out daily or more frequently if clinically indicated. These tests will be repeated at the follow-up visit.

Renal and liver function tests, electrolytes and coagulation profiles, will be carried out at enrolment, 48 hours later, day 5 or 6 of illness and at the follow-up visit. If the ALT measured 48 hours after enrolment is greater than 250 U/l, the study drug will be discontinued. It should, however, be noted that hepatic dysfunction might be secondary to dengue infection and could be positively affected by statin therapy.

Conventional serological and virological tests will be used to confirm dengue infection and identify the infecting serotype. Plasma samples collected at daily intervals until discharge (and daily until day 6 if discharged before day 6) will be assessed for viraemia levels, NS1 levels, and concentrations of various pro- and anti-inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-10).

DNA will be extracted from residual blood samples and genotyped for genetic variants known to be associated with severe dengue, for example, *MICB* and *PLCE1* [22].

Statistical considerations

Sample size

This is an exploratory study focusing primarily on safety and there are no preliminary data regarding the effects of statins in dengue on which to base a sample size calculation. A target sample size of 300 patients in cohort 2, enrolled over two dengue seasons, was chosen based on clinical judgement and feasibility considerations. In a previous randomised trial in dengue, about 10% and 30% of patients experienced at least one serious adverse event or adverse event, respectively [23]. With 300 patients, we will have 80% power to detect an increase of 12% (from 10% to 22%) or 16% (from 30% to 46%) in the frequency of adverse events. In addition, this sample size ensures some power to explore the efficacy of statins. Specifically, this study will have 80% power to detect an increase in the rate of fever clearance by 40% due to statins. Based on simulations, we previously found that 30 patients give approximately 80% power to detect a 0.5 log10-copies/ml per day higher viraemia clearance, a reasonable estimate of what an effective anti-viral might achieve [24]. Thus, with 300 patients (a ten-fold higher sample size), we expect to be able to detect a (hypothetical) 0.16 log10-copies/ml per day higher viraemia clearance due to statins.

Statistical analysis

The primary analysis population will include all patients randomised to placebo from cohort 1 and all patients (regardless of treatment assignment) from cohort 2 according to the intention-to-treat principle. Owing to their low number, patients randomised to low-dose statins from cohort 1 will only be descriptively analysed.

The proportion of patients with any adverse events, any serious adverse events, or specific adverse events will be summarised and compared between the treatment arms based on Fisher's exact test.

Pre-defined secondary endpoints will be compared between the two treatment arms based on linear regression for continuous endpoints, logistic regression for binary endpoints, and Cox regression for time-to-event endpoints. For laboratory markers, comparisons will be adjusted for the pre-dose value of the respective marker and the day of illness at enrolment; plasma viraemia and NS1-endpoints will additionally be adjusted for dengue serotype.

The clinical, virological and immunological findings will also be correlated with *MICB* and *PLCE1* genotype using descriptive statistical methods.

A detailed statistical analysis plan will be finalised prior to unblinding the study data base.

Ethical considerations

Ethical approval

This protocol and both the patient information sheet and the consent form have been reviewed and approved by the Institutional Review Board of the Hospital for Tropical Diseases in Ho Chi Minh City, the Oxford Tropical Research Ethics Committee and the Ethics Committee of the London School of Hygiene and Tropical Medicine.

Informed consent and information sheet

All patients entering the study must give informed consent.

Withdrawal from the trial

Each participant has the right to withdraw from the study at any time. The reason for withdrawal will be recorded in the case report form.

Confidentiality

Patients who enter the trial will be given a unique identification number. This number will be used on both laboratory specimens and case report forms. The study wards and the research unit have the facilities to store study information securely.

The role of the data and safety monitoring board (DSMB)

An independent DSMB will be set up consisting of a biostatistician and senior clinical researchers with expertise in dengue and clinical trials. The DSMB will review the protocol and agree to a data review schedule and reporting requirements before the study commences. All data reviewed by the DSMB will be in the strictest confidence. A DSMB charter will outline its responsibilities and operation.

The DSMB will perform a safety review after day 6 data are available for the first 30 patients enrolled (Cohort 1: 40 mg lovastatin daily). This review will be based on a report created by the DSMB statistician containing unblinded summary tables of baseline demographics, serious adverse effects, adverse effects and disease progressions, as well as viraemia curves. If no safety concerns are identified, the lovastatin dose will be increased to 80 mg daily and recruitment will commence in cohort 2. Enrolment will continue in cohort 1 while awaiting the outcome of the DSMB review. Additional safety reviews will take place after the day 6 data are available for the 30th and 100th patients in cohort 2.

Discussion

Dengue remains a significant global public health challenge with costs to both the infected individual and the struggling health systems of dengue-endemic countries. At present, treatment is limited to supportive care. A therapeutic that can attenuate disease and prevent progression to severe disease would represent a highly significant advance with enormous benefits for both patients and health systems. There is growing observational evidence from the critical care field to suggest that statins may have a beneficial role in a number of conditions, such as sepsis, acute lung injury and pneumonia. Statins have beneficial pleiotropic effects, including stabilizing and anti-inflammatory effects on the endothelium. As endothelial dysfunction is so important in dengue pathogenesis, stabilizing effects at this site may prove to be clinically beneficial. In addition, statins have an excellent safety profile and a low cost. In view of this, we are optimistic about the potential benefit of lovastatin in dengue.

Trial status

We expect that patients will start being recruited to this trial in November 2012.

Abbreviations

ALT: alanine transaminase; CK: creatine kinase; DSMB: data and safety monitoring board; FDA: US Food and Drug Administration; IFN-γ: interferon-gamma; IL-6: interleukin-6; IL-10: interleukin-10; MICB: MHC class I polypeptide-related sequence B; NS1: non-structural protein 1; PLCE1: phospholipase C epsilon 1; TNF-α: tumour necrosis factor-alpha.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JW, NVVC, TTH, RP, CS, BW and JF conceived of the study and participated in its design. JW developed the study protocol with NTT, LTHT, NVH, MW, LM and NTPD. All authors read and approved the final manuscript.

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SECTION A – Student Details

Student	James Whitehorn
Principal Supervisor	Rosanna Peeling and Cameron Simmons
Thesis Title	The pathogenesis and clinical management of dengue

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Lancet Infectious Diseases
Please list the paper's authors in the intended authorship order:	James Whitehorn, Chau Van Vinh Nguyen, Lam Phung Khanh, Duong Thi Hue Kien, Nguyen Thanh Ha Quyen, Nguyen Thi Thanh Tran, Nguyen Thuy Hang, Nguyen Thanh Truong, Luong Thi Hue Tai, Nguyen Thi Cam Huong, Vo Thanh Nhon, Ta Van Tram, Jeremy Farrar, Marcel Wolbers, Cameron Simmons, Bridget Wills
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am the principal investigator of this trial and am the first and corresponding author of this manuscript. I oversaw the conduct of the trial and wrote the trial analysis plan. I wrote the

	first and all subsequent drafts of the manuscript.

Student Signature: _____ **Date:** 4/6/2015

Supervisor Signature: _____ **Date:** 4/6/2015

Lovastatin for the treatment of adult patients with dengue: a randomised, double-blind, placebo-controlled trial

Running title: Lovastatin for dengue

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Summary

Background: Dengue endangers billions of people in the tropical world. There is currently no available therapeutic. The severe manifestations reflect, in part, inflammatory processes affecting the vascular endothelium. In addition to lipid lowering, statins have pleiotropic effects that improve endothelial function. Epidemiological studies suggest outcomes from a range of acute inflammatory syndromes are improved in patients taking statins at presentation.

Methods: We performed a randomised, double-blind, placebo-controlled trial of five days of lovastatin in dengue. The trial was conducted in two phases – the first phase investigated 40mg lovastatin versus visually matched placebo in 30 patients, and the second phase investigated 80mg lovastatin versus visually matched placebo in 300 patients. Adult patients with less than 72 hours fever and a positive dengue NS1 rapid test were eligible for recruitment. The primary outcome was safety. The secondary outcomes were disease progression, fever clearance time, effect on plasma viremia, and quality of life.

Findings: The rates of adverse events were similar in both treatment arms. There was no difference in disease progression, fever clearance time, effect on plasma viremia, and quality of life scores between the treatment arms.

Interpretation: Our findings show that lovastatin was safe and well tolerated in adult patients with dengue. Although not powered to address efficacy, there was no evidence of a beneficial effect on dengue viremia or clinical manifestations of the disease. Our study suggests that continuing statin therapy in patients with dengue is safe.

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Introduction

Dengue exerts an enormous toll on countries of the tropical world, with an estimated 390 million infections annually.[1] While the majority of symptomatic patients recover after a short but often debilitating illness, a small proportion progress to severe disease, in particular development of an unusual vasculopathy characterized by endothelial dysfunction and a plasma leakage syndrome that may result in hypovolaemic shock.[2] Other less common complications include severe bleeding and severe liver/organ involvement.[3] Myalgia and arthralgia are prominent constitutional symptoms associated with dengue, and rhabdomyolysis resulting in renal failure is also occasionally reported.[4] Currently there is no licensed vaccine or therapeutic,[5] and health systems in endemic areas are frequently overwhelmed with patients during the dengue season.[2] It is clear there is a major unmet need for a therapeutic agent that can shorten the duration and severity of symptoms and/or prevent the development of complications.

Inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, also known as statins, have beneficial effects beyond their lipid-lowering properties, including specific effects on endothelial function.[6-10] These beneficial effects are thought to be partly mediated through statin inhibition of the mevalonate pathway, resulting in inhibition of isoprenoid formation and reduced expression of pro-inflammatory cytokines, thereby controlling migration of leucocytes to areas of endothelial inflammation.[11,12] Statins also result in increased nitric oxide bioavailability and thus may improve endothelial function by modulating the production of reactive oxygen species.[13] Endothelial dysfunction is increasingly being recognised as an important factor in the pathogenesis of atherosclerosis,[14,15] and these pleiotropic effects, together

with the lipid-lowering properties, likely explain the prognostic benefits of statin use in cardiovascular disease. There is thought to be some overlap between the inflammatory processes seen in atherosclerosis and sepsis, and several observational studies have suggested that prior statin therapy may be associated with improved outcomes in conditions such as sepsis and pneumonia.[16-20] As a result statins are now being investigated as potential adjunctive therapy for a variety of conditions in which endothelial dysfunction is thought to play a role, including sepsis and acute respiratory distress syndrome although, to date, their adjunctive role has not been substantiated in prospective trials.[21,22]

Although the detailed mechanisms underlying the microvascular endothelial dysfunction seen in severe dengue remain incompletely understood, we hypothesised that the endothelial stabilising effects of statins could favourably modulate the severity of the vasculopathy, especially if used early in the disease course. In addition, *in vitro* work has shown that lovastatin reduces dengue virion assembly, raising the possibility that statins may have some antiviral properties.[23,24] Furthermore, the low cost and ready availability of statins are attractive properties of this class of drug. However, although statins have a generally good safety profile, given the recognised association between statin use and hepatic and muscle dysfunction,[25-27] which are both common features of dengue, our primary objective was to assess the safety of a five-day course of lovastatin administered to adult patients early in the course of dengue illness. In addition, we investigated the potential effects of lovastatin on a variety of clinical and virological parameters.

Methods

Study design and participants

We performed a randomised, placebo-controlled, double-blind, dose escalating trial of lovastatin for adult patients with dengue.[28] In the first phase we assessed 40mg lovastatin versus placebo (30 patients), and in the second phase, conducted after an interim safety review, we assessed 80mg lovastatin versus placebo (300 patients). The study was conducted at two centres in Vietnam: the Hospital for Tropical Diseases in Ho Chi Minh City, and Tien Giang Hospital, My Tho City.

Ethical approval was obtained from the institutional review boards of the Hospital for Tropical Diseases and the Ministry of Health of Vietnam, the London School of Hygiene and Tropical Medicine, and the Oxford University Tropical Research Ethics Committee. The trial was registered with the ISRCTN registry (number: ISRCTN03147572).

The trial protocol has been published,[28] but briefly patients aged 18 or over, presenting within 72 hours of fever onset with an illness consistent with dengue, in whom a rapid test for dengue non-structural protein 1 (NS1) was positive (NS1 Ag-STRIP, Bio-Rad) were eligible for inclusion providing they gave written informed consent. Exclusions included signs or symptoms suggestive of another acute infectious disease, an alanine transaminase (ALT) level > 150 U/L, a creatine kinase (CK) level > 1000 U/L, a platelet count < 50 x 10^9/L, pregnancy or lactation, or a history of cirrhosis or myopathy. Patients were also excluded if they were currently taking statins or had taken them within the week prior to presentation, or were taking any medication contraindicated with statins.

Randomisation and masking

We randomly assigned enrolled patients in a 1:1 ratio to receive lovastatin (40mg (phase one), 80mg (phase two)) or placebo once daily for five days. Randomisation was stratified according to the ward of recruitment, using block randomisation with variable block sizes of four or six. The Vietnamese pharmaceutical company, DOMESCO, provided lovastatin and visually matched placebo without charge. The clinical trials pharmacist was the only person who had access to the computer-generated randomisation list; she pre-packaged bottles containing six doses of either lovastatin or visually matched placebo, including an extra emergency dose in case of vomiting. All other study staff were blinded to treatment allocation until after completion of the study and locking of the database. Each enrolled patient was assigned the next available study code that corresponded to a pre-packaged bottle of study drug. Clinical data were captured on structured case report forms that were then entered into a secure web-based database.

Study Procedures

Potential participants were identified either on the dengue wards or among patients attending the emergency department. Screening took place on the dengue ward and was streamlined to ensure completion within four hours. Upon enrolment history and detailed examination findings were documented by attending physicians specifically trained in all study procedures. Participants received the first dose of study drug as soon as possible after randomisation, with subsequent doses given once daily in the morning; all doses of study drug were directly observed. Standardized clinical information was recorded daily throughout the disease course by the trained physicians. These physicians were also responsible for all clinical management

decisions, but with the following caveats relating to worsening hepatitis, myositis or thrombocytopenia; if the ALT exceeded 250 U/L, the CK exceeded 1000 U/L, or the platelet count fell below 5 x 10^9/L the study drug was stopped. Adverse events and details of their severity and likely relatedness to the study drug were recorded in the case report form and on standardized forms. Serious adverse events were reported to the relevant institutional review boards and to an independent data and safety monitoring committee (DSMB).

Haematocrit, platelet and total cholesterol measurements were performed at enrolment (study day one), then daily to study day six, and thereafter as clinically indicated and at the follow-up visit four weeks after enrolment. Renal and liver function tests, electrolytes, and coagulation profiles were carried out on study days one, three, and five, then as clinically indicated and at the follow-up visit. An ultrasound scan was performed on illness day six to assess plasma leakage. Quality of life was measured using a visual analogue scale daily.

Serological and virological tests were used to confirm the dengue diagnosis and identify the infecting serotype, as outlined in Supplementary Text 1. Plasma samples obtained daily were assessed for viremia levels using an internally controlled, serotype-specific, real-time RT-PCR assay.[29] Serial samples from each patient were assayed at the same time.

The independent DSMB provided study oversight. Interim safety reviews were performed at the end of the first phase, and after the 30th and 100th patients were recruited in phase two.

Outcomes

This was an exploratory study focusing on safety, with the primary outcome defined as a comparison of the proportion of patients with any adverse events, and with any serious adverse events, between the treatment arms. The cut-offs described above for worsening hepatitis (ALT > 250U/L) and myositis (CK > 1000 U/L), and marked thrombocytopenia (platelet count < 5 x 10^9/L), defined certain prespecified adverse events, but in addition we graded all laboratory abnormalities as Grade 1-4 following the established CTCAE system. The secondary outcomes were: (1) disease progression as defined by admission to ICU, development of severe dengue (shock, severe bleeding or neurological involvement), or death; (2) fever clearance time; (3) the area under the $\log_{10}$-transformed curve (AUC) for plasma viraemia between days three and six of illness; and (4) the lowest value of the quality of life score captured on a visual analogue scale. All definitions used in the trial are detailed in Supplementary Text 1.

Additional exploratory outcomes, intended primarily to evaluate vascular leakage severity and other characteristic features of dengue, are described in Supplementary Text 1.

Statistical analysis

The planned sample size of 300 patients in phase two was based on medical and feasibility considerations as well as on power considerations for key endpoints. In a previous randomised trial in dengue, about 10% and 30% of participants experienced at least one serious adverse event (SAE) or adverse event (AE) respectively.[30] The planned sample size guaranteed a power of 80% to detect an increase of 12% in the SAE frequency or 16% in the AE frequency. More extensive power considerations are discussed in the published protocol.[28]

The two study cohorts were analysed separately. Cohort one was analysed descriptively. The main analysis included all patients in cohort two, following the intention-to-treat principle. The proportion of patients with adverse events were summarised and compared between the treatment arms using Fisher's exact test. The pre-defined secondary endpoints were compared between the treatment arms based on linear regression for continuous endpoints, logistic regression for binary endpoints and Cox regression for time-to-event endpoints. The main explanatory variable in the regression models was the treatment assignment, and all analyses were adjusted for day of illness at recruitment. Comparisons of laboratory markers were additionally adjusted for the pre-dose enrolment value, and also for serotype and immune status for plasma viraemia comparisons.

All analyses were performed with the statistical software R v3·1·3 (R Foundation for Statistical Computing, Vienna, Austria).[32]

Role of the funding source

The funder of the study and the drug manufacturer had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Patients were recruited into phase one in November and December 2012. Supplementary Figure 1 shows the trial profile with respect to screening, enrolment and withdrawals. The baseline characteristics at randomisation were similar in the two

study groups. Illness duration and adverse event rates were also similar in the two groups. These data are summarised in Supplementary Tables 1 and 2. All adverse events resolved fully.

Following the initial safety review by the DSMB, phase two commenced in April 2013 and completed recruitment in January 2015. Figure 1 shows the trial profile. Of the 515 subjects with a positive NS1 test who were assessed for eligibility, 339 underwent formal screening and 300 patients were enrolled and randomised. Elevated ALT and/or CK levels were the most common reasons for potential participants to fail screening.

The baseline characteristics were similar in the two study groups and are summarised in Table 1. Treatment was stopped prematurely in 17 patients in the placebo arm and 19 patients in the lovastatin arm. 11 patients in the placebo arm developed laboratory abnormalities on study day 3 where the protocol required the study drug to be stopped – ten with ALT elevations above 250 U/L and one with a CK elevation above 1000 U/L. 12 patients in the lovastatin arm had ALT elevations above 250 U/L that required the study drug to be stopped. However, in neither treatment arm were these laboratory derangements associated with clinical deterioration. The clinical reasons for stopping the study drug were concerns about AST elevations when the ALT level did not meet the stopping criteria.

Treatment allocation was unblinded in only one case (placebo arm, after completion of study drug), where a pregnancy test requested by the patient on study day six was positive; the pregnancy test at enrolment had been negative. Two patients in the lovastatin arm withdrew from the study within 24 hours (one self-discharged and one withdrew consent after the first dose of study drug); no adverse events were

documented and these participants were excluded from all analyses as no data were available. Data from other participants who withdrew from the study later are included in the analyses up to the time of withdrawal.

The number of adverse events did not differ significantly between the groups. 97/151 (64%) of patients in the placebo group and 82/149 (55%) of patients in the lovastatin group had a clinical and/or pre-specified laboratory adverse event (p=0·13). Eight patients in the placebo group and four patients in the lovastatin group experienced a serious adverse event (p=0·38). In addition 112/151 (74%) of patients in the placebo group and 110/149 (75%) of patients in the lovastatin group had at least one grade three or four laboratory adverse event identified (p=1) Details of these adverse events are summarised in Table 2. As expected, biochemical evidence for hepatic dysfunction was common, with a total of 14/151 (9%) and 16/149 (11%) in the placebo and lovastatin groups respectively exceeding the prespecified ALT cutoff of 250 U/L during the acute illness, including those participants in whom the study drug had been stopped early due to worsening hepatitis. In one patient in each group the CK level exceeded 1000 U/L at some time, but less marked CK elevations were common in both groups. No patient developed renal compromise at any time. There was no significant change in the coagulation profile during the study.

Of note, the serious adverse events in this study were all in the category of "prolonged hospitalisation". In 11 patients these prolonged hospital stays were for monitoring of abnormal laboratory tests (nine patients had hepatitis and two had thrombocytopenia) without clinical symptoms, and in one patient (in the placebo arm) the prolonged stay was for diarrhoea and persistent fever. All patients with serious adverse events recovered fully.

Very few patients progressed to severe dengue. Two patients in the placebo group developed hypovolemic shock and one patient in the lovastatin group was admitted to intensive care for close monitoring (OR: 0·51; 95%CI: 0·02-5·38; p=0·57). The fever clearance time did not differ significantly between the study groups (median time to fever clearance four days in both groups; HR: 0·93; 95%CI: 0·72-1·21; p=0·60)) (Table 3 and Supplementary Figure 2). Lovastatin had no observable effect on the dengue viremia kinetics or on the quality of life scores (Figure 2A and 2B).

There were no significant differences between the treatment arms in the various predefined exploratory endpoints (see Table 4), except in the peak percentage change in total cholesterol levels relative to enrolment values; a greater relative reduction was observed in the lovastatin group (30%) compared to the placebo group (23%) (p<0·0001)). Of note, markers for plasma leakage, including percentage haemoconcentration and presence of serosal effusions on ultrasound, were similar between the treatment arms.

Discussion

We hypothesized that the reported benefits associated with statin use in several observational studies of acute inflammatory syndromes might be relevant to dengue, a condition in which endothelial dysfunction is central to pathogenesis. In this randomised, double-blind, placebo-controlled trial in Vietnamese adults with dengue, we found that lovastatin was safe and well tolerated. In particular we found no evidence of adverse effects on hepatic or muscle dysfunction, both characteristic features of acute dengue as well as recognised, albeit rare, complications of statin therapy. However, we also found no evidence of a beneficial effect on any clinical or

virological endpoints.

While our study did not include pharmacokinetic analysis, we used 80 mg lovastatin daily, the upper end of the dose range for adults, and it is reasonable to assume that therapeutic concentrations were achieved as evidenced by the significantly greater reduction in cholesterol in the lovastatin group. The rates of clinical and laboratory adverse events were similar between the treatment arms. Rates of serious adverse events were also similar, and in all cases these events were classified as serious due to prolonged hospitalization for laboratory monitoring rather than on the basis of clinical deterioration. Of note, both liver and muscle abnormalities were observed frequently in both treatment arms, and were no more prevalent in the lovastatin arm; similar abnormalities identified during screening for the study indicate that these are common features of dengue, likely highlighted here by our particular focus on identifying safety issues related to statins.

Progression to severe dengue occurred infrequently in the study population (1%) and there was no difference in the rate of disease progression between the study groups. In view of the small number of events, it is possible that the study missed a small beneficial effect. The frequency of dengue shock syndrome is higher in children and hence children are the preferred patient population for investigation of drugs with immunomodulatory or endothelial stabilising properties.[34] However since we observed no differences in the magnitude of haemoconcentration or in the presence of serosal effusions on ultrasound between the study groups in this adult trial, we do not consider there to be a compelling case for a trial of statin therapy in children at present. We also found no other evidence of the anti-inflammatory properties of statins, in particular fever clearance times were unaffected by statin therapy.

However, more sensitive methods of assessing endothelial function are needed, and could prove useful for future trials of dengue therapeutics. Various noninvasive techniques have been developed and ongoing observational research is assessing the correlation between measurements derived from these techniques and more conventional methods for identifying plasma leakage in dengue.[35,36]

Another limitation of our study is that we obtained relatively sparse data on DENV-2 and DENV-3 due to the dominance of DENV-1 and DENV-4 during the study. While *in vitro* laboratory studies have suggested that statins may reduce dengue virion assembly, potentially through blocking of viral transport from the endoplasmic reticulum to the Golgi apparatus in infected cells, we found no evidence of an antiviral effect.[23,24] It is possible that the effects of statins may differ between the serotypes and it is interesting to note that the *in vitro* work that suggested a potential antiviral effect used DENV-2.

As in other recent trials of antiviral and immunomodulatory agents for dengue,[30,37-39] we administered the study drug within 72 hours of illness onset. However, it is possible that even initiating a therapeutic within this timeframe is already too late to modulate the disease pathways. Peak viraemia typically occurs earlier in the illness and is already waning by 72 hours.[40] In addition, there is evidence that endothelial dysfunction is established by 72 hours.[41,42] Thus to favourably modulate the outcome of infection even earlier intervention may be necessary, a significant practical hurdle in many dengue-endemic regions as patients rarely present to health services so early. It also raises the question of whether dengue therapeutic development should focus more on identifying chemoprophylactic agents that could be used in those at most risk of severe disease, and also on optimising supportive care for those who do develop

severe disease. The ability to distinguish patients at risk of severe disease early in the course of their infection could lead to a more targeted approach to the conduct of clinical trials, by focusing on the subset of patients most likely to benefit. The use of dengue human infection models may also allow streamlining of potential therapeutic candidates through the early rejection of agents that do not demonstrate pre-identified efficacy signals.[43,44]

Following the initial hope raised by the observational studies suggesting that prior statin therapy was associated with improved outcomes in a number of inflammatory conditions, several prospective trials investigating statins as adjunctive therapy have recently been published;[21,22,45] similar to our study findings, none of these trials showed a beneficial role for *de novo* statin therapy. Despite these negative results, this "discovery-in-practice" approach remains important given the high pre-clinical failure rate observed with traditional drug discovery. A dengue therapeutic or chemopropylactic agent remains a desirable component of a dengue control package and, given the ongoing expansion of dengue's geographic footprint, both drug development approaches need to be considered.

In conclusion we have shown that lovastatin therapy is safe in adult patients with dengue although we did not demonstrate any clinical or virological benefit. While the study findings do not endorse adjunctive statin therapy for dengue, the data provide reassurance to clinicians about the safety of continuing established statin therapy in patients who develop dengue. This is a situation that is likely to become increasingly common as the epidemiology of dengue changes, especially in contexts where older age-groups with associated co-morbidities are more likely to be affected, for example in Singapore.[33] Our study further underlines the importance of identifying risk factors

for dengue progression, thus potentially allowing a more personalised approach to dengue clinical trials. Early diagnosis and recognition of severe features together with good supportive care remain central to clinical management.

Footnotes

Author contributions

Literature search: JW, CVVN, JF, CPS, BW

Study design: JA, CVVN, LPK, NTT, LTHT, NTCH, VTN, TVT, JF, MW, CPS, BW

Data collection/study logistics: DTHK, NTHQ, NTTT, NTH, NTT, LTHT, NTCH, VTN, TVT

Data analysis: JW, LPK, DTHK, NTHQ, MW

Data interpretation: JW, CVVN, LPK, DTHK, NTHQ, MW, CPS, BW

Writing: JW, CVVN, LPK, JF, MW, CPS, BW

Conflict of interest statement

JW's institution receives consulting fees on his behalf from Janssen pharmaceuticals related to their work on dengue antiviral development. CPS is an occasional paid consultant to GSK, Tibotec and Unither Virology on the development of antiviral drugs for dengue. BW receives consulting fees for serving on the Data Monitoring Committee for the Takeda Dengue Vaccine Trials program.

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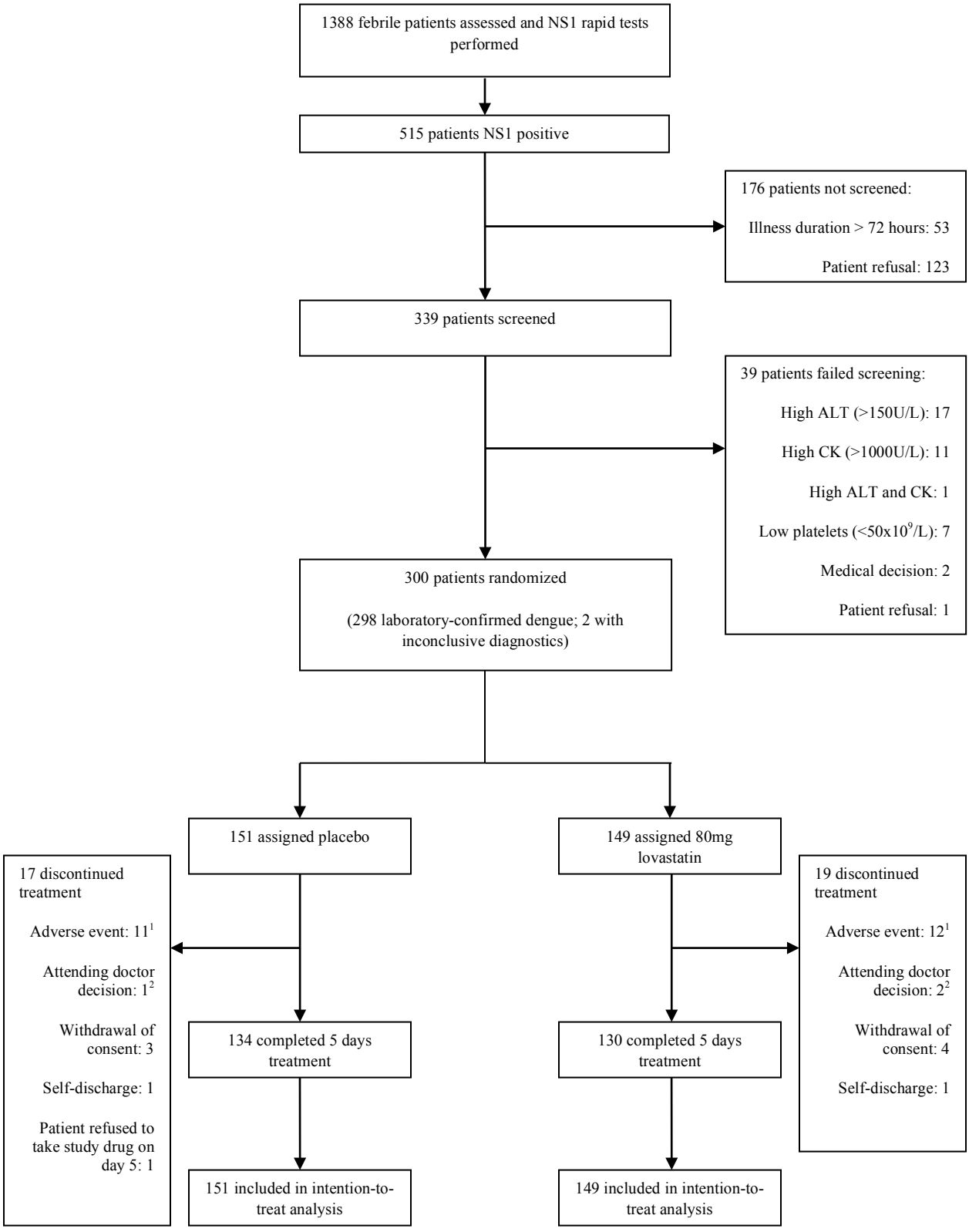
Figure legends

Figure 1: Study enrolment and follow-up for phase two of the study.

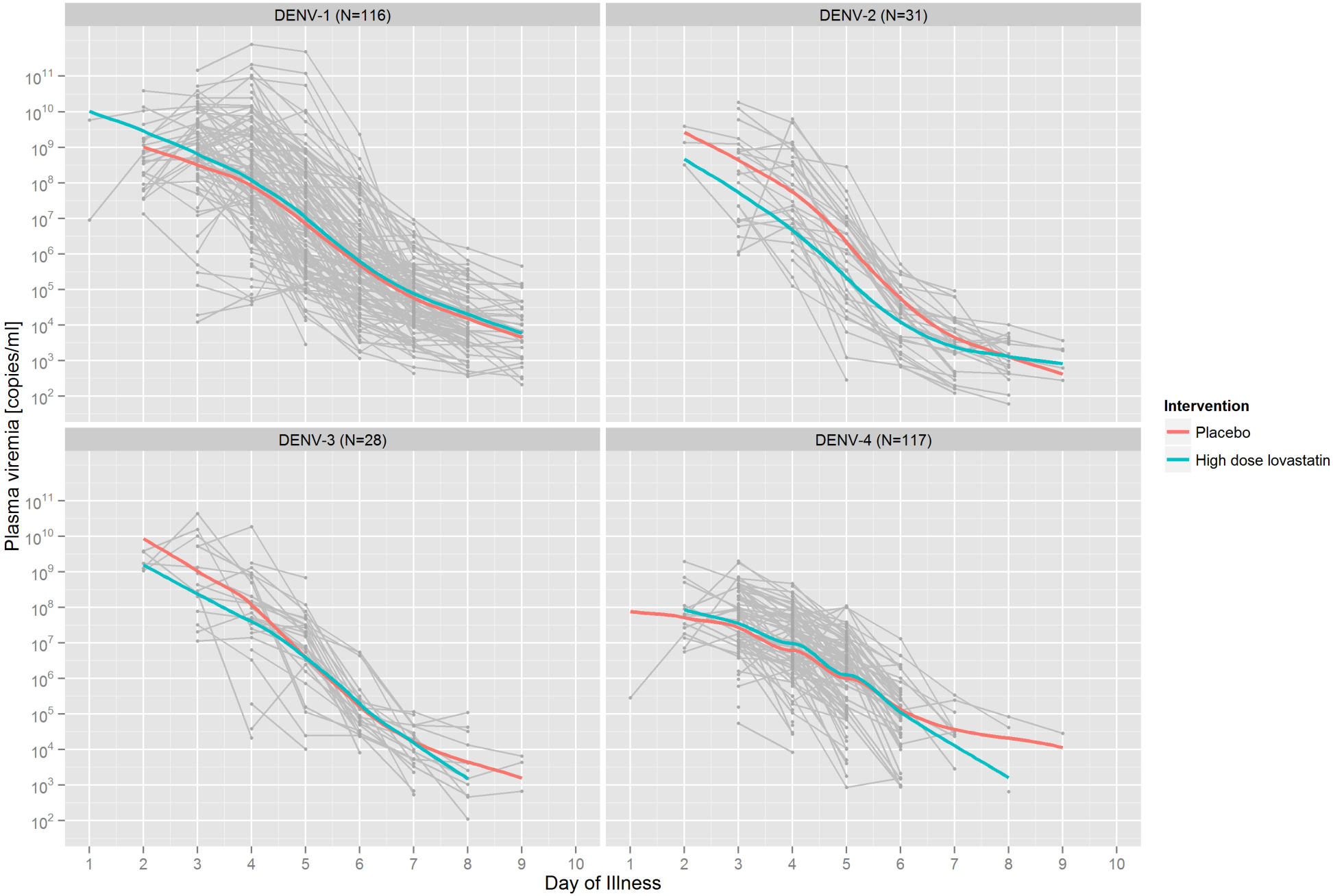
Figure 2: Viraemia levels in lovastatin and placebo-treated patients. Figure 2A shows the viraemia levels by serotype, and Figure 2B shows the viraemia by immune status. The coloured lines in each graph correspond to loess scatterplot smoothers derived from local polynomial regression fitting to data from each treatment arm. The grey background lines represent individual patient data. There was no significant difference in viremia kinetics between the treatment groups.

Supplementary Figure 1: Study enrolment and follow-up for phase one of the study.

Supplementary Figure 2: Kaplan-Meier of fever clearance time in lovastatin and placebo-treated patients. The proportions of patients who remained febrile during inpatient monitoring (study days one to five) are shown. There was no significant difference in fever clearance time between the treatment groups


1388 febrile patients assessed and NS1 rapid tests performed
515 patients NS1 positive
176 patients not screened:
Illness duration > 72 hours: 53
Patient refusal: 123
339 patients screened
39 patients failed screening:
High ALT (>150U/L): 17
High CK (>1000U/L): 11
High ALT and CK: 1
Low platelets (<50x10^9/L): 7
Medical decision: 2
Patient refusal: 1
300 patients randomized
(298 laboratory-confirmed dengue; 2 with inconclusive diagnostics)
151 assigned placebo
149 assigned 80mg lovastatin
17 discontinued treatment
Adverse event: 11[1]
Attending doctor decision: 1[2]
Withdrawal of consent: 3
Self-discharge: 1
Patient refused to take study drug on day 5: 1
19 discontinued treatment
Adverse event: 12[1]
Attending doctor decision: 2[2]
Withdrawal of consent: 4
Self-discharge: 1
134 completed 5 days treatment
130 completed 5 days treatment
151 included in intention-to-treat analysis
149 included in intention-to-treat analysis

1. Exceeded protocol-defined laboratory thresholds for ALT and CK
2. Doctors' decision based on AST elevations (when ALT threshold remained below the stopping threshold)


DENV-1 (N=116)
DENV-2 (N=31)
DENV-3 (N=28)
DENV-4 (N=117)
Plasma viremia [copies/ml]
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10
Day of Illness
Intervention
Placebo
High dose lovastatin

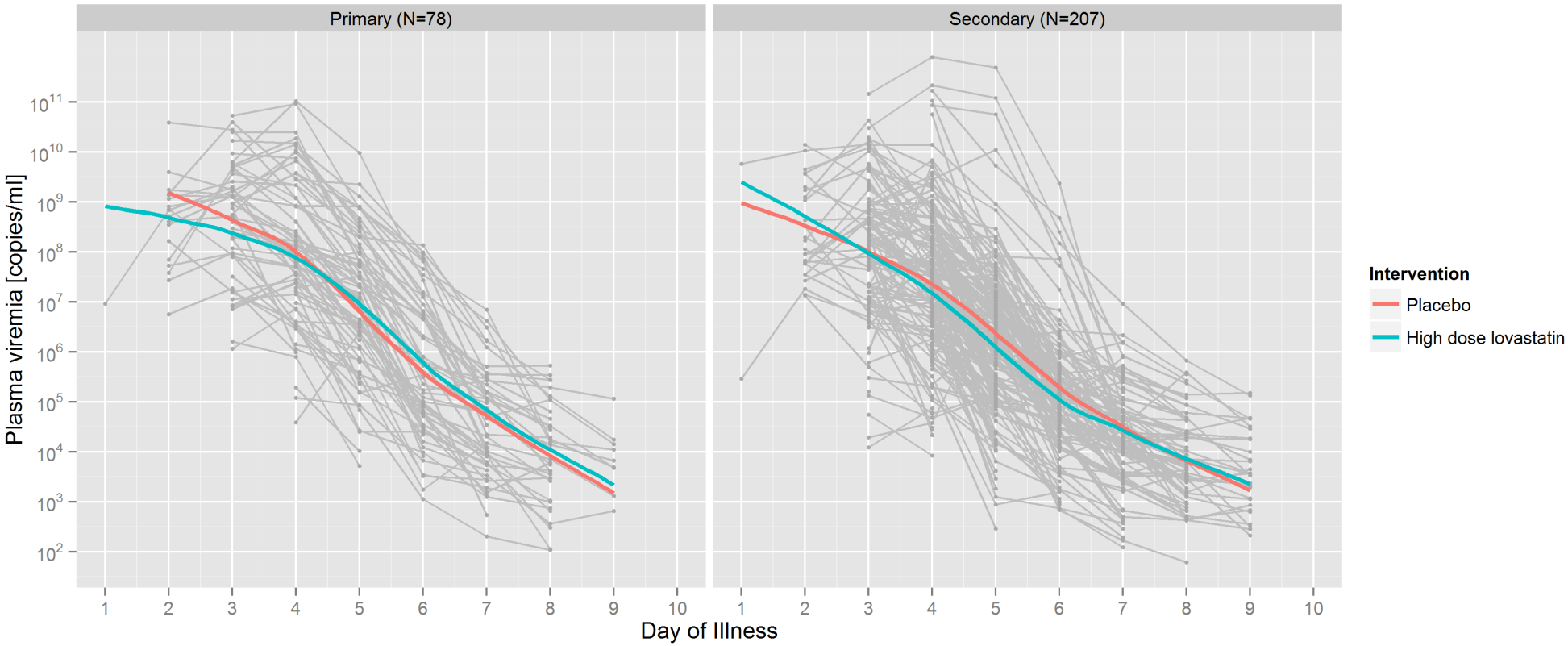

Primary (N=78)
Secondary (N=207)
Plasma viremia [copies/ml]
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10
Day of Illness
Intervention
Placebo
High dose lovastatin

Table 1: Baseline characteristics of the patients in phase two of the study

	Placebo (n=151)	80mg Lovastatin (n=149)
Age (years)	27 (21-33)	25 (21-34)
Sex		
Male	66 (44%)	65 (44%)
Female	85 (56%)	84 (56%)
Temperature (°C)	38·2 (37·7, 38·5)	38·2 (37·7, 38·5)
Day of illness at enrolment		
1	1 (1%)	2 (1%)
2	28 (19%)	20 (13%)
3	65 (43%)	64 (43%)
4	57 (38%)	63 (42%)
DENV serotype		
1	63 (42%)	53 (36%)
2	16 (11%)	15 (10%)
3	13 (9%)	15 (10%)
4	53 (35%)	64 (43%)
Negative	6 (4%)	2 (1%)
Immune status		
Probable primary	35 (23%)	43 (29%)
Probable secondary	111 (74%)	96 (64%)
Inconclusive	5 (3%)	10 (7%)
Plasma viraemia (log_{10} copies/mL)	7·9 (7·1, 8·9)	7·8 (6·9, 8·6)
Haematocrit (%)	41·8 (39·2, 44·8)	41·0 (38·7, 44·2)
Platelet count ($x10^9$/L)	111 (90, 140)	112 (89, 149)
WBC ($x10^6$/dL)	3·2 (2·3, 4·0)	3·1 (2·3, 3·9)
AST (U/L)	51 (36, 79)	45 (32, 72)
ALT (U/L)	38 (25, 67)	31 (23, 60)
CK (U/L)	95 (67, 153)	92 (67, 123)
Cholesterol (mmol/L)	3·8 (3·3, 4·3)	3·8 (3·2, 4·3)

The summary statistic is the absolute count (%) for categorical variables and median (inter-quartile range) for continuous variables. There were 9 patients with negative plasma viraemia at baseline; however, DENV serotype could be determined in one of them based on subsequent plasma viraemia measurements. DENV = dengue virus, WBC = white blood cell count, AST = aspartate transaminase, ALT = alanine transaminase, CK = Creatine kinase

Day of illness was calculated based on the actual date of fever onset (Supplementary Text 1) meaning some patients who were within 72 hours of fever onset were classified as being on day four of illness

Table 2: Primary Outcomes - Adverse Event Details

	Placebo (n=151)	80mg Lovastatin (n=149)[4]	P-value
Adverse events (clinical and/or prespecified laboratory events)			
Any	97 (64%)	82 (55%)	0·13
Abdominal pain	4 (3%)	5 (3%)	0·75
Diarrhoea	39 (26%)	29 (19%)	0·22
Vomiting	27 (18%)	22 (15%)	0·53
Bleeding	51 (34%)	38 (26%)	0·13
Muscle pain	7 (5%)	7 (5%)	1
Worsening hepatitis[1]	14 (9%)	16 (11%)	0·70
Worsening myositis[2]	1 (1%)	1 (1%)	1
Marked thrombocytopenia[3]	1 (1%)	1 (1%)	1
Serious adverse events			
Any	8 (5%)	4 (3%)	0·38
Diarrhoea	1 (1%)	0	1
Hepatitis	6 (4%)	3 (2%)	0·50
Thrombocytopenia	1 (1%)	1 (1%)	1
Grade 3 & 4 laboratory adverse events			
Any	112 (74%)	110 (74%)	1
High ALT (> 200 (M) or 185 (F) U/L)	65 (43%)	65 (44%)	1
High AST (> 200 (M) or 185 (F) U/L)	65 (43%)	65 (44%)	1
High creatine kinase (> 570 U/L)	9 (6%)	6 (4%)	0·60
Low platelet count (< 50 X 10^9 /L)	89 (59%)	84 (56%)	0·73
Low sodium (< 120 mmol/L)	1 (1%)	0	1
Low white cell count (< 1 X 10^9 /L)	1 (1%)	3 (2%)	0·37

P-values derived from Fisher's exact test.

1. Worsening hepatitis defined as ALT > 250 U/L
2. Worsening myositis defined as CK > 1000 U/L
3. Marked thrombocytopenia defined as a platelet count that led to clinical concern. In the cases here it represented a low platelet count that required a prolonged hospital stay for observation. Conventionally in Vietnam patients are kept in hospital until the platelet count is above 50 x 10^9/L.
4. Two patients withdrew on day one in the lovastatin arm meaning the outcome data was not available

Table 3: Secondary Outcomes

Outcome	Placebo (n=151)	80mg Lovastatin (n=149)[1]	Effect (95%CI); p-value
Disease progression	2 (1·3%)	1 (0·7%)	0·53 (0·02, 5·57); p=0·592
Time to fever clearance (days)	4 (3, 5)	4 (3, 5)	0·93 (0·72, 1·21); p=0·604
AUC of viremia between days 3 & 6 of illness (log_{10} copies/mL x days)	16·8 (12·5, 21·6)	16·1 (11·5, 20·2)	-0·17 (-0·88, 0·54); p=0·639
Minimum quality of life score	50 (40, 60)	50 (50, 60)	1·07 (-1·33, 3·46); p=0·381

Summary statistic is events (%) for categorical variables and median (IQR) for continuous data. Median (IQR) for time to fever clearance was based on Kaplan-Meier estimation.

Outcomes of two patients in the lovastatin arm were not available due to early withdrawal. In addition, 3 patients (1 in the lovastatin arm) were excluded from the analysis of fever clearance due to having already defervesced at baseline, 9 patients (2 in the lovastatin arm) were excluded from the analysis of viremia as the initial DENV RT-PCR was negative, 4 patients (2 in the lovastatin arm) were excluded from the analysis of quality of life due to missing data.

The p-value and CI for disease progression were derived from logistic regression and the effect is the odds ratio. The p-value and CI for the AUC of viraemia and minimum quality of life score were derived from linear regression and the effect is the absolute mean difference. The p-value and CI for fever clearance were derived from Cox regression and the effect is the hazard ratio.

All analyses were adjusted for day of illness at baseline. In addition, analysis for fever clearance was adjusted for baseline temperature, analysis for minimum quality of life was adjusted for baseline quality of life, and analysis for AUC of viremia was adjusted for baseline log10 viremia, dengue serotype and immune status.

1. Two patients in the lovastatin arm withdrew on day one of the study meaning the outcome data were not available

Table 4: Exploratory Outcomes

Outcome	Placebo (n=151)	80mg Lovastatin (n=149)[1]	Effect (95%CI); p-value
Platelet nadir (between day 3 & 8 of illness) ($x10^9$/L)	43 (24, 64)	44 (25·5, 71)	2·47 (-3·98, 8·91); p=0·45
Peak haematocrit (between day 3 & 8 of illness) (%)	46 (42·8, 49·3)	44·3 (42, 49)	-0·55 (-1·21, 0·11); p=0·10
Peak percentage change in haematocrit from baseline (between day 3 & 8 of illness) (%)	9·72 (4·46, 15·2)	8·93 (5·01, 12·7)	-1·29 (-2·87, 0·29); p=0·11
Ascites on ultrasound between day 5 & 7 of illness	23 (16%)	17 (12%)	0·69 (0·35, 1·37); P=0.29
Pleural effusion on ultrasound between day 5 & 7 of illness	14 (10%)	11 (8%)	0·79 (0·34, 1·81); P=0.58
Peak ALT (between day 3 & 8 of illness) (U/L)	110 (63·5, 183·8)	88·5 (46, 184·8)	6·02 (-27·56, 39·60); p=0·72
Peak CK (between day 3 & 8 of illness) (U/L)	125 (79·5, 199)	108 (70, 200)	55·76 (-84·48, 196·01); p=0·44
Peak ALT (during study) (U/L)	140 (81·8, 221·8)	133 (67·3, 269)	16·74 (-19·99, 53·47); p=0·37
Peak CK (during study) (U/L)	125 (79·5, 199)	116 (72, 200)	56·81 (-83·43, 197·04); p=0·43
Colloid requirement	1 (0·66%)	0 (0%)	NA
Peak viremia (during study) (log_{10} copies/mL)	8·04 (7·15, 9·16)	8·04 (7·12, 8·78)	0·03 (-0·09, 0·14); p=0·63
Undetectable viremia achieved	70 (49·3%)	87 (60·8%)	1·36 (0·98, 1·89); p=0·07
AUC of viremia during study (log_{10} copies/mL x days)	26·2 (18·2, 31·9)	22·7 (17·6, 30)	-0·54 (-1·64, 0·57); p=0·34
Total number of days in hospital	8 (7, 8)	8 (7, 8)	0·02 (-0·25, 0·28); p=0·89
Peak percentage change in cholesterol from baseline (during study) (%)	-23·1 (-29·4,-15·4)	-30·4 (-38·4,-23·6)	-8·49 (-11·34,-5·63); p=<0·0001

Summary statistic is events (%) for categorical variables and median (IQR) for continuous data.

Treatment effect for colloid requirement cannot be estimated as there was only 1 case in the placebo group who required colloid.

The p-values and CIs for ascites and pleural effusion on ultrasound were derived from logistic regression with adjustment for day of illness at baseline, the effects are odds ratio.

The p-values and CIs for all laboratory variables were derived from linear regression with adjustment for baseline value and the day of illness at baseline, and the effect is an absolute mean difference. Analyses for plasma viremia (peak viremia, AUC of viremia) were additionally adjusted for dengue serotype and immune status.

The reported comparison for “undetectable viremia achieved” was based on a Cox regression of the time from enrolment to the first undetectable viremia with adjustment for baseline viremia, the day of illness at baseline, dengue serotype and immune status, and the effect is a hazard ratio.

1. Two patients in the lovastatin arm withdrew on day one of the study meaning outcome data were not available for these patients

Definitions used in Lovastatin for Dengue Trial

Clinical Definitions

SHOCK: cardiovascular decompensation (indicated by tachycardia, cool peripheries and narrowing of the pulse pressure to less than 20 mmHg) requiring fluid resuscitation and thought to be due to plasma leak.

SEVERE BLEEDING: Bleeding was defined as clinically severe if it resulted in haemodynamic instability and required fluid resuscitation or a blood transfusion. In addition, any bleed that required an intervention to control the bleeding (e.g. nasal packing) was classified as severe. Any intracranial bleed or bleed that resulted in death were classified as severe.

CNS INVOLVEMENT: This was defined as any alteration of consciousness (GCS<15), occurrence of convulsions or any focal neurological deficit even if transient.

DISEASE PROGRESSION: This was present if any of the following occurred: (1) transfer to ICU, (2) shock, (3) severe bleeding, (4) CNS involvement, or (5) death.

FEVER CLEARANCE TIME: This was defined as the time (days) from enrolment to the first time the temperature fell to < 37.5 ^{o}C and remained below this level for 24 hours, or less than 24 hours if the patient was well enough to be discharged home.

DAY OF ILLNESS: This was calculated using the actual date of illness onset and the date of enrolment. This definition meant that some patients who were within 72 hours of fever onset were classified as being on day four of illness at enrolment.

Laboratory Definitions

DENGUE DIAGNOSIS: Serological responses were detected using immunoglobulin M (IgM) and immunoglobulin G (IgG) antibody-capture enzyme-linked immunosorbent assays (Panbio, Australia). DENV plasma viremia levels were measured by a validated quantitative RT-PCR assay. A patient was deemed to have laboratory-confirmed dengue if DENV was detected using the RT-PCR assay or if there was evidence of IgG or IgM seroconversion in paired samples.

All patients had a positive NS1 rapid test at screening. If a patient had a negative RT-PCR result and inconclusive serology they were deemed to have an inconclusive laboratory diagnosis – this occurred in two patients (both in the placebo arm). Nine patients (two in the lovastatin arm) had negative RT-PCR results – these patients were excluded from the analysis of viremia kinetics.

DENGUE IMMUNE STATUS: We classified serological profiles as "probable secondary" when >22 Units of IgG (Panbio's recommended cut-off for a positive result) were detected in either acute or early convalescent samples. If acute and early convalescent samples were IgM positive but IgG negative then we classified as "probable primary". Where IgM or IgG tests results were equivocal or inconsistent we classified the serological profile as "indeterminate".

LABORATORY ADVERSE EVENTS: In additon to the prespecified cutoffs for particular parameters (ALT, CK and platelet count) laboratory adverse events were also graded according to the values in the following table, following the convention of the CTCAE guidelines.[1]

	Grade 1	Grade 2	Grade 3	Grade 4
Haematological				
Haemoglobin	10g/dl – 12.5g/dl	8 – 9.9 g/dl	6.5 –7.9g/dl	<6.5 g/dl
White cell count	2 – 2.94 K/µl	1.0 - 1.99 K/µl	<1.0 K/µl	
Platelets	75 – 200 K/µl	50 – 74 K/µl	20 - 49 K/µl	<20 K/µl
Biochemical				
Sodium (low)	130 – 135 mmol/L	120 - 129 mmol/l	<120 mmol/l	-
Sodium (high)	146 – 150 mmol/L	151 – 155 mmol/L	156 – 160 mmol/l	>160 mmol/l
Potassium (low)	3.0 – 3.5 mmol/l	2.5 – 2.9 mmol/l	<2.5 mmol/l	
Potassium (high)	5 – 5.5 mmol/l	5.6 – 6.0 mmol/l	6.1 – 7.0 mmol/l	>7.0 mmol/l
Creatinine	Male 121 – 180 µmol/L Female 101 – 150 µmol/L	Male 181 – 360 µmol/L Female 151 – 300 µmol/L	Male > 360 µmol/L Female > 300 µmol/L	-
Total Bilirubin	18 – 27 µmol/L	28 – 51 µmol/L	52 – 170 µmol/L	> 170 µmol/L
AST	Male 41 – 120 U/L Female 38 – 111 U/L	Male 121 – 200 U/L Female 112 – 185 U/L	Male 201 – 400 U/L Female 186 – 370 U/L	Male > 400 U/L Female >370 U/L
ALT	Male 41 – 120 U/L Female 38 – 111 U/L	Male 121 – 200 U/L Female 112 – 185 U/L	Male 201 – 400 U/L Female 186 – 370 U/L[1]	Male > 400 U/L Female > 370 U/L

Creatine kinase	191 – 285 U/L	286 – 570 U/L	>570 U/L	-
Cholesterol	5.21 – 7.75 mmol/L	7.76 – 10.34 mmol/L	> 10.34 mmol/L	-
Prothrombin time	13.4 – 20 seconds	20.1 – 33 seconds	>33 seconds	-

Exploratory endpoints

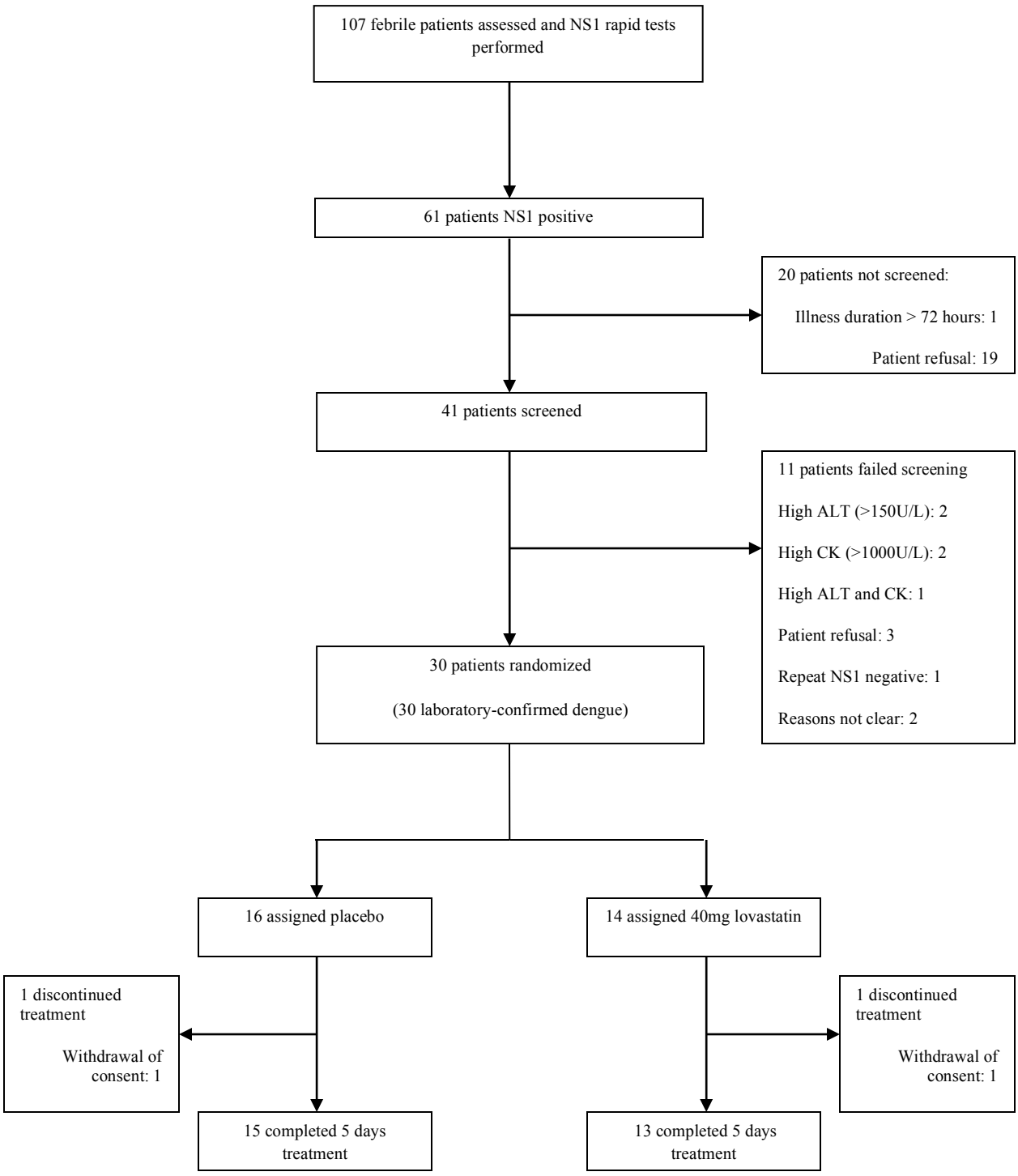
The exploratory outcomes included the following laboratory values observed between days three and eight of illness (i.e. during the time the study protocol required a specific schedule of investigations, when dengue complications typically occur): platelet nadir, maximum haematocrit, peak percentage increase in haematocrit from baseline, and maximum ALT and CK. We also assessed the maximum recorded ALT and CK observed during the whole illness episode, recognising that some laboratory derangements occur later in the evolution of dengue, and the peak percentage decrease in cholesterol during follow-up from baseline.

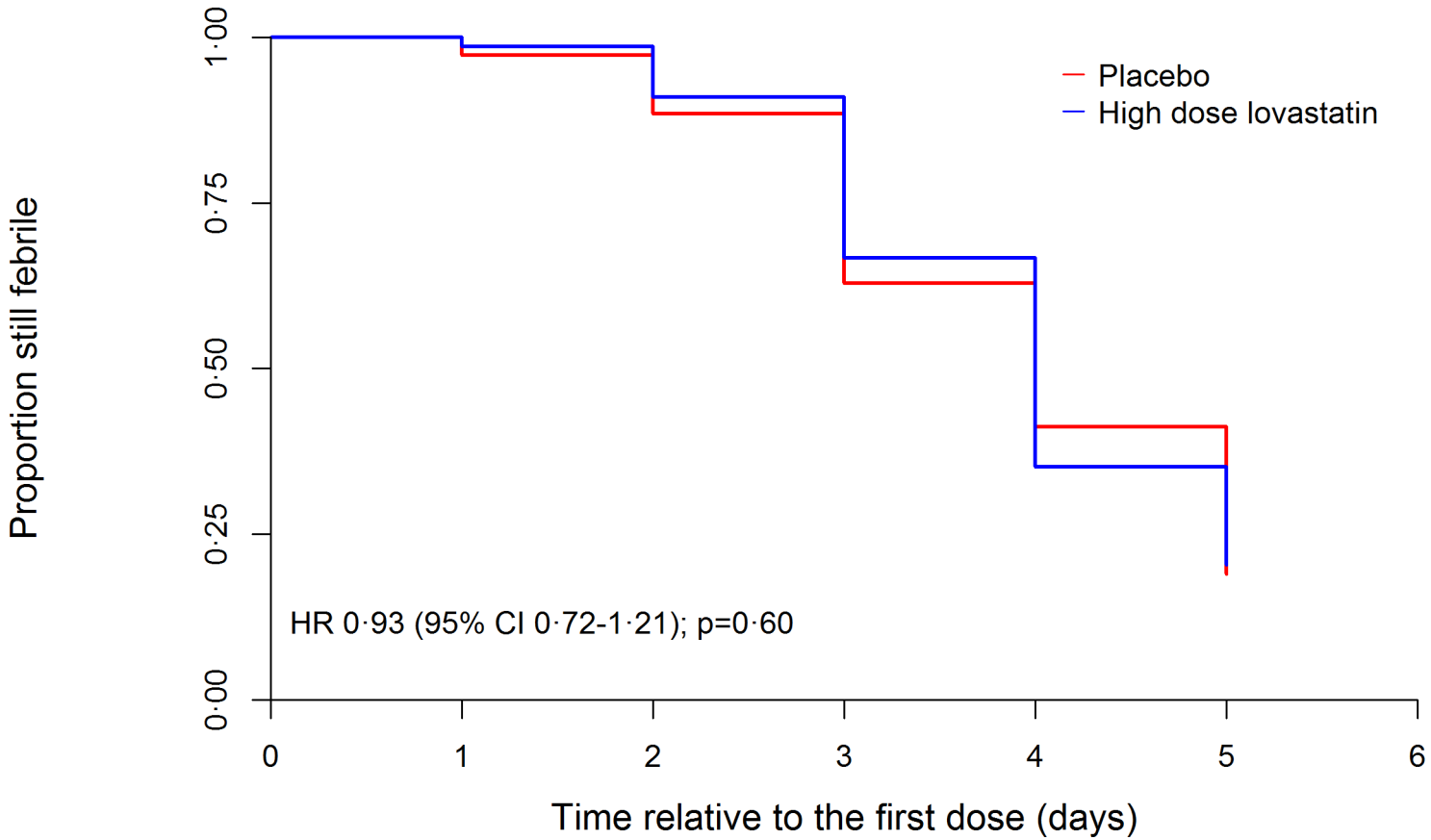
The numbers of patients requiring colloid therapy, and the total number of days in hospital, were also compared between the groups.

Additional pre-specified outcomes were the proportion of patients who developed an undetectable viremia, the peak viremia and the $\log_{10}$-transformed plasma viremia AUC for the whole study period.

References

1. US Department of Health and Human Services. Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0. National Institutes of Health; National Cancer Institute; 2009.


107 febrile patients assessed and NS1 rapid tests performed
61 patients NS1 positive
20 patients not screened:
Illness duration > 72 hours: 1
Patient refusal: 19
41 patients screened
11 patients failed screening
High ALT (>150U/L): 2
High CK (>1000U/L): 2
High ALT and CK: 1
Patient refusal: 3
Repeat NS1 negative: 1
Reasons not clear: 2
30 patients randomized
(30 laboratory-confirmed dengue)
16 assigned placebo
14 assigned 40mg lovastatin
1 discontinued treatment
Withdrawal of consent: 1
1 discontinued treatment
Withdrawal of consent: 1
15 completed 5 days treatment
13 completed 5 days treatment



No. at risk							
Placebo	149	149	144	131	93	61	0
High dose lovastatin	146	146	143	131	95	50	0

Supplementary Table 1: Baseline characteristics of patients in phase one of the study

	Placebo (n=16)	40mg Lovastatin (n=14)
Age (years)	23 (19-28)	26 (21-48)
Sex		
Male	8 (50%)	5 (36%)
Female	8 (50%)	9 (64%)
Temperature (°C)	39 (38·2, 39·5)	39 (38·6, 39·6)
Day of illness at enrolment		
1	0	0
2	6 (38%)	5 (36%)
3	8 (50%)	8 (57%)
4	2 (12%)	1 (7%)
DENV serotype		
1	7 (44%)	5 (36%)
2	2 (12%)	2 (14%)
3	4 (25%)	3 (21%)
4	3 (19%)	4 (29%)
Immune status		
Probable primary	3 (19%)	0
Probable secondary	12 (75%)	12 (86%)
Inconclusive	1 (6%)	2 (14%)
Plasma viraemia (log_{10} copies/mL)	8·6 (7·6, 9·3)	8.4 (7·8, 9·1)
Haematocrit (%)	40·4 (37·9, 42·7)	42·7 (40·0, 45·0)
Platelet count ($x10^9$/L)	130 (108, 171)	106 (87, 147)
WBC ($x10^6$/dL)	3·7 (3·4, 4·5)	3·2 (2·8, 4·1)
AST (U/L)	35 (26, 61)	39 (35, 90)
ALT (U/L)	23 (16, 47)	27 (22, 71)
CK (U/L)	78 (66, 116)	120 (91, 156)

The summary statistic is the absolute count (%) for categorical variables and the median (inter-quartile range) for continuous variables

Day of illness was calculated based on the actual date of fever onset (Supplementary Text 1) meaning some patients who were within 72 hours of fever onset were classified as being on day four of illness

Supplementary Table 2: Details of adverse events in phase one of the study

Characteristic		Placebo (n=16)	40mg Lovastatin (n=14)
Any adverse event			
	Yes	9 (56%)	6 (43%)
	No	7 (44%)	8 (57%)
Any serious adverse event[1]			
	Yes	3 (19%)	2 (14%)
	No	13 (81%)	12 (86%)

The summary statistic is the absolute count (%) for categorical variables and the median (inter-quartile range) for continuous variables.

1. All five serious adverse events were "prolonged hospitalisation". These included two patients with mucosal bleeding (one in each treatment arm), one patient with diarrhoea (in the lovastatin arm), one patient with hepatitis (in the placebo arm), and one patient who developed a urinary tract infection (in the placebo arm).

RESEARCH PAPER 5: PROPHYLACTIC PLATELETS IN DENGUE: SURVEY RESPONSES HIGHLIGHT LACK OF AN EVIDENCE BASE

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SECTION A – Student Details

Student	James Whitehorn
Principal Supervisor	Rosanna Peeling and Cameron Simmons
Thesis Title	The pathogenesis and clinical management of dengue

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	PLOS Neglected Tropical Diseases		
When was the work published?	2012		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	**Yes**	Was the work subject to academic peer review?	**Yes**

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Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am the first and corresponding author of this manuscript. I designed the questionnaire used in the survey. I wrote the first draft of the manuscript. With suggestions from the co-authors I wrote the subsequent versions

Student Signature: _____ **Date:** 4/6/2015

Supervisor Signature: _____ Date: 4/6/2015.

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PLoS NEGLECTED TROPICAL DISEASES

Prophylactic Platelets in Dengue: Survey Responses Highlight Lack of an Evidence Base

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Abstract

Dengue is the most important arboviral infection of humans. Thrombocytopenia is frequently observed in the course of infection and haemorrhage may occur in severe disease. The degree of thrombocytopenia correlates with the severity of infection, and may contribute to the risk of haemorrhage. As a result of this prophylactic platelet transfusions are sometimes advocated for the prevention of haemorrhage. There is currently no evidence to support this practice, and platelet transfusions are costly and sometimes harmful. We conducted a global survey to assess the different approaches to the use of platelets in dengue. Respondents were all physicians involved with the treatment of patients with dengue. Respondents were asked that their answers reflected what they would do if they were the treating physician. We received responses from 306 physicians from 20 different countries. The heterogeneity of the responses highlights the variation in clinical practice and lack of an evidence base in this area and underscores the importance of prospective clinical trials to address this key question in the clinical management of patients with dengue.

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Introduction

Dengue is globally the most important arboviral infection and threatens an estimated 2.5 billion people worldwide [1]. Thrombocytopenia is almost universally observed in dengue infection [2]. This results from both reduced production and increased destruction of platelets [3–5]. It is thought that severe thrombocytopenia correlates with disease severity and may contribute to the risk of developing haemorrhage [6,7]. The 2009 WHO dengue guidelines do not advocate the use of prophylactic platelet transfusions, whereas the 2011 regional WHO guidelines for South East Asia suggest prophylactic platelets may be considered in those with a platelet count less than 10×10^9/L [8,9]. Some dengue-endemic countries support the use of prophylactic platelet transfusions to prevent haemorrhage in patients with thrombocytopenia, for example India ($<10\times10^9$/L), whereas others, such as Brazil, do not [10,11]. However platelet transfusions are costly, potentially dangerous and their use in dengue lacks an evidence base [12–15].

Methods

We conducted a survey among physicians directly involved in the care of dengue patients in order to determine how platelets are used in the clinical management of dengue. The majority of respondents were practicing physicians in dengue-endemic areas. The exceptions to this were respondents from Africa, where dengue is emerging, and the UK where the respondents were infectious disease physicians who regularly see patients who have recently travelled to dengue-endemic areas. A questionnaire containing nine case histories and an additional question about prophylactic platelet transfusion thresholds was emailed to physicians with experience in managing dengue patients and known to us. Respondents were specifically asked that their

Author Summary

A low platelet count is a common feature of dengue infection. It is thought that the platelet count correlates with the severity of the infection and may contribute to the risk of developing haemorrhage, a well-recognised complication of dengue. As a result of this platelet transfusions are used in some settings to reduce the risk of haemorrhage. There is currently no evidence to support this practice, and platelet transfusions are costly and sometimes harmful. We conducted a survey assessing the use of platelets in dengue. Respondents were all physicians involved with the treatment of patients with dengue. Respondents were asked that their answers reflected what they would do if they were the treating physician. We received 306 responses from 20 different countries. The striking feature of the survey responses was the heterogeneity of approaches to the use of platelets in dengue. These findings highlight the variation in clinical practice and lack of an evidence base in this area and underscore the importance of conducting prospective clinical trials to address this key question in dengue clinical management.

responses reflect what they would do if they were the treating physician. Email recipients were invited to further disseminate the questionnaire within their own clinical networks. The complete list of questions is available as a supplementary file (Questionnaire S1).

The case histories were based on real clinical cases seen at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam. Four case histories describe patients with clinically non-severe dengue but varying levels of thrombocytopenia. Case 1 describes an 18-year-old female with platelets of 23×10^9/L and no bleeding. Case 2 describes a 28-year-old male with platelets of 29×10^9/L. He had no bleeding but a past history of a perforated peptic ulcer. Case 3 describes a 29-year-old female with a rapid fall in platelets to 22×10^9/L. She had no bleeding. Case 4 describes a 30-year-old male with platelets of 3×10^9/L and no bleeding. Five case histories describe patients with different manifestations of severe dengue associated with varying levels of thrombocytopenia. Case 5 describes a 19-year-old male with platelets of 18×10^9/L. He had dengue hepatitis but no bleeding. Case 6 describes a 20-year-old female with platelets of 17×10^9/L. She had suspected dengue encephalitis but no bleeding. Case 7 describes a 24-year-old male with platelets of 31×10^9/L. He had hepatic failure thought to be secondary to dengue but no bleeding. Case 8 describes a 23-year-old female with platelets of 8×10^9/L. She had shock, epistaxis and vaginal bleeding. Case 9 describes a 23-year-old male with platelets of 33×10^9/L. He had shock and mucosal bleeding. The final question aimed to determine thresholds at which a physician would consider transfusing platelets as prophylaxis against haemorrhage. Respondents were asked to select a single option.

Results

In total, 306 physicians from 20 different countries responded within a specified time period. The responses from Asia were 52 from Indonesia, 7 from Bangladesh, 5 from the Philippines, 9 from Singapore, 8 from Cambodia, 18 from Malaysia, 3 from Thailand, 20 from Vietnam and 12 from India. The responses from Latin America and the Caribbean were 13 from Cuba, 10 from Brazil, 17 from Paraguay, 6 from Peru, 1 from Mexico, 1 from Bolivia, 1 from Martinique and 81 from Colombia. The responses from Africa were 37 from Nigeria and 2 from South Africa. In addition there were 3 responses from the UK.

Among the 4 case histories describing patients with clinically non-severe dengue associated with varying levels of thrombocytopenia, 16–24% of respondents recommended platelet transfusion at platelet concentrations of $22–29\times10^9$/L., but approximately one-third of the respondents would transfuse platelets if the count fell to 3×10^9/L. (Table 1)

Among the 5 case histories describing patients with different manifestations of severe dengue associated with varying levels of thrombocytopenia, more respondents would transfuse platelets if the patient was in shock and bleeding (case histories 8 and 9). There were substantial differences in the responses from physicians in Africa than those from Asia and America for all 9 cases (Table 1).

The final question aimed to determine thresholds at which a physician would consider transfusing platelets as prophylaxis against haemorrhage. Respondents were asked to select a single option. 31 (10%) respondents would consider a prophylactic platelet transfusion if the platelet count was below 50×10^9/L. 8 (2.6%) respondents would consider a prophylactic platelet transfusion if the platelet count was below 40×10^9. 10 (3.3%) respondents would consider a prophylactic platelet transfusion if the platelet count was below 30×10^9. 17 (5.6%) respondents would consider a prophylactic platelet transfusion if the platelet count was below 20×10^9. 46 (15%) respondents would consider a prophylactic platelet transfusion if the platelet count was below 10×10^9. 190 (62%) respondents would only consider transfusing platelets in patients with signs of haemorrhage.

The responses categorised by global region are summarised in Table 1.

Discussion

Our study has limitations. There is an element of selection bias in the way the survey was conducted, as the physicians who distributed the survey within their countries were known to have an interest in dengue. The survey is subject to response bias meaning that the answers may not accurately reflect clinical practice in the respective countries. In addition, the country representation is not balanced.

Despite these limitations the striking result of this survey is the heterogeneity of approaches to the use of prophylactic platelet transfusions in dengue. 112/306 respondents would consider transfusing platelets prophylactically at various levels of thrombocytopenia. When the responses are categorised by region (Table 1) African respondents would advocate platelet transfusions more frequently, perhaps reflecting more limited experience with dengue and experience with other haemorrhagic fevers. The choice to use prophylactic platelet transfusions may be influenced by cost and availability of platelets, as well as individual experience in managing dengue and other medical conditions that affect the platelet count. There is considerable variability within countries suggesting an individual's practice may differ from recommendations in guidelines. For example 6/12 Indian respondents and 7/10 Brazilian respondents would consider the use of prophylactic platelets. The responses reflect wide variation in clinical practice and are indicative of the paucity of clinical evidence to guide practice in this area.

At present there is limited evidence to support the use of prophylactic platelet transfusions in dengue despite their inclusion in some national guidelines. As the global reach of dengue continues to expand the need to conduct clinical trials to construct

Table 1. Proportion of respondents choosing to transfuse platelets stratified by geographic region (n, (%)); BP = blood pressure; HR = heart rate; HCT = haematocrit.

Clinical case	Asia (n = 134)	Africa (n = 39)	S. America & Caribbean (n = 130)	UK (n = 3)	Total (n = 306)
Case 1 18-year-old female; platelets 23×10^9/L; no haemorrhage; BP 120/80; HR 105; HCT 39%	12 (9)	38 (97.4)	8 (6.2)	0 (0)	58 (19)
Case 2 28-year-old male; platelets 29×10^9/L; no haemorrhage; past history of perforated gastric ulcer; BP 100/75; HR 92; HCT 42%3	20 (14.9)	39 (100)	13 (10)	0 (0)	72 (23.5)
Case 3 29-year-old female; rapid fall in platelets to 22×10^9/L; no haemorrhage; haemodynamically stable	12 (9)	30 (76.9)	6 (4.6)	0 (0)	48 (15.7)
Case 4 30-year-old male; platelets 3×10^9/L; no haemorrhage; haemodynamically stable	57 (42.5)	37 (94.9)	23 (17.7)	3 (100)	120 (39.2)
Case 5 19-year-old male; platelets 18×10^9/L; dengue hepatitis; no haemorrhage; BP 90/60; HR 120; HCT 47%	11 (8.2)	36 (92.3)	7 (5.4)	2 (66.7)	56 (18.3)
Case 6 20-year-old female; platelets 17×10^9/L; dengue encephalitis; no haemorrhage; BP 100/70; HR 100; HCT 40%	25 (18.7)	39 (100)	19 (14.6)	1 (33.3)	84 (27.5)
Case 7 24-year-old male; platelets 31×10^9/L; hepatic failure secondary to dengue; no haemorrhage; BP 125/70; HR 110; HCT 42%	5 (3.7)	39 (100)	9 (6.9)	0 (0)	53 (17.3)
Case 8 23-year-old female; platelets 8×10^9/L; shock, epistaxis and vaginal bleeding; BP 75/50; HR 110; HCT 42%	86 (64.2)	39 (100)	43 (33.1)	3 (100)	171 (55.9)
Case 9 23-year-old male; platelets 33×10^9/L; shock and mucosal bleeding; BP 70/50; HR 120; HCT 46%	57 (42.5)	39 (100)	26 (20)	1 (33.3)	123 (40.2)
Prophylactic platelet transfusion threshold:					
$<50\times10^9$/L	8 (6)	23 (59)	0 (0)	0 (0)	31 (10.1)
$<40\times10^9$/L	1 (0.7)	7 (17.9)	0 (0)	0 (0)	8 (2.6)
$<30\times10^9$/L	1 (0.7)	7 (17.9)	2 (1.5)	0 (0)	10 (3.3)
$<20\times10^9$/L	12 (9)	1 (2.6)	2 (1.5)	2 (66.7)	17 (5.6)
$<10\times10^9$/L	33 (24.6)	0 (0)	12 (9.2)	1 (33.3)	46 (15)
Not in absence of bleeding	75 (56)	1 (2.6)	39 (30)	0 (0)	190 (62.1)

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an evidence base to guide the appropriate use of platelets in dengue becomes ever more pressing.

Supporting Information

Questionnaire S1 Dengue clinical scenarios.
(DOC)

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Author Contributions

Conceived and designed the experiments: JW BW JF TTH. Performed the experiments: RRM MGG EM RRR WVG LN ISL AM LL AF AS JD AB YSL LB DGB AK OH GT HW ML TTH RP. Analyzed the data: JW JF CS. Wrote the paper: JW JF CS RP.

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Dengue clinical scenarios

Case 1:

A 18-year-old woman is seen in the emergency clinic. She has been unwell for 5 days with a high fever, headache and has developed a rash. On examination she is febrile (38.4), her pulse is 105 and her blood pressure is 120/80. Her rapid diagnostic test for dengue is positive. Her other investigations are as follows:

HB = 14.2g/dL	AST = 65 IU/L
WCC = 3.4 $x10^9$/L	ALT = 50 IU/L
PLT = 23 $x10^9$/L	Clotting profile normal
Hematocrit = 39%	

Which of the following would be part of your management plan (tick all that apply)?

- Discharge home with advice and anti-pyretics □
- Admission for observation □
- Platelet transfusion □
- Parenteral fluids □

Case 2:

A 28-year-old man is admitted to the ward with dengue (confirmed serologically). He has had a fever for 7 days. 6 months ago he was admitted to hospital with a severe upper gastrointestinal haemorrhage secondary to a perforated peptic ulcer. Currently he is febrile (38.2), his BP is 100/75 and his pulse is 92. Investigations are as follows:

HB = 15.2g/dL	AST = 62 IU/L
WCC = 5.1 $x10^9$/L	ALT = 45 IU/L
PLT = 29 $x10^9$/L	APTT = 45 seconds PT = 12 seconds
Hematocrit = 42%	

Which of the following would be part of your management plan (tick all that apply)?

- Parenteral fluids □
- Platelets to prevent haemorrhage (given history of peptic ulceration) □
- Upper GI endoscopy □
- Chest x-ray and ultrasound □

Case 3:

A 29-year-old female with confirmed dengue (NS1 and PCR positive) has been admitted for observation on the 3rd day of illness. On the second day of admission she remains febrile (38.8) but is otherwise stable. However the laboratory calls you to inform you that her platelet count has fallen from 102 $x10^9$/L to 22 $x10^9$/L. You re-examine her with this result in mind but

find no evidence of haemorrhage. Her other blood tests, including hematocrit and a clotting profile are normal.

Which of the following would you do (tick all that apply)?

- Discharge home with advice to return if complications develop □
- Continue to observe □
- Administer platelets and observe as an inpatient □
- Arrange imaging to look for evidence of occult haemorrhage □

Case 4:

A 30-year-old man is seen in the clinic with suspected dengue. He has a 5-day history of fever, malaise and headache. On examination he is febrile (38.8) and has a rash that is suggestive of dengue. He is haemodynamically stable and has no evidence of haemorrhage. The haematology laboratory phone you to inform you that his platelet count is 3 $x10^9$/L. Would you consider arranging a platelet transfusion?

- Yes □
- No □

Case 5:

A 19-year-old man with suspected dengue is admitted on the 6th day of illness. He has a rash suggestive of dengue infection and significant bruising at venepuncture sites. He denies any bleeding. He is afebrile (36.2), his blood pressure is 90/60 and his pulse is 120. Investigations are as follows:

HB = 15.2g/dL	AST = 320 IU/L
WCC = 2.1 $x10^9$/L	ALT = 160 IU/L
PLT = 18 $x10^9$/L	APTT = 49 seconds PT = 13 seconds
Hematocrit = 47%	

Which of the following would be part of your management plan (tick all that apply)?

- Administer parenteral fluids □
- Admit to a high dependency area for careful monitoring □
- Ultrasound of liver □
- Platelet transfusion to prevent haemorrhage □

Case 6:

A 20-year-old woman is admitted with fever and a reduced level of consciousness. According to her family she has been unwell for 4 days complaining of a headache in addition to her fever. The day prior to admission she had an episode of vomiting. On examination she has a reduced GCS (11/15), she is febrile (38.5), her pulse is 100 and her blood pressure is 100/70. She has scattered petechiae on her lower limbs but no other evidence of bleeding. She has a CT scan of her brain, which is normal, a blood film for malaria, which is negative and a lumbar puncture that shows 92 white cells (90% lymphocytes) with normal biochemistry. She has a positive NS1 rapid test for dengue and you suspect she may have dengue encephalitis. Her other investigations are as follows:

HB = 12.9 g/dL	AST = 165 IU/L
WCC = 2.9 $x10^9$/L	ALT = 135 IU/L

PLT = 17 x10^9/L	Clotting profile normal
HCT = 40%	

Which of the following form part of your management plan (tick all that apply)?

- Admit for neurological observation □
- Treat with parenteral aciclovir while awaiting CSF PCR result □
- Administer platelets as prophylaxis against haemorrhage □
- Commence parenteral fluids □

Case 7:

A 24-year-old man with suspected dengue is admitted to hospital. He has had a fever for 5 days. On examination he is febrile (38.6), his pulse is 110 and his blood pressure is 125/70. Physical examination reveals some mild bruising and mild hepatomegaly with tenderness on palpation of his right upper quadrant. Dengue is confirmed by PCR. Investigations are as follows:

HB = 13.6 g/dL	AST = 1845 IU/L
WCC = 4.9 x10^9/L	ALT = 1250 IU/L
PLT = 31 x10^9/L	APTT = 72 seconds PT = 19 seconds
HCT = 42%	

Which of the following would be part of your management plan (tick all that apply)?

- Admission for observation □
- Parenteral fluids □
- Institute supportive treatment for pending hepatic failure □
- Platelets □
- Vitamin K □

Case 8:

A 23-year-old woman is admitted on the 7th day of illness. She has dengue as confirmed by an NS1 assay. She reports some epistaxis and abnormal menstrual bleeding. On examination she has scattered petechiae and bruising at venepuncture sites. She has a temperature of 37.9, her blood pressure is 75/50 and her pulse is 110. Investigations are as follows:

HB = 8.9g/dL	AST = 125 IU/L
WCC = 9.2 x10^9/L	ALT = 107 IU/L
PLT = 8 x x10^9/L	APTT = 61 seconds PT = 17 seconds
Hematocrit = 42%	

Which of the following would be part of your management plan (tick all that apply)?

- Administer parenteral fluids □
- Admit to HDU □
- Administer packed red cells □
- Administer platelets □
- Start IV heparin □
- Chest x-ray and ultrasound □
- Nasal packing □

Case 9:

A 23-year-old man is referred to the hospital with confirmed dengue (NS1 positive). He has been unwell for a week. He had been treated with parenteral fluids at the referring health centre. On examination he is afebrile, his pulse is 120 and his blood pressure is 70/50. There is widespread bruising at the site of venepuncture as well as scattered petechiae elsewhere. On examination of his mouth there is evidence of some mucosal bleeding. While examining him he has haemetemesis. His investigations are as follows:

HB = 10.1 g/dL	AST = 324 IU/L
WCC = 7.2 $x10^9$/L	ALT = 127 IU/L
PLT = 33 $x10^9$/L	APTT = 68 seconds; PT = 16 seconds
HCT = 46%	

Which of the following would form part of your management plan (tick all that apply)?

- Parenteral fluids □
- Packed red cells □
- Platelets □
- Insertion of CVP line □
- Admission to intensive care facility □

Question 10:

At what level would you consider prescribing platelets as prophylaxis against haemorrhage (please chose 1 response)?

- <50 $x10^9$/L □
- <40 $x10^9$/L □
- <30 $x10^9$/L □
- <20 $x10^9$/L □
- <10 $x10^9$/L □
- In the absence of haemorrhage I would not prescribe platelets □

RESEARCH PAPER 6: DENGUE HUMAN INFECTION MODELS SUPPORTING DRUG DEVELOPMENT

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Student	James Whitehorn
Principal Supervisor	Rosanna Peeling and Cameron Simmons
Thesis Title	The pathogenesis and clinical management of dengue

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	The Journal of Infectious Diseases		
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Supervisor Signature: _____ **Date:** 4/6/2015

SUPPLEMENT ARTICLE

Dengue Human Infection Models Supporting Drug Development

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Dengue is a arboviral infection that represents a major global health burden. There is an unmet need for effective dengue therapeutics to reduce symptoms, duration of illness and incidence of severe complications. Here, we consider the merits of a dengue human infection model (DHIM) for drug development. A DHIM could allow experimentally controlled studies of candidate therapeutics in preselected susceptible volunteers, potentially using smaller sample sizes than trials that recruited patients with dengue in an endemic country. In addition, the DHIM would assist the conduct of intensive pharmacokinetic and basic research investigations and aid in determining optimal drug dosage. Furthermore, a DHIM could help establish proof of concept that chemoprophylaxis against dengue is feasible. The key challenge in developing the DHIM for drug development is to ensure the model reliably replicates the typical clinical and laboratory features of naturally acquired, symptomatic dengue.

Keywords. dengue; human infection model; clinical trial; drug development.

The 4 serotypes of dengue virus (DENV-1 to DENV-4) are the most important arboviral pathogens of humans. The global burden of dengue, recently estimated at approximately 100 million cases per year, remains unchallenged by licensed vaccines or sustainable vector control strategies [1]. The scale of the dengue burden in the endemic countries of Asia and Latin America has a negative economic impact and places a significant clinical burden on often fragile health care systems [2–4].

Dengue is an acute systemic febrile illness that manifests with abrupt onset as an undifferentiated fever that is difficult to distinguish from other infections without laboratory diagnostic tests [1]. Headache, malaise, myalgia, retro-orbital pain are common symptoms in adults [5]. The clinical features differ by age group, with symptoms such as cough, vomiting and abdominal pain more common in children [6]. Leukopenia and thrombocytopenia are common laboratory features.

In most cases, fever and symptoms resolve uneventfully after 3–7 days with no long-term sequelae [1]. In a subset of cases, a transient vascular leakage syndrome develops after 3–4 days of illness, which, when severe, can lead to life-threatening hypovolemic shock (called dengue shock syndrome) and/or hemorrhage [1, 5]. The vascular leak syndrome may be associated with complement activation and a coagulopathy that can contribute to the risk of hemorrhage [7, 8]. Signs of vascular leakage include an increasing hematocrit, microalbuminuria, or the accumulation of fluids at serosal surfaces (eg, pleural effusions) [5].

Supportive care and the careful titration of minimum volumes of parenteral crystalloid fluids to maintain stable cardiac output during the 1–3-day period of vascular permeability are critical elements in dengue case management [5]. Vasopressors are commonly used in cases with prolonged shock. Clinically significant hemorrhage requiring use of blood products is more common in adults than in children [9].

Improvements in the management of severe dengue cases have seen the case mortality rate decline over the last decade in many but not all endemic settings [10–12]. At present there is no validated way of identifying which patients will progress to more severe disease, meaning that health facilities in endemic areas are often

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overwhelmed with patients with dengue who require ongoing observation as outpatients or are admitted for inpatient observation.

THE CASES FOR DENGUE THERAPEUTIC AND PROPHYLACTIC AGENTS

Antivirals

A drug that can shorten the duration of illness and reduce the risk of disease progression would be a significant advance for both patients and for overstretched health systems in dengue-endemic countries. An obvious approach is to target virus replication with a small molecule antiviral drug that given early in the course of illness has the potential to shorten the duration of infection. Moreover, because high early DENV viremia levels are a risk factor for the development of more severe disease, an antiviral drug might also reduce the incidence of severe complications [13, 14]. In addition, a drug that reduces the magnitude and duration of viremia has the potential to reduce onward DENV transmission by reducing the length of time an individual patient is infectious to mosquitoes [15]. Alternatives to small molecule antiviral drugs include therapeutic monoclonal antibodies; however, high costs and requirement for parenteral administration are currently barriers to their use.

Disease Modulators

Both host and virus factors are important in influencing the outcome of DENV infection. A range of host factors, such as age, sex, genotype, and flavivirus infection history, influence the disease outcome [10, 16, 17]. Because the manifestations of severe dengue are typically observed in secondary DENV infections and when innate and adaptive immune responses have driven steep declines in viremia levels, it is widely held that the immune system plays an important role in disease pathogenesis [18]. This represents the rationale for use of an immunomodulatory drug that can favorably modulate the poorly defined immunopathogenic mechanisms postulated to underlie severe dengue. It is possible that the dichotomous approach of targeting either the virus or the immune response is an oversimplified strategy to the development of dengue therapeutics. Other physiological systems are involved in the control of endothelial integrity, but their role in dengue remains poorly understood [19].

Prophylaxis

A vaccine would be the most effective prophylactic public health intervention for control of dengue, but the development of an efficacious tetravalent dengue vaccine is proving to be a challenge [20, 21]. An alternative or adjunctive approach is the use of drug chemoprophylaxis in the community. Chemoprophylaxis using oseltamivir has been advocated for controlling the spread of influenza within households and was commonly used in some settings during the 2009 H1N1 influenza pandemic, creating a precedent for such an approach for an acute viral infection [22, 23]. The safety profile of a prophylactic dengue drug would have to be exemplary, and there are major questions of whether this approach could be cost-effective and sustainable, given the temporally and spatially heterogenous patterns of DENV transmission in endemic regions [24, 25]. Moreover, in most dengue-endemic regions a significant fraction of the population is already immune and will not benefit from chemoprophylaxis. Consequently, in endemic areas the number needed to treat, and the duration they need to be treated for, may be large for the prevention of a single symptomatic case and very large for the prevention of severe cases. There is a marginally stronger argument for chemoprophylaxis in populations with no immunity to DENV. Examples of this include dengue-naive travelers or military personnel who are transiently present in areas of high DENV transmission. Similarly, chemoprophylaxis could be indicated for communities experiencing "virgin soil" dengue outbreaks because of importation by viremic travelers or infected mosquitoes.

PREVIOUS CLINICAL TRIALS OF THERAPEUTICS FOR DENGUE AND RELEVANCE TO THE DENGUE HUMAN INFECTION MODEL

For a disease with an estimated global burden prevalence of 100 million cases per year, remarkably few clinical trials of treatment approaches have been performed. Early trials tested fluid resuscitation approaches in patients with hypovolemic shock [26–28]. More-recent trials have tested the candidate antiviral agents chloroquine and balapiravir or have targeted the immune response by use of oral corticosteroids [29–31]. Although none of these trials have demonstrated therapeutic efficacy, they have provided a framework for the conduct of such trials and has led to the publication of recommendations for a standardized approach to dengue clinical trial design and conduct [15]. In addition, a trial of lovastatin in dengue is currently underway and further trials are planned [15, 32]. The virological findings in these trials, consistent with previous literature, have underscored the need for antiviral therapy to commence early in the course of illness, because illness resolves and patients are no longer infectious to *Aedes aegypti* mosquitoes by 5–7 days after illness onset [14, 15, 33–35].

POTENTIAL ROLES FOR A HUMAN INFECTION MODEL IN DENGUE DRUG DEVELOPMENT

In principal, a dengue human infection model (DHIM) provides opportunities for fast-tracking dengue drug development, particularly therapeutic and prophylactic antivirals. The overall strength of the DHIM is that it allows for controlled prophylactic or therapeutic studies of DENV infection in susceptible participants who have been preselected for a set of desirable

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Table 1. Early-Phase Clinical Studies of Antiviral Drugs: Strengths and Weaknesses of the DHIM vs Trial Conducted in a Dengue-Endemic Setting

Hurdle	DHIM	Dengue-Endemic Setting	Notes
Dengue case burden	At call	Usually seasonal and with yearly variation in case numbers	In endemic settings dependent on the force of infection
Clinical research capacity	Available	Variable	In endemic settings dependent on local expertise, experience and resources
Efficiency of regulatory system	Variable	Variable	In endemic settings dependent on research infrastructure and local regulatory requirements
Accredited testing laboratories	Available	Variable	In endemic settings dependent on research infrastructure and local regulatory requirements
Ability to ship research specimens across international borders	Yes	Variable	In endemic settings dependent on research infrastructure and local regulatory requirements
Variance in viremia and serotypes	Serotype controlled; possibly less variance in viremia	Variable	In endemic settings dependent on local viral epidemiology
Early therapy possible	Yes	Variable	In endemic settings dependent on patients presenting early
Pharmacokinetic studies	Yes	Yes/Variable	May require international shipping of samples from endemic countries
Intensive pathophysiological monitoring	Yes	Possibly	Ability to conduct intensive monitoring is dependent on the local resources available
Chemoprophylaxis studies	Yes	More difficult	The controlled setting of the DHIM is ideally suited to this kind of study
Provide proof of concept that therapy reduces major clinical complications	Probably not, requires large sample sizes	Yes	Severe clinical events in healthy adults with primary DENV infections are rare
Provide proof of concept that therapy mitigates typical lab features	Probably not, requires large sample sizes	Yes	Demonstrating mitigation would require large sample size
Serious adverse events; clinical experience of managing severe dengue	Variable	Yes	Extensive clinical experience and expertise in endemic settings
Clinical studies of secondary heterotypic DENV infections	Difficult	Yes	Ethical hurdles for the DHIM in view of known risks associated with secondary heterotypic infections
Clinical studies in participants who have received dengue vaccine candidates	Yes	Yes	In the DHIM will depend on the balance of perceived risks and benefits

Abbreviations: DENV, dengue virus; DHIM, dengue human infection model.

characteristics. In contrast, prophylactic or therapeutic studies of DENV infection in an endemic country population are subject to the vagaries of ethnic diversity of the patient population, fluctuating serotype and genotype prevalence, variable viremia levels, and prior flavivirus infection histories. Collectively, these sources of variation in an endemic country population probably boost the required patient sample size required for detection of therapeutic or clinical safety signals.

CRITICAL HURDLES FOR A DHIM

Central to the usefulness of a DHIM is whether it mimics the main features of a symptomatic dengue case. This is a fundamental and critical hurdle in the development of a successful DHIM for use in dengue therapeutic development. Thus, the kinetics of clinical, virological, hematological, and biochemical changes that are typically present in a naturally acquired and clinically uncomplicated adult dengue case should also manifest in the adult DHIM. In the beginning, at least, we assume that the DHIM will involve participants who are flavivirus naive, and thus all experimental infections will be primary infections. In the context of dengue drug development, the characteristics of a symptomatic, naturally acquired DENV infection that a DHIM would need to replicate include acquisition of infection and certain clinical, virological, and laboratory features.

Acquisition of Infection

A DHIM could use DENV-infected mosquitoes to elicit infection in human volunteers. Although this approach may be optimal in replicating the natural history of DENV infection because, for

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example, the immunoregulatory effects of mosquito saliva would be replicated, it may present additional regulatory and logistical hurdles. Alternatively, a DHIM could use subcutaneous inoculation of laboratory-cultured virus, as used in the challenge experiments by Sun and colleagues [36]. This approach bypasses issues surrounding use of mosquitoes, although it remains to be determined if there are material differences between needle-delivered and mosquito-delivered DENV infection.

Clinical Features

Typical signs and symptoms of dengue that a DHIM would need to replicate in otherwise healthy adults include fever, headache, myalgia, musculoskeletal pain, and nausea or vomiting [37]. Minor hemorrhagic features may occur between days 3 and 6 of illness (eg, petechiae) [37]. Prevention or rapid amelioration of these self-limiting symptoms will be a target of dengue therapeutic drug development. Primary infections in otherwise healthy adults are rarely associated with severe clinical complications, but it would be prudent to exclude the elderly and those with comorbid conditions from the volunteer pool, because these groups are at elevated risk of severe complications [38, 39].

Virological Features

The intrinsic incubation period of DENV infection has been estimated to be between 4–10 days [5]. Work that reanalyzed historical mosquito exposure experiments estimated that the intrinsic incubation period in primary infection was approximately 6 days [40]. The duration of viremia varies by serotype and DENV infection history. The peak viremia level is often not observed in naturally occurring infections because it precedes the time point when patients attend health care facilities, but findings suggest that the peak level occurs later in primary infections as compared to secondary [33]. Studies suggest that primary infections with DENV-1 are associated with a longer viremic period and higher viremia levels [33, 41]. An optimal DHIM would replicate the virological features of a naturally acquired DENV infection to enable confident predictions of therapeutic or prophylactic drug efficacy in the field.

Laboratory Features

A DHIM should elicit thrombocytopenia and leukopenia, both typical features in patients with primary or secondary dengue. In addition, many patients have minor elevations in transaminase and creatine kinase levels [9, 42]. Clotting abnormalities are also often observed in severe cases with elevated activated partial thromboplastin times and reduced levels of fibrinogen; however, these occur less frequently in primary infections [43].

STRENGTHS AND WEAKNESSES OF A DHIM FOR DRUG DEVELOPMENT

The strengths and weaknesses of a DHIM for performing early-phase clinical studies compared to performing such studies in naturally acquired cases in an endemic country are summarized in Table 1. In short, the great advantage of the DHIM for early-phase drug development is the ability to perform controlled prophylactic or early therapeutic studies in a setting amenable to intensive pharmacological and basic research investigations.

FOCUS AREAS IN WHICH THE DHIM COULD SUBSTANTIALLY ADVANCE CLINICAL DRUG DEVELOPMENT

A DHIM that mimics natural infection would be particularly well suited to early-phase studies of chemoprophylaxis and accompanying pharmacokinetic studies, in which the timing of treatment and infection can be controlled. In contrast, in a dengue-endemic country, early-phase chemoprophylaxis trials would require large and lengthy community-based cohort studies, as has been used to derive evidence for oseltamivir in the chemoprophylaxis of influenza [22]. The DHIM would thus be a more cost-effective and time-efficient route to acquiring proof of concept evidence that chemoprophylaxis for dengue is possible. For a given antiviral drug, a DHIM would also be well-suited for studies that accurately define the therapeutic window in symptomatic cases, since the time of infection, onset of symptoms and treatment will be prospectively documented. In contrast, in endemic countries enrollment of study participants is dependent on patients seeking health care, who will have various durations of illness history at the time of study enrollment. A DHIM can also aid the development of alternative therapeutic approaches, such as immunomodulatory agents. In addition, a DHIM may aid the development of noninvasive physiological monitoring techniques within clinical trials, perhaps through the identification of appropriate physiological measurements that could be used as end points [15].

In conclusion, the work of Sun and colleagues [36] has shown that a DHIM is possible. This is a significant advance in the field with the potential to contribute to the development of successful dengue therapeutics and much else. The future use of the DHIM in dengue clinical research will require a significant investment from multiple stakeholders. Its contribution in drug development may aid in the selection of appropriate candidate drugs for further development and assist pharmacokinetic studies. The potential significance of these contributions suggests that investing in a DHIM is both appropriate and timely.

Notes

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5. DISCUSSION AND FUTURE DIRECTIONS

The work presented in this thesis expands the knowledge base of dengue clinical management and dengue pathogenesis. I described studies that expand our knowledge of the *MICB* and *PLCE* genes in dengue susceptibility and advance our understanding of pathogenesis. I described experiments that directly compared the susceptibility of *Ae. aegypti* and *Ae. albopictus* to initial and disseminated dengue infection after blood feeding on viremia dengue patients. The insights from this work will allow greater understanding of dengue transmission dynamics, will inform mathematical models of transmission, as well as potentially inform endpoints of various therapeutic and chemoprophylactic interventions. In addition, I have described a randomised clinical trial of lovastatin in adult patients infected with dengue. While the trial did not show a clinical benefit it raised important further questions for future intervention studies.

Discussion

A deeper understanding of dengue susceptibility will provide insights into disease pathogenesis and has the potential to assist vaccine and therapeutic development. Since publication of a GWAS study that identified two susceptibility loci for DSS, a lot of attention has been directed at the genes in which the SNPs are located.[7] The work described in this thesis has extended the finding of the GWAS by confirming the associations with DSS but also demonstrating that these SNPs are also associated with less severe forms of the disease. Given that *MICB* encodes a ligand involved in natural killer cell activation, we speculated that mutations in this gene might be associated with higher plasma viremia levels. Our work did not show this, meaning that the functional basis of these observed associations remains unclear. We analysed the relationship between the mutations and the initial (enrolment) viremia – it is possible that there were different patterns in viral clearance between the host genotypes that could not be explored in this study. While it may be argued that these insights into disease pathogenesis are “small print”, the whole-genome approach removes bias and has the potential to shed light on hitherto unexpected aspects of the host-pathogen interaction process and, thus, to inform potentially novel therapeutic approaches.[60]

The expansion of the geographic range of *Ae. albopictus* has led to concerns about an associated increased arboviral threat to North America and Europe.[61,62] Being able to accurately quantitate the comparative susceptibility of *Ae. albopictus* to dengue infection will lead to the creation of more precise disease transmission models, which may be able to assist public health authorities in planning outbreak responses. In addition, clear data on the levels of viraemia in patients that are infectious to mosquitoes has the potential to influence the design and conduct of therapeutic trials assessing therapeutic and chemoprophylactic agents. The 50% mosquito infectious dose (MID_{50}) estimates presented here could be further refined by pooling data from our team’s previous study, which could further enhance the contribution this work can make to transmission models.[31] The work described in this thesis

has demonstrated that *Ae. albopictus* and *Ae. aegypti* are equally susceptible to the acquisition of an initial dengue infection but that *Ae. albopictus* is overall less susceptible to the acquisition of an infectious phenotype. This difference varied by serotype – *Ae. albopictus* was less likely to acquire an infectious phenotype with DENV-2 and DENV-4 but there was no difference observed between the mosquito types with DENV-1 and DENV-3. Previous vector competence studies were conducted using laboratory experiments – by using direct feeding experiments on viraemic dengue patients our study replicated field conditions and is thus better placed to inform transmission models. Interestingly, our work also showed that *Ae. albopictus* is able to accommodate a higher viral burden in its abdominal tissue. By analysing a subset of DENV-1-infected patients and their associated mosquitoes we showed that this higher viral burden potentially contributes to higher sequence diversity in DENV populations. It would be valuable to extend this work by using larger sample sizes and assessing different serotypes.

Despite dengue's enormous global impact there is still no licensed therapeutic to combat the toll of the disease on patients and overburdened health systems in the tropical world. There is a pressing need for a therapeutic that can shorten the duration of illness and reduce the risk of disease progression.[63] Clinical trials have been conducted in dengue that have used different approaches to influence the disease outcome. These include attempts to reduce dengue viraemia or modulate the host response to dengue infection.[63] Unfortunately, to date, these attempts have not demonstrated a clinical benefit.[64-66] It is important to note that these studies were, in the main, powered for safety as opposed to efficacy. While a dramatic clinical benefit would likely to have been demonstrated, it is possible that a subtle benefit could have been overlooked. However even if this was the case then the number needed to treat would be very large implying that it would probably not reflect a viable treatment option. It was against this backdrop that the lovastatin dengue trial was designed. Statins have received a lot of attention as potential therapeutic adjuncts in a diverse range of conditions such as sepsis and ARDS. It was felt that the potential endothelial stabilising effects of statins could maintain the endothelial integrity thus reducing capillary permeability. Consequently, improvements in capillary permeability should translate to lower levels of haemoconcentration, and a reduced frequency of severe complications. In addition there was a suggestion from some laboratory work that lovastatin may have some antiviral effects in dengue. Furthermore, its low cost and good safety profile made lovastatin an attractive therapeutic candidate. Given interest in its use as an adjunctive agent in critical care medicine, we had hoped to assess whether it had a use in patients with severe dengue but due to concerns about the potential side effects of statins (e.g. hepatitis and muscle inflammation) and their overlap with expected clinical features of dengue, the regulatory authorities in Vietnam did not allow this. It is, however, sobering to note that despite huge initial interest the proposed beneficial role of statins has not been supported in prospective clinical trials in ventilator-associated pneumonia, sepsis and ARDS.[52,53,67] While we were able to show that lovastatin was safe in dengue we did not show any evidence of a clinical or

virological benefit that would support further larger studies. Given the endothelial stabilising effects of statins and the fact that dengue shock is more common in children it is possible that we may have observed more of a clinical benefit if we had targeted a younger age group.[68,69] The peak viraemia occurs very early in the course of clinical illness and there is already evidence of endothelial dysfunction after 72 hours of illness suggesting that the timing of our intervention may have been too late to influence the clinical and virological outcome.[70-72] Conducting intervention studies with patients recruited even earlier in their illness raises significant practical issues as patients rarely present to health facilities so early. While we have not yet tested an antiviral specifically designed for dengue, it is possible that the focus of intervention studies should shift to studying potential chemoprophylactic agents, and, in parallel, studies that aim to optimise supportive care of the critically ill. A chemoprophylactic agent would have to have an excellent safety profile and would probably appeal most to travellers or short-term visitors to high transmission settings. However it is possible that a chemoprophylactic could be used in endemic settings as a reactive ring-fencing approach around an index case or cluster of cases – work from Thailand suggests that dengue transmission is often highly focal.[73] In addition, selectively targeting patients most at risk of severe disease could be an effective strategy in the rollout of a chemoprophylactic agent. Unfortunately we are not yet able to identify patients at risk of disease progression although it is hoped that on-going cohort studies (www.idams.eu) will aid with this.

Future directions

The host susceptibility work described above extends our understanding of the *MICB* and *PLCE1* genes in dengue susceptibility but it raises as many questions as it answers. While the whole-genome approach is an unbiased way of addressing key questions in disease susceptibility, linking findings to functional mechanisms of pathogenesis is always a challenge. Given that *MICB* encodes a ligand involved in NK cell activation we had speculated that mutations in this gene would be associated with higher plasma viraemia. The additional work, also described above, suggests that this is not the case. Our study only looked at enrolment viraemia and not at viral kinetics over the course of dengue illness. It would be intriguing to see whether there are different patterns of viral clearance across the genotypes – prospective studies such as intervention trials are ideally suited to answer this. Defining the functional role of *PLCE1* is more challenging. The fact that mutations in this gene appear to be associated with such a diverse range of conditions raises intriguing questions, but as yet no answers, as to this gene's role in both health and disease. It is likely that further advances in our understanding of dengue pathogenesis will come from prospective studies with well-planned sampling approaches as opposed to the traditional descriptive study approach.

I have presented the first direct comparison of *Ae. aegypti* and *Ae. albopictus* acquisition potentials through direct blood feeding experiments on viraemic dengue patients. This work

showed that overall *Ae. albopictus* was less susceptible to disseminated dengue infection and thus acquisition of an infectious phenotype but that this difference varied by serotype. In addition, the study showed that the abdominal viral burden was higher in *Ae. albopictus*, and in further analysis of a subset of DENV-1-infected mosquitoes was associated with more sequence diversity accumulation. Further questions related to this are important to address – are similar observations seen in other serotypes? How does this sequence variety contribute to viral persistence? What is the nature of the midgut barrier mechanism in *Aedes* mosquitoes? In addition, this work adds to the data confirming the central role of patient plasma viraemia in infecting mosquitoes and maintaining community dengue transmission. Can the administration of a suitable chemoprophylactic agent stop community transmission? It is possible that the use of a DHIM could aid in the identification of an appropriate candidate, and that this kind of approach to disease control could have a role in areas affected by sudden surges of cases. It is important to note that the overall difference in mosquito susceptibility to disseminated DENV infection between mosquito types is small suggesting that many other factors influence the secondary role of *Ae. albopictus* in dengue transmission. Understanding these factors and forming a clearer picture of vector competence would be an important advance.

It is clear that there remain many unanswered challenges in understanding dengue pathogenesis and in the development of therapeutic interventions. One of the issues in designing therapeutic intervention trials in dengue is the relative rarity of severe disease. While this is obviously a good thing for public health it means that trials powered to an endpoint of shock would be large and costly. Perhaps an alternative approach is to design community-based intervention trials with hospital admission as an endpoint? While hospital admission is often a surrogate for the development of dengue with warning signs, the reasons for admission vary greatly between health centres and among different health providers. This kind of trial would require a very standardised approach to clinical management and hospital admission approaches. A move towards community studies would more realistically reflect the burden of dengue, would have potential cost advantages and would allow a larger sample size than is typically used in a hospital-based intervention study. As discussed above, there remain major questions about the optimal timing of administering a therapeutic. Two trials have been conducting with the therapeutic candidate being administered within 48 hours of fever onset.[64,74] The trials of chloroquine, corticosteroids, and lovastatin administered the study drugs within 72 hours of fever onset.[65,66,75] The fact that none of these trials showed an effect on viraemia kinetics or clinical outcome suggest that we may have “missed the boat”. We know that peak viraemia occurs early in illness and that there is already evidence of endothelial dysfunction by 72 hours after fever onset.[70-72] It is possible that the eventual clinical phenotype has already been defined before the patient with dengue presents to health services. Perhaps only a subgroup of patients will benefit from an effective therapeutic? Perhaps the urban centres of the tropical world need to adopt the personalised medicine approach to contribute to the management of dengue cases? We do not yet have the ability

to identify such a subgroup of patients but it is hoped that on-going cohort studies will aid in the identification of features that predict patients most at risk of disease progression. In addition, it is possible that insights into host susceptibility and pathogenesis will aid this process.

As discussed above it is possible that the emphasis in therapeutic trials should be on optimising care of the critically ill. There remain many questions surrounding appropriate supportive care in dengue. Currently clinical management is limited to appropriate fluid resuscitation, which is effective and life-saving.[12] However, what is less clear is how best to resuscitate patients who develop recurrent episodes of shock. Answering this important question would be challenging as it would require a trial involving very unstable patients, but would be an important advance for the field. Thrombocytopenia is a very common feature of dengue and in some settings patients are given prophylactic platelets to prevent haemorrhage. This practice lacks an evidence base – establishing this would assist clinical management. A recently completed trial in 87 patients suggests that platelet transfusions in dengue have no benefit and have an association with harm.[76] It is hoped that the on-going ADEPT trial will better clarify the evidence in this area (ClinicalTrials.gov identifier NCT01030211).

Sun and colleagues used an experimental dengue challenge to assess the efficacy of a live tetravalent vaccine.[59] This approach has the potential to overcome the hurdles that the lack of an animal model that effectively replicates human dengue illness poses. The development and use of a dengue human infection model (DHIM) has the potential to greatly assist the development of therapeutics. This approach would allow the fast track of drug development by allowing controlled studies to be performed in participants pre-selected for a number of favourable characteristics. The controlled approach would also allow detailed pharmacokinetic studies to be performed, which is often not possible in the less controlled setting of a clinical trial in an endemic country. The use of a DHIM would allow the earlier and more efficient identification of therapeutic candidates that can be take on into further phases of clinical development.

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