Pathogenesis of Haemorrhage Associated With Dengue Infection in Adults in Vietnam

Thesis

Pathogenesis of Haemorrhage associated with Dengue Infection in Adults in Vietnam

by

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Abstract

Clinical experience suggests that adults with dengue manifest a pattern of complications different from those observed in children, but direct comparisons among populations experiencing the same exposure have rarely been published. I conducted a large prospective descriptive study of dengue across all age-groups presenting to a single institution in an endemic country during a defined time-period. Vascular leakage was more severe in the paediatric patients and DSS developed much more frequently in this age-group. In contrast haemorrhagic manifestations and severe organ involvement were more common in adults. Similar to the established findings in children, typical coagulation abnormalities were apparent in the adults – i.e. prolonged APTT with reduced fibrinogen levels but without evidence of true disseminated intravascular coagulation. However thrombocytopenia was significantly worse among the adults throughout the evolution of the disease, even after adjusting for the higher rate of secondary infections in this group, and platelet counts after recovery remained lower than in the children. Clinically severe liver involvement was seen only in adults and was infrequent but usually resulted in severe bleeding. Chronic hepatitis B co-infection was associated with modestly but significantly increased levels of alanine aminotransferase, but did not otherwise impact the clinical picture.

To investigate the mechanisms underlying the increase in APTT I carried out APTT Mixing Studies confirming that deficiency of coagulation factors is a major contributory factor. Since there is little evidence for procoagulant activation the most

likely mechanism for this would be leakage of coagulation proteins, many of which are of a similar size to albumin. An additional explanation for the increased APTT could be the presence of a circulating anticoagulant - I found very high levels of heparan sulfate (HS) in the dengue plasma, but was not able to show that the HS exerts an anticoagulant effect. I also used FACS analysis to demonstrate that circulating endothelial cells (CECs) are increased during dengue infections and that percentage CECs correlate with the severity of the coagulopathy and with bleeding. Parallel increases in both CECs and HS levels support the theory that disruption of the endothelial cell/glycocalyx complex occurs during dengue infections - i.e. CECs appear to be shed from the endothelial layer while HS may be shed from the surface glycocalyx. These disruptions likely affect the function of the complex and could contribute to the pathogenesis of the systemic vascular leak syndrome.

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Abbreviations

a2AP	α2 antiplasmin
AICU	Adult Intensive Care Unit
ALT	Alanine aminotransferase
antiHBc	Anti-hepatitis B core antibody
antiHBc IgM	Anti-hepatitis B core IgM
antiHCV	Anti-hepatitis C antibody
APC	Activated protein C
APTT	Activated partial thromboplastin time
AST	Aspartate aminotransferase
AT	Antithrombin
BP	Blood pressure
C protein	Capsid protein
CaCl2	Calcium chloride
CD	Cluster of differentiation
CECs	Circulating endothelial cells
CH1	Children's Hospital No.1
CH2	Children's Hospital No.2
CPCs	Circulating progenitor cells
CRF	Case report form
CV	Coefficient of variation
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DIC	Disseminated intravascular coagulation
DSS	Dengue shock syndrome
E protein	Envelope protein
EDTA	Ethylendiamin Tetraacetic Acid

ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FDPs	Fibrin/fibrinogen degradation products
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FU	Follow up
GAC ELISA	IgG antibody-capture enzyme-linked immunosorbent assay
GAGs	Glycosaminoglycans
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCT	Haematocrit
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HS	Heparan sulfate
HTD	Hospital for Tropical Diseases
ICU	Intensive Care Unit
Ig	Immunoglobulin
INR	International Normalized Ratio
ISI	International Sensitivity Index
IU	International Unit
IV	Intravenous
K/μL	*1000/μL
L	Liter
M protein	Membrane protein
MAC ELISA	IgM antibody-capture enzyme-linked immunosorbent assay
mg	Milligram
mL	Milliliter
mmol	Millimole

mol	Mole
µg	Microgram
µL	Microliter
N/A	Not applicable
NPP	Normal pooled plasma
NS protein	Nonstructural protein
OFI	Other viral febrile illnesses
OUCRU	Oxford University Clinical Research Unit
PAI-1	Plasminogen activator inhibitor-1
PAP complexes	Plasmin - Antiplasmin complexes
PBS	Phosphate buffered saline
PC	Protein C
PCR	Polymerase chain reaction
PE	Phycoerythrin
pI	Isoelectric point
PICU	Paediatric Intensive Care Unit
PP	Pulse pressure
PS	Protein S
PT	Prothrombin time
PTT	Partial thromboplastin time
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SOP	Standard Operating Procedure
SSC	Side scatter
TFPI	Tissue factor pathway inhibitor
TF-VIIa	Tissue Factor-Factor VIIa complex
TM	Thrombomodulin
tPA	Tissue plasminogen activator

vWF	von Willebrand factor
WBC	White blood cell
WHO	World Health Organization
°C	Degree Celcius
95% CI	95% confidence interval

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Chapter 1

Introduction

1.1 Dengue infection - the disease burden

Dengue infection is the most important arthropod-borne viral disease in tropical and sub-tropical countries, not only across Southeast and South Asia but also in Central and South America. Transmission is also seen in Africa, Australia and the Eastern Mediterranean (Figure 1.1) [1].

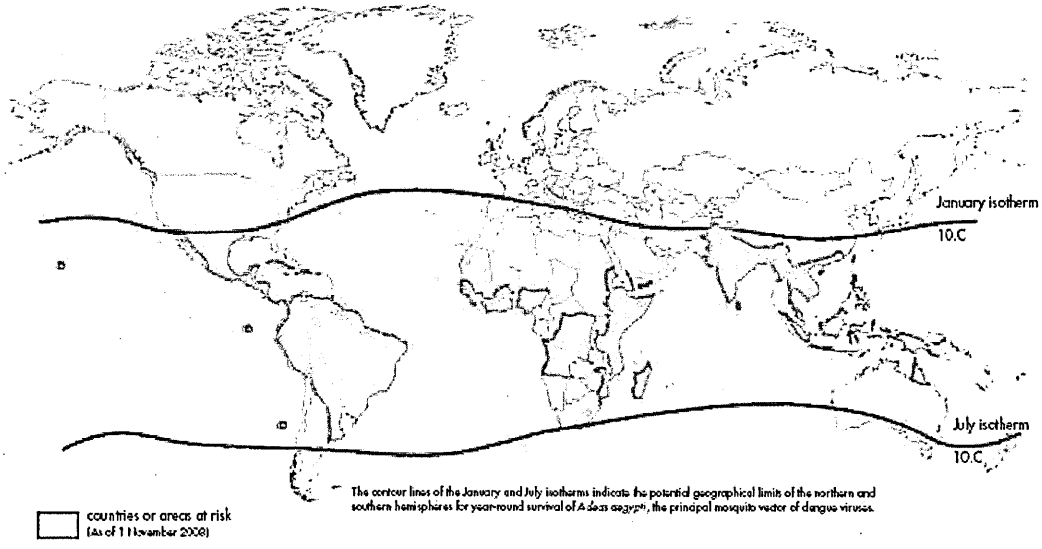


Figure 1-1: Countries/areas at risk of dengue transmission, 2008. *(Taken from World Health Organization (WHO) Guideline, 2009)*

It is estimated that 2.5 billion people are at risk of the disease and annually there are around 50 millions symptomatic dengue cases worldwide. The disease has spread rapidly over the last several decades, with increasing numbers of cases reported, together with ongoing geographic expansion to new countries (Figure 1.2) [1]. The disease occurs mainly in urban areas but is now increasingly occurring in rural areas. Dengue disease has considerable social and economic burden for the population in endemic regions [2].

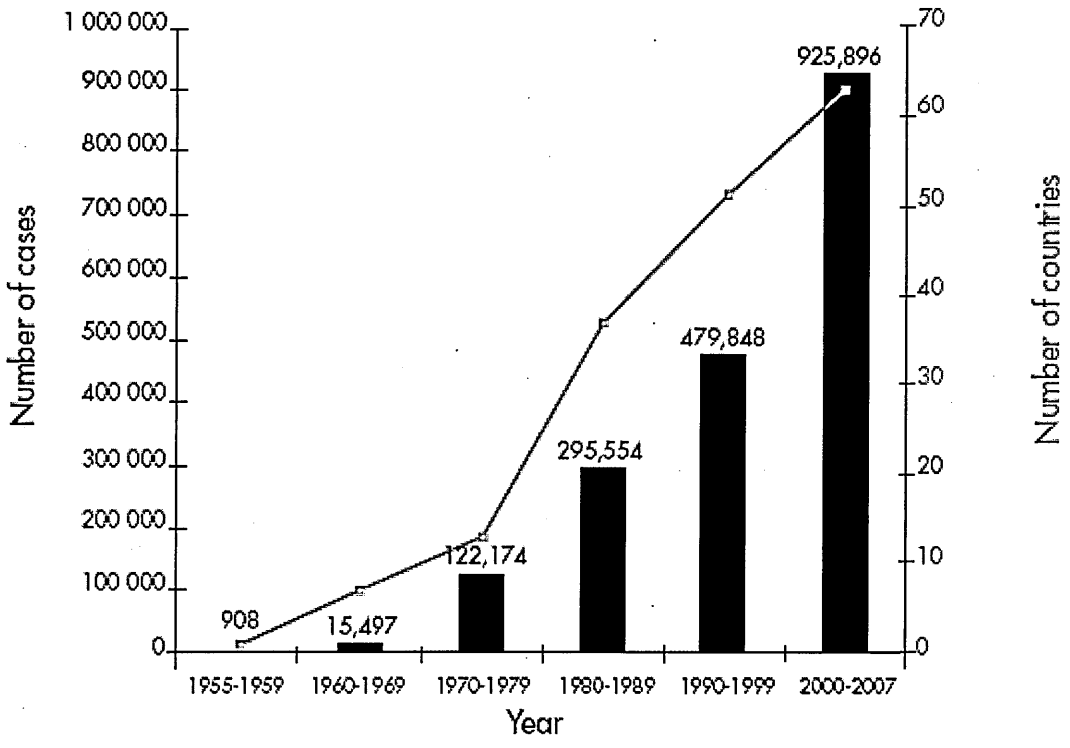


Figure 1-2: Average annual number of dengue cases reported to WHO, and of countries reporting dengue, 1955 to 2007. *(Taken from WHO Guideline, 2009)*

1.2 Dengue – the historical aspect

1.2.1 Dengue-like illness outbreaks

Dengue-like illness were firstly noted in China in 992 and then in the West Indies (the islands of the Caribbean) in 1600s but without detailed information of the disease described. The first detailed description of the disease was made during the Philadelphia outbreak in 1780 by Benjamin Rush with the name "break-heart fever". At the same time, the term "break-bone fever" was used in Spain to describe the outbreak. Dengue-like outbreaks were frequent in North America and tropical regions in Australia and Asia during the 18th and 19th centuries. The term "dengue"

has been commonly used since the outbreak in Cuba in 1828. Later, after World War II when techniques for identifying the causative agent became available, the virus found to be responsible was called the dengue virus [3].

1.2.2 Dengue haemorrhagic fever epidemics

A dengue haemorrhagic fever (DHF)–like disease syndrome with shock and haemorrhage was described in Australia between 1897 and 1902, in Greece in 1928, and in Taiwan in 1931. However, the modern DHF pandemic of this more severe disease form started to emerge in The Philippines in 1954, in Thailand in 1956 and then subsequently in all countries across Southeast Asia. After that, dengue reemerged in Cuba with the first DHF epidemic in 1981 and since then it has spread widely in many countries across Central and South America [3].

1.3 Dengue virus

Dengue virus (DENV) comprises four antigenically distinct serotypes (DENV-1, 2, 3, and 4) and belongs to the family *Flaviviridae*, genus *Flavivirus.* Based on genetic studies of sylvatic dengue strains, four dengue serotypes are thought to have evolved from a common ancestor in non-human primate populations and each entered the urban cycle independently around 500 – 1000 years ago [4]. The virus is a small single stranded, positive-sense RNA virus, containing about 11,000 nucleotides. The virus particles are spherical in shape and are approximately 50 nm in diameter, with a lipid envelope. The virus contain three structural proteins - the small basic capsid (C) protein around the genome of the virus and the envelope (E) and membrane (M)

proteins on the envelope of the virus, and seven nonstructural (NS) proteins named NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Figure 1.3) [1,4].

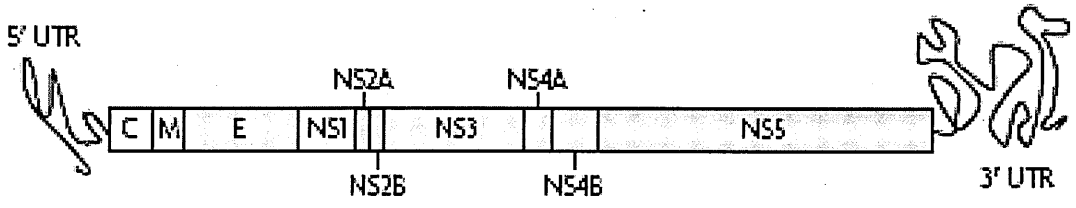


Figure 1-3: The dengue virus genome [4].

The single open reading frame encodes three structural proteins (the C, M and E glycoproteins) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

Each dengue serotype has distinct genotypes, suggesting that DENV has large genetic variation. It is reported that "Asian" genotypes of Dengue 2 and Dengue 3 are more virulent than "American" genotypes [1].

All four dengue serotypes are now circulating in all affected regions in Asia, Africa and America. This is different from the pattern seen 20 to 30 years ago, when serotypes 3 and 4 were not found in America and Africa [4].

1.4 Dengue vector

Aedes aegypti mosquitoes, which are the principle vector of dengue, transmit DENV from infected persons to other persons through their bites. These mosquitoes were firstly identified as the dengue vector in Brisbane in 1904 by Bancroft [5]. Because these mosquitoes need warm temperatures to survive, they are found in tropical and subtropical regions. High seasonal rainfall and high environmental temperature, the

latter more important than the former, are the two main factors that are thought to have contributed to expansion of the *Aedes aegypti* mosquito population and consequently resulted in increased dengue transmission. *Aedes aegypti* lives indoors or around human dwellings and has limited flight range and dispersal. However, the mosquitoes and/or their eggs can be brought to other places a long distance away by transportation vehicles, or as a result of trade in suitable containers such as used vehicle tyres. Mosquitoes usually lay eggs on the walls of natural or artificial water containers, including household water storage containers, and vehicle tyres. *Aedes aegypti* mosquitoes have diurnal feeding, with two peaks – one peak at midmorning and the other in the late afternoon [1,6]. The viruses drawn from infected persons enter the mosquito intestinal tract and undergo various essential life-cycle steps in the mosquito. After an extrinsic incubation period of approximately 10 days, the viruses pass to the salivary glands and can infect healthy persons when these persons are bitten by the mosquito [4].

Dengue virus can also be transmitted by other *Aedes* mosquitoes such as *Aedes albopictus*, *Aedes polynesiensis*. These mosquitoes have particular bionomics, behaviors and geographical distribution, which are thought also to have influenced the expansion of disease although to a lesser extent than *Aedes aegypti* [6].

1.5 The hosts – epidemiological factors associated with infection and disease severity

The disease only affects humans although many genera of non-human primates develop viremia after they are infected with DENV through mosquito bites or during

inoculation experiments, although they remain asymptomatic [3]. As discussed below some host factors can influence the risk of infection and disease severity.

1.5.1 Age

Age is one of the crucial factors influencing the epidemiology of disease. In the past, dengue predominantly affected children, particularly in the school age group. In addition infants under one year may experience symptomatic disease with high risk of severe dengue and death [7,8]. Possible reasons for this are discussed further under the section 1.10.1.

At the present time, although the main disease burden is still in children, increasing numbers of young adults are requiring hospitalization, often with significant associated morbidity and mortality [9,10]. One plausible explanation for this trend could be a reduction in the force of infection due to socioeconomic development and/or improved vector control such that fewer people are exposed during childhood leaving a larger reservoir of susceptible adults [11]. Alternatively lower birth and death rates in transitioning economies may decrease the flow of susceptible individuals into the population and increase the longevity of immune individuals, increasing the likelihood that an infectious mosquito will feed on an immune individual [12]. Differences in clinical presentations between children and adults have been suggested [13-15]. However there is little systematic data available describing the breadth of presentations in different age groups.

1.5.2 Other factors – sex, race, nutritional status, co-morbidities

Based on some national reports and/or hospital activity records, there are higher proportions of dengue cases in male than female patients [8,16]. But interestingly, it has been shown that female patients have a higher risk of developing dengue shock syndrome (DSS) and of dying from this complication than male patients [8,17].

A study in Cuba showed that black persons had a reduced proportion of severe disease than white persons [18]. In addition, serologic surveys showed that the presence of antibody to dengue virus could be demonstrated in a high proportion of Africans and those who are genetically of African origin in Haiti, but the number of symptomatic dengue cases reported among these groups was small [19,20]. One of the plausible explanations is that there could be presence of a resistance gene in black persons.

Several studies have reported that children with malnutrition had a reduced risk of severe disease [21,22]. However, recently one study in El Salvador showed that there was no association between nutritional status and severity of dengue [23].

Studies in Cuba suggested that co-morbidities such as bronchial asthma, sickle cell anaemia and diabetes mellitus could be risk factors for severe disease [18]. Reports focused on fatal cases from Singapore and Puerto Rico also showed that there was a high proportion of patients who had significant co-morbidity diseases such as diabetes mellitus, asthma, etc [24,25].

1.5.3 Genetic markers

Several host genetic markers such as some human leukocyte antigen (HLA) class I alleles, a promoter variant of *CD209* (DCSIGN1-336), some tumour necrosis factor-α and lymphotoxin-α polymorphism genes and AB blood group have been identified as risk factors for severe dengue [26-31]. However, most of the studies to date have been small and the results have not been replicated in second populations so the significance remains uncertain. Recently however a large genome-wide association study from Vietnam has identified susceptibility loci for DSS at MICB and PLCE1, and ongoing studies are investigating whether similar associations can be found in different ethnic groups [32].

1.6 Dengue epidemiology in Vietnam

Historically, a dengue-like illness was first recorded in the north and the central provinces in 1913. In 1929, the southern provinces of Vietnam experienced the first of many dengue fever (DF) epidemics. The first DHF-like outbreak in the country was identified in 1963 in the Mekong Delta region of southern Vietnam. After that, dengue became established as a major public health problem, with tens of thousands of cases reported every year. Between 1963 and 1995, there were 1,518,808 DHF cases and 14,133 deaths reported. Data from the dengue surveillance programme in southern Vietnam showed epidemic peaks of increasing magnitude occurring approximately every five years between 1975 and 1987, with a longer gap of 11 years preceding a large epidemic involving 119,429 DHF cases and 342 fatalities in

1998 [8]. Figure 1-4 illustrates important aspects of dengue epidemiology in Vietnam from 1960 to 2010.

From 1996 to 2008, the annual incidence of hospitalized dengue cases per 100,000 population in the 20 provinces of southern Vietnam peaked at 450 in 1998, then dropped markedly the following year before gradually increasing again over the next decade to reach around 250 cases per 100,000 population in 2008. (Figure 1-5 B). Similar trends were observed among children and adults at three referral hospitals in Ho Chi Minh City – Children's Hospitals No.1 (CH1) and No.2 (CH2) and the Hospital for Tropical Diseases (HTD), with a rapid increase in dengue cases documented in adults. The annual dengue caseload at these hospitals steadily increased between 1996 and 2009, from around 8,000 to 12,000 cases per year in children and from less than 500 to more than 6,000 annual cases in adults (Figure 1-5 A) [8].

All four serotypes of DENV circulate concurrently in the south of Vietnam, but a single serotype generally dominates over multiple dengue seasons. DENV-3 was dominant from 1997 to 1999, followed by DENV-4 from 2000 to 2002. Between 2002 and 2006, DENV-2 was the major serotype detected in hospitalized patients. Currently DENV-1 is the most prevalent serotype, having replacing DENV-2 as the predominant lineage in 2007 [33].

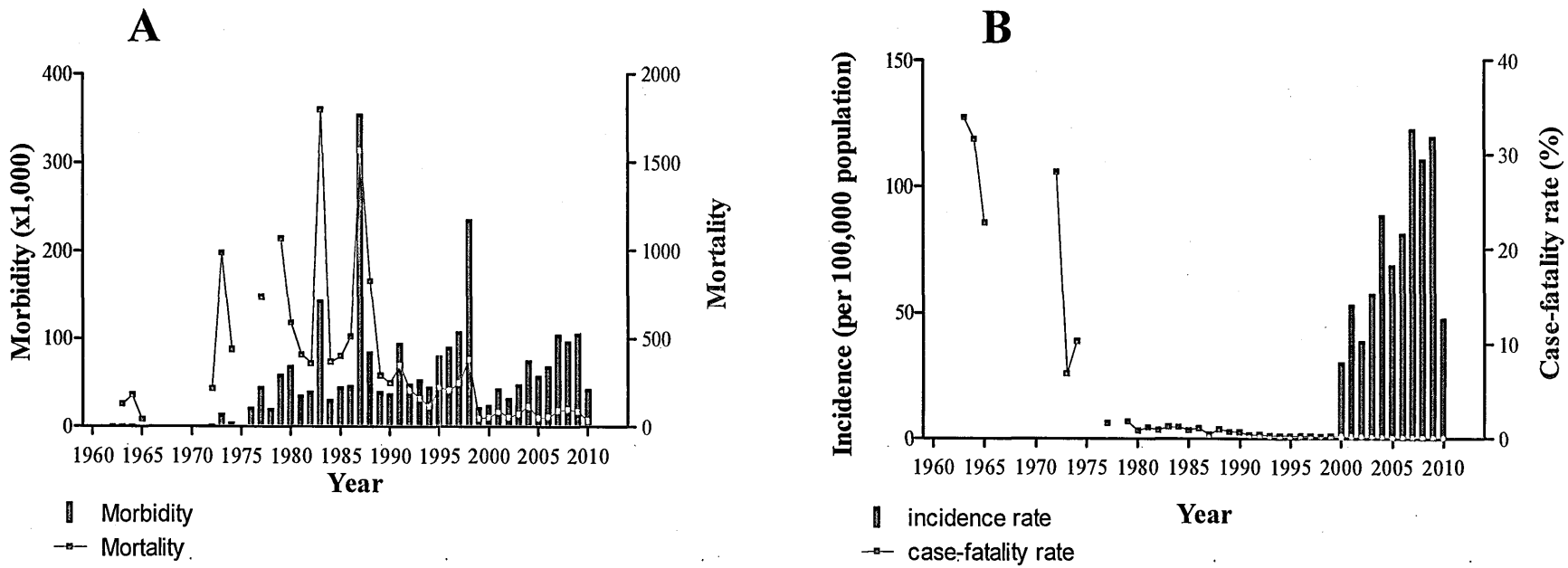


Figure 1-4: Vietnamese dengue cases and deaths officially reported from 1960-2010. *(Source: WHO, Dengue Net).*

(A) Number of dengue (DF/DHF/DSS) cases and deaths in Vietnam.
(B) Case-fatality rate and incidence rate in Vietnam. Incidence data was not available prior 2000

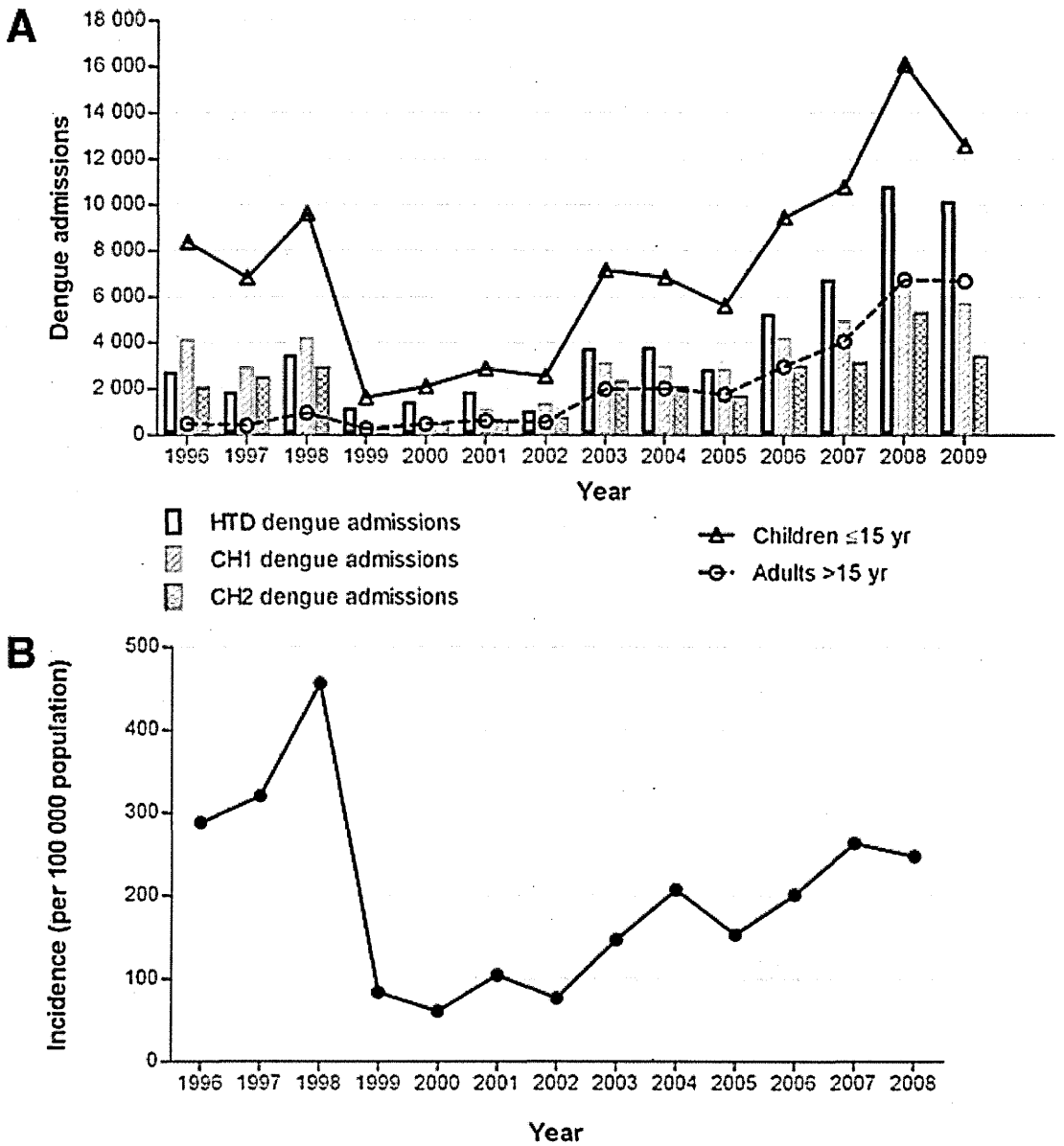


Figure 1-5: Temporal trends in dengue admissions in Ho Chi Minh city, Vietnam, 1996 – 2008 [8].

(**A**) Bars show the number of inpatients with clinically diagnosed dengue each year at the three study hospitals, Hospital for Tropical Diseases (HTD), and Children's Hospitals No.1 (CH1) and No.2 (CH2). Lines show the number of dengue cases in children aged 15 years or less (triangles) and adults over the age of 15 (circles) combined across the study sites. (**B**) Shown for comparison is the annual incidence of hospitalized dengue per 100,000 people in the southern 20 provinces of Vietnam derived from cases reported.

1.7 Clinical manifestations

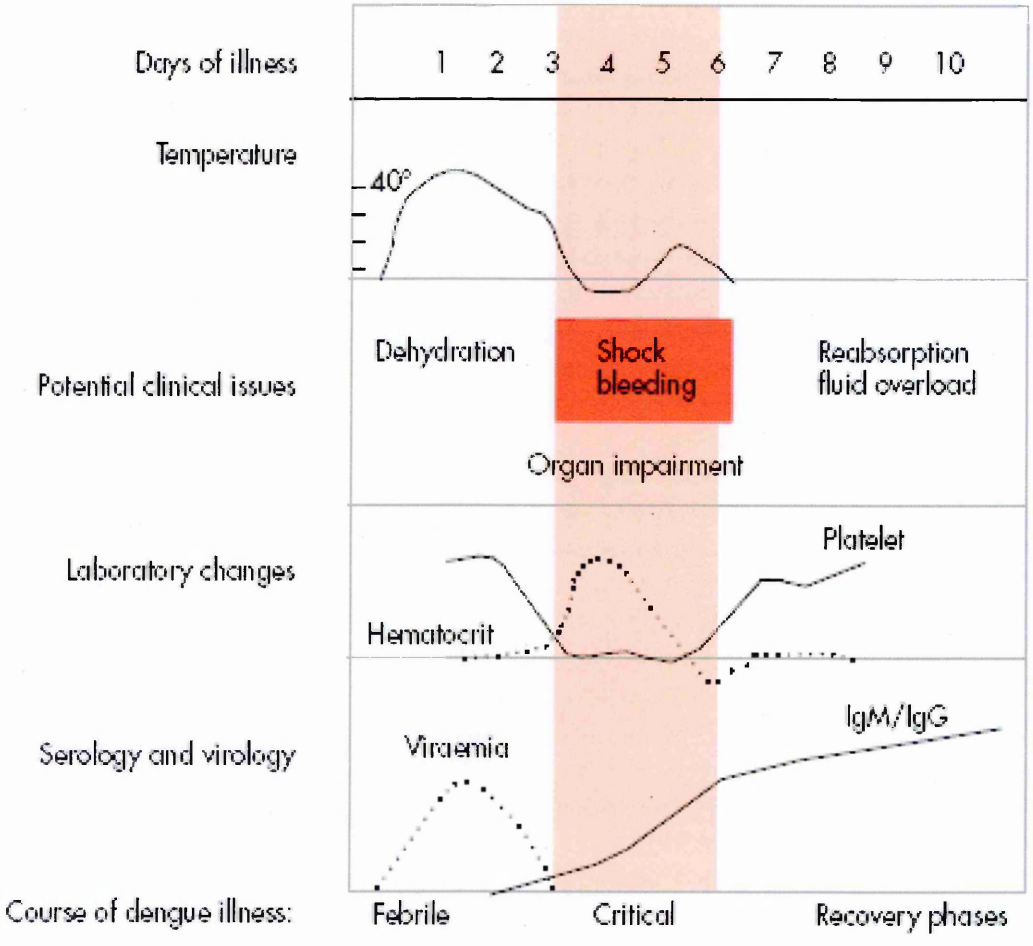


Figure 1-6: The course of dengue illness. *(Taken from WHO Guideline, 2009)*

Dengue infection is asymptomatic in a large proportion of infected persons and symptomatic in the rest. Symptomatic dengue infection has a broad clinical spectrum, ranging from a non-severe acute febrile illness sometimes with evidence of mild vascular leakage and haemostatic derangements, to a severe form of illness with hypovolaemic shock due to plasma leakage, severe bleeding and/or severe organ dysfunction [1,34]. Following an incubation period of around 4 to 10 days from the

bite of an infected mosquito, symptomatic patients may experience 3 clinical stages, usually called the febrile, critical and convalescent periods (Figure 1-6) [1].

1.7.1 Febrile period

Dengue patients have a sudden onset of high fever, which usually lasts for 2 to 7 days. During this time patients often have headache, myalgia, arthralgia, rash, conjunctival injection, and an injected pharynx. Gastrointestinal symptoms such as anorexia, nausea, vomiting or diarrhea occur quite commonly but respiratory symptoms such as sore throat, runny nose and cough are less commonly found. Mild haemorrhage, including skin and mucosal bleeding, may be seen in this period, and the tourniquet test, which assesses the fragility of small vessels, may also be positive at this time. The tourniquet test is performed by inflating a blood pressure cuff applied on the upper arm of the patient to the mid-point between the systolic and diastolic blood pressure for 5 minutes. The test is considered positive when 20 or more petechiae are observed in one inch square on the volar aspect of the forearm just distal to the antecubital fossa [35]. This test has been considered as supportive evidence for a clinical diagnosis of dengue, but since spontaneous petechiae are often present anyway and the test can be positive in other infections, the sensitivity, specificity and diagnostic utility are limited [36]. In terms of laboratory investigations, progressive decreases in leucocyte and platelet counts are usually seen in this period, sometimes with mild increases in hepatic transaminases. Differentiation between dengue infection and other febrile illnesses is not easy at this early stage. Fortunately

complications are unusual at this time and most patients can be managed symptomatically at home and don't need to be hospitalized [1].

1.7.2 Critical period

The majority of patients begin to recover when the fever settles but in a small minority of cases (estimated to be less than 5%) the patient deteriorates around the time of defervescence, which is usually between days 3 – 7 of illness. Signs of vascular leakage including haemoconcentration and ascites/pleural effusions become apparent and may persist for 2 – 3 days. If there is severe vascular leakage, patients will develop hypovolaemic shock, usually preceded by warning signs such as abdominal pain or tenderness, persistent vomiting, and a rapid increase in liver size. Thrombocytopenia and coagulopathy also become worse during this period and as a result skin and mucosal bleeding, still usually mild, are more commonly seen. However, severe bleeding does occur in some patients, often those with prolonged shock, although occasionally also in patients without evidence of vascular leakage. Additionally, in some patients major organ dysfunction such as severe liver involvement, encephalitis/encephalopathy, and myocarditis may be seen at this time, sometimes without signs of significant vascular leakage or shock [1,34].

1.7.3 Convalescent period

After a few days the convalescent period follows with spontaneous reversal of the altered vascular permeability and gradual re-absorption of extravascular fluid over the next 48 to 72 hours. Overall the clinical condition improves, with regained appetite,

resolution of gastrointestinal symptoms, and stable haemodynamic status usually accompanied by a diuresis. Some patients may develop the typical recovery rash, with "isles of white in the sea of red" (Figure 1-7). Bradycardia and electrocardiographic changes may also be seen. The haematocrit (HCT) returns to normal, in many cases after a transient decrease below normal due to the dilutional effect of the re-absorption of leaked fluid back into the intravascular compartment via the lymphatic vessel system. The platelet count also improves rapidly in this period. Some patients may develop respiratory distress due to severe ascites and/or pleural effusions, especially in cases where a large amount of intravenous fluid was given during the critical period. Occasionally pulmonary edema may occur if intravenous fluid is still given in the convalescent period [1].

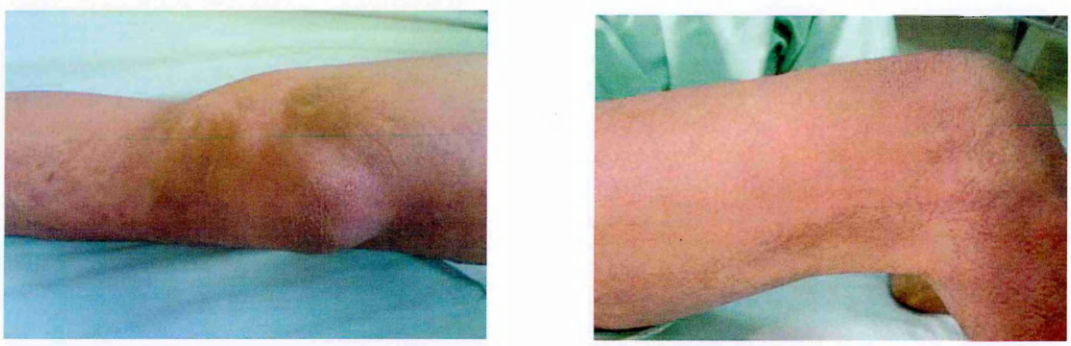


Figure 1-7: Recovery rash in convalescent period in an adult with dengue

1.7.4 Dengue classification

Over the last 20 years there have been many reports describing difficulties in using the traditional DF/DHF classification - including difficulties in applying this classification for clinical and research purposes, and also increasing numbers of reports describing severe cases that were not included within the accepted DHF

classification system [37-39]. In 2008, after conducting a prospective multicentre study across many countries in Southeast Asia and America designed to assess criteria for classification according to clinical severity, a WHO expert group suggested a new classification [1,40]. In this new classification system, dengue infection is divided into 2 categories: dengue and severe dengue. The dengue group is further divided into 2 subgroups: patients with warning signs and those without warning signs (Figure 1-8).

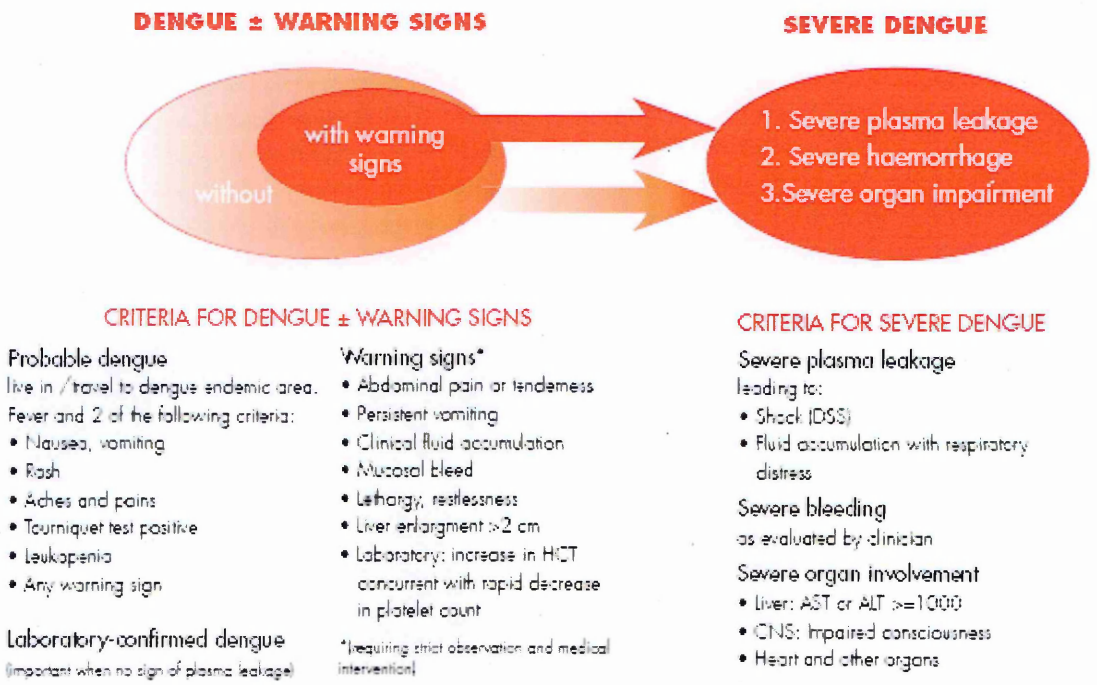


Figure 1-8: Suggested dengue case classification and levels of severity. *(Taken from WHO Guideline, 2009)*

The expert group agreed that "dengue is one disease entity with different clinical presentations and often with unpredictable clinical evolution and outcome". It is

important to remember that non-severe patients may develop to severe dengue whether or not they have warning signs [1].

1.8 Laboratory diagnosis of dengue infection

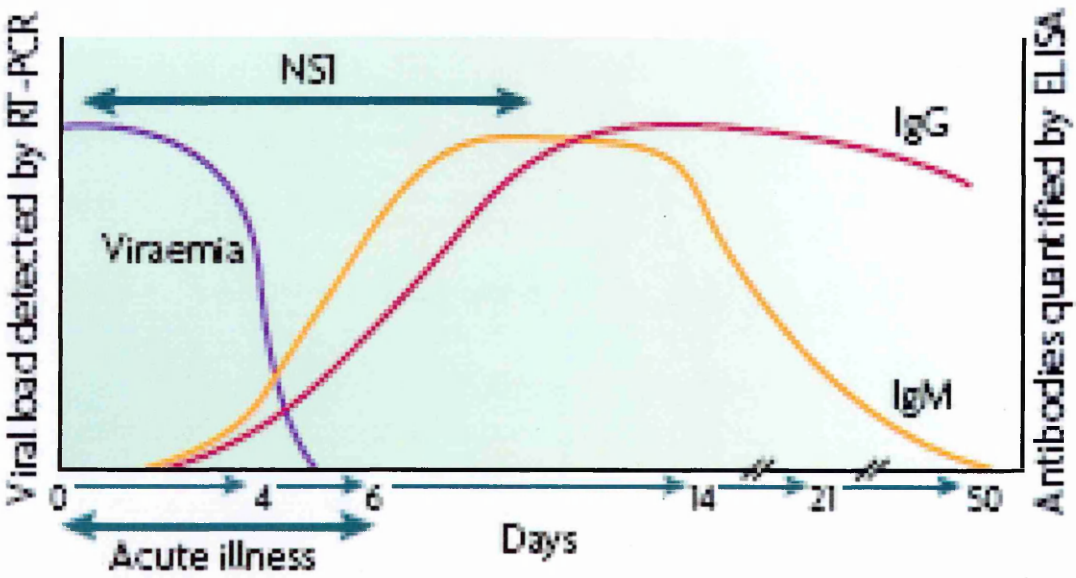


Figure 1-9: Dengue virus, antigen and antibody responses used in diagnosis [4].

Because dengue infection has a wide clinical spectrum, laboratory diagnosis is important to confirm the disease. A variety of different laboratory tests are available for diagnosis – acute dengue infection is usually considered confirmed by any one of the following results a) isolation of virus, b) detection of viral nucleic acid, c) detection of NS1 and d) seroconversion of IgM or IgG. The time frame for a positive result according to these different tests varies (Figure 1-9). Virus isolation, viral nucleic acid and NS1 detection are useful for dengue diagnosis in the early phase up to around day 5 of illness, while serology is useful for dengue diagnosis after the acute phase [1,4]. A positive NS1 result may persist for up to two weeks however,

although the magnitude and kinetics of the response do vary according to the serotype involved and whether the infection is primary or secondary [41]. After the acute phase, when the virus or antigens disappear, IgM and IgG antibodies start to rise, with different patterns seen in primary and secondary infections. These antibodies may persist for several months, so strictly speaking, if seroconversion is not documented, the presence of IgM or high levels of IgG is taken as evidence of recent rather than acute dengue infection [4]. The common laboratory tests for confirmation of dengue infection in clinical practice and in research are described below.

1.8.1 Serological tests

1.8.1.1 IgM antibody-capture enzyme-linked immunosorbent assay (MAC ELISA)

MAC ELISA is a sensitive and relatively specific method for detection of DENV-reactive IgM in serum or plasma. The antigens used in this technique are usually inactivated viral particles. The advantage of this assay is that it is relatively inexpensive and simple. The principle drawbacks of this are a) it is not sensitive in the first few days of illness, b) it is cross-reactive with other *flaviviruses* such as Japanese encephalitis, St Louis encephalitis and yellow fever, but this is not a problem if appropriate specificity controls are included in the assay, and c) there are occasional reports of false positive results with malaria, leptospirosis. Different commercial assays (ELISA and rapid test) are available but they have variable sensitivity and specificity, with ELISA tests better than rapid tests [1,4].

1.8.1.2 IgG antibody-capture enzyme-linked immunosorbent assay (GAC ELISA)

This assay uses similar principles and antigens as the IgM Capture ELISA. In general however, IgG ELISA lacks specificity within the *flavivirus* serocomplex group. Together with MAC ELISA, GAC ELISA on paired sera can be used to confirm acute dengue infection. It is also used to discriminate between primary and secondary infections by assessing the IgM/IgG ratio. However the cut-off for this ratio is variable and not well defined [1,4].

1.8.2 Dengue nucleic acid detection

Since the 1990s it has been possible to diagnose dengue infection by detecting dengue RNA in tissue or plasma samples using reverse transcriptase polymerase chain reaction (RT-PCR) methods, with a variety of different in-house assays. Infecting serotypes of DENV can be detected based on the differences in amplicon sizes. RT-PCR can be single RT-PCR or one-step multiplex RT-PCR. Alternatively, real-time RT-PCR using specific primers and fluorescent probes for each dengue serotype can be used. Real-time RT-PCR assays can either identify one serotype at a time - "singleplex" - or can detect all four serotype from a single sample - "multiplex". RT-PCR and real-time RT-PCR can determine viral titre in the early febrile period, which is considered to be an important predictor of subsequent disease severity. However, this assay is not suitable for routine diagnosis in all settings because it is expensive in terms of specialized training, equipment and reagents [1,4].

1.8.3 DENV NS1 detection

DENV NS1 is a glycoprotein secreted into the peripheral blood. Many tests including antigen-capture ELISA and lateral flow antigen detection systems have been developed to confirm dengue infection by identifying NS1 protein [4]. The specificity of these tests is very high (approximately 100%), but the sensitivity is variable in different studies [42-44]. The sensitivity depends on the day of illness the sample was taken. The highest sensitivity is seen on the first three days of illness [42]. However, NS1 has been detected up to two weeks after the onset of illness, when DENV RNA can no longer be detected by polymerase chain reaction (PCR) [45]. In addition, primary infection has relatively higher sensitivity compared to secondary infection [42]. At present commercial NS1 detection tests can't discriminate the infecting virus serotype, although a serotyping NS1 ELISA that can detect virus serotypes with overall sensitivity of 76.5% and specificity of 100% was developed recently in Thailand [46].

1.9 Clinical management [1,47]

There is no specific drug for treatment of dengue infection at the present time. The general rule for good management is to diagnose patients early, attempt to identify predictors of severe forms of disease as soon as possible, and to give suitable supportive treatment for the patient. The particular management strategy depends on whether patients are diagnosed as dengue (with and without warning signs) or severe dengue.

1.9.1 Dengue without warning signs

For most patients in this group, it is safe to manage them at home in the early febrile period. Hospital admission may be indicated if patients live far away with limited access to health care, or if there is no competent person to care for them at home. Parents or guardian should be instructed clearly about the following things:

- Early recognition of warning signs or evidence of severe dengue. They should return with the patient to the health care system promptly if they have any of these signs.

- To control fever using physical methods such as fans, tepid sponging etc., and to provide antipyretic therapy with paracetamol only. Aspirin and other non-steroidal anti-inflammatory drugs are contraindicated.

- To rehydrate with oral rehydration salts (Oresol) or similar preparation, together with a light diet.

- To return to the health care system with the patient for daily assessments during the critical period until the fever has gone for at least 24 hours.

On daily assessment, health care workers should look carefully for warning signs and/or evidence of severe dengue, and the HCT and platelet count should be checked at each visit. The changing trend of the HCT and platelet count can be very helpful both for diagnosis of dengue infection and to identifying the magnitude and speed of onset of vascular leakage. If patients have warning signs, or a rapid and severe increase in haemoconcentration, they must be admitted. Otherwise most patients can be managed at home provided they can tolerate oral fluids.

1.9.2 Dengue with warning signs

It must be kept in mind that both dengue with and without warning signs may develop to severe dengue, although patients with warning signs do have a higher risk of developing to severe dengue. Thus patients with warning signs must be admitted. Management of these patients includes:

- Close observation to allow early recognition of severe dengue in the critical period

- Antipyretic therapy and oral dehydration should be given as indicated for outpatient management

- Intravenous fluid therapy with isotonic crystalloid solutions is indicated for those patients with repeated vomiting, a very high or rapidly rising HCT or any signs of cardiovascular compromise. The minimum amount of fluid that can maintain hemodynamic status and give good urine volume (at least 0.5 ml/kg/hour) should be prescribed. The intravenous fluid infusion should be stopped if patients can tolerate oral dehydration and the hemodynamic status is stable.

- Careful observation including regular pulse and blood pressure monitoring until fever has gone for at least 24 hours without antipyretics, detailed fluid intake and output. HCT and platelet counts should continue to be checked at least once a day. Other blood investigations may be indicated depending on the clinical picture.

1.9.3 Severe dengue

Management is supportive and depends on the clinical pattern of severe disease seen. The general principles of management of hypovolaemic shock due to plasma leakage,

and of severe bleeding, will be discussed here. Management of severe liver involvement and encephalopathy is similar to the standard treatment of these disorders.

1.9.3.1 Management of hypovolaemic shock due to plasma leakage

Dengue patients with hypovolaemic shock due to vascular leakage require emergency resuscitation. These patients should be admitted to a high dependency or intensive care unit (ICU), staffed by clinicians and nurses experienced in the management of severe dengue. Judicious intravenous fluid resuscitation is the mainstay of the treatment, with most endemic countries establishing formal guidelines for the rate, volume and type of fluid to be used in particular circumstances, in order to try to prevent iatrogenic volume overload. Briefly, plasma losses must be replaced immediately and rapidly with isotonic crystalloid solutions, or with colloid solutions in cases with profound shock (no recordable pulse or blood pressure, or pulse pressure $\leq$ 10 mmHg). A reducing schedule of replacement with crystalloid solutions should then be continued for 24 to 48 hours to counteract ongoing plasma losses and maintain an effective circulation until the re-absorptive phase of the illness begins. Further boluses of colloid resuscitation may be needed for recurrent shock due excessive plasma losses, but must be given with great care and frequent clinical reassessment to prevent fluid overload or pulmonary edema in such patients. The aims of fluid resuscitation are to improve central and peripheral circulation, and to improve organ perfusion until the altered vascular permeability reverts spontaneously to normal. Careful monitoring of vital signs, mental status, and fluid input/output,

together with serial HCT measurements, is essential for assessing the patient's response.

1.9.3.2 Management of severe bleeding

In children, severe bleeding is almost always seen in association with profound or prolonged shock. But in adults, it may be seen in patients without shock. However, particularly in patients with DSS and altered gut motility, internal bleeding may not be apparent for many hours until the first melaena stool is passed. Patients with overt or suspected severe bleeding must be admitted to ICUs with access to blood transfusion services. Fresh whole blood or packed red cells and oxygen are urgently indicated for those patients. Platelet concentrates, fresh frozen plasma and cryoprecipitate may be indicated for some patients with severe bleeding to correct associated coagulation disturbances. However blood products should be given with caution because of the risk of fluid overload and other serious adverse effects. Careful monitoring of bleeding severity, based on frequent haemodynamic monitoring and repeated clinical assessments, together with rapid access to serial haematocrit measurements ideally using an on-site micro-haematocrit centrifuge, is important to assess the patient's response, and to direct therapy.

1.10 Pathogenesis

1.10.1 Pathogenesis of vascular leakage

Systemic vascular leakage is the most important phenomenon in dengue. Although there has been considerable progress in understanding relationships between

immunological derangements and dengue disease, the underlying pathogenic mechanisms responsible for vascular leakage are still poorly understood. The well known "antibody-dependent enhancement" hypothesis suggests that low concentrations of maternal dengue antibodies in infants, or residual heterotypic non-neutralising antibodies from an earlier infection in older patients, bind to a new virus of another serotype introduced in the current infection, and amplify uptake of this virus into cells of the macrophage-monocyte lineage encouraging increased viral replication. The resulting increase in viral load then drives an immunopathogenic cascade that alters microvascular structure or function in some way, thereby resulting in a transient increase in permeability [48,49]. Rapid mobilisation of serotype cross-reactive memory T cells has been proposed as an alternative mechanism to trigger the inflammatory cascade and many other factors such as differences in viral virulence, molecular mimicry, immune complex and/or complement mediated dysregulation, genetic predisposition and other host factors such as age and nutritional status, have been shown to correlate with disease severity in terms of vascular leakage [48,50].

The pathway by which such immunological derangements act on the endothelial barrier and results in increased vascular leakage is not known, at least partly due to lack of understanding surrounding normal microvascular permeability mechanisms. However over the last 20 years there have been considerable advances in understanding the structure and function of the endothelial cells and their associated surface glycocalyx layer with respect to microvascular permeability in health [51,52]. The glycocalyx layer, a highly anionic fibre matrix of proteoglycans,

glycosaminoglycans (GAGs) and adherent plasma proteins, is located on the luminal surface of the vascular endothelium and anchored in the plasma membrane of the endothelial cells. It is now considered to be the primary barrier to the movement of water and molecules, restricting movement of such molecules according to their size, charge and shape, while the underlying endothelial cells appear to function as the secondary barrier [53]. Very few studies have examined structural effects of dengue infection in the human microvasculature and only minor non-specific histological changes have been shown in the microvessels, with no convincing evidence that the virus infects endothelial cells *in vivo* [54-56]. However, visualization of the surface glycocalyx layer is currently not possible using conventional microscopy techniques, so the effects of dengue infection on this layer have never been evaluated.

One way to investigate possible changes in the surface endothelial glycocalyx layer is to study the characteristics of the proteins lost during dengue infections, focusing on the size and charge of the molecules that leak. Previous work from Oxford University Clinical Research Unit (OUCRU) looking at fractional clearance estimations for endogenous proteins has demonstrated firstly that smaller proteins were relatively more affected than larger proteins, and secondly that albumin, normally protected from leakage by its strong negative charge, showed a clearance pattern similar to transferrin - i.e. a neutral molecule of similar size. These results indicate that size selectivity is at least partially retained during acute dengue infections, and suggest that charge selectivity is impaired. In the same study urinary GAG excretion was significantly increased in children with DSS, suggesting a possible role for disruption of the surface glycocalyx in the pathogenesis of the vascular leak [57]. Recently a

more detailed examination of protein clearances using a dextran clearance technique found no differences in dextran fractional clearances at serial time-points (critical period of illness, one and three month follow-up) in dengue patients who had clear evidence of vascular leakage, and also no difference in comparison with the results from a group of healthy persons. One possible explanation could be that during dengue infections endogenous proteins are displaced from the glycocalyx layer altering the permeability characteristics of the endothelial complex – however, during the dextran infusion the dextran molecules may replace the lost proteins and temporarily restore normal permeability [58].

1.10.2 Pathogenesis of haemorrhage

Almost all symptomatic dengue patients have a coagulopathy as well as thrombocytopenia. Although many small studies about the coagulopathy in dengue infection have been carried out during the last 30 years, the pathogenesis remains poorly understood. Debate continues about whether true disseminated intravascular coagulation (DIC) occurs, and about whether fibrinolysis is activated or impaired in dengue infection [59,60].

1.10.2.1 Normal haemostasis

In order to identify and understand the possible coagulation abnormalities that may occur during dengue infections it is necessary to consider the main mechanisms controlling haemostasis in the healthy state.

Normal haemostasis depends on a carefully coordinated balance of three pathways, as shown in Figure 1-10. The pro-coagulant (blue), anti-coagulant (red) and fibrinolytic

(green) pathways act together regulated by proteins expressed on the vascular endothelial surface and on circulating blood components. The figure emphasizes the role of the tissue factor/factor VIIa complex that forms when a blood vessel is injured, in activating the pro-coagulant pathway. This activation results in generation of thrombin, which then acts on fibrinogen forming fibrin and resulting finally in the creation of the fibrin blood clot. Table 1-1 summarizes the principle characteristics of the coagulation factors involved in thrombin generation. As discussed above, two characteristics of plasma proteins that are likely to be influence their capacity for leakage are the molecular size and the charge of individual proteins. The molecular weights of many of the coagulation proteins listed are similar to that of albumin (69,000 Daltons) and therefore they might be expected to leak, especially in patients with increased vascular permeability. With respect to charge, the isoelectric point (pI) is the pH at which a particular molecule carries no net electrical charge; at a pH below their pI proteins carry a net positive charge, whilst above it they carry a net negative charge. Most coagulation proteins for which the pI is known, would therefore carry a negative charge at physiological pH.

The thrombin generation process is carefully regulated by thrombin inhibitors (tissue factor pathway inhibitor and antithrombin) acting together with the different elements of the protein C pathway, to prevent overproduction of thrombin. Finally the fibrinolytic pathway functions to convert plasminogen to plasmin, which then breaks down fibrin to fibrin degradation products (FDPs), thus helping to remove fibrin and balancing the forward action of the procoagulant pathway [61].

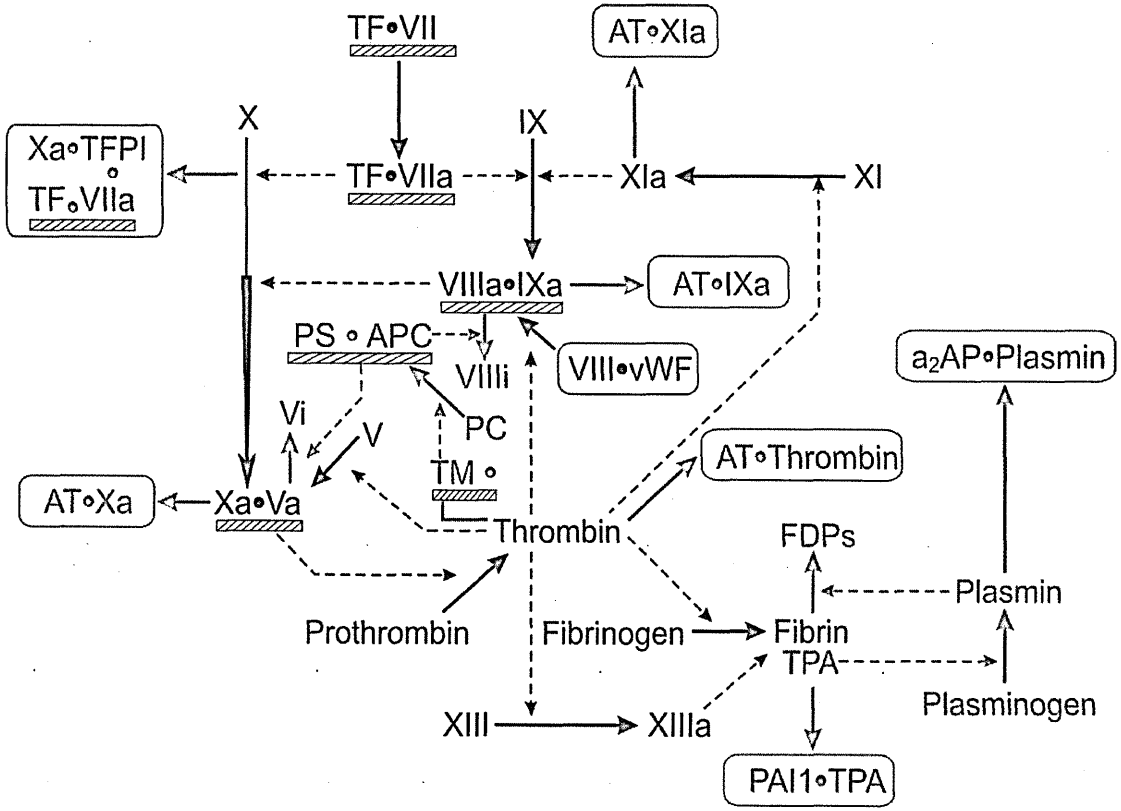


Figure 1-10: The network of haemostasis

(Taken from "Postgraduate Haematology", fourth edition, 1999)

Pro-coagulant pathway: blue components

Anti-coagulant pathway: red components

Fibrinolytic pathway: green components

Inhibited complexes are shown in cartouches.

Dotted arrows = proteolytic activation/inactivation
Solid arrows = change of state

Raised bold points = complexes

The hatched underline represents a phospholipid surface upon which activator complexes assemble.

TF-VIIa: tissue factor-factor VIIa complex

vWF: von Willebrand factor

TFPI: tissue factor pathway inhibitor

AT: antithrombin, TM: thrombomodulin

PS: protein S, PC: protein C, APC: activated protein C

tPA: tissue plasminogen activator

PAI-1: plasminogen activator inhibitor-1

a2AP: α2 antiplasmin

FDPs: fibrin/fibrinogen degradation products.

Table 1-1: Some characteristics of human coagulation factors *(Taken from "Postgraduate Haematology", fourth edition, 1999)*

Traditional name	**Preferred nomenclature**	**Molecular weight** (Dalton)	**Isoelectric point (pI)***	**Plasma concentration** (μg/ml)	**Half-life** (Hours)
Fibrinogen	Factor I	340,000	5.5	$2\text{-}4 \times 10^3$	90
Prothrombin	Factor II	72,000	4.2	120	65
Tissue factor	Factor III	45,000	-	0	-
Calcium	Factor IV	40	-	100	-
Proaccelerin	Factor V	330,000	5.3	10	15
Proconvertin	Factor VII	48,000	-	1	5
Antihaemophilic factor	Factor VIII	360,000	-	0.05	10
Christmas factor	Factor IX	57,500	4 - 5	4	25
Stuart-Prower factor	Factor X	55,000	5.5	12	40
Plasma thromboplastin antecedent	Factor XI	160,000	4 - 5	6	45
Hageman factor	Factor XII	85,000	-	40	50
Fibrin stabilizing factor	Factor XIII	320,000	-	20	200
Fletcher factor	Prekallikrein	90,000	-	40	35
Fitzgerald factor	High molecular weight kininogen	120,000	-	70	150

**: Taken from "Advances in Enzymology and Related Areas of Molecular Biology", 2009 [62]*

Dysregulation of any of the three pathways can result in coagulation abnormalities. Conventionally the first step in assessing possible abnormalities of the coagulation cascade is to perform screening tests such as the prothrombin time (PT), activated partial thromboplastin time (APTT), plasma fibrinogen levels and FDP levels. The PT and APTT are *in vitro* tests that are used to assess two pathways of coagulation activation: the "extrinsic" pathway (activation of coagulation by tissue factor) and the "intrinsic" pathway (activation of coagulation by the contact system), respectively. This division of coagulation activation bears only a limited relationship to the way coagulation is activated *in vivo* (Figure 1-10) but remains a useful concept for interpreting the results of laboratory investigations. The PT measures the time to clot formation in test plasma after addition of an optimal concentration of a tissue extract (thromboplastin) containing tissue factor - it is largely dependent on factors of the "extrinsic" pathway (factor VII) and the common pathway (factor X, V, II and I). The result for an individual sample, measured in seconds, will vary according to the type of thromboplastin used. The International Normalized Ratio (INR) is a standardized way of expressing the results. Each manufacturer gives an International Sensitivity Index (ISI) value for any thromboplastin they produce. The ISI value shows how a particular batch of thromboplastin compares to an international reference thromboplastin. The INR is the ratio of a patient's test result to the log mean normal PT from 20 normal donors, raised to the power of the ISI value for the analytical system used: $INR = (PT\ patient/log\ mean\ normal\ PT)^{ISI}$ [63,64].

On the contrary, the APTT measures the time to clot formation of test plasma after activation of the contact factors, without added tissue thromboplastin. It is dependent on factors of the "intrinsic" pathway (factor XII, XI, IX and VIII) and the common pathway (X, V, II and I) [63,64]. In clinical practice, the APTT is commonly used for monitoring the effect of heparin therapy. However, assays that measure anti-factor Xa activity have become available recently and are generally considered to be more effective for monitoring anticoagulant therapy [65]. As yet this type of test is not readily available in Vietnam.

In addition to the requirement for normal levels of functioning coagulation proteins, since the endothelial surface is an important participant in the haemostatic process, if endothelial cells or other components of the microvascular barrier are lost from the vessel wall following a pathological insult, then haemostasis may be affected. Secondly if endothelial cells are released into the circulation, becoming circulating endothelial cells (CECs), they may themselves participate in the haemostatic process, possibly by the expression of active molecules such as thrombomodulin or tissue factor on the cell surface [66]. For example, it has been shown that CECs isolated from patients with sickle cell disease express tissue factor that is functionally active *in vitro* [67].

Both coagulation and inflammation are essential to an effective host response to infection. Increasingly the molecular links between sepsis induced inflammation, haemostasis and endothelial dysfunction are being elucidated [68,69]. There appears to be an intricate relationship between the systems, whereby inflammation leads to

activation of coagulation, and coagulation also considerably affects inflammatory activity. Inflammation induced activation of coagulation is primarily caused by upregulation of tissue factor expression on monocytes, macrophages and endothelial cells, release of vWF leading to platelet aggregation and adhesion to the sub-endothelial surface, and down regulation of the fibrinolytic and anticoagulant pathways.

1.10.2.2 Pathogenesis of haemorrhage in dengue infection

Early studies (before the year 2000)

After the emergence of DHF in Southeast Asia in the 1950's, many studies about the coagulopathy were conducted in the region, especially in Thailand. However sample sizes were invariably small and the studies were frequently biased towards recruiting particular groups of severe patients. Thrombocytopenia was the major finding and this correlated with disease severity in terms of shock [70]. There was also evidence for increased destruction of platelets demonstrated by shortened duration of survival of transfused platelets [71]. It has been suggested that increased destruction of platelets is related to immune complexes containing dengue antigen found on platelet cell surfaces [72]. Also, evidence of decreased bone marrow production of platelets has been shown, with marked depression of all bone marrow elements and the presence of vacuoles in megakaryocytes in marrow aspirates obtained from day 1 to 4 of illness [73]. Platelet function was also found to be abnormal in dengue infection. In the acute phase of the disease, platelet aggregation in response to adenosine diphosphate was

impaired, with concurrent increases in plasma β-thromboglobulin and platelet factor 4 indicating that platelet secretory activity was increased [74].

In terms of screening coagulation tests, in addition to thrombocytopenia most of the early studies found normal or slightly prolonged PT, significantly prolonged partial thromboplastin time (PTT), mild to moderate reduction of fibrinogen levels and slightly or moderately increased FDPs [70,75,76]. Only a few studies measured specific coagulation factors in small numbers of patients. It was shown that factor II, V, VII, VIII, IX, X and XII levels were slightly decreased [76]. At this time, coagulopathy in dengue was thought of as a mild to moderate DIC although without clear evidence of activation of the pro-coagulant pathway or of the presence of FDPs.

In addition, based mainly on investigation in animals and *in vitro* experiments some authors implicated several possible mechanisms for disturbances in the fibrinolytic pathway in DHF. Antibodies to the dengue E protein that cross-react with plasminogen and thus might influence the fibrinolytic pathway were detected in 75% of Thai patients with dengue [77]. These antibodies were later shown to correlate with the occurrence of haemorrhage but not capillary leak or thrombocytopenia in Tahitian children infected with dengue [78]. Similar antibodies, raised in mice and rabbits, have been demonstrated to inhibit plasmin activity in an *in vitro* system [79]. However the pathogenic role of the antibodies remains uncertain since the animals did not develop any bleeding problems, and the authors also demonstrated that in humans the antibodies persist for several months, by which time the patients had recovered fully. Antibodies to the dengue NS1 protein raised in mice have also been shown to

cross react with human fibrinogen, platelets and endothelial cells and this may have a role in the pathogenesis of DHF [80]. In an *in vitro* system, it has been demonstrated that dengue viral isolates are able to activate plasminogen in the absence of thrombin generation (usually considered a necessary step prior to plasminogen activation), and that the plasmin so generated could degrade both fibrin and fibrinogen [81].

Recent studies

Several more recent studies have examined potential mechanisms underlying the observed platelet abnormalities in more detail. Using an *in vitro* assay, a group in Japan found that during the acute phase of secondary dengue there was a significant increase in phagocytosed platelets within macrophages compared with the findings in healthy persons and this correlated inversely with the platelet count. This suggests that phagocytosis of platelets is one of the mechanisms responsible for thrombocytopenia [82]. Another group demonstrated expression of various dengue proteins within platelets experimentally infected *in vitro* and within platelets isolated from dengue patients, using a variety of different techniques such as flow cytometry, Western blot analysis, double immunofluorescence staining and confocal microscopy. This group also identified dengue viral-like particles within platelets from dengue patients using electron microscopy. These findings suggest that the dengue virus may replicate in platelets and may partly explain the thrombocytopenia and platelet dysfunction that is characteristic during infection [83].

In terms of the coagulopathy recently some groups have focused on looking for evidence of specific derangements in the three major pathways – pro-coagulant, anti-

coagulant and fibrinolytic – during disease evolution through the various different stages [84-90]. Table 1-2 summarizes the main findings of these studies. In the light of these findings there has been much debate as to whether true DIC, with primary activation of the pro-coagulant pathway leading to secondary effects on the other pathways, occurs or whether the primary derangements relate to the anticoagulant and/or fibrinolytic pathways directly.

Work in pediatric patients representing the spectrum of infection from mild to severe disease at OUCRU found that thrombocytopenia, prolongation of the APTT and reduction in plasma fibrinogen levels were the three typical coagulation disturbances, without clear prolongation of PT or presence of FDPs; and all of these typical disturbances were strongly correlated with vascular leakage severity. The findings suggest that the primary abnormality is not likely to represent a true DIC. Given that plasma proteins leak from the circulation one possible mechanism could involve leakage of coagulation proteins, most of which are small in size (see Table 1-1), from the intravascular to the interstitial fluid compartment [86,90]. An alternative or additional explanation could involve disruption of the surface glycocalyx layer during infection, resulting in release of molecules such as the GAG heparan sulfate (HS) into the circulation. HS is a major constituent of the endothelial surface glycocalyx, is a known receptor for dengue virus and is also known to have anticoagulant properties similar to heparin – if released into the circulation it could result in prolongation of the APTT [90]. As mentioned above increased urinary GAG excretion has been demonstrated during acute dengue, although plasma levels have not been measured previously [57].

Table 1-2: Main findings describing the disturbances found in the three coagulation pathways in recent studies of dengue infection

Study	Sample size	Pro-coagulant pathway	Anti-coagulant pathway	Fibrinolytic pathway	Conclusion
1.Krishnamurti et. al., 2001 [84]	- 21 children with DF, 8 with DHF I, 30 with DHF II and 9 with DHF III	- Normal PT, ↑ APTT - No decrease in coagulation factor levels - ↑ prothrombin fragment F1.2		- ↓ Fibrinogen - ↑ D-Dimer - ↓ Plasminogen, ↓ α2 antiplasmin	Haemorrhage is likely due to platelet activation rather than consumptive coagulopathy
2. Huang et. al., 2001 [85]	- 17 children and adults with DF, 8 with DHF/DSS - 17 healthy persons (controls)	- Normal PT , ↑ APTT: DHF worse than DF		- ↑ tPA - PAI-1: normal in acute stage, ↑ in convalescent stage - tPA/PAI-1 in acute stage: DHF/DSS worse than DF	Activation of both coagulation and fibrinolysis: DHF/DSS worse than DF
3. Wills et. al., 2002 [86]	- 167 children with DSS for screening coagulation tests, 48 children of those for specific investigations	- Minor ↑ PT - ↑ APTT - ↑ TF	- ↓ PS, ↓ PC, ↓ AT III, correlated with severity of shock - ↑ TM, normal TFPI	- ↓ Fibrinogen - ↑ PAI-1	Unlikely to be true DIC
4. Van Gorp et. al., 2002 [87]	- 43 children with DHF III and 7 with DHF IV	- ↑ PT (in non survivors), ↑ APTT - ↑ prothrombin fragment F1.2, ↑ thrombin-AT III complex	- ↓ PS, ↓ PC	- ↓ Fibrinogen - ↑ D-Dimer - ↑ tPA, ↑ PAI-1 - ↑ PAP complexes	Activation of both coagulation and fibrinolytic systems but with relative impairment of fibrinolysis

Study	Sample size	Pro-coagulant pathway	Anti-coagulant pathway	Fibrinolytic pathway	Conclusion
5. Carlos et. al., 2005 [88]	- 239 children with DF and 120 with DHF	- Normal PT in almost all patients		- ↓ Fibrinogen in 30 – 40% of patients - ↑ FDP in one fifth of patients	Increased fibrinolysis but not classical DIC
6. Sosothikul et. al., 2007 [89]	- 20 children with DF, 22 with DHF - 38 healthy children (controls)	- Normal PT , ↑ APTT - ↑ thrombin-AT III complex, ↑ TF, ↑ VIIa, and ↓ factor VIII	- ↓ TAFIa levels	- ↓ Fibrinogen - Minor ↑ D-Dimer - ↑ tPA, ↑ PAI-1	Typical DIC as in sepsis
7. Wills et. al., 2009 [90]	- 367 children with dengue infection recruited early in the course of illness, classified into different magnitude of haemoconcentration - 40 OFI patients as controls	- Normal PT (not different with results of OFI group) - ↑ APTT (correlated with vascular leakage severity but not bleeding)		- ↓ Fibrinogen (correlated with vascular leakage severity but not bleeding) - FDPs was negative or weakly positive in the majority of patients	Unlikely to be classical DIC

1.11 Knowledge gaps and objectives of this thesis

As discussed above the pathogenesis of the coagulopathy associated with dengue infection remains poorly understood. In addition to thrombocytopenia, an increase in APTT and a reduction in fibrinogen levels are the two most consistent abnormalities detected. There has been much debate as to whether true DIC occurs and other plausible mechanisms have been suggested, including leakage of coagulation proteins, direct effects of the virus on fibrinogen activation, and/or presence of a circulating anticoagulant.

In addition, data from adults, the group in which bleeding appears to be a more common problem [13-15], are very limited. It is possible that co-morbid conditions, such as pre-existing liver disease or peptic ulcer disease may explain the apparently greater susceptibility to bleeding complications among adult dengue patients. And finally insights from recent advances in our understanding of the complex molecular interactions between haemostasis, sepsis, and vascular endothelial structure and function, have yet to be applied to the problem. For example, in other diseases with endothelial involvement CECs have been shown to be useful biomarkers of disease and are also thought to participate in the pathogenesis of some coagulation disorders, possibly by the expression of active molecules such as thrombomodulin or tissue factor on the surface of the cells [66].

In addition to contributing to the overall body of knowledge about dengue pathogenesis, better understanding of the mechanisms underlying the haemostatic

derangements associated with dengue may be important for several practical reasons. Firstly platelet transfusions are becoming increasingly common in some centres in Asia, without evidence that this is beneficial and with a small but definite associated risk. Detailed knowledge of the natural history of the thrombocytopenia and coagulopathy may help to inform policy on when to transfuse platelets or other blood products, and/or may form the basis for a formal intervention study designed to address these questions. Finally suggestions for novel or alternative management strategies may arise from improved knowledge of interactions occurring at the molecular level – if for example a circulating anticoagulant is identified, this could be a potential target for an inhibitor.

Objectives of this thesis:

1. a) To describe the clinical and basic laboratory features seen in a large cohort of prospectively recruited adults with dengue infection in comparison with the findings observed in children recruited during the same dengue season.

   b) To describe the liver involvement seen in adults with dengue infection and to investigate possible effects of this on the coagulopathy as well as the effect of co-infection with hepatitis B or C viruses on liver enzymes and coagulation tests.

2. To explore the mechanisms underlying the increase in APTT that is typically seen during dengue infections and to address the following specific hypotheses:-

   a) the APTT increase is related to a decrease in coagulation protein levels due to leakage of these proteins from the intravascular compartment

   b) the APTT increase is related to the presence of a circulating anticoagulant, such as the endothelial glycosaminoglycan heparan sulfate

3. To describe the pattern of CECs and to determine whether there is a correlation between numbers of CECs and bleeding manifestations, coagulopathy, and/or the severity of vascular leakage.

Chapter 2

Materials and Methods

This chapter describes the general clinical, laboratory and statistical methods used for the research presented in Chapters 3 – 5 of this thesis. Specific methods used for particular studies will be presented in the relevant chapters.

2.1 Patient cohorts and clinical methods

A series of prospective observational studies of patients presenting with febrile illness consistent with dengue were carried out over several years. All the clinical studies took place at HTD of Ho Chi Minh City. At HTD, patients of 15 years of age or over are admitted to adult wards while those below this age are managed on paediatric wards. The hospital currently admits 7,000 - 10,000 patients with suspected dengue each year, of whom around two-third are aged 15 or over.

2.1.1 The main adult cohort

Adults admitted to HTD with clinically suspected dengue were eligible for enrolment. Recruitment was targeted to include all patients with suspected dengue admitted to the Adult Intensive Care Unit (AICU) during the two-year period between September 2006 and September 2008 as well as a representative sample of male and female patients (200 each) admitted to the infection wards during 2007.

Within 24 hours of admission designated senior doctors on the infection wards tried to recruit all patients with suspected dengue in specific bays on the wards; diagnostic criteria were not specified as we aimed to include the full spectrum of hospitalised cases. I then collected detailed clinical information later that day, and daily for the duration of the hospital stay, using a standard case report form (CRF). A venous

blood sample was obtained each day for clinical and diagnostic investigations as detailed below. Additional investigations and management were at the discretion of the treating physicians. Hospital policy dictates that any patient with suspected dengue on the infection wards about whom there is any concern must be transferred to one of the ICUs for close observation. I continued to observe daily any patients who were transferred from the infection wards to the AICU. After discharge all patients were invited to attend for review 2-4 weeks later, when a final blood sample was obtained.

2.1.2 Paediatric cohorts

Detailed observational studies of children with suspected dengue have been ongoing for several years at the hospital [86,90,91]. During the time-period of my adult studies, paediatric studies with the following enrolment criteria were in progress: a) children 2-15 years old admitted to the Paediatric Intensive Care Unit (PICU) with clinically suspected dengue and severe complications; b) children 5-15 years old admitted to the paediatric infection wards with suspected dengue. As with the adult studies eligibility criteria on the infections wards were deliberately broad to allow the study physicians to invite all potential subjects with suspected dengue to participate. However, unlike the main adult cohort, due to staff workload constraints on PICU, children were only eligible for study recruitment on this ward once major complications developed (i.e. children transferred to PICU for close observation who did not develop shock or other complications were not included). For my comparative

studies I chose all children recruited to these studies during the same calendar period as the adult patients were recruited.

The protocols for the paediatric studies were similar to those of the adult studies. Briefly, after recruitment, demographic information, clinical history and examination details were recorded on standard CRFs and all subjects were followed daily by trained study physicians until discharge. In all cases daily EDTA plasma samples were obtained for platelet count and HCT measurements, but other sampling was kept to the minimum necessary for the particular studies in progress at any time. Management remained in the hands of the ward clinicians following hospital policies. All participants were invited for review around one month after discharge, when final blood samples were obtained.

2.1.3 The adolescent and young adult cohort for the study on circulating endothelial cells

Teenagers and young adults aged between 12 and 25 years admitted to HTD with clinically suspected dengue were eligible for enrolment into this study. Two main groups of patients were eligible – a) patients with suspected dengue admitted to the infection wards on or before day 3 of illness and b) patients admitted to the ICUs with suspected dengue up to and including day 6 of illness. After full dengue diagnostics had been performed (as described below), participants who were subsequently confirmed to have OFI rather than dengue formed one control group. A second disease-control group consisted of patients admitted to the infection wards or ICUs

with smear positive malaria within the first week of illness; malaria was chosen as a disease in which endothelial involvement also occurs and which is seen with reasonable frequency at HTD. However the upper age limit for recruitment was increased to 35 years for the malaria group, to take into account the local disease epidemiology. Finally a group of healthy volunteers aged 18-25 years was also recruited, to allow identification of the normal range for CECs in a Vietnamese population of a similar age.

The clinical protocol for this study was similar to those described above. I saw each patient daily, recorded detailed history and examination findings on a standard CRF, categorised each subject for the severity of vascular leakage and bleeding after discharge, and invited all patients for review several weeks later. As before additional investigations and management were at the discretion of the physicians responsible for patient care. However as well as the standard haematological and biochemical investigations described below for the adult cohort, additional 2 ml EDTA samples were obtained for enumeration of CECs at intervals during the course of the acute illness and at the follow-up visit. To prevent artificial increases in CEC numbers due to traumatic detachment of CECs during venepuncture, a large bore (19G) butterfly needle was used and at least 2 ml of venous blood was first obtained using one syringe, to be used for the full blood count, coagulation tests etc., before the CEC sample was taken into a second separate syringe [92]. All samples were stored on ice

and quickly transferred to the research laboratory for immediate enumeration of CECs.

2.2 Ethics

Ethical approvals were obtained for all studies from the Scientific and Ethical Committee of the HTD of Ho Chi Minh City and the Oxford University Tropical Research Ethics Committee. All subjects or their parent/guardian gave written informed consent before enrolment into the patient cohorts described above. Similarly all the healthy volunteers gave written informed consent for inclusion in the relevant studies. All studies were carried out in accordance with international standards for the ethical conduct of research involving human subjects.

2.3 Definitions for clinical complications, disease severity and clinical periods in dengue infection

Definitions for clinical complication and disease severity are shown in Table 2-1. Following the new WHO guidelines vascular leakage was classified primarily according to whether or not the patient developed hypovolaemic shock [1].

To investigate changes throughout the course of illness, we divided the clinical course of dengue infection into 3 clinical periods: early febrile period (Day 1 – 3 of illness), critical period for complications such as shock (Day 4 - 6), and convalescent period (Day 7 - 10).

Table 2-1: Definitions for complications and disease severity in dengue infection

Complication	Definition
Dengue shock syndrome	Hypotension for age or narrowing of the pulse pressure, with impaired peripheral perfusion [1], if considered to be caused by plasma leakage not bleeding, and requiring volume resuscitation
Bleeding severity	Bleeding severity was coded retrospectively into four categories [90,93]:- - No clinical bleeding detected throughout observation - Minor skin bleeding only (petechiae or bruising at venepuncture sites) - Mild/moderate mucosal bleeding (no intervention required) with or without minor skin bleeding - Severe bleeding - bleeding requiring intervention (eg transfusion, nasal packing), or bleeding into a vital organ (e.g. intracranial bleeding)
Fluid overload	Respiratory distress due to significant ascites and/or pleural effusions, without evidence of any other respiratory pathology such as pneumonia
Encephalopathy	Any degree of mental alteration (Glasgow coma score ≤ 14)
Acute liver failure	Any degree of mental alteration with increased liver enzymes and a coagulopathy (demonstrated by an INR ≥ 1.5) in patients without evidence of pre-existing cirrhosis [94]
Severe liver dysfunction	Either acute liver failure (as above), or new onset of jaundice with significant increase in transaminase levels (> 300 U/L) considered to be due to dengue, without evidence of any other pathology such as acute viral hepatitis
Acute renal failure	Increase in serum creatinine at least 3 times baseline, or serum creatinine ≥ 4 mg/dl with an acute increase of > 0.5 mg/dl [95]

2.4 Laboratory methods

2.4.1 Standard laboratory tests

Haematology: For all patients, a full blood count was performed at least once daily during the course of illness and at the follow-up visits in the routine haematology laboratory of HTD using the ADVIA 120 haematology analyzer (Bayer, Germany). On the ICUs, HCT measurements were also performed on the ward as frequently as required for clinical management. Overall percentage HCT change (haemoconcentration) for each patient was calculated as the percentage difference between the peak HCT and the baseline HCT value, over the baseline value. Peak HCT was chosen from all values recorded between days 3 and 8 of illness if at least 3 days of serial HCT data was available. For the baseline HCT, the lowest value documented before day 3 of illness or at the follow-up visit was used; if neither was available the local population mean for age and sex was used (from a reference database for more than 1000 healthy Vietnamese children and 850 healthy Vietnamese adults, unpublished data). In addition, because HCT values can be markedly affected in patients with severe bleeding overall percentage HCT change was not calculated for those patients. Platelet nadir was chosen from all values between day 3 and 8 of illness if at least 3 days of serial platelet counts were available.

Coagulation tests are performed infrequently in the HTD laboratories. For the first 200 patients recruited to the main adult study and all the patients recruited to the CECs cohort, citrated plasma samples were obtained for screening coagulation tests at specified intervals during the course of disease – febrile, critical and convalescent – but instead of using the

HTD lab the plasma was separated and stored at -80 degrees as soon as possible after sampling. Subsequently coagulation testing was performed in batches in the OUCRU research laboratories using an ST4 Hemostasis Analyser and reagents from Stago (France). The following screening tests were done a) PT using STA NEOPLASTINE CI PLUS, b) APTT using C.K. PREST, c) plasma fibrinogen levels using FIBRIN PREST and d) semi-quantitative D-Dimer using D-DI test. For the data analysis only results from samples without evidence of haemolysis or clot formation, that were separated and frozen within 6 hours, were included.

Biochemistry: For the main adult cohort bilirubin and transaminase levels were also performed at intervals during the course of disease, but using the routine HTD laboratories. In addition, hepatitis B virus (HBV) and hepatitis C virus (HCV) diagnostics were performed on stored convalescent or follow-up samples using Abbott AxSYM reagents (Illinois, USA). Anti-hepatitis B core antibody (antiHBc) was checked as an initial screen for all adult patients, with hepatitis B surface antigen (HBsAg) and anti-hepatitis B core IgM (antiHBc IgM) performed if the test was positive. Anti-hepatitis C antibody (antiHCV) was checked on samples from the first 200 adults enroled in the study.

Quality Control: In accordance with the International Organization for Standards (ISO) quality accreditation, the haematology and biochemistry laboratories of HTD have an ongoing quality assessment program that includes internal quality control every day and external quality assessment every month, run by the Center for Standardization and Quality Control in Medical Laboratories of Ho Chi Minh City. The system operates for all categories of laboratory tests performed at HTD. For the coagulation tests that we performed in the

OUCRU laboratories, we checked internal quality control every day using normal and abnormal control samples from Stago.

2.4.2 Dengue diagnostic tests

Dengue diagnostic capture IgM and IgG ELISA assays were performed using paired enrolment and convalescent specimens and reagents provided by Venture Technologies (Sarawak, Malaysia). The techniques used and interpretation of results were as previously reported (Figure 2 -1) [42]. To define immune-status the ratio of IgM to IgG on or after day 6 of illness was used; a ratio $\geq$ 1.78 defined the infection as primary and one $\leq$ 1.2 as secondary [96]. Patients with ratios between these values, or in whom the results of enrolment and convalescent specimens differed, were considered unclassifiable.

Using the enrolment specimen, DENV real-time RT-PCR was carried out as previously described for a) all patients in the paediatric cohorts (who were generally admitted early) and b) all patients from the main adult cohort who were enrolled within the first 5 days of illness, and who had negative or inconclusive serology [42]. A new one-step real-time multiplex RT-PCR assay had been developed in our research lab by the time the adolescent/young adult cohort was recruited and this new technique was therefore used for the diagnostic work-up in this group of patients [97].

If both serology and PCR were negative or inconclusive, assays for NS1 were performed using Biorad Platelia™ Dengue NS1 Antigen kits following the manufacturer's instructions. Patients in whom all dengue diagnostics were conclusively negative, in whom there was no evidence of any other infectious disease, and who recovered without antibiotic therapy, were considered to have had OFI.

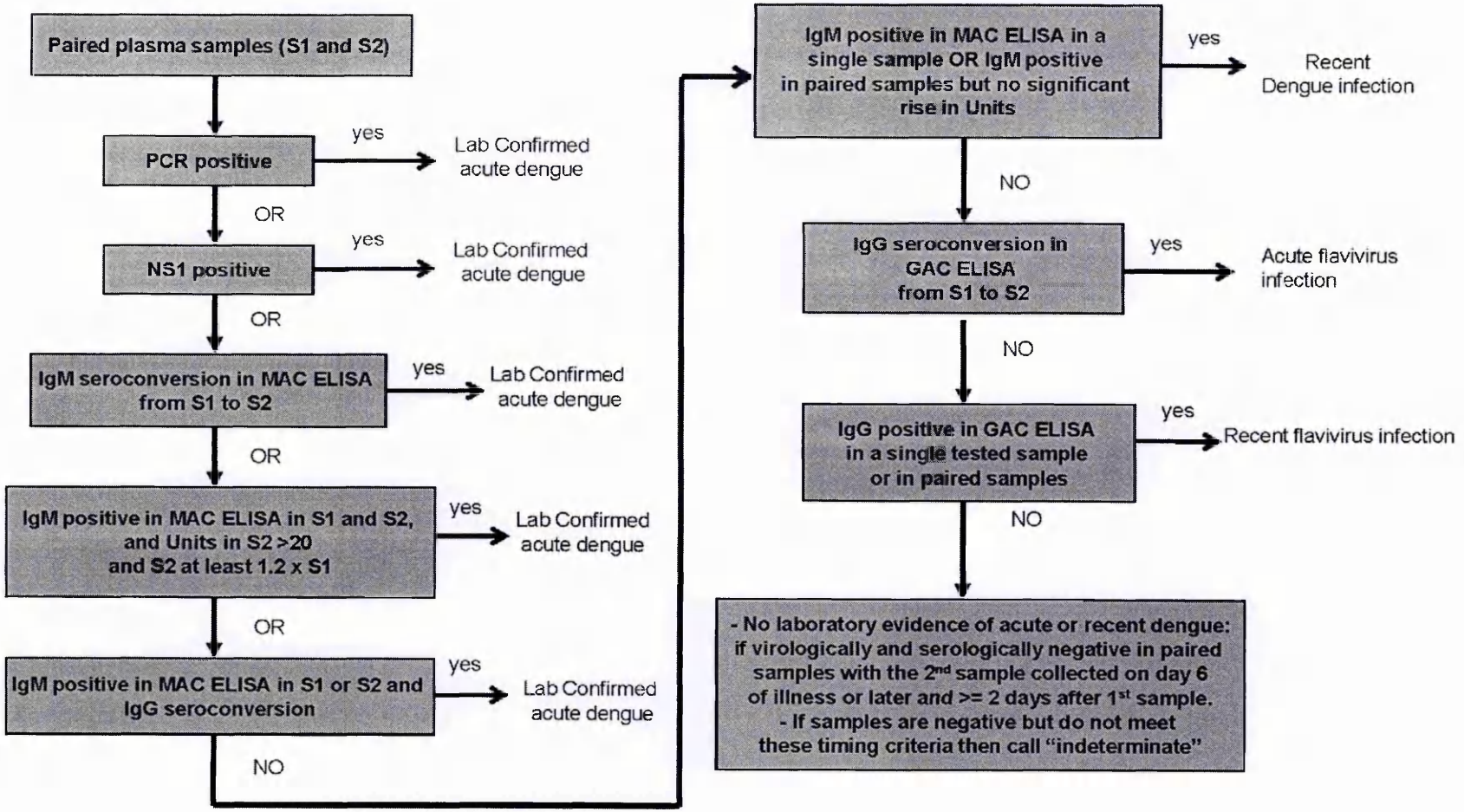


Figure 2-1: Dengue laboratory algorithm

2.5 Statistical Analysis

Throughout the following chapters, data are presented as number (percentage) for categorical variables and median (90% range) for continuous variables. In all "Box and Whisker Plots", boxes represent the median and interquartile values and open circles indicate outlying/extreme values.

Data on clinical characteristics and laboratory results were compared between different patient groups using the Chi-square test or Fisher's exact test for categorical variables, and the Mann Whitney test or the Cuzick test for trend for continuous variables. Within-patient comparisons of different parameters, or of the same parameter measured at different time-points, were performed using the Wilcoxon signed-rank test. Spearman's rank correlation was used to evaluate relationships between continuous variables assessed concurrently. All statistical computations were carried out using SPSS Version 14 (Chicago, Illinois, USA) and Stata-SE Version 8 (Texas, USA).

Chapter 3

Clinical features of dengue infection in adults and children

3.1 Introduction

Reports describing the clinical picture in adults with dengue have only recently started to emerge, and suggest that unusual complications with high mortality such as acute liver failure, encephalopathy, myocarditis and acute renal failure are more common in this group than in children [10,13]. In one study from Nicaragua signs of vascular leakage, shock and marked thrombocytopenia were seen less commonly with increasing age, although internal haemorrhage occurred more frequently in the adult subjects [14]. In a small prospective study from Sri Lanka, evidence of vascular leakage and shock were also seen more frequently in children compared to adults, although bleeding manifestations were similar [9], while in three small studies from Southeast Asia bleeding manifestations, thrombocytopenia and hepatic dysfunction were seen more commonly in young adults with the frequency of shock similar to the paediatric patient group [13,15,98]. In this chapter I will describe the patterns of disease, management strategies, and outcomes for patients recruited into the main adult cohort in comparison with those of paediatric cases recruited into the paediatric studies during the same calendar time at HTD. HTD is the main referral hospital for adults with dengue for southern Vietnam but receives few paediatric referrals. Since referred patients are likely to have more severe disease than direct admissions we considered these two groups separately and structured the comparisons between the age groups to take into account the route of admission.

Secondly I will describe the pattern of liver involvement in the adult cohort in more detail – in particular looking at liver involvement in relation to the severity of vascular leakage and of bleeding. Due to the infrequent development of hepatic complications in children in our setting we do not routinely assess liver function, and limits on the volume of blood taken from the children for research purposes did not allow comparable assessment in the paediatric cohort. One factor that may influence the pattern of disease seen in adults is the greater likelihood of underlying chronic diseases, potentially compounding the effects of the acute infection. Chronic viral hepatitis is common among adults in many tropical and sub-tropical countries where dengue is endemic, and it has been postulated that dengue infection occurring on a background of chronic HBV or HCV infection may result in more severe liver dysfunction and/or haemorrhage than is usual in non-infected individuals. However, the evidence to date is conflicting with two small studies indicating no effect [99,100], while one study suggests that concomitant HBV infection may result in greater hepatic dysfunction [101]. Therefore I also examined the question of whether concomitant infection with HBV or HCV influenced the overall severity of the clinical picture in this cohort.

3.2 Methods

Information relating to the general clinical, laboratory and statistical methods is presented in Chapter 2. A specific point for this chapter is that when comparing platelet counts between children and adults we used multiple linear regression to

assess the effect of covariates including age-group (age < 15 years versus age ≥ 15 years, sex, infecting serotype (DENV 1 - 4 or unknown), and immune status (primary or secondary infection or unknown status).

3.3 Results

During the selected time-periods (September 2006 – September 2008 for the ICUs and the calendar year 2007 for the infection wards) a total of 947 children and 738 adults with suspected dengue were enroled into the paediatric and main adult cohorts. Among them, 881/947 (93%) children and 647/738 (88%) adults were confirmed to have dengue on one or more of the diagnostic assays. In the other patients, 33 children (3%) and 47 adults (6%) were classified as having OFI (5/47 adults were admitted briefly to the AICU for observation during the critical period); 12 children (1%) and 31 adults (4%) had negative dengue diagnostics or an alternative diagnosis (e.g malaria, bacterial sepsis etc.). In the remaining 21 children (2%) and 13 adults (2%) the results of serological and virological diagnostics were inconclusive. Among all patients, 448/947 children (47%) and 295/738 adults (40%) attended for follow-up.

3.3.1 Comparison of the clinical picture between adults and children with dengue

Only patients with laboratory confirmed dengue were included in this comparison. The infecting serotype was identified in 450/881 (51%) children, and in 207/551 (38%) adults in whom DENV RT-PCR was performed. Serotype distribution was similar in the two populations: DENV-1 was detected in 254/450 (56%) children and 108/207 (52%) adults; DENV-2 in 102 children (23%) and 65 adults (31%); DENV-3

in 93 children (21%) and 30 adults (14%); and DENV-4 in 3 children (1%) and 4 adults (2%). Two children had mixed infections. Co-morbidities were uncommon, and were distributed across the severity groups; 1 child and 8 adults had a history of asthma, 2 children had epilepsy and one child had haemophilia, 2 adults had thyroid disease, one had diabetes mellitus and three each had valvular heart disease and hypertension. All patients with co-morbidities survived. Notably, all 3 pregnant adult females in the study developed DSS.

3.3.1.1 Clinical description of patients requiring intensive care

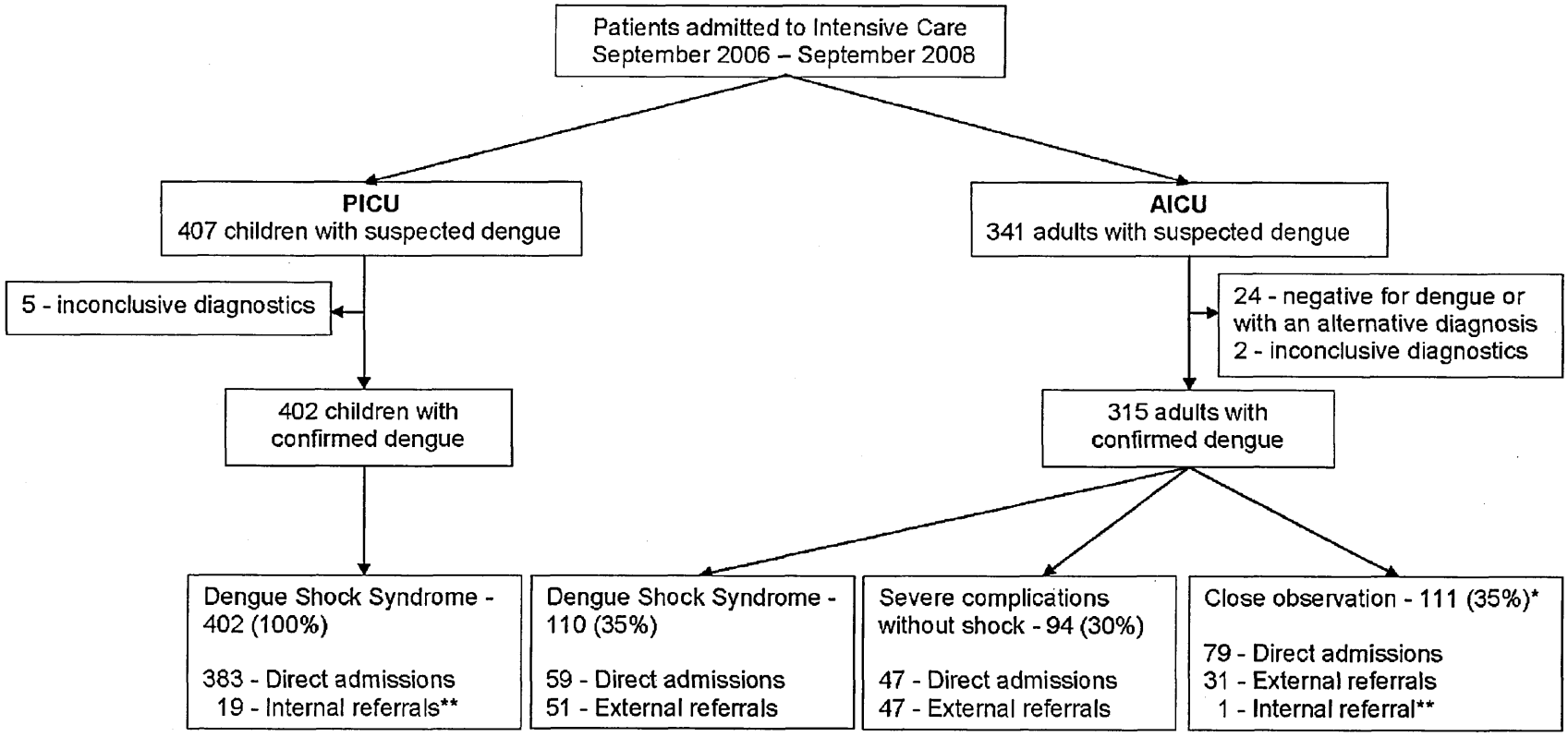
Between September 2006 and September 2008, we recruited 388 children admitted directly to PICU with suspected dengue and hypovolaemic shock (> 95% of all such cases) plus a further 19 children who were recruited into the study in the paediatric infection ward and subsequently transferred to PICU with clinical DSS (Figure 3-1). There were no external referrals from other facilities during the two-year period. Of the 407 children studied, 402 (99%) were confirmed to have dengue. Immune status could be reliably determined in 325/402 (81%) of cases, of whom 18 children with DSS (6%) had primary infections (DENV-1=13; DENV-2=1; unknown serotype=4).

During the same two-year period 341 adults admitted to AICU with suspected dengue were also recruited (90% of all suspected dengue admissions to AICU), of whom 315 (92%) were subsequently confirmed to have dengue. Only 186/315 (59%) of the adults with confirmed dengue were admitted directly to HTD with the remaining 129 (41%) being external referrals from other facilities. 110/315 (35%) of the adults

studied in AICU had DSS (59 direct admissions and 51 external referrals), 94 (30%) experienced a variety of other serious complications (47 direct admissions and 47 external referrals), and 111 (35%) were admitted for close observation (79 direct admissions, 31 external referrals, 1 internal referral) but ultimately recovered without developing any major complications (Figure 3-1). Immune status could be determined in 260/315 (83%) of the patients in AICU, only 16 of whom had primary disease, with only 1 case identified among the patients with shock.

Patients with DSS

Table 3-1 presents a summary of key clinical features, management, and basic laboratory findings, comparing children and adults admitted directly to the two ICUs. Over the two-year period almost 7 times as many children as adults were admitted directly with DSS. The median age for paediatric patients was 9 years. Most adults were in their twenties or early thirties with no patient older than 37 years. Timing of onset of shock was similar in the two groups. Vomiting, abdominal pain and headache were more common among adults at presentation (Chi square test, $p = 0.003$, $p < 0.001$ and $p < 0.001$ respectively). All adults experienced some clinical bleeding, primarily mild/moderate mucosal bleeding, while one third of children had no bleeding and over 50% manifested only minor skin bleeding during the disease course. Eight children and 3 adults had severe bleeding, all in association with profound shock, and 5 children and 1 adult required whole blood/packed cells transfusion.



* These 111 patients were admitted to AICU for close observation but did not go on to develop shock or other complications. Due to workload constraints children admitted to PICU with suspected dengue were only enrolled into the observational study if they developed overt complications.

** Note that these patients were recruited into the study in the infection wards and subsequently transferred to PICU/AICU

Figure 3-1: Profile of patients studied on the intensive care units

Table 3-1: Summary of key clinical features, therapeutic interventions, and laboratory findings, comparing children and adults with dengue shock syndrome studied on the two intensive care units

	Patients with DSS admitted directly		p*	Referred DSS patients - Adults only (n = 51)
	Children (n = 402)	Adults (n = 59)		
Characteristics reported or observed at enrolment**				
Age, years	9 (3 – 14)	19 (15 – 32)	N/A	21 (15 – 34)
Male sex	220 (55)	29 (49)	0.42	20 (39)
Day of illness at shock	5 (4 – 6)	5 (4 – 6)	0.26	5 (3 – 6)[c]
Headache	109 (27)	52 (90)[a]	<0.001	42 (84)[a]
Vomiting	254 (63)	49 (83)	0.003	40 (80)[a]
Abdominal pain	205 (51)	46 (79)[a]	<0.001	40 (82)[b]
Fatigue	362 (90)	58 (98)	0.04	48 (94)
Rash	8 (2)	3 (5)	0.16	2 (4)[a]
BP unrecordable or PP ≤ 10 mmHg at shock	60 (15)	11 (19)	0.46	-
Summary of clinical features and complications observed during disease course				
Hepatomegaly	365 (91)	43 (73)	<0.001	40 (78)
Clinical pleural effusion and/or ascites	147 (37)	22 (37)	0.91	26 (51)
Overall bleeding severity:-				
- No bleeding	131 (33)[a]	0	<0.001***	0
- Skin bleeding only	207 (52)[a]	21 (36)		9 (18)[a]
- Mild/Moderate mucosal bleeding	55 (14)[a]	35 (59)		30 (60)[a]
- Severe bleeding	8 (2)[a]	3 (5)		11 (22)[a]
Recurrent shock	147 (37)[c]	5 (8)	<0.001	27 (61)[d]
Severe liver dysfunction	0	0	-	2 (4)
Acute renal failure	0	0	-	2 (4)
Encephalopathy	1 (<1)	1 (2)	0.24	7 (14)
Length of hospital stay, days	3 (3 – 6)	5 (3 – 8)	<0.001	5 (1 – 16)
Death	3 (<1)	1 (2)	0.42	4 (8)

	Patients with DSS admitted directly		p*	Referred DSS patients - Adults only (n = 51)
	Children (n = 402)	Adults (n = 59)		
Summary of treatment given				
Total IV fluid given for shock (ml/kg)	114 (70 – 158)[c]	75 (45 – 124)	<0.001	91 (41 – 208)[e]
Colloid used	197 (49)[c]	8 (14)	<0.001	27 (61)[d]
Total colloid volume given (ml/kg)	17 (9 – 61)	12 (8 – 24)	0.05	17 (5 – 57)[b]
Whole blood/packed cells transfusion	5 (1)	1 (2)	0.56	8 (16)
Platelet transfusion	0	1 (2)	0.13	7 (14)
Inotropes used	19 (5)	1 (2)	0.49	9 (18)
Diuretics used	79 (20)	3 (5)	0.006	10 (20)
Summary of key laboratory findings				
Day of illness of maximum HCT****	5 (4 – 7)[b]	5 (4 – 6)	0.18	6 (4 – 7)[a]
Maximum HCT****	50 (44 – 56)[b]	51 (44.5 – 64.2)	N/A	47.4 (38.9 – 61.1)[a]
Overall percentage HCT change****§	33 (17 – 58)[f]	26 (9 – 49)	<0.001	22 (2 – 57)[a]
HCT change ≥20%****	351 (92)[f]	39 (70)	<0.001	20 (53)[a]
Platelet nadir, $\times 10^9$/L	32 (11 – 73)	18 (9 – 49)	<0.001	15 (6 – 43)[b]

* p value for comparisons between children and adults admitted directly with shock (Chi-square test or Fisher's exact test for categorical variables, and Mann Whitney test for continuous variables)

** Age-dependent features such as pulse and respiratory rate are not presented.

*** Overall Chi-square test

**** HCT values are likely to be affected by severe bleeding during the acute illness, and thus percentage haemoconcentration was not calculated for patients with severe bleeding. For these variables the denominators are 393, 56 and 39 cases in the three patient groups respectively. Absolute values are age-dependent and were not compared statistically.

§ Percentage change between the maximum recorded HCT between days 3 and 8 of illness compared to a baseline value obtained before day 3 of illness or at the follow up visit. If neither was available the local population mean for age and sex was used as the baseline.

Missing data for [a] = 1 patient, [b] = 2 patients, [c] = 4 patients, [d] = 7 patients, [e] = 8 patients, [f] = 10 patients

Initial shock severity was similar in terms of the proportion with no recordable blood pressure or with very narrow pulse pressure (≤ 10mmHg) at presentation. However adults experienced recurrent shock much less frequently than did children, indicated by the need for colloid resuscitation in only 8 (14%) adults as compared to 197 (49%) children (Chi square test, $p < 0.001$). Adults were usually successfully managed with smaller volumes of fluid than children. Approximately one third of all patients developed clinical pleural effusions or ascites, but only 3 (5%) of adults compared to 79 (20%) of children required diuretics. Three children (2 prolonged shock and severe bleeding, 1 prolonged shock with encephalopathy) and 1 adult (prolonged shock with encephalopathy) died despite full supportive care. All others made a full recovery.

Data for the 51 externally referred adults with DSS are also presented in Table 3-1 and clearly demonstrate that these patients were more severely ill than those admitted directly to AICU. Almost 60% of this group experienced recurrent shock and 11 patients (22%) developed severe bleeding. Clinical pleural effusions and ascites were common with 12/51 (24%) developing respiratory distress. Five patients required assisted ventilation (4 with fluid overload, and 1 with encephalopathy and pneumonia), while the remainder were managed successfully with oxygen and diuretics. Two patients had severe liver dysfunction demonstrated by jaundice and very high transaminase levels, and two developed acute renal failure. A total of 7/51 (14%) of these externally referred adults became encephalopathic, 5 in association with severe bleeding. Four (8%) of the 51 patients died - 3 due to severe bleeding and fluid overload and 1 due to encephalopathy.

Patients with severe complications without shock

Among the 94 patients admitted to AICU without DSS but with other severe complications, the clinical pattern was similar among direct admissions and external referrals, although the latter group tended to be more severely ill. There were 28/47 externally referred patients (60%) with severe bleeding (among them 4 cases associated with severe liver dysfunction, 2 cases with encephalopathy and intracranial bleeding demonstrated on CT scan, 1 case with a bleeding peptic ulcer, and the remaining 21 cases with no associated disorder detected), 14 patients (30%) with mucosal bleeding that settled without intervention, 2 patients with severe liver dysfunction without bleeding, and 1 case each with isolated encephalopathy, haemoglobinuria, and profound thrombocytopenia. Among the 47 direct admissions, there were 12 patients (26%) with severe bleeding (among them 2 cases with associated liver and renal failure, and 1 case with a bleeding peptic ulcer), 27 patients (57%) with mucosal bleeding that settled, 3 patients with syncope, 2 patients with haemoglobinuria and 1 case each with isolated encephalopathy, severe liver dysfunction without bleeding, and profound thrombocytopenia. One of the externally referred patients with acute liver failure and severe bleeding died, but the remaining patients recovered.

Patients admitted for observation

A total of 111 patients were admitted to AICU for close observation, of whom 64 (58%) had transiently narrowed pulse pressure without evidence of impaired

peripheral perfusion. None of these patients went on to develop clear signs of shock and all were discharged well without specific interventions other than maintenance fluid therapy. Compared to the adults managed on the infection wards, these patients had significantly lower platelet nadirs and greater overall haemoconcentration (data not shown). Although 270 children with suspected dengue were admitted to PICU for close observation during the same period, none developed shock or any other complications and thus were not recruited, as per the PICU research protocol.

3.3.1.2 Clinical description of patients managed throughout on the infection wards

During 2007 we recruited 559 children - approximately 25% of all children admitted to the paediatric infection wards with clinical dengue, as judged from the hospital's electronic database - and 398 adults - approximately 10% of all adults admitted to the adult infection wards with clinical dengue (and approximately 50% of those eligible from the designated bays on the relevant infection wards). Of the 559 children, 498 (89%) were confirmed to have dengue, with immune status determined in 419 (84%), 130 primary and 289 secondary infections. Nineteen of these children (4%) developed DSS in hospital (see ICU section) and another 7 children were transferred to PICU for observation but did not develop shock. Of the 398 adults, 333 (84%) had dengue confirmed, among whom immune status was determined in 266 (80%), 30 primary and 236 secondary infections. Within the dengue-confirmed group 286 adults had been admitted directly to HTD while 47 were external referrals. Only 1 adult was

transferred to AICU, a patient with known hyperthyroidism who developed rapid atrial fibrillation that responded to conventional treatment.

Table 3-2 presents the clinical features and basic laboratory findings, comparing the 472 children and 285 adults admitted and managed throughout on the infection wards (hereafter referred to as uncomplicated dengue cases), with findings for the externally referred adults presented separately. Significantly more males than females were recruited in the paediatric ward. This was consistent with the observed male/female ratio observed among all admissions to that ward, but was not apparent in the male/female ratio for PICU admissions. Children were admitted to hospital approximately one day earlier than adults. However, adults were generally more symptomatic than children – most notably 70% complained of muscle pain compared to only 19% of children (Chi square test, $p < 0.001$). Bleeding also occurred more frequently in adults. Almost half the children had no bleeding and minor mucosal bleeding was noted in only 10%, primarily epistaxis, compared to 46% of adults with mainly gum or minor upper gastrointestinal bleeding. In contrast more than 60% of children received some maintenance fluid therapy compared to 29% of the adults, either because of vomiting or high fever with inadequate oral intake.

Table 3-2: Summary of key clinical features, therapeutic interventions, and laboratory findings, comparing children and adults with uncomplicated dengue managed on the infection wards

	Patients with uncomplicated dengue admitted directly		p*	Referred patients with uncomplicated dengue - Adults only (n = 47)
	Children (n = 472)	Adults (n = 285)		
Characteristics reported or observed at enrolment**				
Age, years	12 (7 – 14)	22 (15 – 34)	N/A	21 (16 – 39)
Male sex	303 (64)	132 (46)	<0.001	25 (53)
Day of illness	3 (2 – 5)	4 (2 – 6)	<0.001	4 (2 – 6)
Headache	325 (69)	260 (93)[d]	<0.001	44 (94)
Fatigue	424 (90)	255 (94)[f]	0.07	39 (83)
Muscle pain	90 (19)	190 (70)[f]	<0.001	33 (70)
Cough	76 (16)	66 (24)[c]	0.01	17 (36)
Vomiting	174 (37)	140 (50)[b]	<0.001	28 (60)
Diarrhoea	53 (11)	103 (37)[d]	<0.001	15 (32)
Abdominal pain	96 (20)	112 (41)[e]	<0.001	22 (47)
Rash	33 (7)[a]	37 (13)	0.006	4 (9)
Hepatomegaly	27 (6)	21 (7)[a]	0.36	7 (15)
Summary of subsequent disease course				
Day of illness at defervescence	6 (4 – 8)[a]	6 (4 – 8)[c]	0.35	6 (4 – 9)
Overall bleeding severity:-				
- No bleeding	216 (46)[a]	46 (16)		3 (6)
- Skin bleeding only	209 (44)[a]	107 (38)	<0.001***	13 (28)
- Mild/Moderate mucosal bleeding	46 (10)[a]	132 (46)		31 (66)
Length of hospital stay, days	5 (3 – 7)	5 (2 – 7)	0.04	5 (3 – 8)

	Patients with uncomplicated dengue admitted directly		p*	Referred patients with uncomplicated dengue - Adults only (n = 47)
	Children (n = 472)	Adults (n = 285)		
Summary of treatment given				
Any maintenance IV fluid given	292 (62)	84 (29)	<0.001	16 (34)****
Summary of key laboratory findings				
Day of illness of maximum HCT	6 (3 – 8)[a]	6 (4 – 8)[c]	0.003	6 (4 – 8)
Maximum HCT	43.9 (38.3 – 53.5)[a]	44.9 (38.8 – 53.9)[c]	N/A	45.1 (39.2 – 56.2)
Overall percentage HCT change[§]	14 (-2 – 37)[a]	10 (-3 – 32)[c]	<0.001	11 (-3 – 46)
HCT change ≥ 20%	143 (30)[a]	49 (18)[c]	<0.001	9 (19)
Platelet nadir, $\times 10^9$/L	80 (30 – 178)[a]	42 (12 – 117)[c]	<0.001	40 (10 – 134)

* p value for comparison between children and adults with uncomplicated dengue admitted directly (Chi-square test or Fisher's exact test for categorical variables, and Mann Whitney test for continuous variables)

** Age-dependent features such as pulse and respiratory rate are not presented.

*** Overall Chi-square test

**** no information available prior to HTD admission

[§] Definition for percentage HCT change as in Table 3-1

Missing data for [a] = 1 patient, [b] = 3 patients, [c] = 5 patients, [d] = 6 patients, [e] = 9 patients, [f] = 13 patients.

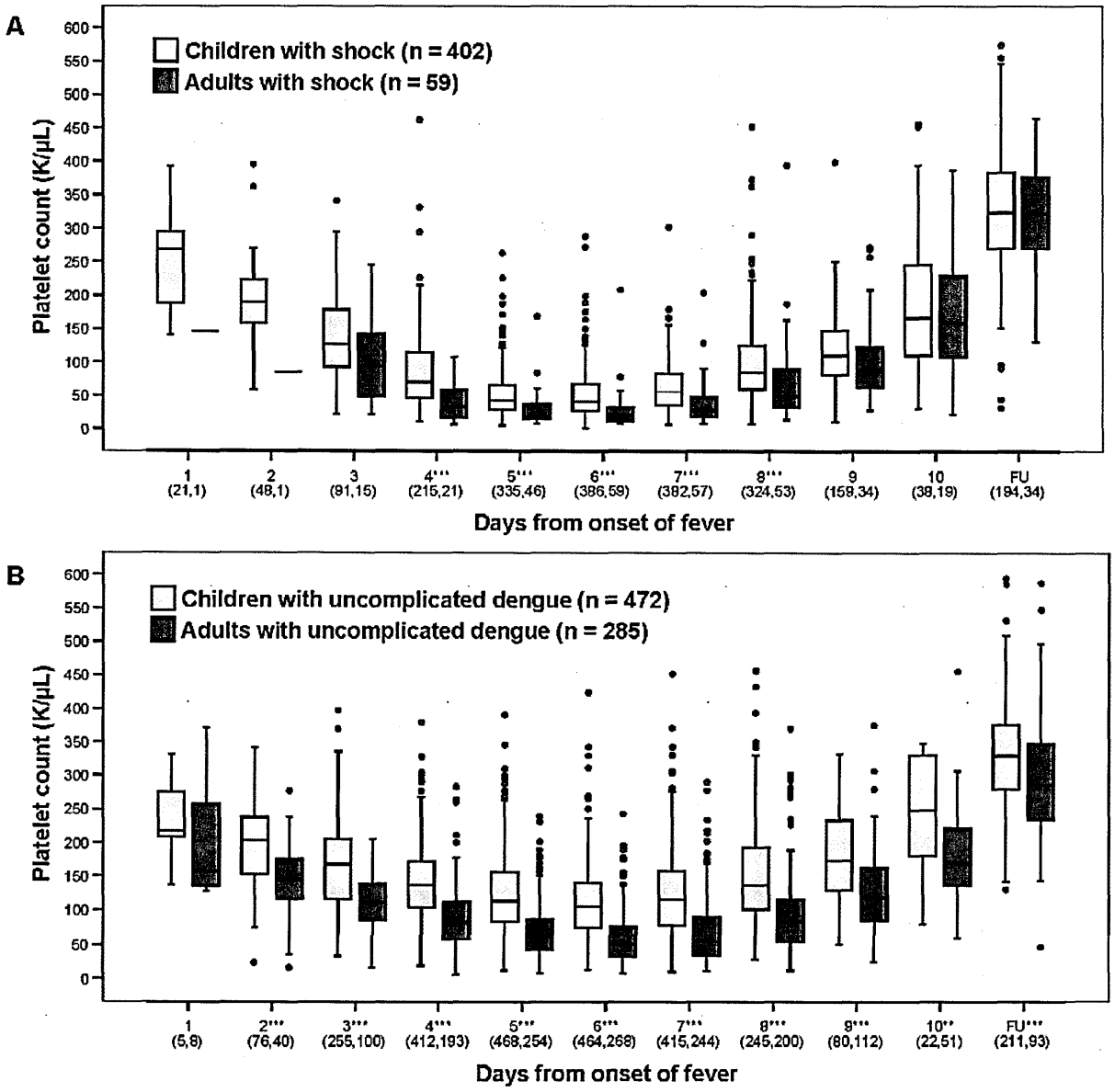
3.3.1.3 Basic laboratory findings

Normal HCT values are age-dependent, but the median maximum values recorded during the acute illness were similar, indicating greater increases in children than adults. Percentage haemoconcentration was also greater in children than adults in both the DSS and the uncomplicated dengue groups (both $p < 0.001$, Mann Whitney test), and consequently the proportion of children with $\geq 20\%$ haemoconcentration was also greater than among the adults (Chi square test, both $p < 0.001$). The median day of illness on which the maximum HCT occurred was slightly later (day 6) in the uncomplicated dengue patients than those with DSS (day 5) (Tables 3-1 and 3-2).

The platelet nadir occurred on day 6 of illness in both age-groups, with levels generally lower in those with shock than uncomplicated dengue (Figure 3-2). Only 8 adults with shock - 4 with severe bleeding, and 4 with counts below 20×10^9/L who required an invasive procedure - and a further 13 adults with severe bleeding without shock received platelet transfusions in addition to other blood products. No paediatric patient received a platelet transfusion. Considering patients directly admitted with shock, daily platelet counts were consistently lower among adults than children (Mann Whitney test, $p<0.001$ for all daily comparisons between days 4 - 8). Similarly among direct admissions with uncomplicated dengue, thrombocytopenia was more severe in adults than children between days 2 - 10 of illness and also at the follow-up visit (Mann Whitney test, all $p<0.01$) (Figure 3-2). Multiple linear regression indicated that serotype and sex had no significant effects on the platelet nadir ($p=0.53$ and 0.67 respectively for patients with uncomplicated dengue; $p=0.12$ and 0.25

respectively for patients with shock), but that there was an association with immune status i.e. secondary infection was an independent predictor of a lower platelet nadir (estimate -22.0×10^9/L (95%CI: –30.6 to –13.4), p<0.001 in the uncomplicated dengue group, and -13.2×10^9/L (95% CI: –23.8 to –2.7), p=0.01 in patients with shock). In addition, age-group had a pronounced effect on the platelet nadir (estimated effect of age ≥ 15 years was -35.5×10^9/L (95% CI: –43.0 to –27.9), p<0.001 in patients with uncomplicated dengue, and -6.7×10^9/L (95% CI: –13.2 to –0.2), p=0.04 in patients with shock). Notably, age also significantly affected the platelet count at follow-up in the uncomplicated dengue group, with a larger effect size compared to that observed during the acute illness; the estimated effect of age ≥ 15 years (assessed by multiple linear regression adjusted for serotype and immune status) on follow-up platelet values was -41.7×10^9/L (95% CI: –64.1 to –19.4), p<0.001. This relationship was not apparent for follow-up platelet values in the DSS group (estimated effect of age ≥ 15 years -6.5×10^9/L (95% CI: –40.1 to 27.2), p=0.7).

Platelet counts significantly correlated with vascular leakage severity in adults during the critical and convalescent periods (Mann Whitney test, both $p < 0.001$), and also with bleeding severity in the early febrile (Cuzick test for trend, $p < 0.05$), critical and convalescent periods (Cuzick test for trend, both $p < 0.01$) (Figure 3-3). At follow-up, platelet counts were significant higher in adults with shock compared to those without shock (Mann Whitney test, $p < 0.05$) (Figure 3-3). Similar patterns were seen in the paediatric cohort except that the platelet counts were similar in the groups with and without shock at the follow-up visit (data not shown).

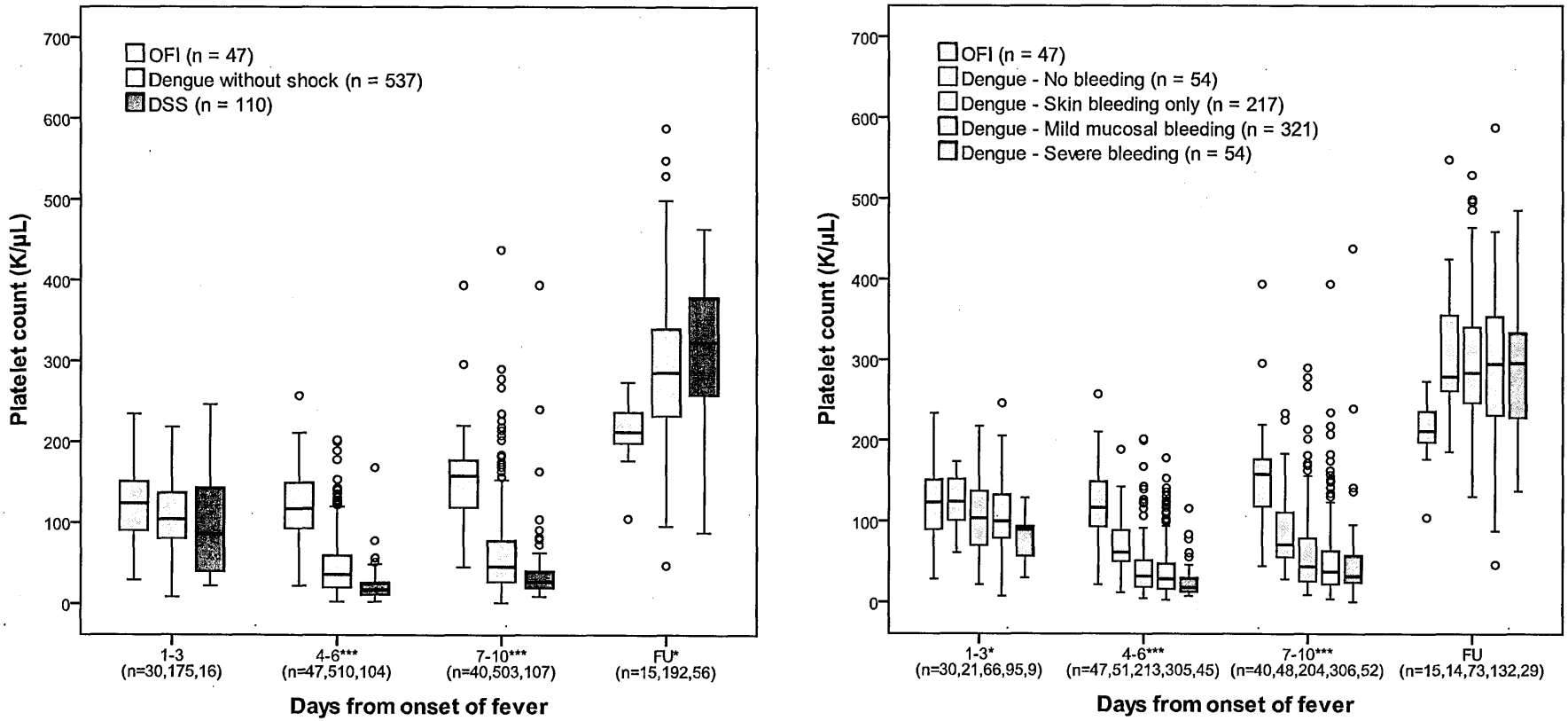


The total number of patients included in each group each day is indicated on the X axis.

The Mann-Whitney test was used to compare the daily platelet counts between children and adults.

*** $p<0.001$ and ** $p<0.01$

Figure 3-2: Box and whisker plots showing daily platelet counts during the course of illness, and at follow-up 2-4 weeks later, for children and adults admitted directly to the relevant ICU with shock (Panel A), and for children and adults with uncomplicated dengue admitted directly to and managed throughout on the relevant infection wards (Panel B).



The Mann Whitney test was used to compare the lowest platelet counts in each time-period between dengue without shock and dengue with shock (left panel), and the Cuzick test for trend was used to compare the lowest platelet counts in each time period among patients with increasing severity of bleeding (right panel). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

Figure 3-3: Box and whisker plots showing associations between platelet count and severity of vascular leakage (left panel) or bleeding (right panel) seen in adults.

3.3.2 Liver involvement associated with dengue infection in adults

This section focuses on adults with confirmed dengue in comparison with OFI patients. Among the dengue patients, hepatomegaly was seen more commonly in patients with shock (83/110 patients, 75%) compared to those without shock (142/536 patients, 26%) (Chi square test, $p < 0.001$). Visible jaundice was noted in only 11/647 (< 2%) dengue patients (9 without shock and 2 with DSS), of whom 8 also experienced severe bleeding. All these patients met the criteria for severe liver dysfunction, and five of them, none of whom had shock, developed acute liver failure and severe coagulopathy. One patient in this group died. Only 5/47 (11%) OFI patients had hepatomegaly, and none developed jaundice.

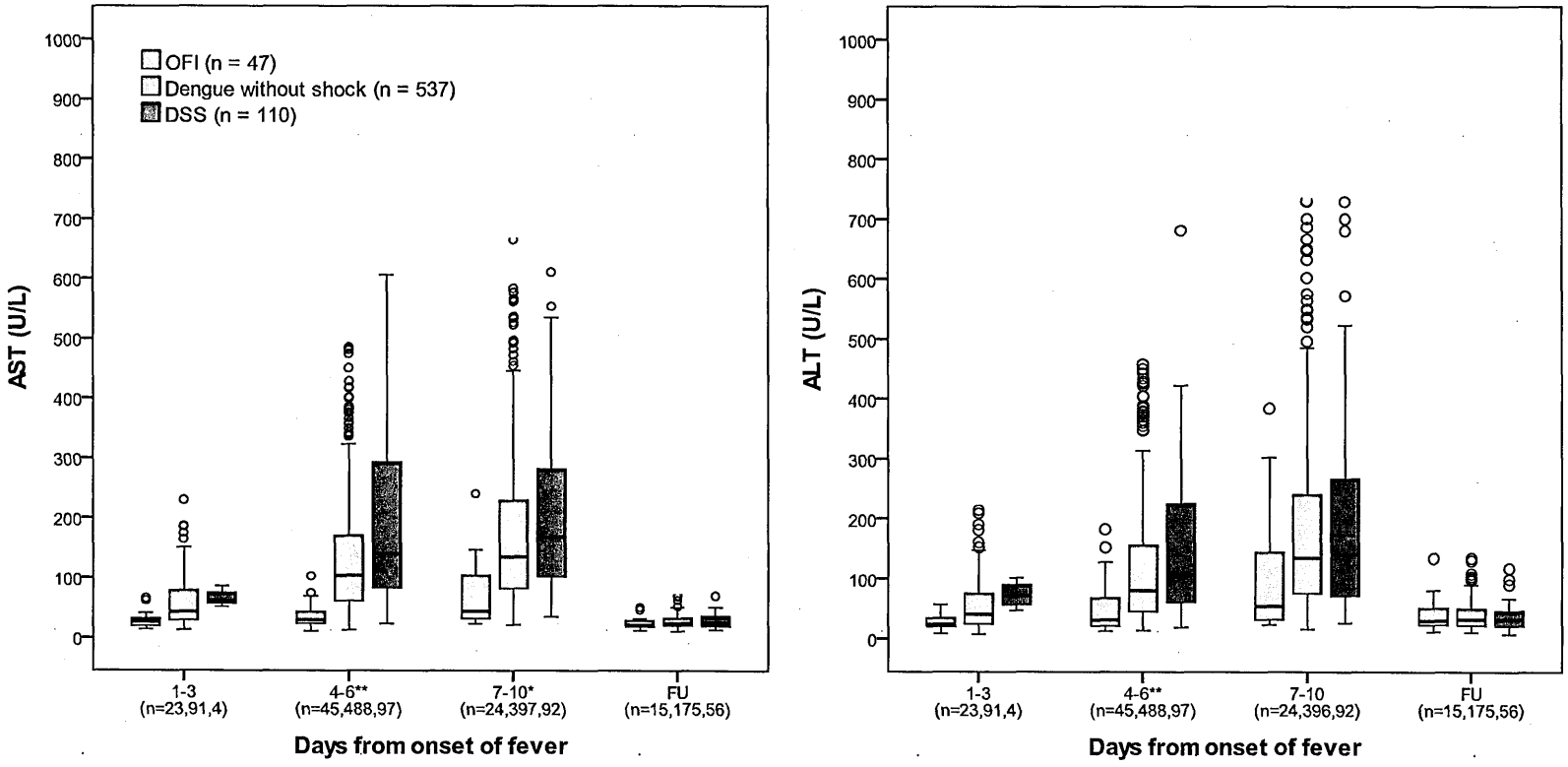
Jaundice appeared relatively late in the dengue patients, commonly during the second week of illness several days after the expected time for shock (median (range) day of onset 7 (6 - 12)), and was minor in all cases except those with acute liver failure. The infecting serotype, DENV-2, was identified in only one case with severe liver dysfunction. Information on ingestion of potentially hepatotoxic medications was limited by the fact that most patients did not know what they had been prescribed before hospital admission. However, more than 90% of patients in the study admitted taking standard doses of paracetamol for symptomatic relief, with the pattern of ingestion similar in those with and without severe liver dysfunction. Although I attempted to gather information on prior alcohol consumption the response rate was poor and the data collected was felt to be unreliable, precluding analysis.

3.3.2.1 Disturbances in liver function tests and associations with disease severity and coagulopathy

Bilirubin levels fell within the normal range for almost all patients except those with visible jaundice. Results of the serial transaminase levels are shown in Figure 3-4, presented according to the severity of vascular leakage, and grouped according to timing during the evolution of the illness. Considering all dengue patients, only 22/647 (3%) had normal aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (below or equal to 40 IU/L) throughout the course of their illness, compared to 23/47 (49%) of the OFI patients. In the dengue group the AST and ALT levels began to increase slightly in the early febrile period (median (90% range) of 43 (18 - 314) for AST and 40 (14 - 236) IU/L for ALT levels, compared to 24 (13 - 68) for AST and 32 (13 - 101) IU/L for ALT at follow-up, Wilcoxon signed-rank test, $p < 0.001$ for AST and $p = 0.02$ for ALT). Both enzyme levels increased significantly in the critical period (107 (30 - 482) for AST and 83 (22- 422) IU/L for ALT), and reached peak concentrations (138 (44 - 545) for AST and 136 (30 - 590) IU/L for ALT) during the convalescent period (Wilcoxon signed-rank test, all $p < 0.001$ for comparisons of both critical and convalescent values with follow-up values and both $p < 0.01$ for comparisons of critical values with convalescent values). The highest enzyme levels were recorded in patients with severe liver dysfunction (median (90% range) of 1663 (320 - 8680) IU/L for maximum AST levels during the disease course and 971 (334 - 9917) IU/L for maximum ALT levels). Levels had returned to the expected normal range at the follow-up visit (around 3 – 4 weeks after onset of illness) in 203 (88%) for AST and 145 (63%) for ALT of the 231 dengue patients reviewed. ALT levels were significantly higher than

AST levels in OFI patients in the critical and convalescent periods (Wilcoxon signed-rank test, both $p < 0.05$). In contrast, AST levels were consistently higher than ALT levels in the critical and convalescent periods in the dengue patients (Wilcoxon signed-rank test, both $p < 0.01$).

In terms of correlation between transaminase levels and markers of disease severity, during the critical period both AST and ALT levels were significantly higher in the dengue patients who experienced shock compared to those without shock (Mann Whitney test, both $p < 0.01$). This association was still apparent during the convalescent period for AST (Mann Whitney test, $p = 0.02$), but ALT levels were similar in the two groups at this time (Figure 3-4). In addition, the AST and ALT levels of the dengue patients correlated significantly with bleeding severity in the critical and convalescent periods (Cuzick test for trend, all $p < 0.001$, data not shown). We also looked for correlations between transaminase levels with results of coagulation tests recorded at the same time in the critical period: among all adults with dengue, both liver enzymes demonstrated weak or moderate negative correlations with the lowest platelet count (Spearman correlation of -0.32 for AST and -0.26 for ALT, both $p < 0.001$, $n = 584$); among patients who had coagulation tests performed, both liver enzymes demonstrated weak or moderate positive correlations with the APTT (Spearman correlation of 0.31 for AST and 0.23 for ALT, both $p < 0.05$, $n = 124$) and moderate negative correlations with plasma fibrinogen level (Spearman correlation of -0.37 for AST and -0.3 for ALT, both $p < 0.01$, $n = 124$). However, the PT showed no significant associations with transaminase levels (Spearman correlation of 0.02 for AST and -0.11 for ALT, both $p > 0.2$, $n = 124$) (data not shown).



- Open circles indicate outlying values but extremes are not shown in the figure because they were very widely distributed.
- Mann Whitney test was used to compare transaminase levels between dengue without shock and dengue with shock in each time-period.
- **: $p < 0.01$ and *: $p < 0.05$

Figure 3-4: Box and whisker plots showing serial measurements of AST (left panel) and ALT (right panel) levels in adults with dengue, presented in groups according to vascular leakage severity, together with results for the group with other febrile illnesses

3.3.2.2 Effect of chronic HBV or HCV co-infection on bleeding severity and laboratory results

Anti-HCV serology was positive in only 3 (2 dengue patients and 1 OFI patient) among the 207 patients tested. Evidence for prior exposure to HBV (antiHBc positive) was found in 344 of 692 patients tested (50%), with evidence for chronic HBV infection (antiHBc positive, HBsAg positive and antiHBc IgM negative) detected in 69/620 (11%) dengue patients and 9/47 (19%) OFI patients. Acute HBV infection (positive antiHBc IgM) was not found in any patient. In the dengue group ALT levels in the critical period were modestly but significantly higher in patients with chronic HBV co-infection than in patients without co-infection (Mann Whitney test, $p = 0.001$). A small increase in AST levels was also observed in the critical period but this was not statistically significant. No association was demonstrated between AST or ALT levels and HBV co-infection by the convalescent period (Table 3-3). In addition HBV infection had no effect on bleeding severity, vascular leakage severity or development of shock, severity of thrombocytopenia, or on any of the coagulation screening tests in the dengue patients. HBV infection was noted in only one of the eleven patients with severe liver dysfunction, and in none of those with acute liver failure.

Table 3-3: Effect of chronic HBV co-infection on bleeding severity and transaminase levels in 620 adults with dengue

Characteristic	Chronic HBV co-infection (n = 69)	No evidence for HBV infection (n = 551)	P value[a]
Severe bleeding	7 (10)	43 (8)	0.5
Mucosal bleeding or any severe bleeding	46 (67)	314 (57)	0.12
AST in critical period[b]	122 (28 – 548)	105 (30 – 483)	0.06
ALT in critical period[b]	118 (29 – 520)	80 (22 – 385)	0.001
AST in convalescent period[c]	152 (58 – 443)	137 (41 – 563)	0.67
ALT in convalescent period[c]	144 (50 – 637)	134 (29 – 575)	0.19

[a] Chi square or Fisher's Exact test, or Mann-Whitney test were used as appropriate.

[b] No of patients with and without chronic HBV co-infection in critical period are 62, 500 respectively

[c] Numbers of patient with and without chronic HBV co-infection in convalescent period are 56, 419 respectively

3.4 Discussion

3.4.1 Comparison of clinical picture between adults and children with dengue

The availability of detailed prospectively-collected observational data on a large number of dengue patients admitted to a single institution in an endemic region permitted this examination of the clinical patterns of dengue disease by age. We

found that although the overall clinical picture was similar in children and adults some clear differences were apparent, particularly in the pattern of complications seen. Admission with shock and development of recurrent shock were more common in children than adults, and plasma leakage was more profound. In contrast bleeding manifestations and organ involvement were more common and more severe in adults. Thrombocytopenia was also more severe in adults, but this may reflect similar disease-associated effects superimposed on intrinsically lower normal platelet values in adults compared to children.

Health-seeking behavior and local admission policies and referral systems may influence observed disease patterns, particularly in a hospitalized population. For example the male excess among children with uncomplicated dengue has been documented previously and may reflect parental health-seeking behavior [8,102]. HTD admits around 7,000 - 10,000 patients with suspected dengue annually. Although it functions as the main provider of inpatient care for local adults with dengue as well as being a referral centre for severe adult disease, local children may be taken preferentially to one of two paediatric hospitals in the city and HTD receives few regional paediatric referrals. We considered that referred patients were more likely to have severe disease than direct admissions, and in fact we confirmed that adults transferred from other hospitals were more severe than those admitted directly to HTD (Table 3-1). However since we had focused the comparative statistical analysis only on direct admissions from the local area, we consider that the main

conclusions about the different disease patterns seen in children and adults are valid and representative of the patterns of symptomatic disease by age in this environment.

The findings support the generally accepted view that severe vascular leakage is more common in children [14,103]. Age is known to influence intrinsic vascular permeability, with children demonstrating a lower threshold for leakage than adults [104]. Similarly, homeostatic mechanisms aimed at minimizing cardiovascular decompensation in the face of increased permeability are less well developed in children. However it is important to note that the population affected by dengue in Vietnam, a hyper-endemic country, is relatively young - the oldest patient in the study was aged 57 years and the oldest DSS case, 37 years. Secondary infection is well recognised as a risk factor for severe disease; 6% of the paediatric shock cases had primary infections compared to 30% of the uncomplicated paediatric cases, while only 6% of adults overall had primary disease with a single patient (aged 18 years) identified among the shock cases. Reports from Taiwan and Singapore, where endemicity is lower than Vietnam and secondary infections likely to occur later in life, indicate that infections occurring in elderly patients with co-morbidities are at greater risk for severe complications and death [105,106].

Skin and mild to moderate mucosal bleeding were significantly more common in adults, and severe bleeding in the absence of DSS occurred only in adults. Although we cannot exclude the possibility that some local children with severe bleeding without shock were admitted to one of the two major local paediatric hospitals,

informal communication with both centres indicates this presentation to be rare. Since coagulation tests were not performed systematically in the children involved in this analysis, no direct comparisons were possible. However, thrombocytopenia was more marked in adults than children, with significantly lower platelet counts documented throughout the evolution of the disease and with clear associations with the severity of leakage and bleeding. The multiple linear analysis indicated that this effect cannot be explained solely by the fact that adults had more secondary infections than children - adult age-group was an independent predictor for lower platelet counts for both severity groups during the acute illness and for the uncomplicated dengue patients at follow-up. The absence of an apparent effect at follow-up in the DSS patients may be explained by differences in the timing of these visits. Most adults attended for review around 2 weeks after discharge, at a time when rebound thrombocytosis was likely still to be a factor, while no children returned for review until 3-4 weeks after discharge when this effect should have settled. Rebound thrombocytosis is normally proportionate to the severity of preceding thrombocytopenia and may have confounded the age effect in the DSS patients (Figure 3-3). Few data are available on platelet changes with age, but one European study that examined platelet counts in 500 healthy subjects also demonstrated a progressive decline in platelet numbers during childhood, leveling off at 18 years [107]. It is therefore likely that the higher frequency and greater severity of bleeding manifestations in adults with dengue reflects the combined effects of several factors: intrinsically lower platelet counts in adults; a greater likelihood for adults in endemic areas to experience secondary

dengue infections which are associated with more severe thrombocytopenia than primary infections; and a higher background rate of underlying diseases such as peptic ulcer disease that may result in compromised tissue integrity [108].

We also found that children were generally less symptomatic than adults in the febrile phase of uncomplicated dengue, although they were more than twice as likely to receive maintenance intravenous fluid therapy. Comparisons of management strategies between the groups are complicated by the fact that both general and dengue-specific fluid management guidelines are tailored to expected age-related requirements. However the favourable outcome in most cases, together with the fact that significantly greater haemoconcentration was demonstrated in the children despite their receiving considerably more fluid therapy overall than the adults, indicates that the interventions were likely proportionate to need. Over the years considerable experience has accumulated regarding fluid management of children with dengue, but as epidemiological patterns begin to change and more adults present with severe and complicated disease, it is crucial that suitable management algorithms are developed that are relevant to the age-groups at risk. Additionally, given that profound but frequently asymptomatic thrombocytopenia is almost universal in adults with dengue, but also that even among young and otherwise healthy adults severe bleeding may occur, the lack of practical guidelines on the use of blood products needs to be addressed. For example, prophylactic platelet transfusions are becoming almost routine in some dengue-endemic countries, using arbitrary thresholds without

evidence of benefit [109,110]. In Vietnam, given the lack of evidence in this area, prophylactic platelet transfusions are not advocated in the national guidelines. Current Vietnamese MOH recommendations are that in the absence of active bleeding platelet transfusions should be considered only when the platelet count falls below 5×10^9/L. However it is well known that individual clinicians, particularly those working in private hospitals, do give prophylactic platelets at higher thresholds up to around 20×10^9/L. A recent survey conducted from OUCRU to assess approaches to the use of platelets in dengue received responses from 306 physicians in 20 different countries; the heterogeneity of the responses highlighted considerable variation in clinical practice both within and between countries [111]. In view of the clinical risks associated with platelet transfusions and the financial cost of such practice it is essential that formal research studies are carried out to provide an evidence base from which to develop clinical practice guidelines for use in dengue-endemic areas in the future.

3.4.2 Liver involvement associated with dengue infection in adults

The main finding was that mild to moderate hepatic inflammation, assessed by serial measurements of liver enzyme levels, was apparent in almost all dengue patients and correlated with other measures of severity, although the most abnormal results were noted rather later than the generally accepted critical period for other complications.

Transaminase levels began to increase from an early stage (day 1 - 3 of illness) and peaked during the second week of illness. Severe liver dysfunction was rare, but also

commonly occurred during the second week of illness. By follow-up, AST levels had returned to normal in the majority of cases, but ALT levels remained slightly increased above the normal range in approximately one-third of patients. This general pattern, with AST increasing more quickly and peaking at a higher level, and then reverting to normal more quickly than ALT levels, is unusual and differs from that commonly seen during acute hepatitis caused by hepatitis viruses [112], but is similar to observations in previous studies of dengue in both children and adults [99,100,113]. In addition, the effect of chronic HBV co-infection was primarily apparent on ALT levels. Although these results indicate that most adults with dengue infection have some degree of hepatic involvement, an additional non-hepatic source of AST could explain the pattern observed; ALT is primarily associated with hepatocytes, with minimal activity in cardiac and skeletal muscle, while AST is present in red blood cells, cardiac and skeletal muscle, and kidney and brain tissue, and is often elevated due to damage to those sources as well as in response to hepatic damage [112]. Given the prominence of musculoskeletal symptoms among adults with dengue, skeletal muscle injury could contribute to the elevation in AST levels. The plasma half-life of AST is shorter than that of ALT [112], but it is possible that the slower improvement in ALT levels simply reflects slower evolution of the hepatic disease than of the musculoskeletal problems. We was unable to investigate specific muscle enzymes or isoforms in this study, but creatine kinase levels are known to be elevated during the acute phase of dengue infection [114]. In combination with other simple laboratory tests transaminase levels, particularly AST levels, have been

suggested as a potential marker to help differentiate dengue from other viral infections during the early febrile phase [115]. However, mild to moderate increases in transaminase levels, with AST higher than ALT levels, are a non-specific finding that may be observed in a number of other infections such as typhoid fever and malaria [116,117], as well as in alcohol related hepatitis [112].

In agreement with previous smaller studies, we found associations between transaminase levels and increasing severity of vascular leakage as well as with bleeding severity [99,100,113,118]. Acute liver failure developed in only a small number of cases, none of whom had severe vascular leakage. Although liver involvement might be expected to contribute to derangements in haemostatic parameters, we only found prolonged PT values in the patients with liver failure. In most other patients PT values fell within the normal range and were similar to the values seen in the OFI group; also we only demonstrated a weak correlation between PT and transaminase levels during the convalescent period, suggesting that liver synthetic function in terms of coagulation factor production was generally well compensated.

The pathogenesis of liver involvement during dengue infections is still poorly understood [119]. Potential mechanisms of hepatic injury involve a variety of potential insults including direct effects of the virus or host immune response on liver cells, circulatory compromise and/or hypoxia due to hypotension or localized vascular leakage inside the liver capsule, hepatotoxic effects of drugs such as acetaminophen

or traditional herbal remedies, and tissue tropism of particular viral serotypes or genotypes. Given the fact that many organ systems are deranged by the time a patient dies, histopathological findings from autopsy studies are of limited value in trying to clarify primary disease mechanisms, and unfortunately suitable biopsy material is rarely available from less severe cases. The magnitude and evolution of the liver enzyme changes demonstrated in this study, and the relationships that we observed with other markers of disease severity, favour an adverse effect of immune dysregulation over a direct viral effect as the likely primary mechanism responsible for the hepatic dysfunction in the majority of patients. Dengue virus is rapidly cleared from plasma during the first few days of symptoms and viral RNA is rarely detectable after the first week of illness [41,120]; a direct viral effect might be expected to be maximal early in the infection coinciding with peak viremia although it is plausible that virus remains sequestered in the liver for some time after clearance from plasma. However, the host immune response is well established by the time the critical phase (day 4 - 6 of illness) is reached, particularly in secondary infections, and is generally considered to be a crucial factor in the development of other complications such as shock and thrombocytopenia [50,60].

Regarding the effects of concomitant infection with other hepatitis viruses common in Asia, we found a significant increase in ALT levels in dengue patients with chronic HBV infection compared to those without infection. The effect was minor however, and did not affect coagulation parameters or impact bleeding severity or clinical signs

of liver disease. In the past, several small studies have shown no effect of HBV infection on acute dengue morbidity [99,100], but our findings are in agreement with a recent study in which some 400 patients with DEN-1 infection were studied with slightly greater increases in ALT levels found among the patients with HBV co-infection [101]. We found no clinically significant effects in this study, but the possibility that repeated dengue infections may alter the rate of progression of the HBV infection needs to be considered. Very few patients with HCV infection were identified and we were unable to assess the effects of co-infection with this virus.

3.4.3 Summary

In summary, this comparison of the clinical features of a large number of adults and children with dengue demonstrates that the complications experienced by the two groups tend to reflect age-related physiological norms. Thus children, who have intrinsically higher microvascular permeability tend to develop vascular leakage and DSS, while adults, with intrinsically lower platelet numbers and a greater likelihood of having concomitant disorders that compromise mucosal integrity or influence specific organ function, tend to experience bleeding complications or severe organ impairment. Secondly it is clear that liver involvement, demonstrated by increases in transaminases levels, occurs in almost all adults with dengue and correlates with disease severity in terms of vascular leakage and bleeding. In most cases the involvement is mild and has little effect on coagulation parameters. Severe liver dysfunction and acute liver failure develop in only a small proportion of patients

(usually without evidence of vascular leakage severe enough to cause shock) but are often associated with severe bleeding. Chronic hepatitis B co-infection was associated with modestly but significantly increased levels of ALT, but did not otherwise impact the clinical picture.

Chapter 4

Exploring the mechanisms responsible for the increase in activated partial thromboplastin time

4.1 Introduction

As described in section 1.11 research to date suggests the increase in APTT seen during dengue infections could be due to one or both of the following mechanisms: a) a decrease in plasma coagulation factor levels following leakage from the intravascular to the interstitial compartment or b) the presence of a circulating anticoagulant such as the endothelial GAG, heparan sulfate.

In this chapter I will describe work done to examine these possibilities using plasma samples collected from the main adult cohort.

4.2 Methods

4.2.1 Patients and clinical methods

For the immediate 50/50 mixing studies and the HS measurements described below, we used serial citrated plasma samples from the first 100 adults recruited in AICU and the first 100 adults recruited on the infection wards. Unfortunately after completion of these studies there was no citrated plasma, and very little EDTA plasma, left from these patients, so for the remaining heparinase experiments we used selected samples from other confirmed dengue patients in the main adult cohort. General methodology relating to the main adult cohort is described in Chapter 2.

To assess differences in HS levels between dengue and other infectious diseases we used residual plasma from other clinical studies carried out at OUCRU in recent years, including studies on severe malaria, Japanese encephalitis, bacterial meningitis,

typhoid fever and tetanus. In each study the diagnosis was confirmed by conventional diagnostic tests according to the particular disease. Residual plasma from 20-30 patients in each disease group, that had been obtained within the first week of illness and stored frozen, was used.

4.2.2 Specific laboratory investigations

4.2.2.1 Immediate APTT 50/50 mixing studies

Standard APTT measurements:

The APTT test involves recalcification of citrated plasma in the presence of a standardized amount of cephalin and a factor XII activator (kaolin) to induce clot formation. We used standard reagents (CK Prest) and an ST4 Hemostasis Analyser from Stago, France. Following their instructions the cephalin/kaolin mixture and a high concentration calcium chloride (CaCl2) solution (0.025mol/L) were first incubated separately at 37^0C for at least 10 minutes. Then 50 μL of the test plasma and 50 μL of the cephalin/kaolin mixture were incubated together in a cuvette at 37^0C for 3 minutes. Then 50 μL of the pre-incubated CaCl2 solution was rapidly added to the mixture and the timer started simultaneously, in order to record the time to clot formation in seconds.

APTT Mixing studies:

Mixing studies can be helpful to investigate the cause of a prolonged APTT result (either coagulation factor deficiency or presence of a circulating anticoagulant). The

study is performed by mixing the patient plasma sample with normal pooled plasma (NPP) and then repeating the APTT test. The principle of this mixing test relies on the fact that if the result corrects to normal a deficiency of one or more coagulation factors is suspected, whereas if the result remains abnormal a circulating anticoagulant is likely to be present [63,121]. The ratio of patient plasma sample to NPP is usually either 1:1 (APTT 50/50 Mix) or 4:1 (APTT 80/20 Mix). The APTT 80/20 Mix allows for partial correction of coagulation factor deficiencies and is recommended if a mild anticoagulant is suspected because the APTT 50/50 Mix (with full correction of coagulation factor deficiencies) can mask the presence of such anticoagulants. In addition, the APTT Mix can be measured either immediately after adding NPP into the test sample (Immediate APTT Mix) or delayed for one to two hours to allow incubation of the mixture at 37^0C (Incubated APTT Mix), when the presence of a time dependent anticoagulant is suspected [122]. The immediate APTT 50/50 mixing test is the most commonly used and we chose to use this test for our initial investigations – thus we added 25μL NPP to 25μL patient plasma, then measured the APTT of this mixture immediately with the same procedure as described above.

4.2.2.2 Heparan sulfate measurements

We used commercial Heparan Sulfate ELISA Kits (Seikagaku, Japan) to measure HS levels in either EDTA or citrated plasma samples from the above selected patient groups, following the manufacturer's instructions. Preliminary investigations

comparing the results from 10 EDTA and 10 citrated plasma samples taken from the same patient during the same venepuncture revealed that percentage differences in HS levels between these two types of specimen were within 10% (data not shown).

4.2.2.3 Heparinase experiments

Having established the presence of high HS levels in the dengue patients (see results section 4.3.3.2), we performed several experiments using heparinase enzymes to investigate whether the HS functions as a circulating anticoagulant – i.e. we wished to degrade any HS present using specific enzymes and then to determine whether the increase in APTT reversed after this treatment. We used recombinant enzymes from Ibex Technologies Inc (Quebec, Canada) - heparinase III that exclusively degrades HS but not unfractionated heparin or low molecular weight heparins, and heparinase I that mainly degrades heparin, and HS to a lesser extent. Heparinase III is the most specific enzyme for degrading HS but since the fractions left may retain some anticoagulant properties, after discussion with experts in the field we elected to carry out preliminary experiments using combinations of the two enzymes in healthy plasma spiked with HS, in order to establish the most suitable protocol to use on the precious remaining patient samples. Thus we first investigated different cocktails of the heparinase enzymes under different experimental conditions for the incubation of plasma samples with heparinases. Experimental conditions applied for enzyme incubation such as temperature, pH and length of incubation as well as calcium concentration in the mixture of samples and enzymes are all parameters that have the

potential to interfere with APTT results irrespective of any enzyme effect. Fortunately the enzymes work well over a wide range of temperature (20 – 37^{0}C) and pH (5.5 – 9) so we maintained standard conditions - room temperature (RT) and physiological pH, for these parameters. However, it was necessary to investigate the effects of the inherent time delay required to incubate plasma samples with the heparinase enzymes - the time delay allows the enzymes time to be effective but it should not be more than one or two hours to prevent the gradual loss of coagulation factors after this time. Secondly, Ca^{2+} is a co-factor for heparinase enzyme activity, with ideal concentrations said to be between 2.5 and 5mmol/L – although this concentration is much lower than the concentration used to initiate clot formation in the standard APTT test, even small amounts of Ca^{2+} may shorten the APTT by triggering the onset of clot formation early. Several experiments are described below in which we assessed the effects of time delays and Ca^{2+} concentrations, in the end choosing the enzyme cocktail and experimental conditions that were most effective for degrading HS but had the least intrinsic effect on APTT results, for the main study.

4.3 Results

4.3.1 Patient information

Among the 200 adults with suspected dengue included in this section of the work, dengue was confirmed in 165 (83%) patients (38 with DSS and 127 without shock) while 21 (11%) patients were classified as having OFI. An alternative diagnosis was confirmed or suspected in 4 (2%) patients, all of whom had negative dengue

diagnostics. In the remaining 10 (5%) patients the results of serological and virological diagnostics were inconclusive.

4.3.2 Screening coagulation tests

4.3.2.1 Prothrombin time

In general, PT values were only marginally affected and improved during the evolution of the illness in both OFI and dengue groups. During the early febrile period, PT values were slightly prolonged but the number of patients assessed at this time was small (and no patients with shock were included until later). In the critical period PT was slightly increased in both dengue groups (Wilcoxon signed rank test, both $p < 0.01$, Table 4-1) but only the results of patients with shock were significantly different from those seen in the OFI group (Mann Whitney test, $p = 0.04$). There was a significant association between PT and vascular leakage severity in the critical period (Mann Whitney test, $p = 0.03$). Also, there were significant associations between PT and bleeding severity in the critical (Cuzick test for trend, $p = 0.04$) and the convalescent periods (Cuzick test for trend, $p = 0.02$) (data not shown).

4.3.2.2 Activated partial thromboplastin time

The APTT was slightly prolonged in the early febrile period, increased further during the critical period and remained slightly prolonged in the convalescent period (Table 4-1). The APTT results significantly correlated with vascular leakage severity during the critical period (Mann Whitney test, $p < 0.001$) and also during convalescent period

(Mann Whitney test, p = 0.02). In general the APTT was more prolonged in those with more severe bleeding during the critical period, but no statistically significant relationship was found (Cuzick test for trend, p = 0.07) (data not shown).

One possible explanation for the increase in APTT that has not been considered previously is that it may be artifactually prolonged due to the haemoconcentration that is a typical feature of many dengue infections. Samples with high HCT values have relatively higher citrate concentrations in the plasma in the collection tubes, and this is recognized to increase both PT and APTT values [123]. Typically however, significant prolongation in APTT but not in PT is seen during dengue infections - i.e. as described above in this study and in previous work in children [86,90]. It is generally suggested that if the HCT value of a blood sample is more than 55%, then both the PT and APTT results may be significantly inaccurate and adjustment of the blood to citrate ratio is recommended [123]. Therefore we repeated the analysis only including APTT results for which the corresponding HCT value was $\leq 55\%$ and found the same pattern for changes in APTT as we had demonstrated when including all APTT results (Table 4-2). The significant correlations with vascular leakage severity during the critical (Mann Whitney test, p = 0.002) and convalescent periods (Mann Whitney test, p = 0.03) remained apparent and we do not believe that haemoconcentration contributed to the overall effects observed.

Table 4-1: Serial results for screening coagulation tests in 165 adults with dengue, presented according to the severity of vascular leakage, and in 21 adults with OFI

	OFI				Dengue without shock				DSS			
	Day 1-3 (n=12)	**Day 4-6** (n=19)	**Day 7-10** (n=9)	**FU** (n=11)	**Day 1-3** (n=14)	**Day 4-6** (n=101)	**Day 7-10** (n=93)	**FU** (n=72)	**Day 1-3**	**Day 4-6** (n=26)	**Day 7-10** (n=37)	**FU** (n=28)
PT (seconds)												
Median	16.6*	15.6	14.9	15	17	15.8***	14.8	14.7		16.7**	15.4**	14.7
90% range	14.9-18.4	13.2-17.6	13.7-15.8	13.6-16.5	12.3-20.9	13.1-20.1	12.4-18.7	13.2-17.4		13.8-47.1	13.3-26.4	12.9-22.4
APTT (seconds)												
Median	35.3*	35*	33.8	33.7	40.8*	44.1***	41.6***	33.4		49.4***	46.1***	35.1
90% range	32-47.9	28-40.8	28.5-39.1	30.8-38.1	34.5-50	33.9-62.7	31.1-57.2	27.9-51		39.8-117	33-120	28.1-66.3
Plasma fibrinogen levels (g/L)												
Median	3.9*	4*	4	3	3.4*	3**	3**	3.3		2.1***	2.5	3.1
90% range	2.5-4.6	2.2-5	2.7-5	2.4-4	2-5	1.8-4	1.7-4.6	1.8-5.4		0.7-3.3	1.2-5.4	1.9-6.3
D-Dimer[δ]												
Negative	8 (66.7)	12 (63.2)	8 (88.9)	7 (63.6)	9 (64.3)	57 (56.4)	52 (55.9)	56 (77.8)		14 (53.8)	13 (35.1)	20 (71.4)
Weakly positive	4 (33.3)	7 (36.8)	1 (11.1)	4 (36.4)	5 (35.7)	40 (39.6)	36 (38.7)	16 (22.2)		12 (46.2)	21 (56.8)	8 (28.6)
Strongly positive	0	0	0	0	0	4 (4)	5 (5.4)	0		0	3 (8.1)	0

- [δ]Negative: < 0.5, Weakly positive: ≥ 0.5 and < 2 and Strongly positive: ≥ 2 μg/mL

- The total number of patients included in each group for each time period is indicated.

- The Wilcoxon signed rank test was used to compare the results in each time period with the corresponding results at follow up (here taken to represent the normal value for that individual). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

Table 4-2: Serial APTT results for the dengue patients, including only results for samples with a corresponding HCT value ≤ 55%

	Day 1-3	Day 4-6	Day 7-10	FU
OFI	34.8 (32 – 47.5) (n=11)	35* (28 – 40.8) (n=19)	33.7 (28.5 – 39.9) (n=8)	33.8 (31.8 – 38.2) (n=10)
Dengue without shock	40.8* (34.5 – 50) (n=14)	44.1*** (33.9 – 62.9) (n= 97)	41.6*** (31.1 – 57.4) (n=89)	33.3 (27.9 – 51.8) (n= 70)
DSS		48.4** (39.3 – 92.6) (n=21)	44*** (32.6 – 120) (n=35)	34.5 (28.1 – 66.6) (n=27)

- Data are expressed as median (90% range).
- The total number of patients included in each group for each time period is indicated.
- The Wilcoxon signed rank test was used to compare the results in each time period with the corresponding results at follow up (here taken to represent the normal value for that individual). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

4.3.2.3 Plasma fibrinogen levels

Plasma fibrinogen levels were slightly increased in the early febrile period in both OFI and dengue patients without shock (Wilcoxon signed rank test, both $p < 0.05$), reflecting the fact that fibrinogen is an acute phase protein. Subsequently fibrinogen levels were significantly lower in dengue patients both with and without shock during the critical period (Wilcoxon signed rank test, both $p < 0.01$) and in dengue patients without shock during the convalescent period (Wilcoxon signed rank test, $p < 0.01$) (Table 4-1). There was a significant inverse association between fibrinogen levels and vascular leakage severity in the critical and convalescent periods (Mann Whitney test,

both $p < 0.01$). Also, fibrinogen levels significantly correlated with bleeding severity in the critical period (Cuzick test for trend, $p = 0.007$) (data not shown).

4.3.2.4 D-Dimer

D-Dimers were either negative or weakly positive in most patients. The percentages for each category were similar between the OFI and dengue patients (Table 4-1). There was no association between D-Dimers and either vascular leakage or bleeding severity (Chi square test, all $p > 0.1$).

4.3.2.5 Summary of screening coagulation tests

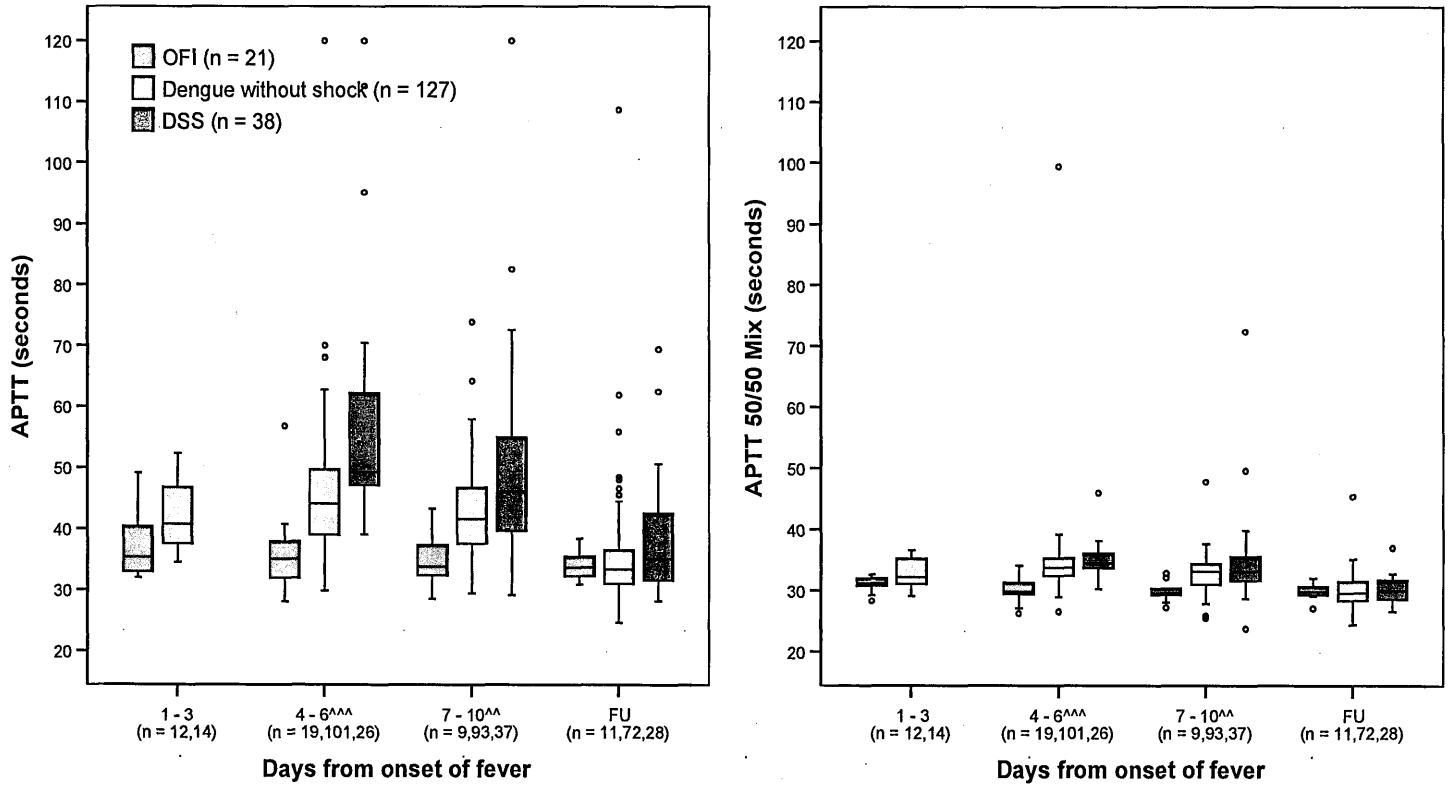
Similar to the results of previous work in children [86,90], the pattern of coagulopathy seen in adults with dengue included mild to moderate increases in the APTT with reduced plasma fibrinogen levels, but only marginal increases in the PT and generally negative D-Dimers. This pattern would be unusual for DIC since there was no real evidence of pro-coagulant activation. Therefore the mechanisms underlying these typical coagulation disturbances need to be explored further.

4.3.3 Specialized investigations to explore the increase in APTT

4.3.3.1 Immediate APTT 50/50 mixing investigations

Following the addition of NPP, there was considerable improvement in the APTT – the median APTT returned to around 34 seconds or lower (Figure 4-1), indicating likely coagulation factor deficiency. However, there was a residual small but significant increase in the APTT in the dengue patient groups compared to OFI in the

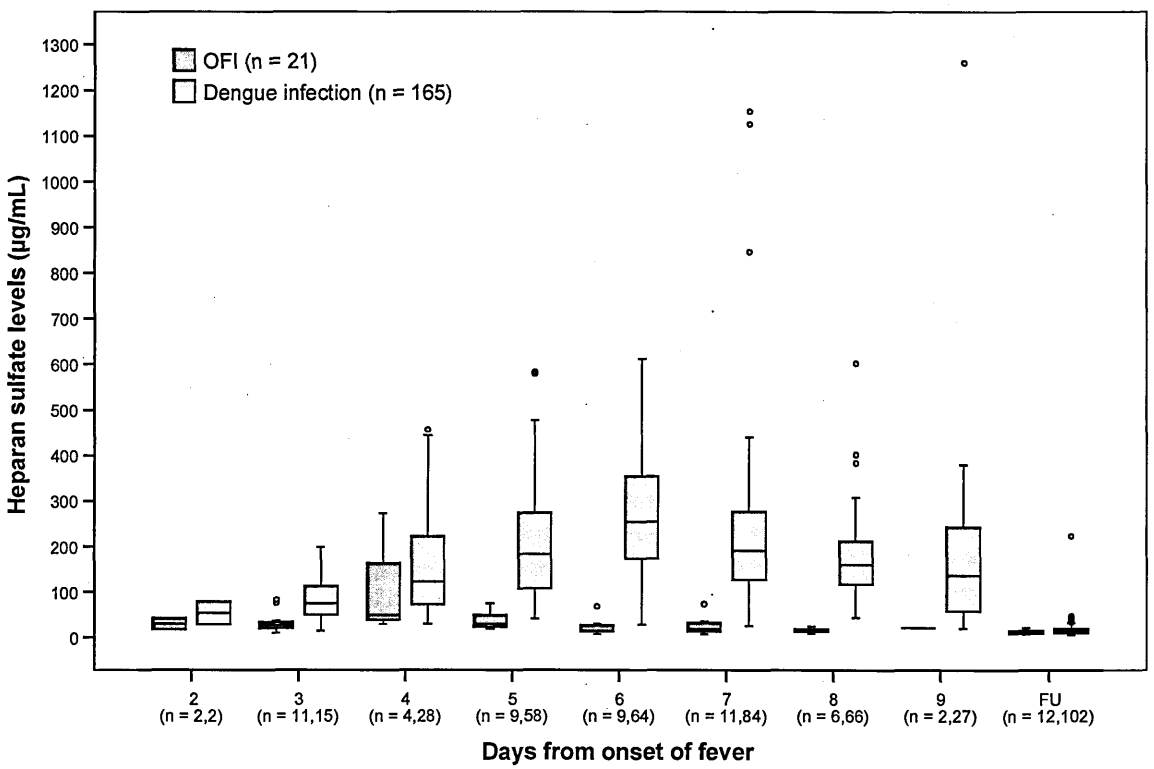
critical and convalescent periods (Mann Whitney test, both $p < 0.01$), and the possibility of a circulating anticoagulant, functioning in a similar way to heparin, remains an important consideration.



- The total number of patients included in each group during each time period is indicated on the X axis.
- The Mann Whitney test was used to compare APTT or APTT 50/50 Mix values between the whole dengue group and the OFI group.
- ^: $p < 0.05$, ^^: $p < 0.01$ and ^^^: $p < 0.001$

Figure 4-1: Box and whisker plots presenting the results of serial standard APTT (left panel) and immediate APTT 50/50 Mix measurements (right panel) for adults with dengue and those with OFI

4.3.3.2 Plasma heparan sulfate levels



The total number of patients included in each group each day is indicated on the X axis.

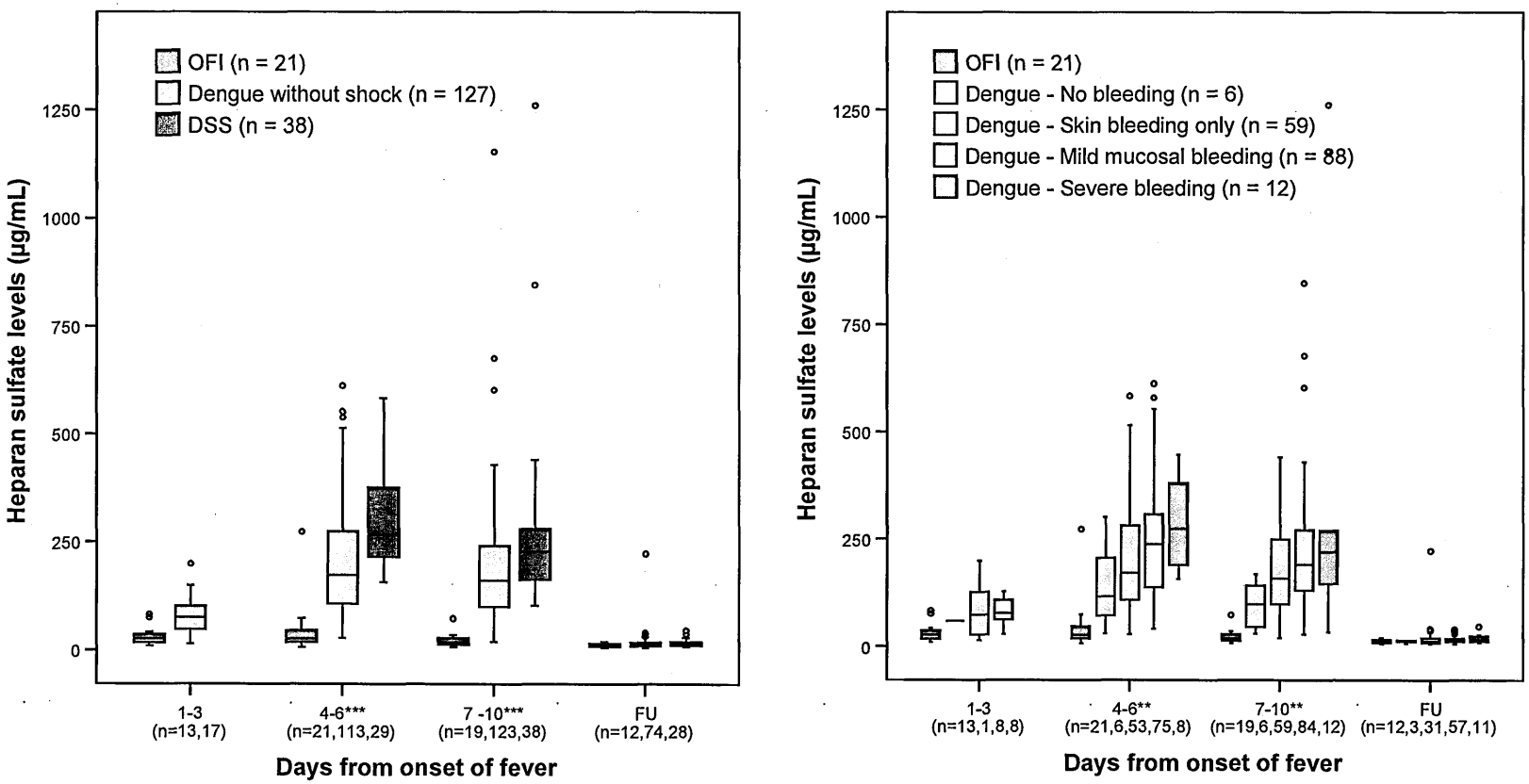
Figure 4-2: Box and whisker plots showing daily heparan sulfate levels in adults with dengue and OFI

We observed that plasma HS levels were markedly increased in the dengue patients with the same time profile as that of vascular leakage (i.e. peaking at day 6 of illness and subsequently improving, Figure 4-2). The levels observed here were considerably higher than those measured with the same kits in patients with mucopolysaccharidoses, the disease in which there is deficiency or dysfunction of lysosomal enzymes needed to degrade GAGs [124].

Figure 4-3 shows that HS levels correlated strongly with both vascular leakage (Mann Whitney test, both $p < 0.001$) and bleeding severity (Cuzick test for trend, both $p <$

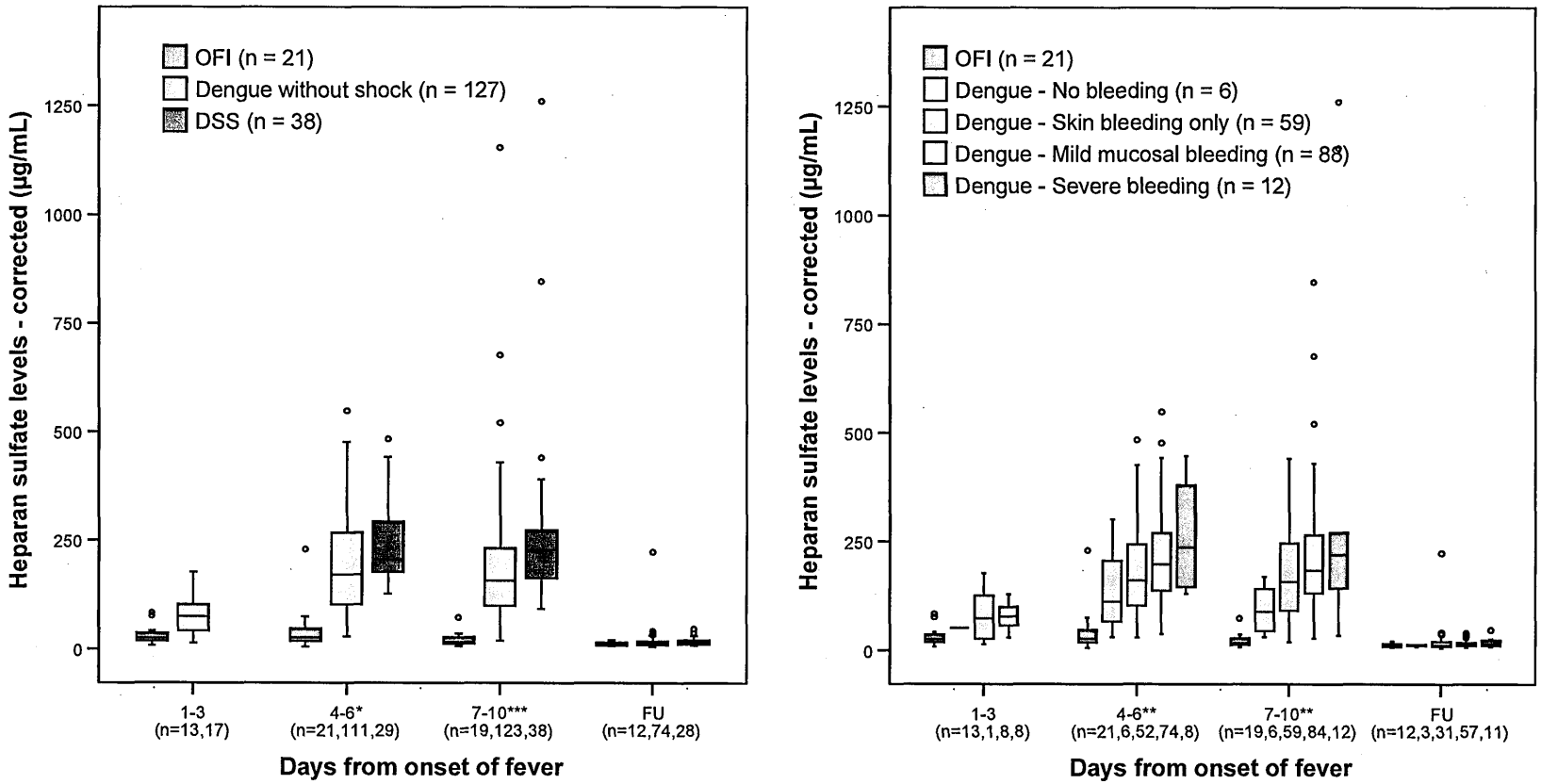
0.01) in the critical and convalescent periods in the dengue patients. Since HS levels may be artificially increased due to the effect of the haemoconcentration phenomenon seen in dengue patients with vascular leakage, we corrected the values using the following method. Firstly, percentage HCT change was calculated for the HCT value on the same day with reference to the baseline value. If the percentage HCT change on this day was $\geq$ 10% (HCT changes of less than 10% from baseline are accepted as within normal daily variability), then the HS level was corrected as follows: corrected HS level = measured HS level/(1 + percentage HCT change). After we corrected HS levels, the correlations with both vascular leakage and bleeding severity in the critical and convalescent periods still existed (Figure 4-4). As expected, we saw the extent of the correction in patients with shock was greater than in those without shock.

We looked for correlations between HS levels and the overall percentage haemoconcentration (as a marker of vascular leakage severity) and with the results of other laboratory markers measured concurrently during the critical period. HS levels demonstrated moderate to strong negative correlations with the platelet count (Spearman correlation of –0.69, $p < 0.001$, $n = 149$) and plasma fibrinogen levels (Spearman correlation of –0.36, $p < 0.001$, $n = 129$); and moderate positive correlations with APTT (Spearman correlation of 0.44, $p < 0.001$, $n = 129$) and percentage haemoconcentration (Spearman correlation of 0.36, $p < 0.001$, $n = 142$) (Figure 4-5). HS levels showed no correlation with PT (Spearman correlation of 0.09, $p = 0.32$, $n = 129$) or D-Dimers (Cuzick test for trend, $p = 0.29$).



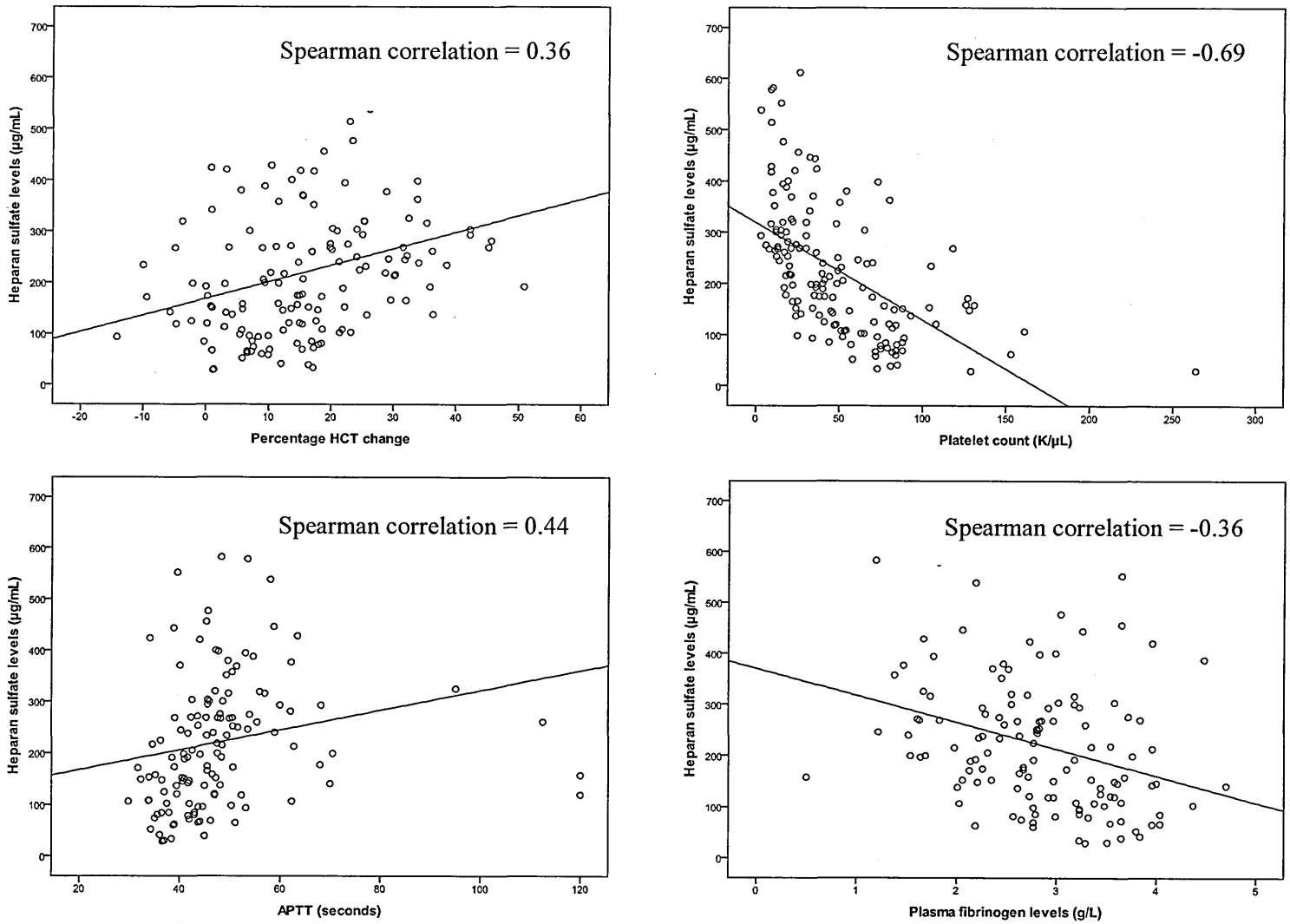
- The total number of patients included in each group during each time period is indicated on the X axis.
- The Mann Whitney test was used to compare HS levels between vascular leakage categories, and the Cuzick test for trend was used to compare HS levels across bleeding severity categories in the dengue patients.
- *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$

Figure 4-3: Box and whisker plots showing relationships between plasma HS levels and the severity of vascular leakage (left panel) and bleeding (right panel)



- The total number of patients included in each group during each time period is indicated on the X axis.
- The Mann Whitney test or Cuzick test for trend were used to compare corrected HS levels across vascular leakage or bleeding severity categories in the dengue patients.
- *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$

Figure 4-4: Box and whisker plots showing relationships between plasma HS levels, corrected for haemoconcentration, and the severity of vascular leakage (left panel) or bleeding (right panel)



Overall percentage HCT change was not calculated for patients with severe bleeding

Figure 4-5: Scatter plots showing correlations between HS levels and overall percentage HCT change, platelet count, APTT, and plasma fibrinogen levels in adults with dengue during the critical period

HS measurements in the non-dengue disease controls showed that although HS levels were increased in some severe malaria patients, the measured values were considerably lower than those seen in the dengue infected patients. In the other disease control groups HS levels were generally low and similar to the findings in the OFI group (Figure 4-6).

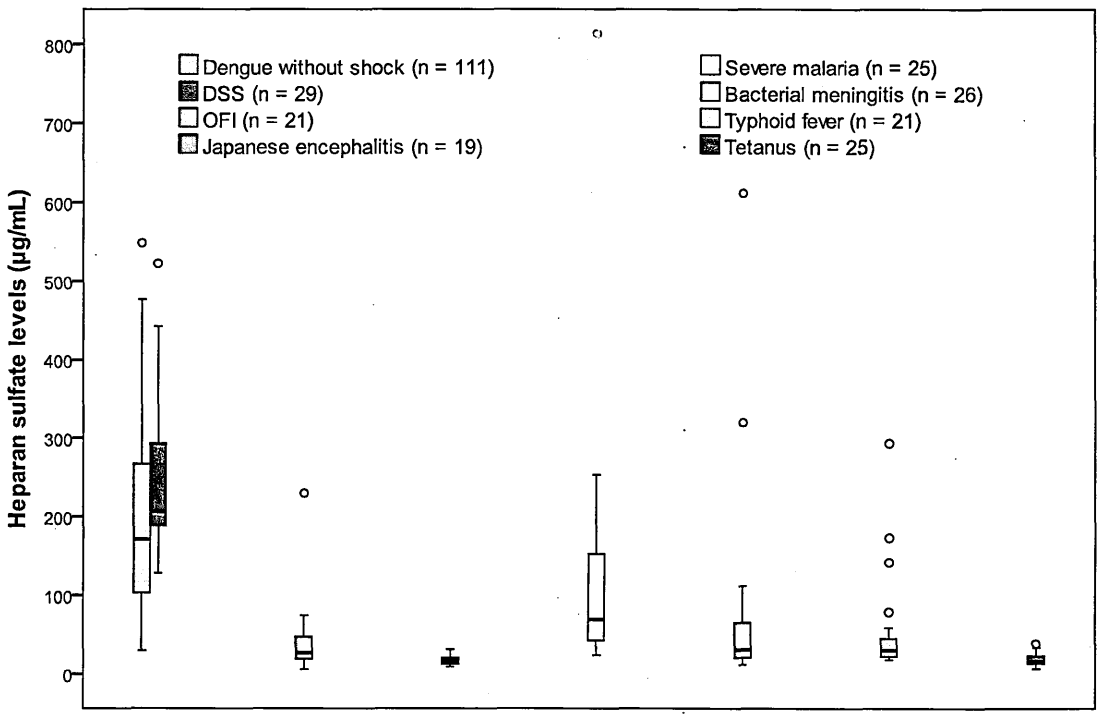


Figure 4-6: Box and whisker plots presenting HS levels for the dengue patients with and without shock (levels from the critical period, corrected for haemoconcentration), and for various non-dengue disease controls

4.3.3.3 Heparinase experiments

Optimization of the conditions: Ca^{2+} concentration and incubation time

To establish the optimal conditions for the subsequent experiments we used NPP from healthy volunteers. To do this we obtained 5 ml venous blood samples from 10 healthy volunteers, taken into standard citrated collection tubes. After separation, we pooled the plasma from all 10 donations to produce a pool of normal plasma. The plasma was then stored at -80 ^{0}C in 1 ml aliquots. We used the following reagents and preparations for these experiments:

a) Heparinase I and III enzymes (both 10 IU/mL) obtained from Ibex in liquid form and stored at -80^{0}C.

b) Purified Heparan Sulfate (extracted from porcine intestinal mucosa) obtained from Sigma (USA) in powder form. Following the manufacturer's instructions 1mg HS powder was dissolved in 100µL sterile water to give a final concentration of 10,000 µg/mL. Then the HS solution was divided and stored in aliquots in the fridge at 2 - 8^{0}C for one week.

c) Standard Calcium Chloride 10% (0.68 mol/L) solution was obtained from the hospital pharmacy, and diluted either 1:2 or 1:4 with sterile water to give the following CaCl2 solutions - solution A (0.34 mol/L) and solution B (0.17 mol/L). Calcium solutions were made fresh for each experiment.

Table 4-3: Optimizing the Ca^{2+} concentration – baseline APTT of NPP for this experiment was 29.5 / 29.4 seconds

Test	NPP volume	Volume of 0.9%saline	Volume of CaCl2	APTT (seconds) – 15 min delay	APTT (seconds) – 30 min delay
1	260 µL	4 µL		29.1 / 29.4	29.2 / 29.6
2	260 µL		4 µL solution A *(5 mmol/L)*	19.7 / 20.0	18.6 / 18.4
3	260 µL		4 µL solution B *(2.5 mmol/L)*	27.6 / 26.7	27.5 / 26.6

The final concentration of the CaCl2 used is indicated in brackets.

Ca^{2+} is necessary as a co-factor for heparinase enzyme activity, with ideal concentrations said to be between 2.5 and 5mmol/L, but Ca^{2+} itself may shorten the APTT. Therefore as a first step we measured the APTT in NPP after incubation with different calcium concentrations and after time delays of 15 and 30 minutes. Since the final concentration of coagulation proteins could also influence the APTT we ensured that the final volume of all samples in each experiment was the same, using 0.9% saline to balance the volume of any other reagents added (Table 4-3). Baseline and test APTT measurements were always performed in duplicate if the volume of plasma available allowed.

As expected, the APTT results were shorter in the presence of CaCl2; with the concentration of 2.5mmol/L and a time delay of 30 minutes the APTT values were only slightly shorter than the baseline values.

Spiking NPP with HS and attempting to reverse the effect on APTT with enzyme cocktails - Experiment 1

As a first step we added purified HS into NPP intending to mimic the high HS levels previously demonstrated in plasma samples from the dengue patients. As before, we corrected the volume of each test sample to a standard so as to ensure similar coagulation protein concentrations in all test samples, and we measured the APTT after incubating all the samples (with and without HS) at RT for 30 minutes. The purified HS clearly prolonged the APTT (Table 4-4, step 1).

In the second step we added different combinations of the heparinase enzymes (final concentration 0.2 IU/mL) with and without CaCl2 (final concentration 2.5 mmol/L) to the remaining mixture of NPP plus purified HS (Table 4-4, step 2). We incubated all the samples at RT for 60 minutes to allow sufficient time for the enzymes to be effective. We then measured a standard APTT twice on each test sample.

When compared to the corresponding APTT results in step 1, the APTT results in step 2 of the test samples without HS or heparinase enzymes (test samples 1 and 2) were slightly longer with a time delay of 60 minutes and with minor dilution with 0.9% saline. In the two test samples that were spiked with HS in step 1 but were incubated without enzymes in step 2 (test samples 3, 4) the APTT results were appreciably prolonged. In test samples that were spiked with HS and treated with enzymes (test samples 5 – 10), the APTT results improved significantly with all 3 enzyme mixtures but the effects were of different magnitude: heparinase III and I in combination (tests

7, 8) was more effective than heparinase I alone (tests 9, 10), which in turn was more effective than heparinase III alone (tests 5, 6). In all cases the difference in APTT results between corresponding test samples with and without Ca^{2+} was around 5 seconds, suggesting that the differences were more likely due to the effect of Ca^{2+} on the intrinsic coagulation process rather than as a direct result of Ca^{2+} on enzyme efficiency.

Spiking NPP with HS and attempting to reverse the effect on APTT with enzyme cocktails - Experiment 2

We went on to perform a similar experiment with NPP spiked with purified HS but without including CaCl2, as suggested by the results of Experiment 1, but tried to see if lower concentrations of the enzymes and a shorter time of action (30 minutes vs 60 minutes) would influence the results (Table 4-5). As before the results showed that the APTT results improved significantly with all 3 enzyme mixtures, again with effects of different magnitude: heparinase III and I (tests 5, 6) > heparinase I alone (test 7) > heparinase III alone (tests 3, 4). In addition, enzyme concentrations of 0.1 IU/mL had similar effects as enzyme concentrations of 0.2 IU/mL.

Table 4-4: NPP spiked with purified HS – Experiment 1

Step 1 – spiking NPP with HS					Step 2 – addition of enzyme cocktails to reverse the effect of HS on the APTT					
Test	**Volume of NPP**	**Volume of 0.9% saline**	**Volume of HS solution**	**APTT (seconds) - 30 minutes delay**	**Remaining sample**	**Volume of 0.9% saline**	**Volume of Heparinase III**	**Volume of Heparinase I**	**Volume of CaCl2**	**APTT (seconds) - 60 minutes delay**
1	180 µL	4 µL		30.3	134 µL	6 µL			2 µL *(2.5 mmol/L)*	31.9 / 32.1
2	180 µL	4 µL		30.4	134 µL	8 µL				34.9 / 35.6
3	180 µL		4 µL *(220µg/mL)*	58.4	134 µL	6 µL			2 µL *(2.5 mmol/L)*	55.4 / 55.8
4	180 µL		4 µL *(220µg/mL)*	58.1	134 µL	8 µL				61.2 / 61.3
5	180 µL		4 µL *(220µg/mL)*	59.4	134 µL	3 µL	3 µL *(0.2 IU/mL)*		2 µL *(2.5 mmol/L)*	45.4 / 45.5
6	180 µL		4 µL *(220µg/mL)*	57.4	134 µL	5 µL	3 µL *(0.2 IU/mL)*			50.8 / 51.5
7	180 µL		4 µL *(220µg/mL)*	57.0	134 µL		3 µL *(0.2 IU/mL)*	3 µL *(0.2 IU/mL)*	2 µL *(2.5 mmol/L)*	37.4 / 37.9
8	180 µL		4 µL *(220µg/mL)*	59.2	134 µL	2 µL	3 µL *(0.2 IU/mL)*	3 µL *(0.2 IU/mL)*		42.8 / 43.5
9	180 µL		4 µL *(220µg/mL)*	56.6	134 µL	3 µL		3 µL *(0.2 IU/mL)*	2 µL *(2.5 mmol/L)*	39.6 / 40.3
10	180 µL		4 µL *(220µg/mL)*	57.4	134 µL	5 µL		3 µL *(0.2 IU/mL)*		46.2 / 45.9

Note that the final sample volume was kept the same in all tests to minimize any effects on coagulation protein concentrations. The final concentration of the different reagents used is indicated in brackets.

Table 4-5: NPP spiked with purified HS – Experiment 2

Step 1 – spiking NPP with HS					Step 2 – addition of enzyme cocktails to reverse the effect of HS on the APTT				
Test	**Volume of NPP**	**Volume of 0.9% saline**	**Volume of HS**	**APTT (seconds) - 30 minutes delay**	**Remaining sample**	**Volume of 0.9% saline**	**Volume of Heparinase III**	**Volume of Heparinase I**	**APTT (seconds) - 30 minutes delay**
1	180 µL	4 µL		30.0	134 µL	6 µL			31.8 / 32.7
2	180 µL		4 µL *(220µg/mL)*	59.2	134 µL	6 µL			60.9 / 61.2
3	180 µL		4 µL *(220µg/mL)*	58.3	134 µL	3 µL	3 µL *(0.2 IU/mL)*		50.5 / 50.6
4	180 µL		4 µL *(220µg/mL)*	57.6	134 µL	4.5 µL	1.5 µL *(0.1 IU/mL)*		50.6 / 51.0
5	180 µL		4 µL *(220µg/mL)*	57.1	134 µL		3 µL *(0.2 IU/mL)*	3 µL *(0.2 IU/mL)*	40.5 / 39.8
6	180 µL		4 µL *(220µg/mL)*	56.9	134 µL	3 µL	1.5 µL *(0.1 IU/mL)*	1.5 µL *(0.1 IU/mL)*	41.0 / 40.8
7	180 µL		4 µL *(220µg/mL)*	55.8	134 µL	3 µL		3 µL *(0.2 IU/mL)*	43.3 / 43.0
8	180 µL		4 µL *(220µg/mL)*	*					

*: A problem occurred with the procedure when measuring APTT in step 1 and therefore step 2 could not be performed.

Note that the final sample volume was kept the same in all tests to minimize any effects on coagulation protein concentrations. The final concentration of the different reagents used is indicated in brackets.

Heparinase experiments with dengue patient samples

We had very limited supplies of the enzymes and the purified HS so were only able to perform a small number of experiments. A citrated plasma sample collected during the critical period from a patient with DSS was selected for this work. Although Experiment 2 above indicated that enzyme concentrations of 0.1 IU/mL for both heparinase III and I were likely to be as effective as concentrations of 0.2 IU/mL we decided to carry out the first experiment at 0.2 IU/mL in order not to miss any potential effects. The mixture of plasma and enzymes shown in Table 4-6 was incubated at RT for 30 minutes and then the APTT was measured twice.

Table 4-6: Heparinase effects on plasma from a dengue patient (DSS) – Experiment 1

Note the baseline APTT before enzyme treatment was 59.3/59.2 seconds

Test	Volume of citrated sample	Volume of 0.9% saline	Volume of Heparinase III	Volume of Heparinase I	APTT (seconds) - 30 minutes delay
1	120 µL	6 µL			61.2 / 61.3
2	120 µL	3 µL	3µL *(0.2 IU/mL)*		61.5 / 60.8
3	120 µL		3µL *(0.2 IU/mL)*	3 µL *(0.2 IU/mL)*	61.6 / 63.3
4	120 µL	3 µL		3 µL *(0.2 IU/mL)*	61.7 / 61.4

The results showed that APTT results in the test samples treated with enzymes (tests 2 – 4) were similar to the result of the test sample without enzymes (test 1). Thus we saw no reversal of the APTT for any combination of enzymes used, different to the responses we had seen in the experiments on NPP spiked with purified HS. One possible explanation could be that reversal of the APTT when HS is degraded by

heparinases may not be apparent if there is also significant deficiency of coagulation factors as might occur in dengue patient samples. Therefore we carried out a further experiment (Table 4-7) in which we added the cocktail of heparinase I and III enzymes and CaCl2 into a citrated plasma sample obtained during the critical period from a confirmed DSS patient, incubated the mixture at RT for 60 minutes, and then went on to perform immediate 50/50 and 80/20 APTT mixing studies on plasma samples with and without enzyme treatment. The results showed that the APTT of the test samples (i.e. with enzymes) were similar to the results of the corresponding samples without enzyme treatment. Finally we repeated a similar experiment (Experiment 3, Table 4-8) with a citrated sample from another dengue patient during the critical period, this time using very high concentrations of the enzyme mixture (0.4 and 0.6 IU/mL). Again, we saw no additional effect of enzyme treatment, over and above the effects of adding NPP in either 50/50 or 80/20 ratios.

Although we had planned to measure plasma HS levels in the dengue samples before and after the various experiments described above we were unable to do so, as Seikagaku, the Japanese manufacturer of the HS ELISA kits, had had to discontinue the research arm of their business after the Japanese tsunami. We were able to perform one small experiment with two plasma samples from DSS patients; we measured HS at very high levels (439 and 572 μg/mL) in these samples. Then we treated the samples with heparinase I and III at concentrations of 0.2 IU/mL and under standard conditions (RT, time incubation of 30 minutes and without CaCl2) and repeating the HS ELISAs. In both cases plasma HS was undetectable after the enzyme treatment indicating that this mixture of enzymes does fully degrade the endogenous HS found in dengue patients' plasma.

Table 4-7: Heparinase effects on plasma from a dengue patient (DSS) – Experiment 2

Note the baseline APTT before enzyme treatment was 46.4/46.1 seconds

Step 1: add enzymes and CaCl2 into test samples and incubate at RT for 60 minutes						Step 2: add NPP and measure APTT immediately	
Test	**Volume of citrated sample**	**Volume of 0.9% saline**	**Volume of Heparinase III**	**Volume of Heparinase I**	**Volume of CaCl2**	**Volume of NPP**	**Immediate APTT Mix (seconds)**
1	60 µL	3 µL			2 µL *(5 mmol/L)*	60 µL	34.3 / 31.9
2	60 µL		1.5 µL *(0.2 IU/mL)*	1.5µL *(0.2 IU/mL)*	2 µL *(5 mmol/L)*	60 µL	31.4 / 32.6
3	96 µL	3 µL			2 µL *(3 mmol/L)*	24 µL	39.2 / 38.9
4	96 µL		1.5 µL *(0.15 IU/mL)*	1.5 µL *(0.15 IU/mL)*	2 µL *(3 mmol/L)*	24 µL	38.2 / 38.1
5	120 µL	3 µL			2 µL *(2.5 mmol/L)*		44.8 / 44.6
6	120 µL		1.5 µL *(0.1 IU/mL)*	1.5 µL *(0.1 IU/mL)*	2 µL *(2.5 mmol/L)*		44.4 / 44.5
7	120 µL	5 µL					46.7 / 45.1

- Test 1: the control for test 2 (ratio between patient sample and NPP = 50/50)
- Test 3: the control for test 4 (ratio between patient sample and NPP = 80/20)
- Test 5: the control for test 6 (no NPP)
- Test 7: dengue patient sample without heparinase enzymes, CaCl2 or NPP

Table 4-8: Heparinase effects on plasma from a dengue patient – Experiment 3

Note the baseline APTT before enzyme treatment was 41.7 / 41.6 seconds

Step 1: add enzymes and CaCl2 into test samples and incubate at RT for 60 minutes						Step 2: add NPP and measure APTT immediately	
Test	**Volume of citrated sample**	**Volume of 0.9% saline**	**Volume of Heparinase III**	**Volume of Heparinase I**	**Volume of CaCl2**	**Volume of NPP**	**Immediate APTT Mix (seconds)**
1	60 µL	8 µL			2 µL *(5 mmol/L)*	60 µL	28.9 / 28.2
2	60 µL		4 µL *(0.6 IU/mL)*	4µL *(0.6 IU/mL)*	2 µL *(5 mmol/L)*	60 µL	28.5 / 28.6
3	96 µL	8 µL			2 µL *(3 mmol/L)*	24 µL	34.8 / 34.7
4	96 µL		4 µL *(0.4 IU/mL)*	4µL *(0.4 IU/mL)*	2 µL *(3 mmol/L)*	24 µL	34.5 / 34.2
5	120 µL	10 µL					41.7 / 39.8

- Test 1: the control for test 2 (ratio between patient sample and NPP = 50/50)
- Test 3: the control for test 4 (ratio between patient sample and NPP = 80/20)
- Test 5: dengue patient sample without heparinase enzymes, CaCl2 or NPP

4.4 Discussion and conclusion

The work presented here supports previous work from Vietnam indicating that the pattern of coagulopathy seen in dengue patients is not typical for DIC. Similar to the paediatric studies this adult study indicates that the main coagulation abnormalities present are an increase in the APTT and a reduction in fibrinogen levels, with little evidence of pro-coagulant activation. Although HS levels were markedly increased in the dengue patients, the APTT mixing studies indicate that the primary mechanism responsible for the prolonged APTT is likely to be coagulation factor deficiency.

We know that many coagulation factors carry negative charge on their surface and are also small in size, with molecular weights typically similar to or less than that of albumin (Table1-1); in normal conditions these characteristics help to protect the proteins from leakage. However, this protection seems to be impaired in dengue infection, as shown in the previous study from the OUCRU group that demonstrated increased clearances of both negatively charged albumin and neutral transferrin, as well as leakage of the similarly sized, negatively charged coagulation protein, antithrombin [57]. Thus leakage of essential coagulation factors is a plausible mechanism for the coagulation factor deficiency seen in dengue.

Another question raised by these results relates to why, if the APTT is prolonged due to leakage of essential coagulation proteins, a similar effect is not apparent for the PT? Coagulation factor VII, one of the proteins crucial for initiating the PT response is also a small molecule (molecular weight 48,000 Daltons) (Table 1-1) and would be

expected to leak alongside other relatively small coagulation proteins. The APTT is dependent on a greater number of coagulation factors (factor XII, XI, IX, VIII, X, V, II and I) than the PT (factor VII, X, V, II and I) [63,121], and thus it is possible that it is more affected by global deficiency of coagulation factors due to leakage. In addition, it is known that the activated form of factor VII (FVIIa) normally circulates in very tiny amounts in plasma (accounting for only approximately 1% of the total factor VII mass), and that the Tissue Factor - Factor VIIa complex that is the starting point for the procoagulant response rapidly triggers an exponentially increasing coagulation cascade. A negative effect on clotting may only become apparent when there is substantial deficiency of total factor VII mass [125]. It seems very likely that Factor VII deficiency does occur in dengue patients with vascular leakage but that in the majority of cases this deficiency is not sufficient to alter the PT.

Further studies could be considered to investigate whether leakage of essential coagulation factors is the main mechanism underlying the prolonged APTT with a normal PT that is the characteristic finding in dengue patients. Since systemic and renal ultrafiltration processes are similar, urine is sometimes used as a surrogate for interstitial fluid when assessing protein leakage [57,126]. The ratio of the concentration of a particular protein to the creatinine concentration in a random urine sample is a simple method that is accepted to assess urinary protein excretion - there is a good correlation between that ratio and the amount of protein excreted in a 24-hour urine collection, provided renal function is stable [127]. Thus one possibility

would be to measure serial plasma concentrations of specific coagulation factors, together with urinary concentrations of these factors, to understand the leakage pattern throughout the course of illness. Secondly, haemostatic balance (and therefore the results of coagulation screening tests such as the PT and APTT) is dependent on the activities of the different coagulation proteins rather than concentrations of these factors per se - although clearly activity and concentration of an individual factor are closely linked. So, serial measurements of coagulation factor activities together with simultaneous plasma concentrations could also be informative. However, given the large number of different coagulation factors involved in normal haemostasis, and the fact that specific investigations are very expensive, the question of which particular factors should be included in a detailed study of this nature becomes important. The APTT is dependent on factors of both the "intrinsic" and common pathways. If there is significant deficiency of coagulation factors of the common pathway alone, both PT and APTT would be expected to be prolonged [63]. Thus the isolated increase in APTT in dengue suggests deficiency of coagulation factors of the "intrinsic" pathway and all factors (XII, XI, IX and VIII) in this pathway should be included. Comparisons of the patterns seen for Factors VIII and XI (large molecules) with those of Factors IX and XII (small molecules) would be interesting. Factor VII, representing the "extrinsic" pathway would also be interesting to measure, as this may suggest why the PT is not prolonged. However a significant drawback to this kind of study relates to the complex nature of the coagulation cascade with multiple interactions and feedback loops, and the wide range of normal values for individual parameters.

Interpretation of results for individual factors is difficult, and statistical analysis is complex given the large number of tests performed and the possible interactions. Samples from very large numbers of patients, carefully collected to minimize activation of coagulation during venesection, would be needed to give reliable results.

The experiments with heparinase enzymes suggest that, although present at very high concentrations, the HS molecules do not have significant anticoagulant properties. However since we were unable to complete the detailed studies that were initially planned due to discontinuation of the ELISA kits, further work is needed before this question can be answered definitively. Although we could not measure HS levels in the patient samples used for the APTT reversal experiments, two of the samples used were obtained from DSS patients in the critical period and in our previous experience samples from these patients at this time generally show marked elevation of HS levels (Figure 4-3). If these samples did indeed have high HS levels, there are several possible explanations for the failure of an additional effect on the APTT following treatment with the heparinase enzymes. One possibility is that the HS molecules present in the dengue patient plasma are structurally different to the purified HS preparation used for the spiking experiments; HS exists in many forms and it is generally thought that a particular antithrombin binding motif must be present for HS to exert an anticoagulant effect. Alternatively, HS molecules carry a strong negative charge and likely interact with many other plasma constituents; in particular HS is known to interact with dengue NS1, a protein often present in large amounts during

the critical phase of dengue infections. If this interaction, or any other interactions with inflammatory or other molecules that may be present in acute dengue plasma, prevent the binding of the heparinase enzymes to the HS molecules, then it is possible that the enzymes might be ineffective.

We are currently engaged in additional studies to investigate these possibilities further. Working in collaboration with specialist biochemists at Harvard MIT we are attempting to extract and characterize the HS molecules from dengue plasma, aiming to see whether the molecules carry binding sites for antithrombin, as well as to identify the likely site of origin of the molecules. HS molecules derived from different sites carry different signatures and we will look particularly to see if the HS molecules carry the signature of endothelial HS, suggesting release from the endothelial surface glycocalyx layer. We are also hoping to develop a new HS ELISA to replace the Seikagaku system, using the same monoclonal antibodies. If this is successful we will then be in a position to look again at whether HS present in dengue patients' plasma is degraded by heparinase enzymes.

Understanding the mechanisms responsible for the typical dengue related coagulopathy is important as there may be clinical implications. Thus dengue-infected patients with severe bleeding should receive fresh frozen plasma promptly to replace the coagulation proteins lost through plasma leakage, and consideration should be given to treatment with cryoprecipitate. Although this study did not focus on leakage of fibrinogen there is evidence from other work that fibrinogen leaks at a similar rate

to much smaller proteins [128], possibly related to its elongated shape, and since severe patients invariably have very low plasma fibrinogen levels replacement therapy may be necessary for patients who bleed. Finally if true anticoagulant properties were to be demonstrated for the HS present in plasma, then consideration might be given to use of agents such as protamine sulfate for dengue patients with severe bleeding; protamine sulfate is a cationic drug that binds negatively charged heparin molecules and is sometimes used to reverse unwanted effects of excessive anticoagulation.

Chapter 5

Circulating endothelial cells in dengue infection

5.1 Introduction

CECs are thought to originate when endothelial cells detach from blood vessel walls following some form of pathological insult. Enumeration of CECs has been developed as a non-invasive technique to explore vascular endothelial dysfunction in various disease states – systemic lupus erythematosus and other inflammatory vasculitides, chronic kidney diseases, thalassaemia, sickle-cell anaemia, rickettsial infections, cancer etc. There is also growing evidence that CECs can be used as biomarkers of vascular disease [66,92,129]. Finally, as well as reflecting vascular damage it appears that CECs may themselves participate in the pathogenesis of some disorders, possibly by the expression of active molecules such as thrombomodulin or tissue factor on the surface of the cells [66].

Different techniques including *in vitro* culture, magnetic bead isolation and fluorescence-activated cell sorting (FACS) have been used to detect these cells, but increasingly FACS analysis has emerged as the preferred option [130,131]. To date there have been only two studies that measured CEC numbers in dengue infection but the results were limited. One small study in Venezuela found that numbers of CECs measured by FACS analysis were increased in four dengue patients compared to healthy subjects [132]. In the other study a group in Thailand measured serial CEC numbers, using the buffy coat smear technique, in 103 children with dengue infection and 8 children with OFI. They found that CEC numbers appeared to correlate with clinical disease severity at the time of defervescence and a few days after that [133].

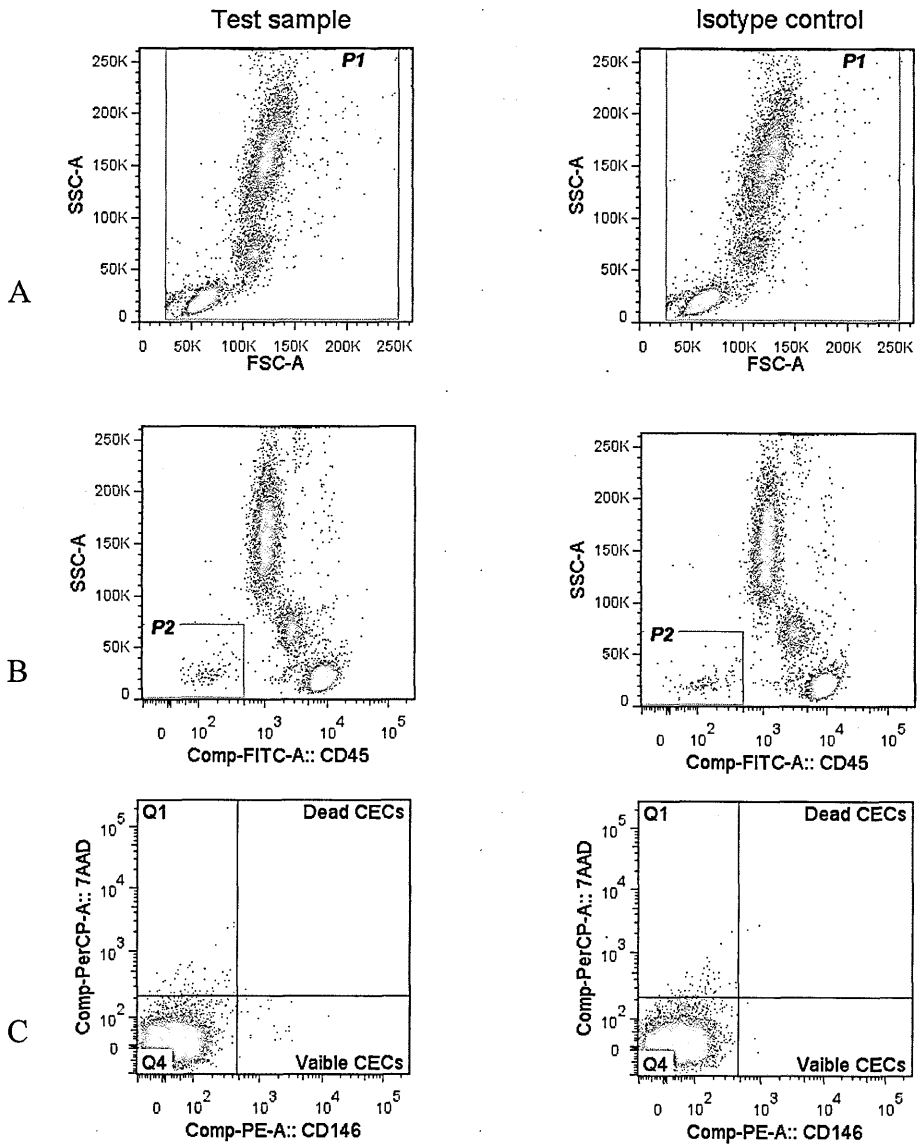
In this chapter I will describe a more detailed study performed to characterize the CEC profile as it evolves in dengue patients from the early febrile phase, and to examine whether there is an association between percentage of CECs detected and disease severity described independently in terms of vascular leakage and bleeding severity. In addition, I will describe the relationship between CEC percentages and concurrent plasma HS levels, used here as an alternative marker of endothelial dysfunction.

5.2 Methods

We chose to focus recruitment for this study on adolescents and young adults, aiming to capture patients within the age range most likely to experience severe vasculopathy/shock during dengue infections. We did not recruit children below 12 years of age as the study protocol required larger blood volumes than is usually considered acceptable for sick children in Vietnam. General clinical, laboratory and statistical methods are presented in Chapter 2. Additional specific methods are presented below.

CEC Enumeration: This was carried out according to the Standard Operating Procedure (SOP) kindly provided by Dr. Andrew Blann from the University Department of Medicine, City Hospital, Birmingham (UK). The technician who performed CEC enumeration for the samples in this study was blind to all clinical information.

We used a BD FACSCanto II flow cytometer and the following reagents from BD Biosciences (USA): FITC Mouse Anti-Human CD45 (555482); PE Mouse Anti-Human CD146 (550315); and 7AAD (559925). Briefly, two aliquots (0.4 ml venous blood per aliquot) were used as the test sample and an isotype control. The test samples were incubated with a mixture of the two fluorochrome-labeled monoclonal anti-human mouse antibodies and 7AAD, a chemical marker that differentiates between dead and live cells, for 30 minutes on ice in the dark. For the isotype controls, PE Mouse IgG_1 κ Isotype Control (555749) was used to replace PE-CD146. Isotype controls are a type of negative control designed to measure the level of non-specific background signal caused by the primary antibodies – the antibody is chosen to be similar to the test antibody but without the specific binding site of interest. After this staining step, test samples and isotype controls were treated similarly. Firstly, red blood cells in the sample were lysed twice using 3ml of pre-diluted BD lysing buffer (349202) for 10 minutes, and then the sample was centrifuged at 2000 rpm for 5 minutes to collect the white blood cells (WBC). The cells were washed with 3ml of phosphate buffered saline (PBS) solution and centrifuged to re-pellet the cells. Then 0.4ml of 2% paraformaldehyde was added to fix the cells before analysis by flow cytometry. Viable CECs were defined as $CD45^-/CD146^+/7AAD^-$ and dead CECs were defined as $CD45^-/CD146^+/7AAD^+$. Full strategy and representative flow cytometer plots are shown in Figure 5-1.



The figure shows the results of the test sample and isotype control from one sample to illustrate the typical findings.

Sequential gating strategy for CECs: (A) Forward (FSC) and side scatter (SSC) plot of all cell events and gating region P1 to include all mononuclear and polymorphonuclear cell events while excluding platelets, dead cells, and microparticles. (B) Dump channel to exclude $CD45^+$ cells and high side scatter events (region P2). (C) Cellular events from gated region P2, with viable CECs as $CD146^+/7AAD^-$ population and dead CECs as $CD146^+/7AAD^+$ population.

Figure 5-1: CEC enumeration by flow cytometry

As CECs are rare events, we followed the recommendations for enumerating rare events by flow cytometry, including thoroughly cleaning the cytometer before data acquisition and also collecting the largest numbers of all events possible (the maximum number was set up at 1.5 million cell events) [130]. In addition, using FlowJo software version 7.6.5 (Oregon, USA), we calculated the minimum number of total events in the blood mononuclear and polymorphonuclear cell population (P1) that should be acquired to ensure adequate reproducibility (aiming for a coefficient of variation (CV) less than 10%) of the results, based on the method described by Donnenberg at al [134]. Based on this initial assessment all samples with total cell events < 450,000 in P1 were excluded from the analysis (see section 5.3.2). CEC results can either be reported as a percentage of CEC events in the P1 population from the FACS analysis, or as an absolute count of CECs/mL. Although absolute counts of CECs relative to a total WBC measurement obtained from a simultaneous full blood count are commonly quoted, there are several reasons why this may not be optimal. Firstly these are rare events and therefore calculation of absolute numbers relative to a measurement obtained on a different blood sample is not ideal; comparison to a marker assessed in the same sample is preferable. Secondly the method assumes that the total WBC is equivalent to the WBC plus CECs which is not strictly speaking correct. As well as taking the two measurements on exactly the same aliquot of blood, since the P1 population represents all nucleated cellular elements of blood, including CECs, white blood cells and fibroblasts, assessing CECs as a percentage of P1 is considered more accurate. Therefore, in developing the analysis plan for this study we chose the percentage of total CECs (viable and dead cells) in P1 in the test samples

examined at intervals during the evolution of the illness as the primary outcome of interest, and the absolute count of total CECs as a secondary outcome.

Heparan Sulfate ELISAs: HS levels were subsequently measured in batches using residual EDTA plasma that was frozen and stored at -80^{0}C after the CEC enumeration. The levels were measured using commercial HS ELISA kits (Seikagaku, Japan) following the manufacturer's instructions. All other laboratory assays were as described in Chapter 2.

5.3 Results

5.3.1 General clinical and laboratory information of the cohort

Between December 2010 and June 2011, 95 patients with suspected dengue were recruited into the study, including 59 patients in the infection wards plus 36 patients in the ICUs. Dengue was confirmed in 73/95 (77%) patients while 15 (16%) patients were classified as OFI. In the remaining 7 patients (7%) the results of serological and virological diagnostics were inconclusive. A single infecting serotype was identified in 52/73 (71%) of the dengue patients – DENV 1 in 21/52 (40%), DENV 2 in 23 (44%), DENV 3 in 4 (8%) and DENV 4 in 4 patients (8%). Four patients had mixed infections. Among the 73 patients with confirmed dengue, only one patient was initially recruited on one of the infection wards and then transferred to the AICU - a 19 year old female with severe bleeding related to a ruptured ectopic pregnancy. All suspected dengue patients enrolled in the study recovered fully and 49 patients (52%) attended the follow-up visit.

We enrolled 13 patients with confirmed malaria, including 10 patients with non-severe malaria (6 plasmodium vivax, 3 plasmodium falciparum and 1 mixed vivax/falciparum infection) and 3 patients with severe malaria, all due to plasmodium falciparum. All malaria patients enrolled in the study recovered fully and 4/13 patients (31%) attended for follow-up visits. Twenty healthy young adults were also recruited, including 10 female and 10 male volunteers with a median (90% range) age of 23 (23 – 25) years.

Table 5-1 presents summary demographic, clinical and laboratory information for the patients with confirmed dengue, OFI and malaria. In this study, more female patients were enrolled than male patients in both dengue infection groups. However, malaria mainly affected male patients, probably as a result of occupational exposure. As expected, dengue patients had more skin and mucosal bleeding while the majority of patients with OFI and malaria had no bleeding. Other than shock, 4 of the dengue patients experienced severe complications (2 with severe bleeding, one with encephalopathy and one with haemophagocytosis). In the malaria group, 3 patients developed jaundice, 2 were encephalopathic and one had renal failure. No complications were seen in the OFI group.

Percentage haemoconcentration was significantly greater in dengue patients with shock than those without shock (Mann Whitney test, $p < 0.001$). In many malaria patients the percentage HCT change was negative, likely reflecting the effects of major haemolysis during the acute illness. We also found typical coagulation abnormalities among the dengue patients including thrombocytopenia, increased APTT and reduced plasma fibrinogen levels, with more severe derangements seen in

the patients with shock. In both dengue severity groups the PT was similar to the results for the OFI patients and the D-Dimer test was clearly positive in less than one third of the patients. Malaria patients also had quite marked thrombocytopenia, but there was no increase in APTT and the fibrinogen levels were increased rather than decreased, consistent with an acute phase response. Similar to Chapter 4, we found HS levels was markedly increased in the dengue patients compared to OFI and malaria patients, with significantly higher levels seen in dengue patients with shock compared to those without shock (Mann Whitney test, $p < 0.001$).

Table 5-1: Summary demographic, clinical and laboratory information for patients with confirmed dengue infection (presented according to vascular leakage severity) and those with other febrile illnesses and malaria

Characteristics	Confirmed dengue		Other febrile illnesses (n = 15)	Confirmed malaria (n = 13)
	No shock (n = 44)	Shock (n = 29)		
Age (years)	21 (14 – 25)	16 (12 – 25)	18 (14 – 24)	25 (21 – 33)
Male sex	17 (39)	13 (45)	7 (47)	12 (92)
Day of illness on enrolment	3 (2 – 6)	6 (4 – 6)	2 (2 – 3)	5 (2 – 7)
Bleeding severity:-				
No bleeding	21 (48)	1 (3)	13 (87)	11 (85)
Skin bleeding only	8 (18)	11 (38)	2 (13)	2 (15)
Mild mucosal bleeding	13 (30)	12 (41)	0	0
Severe bleeding	2 (5)	5 (17)	0	0
Percentage haematocrit change[§*]	8.2 (-1.7 – 20.3)	29.4 (11.6 – 49.8)	5.3 (-7.7 – 8.0)	-14.4 (-29.5 – -4.1)
Lowest platelet count ($\times 10^9$/L)[£*]	47 (12 – 153)	12 (6 – 35)	140 (46 – 241)	48 (4 – 134)
Highest PT value (seconds)[£*]	14 (11.9 – 20.5)	13.9 (12 – 21.7)	14.9 (11.7 – 24.4)	15.5 (12.9 – 17.9)
Highest APTT value (seconds)[£*]	38.4 (31.7 – 53.2)	43.1 (36.1 – 91)	36 (29.6 – 47.6)	36.3 (26.7 – 45.7)
Lowest fibrinogen levels (g/L)[£*]	3.7 (1.8 – 6.1)	2.8 (1.4 – 4.6)	4.5 (2.9 – 7.8)	5.3 (2.7 – 7.3)
D-Dimer $\geq$ 2 µg/mL (No and %)[£*]	8 (19)	8 (28)	2 (13)	5 (38)
Highest HS levels (µg/mL)[£**]	179 (15 – 588)	471 (238 – 1039)	29 (10 – 165)	64 (10 – 151)

[§] Because peak HCT values are markedly affected in patients with major bleeding, severe hemolysis or haemophagocytosis requiring blood transfusion so percentage HCT change was not calculated for those patients. In addition, percentage HCT change was not calculated in one patient with a history of chronic anemia for whom no follow-up value was available to act as the baseline. For this variable the denominators are 38, 24, 15 and 13 cases in the confirmed dengue without shock, confirmed dengue with shock, OFI and malaria groups, respectively.

[£] The most abnormal results recorded between days 3 and 8 of illness

[*] Missing data for one patient in the confirmed dengue group without shock

[**] Missing data for 4 patients in the confirmed dengue group without shock and 2 patients in the OFI group

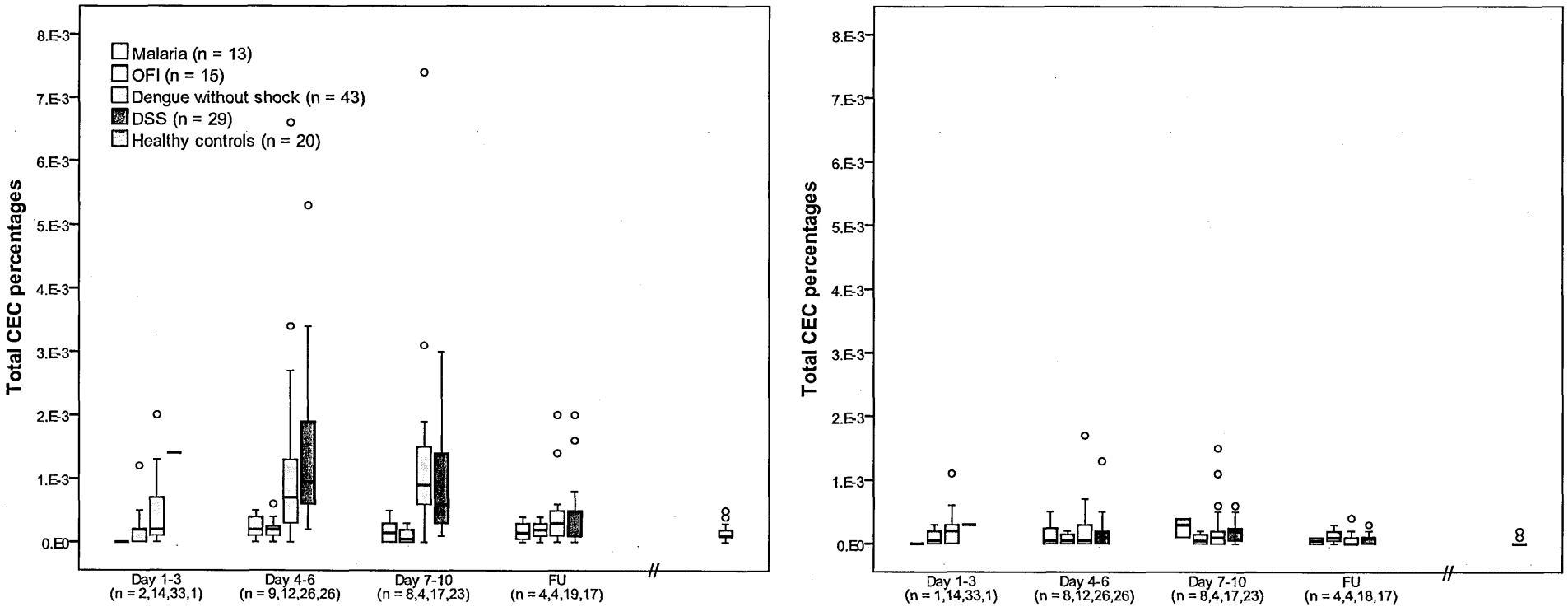
5.3.2 Optimization of the CEC analysis

We performed CEC enumeration for 283 samples in total. To determine the minimum number for total cell events in P1, we randomly chose three samples with a large number of total cell events (at least 1,300,000 events). Then we analyzed the variation in the percentages of CECs ($CD45^-/CD146^+$ population) identified in 9 different gating plots with increasing numbers of total cell events in P1 (7,000 increasing to 640,000 events). Each gate was repeated three times and the CV calculated; a CV of < 10% was achieved when approximately 450,000 cells per replicate were analyzed, a cut-off that is quite similar to the suggested number of total cell events indicated in a review article about CEC enumeration by flow cytometry [130]. We therefore included only the 259 samples (92%) which had a total event number in P1 $\geq$ 450,000 for the analysis. In addition one dengue patient on the AICU also had thalassemia, a condition known to be associated with very high CEC numbers [135], so we also excluded this patient from the analysis.

5.3.2.1 Comparison of CEC results in the test samples and isotype controls

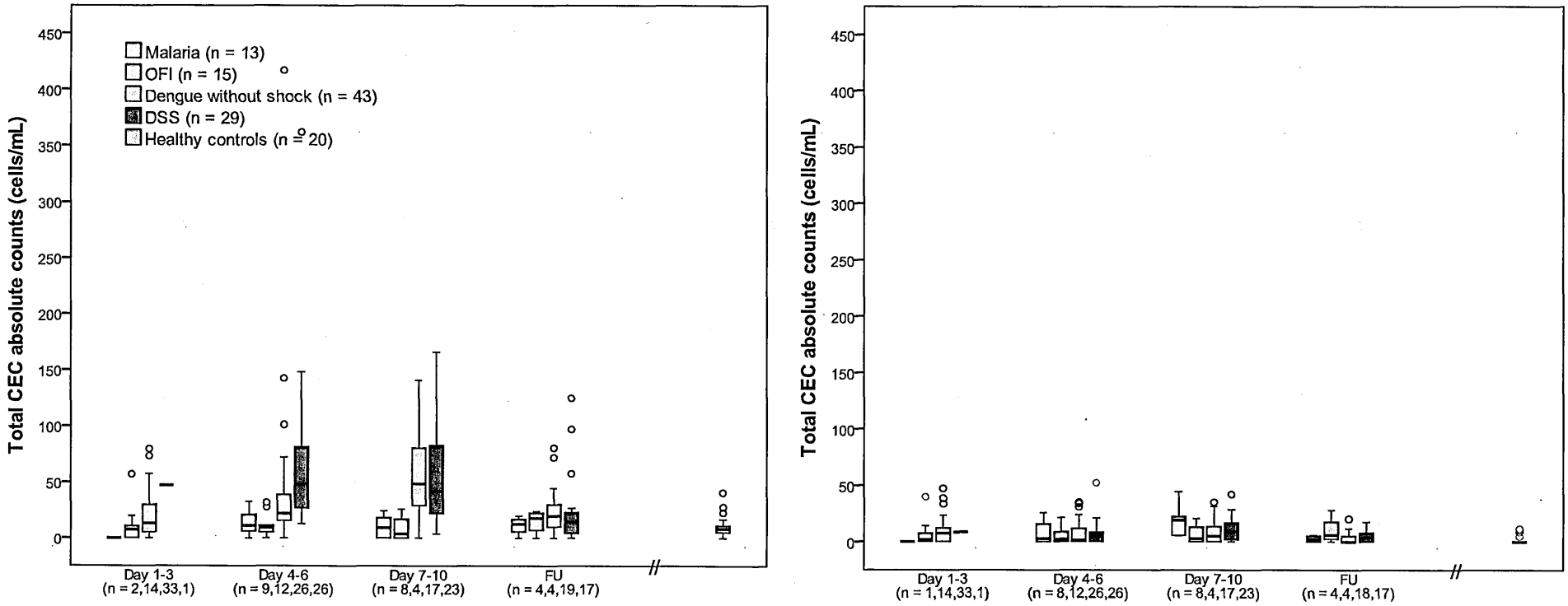
For all samples eventually included in the analysis, serial results for the total CEC percentage (of both viable and dead cells) in the P1 population in the test samples and the isotype controls are shown in Figure 5-2. Serial results for the total CEC absolute counts are shown in Figure 5-3. As the pattern and evolution of changes was very similar to the percentage CEC results we present statistical comparisons for the percentage CEC data only.

The findings confirm that CECs are very rare events. Despite this we were able to detect significant changes in the CEC results over time and in comparison with the different control groups. In the healthy volunteer group the median (90% range) for the total CEC percentage in P1 in the test samples was 0.0001 (0 – 0.0005)%, equivalent to a median (90% range) for the absolute CEC count of 8 (0 – 40) cells/mL. In the isotype control samples, CECs were undetectable in almost all the healthy volunteers although small numbers were found in the patient groups with dengue, OFI and malaria. This suggests that some non-specific cellular binding of the markers occurs during acute infections, when many inflammatory pathways are upregulated. In the OFI and malaria groups, the results for CECs detected in the test samples were similar to the results seen in the corresponding isotype control samples. By contrast the CEC results in both dengue patient groups were substantially higher in the test samples than the isotype controls.



- One dengue patient without shock who had thalassemia was not included.
- The total number of patients included in each group at each time period is indicated on the X axis.

Figure 5-2: Box and whisker plots showing changes in the percentage of total CECs (viable and dead cells) identified in the P1 population in the test samples (left panel) and in the corresponding isotype control samples (right panel) for patients with dengue (with and without shock), malaria, and OFI during the course of illness. Results for the healthy controls are also shown in the two panels.



- One dengue patient without shock who had thalassemia was not included.
- The total number of patients included in each group at each time period is indicated on the X axis.

Figure 5-3: Box and whisker plots showing changes in the absolute counts of total CECs (viable and dead cells) in the test samples (left panel) and in the corresponding isotype control samples (right panel) for patients with dengue (with and without shock), malaria, and OFI during the course of illness. Resnlts for the healthy controls are also shown in the two panels.

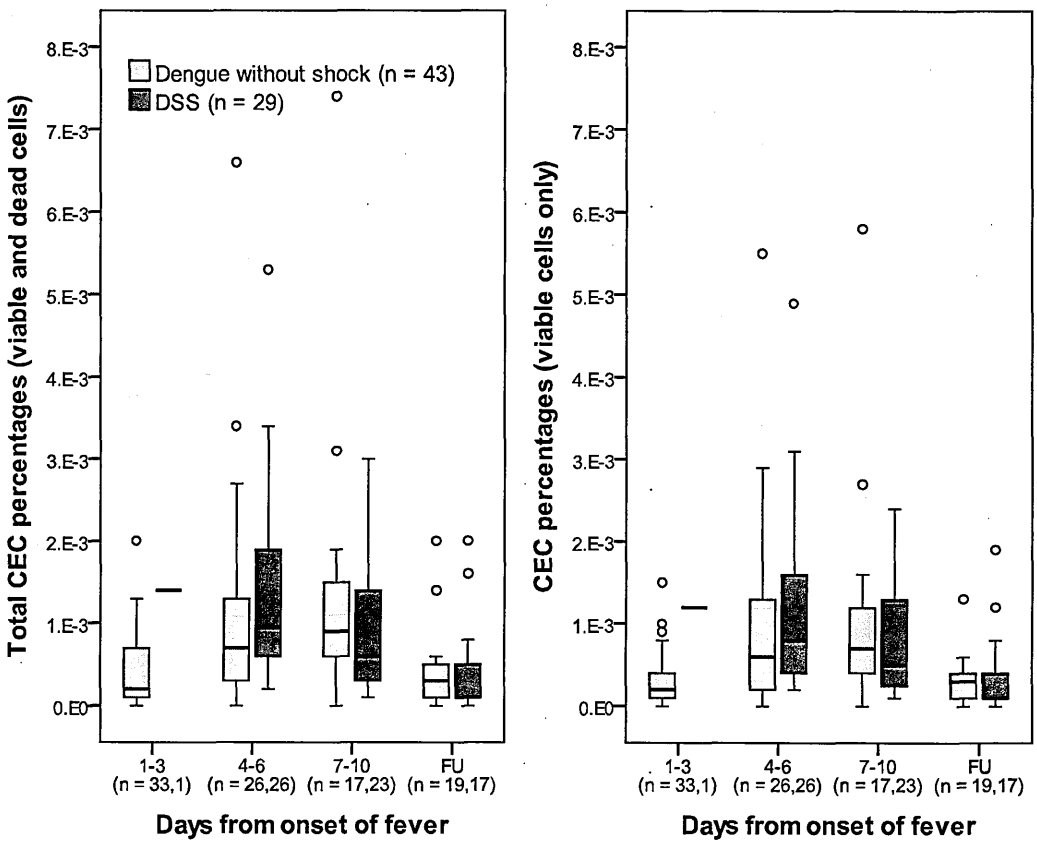
5.3.2.2 Evolving changes in CEC percentages in the dengue patients and the disease controls.

As shown in the left panel of Figure 5-2, there was no apparent change over time in the total CEC percentages in the test samples from the OFI and malaria patients. In the group of dengue patients without shock, the total CEC percentages in the early febrile period were similar to the values at follow-up (median (90% range) of 0.0002 (0 – 0.0015)% compared to 0.0003 (0 – 0.0014)%, p = 0.16), but increased significantly in the critical period (median (90% range) of 0.0007 (0.0001 – 0.0055)%, p = 0.02), and remained elevated during the convalescent period (median (90% range) of 0.0009 (0 – 0.004)%, p = 0.01), all comparisons being with the follow-up values in the same patient using the Wilcoxon signed-rank test. Only one patient with DSS was captured in the early febrile period (CECs = 0.014% of P1); otherwise this group showed a similar evolution in CEC percentages during the course of the illness to the dengue patients without shock, although with a more marked increase during the critical period. Thus the median (90% range) during the critical period was 0.001 (0.0002 – 0.0046)% and during the convalescent period was 0.0006 (0.0001 – 0.003)% compared to 0.0001 (0 – 0.0017)% at follow-up (both $p \leq 0.01$ for comparisons of critical and convalescent values with follow-up values, Wilcoxon signed-rank test).

In addition, during both the critical and convalescent periods the total CEC percentages detected in the dengue patients without shock were significantly higher

than those seen in the OFI or malaria patients ($p < 0.01$ for all comparisons, Mann Whitney test).

5.3.2.3 Correlations between CEC percentages with clinical and laboratory markers of disease severity in the dengue patients



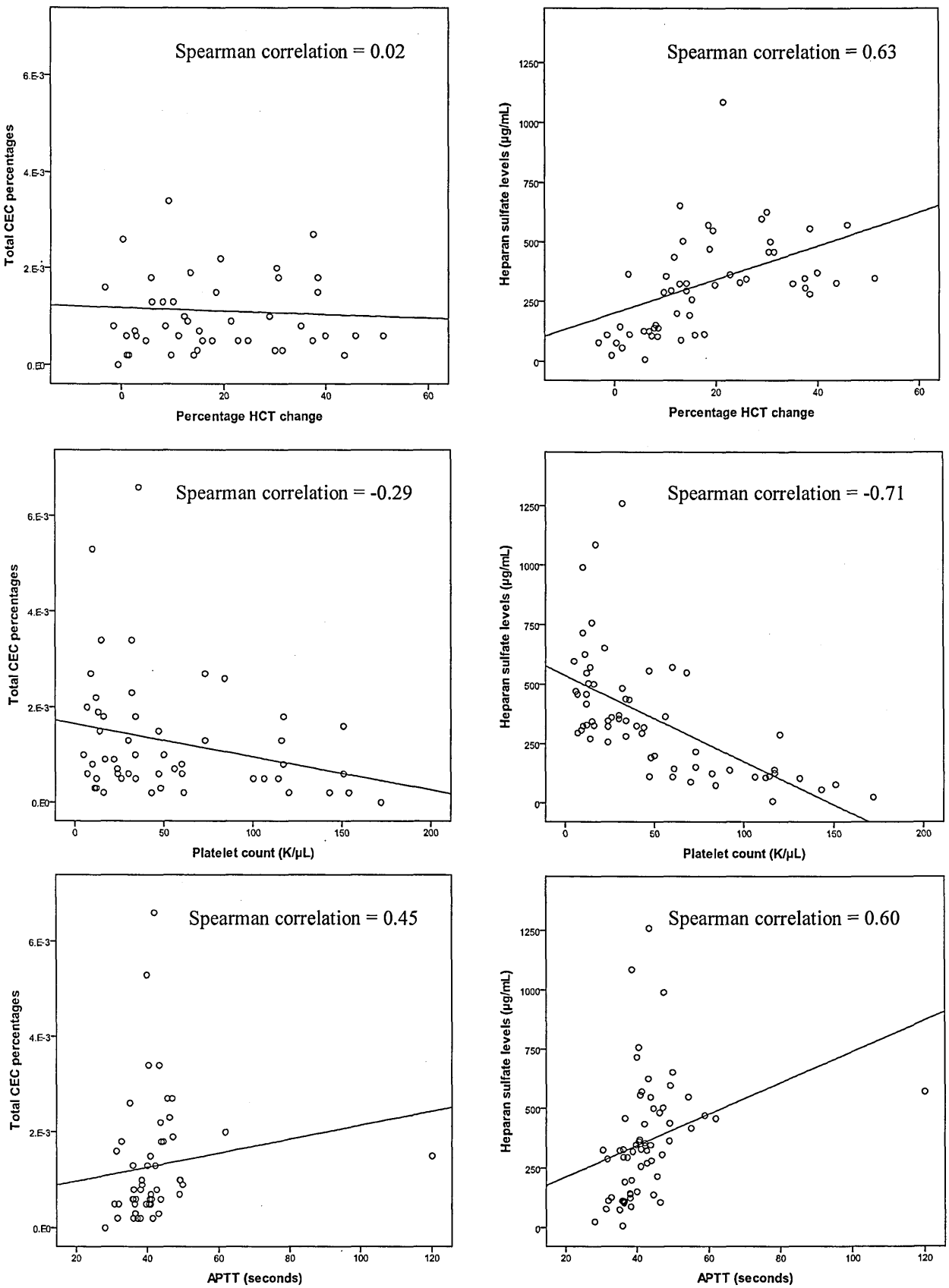
- One dengue patient without shock who had thalassemia was not included.
- The total number of patients included in each group each time period is indicated on the X axis.

Figure 5-4: Box and whisker plots showing changes in total CEC percentage (left panel) and in viable CEC percentage (right panel) in the test samples during the course of illness and at follow-up, in confirmed dengue patients with and without shock.

Figure 5-4 shows results for total CEC percentages (viable and dead cells) and for viable CEC percentages alone, in the test samples of the dengue patients, presented according to vascular leakage severity. The figure shows that viable CECs accounted for the majority of total CECs at all time-periods. Although apparently more elevated in the DSS cases during the critical period, neither total CEC percentages nor viable CEC percentages were statistically significantly different between the dengue patients with and without shock (all $p > 0.1$ in both the critical and convalescent periods, Mann Whitney test). In the four dengue patients with severe complications other than shock total CEC percentages were also markedly elevated, ranging from 0.001 - 0.0034% in the critical period.

In addition, both total CEC and viable CEC percentages were significantly correlated with bleeding severity in the critical period (Cuzick test for trend, both $p < 0.05$) but not in the convalescent period (data not shown). In terms of correlations between CEC percentages and other key laboratory results, during the critical period total CEC percentages demonstrated mild or moderate negative correlations with concurrent platelet counts and plasma fibrinogen levels (Spearman correlations of – 0.29 and – 0.39, both $p < 0.05$, $n = 52$ and 51, respectively) and moderate positive correlations with concurrent PT, APTT and HS levels (Spearman correlations of 0.34 to 0.45, all $p < 0.05$, $n = 51$, 51 and 49, respectively) but no correlation with overall percentage haemoconcentration (Spearman correlation of 0.02, $p = 0.88$, $n = 46$). However, HS levels demonstrated a strong positive correlation with overall percentage

haemoconcentration (Spearman correlation of 0.63, $p < 0.001$, $n = 54$) and stronger correlations with the other key laboratory values measure during the critical period. Thus HS levels demonstrated strong negative correlations with concurrent platelet counts and plasma fibrinogen levels (Spearman correlations of – 0.71 and – 0.55, both $p < 0.001$, $n = 63$ and 62, respectively), a moderate positive correlation with the concurrent PT (Spearman correlation of 0.42, $p = 0.001$, $n = 62$), and a strong positive correlation with concurrent APTT values (Spearman correlation of 0.60, $p < 0.001$, $n = 62$). Figure 5-5 shows the correlations between the total CEC percentage with percentage haemoconcentration, concurrent platelet count and APTT (as one example of the different coagulation tests performed) and indicates that for each parameter the correlations were stronger with plasma HS levels than with CEC percentages.



Note that overall percentage HCT change was not calculated for patients with severe bleeding. All individual lab values represented were taken during the critical period.

Figure 5-5: Scatter plots showing correlations between total CEC percentage (left panels) and plasma HS levels (right panels), with key lab values associated with dengue disease severity.

5.4 Discussion

This study has shown that CEC percentages clearly increase during the critical and convalescent periods of dengue infection and then return to the normal range within one month after the illness. Very similar patterns were observed using the absolute CEC count/mL as the outcome of interest. The pattern of a transient increase that follows the expected clinical evolution of the disease was not seen in the non-dengue disease controls, either in the OFI or the malaria groups. CEC percentages correlated significantly with the majority of clinical and laboratory markers of dengue disease severity, although no clear relationship was demonstrated with respect to vascular leakage severity assessed in terms of development of DSS and overall percentage haemoconcentration. We were unable to investigate whether increases in CECs during the early febrile period might be a useful predictor for the subsequent development of shock, because only one patient recruited during this time went on to develop DSS.

CECs are rare events in blood and enumeration is challenging. Debate continues as to the best method for measurement of these cells and a standard protocol using flow cytometry remains to be agreed upon [130,131]. Problems include concerns that CECs detected in this way might in fact be large platelets, activated lymphocytes or circulating progenitor cells (CPCs). CPCs are rare cells, derived from bone marrow, that circulate in the blood and can differentiate into endothelial cells, thus playing an important role in vascular repair. For this study we used the protocol from a research institution in Birmingham (UK) that has established a reputation in the field and

published several studies about CECs in cancer, vascular diseases etc. The Birmingham protocol uses CD146 and CD45, by which we can exclude platelets, which do not express CD146, and activated lymphocytes, which do express CD45. However, CEC events detected by this method could include some CPCs because they have the same CD45-/CD146+ profile as CECs and the protocol does not include specific markers for progenitor cells, such as CD133 or CD34. Some authors do suggest, however, that CD146 positive cells are all CD133 negative [129], but even so it is possible that the population of CECs we identified did include some CPCs. We were also careful to follow general guidelines for enumeration of rare events by FACS, thereby minimizing contamination and ensuring an adequate population of cells in P1. Finally in support of the technique we used, the results for CEC absolute counts in healthy volunteers in this study were low (median (90% range) of 8 (0 – 40) cells/mL), and in keeping with the ranges previously published for healthy persons by the Birmingham group using similar techniques [92,136]. Compared to results from other studies investigating endothelial involvement in conditions such as cardiovascular disease, cancer, vasculitis and septic shock, the magnitude of the increase in CECs in the dengue patients was similar or slightly higher than the findings in these diseases [66,129]. We believe that these results do reflect relative changes in the CEC population in these patients, but it should be emphasized that such rare event analysis is difficult and it is the evolution over time that is most convincing rather than changes in the CEC percentages measured. By contrast HS plasma levels are much more easily measured, requiring only standard ELISA

methodology, and varied between <10μg/mL to >1mg/mL in the dengue patients in this study. Thus clear differences between patient groups and over time are considerably easier to identify.

Previous studies showing increases in plasma levels of soluble cell adhesion molecules, and several endothelial related coagulation factors such as tissue plasminogen activator, thrombomodulin and von Willebrand factor [86,89,132,133], have confirmed that the vascular endothelium is affected during dengue infections, but our results provide direct evidence of damage sufficient to cause shedding of endothelial cells, albeit in small numbers. The majority of the CECs identified were viable cells and this suggests that the endothelial cells were recently shed during the course of illness.

The parallel increases in both CECs and HS levels support the theory that disruption of the endothelial cell/glycocalyx complex occurs during dengue infections - i.e. CECs appear to be shed from the endothelial layer while HS may be shed from the surface glycocalyx. These disruptions likely affect the function of the complex and could contribute to the pathogenesis of systemic vascular leak syndrome and haemostatic derangement. Although clearly elevated, we did not show strong correlations between CEC percentages and severity of vascular leakage assessed in terms of development of DSS or overall percentage haemoconcentration – however, HS levels correlated strongly with both these parameters. Accepting the caveat that CEC changes are more difficult to measure, these results could indicate that

disruption of the surface glycocalyx layer during dengue infections is more pronounced than the effects on the underlying endothelial layer.

CEC percentages correlated significantly with bleeding severity and laboratory markers of coagulation derangement, such as the platelet count, PT, APTT and plasma fibrinogen levels, and it may be that CECs themselves participate in the coagulopathy through expression of molecules such as tissue factor or thrombomodulin that are active in the procoagulant or anticoagulant pathways. In order to characterize whether the CECs express these active molecules further studies would be needed. For example CD 142 is an established marker for tissue factor expressions and it should be possible to design a protocol using this marker together with the markers used for CEC enumeration to identify whether the CECs express tissue factor.

In summary, serial enumeration of CECs among 73 patients with dengue, 15 with OFI and 13 with malaria showed that CECs were significantly increased in the dengue patients but not in the patients with OFI or malaria. CEC percentages correlated with several markers of disease severity, following a similar pattern during the course of the illness, but we did not demonstrate a clear association with development of shock. Other studies enrolling a larger number of patients early in the illness course, and including additional characterization of the CECs identified, could help to give more information on the role of CECs in dengue pathogenesis and also to answer the question whether early increases in CECs can be used as a prognostic factor for subsequent disease outcome.

Chapter 6

Conclusion

6.1 Contributions of the thesis

The work described in this thesis provides several important contributions to our knowledge about clinical dengue disease, as well as filling significant gaps in our understanding about the pathogenesis of haemorrhage associated with dengue. Firstly, the comprehensive clinical studies performed as part of this work have confirmed the empirical view that vascular leakage is more severe in children than adults and that DSS occurs more frequently in this age group, while haemorrhagic manifestations and organ impairment are more commonly seen in adults. Previous reports describing the complications seen have generally focused on either paediatric or adult populations, depending on the local epidemiological pattern of disease, but have rarely looked across the age spectrum in a particular geographical location. In this study we assessed the rate and severity of complications among direct admissions of all ages to a single hospital in a hyper-endemic region. Although broad-based recruitment at a community level would likely have provided a clearer picture of the patterns of disease by age, such a study would not be feasible in our setting and we are confident that the pattern of complications documented here is representative of true differences in response to dengue infection by age. Secondary infections clearly play a role in determining disease phenotype, but age-related intrinsic physiological responses are also important. Thus children, who have intrinsically higher microvascular permeability tend to develop vascular leakage and DSS, while adults, with intrinsically lower platelet numbers [107], and a greater likelihood of having

concomitant disorders that compromise mucosal integrity such as peptic ulcers [108], tend to experience bleeding complications.

The clinical studies also revealed that liver involvement, demonstrated by increased transaminase levels, occurs in almost all adults with dengue but is rarely associated with severe complications. PT values were normal in the majority of patients (except for the rare cases with acute liver failure) suggesting that generally the liver involvement does not contribute to the haemostatic derangements that are a frequent feature of infection.

The finding that thrombocytopenia was not only more severe in adults during the evolution of dengue disease, but that platelet counts were also significantly lower in adults than children after recovery, is novel. Only one published study has examined platelet counts by age in a healthy population [107], with similar results to our findings. Our results indicate that the more pronounced thrombocytopenia seen in adults with dengue likely reflects similar disease-associated effects superimposed on intrinsically lower normal platelet values in adults compared to children. Work by other authors has shown that in patients with dengue the residual platelets are also dysfunctional [74], and there has been some recent evidence that the dengue virus may actually replicate within platelets [83].

The pattern of coagulopathy seen in adults included mild to moderate prolongation of the APTT with reduction of plasma fibrinogen levels but only marginal prolongation of the PT and generally negative D-Dimers, very similar to the findings previously

published on paediatric dengue [86,90]. This pattern would be unusual for DIC and the various experiments described here suggest that the increase in APTT is likely to be due in large part to deficiency of coagulation factors, most likely due to leakage. Antithrombin, a coagulation factor with a molecular weight of 58,000 Daltons (similar to that of albumin) and carrying a negative charge was shown to be markedly increased in urine from children with DSS [57], and it is likely that many other coagulation proteins with similar size and charge characteristics also leak. Although HS levels were markedly increased in the dengue patients, and the molecule is closely related to the synthetic anticoagulant heparin, we were unable to demonstrate anticoagulant activity in the dengue plasma; however work is ongoing to try to identify whether the HS carries the specific antithrombin binding sites thought to be necessary for anticoagulant function. Lastly, we examined changes in the percentage of CECs present in plasma at different stages during the evolution of dengue infection. CEC percentages were clearly increased in the dengue patients but not in those with OFI or malaria, and the CEC percentages correlated significantly with bleeding severity and the majority of the screening coagulation tests.

In summary, the pathogenesis of the haemostatic derangement associated with dengue infections likely reflects a combination of several mechanisms including a) leakage of essential coagulation proteins as part of the general systemic vascular leakage process resulting in an imbalance of the coagulation system tipped towards anticoagulation and b) thrombocytopenia with dysfunction of the remaining platelets. These factors are together potentially exacerbated by high plasma levels of HS (that may exert

intrinsic anticoagulant activity), and/or the presence of CECs (that may carry molecules that are important for regulation of the different coagulation pathways). In rare cases with acute liver failure, the haemostatic problems may be compounded by impaired coagulation factor synthesis in the liver. However, in the majority of dengue infections bleeding is very mild (skin petechiaie or bruising only) and thrombocytopenia is likely to be the dominant factor responsible. With increasing severity of disease the coagulopathy worsens and if tissue integrity is breached for any reason significant bleeding may result. Adults are much more likely to have underlying peptic ulcer disease or to experience gastritis with their dengue infection and this probably contributes to the greater risk for gastrointestinal bleeding in this group.

From a clinical perspective this thesis makes some important contributions towards improving patient care. Firstly, knowledge of the patterns of disease and likely complications in different age-groups is helpful to facilitate development of suitable management guidelines that are relevant to the age-groups at risk. For example, considerable efforts have been put into the development of fluid management guidelines for children while as yet there is little advice on how to manage adults, at least in part because until recently fluid resuscitation was not viewed as a clinical problem relevant to this patient group. In general adults need smaller volumes of intravenous fluid per kilogram body weight compared to children, and it is not appropriate to simply extrapolate from the current paediatric guidelines. Thus although DSS occurs less frequently in adults than children, as the overall number of

adult cases is increasing globally, more attention will need to be addressed to developing appropriate guidelines. Secondly, in the small number of adults with severe shock or bleeding, liver failure etc. there may be a requirement for invasive procedures such as insertion of central lines or chest drains. Extra care must be taken with these patients since they usually also have profound thrombocytopenia and a serious coagulopathy; only very experienced staff in specialized centres should perform these procedures and serious consideration should be given as to whether the procedure is really necessary. Use of prophylactic platelet transfusions, fresh frozen plasma, cryoprecipitate etc should all be considered and an effective blood transfusion service is essential for management of severe dengue cases. These situations are very rare however, and it is important to remember that in the great majority of adults with dengue, even those with profound thrombocytopenia and significant coagulopathy, serious bleeding does not occur and watchful waiting is all that is required since the abnormalities are transient and generally improve spontaneously. The question of intervention with platelet transfusions is discussed below.

6.2 Limitations of the thesis

The clinical studies described were all hospital based and thus care must be taken when extrapolating the findings to a population level. Although we do not think we missed many patients from our hospital catchment area, it is possible that some were seen at other local centres. Secondly although patients were seen daily, for the analysis bleeding severity was described in terms of an overall score for each patient,

including events that occurred prior to hospitalisation. Thus we were not able to examine relationships between development/deterioration of bleeding and concurrent specific haemostatic abnormalities. Also we did not have comparable data on the coagulopathy from the paediatric patient cohort – the patterns observed in the adult cohort were very similar to our previous findings in children, but we were not able to make direct comparisons to assess age-related differences in the magnitude of the abnormalities detected. Finally for this work we concentrated on investigating potential mechanisms responsible for the prolonged APTT in dengue patients. Understanding the mechanisms responsible for the other typical haemostatic derangements, such as thrombocytopenia and low plasma fibrinogen levels, is equally important and we are currently developing these areas of research.

6.3 Further studies

To investigate further the observed APTT changes we have developed a collaboration with Professor Ram Sasisekharan's group at MIT, aiming to extract and characterize the HS present in dengue plasma using a variety of techniques. In particular we wish to establish whether these molecules contain binding sites for antithrombin and whether the surface glycocalyx layer is the likely origin of the HS molecules. In this way we hope to answer not only the question of whether the HS molecules contribute to the coagulopathy by functioning as anticoagulants but also to give more insight into the pathogenesis of vascular leakage. Secondly up to now no prognostic markers for the likely development of vascular leakage have been identified. However plasma HS

levels proved to be easy to measure by ELISA, and we have shown good correlations with clinical disease severity during the critical and convalescent periods. A study to determine whether early increases in HS levels predict the subsequent development of severe vascular leakage or DSS would be very useful, but would likely require very large numbers of participants to be enrolled, given the overall rate of progression to DSS in our setting is around 5%. Also at present, with the withdrawal of Seikagaku's ELISA kit after the Japanese tsunami, a simple and reliable method to measure HS is no longer available. We are working with the MIT group to try to develop a new ELISA, using the same monoclonal antibodies as are in the Seikagaku kit, and will then consider further studies to look at HS levels as a prognostic factor.

As discussed in Chapter 4, a detailed study measuring serial plasma concentrations and activities of coagulation factors of the "intrinsic" and "extrinsic" pathways, as well as urinary excretion of these factors throughout the course of illness, could be helpful in investigating the mechanisms responsible for the prolonged APTT with a relatively normal PT. An alternative method to look at possible leakage of coagulation factors would be to examine the microvasculature directly, through examination of skin biopsies. Therefore we have established a study to obtain small skin biopsies (punch or shave) from patients within 24 hours of admission with DSS, provided the subject is clinically stable. Immunohistochemistry and EM studies of these biopsies, together with similar biopsies from healthy volunteers, are ongoing but the preliminary findings do support the theory that coagulation proteins leak to the interstitial space. Fibrinogen is of particular interest as plasma levels can be extremely

low in children with DSS, but without good evidence for consumption/DIC. Although it is a relatively large molecule (molecular weight = 340,000 Daltons), animal studies indicate that fibrinogen passes though the endothelial surface glycocalyx at a similar rate to albumin, possibly related to its elongated shape [128]. We are currently carrying out a specific study measuring serial plasma fibrinogen levels and urine fibrinogen to creatinine ratios, together with immunohistochemistry for fibrin and fibrinogen on skin biopsy samples from the same patients obtained at the peak of plasma leakage. This may help to confirm whether leakage of fibrinogen is the primary mechanism for the low plasma fibrinogen levels observed.

Finally dengue patients, particularly adults, often present to medical attention during the critical period with significant thrombocytopenia but without clinical evidence of bleeding. The management strategy for such patients is not well established; in some countries prophylactic platelet transfusion has become established as the standard of care although there is no evidence that such transfusions influence outcome [110]. At HTD, we very rarely transfuse platelets to these patients, and in almost all cases the platelet count rises promptly during the second week of illness without development of any bleeding, other than minor skin bleeding at venepuncture sites. Before use of platelet transfusions for patients with dengue becomes widespread across endemic countries it is important that formal research studies are conducted to look at potential benefits, associated clinical risks and the cost implications of such transfusions.

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Publications

Publications arising from this thesis:

1. **Trung DT**, Wills B. Systemic vascular leakage associated with dengue infections - the clinical perspective. Curr Top Microbiol Immunol 2010;338:57-66.

2. **Trung DT**, Thao le TT, Hien TT, Hung NT, Vinh NN, Hien PT, Chinh NT, Simmons C, Wills B. Liver involvement associated with dengue infection in adults in Vietnam. Am J Trop Med Hyg 2010;83 (4):774-80.

3. **Dinh The T**, Le Thi Thu T, Nguyen Minh D, Tran Van N, Tran Tinh H, Nguyen VanVinh C, Wolbers M, Dong Thi Hoai T, Farrar J, Simmons C, Wills B. Clinical features of dengue in a large vietnamese cohort: intrinsically lower platelet counts and greater risk for bleeding in adults than children. PLoS Negl Trop Dis. 2012 Jun;6(6):e1679.

Publications arising from this thesis currently in preparation:

1. **Dinh The Trung**, Huynh Thi Le Duyen, Nguyen Than Ha Quyen, Tran Tinh Hien, Jeremy Farrar, Cameron Simmons, Bridget Wills. Circulating endothelial cells in dengue infection. (Manuscript in preparation)

Other related publications:

1. Hang VT, Nguyet NM, **Trung DT**, Tricou V, Yoksan S, Dung NM, Van Ngoc T, Hien TT, Farrar J, Wills B, Simmons CP. Diagnostic Accuracy of NS1 ELISA and Lateral Flow Rapid Tests for Dengue Sensitivity, Specificity and Relationship to Viraemia and Antibody Responses. PLoS Negl Trop Dis 2009;3 (1):e360.

2. Wills B, Tran VN, Nguyen TH, Truong TT, Tran TN, Nguyen MD, Tran VD, Nguyen VV, **Dinh TT**, Farrar J. Hemostatic changes in Vietnamese children with mild dengue correlate with the severity of vascular leakage rather than bleeding. Am J Trop Med Hyg 2009;81 (4):638-44.

3. Nguyen-Pouplin J, Pouplin T, Van TP, **Dinh The T**, Thi DN, Farrar J, Tinh HT, Wills B. Dextran fractional clearance studies in acute dengue infection. PLoS Negl Trop Dis 2011;5 (8):e1282.