Factors that influence the severity of Dengue infection

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PhD thesis

Declaration of originality

I declare that this thesis was entirely written on my own. The paper 'Complement alternative pathway genetic variation and Dengue infection in the Thai population' published in Clin. Exp. Immunol. 2013 Nov; 174(2):326-34 is part of my thesis and all other referenced works have been appropriately cited and given in the list of sources, to the best of my knowledge.

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Abstract

Dengue disease is a mosquito-borne condition that has become a major public health concern. Dengue severity can be classified into mild Dengue fever (DF) and severe Dengue or Dengue hemorrhagic fever (DHF). Genetic polymorphisms and variations in the host immune response are believed to contribute to different degrees to the disease progression. In this study, factors that have an influence on Dengue infection were identified. Genetic variants in the complement system, of which the degree of activation increases with Dengue severity, were identified. There was no association between the disease and complement genetic variants (complement factor H [CFH] rs3753394, CFH rs1061170, CFH rs800292, complement factor B (FB) rs12614/rs641153 and complement C3 rs2230199) in Thai cohorts. By contrast, with multiplex bead immunoassay, numerous biological markers measured in the plasma of Dengue patients were shown to increase the risk of Dengue severity. Up-regulation of plasma matrix metalloproteinase 3 (MMP-3) was shown to be associated with the risk of developing DHF even though recombinant MMP-3 (rMMP-3) did not affect endothelial permeability *in vitro*. Finally, an early predictive model to predict DHF was examined by using binary logistic regression. Low platelet cell count and high plasma levels of Monocyte chemotactic protein 2 (MCP2) were identified as early indicators. Sensitivity, specificity, and accuracy of the test were 73.17%, 83.13% and 82%, respectively. These data might be useful in the development of therapeutic agents and predictive markers for Dengue infection.

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Abbreviations

ADE	Antibody Dependent Enhancement
AST	Aminotransaminases
ALT	Alanine aminotransaminases
APS	Ammonium Persulfate
AUC	Area under ROC curve
BCA1	B Cell Attractant Chemokine1
BSA	Bovine Serum Albumin
°C	Celcius degree
CART	Classification and Regression Tree
CFH	Complement Factor H
CFHR	Complement Factor H-Related protein
CMC	Carboxymethylcellulos
CTACK	Cutaneous T cell Attracting Chemokine
DAB	3, 3′-Diaminobenzidine tetrahydrochloride
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (CD209)
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
DV	Dengue virus
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGM	Endothelial cell Growh media
ENA78	Epithelial Neutrophil Activating Peptide 78
ELISA	Enzyme Link Immunosorbent Assay
FACS	Fluorescence-activated cell sorting,

Abbreviations (continued)

FB	Factor B
FBS	Fetal bovine Serum
FGF	Fibroblast Growth Factor
fHbp	Factor H binding protein
ffu	Foci Forming Unit
FITC	Fluorescein isothiocyanate
g	Gravity force
GCSF	Granulocyte Colony-Stimulating Factor
GCP2	Granuloctye Chemotactic Protein 2
GMCSF	Granulocyte/Macrophage Colony-Stimulating Factor
gp130	Glycoprotein 130
HBSS	Hank's Balanced Salt Solution
HRP	Horseradish Peroxidase
Hct	Hematocrit
HGF	Hepatocyte Growth Factor
HUVEC	Human Umbilical Vein Endothelial Cell
IFN	Interferon
IL	Interleukin
IP10	Interferon gamma-induced protein 10
ITAC	Interferon-inducible T-cell Alpha Chemoattractant
JEV	Japan Encephalitis Virus
lft_protein	Liver function protein
lft_albumin	Liver function albumin
logit	Log odds of the probability
M	Molar
MCP	Monocyte Chemoattractant protein
MCSF	Macrophage Colony-Stimulating Factor
MEM	Minimal Essential Media
MIG	Monokine Induced by Gamma interferon
MIP	Macrophage Inflammatory Protein
MLPA	Multiplex Ligation-dependent Probe Amplification assay

Abbreviations (continued)

ml	Milli-Litre
µl	Micro-Litre
MMP	Matrix Metalloproteinase
MOI	Multiplicity of Infection
n	Number
nm	Nanometre
NS1	Non-structural protein-1
OFIs	Other Febrile illnesses
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered saline
PBS-T	1X Phosphate Buffered Saline Tween-20
PE	Phycoerythrin
PMA	phorbol 12-myristate 13-acetate
PMN	Polymorphic Mononuclear Cells
PMSF	Phenylmethanesulfonyl Fluoride
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RFU	Relative fluorescence unit
rGM-CSF	recombinant Granulocyte-Macrophage Colony-Stimulating Factor
ROC curve	Receiver operating characteristic curve
rpm	revolutions per minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDF	Stromal Cell-Derived Factor
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
SNP	Single Nucleotide Polymorphism
sVEGFR	soluble Vascular Endothelial Growth Factor Receptor
TARC	Thymus and Activation Regulated Chemokine
TEMED	Tetramethylethylenediamine
TNF	Tumor Necrosis Factor
TLR	Toll-like receptor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TPB	Tryptose-Phosphate-Broth

Abbreviations (continued)

TPO	Thrombopoietin
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell
WNV	West Nile Virus
WHO	World Health Organization

Chapter 1

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1.1 Dengue disease

1.1.1 History

Dengue disease was first identified during World War II, but Dengue-like disease had been previously described in the ancient Eastern hemisphere. The well-described Dengue symptoms were reported in the Philadelphia outbreak of 1780 (Halstead, 2008). Dengue disease has been documented in all continents except Antarctica. It is mostly found in tropical and subtropical areas, but rarely reported in Africa (Halstead, 1980). It is possible that black Africans are genetically resistant and rarely express severe disease, even though a high prevalence of antibodies has been found (Halstead et al., 2001).

In the 1900s, the first vector, *Aedes aegypti*, was identified. *Aedes agypti* and *Aedes albopictus* are urban mosquitoes carrying Dengue viruses that readily enter into the urban cycle and transmit virus to humans (Simmons et al., 2012). These mosquitoes poorly transmit sylvatic viruses, suggesting that zoonotic viruses do not readily enter into the human community (Moncayo AC, 2004). By contrast, wild mosquitoes (e.g. *Aedes leutocephalus* and *Aedes furcifer*) probably carry urban virus (Diallo et al., 2005). As yet, urban virus has not been isolated from infected sub-primates in Africa (Halstead, 2008).

Dengue virus was isolated in a laboratory in 1943-1944 (Gubler, 2002). Inoculation of virus known to be harmful to humans does not produce infection in several animals, including chickens, guinea pigs, rabbits, hamsters, rats, but does infect primates (Halstead, 2008). Infection of primates may be asymptomatic but with viremia occurring at levels sufficient to infect mosquitoes.

1.1.2 Infectious agents

Classification:

Dengue virus belongs to the *Flaviviridae* family within the Flavivirus genus (Flavi= yellow). Serological relationships between flaviviruses have been determined using antibodies associated with hemagglutination-inhibition (HAI) and neutralization (WHO, 1997). Complement fixation activity has also been associated with the viral NS1 protein (Muller and Young, 2013). Dengue serogroups are composed of 4 serotypes (Den1-4) and nucleotide sequencing has revealed that NS3 and NS5 proteins are highly conserved between Dengue viruses, suggesting a genetic relationship (Perera and Kuhn, 2008).

Virion structure and morphology:

The Dengue virion is composed of three structural proteins: the smallest protein, capsid (C), which covers the viral genome, the envelope (E) protein and the membrane (M) protein (Perera and Kuhn, 2008). Two types of particles are recognized as 1) mature extracellular virion with M protein and 2) immature intracellular virion containing precursor M (prM), which is proteolytically cleaved during the process of maturation to yield M protein. However, partially mature virions have also been detected in the extracellular environment.

Virus particles are spherical with a lipid envelope derived from the host cell membrane. Carbohydrate, which comprises about 10% of the virion, presents as glycolipids and glycoproteins. The virus particle is about 50 nM in diameter in the mature particle and 60 nm in the immature form, according to the different arrangement of PrM/E heterodimers (Perera and Kuhn, 2008). In the immature virion, E and prM proteins form 60 trimeric spikes protruding outward from the virion surface. During the maturation process, Pr is cleaved from prM under acidic conditions (pH 5.8-6.0) in the trans-golgi network, generating the smooth mature particle with 90 homodimers of E proteins (Figure1.1) ((Perera and Kuhn, 2008).

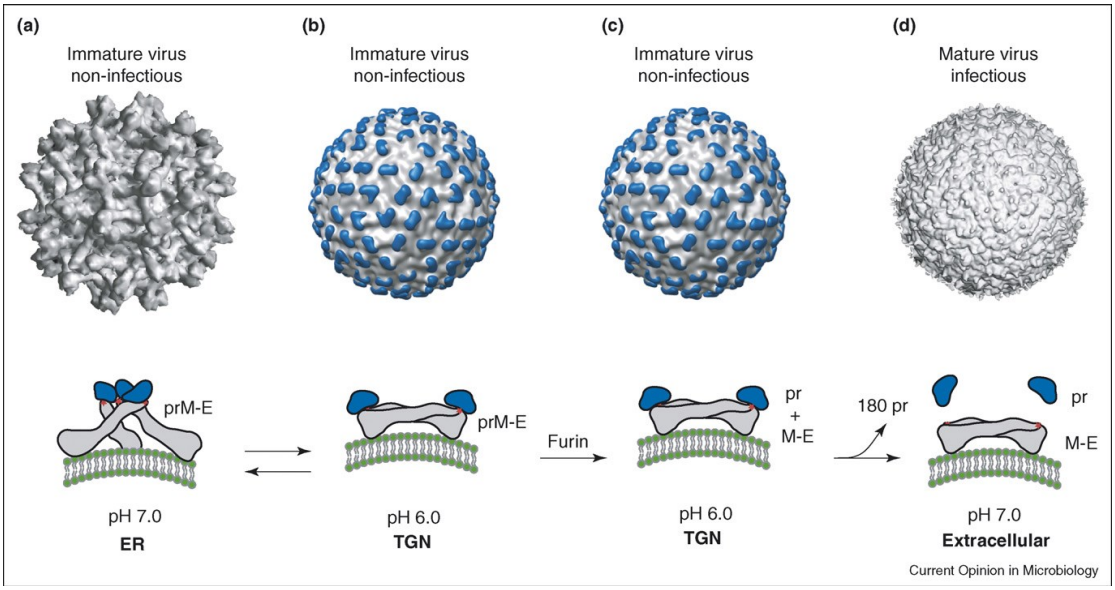

Figure 1.1: Structure of Dengue virus (Perera and Kuhn, 2008): a) A 60 prM-E trimeric form of the immature Dengue virus generates a spiky virion in the endoplasmic reticulum (ER) at neutral pH. B) The prM-E is dissociated and changed to 90 dimers that lie flat under acidic conditions in the trans-golgi network (TGN). This conformational change can be reversible upon raising the pH. C) The pr protein is cleaved by the host serine protease Furin, in the TGN and forms a 'cap' on the homodimers. D) The mature virion is secreted into the extracellular environment, releasing pr proteins from the surface.

The genome is single-stranded, positive-sense RNA about 11 kbp, including a 5'type I cap (m7 Gppp), and a 3' terminus lacking a poly-A tail (Halstead, 2008). It contains a single open reading frame encoding 10 proteins: 3 structural proteins (C, prM and E) and 7 non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (Figure 1.2). This single polyprotein is subsequently cleaved by host and viral enzymes.

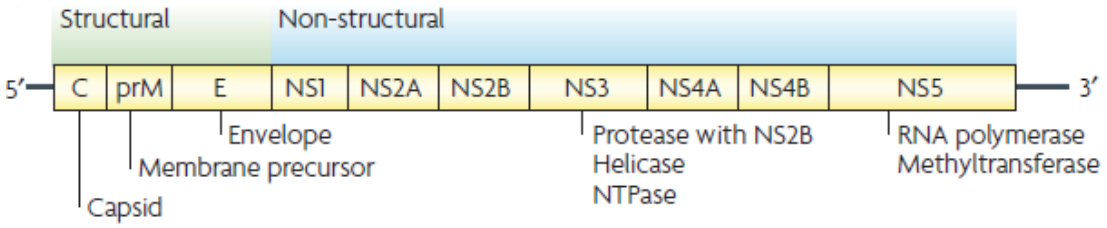

Figure 1.2: Dengue viral genome (Whitehead et al., 2007)

Proteins encoded by the virus:

Structural proteins

Capsid protein (C) is about 113 amino acids (aa) with two highly basic domains at the N and C termini. It is found in the nucleus, nucleoli and cytoplasm of infected cells. Capsid is important in assembly of the virion and it also interacts with ribonucleoprotein suggesting a role in viral replication (Halstead, 2008).

Matrix protein (M) has 2 forms depending on the maturity of the virus: immature PrM, and mature M protein. Co-synthesis of prM and E proteins is essential for protein folding, assembly of E protein and fusion of the mature virion (Halstead, 2008). Immature virus has been reported to be more acid resistant than the mature form, which is important for transporting the virion to the cell surface. PrM contains 6 cysteine residues that form disulfide bridges. During virus release, prM is cleaved by Furin within the trans-Golgi network, generating M protein (Perera and Kuhn, 2008). However, some immature virions, containing prM/E heterodimer have been found in the extracellular environment.

Envelope protein (E) (495aa) is important in virus attachment to cells and fusion to the membrane. It is also a major target for neutralizing antibodies (Whitehead et al., 2007). By crystallization, 3 domains of the E protein have been identified: domains I, II, and III (Rothman, 2011).

Non-structural proteins

NS1 protein is a glycosylated protein that forms dimers in the endoplasmic reticulum (ER). At the cell membrane, NS1 is still found in its dimeric form; however in the extracellular environment, NS1 (soluble NS1; sNS1) appears to be a hexameric form of three dimeric subunits (Muller and Young, 2013). Circulating sNS1 can bind to the surface of both infected and uninfected cells via interaction with glycosaminoglycans (GAGs) (Muller and Young, 2013, Avirutnan et al., 2007). The sNS1 is able to activate complement directly or by binding to anti-NS1 antibodies (Avirutnan et al., 2006). Viewed by immunolocalization, intracellular NS1 is found with a double stranded replicative form, suggesting an involvement in viral replication during the early stage of infection (Muller and Young, 2013).

NS3 protein is a viral multifunctional protease, whose function involves cleavage of the viral polyprotein as well as playing a role in viral replication (Perera and Kuhn, 2008). NS3 does not function individually, but requires the presence of NS2B for efficient activity (Perera and Kuhn, 2008).

NS5 protein is the largest and the most conserved sequence between Dengue viruses (67% sequence identity) (Perera and Kuhn, 2008). It has two activities; the C terminus encodes the viral RNA-dependent RNA polymerase (RdRp), while the N terminus contains a methyltransferase motif, suggesting a role in viral replication (Perera and Kuhn, 2008).

Other NS proteins (NS2A, NS2B, NS4a, and NS4b) have been identified in close association with other molecules to form a viral replicating complex, playing a role in viral replication.

1.1.3 Replication strategy

Dengue virus attaches to receptors on the surface of target cells via receptor-mediated endocytosis and the virus fuses with a vesicle membrane in a pH-dependent manner, followed by uncoating of the viral genomic complex. Several cellular receptors specific to viral binding have been identified. The presence of

glycosaminoglycans (GAGs), especially heparan sulfate, is important for viral binding *in vitro* (Germi et al., 2002). However, this attachment is just an initial step since other receptors are required for binding and entry. For example, heat-shock protein 70 (HSP70), and HSP90 are potential receptors on monocytes and macrophages (Reyes-Del Valle et al., 2005). An important receptor on immature dendritic cells is DC-SIGN (Dendritic Cell-Specific Intracellular adhesion molecule-3-Grabbing Non-integrin; CD209), which is able to bind high mannose oligosaccharides found on the envelope protein of Dengue virus (Tassaneetrithep et al., 2003). In addition, virion-antibody complexes can bind to the Fc receptor or complement receptor on the surface of some immune cells, such as monocytes and macrophages (Cardosa et al., 1983, Nielsen, 2009). This antibody-mediated infection is specifically called "antibody dependent enhancement" (ADE) and it is believed to contribute to the development of severe Dengue hemorrhagic fever (DHF) (Halstead, 2003). After uncoating, viral RNA is translated into a single polyprotein, which is then cleaved by proteases to produce structural and non-structural proteins. With the host machinery and the viral proteins, replication occurs. Assembly of the virus takes place at the intracellular membrane. Capsid protein associates with the viral genome at the ER membrane, while viral prM and E proteins move to the ER and Golgi apparatus to be modified by glycosylation. The virion is subsequently transported through exocytic vesicles, where prM protein is cleaved by Furin to form mature virus prior to release. The process of replication is summarized in Figure 1.3.

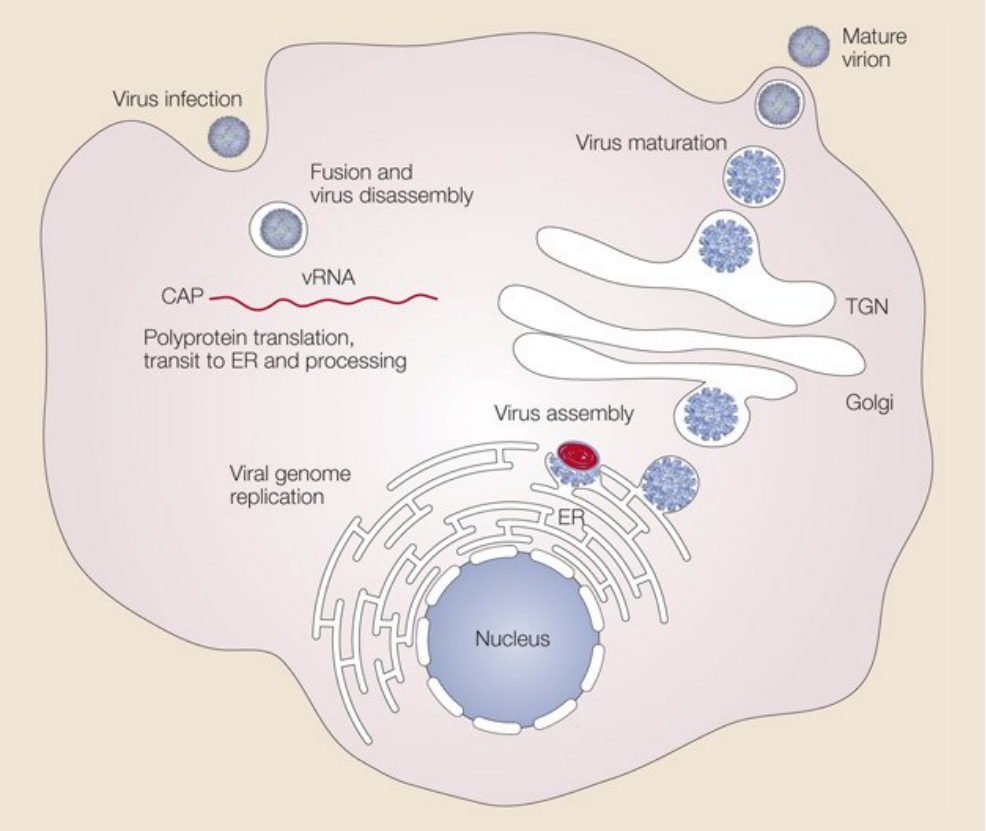

Figure 1.3: Dengue replication cycle (Mukhopadhyay et al., 2005).

1.1.4 Clinical features of Dengue disease

Dengue infection has a broad spectrum of clinical manifestation. Most patients infected with Dengue show few signs of infection and recover without a severe clinical presentation. Nevertheless, a small percentage will present with clinical symptoms and progress to severe illness with plasma leakage. Despite the small proportion who develop severe disease, Dengue infection is a life-threatening disease especially in children and the number of infected individuals tends to increase every year.

The course of infection:

Dengue disease is a systemic infection and the time course is largely divided into the febrile, critical, and recovery phases (Figure 1.4).

Febrile phase; lasts for 2-7 days. Patients typically have a high fever (≥ 38.5°C) accompanied by headache, myalgia, and arthralgia. The liver is often enlarged after a few days of fever. The total white blood cell (WBC) count progressively decreases. The symptoms at the early stage are difficult to discriminate from other febrile illnesses (OFIs), and also are indistinguishable between mild and severe Dengue.

Critical phase; (defervescense); usually occurs on day 3-7 of illness. At this period, the fever drops to 37.5-38 °C. Increases in haematocrit and vascular permeability, together with a progressive decline in WBC count and platelet count may occur. Patients with a high vascular permeability may develop plasma leakage. Pleural effusion and shock may be detectable depending on the degree of leakage. Following a long period of shock, organ impairment and metabolic acidosis may present and contribute to severe hemorrhage.

Recovery phase; occurs 24-48 hours after the critical period. General well-being gradually returns to normal. The WBC count starts to increase whereas the platelet count recovers later.

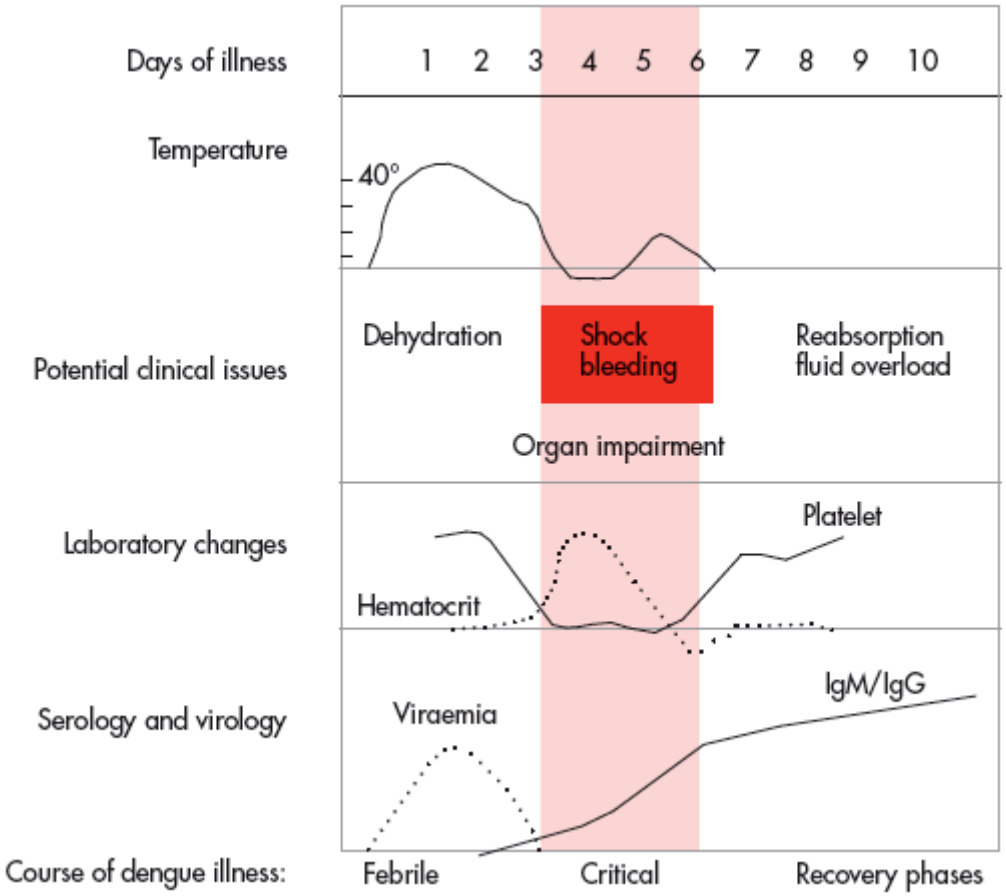

Figure 1.4: Course of Dengue infection (Organization, 2009).

Classification of Dengue severity:

The clinical features of Dengue disease are classified based on its severity into three categories: undifferentiated fever, Dengue fever (DF) and Dengue hemorrhagic fever (DHF) (Organization, 2009). DHF can be further divided into 4 severity grades. DHF grade III and IV is also named Dengue shock syndrome (DSS). Even though there have been many reports of difficulties in the use of this classification, it is widely used at the present time (Simmons et al., 2012).

Dengue Fever (DF)

The clinical features of DF are dependent on the age of infected patients. Infants and young children may have undifferentiated fever with maculopapular rash. Older children and adults may present with fever, headache, muscle/joint/bone pains, rash, nausea and vomiting. Leucopenia is usually detected. Hemorrhage is uncommon.

Dengue hemorrhagic fever (DHF)

The major patho-physiological change that determines DHF is plasma leakage. Thrombocytopenia, hemoconcentration, and coagulation abnormality generally present in DHF grade I and II. When accompanied by hypotension, it is designated as DHF grade III, and IV or DSS. Complications in DSS may include pleural effusions, liver dysfunction, shock, and severe bleeding. The spectrum of DHF is shown in Figure 1.5.

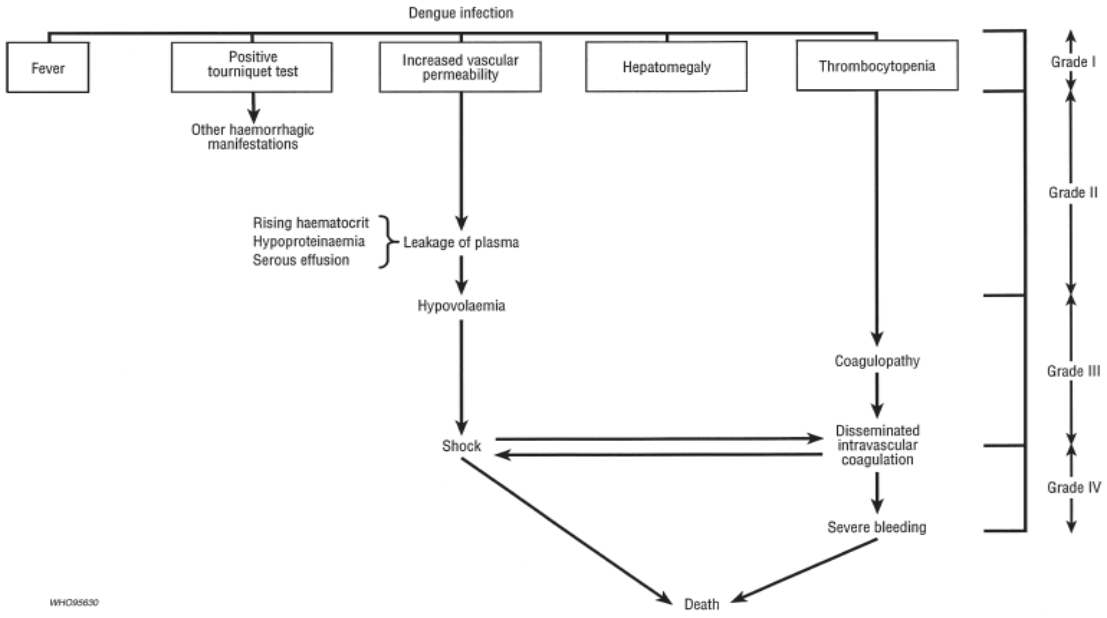

Figure 1.5: The spectrum of DHF (WHO, 1997).

Type of Infection:

There are two types of Dengue infection: primary and secondary infection. Primary infection is defined as the first infection with a slow and low titer of antibody response. Between days 3 and 5 after the onset of infection, seroconversion from negative to positive IgM anti-Dengue antibody together with a decline in viremia is detectable. IgG titers are low at the end of the first week and slowly increase. The secondary infection is characterized by re-infection with Dengue virus which can be either a homologous or heterologous serotype. The antibody titers are extremely high and may cross-react with different Dengue serotypes as well as other flaviviruses. IgG antibody is detectable during the acute phase and may rise after two weeks of infection. The peak IgM titer in secondary infection is usually lower than that in primary infection.

Lab diagnostic tests:

A lab diagnostic test for Dengue infection is essential in order that appropriate treatments may be administered. The ideal test should be cheap, sensitive (few false-negatives), specific (few false-positives), rapid, stable at room temperature, easy to use, machine-less, as well as delivered to those who need it (Blacksell, 2012).

So far, lab diagnostic tests to confirm Dengue infection include virus isolation, detection of viral nucleic acid (RNA) and viral antigens as well as anti-Dengue antibodies (Simmons et al., 2012). During the early stage of infection (within 5 days of initial onset), virus and viral products may be detected in plasma, serum or tissues. Virus isolation is a very effective technique to confirm the disease; however, this technique is laborious, expensive and usually takes several days (Peeling et al., 2010). Alternatively, detection of viral nucleic acid is a reliable test and only takes about 2 days to get the results; however, this method still requires expensive machines and reagents (Peeling et al., 2010). NS1 antigen ELISA is promising since it can be detected within one day. Sensitivity of NS1 detection can exceed 90% in persons with primary infection during febrile phase, and NS1 antigen may be detected after fever for several days (Simmons et al., 2012). Still, the sensitivity and the accuracy are lower in the secondary infection, probably due to the interference of the serological

response to previous infection (Ty Hang et al., 2009, Tricou et al., 2010, Felix et al., 2012). NS1 detection may become more powerful if the test is used in combination with serological methods (Blacksell, 2012).

At the end of the acute phase, when viremia and viral products are undetectable, serological tests detecting IgM and IgG levels become meaningful. IgM can be detected in the early days (4 days after a fever). Primary and secondary infection can also be determined by this IgM/IgG capture ELISA (Simmons et al., 2012). IgM titer in the primary infection is higher than that in the secondary infection. In contrast, IgG titer in the secondary infection is very high. However, recent vaccination/infection of antigenically-related flaviviruses such as Japanese encephalitis virus can interfere with the diagnosis (Simmons et al., 2012). The diagnostic tests for Dengue infection are depicted in Figure 1.6.

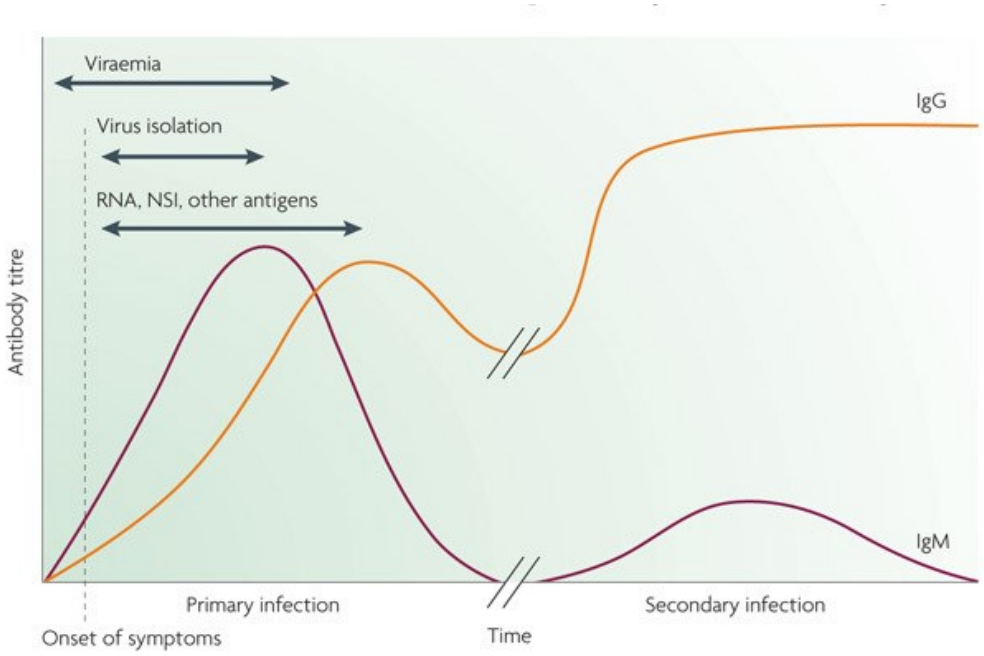

Figure 1.6: Diagnostic tests for Dengue infection (Peeling et al., 2010).

1.1.5 Immune responses to Dengue infection

Elimination of viral infection requires both innate and adaptive immune responses. Innate immunity refers to non-specific responses and does not require previous antigenic exposure. This develops rapidly after infection. It comprises non-specific barriers including skin, phagocytic cells, natural killer (NK) cells, acute phase proteins such as complement factors, and cytokines. In Dengue infection, increases in complement activation, NK cell activation, and inflammatory cytokines have been described. Adaptive immunity refers to the response to the previous antigenic exposure. This system mainly refers to T and B lymphocytes. In contrast to the innate immune response, the adaptive immune response develops slowly, but more specifically, and effectively. Activation of T cells and B cells has been demonstrated in Dengue infection. However, these observations only represent the immune response against infection and do not provide insight about protective immunity.

Innate Immunity:

Soluble factors

- Interferon

The interferon-dependent innate immune response is necessary for protection against dengue infection. Type I IFNs (IFN-α/β) are produced promptly, within an hour, after infection and induce antiviral activity. *In vitro* studies have revealed that Dengue infection stimulates interferon secretion (Kurane et al., 1992, Kurane and Ennis, 1988) and pretreatment with inteferon is able to control Dengue infection (Diamond et al., 2000). IFN-receptor knock-out mice are also susceptible to Dengue infection (Shresta et al., 2004). The mechanism by which interferon blocks Dengue infection is thought to involve inhibition of the RNA replication step (Diamond and Harris, 2001). *In vivo* studies have revealed that these IFNs are highly activated in uncomplicated cases, yet they are less effective in severe disease [reviewed in (Espada-Murao and Morita, 2011)]. The underlying mechanisms that suppress IFN activation are not fully understood. One explanation is that the virus has an ability to counteract these molecules. NS2A and NS4B may be key factors that block IFN

signaling pathways (Jones et al., 2005). A restricted IFN response therefore, could enhance infection associated with severe disease. In addition, an altered profile of IFN-producing cells may account for a differential immune response. For example, levels of plasmacytoid dendritic cells, the potent IFN-α producing cells, are decreased in DHF compared to DF cases (Pichyangkul et al., 2003).

Type II interferon (IFN-γ) is mainly produced by T cells and NK cells. The IFN-γ receptors are highly expressed on monocytes and macrophages, as well as endothelial cells. IFN- γ production by Dengue virus-specific T lymphocytes has been revealed *in vitro* (Kurane et al., 1989a, Kurane et al., 1989b). The effect of IFN- γ on Dengue infection *in vitro* remains obscure since both stimulatory and inhibitory consequences have been reported. Pre-treatment of IFN-γ can inhibit infection in HepG2 cells, foreskin fibroblasts, and monocytes (Diamond et al., 2000, Sittisombut et al., 1995). On the other hand, IFN-γ can also induce infection in mononuclear cells in the presence of enhancing antibody (Diamond et al., 2000, Kontny et al., 1988). In addition the relationship between IFN-γ response and disease severity is not clear (Srikiatkhachorn and Green, 2010). Some publications have shown high levels of IFN-γ in DHF cases, but not in others [reviewed in (Espada-Murao and Morita, 2011)]. Inconsistency may be due to experimental design, time to sample collection and population sampling (Srikiatkhachorn and Green, 2010). However, time course studies show that IFN- γ level peaks at an early time point, and this increased level has been observed in DHF cases with plasma leakage (Libraty et al., 2002).

- Cytokines

The cytokine storm theory has been proposed to contribute to Dengue severity. Massive production of cytokines is believed to be a product of T cell activation. However, this does not explain cytokine production in infants with primary infection, indicating that other determinants may be involved in this process (Nguyen et al., 2004). It has been hypothesized that maternal antibodies enhance infection and these may be responsible for the increased cytokine response in this situation. *In vitro* studies have also shown that Dengue infected cells such as keratinocytes, dendritic cells and endothelial cells can release cytokines (Surasombatpattana et al., 2011, Dejnirattisai et al., 2008, Dalrymple and Mackow, 2012, Avirutnan et al., 1998). Variations in viral load also have an influence on cytokine induction. For example,

expression of IL8, MIP-1α, RANTES by monocytes could be up-regulated by a small amount of virus, whereas production of TNF-α and IL1β requires higher viral loads (Chen and Wang, 2002).

Complement system

Complement appears to have both protective and pathogenic roles in viral infection. On one hand, complement can attack infected cells, inhibiting viral production. C1q, the complement component is able to bind avidly with IgG, restricting "antibody-dependent enhancement" (ADE) in *in vitro* studies and in mice (Mehlhop et al., 2007, Yamanaka et al., 2008). Mannan binding lectin (MBL) also neutralizes Dengue infection through complement activation-dependent and independent pathways (Avirutnan et al., 2011a). An experiment in immunodeficient mice has confirmed that complement is able to suppress viral infection since $C3^{-/-}$ mice are more susceptible to West Nile virus infection, another virus in the *Flaviviridae* family (Mehlhop et al., 2005).

Excessive complement activation, on the other hand, could exacerbate the disease. High plasma levels of sC5b9 complex, C3a and C5a are associated with severe disease (Nascimento et al., 2009, Avirutnan et al., 2006, Bokisch et al., 1973). Moreover, *in vitro* studies have shown that IgM-virus-complement complexes could enhance viral infection in macrophages through complement receptor 3 (CR3) (Cardosa et al., 1983). NS1 is also able to activate the complement, exacerbating the disease (Muller and Young, 2013).

Natural Killer (NK) cells

NK cells are capable of killing virus-infected cells rapidly within an hour. Lysis of target cells by NK cells is not MHC-restricted and does not require the specific recognition of viral products. NK cells recognize target cells via a variety of receptors and lyse these cells by release of cytotoxic granules. The role of NK cells in Dengue infection is not well understood. Activation of NK cells has been reported during acute Dengue infection and is more profound in DHF (Green et al., 1999a).

Adaptive immunity:

Antibody responses

Interpretation of antibody responses to Dengue infection needs knowledge of previous exposure. Pre-existing antibodies from previous infection or pre-immunization with flaviviruses are commonly detected. In primary infection, IgM antibodies increase after the decline of viremia and have the highest activity in a primary infection, whereas IgG antibodies exceed IgM production during secondary infection (Peeling et al., 2010). The role of antibody in Dengue infections have been demonstrated in several mechanisms such as neutralization, antibody-dependent enhancement (ADE), antibody-dependent complement activation, opsonization, and antibody-dependent cellular cytotoxicity (ADCC). In neutralization, antibodies interact with the viral structural E proteins, inhibiting viral entry and uncoating of the viral genome (Halstead, 2003). Neutralizing IgG antibodies persist for many years and are able to protect against re-infection with the same serotype. Non neutralizing antibodies, on the other hand, may be responsible for immune enhancement of Dengue infection (ADE). In addition, antibodies specific to the NS1 protein could bind the NS1 on the cell surface, triggering complement lysis (Muller and Young, 2013). Phagocytic cells, such as macrophages, can opsonize and kill virions marked by antibodies. Antibodies can also attach to antigens on the cell surface, followed by the recruitment of Fc-receptor bearing cells to kill these cells by means of ADCC. The mechanisms of neutralization, ADE, and antibody-dependent complement lysis are summarized in Figure 1.7.

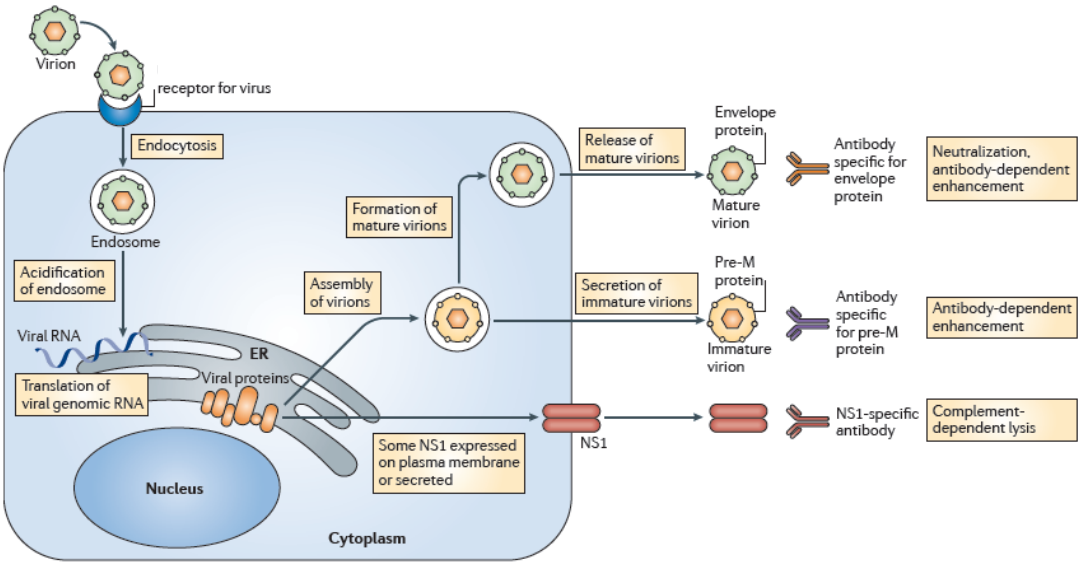

Figure 1.7: Antibody responses to Dengue infection [modified from (Rothman, 2011)].

T lymphocytes

Dengue-specific T cells target Dengue-infected cells and subsequently proliferate, and produce cytokines. T cell epitopes are found throughout the Dengue polyprotein, but most identified epitopes reside on the NS3 protein. In primary infection, T cell responses to the infecting serotype are characterized, even though some cross-reactive T cell responses are also identified (Kurane et al., 1989a, Kurane et al., 2011). These cross-reactive T cell responses may enhance the severity of the disease. An experiment to determine the heterogeneity among virus-specific T cells has found that most T cells produce TNF-α, which may lead to an increased risk of DHF during secondary infection (Rothman, 2011). Recognition of the heterologous serotype may stimulate different degrees of T cell activation. Recently, it has been demonstrated that Dengue-specific memory T cells in secondary infection show low affinity to the current virus, but higher affinity to others, which may be strains from previous infections (Mongkolsapaya et al., 2003). This partial recognition of infecting virus by T cells may reduce viral elimination, and profound T cell activation may alter the cytokine profiles, increasing immunopathology.

1.1.6 Pathogenesis of the disease

To better understand the pathogenesis of Dengue infection, several models have been developed. The immune response against Dengue infection has been generally studied in clinical experiments due to the lack of appropriate animal models. The interaction between innate and adaptive immunity has also been studied in *vitro*, but this does not totally reflect the complex interactions between the different arms of the immune systems. Infection of laboratory strains of Dengue virus into immortal cell lines may not fully explain the *in vivo* mechanism. Overall, interpretation of the results has to be considered a weakness of each model.

Epidemiological and clinical observations:

In the clinical studies of Dengue infection, many observations have been drawn. Primary infection with any one of four serotypes generates lifelong antibodies to the homologous serotype. Almost all cases of primary infection show either no sign of symptoms or mild disease, whereas a small percentage are severe cases, most of which are seen in infants (Halstead, 2003). It has been hypothesized that maternal anti-Dengue antibodies are transferred to those infants and these antibodies may not neutralize virus but enhance infection via ADE (Halstead, 2003). Secondary infection with heterologous serotypes is more complex than primary infection since nearly all cases re-infected with a different serotype show severe DHF. In addition, plasma leakage, a hallmark of DHF, occurs during defervescence (when viremia is declining), suggesting that this mechanism is probably mediated by the host immune response (Simmons et al., 2012).

Mechanisms of Dengue disease:

The mechanisms that determine Dengue severity are not fully understood but are likely to be a combination of multifactorial processes (Figure 1.8). After Dengue virus is injected into the dermis, immature Langerhans cells (LCs) and keratinocytes are infected. LCs migrate from the site of infection and subsequently spread through the lymphatic system. Viral antigens have been isolated in many cells such as DCs, monocytes, and macrophages (Martina et al., 2009, Noisakran et al., 2010). In the

post-mortem tissues, viral antigens have also been detected in endothelial cells in several organs, particularly in the liver (Martina et al., 2009). The viral replication in these cells could reflect the viral load in the blood, which is correlated to the disease severity. In addition to high viremia, thrombocytopenia and platelet dysfunction may account for the fragile capillary, resulting in hemorrhage (Noisakran and Perng, 2008). Infection also activates immune responses, which may enhance rather than reduce the disease. NS1 proteins can activate the complement pathway, leading to vascular leakage and inflammation (Avirutnan et al., 2006). In secondary infection, pre-existing antibodies can enhance infection via ADE (Halstead, 2003). Anti-NS1 antibodies also act as auto-antibodies, resulting in release of inflammatory cytokines such as IL6 and IL8 leading to inflammation and/or apoptosis (Martina et al., 2009). Over-produced, low-avidity, cross-reactive T cells might delay viral clearance, and produce massive amounts of cytokines (Rothman, 2011). Other soluble mediators can also change the function of endothelial cells, leading to plasma leakage (Martina et al., 2009).

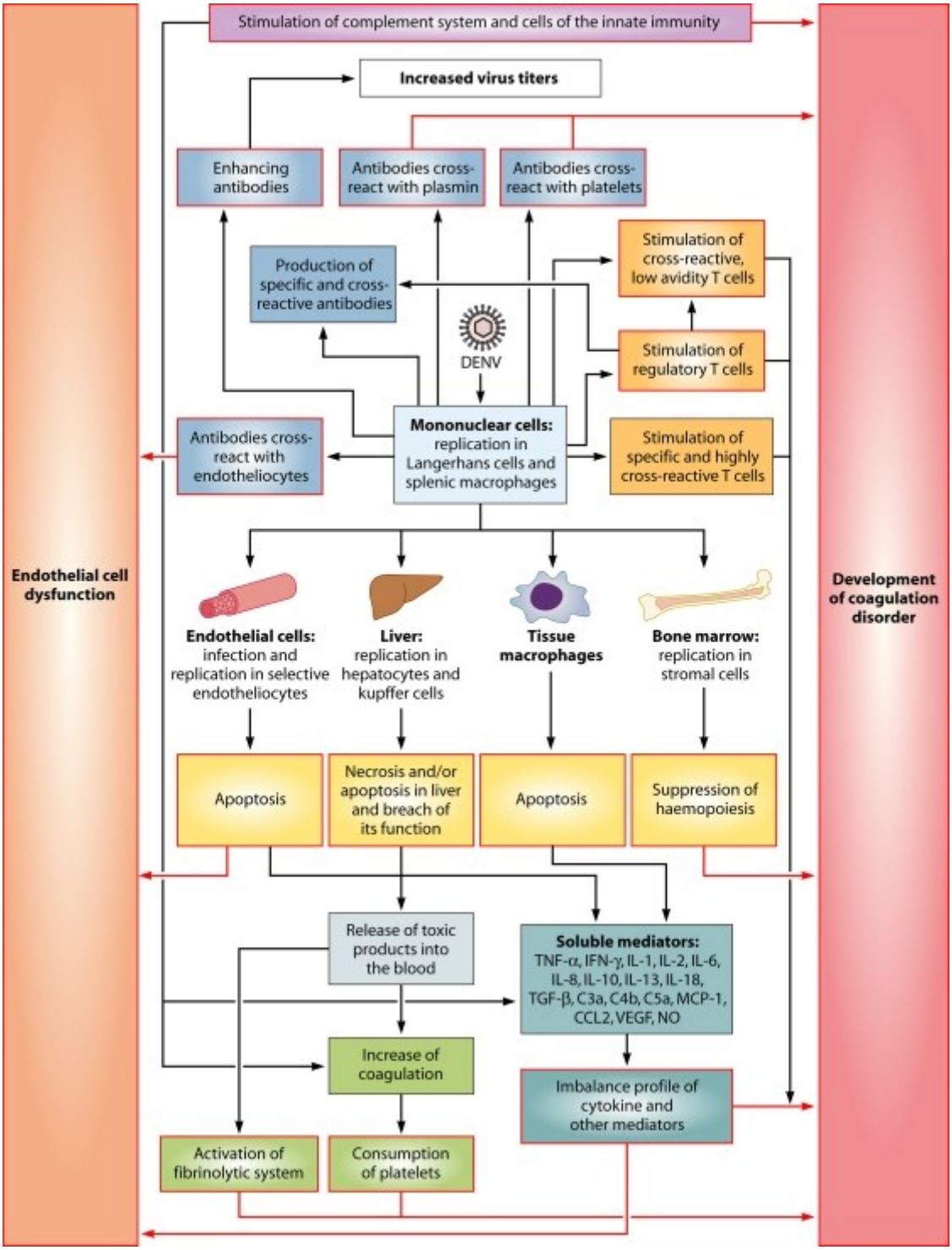

Figure 1.8: Proposed mechanisms of Dengue infection (Martina et al., 2009).

Determinants of susceptibility to Dengue infection and severity:

Viral determinants

Different viral genotypes may determine Dengue severity. Well established examples have been reported in the Western hemisphere. After Dengue 1 was introduced into the Caribbean region in 1977, an introduction of Asian genotype Dengue 2 in Cuba in 1981 was accompanied with the occurrence of DHF; however, similar introductions of Dengue 1 in 1990 and an American strain Dengue 2 in 1995 in Peru did not result in DHF (Halstead, 2003). Sequence analysis of these two strains of Dengue 2 revealed that six different amino acids were found, contributing to structural changes and differences in virulence (Halstead, 2003).

Host determinants

1. Genetic factors

There are some ethnic groups that show tendency to develop DHF while others may have mild DF. In 1981, the DHF/DSS risk ratio in whites: blacks: mixed in Cuba was reported as 5.5:1:1.8 (Coffey et al., 2009). Similar data from 1997 and 2001 Cuban Dengue epidemics were observed and confirmed the 1981 data that blacks are more resistant to develop DHF than whites (de la et al., 2007). However, interpretation of these findings must be done with caution since other factors, such as viral genotype and vector transmission, could also be determinants of disease outcome.

In addition to ethnicity, several gene polymorphisms have been shown to influence susceptibility to Dengue infection. Human Leukocyte Antigens (HLAs) show great variability and some of these variations are associated with Dengue severity. For example, HLA-A2 and HLA*0207 correlate with susceptibility to infection in the Thai population, whereas HLA-A24 and HLA*0203 are protective (Wagenaar et al., 2004). Association between Dengue susceptibility and polymorphisms have also been characterized in several other genes such as Fc

receptors, Vitamin D receptor, IL4, IL1-RA (Loke et al., 2002), TNF-α (Vejbaesya et al., 2009), and DC-SIGN(Sakuntabhai et al., 2005, Wang et al., 2011).

2. Immunological factors

The immune status in the Dengue-infected individuals may develop in two directions—protective or pathogenetic. In most instances, immunopathogenesis occurs in secondary infection with a heterologous serotype. Several mechanisms in controlling the disease severity have been proposed.

- Humoral immune response
    a. Pre-existing antibodies

Immune enhancement of Dengue infection was first described in the 1970s. Increased levels of viremia were observed in rhesus monkeys during secondary infection as compared to the primary infection. It was not known how the immune system enhanced infection until 1977, when it was discovered that antibodies were the mediators (Halstead, 2008). Pre-existing heterotypic antibodies are unsuccessful at neutralizing the currently infecting virus, but enhance viral entry into Fc receptor-bearing cells through the process of ADE. In humans, IgG1 and IgG3 subclasses are responsible for ADE and peripheral blood mononuclear cells (PBMCs) have Fc receptors on their surfaces. It is well-established that ADE occurs when antibodies are directed against viral epitopes that are not involved in the viral attachment and uncoating process. However, a great number of studies have also demonstrated that homotypic neutralizing antibodies are able to produce ADE at a dilution above the neutralization level (sub-neutralizing concentration) (Halstead, 2003). Recently, it has been discovered that anti-prM antibodies fail to neutralize the virus even at high titers (Dejnirattisai et al., 2010). The prM proteins, in general, are cleaved during the process of maturation, implying that anti-prM antibodies are unable to bind most viral particles. Cleavage of prM; however, is not complete, contributing to the release of partially mature virions which contain some prM molecules on their surfaces. These prM components are enough for anti-prM antibodies to recognize and drive ADE (Dejnirattisai et al., 2010).

Dengue-antibody complexes not only enhance viral infection via ADE, but also suppress IFN-mediated antiviral responses. Dengue infection of the human monocytic cell line THP-1 via ADE can disrupt interferon-related proteins, the retinoic acid inducible gene I (RIG-I) and the melanoma differentiation associated gene 5 (MDA-5), as well as enhance the expression of anti-inflammatory cytokines such as IL6 and IL10 (Ubol et al., 2010, Chareonsirisuthigul et al., 2007).

Although ADE can increase viral titer, there are some questions that remain unknown. First, DHF can appear in primary infection, albeit at a low percentage, in older children and adults. Second, although high viremia is associated with Dengue severity, viral titer at the time of leakage is well below the peak level. In addition, there are some patients who have high viremia but do not develop plasma leakage. These findings suggest that other mechanisms may be at work in these cases [reviewed in (Rothman, 2011)].

b. Autoantibodies

Anti-NS1 antibodies are able to cross-react with several host cell components including blood clotting factors, integrin/adhesion molecules, extracellular matrix (ECM), etc (Muller and Young, 2013). Not only are anti NS1 antibodies cross-reactive to several tissues in mice (Nielsen, 2009), these antibodies can also bind to the surface of human platelets and endothelial cells and subsequently induce inflammatory cytokines such as IL8, IL6 and MCP-1 as well as ICAM-1 expression, followed by an increased adhesion of PBMCs to endothelial cells (Muller and Young, Lin et al., 2005). Moreover, cells exposed to anti-NS1 antibodies may undergo 'programmed cell death' since DNA fragmentation and phosphatidylserine exposed on the cell surface are found in these cells (Lin et al., 2002). These findings suggest a possible role for these antibodies in the endothelial dysfunction and vascular damage that is an underlying mechanism of DHF/DSS.

- Cross-reactive T cell responses

Cross reactive T cells have been proposed to contribute to Dengue severity during secondary infection. Generally, the efficiency to eradicate infected cells depends on the avidity of activated T cells. Expansion of $CD8^+$ T cells with low avidity to the currently infecting virus but high avidity to a presumed previous

serotype has been demonstrated (Mongkolsapaya et al., 2003). This memory T cell repertoire may out-compete the naïve T cells contributing to incomplete viral elimination and produce high levels of pro-inflammatory cytokines such as TNF-α, leading to apoptosis and inflammation (Mongkolsapaya et al., 2003).

- Complement activation

Imbalance of complement activation has been observed in Dengue disease. For example, high levels of C3a and C5a have been found to correlate with Dengue severity (Nascimento et al., 2009). These anaphylatoxins are known to have an effect on vasodilatation and promote degranulation of polymorphonuclear cells such as neutrophils. Mediators released from the degranulation can induce local inflammation and systemic disturbance, if generated in a high concentration. In addition, there is a correlation between increased disease severity and an imbalance in the levels of complement factor H (CFH) leading to abnormal regulation of the alternative complement pathway (Nascimento et al., 2009). The association between dysregulation of complement components and Dengue disease is summarized in table 1.1.

During secondary infection, anti-NS1 antibodies are able to bind NS1 proteins, followed by formation of an immune complex which activates complement and generates C3a and C5a (Avirutnan et al., 2006). In the absence of anti-NS1 antibodies, NS1 proteins can interact with Clusterin, a complement regulator, increasing the formation of the C5b9 complex on the cell surface, followed by cell lysis (Kurosu et al., 2007). This C5b9 complex is correlated with disease severity (Avirutnan et al., 2006). Soluble NS1 has also been shown to function in immune evasion since it can inhibit the classical and lectin pathways by interacting with C1s and C4BP, leading to a decrease in the formation of the C5b9 complex on infected cells and an increase in viral replication (Avirutnan et al., 2010, Avirutnan et al., 2011b).

- Soluble mediators

It is believed that high viral loads and activation of low-avidity cross-reactive T cells are responsible for the cytokine storm as well as secretion of several mediators, leading to increased plasma leakage. Differential viral replication and T

cell activation may account for different cytokine profiles and different vascular permeability patterns. The soluble mediators, either alone or in combination, are able to deregulate the function of immune and non-immune cells. For example, Dengue virus is able to increase the cell surface expression of vascular endothelial growth factor receptors (VEGFR) on endothelial cells, but suppresses expression of the soluble form (sVEGFR2) *in vitro* (Srikiatkhachorn et al., 2007). Up-regulation of surface VEGFR promotes binding of VEGF on endothelial cells, contributing to changes in endothelial morphology and increases in cell permeability (Srikiatkhachorn et al., 2007). In clinical observation, disease severity is correlated with an increased plasma level of vascular VEGF-A together with a decline in sVEGFR2 and the VEGF-sVEGFR2 complex (Srikiatkhachorn et al., 2007).

3. Others host factors

Other possible risk factors for development of DHF/DSS include sex and age. Retrospective studies in Vietnam have revealed that most Dengue cases admitted to hospital between 1996 and 2009 were males, yet females had a higher risk of developing DSS and death. In addition, the mortality rate was high in young children but declined with increasing age (Anders et al., 2011, Guzman et al., 2002). The underlying mechanisms for the association between these factors and disease severity however, remain unknown.

1.1.7 The role of endothelial cells in Dengue infection

Endothelial cells are the fluid barriers of blood vessels that play an important role in controlling coagulation as well as the extravasation of fluids, platelets and blood cells. Endothelial cells can become activated by inflammatory cytokines and regulate the recruitment of leukocytes to the site of inflammation by expressing cell adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 (van Hinsbergh, 2012). Transportation of solutes and plasma proteins across the endothelium through inter-endothelial junctions is generally well-orchestrated; however, external stimuli and inflammatory mediators can change endothelial cell function, prolonging permeability (Komarova and Malik, 2010).

In vitro experiments have shown that Dengue virus, TNF-α, or a combination of both can affect endothelial permeability (Dewi et al., 2004). Addition of peripheral blood mononuclear cells (PBMCs) to infected endothelial cells also enhances this phenomenon (Dewi et al., 2008). PBMCs are able to induce morphological changes in endothelial cells and decrease the expression of VE-cadherin (vascular endothelial cadherin), the adhesion molecules that maintain endothelial cell integrity. Moreover, Dengue infection of endothelial cells in the presence of non-neutralizing antibodies can activate complement (Avirutnan et al., 1998). Infected endothelial cells also release RANTES and IL8, which are able to recruit immune cells, particularly neutrophils (Avirutnan et al., 1998). Cell death was also demonstrated in the same study. These evidences suggest that Dengue virus might damage endothelial cell integrity.

Although *in vitro* infection of endothelial cells has been demonstrated, infection of endothelial cells *in vivo* has not been elucidated. In post mortem studies, Dengue antigens (structural proteins) but not its genome were detected in endothelial cells in the liver, lung and heart without evidence of endothelial cell damage (Jessie et al., 2004) (Salgado et al., 2010). It is noteworthy that detection of viral antigen is not proof of viral replication since it might be the product of killed virus or degraded immune complexes (Jessie et al., 2004). Nevertheless, another study has shown that NS3 protein could be detected in the splenetic endothelial cells but not in other tissues, implying that endothelial cells in the spleen may be supportive for viral propagation (Balsitis et al., 2009). In IFN-receptor deficient mice, ADE could enhance infection in liver sinusoidal endothelial cells and correlated to disease severity (Zellweger et al., 2010). Although this finding provides an important role for endothelial cells in Dengue pathogenesis in mice, there is no evidence to support ADE-mediated infection in primary human endothelial cells *in vitro* (Arevalo et al., 2009).

As a whole, it is difficult to conclude if Dengue actively replicates in endothelial cells *in vivo*. Limitations in studies of human specimens, which were usually collected after the peak of viremia, and the lack of suitable animal models makes it difficult to completely understand Dengue pathogenesis.

1.1.8 Markers of Dengue disease

Dengue infection ranges from asymptomatic infection, mild DF, to four-grade DHF. During the early stage of infection, the signs and symptoms are indistinguishable between mild and severe disease and there are no accurate indicators to predict the development of DHF currently. Although attempts to find the predictive indicators are ongoing, many markers associated with Dengue severity have been characterized.

Clinical markers:

Platelet count and haemoconcentration level

Thrombocytopenia and haematocrit are recognized in Dengue infection. A platelet count below 100,000 cells per cu mm, together with an increase in haematocrit of 20%, is a criterion to determine DHF (1997). These changes are usually found during day 3 to day 8 and appear to reach their peak at the defervescence stage. Platelet cell counts and haemoconcentration level could serve as monitoring indicators for disease progression.

White blood cell count

The white blood cell (WBC) count in DHF varies during the onset of the illness since leucopenia or mild leucocytosis may be present. A drop in WBC count due to a reduced number of neutrophils however, is usually observed at the end of the febrile phase while elevated lymphocyte numbers and the presence of atypical lymphocytes (enlarged lymphocytes) is commonly detected before defervescence (1997).

Liver proteins

Detection of liver dysfunction is a common finding in DF but more pronounced in DHF. A reduction in proteins due to the loss of albumin can be observed. Elevated plasma levels of liver aminotransferases, which include aspartate

aminotransaminases (AST) and alanine aminotransaminases (ALT), are detected (1997).

Viral load and NS1 products:

High viral nucleic acid and NS1 protein levels in plasma appear to correlate with disease severity (Srikiatkhachorn and Green, 2010). The peak of viral titer and NS1 levels occurs during the early stage of febrile illness before declining during defervescence (first 72 hours). In primary infection, NS1 can be detected in plasma taken as much as 12 days after disease. Nevertheless, detection of NS1, although a promising marker, may be more complicated in a secondary infection when anti-NS1 antibodies are present. Beyond 5-7 days of illness, NS1 is rarely detected [reviewed in (Muller and Young, 2013)].

Biological markers:

Several studies have demonstrated a great number of plasma protein markers associated with Dengue severity. These markers are summarized below and in the table 1.1. However, heterogeneity in the timing of sample collections in each study makes it difficult to compare some observations. Interpretation of these results therefore, must be done with caution since Dengue disease is a time-course dependent illness.

Complement system

Abnormal levels of complement components are found in Dengue disease and seem to associate with the severity. For example, an increase in soluble C5b9 complex, C3a, C4a and C5a, and MBL together with a decrease in plasma factor H was reported in DHF patients (Nascimento et al., 2009, Avirutnan et al., 2006).

Cytokines and Chemokines

In vitro studies have demonstrated that infected cells (dendritic cells, keratinocytes, endothelial cells etc.) can induce production of interferons,

proinflammatory cytokines and chemokines including TNF-α, IL8, RANTES, MIG, IP10, ITAC etc, depending on the cell type (Dejnirattisai et al., 2008, Avirutnan et al., 1998, Surasombatpattana et al., 2011, Dalrymple and Mackow, 2012, Ho et al., 2001). This postulates that several cytokines might be released to eliminate the infection during the early stage of illness; however, there are some conflicting results in some cytokines measured in the plasma of Dengue patients. This might be due to the timing of sample collection. More information about the timing of these cytokine profiles is required.

During the late stage, cellular immune responses have a greater influence on disease than the innate cytokines. It has been postulated that activation of cross-reactive Dengue-specific $CD8^+$ T cells during a secondary infection may play a role in the massive production of cytokines, leading to DHF (Mongkolsapaya et al., 2003). Infection of peripheral blood mononuclear cells (PBMC) *in vitro* produces Th1 cytokines, such as IFN-γ, IL2, and IL6, in the early stages but induces Th2 cytokine production (IL4, IL5, and IL10) in the later stages (Chaturvedi et al., 1999). In viral infection such as HIV, a shift from Th1 to Th2 tends to lead to severe pathogenesis. This has also been described in Dengue infection and appears to be associated with the severity of disease (Chaturvedi, 2009).

Soluble receptors

Association between soluble cellular receptors of ligands, including cytokines, and disease severity has been reported in several studies. Up-regulation of soluble receptors associated with T cell activation, such as sIL2R, sCD4, and sCD8 has been reported in plasma of DHF patients on day 3-4 after the onset (Kurane et al., 1991). In one study, sIL2R was correlated with the increase in liver enzymes (Libraty et al., 2002). A progressive decline of sVEGFR2 (soluble vascular endothelial growth factor receptor 2) accompanied with a decline in sVEGFR2-VEGF complexes and a rise in free VEGF was correlated to DHF (Srikiatkhachorn et al., 2007). Because VEGF is important in vascular permeability, changes in sVEGFR2 may regulate the plasma leakage, a hallmark of DHF, *in vivo*. Elevations in the soluble form of TNF-α receptors have also been reported in DHF (Libraty et al., 2002), even though there have been conflicting results in some studies (Wang et al., 2007, Braga et al., 2001).

Coagulation and Endothelial cell activation and damage

Imbalance in coagulation and fibrinolytics is found in Dengue disease. In DHF patients, Von Willebrand factor, tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), tissue factor, and thrombomodulin were elevated(Sosothikul et al., 2007, Huang et al., 2001). In addition, thrombopoietin (TPO), which is a regulator of platelet production, was also increased in DHF patients and conversely correlated with the platelet counts (Matondang et al., 2004, Cardier et al., 2006).

Activation of endothelial cells contributes to changes in permeability. Increased levels of soluble endothelial surface molecules (sICAM1 and sVCAM) have been reported in the plasma of DHF patients (Cardier et al., 2006). An association between Dengue severity and elevated levels of matrix metalloproteinase 9 (MMP9), which is able to increase endothelial permeability, has been published (Kubelka et al., 2010). However another study showed no difference in MMP9 levels between DF and DHF in any stage of illness (Voraphani et al., 2010).

Table 1.1: Soluble factors associated with Dengue disease

Soluble factors	Stage of detection	Findings	References
Complement system			
sC5b-9	Acute	DHF > DF	(Avirutnan et al., 2006)
C3a C4a and C5a	Acute	DHF > DF	(Nascimento et al., 2009)
C1q	Acute	DHF = DF	(Nascimento et al., 2009)
Mannose binding lectin (MBL)	Acute	DHF > DF	(Nascimento et al., 2009)
Factor D	Acute	DHF = healthy control > DF	(Nascimento et al., 2009)
Factor H	Acute	DF = healthy control > DHF	(Nascimento et al., 2009)
Cytokines			
IFN-α	Acute, Febrile	DHF, DF > healthy control	(Kurane et al., 1993)
IFN-γ	Not specified	severe > mild	(Bozza et al., 2008)
	Not specified	DHF = DF	(Braga et al., 2001)
	Acute	Dengue > healthy control	(Azeredo et al., 2001)
	Acute, Febrile	DHF > healthy control; DF > healthy control	(Kurane et al., 1991)
TNF-α	Not specified	DHF, DF with hemorrhage > DF	(Braga et al., 2001)
	Acute	Dengue > healthy control	(Azeredo et al., 2001)
	Acute, Febrile	DHF3, DHF4 > DHF1, DHF2	(Hober et al., 1993)
Macrophage inhibitory factor (MIF)	Acute, Febrile	DHF-survivors > DF; DF > healthy control	(Chen et al., 2006)
		DHF-non survivors > DHF-survivors; DHF-non survivors > DF	
IL1β	Not specified	severe > mild	(Bozza et al., 2008)
IL2	Acute, Febrile	DHF > healthy control; DF > healthy control	(Kurane et al., 1991)
IL4	Not specified	severe > mild	(Bozza et al., 2008)
IL6	Acute, Febrile	DHF-non survivors > DF; DF > healthy control	(Chen et al., 2006)
		DHF-non survivors > DHF-survivors	
	Not specified	severe > mild	(Bozza et al., 2008)
IL7	Not specified	severe > mild	(Bozza et al., 2008)
IL10	Acute, Febrile	DHF-non survivors > DHF-survivors; DHF-non survivors > DF	(Chen et al., 2006)
	Acute	Dengue > healthy control	(Azeredo et al., 2001, Pérez et al., 2004)
	Acute, Febrile	DHF > DF; Dengue > OFI	(Green et al., 1999c)
IL12	Acute, Febrile	Dengue = healthy control	(Pérez et al., 2004)
	Acute, Febrile	DHF > DF; Dengue > OFI	(Green et al., 1999c)
	Acute, Febrile	DF > DHF1 > DHF2	(Pacsa et al., 2000)
IL13	Not specified	severe > mild	(Bozza et al., 2008)
IL1 receptor antagonist (IL1Ra)	Febrile	DHF-non survivor > DHF-survivor	(Suharti et al., 2003)

Chemokines			
CCL4 (MIP1-β)	Not specified	severe < mild	(Bozza et al., 2008)
	Acute, Febrile	Dengue > OFI	(Becerra et al., 2009)
CCL5 (RANTES)	Acute, Febrile	DF = healthy control > DHF	(Pérez et al., 2004)
CCL2 (MCP1)	Not specified	DSS > healthy controls	(Avirutnan et al., 1998)
CCL8 (MCP2)	Febrile & Defervescence	Dengue > OFI	(Becerra et al., 2009)
CXCL8 (IL8)	Acute	DHF3, DHF4 > DHF1, DHF2 and DF	(Raghupathy et al., 1998)
	Not specified	DSS > healthy controls	(Avirutnan et al., 1998)
CXCL9 (MIG)	Febrile	DHF > DF	(Dejnirattisai et al., 2008)
CXCL10 (IP10)	Febrile	DHF > DF	(Dejnirattisai et al., 2008)
	Febrile & Defervescence	Dengue > OFI	(Becerra et al., 2009)
CXCL11 (ITAC)	Febrile	DHF > DF	(Dejnirattisai et al., 2008)
Soluble receptors			
sCD4	Acute, Febrile	DHF > DF; DHF > healthy control; DF > healthy control	(Kurane et al., 1991)
sCD8	Acute, Febrile	DHF > DF; DHF > healthy control	
	Defervescence	DHF > DF; Dengue > OFI	(Green et al., 1999b)
sIL2Ra	Acute, Febrile	DHF = DF > healthy control	(Valero et al., 2008)
	Acute, Febrile	DHF3 > DHF1, DHF2	
	Acute, Febrile	DHF > DF; DHF > healthy control; DF > healthy control	(Kurane et al., 1991)
	Defervescence	DHF > DF; Dengue > OFI	(Green et al., 1999b)
sVEGFRII	Febrile & Defervescence	DHF < DF	(Srikiatkhachorn et al., 2007)
sTNFRI	Febrile & Defervescence	Dengue > OFI	(Green et al., 1999b)
sTNFRII (p75)	Acute	Dengue > healthy control	(Azeredo et al., 2001)
	Febrile & Defervescence	DHF > DF; Dengue > OFI	(Green et al., 1999b)
	not specified	DHF = DF > healthy control	(Braga et al., 2001)
Coagulation and endothelial function			
Von Willebrand factor (VWF)	Febrile	DHF > DF	(Sosothikul et al., 2007)
Tissue plasminogen activator (tPA)	Acute	DHF, DSS > DF; DHF, DSS > healthy control	(Huang et al., 2001)
	Defervescence	DHF > DF	(Sosothikul et al., 2007)
Plasminogen activator inhibitor	Febrile & Defervescence	DHF > DF	(Sosothikul et al., 2007)
Tissue factor (TF)	Febrile	DHF > DF	(Sosothikul et al., 2007)
Soluble thrombomodulin (sTM)	Febrile & Defervescence	DHF > DF	(Sosothikul et al., 2007)
TPO	Thrombocytopenia phase	DHF > DF	(Matondang et al., 2004)
	Acute, Febrile	DHF > DF; DF > healthy control	(Cardier et al., 2006)

MMP-9	Not specified	severe > mild = control	(Kubelka et al., 2010)
	Febrile, and Toxic stage	control > Dengue	(Voraphani et al., 2010)
ADAMTS13	Febrile	DHF < DF	(Sosothikul et al., 2007)
VEGF	Defervescence	DHF > DF	(Srikiatkhachorn et al., 2007)
	Acute, Febrile	DHF > DF = OFI	(Tseng et al., 2005)
sICAM1	Acute, Febrile	DHF = DF > healthy control	(Valero et al., 2008)
sVCAM	Acute, Febrile	DHF3, DHF4 > DHF1, DHF2, DF	(Koraka et al., 2004)
Others			
GMCSF	Not specified	severe > mild	(Bozza et al., 2008)
TRAIL	Acute, Febrile	Dengue > OFI	(Becerra et al., 2009)

1.1.9 Treatment, vaccine development, prevention and control

Treatment of disease

So far, there is no specific treatment for Dengue disease. Supportive care and fluid replacement are suggested. Blood transfusion may be required if persons have severe bleeding. Paracetamol is sufficient for high fever. Aspirin, Ibuprofen, and other non-steroidal anti-inflammatory agents (NSAIDs) should be avoided .

Vaccine development

Due to an increased number of severe Dengue cases, particularly in children around the world, the development of an effective vaccine against all Dengue serotypes is urgently needed. The ideal vaccine must induce anti-Dengue antibodies to the level of protection against any of the four serotypes, provide life-long protection, require minimal booster immunization and be low cost (Whitehead et al., 2007).

Impediments to the development of a Dengue vaccine are the immune responses that enhance Dengue infection and a lack of animal models. Pre-existing, non-neutralizing heterotypic antibodies and cross-reactive T cell responses increase the risk of developing DHF/DSS, and these features must be considered in a vaccine design. Animal models have been limited to develop immunogenicity in mice and monkeys. Immunocompromized models have been useful but only in some aspects of pathogenesis (Mathew and Rothman, 2008). So far, a licensed vaccine is not available. Current vaccines are mainly focused on lived-attenuated virus vaccines. The most promising candidate is a live-attenuated tetravalent vaccine based on a chimeric yellow fever-Dengue virus (CYD-TDV) (Guy et al., 2010). This vaccine is composed of four vaccines using a backbone of yellow fever vaccine 17D (YFV 17D) expressing E and prM proteins of each Dengue virus serotype (Guy et al., 2010). So far, CYD-TDV has been under evaluation in phase IIb to phase III trials (Sabchareon et al., 2012). Other candidates in the early phase of vaccine development include live attenuated Dengue and recombinant subunit vaccines (Whitehead et al., 2007).

Prevention and control

Preventing Dengue transmission depends on controlling mosquito vectors. Mosquitoes' habitats such as water storage containers should be emptied or covered to prevent access by mosquitoes. Insecticides should be used to kill adult mosquitoes. Active monitoring and surveillance of vectors should be performed.

Recently, there are new attempts to control the vector since the current approaches are inefficient to prevent transmission. For example, genetically modified male mosquitoes have been generated that fertilize the wild-type female mosquitoes in order to reduce the number of eggs (Simmons et al., 2012). Introduction of the bacterium *Wolbachia spp.* into mosquitoes can partially limit Dengue infection and may compete with the natural mosquito populations (Simmons et al., 2012).

1.2 Complement system

1.2.1 Complement system

Several lines of evidence show that Dengue disease is mediated by immunological processes, including the complement system. The complement system comprises three pathways: classical, mannose-binding lectin (MBL), and alternative (Figure 1.9) (Zipfel and Skerka, 2009). The classical pathway is triggered by an interaction of C1q with antigen-antibody complexes, whereas the lectin pathway begins with a binding of MBL with mannose on the surface of pathogens. The alternative pathway (AP) is spontaneously and constantly activated through the hydrolysis of C3 and serves as an amplification loop for the classical and the lectin pathways. The hydrolyzed C3 molecules then combine with factor B and factor D to produce C3 convertase (C3bBb). The other two pathways also generate C3 convertase however, in a different form (C4bC2a). These enzymes are necessary for cleaving C3, a central component of all complement pathways, to C3a, and C3b. The C3a is known as an anaphylactic molecule, whereas the C3b molecule can either be deposited on nearby surfaces, or combine with additional C3 convertases and subsequently function as C5 convertases. These enzymes cleave C5 to C5a, another anaphylactic peptide, and C5b, which recruits other components (C6-9) to a target surface and promote formation of the C5b-9 membrane attack complex (MAC), generating pores on the surface. Additionally, the C3b molecules function as opsonins which bind covalently to pathogens and thereby target them for destruction by phagocytic cells that express C3b receptors on their surfaces (Zipfel and Skerka, 2009).

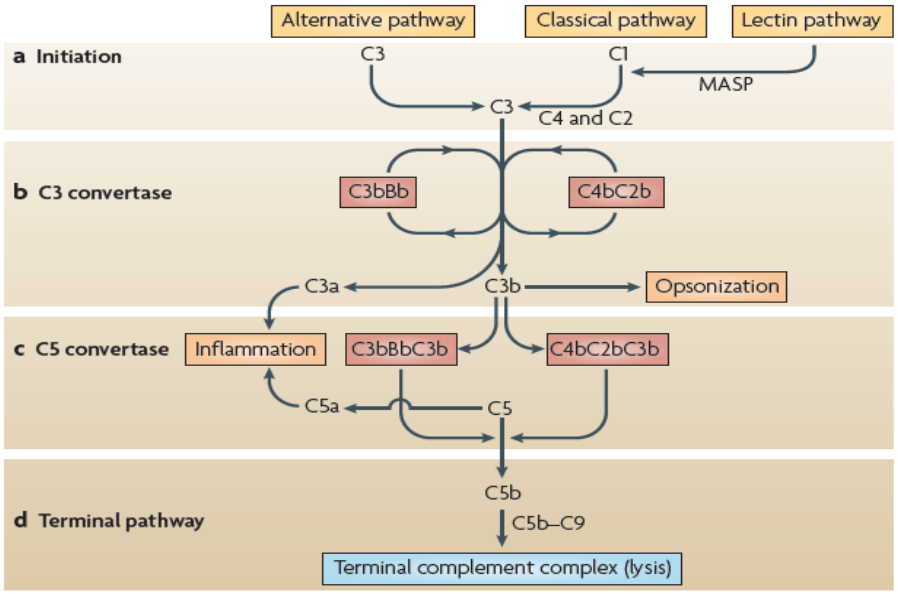

Figure 1.9: The complement pathways: Classical, Mannan-binding Lectin, and Alternative (Zipfel and Skerka, 2009).

1.2.2 Dysregulation of complement regulators and diseases

Complement, which lacks the ability to discriminate between self and non-self requires complement regulators to protect host surfaces from damage during complement activation. The complement system, as a result, gets rid of only modified host cells, or microbial agents. With the cooperation of multiple regulators, activation and inhibition of complement is well-regulated *in vivo*. However, dysfunctional complement regulators (mutation and polymorphisms) may lead to uncontrolled activation and non-specific damage to cells (Zipfel and Skerka, 2009). These defects contribute to several diseases such as the kidney diseases (atypical Haemolytic Uraemic Syndrome [aHUS] and Membranoproliferative Glomerulonephritis Type II [MPGN II]), the retinal disease (Age-related Macular Degeneration [AMD]), Systemic Lupus Erythomatosus (SLE), infectious diseases, etc. (Zipfel and Skerka, 2009). A critical step to control complement activation appears to be in the AP since it is regularly activated. Factor H (CFH) is a major regulator in this pathway and can regulate both in the fluid phase and on cellular membrane surfaces.

The complement factor H family:

The factor H family is composed of CFH protein, CFH-like protein 1 (CFHL1), and CFH-related proteins (CFHRs). Genes encoding for these proteins are located on human chromosome 1q32 within the RCA (the regulator of complement activation) cluster. The gene cluster and the structure of these proteins are shown in figure 1.10.

CFH protein is an abundant plasma protein, the level of which varies widely (116-562 μg/ml) in the population (de Cordoba and de Jorge, 2008). It is constitutively produced by the liver, but also synthesized by epithelial cells, lymphocytes and umbilical vein endothelial cells (Rodriguez de Cordoba et al., 2004). This protein is a 155-kDa single chain glycoprotein, composed of 20 short consensus repeat domains (SCR), each of which contains about 60 amino acids (Rodriguez de Cordoba et al., 2004). The N-terminal end of the protein (SCR 1-4) is a site for co-factor activity of factor I mediated C3b cleavage and decay accelerating activity against C3bBb, whereas the surface binding/cell surface regulation is found in the C-terminal region (SCR 19-20) (Zipfel et al., 1999). CFH protein blocks the formation of C3 convertase by sequestering C3b from factor B and promoting dissociation of these enzymes once formed. It also acts as a co-factor for the factor I-mediated proteolytic cleavage of C3b to inactive C3b (iC3b). This is then fragmented to C3dg and C3c (Pickering and Cook, 2008).

CFHL1 is the product of alternative splicing of CFH gene and produced in the liver similar to CFH. It comprises the first seven N-terminal domains of CFH indicating an ability to regulate complement activity and four amino acids specific to CFHL1 at the C terminus (Jozsi and Zipfel, 2008).

CFHRs are encoded by CFH-related genes which share sequence similarity to CFH in both amino acids and nucleotides (Jozsi and Zipfel, 2008). There are five similar CFHR proteins (CFHR1-5) whose functions are supposed to be similar to CFH, suggesting an overlapping function in the immune system and regulation. A homology alignment shows that CFHR proteins have two major conserved regions located in the N-, and C- termini. The N-termini are related to SCRs 6-9 of CFH,

which mediates binding to heparin and C-reactive protein (CRP). The C-termini of these proteins show an identity in a different degree to the SCRs 19-20 of CFH (Jozsi and Zipfel, 2008). This homology suggests that the C-terminus may be involved in cell surface binding.

CFHR1 contains 5 SCRs and is identified in two glycosylated forms (CFHR1α and CFHR1β). The plasma concentration is about 70-100 μg/ml (Skerka and Zipfel, 2008). CFHR1 is able to bind C3b, suggesting a role in the complement regulation (Skerka and Zipfel, 2008). It can also bind to surfaces of pathogens such as *Borrelia burgdorferi*, *Candida albicans*, *Psuedomonas aeruginosa* and Group A *streptococci* (Skerka and Zipfel, 2008, Jozsi and Zipfel, 2008). In addition, CFHR1 has an ability to block C5a-mediated chemoattraction of neutrophils (Fritsche et al., 2010).

CFHR2 contains 4 SCRs and is identified in two glycosylated forms. The plasma concentration is unidentified and the function has not been determined (Skerka and Zipfel, 2008).

CFHR3 contains 5 SCRs. Multiple glycosylated forms are detected in plasma. The concentration is unidentified. A role of CFHR3 may involve binding to C3b and C3d molecules as well as to heparin (Jozsi and Zipfel, 2008, Skerka and Zipfel, 2008). It is capable of inhibiting activity of C3 convertase and blocking C5a-mediated chemoattraction of neutrophils (Fritsche et al., 2010).

CFHR4 has two separate forms (CFHR4a and CFHR4b). The CHFR4a contains 9 SCRs and the CFHR4b includes 5 SCRs. The plasma concentration is not determined. It may function in complement regulation in the form of a CFH cofactor (Skerka and Zipfel, 2008). It can also bind to C3b and C3d (Jozsi and Zipfel, 2008).

CFHR5 is composed of 9 SCRs. The concentration is undetermined. It is able to bind to heparin, C3b, and CRP (Skerka and Zipfel, 2008, Jozsi and Zipfel, 2008).

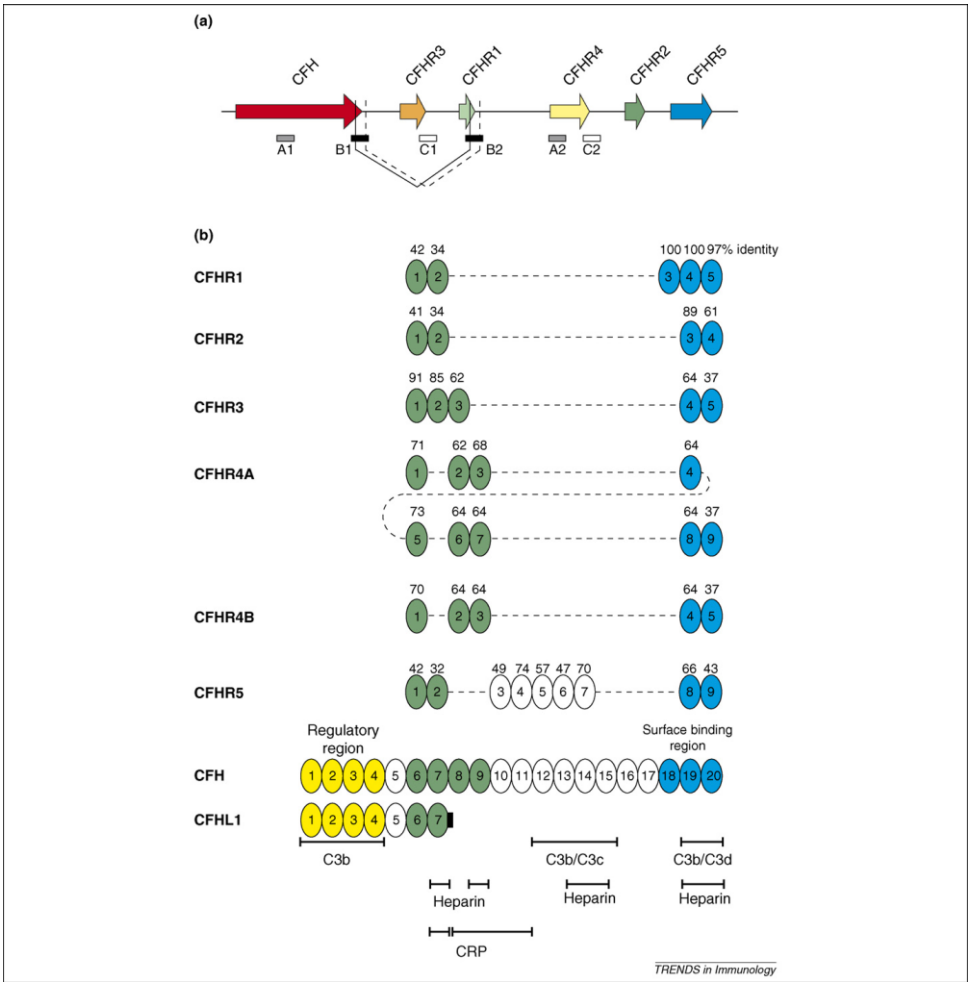

Figure 1.10: The CFH protein family (Jozsi and Zipfel, 2008). A) Arrangement of the CFH and the CFHRs genes. The CFH gene encodes for CFH and CFHL1 whereas the CFHR4 gene encodes for CFHR4a and CFHR4b. The sites of non-allelic recombination are marked by the dot and the solid lines, resulting in a CFH: CFHR1 hybrid gene or a large deletion of CFHR3 and CFHR1 respectively. B) Short consensus repeats (SCRs) of the CFH protein family. The SCRs of CFH share homologous domains with other proteins in the family. The numbers indicated in the SCRs are the numbers of the SCR domain and the numbers indicated above are the identity of the domains compared to those of CFH protein. The protein binding domains of CFH are specified below.

1.2.3 Polymorphisms in the CFH genes associated with diseases

CFH gene cluster

Several polymorphisms/mutations have been identified in the CFH gene. Single nucleotide polymorphisms (SNPs) in exon 2 (CFH-62 V/I, G184A; rs800292) and in exon 9 (CFH-402 Y/H, T1204C; rs1061170) are associated with AMD (de Cordoba and de Jorge, 2008). A SNP in the promoter (CFH C-332T; rs3753394) is associated with Meningococcal disease (MD) (Rodriguez de Cordoba et al., 2004). Recently, CFH C-332/T and CFH-62 V/I have been found to associate with Dengue disease (Pastor et al., 2013). Moreover, missense mutations that cluster within SCR 19-20 are identified in individuals with aHUS (Rodriguez de Cordoba et al., 2004).

So far, the biological function has been largely studied in CFH C-332T, CFH-62 V/I, and CFH-402 Y/H. CFH C-332T is located in the NF-KB binding site, implying that this polymorphism may affect transcriptional processes (Warwicker et al., 1997, Caprioli et al., 2003). CFH-62 I binds to C3b more effectively than CFH-62 V; therefore, it can better compete with FB, leading to reduced complement activation (Tortajada et al., 2009). CFH-402 H has an impaired binding to heparin and C-reactive protein, affecting cell surface binding and inflammation (Laine et al., 2007).

In addition to SNPs, sequence analysis shows repetitive regions between the CFH and the CFHRs, resulting in DNA rearrangement, gene conversion or DNA duplication/deletion. Non-allelic homologous recombination between CFHR1 and CHFR3 causes a deletion between these two genes (ΔCFHR3-1) (Hageman et al., 2006, Spencer et al., 2008) (Figure 1.10 a). An association between this deletion and diseases has been largely studied in aHUS and AMD. This deletion increases the risk of aHUS, while it is protective in AMD (Spencer et al., 2008); however, the reason why the CFHR3-1 deletion differently functions in these two diseases is still ambiguous (Skerka and Zipfel, 2008). Moreover, a novel polymorphic deletion affecting CFHR1 and CFHR4 has been detected in healthy individuals and among patients with aHUS (Moore et al., 2010).

Combination of polymorphisms in complement proteins CFH, Factor B (FB), and C3

An association between diseases and genetic variation in the complement is not only focused on an individual gene, but also in a combination of polymorphisms. Recently, it has been shown that polymorphisms in CFH, FB and C3 (collectively called 'complotype') are associated with a 6-fold increased complement-dependent hemolytic activity *in vitro* (Heurich et al., 2011). FB and C3 are complement activators in the AP while CFH is a regulator. Therefore, alternative complement activation can be influenced by genetic determinants and this evidence has been shown in AMD (Hageman et al., 2005, Gold et al., 2006, Yates et al., 2007). Individuals who have increased susceptibility to AMD show high activation of complotype (FB-32 R/R: rs12614 [C] and rs641153 [G]; CFH-62 V/V: rs800292 [G]; and C3-102 F/F where F is denoted as 'fast electrophoresis allele': rs2230199 [G]) while individuals with the low activation of complotype have a reduced risk of AMD (FB-32 Q/Q rs12614 [C] and rs641153 [A]; CFH-62 I/I: rs800292 [A]; and C3-102 S/S where S is denoted as 'slow electrophoresis allele': rs2230199 [C]). Consistent with these data were the findings that CFH-32 I, FB-32 R can bind to C3b effectively and C3-102 F can increase target cell lysis *in vitro* (Tortajada et al., 2009, Montes et al., 2009, Heurich et al., 2011).

1.2.4 Utilization of CFH by microorganisms

Microbes including bacteria, fungi, and viruses have strategies to evade the immune responses. Recruiting host complement regulators such as CFH is a mechanism which microbes exploit to defend against complement activation. Binding of microbial molecules to CFH have been explored in numerous studies (Figure 1.11).

Several bacteria have been found to bind CFH, restricting killing by human complement. For example, *Borrelia* species, the bacteria that causes Lyme borreliosis, express complement regulator-acquiring surface proteins (CRASP1-5), all of which bind to CFH, CFHL1 and CFHR1 but with different affinity (Jozsi and Zipfel, 2008). Ligands of the *Neisseria* family including porin 1 A (Por1A) and factor H binding protein (fHbp), M protein of *Streptococci*, LenA and LfhA of *Leptospira interrogans* can also interact with CFH (Ngampasutadol et al., 2008, Schneider et al., 2009, Jozsi and Zipfel, 2008). In addition to bacterial ligands, non-structural protein 1 (NS1) of West Nile virus (WNV), a member of the *Flaviviridae* family, can recruit CFH to the surface of infected cells, preventing lysis from complement activation (Chung et al., 2006). Interaction of CFH to glycoproteins (gp120 and gp41) on HIV particles has also been demonstrated (Pinter et al., 1995a, Pinter et al., 1995b).

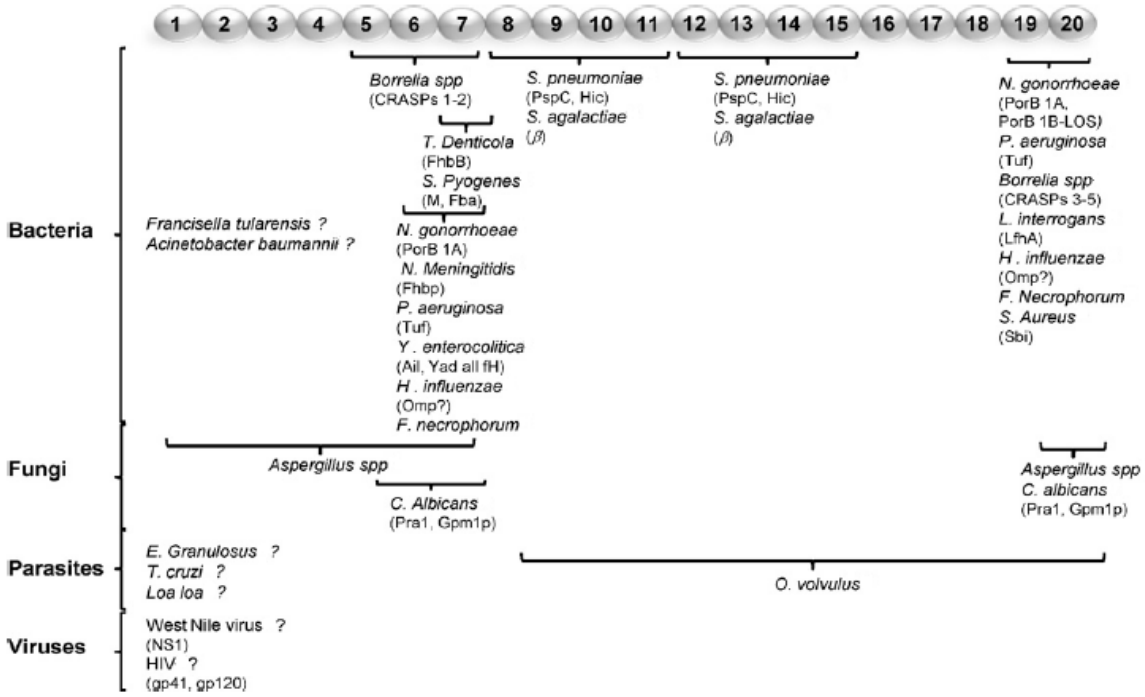

Figure 1.11: Binding of CFH to pathogens and their ligands [Modified from (Ferreira et al., 2010)]. SCR domains of CFH are shown in the numbers 1 to 20. Names in parentheses are the microbes' ligands known to interact with CFH. Unknown interacting domains are indicated by question marks.

1.3 Matrix Metalloproteinases

1.3.1 Matrix Metalloproteinases (MMPs): overview

Matrix metalloproteinases (MMPs) are metallo-endopeptidases (also called matrixins) that require zinc ions in their active sites (Birkedal-Hansen et al., 1993, Parks et al., 2004). The first MMP was discovered in Xenopus' tail in 1961 and the latest MMP was discovered over 40 years later (Greenlee et al., 2007). Mammalian MMPs comprise 24 members and were originally identified based on their substrate specificity such as collagenases (MMP-1, -8, and -13), gelatinases (MMP-2, and -9), stromelysins (MMP-3, -10 and -11), elastases (MMP-7, and -12), and membrane-typed MMPs (MT-MMPs; MMP-14, -15, -16, and -17) (Nagase et al., 2006). The additional number system based on the order of discovery and structural classification were sequentially applied (Greenlee et al., 2007). The list of MMPs is shown in the Figure 1.12.

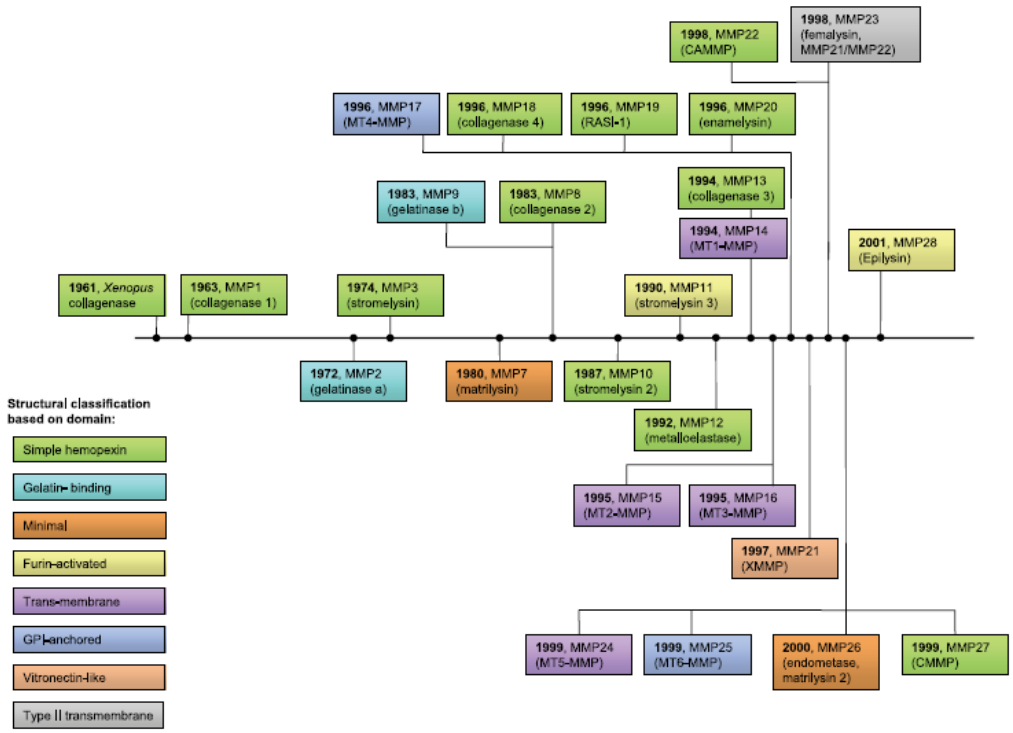

Figure 1.12: The discovery of MMPs (Greenlee et al., 2007). MT-MMP (membrane-type MMP), XMMP (Xenopus MMP), CMMP (Chicken MMP)

1.3.2 General structures of MMPs

The basic structure of MMPs includes a propeptide domain (about 80 amino acids), a catalytic domain (about 170 amino acids), and a C-terminal hemopexin-like domain (about 200 amino acids) (Nagase and Woessner, 1999). The schematic picture of MMPs is shown in Figure 1.13.

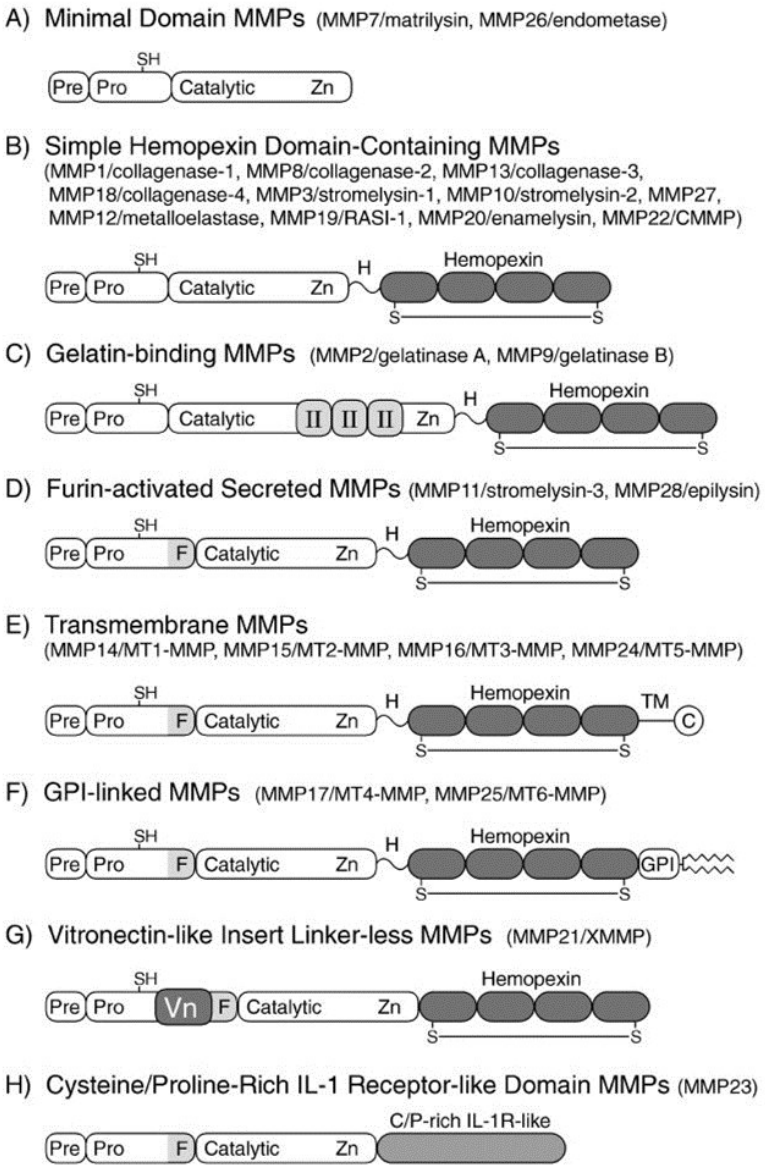

Figure 1.13: Structures of MMPs (Sternlicht and Werb, 2001). Pre, N-terminal signal sequence that is removed after synthesis; Pro, propeptide domain with a zinc-ligating thiol group (SH); F, Furin binding site; Vn, Vitronectin-like domain; catalytic, catalytic domain; Zn, zinc binding domain; II, fibronectin type II domain; H, hinge

region; hemopexin, hemopexin-like domain containing four repeats with the first and last linked by a disulfide bond; TM, transmembrane domain with cytoplasmic tail (C); GPI, a glycophosphatidyl inositol membrane-anchoring signal; C/P, cysteine/proline rich domain; IL-1R-like, interleukin-1 type II receptor-like domain.

The propeptide domain contains a conserved region called 'Cysteine switch motif' (PRCG [V/N] PD). Cysteine in this region can interact with the zinc ion at the zinc binding motif in the catalytic domain, keeping MMP inactive (Nagase et al., 2006). In addition, MMP-11, MMP-21, MMP-23, MMP-28, and MT-MMPs have the conserved sequence (RX [K/R]R) recognized by Furin, leading to activation of MMPs intracellularly (Nagase and Woessner, 1999).

The catalytic domain contains the zinc-binding motif (HEXXHXXGXXH; where X is a variable amino acid) and the "methionine turn" (XXMXP), which is eight residues after the zinc binding motif (Nagase and Woessner, 1999). This methionine is thought to maintain the structure around the zinc-binding site (Nagase et al., 2006). Also, MMP-2 and MMP-9 have an additional three repeats of fibronectin type II (gelatin) domain essential for digestion of collagens and gelatins (Nagase et al., 2006).

The hemopexin-like domain is at the C-terminus of the enzymes. This domain is essential for cleaving triple helical collagens (Nagase et al., 2006). It is also necessary for cell surface activation of pro MMP-2 by MT1-MMP (Nagase et al., 2006). Matrilysins (MMP-7, and MMP-26) and MMP-23 lack this domain (Nagase et al., 2006).

In addition to these three basic structures, there are additional domains in some MMPs such as a transmembrane domain which is important for anchoring the enzyme to the cell surface (Sternlicht and Werb, 2001).

1.3.4 Regulation of MMPs

MMPs activities are tightly regulated in transcriptional and post-transcriptional levels. They are controlled by several factors including growth

hormone, cytokines/chemokines, cell adhesion molecules as well as extracellular matrix (ECM) (Nagase et al., 2006). MMPs are generally expressed low levels in normal conditions and are synthesized as inactive proenzymes but can be transcriptionally induced by inflammatory cytokines e.g. TNF-α, and IL1-β, as well as by cell-cell interaction (R Hanemaaijer, 1993, Cornelius et al., 1995, François Mach, 1999, Lacraz et al., 1994). Exceptions to this are proMMP-8, proMMP-9, and proMMP-25 that are stored in the granules within the neutrophils (Greenlee et al., 2007).

The secreted form of proMMPs can be activated by tissue and plasma proteinases. They can also be activated by organic mercury compounds, low pH, heat and sodium dodecyl sulfate (SDS) *in vitro* (Nagase et al., 1990, Greenlee et al., 2007). These biological and chemical compounds as well as physiological treatments lead to disruption of the cysteine-zinc interaction exposing the catalytic active site, which is further cleaved by MMPs themselves (Nagase et al., 1990, Nagase et al., 1992, Suzuki et al., 1995, Greenlee et al., 2007). Exceptions to this are some MMPs including MMP-11, MMP-28 and MT-MMPs. These MMPs contain Furin recognition sequences and are activated in the trans-golgi network (TGN) by Furin before they reach to the cell surface (Nagase et al., 2006).

Regulation of MMPs is also controlled through compartmentalization (Greenlee et al., 2007). Activation of MMP-2 generally occurs on the cell surface and depends on the expression of MT1-MMP (MMP-14) and TIMP2 (tissue inhibitors of matrix metalloproteinase 2) (Nagase et al., 2006). Hemopexin-like domain of pro-MMP2 binds to the C-terminus of TIMP2. This enzyme-inhibitor complex interacts with two active MT1-MMPs on the cell surface: one MT-MMP acts as a receptor and another one acts as an activator of pro-MMP-2. However, excessive TIMP2 can prevent the binding of the second MT1-MMP, inhibiting the activation process.

Finally, their activities are controlled by two types of endogenous inhibitors: α2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs) (Greenlee et al., 2007). The α2-macroglobulin is an abundant plasma glycoprotein which is able to irreversibly inhibit several types of proteinases including MMPs. By contrast, TIMPs (TIMP1-4) can reversibly inhibit all MMPs tested but with different efficiencies

(Nagase et al., 2006). TIMPs form a non-covalent complex to the catalytic domain of MMP in a 1:1 ratio (Nagase et al., 2006).

1.3.5 MMP substrates

MMPs are able to degrade not only ECMs but also target non-ECM substrates such as growth factors/receptors, cytokines/chemokines, and adhesion molecules (Manicone and McGuire, 2008). Since MMPs can target multiple kinds of proteins, they can influence the abilities, functions, and localization of several proteins. Therefore, abnormalities due to impaired MMPs can be seen in MMP-null mouse strains (Table 1.2; modified from (Manicone and McGuire, 2008)).

Table 1.2: Abnormalities in MMP-null mice

MMP-null mice	Abnormalities
MMP2-null	Impaired eosinophil egression in allergic lung inflammation
	Increased immune-mediated arthritis
	Enhanced myocardial inflammation in TNF-α induced cardiomyopathy
	Enhanced autoimmune encephalomyelitis
	Enhanced experimental colitis
MMP3-null	Decreased neutrophil recruitment in model of acute lung injury
	Decreased neutrophil recruitment in LPS-induced neuroinflammation
	Impaired contact hypersensitivity
	Enhanced collagen-induced arthritis
	Reduced accumulation of macrophages in atherosclerotic plaques
	Reduced macrophage-chemoattractant activity
	Reduced recruitment of CD4+ Lymphocytes into intestinal mucosa
MMP7-null	Reduced neutrophil efflux in bleomycin-induced lung injury
	Lack of activation of TNF-α on macrophages
MMP8-null	Delayed neutrophil recruitment and resolution. Reduced neutrophil apoptosis
	Decreased neutrophil recruitment to dermis surrounding chemical-induced skin tumors
MMP9-null	Reduced inflammation in experimental colitis
	Diminished egression of leukocytes in allergic lung inflammation
	Enhanced airway inflammation in allergic lung inflammation
	Decreased lymphocyte recruitment and mononuclear inflammation in allergic lung inflammation
	Decreased immune-mediated arthritis
	Protected from lethal endotoxic shock
	Prolonged contact hypersensitivity
	Reduced experimental autoimmune encephalomyelitis
	Diminished Dendritic cell migration
	Increased neutrophil recruitment in IL13-induced lung injury
	Impaired macrophage infiltration in atherosclerosis
	Reduced neutrophil influx, mortality and bacterial burden during infection with Francisella Tularensis
MMP10-null	Altered inflammatory responses
MMP12-null	Decreased eosinophil and macrophage recruitment in IL13-induced lung injury
	Reduced macrophage migration and influx in smoke-induced emphysema
	Reduced macrophage recruitment and improved function in spinal cord injury
	Reduced neutrophil influx to alveolar space in acute lung injury
	Reduced release of TNF-α from macrophages after smoke exposure
MMP13-null	Attenuated inflammatory reaction during cholestasis

1.3.5 Roles of MMPs

The role of MMPs in normal conditions has been described in ECM remodeling, embryonic development and morphogenesis (Greenlee et al., 2007). For example, *Drosophila melanogaster1*-MMP (DM1-MMP1) is necessary for fly development as an experiment has shown that flies with mutant DM1-MMP1 protein

are smaller and irregularly shaped (Greenlee et al., 2007). Embryonic development of zebrafish requires MMP-2 (Greenlee et al., 2007). Xenopus MMP-7 (XMMP-7), which is homolog to human MMP-7, and XMMP-9 have been found during embryonic development (Greenlee et al., 2007).

MMPs also function in tissue and wound repair (Gill and Parks, 2008), inflammation and immune response to infection (Manicone and McGuire, 2008, Elkington et al., 2005, Parks et al., 2004). For example, soon after injury IL8 and MMP-7 are secreted from injured epithelial cells. IL-8 then accumulates on the heparan sulfate of syndecan-1, a transmembrane proteoglycan on the surface of epithelial cells, whilst MMP-7 sheds the ectodomain of syndecan-1, releasing the syndecan-1/IL-8 complex leading to recruitment of neutrophils to the site of injury (Parks et al., 2004). MMP-7 is able to cleave Epithelial (E)-cadherin facilitating cell migration and also shed the membrane-bound FAS ligand on the cell surface, inducing apoptosis (Parks et al., 2004). Neutrophil elastase together with MMP-9 is released from activated neutrophils to fight against pathogens such as Gram negative bacteria (Parks et al., 2004). In addition, several MMPs are up-regulated during angiogenesis, suggesting a role in blood vessel formation (Greenlee et al., 2007). Blocking activities of MMPs with the MMP inhibitor also affects organ regeneration in newts, hydra, and zebrafish (Greenlee et al., 2007).

MMPs can be found either in a soluble form or at the cell surface. It has been hypothesized that these two forms may play different roles in their regulating activities. For example, the active cell membrane-bound MMP-9 on the surface of neutrophils resists inhibition by TIMPs although it can cleave ECMs *in vitro* as the soluble MMP-9 (Greenlee et al., 2007). MMP-8 on the surface of activated neutrophils also resists inhibition by TIMPs (Greenlee et al., 2007).

Collectively, MMPs can facilitate migration of immune cells to the sites of infection, regulate permeability, modulate activity of cytokines and chemokines, as well as generate a chemokine gradient (Gill and Parks, 2008, Nagase et al., 2006, Sharma et al., 1996).

1.3.6 MMPs in infectious diseases

Recently, many publications have demonstrated MMPs activities in infectious diseases. The cerebrospinal fluid (CSF) levels of MMP-9 relate to HIV and HTLV-1 infection associated neurological diseases (Sporer et al., 1998, Giraudon et al., 1998). HIV-infected macrophages also increase MMP-2 secretion, which then cleaves CXCL12 (stromal cell derived factor -1; SDF-1) to a neurotoxin (Zhang et al., 2003). MMP-9 mediated blood brain barrier (BBB) permeability can facilitate West Nile virus (WNV) entry to the brain of infected mice (Wang et al., 2008). Up-regulation of MMP-3 and MMP-9 promote cell invasion induced by Epstein Barr virus and Hepatitis B virus (Lan et al., 2013, Balasubramanian et al., 2011, Chung et al., 2004). An increase of plasma MMP-9 is detected in Dengue patients and associated with disease severity (Kubelka et al., 2010). In addition, soluble factors secreted from Dengue infected-dendritic cells increase vascular endothelial cell permeability in an MMP-9 dependent manner and decrease the expression of VE-cadherin and Pecam-1 on endothelial cells (Luplertlop et al., 2006).

Chapter 2

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2.1 Buffers and reagents

3% carboxymethylcellulos (3% CMC; Sigma-Aldrich, USA)

3 g of carboxymethylcellulos in water 100 ml

1.5% CMC

3% CMC 50 ml, 2xMEM (Invitrogen, USA) 45 ml, Fetal Bovine Serum (FBS) 3 ml, streptomycin (10x) 1ml and penicillin (10x) 1ml

3, 3′-Diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, USA)

DAB 0.6 mg, H_2O_2 20µl, and 8% $NiCl_2$ 100 µl in PBS 10 ml

MACS buffer

0.5% FBS, 2 mM EDTA in PBS

FACS wash

2% FBS, 1% Human serum (PAA, Austria) 0.5% BSA (Sigma-Aldrich, USA), 0.5% NaN_3 in PBS

FACS fix solution

1% formaldehyde in PBS

Assay Buffer

50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) Brij-35, pH 7.5 (Filtered with 0.2 micron filter)

Phenylmethanesulfonyl Fluoride (PMSF, M.W. 174.2)

0.2 M stock in 2-propanol

MMP-3 substrate (R&D systems, USA)

2 mM stock in DMSO

Chymotrypsin (Sigma-Aldrich, USA).)
10 mg/ml stock in 1 mM HCl

FITC-dextran (Sigma-Aldrich, USA)
25 mg/ml stock in DMSO

SDS-PAGE gel
10% acrylamide resolving gel: water 1.9 ml, 30% acrylamide 1.7 ml, 1.5 M Tris (pH 8.8) 1.3 ml, 10% SDS 0.05 ml, 10% APS 0.05 ml, TEMED 0.002 ml
5% stacking gel: water 0.68 ml, 30% acrylamide 0.17 ml, 1 M Tris (pH 6.8) 0.13 ml, 10% SDS 0.01 ml, 10% APS 0.01 ml, TEMED 0.001 ml

SDS-PAGE gel loading buffer (4x)
Water 4 ml, 0.5 M Tris-HCl (pH 6.8) 8 ml, SDS 1.6 g, Glycerol 8 ml, bromophenol blue 4 mg

SDS-PAGE running buffer (5x)
Tris 15.1 g, Glycine 94 g, 10% SDS 50 ml, made up to 1L with water

Western blot transfer buffer
24 mM Tris-base, 192 mM Glycine, 20% Methanol

4x non-reducing loading dye
0.02 % Bromphenol blue, 40% Glycerol, 8% SDS, and 250 mM Tris-HCl (pH 6.8)

0.1% PBS-T
1 ml of tween-20 in 1xPBS 1L

ELISA coating buffer
50 mM sodium bicarbonate pH 9.5 (1 tablet of bicarbonate; Sigma Aldrich, USA) in 100 ml water

2N H_2SO_4
1.96 ml of 99% H_2SO_4 made up to 10 ml with water

2.2 Patients and Controls

Patients with Dengue disease were children admitted during acute febrile illness and recruited from Songkla and Khonkan hospitals in Thailand during 2001-2008. Clinical biochemical analysis was conducted on the day of presentation, and follow-up. Viral diagnosis was established using RT-PCR and disease severity was graded according to WHO guidelines: DF and DHF grades 1 to 4. Primary and secondary infection was determined by IgM/IgG ELISA. Patients with other febrile illnesses were those presenting with fever and having undetectable dengue viral genome and an anti-dengue antibody titre lower than the cut off value for an acute-dengue infection. Healthy volunteers were recruited from Siriraj Hospital, Thailand. Heparin and EDTA plasma were aliquotted and kept at -70 °C. Written informed consent was obtained and sample collection approved by the Institutional review board (IRB).

Clinical biochemical analysis was conducted on the day of presentation. The markers assessed included Hematocrit (Hct), white blood cell count (WBC), polymorphic mononuclear cells (PMN); lymphocyte, atypical lymphocyte, monocyte, basophil and eosinophil numbers; platelet count, liver aspartate aminotransaminases (ast), liver alanine aminotransaminases (alt), liver total protein (lft_protein), and liver_albumin (lft_albumin).

The processes of genomic DNA extraction, viral diagnosis, severity classification, identification of primary/secondary infection, and clinical biochemical analysis were performed by staff in Thailand.

2.3 Dengue virus infection *in vitro*

C6/36 cell lines were cultured in 10% FBS, 10% TPB (Sigma-Aldrich, USA) Leibovitz L-15 (Invitrogen, USA) supplemented with streptomycin and penicillin at 28°C. Vero cell lines were maintained in MEM (Invitrogen, USA) supplemented with 10% FBS at 37°C in a CO_2 incubator. Dengue virus serotype 2 strain 16681 (DV2) was propagated in C6/36 cells. Cells were plated for 2 days until 90% confluent. At

the day of infection, cells were maintained in 1.5% FBS, 10% TPB L-15 without antibiotics, and infected with the virus at a multiplicity of infection of 0.01 for 3 hours, then topped up with 1.5% FBS, 10% TPB L-15 to 10 ml in a T75 flask. Infected cells were incubated at 28°C until cytopathic effect (CPE) could be seen. Mock infected cells were used as a negative control.

To titrate the virus in the supernatant, Vero cells ($2.5x10^4$) in a volume of 125µl were plated on each well of a 96-well plate and incubated for 2 days in a CO_2 incubator. On the day of infection, 70µl of media was discarded from the cells. The viral supernatant was 10-fold serially diluted with MEM supplemented with 3% FBS and added into the cells in a volume of 50µl for 2 hours at 37 °C. The infected cells were then overlaid with 125µl of MEM containing 1.5% carboxymethylcellulos (Sigma-Aldrich, USA) and 3% FBS, and incubated for a further 2 days. Infected foci were observed by immunoperoxidase staining. Briefly, the infected cells were washed with PBS three times, fixed with 100 µl of 3.7% formaldehyde for 10 minutes and permeabilized with 100 µl of 2% triton-X 100 for 15 minutes at room temperature. Following washing with PBS three times, 50µl of 4G2 (monoclonal antibody supernatant against Dengue E protein) was added for 1 hour at 37°C. Following washing with PBS three times, 50µl of rabbit anti mouse-HRP (1:1000; DAKO, Denmark) in PBS containing 2% FBS and 0.05% tween-20 were added for 1 hour at 37°C. Following washing with PBS three times, 50µl of DAB (Sigma-Aldrich, USA) was added for 5-10 minutes in the dark or until the infectious foci were clearly visible. The reaction was stopped by discarding the solution and washing with distilled water. Number of foci were counted by ELISpot reader (AID EliSpot Reader Systems, USA) and reported as foci forming units per ml. All the experiments with live virus were performed in the Biosafety level 3 facilities.

2.4 Genotyping

Genomic DNA (25 ng) was amplified by PCR using CERTAMP high fidelity polymerase (Biotools, Spain). Single nucleotide polymorphisms (SNPs), primer sequences, and PCR product sizes are listed in appendix A1. PCR cycling for the factor H (CFH) rs3753394, rs1061170 and rs6677604 variants was 94°C for 3

minutes and 35 cycles of 94°C for 30 seconds, 57°C for 1 minute, and 72°C for 1 minute, followed by 72°C for another 10 minutes. For Factor B (rs12614 and rs641153), the conditions were 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 2 minutes, followed by 72°C for 10 minutes. The PCR conditions for C3 (rs2230199) were 94°C for 2 minutes, 20 cycles of 94°C for 30 seconds, and 1 minute at 65°C which was decreased by 0.5°C every cycle, and another 20 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute, followed by 72°C for another 5 minutes. Following purification using the ChargeSwitch PCR Clean-up kit, (Invitrogen, USA) amplicons were sequenced and the SNP genotype recorded.

The CFH rs800292 variant was determined using a Taqman SNP genotyping assay (#4351379, Applied Bioscience, USA). The reaction was performed by real-time PCR (Applied Bioscience, USA) and the condition used were 95°C for 10 minutes and 40 cycles of 92°C for 15 seconds, and 60°C for 1 minute. Results were analyzed by the Sequence Detection System Software version 2.3 (Applied Bioscience, USA).

2.5 Copy number assays

ΔCFHR3-1 copy number was detected using a combination of (i) Taqman copy number assay probes (Applied Bioscience, USA), (ii) multiplex ligation-dependent probe amplification assay (MLPA, MRC Holland, Netherlands) and (iii) genotyping of rs6677604 (a SNP within intron 12 of the CFH gene that has been shown to tag the ΔCFHR3-1 deletion).

Taqman copy number assay: 40 ng of genomic DNA samples were mixed with Taqman genotyping master mix (Applied Bioscience, USA), Taqman copy number reference RNaseP (#4403328, Applied Bioscience, USA), and Taqman probes specific to either CFHR1 or CFHR3 (Applied Bioscience, USA). The reaction was performed by real-time PCR (Applied Bioscience, USA) and the conditions used were 95°C for 10 minutes and 40 cycles of 92°C for 15 seconds, and 60°C for 1 minute. Results were analyzed by the CopyCaller software (Applied Bioscience, USA). DNA

samples with known copy number were used as the reference samples. Duplicates for each DNA sample were performed.

MLPA: 200 ng of genomic DNA samples were denatured at 98°C for 5 minutes, and hybridized with SALSA MLPA P236 ARMD mix-1 probemix covering for CFH, CFHR3, CFHR1, and CFHR2 (MRC Holland, Netherlands) at 60°C for 16 hours. The hybridized DNA samples were ligated at 54°C for 15 minutes and the temperature was increased to 98°C for 5 minutes. Following the ligation reaction, 10 µl of ligation products were added into the PCR polymerase mix at 60°C and the PCR reaction was started immediately. PCR condition was 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds, followed by 72 °C for 20 minutes. PCR products were mixed with the GeneScan 500 ROX Size Standard (Applied Bioscience, USA) in Hi-Di Formamide (Applied Bioscience, USA) and separated using capillary electrophoresis (3730xl DNA Analyser, Applied Biosystems, USA).

Results were analyzed by Gene Mapper software (Applied Bioscience, USA). The peak heights of the probes amplified in each sample were exported to EXCEL. The heights were normalized by dividing each peak with the total heights of all house keeping probes in each sample. Each normalized height was divided by the average of the normalized heights in the three control samples, which are known to contain two copies of CFHR3 and CFHR1. Copy number variation was determined by these relative heights to the controls. The presence of two copies of genes gave the approximate relative height of 1. Heterozygous deletion (1 copy) gave a relative height of 0.35-0.50, while homologous deletion (0 copies) gave less than 0.35.

2.6 CFH and NS1 interaction assays

To determine if CFH interacted with NS1, ELISA, Far Western and immunopreciptiation techniques were utilized.

ELISA: 5 µg/ml human CFH (QUIDEL, USA) in carbonate buffer was coated on a 96-well MaxiSorp plate (NUNC, Denmark) at 4°C overnight and each well was

washed with 0.1% PBS-T for three times. Following blocking with 5% (w/v) skimmed milk for 2 hour at 37°C and washing with 0.1% PBST for three times, either mock or DV2 supernatant was then added to the wells and incubated at 37°C for one hour. Following washing with 0.1% PBS-T for three times, NS1 was detected by adding 5 µg/ml 1F11 (monoclonal mouse anti-NS1 antibody) for 1 hour at 37°C followed by alkaline phosphatase-conjugated goat anti-mouse antibody (1:10000; Sigma-Aldrich, USA) for 1 hour at 37°C and detection substrate (0.001 mg/ml p-nitrophenylphosphate, BioRad, USA) for 1 hour at room temperature in the dark. The absorbance was measured at 405 nm on the ELISA reader (TECAN, Switzerland).

The reciprocal approach comprised of coating plates with 1F11 (10 µg/ml) in carbonate buffer at 4°C over night. Following washing with 0.1% PBS-T for three times and blocking with 3% BSA for 1 hour at 37°C, either mock or DV2 supernatant was then added to the wells and incubated for one hour at 37°C. Following washing with 0.1% PBS-T for three times, 5µg/ml CFH was added and incubated for one hour at 37°C. CFH was detected using goat anti-human CFH at a dilution of 1:2000 (Quidel, USA) followed by horseradish peroxidase-conjugated mouse anti-goat antibody at 1:30000 (Sigma-Aldrich, USA). Detection substrate (3,3′,5,5′-tetramethylbenzidine; R&D systems, USA) for 30 minutes. Signal development was stopped by addition of 2N H_2SO_4. The absorbance was measured at 450/620 nm on the ELISA reader (TECAN, Switzerland).

Far western blotting: Supernatants from mock treated- or DV2 infected cells, 500 ng of recombinant NS1 (rNS1; Abcams, UK), 500 ng of CFH, or 500 ng of Neisseria Factor H binding protein (fHbp; a gift from Prof. Chris Tang, University of Oxford, UK) were added to non-reducing loading dye, and loaded onto a 10% SDS-PAGE (buffer and reagents section). The gel was run at 100V for 30 minutes and then the voltage was increased to 120V for 1 hour. The gel was transferred onto a nitrocellulose membrane in a semi-dry blotting unit with western blot transfer buffer. After transfer at 0.05A for 1 hour, the membrane was blocked with 5% skimmed milk for 1 hour at room temperature and rinsed with PBS. The membrane was cut into strips before processing further.

The membrane strips of supernatants, rNS1 or fHbp were rinsed with PBS and incubated with 200µl of CFH (5 µg/ml) at 4°C overnight. The membrane strips of

CFH were also applied with 200µl of mock supernatant, DV2 supernatants, or rNS1 (5 µg/ml) at 4°C overnight. Following washing with 0.1% PBS-T for 5 minutes three times, goat anti-human CFH (Quidel, USA) at a dilution of 1:1000, 1F11 (neat supernatant) and mouse anti-fHbp (a gift from Prof. Chris Tang, University of Oxford, UK) at a dilution of 1:1000 were added onto the strips at 4°C overnight in order to detect CFH, NS1 or fHbp, respectively. Following washing with 0.1% PBS-T for 5 minutes three times, either anti goat-HRP (1:10000; Dako, Denmark) or anti mouse-HRP (1:1000; Dako, Denmark) was added onto the strips at room temperature in the dark for 1 hour. Following washing with 0.1% PBS-T for 5 minutes three times, the strips were incubated with western HRP substrate (Millipore, USA) for 5 minutes. Photographic film (Sigma-Aldrich, USA) was used to visualize the signal.

Co-immunoprecipitation: mock and DV2 supernatant were incubated with protein A (Roche, USA), followed by rotating at 4 °C for one hour and centrifuging at 10,000 rpm for 2 minutes. Supernatants were then incubated with 5 µg/ml CFH for 3 hours at 4°C followed by 1F11 (5 µg/ml) for 1 hour. Protein A was then added to the mixture and rotated overnight (4°C). After centrifuging, beads were washed for 5 minutes three times using 0.1% PBS-T and the bound protein eluted using 10µl of 2x non-reducing loading buffer. The eluate was analysed by 12% SDS-PAGE. NS1 and CFH were detected using 1F11 and goat anti-human CFH antibody respectively.

2.7 Multiplex bead assay

Cytokines, chemokines, soluble receptors, growth factors, and matrix metalloproteinases in this study are listed in appendix A2 and measured according to the manufacturer's protocols. Briefly, heparin/EDTA plasma samples as well as cell culture supernatant were diluted as recommended. Any samples that were above the upper standard range were further diluted. Diluted samples, controls, and standards were added to the microparticle mixture for 2 hours at room temperature in the dark on a microplate shaker. Following washing, biotin antibody cocktail was added, and further incubated for 1 hour. Following washing with washing buffer, Streptavidin conjugated with phycoerythrin was added for 30 minutes. Analytes were measured by

Bioplex 200 (Bio-Rad, USA). Data were analyzed by Bioplex manager 4.1.1 software (Bio-Rad, USA).

2.8 Peripheral blood mononuclear cells (PBMCs) isolation and activation

50 ml bloods taken from healthy donors were diluted with plain RPMI 1640 (PAA, Austria) in a 1:1 ratio. 35 ml diluted blood was gently overlaid on top of 15 ml lymphoprep (Nycomed, Norway) in a 50 ml falcon tube, followed by centrifuging at 2000 rpm for 30 minutes at room temperature with no break. PBMCs were drawn off at the interface layer between the serum and lymphoprep using a pastette. They were transferred to a new 50 ml tube, and washed three times with plain RPMI 1640 for 10 minutes at 2000 rpm, 1000 rpm and 1500 rpm, respectively. Cells were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (R10) and counted.

Activated PBMCs were generated by stimulating cells (1-2 x10^6 cells/ml) with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin for 24 hours. Cells were washed with R10 three times before performing an experiment. Activated PBMCs were confirmed with anti-CD69 antibody conjugated with fluorescence (Dako, Denmark) by flow cytometry using a FACSCalibur (BD Biosciences, USA).

2.9 Dendritic cells preparation, infection and co-culture

$CD14^+$ cells were isolated by positive selection from PBMCs using MACS CD14 microbeads (Miltenyi Biotec, Germany). Briefly, PBMCs were centrifuged at 300 g for 10 minutes. Cell pellet was resuspended in 80µl of MACS buffer per 10µl of CD14 microbeads per 10^7 total cells. Cell suspension was rotated at 4 °C for 30 minutes and washed by adding 2 ml buffer per 10^7 total cells prior to centrifugation at 300 g for 10 minutes. Cell pellet was resuspended up to 10^8 in 500µl of buffer, applied on to the column and washed three times with buffer. The magnetically labeled cells were flushed out by adding the appropriate amount of R10. $CD14^+$ cells

were counted and the purity of $CD14^+$ cells was determined by flow cytometry using anti CD14-PE antibody (eBioscience, USA). $CD14^+$ cells at $5x10^5$ cells/ml were cultured in R10 with 20 ng/ml human rGM-CSF (First link, UK), and 25 ng/ml human rIL-4 (eBioscience, USA) for 5 days at 37 ºC in a 5% CO_2 incubator. On day 3, half of the culture media was carefully discarded and replaced with fresh media with GM-CSF at 40ng/ml and IL-4 at 50ng/ml. Immature dendritic cells (DCs) were harvested, counted and confirmed with anti CD83-FITC (BD Biosciences, USA), anti CD14-PE, and anti CD209 (DC-SIGN; R&D systems, USA) followed by anti mouse IgG conjugated with phycoerythrin (anti mouse-PE; Dako, Denmark) by flow cytometry using the FACSCalibur.

For infection of DCs, cells were treated with mock or infected with DV2 for 2 hours at a multiplicity of infection (MOI) of 2. Cells were washed twice with R10 before performing an experiment. Infectivity was determined at 24 hours after infection by the FACSCalibur using 4G2 antibody (1 µg/staining) and anti mouse-PE (0.5 µg/staining). For the kinetic experiment, mock treated- and DV2 infected-cells supernatants were collected at 6, 12, 24, 48, and 72 hours after infection.

Resting/activated PBMCs and/or HUVECs were co-cultured with mock treated or DV2-infected DCs (cell numbers - PBMCs: HUVECs: DCs $2x10^5$: $1x10^5$: $2x10^4$) in a 96-well plate. Cell supernatants were collected at 24 hours after coculture. Infectivity was determined at 24 hours after infection by flow cytometry using 4G2 antibody. For the kinetic experiment, mock treated- and DV2 infected-cells supernatants were collected at 6, 12, 24, 48, and 72 hours after infection.

2.10 HUVEC cell culture and infection

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Lonza, Switzerland) and grown using the EGM bullet kit (Lonza, Switzerland). For infection of HUVECs, $2x10^4$ cells were seeded on a 96-well plate and infected with DV2 at a MOI of 5 for 2 hours. Cells were washed with 100µl of 1x Hank's balanced salt solution (HBSS, Lonza, Switzerland) twice before adding 100µl

of EGM media. Cell supernatants were collected at 24 and 48 hours after infection. Infected cells were observed by immunoperoxidase staining as described above.

2.11 Transwell experiment

HUVECs ($2x10^5$ cells) were seeded in a volume of 500µl on a lower chamber of a 24-well transwell plate (Transwell permeable support 0.4µm; Corning, USA). Activated PBMCs were stimulated with 100 ng/ml PMA and 500 ng/ml ionomycin for 24 hours. On the day of co-incubation, EGM was discarded and replaced with 700µl of R10. $1x10^6$ cells of activated PBMCs or resting PBMCs in 200µl of R10 were added to an upper chamber of the transwell. Supernatants from the lower chamber were collected at 24 hours after incubation. Mixtures of HUVECs and resting PBMCs/activated PBMCs were used as controls.

2.12 Flow cytometry

Cells were harvested from containers and centrifuged at 1500 rpm for 5 minutes to remove supernatant. Following washing once with PBS, antibodies (0.5µg) against CD14-PE (eBioscience, USA), CD83-FITC (BD Biosciences, USA), CD69-FITC (Dako, Denmark), were added directly to the cells for 30 minutes at 4 °C. Isotype controls were used as negative controls. For the CD209 staining, cells were incubated with 1µg mouse anti CD209 for 30 minutes at 4 °C. Following washing with PBS 1ml, anti mouse-PE (0.5 µg/staining) was added to the cells for 1 hour at 4 °C. Cells were washed with PBS and fixed with FACS fix solution.

For intracellular staining of infection, cells were fixed with 3.7% formaldehyde for 30 minutes, washed once with 1x PBS, and permeablized with 0.5% saponin/FACS wash for 15 minutes at room temperature. Following washing, cells were stained with 4G2 (1 µg/staining) for 1 hour at 4 °C and washed with 0.5% saponin/FACS wash. Anti mouse-PE (0.5µg/staining) was subsequently added and incubated for 30 minutes at 4 °C before washing. Cells were analyzed on a FACSCalibur (BD Biosciences, USA).

2.13 Activation of MMP-3

Recombinant human MMP-3 (R&D systems, USA) was activated at 20 μg/ml in an assay buffer containing 5μg/ml chymotrypsin. The reaction was incubated for 30 minutes at 37 ºC, and stopped with PMSF (2mM). Assay buffer without MMP-3 was used as a negative control. Following the activation, MMP-3 was purified with a Zeba spin desalting column (7K MWCO; Thermo Fisher scientific, USA) and eluted with the assay buffer without Brij-35.

To test its activity, 50μl of MMP-3 at 2.5 ng/μl was mixed with 50μl of 20 μM fluorogenic MMP-3 substrate (MCA-Arg-Pro-Lys-Pro-Val-Glu-NVAL-Trp-Arg-Lys(DNP)-NH_2) in assay buffer. Fluorescence signal was read with a fluorescent plate reader (SpectaMax M2; Molecular Devices, USA) in a kinetic mode for 5 minutes at 320 and 405 nm excitation and emission, respectively. Specific activity (pmol/min/μg) was calculated.

$$\text{Specific Activity} = \frac{\text{Rate (RFU/sec) x Conversion factor (pmole/RFU) x 60 sec/min}}{\text{MMP-3 amount (μg)}}$$

To determine the conversion factor (CF), a standard curve was constructed using a peptide fragment linked with only the fluorescence group (MCA-Pro-Leu-OH; Enzo Life Sciences, USA). The peptide fragments were added into a 96-well black well plate at different amounts in assay buffer (0, 40, 80, 120, 160, and 200 pmoles). Relative fluorescence unit (RFU) was read at 320/405 nm. RFU (y-axis) was plotted against the amount of peptide (x-axis) and fitted by linear regression ($y = mx+c$). The slope of the line (m) represents RFU at 1 pmole.

The active form of MMP-3 was confirmed by Western blot. 100 ng of proMMP-3, active MMP-3 and buffer was heated at 95 ºC for 5 minutes before loading onto a 10% reducing SDS-PAGE. Following electrophoresis, proteins on the gel were transferred to the nitrocellulose membrane, which was then blocked with 5% skimmed milk. MMP-3 was detected by mouse anti-human MMP-3 (Abcam, UK) at

a dilution 1:1000 at 4 °C overnight, and anti mouse-HRP (1:1000) for 1 hour at room temperature.

2.14 *In vitro* Endothelial Permeability assay

100µl of HUVECs ($2x10^4$ cells) were seeded on an upper insert assembly of a 96-well polyester membrane transwell plate (Corning, USA), which was pre-coated with 10 µg/cm^2 type I rat tail collagen (Sigma-Aldrich, USA) and 250µl of EGM was added in a lower receiver chamber of the plate. Cells were incubated in a moisture chamber for 3 days. The confluence of the HUVECs monolayer was examined by crystal violet staining. Stained insert was not used for permeability testing. The media from both chambers was carefully removed. Media containing TNF-α at 100 ng/ml (R&D systems, USA), active MMP-3 at the initial concentration of 1 µg/ml, and the assay buffer were added to the cells for 24 hours. EGM media only was added into control wells; negative control, background, and no monolayer. Following completion of permeability treatment, 100µl of 1 mg/ml 70 kDa FITC-dextran diluted in growth media was added in an upper chamber and 250µl of growth medium was added in the receiver plate. The FITC-dextran was allowed to permeate at room temperature for 30 minutes, protected from light. 100µl of media in the lower chamber was removed and transferred to a black 96-well plate. Fluorescence was read at 485 nm and 538 nm excitation and emission, respectively by a fluorescent plate reader. After completion of permeability testing, the cell monolayer was stained for imaging of cell integrity.

2.15 Statistical Analyses

Complement genetics

Hardy-Weinberg equilibrium and Association tests were carried out by using the χ^2 test. The formula is $\chi^2 = \Sigma$ (observed – expected)^2/expected. Observed values were obtained from the numbers of genotypes counted in the experiment, and expected values were calculated according to the Hardy-Weinberg law ($p^2+2pq+q^2$). Logistic regression model was used to adjust odds ratio with sex and age.

Bonferroni's correction was performed by dividing α-level with the total number of polymorphisms compared (n=6) to obtain an adjusted p-value of <0.05/6 = 0.008. The analyses were performed on SPSS version 19.0 (SPSS, Inc, USA).

Biomarkers

Analysis was performed using the R statistical programming package [R Development Core Team 2011 (Team, 2011)] and SPSS version 19 (SPSS, Inc., USA). Mann-Whitney U test and Kruskal-Wallis were used to compare univariates in two and three groups, respectively. To avoid the false positive from multiple-comparison, Bonferroni's correction was performed by dividing α-level with the total number of markers compared (n=66) to obtain an adjusted p value of <0.05/66 = 7.6 x 10^{-3}.

Multivariate analysis was performed by binary logistic regression and classification and regression tree (CART). Of the 66 biomarkers, 35 had > 10% of data missing or fell below the lower detection limit and were subsequently removed from the multivariate analysis. Data below the limitation were imputed to the low limit/2. The dataset was randomly divided by the computer program into a 2/3 training data set and a 1/3 test data set. The use of separate test and training datasets was to avoid the well-known phenomenon of "modeling noise" whereby an overoptimistic estimation would be obtained if predictiveness of a model is assessed using the same data that was used to obtain the model. A predictive model was estimated for each of the two techniques using the training data set and predictions obtained using the model for the test dataset. The predictive ability of the two methods was then compared by using ROC curve methodology on the predicted values.

Matrix Metalloproteinases

Pearson's correlation was performed to test an association between MMPs and peripheral blood cells. Student's T test was performed for *in vitro* studies. Statistical significance was considered at a p-value less than 0.01 as the total number of proteins

interested was 5 (0.05/5 = 0.01). Analysis was performed using SPSS version 19 (SPSS, Inc., USA).

2.16 Binary logistic Regression

The markers with a skewness greater than one, according to the R package "moments" (Novomestky, 2012), were log transformed to achieve roughly symmetric covariate values (KAY, 1987, Grund, 2010 #521) and to avoid the potential influence of outliers. One was added to each biomarker before transforming to avoid zero values being discarded. Logistic regression was employed with DHF/DF as the binary outcome variable. This approximates the log odds of the probability of DHF status using a weighted combination of a subset of predictive biomarkers by backward elimination of non-significant biomarkers using the "lrm" and "fastbw" functions from the R package rms (Jr, 2012). The goodness of fit was assessed by the Le Cessie-van Houwleningen-Copas-Hosmer test (Copas, 1989, Cole, 1991 #524). The model was refitted using ordinary generalized linear model (glm) and the fit assessed using the Hosmer-Lemeshow function.

2.17 Classification and Regression tree modeling (CART)

Classification tree was performed for categorical outcome using R package "rpart"(Ripley, 2011). To measure the purity of the split in which the values are predominately of one outcome, the Gini index was used. The minimal number of observations in a node was set at 10. The tree output for the training data set gave the numbers and proportions of each outcome group in each split. The tree was plotted using the "partykit" package (Zeileis, 2012).

2.18 Receiver operating characteristic (ROC)

ROC curve was performed in R package "ROCR" (Lengauer, 2009). Area under ROC curve (AUC) was used to evaluate the accuracy of the model. The

sensitivity, the specificity and the optimal cutoff of the estimated probability of DHF were obtained by Youden's index which maximizes sensitivity plus specificity.

Chapter3

Results

Complement alternative pathway genetic variation and Dengue infection in the Thai population

3.1 Introduction

3.2 Results

3.2.1 Study population

3.2.2 Complement gene polymorphisms between patients with severe and non-severe Dengue infection in the Thai population

3.2.3 The CFHR3-1 deletion polymorphism and Dengue infection severity

3.2.4 An interaction between Factor H and Dengue NS1

3.3 Discussion

3.1 Introduction

The complement system is an essential part of host defense against flavivirus infection and complement activation occurs during a course of infection. There has been evidence showing that mice knocking out complement elements (C3, C1q, C4, or factor B [FB]) are at risk to get West Nile virus (WNV) infection (Avirutnan et al., 2008). C1q is able to reduce the antibody-dependent enhancement (ADE) of both WNV and Dengue infection, as a result of increase in neutralization activity (Mehlhop et al., 2007). In DHF patients, C3 activation is increased while levels of C4 and FB are reduced (Bokisch et al., 1973). When compared to DF, levels of C3 are decreased while levels of C3a and C3b, fragments of C3 cleavage, are increased in DHF (Nascimento et al., 2009). C5b-9 complexes, together with high levels of Dengue NS1 protein, also correlate with Dengue severity (Avirutnan et al., 2006).

The mechanism by which complement regulates flavivirus infection is not completely known. Complement C4, a classical pathway activation protein, is able to bind Dengue NS1, leading to C4 degradation (Avirutnan et al., 2010). In addition, complement regulatory protein, complement factor H (CFH) can bind to WNV NS1, an interaction that prevent complement activation in a solution and complement-mediated lysis on infected cells (Chung et al., 2006). In contrast, Japanese Encephalitis virus (JEV) NS1 does not interact to CFH and does not influence the host innate immune response (Krishna et al., 2009).

Activation of alternative complement pathway can be influenced by genetic variations in complement proteins C3, FB and CFH (C3-102: rs2230199; FB-32: rs12614 and rs641153; CFH-62: rs800292; collectively called the ‘complotype’); this complotype is associated with a 6-fold variation in complement haemolytic activity *in vitro* (Heurich et al., 2011). That the variation in lytic activity governed by these polymorphisms is biologically relevant was supported by the association of the complotype with age-related macular degeneration (AMD, (Hageman et al., 2005, Gold et al., 2006, Yates et al., 2007)). Increased susceptibility to AMD are found in individuals with the ‘high’ activation’ complotype (FB-32 R/R: rs12614 [C] and rs641153 [G]; CFH-62 V/V: rs800292 [G]; and C3-102 F/F: rs2230199 [G]) whereas

those with the ‘low activation’ complotype have decreased risk (FB-32 Q/Q rs12614 [C] and rs641153 [A]; CFH-62 I/I: rs800292 [A]; and C3-102 S/S: rs2230199 [C]). These data support the findings that serum complement activation can be enhanced in AMD (Reynolds et al., 2009).

Genetic polymorphisms across the region encoding CFH and CFH-related proteins (CFHR) also influence on AMD. These are a polymorphism within CFH (CFH-402 Y/H: rs1061170, (Gold et al., 2006)) and a common deletion polymorphism that affects CFH-related proteins 1 and 3 (ΔCFHR3-1, (Spencer et al., 2008)). Recently, polymorphisms in CFH promoter (-332 C/T, rs3753394) and in CFH-62 (V/V: rs800292 [G]) have been linked to Dengue disease although the function of these polymorphism is not well understood (Pastor et al., 2013). In this study, it was hypothesized that Dengue severity during secondary infection is influenced by these complement genetic polymorphisms. Since the variation in the complotype (CFH rs800292, FB rs12614/rs641153 and C3 rs2230199) has been associated with different degree of complement activation, the relationship between Dengue infection and the complotype was specifically observed. The AMD-associated CFH rs1061170 variant, the CFH promoter rs3753394 variant and the ΔCFHR3-1 deletion polymorphism were also included. Besides, the interaction between Dengue NS1 and CFH protein was investigated. Because both NS1 and CFH have been identified in the extracellular space and on the cell membrane, the interaction between these two proteins was tested by three methods: ELISA, Far western blot, and Co-immunoprecipitation.

Except for CFH rs800292, all single nucleotide polymorphisms (SNPs) in this study were identified by PCR amplification using specific primers and sequencing. The genotypes were then recorded. To detect the CFH rs800292 (CFH-62 V/I: G/A) polymorphism, Taqman SNP genotyping assay was used. The principal of Taqman SNP genotyping is shown in Figure 3.1. Briefly, the first step is PCR amplification using specific primers and two Taqman probes for distinguishing between two different alleles. Each probe contains a reporter dye at the 5’ end (VIC® dye is linked to the allele 1 [A], and FAM™ dye is linked to the allele 2 [G]), a non-fluorescence quencher at the 3’ end and a minor groove binder, which allows greater specificity of

the probe. During PCR, probes specifically anneal to complementary sequences between the primer sites. DNA polymerase, which has 5' nuclease activity, cleaves probes that are hybridized to the target and the reporter dye is therefore separated from the quencher, resulting in increased fluorescence signals. A VIC-dye only represents homozygosity for allele 1 (A/A), a FAM-dye only represents homozygosity for allele 2 (G/G), and both VIC and FAM signals indicate heterozygosity (G/A).

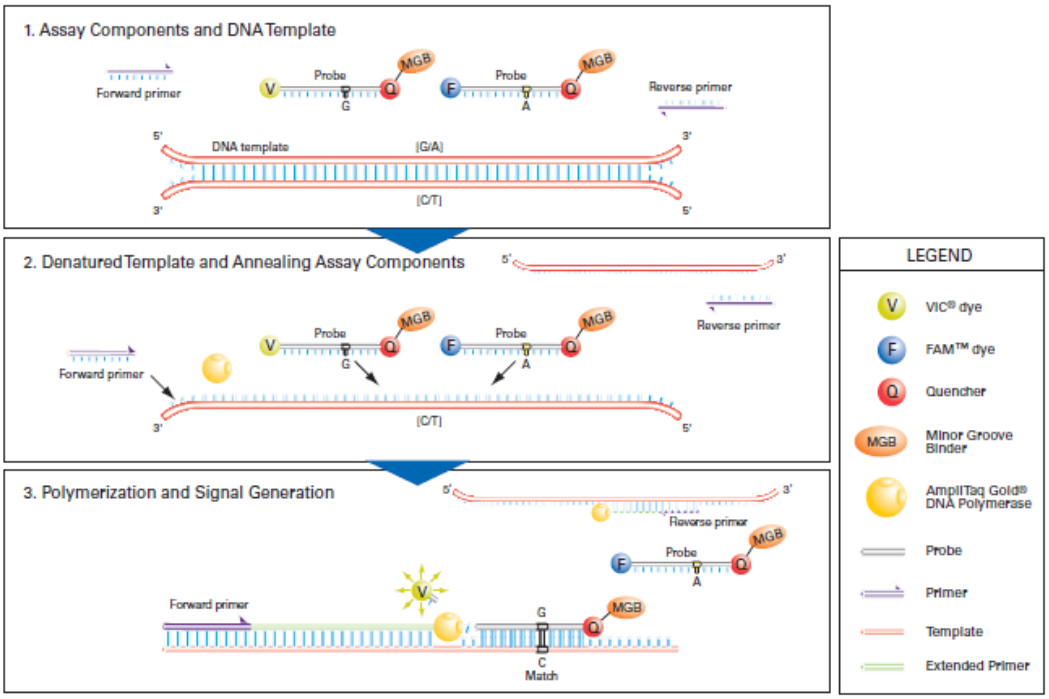


Figure 3.1: The principal of Taqman SNP genotyping assay (Applied Bioscience)

To detect the ΔCFHR3-1 polymorphism, a combination of (i) Taqman copy number assay probes, (ii) genotyping of CFH rs6677604, and (iii) multiplex ligation-dependent probe amplification assay (MLPA) was used.

Taqman copy number assay is a duplex real-time PCR, simultaneously detecting a target gene of interest (CFHR1 or CFHR3) and a reference gene which is known to have a diploid genome (RNAse P) (Figure 3.2). The copy number of the target gene is determined by relative quantitation using the comparative cycle

threshold ($\Delta\Delta C_T$). The C_T is the point where fluorescence signal exceeds the background level. This method measures the C_T difference between the target and the reference gene (ΔC_T), and then compares the ΔC_T in the test samples to the reference samples in which the target genes are known to have 2 copies. A FAM™ dye probe is used to detect the target gene while a VIC® dye-labeled TAMRA™ probe detects the reference gene.

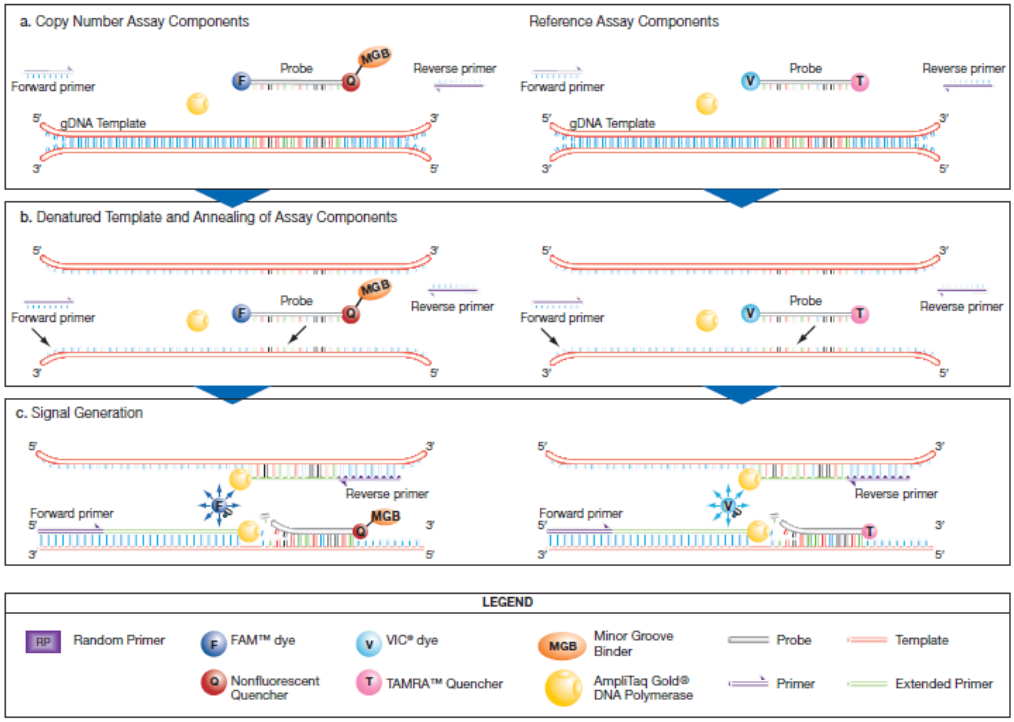


Figure 3.2: The principal of Taqman copy number assay (Applied Bioscience)

The CFH rs6677604 is the SNP within intron 12 of the CFH gene. The allele 'A' has been shown to tag the ΔCFHR3-1 deletion allele and the allele 'G' has been linked to the common CFHR3-1 allele (Gharavi et al., 2011).

The MLPA technology is a multiplex PCR method detecting copy number changes (Figure 3.3). MLPA reactions are divided into five steps: 1) DNA denaturation and MLPA probes hybridization, 2) ligation reaction, 3) PCR amplification, 4) capillary electrophoresis, and 5) data analysis. First, genomic DNA is denatured and hybridized with a mixture of MLPA probes. One set of MLPA

probes includes two oligonucleotides, each carrying the PCR primer sequences (primer X and primer Y; black labelled) and hybridizing sequences (blue labeled). In addition, one oligonucleotide also contains a stuffer sequence (green labeled) which varies in length in order that PCR fragments can be separated by electrophoresis. The two oligonucleotides hybridize to adjacent DNA sequences and can be ligated during the ligation reaction. The ligation products are amplified using only one set of primers and the number of probes ligated reflects the number of targets in the samples. By capillary electrophoresis, the PCR products having unique lengths (113-454 bp) are separated. Finally the peak patterns are generated. The relative peak heights are compared.

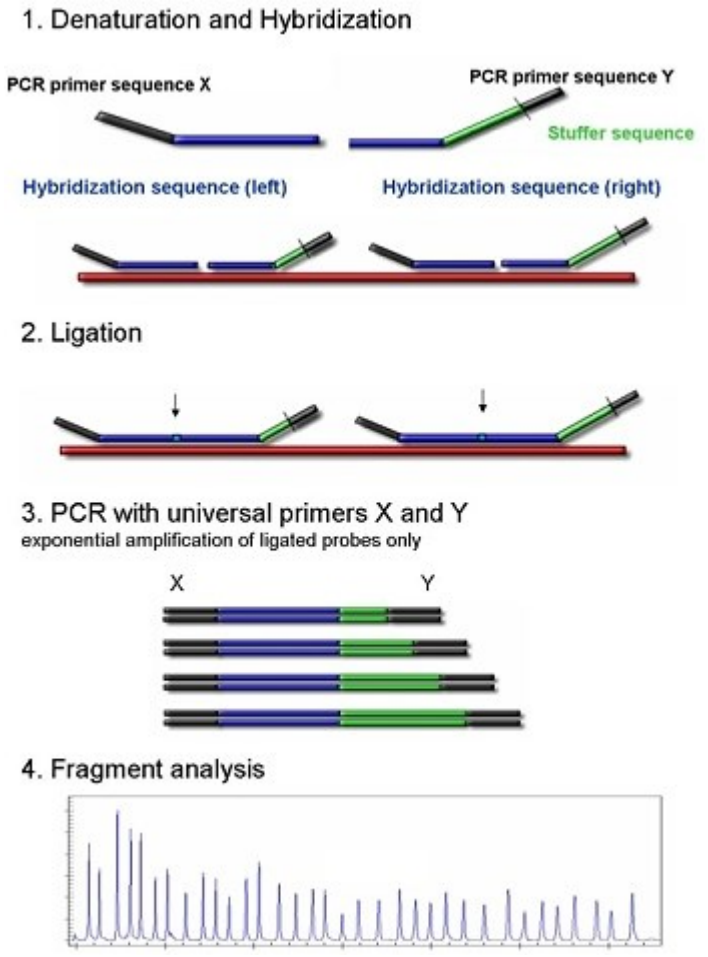


Figure 3.3: Multiplex ligation-dependent probe amplification assay (MLPA) technique (MRC-Holland)

3.2 Results

3.2.1 Study population

The demographics of the study population are shown in Table 3.1. The age of patients with Dengue infection and other febrile infection (OFI) was between 2-15 years while that of healthy controls was 19-47 years. The number of males and females within all groups was somewhat equivalent with the exception of the two females in the DHF4 sub-group. Only patients with secondary Dengue infection were included in this study.

Table 3.1: Study population demographics

		Sex		Age	
Group	**N**	**Male**	**Female**	**Median**	**Range**
Healthy	22	9	13	21	19-47
Other febrile illnesses (OFI)	63	28	35	8	2-15
DF	121	61	60	10	3-15
DHF1	68	35	33	9.5	2-15
DHF2	76	47	29	11	5-15
DHF3	41	19	22	10	4-15
DHF4	2	0	2	7.5	5-10

Healthy and other febrile illness (OFI) sub-groups were utilised as non-Dengue controls.

DF= Dengue fever; DHF1-4 = Dengue Hemorrhagic fever 1-4

3.2.2 Complement gene polymorphisms between patients with severe and non-severe Dengue infection in the Thai population

The individual SNP frequencies in FB (rs12614, rs641153), C3 (rs2230199) and CFH (rs3753394, rs1061170, and rs800292) were identified. The SNP frequencies did not differ between the patients with Dengue infection and the non-Dengue controls (healthy controls and individuals with other febrile illness [OFI]) (Table 3.2). Next, the SNP frequencies between Dengue infection sub-groups defined as those with DF (n = 121) and those with DHF (n = 187) were examined (Table 3.3). The risk of patients with the genotype TT (the CFH rs1061170) getting DHF was about two times the risk of patients with the other genotypes (CT + CC) getting DHF (adjusted OR =2.03; 95% CI = 1.15-3.60; Table 3.3). Although the odd ratio and the 95% CI suggested an association between this genotype and getting DHF but the allele frequencies at the CFH rs1061170 in patients with DF or those with DHF were not significantly different at p-value of 0.008 (p-value = 0.01; Table 3.3; see section 2.15 in Materials and Methods for the adjusted p-value) suggesting that the allele T was not associated with DHF. No differences in other SNPs were associated with the Dengue infection (Table 3.3). Nor did genotypes and alleles between patients with shock (n = 43, defined as DHF3 and DHF4) and without shock (n= 144, defined as DHF1 and DHF2; Table 3.3) differ.

Of note, the minor allele frequencies of C3 rs2230199 and the adjacent rs12614 and rs641153 FB variants were low (C3: 2% and FB: <10%). This is in contrast to the minor allele frequency of the CFH rs800292 polymorphism which was about 42% in this population but its frequency was similar in Dengue sup-groups. Since variations at the C3 rs2230199, FB rs12614 and FB rs641153 were rare, patients with either the most (C3F/F, FB-32R [C/G at the adjacent rs12614/rs641153] and CFH 62V) or least (C3S/S, FB-32Q [C/A at the adjacent rs12614/rs641153] and CFH 62I) active complotype described *in vitro* could not be observed (Heurich et al., 2011) (Table 3.4).

Table 3.2: Relationship of complement gene polymorphisms between Dengue infection and controls

Locus of SNP	Genotype	Phenotype[a]	All Dengue infection	Controls	OFI	Healthy	P-value[b]
CFH rs1061170	TT	402 Tyr	247 (80.2)	68 (80.0)	51 (81.0)	17 (77.3)	
	CT	402 Tyr/His	57 (18.5)	16 (18.8)	11 (17.5)	5 (22.7)	
	CC	402 His	4 (1.3)	1 (1.2)	1 (1.6)	0 (0.0)	
	Frequency T:C		0.89:0.11 N = 308	0.89:0.11 N = 85	0.90:0.10 N = 63	0.89:0.11 N = 22	1
CFH rs3753394	CC	-	84 (27.4)	22 (25.9)	16 (25.4)	6 (27.3)	
	CT	-	154 (50.2)	41 (48.2)	30 (47.6)	11 (50.0)	
	TT	-	69 (22.5)	22 (25.9)	17 (27.0)	5 (22.7)	
	Frequency C:T		0.52:0.48 N = 307	0.50:0.50 N = 85	0.49:0.51 N = 63	0.52:0.48 N = 22	0.60
CFH rs800292	GG	62 Val	104 (34)	32 (37.6)	22 (34.9)	10 (45.5)	
	GA	62 Val/Ile	150 (49)	41 (48.2)	32 (50.8)	9 (40.9)	
	AA	62 Ile	52 (17)	12 (14.1)	9 (14.3)	3 (13.6)	
	Frequency G:A		0.58:0.42 N = 306	0.62:0.38 N = 85	0.60:0.40 N = 63	0.66:0.34 N = 22	0.48
FB rs12614	CC	-	270 (88.8)	77 (90.6)	57 (90.5)	20 (90.9)	
	CT	-	33 (10.9)	8 (9.4)	6 (9.5)	2 (9.1)	
	TT	-	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	
	Frequency C:T		0.94:0.06 N = 304	0.95:0.05 N = 85	0.95:0.05 N =63	0.95:0.05 N = 22	0.71
FB rs641153	GG	-	252 (83.2)	69 (81.2)	50 (79.4)	19 (86.4)	
	GA	-	50 (16.5)	16 (18.8)	13 (20.6)	3 (13.6)	
	AA	-	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	
	Frequency G:A		0.91:0.09 N = 303	0.91:0.09 N = 85	0.90:0.10 N = 63	0.93:0.07 N = 22	0.64
C3 rs2230199	CC	102 Arg (C3S)	300 (98)	83 (97.6)	61 (96.8)	22 (100.0)	
	CG	102 Arg/Gly (C3S/C3F)	2 (0.7	2 (2.4)	2 (3.2)	0 (0.0)	
	GG	102 Gly (C3F)	4 (1.3)	0 (0.0)	0 (0.0)	0 (0.0)	
	Frequency C:G		0.98:0.02 N = 306	0.98:0.02 N =85	0.98:0.20 N = 63	1.00:0.00 N = 22	1

Data shown are the number (percentage) of patients with Dengue fever (DF) or Dengue Hemorrhagic fever (DHF), with genotypic variations and allelic frequency ratio of each complement gene.; SNP, single nucleotide polymorphism

a) number e.g. 402 refers to amino acid number where methionine = 1, C3S and C3F refer to 'slow' and 'fast' alleles; b) Dengue vs controls

Table 3.3: Relationship between complement gene polymorphisms and Dengue infection severity

Locus of SNP	Genotype	Phenotype[a]	All Dengue infection	DF	DHF	P-value[c]	OR (95%CI)	Adjusted OR (95%CI)[b]	DHF without shock	DHF with shock	P-value[d]	OR (95%CI)	Adjusted OR (95%CI)[b]
CFH rs1061170	TT	402 Tyr	247 (80.2)	89 (73.6)	158 (84.5)		1.96 (1.11-3.45)	2.03 (1.15-3.60)	121 (84.0)	37 (86.0)		1.17 (0.44-3.10)	0.99 (0.36-2.70)
	CT	402 Tyr/His	57 (18.5)	29 (24.0)	28 (15.0)		0.56 (0.31-1.00)	0.53 (0.30-0.96)	22 (15.3)	6 (14.0)		0.90 (0.34-2.38)	1.06 (0.39-2.1)
	CC	402 His	4 (1.3)	3 (2.5)	1 (0.5)		0.21 (0.02-2.06)	0.24 (0.02-2.32)	1 (0.7)	0 (0.0)		-	-
	Frequency T:C		0.89:0.11 N =308	0.86:0.14 N =121	0.92:0.08 N =187	0.01	2.01 (1.19-3.38)	-	0.92:0.08 N =144	0.93:0.07 N =43	1	1.16 (0.46-2.95)	-
CFH rs3753394	CC	-	84 (27.4)	36 (29.8)	48 (25.8)		0.82 (0.49-1.37)	0.79 (1.47-1.32)	34 (23.8)	14 (32.6)		1.55 (0.74-3.26)	1.66 (0.77-3.57)
	CT	-	154 (50.2)	65 (54.2)	89 (47.8)		0.79 (0.50-1.25)	0.80 (0.51-1.28)	69 (48.3)	20 (46.5)		0.93 (0.47-1.85)	0.92 (0.46-1.84)
	TT	-	69 (22.5)	20 (16.7)	49 (26.3)		1.81 (1.01-3.23)	1.83 (1.02-3.28)	40 (27.9)	9 (20.9)		0.68 (0.30-1.55)	0.66 (0.29-1.50)
	Frequency C:T		0.52:0.48 N =307	0.57:0.43 N =121	0.50:0.50 N =186	0.1	0.76 (0.55-1.05)	-	0.48:0.52 N =143	0.56:0.44 N =43	0.22	1.37 (0.85-2.23)	-
CFH rs800292	GG	62 Val	104 (34)	33 (27.7)	71 (38.0)		1.60 (0.97-2.63)	1.61 (0.98-2.66)	59 (41.0)	12 (27.9)		0.56 (0.27-1.17)	0.57 (0.27-1.22)
	GA	62 Val/Ile	150 (49)	65 (54.6)	85 (45.5)		0.69 (0.44-1.10)	0.70 (0.44-1.11)	63 (43.8)	22 (51.2)		0.74 (0.38-1.47)	0.78 (0.39-1.56)
	AA	62 Ile	52 (17)	21 (17.6)	31 (16.6)		0.93 (0.50-1.70)	0.90 (0.49-1.66)	22 (15.3)	9 (20.9)		1.47 (0.62-3.48)	1.54 (0.64-3.71)
	Frequency G:A		0.58:0.42 N =306	0.55:0.45 N =119	0.61:0.39 N =187	0.18	1.26 (0.91-1.75)	-	0.63:0.37 N =144	0.53:0.47 N =43	0.13	0.68 (0.42-1.11)	-
FB rs12614	CC	-	270 (88.8)	105 (87.5)	165 (89.7)		1.24 (0.60-2.55)	1.21 (0.59-2.50)	126 (88.7)	39 (92.9)		1.65 (0.46-5.96)	1.64 (0.45-6.02)
	CT	-	33 (10.9)	15 (12.5)	18 (9.8)		0.76 (0.37-1.57)	0.78 (0.37-1.61)	15 (10.6)	3 (7.1)		0.65 (0.18-2.37)	0.67 (0.18-2.46)
	TT	-	1 (0.3)	0 (0.0)	1 (0.5)		-	-	1 (0.7)	0 (0.0)		-	-
	Frequency C:T	-	0.94:0.06 N = 304	0.94:0.06 N =120	0.95:0.05 N = 184	0.72	1.16 (0.58-2.31)	-	0.94:0.06 N =142	0.96:0.04 N =42	0.58	1.72 (0.49-6.01)	-
FB rs641153	GG	-	252 (83.2)	99 (83.2)	153 (83.2)		1.00 (0.54-1.85)	1.04 (0.56-1.93)	120 (84.5)	33 (78.6)		0.67 (0.28-1.60)	0.65 (0.27-1.56)
	GA	-	50 (16.5)	20 (16.8)	30 (16.3)		0.96 (0.52-1.79)	0.92 (0.50-1.73)	22 (15.5)	8 (19.0)		1.28 (0.53-3.14)	1.34 (0.54-3.31)
	AA	-	1 (0.3)	0 (0.0)	1 (0.5)		-	-	0 (0.0)	1 (2.4)		-	-
	Frequency G:A		0.91:0.09 N =303	0.92:0.08 N =119	0.91:0.09 N =184	1	0.96 (0.54-1.73)	-	0.92:0.08 N =142	0.88:0.12 N =42	0.27	0.62 (0.28-1.37)	-
C3 rs2230199	CC	102 Arg (C3S)	300 (98)	118 (99.2)	182(97.3)		0.31 (0.04-2.67)	0.30 (0.03-2.63)	139 (96.5)	43 (100)		-	-
	CG	102 Arg/Gly (C3S/C3F)	2 (0.7)	1 (0.8)	1 (0.5)		0.63 (0.04-10.24)	0.55 (0.03-9.08)	1 (0.7)	0 (0.0)		-	-
	GG	102 Gly (C3F)	4 (1.3)	0 (0.0)	4 (2.2)		-	-	4 (2.8)	0 (0.0)		-	-
	Frequency C:G		0.98:0.02 N =306	1.00:0.00 N =119	0.98:0.02 N =187	0.1	0.17 (0.02-1.36)	-	0.97:0.03 N =144	1.00:0.00 N =43	0.13	-	-

Data shown are the number (percentage) of patients with Dengue fever (DF) or Dengue Hemorrhagic fever (DHF), with genotypic variations and allelic frequency ratio of each complement gene.; OR, Odds ratio; SNP, single nucleotide polymorphism

a) Number e.g. 402 refers to amino acid number where Methionine = 1, C3S and C3F refer to 'slow' and 'fast' alleles; b) Adjusted for Sex, and Age at the presence of Dengue disease by Logistic Regression; c) DF vs DHF; d) no shock vs shock;

Table 3.4: Relationship between complotype and Dengue infection severity

Complotype	Complement activation	DF (n = 115)	DHF (n = 184)	P-value[c]	OR (95%CI)	Adjusted OR[b] (95%CI)	DHF without shock (n=142)	DHF with shock (n=42)	P-value[d]	OR (95%CI)	Adjusted OR[b] (95%CI)
Combined polymorphism[a]											
FB32-R/FH62-V/C3F	High	0 (0.0)	0 (0.0)	-	-	-	0 (0.0)	0 (0.0)	-	-	-
FB-32R/FH-62V	High	25 (21.7)	53 (28.8)	0.22	1.46 (0.84-2.51)	1.46 (0.84-2.52)	45 (31.7)	8 (19.0)	0.12	0.51 (0.22-1.18)	0.51 (0.22-1.20)
FB32-R/C3F	High	0 (0.0)	4 (2.2)	0.16	-	-	4 (2.8)	0 (0.0)	0.56	-	-
FB32-Q/FH62-I/C3S	Low	0 (0.0)	0 (0.0)	-	-	-	0 (0.0)	0 (0.0)	-	-	-
FH62-I/C3S	Low	20 (17.4)	29 (15.8)	0.75	0.89 (0.48-1.66)	0.87 (0.46-1.63)	20 (14.1)	8 (19.0)	0.47	1.46 (0.84-2.51)	1.46 (0.84-2.52)
FB32-Q/C3S	Low	0 (0.0)	1 (0.5)	1	-	-	0 (0.0)	1 (2.3)	0.23	-	-

Data are expressed as number (percentage) of patients, DF - Dengue fever, DHF - Dengue Hemorrhagic fever, OR - odds ratio. a) Number refers to amino acid number where Methionine = 1.

FB32-R and FB32-Q refer to 'Arginine' and 'Glutamine'. FH62-V and FH62-I refer to 'Valine' and 'Isoleucine'. C3S and C3F refer to 'slow' and 'fast' alleles. b) Adjusted for sex and age at presentation using logistic regression, c DF vs. DHF and d no shock vs. shock.

Next, to determine if the SNPs satisfy the Hardy-Weinberg equilibrium (HWE), the condition that the genotype frequencies in a population remain constant over the generations, a chi-square test was performed. The observed and expected genotype frequencies were detected. The observed and expected counts of all SNPs, except the C3 rs2230199, were equivalent, indicating that these SNPs were in HWE (Table 3.5).

Table 3.5: Hardy Weinberg Equilibrium (HWE)

SNPs	Genotype	Observed counts	Expected counts	X^2 value	HWE P-value
CFH rs1061170	CC	5	4.38		
	CT	73	74.24		
	TT	315	314.38		
	Frequency T:C	0.89:0.11		0.11	0.74
CFH rs3753394	CC	106	105.64		
	CT	195	195.71		
	TT	91	90.64		
	Frequency C:T	0.52:0.48		0.01	0.94
CFH rs800292	GG	136	137.06		
	GA	191	188.87		
	AA	64	65.06		
	Frequency G:A	0.59:0.41		0.05	0.82
FB rs12614	CC	347	347.19		
	CT	41	40.62		
	TT	1	1.19		
	Frequency C:T	0.94:0.06		0.03	0.85
FB rs641153	GG	321	322.98		
	GA	66	62.04		
	AA	1	2.98		
	Frequency G:A	0.91:0.09		1.58	0.21
C3 rs2230199	CC	383	379.09		
	CG	4	11.82		
	GG	4	0.09		
	Frequency C:G	0.98:0.02		171.08	< 0.0001

The expected genotype frequencies are computed from Hardy Weinberg equation $n(p^2+2pq+q^2)$

where p and q are the allele frequencies and n is the total number of genotypes

$x^2 = \Sigma$ (observed counts– expected counts)^2 /expected counts

H_0: the population is in Hardy–Weinberg equillibrium (HWE); H_1: the population is not in HWE

H_0 is rejected when p-value < 0.05 at degree of freedom 1

3.2.3 The CFHR3-1 deletion polymorphism and Dengue infection severity

Since genetic polymorphisms across the region encoding CFH and CFH-related proteins (CFHR) influence on AMD, it was interesting to know if the CFHR3-1 deletion polymorphim was associated with Dengue infection. The CFHR3-1 deletion polymorphism was identified using Taqman CNV assay. The unusual polymorphisms were confirmed by tag SNP (CFH rs6677604) and MLPA. The result is shown in Table 3.6. Most individuals contained two copies of CHFR3-1 (n= 321). In this analysis one individual with 3 copies of CFHR3-1 was noted, suggesting duplication of these genes on one allele. Moreover, there were nine individuals presenting 2 copies of the *CFHR3* gene with heterozygous deletion of the *CFHR1* gene. This might be due to the presence of an allele in which there is deletion of both *CFHR1* and *CFHR4* genes (Δ*CFHR4-1*). Individuals with either 3 copies of CFHR3-1 or CFHR1 heterozygote showed the G/G genotype by the tag SNP (the CFH rs6677604).

The CFHR3-1 deletion homozygote (0 copies) was detected in 3 individuals whilst the single copy of CFHR3-1 was present in 45 individuals. The A/A genotype occurred in 3 of 3 (100%) subjects with the Δ*CFHR3-1* deletion and the G/A genotype was found in all 45 individuals (100%) with heterozygosity for the combined deletion. However, there was one subject in whom a single copy of CFHR3-1 was detected by both Taqman CNV assay and MLPA; however the tag SNP (the CFH rs6677604) showed the G/G genotype. This subject was excluded from the analysis.

The Δ*CFHR3-1* allele frequency in the controls (healthy and OFIs) and the patients with Dengue infection was 6.79% and 6.94% respectively and did not significantly differ between these two groups or between sub-groupings of Dengue infection. This means that a copy number variation in CFHRs is not associated with Dengue infection.

Table 3.6: Copy number variation in *CFHR3* and *CFHR1* genes and Dengue virus infection severity

Sample:	N	*CFHR3-1* 100% (2 copies)	*CFHR3-1* 50% (1 copy)	*CFHR3-1* 0% (0 copy)	P-value	*CHFR1-CFHR3* deletion *ΔCFHR3-1* allele frequency (%)	*CHFR3-1* 150% (3 copies)	*CFHR1* 50% (1 copy)
Controls	82	70	11	0		6.79	0	1
Dengue	297	251	34	3	1.00[a]	6.94	1	8
DF	113	95	14	0		6.42	0	4
DHFs	184	156	20	3	0.74[b]	7.26	1	4
No Shock	142	118	16	3		8.03	1	4
Shock	42	38	4	0	0.47[c]	4.76	0	0

Controls include healthy controls and other febrile illness (OFI)

150% = 3 copies of *CFHR3-1*, 100% = 2 copies of *CHFR3-1*; 50% = 1 copy of *CHFR3*-1; 0% = no copies of the *CFHR3-1* genes

ΔCFHR3-1 allele frequency is the percentage of *ΔCFHR3-1* alleles divided by total *CFHR3-1* alleles

[a] Dengue vs. Controls; [b] DF vs. DHF; [c] No Shock vs. Shock

3.2.4 An interaction between Factor H and Dengue NS1

An interaction between Dengue NS1 protein and Factor H (CFH) was examined. First, the interaction was tested by ELISA. CFH protein was coated onto the plate, and incubated with either mock infected or Dengue virus 2 (DV2) supernatant, the latter of which contains soluble NS1 protein. The CFH-NS1 association was detected by anti-NS1 antibody. As shown in Figure 3.4A, the signal was low to undetectable in both mock, and DV2 supernatant, implying that there is no interaction between NS1 and CFH. The result was similar in a reciprocal way when anti-NS1 antibody was coated onto the plate to capture NS1, followed by the addition of CFH protein, which was then detected by anti-CFH antibody (Figure 3.4B).

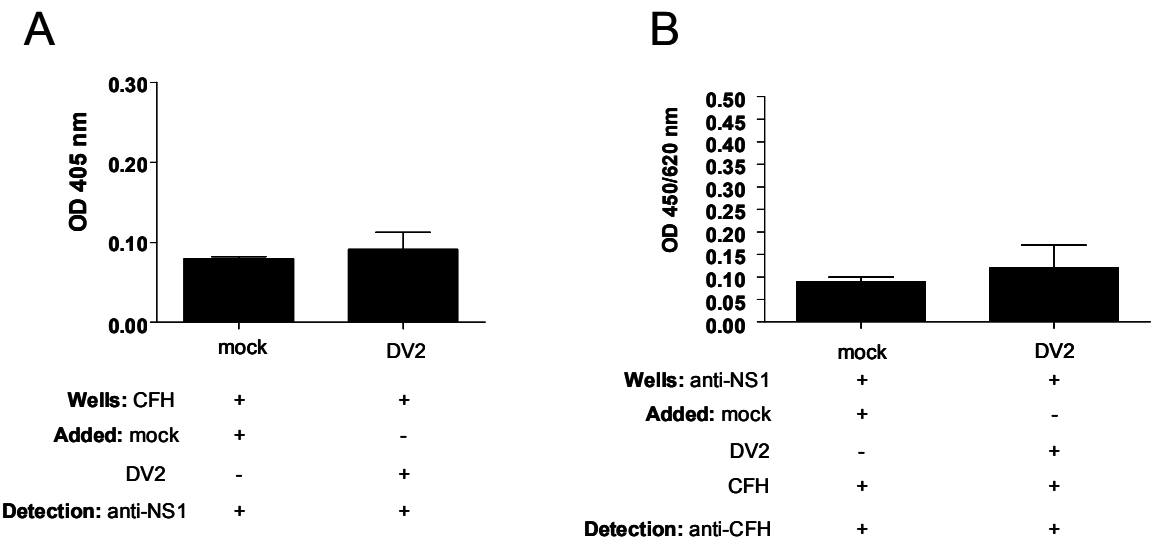


Figure 3.4: An interaction between CFH and NS1 A) ELISA: supernatants were added onto the plated coated with human CFH, and detected with anti-NS1 antibody. B) anti-NS1 antibody was coated onto the ELISA plate; supernatants and CFH were sequentially added and anti-CFH antibody was used as a detection antibody. The experiments were performed three times independently.

Next an interaction between these two proteins was investigated by Far western blot. The utility of the system was confirmed by using the binding between CFH and *Neisseria* factor H binding protein (fHBp). The fHBp was loaded onto the gel and blotted onto the membrane, which was then incubated with CFH followed by anti-CFH antibody. An interaction of these two proteins was detected (Figure 3.5). Next, an interaction between CFH and Dengue NS1 was tested (Figure 3.6). CFH proteins were loaded onto the gel, transferred to the membrane, which was incubated with either supernatants or recombinant NS1 protein and detected with anti-NS1 antibody (Figure 3.6A; lane 1-4) or anti-CFH antibody (Figure 3.6A; lane 5-8). The results showed that CFH could not bind to either supernatants or rNS1. Similar results were shown in a reciprocal way (Figure 3.6B and 3.6C).

Finally, testing for binding between NS1 and CFH was performed by co-immunoprecipitation (Figure 3.7). Supernatants were incubated with CFH, and pulled down by anti-NS1 antibody. Anti-CFH antibody was used to detect CFH in the Western blot. To ensure that any NS1 protein was the result of the pull-down, the membrane was probed with anti-NS1 antibody. Yet again, no association between these two proteins was found.

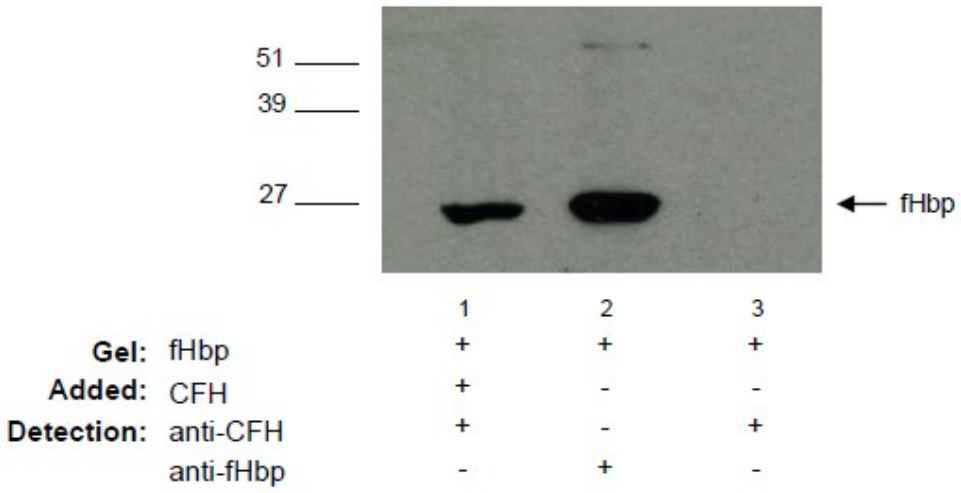


Figure 3.5: Far western blot between *Neisseria* complement factor H binding protein (fHbp) and complement factor H (CFH). The fHbp protein was loaded onto the gel and transferred to a membrane which was then incubated with human CFH (lane 1) or buffer (lanes 2 and 3). The interaction between gel-immobilised fHbp and the added CFH was detected using an anti-CFH antibody (lane 1). Gel-immobilised fHbp was visualized using an anti-fHbp antibody (lane 2). The anti-CFH antibody did not recognize gel-immobilised fHbp in the absence of CFH (lane 3).

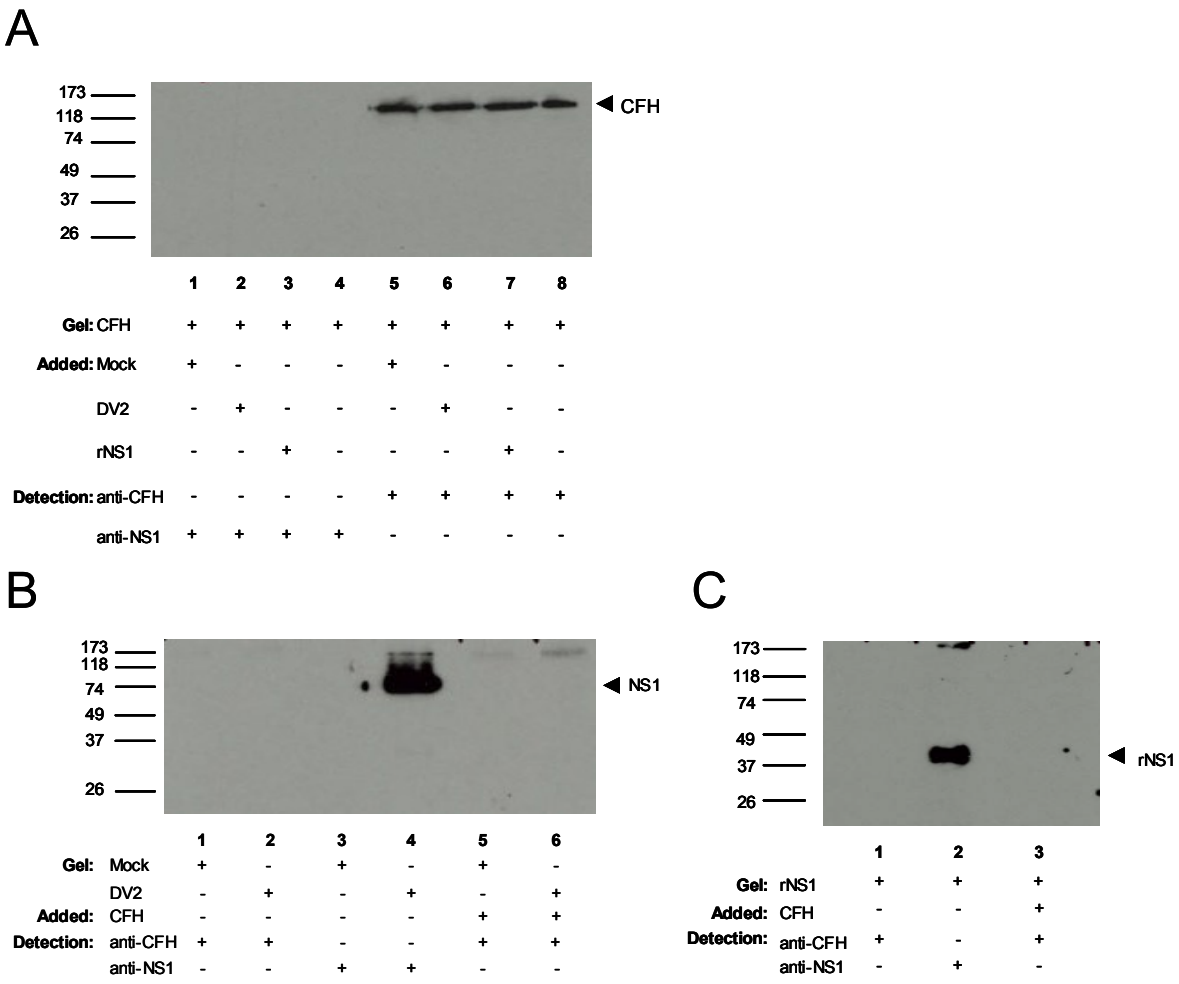


Figure 3.6: Far western blot between NS1 of DV2 and CFH: A) CFH proteins were loaded onto the gel, transferred to the membrane, which was incubated with either supernatants or rNS1 and detected with anti-NS1 antibody (lane 1-4) or anti-CFH antibody (lane 6-8). B) Supernatants and C) rNS1 were loaded onto the SDS-PAGE gel, transferred to a membrane which was then incubated with CFH and the interaction was detected with anti-CFH antibody. Positive controls were DV2 supernatant and rNS1 detected with anti-NS1 antibody.

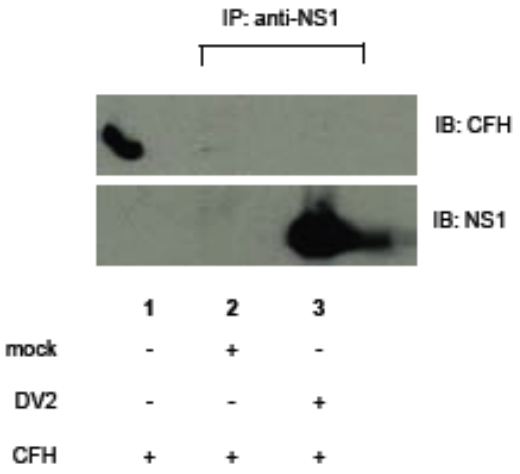


Figure 3.7: Co-immunoprecipitation between NS1 of DV2 and CFH: both mock and Dengue supernatants were incubated with CFH and sequentially pulled down with anti-NS1 antibody (IP), and protein A. Anti-CFH antibody was used to detect CFH in western blot (IB). Purified CFH protein was used as a positive control (lane 1). After CFH was detected, the membrane was washed with PBS and incubated with anti-NS1 antibody to confirm that the proteins were pull-down (panel below).

3.3 DISCUSSION

In this study the relationship between selected complement gene polymorphisms and Dengue infection severity was assessed. The polymorphisms included variation within four nucleotides (two adjacent polymorphisms within FB and one in each of C3 and CFH) that have been shown to be associated with a 6-fold variation in complement haemolytic activity *in vitro* (Heurich et al., 2011). Due to the low minor allele frequency at the C3 and FB polymorphisms (Table 3.2), it would be difficult to see the effect of variation at this locus. Indeed, reported minor allele frequency at the C3 polymorphism in Asian population is 0% (international human haplotype map project; HapMap), which is similar to this result and therefore this is considered as 'rare allele'. The rare allele is generally difficult to interpret in analysis since it requires a very large population in order to get strong statistic power to make a meaningful statement. As a result most genetic studies exclude single nucleotide polymorphisms with the minor allele frequency below 5%. Consequently this rare allele does not affect Dengue infection since almost all people contain the same allele. Although the minor allele frequency at the CFH rs800292 polymorphism was more than 10%, the variation in this genotype did not differ between Dengue sup-groupings (Table 3.3). In addition, by using the χ^2 test, the C3 polymorphism was not in the Hardy-Weinberg equilibrium (HWE) meaning that the observed frequency is deviated from the expected frequency (Table 3.5). The HWE is that the genotype and/or allele frequency proportion in a population remains constant over the generations. A departure from HWE might be due to evolutionary influences such as migration, inbreeding, and small population size, leading to increased homozygosity in the population.

A correlation between Dengue infection severity and complement activation has been studied (Bokisch et al., 1973, Nascimento et al., 2009). In DHF, a decrease in plasma CFH during the acute phase of infection was detected and its levels returned to normal during convalescence (Nascimento et al., 2009). This was in contrast to DF patients in whom plasma levels of CFH did not differ throughout the Dengue course (Nascimento et al., 2009). The different plasma CFH levels in patients with Dengue infection might be due to polymorphisms in the CFH promoter. Pastor *et.al*. has

recently reported an association between the CFH rs3753394 polymorphism and Dengue severity (Pastor et al., 2013). However, a significant association between Dengue severity and this SNP as well as the AMD-associated CFH rs1061170 SNP was not found in this study (p-value> 0.05; Table 3.3).

The deletion of the *CFHR1* gene and *CFHR3* gene *(ΔCFHR3-1*) is a copy number variation generated from non-allelic homologous recombination within the CFHR locus (Gold et al., 2006, Spencer et al., 2008). The absence of CFHR1 and CFHR3 is thought to decrease the risk of getting AMD and IgA nephropathy (Spencer et al., 2008, Gharavi et al., 2011). Here is the first report investigating the relationship between the copy number variation within the CHFR locus and Dengue susceptibility. The frequency of the *ΔCFHR3-1* allele in the Dengue population was approximately 7% (Table 3.6) while the reported allele frequencies in Asians, European Caucasians, and the African population are 2%, 4.7%, and 16%, respectively (Gold et al., 2006). However, there was no evidence that this variation is associated with Dengue infection (Table 3.6). Notably, uncommon variations were observed during analysis. There was one individual with three copies of the *CFHR3* and *CFHR1*, probably due to a duplication of these genes in one allele (Fritsche et al., 2010, Schmid-Kubista et al., 2009). Unfortunately, the effect of the three copies of the CFHR3 and CFHR1 gene has not been demonstrated elsewhere. Nine individuals with the heterozygous deletion of the *CFHR1* gene but the presence of two copies of the *CFHR3* genes were also detected. One possible explanation for these findings is the presence of the deletion in the *CFHR4* and *CFHR1* genes (*ΔCFHR4-1*). This novel deletion was first described in atypical hemolytic uremic syndrome (aHUS) in European Caucasians where the allele frequency of the *CFHR4* and *CFHR1* deletion in aHUS patients is about 1.4% and that in the control is about 0.9% (Moore et al., 2010). However, this has no significance due to the small number of subjects. Similarly the *CFHR4* and *CFHR1* deletion is observed in AMD but this is also insufficient to have statistical detection (Kubista et al., 2011). An isolated deletion of the *CFHR1* gene with the presence of two copies of other *CFHR* genes might be another explanation for these findings (Moore et al., 2010, Kubista et al., 2011). Since there was no probe for *CFHR4* gene in this study, this remained inconclusive.

The allele 'A' in the tag SNP (the CFH rs6677604) is linked to the Δ*CFHR3-1* deletion allele (Zhao et al., 2011). However, there was one individual containing one copy of *CFHR3-1* detected by Taqman CNV assay and MLPA in whom the 'A' allele could not be confirmed by the CFH rs6677604 genotyping. This may suggest that the CFH rs6677604 was not in complete linkage disequilibrium (LD) in the Thai population. Zhao *et. al* has reported that 10 in 106 people of Amerindian-European descent and 7 in 196 people of African-American descent also present the GG genotype with the heterozygote *CFHR3-1* deletion (Zhao et al., 2011).

It should be noted that Dengue patients with primary infection were not included in this study since most of them have asymptomatic infection. Besides, the small sample size for DHF, particularly DHF3 and DHF4, would affect a statistical power. To increase a sufficient power, the number of a sample size should be larger. Considering the number of minor allele frequencies particularly in C3 in this study, the number of DF and DHF should be at least 390 each group (calculated from an online sample size estimator for a case-control study; http://osse.bii.a-star.edu.sg). When compared between Dengue with shock and without shock, the sample sizes needed for each group should be up to 260. This large number of subjects would limit the analysis.

Dengue soluble NS1 can modulate complement activation and the levels of NS1 protein are correlated with disease severity (Libraty et al., 2002, Avirutnan et al., 2006). In the presence of anti-Dengue antibodies, complement activation is enhanced (Avirutnan et al., 2006). Dengue NS1 has been shown to bind to complement proteins. Interaction between NS1 and C4 leads to degradation of C4, resulting in a viral evasion of complement-dependent destruction (Avirutnan et al., 2010)**.** Dengue NS1 is also able to interact with Clusterin, a complement regulatory protein inhibiting formation of the terminal complement complex (Kurosu et al., 2007). This interaction may contribute to the progression of DHF. Recently, it has been shown that WNV NS1, but not JEV, can interact with CFH, reducing complement activities *in vitro* (Chung et al., 2006, Krishna et al., 2009). A direct interaction between CFH and Dengue NS1 protein was not detected by ELISA, far-Western blotting or co-immunoprecipitation (Figure 3.5, 3.6 and 3.7). It might be that interaction between

NS1 protein and CFH requires other components. Glycosaminoglycans (GAGs) are known to bind CFH and NS1 and might be mediators linking between NS1 and CFH (Muller and Young, 2013, Avirutnan et al., 2007, Zipfel et al., 1999). These carbohydrate ligands are generally found in close proximity to cell surfaces or extracellular matrixes. CFH can bind to these ligands which are rich on healthy cells and prevents complement attack. Since NS1 is able to bind to uninfected healthy cells via an interaction with GAGs, NS1 might interact with CFH on the cell surface or even compete with CFH to bind to the cells, interfering with CFH activity leading to vascular leakage in Dengue infection. To test this hypothesis, an interaction of CFH and NS1 together with GAGs should be performed. Staining of CFH and NS1 proteins on the cell surface as well as complement lysis might be tested. In addition, GAG expression on a surface of both infected and non-infected cells should be observed. Alternatively, CFH may bind to other viral molecules modulating CFH activities. As shown in HIV, CFH can bind to glycoproteins (gp120 and gp41) on HIV particles (Pinter et al., 1995a, Pinter et al., 1995b). Hence, interaction between CFH and Dengue viral particles should be studied.

Chapter 4

Results

Early predictive markers to classify Dengue severity

4.1 Introduction

4.2 Results

4.3 Discussion

4.1 Introduction

Dengue disease is one of the most concerning public health problems on account of there being no effective vaccine, drugs or specific treatment. Suspected patients with Dengue infection may need to be hospitalized until the critical period as fatal complications may rapidly occur 3 to 7 days after fever drops. The symptoms of Dengue disease at an early stage are difficult to discriminate between non-severe and severe dengue (DF and DHF respectively). However, with medical care, the mortality rate can decrease from 20% to less than 1% . Early prediction/diagnosis can help physicians to intensively care for patients with DHF, and reduce the number of patients with DF in a hospital, thus reducing the financial burden on the hospital.

Due to a lack of appropriate animal models, clinical studies have been important instruments in investigating the pathogenesis of Dengue infection. Several studies have identified markers for disease severity as reviewed in the chapter 1 (section 1.1.8). Platelet counts < 100,000 cells per cu mm together with an increase in haematocrit equal to or greater than 20% above average (1997) are currently used to monitor the progression of the disease; however they are not accurate enough to use as early predictors for the disease severity (Potts et al., 2010). The plasma level of Dengue soluble NS1 antigen is higher in DHF cases than those with DF during the febrile phrase (Libraty et al., 2002). ELISA detected NS1 antigen would be useful in the early days of illness even after fever for several days. The sensitivity of the test for Dengue primary infection is more than 90% (Simmons et al., 2012) and the test can be more powerful when it is used with a combination of serological methods (Blacksell, 2012). However, the sensitivity and the accuracy of the test are lower when it is used for the secondary infection (Ty Hang et al., 2009, Tricou et al., 2010, Felix et al., 2012). NS1 antigen therefore, might be a promising marker if there is a reliable and practical diagnostic test available. In addition, several circulating cytokines and chemokines including TNFα, IL8, sVEGFRII, IP10, ITAC, RANTES, to name a few have been detected. There are however, conflicting results for some of these cytokines (Potts et al., 2010). For example, the levels of IFNγ in severe Dengue was higher than those in mild Dengue in one study whereas conflicting evidence has been reported in another study showing no significant difference between these two groups (Bozza et

al., 2008, Braga et al., 2001). This might be due to numerous factors such as differences in the timing of sample collection and sample processing, methods in grading the disease severity, and the number of samples used in the study. The published soluble markers associated with Dengue illness are summarized in the chapter 1 (Table 1.1).

In this study, predictive markers to identify DHF during the early days of infection were defined. Clinical markers and biomarkers from a large scale of samples collected in the same way were assembled and plasma proteins were measured by multiplex assays and analyzed by statistical methods.

4.2 Results

4.2.1 Study population

Subjects in this study appeared with a fever, signs and symptoms of Dengue disease and they were hospitalized at Songkla and Khonkan hospitals in the southern and northeastern provinces in Thailand during 2001 to 2008. The process of sample collection, grading the disease severity and confirmation of Dengue serotype were performed by staff in Thailand (see in Materials and Methods, section 2.2: patients and controls,). Plasma samples in this experiment were collected from children clinically presenting with Dengue disease during the acute stage before fever subsided. Plasma samples from children clinically presenting with other febrile illnesses (OFI) were used as control and baseline.

Demographic information of the individuals in this study is shown in Table 4.1. The total number of Dengue patients in this study was 453 (DF = 226 and DHF = 227). DHF was classified according to the WHO protocol into 4 grades and the numbers of subjects in DHF1 to 4 were 73, 119, 33 and 2 respectively. Patients with mild disease (DF) and with severe Dengue Hemorrhagic Fever (DHF) had an average age of 9.67 and 10.20 years olds respectively (range 3-15 years old). The number of female and male patients in both groups was somewhat equivalent. Almost all patients had a secondary infection and appeared at the hospitals between the 3rd and 5th day following the onset of illness. Those with other febrile illnesses (OFI) were used as controls (n = 69). Average age in OFI group was 8.43 years (range 2-14 years old). Dengue serotype was confirmed by RT-PCR. The numbers of Dengue serotypes 1 to 4 were 174, 102, 15, and 100 respectively. There were 60 samples in which viral genome could not be detected.

Table 4.1: Demographic information of the study population

Disease severity	Age (yr)*	Gender (n)		Type of infection		Day since onset								
		Males	Females	Primary	Secondary	1	2	3	4	5	6	7	8	10
DF (n = 226)	9.67± 2.6	106	120	11	215	4	25	57	81	43	10	4	1	1
DHF (n= 227)	10.20±2.7	123	104	3	224	1	15	67	80	51	9	4	0	0
OFI (n = 69)	8.43±2.9	37	32	-	-	-	-	-	-	-	-	-	-	-

DF: mild Dengue fever, DHF: Dengue hemorrhagic fever, OFI: other febrile illnesses; * mean± SD

4.2.2 Clinical and biological characteristics during the acute stage of infection

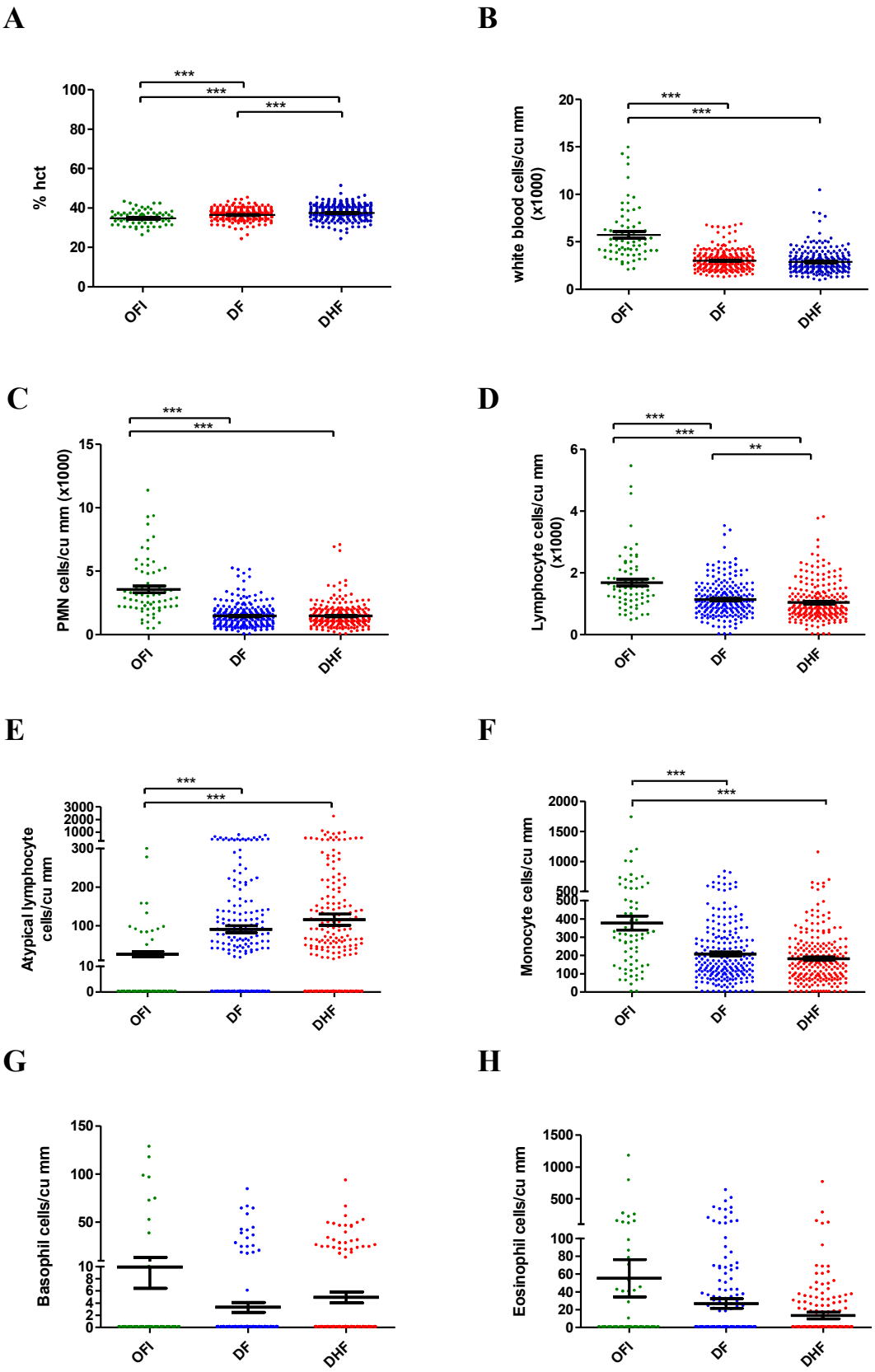
4.2.2.1 Clinical characteristics

The assessment of clinical markers was done by staff in Thailand, as described in Materials and Methods (Section 2.2). Clinical markers included hematocrit (hct), total white blood cell (wbc), polymorphic mononuclear cell (pmn), lymphocyte, atypical lymphocyte, monocyte, basophil, eosinophil, platelet cell, liver aspartate aminotransaminases (ast), liver alanine aminotransaminases (alt), liver total protein (lft_protein), and liver albumin (lft_albumin). The clinical profiles are shown in Table 4.2 and Figure 4.1. A reduction of platelet cell count, together with an induction of hct and of liver transaminases (ast and alt), was observed in the plasma of Dengue patients and was associated with disease severity (Figures 4.1A, I, L and M). Although elevated liver enzymes were detected in the plasma, levels of albumin appeared to be reduced in Dengue plasma particularly in DHF (Figure 4.1K). A decrease in the total liver protein was also shown only in DHF (Figure 4.1J). A lower wbc count in individuals with Dengue infection was confirmed (Figure 4.1B). When measuring each white blood cell by the differential count method, the numbers of the polymorphonuclear cells (PMN), the lymphocytes and the monocytes were reduced while those of the atypical lymphocyte were elevated in Dengue group (Figures 4.1C, D, E and F). In addition, the number of the lymphocytes was much lower in DHF (Figure 4.1D). No differences in the frequency of basophils and eosinophils were shown in any group (Figures 4.1G and H).

Table 4.2: Clinical characteristics of the study population

Clinical characteristics	DF	DHF	OFI	p-value[a]	p-value[b]	p-value[c]
Hematocrit (hct) (mean % ±SD)	36.45±3.00	37.57±3.83	34.79±3.62	**2.79×10^{-4}**	**2.00×10^{-4}**	**4.50×10^{-7}**
White blood cell (wbc) (x1000) (mean cells/cu mm ±SD)	2.99±1.11	2.87±1.26	5.72±2.88	0.78	**1.20×10^{-19}**	**3.70×10^{-21}**
PMN (x1000) (mean cells/cu mm ±SD)	1.48±0.92	1.48±0.97	3.56±2.24	0.99	**1.80×10^{-18}**	**6.25×10^{-19}**
Lymphocyte (x1000) (mean cells/cu mm ±SD)	1.14±0.56	1.05±0.60	1.68±0.95	**4.80×10^{-3}**	**3.89×10^{-7}**	**5.38×10^{-10}**
Atypical lymphocyte (mean cells/cu mm ±SD)	91.26±136.87	116.76±222.70	25.80±60.32	0.30	**2.24×10^{-6}**	**8.89×10^{-8}**
Monocyte (x1000) (mean cells/cu mm ±SD)	0.21±0.16	0.19±0.15	0.38±0.32	0.12	**4.47×10^{-8}**	**3.77×10^{-8}**
Basophil (mean cells/cu mm ±SD)	3.35±12.28	4.99±13.79	9.93±28.96	0.08	0.23	0.97
Eosinophil (mean cells/cu mm ±SD)	27.19±81.90	13.63±57.18	55.35±173.78	0.38	0.20	0.06
Platelet count (x1000) (mean cells/cu mm ± SD)	149.53±60.78	94.53±50.77	210.38±64.53	**1.15×10^{-21}**	**2.80×10^{-12}**	**2.80×10^{-27}**
Liver total protein (lft_protein) (mean g/dl ± SD)	6.96±0.56	6.59±0.76	7.08±0.60	**9.20×10^{-7}**	0.14	**5.20×10^{-6}**
Albumin (lft_albumin) (mean g/dl ± SD)	3.69±0.35	3.49±0.50	3.79±0.44	**2.78×10^{-5}**	**0.02**	**3.70×10^{-6}**
Liver aspartate aminotransaminases (ast) (mean IU/L ± SD)	81.07±76.40	155.28±169.81	46.09±28.87	**3.30×10^{-13}**	**6.40×10^{-10}**	**4.00×10^{-21}**
Liver alanine aminotransaminases (alt) (mean IU/L ± SD)	53.27±38.43	82.01±78.77	35.77±23.47	**1.90×10^{-10}**	**1.60×10^{-9}**	**1.10×10^{-17}**

DF: mild Dengue fever, DHF: Dengue hemorrhagic fever, OFI: other febrile illnesses; [a] DF vs DHF [b] OFI vs DF [c] OFI vs DHF


A
% hct
***
***
***
OFI
DF
DHF
B
white blood cells/cu mm (x1000)
***
***
C
PMN cells/cu mm (x1000)
***
***
D
Lymphocyte cells/cu mm (x1000)
***
***
**
E
Atypical lymphocyte cells/cu mm
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F
Monocyte cells/cu mm
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Basophil cells/cu mm
H
Eosinophil cells/cu mm

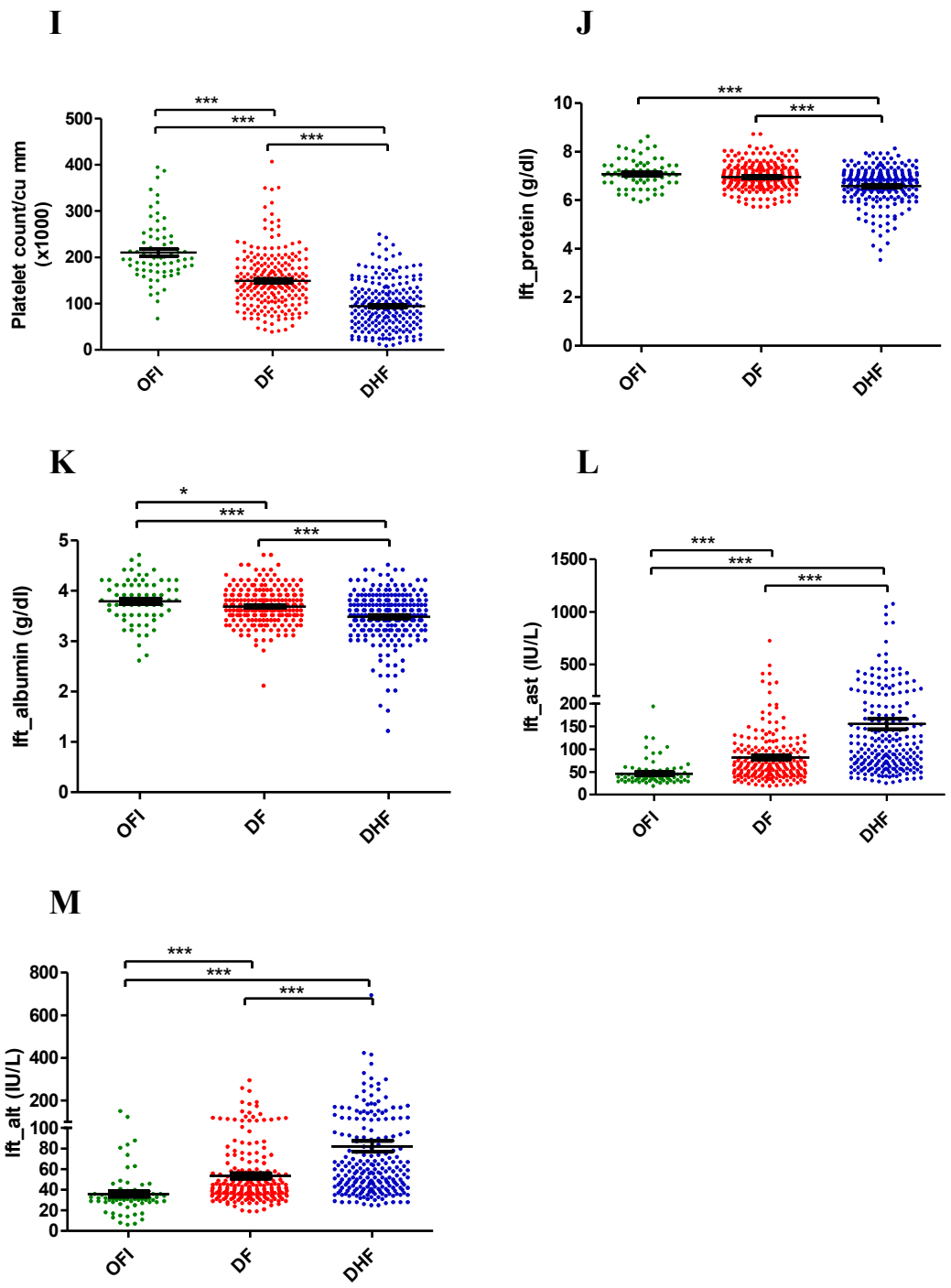


Figure 4.1: Clinical characteristics of patients with OFI, DF and DHF during the acute stage of disease. Horizontal lines represent mean values with SEM. Statistical significance was determined by the Kruskal-Wallis test for 3 groups and the Mann-Whitney test for 2 groups (*, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$).

4.2.2.2 Biological characteristics

Cytokines, chemokines, soluble receptors, growth factors, and matrix metalloproteinases (MMPs) in the plasma were measured by multiplex bead immunoassay as described in the Materials and Methods (section 2.7). Of 66 markers, nineteen markers were below the limit of detection in most samples. These were MMP7, IL1β, IL13, IL4, IL5, IL7, IL17, Eotaxin3, SDF1, TNFα, IFNα, IFNγ, MIP1α, HGF, EGF, VEGF, FGF, GCSF, and GMCSF. The number of measurable samples in this group of markers was too low to compare and therefore excluded from further analysis. However, IL1β, IL13, IL4, IL7, TNFα, IFNα, IFNγ, VEGF and GMCSF have been shown to associate with DHF elsewhere (Table 1.1). Sixteen markers that contained missing data greater than 10% were MCSF, ENA78, IL1RA, IL2, IL6, IL15, IL16, IL23, IL33, Eotaxin, MIG, I309, TARC, IL2R, 6Ckine, and GCP2. The markers that have previously been reported are IL1Ra, IL2, IL6 and MIG (Table 1.1). Data missing in this group was due to a combination of insufficient samples and/or no samples and technical issues during experiments such as pipetting error, plate-to-plate variation as well as protein stability. It is noteworthy that in these markers, plasma protein concentrations in many samples fell below or at the lowest limit of quantitation. For MCSF, although the number of samples was sufficient, the quality of the data was not great since half of the samples had protein concentrations below the lowest limit of detection. In addition, due to missing data in the control group, IL2, IL6, IL23 and MIG were compared only between DF and DHF. The percentage of markers classified as detectable and undetectable markers, as well as markers with >10% missing data are shown in Figure 4.2.

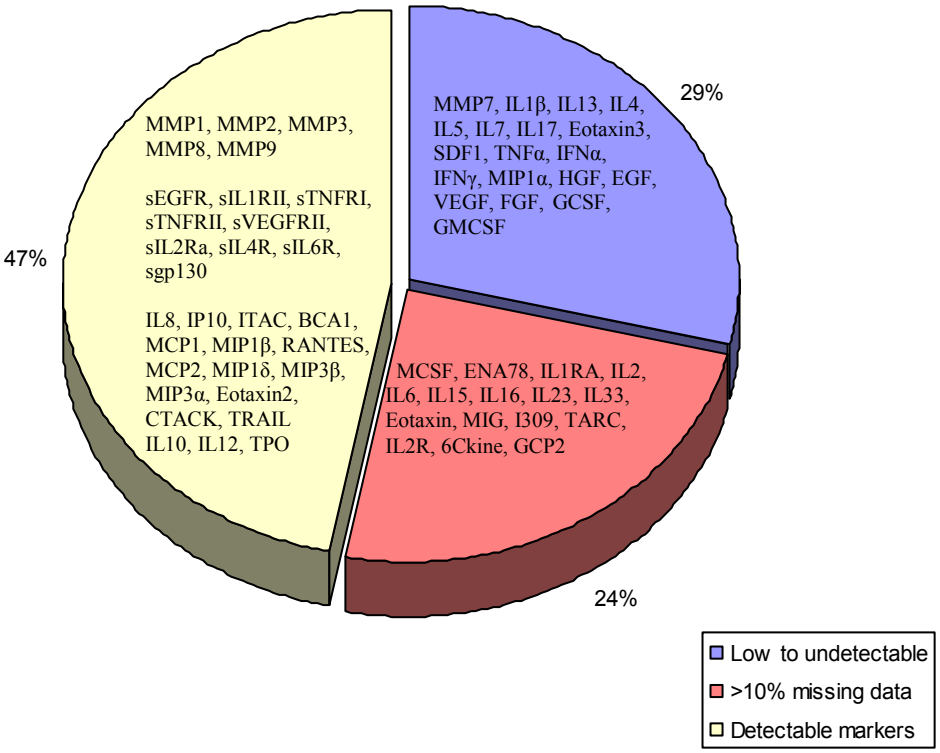


Figure 4.2: Classification of biological markers into three groups: detectable, low to undetectable and missing data greater than 10%. Percentage of markers in each group is shown. Total biological markers in the experiment were 66 markers (100%).

When compared to the control group, elevated plasma levels of sTNFRII, IP10, ITAC, BCA1, MCP2, MCSF and IL15 were detected in Dengue patients and appeared to be associated with Dengue severity (DHF > DF > OFI) (Tables 4.3, 4.4 and 4.5 and Figures 4.3.2D, 4.3.3B-D, 4.3.4D, 4.3.7A and 4.3.7D). Conversely, levels of RANTES decreased in Dengue groups (DF and DHF), particularly in DHF (OFI > DF > DHF) (Tables 4.3 and4.5 and Figure 4.3.4C). There were eleven markers in which plasma levels differed between the control and Dengue groups but this difference did not relate to the severity. Of 11 markers, plasma MMP8 and MMP9 were lower in Dengue groups than the control group (OFI > Dengue) (Tables 4.3 and 4.5, Figures 4.3.1D and 4.3.1.E) while increased concentrations of sIL2Ra, sIL6R, MCP1, MIP1β, MIP3β, IL10, TRAIL, IL33 and IL2R were detected (Dengue > OFI) (Tables 4.3, 4.4 and 4.5 and Figures 4.3.2F, 4.3.2H, 4.3.4A, 4.3.4B, 4.3.4F, 4.3.5A, 4.3.5C, 4.3.7F and 4.3.7J). Moreover, increases in plasma levels of MMP3, sTNFRI,

IL8, MIP3α, CTACK, TPO and IL16 were observed in DHF (DHF > DF and OFI) (Tables 4.3, 4.4 and 4.5 and Figures 4.3.1C, 4.3.2C, 4.3.3A, 4.3.4G, 4.3.4I, 4.3.6 and 4.3.7E) while sVEGFRII and IL12 markedly decreased (DF and OFI > DHF) (Tables 4.3 and 4.5 and Figures 4.3.2E and 4.3.5B). In contrast to these proteins, plasma levels of I309 were elevated only in DF (DF > DHF and OFI) (Tables 4.4 and 4.5 and Figure 4.3.7H). In addition, decreased Eotaxin2 levels were detected in DF (DHF and OFI > DF) (Tables 4.3 and 4.5 and Figure 4.3.4H). Elevated MIP1δ proteins were detected in DHF plasma when compared to the control (DHF > OFI) (Tables 4.3, and 4.5 and Figure 4.3.4E) whereas MMP1, sIL4R, and ENA78 levels were significantly down-regulated in DHF (OFI > DHF) (Tables 4.3, 4.4 and 4.5 and Figures 4.3.1A, 4.3.2G, and 4.3.7B). Increased IL6 levels were detected in DHF when compared to DF (DHF > DF) (Tables 4.4 and 4.5 and Figure 4.3.7N) but there was no significant difference in plasma levels of IL2, IL23 and MIG between these two groups (Tables 4.4 and 4.5 and Figures 4.3.7M, 4.3.7O and 4.3.7P). Among three groups, no significant differences were found in nine markers including MMP2, sEGFR, sIL1RII, sgp130, IL1Ra, Eotaxin, TARC, 6Ckine and GCP2 (Tables 4.3, 4.4 and 4.5 and Figures 4.3.1B, 4.3.2A, 4.3.2B, 4.3.2I, 4.3.7C, 4.3.7G, 4.3.7I, 4.3.7K and 4.3.7L). The descriptive statistical analysis for all biological markers is shown in Tables 4.3 and 4.4 and Figures 4.3.1- 4.3.7.

Table 4.3: Statistical analysis of the detectable biological markers in DF, DHF and OFI

	DF				DHF					Other Febrile Illness (OFI)				
Bio-Markers	**N**	**Mean±SD**	**Median**	**Range**	**N**	**Mean±SD**	**Median**	**Range**	**p-value[a]**	**N**	**Mean±SD**	**Median**	**Range**	**p-value[b]**
Matrix metalloproteinases (MMPs):														
MMP1 (pg/ml)	225	486.52±470.01	332.30	3294.93	226	529.73±1297.32	352.10	18668.35	0.56	69	653.20±567.42	519.86	2863.00	0.0013
MMP2 (pg/ml)	211	170682.49±37415.54	165553.94	203509.25	216	172109.83±35408.03	170471.63	205141.74	0.63	69	159712.72±41197.33	161271.48	193882.50	0.1272
MMP3 (pg/ml)	225	3149.37±1951.17	2874.92	16782.81	226	4462.11±2938.73	4003.35	27325.27	**$1.1x10^{-9}$**	69	3171.85±3198.16	2340.36	18765.90	**$3.00x10^{-11}$**
MMP8 (pg/ml)	211	3479.90±3925.80	2500.40	37352.56	216	3600.66±3322.96	2557.29	22687.79	0.31	69	15300.53±23915.04	6869.82	117735.06	**$3.54x10^{-14}$**
MMP9 (pg/ml)	211	23843.42±15973.25	19936.29	108499.42	216	27000.58±24849.13	22039.55	306779.64	0.03	69	75315.09±99454.20	39579.22	556554.41	**$3.10x10^{-15}$**
Soluble cytokine receptors:														
sEGFR (pg/ml)	224	40704.80±10632.76	38750.04	74400.18	227	44270.27±13448.30	41385.22	80704.36	0.0057	69	38063.33±5709.65	38009.33	34814.91	0.0014
sIL1RII (pg/ml)	224	4194.20±1355.33	3972.45	14300.39	227	4589.49±3062.07	4275.92	44627.45	0.02	69	4046.00±959.84	4020.25	5062.79	0.0268
sTNFRI (pg/ml)	224	1258.83±355.33	1209.62	2671.08	227	1435.59±414.87	1403.93	3197.50	**$6.36x10^{-8}$**	69	1190.45±498.50	1096.20	2472.94	**$5.11x10^{-10}$**
sTNFRII (pg/ml)	224	15704.69±5415.28	15345.15	30185.94	227	19156.49±6094.68	18519.54	35839.80	**$6.49x10^{-10}$**	69	8804.71±4815.25	7562.88	27120.32	**$2.02x10^{-31}$**
sVEGFRII (pg/ml)	207	19003.52±3231.21	19177.23	20638.29	204	16376.35±3889.52	16476.45	21928.39	**$1.14x10^{-12}$**	69	20498.44±6537.77	20020.80	26903.45	**$9.98x10^{-13}$**
sIL2Ra (pg/ml)	224	1545.23±690.79	1388.98	3853.55	226	1608.31±677.05	1487.94	4236.74	0.20	69	1073.16±569.34	945.57	2944.10	**$1.26x10^{-9}$**
sIL4R (pg/ml)	223	1587.43±174.17	1564.38	1391.86	227	1556.34±164.01	1540.93	1390.44	0.05	69	1657.40±179.40	1628.38	1047.09	**$5.79x10^{-5}$**
sIL6R (pg/ml)	224	22873.96±6681.72	21738.93	42992.42	226	23304.81±6161.84	22184.45	38072.09	0.38	69	19270.67±6982.47	17165.05	34998.00	**$4.51x10^{-7}$**
sgp130 (pg/ml)	224	197473.75±28057.87	198057.15	202620.61	226	194993.36±26114.59	197855.10	156888.70	0.66	69	194901.51±19075.76	193371.20	111985.40	0.6969
Chemokines:														
CXC family:														
IL8 (CXCL8) (pg/ml)	225	34.54±16.28	31.58	121.89	226	46.60±33.57	38.03	314.04	**$4.67x10^{-6}$**	69	29.64±12.41	27.78	60.22	**$4.12x10^{-8}$**
IP10 (CXL10) (pg/ml)	225	4076.82±4083.07	2988.66	38758.84	226	6532.80±6079.57	4096.61	42170.39	**$3.34x10^{-10}$**	69	2980.47±6700.51	209.55	29508.38	**$2.57x10^{-24}$**
ITAC (CXCL11) (pg/ml)	225	1064.58±1244.00	608.90	7683.84	227	2046.73±4227.77	1167.74	52349.29	**$6.45x10^{-8}$**	69	421.68±649.28	81.30	6631.92	**$2.54x10^{-23}$**
BCA1 (CXCL13) (pg/ml)	221	104.26±57.25	94.73	460.86	217	159.86±117.03	122.18	700.57	**$1.94x10^{-9}$**	69	78.34±31.07	76.58	223.16	**$1.53x10^{-15}$**

Cut-off of adjusted P-value = $7.6x10^{-4}$ (0.05/66); the bold and underlined values mean significant difference between groups at p-value < $7.6x10^{-4}$

Range means maximal value-minimal value

a: compared between DF and DHF and analyzed by Mann-Whitney U test;

b: compared among OFI, DF and DHF and analyzed by Kruskal-Wallis test

Bio-Markers	DF N	DF Mean±SD	DF Median	DF Range	DHF N	DHF Mean±SD	DHF Median	DHF Range	p-value[a]	Other Febrile Illness (OFI) N	OFI Mean±SD	OFI Median	OFI Range	p-value[b]
CC family:														
MCP-1 (CCL2) (pg/ml)	225	611.30±299.10	622.90	2557.00	226	810.7±447.20	706.90	2662.70	$5.30x10^{-3}$	69	611.28±375.08	503.83	2556.87	**$6.78x10^{-7}$**
MIP-1β (CCL4) (pg/ml)	225	127.95±104.46	96.39	774.96	226	133.04±91.69	117.38	870.92	0.04	69	135.03±231.27	40.91	1189.26	**$1.12x10^{-8}$**
RANTES (CCL5) (pg/ml)	225	3090.33±5281.21	1082.37	49539.19	226	2400.98±5840.98	720.21	61899.19	**$2.15x10^{-4}$**	69	3673.27±3425.05	2080.09	12342.04	**$6.27x10^{-11}$**
MCP-2 (CCL8) (pg/ml)	221	205.43±111.42	187.03	928.61	217	265.36±150.47	249.19	827.04	**$8.47x10^{-6}$**	69	149.05±138.77	111.70	703.38	**$4.92x10^{-12}$**
MIP-1δ (CCL15) (pg/ml)	221	4458.63±2767.35	3881.90	16071.17	217	5338.96±3340.13	4699.02	21982.91	$6.00x10^{-3}$	69	3625.48±2638.44	2704.76	10760.49	**$1.69x10^{-5}$**
MIP3β (CCL19) (pg/ml)	225	302.17±142.74	260.63	988.57	226	319.20±158.99	285.10	1042.29	0.22	69	118.69±108.00	98.08	610.11	**$6.31x10^{-25}$**
MIP3α (CCL20) (pg/ml)	225	30.09±18.19	25.97	159.38	227	45.88±47.04	36.57	553.57	**$6.08x10^{-8}$**	69	26.64±20.74	21.00	112.42	**$5.03x10^{-10}$**
Eotaxin-2 (CCL24) (pg/ml)	221	63.32±50.15	48.48	362.44	216	91.12±89.44	63.34	653.98	**$4.01x10^{-5}$**	69	69.13±57.33	61.22	355.25	**$2x10^{-4}$**
CTACK (CCL27) (pg/ml)	221	922.44±344.33	919.76	2201.59	217	1074.43±406.29	1031.32	2523.03	**$8.75x10^{-5}$**	69	767.63±331.19	747.15	1692.24	**$7.98x10^{-9}$**
Cytokines:														
IL family:														
IL10 (pg/ml)	225	139.37±127.81	103.63	560.48	226	146.61±136.66	114.60	771.59	0.39	69	20.35±41.45	4.6	253.31	**$2.1x10^{-26}$**
IL12 (pg/ml)	225	324.89±112.30	315.51	659.14	226	291.15±124.48	268.96	1074.39	**$2.25x10^{-4}$**	66	484.93±559.44	376.76	3496.34	**$1.5x10^{-7}$**
Others:														
TRAIL (pg/ml)	221	98.29±59.79	88.43	383.61	217	97.01±64.96	89.18	402.64	0.83	69	69.67±46.76	50.95	196.26	**$7.33x10^{-5}$**
Growth factor:														
Thrombopoietin (TPO) (pg/ml)	221	701.78±758.00	493.37	6674.55	217	1062.04±2085.94	635.99	22965.96	**$2.50x10^{-4}$**	69	557.36±544.47	426.99	2678.66	**$8.28x10^{-7}$**

Cut-off of adjusted P-value = $7.6x10^{-4}$ (0.05/66); the bold and underlined values mean significant difference between groups at p-value < $7.6x10^{-4}$

Range means maximal value-minimal value

a: compared between DF and DHF and analyzed by Mann-Whitney U test;

b: compared among OFI, DF and DHF and analyzed by Kruskal-Wallis test

Table 4.4: Statistical analysis of the biological markers with missing data greater than 10% in DF, DHF and OFI

	DF				DHF					Other Febrile Illness (OFI)				
Bio-Markers	**N**	**Mean±SD**	**Median**	**Range**	**N**	**Mean±SD**	**Median**	**Range**	**p-value[a]**	**N**	**Mean±SD**	**Median**	**Range**	**p-value[b]**
MCSF (pg/ml)	223	820.72±1251.84	390.76	9459.52	227	2051.28±3394.76	1136.57	26557.82	**$2.73x10^{-6}$**	69	334.70±1077.81	0	8340.503	**$3.20x10^{-12}$**
ENA78 (pg/ml)	193	121.66±197.35	60.87	1178.53	185	103.47±272.13	43.15	3325.99	0.01	67	176.21±285.53	73.37	1874.36	**$1.10x10^{-4}$**
IL1Ra (pg/ml)	196	992.83±374.40	953.32	2373.30	194	1096.59±1350.81	956.32	18564.57	0.94	69	1259.22±3277.66	768.62	27542.398	$5x10^{-3}$
IL2 (pg/ml)	115	12.11±14.96	8.78	113.27	109	19.26±95.83	8.83	1007.78	0.97	-	-	-	-	-
IL6 (pg/ml)	158	12.94±14.62	9.98	115.00	158	16.45±17.06	12.21	146.78	**$5.00x10^{-4}$**	-	-	-	-	-
IL15 (pg/ml)	177	525.90±205.29	502.86	1130.43	161	681.31±353.98	619.41	2749.70	**$7.90x10^{-15}$**	66	293.81±337.43	158.57	1982.18	**$1.89x10^{-6}$**
IL16 (pg/ml)	165	159.03±154.84	113.11	1139.31	154	199.08±159.30	149.53	1162.98	**$3.00x10^{-4}$**	66	137.90±147.09	98.09489	850.178	**$4.04x10^{-5}$**
IL23 (pg/ml)	159	144.18±259.97	77.58	2256.72	159	229.27±842.69	71.04	8134.62	0.93	-	-	-	-	
IL33 (pg/ml)	165	48.40±47.92	33.44	337.70	154	77.37±176.1	38.24	1703.00	0.26	66	34.75±39.11	21.72	176.1	**$1.00x10^{-4}$**
Eotaxin (pg/ml)	186	32.50±15.47	28.53	101.45	203	35.62±34.67	29.63	413.24	0.70	68	42.45±58.51	29.22	464.12	0.93
MIG (pg/ml)	192	187.20±125.23	160.35	1033.36	199	194.85±225.46	153.26	2541.66	0.22	-	-	-	-	-
I309 (pg/ml)	193	6.54±5.54	4.77	35.93	186	5.25±8.01	3.62	92.07	**$7.83x10^{-5}$**	68	4.11±2.78	3.70	13.53	**$3.70x10^{-5}$**
TARC (pg/ml)	193	11.58±13.04	7.22	90.65	186	8.72±8.64	6.73	69.98	0.03	68	12.27±10.84	7.892096	43.823931	0.03
IL2R (pg/ml)	206	609.91±318.85	512.07	1779.77	193	657.18±395.76	599.61	3875.31	0.05	66	484.93±559.44	376.76	3496.34	**$7.50x10^{-9}$**
6Ckine (pg/ml)	165	351.33±168.84	349.63	897.24	154	386.41±207.90	366.18	1116.76	0.36	66	379.67±192.15	350.8098	994.2249	0.59
GCP2 (pg/ml)	182	31.42±18.45	26.70	136.86	195	35.32±22.01	29.72	164.61	0.09	69	29.69±17.72	26.92	101.91	0.10

Cut-off of adjusted P-value = $7.6x10^{-4}$ (0.05/66); the bold and underlined values mean significant difference between groups at p-value < $7.6x10^{-4}$

Range means maximal value-minimal value

a: compare between DF and DHF and analyzed by Mann-Whitney U test;

b: compare among OFI, DF and DHF and analyzed by Kruskal-Wallis test

Table 4.5: Summary of the findings for biological markers in DF, DHF and OFI during acute febrile stage

Findings	Soluble factors
DHF > DF > OFI	sTNFRII, IP10, ITAC, BCA1, MCP2, IL15, and MCSF
OFI > DF > DHF	RANTES
OFI > Dengue	MMP8 and MMP9
Dengue > OFI	sIL2Ra, sIL6R, IL2R, IL10, MCP1, MIP1β, MIP3β, TRAIL, and IL33
DHF > DF and OFI	MMP3, sTNFRI, IL8, MIP3α, CTACK, TPO and IL16
DF and OFI > DHF	sVEGFRII and IL12
DF > DHF and OFI	I309
DHF and OFI > DF	Eotaxin2
DHF > OFI	MIP1δ
OFI > DHF	MMP1, sIL4R, and ENA78
DHF > DF	IL6
DHF ≈ DF	IL2, IL23 and MIG
DHF ≈ DF ≈ OFI	MMP2, sEGFR, sgp130, sIL1RII, Eotaxin, TARC, 6Ckine, IL1Ra and GCP2

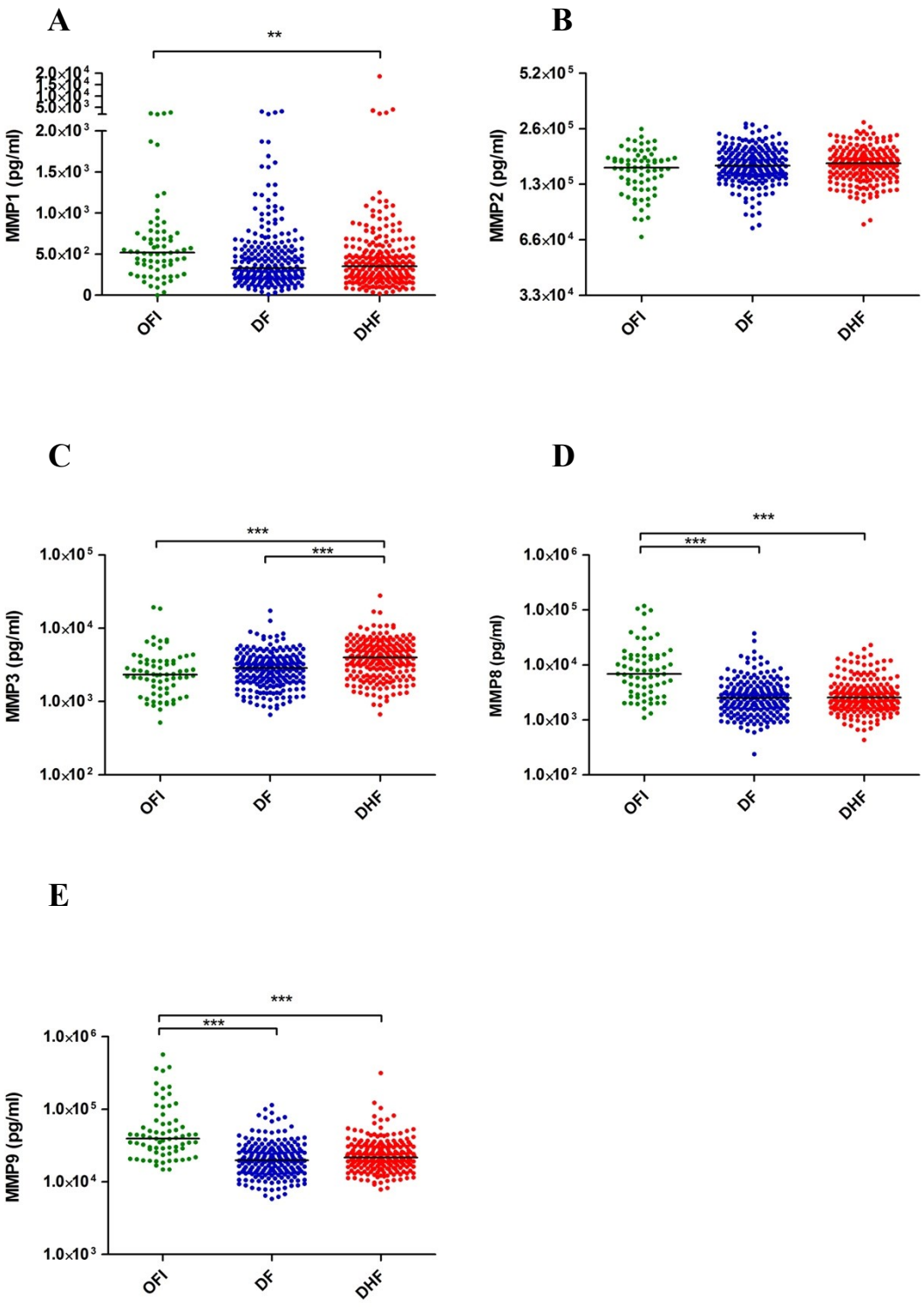
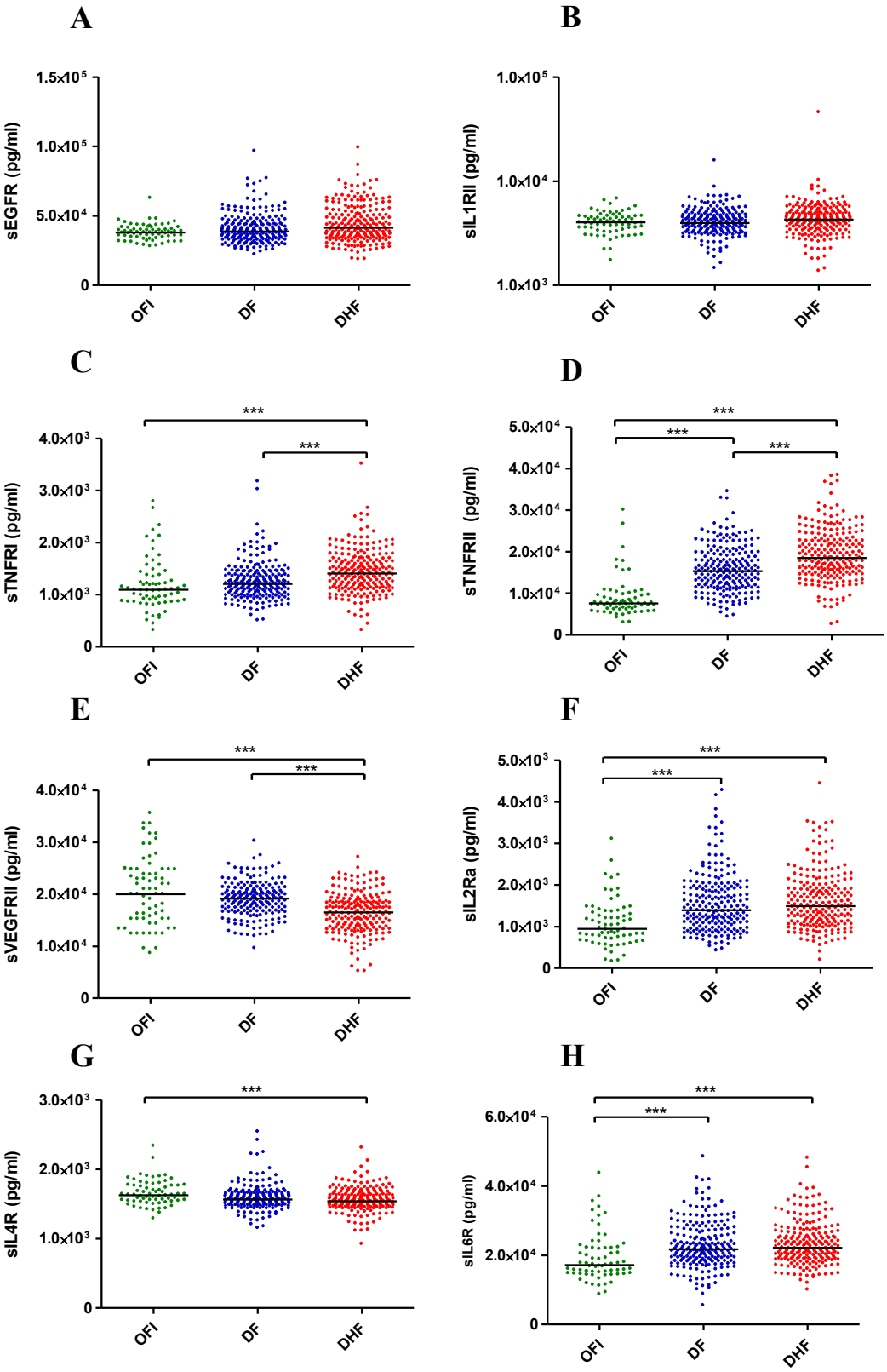


Figure 4.3.1: Plasma concentrations of matrix metalloproteinases (MMPs) in OFI, DF and DHF groups during the acute stage of the disease. Horizontal lines represent median values. Statistical significance was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).


A
sEGFR (pg/ml)
1.5x10^5
1.0x10^5
5.0x10^4
0
OFI
DF
DHF
B
sIL1RII (pg/ml)
1.0x10^5
1.0x10^4
1.0x10^3
OFI
DF
DHF
C
sTNFRI (pg/ml)
***
***
4.0x10^3
3.0x10^3
2.0x10^3
1.0x10^3
0
OFI
DF
DHF
D
sTNFRII (pg/ml)
***
***
***
5.0x10^4
4.0x10^4
3.0x10^4
2.0x10^4
1.0x10^4
0
OFI
DF
DHF
E
sVEGFRII (pg/ml)
***
***
4.0x10^4
3.0x10^4
2.0x10^4
1.0x10^4
0
OFI
DF
DHF
F
sIL2Ra (pg/ml)
***
***
5.0x10^3
4.0x10^3
3.0x10^3
2.0x10^3
1.0x10^3
0
OFI
DF
DHF
G
sIL4R (pg/ml)
***
3.0x10^3
2.0x10^3
1.0x10^3
0
OFI
DF
DHF
H
sIL6R (pg/ml)
***
***
6.0x10^4
4.0x10^4
2.0x10^4
0
OFI
DF
DHF

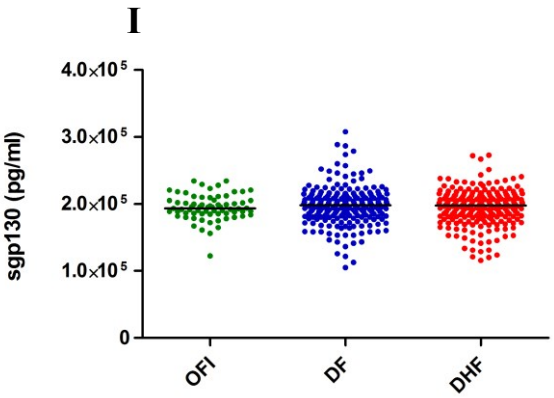


Figure 4.3.2: Plasma concentrations of soluble cytokine receptors in OFI, DF and DHF groups during the acute stage of the disease. Horizontal lines represent median values. Statistical significance was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).

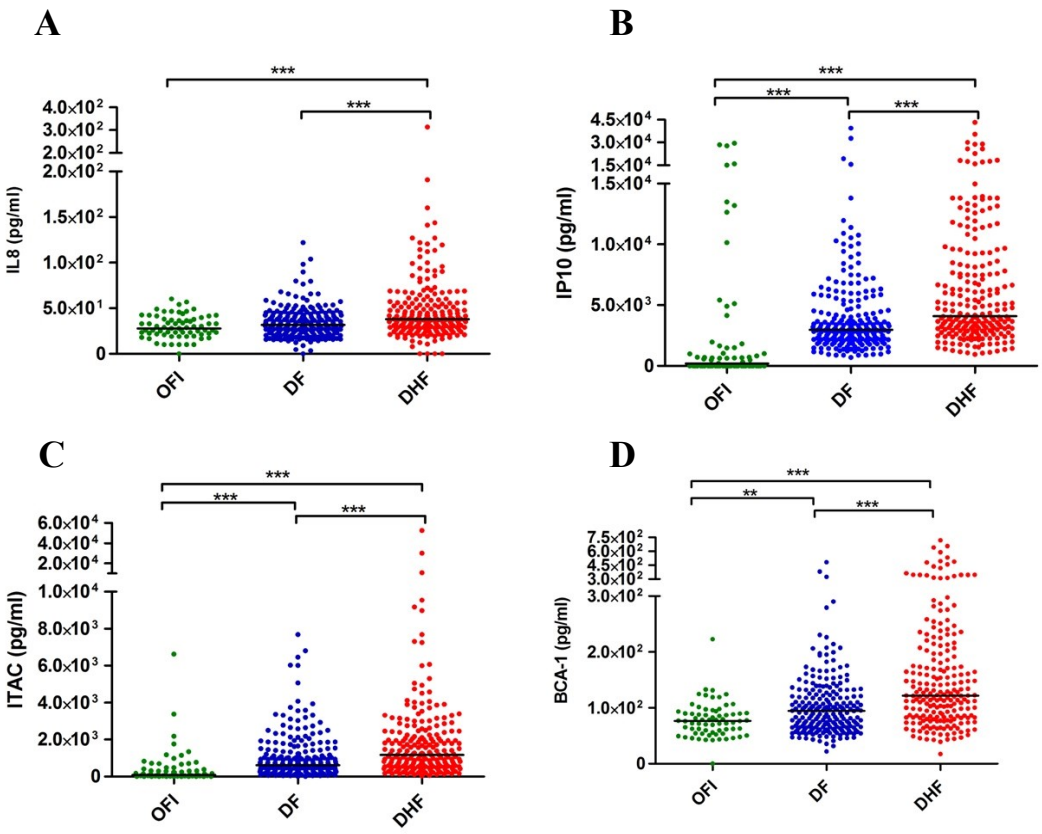
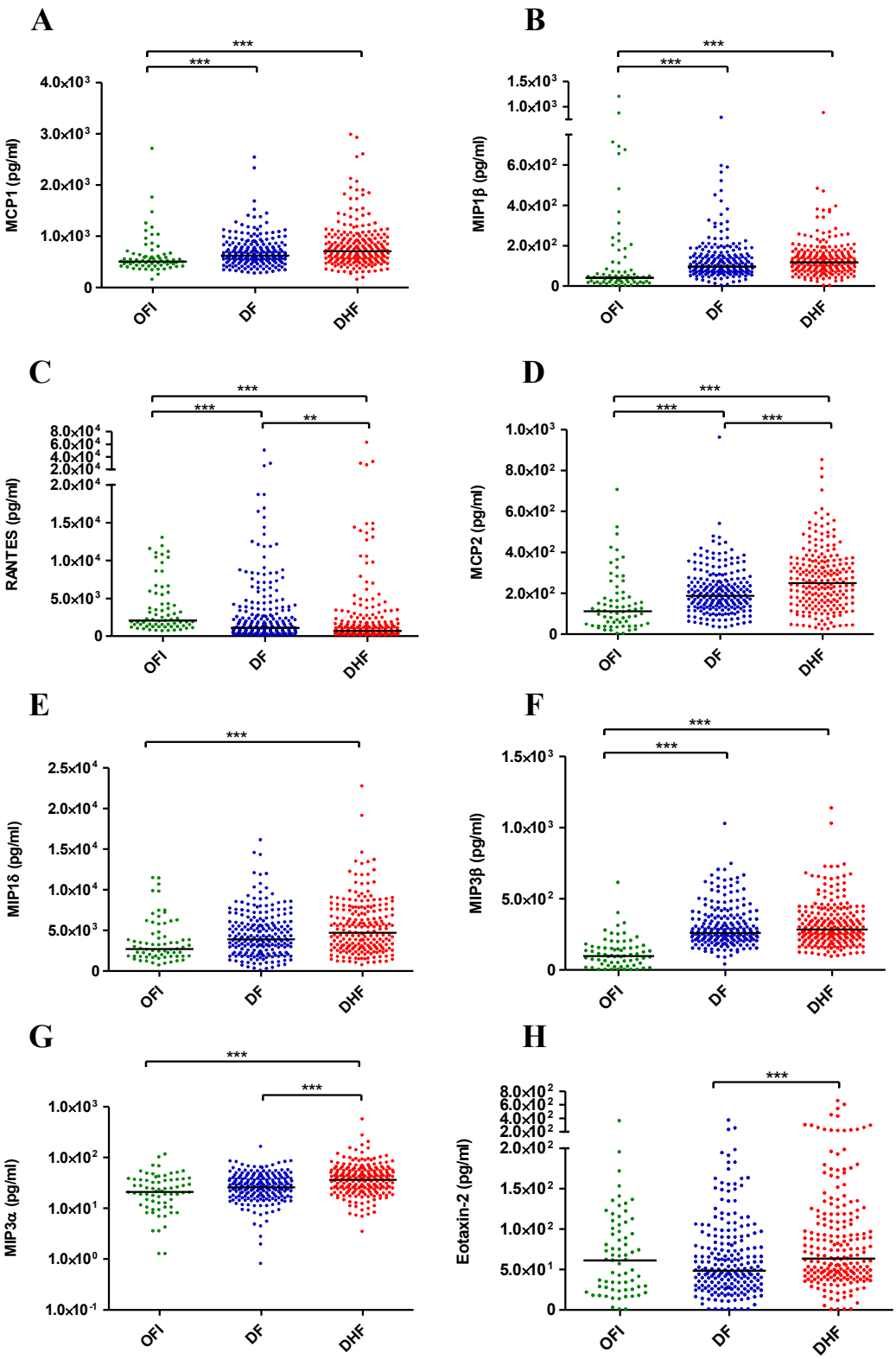


Figure 4.3.3: Plasma concentrations of chemokines (CXC family) in OFI, DF and DHF groups during the acute stage of the disease. Horizontal lines represent median values. Statistical significance was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).


A
MCP1 (pg/ml)
4.0x10³
3.0x10³
2.0x10³
1.0x10³
0
***
***
OFI
DF
DHF
B
MIP1β (pg/ml)
1.5x10³
1.0x10³
6.0x10²
4.0x10²
2.0x10²
0
***
***
OFI
DF
DHF
C
RANTES (pg/ml)
8.0x10⁴
6.0x10⁴
4.0x10⁴
2.0x10⁴
2.0x10⁴
1.5x10⁴
1.0x10⁴
5.0x10³
0
***
***
**
OFI
DF
DHF
D
MCP2 (pg/ml)
1.0x10³
8.0x10²
6.0x10²
4.0x10²
2.0x10²
0
***
***
***
OFI
DF
DHF
E
MIP1δ (pg/ml)
2.5x10⁴
2.0x10⁴
1.5x10⁴
1.0x10⁴
5.0x10³
0
***
OFI
DF
DHF
F
MIP3β (pg/ml)
1.5x10³
1.0x10³
5.0x10²
0
***
***
OFI
DF
DHF
G
MIP3α (pg/ml)
1.0x10³
1.0x10²
1.0x10¹
1.0x10⁰
1.0x10⁻¹
***
***
OFI
DF
DHF
H
Eotaxin-2 (pg/ml)
8.0x10²
6.0x10²
4.0x10²
2.0x10²
2.0x10²
1.5x10²
1.0x10²
5.0x10¹
0
***
OFI
DF
DHF

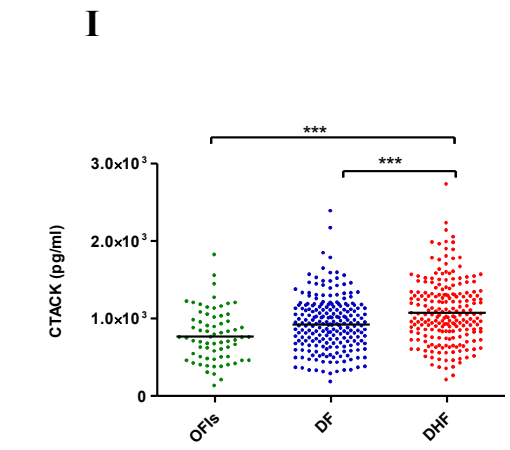


Figure 4.3.4: Plasma concentrations of chemokines (CC family) in OFI, DF and DHF groups during the acute stage of the disease. Horizontal lines represent median values. Statistical significance was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).

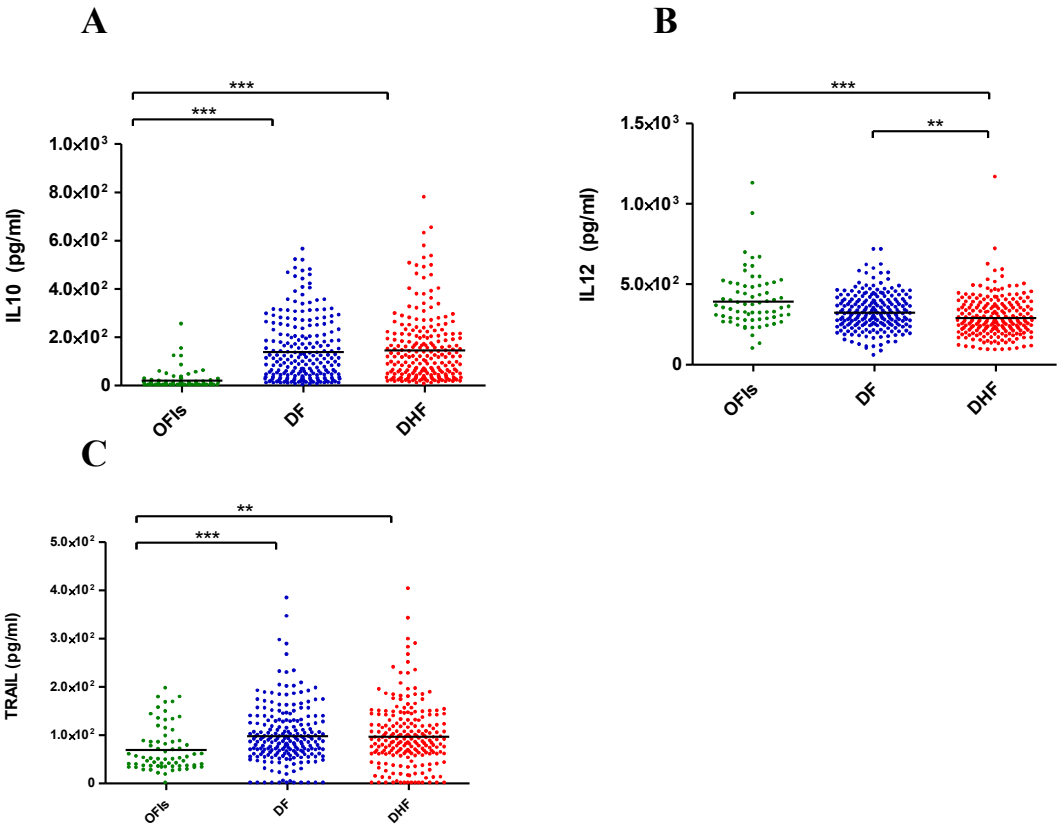


Figure 4.3.5: Plasma concentrations of cytokines in OFI, DF and DHF groups during the acute stage of the disease. Horizontal lines represent median values. Statistical significance was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).

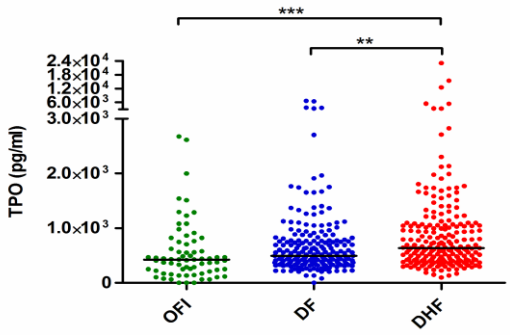
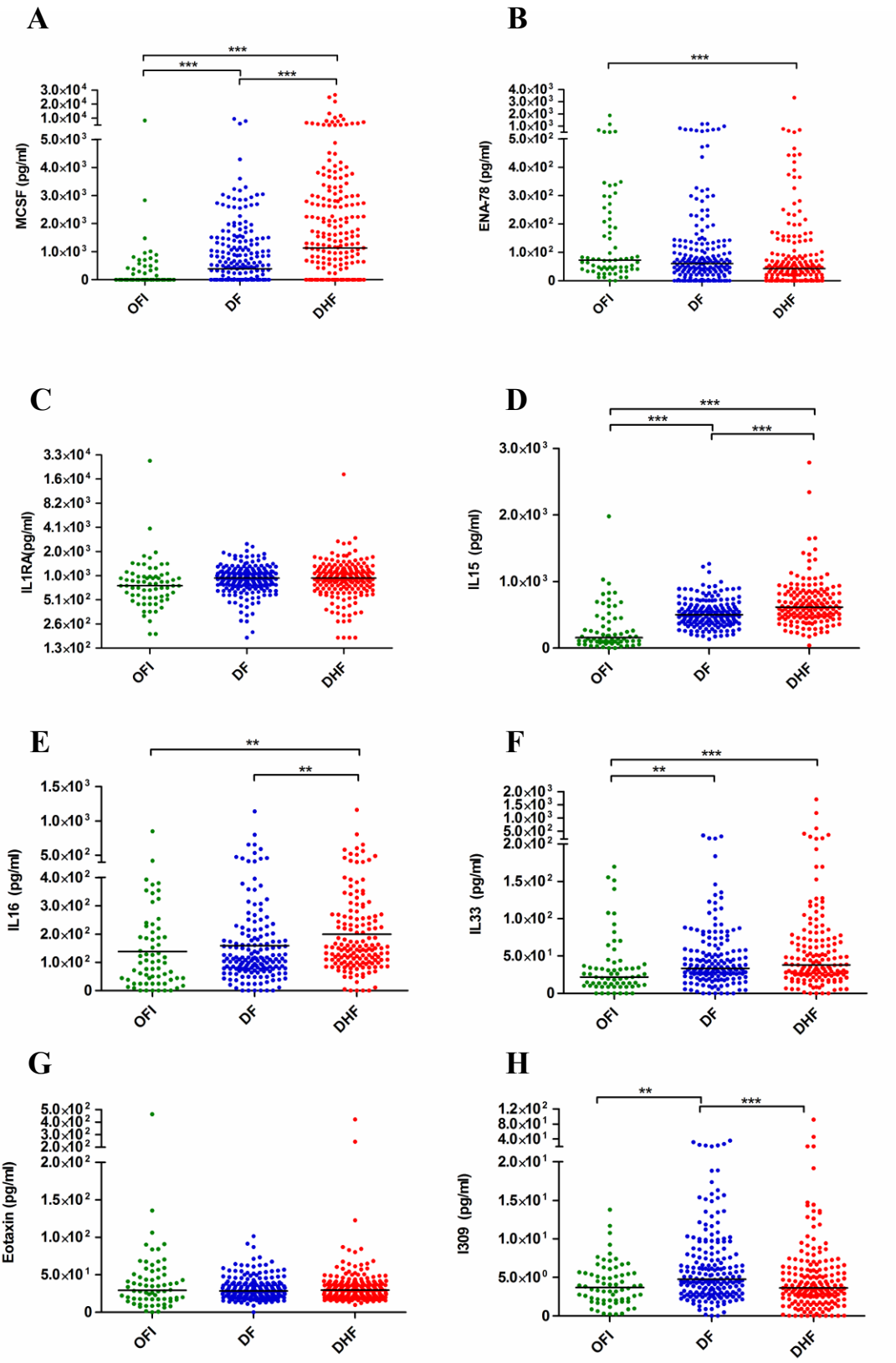


Figure 4.3.6: Plasma concentrations of growth factor in OFI, DF and DHF groups during the acute stage of the disease. Horizontal lines represent median values. Statistical significance was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).


A
MCSF (pg/ml)
3.0x10^4
2.0x10^4
1.0x10^4
5.0x10^3
4.0x10^3
3.0x10^3
2.0x10^3
1.0x10^3
0
***
***
***
OFI
DF
DHF
B
ENA-78 (pg/ml)
4.0x10^3
3.0x10^3
2.0x10^3
1.0x10^3
5.0x10^2
4.0x10^2
3.0x10^2
2.0x10^2
1.0x10^2
0
***
OFI
DF
DHF
C
IL1RA(pg/ml)
3.3x10^4
1.6x10^4
8.2x10^3
4.1x10^3
2.0x10^3
1.0x10^3
5.1x10^2
2.6x10^2
1.3x10^2
OFI
DF
DHF
D
IL15 (pg/ml)
3.0x10^3
2.0x10^3
1.0x10^3
0
***
***
***
OFI
DF
DHF
E
IL16 (pg/ml)
1.5x10^3
1.0x10^3
5.0x10^2
4.0x10^2
3.0x10^2
2.0x10^2
1.0x10^2
0
**
**
OFI
DF
DHF
F
IL33 (pg/ml)
2.0x10^3
1.5x10^3
1.0x10^3
5.0x10^2
2.0x10^2
1.5x10^2
1.0x10^2
5.0x10^1
0
***
**
OFI
DF
DHF
G
Eotaxin (pg/ml)
5.0x10^2
4.0x10^2
3.0x10^2
2.0x10^2
2.0x10^2
1.5x10^2
1.0x10^2
5.0x10^1
0
OFI
DF
DHF
H
I309 (pg/ml)
1.2x10^2
8.0x10^1
4.0x10^1
2.0x10^1
1.5x10^1
1.0x10^1
5.0x10^0
0
**
***
OFI
DF
DHF

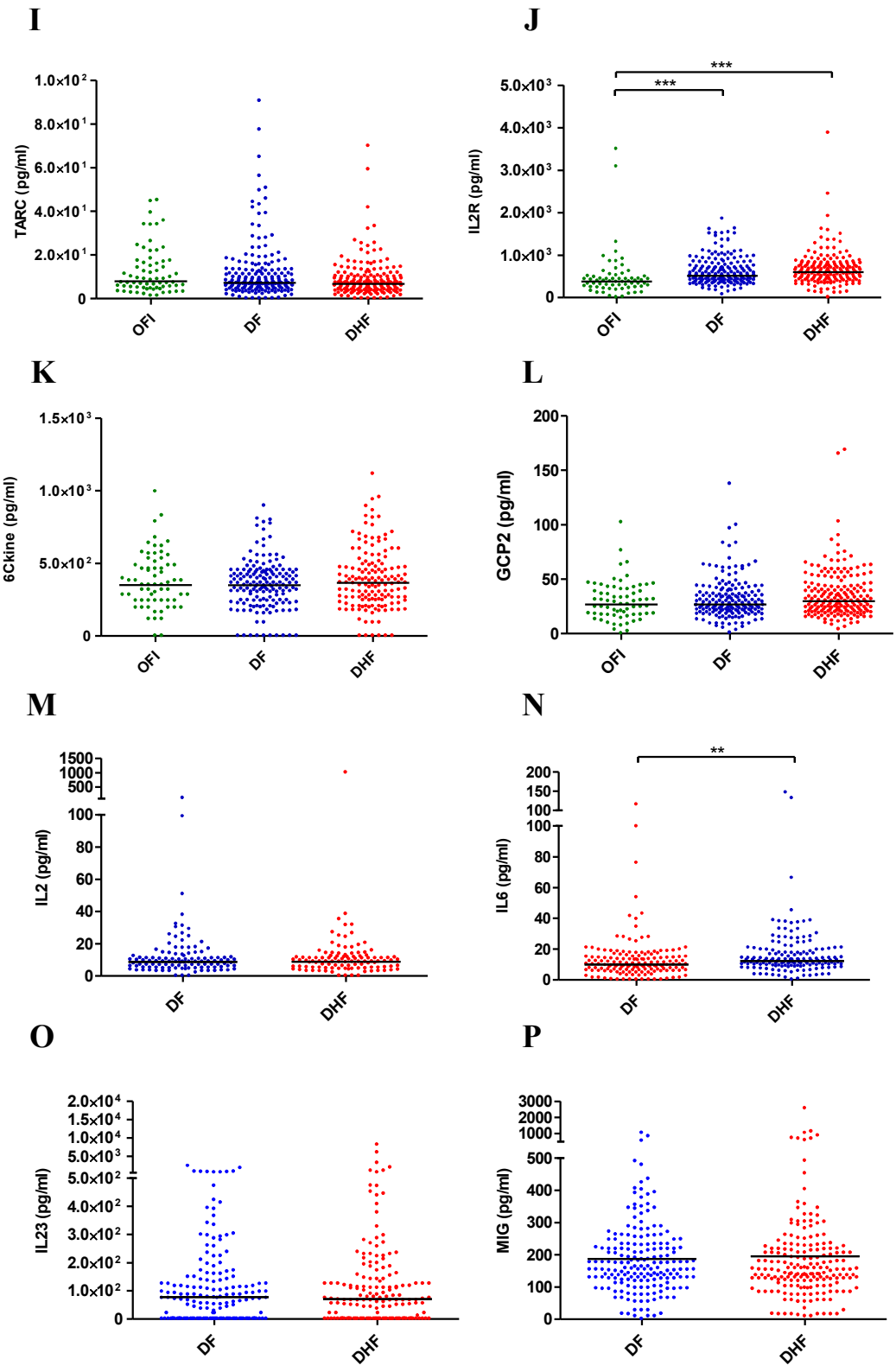


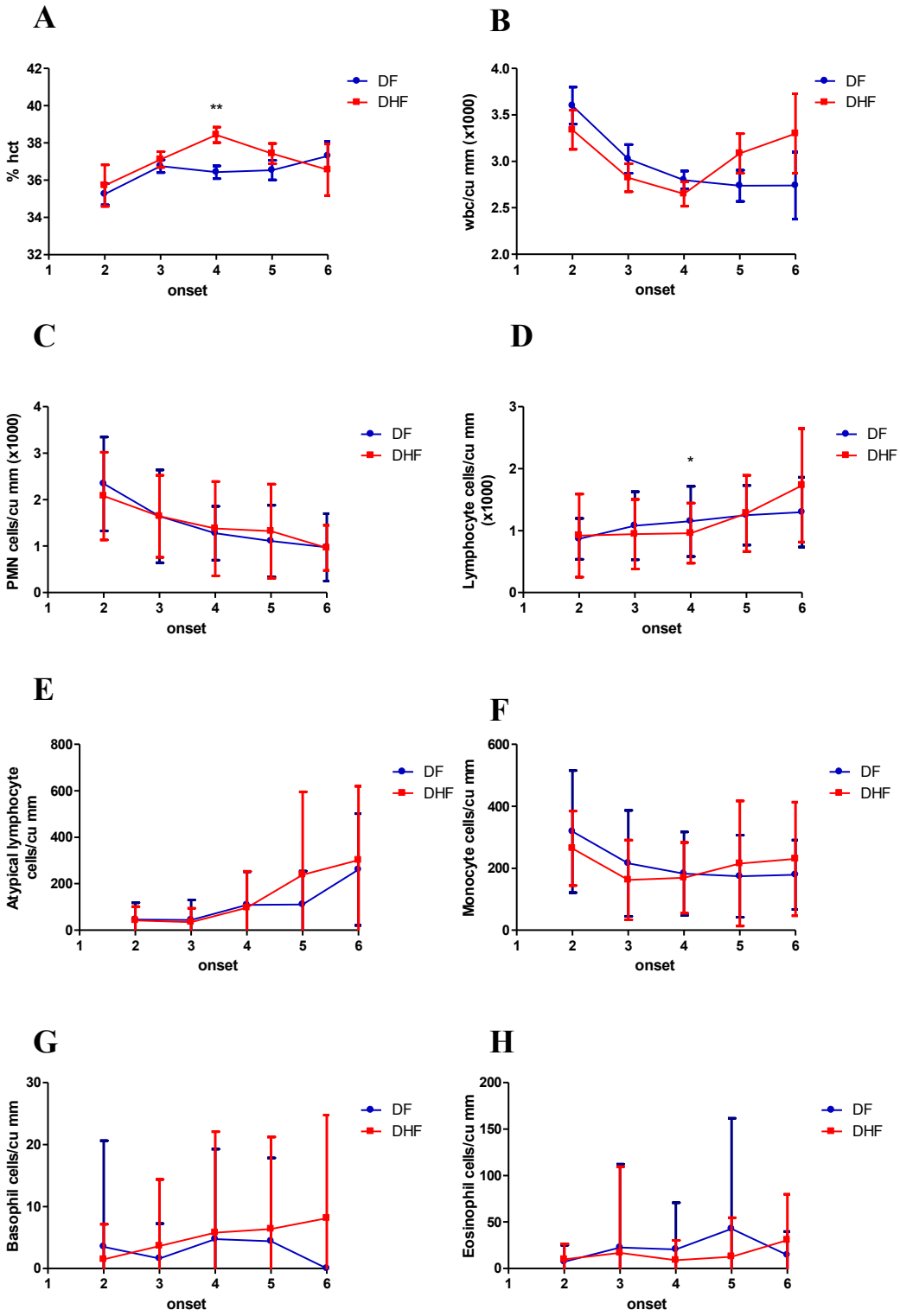
Figure 4.3.7: Plasma concentrations of biological characteristics with missing data greater than 10% in OFI, DF and DHF groups during the acute stage of disease. Horizontal lines represent median values. Statistical significance was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).

4.2.3 Levels of clinical and biological characteristics over the time course of illness

4.2.3.1 Clinical characteristics

Since Dengue disease is a time course-dependent illness, kinetics of biological markers were determined over the onset and during the acute phase in DF and DHF patients. Only plasma samples of the Dengue patients collected between day 2 and 6 were observed due to the small sample size on day 1, 7, 8 and 10 (Table 4.1 and Figure 4.4). Since there are different numbers of samples in each day, interpretation in term of symptom must be careful.

Over the time course of disease, an increase in the hct readings was found in DHF on day 4 of the illness while the wbc count was not significant different in both groups (Figures 4.4.A and B). When measuring the number of each white blood cell, trends in PMN, atypical lymphocyte, monocyte, basophil, and eosinophil numbers were similar and showed no significant difference in two groups (Figures 4.4C, E, F, G and H). Only lymphocytes slightly decreased in DHF on day 4; however there was no difference found on day 5 and 6 (Figure 4.4D). The platelet cell count, particularly in DHF, progressively declined (Figure 4.4I). At day 4, the frequency of platelet cells in DHF was below 100,000 cells per cu mm, which is the criterion to determine DHF. Decreases in the liver proteins and albumin, together with increases in the liver enzymes (ast and alt) were pronounced in DHF at days 3, 4 and 5 after the onset of disease (Figures 4.4J-M).


A
% hct
onset
DF
DHF
**
B
wbc/cu mm (x1000)
onset
DF
DHF
C
PMN cells/cu mm (x1000)
onset
DF
DHF
D
Lymphocyte cells/cu mm (x1000)
onset
DF
DHF
*
E
Atypical lymphocyte cells/cu mm
onset
DF
DHF
F
Monocyte cells/cu mm
onset
DF
DHF
G
Basophil cells/cu mm
onset
DF
DHF
H
Eosinophil cells/cu mm
onset
DF
DHF

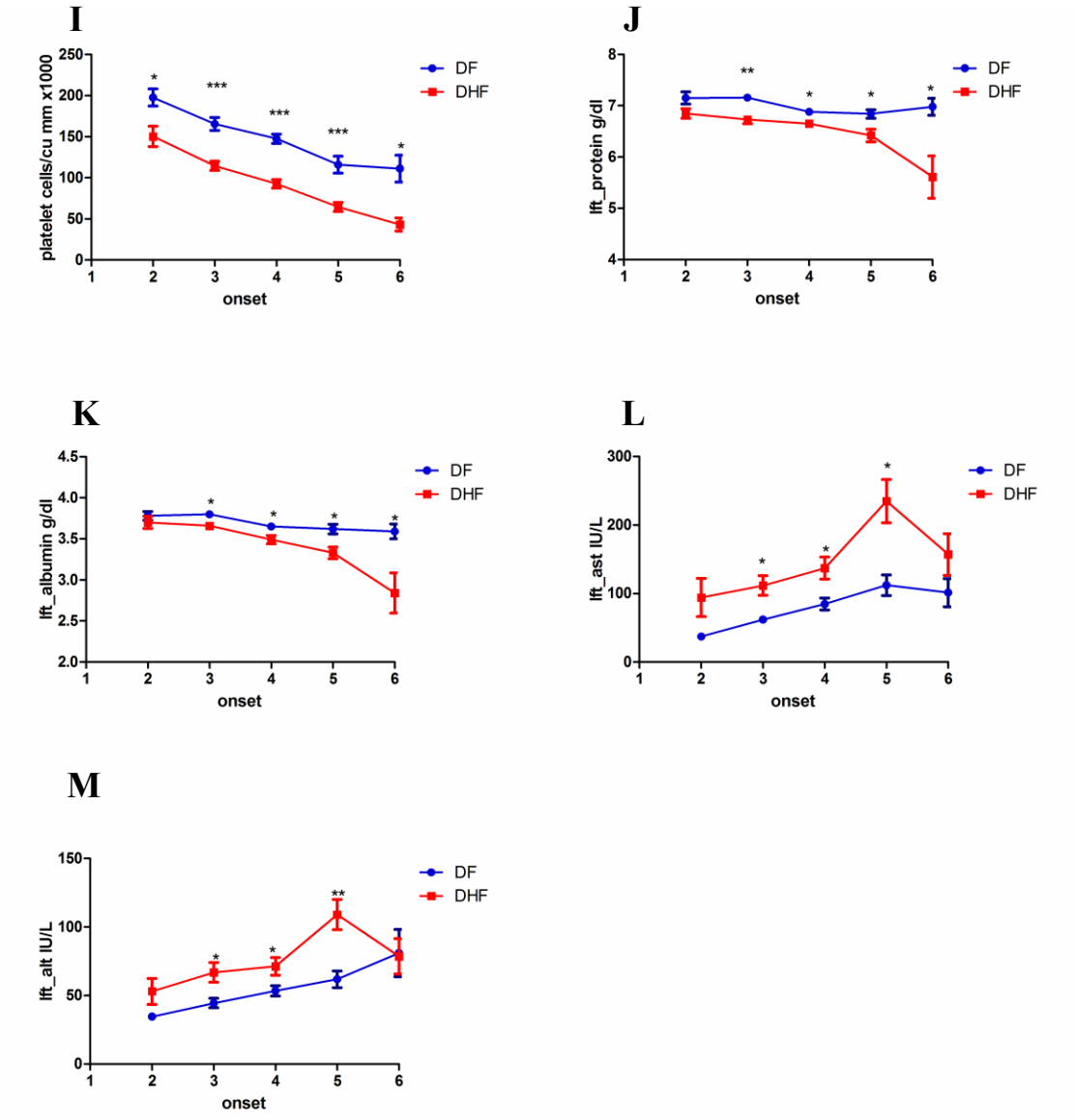


Figure 4.4: Clinical characteristics of patients with DF and DHF between day 2 and 6 after disease onset. The lines represent mean values with SEM (Standard error of mean). Statistical difference between DF and DHF at each time point was determined by Mann-Whitney test (*, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$).

4.2.3.2 Biological characteristics

Biological characteristics between DF and DHF were observed during day 2 to day 6 of the illness and the time course was constructed with different numbers in each day (Table 4.1 and Figures 4.5.1-4.5.6). Similar to clinical experiment, interpretation in term of symptom must be careful. Elevated plasma levels of MMP3 were detected in DHF between day 2 and day 6 and a significant difference was found on day 4 while other MMPs had no difference throughout this period (Figures 4.5.1A-E). Although there was no significant difference in these MMPs, patterns of their concentrations were quite similar in both DF and DHF. The levels of MMP1 and MMP8, which are collagenases, declined greatly on day 4 and increased afterwards (Figures 4.5.1A and 4.5.1D). MMP2 secretion increased in both groups in the first five days (Figure 4.5.1B). On day 6, the secretion of MMP2 in DF declined (Figure 4.5.1B). Unlike MMP2, MMP9 levels gradually decreased until day 5 (Figure 4.5.1E). On day 6, MMP9 concentration was increased in DHF whereas it was still low in DF (Figure 4.5.1E).

Next, the plasma levels of soluble receptor proteins were observed. sVEGFRII levels were markedly decreased in DHF and a significant difference was detected on day 4 and day 6 (Figure 4.5.2E). Higher levels of sTNFRI and sTNFRII were detected in DHF throughout the period; this was significant only at an early time-point during the disease (day 3) (Figures 4.5.2C and 4.5.2D). Apart from these three molecules, other soluble proteins showed no difference between DF and DHF. In general, concentrations of sEGFR and sIL6R levels increased in both groups (Figures 4.5.2A and 4.5.2H). The levels of sIL2Ra in DHF were constantly high over the time course (Figure 4.5.2F) while the levels of this protein in DF gradually increased; as seen in Figure 4.3.2F, sIL2Ra levels in the Dengue group were higher than those in the control group. Also, similar trends in sIL1RII and sgp130 levels were observed in DF and DHF (Figures 4.5.2B and 4.5.2I). Their concentrations were quite constant until day 6 when a decrease in these protein levels was detected in DHF. Lastly, patterns of sIL4R expression fluctuated in both groups (Figure 4.5.2G).

For the neutrophil chemotactic factors (IL8, ENA78 and GCP2), none of these markers showed a significant difference between the 2 groups from day 2 to day 6

(Figures 4.5.3A, 4.5.7B and 4.5.7P). IL8 levels in DHF were high throughout this period while those in DF gradually increased before declining again on day 6 (Figure 4.5.3A). Concentrations of GCP2 increased in both groups and the trend was similar (Figure 4.5.7P). For ENA78, levels were quite low and the number of samples was small; therefore, the pattern of expression was not clearly seen (Figure 4.5.7B).

Furthermore, three members in the CXCR3 family (IP10, ITAC and MIG) and BCA1, a chemokine that attracts B cells, were observed. Both IP10 and ITAC are known to chemoattract activated T cells and NK cells. Plasma levels of IP10 and ITAC were higher in DHF in the early days of the illness and significance was detected on day 3 (Figures 4.5.3B-C). A rapid decline of these chemokines was clearly seen in DHF after the 4^{th} day. Unlike IP10 and ITAC, plasma levels of MIG were slightly elevated throughout this period in both groups and presented the same patterns (Figure 4.5.7K). However, on day 6, the level of MIG in DHF decreased. An increase in BCA1 concentration was inclined similarly in both groups but a profound rise in BCA1 was found in DHF only on days 5 and 6 (significant on day 5) (Figure 4.5.3D). Similar to IP10 and ITAC, plasma levels of MCP1 and MCP2, monocyte attracting chemokines, were high in the early days, particularly in DHF, and then declined after day 4 (Figures 4.5.4A and 4.5.4D). Only MCP2 levels showed statistical significance on day 3 (Figure 4.5.4D). Likewise, high levels of MIP1β in DHF were seen during the early days and declined later while the levels of RANTES in DHF were lower than those in DF throughout the acute stage (Figures 4.5.4B and 4.5.4C). Next, a rise in MIP1δ, MIP3β and MIP3α levels was detected in DHF but the levels of these proteins subsequently dropped afterwards (Figures 4.5.4E, 4.5.4F and 4.5.4G). Increases in Eotaxin2 and TPO levels were also observed in both DF and DHF but their concentrations in DF declined after day 5 (Figures 4.5.4H and 4.5.6). In contrast to these two markers, concentrations of CTACK continuously increased throughout this stage in both groups (Figure 4.5.4I). Induction of IL10 was also detected in the plasma of Dengue patients and peaked on the 5^{th} day of the illness before declining (Figure 4.5.5A). In contrast to IL10, high levels of TRAIL were detected early and progressively dropped up to day 6 (Figure 4.5.5C). The levels of IL12 and I309 in DHF, similar to RANTES, were lower than those in DF (Figures 4.5.5B, and 4.5.7L).

Kinetic patterns of MCSF and TARC levels changed drastically over time in the DHF group. An induction of these proteins was found and peaked between day 3 and day 5 but levels greatly decreased thereafter to a very low level by day 6 (Figures 4.5.7A and 4.5.7M). Similarly, plasma levels of IL23 in DHF were markedly induced early during the disease (day 3) but declined afterwards (Figure 4.5.7H). In addition, reductions of IL1Ra and IL15 concentrations, together with inductions of IL16 and IL33, were observed in DHF while the concentrations of these proteins did not present a noticeable change in DF (Figures 4.5.7C, 4.5.7F, 4.5.7G and 4.5.7I). High concentrations of IL6 were also detected in DHF from the 2nd to the 4th day of illness and considerably decreased during the late days (Figure 4.5.7E). A change of IL6 levels in DF was not noticeably seen (Figure 4.5.7E). Finally, a rise in IL2R appeared in both groups (Figure 4.5.7N) whereas patterns of IL2, Eotaxin and 6Ckine were similar in both groups at every time point and there were no drastic changes in these markers (Figures 4.5.7D, 4.5.7J and 4.5.7O).

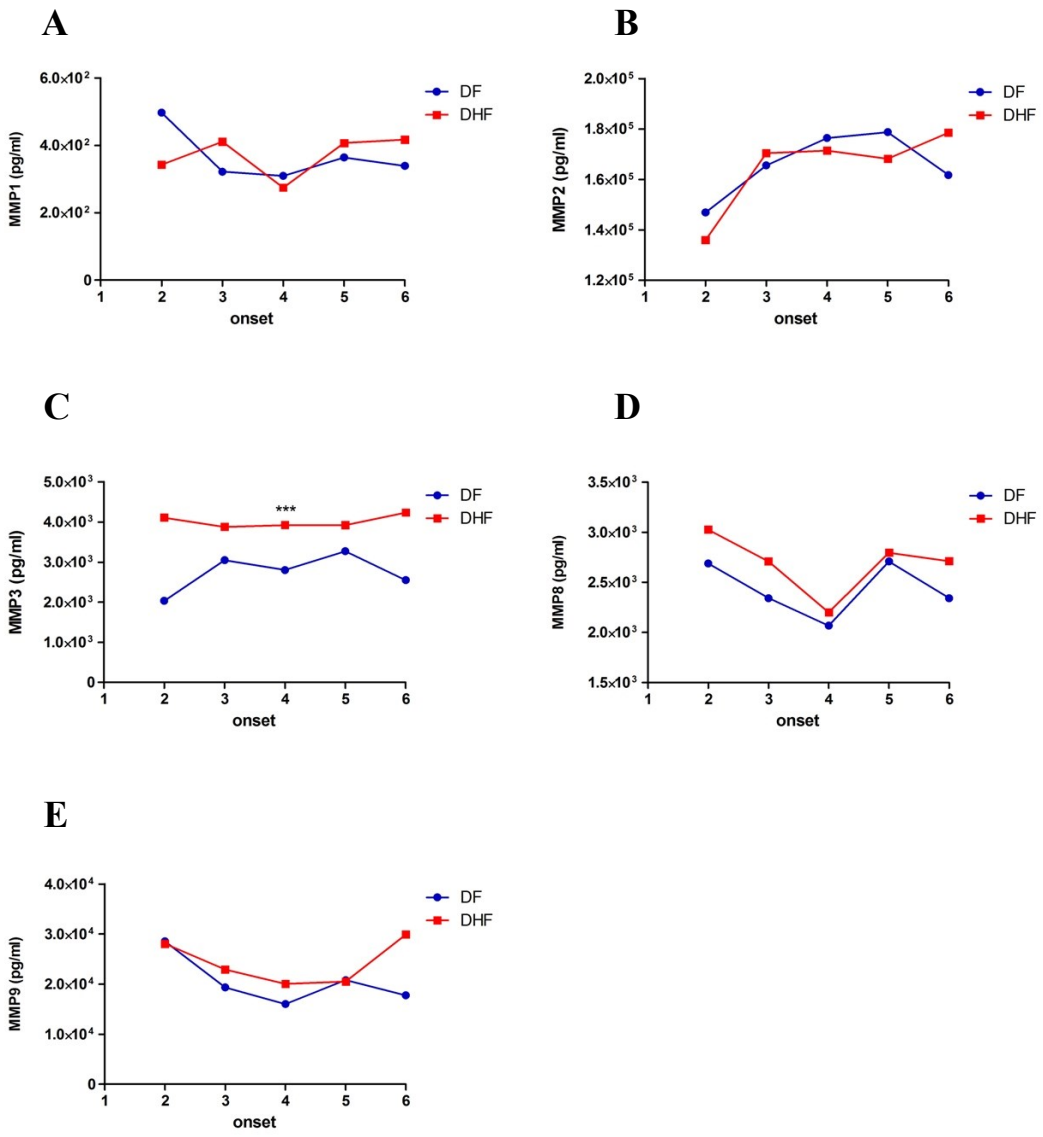
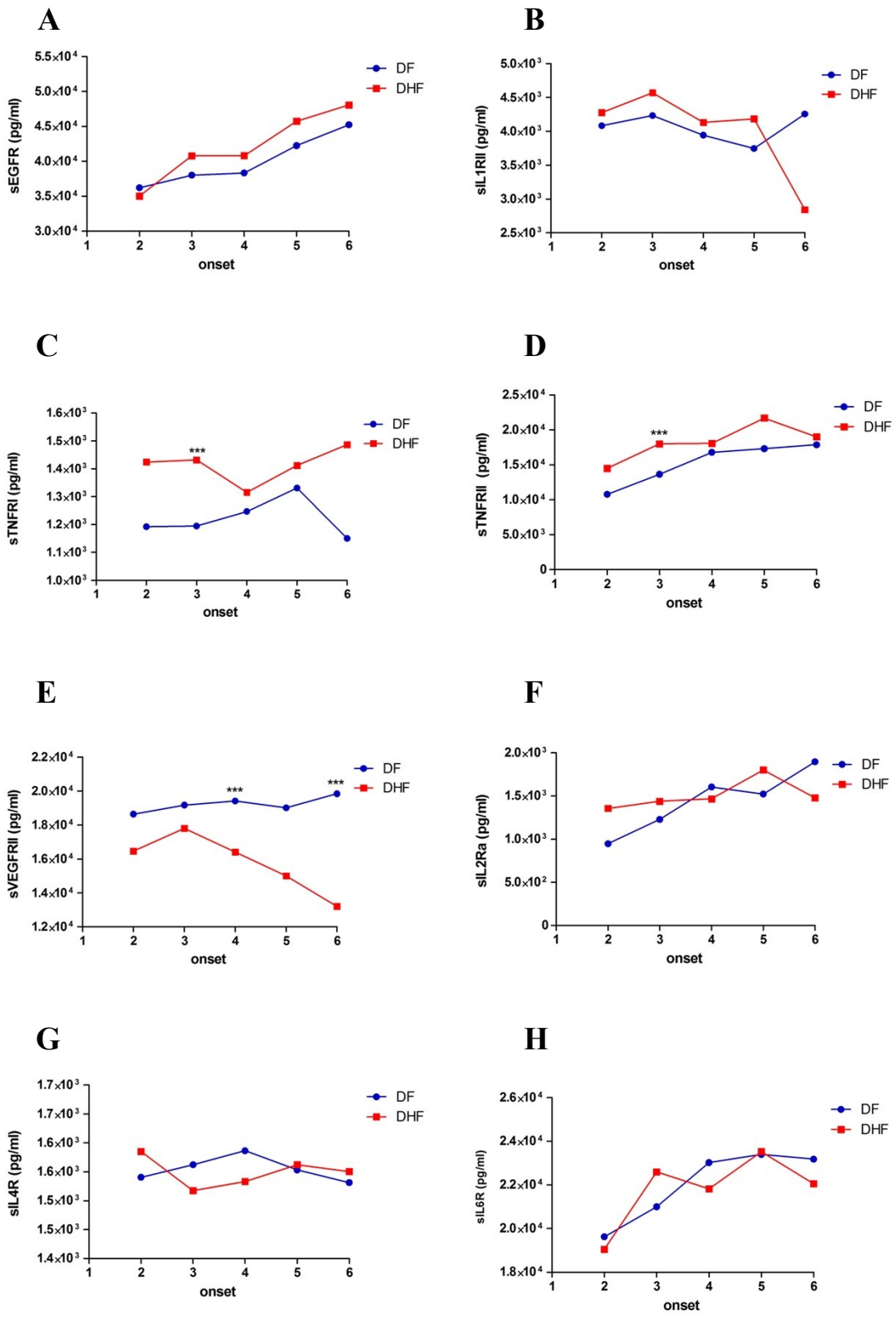


Figure 4.5.1: Comparison of plasma MMPs of patients with DF and DHF between day 2 and 6 of disease. The lines represent median values. Statistical difference between DF and DHF at each time point was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).


A
sEGFR (pg/ml)
5.5x10^4
5.0x10^4
4.5x10^4
4.0x10^4
3.5x10^4
3.0x10^4
1 2 3 4 5 6
onset
DF
DHF
B
sIL1RII (pg/ml)
5.0x10^3
4.5x10^3
4.0x10^3
3.5x10^3
3.0x10^3
2.5x10^3
1 2 3 4 5 6
onset
DF
DHF
C
sTNFRI (pg/ml)
1.6x10^3
1.5x10^3
1.4x10^3
1.3x10^3
1.2x10^3
1.1x10^3
1.0x10^3
***
1 2 3 4 5 6
onset
DF
DHF
D
sTNFRII (pg/ml)
2.5x10^4
2.0x10^4
1.5x10^4
1.0x10^4
5.0x10^3
0
***
1 2 3 4 5 6
onset
DF
DHF
E
sVEGFRII (pg/ml)
2.2x10^4
2.0x10^4
1.8x10^4
1.6x10^4
1.4x10^4
1.2x10^4
***
***
1 2 3 4 5 6
onset
DF
DHF
F
sIL2Ra (pg/ml)
2.0x10^3
1.5x10^3
1.0x10^3
5.0x10^2
0
1 2 3 4 5 6
onset
DF
DHF
G
sIL4R (pg/ml)
1.7x10^3
1.7x10^3
1.6x10^3
1.6x10^3
1.5x10^3
1.5x10^3
1.4x10^3
1 2 3 4 5 6
onset
DF
DHF
H
sIL6R (pg/ml)
2.6x10^4
2.4x10^4
2.2x10^4
2.0x10^4
1.8x10^4
1 2 3 4 5 6
onset
DF
DHF

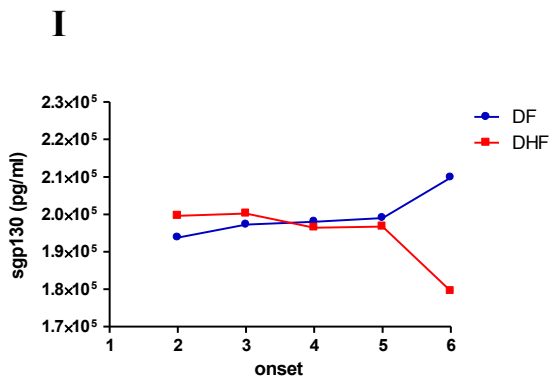


Figure 4.5.2: Comparison of plasma soluble receptors of patients with DF and DHF between day 2 and 6 of disease. The lines represent median values. Statistical difference between DF and DHF at each time point was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).

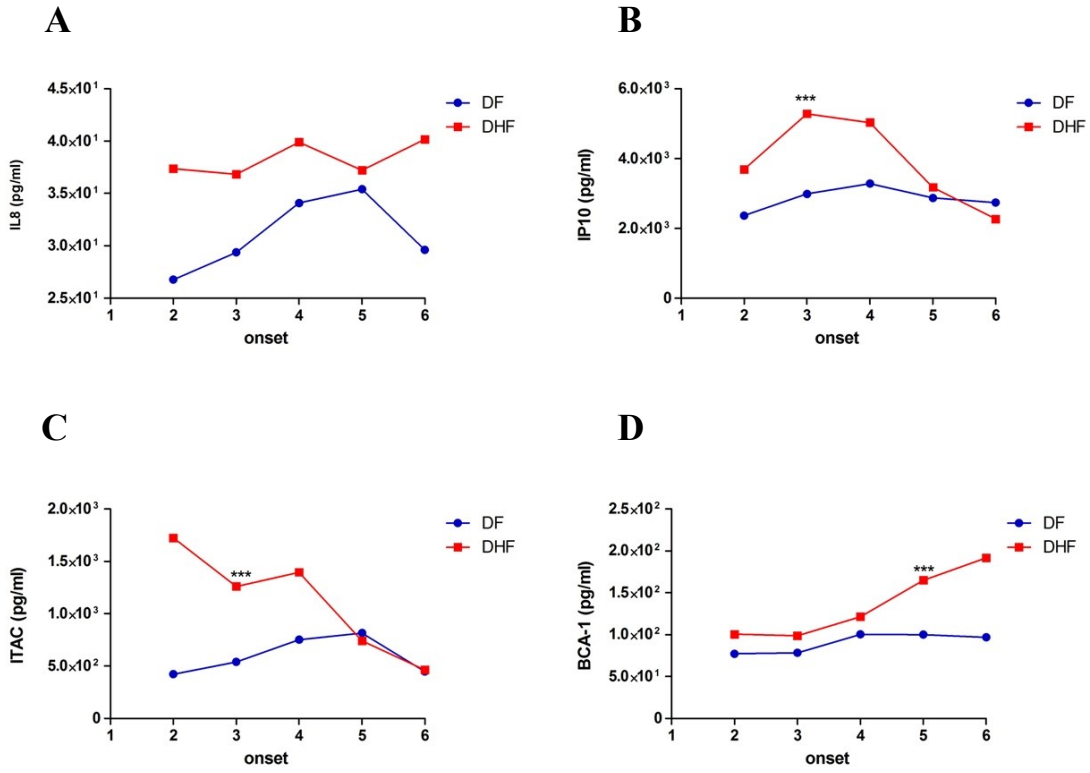
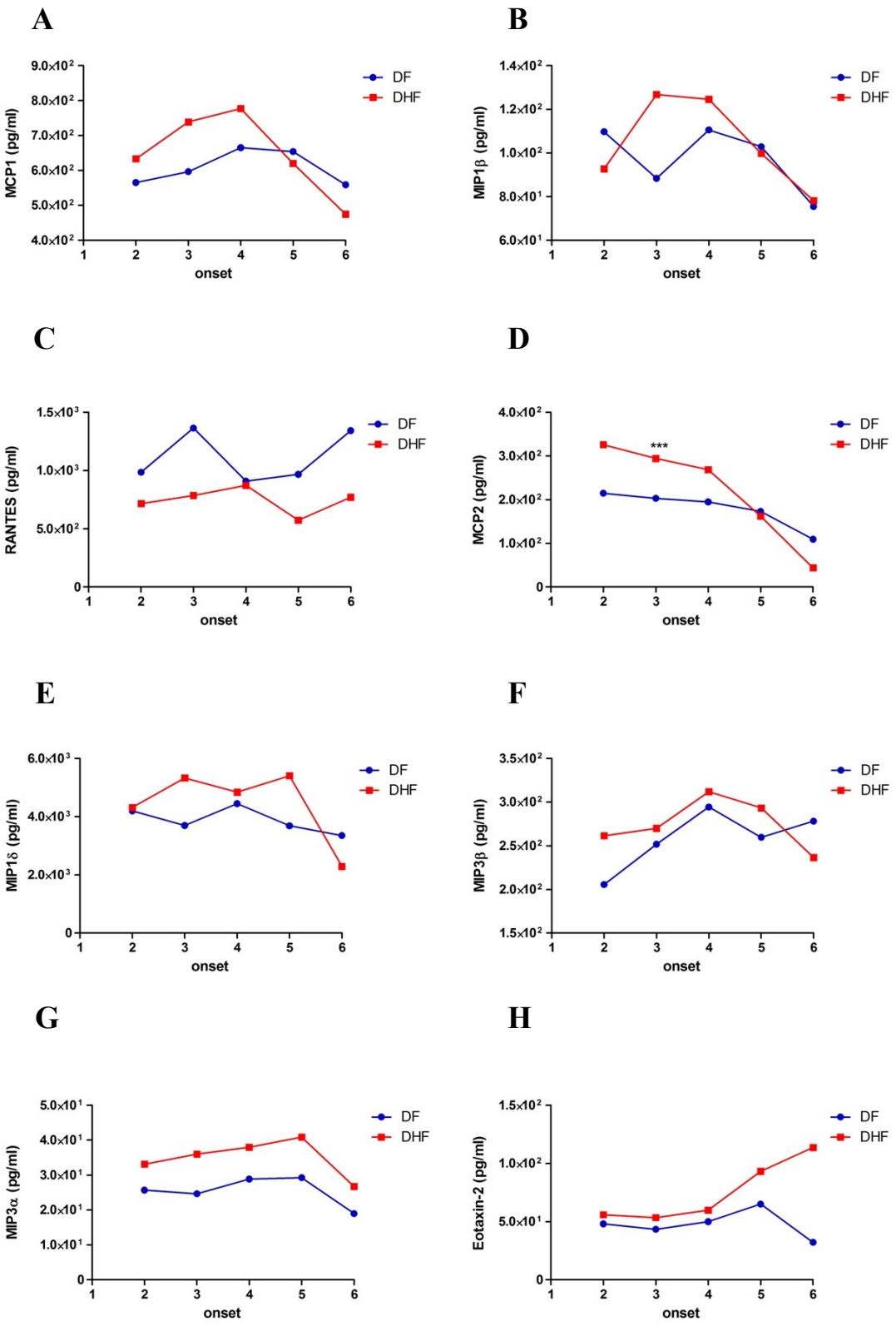


Figure 4.5.3: Comparison of plasma chemokines (CXC family) of patients with DF and DHF between day 2 and 6 of disease. The lines represent median values. Statistical difference between DF and DHF at each time point was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).


A
MCP1 (pg/ml)
9.0x10²
8.0x10²
7.0x10²
6.0x10²
5.0x10²
4.0x10²
1 2 3 4 5 6
onset
DF
DHF
B
MIP1β (pg/ml)
1.4x10²
1.2x10²
1.0x10²
8.0x10¹
6.0x10¹
1 2 3 4 5 6
onset
DF
DHF
C
RANTES (pg/ml)
1.5x10³
1.0x10³
5.0x10²
0
1 2 3 4 5 6
onset
DF
DHF
D
MCP2 (pg/ml)
4.0x10²
3.0x10²
2.0x10²
1.0x10²
0
***
1 2 3 4 5 6
onset
DF
DHF
E
MIP1δ (pg/ml)
6.0x10³
4.0x10³
2.0x10³
0
1 2 3 4 5 6
onset
DF
DHF
F
MIP3β (pg/ml)
3.5x10²
3.0x10²
2.5x10²
2.0x10²
1.5x10²
1 2 3 4 5 6
onset
DF
DHF
G
MIP3α (pg/ml)
5.0x10¹
4.0x10¹
3.0x10¹
2.0x10¹
1.0x10¹
0
1 2 3 4 5 6
onset
DF
DHF
H
Eotaxin-2 (pg/ml)
1.5x10²
1.0x10²
5.0x10¹
0
1 2 3 4 5 6
onset
DF
DHF

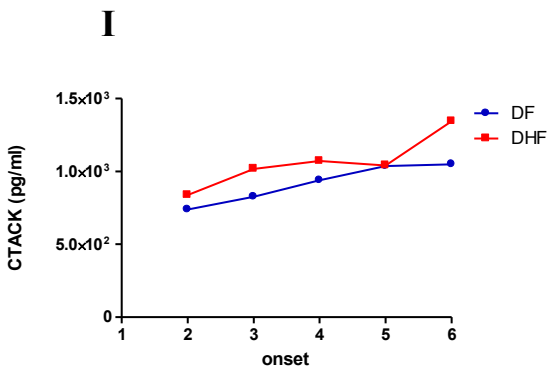


Figure 4.5.4: Comparison of plasma chemokines (CC family) of patients with DF and DHF between day 2 and 6 of disease. The lines represent median values. Statistical difference between DF and DHF at each time point was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).

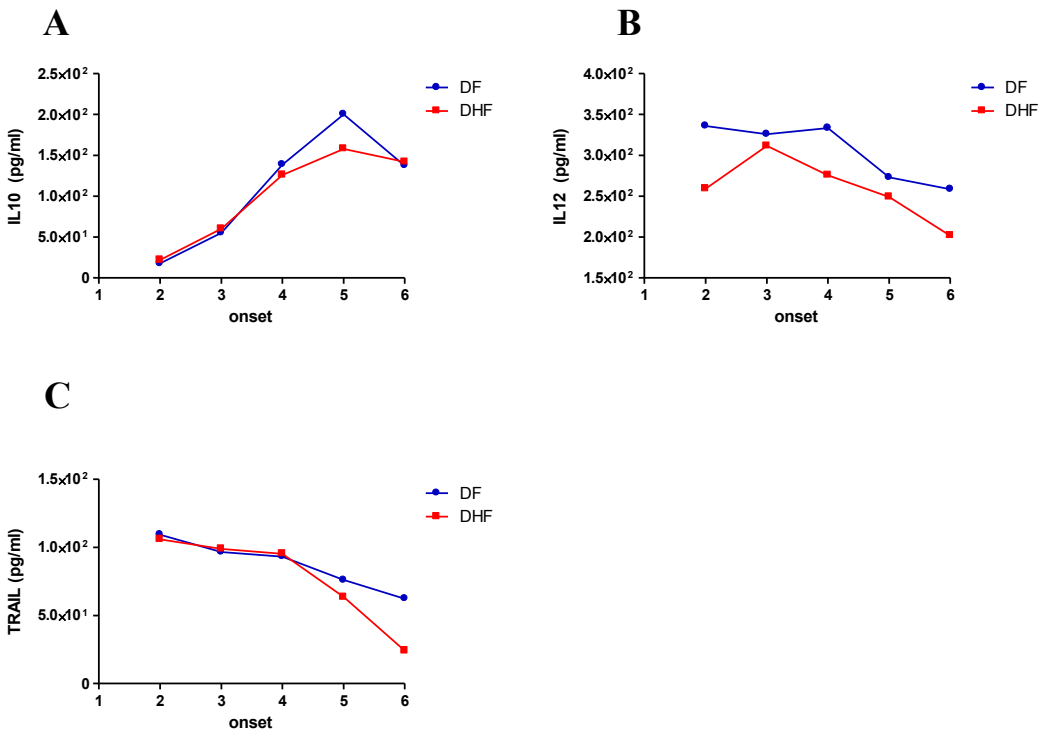


Figure 4.5.5: Comparison of plasma cytokines of patients with DF and DHF between day 2 and 6 of disease. The lines represent median values. Statistical difference between DF and DHF at each time point was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).

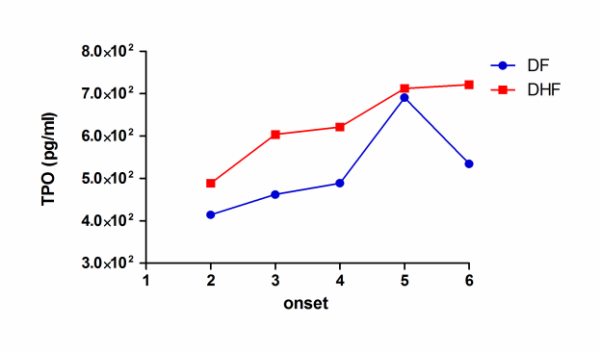
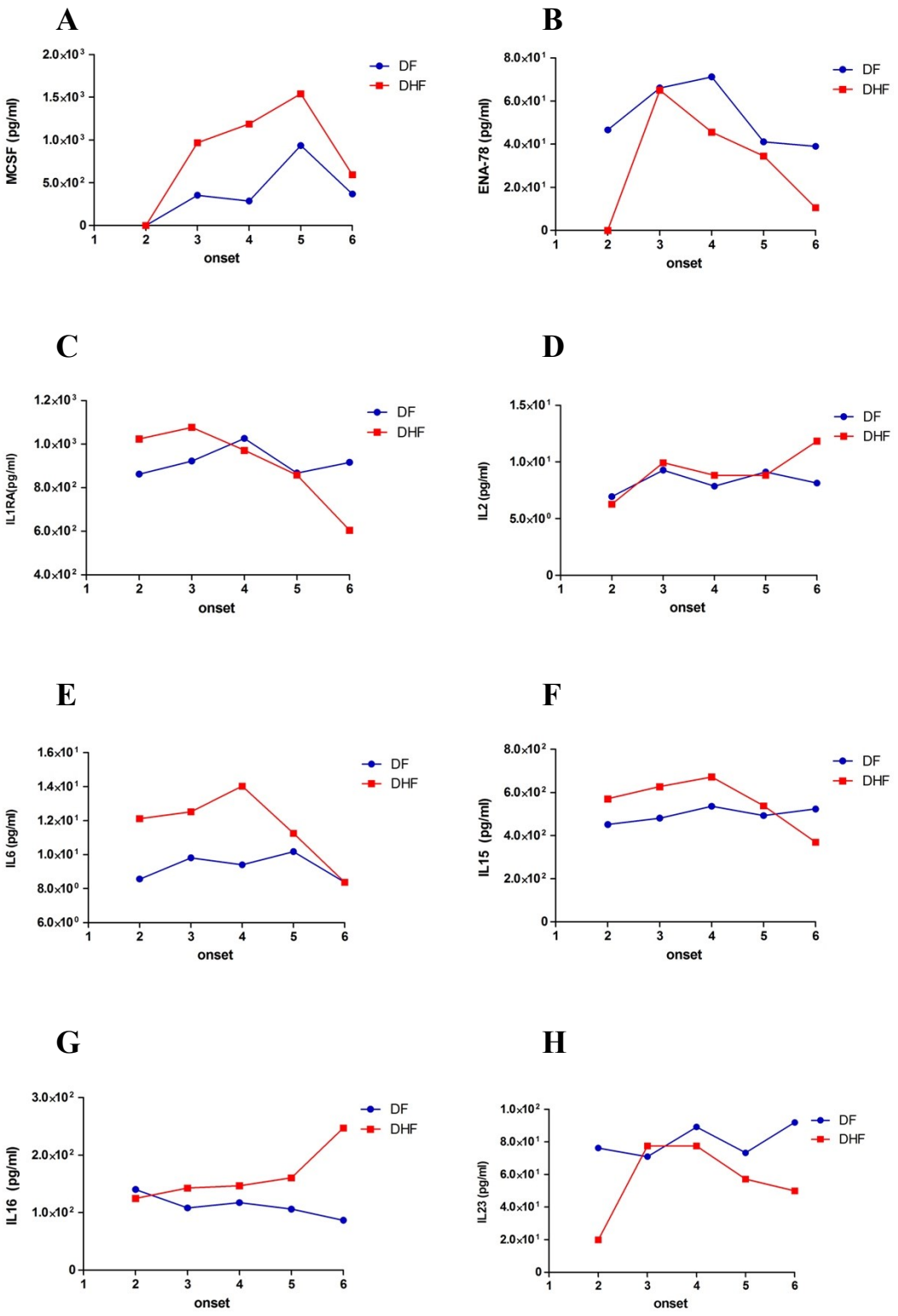


Figure 4.5.6: Comparison of plasma cytokines of patients with DF and DHF between day 2 and 6 of disease. The lines represent median values. Statistical difference between DF and DHF at each time point was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).


A
MCSF (pg/ml)
2.0x10³
1.5x10³
1.0x10³
5.0x10²
0
1 2 3 4 5 6
onset
DF
DHF
B
ENA-78 (pg/ml)
8.0x10¹
6.0x10¹
4.0x10¹
2.0x10¹
0
1 2 3 4 5 6
onset
DF
DHF
C
IL1RA(pg/ml)
1.2x10³
1.0x10³
8.0x10²
6.0x10²
4.0x10²
1 2 3 4 5 6
onset
DF
DHF
D
IL2 (pg/ml)
1.5x10¹
1.0x10¹
5.0x10⁰
0
1 2 3 4 5 6
onset
DF
DHF
E
IL6 (pg/ml)
1.6x10¹
1.4x10¹
1.2x10¹
1.0x10¹
8.0x10⁰
6.0x10⁰
1 2 3 4 5 6
onset
DF
DHF
F
IL15 (pg/ml)
8.0x10²
6.0x10²
4.0x10²
2.0x10²
0
1 2 3 4 5 6
onset
DF
DHF
G
IL16 (pg/ml)
3.0x10²
2.0x10²
1.0x10²
0
1 2 3 4 5 6
onset
DF
DHF
H
IL23 (pg/ml)
1.0x10²
8.0x10¹
6.0x10¹
4.0x10¹
2.0x10¹
0
1 2 3 4 5 6
onset
DF
DHF

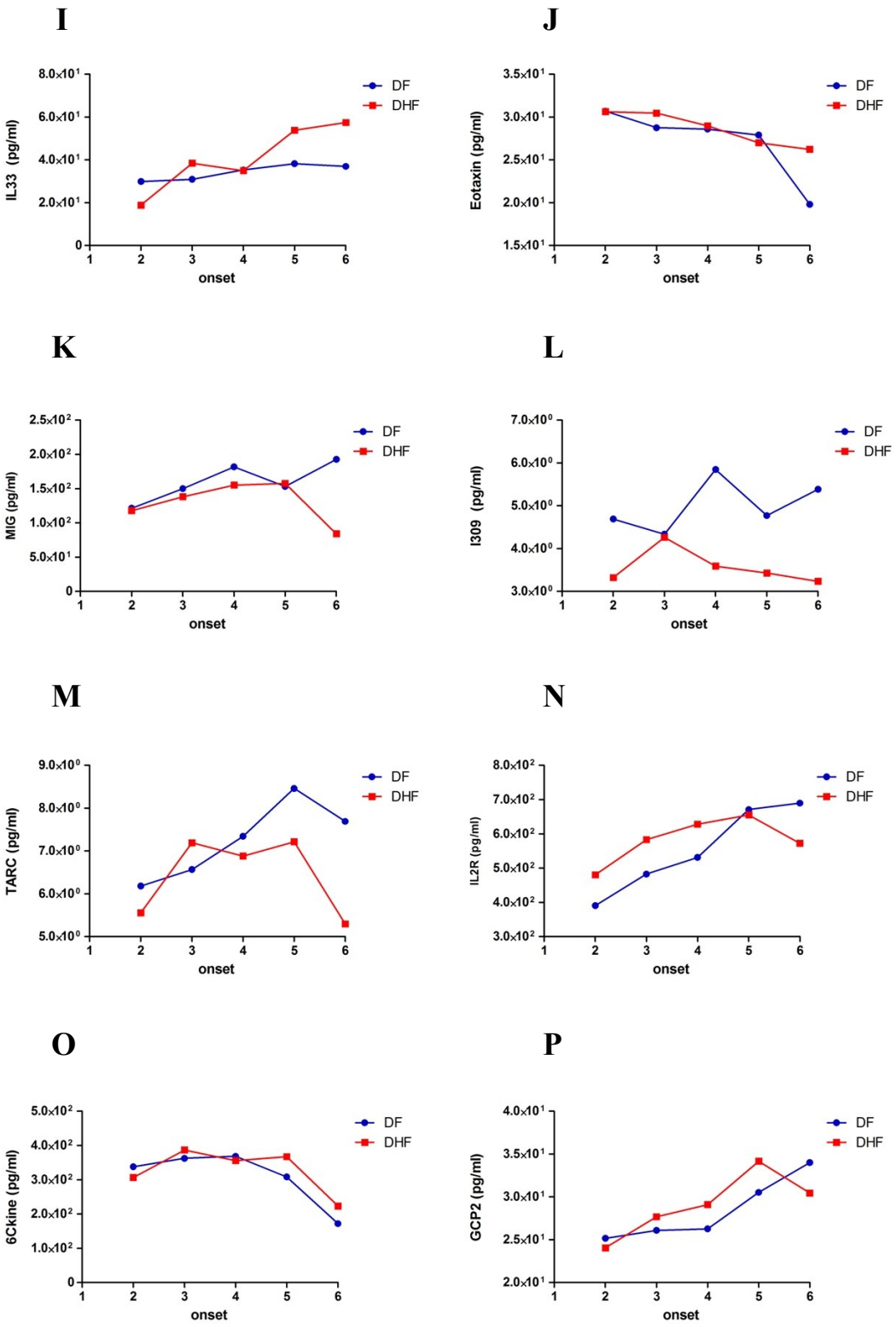


Figure 4.5.7: Comparison of biological markers with missing data greater than 10% of patients with DF and DHF between day 2 and 6 of disease. The lines represent median values. Statistical difference between DF and DHF at each time point was determined by the Mann-Whitney test (**, $p < 0.00076$; ***, $p < 0.0001$).

4.2.4 The relationship between soluble mediators in Dengue disease

To determine if soluble proteins induced during Dengue disease are associated, the correlation between markers was tested in Dengue groups. As seen in Table 4.6, a strong relationship was seen between IP10 and IL15 (r = 0.86) and ast and alt (r = 0.82). A high correlation was also observed in a pair of IL23 and IL33 (r = 0.74) and a pair of wbc and PMN (r = 0.73). Moreover, moderate correlations ($0.5 \leq r < 0.7$) were found between MMP8 and MMP9, IL12 and IL1Ra, MCP1and IL15, MCP1 and Eotaxin, MCP1 and IP10, MCP1 and ITAC, MCP1 and MCP2, sIL2Ra and IL2R, sIL2Ra and sTNFRII, sTNFRII and IL10, sTNFRII and sTNFRI, sTNFRII and platelets, sTNFRII and ast, ENA78 and RANTES, ENA78 and TARC, Trail and MCP2, ITAC and IL15, ITAC and IP10, ITAC and MCP2, IL33 and TPO, BCA1 and platelets, lft_albumin and lft_protein, ast and platelets, and alt and platelets. In addition, a weak correlation was found between several pairs of markers such as IL6 and IL8, MMP9 and IL10, to name a few. Of note, there were some negative correlations found. For example, a reduction of platelet cells was associated with increased levels of sTNFRII, BCA1, ast, and alt. The strong relationship between soluble mediators is drawn in Figure 4.6.

Table 4.6 Correlation between markers

	MMP1	MMP2	MMP3	MMP8	MMP9	IL-6	IL-8	IL-1RA	IL-10	IL-2	IL-2R	IL-12	IL-15	EOTOXIN	IP-10	MCP-1	MIG	MIP-1b	RANTES
MMP1	1.00																		
MMP2	0.01	1.00																	
MMP3	0.03	0.26	1.00																
MMP8	0.12	-0.11	0.01	1.00															
MMP9	0.11	0.02	0.06	0.66	1.00														
IL-6	-0.04	-0.01	0.03	0.01	0.09	1.00													
IL-8	0.00	0.08	0.11	-0.05	-0.07	0.38	1.00												
IL-1RA	-0.06	-0.08	-0.08	-0.02	-0.06	0.40	0.25	1.00											
IL-10	-0.13	0.14	0.03	-0.18	-0.30	0.20	0.36	0.11	1.00										
IL-2	-0.02	0.04	-0.01	0.12	0.16	0.34	0.12	0.35	-0.01	1.00									
IL-2R	0.02	0.18	0.12	0.06	-0.06	0.25	0.29	0.21	0.42	0.18	1.00								
IL-12	-0.02	-0.13	-0.17	0.04	-0.10	0.10	0.02	0.60	-0.06	0.12	0.12	1.00							
IL-15	-0.05	0.06	0.19	0.03	-0.02	0.41	0.44	0.36	0.23	0.18	0.33	0.12	1.00						
EOTOXIN	-0.02	0.19	0.08	0.01	0.04	0.14	0.22	0.30	-0.05	0.18	0.18	0.24	0.40	1.00					
IP-10	-0.11	0.13	0.22	-0.09	-0.05	0.29	0.42	0.31	0.23	0.11	0.27	0.03	0.86	0.33	1.00				
MCP-1	-0.10	0.17	0.11	-0.12	-0.07	0.45	0.37	0.34	0.15	0.12	0.20	0.15	0.64	0.51	0.58	1.00			
MIG	-0.04	-0.03	-0.07	0.02	-0.15	0.24	0.34	0.40	0.39	0.18	0.42	0.30	0.46	0.22	0.38	0.23	1.00		
MIP-1b	-0.08	-0.09	-0.05	0.06	-0.03	0.45	0.25	0.42	0.18	0.31	0.22	0.38	0.34	0.28	0.16	0.36	0.40	1.00	
RANTES	-0.02	-0.05	-0.06	0.12	0.06	-0.14	-0.25	0.08	-0.21	0.13	-0.03	0.39	-0.10	0.19	-0.20	-0.02	0.05	0.26	1.00
sEGFR	-0.06	0.08	0.15	0.05	0.00	0.03	0.16	0.09	0.16	0.10	0.24	-0.04	0.33	0.08	0.29	0.12	0.15	0.03	-0.01
sgp130	0.07	0.13	-0.05	-0.07	-0.04	0.03	0.15	0.20	-0.01	0.00	0.03	0.10	0.15	0.21	0.16	0.18	0.08	0.00	-0.06
sIL1RII	0.02	-0.01	0.23	0.06	0.07	-0.01	0.14	0.04	-0.08	-0.04	0.00	-0.05	0.21	0.11	0.20	0.13	0.00	0.01	-0.04
sIL-2Ra	-0.02	0.22	0.15	0.04	-0.05	0.13	0.26	0.18	0.43	0.13	0.64	0.01	0.27	0.14	0.30	0.13	0.35	0.02	-0.10
sIL-4R	-0.12	0.17	0.05	-0.04	-0.02	-0.10	-0.07	0.04	0.06	0.09	0.04	-0.01	0.03	0.11	0.04	0.06	0.09	-0.07	0.08
sIL-6R	0.01	0.29	0.09	-0.12	-0.11	0.03	0.08	-0.05	0.15	-0.02	0.13	-0.09	0.08	0.00	0.12	-0.01	0.08	-0.04	-0.04
sTNFRI	0.07	-0.02	0.29	0.05	0.02	0.25	0.37	0.26	0.26	0.07	0.33	0.03	0.35	0.05	0.36	0.28	0.26	0.19	-0.21
sTNFRII	-0.02	0.20	0.27	-0.05	-0.09	0.27	0.40	0.11	0.51	0.04	0.42	-0.09	0.37	0.02	0.42	0.26	0.27	0.15	-0.28
sVEGFRII	-0.03	-0.02	-0.16	-0.08	-0.17	-0.10	-0.11	0.21	-0.05	0.06	-0.07	0.20	-0.05	0.10	-0.06	0.11	0.03	0.00	0.22
MCP-2	-0.06	-0.12	0.03	-0.04	0.03	0.34	0.27	0.33	-0.03	0.02	0.06	0.18	0.41	0.29	0.40	0.61	0.11	0.37	0.02
ENA-78	0.07	-0.11	-0.07	-0.02	-0.07	0.01	-0.16	0.18	-0.09	0.11	0.02	0.31	-0.03	0.20	-0.10	-0.01	0.15	0.26	0.66
BCA-1	-0.03	0.07	0.17	0.07	0.01	0.14	0.34	0.06	0.29	0.00	0.33	-0.10	0.34	0.09	0.35	0.10	0.31	0.11	-0.18
I-309	0.01	0.17	-0.07	-0.02	-0.10	-0.11	-0.12	0.11	0.01	-0.05	0.07	0.25	0.05	0.19	0.06	0.12	0.11	0.02	0.16
IL-16	0.07	-0.01	0.08	-0.01	-0.08	-0.01	0.26	0.05	0.16	0.03	0.17	0.12	0.17	0.13	0.22	0.12	0.09	0.15	-0.02
MIP-1d	-0.01	0.09	0.08	0.03	-0.02	0.05	0.06	-0.01	0.04	-0.05	0.10	0.06	0.05	0.07	0.04	0.09	0.06	0.10	0.00
TARC	-0.01	-0.02	-0.10	0.09	-0.03	-0.02	-0.03	0.15	0.08	0.11	0.09	0.33	0.06	0.18	-0.01	0.04	0.20	0.25	0.48
6Ckine	0.03	0.04	-0.01	-0.01	-0.12	0.14	0.19	0.19	0.20	0.06	0.24	0.25	0.24	0.23	0.26	0.20	0.27	0.24	0.06
Eotaxin-2	0.05	0.10	0.15	-0.02	-0.03	0.09	0.31	0.03	0.21	-0.09	0.25	-0.06	0.37	0.16	0.36	0.18	0.22	0.03	-0.21
CTACK	-0.03	0.10	0.25	0.04	-0.01	0.12	0.15	-0.02	0.07	0.05	0.23	-0.01	0.27	0.14	0.19	0.15	0.12	0.09	-0.05
IL-23	-0.02	0.10	-0.02	-0.17	-0.14	0.01	0.06	0.13	0.15	0.02	0.15	0.09	0.19	0.16	0.19	0.14	0.22	0.11	0.20
TPO	-0.07	0.05	0.08	0.02	-0.09	0.19	0.20	-0.04	0.23	0.09	0.21	-0.03	0.22	0.06	0.09	0.10	0.15	0.17	-0.10
TRAIL	0.04	-0.03	-0.03	-0.05	0.00	0.13	0.07	0.25	-0.16	-0.09	-0.11	0.18	0.28	0.17	0.25	0.39	0.04	0.11	-0.10
IL-33	-0.11	-0.02	-0.01	-0.05	-0.14	0.16	0.24	0.22	0.35	0.09	0.26	0.10	0.31	0.06	0.28	0.19	0.42	0.18	0.00
M-CSF	0.01	-0.03	0.10	-0.04	-0.03	0.24	0.21	0.04	0.13	0.04	0.09	-0.04	0.16	-0.14	0.19	0.12	0.15	0.07	-0.19
GCP2	-0.13	0.02	0.06	-0.10	-0.14	0.12	0.34	0.18	0.33	0.04	0.21	-0.01	0.32	0.14	0.36	0.17	0.34	0.09	-0.15
I-TAC	-0.06	0.00	0.08	-0.08	-0.10	0.24	0.41	0.23	0.21	-0.05	0.14	0.00	0.53	0.27	0.58	0.52	0.34	0.19	-0.32
MIP3b	-0.09	0.19	0.00	0.00	-0.11	0.09	0.13	0.15	0.22	0.03	0.12	0.11	0.18	0.25	0.24	0.25	0.24	0.14	-0.02
MIP3a	-0.01	0.03	0.17	0.02	-0.05	0.28	0.35	0.15	0.17	0.08	0.18	0.05	0.34	0.23	0.36	0.29	0.22	0.18	-0.15
hct	0.09	0.16	0.22	0.07	0.05	-0.07	0.08	-0.11	0.11	-0.06	0.14	-0.17	0.10	-0.07	0.10	-0.07	0.05	-0.04	-0.06
wbc	0.11	-0.11	-0.03	0.35	0.31	-0.05	-0.18	0.04	-0.23	0.06	-0.01	0.00	-0.11	0.04	-0.14	-0.20	-0.01	-0.10	0.16
pmn	0.10	-0.24	-0.05	0.41	0.34	0.08	-0.17	0.08	-0.30	0.07	-0.12	-0.02	0.00	-0.03	-0.04	-0.05	-0.03	0.00	0.11
lymphocyte	0.01	0.20	0.05	0.03	0.01	-0.12	-0.03	-0.03	0.08	-0.02	0.11	0.00	-0.11	0.10	-0.12	-0.20	0.03	-0.15	0.06
atyp. lymphocyte	0.04	-0.01	-0.01	-0.04	-0.06	-0.08	0.03	-0.14	0.18	0.05	0.05	-0.16	-0.02	-0.06	-0.02	-0.20	0.09	-0.11	-0.07
monocyte	0.06	-0.05	0.03	0.17	0.20	-0.08	-0.14	-0.03	-0.17	-0.09	-0.06	-0.01	-0.22	0.04	-0.27	-0.22	-0.22	-0.10	0.18
basophil	0.04	-0.03	0.03	0.05	0.01	0.09	0.07	0.03	0.05	0.11	0.05	0.07	0.01	0.00	0.01	0.03	0.08	0.11	0.00
eosinophil	0.12	0.06	0.02	0.05	0.04	0.03	-0.03	-0.10	0.02	0.01	0.13	-0.02	-0.01	0.06	-0.08	-0.08	-0.03	0.01	0.04
platelet	0.11	-0.23	-0.28	0.00	-0.03	-0.14	-0.29	0.15	-0.36	-0.03	-0.27	0.35	-0.21	0.07	-0.28	-0.01	-0.08	0.02	0.37
lft_protein	0.08	-0.11	-0.10	-0.01	-0.07	-0.07	-0.19	0.13	-0.12	0.07	-0.17	0.16	-0.01	0.02	-0.10	-0.07	0.10	0.10	0.23
lft_albumin	0.02	-0.14	-0.12	-0.05	-0.10	-0.03	-0.17	0.19	-0.14	0.02	-0.16	0.25	-0.08	0.07	-0.09	0.02	0.10	0.14	0.16
lft_ast	-0.03	0.22	0.24	-0.02	-0.06	0.15	0.38	-0.01	0.36	0.07	0.35	-0.20	0.32	-0.01	0.33	0.09	0.22	0.03	-0.24
lft_alt	0.02	0.17	0.22	0.02	0.02	0.10	0.29	-0.05	0.27	0.03	0.23	-0.20	0.23	-0.02	0.24	0.04	0.14	-0.01	-0.17

	sEGFR	sgp130	sIL1RII	sIL-2Ra	sIL-4R	sIL-6R	sTNFRI	sTNFRII	sVEGFRII	MCP-2	ENA-78	BCA-1	I-309	IL-16	MIP-1d	TARC	6Ckine	Eotaxin-2	CTACK	IL-23	TPO
sEGFR	1.00																				
sgp130	0.12	1.00																			
sIL1RII	0.19	0.22	1.00																		
sIL-2Ra	0.33	0.16	0.11	1.00																	
sIL-4R	0.04	0.10	0.00	0.09	1.00																
sIL-6R	0.17	0.14	0.11	0.26	0.14	1.00															
sTNFRI	0.27	0.22	0.32	0.48	0.00	0.10	1.00														
sTNFRII	0.35	0.14	0.16	0.60	-0.02	0.30	0.64	1.00													
sVEGFRII	0.11	0.15	0.07	0.06	0.14	0.06	-0.08	-0.18	1.00												
MCP-2	0.00	0.19	0.23	0.01	-0.10	-0.14	0.31	0.12	0.03	1.00											
ENA-78	0.07	0.00	-0.01	-0.10	-0.01	-0.03	-0.16	-0.26	0.21	0.08	1.00										
BCA-1	0.32	0.06	0.10	0.43	0.00	0.16	0.33	0.48	-0.25	0.04	-0.07	1.00									
I-309	-0.03	0.05	-0.07	0.20	0.08	0.10	-0.09	-0.05	0.20	0.07	0.12	-0.03	1.00								
IL-16	0.16	0.09	0.06	0.16	-0.07	0.08	0.25	0.31	-0.07	0.15	0.09	0.24	0.08	1.00							
MIP-1d	0.04	0.05	0.08	0.02	-0.03	0.13	0.10	0.13	-0.06	0.12	-0.01	0.16	0.11	0.14	1.00						
TARC	0.12	-0.04	-0.08	0.04	-0.03	-0.01	-0.12	-0.11	0.12	0.06	0.62	0.10	0.36	0.10	0.01	1.00					
6Ckine	0.12	0.01	0.11	0.24	-0.09	0.13	0.22	0.21	0.00	0.24	0.20	0.28	0.23	0.35	0.26	0.23	1.00				
Eotaxin-2	0.32	0.15	0.25	0.35	0.01	0.20	0.34	0.43	-0.11	0.11	-0.14	0.37	0.10	0.23	0.12	0.07	0.26	1.00			
CTACK	0.20	-0.01	0.06	0.25	-0.03	0.07	0.18	0.27	-0.15	0.17	-0.12	0.27	0.16	0.17	0.18	0.06	0.12	0.25	1.00		
IL-23	0.22	0.00	-0.02	0.14	0.17	0.10	0.02	0.03	0.17	0.07	0.41	0.18	0.15	0.38	0.00	0.37	0.33	0.12	-0.05	1.00	
TPO	0.10	-0.06	-0.10	0.16	-0.02	0.08	0.17	0.26	-0.11	0.05	-0.23	0.27	0.06	0.34	0.13	0.06	0.26	0.18	0.28	0.21	1.00
TRAIL	-0.11	0.22	0.09	-0.14	-0.05	-0.07	0.11	-0.08	0.13	0.57	0.00	-0.12	0.18	0.08	0.05	-0.05	0.05	0.04	0.09	0.05	-0.10
IL-33	0.31	-0.05	-0.11	0.27	0.08	0.05	0.20	0.30	0.08	0.09	0.09	0.38	0.06	0.38	0.04	0.21	0.29	0.21	0.13	0.74	0.67
M-CSF	0.20	-0.06	0.04	0.07	-0.25	-0.02	0.21	0.30	-0.13	0.17	-0.02	0.20	-0.20	0.01	0.13	-0.03	-0.02	0.08	0.24	0.03	0.09
GCP2	0.33	0.12	0.11	0.32	0.21	0.13	0.33	0.39	-0.02	0.13	0.02	0.37	0.00	0.25	0.08	0.13	0.28	0.39	0.03	0.25	0.27
I-TAC	0.08	0.25	0.17	0.25	0.04	-0.01	0.39	0.38	-0.09	0.55	-0.24	0.29	0.07	0.27	0.13	-0.13	0.24	0.35	0.18	0.13	0.20
MIP3b	-0.05	0.14	-0.04	0.26	0.16	0.10	0.13	0.19	0.00	0.17	-0.13	0.24	0.24	0.12	0.16	0.05	0.23	0.17	0.16	0.09	0.23
MIP3a	0.24	0.06	0.13	0.26	-0.06	0.07	0.31	0.37	-0.07	0.22	-0.06	0.44	-0.03	0.27	0.15	0.06	0.32	0.27	0.24	0.12	0.20
hct	0.13	0.13	0.19	0.09	0.08	0.24	0.09	0.18	-0.03	-0.07	-0.06	0.14	-0.05	-0.02	0.03	-0.01	0.00	0.14	0.01	-0.01	-0.01
wbc	-0.02	-0.10	-0.02	-0.01	0.08	-0.01	-0.09	-0.15	0.04	-0.17	0.12	-0.02	0.08	-0.08	-0.05	0.10	0.09	0.02	-0.12	0.00	-0.14
pmn	-0.09	-0.05	0.10	-0.18	0.06	-0.17	-0.04	-0.24	0.05	0.07	0.10	-0.06	-0.02	-0.12	-0.01	0.03	0.03	-0.09	-0.20	-0.06	-0.14
lymphocyte	0.11	-0.06	-0.14	0.22	0.07	0.21	-0.07	0.11	0.02	-0.30	0.04	0.04	0.20	0.01	0.00	0.10	0.08	0.18	0.16	0.09	0.03
atyp. lymphocyte	0.16	-0.10	-0.07	0.10	-0.01	0.22	-0.05	0.20	-0.15	-0.28	-0.08	0.13	-0.01	0.07	-0.03	-0.02	-0.08	0.18	0.08	0.03	0.00
monocyte	-0.11	0.02	0.06	-0.11	0.13	-0.02	-0.13	-0.25	0.15	-0.14	0.11	-0.06	0.03	-0.17	0.00	-0.02	0.03	-0.12	-0.17	-0.10	-0.15
basophil	-0.05	0.02	-0.06	0.08	-0.01	0.05	0.08	0.06	0.02	0.01	0.02	0.06	0.05	-0.03	0.00	0.05	0.03	0.10	0.00	-0.02	0.04
eosinophil	-0.10	-0.04	-0.04	0.13	-0.02	0.01	-0.02	0.01	-0.02	-0.09	-0.05	0.06	0.04	-0.01	-0.03	0.01	0.07	0.00	0.01	-0.09	0.05
platelet	-0.31	0.10	-0.05	-0.30	0.06	-0.18	-0.26	-0.56	0.47	0.10	0.33	-0.50	0.17	-0.20	-0.05	0.14	-0.02	-0.35	-0.26	0.02	-0.31
lft_protein	-0.08	0.12	-0.03	-0.19	0.14	0.02	-0.18	-0.34	0.35	-0.05	0.25	-0.14	0.03	-0.12	-0.07	0.12	-0.02	-0.20	-0.20	0.12	-0.10
lft_albumin	-0.10	0.15	0.04	-0.26	0.10	-0.04	-0.16	-0.34	0.33	0.08	0.17	-0.22	0.08	-0.21	-0.02	0.06	-0.02	-0.20	-0.19	0.03	-0.12
lft_ast	0.46	0.11	0.20	0.41	0.03	0.26	0.38	0.61	-0.27	0.02	-0.15	0.49	-0.11	0.29	0.15	-0.06	0.20	0.42	0.29	0.13	0.28
lft_alt	0.37	0.11	0.23	0.29	0.01	0.23	0.31	0.44	-0.22	-0.02	-0.09	0.33	-0.13	0.22	0.11	-0.05	0.11	0.33	0.24	0.06	0.19

	TRAIL	IL-33	M-CSF	GCP2	I-TAC	MIP3b	MIP3a	hct	wbc	pmn	lymphocyte	atyp. lymphocyte	monocyte	basophil	eosinophil	platelet	lft_protein	lft_albumin	lft_ast	lft_alt
TRAIL	1.00																			
IL-33	-0.01	1.00																		
M-CSF	0.13	0.19	1.00																	
GCP2	-0.08	0.40	0.04	1.00																
I-TAC	0.41	0.23	0.14	0.42	1.00															
MIP3b	0.13	0.12	-0.12	0.34	0.44	1.00														
MIP3a	0.08	0.29	0.20	0.30	0.36	0.31	1.00													
hct	-0.02	0.00	-0.06	0.05	0.08	0.06	-0.02	1.00												
wbc	-0.15	-0.07	-0.20	-0.06	-0.22	-0.14	-0.04	-0.01	1.00											
pmn	-0.01	-0.12	-0.13	-0.04	-0.08	-0.08	0.00	0.02	0.73	1.00										
lymphocyte	-0.14	0.11	-0.06	0.01	-0.21	-0.05	-0.05	-0.05	0.47	-0.13	1.00									
atyp. lymphocyte	-0.18	0.09	-0.01	0.06	-0.07	-0.04	0.06	0.07	0.08	-0.21	0.24	1.00								
monocyte	-0.11	-0.20	-0.23	-0.14	-0.25	-0.14	-0.10	0.14	0.44	0.32	0.09	-0.15	1.00							
basophil	0.02	0.05	0.02	0.01	0.00	0.08	0.05	0.01	0.01	-0.03	0.03	-0.01	0.04	1.00						
eosinophil	-0.04	-0.05	-0.12	-0.04	-0.06	0.07	-0.02	0.05	0.07	-0.02	0.09	-0.03	0.16	0.21	1.00					
platelet	0.24	-0.23	-0.27	-0.28	-0.21	-0.08	-0.23	-0.24	0.18	0.26	-0.05	-0.36	0.21	0.02	0.02	1.00				
lft_protein	0.08	0.04	-0.22	-0.08	-0.09	0.00	-0.06	0.14	0.12	0.19	-0.09	-0.12	0.21	0.00	0.01	0.42	1.00			
lft_albumin	0.19	-0.08	-0.17	-0.12	-0.03	0.01	-0.16	0.09	0.01	0.15	-0.16	-0.24	0.14	0.01	0.01	0.40	0.65	1.00		
lft_ast	-0.19	0.29	0.34	0.39	0.26	0.11	0.29	0.16	-0.09	-0.18	0.13	0.29	-0.20	-0.01	-0.07	-0.65	-0.29	-0.29	1.00	
lft_alt	-0.20	0.17	0.24	0.26	0.15	0.04	0.18	0.25	-0.05	-0.11	0.06	0.25	-0.10	0.02	-0.06	-0.51	-0.18	-0.21	0.82	1.00

<0.3	0.3≤r<0.5	0.5≤r<0.7	≥0.7

The color coding for a correlation: strong (r≥ 0.7, red), moderate (0.5≤r<0.7, yellow), fair (0.3 ≤r<0.5, green) and weak (r<0.3, white). The grey color is an identity of the same marker. The spearman's correlation coefficient (r) was calculated from Dengue groups.

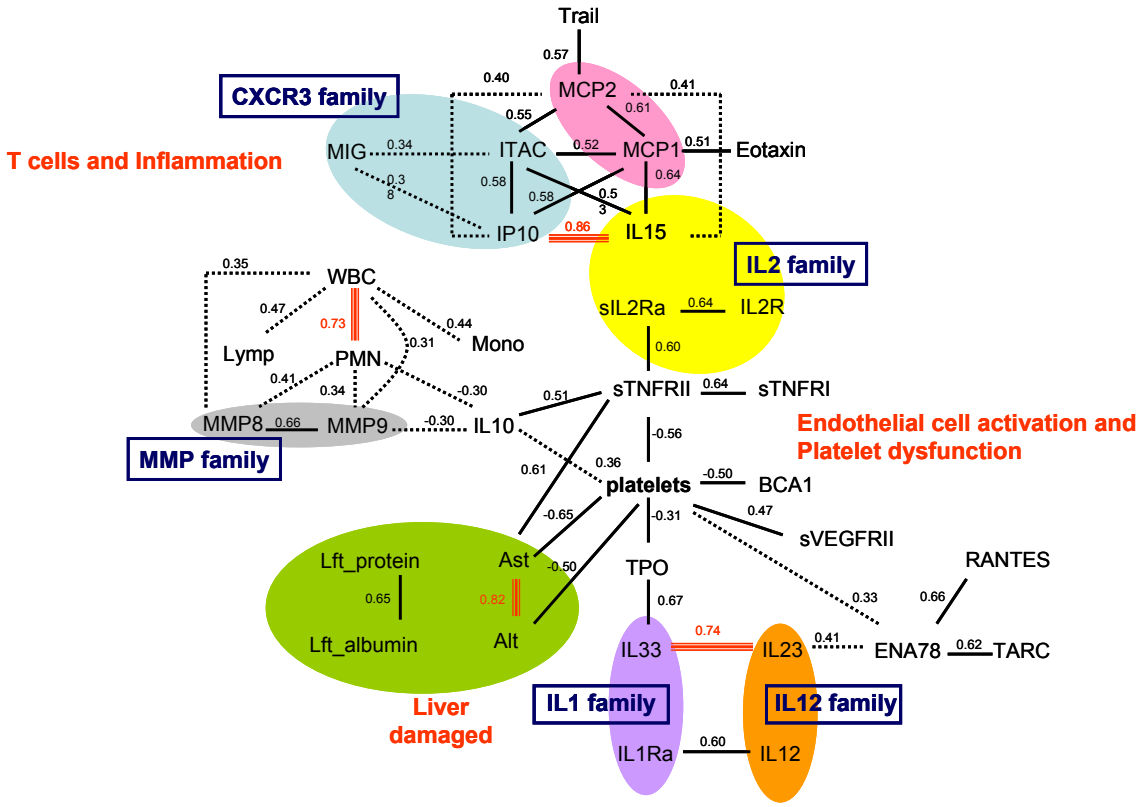


Figure 4.6: The relationship between soluble mediators in Dengue infection. The numbers presented in the figure are the correlation coefficient ($-1 \leq r \leq 1$).

4.2.5 Identification of predictive markers

4.2.5.1 Binary logistic regression

To identify predictive markers for discriminating between DHF and DF, binary logistic regression was performed as described in Materials and Methods (section 2.16) under the suggestion of Dr. Bernard North, the Imperial College Statistical Advisory Service. Logistic regression was employed with DHF and DF as a binary outcome, approximating the log odds of the probability of DHF status (logit of DHF) by a weighted combination of a subset of predictive biomarkers from the data set by backward elimination of non-significant biomarkers.

First, the markers which were associated with Dengue severity (Table 4.2, p-value[a] <0.05; Table 4.3, p-value[a] $<7.6 \times 10^{-4}$, and Figure 4.2) and contained fewer than 50 missing values (<10% missing value) were chosen. These markers consisted of 7 clinical parameters and 15 biomarkers including hct, lymphocyte platelet, lft albumin, lft protein, ast, alt, MMP3, sTNFRI, sTNFRII, sVEGFRII, IL8, IP10, ITAC, BCA1, RANTES, MCP2, MIP3α, Eotaxin2, CTACK, IL12 and TPO. Of these markers, MMP3, BCA1, MIP3α, Eotaxin2, and CTACK are newly identified. Before using the logistic regression for analysis, the concentrations of these markers should be in a normal distribution; otherwise the model obtained might be skewed. Any markers with skewed distribution were log-transformed. These markers were ast, alt, MMP3, sTNFRI, IL8, IP10, ITAC, BCA1, RANTES, MCP2, MIP3α, Eotaxin2, IL12 and TPO.

The data set was divided into a training set (2/3 of the whole data) and a testing set (1/3 of the whole data) in order to avoid over-fitting of the data. Next, to test how well a statistical model fits the set of observed data, the goodness of fit test was performed in the training data set. The goodness of fit is used for measuring how far the observed data deviate from the expected data under the null hypothesis that a model is fit. The p-value was 0.08, which indicated that there was no lack of fit (p-value > 0.05), suggesting the model would be accurate enough. Then, a final model was obtained from the training set. Platelets and MCP2 were found to be predictive with low platelet cell count and high concentration of MCP2 associated with DHF. The model generated was shown in Figure 4.7.

Predicted logit of DHF = -1.7601 - 0.0203 (platelet counts) + 0.8119($\log_e$ MCP2)

Figure 4.7: The predictive model generated from the binary logistic regression. A 1 unit change of the platelet cell count (platelet cell counts per cu mm x1000) can decrease by -0.0203 unit the logit of DHF while 1 unit change of $\log_e$MCP2 (pg/ml) can increase by 0.8119 unit the logit of DHF.

The model was refitted using ordinary GLM and the Hosmer-Lemeshow function was also used to assess the fit as described in Material and Methods (section 2.16). The result showed that it gave an adequate fit as p-value is greater than 0.05 (p-value = 0.31). Next, the model was evaluated by ROC curve in the test data set, as described in Materials and Methods (section 2.18) (Figure 4.8). Briefly, an accuracy of the model can be evaluated by an AUC (area under the curve). Sensitivity, specificity and optimal cutoff of the estimated probability of DHF were obtained by Youden's index. The accuracy of the model estimated by the AUC was 0.82. In general, the AUC of 0.5 (below the diagonal line) means random guessing while the AUC of 1 reflects the best forecast. In this result, the AUC of 0.82 is considered good enough for prediction. Sensitivity of the test (the probability that a test result will indicate DHF among those with the DHF) was 0.7317 (73.17%) while specificity (the probability that a test result will indicate DF among those with the DF) was 0.8313 (83.13%). In other words, a test of 73.17% sensitivity correctly detects 73.17% of people with DHF but 26.83% of those with DHF present undetected (false negatives) while a test with 83.13% specificity correctly identifies 83.13% of those with DF but 16.87% of those with DF are reported as DHF (false positives). In addition, an optimal cutoff of the estimated probability of DHF was considered by 1) Youden's index, which maximizes the sensitivity plus the specificity and 2) the shortest distance from the concave line to the upper-left corner of the graph (x,y: 0, 1) (Figure 4.8). From the graph the Youden's index is 0.5647 ($0 \leq$ Youden's index ≤ 1) and Youden cut-off of the probability was at 0.5919 ($0 \leq$ Youden cut-off ≤ 1).

The predicted logit of DHF can be changed to the probability of DHF and compared to the Youden cut-off as a formula below:

$$P(DHF) = \frac{1}{1+e^{-(-1.7601-0.0203\,\text{platelet count}+0.8119\log_e \text{MCP2})}}$$

P (DHF) that presents equal or over 0.5919 will be reported as 'DHF' while it will be reported as 'DF' when P (DHF) falls below 0.5919.

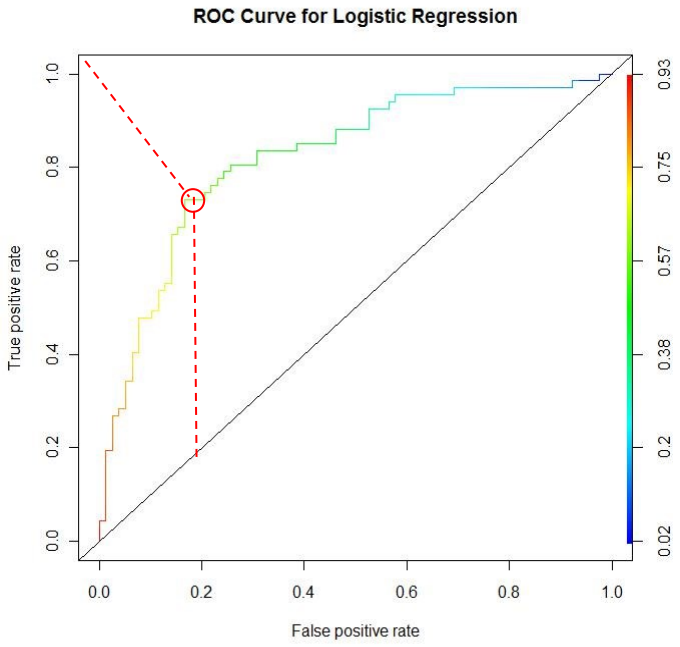


Figure 4.8: The receiver operator characteristic (ROC) curve showing a true positive rate (sensitivity) and a false positive rate (1-specificity) of the model obtained from the logistic regression. Each point of the ROC curve represents a pair of sensitivity and specificity corresponding to the probability of DHF. The black diagonal line connecting (0, 0) and (1, 1) corresponds to random guessing and the area under this line is 0.5. The red vertical line is Youden's index and the red diagonal line is the shortest distance from the ROC line to the point (0, 1). The red circle is the optimal cut-off of the probability obtained from the analysis.

Since high sensitivity is much more important where the test is used to screen a serious but preventable illness, 90% or greater sensitivity was therefore computed from the ROC curve. A cut-off of the estimated probability with 90% sensitivity was shifted from 0.5919 to 0.3485. The sensitivity of the test was increased to 0.91 (91%) but the specificity dropped to 0.47 (47%).

4.2.5.2 Classification and Regression Tree (CART) model

Since CART is easy to interpret and nonlinear relationship between variables does not affect an analyze of a tree model, this method is therefore used for identifying predictive markers. To compare a predictive model with the binary logistic regression, CART was computed in the same training data set, as described in Materials and Methods. CART is a binary recursive partitioning based on individual markers. To construct the classification tree, selection of predictors depends on how much purity those markers have to classify the class (in this case DF or DHF). Moreover, the decision to split a new tree node is based on the minimal number of observations that must exist in the node. The default minimal number is set at 10 (the number of observations in a node must be 10 before attempting a split). The terminal nodes can be assigned after the tree grows large and is pruned back by considering the minimal error in a cross validation. Nodes with predictors that do not improve the tree's purity are trimmed.

The output of the training data gave the cut-offs of predictors, the numbers and proportions of DHF and DF as shown in Figure 4.9. The predictive marker obtained from the analysis was only the platelet cell count and the cut-off was assigned at 125.5 cells per cu mm (x1000). High platelet cell count was associated with DF ($\geq$ 125.5). The number of total observations in the left node was 136 and that in the right node was 166. The class on the left node was classified as DF where the true DF count was 97 and the probability of DF was 0.713 (the DF class count = 97/136) while the class on the right node was classified as DHF where the true DHF count was 116 and the probability of DHF was 0.699 (the DHF class count = 116/166). The tree model was estimated by the ROC curve in the testing data set (Figure 4.10). The AUC of the model was 0.72 which was fair. The sensitivity was 77.77% and the specificity was 67.09%. This means a test of 77.77% of people with DHF but 22.23% of those with DHF present undetected (false

negatives) while a test with 67.09% specificity correctly identifies 67.09% of those with DF but 32.91% of those with DF are reported as DHF (false positives).

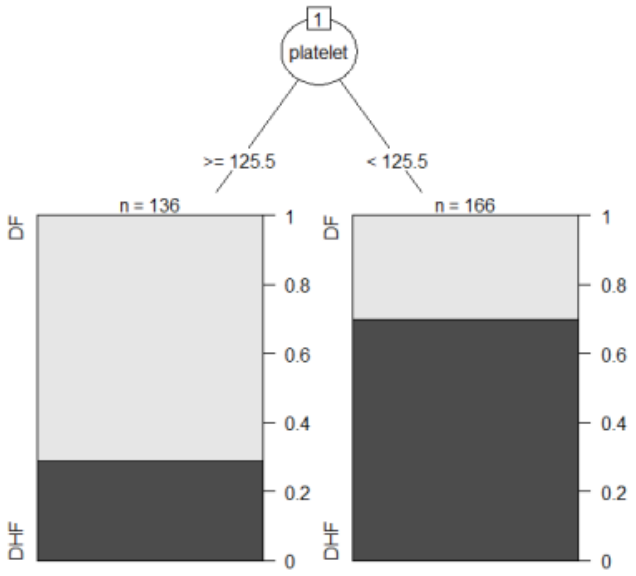


Figure 4.9: Classification tree of Dengue severity. Platelet cell count was a predictive marker to classify DF and DHF and the cut-off was 125.5. The class count was shown as the probability whose value is between 0 and 1. The light grey area is the probability of DF while the dark grey area is the probability of DHF. The number of observations is shown on the top of each node.

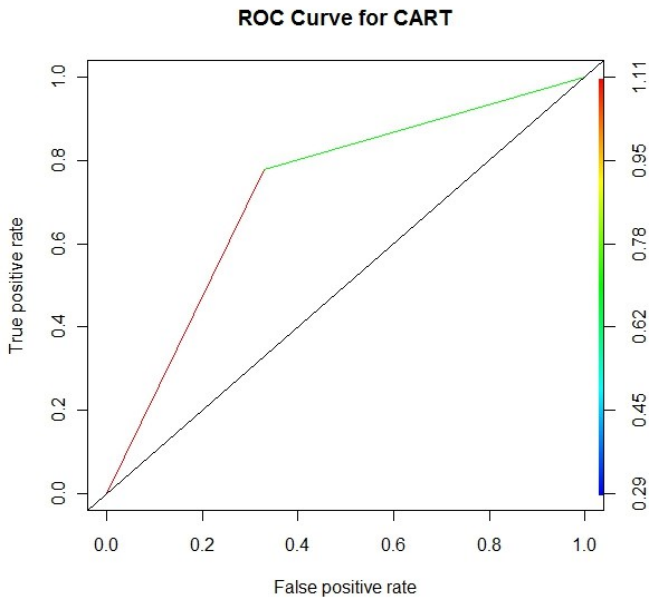


Figure 4.10: The ROC curve showing a true positive rate (sensitivity) and a false positive rate (1-specificity) of the model obtained from the CART.

4.3 Discussion

In this experiment, clinical and biological markers were identified in plasma samples from Dengue infected children during the acute phase. The concentration of these plasma proteins as well as the number of peripheral blood cells were determined and compared. The correlation between these markers was also observed and predictive models analyzed by binary logistic regression and CART was used to discriminate DF and DHF. Platelet cells together with MCP2 were identified as predictive markers for Dengue severity.

When clinical features were observed among DHF, DF and OFI groups, the increase in haematocrit (hct) was present in the Dengue groups but was less than 20% higher than the control (OFI) group (Table 4.2 and Figure 4.1A), based on WHO criteria to determine DHF (Organization, 2009). The hct values found in this study also remained within the average (mean) range of hct found in Thai children aged between 2 and 10 (35-40%). This different result might be due to the small number of samples in the experiment and the duration of sample collection. The platelet cell count, as expected, was below 100,000 cells per cu mm in DHF patients (Table 4.2 and Figure 4.1I). A reduction in platelet cells was also observed in DF patients and this was below the normal range of the platelet cell count (150,000–400,000 cells per cu mm; (Torcharus)). A decrease in white blood cell count (wbc) was also observed in Dengue patients when compared to the control group (Table 4.2 and Figure 4.1B) and the normal range of wbc count in Thai children (4.5-15 x1000 cells per cu mm; (Torcharus)). When viewing the longitudinal experiment, the hct values in DHF peaked at day 4 when the number of wbc was lowest (Figures 4.4A and 4.4B). This day might be a critical period of Dengue illness. Moreover, this trend was similar to the trend described in WHO literature (Figure 1.4, (Organization, 2009)). Like wbc, the number of polymorphonuclear cells (PMN or neutrophils) was reduced in Dengue patients (Table 4.2 and Figure 4.1C). Possible mechanisms to explain the low number of PMN are: recruitment of these cells to the site of infection, neutrophil adherence to the endothelium, and neutrophil cell death due to phagocytosis (Avirutnan et al., 1998, DiStasi and Ley, 2009). Next, reduced lymphocyte numbers were detected in Dengue patients and the number of lymphocytes in DHF was lower than those in DF (Table 4.2 and Figure 4.1D). This result was confirmed by other

reports showing that fewer lymphocytes are found during acute DHF (Kumar et al., 2012, Green et al., 1999a). Apoptosis of activated lymphocytes can contribute to this phenomenon. Activated cells may migrate to infected tissues and undergo apoptosis after eradicating virus-infected cells (Mongkolsapaya et al., 2003). Likewise, elevated atypical lymphocytes were observed in Dengue patients, particularly in DHF (Table 4.2 and Figure 4.1E). A reduction in liver proteins and albumin together with an increase in liver aminotransferases (ast and alt) was observed as similar to WHO criteria (Table 4.2 and Figure 4.1J-M). Low protein levels as well as increased liver enzymes in plasma indicate impaired liver function in Dengue illness (Seneviratne et al., 2006).

When observing biological features, there were several indicators associated with Dengue disease (Table 4.3 and Figure 4.3). Some of these markers have been previously reported and replicated in other studies (Table 1.1). High levels of T cell activation markers, soluble IL2Rs, for example, were detected in Dengue patients when compared to the control group, although no significant difference between DF and DHF was detected as earlier reported (Figure 4.3.2F) (Kurane et al., 1991, Green et al., 1999b). High plasma levels of MIP1β, IL10 and TRAIL were found in Dengue patients and these results match to the other studies (Figure 4.3.4B, 4.3.5A and 4.3.5C; (Azeredo et al., 2001, Becerra et al., 2009)). Similarly, over-production of soluble TNF receptors (sTNFRI and sTNFRII), TPO and inflammatory chemokines including IL6, IL8, IP10, ITAC, MCP1 and MCP2 were also detected in Dengue patients (Figure 4.3.2C, 4.3.2D, 4.3.3A-C, 4.3.4A, 4.3.4D, 4.3.6 and 4.3.7N; (Green et al., 1999b, Dejnirattisai et al., 2008, Avirutnan et al., 1998, Becerra et al., 2009, Chen et al., 2006, Matondang et al., 2004, Cardier et al., 2006)). Moreover, some of these markers including sTNFRII, TPO, IL6, IL8, IP10 and ITAC were associated with disease severity as previously shown (Green et al., 1999b, Dejnirattisai et al., 2008, Chen et al., 2006, Matondang et al., 2004, Cardier et al., 2006). Decreases in plasma levels of sVEGFRII, IL12 and RANTES were detected in DHF patients and the lower plasma levels of neutrophil-secreted MMP9 was found in Dengue patients (Figures 4.3.1E, 4.3.2E, 4.3.4C and 4.3.5B). These results matched to the previous reports (Srikiatkhachorn et al., 2007, Pacsa et al., 2000, Pérez et al., 2004, Voraphani et al., 2010). There was one marker, MIG, that the result was different from the previous experiment. An increase in plasma levels of MIG was not detected here (Figure 4.3.7P) (Dejnirattisai et al., 2008). Supposing that this observation was not certain, a possible cause of the different result would have been a great number

of missing value in MIG, generating unreliable data due to a random sampling error (Table 4.4).

Although these soluble proteins have been shown to be associated with Dengue infection, they are still not defined as good early indicators for classifying Dengue severity and only a few reports have shown a function for these proteins in disease such as IP10, ITAC and sVEGFRII (Dejnirattisai et al., 2008, Srikiatkhachorn et al., 2007). So far, attempts to find better early indicators of Dengue severity are still ongoing. In the present study, many biomarkers associated with Dengue infection were newly identified. Increases in plasma levels of MMP3, BCA1, MIP3α, and CTACK were found in Dengue patients (Figures 4.3.1C, 4.3.3D, 4.3.4G, and 4.3.4I) whereas Eotaxin2 seemed to be lower in DF (Figure 4.3.4H). MMP1, MMP8, sIL4R, sIL6R, MIP1δ and MIP3β were also associated with Dengue infection when compared to the control (Figures 4.3.1A, 4.3.1D, 4.3.2G, 4.3.2H, 4.3.4E and 4.3.4F).

Looking at the kinetics of soluble mediator expression in DF and DHF patients, there were some markers for which the plasma levels were significantly different although interpretation in term of symptom must be careful since the time course were constructed between day 2 and 6 of disease and the number of samples in each day are small and different in each day. Plasma levels of five markers including sTNFRI, sTNFRII, IP10, ITAC and MCP2 were highly increased in DHF on day 3 (Figures 4.5.2C, 4.5.2D, 4.5.3B, 4.5.3C and 4.5.4D). Plasma levels of MMP3 and sVEGFRII were significantly different between these two groups on day 4 where increased hct and reduced wbc were also detected (Figures 4.5.1C and 4.5.2E). Increased plasma BCA1 was also detected in DHF later on day 5 (Figure 4.5.3D). Differential expression of these soluble mediators may give clues as to how proteins interplay in Dengue infection. Over-produced soluble TNF receptors may respond to TNFα production since both type I and II receptors act as TNF antagonists, inhibiting TNFα activities (Chizzolini et al., 2009). The sVEGFRIIs are soluble receptors for VEGF, a vascular permeability factor, and a decline in these receptors is believed to be associated with vascular leakage occurring during the critical period in Dengue illness (Srikiatkhachorn et al., 2007).

Apart from these mediators, several markers are likely to be associated with the Dengue illness, such as MCSF, IL15, IL16 and I309 (Table 4.4 and Figure 4.3.7).

Unfortunately, it could not be absolutely confirmed whether these markers are related to Dengue infection due to a large amount of missing data. Data can be missing on account of the quality and quantity of the proteins since some plasma proteins have short half-lives and some are degraded easily. Differences in the time from onset day can also affect the amount of these proteins in the plasma since it cannot exactly determine when patients became developed illness. Lot-to-lot variation of multiplex assay kits and their sensitivity can also cause a deviation of the sample median. Technical difficulties in measuring protein concentrations in the plasma samples during the experiment can further lead to inaccurate analysis.

When the relationship between markers was tested (Table 4.6 and Figure 4.6), a high degree of correlation was seen between liver enzymes ($r = 0.82$). Liver enzymes may share similar biological activities; both of them are leaked from the liver to the blood stream and are used as liver-injury markers. Liver protein and liver albumin were also moderately correlated. Indeed, albumin is a major subset of the liver protein. A correlation between wbc and PMN was also high ($r = 0.74$). This is due to the fact that neutrophils compose about 40-60% of total white blood cells.

A strong relationship was seen between IP10 and IL15 ($r = 0.86$; Table 4.6 and Figure 4.6). IL15 can stimulate T cell proliferation and activation while IP10 functions in T cell and monocyte recruitment. Besides, IL15 and IP10 were moderately correlated with MCP1 and ITAC, both of which have a similar function to IP10. In addition, MCP2 were correlated with both MCP1 and ITAC. Eotaxin, a cytokine inducing eosinophil recruitment, seemed to be associated with MCP1 as well. Nevertheless, it was quite unexpected that MIG did not have a strong correlation with IP10 and ITAC since it is in the same CXC3 family and shares similar biological activities. It might be due to a different spatial and temporal expression, regulating by different cell sources and stimuli (Groom and Luster, 2011a, Groom and Luster, 2011b). For example, IP10 and ITAC are mostly triggered by innate stimuli which can induce type I and II IFNγ as well as TNF-α wheras MIG is restricted to IFNγ (Groom and Luster, 2011b). Moreover, IP-10 can be induced by innate immune sensors such as TLRs (Toll like receptors) and RIG-I (Retinoic acid inducible gene I) (Groom and Luster, 2011b). Additionally, in the murine cerebral model, MIG is mostly expressed in brain endothelial cells whereas IP10 is expressed by neuron (Campanella et al., 2008). Similarly, IP10 is produced in hepatic

dendritic cells in a murine model of liver disease and MIG is produced in parenchyma cells (Yoneyama et al., 2002). Again, the weak correlation might also be due to the number of missing values in MIG. What's more, IL15 generally has a similar structure and function to IL2 (http://www.ncbi.nlm.nih.gov/gene/3600) but these molecules were not correlated. In Dengue illness, up-regulation of CXCR3-reactive chemokines (MIG, IP10 and ITAC) may recruit T cells to the site of infection, inducing other soluble mediators such as TNFα and IFNγ while MCP-1 may recruit monocytes (Dejnirattisai et al., 2008, Avirutnan et al., 1998, Antony et al., 1993). Although roles of IL15, MCP2 and Eotaxin have not been characterized in Dengue illness, with similar structures, functions as well as close correlation between these molecules, they may serve similar activities as CXCR3-reactive chemokines and MCP1. However, it cannot overlook the effects of their receptors that express on different cell sources, causing different outcome.

Another strong relationship was observed between IL23 and IL33 ($r = 0.74$). IL23 functions in the differentiation of naïve T helper cells to Th17 cells which can produce IL17, IL6 and TNFα but not IFNγ and IL4; it also acts on dendritic cells and macrophage to produce IL1, IL6 and TNFα (Iwakura and Ishigame, 2006). Although plasma IL23 was not associated with Dengue severity in this study and it could not be detected in the control group due to the very low concentration in the plasma (Table 4.4), it is still interesting that this cytokine might locally accumulate at the site of infection, inducing numerous proinflammatory cytokines such as TNFα. IL33 is a potent activator of eosinophils, mast cells and basophils which are important in preventing against parasite infection (Miller, 2011). Nevertheless, IL33 can also stimulate type 2 cytokine secretion such as IL5 and IL13 as well as induce a LPS-stimulated production of TNFα in macrophages (Miller, 2011, Espinassous et al., 2009). Since TNFα is one of a key factor in Dengue illness, IL33 may also play a role in TNFα production in this disease. In addition, IL23 is a new member of the IL12 family; however an association was not found between this pair. IL12 seems to be important for the induction of IFNγ and T cell regulation (Langrish et al., 2004, Gee et al., 2009). It was found to be moderately correlated with IL1Ra, which is an antagonist for IL1α. Interestingly, both IL33 and IL1Ra are members of the interleukin 1 cytokine family.

IL33 was also associated with TPO (thrombopoietin). TPO has a role in stimulating the proliferation and maturation of megakaryocytes, regulating the number of

circulating platelets (Kuter, 1996). It was, nevertheless, not know a relationship between these two markers while a negative correlation between TPO and platelet was found in this study. The levels of TPO increased in the DHF group where the platelet cell count was much reduced when compared to the DF group as previously reported (Figure 4.3.6) (Matondang et al., 2004, Cardier et al., 2006). A decrease in the platelet cell count might stimulate TPO secretion as to induce platelet production during the early stage of infection (Matondang et al., 2004). Furthermore, a moderate correlation was observed in many pairs of predictors such as MMP8 and MMP9, sIL2Ra, and IL2R, sTNFRI and sTNFRII, to name a few.

MMP8 and MMP9 are released from neutrophils and play roles in inflammation, including the regulation of cytokine activities, neutrophil migration and degradation of the extracellular matrix (Manicone and McGuire, 2008, Van den Steen et al., 2003). A previous study has demonstrated that MMP9 may involve in endothelial leakage since anti-MMP9 antibody and an MMP inhibitor are able to enhance endothelial permeability *in vitro* and reduce expressions of PECAM1 and VE-cadherin, adhesion molecules on endothelial cells (Luplertlop et al., 2006). Seeing that infected endothelial cells can recruit neutrophils to the site of infection (Avirutnan et al., 1998), secreted MMPs may be mediators for the vascular leakage in Dengue infection.

sIL2Ra and IL2R are different forms of IL2 receptors; they have the same ligand which is IL2. sTNFRII was associated with many markers including sIL2Ra, sTNFRI, IL10, platelet, and ast. The relationship between these markers may involve several mechanisms. For example, sTNFRs can mediate TNFα-induced apoptosis while IL10 can regulate TNFα production (Micheau and Tschopp, 2003, Shibata et al., 1999, Armstrong et al., 1996). Additionally, an association was found between the following pairs; MCP2 and TPO, BCA1 and platelet, ENA78 and RANTES as well as ENA78 and TARC. Although several correlations were detected in this study, the obvious link between these markers is not known. It might be either causality or presumably occur at the same time but different pathway.

Predictive markers in this experiment were defined by two different methods: binary logistic regression and CART (Figures 4.7 and 4.9). By the logistic regression analysis the platelet cell count and the plasma MCP2 levels were defined as predictive

markers. Low platelet cells and high levels of MCP2 generated an increased predicted probability of DHF. When the data was analyzed by CART, only the platelet cell count was obtained as a predictive marker and the cut-off discriminating between mild and severe Dengue was 125,500 cells per cu mm. The CART result did not follow the WHO protocol in which a platelet cell count below 100,000 cells per cu mm is a criterion for DHF (1997). Samples collected at different time points may lead to a deviation in the results. In this study, samples were collected during the 1st day to 10th day of illness (Table 4.1) but the platelet cell count below 100,000 cells per cu mm in the DHF group was observed after day 4 (Figure 4.4I). Since the objective of the study is to identify early predictive indicators, it could be possible that the platelet cell count is greater than that established by the WHO criterion. However, there are some difficulties to collect samples during the early stage of infection particularly during the first three days. These are undifferentiated fever during the early febrile stage, variation in incubation period of the disease and unease to access to hospitals. To overcome these problems, surveillance of Dengue disease and early warnings may require.

From the analyses, the sensitivities of the diagnostic models were not very high and somewhat equivalent (regression: 73%; CART: 78%) while the specificity and the accuracy of the logistic regression was much greater than those of the CART (regression: 83% specificity and AUC of 0.82; CART: 67% specificity and AUC of 0.72). When comparing these two methods, the logistic regression gave greater accuracy and better specificity and was found to be suitable as a predictive test. However, the sensitivity is generally more important than the specificity in a screening test since false negative (actual DHF but defined as DF) is more dangerous than false positive (actual DF but defined as DHF). Patients who are predicted to develop DHF will stay in a hospital while patients who are considered as DF will be sent back home. There is a higher risk for any patient who develops DHF at home, away from intensive medical care. Attempts to increase the sensitivity of the logistic regression analysis may reduce the number of false negatives but the specificity also dropped to 47%. Decreased specificity made the model unpromising since it cannot actually reduce the number of patients with DF in a hospital. Apart from the sensitivity, the regression analysis is quite difficult to interpret and use since the MCP2 concentration and the platelet cell count need to be converted to the probability before comparing to the cut-off. As a result, to understand how to interpret the analysis users may need to know some basic statistics.

Many studies have tried to define early predictive markers for Dengue severity. Brasier *et. al.* have shown that IL10, platelet and lymphocytes were possible predictors by using logistic regression and CART analysis (Brasier et al., 2012). The accuracy of the logistic regression was greater than CART since the AUC of the regression gave 0.96 whereas the CART analysis gave a value of 0.85. However, the number of subjects was somewhat low (total Dengue =51, DF = 38 and DHF =11) while they used 11 parameters including 8 clinical analyzes, gender, day of fever and diarrhea. In addition, they did not provide information about type of infection. The model obtained therefore might not be reliable due to an overfitting ["an overly optimistic model results" (Babyak, 2004)]. Another study has developed a diagnostic model analyzed by CART (Potts et al., 2010). They collected samples during the first 72 hours from the onset of disease. Clinical features, age and gender were included in this study. The subjects in this study were comprised of 208 DHF, 374 DF and 648 OFI. However, they did not categorize DHF and DF as severe and mild Dengue respectively, but they did classify the disease using several criteria such as Dengue shock syndrome (as defined by WHO), DSS or pleural effusion index (PEI) >15, DSS or received intravenous fluid etc. They also combined non-severe Dengue with OFI. Their model was obtained from wbc, monocyte and platelet cell count, and hct. They claimed that the sensitivity of the model to identify severe Dengue was 97% but only 48% of non-severe Dengue was classified correctly. Hence, their model might not truly discriminate DF and DHF. Moreover, a recent experiment has identified predictive markers from the serum proteome, inflammatory markers and cytokines in 62 adult Dengue patients (44 DF and 18 DHF) at an early febrile stage (Kumar et al., 2012). They collected samples 48-72 hours after the onset of disease and selected samples with primary infection. Their analysis combined several statistical methods to select predictors and find the model for classifying Dengue severity. They proposed 4 different models with >70% sensitivity and the AUCs gave values about 0.86-0.92. However, the small number of samples and the large number of predictors used might generate an overfitted model. In addition, since they used both a multiplex immunoassay and a multiple mass spectrometry to identify markers, it is quite expensive to use these assays, particularly in developing countries. When compared to their experiments, only complete blood count test (CBC) and MCP2 singleplex bead assay are required in this study.

There are some suggestions to improve the model. Firstly, to obtain a model that will replicate in a new sample, the sample size should be big enough to generate the estimates of the model. It would have been more accurate if the sample size had been larger, particularly for those samples collected between the 3rd and 5th day of the illness. Indeed, analysis of samples collected on the same period might give a more precise prediction. Secondly, the number of markers should not exceed the sample size. Ideally, one marker requires about 10 to 15 samples (Babyak, 2004). Therefore, the number of markers should be less than 45 markers because the number of samples was about 450. Herein this study, 31 markers were used for the analysis, which was in this criterion. However, the greater the number of markers in the model, the greater the difficulty in generating the model since high dimensional data generates complex correlations among the markers. The highly correlated markers (high degree of correlation among predictors; multicolinearity; $r > 0.8$) can violate the regression assumption that the predictors in the regression must be linearly independent. Thus, those markers that highly correlated among each other should be excluded. Lastly, an approach that statisticians propose to avoid overly optimistic modelling, a technique called *bootstrapping*, could be employed. Bootstrapping is a random resampling method with replacement from the data at hand multiple times, creating several new datasets. Bootstrapping was shown to be superior to the use of training and testing sets to validate a model (Babyak, 2004). This method can treat the data as a population from which smaller samples are taken and the descriptive statistics can be calculated from the bootstrap samples.

There are some considerations when deciding which predictors should be included in the regression model. Generally, markers with a good theoretical grounding are chosen and maintained in the model. In the review of Babyak, several papers chose markers associated with the disease by prescreeining with univariate analysis such as Mann-Whitney U test; yet many statisticians do not recommend using univariate prescreening to select the markers since it can increase the number of errors (Babyak, 2004). The relationship between markers and the outcome might not be associated in the univariate test but may appear important after adjusting with other markers in simultaneous screenings. As a result, limiting the number of predictors by univariate pretesting might be inappropriate. Unfortunately, without awareness of this issue at the very first time, pretesting with Mann-Whitney U test was also done in this study; the results therefore, might not be accurate. It is quite difficult to choose the reasonable *a*

priori choices from the data at hand since there is limited biological information. One approach that might solve this problem is to combine closely related markers, based on good theoretical grounding, into a new variable using a clustering algorithm such as factor analysis (Babyak, 2004). This strategy may ideally limit the number of predictors but is in fact a more sophisticated approach and requires prior knowledge of data mining. In addition, many predictors are still required for the prediction. Therefore, this might not be a good strategy for this study.

Some limitations of the diagnostic tests in this study were shown. Since this model was developed from screening of Thai children, it might not be applied to other ethnicities or to patients of a different age. The predictive model therefore needs to be verified in another cohort. Sensitivity of the test was not very high and this could affect the ability of the test to screen severe Dengue infection.

In conclusion, platelet cells and MCP2 were defined as the markers for classifying DHF and DF in an early prediction model. With these predictors, physicians may identify people who have mild DF, decreasing the number of in-patients requiring hospitalization. In addition, the kinetic study of plasma proteins and the pair-wise correlation among predictors may be useful in other studies such as protein pathways/networks, and drug design.

Chapter 5

Results

Matrix Metalloproteinases in Dengue disease

5.1 Introduction

5.2 Results

5.2.1 Patients' characteristics

5.2.2 MMPs profiles in Dengue disease

5.2.3 Correlation between peripheral blood cells and MMPs

5.2.4 Influence of Dengue virus, immune cells and non-immune cells on MMPs secretion *in vitro*

5.2.5 MMP3 and endothelial permeability

5.3 Discussion

5.1 Introduction

Matrix metalloproteinases (MMPs) have important roles in several systems including the immune response to infection. MMPs are involved in the process of leukocyte extravasation by degrading extracellular matrix as well as regulating cytokine/chemokine activities (Parks et al., 2004). MMPs are required for migration of immune cells such as T lymphocytes, neutrophils, and dendritic cells (Parks et al., 2004, Warner et al., 2001, Wang et al., 1999, Chabot et al., 2006, Saalbach et al., 2010). They are also important for eradicating pathogens. For example, MMP-3 is necessary for infiltration of T lymphocytes and clearance of bacteria in the gut (Li et al., 2004). However, excess MMPs activity may contribute to tissue damage. Over-production of MMP-3, as a consequence of T cell activation by poke-weed mitogen, causes injury to small intestine explants (Pender et al., 1997). Inhibition of the gelatinases (MMP-2 and MMP-9) by specific inhibitors reduced T cell proliferation and activation *in vitro* and abolished T cell-mediated lung injury *in vivo* (Benson et al., 2011). Moreover, MMPs can benefit pathogens and that may lead to immunopathology. MMP-9 facilitates West Nile virus (WNV) to enter into the brain by enhancing blood-brain barrier (BBB) permeability (Wang et al., 2008). Infection of endothelial cells and dendritic cells with Dengue virus leads to over-production of MMP-2 and MMP-9, resulting in enhanced endothelial leakage (Luplertlop et al., 2006). MMP-3 has an effect on the rat glomerular barrier, increasing albumin permeability which may lead to proteinuria in kidney diseases (Sharma et al., 1996).

The picture of Dengue pathogenesis is not complete. Several host factors including MMPs have been reported to determine Dengue severity. Up-regulation of circulating MMP-9 has been shown in Dengue disease and associated with the disease severity (Kubelka et al., 2010). However, another study has reported no association between plasma levels of MMP-9 and Dengue infection during the acute phase (Voraphani et al., 2010). Indeed, this latter study showed that MMP-9 levels were reduced when compared to the follow-up stage as well as in the control group (Voraphani et al., 2010). Since the association between circulating MMPs and Dengue is not well defined, the plasma levels of MMPs (MMP-1, -2, -3, -7, -8, and -9) in Thai children infected with Dengue virus were investigated to find out if they are associated with

disease severity. It is also hypothesized that MMPs may play a role in Dengue pathogenesis; therefore, an interplay between Dengue virus, immune cells and non-immune cells can also influence MMPs secretion *in vitro* was also explored.

To investigate MMPs, a multiplex assay was used since this can simultaneously detect multiple analytes in a small sample volume. In principle, fluorophore-coded beads, precoated with specific antibodies for the proteins of interest (in this case MMPs), capture the analytes in the sample, followed by binding of biotinylated detection antibodies and phycoerythrin (PE)-conjugated streptavidin (Figure 5.1). The beads are read with dual lasers: one determines the analytes and another detects the magnitude of PE signal, which is directly proportional to the amount of protein.

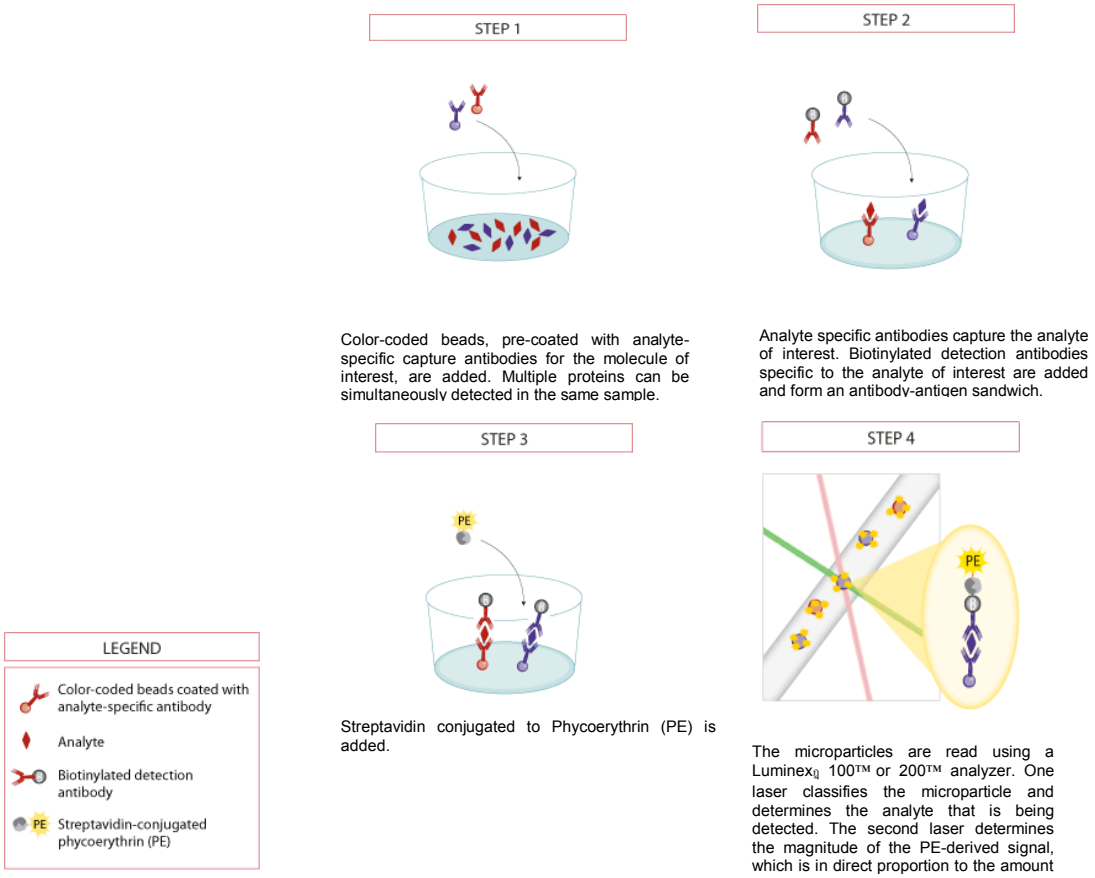


Figure 5.1: Principle of the multiplex assay (R&D systems)

5.2 Results

5.2.1 Patients' characteristics

To examine the circulating MMPs, plasma samples were collected from children clinically presenting with Dengue disease or other febrile illnesses (OFI). Samples were collected during the acute and convalescent phases. Samples during the acute phase were analyzed from the first hospital day in order to get a predictive test. Demographics and clinical profiles during the acute stage are summarized in Tables 4.1- 4.2 and Figure 4.1

5.2.2 MMPs profiles in Dengue disease

Plasma levels of MMPs (MMP-1, -2, -3, -7, -8, and -9) during acute phase were measured (Figure 5.2). Levels of MMP1, MMP-8 and -9 in Dengue patients (DF and DHF) were significantly lower than those in patients with OFI while MMP-3 levels were up-regulated. Levels of MMP-2 were comparable among three groups. MMP-7 was below the level of detection. When comparing results within Dengue groups (DF = 226, DHF1 = 73, DHF2 = 119, and DHF3 = 33; Figure 5.3), MMP-3 levels were higher in DHFs than in DF. The plasma levels of MMP-9 in DF and DHF1 were low and showed significance when compared to DHF3. No significant differences were detected within other MMPs. Secreted plasma MMPs were also compared between acute and convalescent stages (Table 5.1 and Figure 5.2). Increases of plasma MMP-1, -2, -8 and -9 levels were observed during the convalescent phase whereas plasma MMP-3 declined.

Next, the levels of MMPs over the time course of infection were investigated (DF = 24, DFH = 32; Figure 5.4). Surprisingly, the levels of MMP-1, -8, and -9 during the febrile stage were lower in DHF than those in DF and increased in the convalescent stage even though significance was found only in MMP-1 and MMP-8 on day -2. The levels of MMP-2 increased in both groups between fever day -3 and the critical day 0 (the day of defervescence); however the peak occurred on day -1, during which time higher MMP-2 was observed in DHF. No significant difference was found after day 0 in both DF and DHF. In contrast, a significant difference in the MMP-3 levels could not be detected,

even though it was higher in DHF. Elevated MMP-3 was found throughout the febrile period, peaked at day -1 and returned to normal after recovery.

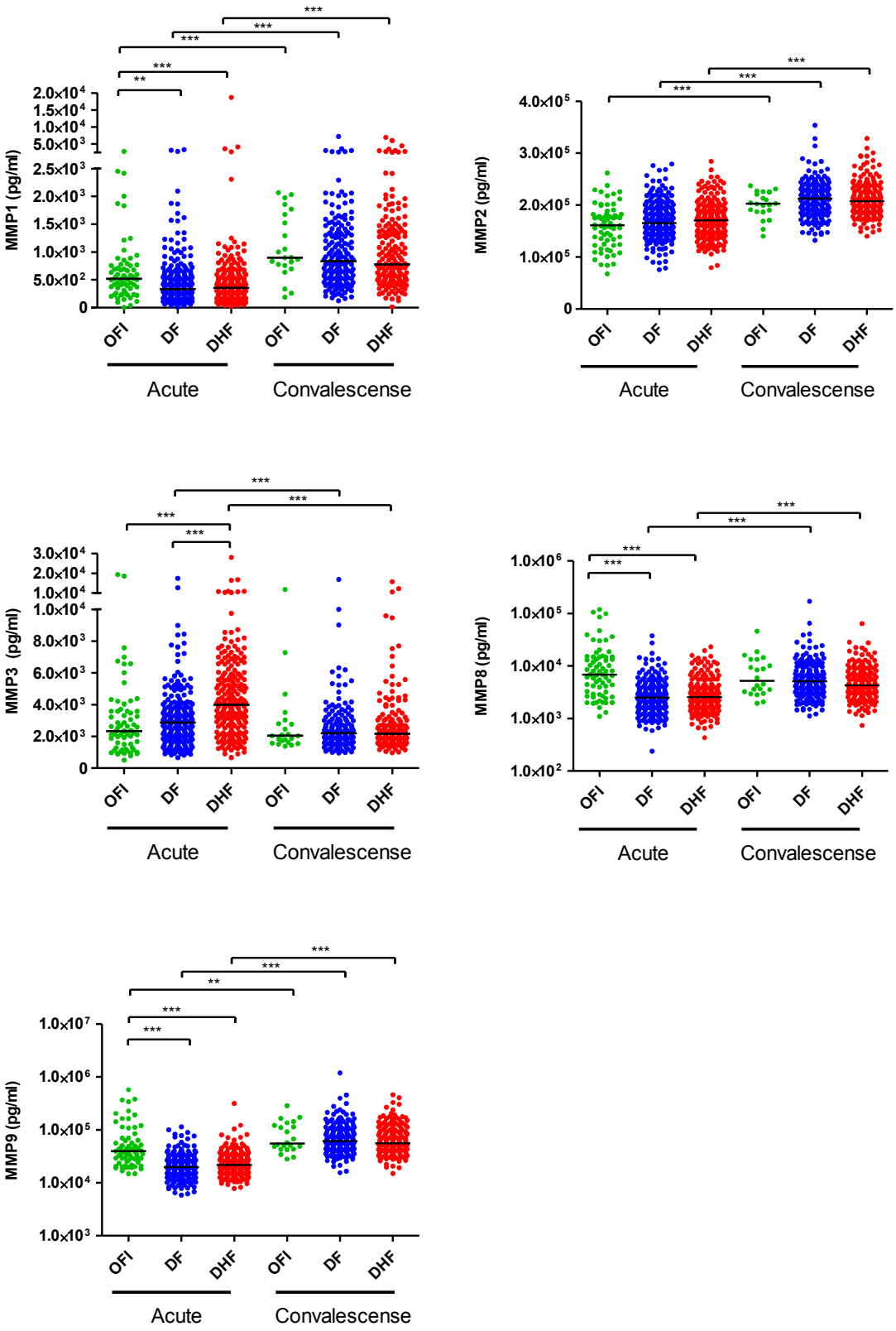


Figure 5.2: Plasma levels of MMPs during the acute and convalescent stages: Horizontal lines represent median values. Statistical analysis was determined by the Kruskal-Wallis test for 3 groups and Mann-Whitney test for 2 groups (**, $p < 0.01$; ***, $p < 0.001$).

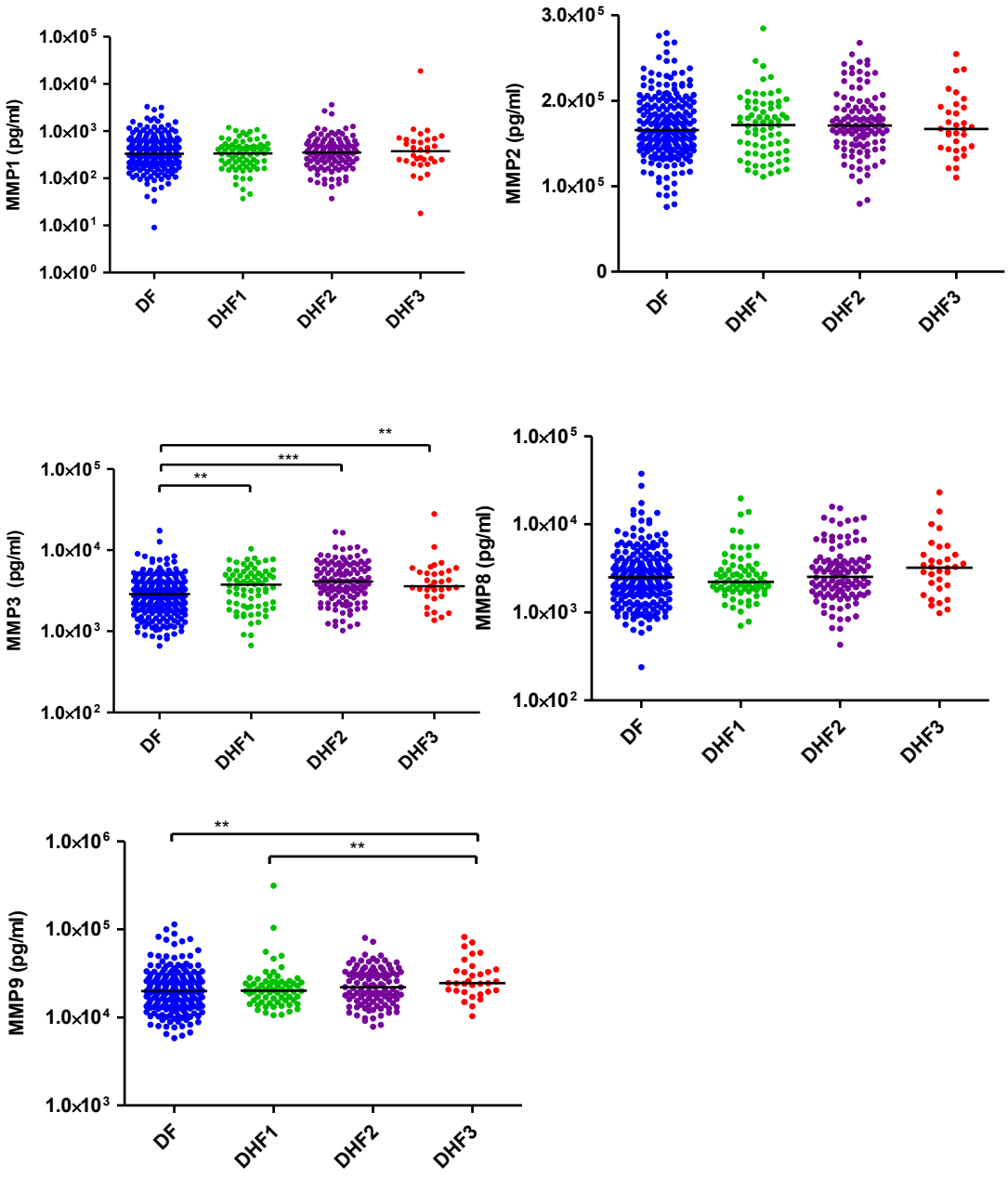


Figure 5.3: Plasma levels of MMPs graded according to disease severity during the acute phase: Horizontal lines represent median values. Statistical analysis was determined by the Kruskal-Wallis test for 3 groups and Mann-Whitney test for 2 groups (**, $p < 0.01$; ***, $p < 0.001$).

Table 5.1: Plasma MMPs in acute and convalescent samples

MMPs (mean pg/ml ± SD)	DF		DHF		OFI	
	Acute (n =226)	Convalescent (n =175)	Acute (n =227)	Convalescent (n =176)	Acute (n=69)	Convalescent (n =22)
MMP1	486.52±470.01	1021.92±778.54	529.73±1297.32	1056.28±903.35	653.20±567.42	1100.75±583.51
MMP2	170682.49±37415.54	212326.54±35568.58	172109.83±35408.03	212132.76±34044.49	159712.72±41197.33	200831.60±25766.51
MMP3	3149.37±1951.17	2607.87±1688.90	4462.11±2938.73	2757.61±1954.2	3171.85±3198.16	2861.22±88345.56
MMP8	3479.90±3925.80	8242.75±14500.14	3600.66±3322.96	6337.59±6532.66	15300.53±23915.04	9101.89±65012.79
MMP9	23843.42±15973.25	89096.33±105851.34	27000.58±24849.13	80523.87±65017.29	75315.09±99454.20	88345.56±63764.33

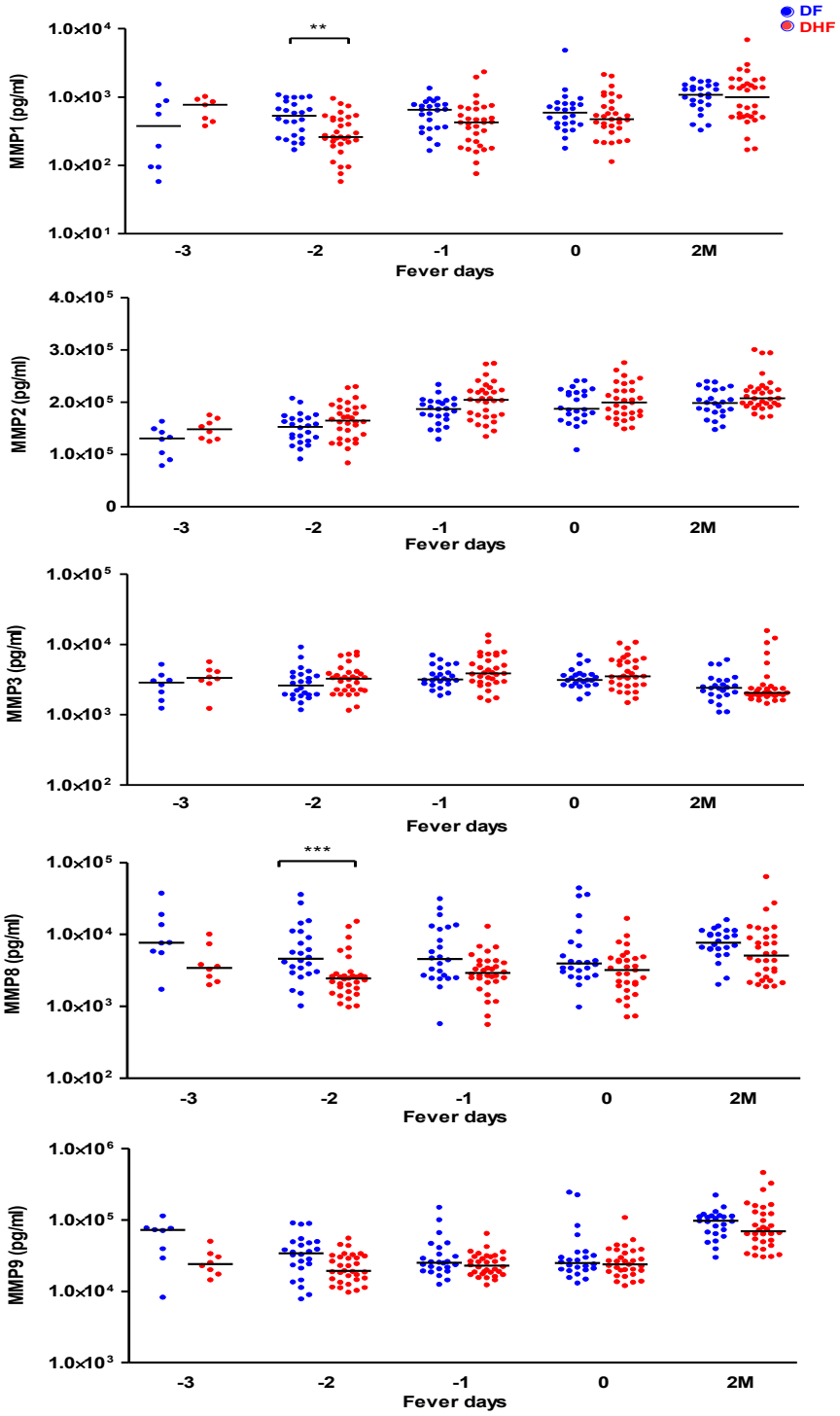


Figure 5.4: Plasma levels of MMPs over the time course: Day 0 is defined as the day that fever subsides, and 2M is the convalescent stage. Blue dots and red dots are represented for DF and DHF, respectively. Horizontal lines represent median values. Statistical analysis was determined by Mann-Whitney test for 2 groups (**, $p < 0.01$; ***, $p < 0.001$).

5.2.3 Correlation between peripheral blood cells and MMPs

There are several cellular sources of MMPs in plasma. Peripheral blood mononuclear cells (PBMCs) are known to release MMPs (Bar-Or et al., 2003, Matache et al., 2003). In activated neutrophils, MMP-8 and -9 are released from the granules into the extracellular space (Faurschou and Borregaard, 2003). Recent studies have identified that MMPs are stored in resting platelets and can be released during platelet activation (Jurasz et al., 2002). Therefore the association between plasma levels of MMPs and peripheral blood cells was examined. Positive correlations between MMP-8, MMP-9, white blood cells (wbc) and neutrophils (pmn) during the acute phase ($r > 0.3$, Table 5.2) were found; however, there were no correlation to lymphocytes and platelet counts. MMP-9 was also partially correlated with monocytes. The correlation between MMP-1 and peripheral blood cells was very small while MMP-2 was partially correlated with platelet cells, lymphocytes and neutrophils and MMP-3 was moderately associated with platelet counts. Association between MMPs and basophils, or eosinophils was not observed.

Table 5.2: Pearson's correlation (r) between MMPs and peripheral blood cells in Dengue disease

MMPs	Platelet counts	log (WBC)	Lymphocytes	Monocytes	Neutrophils
log (MMP1)	0.14	0.14	0.03	0.10	0.13
MMP2	-0.23	-0.11	0.22	-0.07	-0.27
log (MMP3)	-0.31	0.00	0.06	-0.01	-0.03
log (MMP8)	0.00	0.38	0.04	0.17	0.40
log (MMP9)	-0.03	0.37	-0.05	0.21	0.32

5.2.4 Influence of Dengue virus, immune cells and non-immune cells on MMPs secretion *in vitro*

Although the immune responses against Dengue infection are generally focused on clinical experiments due to the lack of appropriate animal models, *in vitro* studies are also important. However, most studies have been carried out by infection of a single cell type which cannot fully correlate with and explain Dengue pathogenesis. Therefore, an experiment was set up to co-culture dendritic cells, which are the primary target of Dengue virus, endothelial cells, the physical barrier between peripheral tissues and the blood stream, and PBMCs.

To test if Dengue virus *per se* can induce MMP secretion, monocyte-derived immature dendritic cells (DCs) were infected with Dengue virus type 2 (DV2) and cell supernatant was collected at 6, 12, 24, 48 and 72 hours after infection. Mock infected supernatant was used as a control. First, the characteristics of the immature DCs and infectivity levels were tested. The features of immature DCs were $CD14^-$, $CD83^-$ and $CD209^+$ (Figure 5.5a) and infectivity was about 33-79% (Figure 5.5b). Next, the levels of MMP-1, -2, -3, and -9 in the supernatant were determined by multiplex assay. MMP-7 and -8 levels were low or undetectable in the cell supernatant. DCs constitutively express MMP-9 and MMP-1; however, induction of MMPs from infected DCs could not be detected (Figure 5.6). Endothelial cells, which are susceptible to Dengue virus, were also infected. Infection on HUVECs was confirmed by immunostaining at 48 hours after infection (Figure 5.7a). Supernatants were collected at 24 and 48 hours after infection. Up-regulation of MMP-1, and MMP-2, which are mainly produced by endothelial cells, was not found, nor was the induction of any other MMPs (Figure 5.7b).

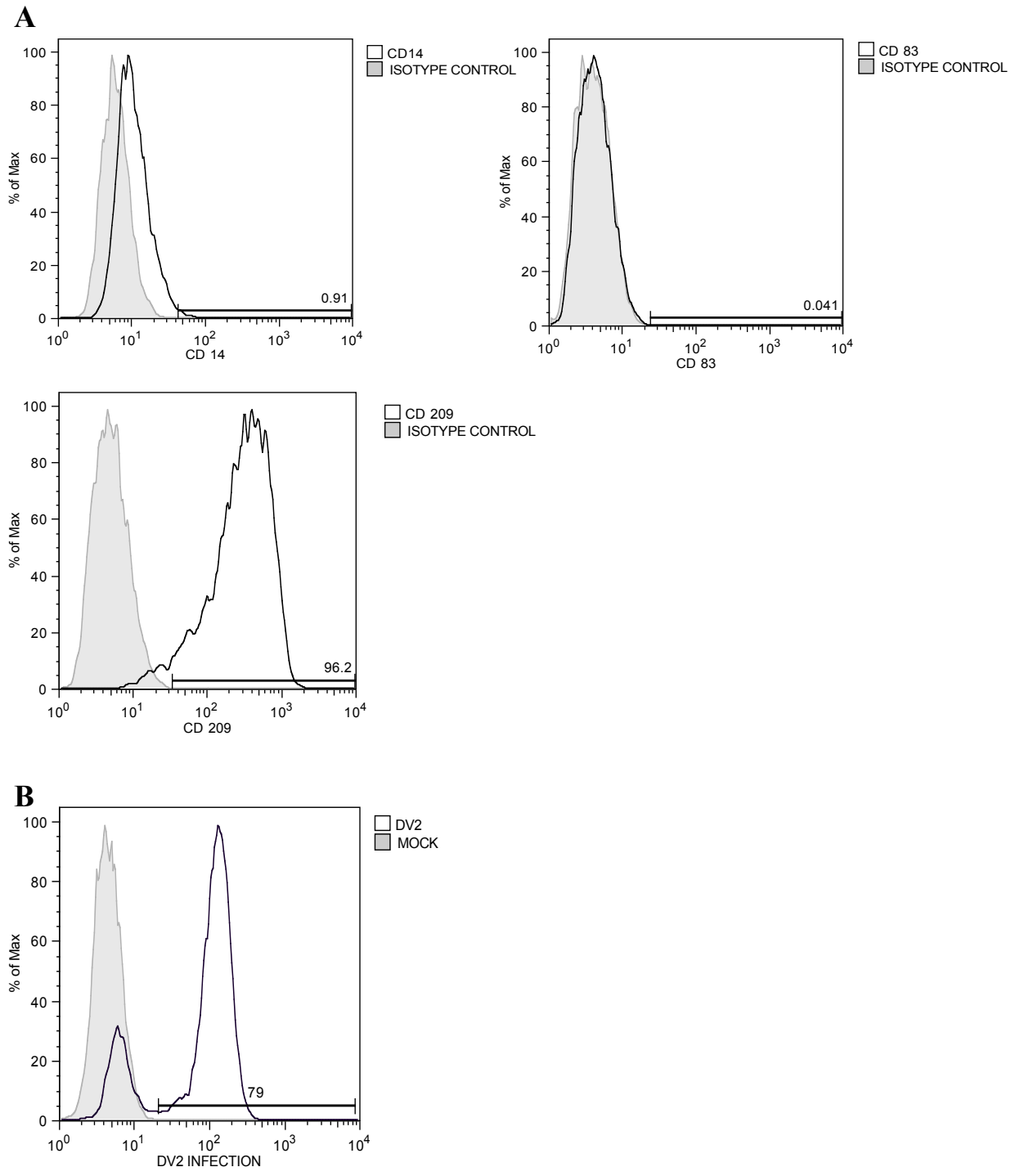


Figure 5.5: Cell surface expression and DV2 infection on Dendritic cells (DCs). $CD14^{+}$ cells isolated from PBMCs were harvested and cultured in RPMI with GM-CSF and IL4 for 5 days to generate DCs. Dendritic cells were mock infected or infected with DV2 for 24 hours. A) Cell surface staining against CD14, CD83 and CD209 on the infected cells were tested. B) Infectivity at 24 hours post infection was confirmed by flow cytometry. This figure is representative of three independent experiments.

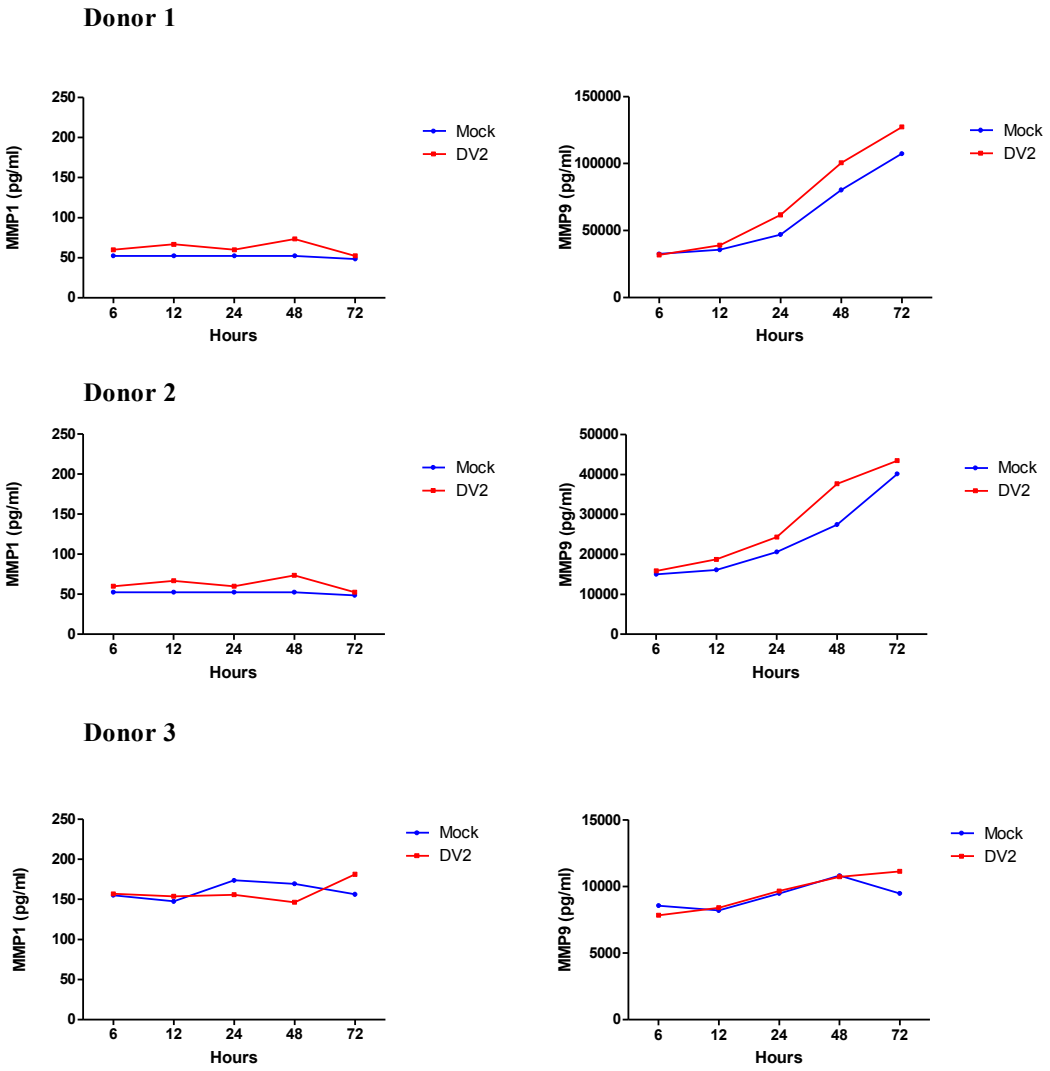
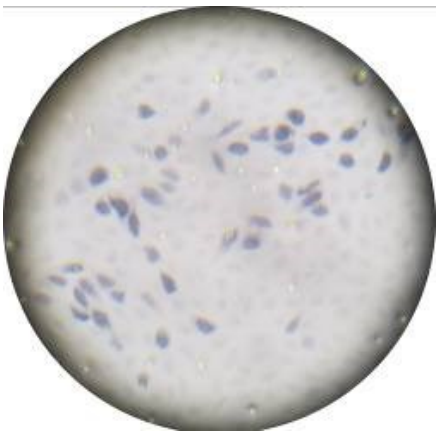


Figure 5.6: MMPs secretion in the supernatant of dendritic cells (DCs). $CD14^{+}$ cells isolated from PBMCs of three different donors were harvested and cultured in RPMI with GM-CSF and IL4 for 5 days to generate Dendritic cells. Dendritic cells were mock infected or infected with DV2 for 24 hours. Supernatants were collected at the indicated time points after infection (Hours) to measure MMP levels. Only MMP-1 and -9 were detectable. Results are representative of three independent experiments.

A



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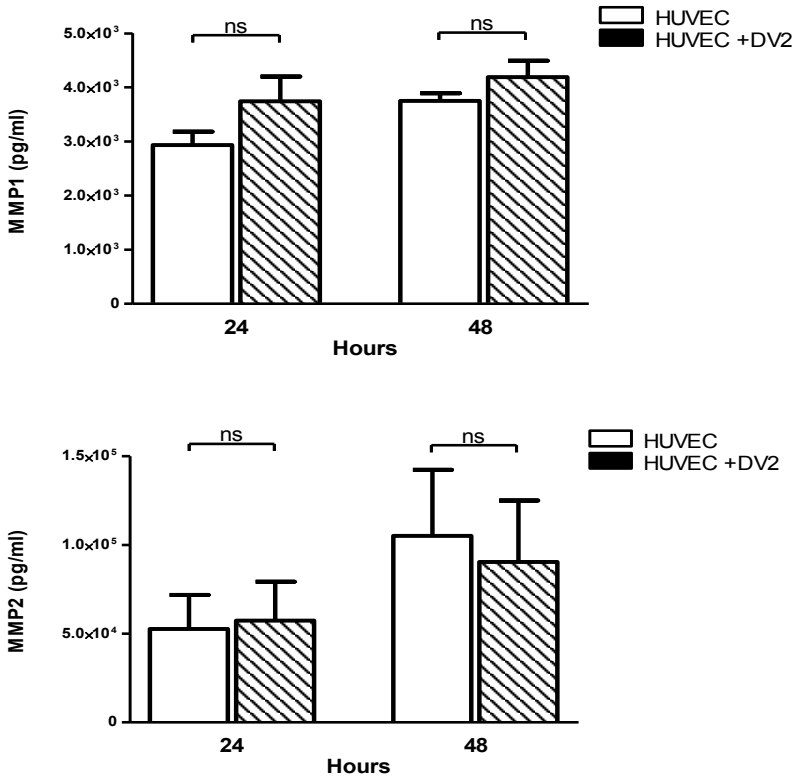
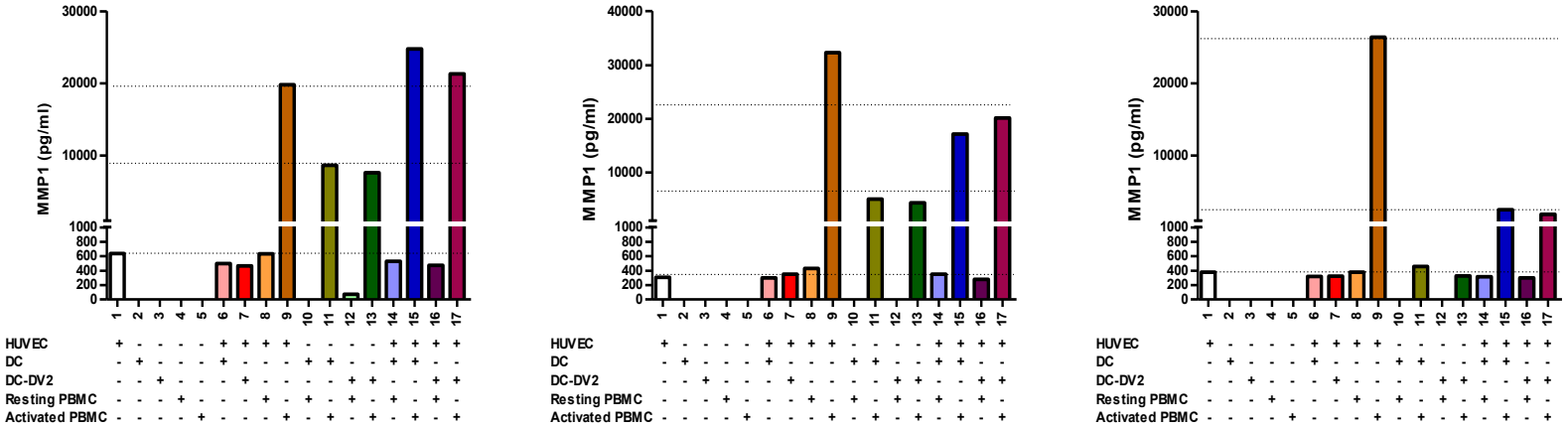


Figure 5.7: MMP secretion in the supernatant of HUVECs. HUVEC were mock infected or infected with DV2 at MOI of 5. A) Infection of HUVECs was confirmed by immunoperoxidase staining against the viral E protein. Infected cells are the darkly stained cells. B) Cell supernatants were collected at the indicated time points after infection to measure MMP levels. Only MMP-1 and -2 were detectable. Results from 3-independent experiments are shown as mean±SEM.

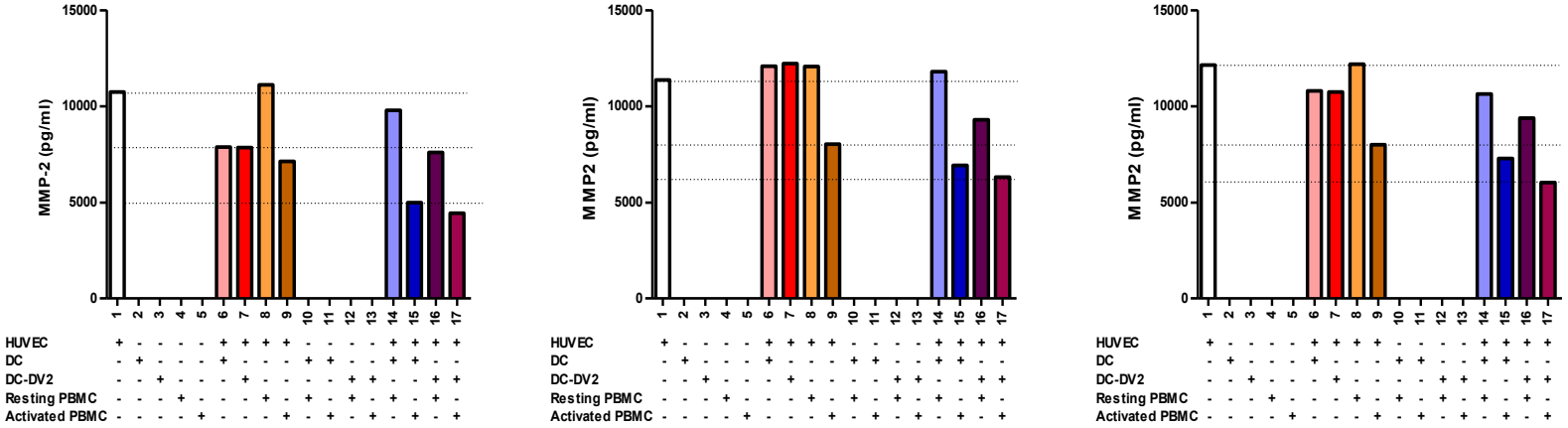
Next, secretion of MMPs from DCs, HUVECs, resting PBMCs and activated PBMCs, as well as from co-culture between these cells, was measured (Figure 5.8). HUVECs secreted MMP-1, -2 and a small amount of MMP-3 while DCs mainly released MMP-9. Only a small amount of MMP-9 from resting and activated PBMCs could be detected. Activation of PBMCs was confirmed by the expression of surface CD69 (Figure 5.9). Co-culture of HUVECs and DCs (mock-treated and DV2-infected cells) did not affect MMP-1 and -3 secretions while expression of MMP-2 and -9 varied a lot. Co-culture of resting PBMCs and HUVECs had no effect on MMP levels. On the other hand, when either HUVECs or DCs were mixed with activated PBMCs, MMP levels considerably increased, suggesting that activated PBMCs were able to induce secretion of MMP-1, -3, and -9 from both HUVECs and DCs (Figure 5.8a, c and d). In contrast, MMP-2 levels were moderately reduced when HUVECs were incubated with activated PBMCs. There was no effect of activated PBMCs on MMP-2 secretion from DCs. Since PMA/I, which is used to activate PBMCs, can change MMP expression (R Hanemaaijer, 1993), the enhanced MMP levels in this experiment might be an effect of this stimulator. To test this possibility, the last wash of activated PBMC was added to HUVECs for 24 hours; the supernatant was harvested to measure MMP levels. No change of MMP expressions was observed (Figure 5.10). Therefore, altered MMPs levels in the presence of activated PBMCs were not the result of PMA/I. Finally, there was no synergistic effect on MMP-1, -3, and -9 secretions when activated PBMCs, DCs and HUVECs were co-cultured; however, this interaction greatly reduced MMP-2 levels (Figure 5.8).

To investigate whether the effect of activated PBMCs on MMPs secretion is mediated by soluble factors, activated PBMCs were coincubated in a transwell system in which HUVECs were separated from activated PBMCs. Soluble factors were found to partially alter MMPs secretion (Figure 5.11).

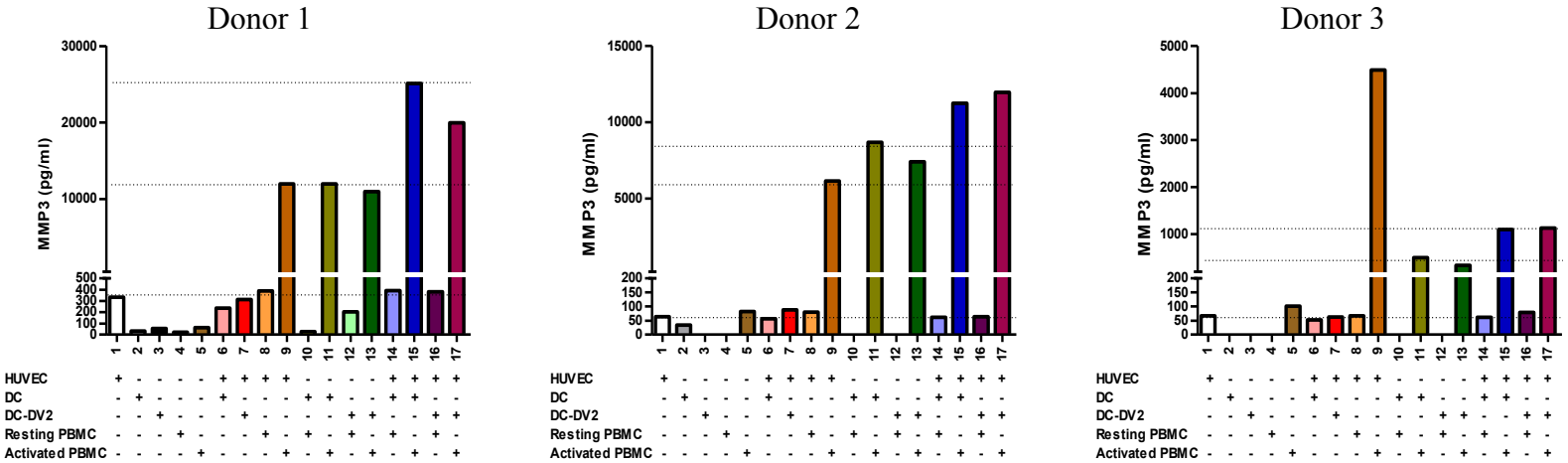
A


Donor 1
Donor 2
Donor 3
MMP1 (pg/ml)
HUVEC
DC
DC-DV2
Resting PBMC
Activated PBMC

B


Donor 1
Donor 2
Donor 3
MMP-2 (pg/ml)
MMP2 (pg/ml)
HUVEC
DC
DC-DV2
Resting PBMC
Activated PBMC

C


Donor 1
Donor 2
Donor 3
MMP3 (pg/ml)
HUVEC
DC
DC-DV2
Resting PBMC
Activated PBMC

D

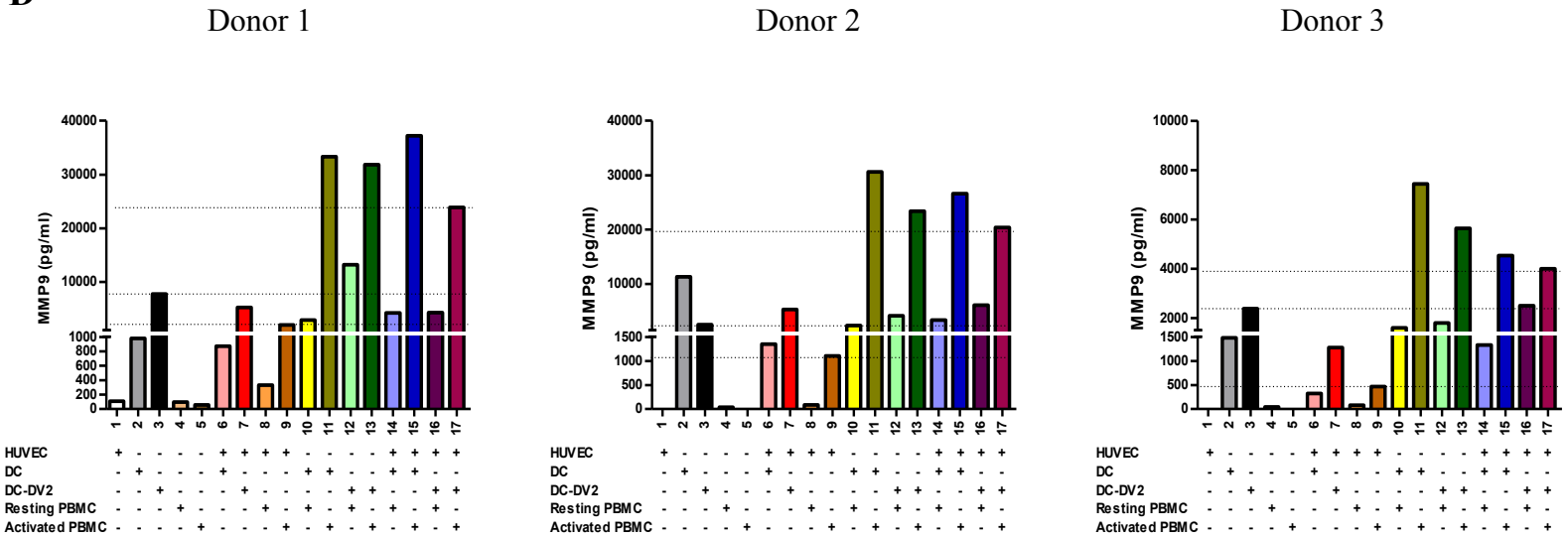

Donor 1
Donor 2
Donor 3
MMP9 (pg/ml)
HUVEC
DC
DC-DV2
Resting PBMC
Activated PBMC

Figure 5.8: Secreted MMPs in the supernatant of HUVECs, DCs (mock treated or DV2 infected) and resting PBMCs or PMA/I-activated PBMCs. Resting/activated PBMCs and/or HUVECs were co-cultured with mock treated or DV2-infected DCs. Cell supernatants were collected at 24 hours after coculture. A) MMP-1, B) MMP-2, C) MMP-3, and D) MMP-9 in the supernatants were measured at 24 hours after incubation. Results are from 3-independent experiments. DC-M: mock-infected dendritic cells, DC-DV2: DV2-infected dendritic cells.

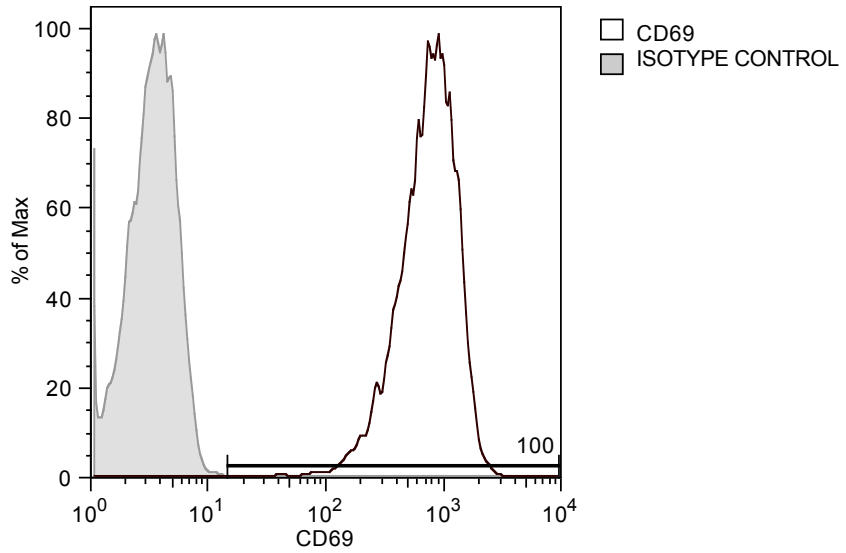


Figure 5.9: Cell surface expression on activated PBMCs. PBMCs were activated with PMA/I for 24 hours and washed for 3 times. Cell surface staining against CD69, the early activation marker, was performed by flow cytometry. Isotype control was used as a negative control.

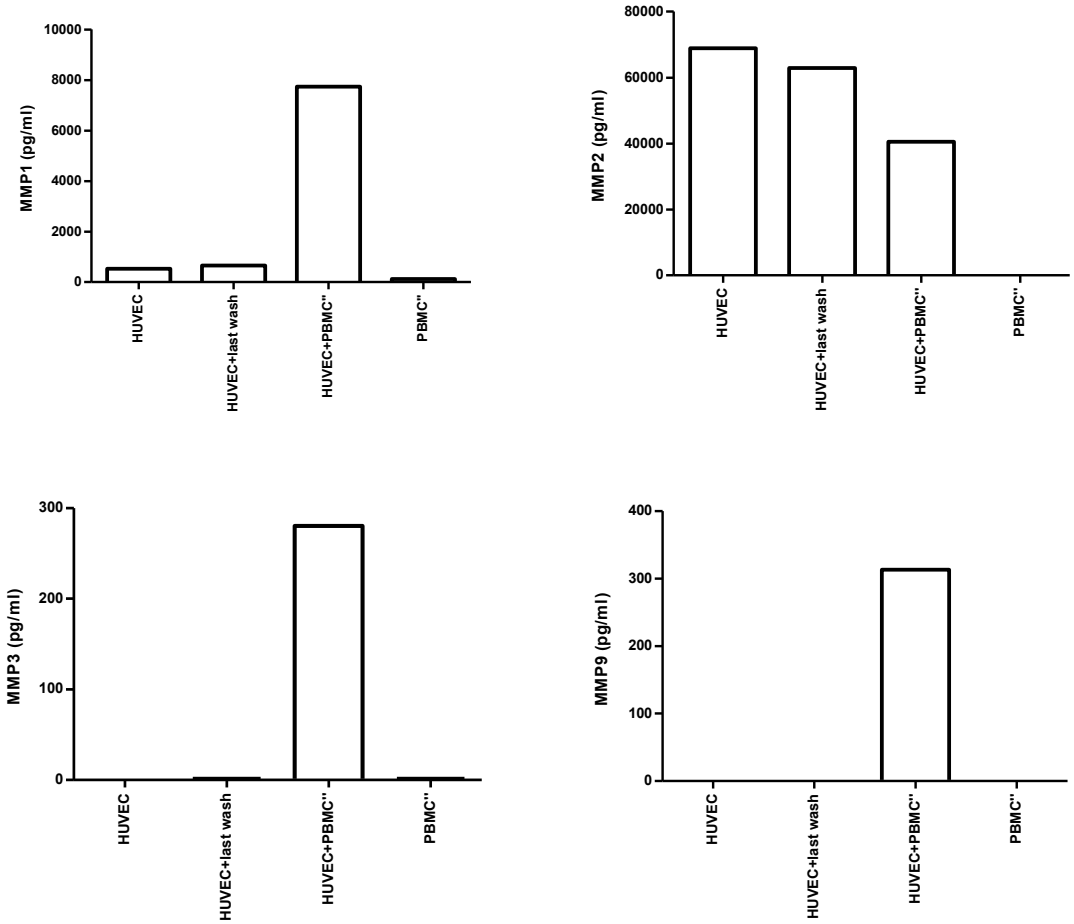


Figure 5.10: MMP levels measured in the supernatant of HUVECs treated with the last wash of activated PBMCs. Activated PBMCs were washed in R10 for three times. The supernatant of the last wash of activated PBMCs was collected. HUVECs were incubated with the last wash for 24 hours and the supernatant was harvested. Co-culture between activated PBMCs (PBMC'') and HUVECs was used as a control. MMP-1, -2, -3, and -9 were measured. The result is representative of 2-independent experiments.

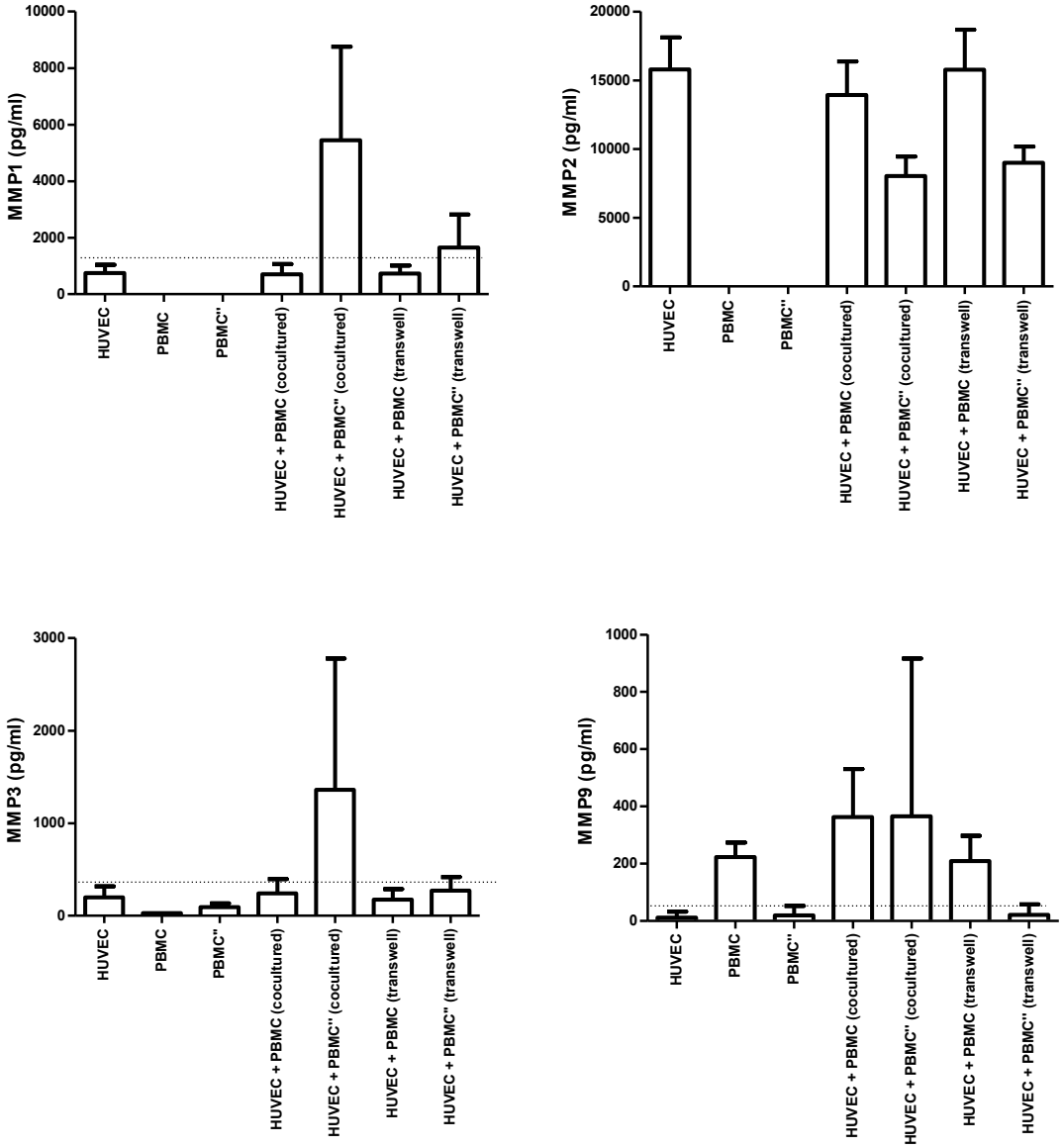
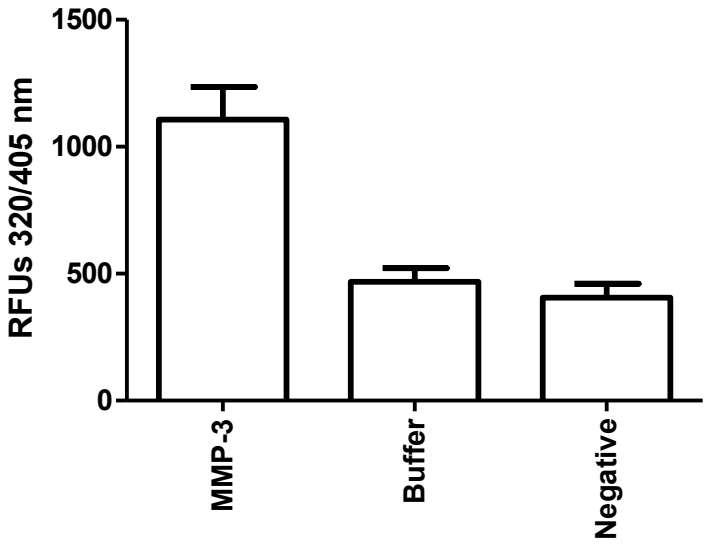


Figure 5.11: Secreted MMPs in the supernatant of HUVECs incubated with resting PBMCs (PBMC) or activated PBMCs (PBMC'') on the transwell. HUVECs were plated on a lower chamber of the transwell, whereas PBMCs or activated PBMCs were seeded on an upper chamber. Co-culture between HUVECs and either PBMCs or activated PBMCs were used as the controls. Incubation was performed for 24 hours and the supernatant in the lower chamber was collected to measure MMPs. The results from 3-independent experiments are shown as mean±SD.

5.2.5 MMP-3 and endothelial permeability

The hallmark of DHF is vascular permeability which is regulated by several factors. There is evidence showing that MMP-3 is capable of increasing albumin permeability of isolated rat glomeruli (Sharma et al., 1996). Thereby, it is hypothesized that increased plasma MMP-3 may be involved in the vascular leakage seen in Dengue infection. First, an inactive form of recombinant MMP-3 (rMMP-3) was activated to get an active enzyme. To test its activity, the active rMMP-3 was mixed with the fluorogenic peptide substrate containing a cleavage site specific for MMP-3, causing an increase in fluorescence signal (Figure 5.12a). The rMMP-3 can specifically cleave its substrate. The specific activity (pmol/min/μg) was >150 pmol/min/μg as measured under the conditions described in the manufacturer's protocol (see section 2.13 in Materials and Methods). Active rMMP-3 was also detected as a 45 kDa species by western blot (Figure 4.12b). Next, the activity of active rMMP-3 on endothelial permeability was tested. HUVECs were seeded on an upper insert assembly of a transwell plate for 3 days. Media containing TNF-α at 100 ng/ml, active MMP-3 at the initial concentration of 1 μg/ml, and the assay buffer were added to the cells for 24 hours. FITC-dextran was added in an upper chamber and growth medium was added in the receiver plate. The FITC-dextran was allowed to permeate for 30 minutes and media in the lower chamber was removed and transferred to a black 96-well plate. Fluorescence was read at 485 nm and 538 nm excitation and emission, respectively by a fluorescent plate reader. Although the initial concentration of rMMP-3 used was about one thousand times higher than that found *in vivo,* neither rMMP-3 at 1 μg/ml nor the buffer induced endothelial permeability (Figure 5.13).

A



B

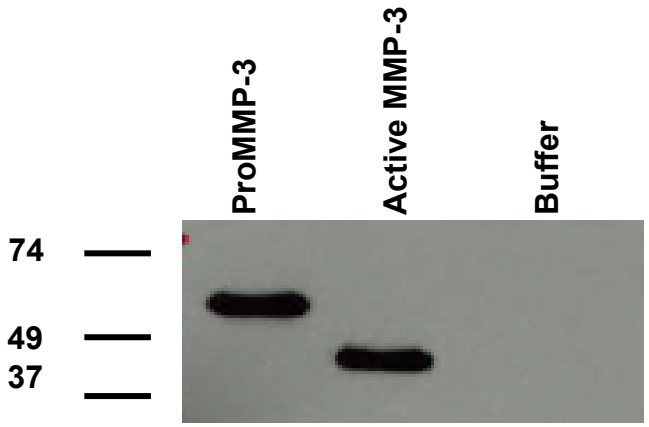


Figure 5.12: Characteristics of active rMMP-3. A) rMMP-3 at 2.5 ng/µl or buffer was mixed with the fluorogenic peptide substrate. A negative control contained only the substrate. Fluorescence signal was read after 5 minutes at 320 and 405 nm. The 3-independent experiments are shown as mean±SD. B) Active form of rMMP-3 was confirmed by Western blot. The 52 kDa proform and the 45 kDa active form were detected. Mouse anti-MMP-3 and anti-mouse HRP were used as a primary antibody and a secondary antibody respectively, at a dilution 1:1000.

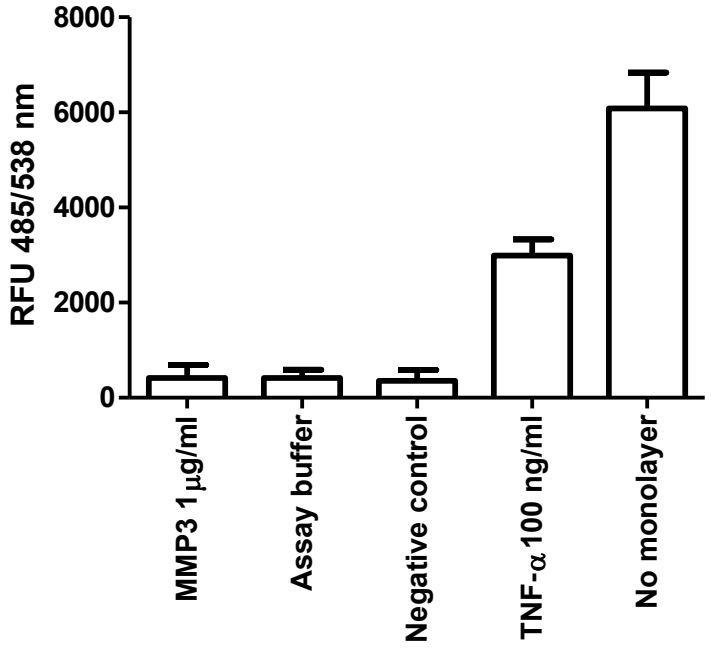


Figure 5.13: Endothelial permeability. rMMP-3 at the initial concentration of 1 μg/ml or buffer was added to the HUVECs on a transwell for 24 hours. HUVECs treated with TNF-α (100 ng/ml) and cell monolayers without treatment (only growth medium) were used as a positive and a negative control, respectively. No monolayer was demonstrated to be 100% permeable. FITC-dextran was added in an upper chamber for 30 minutes and the media in a lower chamber was transferred to a black 96-well plate. The fluorescence signal was measured at the wavelength of 485/538 nm. The 3-independent experiments are shown as mean±SD.

5.3 Discussion

In this study the plasma levels of MMPs in Thai children infected with Dengue virus were investigated. The data showed that up-regulation of MMP-3 was associated with dengue disease severity during the acute phase and returned to normal during the convalescent phase. In contrast to MMP-3, other MMPs were down-regulated. However, when the level of these MMPs was observed over the time course of the disease, a significant difference in MMP-3 levels between DF and DHF was not observed. The unexpected results may be due to a small sample size (DF =24 and DHF =32). An adequate sample size should be at least 10-15 times greater than the number of markers tested (Babyak, 2004).

Previous studies have investigated the relationship between plasma MMPs and Dengue disease (Kubelka et al., 2010, Voraphani et al., 2010). Lower plasma MMP-9 levels are observed during the febrile stage, compared to the convalescent phase. This does not correlate with the severity of disease in Thai children (Voraphani et al., 2010). This study confirms our MMP-9 results. In contrast, another study has found that an induction of plasma MMP-9 is associated with Dengue severity (Kubelka et al., 2010). Also, they have found increases of MMP-12 and -13 in Dengue patients when compared to the healthy controls, whereas MMP-2 did not change (Kubelka et al., 2010). This study was conducted in Brazilian adult patients whose levels of plasma MMPs may differ from the levels in children. In addition, they used a new World Health Organization guideline which provides a different strategy to discriminate between severe and mild Dengue (with or without warning signs, respectively; new edition in 2009 (2009)), whereas our study classified Dengue cases by the existing classification using DF and DHF as non-severe and severe Dengue respectively. Moreover, Kubelka et al. did not report when the samples were collected; this could affect the levels of plasma MMPs. These contradictory observations in the Brazilian population may also be influenced by ethnicity, age, criteria to grade the disease severity as well as the virus strain.

This is the first report showing that MMP-1, -2, -8 and -9 decreased whereas MMP-3 increased in Dengue infection. The reason for the low levels of MMPs during

the febrile stage is not clear. One of the possible explanations is that the number of MMPs-producing cells is reduced. Peripheral blood cells are known as sources of several MMPs; however in Dengue disease, both leucopenia and thrombocytopenia are usually detected. Only a moderate correlation between MMP-8, -9, WBC count and neutrophils ($r > 0.3$), but not the platelet count, was observed. This result does not fully explain the whole story of low MMPs. Alternative explanations can be that 1) synthesis of MMPs might be inhibited, 2) MMPs might be rapidly consumed, 3) MMPs may anchor to the cell membrane as membrane-bound forms, as a result of decreasing soluble forms (review in (Parks et al., 2004, Greenlee et al., 2007)), 4) MMP expression may be induced later at the time of plasma leakage, and 5) proMMPs may locally bind to their substrates in tissues and accumulate there. An example of localized expression of MMPs is reported in inflammatory bowel disease (IBD). MMP-8 is predominantly localized at the surface of epithelial cells during IBD and also expressed in the ulcer base of both ulcerative colitis and Crohn's disease, mediating tissue destruction (Ravi et al., 2007). With these possibilities, measuring plasma MMPs might not reflect total MMPs. To investigate these possibilities, mRNA, intracellular and membrane-bound forms of MMPs from PBMCs as well as tissue staining might be tested. Besides this, measurement of samples after defervescence may be required.

Altered levels of MMPs may be influenced by virus, soluble factors, or cell-cell contact. Recent experiments have shown that Dengue infected DCs and endothelial cells overproduce MMP-9 and MMP-2 (Luplertlop et al., 2006, Luplertlop and Misse, 2008). Their studies have also shown that anti-MMP antibodies and an MMP inhibitor decreased endothelial permeability and retained expression of vascular endothelial-cadherin (VE-cadherin) and platelet endothelial adhesion molecule-1 (PECAM-1), suggesting that MMPs may be involved in the vascular leakage observed. Our study, indeed, did not find a significant change of MMPs production from infected cells at any time point. However, activated PBMCs were found to affect MMPs secretion from DCs and endothelial cells. It is not surprising that activated PBMCs induce MMP-1, -3, and -9 secretions from endothelial cells since previous studies have also observed this result (François Mach, 1999, Mitola et al., 2003). Yet, none has shown that these cells also affect MMPs production from DCs. While MMP-

1, -3, and -9 were augmented in the co-culture system, secreted MMP-2 was surprisingly reduced.

In Dengue infection, massive numbers of activated T cells are produced *in vivo* during secondary infection (Mathew and Rothman, 2008, Rothman, 2011). These T cells can interact with both dendritic cells and endothelial cells lining the blood stream, inducing MMPs as well as several kinds of soluble proteins. Uncontrolled induction and activation of MMPs, despite virus-independence, can contribute to immunopathogenesis. They can degrade extracellular matrix (ECM) components such as collagen, gelatin, and stromelysin, causing tissue destruction such as the capillary leakage commonly found in Dengue disease. MMPs can also cleave cytokine/chemokine molecules, resulting in either their activation or inactivation and possibly leading to a cytokine storm. For example, MMP-9 is able to increase the activity of IL-8 (Manicone and McGuire, 2008), which is known as a potent neutrophil chemoattractant. Elevated IL-8 has been detected in plasma of Dengue patients and it is associated with disease severity (see chapter 4 and (Raghupathy et al., 1998). Since activated PBMCs can induce MMP-9 from endothelial cells, this incidence may trigger IL-8 activity in Dengue disease. In addition, MMPs can cleave VEGF, latent TNF-α, TGF-β, IL1-β, and MCPs etc., all of which are detected in Dengue disease (Manicone and McGuire, 2008, Parks et al., 2004, Srikiatkhachorn and Green, 2010). Cytokines such as TNF-α, IL-6 and IL-8, on the other hand, can also induce MMPs production (R Hanemaaijer, 1993, Kossakowska et al., 1999, Kothari et al., 2014, Li et al., 2003, Chakrabarti and Patel, 2005). Moreover, MMPs might facilitate leucocyte migration to the site of infection and inflammation. For example, MMP-3 null mice have impaired neutrophil recruitment to the lung in an acute lung injury model (Warner et al., 2001).

In these experiments soluble factors from activated PBMCs were not powerful enough to modulate MMPs production. A previous report has shown that activation of endothelial cells by interaction with T cells increases MMP-1 and -3 secretions, induces *de novo* expression of MMP-9, and activates MMP-2 through CD40-CD40L interaction (François Mach, 1999). A decrease in levels of MMP-2 was not present in this study. Although a great effect of these soluble factors *in vitro* was not seen, they may trigger MMPs production/activation *in vivo*. In addition, these *in vitro*

experiments showed that MMP production varied greatly, particularly in MMP-2 and -9 levels. Despite the fact that technical errors during experiments cannot be excluded, this variation might be due to genetic polymorphisms in each donor. Also, activated PBMCs in different individuals may show variability in secretion of cytokines such as TNF-α and IFN-γ. This may induce different degrees of MMP production since IFN-γ is able to inhibit TNF-α induction of MMPs (R Hanemaaijer, 1993, Balasubramanian et al., 2011, Cornelius et al., 1995). In Dengue cases, it has been proposed that individuals with activated T cells that produce high TNF-α but low IFN- γ levels are at risk of developing DHF; however those with greater levels of IFN- γ than the levels of TNF-α seem to have mild DF (Mathew and Rothman, 2008). This phenomenon might be explained by the fact that TNF-α can induce MMP secretion, damaging host tissues, while IFN- γ is able to suppress TNF-α activity. To test this possibility, cytokines from supernatants of the co-culture system might be measured.

Up-regulation of plasma MMP-3 may be involved with the immunopathogenesis of Dengue infection. The ability of MMP-3 to induce endothelial leakage was tested. High concentrations of rMMP-3 were added directly to the endothelial cell *in vitro*. Disappointingly, rMMP-3 did not lead to enhanced permeability. It is possible that rMMP-3 might be degraded either by some proteases in the endothelial media or by autocatalytic cleavage. Activity of rMMP-3 after cell culture for 24 hours might be tested. Moreover, MMP-3 might require interplay with other factors to mediate such an effect. A study of metastasis has reported that angiopoietin-2, MMP-3, and MMP-10 synergistically increase vascular permeability, contributing to extravasation of circulating tumor cells and the increased permeability of pulmonary vasculatures (Huang et al., 2009). In addition, MMP-3 might be involved in other mechanisms. For example, MMP-3 knock-out mice infected with intestinal bacteria show delayed clearance of bacteria and infiltration of CD4 T cells in the lamina propria, suggesting a role in T cell migration (Babyak, 2004). MMP-3 production in human fetal small intestine explants is a consequence of T cell activation with pokeweed mitogen (Pender et al., 1997) and increased production of MMP-3 could degrade the lamina propria matrix, leading to mucosal inflammation and gut injury (SCHUPPAN and HAHN, 2000). They also found that nanomolar concentrations of recombinant MMP-3 are able to induce severe tissue damage when added to the fetal explants. Since intestinal fibroblasts are a source of MMP-3 and

TNF-α released by T lymphocytes is a powerful inducer of this protease, this could provide a link between mucosal inflammation and tissue destruction. Therefore, it is possible that MMP-3 might play a role in intestinal mucosal injury in Dengue infection. Vejchapipat et. al. have demonstrated an association between Dengue infection and intestinal mucosal injury (Vejchapipat et al., 2006). They found that serum levels of intestinal fatty acid binding protein, which is a specific marker for mucosal injury, increased in the Dengue patients, particularly in DHF grade 4 compared to the control group (Vejchapipat et al., 2006). In addition, Tsai et. al. have shown that most of the Dengue patients with upper gastrointestinal bleeding had gastric ulcers or duodenal ulcers as well as hemorrhagic gastritis (Tsai et al., 1991); these wounds might be the result of MMP-3 activity. To test this possibility, expression of MMP-3 might be tested by staining of the intestinal tissue of Dengue patients with gut injury; intestinal fibroblasts as well as T lymphocytes in the gut should be the targets. Additionally, testing MMP-3 activity might be performed.

In summary, up-regulation of MMP-3, together with low levels of other MMPs, was associated with Dengue infection in a Thai cohort. MMPs production was not directly influenced by Dengue virus, but by interaction between immune and non-immune cells. Finally, MMP-3 did not play a direct role in endothelial permeability *in vitro*. Although the roles and mechanisms of MMPs in Dengue disease are not well understood, targeting MMPs as therapeutic agents might be of interest. Since excess MMPs activity can contribute to host tissue damage, regulating the over-production of MMPs might relieve the symptoms of the disease. In addition, MMPs might be useful as biomarkers to discriminate between DF and DHF.

Chapter 6

Discussion

6.1 Introduction

6.2 Complement alternative pathway genetic variation and Dengue infection in the Thai population

6.3 Matrix Metalloproteinases in Dengue disease

6.4 Early predictive markers to classify Dengue severity

6.5 Conclusion

6.1 Introduction

Dengue disease is a life threatening disease particularly in children. Mild and severe illness is graded on account of the symptoms of the disease. Pathogenesis of Dengue infection is not well understood. It is believed that variations in genetics as well as host immune responses are important factors contributing to Dengue severity. So far, there have been no good early predictive markers to classify mild DF and severe DHF. Attempts to find these markers are still ongoing and both genetic polymorphisms and biomarkers have been studied. With powerful early predictive mediators, physicians can precisely identify and treat those who are susceptible to developing severe DHF, thus reducing the mortality rate. In addition, knowing the function of these mediators could help in understanding the pathogenesis of the disease and may help generate therapeutic agents in the future.

6.2 Complement alternative pathway genetic variation and Dengue infection in the Thai population

The complement system appears to respond during an early stage of Dengue infection. A correlation between complement activation and severe DHF has been reported previously (Nascimento et al., 2009, Avirutnan et al., 2006, Bokisch et al., 1973). Nascimento *et. al*. have shown that plasma complement factor H (CFH) was lower in patients with DHF during the acute phase of the illness, returning to normal during convalescence while CFH levels in DF did not change (Nascimento et al., 2009). A reduced CFH is able to generate nonspecifically uncontrolled complement activation. This can cause tissue damage in Dengue illness. Recently, a polymorphism in the CFH promoter [CFH promoter (-332 C/T: rs375339)] was associated with Dengue susceptibility (Pastor et al., 2013). The authors proposed that this variant might affect expression of CFH. However, in this present study, association between this variant and Dengue severity was not found (Table 3.2 and 3.3). Polymorphisms in a group of complement proteins called the 'complotype' (CFH-62: rs800292; FB-32: rs12614 and rs12615; and C3-102: rs2230199) were also tested. The complotype has been previously described to play a role in age-

related macular degenerative disease (AMD) (Hageman et al., 2005, Gold et al., 2006, Yates et al., 2007). Individuals with the 'high activation' complotype have an increased susceptibility to develop AMD (FB-32 R/R: rs12614 [C] and rs641153 [G]; CFH-62 V/V: rs800292 [G]; and C3-102 F/F: rs2230199 [G]) whereas the 'low activation' complotype confers a reduced risk of developing AMD (FB-32 Q/Q rs12614 [C] and rs641153 [A]; CFH-62 I/I: rs800292 [A]; and C3-102 S/S: rs2230199 [C]). This evidence was supported by an *in vitro* study showing that the high activation complotype generates a high complement hemolytic activity (Heurich et al., 2011). However, a relationship between these variants and Dengue susceptibility could not be detected here (Tables 3.3-3.4). Similarly, CFH-402 (Y/H; rs1061170), the AMD-associated polymorphism, was not correlated with Dengue (Tables 3.2-3.3). In addition to these single nucleotide polymorphisms, a copy number variation in the complement related proteins (CFHRs) is able to affect complement regulation (Jozsi and Zipfel, 2008). Although the absence of CFHR1 and CFHR3 (ΔCFHR3-1) is associated with protection against AMD (Spencer et al., 2008), there was no evidence that this variation conferred protection against Dengue infection or even its severity (Table 3.6). These data demonstrate that the severity of Dengue infection is not strongly influenced by complement alternative genetic variation in the Thai population. However, we cannot exclude this possibility in other different ethnicities. In addition, other polymorphisms in complement genes could influence Dengue infection. This possibility might be tested by genome-wide association studies (GWAS).

Many pathogens are able to modulate complement mediated responses by binding to CFH (Ferreira et al., 2010). NS1 of West Nile virus (WNV) is able to recruit CFH, suppressing complement activation in solution and complement-dependent lysis of infected cells (Chung et al., 2006). However, this phenomenon does not happen with NS1 of Japanese encephalitis virus (JEV) (Krishna et al., 2009). In this study, the direct binding between NS1 of Dengue virus and CFH could not be detected (Figures 3.5-3.7). The interaction between these two molecules may require other components. For example, NS1 might bind to CFH via an interaction of carbohydrate ligands such as heparin sulfate (Muller and Young, 2013, Avirutnan et al., 2007, Zipfel et al., 1999). NS1 might also compete with

CFH for binding to ligands on the surface of healthy, uninfected cells, leading to complement activation and complement-induced vascular leakage. It is also possible that NS1 might not be a key component in the modulatation of CFH activities. Nor do other non-structural proteins fulfill this role since these proteins present inside the infected cells. Numerous microbes can recruit CFH to their surface to avoid complement attack (Ferreira et al., 2010). This might happen with Dengue virus particles. To test these hypotheses, a binding between Dengue particles and CFH might be performed.

6.3 Early predictive markers to classify Dengue severity

Not only are genetic polymorphisms studied to identify markers of Dengue severity, peripheral blood cell counts and plasma protein concentrations of Dengue patients are also widely explored. Clinical profiles found in this study were similar to those described in the WHO criteria: reductions in platelet cell, wbc, neutrophil counts, lymphocytes, liver protein and albumin levels as well as increases in hct, atypical lymphocytes, and liver aminotransferases (ast and alt) (Table 4.2). In addition to these clinical features, many soluble proteins have been correlated with Dengue susceptibility (Tables 4.3-4.4). Some of them have been previously reported and confirmed in this study whereas others have been newly identified by this study. These newly identified markers that associated with Dengue severity are MMP3, BCA1, MIP3α, CTACK and Eotaxin2; those that associated with Dengue susceptibility are MMP1, MMP8, sIL4R, sIL6R, MIP1δ and MIP3β (Table 4.3).

Although numerous publications have proposed early predictive markers, none of them can discriminate DF and DHF accurately. This may be due to variations in the duration/time of sample collection, sample collection method, age of the patients, etc. Attempts to define biomarkers discriminating DF and DHF were also performed in this study. Binary logistic regression and classification tree (CART) were applied to search for good indicators or predictors of disease severity. With the binary regression, platelet cell count and MCP2 were defined as the markers for classifying DHF and DF in early stages of the disease while CART

gave only the platelet cell count as an early predictive marker (Figures 4.7 and 4.9). When considering the sensitivity, specificity and accuracy of the models from these two methods, the binary logistic regression gave the better prediction and accuracy scores although there are some difficulties using this method (Figures 4.8 and 4.10). Importantly, the sensitivity of the test should be high enough to reduce the number of false negative results (true DHF but defined as DF) since there is a greater risk that patients will develop DHF at home, where they cannot access the intensive medical attention that is required. However, when attempting to improve the sensitivity, the number of false positives (true DF but defined as DHF) was also highly increased. A model with high false positives is unpromising as well since it cannot reduce the number of in-patients, representing a considerable burden for hospitals. In future experiments, biomarkers defined here should be verified in another cohort to confirm their utility in defining DF from DHF at early stages of the disease.

6.4 Matrix Metalloproteinases in Dengue disease

Plasma leakage in Dengue infection is a hallmark of severe DHF but a mechanism for this phenomenon is not well defined. Matrix metalloproteinases (MMP), zinc-dependent endopeptidases, may be involved in this process since they can degrade extracellular matrices, regulate cytokine/chemokine activities, induce T cell migration and activation, etc. (Parks et al., 2004, Pender et al., 1997). Recently, correlations between plasma levels of MMPs and Dengue susceptibility have been observed. Up-regulation of circulating MMP9 was correlated with the disease severity, but this could not be replicated in another study showing that decreasing MMP9 levels were detected during the acute stage of infection when compared to the follow-up stage as well as to the control group (Kubelka et al., 2010, Voraphani et al., 2010). In this study, a reduction in plasma MMP1, -8, and -9 during the acute stage were correlated with Dengue susceptibility but these were not associated with the disease severity (Figure 5.2 and 5.3). The levels of MMP2 were not correlated with Dengue infection since the concentrations of this enzyme were comparable in both the Dengue and control groups (Figure 5.2 and 5.3).

However, it is of interest that the levels of MMP2 were low during the acute stage even in the control group, increasing during convalescence. While it remains uncertain why these plasma MMP1, -2, -8, and -9 levels were reduced during the acute stage, up-regulation of MMP3 could be observed in Dengue patients and correlated with the disease severity (Figure 5.2 and 5.3). In future experiments, the number of Dengue samples observed over the time course should be bigger in order to increase the statistical power of the analysis.

It is postulated that local expression of uncontrolled MMPs could mediate tissue destruction as well as vascular leakage. Previous *in vitro* experiments have demonstrated that MMP2 and -9 were over-produced in infected cells (Luplertlop et al., 2006, Luplertlop and Misse, 2008). They also showed that these over-produced MMPs were able to increase endothelial permeability and were associated with a reduced expression of adhesion molecules on the cell surface (Luplertlop et al., 2006). This permeability is reduced when MMP specific inhibitors and an anti-MMP9 antibody are used. However, in this present study, over-production of MMPs from infected cells could not be detected (Figure 5.6 and 5.7). Although MMPs production was not directly changed by the virus, it was confirmed that activated PBMCs could alter production of MMP1, -3, and -9 in both dendritic cells and endothelial cells *in vitro* (Figure 5.8). These results were supported by previous studies (François Mach, 1999, Mitola et al., 2003). Moreover, MMP levels, particularly MMP-9, secreted from activated PBMCs could be different from those of resting PBMCs (Figure 5.11). In addition, a reduction of MMP2 in the supernatant was surprisingly found when activated PBMCs were added into endothelial cells. A decrease in plasma MMP2 might be inferred from this experiment (Figure 5.2). Massive activation of T cells is generally observed in Dengue illness especially in severe Dengue cases (Mongkolsapaya et al., 2003). These activated T cells can migrate to the site of infection and interact with dendritic cells and endothelial cells lining the blood vessels (Martina et al., 2009, Dejnirattisai et al., 2008). They may be key cells contributing to immunopathogenesis by triggering the release of many soluble factors including MMPs. Even though attempts to mimic an interaction between activated PBMCs, endothelial cells and infected dendritic cells were not successful due to variations in

the results (Figure 5.8), it is still possible that the interaction of these cells might induce production and activation of MMP *in vivo*.

Up-regulation of MMP3 during Dengue infection is a new insight but the role of MMP3 in the illness is still unknown. MMP3 is generally able to degrade extracellular matrices such as collagen, fibronectin, elastin, and laminin (http://www.ncbi.nlm.nih.gov/gene/4314). This enzyme is also involved in the activation of other MMPs, tissue remodeling, and T cell migration and activation (Li et al., 2004, SCHUPPAN and HAHN, 2000). In addition, there is evidence showing that MMP3 has an effect on the glomerular barrier in rats, increasing albumin leakage (Sharma et al., 1996). With these myriad abilities, MMP3 may have multiple functions in Dengue infection. Since a recent study has shown that MMP9 can increase endothelial leakage *in vitro* (Luplertlop et al., 2006), it was of interest to see if MMP3 had the ability to cause vascular leakage. Unfortunately, MMP3 alone appeared not to have a direct effect on endothelial permeability (Figure 5.12). A study in tumor extravasation has shown that a combination of MMP3, angiopoietin-2 and MMP10 can increase the permeability of the pulmonary vasculature, leading to tumor cell migration across endothelial barriers (Huang et al., 2009). This evidence suggests that MMP3 might be able to cause endothelial permeability in Dengue infection but may require companions to do so. However, it is uneasy to investigate companion proteins since MMP3 can bind to numerous proteins such as MCP1-4 (monocyte chemotactic proteins), CXCL7, CXCL12, and other MMPs (Parks et al., 2004).

6.5 Conclusion

All in all, factors contributing to Dengue severity were investigated in this study. Genetic polymorphisms in the alternative complement pathway were not associated with Dengue illness in a Thai population. In contrast, several plasma mediators were found to increase the risk of developing DHF. Up-regulation of plasma MMP3 was associated with Dengue severity although the role of MMP3 in Dengue pathogenesis remains uncertain and should be investigated further. Finally

a predictive model to predict DHF identified low platelet cell count and high concentration of MCP2 as potential markers. These data help to broaden our knowledge of Dengue pathogenesis and may prove helpful in the development of therapeutic agents as well as predictive indicators for Dengue disease severity.

Appendices

Appendix 1: SNPs studied, primers sequences and product sizes

Gene	Location	ID Number	Primer Sequence (5'--3')	Product size (bp)
Factor H	Promoter	rs3753394	TCTTTACCTTCTCAATATCCAGC	867
			ACTCCTGTGAAAAGCATCATTAG	
	Exon 2	rs800292	Pre-designed by applied bioscience	-
			Pre-designed by applied bioscience	
	Exon 9	rs1061170	CCTTTGTTAGTAACTTTAGTTCGTC	490
			GGTCCATTGGTAAAACAAGG	
	Intron 12	rs6677604	TTGCTTGAGGAAAGTTGTGC	707
			TGCTCATTTTCCTTCCCATC	
Factor B	Exon 2	rs12614, rs641153	CAAGCCAGGACACACCATC	1800
			TCTTCCGCTTCTGTTGTTCC	
C3	Exon 3	rs2230199	GAAGACCAAGAATAATGGGCA	511
			TCTTTCTCTGTCTCTCTCGAT	

Appendix 2: Biological markers

Matrix Metalloproteinases (MMPs) (R&D systems, USA)

MMP1, MMP2, MMP3, MMP7, MMP8, and MMP9

Soluble cytokine receptors (Millipore, USA)

sTNFRI, sTNFRII, sVEGFRII, sEGFR, sgp130, sIL1RII, sIL2Ra, sIL4R, and sIL6R.

Cytokine/chemokine panel II (Millipore, USA)

MCP2, ENA78, SDF1α+β, BCA1, I309, IL16, MIP1δ, TARC, 6Ckine, Eotaxin2, CTACK, IL23, TPO, TRAIL, IL33, and Eotaxin3

Cytokine/chemokine panel III (Millipore, USA)

ITAC, MIP3α, MCSF, GCP2, and MIP3β

Human cytokine 30-plex (Invitrogen, USA)

TNFα, IFNγ, IL6, IL8, IL1β, IL10, IL13, IL2, IL2R, IL12, IL15, IL4, IL5, IL7, IL17, Eotaxin, IP10, MCP1, MIG, MIP1α, MIP1β, RANTES, HGF, FGF, GCSF, EGF, VEGF, GMCSF

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