Immune Activation in the Pathogenesis of Dengue Virus Infection

Cornelia A.M. van de Weg

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The greatest glory in living lies not in never falling, but in rising every time we fall.

Nelson Mandela

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General Introduction

General Introduction

Clinical Aspects of Dengue Virus Infection

Dengue virus (DENV) is a positive-stranded RNA virus and belongs to the *Flaviviridae* family. The virus is transmitted by the bite of an infected *Aedes*-mosquito and circulates in tropical and subtropical areas around the world. The incidence of dengue has risen dramatically over the past decades. For years, the World Health Organization (WHO) estimated a worldwide incidence of 50-100 million infections per year [1]. However, this estimation was based on a rather simplified model. A recent study using cartographic approaches suggested that 390 million infections occur annually [2]. The majority of the infected population (75%) will not show any clinical symptoms, but they are a potential reservoir from which other people can get infected [3]. It is estimated that 70% of the clinical apparent DENV infections occur in South-East Asia [2], most probably due to the high grade of urbanization in this area, providing a lot of breeding places for mosquitos [4]. Approximately 14% of the DENV infections with clinical symptoms occur in the Americas and 16% on the African continent [2].

Clinical symptoms

DENV belongs to the viruses that cause hemorrhagic fevers, indicating that dengue is a febrile disease that may present with hemorrhagic manifestations.

The majority of symptomatic patients present with an undifferentiated febrile disease accompanied by general symptoms, such as headache, myalgia, arthralgia, skin rash and retro-orbital pain. Patients may also suffer from gastro-intestinal symptoms, such as nausea, vomiting and abdominal pain. Children with dengue are more likely to report anorexia, nausea, vomiting, and abdominal pain, than children with other febrile illnesses [5,6].

The tourniquet test has been commonly used as a diagnostic criterion for DENV infection. In this test a blood pressure cuff is applied to the upper arm and inflated to the midpoint between the systolic and diastolic blood pressure for five minutes. If more than 20 petechiae appear in an area of 2,5 cm^2 on the arm, the test is considered to be positive. A positive test result indicates an increase in capillary fragility. This test has high specificity, indicating that a negative result is a useful tool to rule out the diagnosis of DENV infection [5-7]. Many DENV infected patients show spontaneous petechiae and this clinical symptom has been considered an early warning sign and positive predictor for the diagnosis of DENV infection [6].

A significant number of patients will present with spontaneous bleeding, such as epistaxis, gum bleeding or prolonged bleeding from a venipuncture site. In severe cases, patients may present with extensive gastro-intestinal hemorrhage, such as hematemesis and melena [7]. It is proposed that patients with severe dengue suffer from disseminated intravascular coagulation (reviewed in [8]), which may cause extensive hemorrhage in the gastro-intestinal tract.

A prominent feature of DENV infection is plasma leakage. Interestingly, the leakage is confined to the pleural and peritoneal cavity and therefore many patients present with pleural effusion or ascites. Extensive plasma leakage may eventually result in shock. Respiratory distress due to fluid accumulation has also been reported, but is not common [9].

Especially in severe cases the liver can be highly affected as shown by an increase in levels of liver en-

zymes [5,9,10]. But even in uncomplicated dengue an increase in liver enzymes and hepatomegaly have been reported [6]. DENV infection causing organ impairment does not occur often, but liver and renal failure and severe neurological disease have been reported in fatal disease [11,12].
Certain laboratory markers are characteristic for DENV infection. Already in the early phase, DENV infected patients may show signs of thrombocytopenia (platelet count ≤ 100.000 cells/mm^3), leukopenia, and elevated levels of SGOT [5]. These clinical parameters can be useful to discriminate DENV infected patients from patients with other febrile illnesses. Hematocrit is a marker of hemoconcentration and its rapid increase is indicative for severe plasma leakage, which may eventually result in shock. A peak hematocrit of more than 50% has been associated with shock [6,9].
It has been shown that there is a difference in disease manifestation between adults and children. Adults are more likely to develop hemorrhage and severe organ impairment, while children are more prone to develop shock [13,14]. Moreover, adults generally present with more profound thrombocytopenia than children [13].

Time course of clinical symptoms

The time course of a DENV infection shows a typical pattern, which has been described in the 2009 WHO dengue case classification [1]. The first 3-5 days after onset of symptoms are characterized as a febrile phase (Figure 1). As the name suggests this phase is characterized by fever and general symptoms. At this time, virus titers usually reach peak levels. Patients in the febrile phase can already present with warning signs, which may precede the development of severe disease. Abdominal pain or tenderness, persistent vomiting, clinically manifest fluid accumulation, mucosal bleeding, lethargy and restlessness, hepatomegaly and an increase in hematocrit with a drop in platelet count are all listed as warning signs in the 2009 WHO dengue case classification [1].
The time of defervescence is the start of the critical phase. The transition from the febrile to the critical phase may develop within a few hours. During this phase, patients may develop severe symptoms, such as shock, respiratory distress, severe hemorrhage and organ impairment. Interestingly, the virus is often no longer detectable in the circulation, suggesting that the immune response against the virus is involved in causing the clinical symptoms at this time point. A decrease in platelet count and a rapid increase in the levels of hematocrit can also precede the critical phase. The critical phase usually lasts no longer than 24-48 hours after which patients enter the recovery phase, during which the clinical condition of the patients improves. However, in this phase patients are at risk for the development of pulmonary edema or heart failure due to fluid overload.

Clinical classification

To achieve universal consensus about the clinical case classification of DENV infected patients, WHO released guidelines in 1974. Although these guidelines were updated several times the utility and accuracy of classifying patients according to disease severity criteria have continued to be a matter of debate. Therefore, several study groups have proposed a reassessment of the classification criteria [6,7,10,15,16]. In the guidelines from 1997, patients were classified in three categories, i.e. dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [17]. DF was defined as an acute febrile illness with general complaints and a positive laboratory confirmation of DENV infection. To be diagnosed with DHF, patients had to fulfill the following criteria: fever or a history of fever, hemorrhagic tendency (at least a positive tourniquet test), thrombocytopenia and signs of plasma leakage (at least a 20% increase in hematocrit). DHF with signs of shock was classified as DSS.

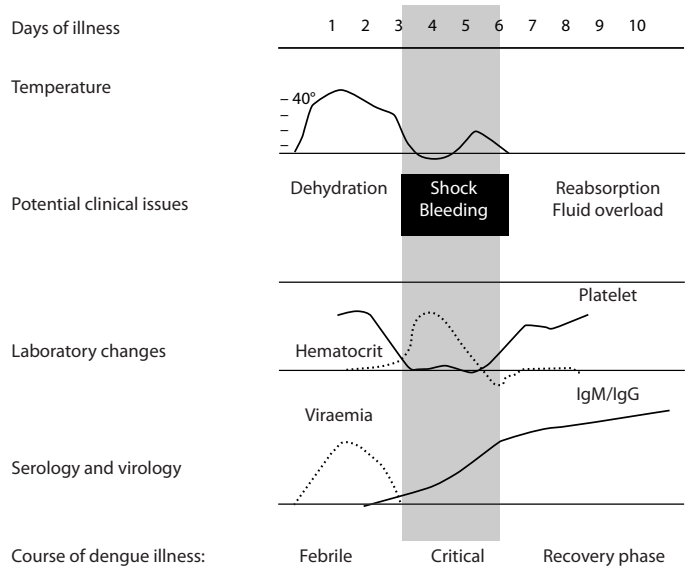

Figure 1 | Characteristic signs and symptoms during the course of a dengue virus infection. Source: Yip, Medical Progress, October 1980 [19]

The problem with this classification was the stringency. A recent study showed once again that almost one third of the patients with DSS did not fulfill all four criteria of the 1997 WHO classification [9].
The 2009 WHO guidelines distinguish between severe and non-severe dengue [1]. Severe dengue is defined by the occurrence of plasma leakage leading to shock and/or fluid accumulation leading to respiratory distress and/or severe bleeding and/or severe organ impairment. The non-severe dengue group is divided in patients with and without warning signs. The 2009 WHO dengue case classification is less stringent than the 1997 WHO classification, but leaves more room for interpretation and patients are more easily classified as having severe disease. Therefore, the debate about the most optimal classification for DENV infected patients is still ongoing [18].

Laboratory diagnostics

As has been described previously it is not possible to discriminate DENV infected patients from those with other febrile illnesses, based on clinical signs and symptoms. A positive result in at least one of the laboratory assays is needed to confirm the diagnosis. In order to choose an appropriate assay, the time of sampling after the onset of disease is important.
Viremia in DENV infected patients lasts for an average of 4-5 days and the highest DENV titers are reached very early after the onset of symptoms [20]. During this period viral RNA can be detected with reverse-transcriptase polymerase chain reaction (RT-PCR) (Figure 2). This method is very sensitive and specific and can also be used to determine the infecting serotype [21]. The major drawback is that it can only be used in the acute phase and that this method requires specialized laboratory equipment and personnel.
DENV infected cells produce significant amounts of the non-structural protein 1 (NS1), which is re-

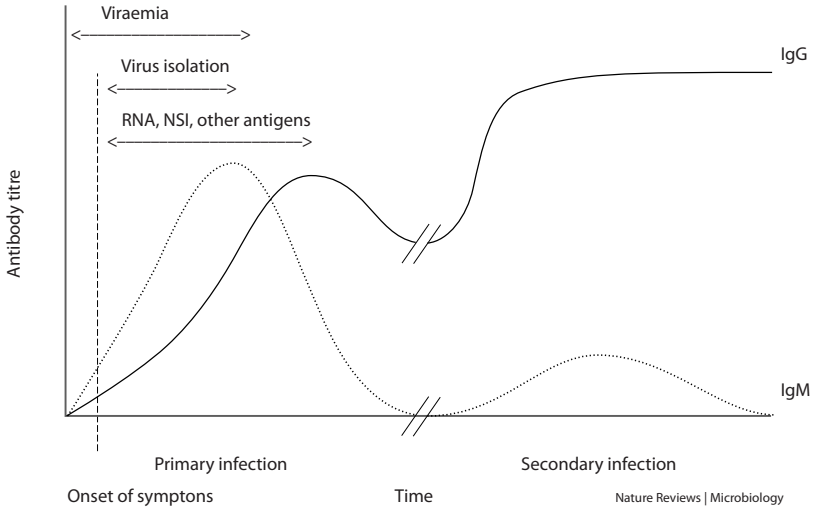

Figure 2 | Possible laboratory diagnostic methods during the course of a DENV infection. Reproduced with permission. Source: Peeling et al. Nature Reviews Microbiology. 2010 [23]

leased in the circulation. This protein can be detected with enzyme-linked immunosorbent assay (ELISA) and rapid immunochromographic assay. It has been shown that NS1 can be detected from one till nine days after the onset of symptoms [22]. Moreover, these assays are cheap and easy to use.
The human host will respond to DENV infection with the production of IgM and IgG antibodies directed against the virus. IgM is usually detectable at 3-5 days after the onset of fever. The titers are usually higher in patients with primary than in patients with secondary infection. Patients with primary infection are negative for IgG in the acute phase of disease. In contrast to patients with secondary infection, whose levels of IgG increase rapidly in the first days of infection. IgM antibodies can be detected in the circulation for up to three months and IgG antibodies much longer. In order to get a conclusive diagnosis of an acute DENV infection it is required to test paired samples to demonstrate seroconversion. With paired samples it is usually possible to determine whether the patient suffers from primary or secondary DENV infection (Reviewed in [23]). The avidity of IgG antibodies can be used to discriminate between primary and secondary DENV infection, because it is low in primary and matures to high avidity antibodies in secondary infection [24].
Altogether, the main advantage of RT-PCR is that it directly detects the presence of the virus and is therefore very specific. However, viral RNA can only be detected in the acute phase. The IgM and IgG assays are cheaper and easier to perform, but they become positive later after the onset of symptoms and paired samples may be needed. Moreover, cross reactivity of antibodies induced by other flaviviruses complicate serological diagnosis, largely depending on the geographical area involved. The NS1 antigen test is positive in the acute and part of the convalescent phase and is cheap and easy to perform. However, it has been reported that the performance is poor in certain populations [25].

Treatment

At this moment no specific antiviral treatment is available for DENV infection and thereby therapy is symptomatic consisting of intense monitoring combined with supportive care. Initially, patients with non-severe dengue should be assessed carefully for the presence of warning signs or risk factors for severe dengue, i.e. co-morbidity and pregnancy [1]. Patients with warning signs and/or risk factors and patients with severe dengue should be admitted to the hospital. All patients should be given oral rehydration fluids to maintain the fluid balance and prevent dehydration. Because of gastro-intestinal symptoms and the increased bleeding tendency during DENV infection, it is contra-indicated to take aspirin, ibuprofen or other non-steriodal anti-inflammatory agents (NSAIDs). Patients with shock should initially be treated with an isotonic crystalloid fluid regimen. Treatment with colloid IV fluids is only indicated if the patient suffers from severe shock, which does not respond to the standard therapy. A randomized controlled trial showed that the crystalloid solution Ringer's lactate was as effective as two different colloids for initial resuscitation of children with moderately severe shock [26]. A 10-year prospective study about patients with dengue shock syndrome showed that treatment with colloids, inotropic support and/or blood products is only rarely indicated [9]. In this study the case fatality rate was only 0.5%, suggesting that extensive supportive care is not really needed in the majority of cases. In order to prevent fluid overload in the recovery phase it is also important to be cautious with IV fluid therapy during the critical phase, especially because this phase usually does not take longer than 48 hours. Blood transfusion is only indicated in case of severe bleeding.

Pathogenesis

Viral genome structure

DENV belongs to the family *Flaviviridae*, genus *Flavivirus*. The genome is approximately 11 kb in length. DENV consists of four antigenically distinct serotypes (DENV 1-4). The DENV genome encodes three structural proteins, the capsid (C), the (pre-)membrane (prM/M) and the envelope (E) and seven non-structural proteins, e.g. NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.

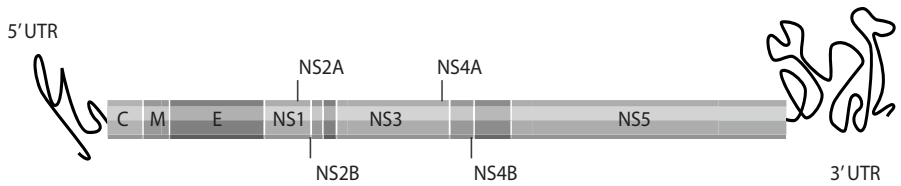

Figure 3 | The molecular structure of a DENV. C = capsid. M = membrane. E= envelope. NS = non-structural. Reproduced with permission. Source: Guzman et al. Nature Reviews Microbiology. 2010 [27]

Virulence

There are two in vivo criteria that are indicative for the virulence of DENV: the disease severity or the level of viremia it causes in a patient. The main in vitro indicator for DENV virulence is the replicating and transmission fitness of the virus (reviewed in [28-30].

DENV consists of four antigenically distinct serotypes, DENV-1, DENV-2, DENV-3 and DENV-4. It is generally believed that DENV-2 is the most virulent serotype, but all other serotypes are also capable of causing severe disease [20]. Moreover, genetic variants within one serotype can also differ in virulence. The South-East Asian genotype of DENV-2 is considered to be more virulent than the American genotype. For example, the first outbreak in Cuba with severe cases was indeed caused by a DENV-2 with a South-East Asian genotype [31]. Leitmeyer and others compared the molecular structure of South-East Asian DENV-2 strains with American DENV-2 strains [32]. They found differences in the envelope region for which they speculated that it would alter virion binding to the host cell. Moreover, the differences found in the 5' and 3' UTR were proposed to alter the initiation of translation and the viral replication, respectively. In an experiment with recombinant viruses it was shown that substitution of the amino acid Asn (Asian genotype) with Asp (American genotype) at position 390 in the envelope region resulted in a decreased ability of the virus to replicate in macrophages [33]. Cologna et al. compared the replication kinetics of South-East Asian DENV-2 strains with American strains in immature Dendritic Cells [34]. They showed that the South-East Asian strains had a higher viral output per infected cell. Moreover, in a competition model in mosquitoes they found that the South-East Asian strains outcompeted the American strains, underlining their epidemic potential.
It has been shown that the 3' UTR region plays an important role in virus replication and translation [35]. The 3' UTR region of the virus consists of a proximal and a distal region. The proximal region has the highest variability in primary sequence and secondary structure, whereas the distal region is highly conserved in secondary structure among flaviviruses [36]. It is predicted that the distal region forms secondary stem-loop structures, which may play an important role in virus replication [37-39]. However, although several studies have been conducted, the importance of the variable region is still unclear [40].

Immune activation

Severe disease during DENV infection usually occurs around the time of defervescence when the virus is usually no longer detectable in the circulation. This indicates that severe disease is caused by extensive activation of the immune system of the host. One of the main research questions is which mechanisms are responsible for this full-blown activation.

Antibody-dependent enhancement and original antigenic sin

One of the first and most supported theories for mechanisms underlying the development of severe disease during DENV infection is antibody-dependent enhancement. This theory is based on the observation that patients with a secondary DENV infection have a higher risk to suffer from severe disease. The primary target cells of a DENV infection are monocytes and macrophages [41]. The theory of antibody-dependent enhancement hypothesizes that non-neutralizing antibodies directed against another serotype than the infecting one, are facilitating the uptake of virus by monocytic cells (Reviewed in [42]). In vitro evidence supports this hypothesis by showing that DENV infection of macrophages in the presence of antibodies resulted in an increase of the amount of infected cells and an increase in the production of certain pro-inflammatory cytokines [43]. In vivo, it has been shown that a higher viral load in the acute phase of disease will result in the occurrence of more extensive plasma leakage around the time of defervescence [44].
Another theory about how DENV infection may lead to immune activation is original antigenic sin [45]. This theory was based on the observation that influenza vaccinated humans elicited antibodies

against the vaccine strain, but at the same time even higher titers of antibodies against influenza strains they had been exposed to during childhood. Original antigenic sin in the dengue field postulates that low-avidity T-cells, also directed against another serotype than the infecting one, fail to clear the virus from the circulation, but still induce extensive cytokine production (Reviewed in [46]). It has indeed been shown that secondary DENV infection causes expansion of T cells with low avidity against the infecting serotype [47]. However, a very recent study showed that a strong immune response with multifunctional CD8+ T cells was not harmful, but associated with protection against severe dengue [48]. They showed that the HLA alleles had a higher impact on the susceptibility for severe disease.

Cytokine storm

It has been hypothesized that severe DENV infection is associated with a cytokine storm (reviewed in [29,49]). The exact definition of a cytokine storm is still a matter of debate. In general it is assumed that a cytokine storm starts with an excessive release of pro-inflammatory cytokines (e.g. TNF-α and IL-1β). These cytokines then induce other pro-inflammatory (e.g. IL-6), but also anti-inflammatory cytokines (e.g. IL-10). This augmented immune response could therefore be the result of a disturbed balance between pro- and anti-inflammatory cytokines. During severe DENV infection a cytokine storm has been proposed to be responsible for the increased vascular permeability and coagulation disturbances (reviewed in [49]).
The acute phase of DENV infection is characterized by viremia inducing a strong Th1 response with peak levels of IFN-γ [44,50-53], IFN-α [53,54], IL-12 [55-57], MCP-1 [58-60], MIG and MIP-1β [58]. The Th1 response starts with the chemokine MIP-1β, which can stimulate dendritic cells to produce IL-12 [61]. Moreover, IL-12 is known to induce the production of IFN-γ by Th1-cells and subsequently IFN-γ can induce the production of MIG [61]. IFN-α and IFN-γ have strong antiviral properties, suggesting that they play an important role in reducing the viral load.
Increased levels of IL-4, IL-5 and IL-13 are indicative of a Th2 response. It is hypothesized that during the course of a DENV infection a switch from a Th1 to a Th2 response takes place [62], because it was shown that levels of IL-13 peaked at more than 9 days after the onset of fever. However, the majority of studies provide no evidence for increased levels of IL-4 [50,52,63,64] and IL-5 [58,63,64] in DENV infected patients. IL-10 is an anti-inflammatory cytokine and it has been hypothesized that high levels of IL-10 have an inhibitory effect on dendritic cells and macrophages (reviewed in [65]). High levels of IL-10 have been described in severe dengue, especially at the day of defervescence [44,51,55,56].

In DENV infected patients increased levels of pro- and anti-inflammatory mediators have been found. The classic pro-inflammatory cytokines are IL-6, IL-8, TNF-α and IL-1β. High levels of IL-6 and IL-8 were reported in dengue cases with severe plasma leakage and shock [66] and in non-survivors [52,67,68]. IL-6 production is induced by TNF-α and IL-1β [61]. However, evidence about increased levels of TNF-α and IL-1β are conflicting and in many studies no increased levels of these mediators were found [50,51,58,66,68]. This can be explained by the observation that TNF-α and IL-1β are produced early after infection and are removed quickly from the circulation.

Temporal pattern of the immune response

To investigate the host reponse of a DENV infection, several studies have applied transcriptome profiling [69-79]. It has been shown that the time after the onset of disease has a major impact on the transcriptome profile of the patient [69,79]. The acute phase of disease is characterized by an increased

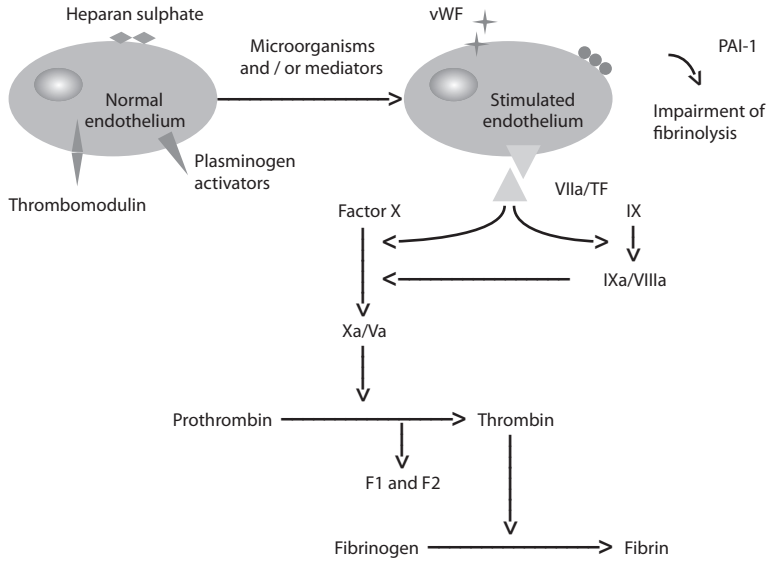

Figure 4 | Activation of the coagulation cascade and the fibrinolytic system during DENV infection due to stimulation of endothelial cells. Reproduced with permission. Source: Mairuhu et al. Lancet Infectious Diseases. 2003 [8].

expression of genes involved in immunity and inflammation [73,78]. In this phase transcripts involved in the innate immune response, in particular interferon induced genes and complement, are highly upregulated [73,78]. The convalescent phase is characterized by increased abundance of transcripts involved in cell cycle and cell repair mechanisms [70,79].

Vascular permeability

One of the most prominent symptoms of DENV infected patients is the occurrence of pleural effusion and ascites. The occurrence of these symptoms suggests an increase in the permeability of endothelial cells. Whether this effect is caused directly by infection or indirectly by inflammatory mediators is still not clear.

As a model to study vascular permeability Human Umbilical Vein Endothelial Cells (HUVECs) can be used. It has been shown that HUVECs are permissive for DENV infection and that infection results in the production of cytokines, chemokines and complement, suggesting that infection of endothelial cells may also contribute to the postulated cytokine storm [80].

The hypothesis is that increased vascular permeability during DENV infection occurs as a result of disturbance of the tight and adherens junctions, which join the cytoskeletons of adjacent cells. It has been shown that DENV infection in endothelial cells induced rearrangements in the actin cytoskeleton and a decreased expression of the adherens junction protein VE-cadherin and tight junction protein zonulin-1 [81,82].

Two studies with endothelial cell models showed that direct infection did not result in an increase in permeability of the cells [83,84]. However, it was shown that co-incubation of HUVECs with PBMCs [83]

or the supernatant of DENV infected THP-1 cells [84] did increase the permeability of endothelial cells. In both studies it was suggested that TNF-α release by these cells was responsible for the increase in permability. IL-8, IL-6 and IL-12p70 have also been shown to induce an increase in vascular permeability in vitro [43,84].

Other mediators than the classical cytokines have also been associated with the occurrence of increased endothelial cell permeability and the occurrence of plasma leakage. Srikiatkhachorn et al. found a direct correlation between VEGF and the pleural effusion index and an inverse correlation between sVEGFR-2 and this index in DENV infected patients [85]. VEGF can be bound by sVEGFR-2, which will inhibit its vasoactive properties and therefore low levels of this receptor are probably associated with plasma leakage. MCP-1 was also found to be associated with the occurrence of plasma leakage in patients [60]. It is produced by infected monocytes and can also induce endothelial-cell permeability changes in vitro [60]. Another study showed that DENV infected immature Dendritic Cells (DCs) could induce vascular permeability in HUVECs by secreting MMP-9 and to a lesser extent MMP-2 [86]. In DENV infected patients significantly increased levels of MMP-9 have been found, especially in case of severe disease [87]. However, no significant differences in MMP-2 levels between dengue patients and healthy controls were found. It was also shown that decreased angiopoietin-1 and increased angiopoietin-2 levels correlated with plasma leakage in dengue patients [88]. Angiopoietin-1 is stored in platelets and has a modulatory effect on the vascular barrier. In contrast, angiopoietin-2 is synthesized by endothelial cells and can cause vascular permeability.

Altogether, it may be concluded that vascular permeability during DENV infection is most probably caused due to the release of vasoactive mediators of infected cells.

Coagulation disturbances

Hemostasis is generally described in terms of primary and secondary hemostasis. Primary hemostasis is the formation of a hemostatic plug at a site of endothelial injury, often because platelets are exposed to proteins such as tissue factor. During secondary hemostasis the coagulation cascade is switched on, which eventually results in fibrin generation. During DENV infection both hemostatic systems are activated.

The prothrombin time (PT) is a marker of activation of primary hemostasis. A prolongation has only been reported in non-survivors or patients with very severe shock, suggesting that activation of the contact pathway (tissue factor (TF)) only occurs in severely DENV infected patients [89-91]. However, there is clinical and in vitro evidence that the contact pathway is activated. Increased TF expression was detected in monocytes of DENV infected patients in the acute phase of disease [92]. Moreover, TF expression was also shown to be upregulated by endothelial cells upon DENV infection [93]. The majority of patients with DENV infection suffer from thrombocytopenia, which compromises the primary hemostasis. In severe disease, the platelet count can reach very low levels. One hypothesis is that DENV infection induces bone marrow suppression. Already in 1964, bone marrow aspirations in DENV infected patients showed hypocellularity in the first 4 days after onset of fever [94]. Murgue et al. showed that DENV replication in Cord Blood Mononuclear Cells (CBMNCs) reduced the number of viable cells and inhibited the number of progenitors [95]. Moreover, incubation of CBMNCs with supernatant from DENV infected cells also resulted in inhibition, suggesting that other factors than direct viral replication cause inhibition of hematopoiesis during DENV infection [96].

Another hypothesis is that thrombocytopenia is caused by complement induced lysis of platelets through the binding of autoantibodies. In line with this hypothesis, dengue antigen-antibody complexes were detected on the surface of platelets [97]. Moreover, it was shown that platelets derived from DENV infected patients had a shorter survival time [98]. In another study, platelet associated IgM

and IgG antibodies were detected in a cohort with DENV infected patients [99].
An alternative hypothesis to be mentioned is that platelets are bound to activated endothelial cells. Funahara et al. already observed that incubation of platelets with DENV infected HUVECs decreased the amount of free platelets [100]. Most probably, platelet adherence to DENV infected endothelial cells is caused by the release of von Willebrand Factor (vWF) by activated endothelial cells. vWF multimers can bind to platelets and form a hemostatic plug at a site of endothelial cell injury. Clinical studies showed increased levels of vWF antigen in DENV infected patients [101-103].
Besides thrombocytopenia, impaired function of platelets may have deleterious effects on the host. An increased soluble P-selectin/platelet ratio in severely DENV infected patients indicated that these platelets were highly activated [104]. In these patients an inverse correlation between plasma leakage and platelet count was found. It was shown that anti-NS1 immunoglobulins could activate human platelets, which may eventually exhaust them and impair their function [105]. Another clinical study showed that platelets from DENV infected patients had a defect in their ADP-releasing ability [98]. Platelets play an important role in maintaining the vascular integrity of endothelial cells and therefore either thrombocytopenia as well as thrombocytopathy may result in increased vascular permeability and plasma leakage (reviewed in [106]).
The majority of studies show a prolongation of the activated partial thromboplastin time (APTT) in DENV infected patients suggesting that the intrinsic pathway (secondary hemostasis) is highly activated [89,90,103]. Activation of secondary hemostasis induces thrombin generation. Thrombin generation during severe dengue is shown by decreased levels of fibrinogen [90,91,98,107] and antithrombin [91] and increased levels of prothrombin fragments 1+2 (F1+2) and the thrombin-antithrombin complex (TAT) [90]. Moreover, decreased levels of the anticoagulant protein C and S in DENV infected patients most probably fail to counteract the process of thrombin generation [90,91].
Thrombin generation induces fibrinolysis. Increased fibrinolysis results in elevated levels of its final products, such as D-dimer and fibrin degradation products [90,107]. Increased levels of tissue plasminogen activator and decreased levels of thrombin-activatible fibrinolysis inhibitor also indicate that the fibrinolytic system is highly activated during DENV infection in humans [89,90,103,108]. In severe dengue, activation of the fibrinolytic system resulted in increased levels of its main inhibitor plasminogen activator inhibitor 1 (PAI-1) [90,91,103].
Altogether, it may be concluded that DENV infection is characterized by extensive activation of the primary and secondary hemostatic system, although primary hemostasis is compromised by thrombocytopenia.

Outline of the thesis

In this thesis mechanisms of immune activation that may contribute to pathogenesis, and clinical markers detecting immune activation and disease progression, were investigated. Clinical classification based on disease severity is fundamental for every observational study. In **chapter 2** the newly introduced 2009 WHO dengue case classification is compared with the previously used 1997 classification. The utility and applicability of both classifications for research and clinical practice are discussed. In many infectious diseases and inflammatory conditions the translocation of lipopolysaccharide (LPS) to the circulation has been described. We investigated to what extent this phenomenon also occurred in DENV infected patients. **Chapter 3** describes the association between microbial translocation and disease severity in Indonesian children with dengue virus infection. The findings in this study are confirmed in **chapter 4**, which describes microbial translocation in Brazilian adults with acute dengue virus infection. LPS is a very potent immune stimulator and in the Brazilian cohort levels of LPS could be linked to patients with a pro-inflammatory cytokine profile. Endothelial cells play a crucial role in the development of plasma leakage during DENV infection. Activation of these cells results in the release of several markers. We investigated nine endothelial markers and their relation to plasma leakage in DENV infected patients in Brazil. **Chapter 5** describes that two of these proteins can serve as surrogate markers for plasma leakage in patients with acute DENV infection. Another marker, generally expressed in patients with infectious diseases, is ferritin. In **chapter 6** we investigated ferritin expression in two cohorts of DENV infected patients from Aruba and Brazil. We found that ferritin can discriminate between DENV infection and other febrile illnesses. Moreover, increased levels of ferritin were associated with clinical severe disease, immune activation and coagulation disturbances, suggesting that ferritin is a marker for disease activity. The immune response towards DENV infection follows a specific pattern over time. In **chapter 7** we confirm other studies that time after the onset of disease is the main determinant of gene expression patterns in DENV infected patients. Moreover, using an unsupervised network analysis certain clinical markers were linked to gene clusters, suggesting that they could serve as markers for disease activity. In the summarizing discussion the importance of the previously described mechanisms contributing to immune activation, and clinical markers indicative for these processes, are addressed with suggested directions for future research.

2

Evaluation of the 2009 WHO Dengue Case Classification in an Indonesian Pediatric Cohort

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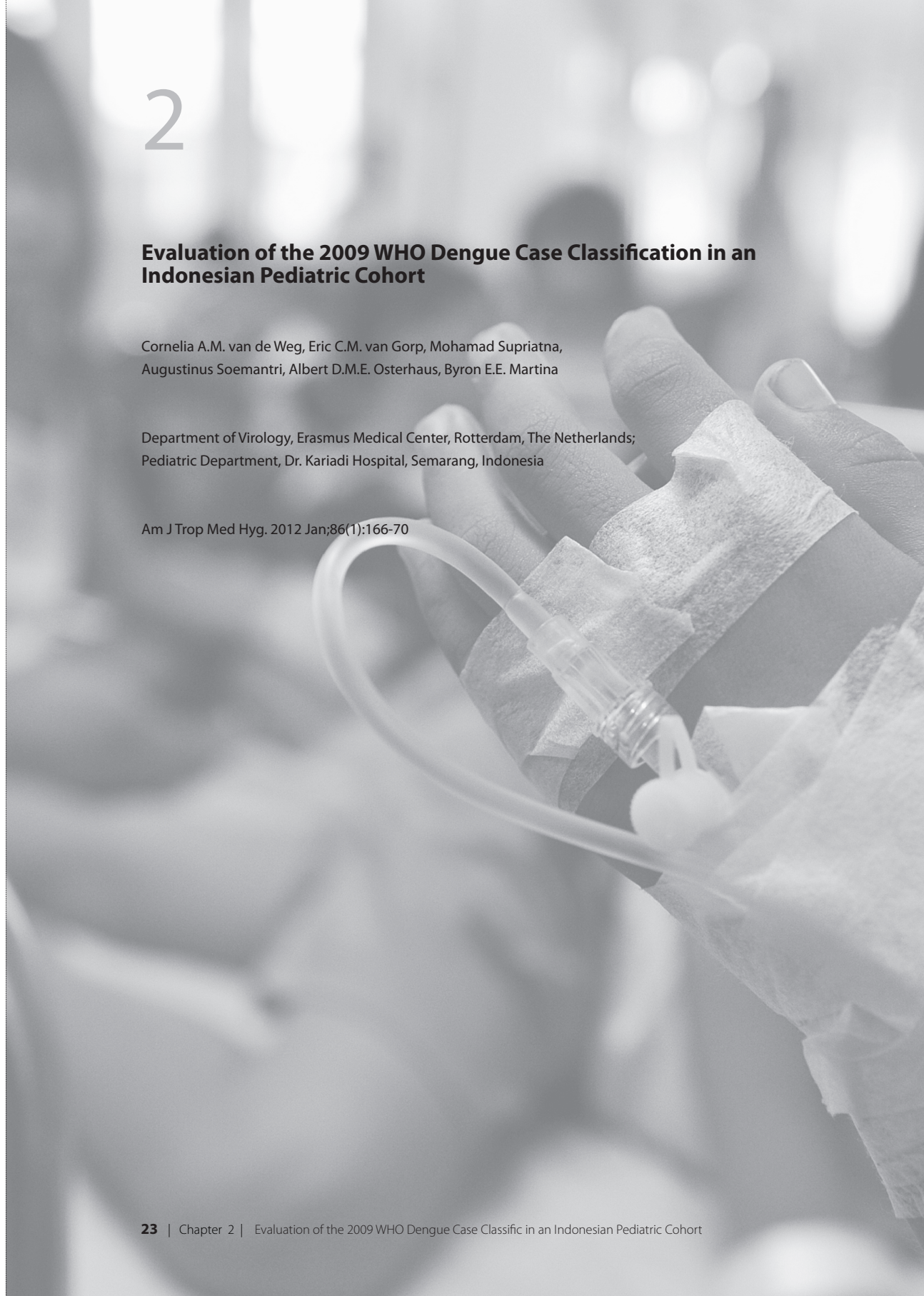

Abstract

The classification of dengue virus infected patients continues to be a challenge to researchers and clinicians in the field. The accuracy of the 1997 World Health Organization (WHO) dengue case definition has been debated for a decade, because the definition was very stringent, for instance, several researchers showed that apparently severe cases were misclassified as not severe. Therefore the WHO issued revised guidelines in 2009. Here we retrospectively compared the performance of the WHO case definition of 2009 with the WHO case definition of 1997 in a detailed documented pediatric cohort from Indonesia. Intensive treatment intervention was used as an indicator of severity of disease. In line with our expectations, the 2009 WHO case classification proved to be a lot more specific, albeit less sensitive than the WHO case classification of 1997. We conclude that the revised classification is promising both from research and clinical perspectives, but validation of the classification criteria still needs to be addressed.

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Introduction

To achieve universal consensus about the clinical case classification of dengue virus (DENV) infected patients the World Health Organization (WHO) released guidelines in 1974. Although these guidelines were updated several times the utility and accuracy of classifying patients according to disease severity criteria have continued to be a matter of debate. Therefore, reassessment of the classification criteria has been proposed by several study groups [6,7,10,15,16], prompting the WHO to issue a revised classification in 2009 [1].

In the original guidelines from 1997 patients are classified in three separate categories, i.e. dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [17]. DF was defined as an acute febrile illness with general complaints and a positive laboratory confirmation of DENV infection. The diagnosis DHF was restricted for patients with the collective presence of fever, hemorrhagic tendency, thrombocytopenia and signs of plasma leakage. DHF with signs of shock was classified as DSS.

The 2009 WHO guidelines distinguish between severe and non-severe dengue [1]. Severe dengue is defined by the occurrence of plasma leakage and/or fluid accumulation leading to shock or respiratory distress; and/or severe bleeding; and/or severe organ impairment. The non-severe dengue group is divided in patients with and without warning signs. Abdominal pain or tenderness, persistent vomiting, clinically manifest fluid accumulation, mucosal bleeding, lethargy and restlessness, hepatomegaly and an increase in hematocrit with a drop in platelet count are all listed as warning signs. Moreover, the clinical course of a DENV infection is divided in three phases, i.e. the febrile, the critical and the recovery phase. Patients in the febrile phase can already present with warning signs, which may precede the development of severe disease [12]. The critical phase usually starts around the time of defervescence and is characterized by progressive leukopenia together with a drop in the platelet count followed by plasma leakage and/or hemorrhage.

To evaluate the performance of the 2009 WHO case definition compared with the performance of the 1997 WHO case definition, we reassessed the clinical diagnosis of a cohort of DENV infected children in Indonesia according to these criteria. Moreover, the utility and accuracy of both classification systems were assessed using the treatment received during admission.

Methods

From February 2001 till April 2003 this study was conducted at the Dr. Kariadi Hospital in Semarang, Indonesia [16]. In this area of central Java dengue is endemic and all four serotypes are circulating. Children aged 2-14 years admitted to the pediatric ward or the pediatric intensive care unit with a clinical suspicion of DENV infection were included. Written informed consent was obtained from a parent or legal guardian before inclusion. The ethical committee of the Dr. Kariadi hospital had approved this study. Signs and symptoms, findings on physical examination and routine laboratory test data were obtained at admission and during the stay in the hospital with a standardized case report form. The platelet count was determined daily. Moreover, at admission the hematocrit was measured every

2 hours for the first 6 hours and then every 6 hours until stable. For diagnostic purposes a blood sample was obtained at the first and seventh day of admission. DENV infection was diagnosed by serotype-specific reverse-transcription polymerase chain reaction (RT-PCR) [109] carried out on samples obtained at the day of admission, and/or detection of DENV specific IgM serum antibodies (Focus technologies, Cypress, Calif., USA) in the acute phase sample and/or by detection of a four-fold increase in the titer of IgG antibodies (Focus technologies, Cypress, Calif., USA) in paired acute and convalescent sera. Patients with a positive laboratory diagnosis of DENV infection and a complete clinical data set were selected for further evaluation.

In terms of disease severity patients were retrospectively classified according to both the 1997 and 2009 WHO case classification. According to the 1997 WHO case definition the patients were classified in three groups [17]:

1. DF: Presence of two or more of the following symptoms: headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, and leukopenia.
2. DHF: all of the following symptoms should be present: fever, a hemorrhagic tendency (at least a positive tourniquet test); thrombocytopenia (≤ 100.000 cells/mm^3); evidence of plasma leakage (at least a rise in hematocrit of ≥ 20% compared with the baseline value of the patient) or other signs of plasma leakage (such as pleural effusion and/or ascites).
3. DSS: All four criteria of DHF should be met plus signs of shock such as hypotension for age (age < 5 years: < 80 mmHg , age ≥ 5 years: < 90 mmHg) and/or narrow pulse pressure (systolic minus diastolic blood pressure < 20 mmHg).

The following criteria were used to classify patients as having severe DENV infection according to the 2009 WHO case definition [1]:

1. Severe plasma leakage and/or fluid accumulation leading to shock and/or respiratory distress: Presence of one of the following signs of shock: hypotension for age, narrow pulse pressure or signs of respiratory distress: (dyspnoe (reported by physician), tachypnoe (respiratory rate of > 40/min), PaO_2:FiO_2 < 200 mmHg, and/or signs of respiratory acidosis in the astrup (PaO_2:FiO_2 and astrup data were only available if the patient was admitted to the pediatric intensive care unit)).
2. Significant bleeding: signs of internal hemorrhage like hemoptoe, hematemesis, melaena, hematuria.
3. Severe organ impairment: pulmonary edema, disseminated intravascular coagulation, encephalopathy, liver failure and/or renal failure.

Table 1 | Characteristics of the patients classified according to the 1997 and 2009 WHO dengue case definition at the day of admission

	1997 WHO classification			2009 WHO classification	
	DF (N=24)	DHF (N=83)	DSS (N=66)	Non-severe (N=69)	Severe (N=104)
General manifestations:					
Day of fever*	3 (2,3-4,0)	4 (3,0-4,0)	4 (3,0-5,0)	3 (3,0-4,0)	4 (3,0-5,0)
Male: female ratio	10 (41,7%) male	39 (47,0%) male	34 (51,5%) male	29 (42,0% male)	54 (51,9%) male
Age (years)*	7 (4,0-10,5)	7 (5,0-10,0)	6 (5,0-7,8)	7 (6,0-10,5)	6 (4,0-8,0)
Temperature (°C)*	37,8 (37,3-38,2)	37,7 (37,3-38,3)	37,8 (37,1-38,3)	37,9 (37,3-38,4)	37,5 (37,2-38,0)
Skin manifestations:					
Ecchymosis	0	3 (3,6%)	6 (9,1%)	1 (1,4%)	8 (7,7%)
Exanthem	1 (4,2%)	0	3 (4,5%)	0	4 (3,8%)
Purpura	1 (4,2%)	0	4 (6,1%)	1 (1,4%)	4 (3,8%)
Petechiae	7 (29,2%)	40 (48,2%)	39 (59,1%)	33 (47,8%)	53 (51,0%)
Hemorrhagic manifestations:					
Positive tourniquet test	14 (58,3%)	74 (89,2%)	30 (45,5%)	66 (95,7%)	52 (50,0%)
Gum bleeding #	0	1 (1,2%)	3 (4,5%)	0	4 (3,8%)
Epistaxis #	2 (8,3%)	13 (15,7%)	9 (13,6%)	12 (17,4%)	12 (11,5%)
Bleeding from venipuncture sites	1 (4,2%)	8 (9,6%)	18 (27,3%)	2 (2,9%)	25 (24,0%)
Hematemesis	0	6 (7,2%)	14 (21,2%)	0	20 (19,2%)
Melaena	0	5 (6,0%)	11 (16,7%)	0	16 (15,4%)
Hemoptoe	0	0	2 (3,0%)	0	2 (1,9%)
Hematuria	0	1 (1,2%)	1 (1,5%)	0	2 (1,9%)
Gastro-intestinal manifestations:					
Abdominal Pain #	18 (75,0%)	65 (78,3%)	62 (93,9%)	48 (69,6%)	97 (93,3%)
Nausea	18 (75,0%)	63 (75,9%)	55 (83,3%)	51 (73,9%)	85 (81,7%)
Diarrhea	0	2 (2,4%)	2 (3,0%)	1 (1,4%)	3 (2,9%)
Vomiting #	14 (58,3%)	46 (55,4%)	40 (60,6%)	38 (55,1%)	62 (59,6%)
Hepatomegaly #	15 (62,5%)	53 (63,9%)	62 (93,9%)	39 (56,5%)	91 (87,5%)
Neurological manifestations:					
Delirium #	0	2 (2,4%)	4 (6,1%)	0	6 (5,8%)
Irritability #	1 (4,2%)	1 (1,2%)	5 (7,6%)	0	7 (6,7%)
Decreased level of consciousness #	0	2 (2,4%)	7 (10,6%)	0	9 (8,7%)
Signs of plasma leakage:					
Pleural effusion #	8 (33,3%)	35 (42,2%)	54 (81,8%)	16 (23,2%)	81 (77,9%)
Ascites #	0	3 (3,6%)	6 (9,1%)	0	9 (8,7%)
Pulse rate*#	112 (101-129)	116 (100-120)	120 (117-137)	110 (100-120)	120 (115-132)
Systolic blood pressure (mmHg)*	98 (80-100)	100 (90-105)	80 (70-89)	100 (98-100)	80 (70-90)
Respiratory manifestations:					
Dyspnoe	1 (4,2%)	3 (3,6%)	12 (18,2%)	1 (1,4%)	15 (14,4%)
Respiratory rate*	28 (24-28)	28 (24-28)	30 (28-34)	28 (24-28)	30 (28-32)
Laboratory values:					
Hematocrit (%)* #	37,5 (33,2-40,1)	41,2 (37,1-44,7)	42,7 (39,7-47,4)	40,1 (35,1-43,0)	42,6 (37,4-46,7)
Platelet count (/mm^3)* #	115500 (55.500-142.750)	67000 (42.000-90.000)	42500 (29.000-65.750)	85000 (57.500-120.000)	49000 (31.000-69.000)
Leukocyte count (X 10^9/L)*	4,3 (3,0-5,8)	4,6 (3,2-6,6)	5,2 (3,9-7,1)	4,2 (3,2-5,9)	5,2 (3,8-7,9)

** values in median, interquartile range, # listed as warning sign in the 2009 classification, DF= dengue fever, DHF = dengue hemorrhagic fever, DSS = dengue shock syndrome*

Patients, who did not meet any of the criteria of a severe DENV infection according to the 2009 WHO case definition, were classified as non-severe dengue. We did not distinguish between non-severe dengue with and without warning signs, because the clinical data related to warning signs were not complete; we feared that using an incomplete dataset would result in a biased picture of disease severity in the non-severe dengue patients.

In addition, we determined the phase of infection in which patients were admitted according to the 2009 WHO criteria. The characteristics of the critical phase are defervescence (temperature below 38°C), progressive leukopenia, drop in platelet count, and plasma leakage. Patients were classified as being in the critical phase if their temperature was below 38°C or they had very severe thrombocytopenia (platelet count ≤ 50.000 /mm^3) [110]. If these two conditions were not fulfilled, patients were still classified as being admitted in the critical phase if at least two of three of the following conditions were present: rise in hematocrit (≥ 20% increase compared with the baseline value of the patient), leukopenia (2-6 years: < 5.000/ µl and > 6 years: < 4.500/µl), and/or thrombocytopenia (≤ 100.000 cells/mm^3).

To determine the utility and accuracy of the 1997 and 2009 classification systems in this setting, the treatment received during admission was assessed. A distinction was made between minor and intensive treatment intervention. The definition of intensive treatment intervention consisted of fluid replacement therapy distributed in a higher dose than the maintenance values as described in the 2009 WHO criteria (0-10 kg: > 4 ml/kg/hr, 10-20 kg: 40 + 2* (weight patient -10) ml/kg/hr, > 20 kg: 60 + 1*(weight patient -20) ml/kg/hr); coagulation support (platelet infusion, fresh-frozen plasma, and/or fresh plasma); and/or circulatory support (dopamine) [1].

Whether patients received an intensive treatment intervention during admission was set as the condition (gold standard) to calculate sensitivity and specificity for both case classifications. The sensitivity was determined by dividing the number of patients with intensive treatment intervention and severe dengue or DHF/DSS by the total number of patients with an intensive treatment intervention. In addition, the specificity was calculated by dividing the true negatives (non-severe dengue or DF without treatment intervention) by the total number of patients without treatment intervention.

Results

Patients (173) were selected from the cohort with a laboratory confirmed diagnosis of DENV infection.

According to the 2009 classification, 69 patients (39,9%) suffered from non-severe and 104 patients (60,1%) suffered from severe DENV infection, whereas the 1997 WHO guidelines classified 24 patients (13,9%) as DF and 149 patients (86,1%) as DHF/DSS (Table 1). In the group diagnosed with severe DENV infection, 64 patients showed severe plasma leakage, 6 patients suffered from severe bleeding, 18 patients showed plasma leakage and bleeding, and 16 patients had signs of severe organ impairment. Table 1 describes the baseline characteristics of the patients at the day of admission in both classification systems. Many of the signs and symptoms listed are considered warning signs in the revised classification. Interestingly, the distribution of the signs and symptoms of the DF and non-severe group and the DSS and severe group are quite similar, whereas the DHF group seems to have a mixture of severe and non-severe patients.

Signs of shock, respiratory distress, internal hemorrhage, and organ impairment were used to classify

Table 2 | The occurrence of signs and symptoms of severe dengue in the 1997 WHO dengue case definition

	1997 WHO classification		
	DF (N=24)	DHF (N=83)	DSS (N=66)
Signs of shock (Total)	**8**	**22**	**58**
Hypotension for age	7	0	48
Narrow pulse pressure	3	0	10
Compensated shock	1	22	0
Respiratory distress (Total)	**0**	**3**	**22**
Dyspnoe	1	3	19
Tachypnoe	0	2	14
$PaO_2 : FiO_2$ < 200 mmHg	1	2	9
Hemorrhage (Total)	**0**	**10**	**28**
Melaena	0	7	22
Hematemesis	0	6	21
Hematuria	0	1	4
Hemoptoe	0	0	6
Organ impairment (Total)	**1**	**3**	**12**
Dissemenated intravascular coagulation	1	1	9
Liver failure	0	0	0
Renal failure	0	0	0
Encephalopathy	0	2	4

DF= dengue fever, DHF = dengue hemorrhagic fever, DSS = dengue shock syndrome

patients as severe dengue according to the 2009 WHO case definition. Table 2 shows how often these severe symptoms appear in the three different patient groups of the 1997 WHO guidelines. Eight patients in the DF group present themselves with signs of shock. These patients failed to meet all four criteria of the 1997 WHO criteria, like thrombocytopenia or hemorrhagic tendency, and were therefore not classified as DHF/DSS.

The majority of the patients in this cohort were admitted during the critical phase of their DENV infection (Table 3). Patients with non-severe dengue or DF were more likely to get admitted during the febrile phase. Patients admitted in the critical phase had a lower temperature and platelet count and an increased hematocrit and leukocyte count compared with patients admitted in the febrile phase. Because the number of people admitted in the febrile phase of their disease was low (N=22), we could not investigate the predictive value of warning signs with which patients may present in the febrile phase.

As a measure of disease severity, we also scored whether intensive treatment intervention was initiated during admission (Table 4). Of the patients in the non-severe dengue group according to the 2009 guidelines, 38 patients (55,1 %) had received intensive treatment intervention compared with 13 patients (54,2%) classified as DF. Of the severe dengue group, 91 patients (87,5%) had received intensive treatment intervention, a slightly higher number than the 116 patients (77,9%) in the DHF/DSS group. It should be noted that, in the revised classification of 2009, all patients with plasma leakage combined with bleeding or organ impairment received an intensive treatment intervention. These results in-

Table 3 | Characteristics of patients admitted in the febrile and critical phase

		1997 WHO classification			2009 WHO classification			
Disease severity		*DF (N=24)*	*DHF (N=83)*	*DSS (N=66)*	*Non-severe (N=69)*	*Plasma leakage or bleeding (N=70)*	*Plasma leakage and bleeding (N=18)*	*Organ impairment (N=16)*
Phase at admission	Febrile (N=21)	7	12	2	17	1	0	3
	Critical (N=152)	17	71	64	52	69	18	13
Phase at admission		Febrile (N=21)			Critical (N=152)			
Temperature (°C) *		38,5 (38,3-38,8)			37,5 (37,2-38,0)			
Hematocrit (%)*		37,3 (34,1-42,0)			42,1 (37,5-46,0)			
Platelet count (/mm^3)*		120.000 (95.000-137.500)			56.500 (34.000-78.000)			
Leukocyte count (X 10^9 /L)*		4,5 (3,5-5,1)			4,8 (3,4-7,0)			

** values in median, interquartile range, DF= dengue fever, DHF = dengue hemorrhagic fever, DSS = dengue shock syndrome*

dicate that the 2009 classification system is more specific than the 1997 classification system, with specificities of 70,5 % and 25,0 %, respectively. Not unexpectedly, the 2009 guidelines proved to be less sensitive than the 1997 guidelines, with sensitivities of 70,5 % and 89,9%, respectively.

Discussion

In the present study, we compared the utility and accuracy of the 1997 and 2009 WHO clinical case classifications for dengue in a cohort of Indonesian children. Taking intensive treatment intervention as an indicator of severe disease, we conclude that the latter classification is more specific, albeit at the cost of a lower sensitivity.

A major concern about the 1997 WHO case definition was that the criteria were too stringent, and therefore, patients with severe disease manifestations were misclassified as DF cases [6,10,14,110]. This problem also becomes evident in our study in which eight patients diagnosed as DF according to the 1997 classification do present themselves with signs of shock. With the revised classification, these patients are apparently accurately diagnosed as having severe dengue. However, a concern about the 2009 classification is that loosening the case definition may result in more hospital admissions, because more patients will be classified as severe dengue cases. Nevertheless, because misclassification of patients with a life-threatening condition is less acceptable, revision of the 1997 WHO case definition was indeed warranted.

Another problem of the 1997 WHO case classification is that the platelet count and the hematocrit levels play a pivotal role in establishing the diagnosis. In this study, all patients included were monitored carefully and therefore no data were lacking. However, in daily clinical practice, it may be too compli-

Table 4 | Intensive treatment intervention received by patients classified according to the 2009 and 1997 WHO case definition

	Treatment intervention			
1997 WHO classification:	FRT > baseline level	Coagulation support	Circulation support	**Intensive treatment intervention**
DF (N=24)	11	5	1	**13**
DHF (N=83)	51	19	3	**56**
DSS (N=66)	53	35	17	**60**
2009 WHO classification:				
Non-severe (N=69)	38	6	0	**38**
Plasma leakage (N=64)	43	25	4	**53**
Bleeding (N=6)	4	2	0	**4**
Plasma leakage + bleeding (N=18)	15	12	6	**18**
Organ impairment (N=16)	15	14	11	**16**

FRT = fluid replacement therapy, DF= dengue fever, DHF = dengue hemorrhagic fever, DSS = dengue shock syndrome

cated and expensive to monitor every patient this closely. Therefore, an advantage of the 2009 classification over the 1997 classification is that extensive laboratory evaluation is not needed to reach a conclusion about the condition of the patient.
Recently, a large prospective and retrospective multicenter study to investigate the usefulness and applicability of the 2009 case classification in clinical practice and surveillance was carried out under the auspices of the WHO [111]. Comparison of the outcomes of the 1997 and 2009 classification results in data that are similar to our study. However, in our cohort more DF cases were eventually classified as suffering from severe dengue. Moreover, the WHO study reports that it is difficult to obtain information about the occurrence of warning signs from a retrospective analysis. We encountered a similar problem in our study in which data had been collected when the 1997 classification was still commonly used.

Srikiathachorn et others also tested the 1997 WHO classification with treatment intervention as an indicator of severe disease [7]. They found a sensitivity of 62% and a specificity of 92% of the WHO case definition for DHF. In our cohort, we found a higher sensitivity and a much lower specificity using the 1997 classification. The most important difference between our study and the study by Srikiathachorn and others is that they had included a large group of patients with other febrile illnesses in their analysis. This addition increased the specificity in their study, because the signs and symptoms used in the 1997 WHO dengue case classification are quite specific for dengue compared with other febrile diseases. In contrast, our study is more focused on the distinction between severe and non-severe disease in a population that has already been diagnosed with DENV infection.

An important advantage of the 2009 classification is that DENV infection is clearly described as a triphasic disease. This is an important clinical indicator and may also be an important fact for pathogenesis studies, because it will most probably make the comparison of patient groups more accurate.

Until this time, patients were usually classified on the day of admission or day of fever, which according to the revised classification, does not necessarily mean that patients are in the same phase of disease. Moreover, for clinicians, it is also important to realize that patients admitted in the febrile phase are at risk to develop severe disease and should be monitored carefully.

A major drawback of the 2009 classification compared with the 1997 classification is that the criteria are less strictly defined, leaving room for arbitrary interpretation by the clinician or researcher. For example, in the 2009 case definition, the occurrence of severe bleeding has to be evaluated by the physician. Physicians may have different opinions about what kind of bleeding is severe, and therefore, this criterion may complicate the comparison of research results from different study settings. Moreover, because it is hard to obtain information about the occurrence of warning signs retrospectively, the comparison between research before and after the revised classification will be a challenge.

Taken together, we conclude that, both in clinical and research settings, the performance of the 2009 WHO case classification proves to be an improvement over the performance of the 1997 WHO case classification, although more validated and detailed classification criteria need to be defined.

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3

Lipopolysaccharide Levels are Elevated in Dengue Virus Infected Patients and Correlate with Disease Severity

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Abstract

Background

Although in the majority of cases dengue virus (DENV) infection results in a self-limiting febrile disease, it can cause severe plasma leakage in a minority of patients. The appearance of plasma leakage indicates an increased permeability of the vascular wall. In this study we investigated if DENV infection can lead to leakage of lipopolysaccharide (LPS) from the intestine into the blood of the patient, indicative of an increased permeability of the intestinal mucosal barrier.

Objectives

The aim of this study was to investigate if LPS levels were elevated in DENV infected patients and if these levels correlated with disease severity.

Study design

LPS levels in the blood of DENV infected children were determined using the Limulus Amebocyte Lysate assay. To determine disease severity we used the 1997-WHO criteria, the expert physician's judgement and a score that focused on plasma leakage in particular. Furthermore, the modulatory factors LPS binding protein (LBP) and sCD14, as well as the immune activation marker neopterin were determined.

Results

We showed significantly elevated LPS levels in plasma of DENV infected children compared to healthy controls. The plasma leakage severity score had the strongest correlation with levels of LPS. Levels of LBP, sCD14 and neopterin were elevated compared to healthy controls.

Conclusion

In this study we show evidence of elevated LPS levels during DENV infection. Moreover, a correlation between LPS levels and disease severity was found, especially when disease severity was determined in terms of plasma leakage.

Key words

Dengue virus, microbial translocation LPS, LBP, sCD14, neopterin

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Background

Dengue Virus (DENV) belongs to the family Flaviviridae and consists of four serotypes (DENV 1-4). Annually an estimated number of 50 – 100 million infections occur in tropical and subtropical countries around the world. DENV infection usually results in a subclinical or self-limiting febrile disease, but may also lead to severe disease, previously known as Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS). Severe disease is characterised by thrombocytopenia, haemorrhagic manifestations, liver disturbances and a sudden onset of vascular permeability, believed to be caused by a cytokine storm (reviewed in [29]).

The primary target cells of DENV are monocytes and tissue macrophages. In humans the gut-associated lymphoid tissue (GALT) contains the largest pool of macrophages and memory T cells. Another virus that targets monocytes is human immunodeficiency virus (HIV). Recently, it was shown that replication of HIV in the GALT was associated with elevated LPS levels, also known as microbial translocation (MT) [112].

MT and its effects have extensively been studied in diseases where the intestinal mucosal barrier is severely damaged or inflamed, e.g. in graft versus host disease after hematopoietic transplantation [113] and inflammatory bowel disease [114]. In these diseases it has been found that MT contributes significantly to the inflammatory response. Moreover, it has been shown that in Gram-negative sepsis, a disease with generalized vascular permeability caused by an exaggerated immune response, MT occurs and correlates with disease severity and clinical outcome [115].

Like sepsis, the hallmarks of severe DENV infection are plasma leakage and a highly activated immune system [29]. Moreover, like HIV, DENV predominantly replicates in mononuclear cells of which many reside in the GALT. Taken together, we hypothesize that DENV infection may cause MT and that LPS levels may correlate with disease severity.

Objectives

The objective of this study was to investigate whether elevated LPS levels can be found in DENV infected patients and whether these levels correlate with disease severity.

Study design

Clinical cohort

Following written parental consent, serial blood samples from 200 children aged 3 – 14 years with a clinical suspicion on DENV infection were collected at the Dr. Kariadi Hospital in Semarang, Indonesia in the period of February 2001 till March 2003 [16]. This study was approved by the ethics committee of the Dr. Kariadi Hospital.

DENV infection was confirmed by serotype-specific reverse transcription-PCR (RT-PCR) from the samples obtained at day of admission [109] or by detection of DENV specific IgM antibodies (Focus tech-

Classification	WHO classification			MD classification		
	DF N = 18	DHF N = 45	DSS N = 26	DF N = 10	DHF N = 35	DSS N = 44
Sex	50.0 % male	51.1 % male	46.2 % male	50.0 % male	48.6 % male	50.0 % male
Age (years)	7.5 (4 – 11)	8 (5 – 10)	7 (5 – 10)	8 (6.25 - 11.5)	8 (6 – 10)	7 (5 – 10)
Day of fever	3 (2 - 3.25)	3 (3 – 4)	4 (3.75 – 5)	3 (2 – 4)	4 (3 – 4)	4 (3 – 4)
Haemoglobin (g/dl)	11.8 (10.9 - 13.1)	13.2 (12.0 - 14.3)	14.1 (12.2 - 14.9)	11.9 (11.4 - 13.1)	12.9 (12.0 - 13.9)	13.8 (11.9 - 15.1)
Haematocrit (%)	35.7 (23.1 - 36.7)	40.9 (36.9 - 43.6)	43.0 (40.4 - 47.9)	36.7 (33.3 - 40.0)	40.7 (36.8 - 43.0)	42.5 (37.2 - 47.0)
Tachycardia (pulse >120/min)	22.2% N = 4	31.1 % N = 14	65.4 % N = 17	10.0 % N = 1	28.6 % N = 10	54.5 % N = 24
Hypotension (systolic blood pressure < 80 mmHg)	22.2% N = 4	0 % N = 0	46.2 % N = 12	10.0 % N = 1	0 % N = 0	34.1 % N = 15
Narrow pulse pressure (diff < 20 mmHg)	16.7 % N = 3	0 % N = 0	23.1 % N = 6	0 % N = 0	0 % N = 0	20.5 % N = 9
Pleural effusion (X-ray)	38.9 % N = 7	40.0 % N = 18	80.8 % N = 21	0 % N = 0	28.6 % N = 10	81.8 % N = 36
Ascites (physical examination)	0 % N = 0	2.2 % N = 1	3.8 % N = 1	0 % N = 0	0 % N = 0	4.5 % N = 2
Plasma leakage severity score	1 (0 – 2)	1 (0 – 1)	2 (2 - 3.25)	0 (0 - 0.25)	1 (0 – 1)	2 (2 – 3)

Table 1 | Patient characteristics of the Indonesian cohort classified according to the 1997 WHO criteria and the physician's judgement (MD diagnosis). Age, day of fever, Hb, Ht and plasma leakage severity score are expressed as median (interquartile range).

nologies) in acute and/or convalescent samples. Thirty-seven children from the same area who had the same genetic background but a negative serology for DENV infection served as healthy controls. Patients were classified using three classification systems. The first two were the stringent 1997-WHO-criteria [17] and the expert physician's judgement of disease severity (MD classification). The MD classification is the diagnosis given by the treating physician and recorded in the patient's chart. The possible diagnoses are: DF, DHF and DSS –intuitively based on physical examination, clinical course and laboratory data.

The third classification system was based on a plasma leakage severity (PLS) score that takes the following signs into account : Ht > 45.0%, tachycardia (pulse > 120/min), hypotension (systolic blood pressure < 80 mmHg), narrow pulse pressure (difference between systolic and diastolic blood pressure < 20 mmHg), pleural effusion (proven by X-ray), and signs of ascites (shown by physical examination). Each sign present is assigned a value 1; the PLS score was defined as the sum score, ranging from 0 to 6. Haemoglobin and haematocrit levels, as well as any signs of plasma leakage were recorded daily.

Laboratory Determinations

Samples have been stored at -80°C and repetitive freeze-thaw cycles have been avoided. LPS was determined with a commercially available Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod Incorporated). Because components in plasma may interfere with this assay [116], plasma samples were diluted 1:5 with LAL reagent water and heat-inactivated at 60°C for 30 minutes. Known concentrations of Escherichia Coli LPS were diluted in heat-inactivated negative plasma (1:5 dilution) and served as standard values. The standard and plasma samples were incubated with the Pyrochrome for 70 minutes at 37°C. After the reaction was stopped the test was read at an absorbence of 405 nm.

Soluble CD14 (sCD14; 'Quantikine' ELISA, R&D Systems), LPS binding protein (LBP; 'HK315' ELISA, Hycult Biotech), and neopterin (IBL ImmunoBiological Laboratories) were determined using commercially available assays. To this end, samples were diluted 1:200 (sCD14), 1:1000 (LBP), and used undiluted (neopterin). The assays were performed according to the manufacturer's instructions.

Statistical analysis

The Kruskal Wallis H test was used for comparison of more than two groups. Statistical significance between individual groups was determined with the Mann Whitney U test. Correlations between continuous variables were determined with the Spearman correlation coefficient test. P values ≤ 0.05 were considered significant.

Results

Eighty-nine plasma samples from Indonesian children with laboratory confirmed DENV infection were selected for this analysis. Clinical characteristics of the cohort are summarized in table 1. Based on the MD classification, more patients had severe disease (DHF/DSS) than those diagnosed with the 1997 WHO classification system (Table 1). The application of the PLS score to both classifications supported the MD classification of disease severity. The median PLS score of patients classified with DF, DHF and DSS according to the WHO system was 1, 1, and 2 respectively. In contrast, the median PLS score in the MD classification for DF, DHF, and DSS was 0, 1 and 2 respectively. It has been reported previously [7] that due to the stringency of the 1997 WHO classification patients with severe disease according to the expert physician's judgement may be classified as having non-severe disease.

To investigate whether MT occurred during DENV infections, LPS levels were measured. Significantly

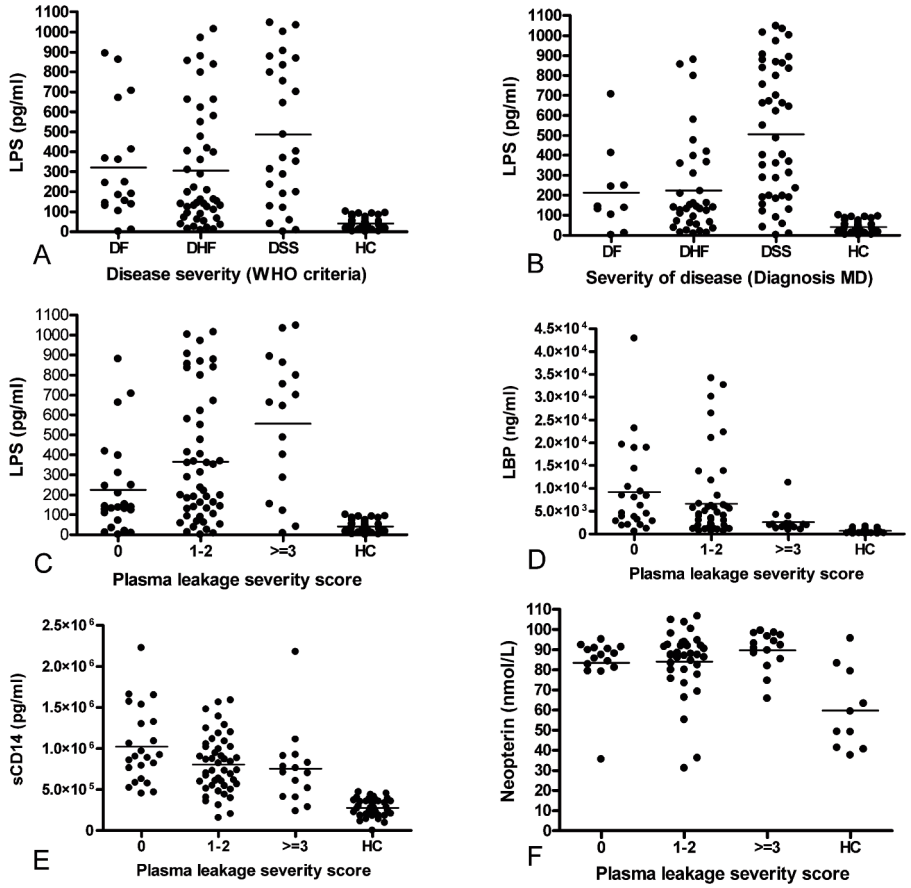

Figure 1 | *LPS, LBP, sCD14 and neopterin in relation to severity of disease. A: correlation between LPS levels and severity of disease based on 1997 WHO criteria. Significant differences were found for DHF vs DSS (P=0,04); DF, DHF and DSS vs healthy controls (HC) (all P < 0,0001). B: Correlation between LPS levels and severity of disease based on MD classification. Significant differences for DF vs DSS (P=0,01); DHF vs DSS (P=0,0001); DF vs HC (P=0,003); DHF and DSS vs HC (P < 0,0001). C: Correlation between LPS levels and severity of disease based on Plasma Leakage Severity (PLS) score. Significant differences for 0 vs ≥ 3 (P=0,006); 0, 1-2 and ≥ 3 vs HC (P < 0,0001). D: Correlation between LBP levels and severity of disease based on PLS score. Significant differences for 0 vs ≥ 3 (P=0,002); 0, 1-2 and ≥ 3 vs HC (P < 0,0001). E: Correlation between sCD14 levels and severity of disease based on PLS score. Significant differences for 0 vs ≥ 3 (P=0,046); 0, 1-2 and ≥3 vs HC (P < 0,0001). F: Correlation between neopterin and severity of disease based on PLS score. Significant differences for 0 vs HC (P=0,001); 1-2 vs HC (P=0,003); ≥ 3 vs HC (P=0,001). Horizontal lines indicate the median of the groups. Underlined * means significant difference between two groups. */* means that this group is significantly different from all other groups.*

elevated LPS levels were detected in the plasma of all DENV infected patients compared to healthy controls (Figure 1A-C). LPS levels were subsequently analyzed in relation to disease severity. Using the 1997 WHO classification only a significant difference was found between DHF and DSS patients (P = 0,04) (Figure 1A). However, when patients were divided according to the MD classification, LPS levels in patients with DSS differed significantly from those with DHF (P < 0,001) and DF (P=0,01).
When plasma LPS levels were compared in patients classified according to the PLS score, a significant

difference was observed between the group with no signs and the group with three and more signs (P=0,006) (Figure 1C). A trend was shown in which a higher PLS score correlated with increased LPS levels.
As LBP and sCD14 play an important role in the modulation of the LPS response we measured their levels in relation to the PLS score. As shown in Figure 1D, the LBP levels in the group with three and more symptoms were significantly lower than the levels in the group with no symptoms (P=0,002). The levels of the healthy controls were significantly lower than in all other groups (P<0,0001). Similarly, the levels of sCD14 in the group with three and more symptoms were slightly lower than the group with no symptoms (P=0,046) (Figure 1E). The levels in all groups were elevated compared to the healthy controls (P<0,001).
To investigate whether MT was associated with activation of monocytes/macrophages, the levels of neopterin were determined. The elevated neopterin levels indicated that monocytes/macrophages were significantly activated in DENV infected patients compared to healthy controls (P ≤ 0,01). Consistently, a weak correlation between levels of LPS and levels of neopterin was observed (P=0,023, r=0,29) (data not shown). Moreover, there was a slightly stronger correlation between LBP and sCD14 levels (P=0,0002, r=0,40) (data not shown).

Discussion

In this study we found elevated levels of LPS in DENV infected patients compared to healthy controls. Moreover, the levels of LPS showed to have a correlation with disease severity, indicated by an increased PLS score.
Classifying DENV infected patient in terms of disease severity has always been a challenge to physicians and researchers in the field. The application of our PLS score to the 1997 WHO classification and the MD classification suggests that the MD classification reflects disease severity more accurately.
Plasma leakage is a hallmark of severe DENV infection and may be indicative of increased permeability of the vascular wall. Therefore we hypothesized that a similar mechanism causing plasma leakage may be in part responsible for MT. In agreement with this, the PLS score showed the best correlation with LPS levels compared with the 1997 WHO and MD-classification. However, the PLS score only accounts for plasma leakage and shock, while haemorrhagic, gastrointestinal and liver manifestations are not taken into account. Nevertheless, LPS levels did not correlate with the occurrence of haemorrhage and the levels of liver enzymes (data not shown). From the gastrointestinal manifestations diarrhea is most likely to be associated with MT. However, only four patients were reported to suffer from diarrhea in our study population.
Taken together, our data suggest that in order to investigate certain specific pathogenetic pathways in humans it might be useful to classify patients according to specific clinical symptoms and laboratory markers.
Because of the interference with plasma components the LAL assay is known to be very challenging and therefore several strategies have been described [116]. Interestingly, the absolute values of LPS in our study were higher compared to those reported in other studies [112,114,115]. The difference between this study and others is that the standard was diluted in a plasma dilution comparable with the dilution of the patient samples. Because of the inhibitory activity of plasma the standard should contain the same amount of plasma as the diluted samples.
LPS is an important immune stimulator, because it can activate the innate immune system by signalling through a membrane-bound CD14 (mCD14)/Toll-like receptor 4 complex. The systemic response upon the release of LPS in the circulation is modulated by LBP and sCD14. LBP is synthesized by the liver upon stimulation with LPS and can accelerate the transfer of LPS to membrane-bound CD14. On

the other hand LBP can also shuttle LPS to lipoproteins [117]. The LBP concentrations in patients with a PLS score ≥ 3 were very low, suggesting that severely ill patients fail to produce enough LBP to neutralize LPS. These results are comparable with those from studies about MT and septic shock, in which non-survivors had lower LBP values than survivors [115].

sCD14 is produced by monocytes upon activation by LPS and has been suggested as a marker of disease severity [118]. In our study population the levels of sCD14 were significantly elevated in all dengue groups compared to healthy controls. Moreover, the group with the highest PLS score also showed decreased levels of sCD14, but to a lesser extent than the LBP levels. This was also found in patients with sepsis [119]. A dual mechanism may be involved here. On the one hand, high concentrations of sCD14 prevent an exaggerated immune response by competing with LPS for mCD14. On the other hand, low amounts of sCD14-LPS complexes can activate endothelial cells [120].

In HIV infected individuals MT has also been found and shown to correlate with the activity of disease [112]. During active HIV disease infection and depletion of CD4+ T cells contributes significantly to disease pathogenesis. A substantial part of these CD4+ T cells reside in the GALT and therefore the GALT is probably highly affected in these individuals, which eventually can result in impaired mucosal integrity [121]. It is not clear whether DENV replicates in cells of the GALT. However, it is likely because intestinal macrophages represent the largest pool of tissue macrophages in humans. We hypothesize that DENV replication in intestinal macrophages may lead to a pro-inflammatory environment in which the epithelial cells are disrupted. In agreement with other studies [122,123] we found increased levels of neopterin in DENV infected patients indicative of monocyte activation.

Taken together, this is the first study suggesting that DENV infection may be associated with MT, as measured by plasma levels of LPS. Furthermore, levels of LPS seemed to correlate with disease severity. Further studies are needed to elucidate the implications of this finding.

Conflict of interest

None of the authors declare conflict of interest apart from Albert Osterhaus who is a part time employee of Viroclinics BV (for details go to www.erasmusmc.nl). The stated competing interest does not alter the author's adherence to the policies on sharing data and materials.

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Ethical approval

This study was approved by the ethics committee of the Dr. Kariadi Hospital.

4

Microbial Translocation is Associated with Extensive Immune Activation in Dengue Virus Infected Patients with Severe Disease

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Abstract

Background

Severe dengue virus (DENV) disease is associated with extensive immune activation, characterized by a cytokine storm. Previously, elevated lipopolysaccharide (LPS) levels in dengue were found to correlate with clinical disease severity. In the present cross-sectional study, we identified markers of microbial translocation and immune activation, which are associated with severe manifestations of DENV infection.

Methods

Serum samples from DENV infected patients were collected during the outbreak in 2010 in the State of São Paulo, Brazil. Levels of LPS, lipopolysaccharide binding protein (LBP), soluble CD14 (sCD14) and IgM and IgG endotoxin core antibodies were determined by ELISA. Thirty cytokines were quantified using a multiplex luminex system. Patients were classified according to the 2009 WHO classification and the occurrence of plasma leakage/shock and hemorrhage. Moreover, a (non-supervised) cluster analysis based on the expression of the quantified cytokines was applied to identify groups of patients with similar cytokine profiles. Markers of microbial translocation were linked to groups with similar clinical disease severity and clusters with similar cytokine profiles.

Results

Cluster analysis indicated that LPS levels were significantly increased in patients with a profound pro-inflammatory cytokine profile. LBP and sCD14 showed significantly increased levels in patients with severe disease in the clinical classification and in patients with severe inflammation in the cluster analysis. With both the clinical classification and the cluster analysis, levels of IL-6, IL-8, sIL-2R, MCP-1, RANTES, HGF, G-CSF and EGF were associated with severe disease.

Conclusions

The present study provides evidence that both microbial translocation and extensive immune activation occur during severe DENV infection and may play an important role in the pathogenesis.

Keywords

dengue virus, microbial translocation, cytokine storm, immune activation, LPS

Microbial Translocation is Associated with Extensive Immune Activation in Dengue Virus Infected Patients with Severe Disease

Author summary

The pathogenesis of severe dengue virus (DENV) infection is still not fully understood. It is hypothesized that it is caused by a cytokine storm as is described in severe sepsis. In the sepsis field, the potent immunostimulator lipopolysaccharide (LPS) is proposed to play an important role in the development of a cytokine storm. In a previous study we have found elevated levels of LPS in children with severe DENV infection. In this study we have investigated if we could confirm that microbial translocation occurs in DENV infected patients. Moreover, we have determined the levels of thirty cytokines to get more insight in the cytokine storm during DENV infections and we have investigated whether microbial translocation is associated with immune activation. The patients in this cohort were classified according to their clinical presentation. Furthermore, a cluster analysis based on the expression of the determined cytokines was applied to identify patients with similar cytokine profiles. With these two techniques we identified cytokines that may contribute significantly to the cytokine storm and we could relate elevated levels of LPS to patients with a pro-inflammatory cytokine profile.

Introduction

Dengue virus (DENV) infection has been emerging in the American and Caribbean region in the past decade. During a DENV-2 outbreak in 2010 in the State of São Paulo, Brazil, more than 34.000 cases and 64 deaths were reported by the Health Department [124]. Symptoms of severe DENV infection range from shock and respiratory distress to major hemorrhagic manifestations and organ failure. The majority of these symptoms are manifest around the time of defervescence. In the early febrile phase, DENV infection is characterized by a high viral load and extensive activation of the Th1 response [44]. Around the time of defervescence, virus titres often decrease below the limit of detection. This critical phase is characterized by extensive immune activation and a so-called cytokine storm (reviewed in [29]) that is characterised by high levels of cytokines with mostly pro-inflammatory properties. The mechanism underlying this cytokine storm is still a matter of debate. Evidence points towards antibody-dependent enhancement in which cross-reactive non-neutralizing antibodies enhance the uptake of virus by monocytic cells (reviewed in [125]). Moreover, it has been proposed that 'original antigenic sin' is important in the pathogenesis of dengue (reviewed in [46]), postulating that low-avidity T cells are activated by the virus, but fail to clear it.

During HIV infection, elevated levels of lipopolysaccharide (LPS) were detected in the circulation and correlated with immune activation [112]. There is evidence that a local pro-inflammatory environment in the gut causes disruption of the intestinal barrier, which may eventually result in microbial translocation (MT) (reviewed in [126]). We recently showed that elevated LPS levels are present during DENV infection and correlate with disease severity [127]. DENV replicates in monocytes/macrophages, of which many reside in the gut-associated lymphoid tissue (GALT). Therefore, we hypothesized that this may cause a local pro-inflammatory environment in the bowel, eventually affecting the integrity of the intestinal barrier and resulting in MT. Consequently, because LPS is known to be a potent immune stimulator, elevated LPS levels may contribute to the cytokine storm during severe DENV infection.

In the present study, we studied markers of MT and immune activation in DENV infected patients. Using clinical classification and a cluster analysis we have identified cytokines that correlate with disease severity. Moreover, significantly increased LPS levels were found in a cluster of patients with a pronounced pro-inflammatory cytokine profile.

Materials and methods

Ethics Statement

All procedures adopted in this study were performed according to the terms agreed by the Institutional Review Board from the Hospital das Clínicas, University of São Paulo (CAPPesq - Research Projects Ethics Committee). This study was approved by CAPPesq under protocol 0652/09. Written informed consent was obtained from all study volunteers. All included study participants were anonymized with a study number.

Clinical cohort

This cohort has been described previously [124]. Briefly, during the 2010 outbreak samples were collected from patients with clinical suspected dengue fever presenting at the emergency department, department of internal medicine or the intensive care unit at the Ana Costa Hospital, Santos, State of São Paulo. Patients were diagnosed with DENV infection by detection of DENV NS1 antigen and/or IgM-specific antibodies using a commercially available rapid test (Dengue duo test bioeasy, Standard Diagnostic Inc. 575-34, Korea) or by detection of DENV RNA by real time PCR (RT-PCR). Details concerning the day of onset of fever (day of fever), clinical signs and symptoms and the final diagnosis were recorded by the treating physician. Serum samples were withdrawn and stored at -80°C. Patients were classified according to the 2009 WHO classification [1,128] and the occurrence of hemorrhagic manifestations and the occurrence of plasma leakage and shock. Hemorrhagic manifestations were observed by the treating physician. The occurrence of plasma leakage was detected by ultrasound or X-ray examination. The diagnosis shock was made by the treating physician based on symptoms such as hypotension, narrow pulse pressure, tachycardia and cold extremities. Age-matched healthy volunteers with a similar socio-economic background were used as controls.

IgG avidity ELISA and viral load

The IgG avidity test was used to determine primary or secondary DENV infection [24]. Samples with low avidity IgG antibodies were classified as primary DENV infection, whereas samples with high avidity IgG antibodies were classified as secondary. Samples in which IgG antibodies were not detected could not be classified, although the majority was probably primary DENV infection.

Viral load was determined by an "in-house" RT-PCR method and virus serotype was determined by a multiplex PCR. Both methods have been described in detail previously [25]. For both assays RNA was extracted from plasma using the Qiagen Viral RNA kit (Qiagen, Germany). RT-PCRs were conducted in duplicate. For the viral load SuperScript III Platinum SYBR Green One-Step qRT-PCR kit with ROX (Invitrogen, Inc., EUA) and for the dengue serotype multiplex PCR Platinum Taq polymerase (Invitrogen, Brazil) was used. In both RT-PCRs primers covering all four DENV serotypes were used [21]. Sequences of the primers were the following: D1, 5′-TCA ATA TGC TGA AAC GCG CGA GAA ACC G; TS1, 5′- CGT CTC AGT GAT CCG GGG G; TS2, 5′- CGC CAC AAG GGC CAT GAA CAG; TS3, 5′-TAA CAT CAT CAT GAG ACA GAG C; and DENV-4, 5′-TGT TGT CTT AAA CAA GAG AGG TC.

Table 1 | Baseline characteristics of the clinical classifications

2009 WHO dengue case classification			
	WS- (N=50)	WS+ (N=49)	Severe (N=33)
Sex	52% male	61,2% male	39,4% male
Age*	44 (28-57,5)	13,5 (9,25-30,25)	35,5 (15-58,5)
Day of fever*	3 (3-5)	5 (4-7)	6 (4-7)
Plasma leakage and shock			
	No (N=74)	Plasma leakage (N=33)	Shock (N=25)
Sex	52,7% male	66,7% male	32,0% male
Age*	38 (23-55,25)	13 (8-26)	42 (12-62,5)
Day of fever*	4 (3-6)	5 (3-8)	5,5 (4-7)
Hemorrhage			
	No (N=87)	Minor bleeding (N=29)	Severe bleeding (N=16)
Sex	54,0% male	51,7% male	43,8% male
Age*	38 (16-55)	22 (12-59)	31 (9-45)
Day of fever*	4 (3-6)	6 (4-9)	4 (3,5-7)

*Baseline characteristics of the cohort when the patients are divided according to the 2009 WHO dengue case classification, the occurrence of plasma leakage and shock and the occurrence of hemorrhagic manifestations. Abbreviations: WS-: non-severe dengue without warning signs, WS+: non-severe dengue with warning signs. * values are given in median (inter-quartile range).*

Markers of MT

Samples were aliquoted and stored at -80° C. Repetitive freeze-thaw cycles were avoided.
LPS was determined with a commercially available Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod Incorporated, USA). Samples were diluted 1:20 with LAL Reagent Water and heat-inactivated at 60°C for 30 minutes. Depyrogenated glassware was used to prevent contamination (Pyrotubes, Associates of Cape Cod Incorporated, USA). Hereafter, 50 µl of LAL was added and samples were incubated in the Pyros Kinetix Flex Machine (Associates of Cape Cod Incorporated, USA). Escherichia coli endotoxin was used to prepare the standard curve. Soluble CD14 (sCD14; 'Quantikine' ELISA, R&D Systems, UK), LPS binding protein (LBP ELISA, Hycult Biotech, USA) and IgM and IgG endotoxin core antibodies (EndoCab ELISA, Hycult Biotech, USA) were determined using commercially available assays. The assays were performed according to the manufacturer's instructions and every sample was measured in duplicate. One patient was excluded from the LPS analysis due to extremely high levels of LPS (56504 pg/ml) and therefore a secondary bacteremia could not be excluded.

Cytokine measurements

Cytokines were measured using a multiplex immunoassay kit with spectrally encoded antibody-conjugated beads (Human Cytokine 30-plex panel, Invitrogen, USA). The following cytokines were measured (Sensitivity limits (pg/ml): EGF (<18,8), Eotaxin (<0), FGF-basic (<12,3), G-CSF (<38,5), GM-CSF (<40), HGF (<0), IFN-α (<116), IFN-γ (<34), IL-1RA (<116), IL-1β (<20), IL-2 (<33), sIL-2R (<40), IL-4 (<108), IL-5 (<40), IL-6 (<13,5), IL-7 (<60), IL-8 (<20), IL-10 (<47), IL-12 (p40/p70) (<40), IL-13 (<60), IL-15 (<58), IL-17

(<80), IP-10 (<640), MCP-1 (<60), MIG (<20), MIP-1α (<17), MIP-1β (<18), RANTES (<20), TNF-α (<21) and VEGF (<823). Serum samples were diluted 1:2. The test was performed according to the manufacturer's instructions and was run on a Luminex 200 dual laser detection system.

Cluster analysis

The cluster analysis procedure was adapted from van den Ham et al. [129]. Briefly, cytokine values were log-transformed and subjected to hierarchical correlation clustering (i.e., with distance measure 1 – pearson's pairwise correlation value) using Ward's method that minimizes within-cluster variance. Both patients and cytokines were clustered to obtain a heatmap. Cytokines that had more than 5% of values missing (FGF-basic, GM-CSF, IL-1β, IL-5, IL-7, IL-13 and IL-17) were excluded from the analyses. Three serum samples were excluded from the cytokine analysis, because their levels were out of range for most of the cytokines evaluated and therefore the quality of the sample was most likely compromised. Cluster analysis was performed in R 2.15 (R Development Core Team [R Foundation for Statistical Computing], 2012, www.r-project.org). R scripts used to construct the trees and heatmaps are available upon request.

Statistical analysis

The Kruskal-Wallis H test was used for comparison of more than two groups. Statistical significance between individual groups was determined with the Mann-Whitney U test. Using the Bonferroni correction a p-value cut-off of ≤ 0.0083 for cytokine analyses was applied. For testing the significance of LPS, LBP and sCD14 levels associated with the clinical classifications and the clusters a p-value cut-off ≤ 0.05 was used. Correlations were calculated using the Spearman's correlation coefficient. To calculate the association of severe disease with the three main clusters the Fisher's exact test was used. For this test we used a p-value cut-off ≤ 0,05 to reach significance.

Results

Cohort

During the 2010 outbreak serum samples were obtained from 811 patients with laboratory confirmed acute DENV infection. From this cohort, 99 patients with non-severe dengue were randomly selected based on the availability of samples and clinical data. Moreover, patients with severe co-morbidity were excluded. Eventually, 50 patients without warning signs (WS-) and 49 with warning signs (WS+) were selected. Only 33 patients presented with severe dengue according to the 2009 WHO case classification [1] and they were all included in this analysis. Among patients with warning signs, 29/49 (59.2%) showed plasma leakage diagnosed by ultrasound/X-rays (pleural and peritoneal 12; peritoneal 12; pleural 5), 23 (46.9%) showed mucosal bleeding, 14 (28.6%) persistent vomiting, 5 (10,2%) abdominal pain and 3 (6,1%) lethargy. Among patients with severe dengue, 27/33 (81,8%) showed signs of severe plasma leakage (25 shock, 2 fluid accumulation leading to respiratory distress), 14 (42,4%) showed severe bleeding and one (3,0%) severe liver involvement (AST and ALT > 1000). The clinical presentation and general characteristics of the cohort are described in Table 1.

Of the 132 patients included, three had a primary and 113 had a secondary infection based on the IgG avidity test [24]. In 16 patients IgG antibodies could not be detected.

Viral load and dengue serotype

Viral RNA was detected in 120 of 132 samples. Significantly higher DENV RNA load was detected in samples collected 1-3 days after onset of fever compared to day 4-7 and day >7 (Figure 1). Moreover, DENV RNA levels were significantly higher in WS- patients compared to WS+ and severe patients, but this difference occurred most likely because they presented earlier after the onset of fever (Figure 1, Table 1). Dengue serotype could be determined in 126/811 (15.5%) patients with laboratory confirmed acute DENV infection during the 2010 Santos outbreak. From these, 118/126 (93.7%) typed as DENV-2, 4 (3.2%) as DENV-1 and 4 (3.2%) as DENV-3. Among the 132 patients included in this study, DENV serotype could be determined in 20 (15.2%) patients. 19 out of 20 patients were typed as DENV-2 and the remaining one as DENV-3.

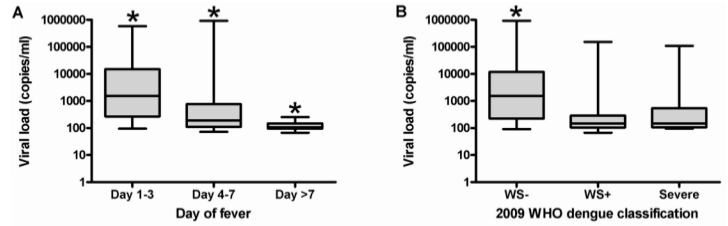

Figure 1 | Viral load

A: Day of fever: All three groups differed significantly from each other with the highest levels at day 1-3 and the lowest levels at day>7 (Day 1-3 vs day 4-7 P=0,001; day 1-3 vs day>7 P<0,0001; day 4-7 vs day>7 P=0,006). B: 2009 WHO dengue case classification: levels in WS- patients were significantly increased compared to WS+ and severe patients (WS- vs WS+ P<0,0001; WS- vs severe P=0,001). Abbreviations: WS-: non-severe dengue without warning signs, WS+: non-severe dengue with warning signs. Horizontal bars inside the boxplot indicate the median. The box indicates the interquartile range. Black asterisk = significantly different from all other groups. The Mann-Whitney U test was used to compare the groups with each other.

Association of levels of circulating cytokines with clinical disease severity

Eotaxin, IL-2, IL-4, IL-1RA and IFN-γ were detected at very low levels in DENV infected patients and healthy controls and did not show any significant differences between groups when patients were classified according to the 2009 WHO classification or the occurrence of plasma leakage/shock and hemorrhage (Table 2). In the majority of samples MIP-1α and TNF-α also showed values below the detection limit. However, some healthy controls showed extremely elevated levels and therefore a significant difference between healthy controls and dengue patients was shown (Table 2).

IFN-α, IL-10, IL-12 IL-15, IP-10, MIG and MIP-1β were significantly increased or decreased in dengue patients compared to healthy controls if patients were classified according to the 2009 WHO classification (Table 2, Figure S1). These cytokines did not show significant differences among the disease severity groups.

Some cytokines showed significant differences in levels between dengue disease severity groups. Using the 2009 WHO dengue case classification, levels of RANTES and MCP-1 were significantly increased in WS- patients compared to patients with severe and WS+ dengue respectively. In contrast, levels of IL-6, IL-8, HGF and G-CSF were significantly increased in severe dengue compared to uncomplicated disease (Table 2, Figure 2).

When these cytokines were determined in patients classified according to the occurrence of plasma leakage and shock, levels of RANTES and EGF were significantly decreased in patients with shock compared to patients with uncomplicated dengue. Moreover, levels of IL-6, HGF and G-CSF were significantly increased in shock patients compared to patients with uncomplicated disease (Table 2, Figure 3).

Patients were also classified according to the occurrence of hemorrhage. Levels of sIL-2R, IL-6, IL-8, IL-15 and G-CSF were significantly increased in patients with severe bleeding compared to patients with no bleeding (Table 2, Figure S2).

Nine out of 132 patients died within 14 days after the onset of fever. In these patients IL-6, G-CSF and

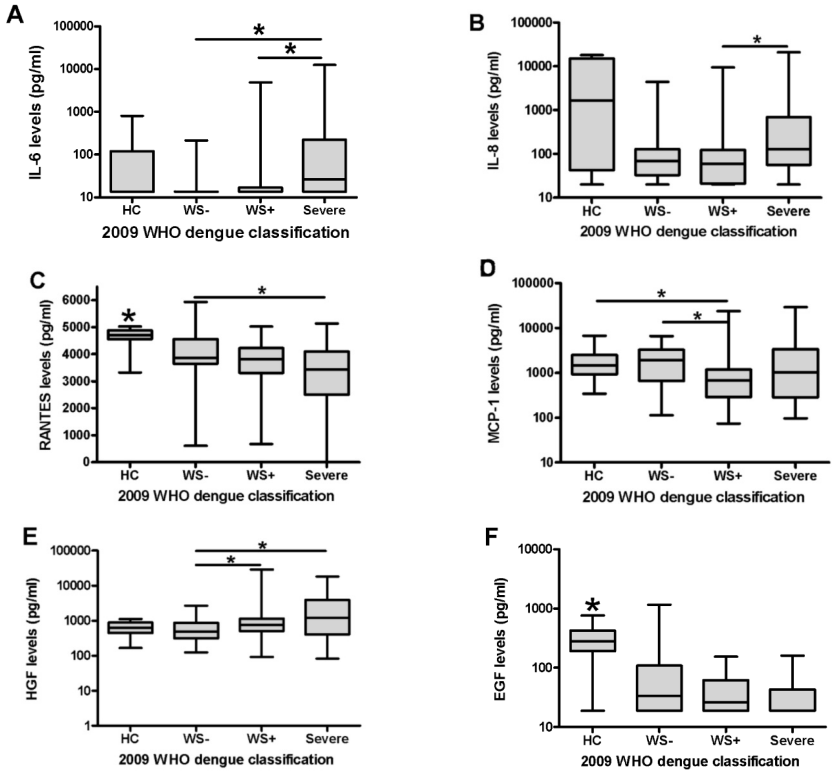

Figure 2 | **Cytokine levels in dengue virus infected patients classified according to the 2009 WHO dengue classification.**

A: IL-6: Levels in severe patients are significantly increased compared WS- (P<0,0001) and WS+ (P<0,0001) patients (KW P<0,0001, KW dengue groups P<0,0001). B: IL-8: Levels in severe patients are significantly elevated compared to WS+ patients (P=0,004) (KW P=0,004, KW dengue groups P=0,011). C: RANTES: Levels in severe (P=0,002) patients are significantly decreased compared to WS- patients. Levels in all patient groups are significantly decreased compared to HC (WS- vs HC P=0,001, WS+ and severe vs HC P<0,0001), (KW P<0,0001, KW dengue groups P=0,006). D: MCP-1: Levels in WS+ patients are significantly decreased compared to WS- (P=0,001) patients and HC (P=0,008) (KW P=0,006, KW dengue groups P=0,006). E: HGF: Levels in severe (P=0,001) and WS+ (P=0,005) patients are significantly increased compared to WS-patients (KW P=0,001, KW dengue groups P=0,001). F: EGF: Levels in all patient groups are significantly decreased compared to HC (WS-, WS+ and severe vs HC P<0,0001, KW P<0,0001, KW dengue groups P=0,03). G: G-CSF: Levels in severe patients are significantly increased compared to WS- patients (P= 0,003, KW P= 0,02, KW dengue groups P= 0,008). Legend: HC = healthy control, WS- = non-severe dengue without warning signs. WS+ = non-severe dengue with warning signs, KW = kruskal wallis. Horizontal bars inside the boxplot indicate the median. The box indicates the interquartile range. Black asterisk = significantly different from all other groups. Underlined black asterisk= significant difference between two groups. The Mann-Whitney U test was used to compare the groups with each other.

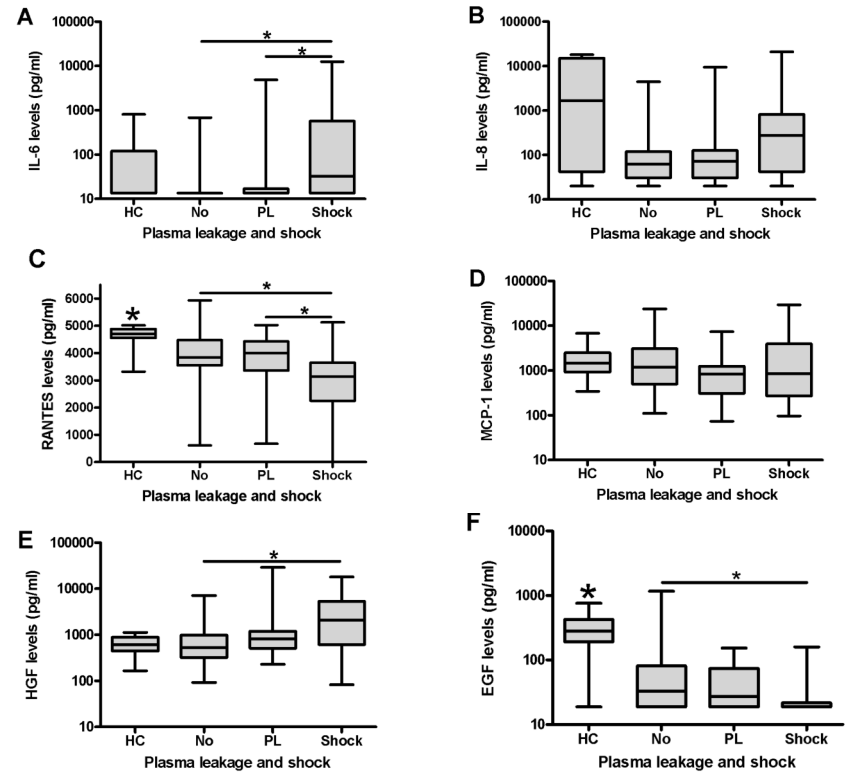

Figure 3 | **Cytokine levels in dengue virus infected patients classified according to the occurrence of plasma leakage/shock.**

A: IL-6: Levels in shock patients are significantly increased compared to patients with (P=0,001) and without (P<0,0001) plasma leakage (KW P<0,0001, KW dengue groups P<0,0001). B: IL-8: No significant differences (KW P=0,01, KW dengue groups P=0,04). C: RANTES: Levels in shock patients are significantly decreased compared to patients with (P=0,002) and without (P<0,0001) plasma leakage. Levels in all patient groups are significantly decreased compared to HC (No, PL and shock vs HC P<0,0001, KW P<0,0001, KW dengue groups P=0,001). D: MCP-1: No significant differences (KW P=0,162, KW dengue groups P=0,17). E: HGF: Levels in patients with shock are significantly increased compared to patients with no plasma leakage (P=0,001) (KW P=0,001, KW dengue groups P=0,001). F: EGF: Levels in shock patients are significantly decreased compared to patients without (P=0,004) plasma leakage. Levels in all patient groups are significantly decreased compared to HC (No, PL and shock vs HC P<0,0001, KW P<0,0001, KW dengue groups P=0,015). G: G-CSF: Levels in patients with shock are significantly elevated compared to patients without plasma leakage (P=0,002, KW P=0,01, KW dengue groups P=0,004). Abbreviations: HC= healthy control, No= no occurrence of plasma leakage, PL= plasma leakage, KW= kruskal wallis. Horizontal bars inside the boxplot indicate the median. The box indicates the interquartile range. Black asterisk = significantly different from all other groups. Underlined black asterisk= significant difference between two groups. The Mann-Whitney U test was used to compare the groups with each other.

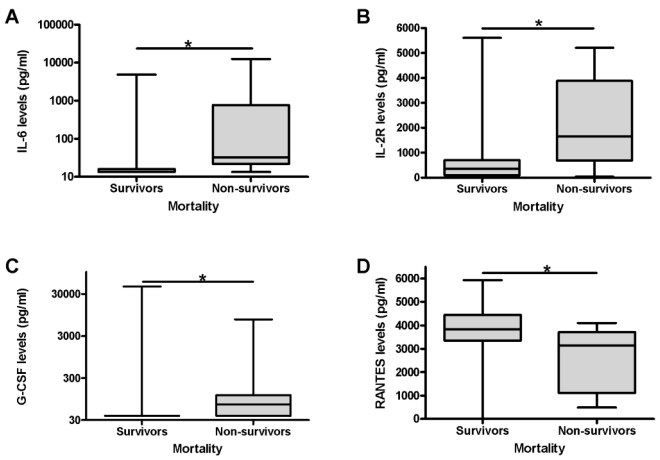

Figure 4 | IL-6, IL-8, IL-2R and RANTES are associated with mortality.
A: IL-6: Levels are significantly elevated in non-survivors compared to survivors (P<,0,0001). B: IL-2R: Levels are significantly elevated in non-survivors compared to survivors (P=0,005). C: G-CSF: Levels are significantly elevated in non-survivors compared to survivors (P=0,001). D: RANTES: Levels are significantly decreased in non-survivors compared to survivors (P=0,005). Underlined black asterisk= significant difference between two groups. The Mann-Whitney U test was used to compare the groups with each other.

sIL-2R were significantly increased and RANTES significantly decreased in non-survivors compared to survivors (Table 2, Figure 4).

IFN-α, IL-12, MCP-1, MIG, MIP-1β showed a dynamic temporal pattern during the course of disease. They were significantly increased at day 1-3 after the onset of fever compared to day 4-7 and day>7 (Table 2, Figure S3). This may explain why the levels of MCP-1 were significantly higher in uncomplicated than in more severe dengue in patients classified according to the 2009 WHO dengue case classification, since patients with non-severe dengue presented earlier in their course of disease (Table 1). Interestingly, the mediators IFN-α (P=0.001), IL-12 (P=0.01), MCP-1 P<0.0001), MIG (P=0,01) and MIP-1β (P<0.0001) showed to have a significant positive correlation with the viral load (data not shown).

Cluster analysis identifies a group of patients with a pro-inflammatory cytokine profile

The cluster analysis groups samples or cytokines based on cytokine levels only, and not based on clinical presentation (non-supervised analysis). The sample and cytokine cluster analyses can be combined and visualized as a heatmap (Figure 5). A dendrogram shows the similarity between samples (left side of figure 5), where samples in the same branch are more similar regarding their cytokine profiles to each other than to samples in other branches. The sample dendrogram can be divided into three principle clusters that largely segregate healthy controls (cluster A), mild to moderately ill DENV infected patients (cluster B), and severely ill DENV infected patients (cluster C). Clinical disease was more severe in cluster C than in clusters A and B, illustrated by a statistically significant higher incidence of severe disease (P= 2,2 X 10^{-16}), shock (3,4 X 10^{-5}), severe hemorrhage (P=0,007) and death (P=0,03) in this cluster compared to cluster A and B (Table 3).

A subgroup of 'healthy control' cluster A displayed elevated levels of inflammatory cytokines when compared to other control samples, including MIP-1α, MIP-1β, TNF-α, EGF and IL-8. These controls are likely to have suffered from an underlying unidentified inflammatory condition (Table 2, Figure 5).

The majority of dengue cases were part of cluster B. In cluster B 40% of patients suffered from WS-, 39 % from WS+ and 21% from severe dengue (Table 3). This distribution resembles the whole cohort, which consisted of 38% WS-, 37% WS+ and 25% severe dengue. The cytokine pattern in this cluster shows a rather diffuse pattern. A few patients show increased concomitant expression of IFN-γ, IFN-α, MCP-1, MIG and IL-12, which is indicative for an early antiviral response.

Cluster C shows a strong pro-inflammatory cytokine pattern. RANTES and EGF are downregulated,

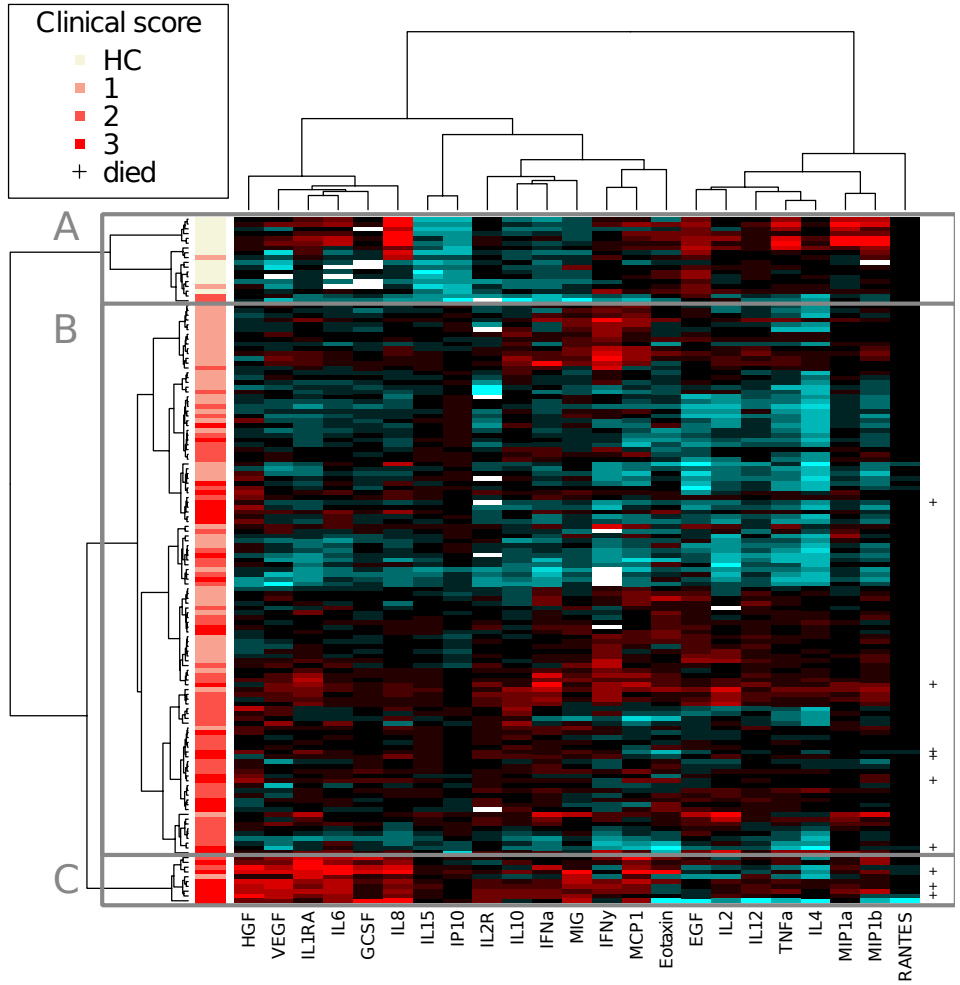

Figure 5 | Heatmap of cluster analysis.
A cluster analysis was performed with 23 cytokines, which resulted in a dendrogram indicated on the left of the heatmap. Every horizontal line indicates one patients. The vertical bar on the left of the heatmap indicates the disease severity of the patient. A: Cluster with mainly healthy controls and four dengue patients. B: Cluster with mild to moderately ill dengue virus infected patients. C: Cluster with severely ill dengue virus infected patients. Abbreviations: 1: non-severe dengue without warning signs. 2: non-severe dengue with warning signs. 3: severe dengue. +: patient died within 14 days after the onset of fever.

whereas IL-6, IL-8, IL-10, IL-15, IL-1RA, sIL-2R, HGF, VEGF, G-CSF, MCP-1, IP-10, and MIG are upregulated compared to other clusters. Interestingly, IL-1RA, IL-10, IL-15, IP-10, MIG and VEGF are associated with severe disease in the cluster analysis, but not with severe disease using the clinical classifications. Using for example the 2009 WHO classification IL-10, IL-15, IP-10 and MIG were significantly elevated in dengue patients compared to healthy controls, but levels were not significantly elevated in severe compared to uncomplicated dengue. Cluster C identifies a group of patients with an extensive pro-inflammatory cytokine profile, suggestive for a cytokine storm. Moreover, severe clinical symptoms occurred significantly more often in cluster C compared to the other clusters.

Table 2 | Overview of cytokine analyses

Cytokine	Accession number	2009 WHO classification	Plasma leakage/ shock	Hemorrhage	Mortality	Temporal pattern	Cluster analysis	Conclusion
Eotaxin	P51671	–	–	–	–	–	–	No association
MIP-1α (CCL3)	P10147	WS- vs HC P=0,001 WS+ vs HC P=0,004	No vs HC P=0,001 PL vs HC P=0,006	No vs HC P=0,001	–	–	↑ cluster A*	Background HC
TNF-α	P01375	WS- vs HC P<0,0001 WS+ vs HC P<0,0001 Sev vs HC P=0,002	No vs HC P<0,0001 PL vs HC P=0,002	No vs HC P<0,0001 Minor vs HC P=0,003	–	–	↑ cluster A*	Background HC
IL-2	P60568	–	–	–	–	–	–	No association
IL-4	P05112	–	–	–	–	–	–	No association
IFN-γ	P01579	–	–	–	–	–	↑ cluster B*	Early antiviral response
IL-1RA	P18510	–	–	–	–	–	↑ cluster C	Association severity in CA
IFN-α	P01562	WS- vs HC P=0,001 WS+ vs HC P=0,003 Sev vs HC P=0,001	No vs HC P=0,001 PL vs HC P=0,001 Shock vs HC P=0,003	No vs HC P=0,001 Sev vs HC P<0,0001	–	D1-3 vs D4-7 P=0,002 D1-3 vs D>7 P=0,003	↑ cluster B*	Early antiviral response
IL-15	P40933	WS- vs HC P<0,0001 WS+ vs HC P<0,0001 Sev vs HC P<0,0001	No vs HC P<0,0001 PL vs HC P<0,0001 Shock vs HC P<0,0001	No vs HC P<0,0001 Minor vs HC P<0,0001 Sev vs HC P<0,0001 No vs Sev P=0,003	–	–	↓ cluster A ↑ cluster C	Association with dengue in CC and severe disease in CA
IP-10 (CXCL10)	P02778	WS- vs HC P<0,0001 WS+ vs HC P<0,0001 Sev vs HC P<0,0001	No vs HC P<0,0001 PL vs HC P<0,0001 Shock vs HC P<0,0001	No vs HC P<0,0001 Minor vs HC P<0,0001 Sev vs HC P<0,0001	–	–	↓ cluster A ↑ cluster C	Association with dengue in CC and severe disease in CA
MIP-1β (CCL4)	P13236	WS- vs HC P=0,002 WS+ vs HC P<0,0001 Sev vs HC P=0,002	No vs HC P=0,001 PL vs HC P=0,001 Shock vs HC P=0,004	No vs HC P<0,0001 Minor vs HC P<0,0001	–	D1-3 vs D4-7 P<0,0001 D1-3 vs D>7 P<0,0001	↑ cluster A*	Association dengue in CC. Early antiviral response. Background HC.
MIG (CXCL9)	Q07325	WS- vs HC P<0,0001 Sev vs HC P=0,002	No vs HC P<0,0001 Shock vs HC P=0,005	No vs HC P=0,001 Sev vs HC P<0,0001	–	D1-3 vs D4-7 P=0,008 D1-3 vs D>7 P=0,001	↑ cluster B* ↑ cluster C	Early antiviral response. Association severe disease in CA
IL-10	P22301	Sev vs HC P=0,008	-	–	–	–	↑ cluster C	Association dengue in CC and severe disease in CA.
IL-12	P29459 P29460	WS+ vs HC P=0,002	–	–	–	D1-3 vs D4-7 P=0,001 D1-3 vs D>7 P=0,003	↑ cluster B*	Early antiviral response

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*Overview of the significant associations of all analyses performed and specified per cytokine. The Mann-Whitney U test was used to compare the groups with each other. Abbreviations: HC: healthy control. 2009 WHO classification: WS-: non-severe dengue without warning signs, WS+: non-severe dengue with warning signs, Sev: severe dengue. Plasma leakage/shock: No : no plasma leakage occured, PL: plasma leakage detected, Hemorrhage: No : no hemorrhagic symptoms, Minor: minor hemorrhage, Sev: severe hemorrhage. Temporal pattern: D1-3: day 1-3 after the onset of fever, D4-7: day 4-7 after the onset of fever, D>7: more than 7 days after the onset of fever. Cluster analysis: * only in a part of the cluster this cytokine is up- or downregulated. Conclusions: CC: clinical classification, CA: cluster analysis.*

MT is associated with severe dengue

Statistically significant elevated LPS levels were found in cluster C compared to 'dengue' cluster B and 'healthy control' cluster A (Figure 6). In the 2009 classification there proved to be a trend towards higher LPS levels in severe dengue, although these differences were not statistically significant. However, in the plasma leakage/shock classification LPS levels were significantly increased in patients with shock compared to patients with no plasma leakage. In the 2009 classification, LBP levels were significantly increased in dengue patients compared to healthy controls. Moreover, levels in severe patients were significantly increased compared to WS- patients. In the plasma leakage/shock classification le-

Table 2 (continue) *Overview of cytokine analyses*

Cytokine	Accession number	2009 WHO classification	Plasma leakage/ shock	Hemorrhage	Mortality	Temporal pattern	Cluster analysis	Conclusion
G-CSF	P09919	WS- vs Sev P=0,003	No vs Shock P=0,002	No vs Sev P<0,0001	P=0,001	–	↑ cluster C	Association with severe disease in CC and CA
IL-6	P05231	WS- vs Sev P<0,0001 WS+ vs Sev P<0,0001	No vs Shock P<0,0001 PL vs Shock P=0,001	No vs Sev P=0,001	P<0,0001	–	↑ cluster C	Highly associated with severe disease
IL-8	P10145	WS+ vs Sev P=0,004	–	No vs Sev P<0,0001 Minor vs Sev P=0,002	–	D1-3 vs D4-7 P=0,008	↑ cluster A* ↑ cluster C	Association with severe disease in CC and CA
RANTES (CCL5)	P13501	WS- vs HC P=0,001 WS+ vs HC P<0,0001 Sev vs HC P<0,0001 WS- vs Sev P=0,002	No vs HC P<0,0001 PL vs HC P<0,0001 Shock vs HC P<0,0001 No vs Shock P<0,0001 PL vs Shock P=0,002	No vs HC P<0,0001 Minor vs HC P<0,0001 Sev vs HC P<0,0001	P=0,005	–	↓ cluster C	Highly associated with severe disease
EGF	P01133	WS- vs HC P<0,0001 WS+ vs HC P<0,0001 Sev vs HC P<0,0001	No vs HC P<0,0001 PL vs HC P<0,0001 Shock vs HC P<0,0001 No vs Shock P=0,004	No vs HC P<0,0001 Minor vs HC P<0,0001 Sev vs HC P<0,0001	–	–	↑ cluster A ↓ cluster C	Increased levels background HC. Decreased levels associated with severe disease in CC and CA
HGF	P14210	WS- vs WS+ P=0,005 WS- vs Sev P=0,001	No vs Shock P=0,001	–	–		↑ cluster C	Associated with severe disease in CC and CA
MCP-1 (CCL2)	P13500	WS+ vs HC P=0,008 WS- vs WS+ P=0,001	–	–	–	D1-3 vs D4-7 P<0,0001 D1-3 vs D>7 P<0,0001	↑ cluster B* ↑ cluster C	Early antiviral response. Association severe disease in CC and CA
VEGF	P15692	–	–	–	–	–	↑ cluster C	Association with severe disease in CA
IL-2R	A2N4P8	–	–	Sev vs HC P=0,003 No vs Sev P=0,002 Minor vs Sev P=0,008	P=0,005	–	↑ cluster C	Highly associated with severe disease, hemorrhage in particular.

vels were significantly increased in patients with shock and no plasma leakage compared to healthy controls. Moreover, levels in patients with shock were significantly increased compared to patients with plasma leakage. In the cluster analysis LBP levels in all three clusters differed significantly from each other. sCD14 levels were significantly increased in DENV infected patients compared to healthy controls in the 2009 and the plasma leakage/shock classification and in the 'dengue' clusters B and C compared to the 'healthy control' cluster A. Moreover, in the 2009 classification levels in WS+ patients were significantly increased compared to WS- patients. When patients were classified according to the occurrence of hemorrhagic manifestations, LPS levels were not significantly different, and sCD14 and LBP again showed to be significantly elevated in DENV infected patients compared to controls (Data not shown). No significant differences in IgM- and IgG-specific endotoxin core antibodies were found among the groups classified according to the 2009 classification or the occurrence of plasma leakage and shock (Data not shown).

Discussion

In this study, we have examined a cohort of dengue patients and healthy controls to investigate the role of immune activation and MT in DENV pathogenesis. We found evidence for the occurrence of MT during DENV infection. Furthermore, in the cluster analysis, we showed that the cluster of patients with the highest LPS levels appeared to suffer from a cytokine storm.

The two complementary analysis techniques applied in this study yielded similar results. However, the cluster analysis identified more markers associated with severe disease than the clinical classification system. The cluster analysis groups patients based on the occurrence of identical inflammatory processes, overcoming the potential clinical classification biases that may occur due to the fact that disease presentation of patients can be quite variable and the severity of disease is subject to clinical interpretation. In the cluster analysis, levels of cytokines determined the outcome of the clusters. Therefore this technique cannot be used to relate absolute values of cytokines to the clusters with pa-

Table 3 Clinical characteristics of the cluster analysis

Cluster	A			B				C				Fisher's exact test
	N=18			N=115				N=10				
Age (years)*	26,5 (22-35)			31,5 (13-49)				45 (14-63)				
Sex	38,9% male	53,0 % male	30,0% male									
Day of fever*	6 (4-11)			4 (3-6)				4 (3-5)				
2009 WHO dengue case classification	77,8,0% (N=14) HC	11,1% (N=2) WS-	11,1% (N=2) WS+		40,0% (N=46) WS-	39,1% (N=45) WS+	20,9% (N=24) Severe		10,0% (N=1) WS-	20,0% (N=2) WS+	70,0% (N=7) Severe	Severe dengue $P= 2,2 X 10^{-16}$
Survival	77,8,0% (N=14) HC	22,2% (N=4) Survived			94,8% (N=109) Survived	5,2% (N=6) Died			70,0% (N=7) Survived	30,0% (N=3) Died		Death P=0,03
Hemorrhage	77,8,0% (N=14) HC	16,7% (N=3) NO	5,6% (N=1) Minor		68,7% (N=79) NO	22,6% (N=26) Minor	8,7% (N=10) Severe		40,0% (N=4) NO	20,0% (N=2) Minor	40,0% (N=4) Severe	Severe hemorrhage P=0,007
Plasma leakage and shock	77,8,0% (N=14) HC	16,7% (N=3) NO	5,6% (N=1) PL		57,4% (N=66) NO	27,0% (N=31) PL	15,7 % (N=18) Shock		10,0% (N=1) NO	20,0% (N=2) PL	70,0% (N=7) Shock	Shock $P= 3,4 X 10^{-5}$

*Clinical manifestations of patients divided in the three clusters. Abbreviations: HC: healthy control, WS-: non-severe dengue without warning signs, WS+: non-severe dengue with warning signs, PL: plasma leakage. * values are given in median (interquartile range).*

tients. Altogether, the strength of our approach is the use of both clinical classification and cluster analysis in order to increase the sensitivity to find markers of disease severity.

One limitation of this study is the cross-sectional study design. We have recorded the disease severity of the patient at the time of inclusion and at the same moment the samples for LPS and cytokine analysis were drawn, so the levels of LPS and cytokines were related to signs and symptoms that were present at that same time.

Both in a previous [127] and in the present study, we have shown that elevated levels of LPS are associated with severe dengue. Moreover, MT was indirectly confirmed by increased levels of LBP and sCD14 as observed in sepsis patients [115,130]. In contrast to our previous study, the association between LPS levels and clinical disease severity was less strong. However, also in this study there proved to be a significant association in patients classified according to the occurrence of plasma leakage and shock. Moreover, LPS levels were significantly increased in the cluster with the highest incidence of shock (cluster C) and levels of LBP did also show a direct association with disease severity in the 2009 and the plasma leakage/shock classification. This cohort differed from our previous study in several ways: age of the population (children vs. children and adults), the geographical location (Indonesia vs. Brazil) and the samples used (plasma vs. serum). Whether age or different pathogen pressures at different geographical locations may account for the observed differences remain to be established. IgM and IgG endotoxin core antibodies were determined, but no strong association with disease severity was found. This is in agreement with studies in HIV and sepsis patients, which show conflicting results with regards to the association between endotoxin core antibodies and disease severity [112,131,132].

It has been hypothesized that severe DENV infection is caused by an exaggerated immune response, associated with a cytokine storm (reviewed in [29,49]). The exact definition of a cytokine storm is still a matter of debate. In general it is assumed that a cytokine storm starts with an excessive release of pro-inflammatory cytokines (e.g. TNF-α and IL-1β). These cytokines then induce other pro-inflammatory (e.g. IL-6), but also anti-inflammatory cytokines (e.g. IL-10). This augmented immune response could therefore be the result of a disturbed balance between pro- and anti-inflammatory cytokines. During severe DENV infections a cytokine storm has been proposed to be responsible for the increased vascular permeability and coagulation disturbances (reviewed in [49]).

Studies in patients with HIV and visceral leishmaniasis showed that MT may contribute to severe disease through excessive immune activation [112,133]. It is known that LPS stimulation can induce the production of IL-6, IL-8, TNF-α and IL-1β [134] and the growth factors VEGF and HGF [135]. Interestingly, in the present study high levels of four of these markers were found in the pro-inflammatory cluster C. This suggests that MT may play a role in the cytokine storm in severe dengue. Moreover, Bosisio et al. [136] showed that priming of mononuclear cells with IFN-γ increased the expression of the TLR4

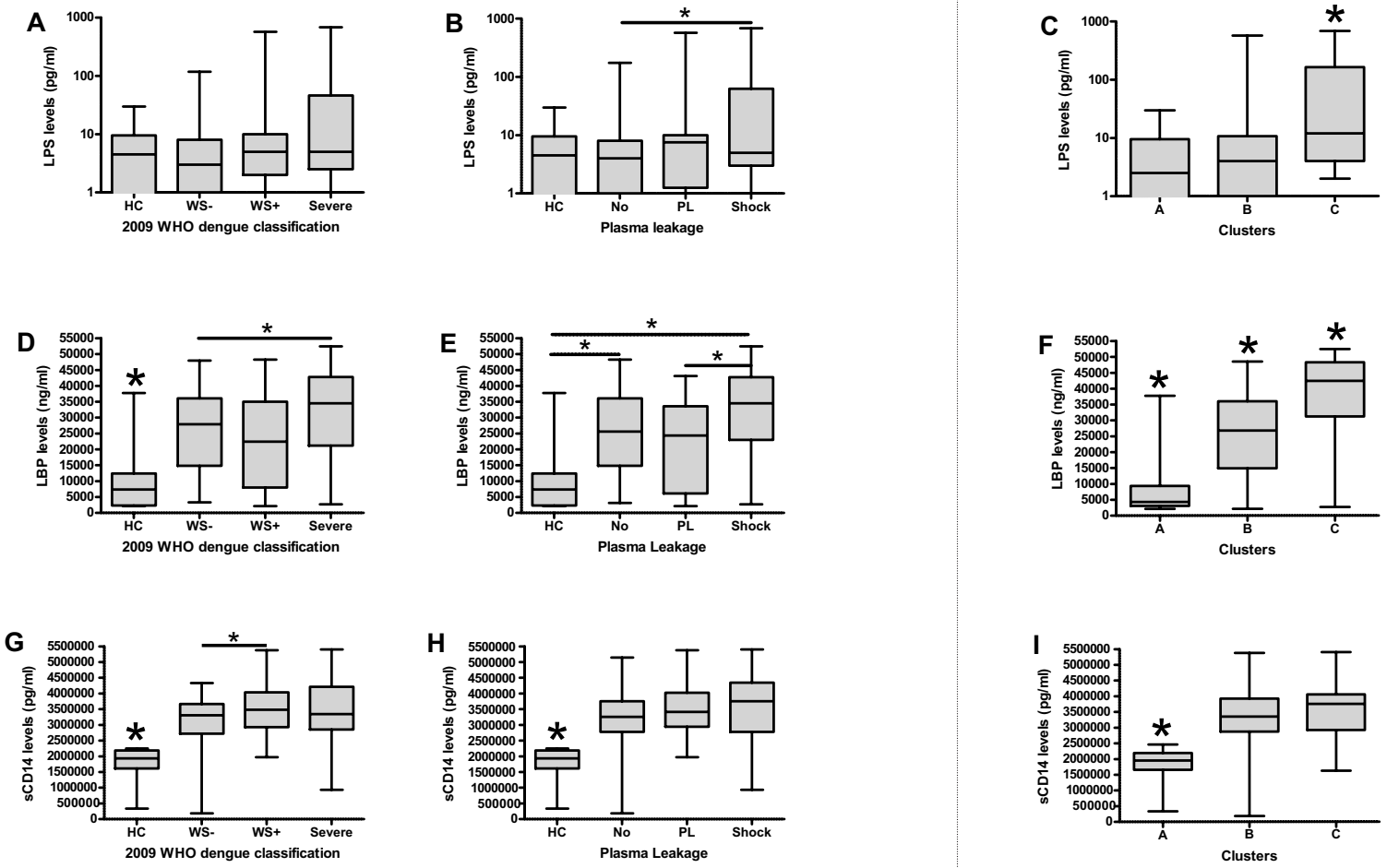

Figure 6 | LPS, LBP and sCD14 levels in dengue virus infected patients

LPS: A: No significant differences in the 2009 WHO dengue case classification. B: Levels in patients with shock were significantly increased compared to patients without plasma leakage (P=0,04). C: Cluster analysis: cluster C was significantly elevated compared to cluster A (P=0,01), and B (P=0,02). LBP: D: Levels were significantly elevated in all dengue patients compared to HC (WS- vs HC P=0,009, WS+ vs HC P=0,03, Severe vs HC P=0,01). Levels in patients with severe dengue were significantly elevated compared to WS- dengue (P=0,03). E: Levels were elevated in patients with shock (P=0,008) and no plasma leakage (P=0,008) compared to HC. Levels were also elevated in patients with shock compared to patients with plasma leakage (P=0,03). F: In the cluster analysis levels in cluster C were significantly elevated compared to cluster A (P=0,002) and B (P=0,007). Moreover, cluster B was significantly elevated compared to cluster A (P<0,0001). sCD14: G: In the 2009 classification levels of sCD14 in DENV infected patients were significantly elevated compared to HC (WS-, WS+ and severe vs HC P<0,0001). Levels were significantly increased in WS+ compared to WS- patients (P=0,04). H: In the plasma leakage/shock classification levels of sCD14 in DENV infected patients were significantly elevated compared to HC (No, PL and shock vs HC P<0,0001). I: In the cluster analysis cluster B (P<0,0001) and C (P=0,002) were significantly elevated compared to cluster A. Abbreviations: HC : healthy control, WS-: non-severe dengue without warning signs. WS+: non-severe dengue with warning signs, No : No occurrence of plasma leakage, PL: occurrence of plasma leakage. Horizontal bars inside the boxplot indicate the median. The box indicates the interquartile range. Black asterisk = significantly different from all other groups. Underlined black asterisk= significant difference between two groups. The Mann-Whitney U test was used to compare the groups with each other.

receptor and subsequent LPS-induced cytokine production. This would suggest that DENV induced IFN-γ production could enhance the pro-inflammatory LPS signaling pathway. In addition, Chen et al. [137,138] showed that LPS could prolong DENV infection of monocytes and macrophages. A sustained DENV infection due to MT may also contribute to the cytokine storm during DENV infection. All these studies suggest that MT may play an important role in the initiation and perpetuation of the cytokine storm during severe DENV infection. However, in this study MT was associated with extensive immune activation, but to investigate whether there is a causal relationship between MT and the cytokine storm further studies are warranted.

Our cohort confirms several known associations for dengue. In agreement with previous work, our study showed evidence of a strong Th1 response in the early phase of disease with peak levels of IFN-α [53,139], IL-12 [55-57], MCP-1 [58-60], MIG and MIP-1β [58]. All these Th1 cytokines correlated significantly with viral load, suggesting that they are associated with a host response aiming at reducing the viral load.

In the present study we have quantified pro- and anti-inflammatory mediators to provide evidence for a role of a cytokine storm in severe dengue patients. Levels of IL-10, IL-15, VEGF, G-CSF and IP-10 were increased in 'severe dengue' cluster C in the cluster analysis. High levels of IL-10 and VEGF have been described in severe dengue, especially at the day of defervescence [44,51,55,56,85]. Interestingly, patients in severe cluster C presented around this time (day 3-5 after onset of fever). The high incidence of shock in cluster C could be partly explained by high levels of VEGF and MCP-1, which are proposed to be important contributors to plasma leakage [60,85]. IL-15 and IP-10 [140] were reported to play an important role in the NK cell response, whereas G-CSF [61] stimulates neutrophil development and differentiation. High levels of IL-15, IP-10 and G-CSF in cluster C suggest that extensive activation of the innate immune system may contribute to the cytokine storm in severe dengue. In contrast, high levels of IL-10 have an inhibitory effect on dendritic cells and macrophages (reviewed in [65]).

In both the clinical classifications and the cluster analysis, IL-6, IL-8, sIL-2R, RANTES, HGF and EGF were strongly associated with severe disease. High levels of IL-6 and IL-8 were reported in dengue cases with severe plasma leakage and shock [66] and in non-survivors [52,67,68]. IL-6 production

is induced by TNF-α and IL-1β [61]. In our study no increased levels of TNF-α and IL-1β were found. This is in agreement with earlier reports [50,51,58,66,68] and can be explained by the observation that TNF-α and IL-1β are produced early after infection and are removed quickly from the circulation. In addition, sIL-2R has been associated with severe dengue [44,50,139] and is proposed to serve as a marker of immune activation (reviewed in [141]). Thrombocytopenia is a hallmark of DENV infection and since thrombocytes are an important source of RANTES and EGF, severe thrombocytopenia may explain the depletion of these two markers. This has been described previously in severe dengue [55] and cerebral malaria [142].

In summary, we provide evidence that MT is associated with extensive immune activation during severe dengue. LPS may play an important role in the development of the cytokine storm. Besides the classical mediators (e.g. IL-6, IL-8, IL-10), we identified cytokines (IL-1RA, sIL-2R), chemokines (MCP-1, IP-10, MIG, RANTES) and growth factors (HGF, EGF, G-CSF, VEGF) that may play an important role in the cytokine storm during severe DENV infection.

Acknowledgements

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Supporting information

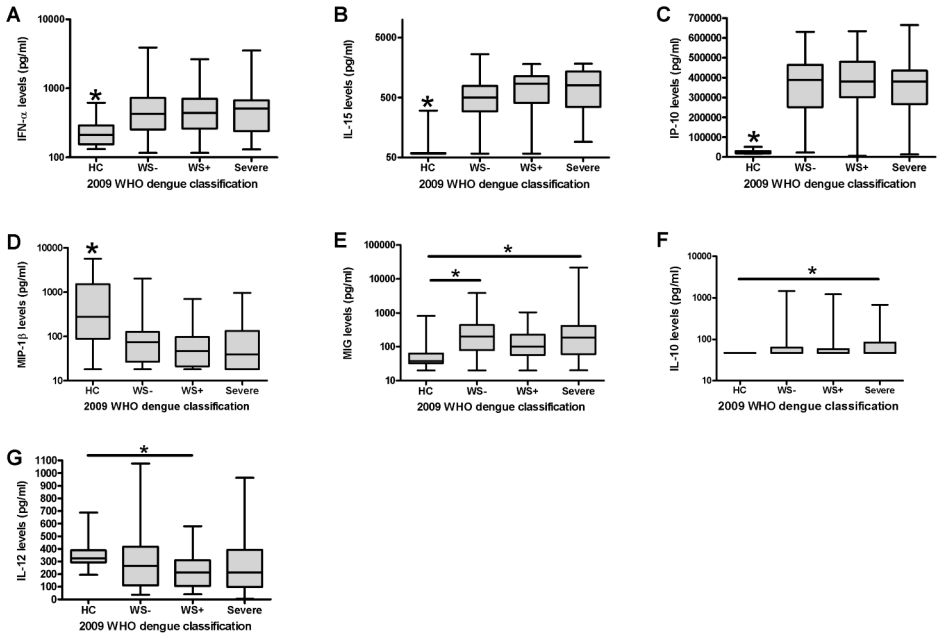

Figure S1 | Cytokine levels in dengue virus infected patients are significantly different compared to healthy controls.

A: IFN-a: Levels in DENV infected patients are significantly elevated compared to HC (WS- vs HC P=0,001, WS+ vs HC P=0,003 and severe vs HC P=0,001, KW P=0,005, KW dengue groups P=0,93). B: IL-15: Levels in DENV infected patients are significantly elevated compared to HC (WS-, WS+ and severe vs HC P<0,0001, KW P<0,0001, KW dengue groups P=0,08). C: IP-10: Levels in DENV infected patients are significantly elevated compared to HC (WS-, WS+ and severe vs HC P<0,0001, KW P<0,0001, KW dengue groups P=0,82). D: MIP-1β: Levels in dengue patients are significantly decreased compared to HC (WS- vs HC P=0,002, WS+ vs HC P<0,0001, Sev vs HC P=0,002, KW P=0,002, KW dengue groups P=0,31). E: MIG: Levels in WS- (P<0,0001) and severe (P=0,002) patients are significantly elevated compared to HC (KW P<0,0001, KW dengue groups P=0,03). F: IL-10: Levels in severe patients are significantly elevated compared to HC (P=0,008, KW P=0,08, KW dengue groups P=0,54). G: IL-12: Levels in WS+ patients are significantly decreased compared to HC (P=0,002, KW P=0,05, KW dengue groups P=0,26). Abbreviations: HC = healthy control, WS- = non-severe dengue without warning signs. WS+ = non-severe dengue with warning signs. Horizontal bars inside the boxplot indicate the median. The box indicates the interquartile range. Black asterisk = significantly different from all other groups. Underlined black asterisk= significant difference between two groups. The Mann-Whitney U test was used to compare the groups with each other.

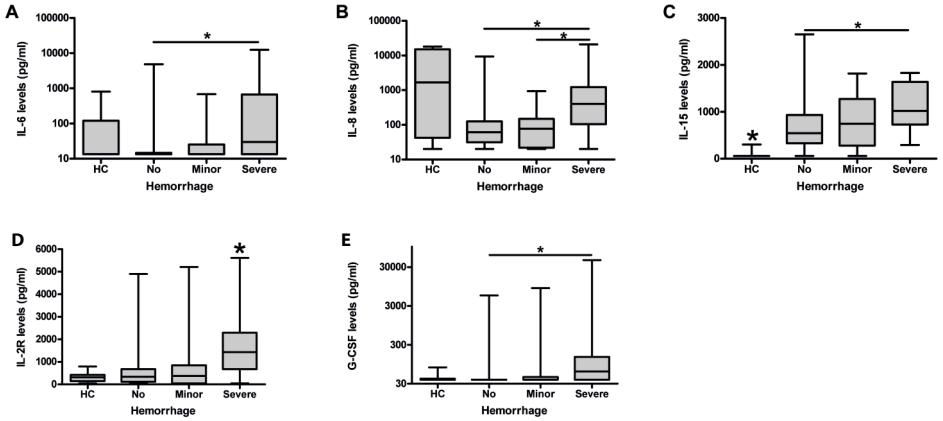

Figure S2 | Cytokine levels in dengue virus infected patients classified according to the occurrence of hemorrhage.

A: IL-6: Levels are significantly elevated in patients with severe bleeding compared to patients with no hemorrhage (P=0,001) (KW P=0,007, KW dengue groups P=0,003). B: IL-8: Levels in patients with severe bleeding are significantly elevated compared to patients with minor (P=0,002) and no (P<0,0001) hemorrhage (KW P=0,001, KW dengue groups P=0,001). C: IL-15: Levels in patients with severe hemorrhage are significantly elevated compared to patients with no hemorrhage (P=0,003). Levels in HC are significantly decreased compared to all other dengue groups (WS-, WS+ and severe vs HC P<0,0001) (KW P<0,0001 , KW dengue groups P=0,009). D: sIL-2R: Levels are significantly elevated in patients with severe bleeding compared to HC (P=0,003) or patients with minor (P=0,008) or no hemorrhage (P=0,002) (KW P=0,013, KW dengue groups P=0,007). E: G-CSF: Levels in patients with severe hemorrhage are significantly increased compared to patients with no hemorrhage (P<0,0001) (KW P=0,005, KW dengue groups P=0,002). Abbreviations: HC = healthy control, No = No occurrence of hemorrhage. KW = kruskal wallis. Horizontal bars inside the boxplot indicate the median. The box indicates the interquartile range. Black asterisk= significantly different from all other groups. Underlined black asterisk = significant difference between two groups. The Mann-Whitney U test was used to compare the groups with each other.

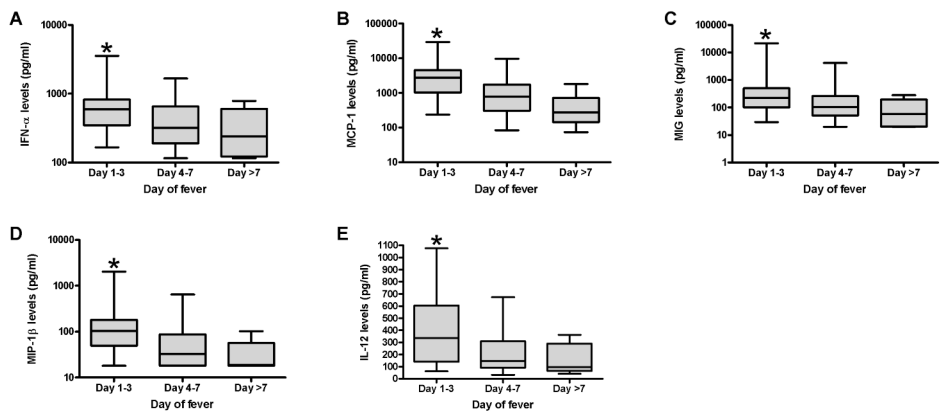

Figure S3 | Levels of cytokines during the course of disease.

A: IFN-α: Levels at day 1-3 were significantly increased compared to day 4-7 (P=0,002) and day>7 (P=0,003) (KW P=0,001). B: MCP-1: Levels at day 1-3 were significantly increased compared to day 4-7 (P<0,0001) and day>7 (P<0,0001) (KW P<0,0001). C: MIG: Levels at day 1-3 were significantly increased compared to day 4-7 (P=0,008) and day>7 (P=0,001) (KW P=0,001). D: MIP-1β: Levels at day 1-3 were significantly increased compared to day 4-7 (P<0,0001) and day >7 (P<0,0001) (KW P<0,0001). E: IL-12: Levels at day 1-3 were significantly increased compared to day 4-7 (P=0,001) and day >7 (P=0,003) (KW P=0,001). Abbreviations: KW = kruskal wallis. Black asterisk= significantly different from all other groups. The Mann-Whitney U test was used to compare the groups with each other.

5

Serum Angiopoietin-2 and Soluble VEGF Receptor 2 are Surrogate Markers for Plasma Leakage in Patients with Acute Dengue Virus Infection.

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Abstract

Background

Endothelial cell dysfunction is believed to play an important role in the pathogenesis of plasma leakage in patients with acute dengue virus (DENV) infection. Several factors, produced by activated endothelial cells, have been associated with plasma leakage or severe disease in patients with infectious diseases.

Objectives

The aim of this study was to investigate which of these markers could serve as a surrogate marker for the occurrence of plasma leakage in patients with acute DENV infection.

Study design

A case-control study was performed in patients with acute DENV infection in Santos, Brazil. Plasma leakage was detected with X-ray and/or ultrasound examination at admission. Serum levels of soluble endoglin, endothelin-1, angiopoietin-2, VEGF, soluble VEGFR-2, MMP-2, MMP-9, TIMP-1 and TIMP-2 were determined using commercially available ELISAs.

Results

Increased levels of angiopoietin-2, endothelin-1 and MMP-2 and decreased levels of soluble VEGFR-2 were significantly associated with the occurrence of plasma leakage. An unsupervised cluster analysis confirmed that angiopoietin-2 and soluble VEGFR-2 were strongly associated with clinical apparent vascular leakage.

Conclusion

Angiopoietin-2 and soluble VEGFR-2 can serve as surrogate markers for the occurrence of plasma leakage in patients with acute DENV infection.

Key words

dengue virus, plasma leakage, vascular permeability, angiopoietin-2, soluble VEGFR-2

Serum Angiopoietin-2 and Soluble VEGF Receptor 2 are Surrogate Markers for Plasma Leakage in Patients with Acute Dengue Virus Infection.

Background

Dengue virus (DENV) is a flavivirus, which is transmitted by the bite of a mosquito. A recent study showed that 390 million persons are infected with DENV each year, of which 96 million develop clinical symptoms [2]. A hallmark of dengue disease is an increase in vascular permeability, presented as pleural effusion and/or ascitis. In severe cases, extensive plasma leakage may lead to the development of hypotension and shock [9].

Endothelial cells play a crucial role in the development of plasma leakage during DENV infection. DENV can infect endothelial cells in vitro, but whether this also occurs in vivo, is still a matter of debate [41,80]. Moreover, it is not clear whether DENV causes vascular permeability by direct infection of endothelial cells or through the release of vasoactive agents by infected monocytes and macrophages, which are the primary target cells of DENV infection [41]. In vitro, direct infection of endothelial cells did not lead to an increase in permeability, while co-incubation of endothelial cells with mononuclear cells or the supernatant from DENV infected monocytic cells did result in an increase [83,84]. This suggests that mechanisms other than direct infection may activate endothelial cells, resulting in an increase in vascular permeability. It is believed that uncontrolled endothelial activation and subsequent dysfunction contributes to the severity of dengue (reviewed in [143]).

Vascular endothelial growth factor (VEGF), initially identified as vascular permeability factor, promotes the growth, proliferation and migration of endothelial cells. VEGF is increased in DENV infected patients with plasma leakage, especially around the time of defervescence [85,144]. VEGF can be bound to sVEGFR-1 and sVEGFR-2, which are expressed predominantly on endothelial cells [145]. Levels of sVEGFR-1 were increased in patients with severe dengue, contrasting with decreased levels of sVEGFR-2 [85].

Matrix metalloproteinases (MMPs) are proteolytic enzymes that can cleave proteins of the extracellular matrix [146]. The activity of these enzymes is regulated by tissue inhibitors of matrix metalloproteinases (TIMPs). Endothelial cells produce MMP-2 and MMP-9 and also TIMP-1 and TIMP-2 [147]. Increased levels of MMP-9 were detected in patients with severe DF compared to mild DF [87]. In the same study, no significant differences were detected in MMP-2 levels between dengue fever patients and healthy controls.

Angiopoietin-1 (Ang-1) is produced by perivascular cells and has a stabilizing effect on the vascular barrier [148]. Angiopoietin-2 (Ang-2) is synthesized by endothelial cells and is a potent inducer of vascular permeability by counteracting the barrier stabilizing effects of Ang-1 [149]. Decreased levels of Ang-1 and increased levels of Ang-2 were correlated with the occurrence of plasma leakage in DENV infected patients, suggesting that an imbalance between these two proteins may be involved in endothelial dysfunction [88].

Activated endothelial cells produce a number of other proteins, including soluble endoglin (sEng) and endothelin-1 (ET-1). Upon inflammation, Eng is cleaved by MMPs and released in the circulation as

sEng. sEng binds to TGF-β1 and abrogates its anti-inflammatory effects. Levels of sEng were increased in children with severe malaria [150]. ET-1 is produced by endothelial cells and is a potent vasoconstrictor and has inotropic, chemotactic and mitogenic properties [151]. Increased levels have been detected in patients with sepsis and malaria [152,153].

Objectives

The aim of this study was to investigate which of the following markers sEng, ET-1, MMP-2, MMP-9, TIMP-1, TIMP-2, Ang-2, VEGF and sVEGFR-2, all produced by activated endothelial cells, could serve as a surrogate marker for the increase in vascular permeability during DENV infection.

Study design

Clinical cohort

This cohort has been previously described [25,124,154,155]. Briefly, during the 2010 outbreak, samples were collected from patients with clinical suspected dengue presenting at the Ana Costa Hospital, Santos, State of São Paulo. Patients were diagnosed with DENV infection by detection of DENV NS1 antigen and/or IgM-specific antibodies using a commercially available rapid test (Dengue duo test bioeasy, Standard Diagnostic Inc., 575-34, Korea) or by detection of DENV RNA by real time PCR (RT-PCR). Serum samples were drawn and stored at -80°C. Patients were classified according to the 2009 WHO classification [1,128] and the occurrence of plasma leakage. The occurrence of plasma leakage was detected by ultrasound or X-ray examination performed upon a clinical suspicion for plasma leakage, such as hemoconcentration, profound thrombocytopenia, tachycardia, hypotension or dehydration. Healthy volunteers with a similar socio-economic background were used as the reference group.

IgG avidity ELISA

The IgG avidity test was used to determine primary or secondary DENV infection [24]. Samples with low avidity IgG antibodies were classified as primary DENV infection, whereas samples with high avidity IgG antibodies were classified as secondary. Samples in which IgG antibodies were not detected could not be classified, although the majority was probably primary DENV infection.

Determination of viral load

Viral load was determined by an "in-house" RT-PCR method. This method has been previously described in detail [25]. RNA was extracted from plasma using the Qiagen Viral RNA kit (Qiagen, Germany).

Table 1 | Characteristics of patients with and without plasma leakage

	No plasma leakage (N=56)	Plasma leakage (N=49)	Healthy controls (N=15)	Significance
Sex	59% male (N=33)	63% male (N=31)	60% male (N=9)	p=0.9 (Chi)
Age*	21 (11-45)	12 (8-29)	25 (24-28)	p=0.001 (KW)
Day of fever*	5 (4-6)	5 (4-7) MV=1	NA	p=0.9 (MWU)
2009 WHO dengue case classification	64% (N=36) WS- 34% (N=19) WS+ 2% (N=1) Severe	100% (N=49) WS+	NA	p<0.0001 (Chi)
Admission	52% (N=29) MV=2	96% (N=47) MV=1	NA	p<0.0001 (F)
Type plasma leakage	–	Ascites: 29% (N=14) Pleural: 16% (N=8) Pleural and pericardium: 55% (N=27)	NA	
Hemorrhagic manifestations	30% (N=17)	47% (N=23)	NA	p=0.1 (F)
Type hemorrhagic manifestation	70% (N=39) No 25% (N=14) Minor mucosal 5% (N=3) Petechiae	53% (N=26) No 31% (N=15) Minor mucosal	NA	
Platelet count*	122.500 (57.250-166.500) MV=2	42.000 (33.000-73.000)	NA	p<0.0001 (MWU)
Viremic	73% (N=41)	59% (N=29)	NA	p=0.2 (F)
Viral copy number in viremic patients (copies/ml)*	198 (122-950)	126 (100-256)	NA	p=0.01 (MWU)
IgG avidity*	21% (N=12) Not detectable 2% (N=1) Primary 77% (N=43) Secondary	2% (N=1) Not detectable 98% (N=48) Secondary	NA	p=0.006 (Chi)

**: Values are in median (interquartile range)*

Abbreviations: Statistical test used is the: Chi = Chi-squared test, KW = Kruskal Wallis test, MWU= Mann-Whitney U test, F= Fisher's exact test. MV= missing value. WS- = non-severe dengue without warning signs. WS+ = non-severe dengue with warning signs. NA= not applicable.

The RT-PCR was conducted in duplicate. SuperScript III Platinum SYBR Green One-Step qRT-PCR kit with ROX (Invitrogen, Inc., EUA) was used. Primers covering all four DENV serotypes were used of which the sequences have been published [21].

Endothelial cell markers

Levels of sEng, ET-1, MMP-2, MMP-9, TIMP-1, TIMP-2, Ang-2, VEGF and sVEGFR-2 were measured using commercially available ELISA kits ('Quantikine', R&D systems, USA). The sensitivity limits in the diluted samples were sEng (0,03 ng/ml), ET-1 (0,207 pg/ml), MMP-2 (0,082 ng/ml), MMP-9 (0,156 ng/ml), TIMP-1 (0,08 ng/ml), TIMP-2 (0,064 ng/ml), Ang-2 (21,3 pg/ml), VEGF (9 pg/ml) and sVEGFR-2 (11,4 pg/ml). The assays were performed according to the manufacturer's instructions. Every sample was run in duplicates. Repetitive freeze-thaw cycles were avoided.

Cluster analysis

The cluster analysis procedure was adapted from van den Ham et al. [129]. Briefly, permeability marker values were log-transformed and subjected to hierarchical correlation clustering (i.e., with distance measure 1 – pearson's pairwise correlation value) using Ward's method that minimizes within-cluster variance. Both patients and permeability markers were clustered to obtain a heatmap. VEGF was excluded and one patient as well, because too many values were missing. Cluster analysis was performed in R 2.15 (R Development Core Team [R Foundation for Statistical Computing], 2012, www.r-project.org). R scripts used to construct the trees and heatmaps are available upon request.

Statistical analysis

The Kruskal Wallis H and the Mann Whitney U test were used for comparisons of more than two or between two groups, respectively. Correlations were determined using the Spearman's correlation coefficient. The Chi-squared test was used to calculate whether the distribution was significantly different between more than two groups and/or conditions and the Fisher's exact test in case of two groups/conditions. P values ≤ 0.05 were considered significant. Using the Bonferroni correction, a p-value cut-off of ≤ 0.02 for endothelial cell marker analyses was applied.

Results

For this study, 105 out of 811 patients with laboratory confirmed acute DENV infection were selected based on the absence or presence of plasma leakage and the availability of sample and clinical data. Forty-nine patients with plasma leakage and fifty-six without were included and they were stratified based on gender and day after the onset of fever. Patients with severe co-morbidity were excluded

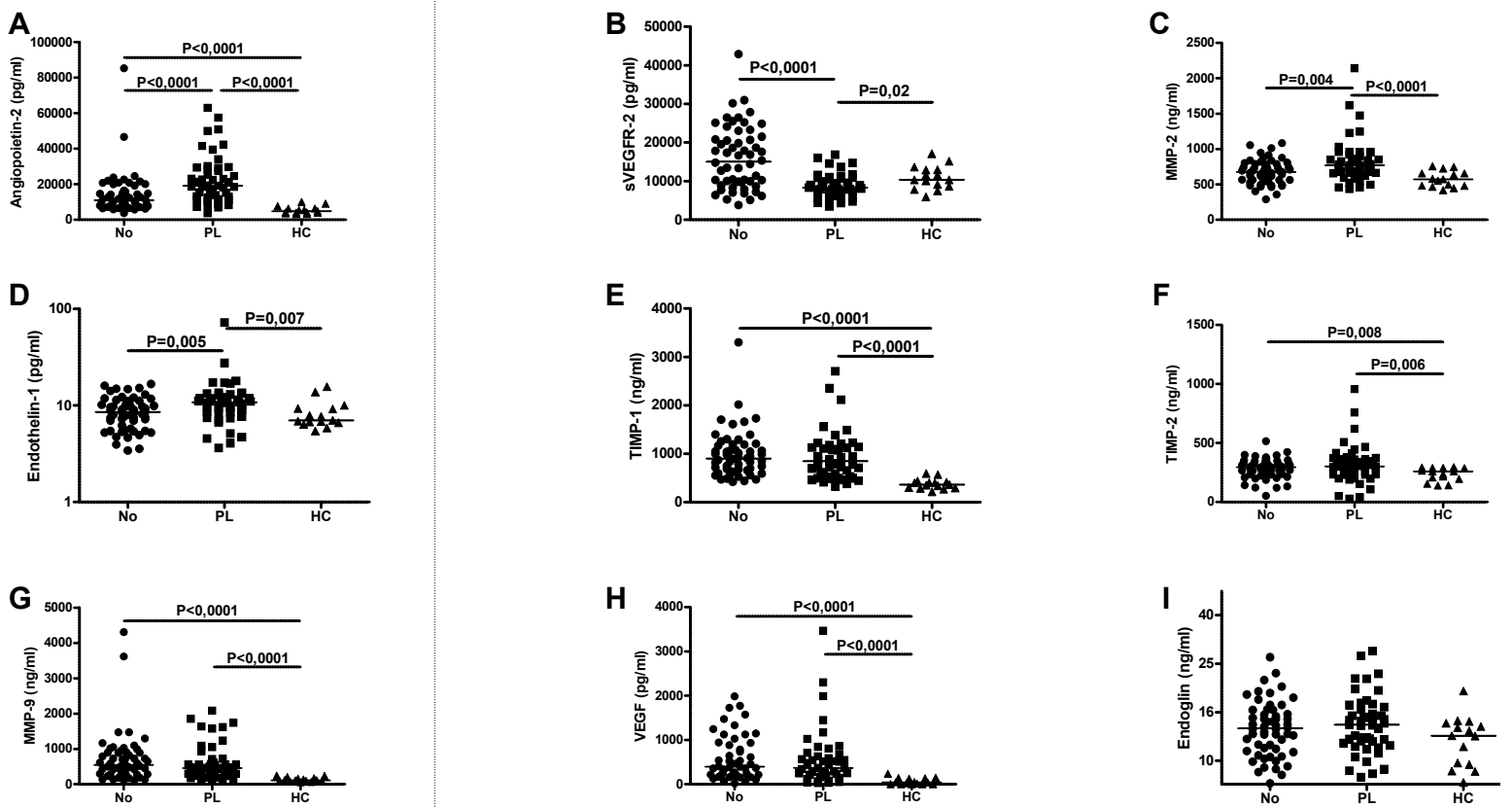


Figure 1 | The association of endothelial markers with the occurrence of plasma leakage.
Levels of Ang-2 (A), MMP-2 (C) and ET-1 (D) were significantly increased and levels of sVEGFR-2 (B) were significantly decreased in patients with plasma leakage compared to patients with no plasma leakage. Levels of Ang-2 (A), TIMP-1 (E), TIMP-2 (F), MMP-9 (G) and VEGF (H) were significantly elevated in dengue patients compared to healthy controls. Levels of sEng (I) were not significantly different in any of the groups. Abbreviations: No = no plasma leakage. PL = plasma leakage. HC = healthy control. Missing values: Ang-2, sVEGFR-2: PL (N=2); MMP-2, ET-1, MMP-9: PL (N=1); TIMP-1, TIMP-2, sEng: no missing values; VEGF: No (N=4), PL (N=5).

from this analysis. The clinical characteristics of the patients are depicted in Table 1.

Patients with plasma leakage were significantly younger (p=0.001), had significantly lower levels of platelets (p<0.0001) and a lower viral load (p=0,01). Patients with plasma leakage were also less often viraemic, although this difference was not significant. The IgG avidity test indicated that 98% of patients with plasma leakage suffered from a secondary DENV infection. Of the patients without plasma leakage, 21% had undetectable IgG antibodies, suggesting that these patients presented with their first DENV episode. When patients were classified according to the 2009 WHO classification 69 % from the WS- and 96 % from the WS+ patients suffered from a secondary DENV infection.

To determine the association of endothelial cell markers with the occurrence of plasma leakage, we have measured the levels of nine endothelial cell markers in the serum of DENV infected patients and healthy controls. Levels of Ang-2, MMP-2 and ET-1 were significantly increased and levels of sVEGFR-2 significantly decreased in patients with plasma leakage compared to patients without plasma leakage (Figure 1). The other markers, TIMP-1, TIMP-2, MMP-9 and VEGF, were significantly elevated in DENV infected patients compared to healthy controls. Levels of sEng were not significantly different in any of the groups.
When patients were classified according to the 2009 WHO dengue case classification, levels of Ang-2 and ET-1 were also significantly elevated and levels of sVEGFR-2 significantly decreased in patients with WS+ compared to WS- dengue (Figure 2).

To investigate whether time after the onset of disease had an impact on the expression of these nine markers, patients were divided into three groups consisting of 2-3 days, 4-6 days and ≥ 7 days after onset of symptoms (Supplementary Figure 1). There were no significant differences between the groups, suggesting that time after the onset of disease, independent from the occurrence of plasma leakage, did not have a major impact on the expression of these markers. Moreover, the Spearman's correlation coefficient was also not significant when the endothelial markers were directly correlated with the day after the onset of disease (Data not shown).

The cluster analysis groups patients based on similarities in the expression of the determined markers (Figure 3). In the resulting heatmap, a dendrogram shows the similarity between the subjects (left side of Figure 3), where subjects in the same branch are more similar to each other than to subjects in other branches. The subject dendrogram was divided into three main clusters of which cluster A contained the largest proportion of DENV infected patients with plasma leakage and cluster B contained the largest proportion of healthy controls (Table 2). Levels of sVEGFR-2 were clearly decreased and levels of Ang-2 increased in cluster A. Plasma leakage occurred significantly more often in cluster A than the other clusters (Chi-squared test, p<0.0001). Levels of MMP-9 were increased in cluster C compared to the other clusters.

The Spearman's correlation coefficient was used to correlate the endothelial permeability markers with the platelet count and with each other. Ang-2 and sVEGFR-2 correlated strongly with the platelet count (Ang-2: ρ = -0.63 (p<0.0001), sVEGFR-2 ρ =0.74 (p<0.0001)) (Figure 4A and 4B). Ang-2 and sVEGFR-2 also correlated strongly with each other (ρ =-0.52 (p<0.0001)) (Figure 4C). Moreover, a strong

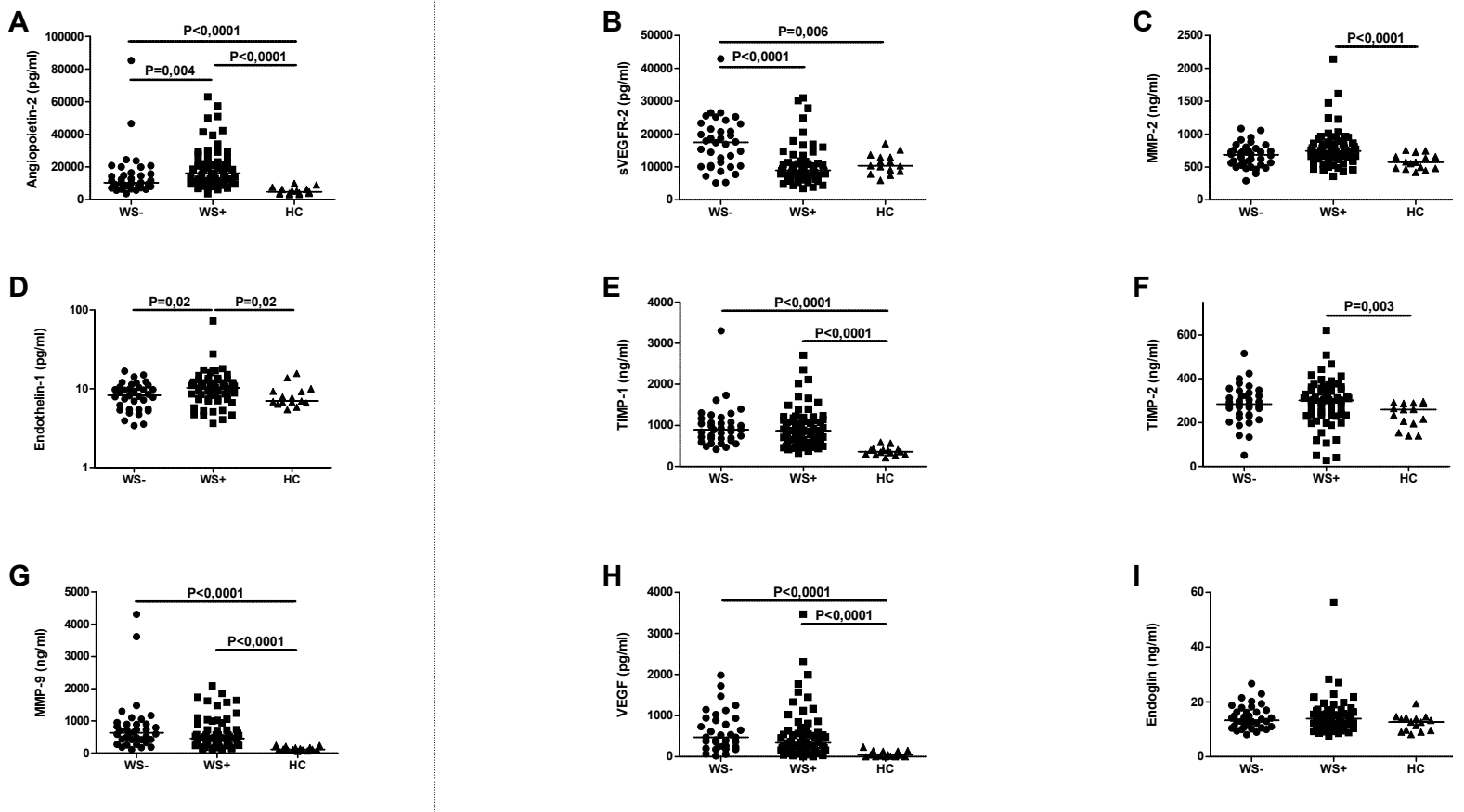


Figure 2 | **The association of endothelial markers with the 2009 WHO dengue case classification.**
Levels of Ang-2 (A) and ET-1 (D) were significantly increased and levels of sVEGFR-2 (B) were significantly decreased in patients with WS+ compared to patients with WS- dengue. Levels of Ang-2 (A), TIMP-1 (E), MMP-9 (G) and VEGF (H) were significantly elevated in dengue patients compared to healthy controls. Levels of MMP-2 (C) and TIMP-2 (F) were significantly increased in WS+ compared to healthy controls. Levels of sEng (I) were not significantly different in any of the groups. Abbreviations: WS- = non-severe dengue without warning signs. WS+ = non-severe dengue with warning signs. HC = healthy control. Missing values: Ang-2, sVEGFR-2: WS+ (N=2); MMP-2, ET-1, MMP-9: WS+ (N=1); TIMP-1, TIMP-2, sEng: no missing values; VEGF: WS- (N=2), WS+ (N=7).

correlation was found between MMP-2 and TIMP-2 (ρ =0.60 (p<0.0001)) (Figure 4D). Interestingly, TIMP-2 has been described to play a central role in modulating MMP-2 activity [156].

Discussion

In this study, we have shown increased levels of Ang-2, MMP-2 and ET-1 and decreased levels of sVEGFR-2 in patients with plasma leakage. Moreover, cluster analysis confirmed that increased expres-

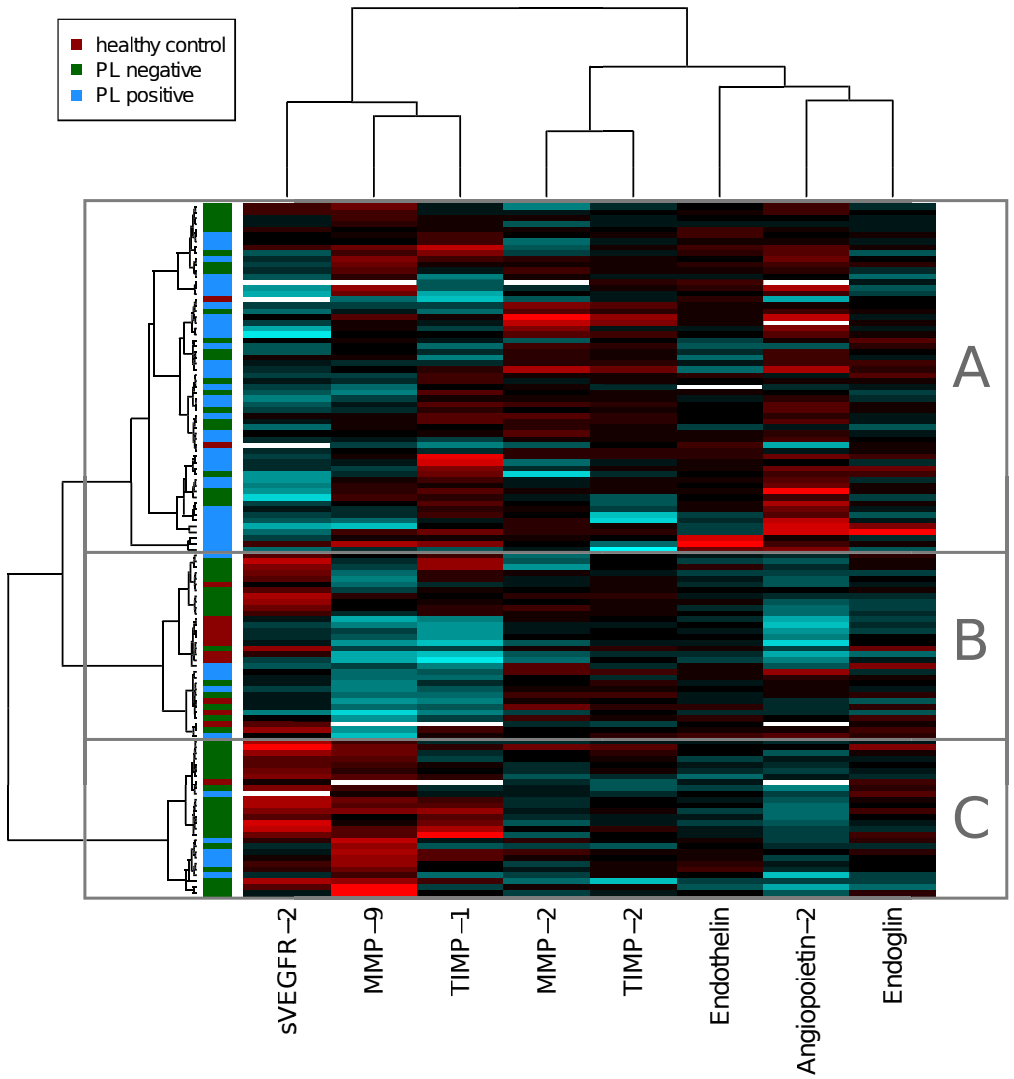


Figure 3 | **Heatmap of the cluster analysis**

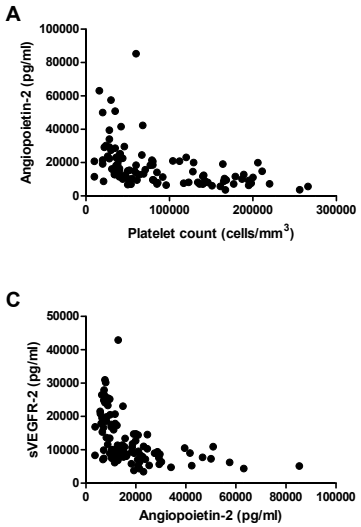
A cluster analysis was performed with eight endothelial markers, which resulted in a dendrogram indicated on the left of the heatmap. Every horizontal line indicates one patient. The vertical bar on the left of the heatmap indicates the occurrence of plasma leakage in the patient. Cluster A had the highest proportion of patients with plasma leakage. Cluster B contained the majority of the healthy controls. Cluster C consisted of mainly DENV infected patients and had a lower proportion of patients with plasma leakage than cluster A.

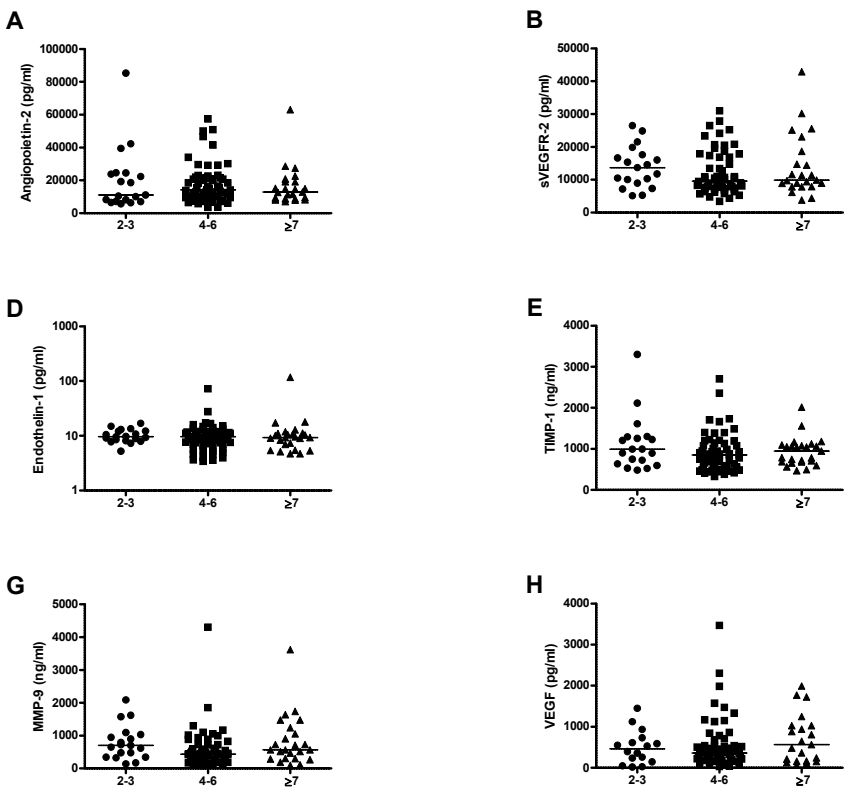
Table 2 | **Characteristics of the three clusters in the cluster analysis**

	Cluster A (N=60)	**Cluster B** (N=32)	**Cluster C** (N=27)	**Statistics**
Age*	15 (11-46)	11 (9-17)	18 (9-33)	p=0.6 (KW)
Gender	65% (N=39) male	53% (N=17) male	63% (N=17) male	p=0.53 (Chi)
2009 WHO dengue case classification	20% (N=12) WS- 75% (N=45) WS+ 2% (N=1) Severe 3% (N=2) HC	31% (N=10) WS- 34% (N=11) WS+ 34% (N=11) HC	52% (N=14) WS- 44% (N=12) WS+ 4% (N=1) HC	p<0.0001 (Chi)
Plasma leakage	62% (N=37)	19% (N=6)	22% (N=6)	p<0.0001 Chi)
Hemorrhage	42% (N=25)	13% (N=4)	41% (N=11)	p<0.0001 (Chi)
Angiopoietin-2* (pg/ml)	20.751 (14.851-27.775) MV=2	8.806 (6.463-12.524) MV=1	8.210 (6.926-9.703) MV=1	NA
Endoglin* (ng/ml)	13,5 (11,5-15,2)	13,4 (10,2-16,0)	14,1 (12,2-17,1)	NA
Endothelin-1* (pg/ml)	10,4 (8,2-12,8) MV=1	8,2 (6,8-11,4)	7,8 (5,5-9,6)	NA
MMP-2* (ng/ml)	756 (645-851) MV=1	685 (587-779)	589 (539-696)	NA
MMP-9* (ng/ml)	470 (297-648) MV=1	170 (115-253) MV=1	982 (721-1511) MV=1	NA
sVEGF-R2* (pg/ml)	7.893 (6.394-10.168) MV=3	10.769 (9.525-18.341)	18.277 (13.342-25.234) MV=1	NA
TIMP-1* (ng/ml)	874 (641-1137)	529 (397-986) MV=1	861 (625-1199) MV=1	NA
TIMP-2* (ng/ml)	302 (233-354)	291 (260-347)	275 (207-304)	NA

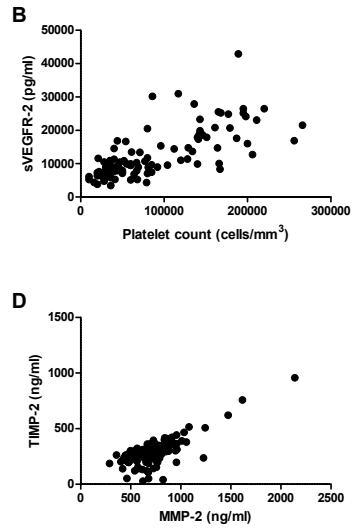
**: Values are in median (interquartile range). Abbreviations: MV=missing value (due to failure in the assay, e.g. variation in duplo too large). KW= Kruskal Wallis test. Chi = Chi-squared test. WS- = non-severe dengue without warning signs. WS+ = non-severe dengue with warning signs. HC = healthy control. NA = not applicable, e.g. it is not permitted to perform statistics on values that determine the formation of the clusters.*

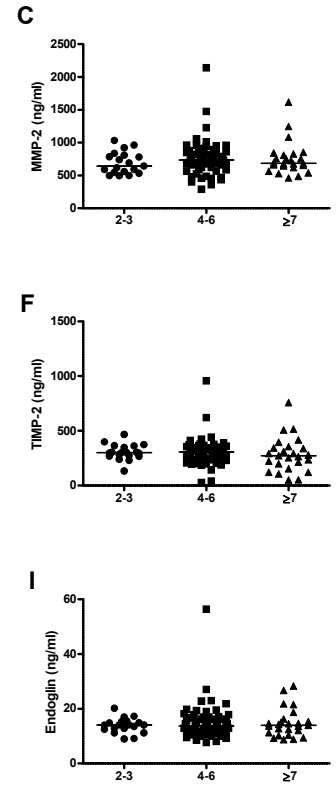
Figure 4 | Angiopoietin-2 and sVEGFR-2 are correlated with the platelet count and each other.
A. Ang-2 shows a significant inverse correlation with the platelet count (ρ = -0,63 (p<0,0001). B. sVEGFR-2 shows a significant direct correlation with the platelet count (ρ =0,74 (p<0,0001)). C. Ang-2 showed a significant inverse correlation with sVEGFR-2 (ρ = -0,52 (p<0,0001)). D. MMP-2 shows a significant direct correlation with TIMP-2 (ρ =0,60 (p<0,0001)). Missing values: platelet count (N=2), Ang-2 (N=2), sVEGFR-2 (N=2), MMP-2 (N=1).





Supplementary figure 1 | Time after the onset of disease does not associate with the endothelial markers.
Number of patients in each group: day 2-3 = 25, day 4-6 = 60, day ≥7 = 20. Levels of the endothelial markers did not associate with time after the onset of disease. Missing values: Ang-2, sVEGFR-2: Day 2-3 (N=2); MMP-2, MMP-9: Day 2-3 (N=1); ET-1 Day 4-6 (N=1); TIMP-1, TIMP-2, sEng: No missing values; VEGF: Day 2-3 (N=4), Day 4-6 (N=2), Day ≥7 (N=3).





sion of Ang-2 and decreased expression of sVEGFR-2 was strongly associated with plasma leakage.

Patients with plasma leakage were significantly younger than patients without plasma leakage, which is in line with epidemiological studies that have shown higher frequencies of plasma leakage and a propensity to develop dengue shock syndrome in children relative to adults [13,14,157]. It has been hypothesized that the capillaries in growing children are more fragile and more sensitive to vasoactive agents than the capillaries of adults [158]. In this study, patients were most frequently included on day 5 after the onset of fever. It has been shown that DENV infected patients are viraemic for five days on average [20]. Moreover, patients with secondary DENV infection showed higher viral titres, but a shorter duration of their viraemia than patients with primary DENV infection [20]. This may explain why patients with plasma leakage in our study had a lower viral load and were more often experiencing a secondary infection compared to patients without plasma leakage.

This is the first time that increased levels of ET-1 have been detected in DENV infected patients with plasma leakage. ET-1 was first isolated from porcine aortic endothelial cells and is known to be a potent inducer of vasoconstriction [151]. In patients with septic shock, elevated levels of ET-1 were associated with disease severity and correlated significantly with a lower cardiac index, most probably due to suppression of the cardiac function by ET-1 [153]. Interestingly, a low cardiac index was also reported in patients with dengue shock syndrome [159].

MMPs can lyse the subendothelial basement membrane and could therefore contribute to the development of hyperpermeability. MMP-2 was significantly elevated in patients with plasma leakage compared to patients without plasma leakage, while MMP-9 was significantly elevated in DENV infected patients compared to healthy controls. It has been shown that DENV infection of microvascular endothelial cells induced secretion of MMP-2 more strongly than secretion of MMP-9 [160]. In contrast, DENV infected dendritic cells secreted higher levels of MMP-9 than MMP-2 [86]. The association between MMP-2 (but not MMP-9) and the occurrence of plasma leakage suggests that endothelial cell activation plays an important role in the development of vascular leakage.

No significant differences in VEGF levels in patients with and without plasma leakage were detected. Some studies have reported increased levels of plasma VEGF in severe versus uncomplicated dengue [85,144,161], while others did not detect any differences in levels between severity groups [162,163]. Both in the supervised clinical classification and in the unsupervised cluster analysis, Ang-2 and sVEGFR-2 were strongly associated with the occurrence of plasma leakage, which is in line with other clinical studies [85,88]. In vitro studies also showed that DENV infection of endothelial cells resulted in decreased levels of sVEGFR-2 and increased Ang-2 expression [85,164]. Ang-2 is synthesized by endothelial cells and quickly released in the circulation upon activation [165]. Ang-1 and Ang-2 are antagonistic ligands of the Tie-2 receptor, a vascular specific tyrosine kinase receptor. Ang-1/Tie-2 signalling brings the endothelium in a quiescent state, while Ang-2/Tie-2 signalling results in endothelial detachment [149]. In line with this study, increased levels of circulating endothelial cells have been detected in patients with DHF [166]. Another study showed that Ang-2 stimulation of endothelial cells resulted in an increase in vascular permeability due to downregulation of proteins in the adherent and tight junctions between the cells [164]. Interestingly, VEGF-sVEGFR-2-signalling also affects the adherens junction, because it causes endocytosis of VE-cadherin in the endothelial cell [167].
Because of the cross-sectional study design we could not draw any conclusions about the predictive value of Ang-2 and sVEGFR-2 before the onset of plasma leakage. However, different analysis techniques all indicated that Ang-2 and sVEGFR-2 are strongly associated with the presence of plasma leakage and can therefore be considered as surrogate markers.

Patients with plasma leakage had significantly lower levels of platelets than those without plasma leakage. Moreover, the platelet count showed a strong correlation with Ang-2 and sVEGFR-2. Thrombocytopenia is one of the hallmarks of a DENV infection and has been shown to correlate inversely with the occurrence of plasma leakage [104]. Besides the low number, it is hypothesized that the function of platelets is also impaired in DENV infected patients [104]. Under normal circumstances, platelets play a crucial role in stabilizing the vascular barrier and therefore, the presence of thrombocytopenia and thrombocytopathy, may contribute highly to endothelial cell dysfunction (reviewed in reference [106]).

In summary, we conclude that Ang-2 and sVEGFR-2 showed a strong correlation with the occurrence of plasma leakage in DENV infected patients. It is important to note that these markers did not suffer interference by the days after the onset of symptoms, suggesting they could serve as surrogate markers for plasma leakage in patients with acute DENV infection.

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Competing interests

None of the listed authors declare conflict of interest apart from Albert Osterhaus who is a part time employee of Viroclinics BV (for details go to www.erasmusmc.nl). The stated competing interest does not alter the author's adherence to the policies on sharing data and materials.

Ethical approval

All procedures adopted in this study were performed according to the terms agreed by the Institutional Review Board from Hospital das Clínicas, University of São Paulo (CAPPesq - Research Projects Ethics Committee). This study was approved by CAPPesq under protocol 0652/09. Written informed consent was obtained from all study volunteers. All included study participants were deidentified and a study number was assigned to guarantee confidentiality.

6

Hyperferritinaemia in Dengue Virus Infected Patients is Associated with Immune Activation and Coagulation Disturbances

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Submitted

Abstract

Background

During a dengue outbreak on the Caribbean island Aruba, highly elevated levels of ferritin were detected in dengue virus infected patients. Ferritin is an acute-phase reactant and hyperferritinaemia is a hallmark of diseases caused by extensive immune activation, such as haemophagocytic lymphohistiocytosis. The aim of this study was to investigate whether hyperferritinaemia in dengue patients was associated with clinical markers of extensive immune activation and coagulation disturbances.

Methodology/principal findings

Levels of ferritin, standard laboratory markers, sIL-2R, IL-18 and coagulation and fibrinolytic markers were determined in samples from patients with uncomplicated dengue in Aruba. Levels of ferritin were significantly increased in dengue patients compared to patients with other febrile illnesses. Moreover, levels of ferritin associated significantly with the occurrence of viraemia. Hyperferritinaemia was also significantly associated with thrombocytopenia, elevated liver enzymes and coagulation disturbances. The results were validated in a cohort of dengue virus infected patients in Brazil. In this cohort levels of ferritin and cytokine profiles were determined. Increased levels of ferritin in dengue virus infected patients in Brazil were associated with disease severity and a pro-inflammatory cytokine profile.

Conclusions/significance

Altogether, we can conclude that ferritin can be used as a clinical marker to discriminate between dengue and other febrile illnesses. The occurrence of hyperferritinaemia in dengue virus infected patients is indicative for highly active disease resulting in immune activation and coagulation disturbances. Therefore, we suggest that patients with hyperferritinaemia should be monitored carefully.

Keywords

dengue virus infection, ferritin, immune activation, cytokine, coagulation

Hyperferritinaemia in Dengue Virus Infected Patients is Associated with Immune Activation and Coagulation Disturbances

Author summary

Ferritin is an acute-phase reactant and produced by reticulo-endothelial cells in response to inflammation and infection. In general, ferritin levels are increased in inflammatory conditions, but in this study we found that ferritin levels were much higher in dengue virus infected patients than in patients with other febrile illnesses. This indicates that ferritin could be used as a marker to discriminate between dengue and other febrile diseases. Moreover, the presence of hyperferritinaemia (ferritin levels≥ 500 μg/L) was associated with markers of immune activation and coagulation disturbances and clinical disease severity, suggesting that it could serve as a marker of activity of disease. Clinical markers to determine the presence and severity of dengue virus infection are important for diagnostic and treatment purposes. Our results indicate that increased ferritin levels could be used to increase the likelihood on a positive dengue diagnosis. Moreover, patients with hyperferritinaemia should be monitored carefully, because they are at risk to develop severe disease due to extensive immune activation.

Introduction

Outbreaks of dengue virus (DENV) infection have become more frequent in the American and Caribbean region, even threatening to spread in the United States [168]. DENV is a flavivirus, which is transmitted by the bite of an Aedes mosquito. Brazil is the country with most reported dengue cases in the Americas. A large DENV-2 outbreak in 2010 caused more than 34.000 cases and 64 deaths in the State of São Paulo, Brazil [124]. On the Caribbean island Aruba, there was an epidemic from September 2011 till April 2012, in which DENV-1 and DENV-4 were both co-circulating.

The symptoms of DENV infection are mild and self-limiting in the majority of cases, consisting of fever, headache, retro-orbital pain, myalgia, arthralgia, thrombocytopenia, minor mucosal bleeding and skin manifestations. Some patients develop severe symptoms, such as shock, severe bleeding or organ impairment. These symptoms usually develop three to five days after the onset of disease around the time of defervescence. It has been hypothesized that severe dengue is caused by a cytokine storm inducing systemic inflammatory effects (Reviewed in [29]). The pathophysiological mechanisms that cause this cytokine storm are not fully unravelled and represent an important focus for dengue research.

In addition to the current laboratory markers for dengue, ferritin levels were described to be associated with clinical disease severity in children [169]. In many cases ferritin levels higher than 500 μg/L were detected, defined as hyperferritinaemia [170]. Ferritin is an acute-phase reactant and highly expressed by cells of the reticulo-endothelial system in response to infection and inflammation. Ferritin binds iron, limiting its availability in the circulation. Because many pathogenic microorganisms need iron for their proliferation, this mechanism is favourable for the host.

Moreover, iron deficiency enhances the immunological performance of lymphocytes, neutrophils and macrophages (reviewed in [171]).
Hyperferritinaemia is a hallmark of diseases, characterized by extensive immune activation, including haemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS). HLH can be congenital or triggered by an external stimulus, such as malignancy or viral infection, including dengue [172]. The cytotoxic function of NK cells and CD8+ T lymphocytes is impaired in patients with HLH, which results in reduced clearance of infected and antigen-presenting cells from the circulation. This may lead to an exaggerated immune response with proliferation of dendritic cells, tissue macrophages and T-cells, contributing to a cytokine storm (reviewed in [173]). The symptoms of HLH consist of ongoing fever, hepatosplenomegaly, cytopenia (affecting more than 2 cell lineages), hypofibrinogenaemia, hypertriglyceridaemia, hyperferritinaemia, increased levels of sIL-2R and coagulopathy (reviewed in [170]).
Although clinical symptoms of DENV infection are usually rather mild compared to HLH, some patients develop severe symptoms. These are most probably caused by extensive immune activation and show similarities with the clinical hallmarks of HLH and MAS, suggesting similar pathophysiology.

The aim of this study was to investigate the association between hyperferritinaemia, immune activation and coagulation disturbances in DENV infected patients. We showed that the presence of hyperferritinaemia could discriminate between dengue and other febrile diseases. Moreover, we found an association between increased ferritin levels and severe clinical disease, thrombocytopenia, liver enzyme and coagulation disturbances and a pro-inflammatory cytokine profile.

*In one patient ferritin levels were not determined. Abbreviations: WS- = non-severe dengue without warning signs. WS+ = non-severe dengue with warning signs, OFI= other febrile illness, DENV= dengue virus. *= Values are in median (interquartile range)*

Table 1 | General characteristics cohort

	2009 WHO classification			Presence of hyperferritinaemia			
	WS-	**WS+**	**Fisher's exact test**	**No hyperferritinaemia**	**Hyperferritinaemia**	**Fisher's exact test**	**OFI**
Number of patients	17	27		24	19		17
Age*	49 (30-57)	42 (31-63)		40 (30-55)	49 (32-64)		35 (29-52)
Sex	8 male (47%)	10 male (37%)	P=0.6	7 male (29%)	11 male (58%)	P=0.07	6 male (35%)
Viraemic	10 (59%)	18 (67%)	P=1.0	12 (50%)	16 (84%)	P=0.05	NA
Serotype	5 DENV-1 3 DENV-4	3 DENV-1 1 DENV-2 8 DENV-4		5 DENV-1 2 DENV-4	3 DENV-1 1 DENV-2 9 DENV-4		NA
Hospitalization rate	4 (24%)	11 (41%)	P=0.3	6 (25%)	8 (42%)	P=0.3	NA

Materials and methods

Cohort Aruba and Brazil

Ethics statement

The study in Aruba was approved by the Institutional Review Board of the Dr. Horacio Oduber Hospitaal in Aruba. The study in Brazil was approved by the Institutional Review Board from Hospital das Clínicas, University of São Paulo (CAPPesq - Research Projects Ethics Committee) under protocol 0652/09. Patients in both studies were included after written informed consent was obtained. In case of participants younger than 18 years informed consent was obtained from their parent or legal guardian. Clinical data and blood samples were anonymized with a study number.

In Aruba, all patients of 16 years and older with a clinical suspicion of dengue, presenting at the emergency room of the Dr. Horacio Oduber Hospitaal and one clinical practice between September 2011 and April 2012, were included. Pregnant women and patients with no available data about clinical symptoms were excluded. After obtaining informed consent, the treating physician filled out a standard case report form (CRF). Patients were admitted to the hospital when at least one of the following symptoms was present: systolic blood pressure lower than 90 mm Hg, diastolic blood pressure lower than 60 mmHg, pulse rate higher than 100/min, signs of dehydration, platelet count lower than 50.000 cells/mm^3, increase in haematocrit above 50%, or signs of mucosal bleeding. Patients were classified according to the 2009 WHO dengue case classification [1,128]. Briefly, patients with fever and general symptoms were classified as non-severe dengue without warning signs (WS-). Patients with one of the following warning signs were classified as non-severe dengue with warning signs (WS+): abdominal pain, vomiting, minor mucosal bleeding, pleural effusion, ascites and hepatomegaly. Patients with shock, respiratory distress, severe bleeding and/or organ impairment were classified as severe dengue.
Serum and plasma samples were drawn at day 2-3, 4-5 and 6-8 and 28 days after the onset of fever. Samples not immediately used, were stored at -80°C and shipped to The Netherlands on dry ice. Repetitive freeze-thaw cycles were avoided. The samples from day 28 were used as an autologous control group.
The clinical diagnosis of dengue was confirmed either by a positive NS1 antigen test ('Platelia', Bio-Rad,

France) at day 2-3 and/or a positive IgM enzyme-linked immunosorbent assay (ELISA) ('IgM Capture DxSelect', Focus Diagnostics, USA) at day 4-5 and/or an increase in IgG titre ('IgG Capture DxSelect', Focus Diagnostics, USA) between the acute and convalescent sample. Patients with a negative NS1, IgM and IgG in the acute phase sample and a negative IgG in the convalescent sample were diagnosed as Other Febrile Illness (OFI). If the IgG was positive in the acute phase sample, but the convalescent sample was missing, patients were considered inconclusive and excluded from this analysis.

The cohort in Brazil was described previously [155]. Briefly, during the 2010 DENV-2 outbreak, patients with clinical suspected dengue fever presenting at the Ana Costa Hospital, Santos, State of São Paulo were included. Patients were diagnosed with DENV infection by detection of DENV NS1 antigen and/or IgM-specific antibodies using a commercially available rapid test (Dengue Duo Test Bioeasy, Standard Diagnostic Inc. 575-34, Korea) or by detection of DENV RNA by real time PCR (RT-PCR). Serum samples were drawn and stored at -80°C. Patients were classified according to the 2009 WHO classification and the occurrence of haemorrhagic manifestations and the occurrence of plasma leakage and shock [1,128]. Age-matched healthy volunteers with a similar socio-economic background were used as controls.

Serotype- and copy number quantitative reverse transcriptase PCR (RT-PCR) on serum samples from Aruba

In order to determine the infecting serotype a semi-quantitative RT-PCR (Taqman) was performed. Primers and probes directed against the capsid were derived from Sadon et al. [174]. Briefly, 4X TaqMan Fast Virus 1-step Master Mix (Invitrogen) was used with 20 pmol of primers and 10 pmol of probes. The cycling program consisted of 5 minutes at 50°C, then 20 seconds at 95°C followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C.

Another quantitative RT-PCR was performed to determine the viral copy number. The primers and probes directed against the 3'UTR were derived from Drosten et al. [175]. Briefly, 4X TaqMan Fast Virus 1-step Master Mix was used with 15 pmol of primers and 10 pmol of probes and an additional 25 mM of $MgCl_2$ was added [176]. The cycling program was similar to the serotype quantitative RT-PCR.

Ferritin and cytokines Aruba

Plasma ferritin concentrations were determined at the Landslaboratorium in Aruba within a few hours after blood sampling. The assay was performed using the 'Access' (Beckman Coultier, USA) under standardized conditions.

Serum sIL-2Rα and IL-18 levels were determined at the department of experimental internal medicine from the Radboud University. sIL-2Rα was measured using a commercially available luminex kit ('Milliplex', Merck Millipore, Germany). Samples were diluted 1:5 and the assay was performed according to the manufacturer's instructions and run on a Luminex 200 dual laser detection system. The sensitivity limit was 15 pg/ml. Levels of IL-18 were measured using a commercially available ELISA kit (MBL, Japan) according to the manufacturer's instructions.

Markers of coagulation Aruba

All markers of coagulation were determined in citrate samples.

All markers of coagulation were determined in citrate samples.

Activated partial thromboplastin time (APTT) and prothrombin time (PT) were determined at the Landslaboratorium in Aruba within a few hours after blood withdrawal. PT (Dade Innovin) and APTT (Dade Actin FSL) were determined on a Sysmex CA-1500 System (Siemens Healthcare Diagnostics, USA).

All other coagulation parameters were determined at the department of Experimental Vascular Medicine from the Academic Medical Centre. Von Willebrand Factor (vWF) was measured using a homemade ELISA with antibodies from DAKO (Glostrup, Denmark). In vitro thrombin generation was assayed by measuring peak thrombin levels with the Calibrated Automated Thrombography (CAT) as described previously [177]. In vivo thrombin generation was determined by detecting thrombin-antithrombin complexes (TAT) using a commercially available ELISA (Enzygnost). Levels of the fibrinolytic markers plasminogen activator inhibitor type 1 (PAI-1) and plasmin-α2-antiplasmin (PAP) complexes were measured with commercially available ELISAs according to the manufacturer's instructions (PAI-1, Hyphen BioMed; PAP complexes, DRG Diagnostics). D-dimer levels were determined with a particle-enhanced immunoturbidimetric assay (Innovance D-dimer, Siemens Healthcare Diagnostics).

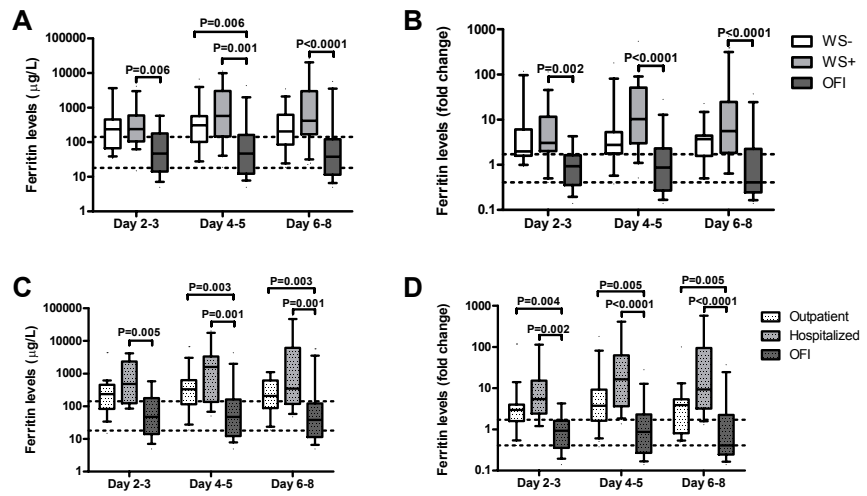

Figure 1 | Ferritin levels and fold change are increased in dengue patients

Absolute levels of ferritin were significantly increased in WS+ patients compared to OFI at each time point and in WS- patients compared to OFI at day 4-5 (A). Absolute levels were also elevated in hospitalized and outpatients compared to OFI (C) at almost each time point. The ferritin fold change was significantly elevated in WS+ patients compared to OFI (B) and in hospitalized and outpatients compared to OFI (D) at each time point. Area between two dotted horizontal lines: interquartile range from the autologous control group. Boxplots indicate the interquartile range, the horizontal line inside the box indicates the median. The whiskers reach from the 10th till the 90th percentile. P-value ≤ 0.006 is considered significant.

Ferritin and cytokine assays Brazil

Serum ferritin levels were determined with a commercially available ELISA (Biolisa ferritina, Bioclin, Brazil) performed according to the manufacturer's instructions.

The measurement of cytokines and the cluster analysis have been described in a previous publication [155]. Briefly, levels of thirty cytokines were measured using a multiplex immunoassay kit with spectrally encoded antibody-conjugated beads (Human Cytokine 30-plex panel, Invitrogen, USA). Twenty-three cytokines were used in a cluster analysis procedure, which was adapted from van den Ham et al. [129]. Briefly, cytokine values were log-transformed and subjected to hierarchical correlation clustering (i.e., with distance measure 1 – pearson's pairwise correlation value) using Ward's method.

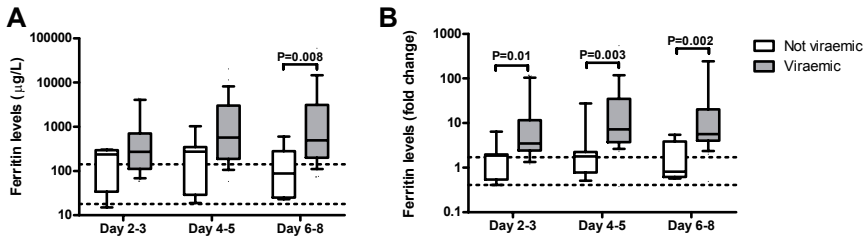

Figure 2 | **Ferritin levels and fold change are associated with viraemia**

The absolute ferritin levels were significantly increased in viraemic patients compared to non-viraemic patients at day 6-8 (A) and the ferritin fold change was significantly elevated in viraemic patient at each time point (B). Area between two dotted horizontal lines: interquartile range from the autologous control group. Boxplots indicate the interquartile range. The horizontal line inside the box indicates the median. The whiskers reach from the 10th till the 90th percentile. P-value ≤ 0.02 is considered significant.

Statistics

IBM SPSS Statistics v.20 was used to calculate statistical significance. The Mann Whitney U test was used to compare the difference between two groups. The Spearman's correlation coefficient was applied to calculate correlations. The Chi-Squared test was used to calculate differences in proportions between groups and the Fisher's exact to determine whether one distribution was unequally distributed over the groups. Using bonferroni correction the p-value was adjusted for multiple testing.

Results

Seventy-three patients were included in Aruba between September 2011 and April 2012. The clinical diagnosis of forty-four patients could be confirmed by serology and/or RT-PCR (Table 1). Seventeen patients tested negative for DENV infection and were included in the OFI group. Twelve patients were excluded with an inconclusive diagnosis. In that particular season, both DENV-1 and DENV-4 were circulating. One patient tested positive for DENV-2, but this patient was probably infected in Suriname. The epidemic was rather mild and only one case of severe dengue was recorded according to the 2009 WHO dengue case classification. This patient presented with melaena and was included in the WS+ group. From the other dengue positive patients, seventeen were classified as WS- and twenty-six as WS+ dengue. The most common warning sign was abdominal pain (20/44, 45%) followed by vomiting (10/44, 23%) and in a few patients epistaxis (2/44, 5%) and hepatomegaly (1/44, 2%) was reported. Pleural effusion and ascites were not reported, probably because ultrasound and/or X-ray examination were performed on a limited basis. Fifteen dengue patients were admitted to the hospital.
A total of 191 samples were included in this analysis collected at day 2-8 after the onset of fever (sample size in supplementary table 1). Follow-up samples collected at day 28 from dengue and OFI patients served as an autologous control group.

Ferritin expression is associated with dengue virus infection and viraemia

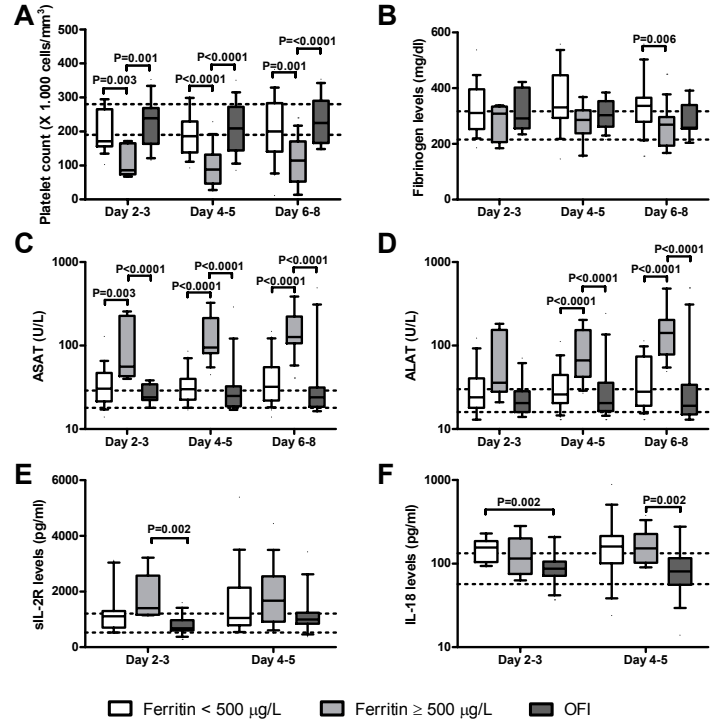

Figure 3 | **Hyperferritinaemia is associated with certain markers of HLH and MAS**

The platelet count (A) was significantly decreased and the liver enzyme ASAT (C) significantly increased in patients with hyperferritinaemia compared to patients with no hyperferritinaemia and OFI at each time point. Levels of fibrinogen were significantly decreased in patients with hyperferritinaemia compared to patients without hyperferritinaemia at day 6-8 (B). Levels of ALAT were significantly increased in patients with hyperferritinaemia at day 4-5 and 6-8 (D). Levels of sIL-2R were significantly increased in patients with hyperferritinaemia compared to OFI at day 2-3 (E). Levels of IL-18 were significantly increased in patients with no hyperferritinaemia compared to OFI at day 2-3 and in patients with hyperferritinaemia compared to OFI at day 4-5 (F). Missing values: Platelet count, ASAT, ALAT: no missing values. sIL-2R and IL-18: Day 2-3: No HF (N=3), HF (N=2). Day 4-5: No HF (N=5), HF (N=3). Area between two dotted horizontal lines: interquartile range from the autologous control group. Boxplots indicate the interquartile range, the horizontal line inside the box indicates the median. The whiskers reach from the 10th till the 90th percentile. P-value ≤ 0.006 is considered significant.

Ferritin levels were determined in patients with dengue and OFI to identify any association with disease severity. Using the 2009 WHO dengue case classification, ferritin levels were significantly increased in WS+ patients compared to OFI at each time point and in WS- patients compared to OFI at day 4-5 (Figure 1A). At day 4-5 and 6-8 the highest ferritin levels were observed and a tendency was shown towards higher ferritin levels in WS+ patients compared to WS-, although these differences were not statistically significant.

In clinical practice and according to the official HLH-criteria, ferritin levels ≥ 500 µg/L are considered hyperferritinaemia [170]. A larger proportion of males showed hyperferritinaemia compared to females in this cohort, which approached statistical significance (Table 1). It is known that baseline ferritin

levels are higher in the male than in the female population [178]. We calculated a fold change by dividing the absolute values of ferritin by the median ferritin levels for males and females from the autologous control group (Females: /37 µg/L and males: /154 µg/L), which were similar to levels previously described [178]. The ferritin fold change was significantly increased in WS+ dengue patients compared to OFI at each time point (Figure 1B).

Another marker of disease severity is the hospitalization rate. Absolute levels and the ferritin fold change were significantly increased in hospitalized and outpatients compared to OFI at almost all time points (Figure 1C and 1D). The absolute ferritin levels as well as the fold change showed a tendency of increased values in hospitalized patients compared to outpatients.
Because the difference in ferritin levels between patients with dengue and OFI was significant, we calculated an odds ratio for the occurrence of hyperferritinaemia and a confirmed diagnosis of DENV infection. In dengue patients, 19 out of 43 had hyperferritinaemia compared to two out of 17 patients with OFI. This resulted in a sensitivity of 44%, a specificity of 88% and an odds ratio of 6, suggesting that the occurrence of hyperferritinaemia could serve as a discriminatory marker between dengue and OFI.

The presence or absence of viraemia in the early phase was linked to ferritin levels during the course of disease. Patients were considered viraemic if they had detectable virus titres at day 2-3 and day 4-5. Patients with undetectable levels at these days were considered non-viraemic.
The absolute ferritin levels were significantly elevated in viraemic patients compared to non-viraemic patients at day 6-8 (Figure 2A). The ferritin fold change was significantly elevated in viraemic patients at all time points (Figure 2B). There were no strong correlations between the viral load and the levels of ferritin at the same day of disease. However, absolute levels of ferritin at day 6-8 correlated significantly with the viral copy number at day 2-3 (ρ=0.5; P=0.008) and day 4-5 (ρ=0.5; P=0.002). The ferritin fold change at day 6-8 also showed a significant correlation with the viral load at day 2-3 (ρ=0.5; P=0.003) and day 4-5 (ρ=0.6; P<0.0001). This suggests that viral replication in the early phase of disease may cause an increase in ferritin levels in the convalescent phase.

Hyperferritinaemia in dengue is associated with thrombocytopenia and elevated liver enzymes

Hyperferritinaemia is a prominent symptom of patients with HLH. To investigate whether the clinical picture of DENV infection shows more similarities, the official diagnostic criteria for HLH [170] were linked to hyperferritinaemia in dengue patients. In each patient the occurrence of hyperferritinaemia was evaluated at each time point.
Severe cytopenia in at least two cell lineages is a prominent feature of HLH due to the increased phagocytic activity of macrophages. In our cohort the platelet count was significantly decreased in patients with hyperferritinaemia compared to patients with no hyperferritinaemia and OFI at each time point (Figure 3A). No significant differences in the leukocyte count were detected (Data not shown).
Another criterium is the presence of hypertriglyceridaemia and/or hypofibrinogenaemia. Levels of fibrinogen were significantly decreased in patients with hyperferritinaemia compared to patients without hyperferritinaemia at day 6-8, but levels were still in the range of the autologous control group (Figure 3B). The triglyceride levels were in the normal range of the autologous control group in both dengue as well as OFI patients (data not shown).
MAS is characterized by hepatosplenomegaly and liver dysfunction. The liver also plays an important role in the pathogenesis of DENV infection. Levels of the liver enzyme ASAT were significantly increased in patients with hyperferritinaemia compared to patients with no hyperferritinaemia and OFI at each time point (Figure 3C). ALAT levels were also significantly increased in patients with hyperferritinaemia at day 4-5 and 6-8 (Figure 3D).
sIL-2R is a marker of T-cell activation and IL-18 of macrophage activation. sIL-2R was significantly increased in patients with hyperferritinaemia compared to OFI at day 2-3 (Figure 3E). Levels of IL-18 were significantly elevated in patients with no hyperferritinaemia compared to OFI at day 2-3 and in patients with hyperferritinaemia compared to OFI at day 4-5 (Figure 3E).

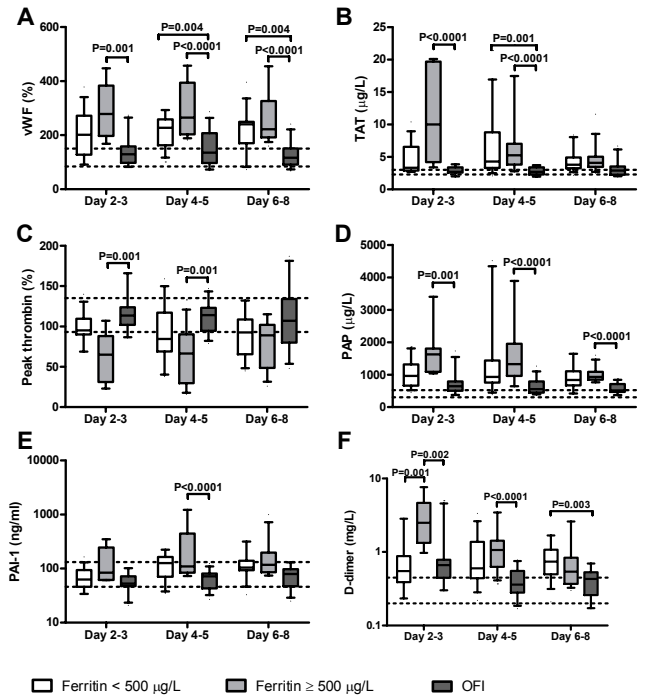

Figure 4 | **Hyperferritinaemia is associated with markers of coagulation and fibrinolysis**
Levels of vWF were significantly increased in patients with hyperferritinaemia compared to OFI at each time point and significantly increased in patients without hyperferritinaemia compared to OFI at day 4-5 and 6-8 (A). Levels of TAT (B) were significantly increased and levels of peak thrombin (C) significantly decreased in patients with hyperferritinaemia at day 2-3 and 4-5. Levels of PAP were significantly increased in patients with hyperferritinaemia compared to OFI (D). Levels of PAI-1 were significantly increased in patients in patients with hyperferritinaemia compared to OFI at day 4-5 (E). Levels of D-dimer were significantly increased in patients with hyperferritinaemia compared to OFI at day 2-3 and 4-5 and in patients with hyperferritinaemia compared to patients without hyperferritinaemia at day 2-3 (F). Missing values vWF, PAI-1: Day 2-3: OFI (N=1). Day 4-5: HF (N=1); TAT, PAP, D-dimer: No missing values; Peak thrombin: Day 2-3: No (N=1), OFI (N=1). Day 4-5: No (N=1), HF (N=4), OFI (N=2). Day 6-8: No (N=2), HF (N=1), OFI (N=2).
Area between two dotted horizontal lines: interquartile range from the autologous control group. Boxplots indicate the interquartile range, the horizontal line inside the box indicates the median. The whiskers reach from the 10th till the 90th percentile. P-value ≤ 0.006 is considered significant.

Altogether, we can conclude that hyperferritinaemia in uncomplicated dengue patients is strongly associated with thrombocytopenia and elevated liver enzymes, but these patients had no hypertriglyceridaemia, hypofibrinogenaemia or cytopenia in another lineage than the platelets.

Hyperferritinaemia is associated with activation of coagulation and fibrinolysis

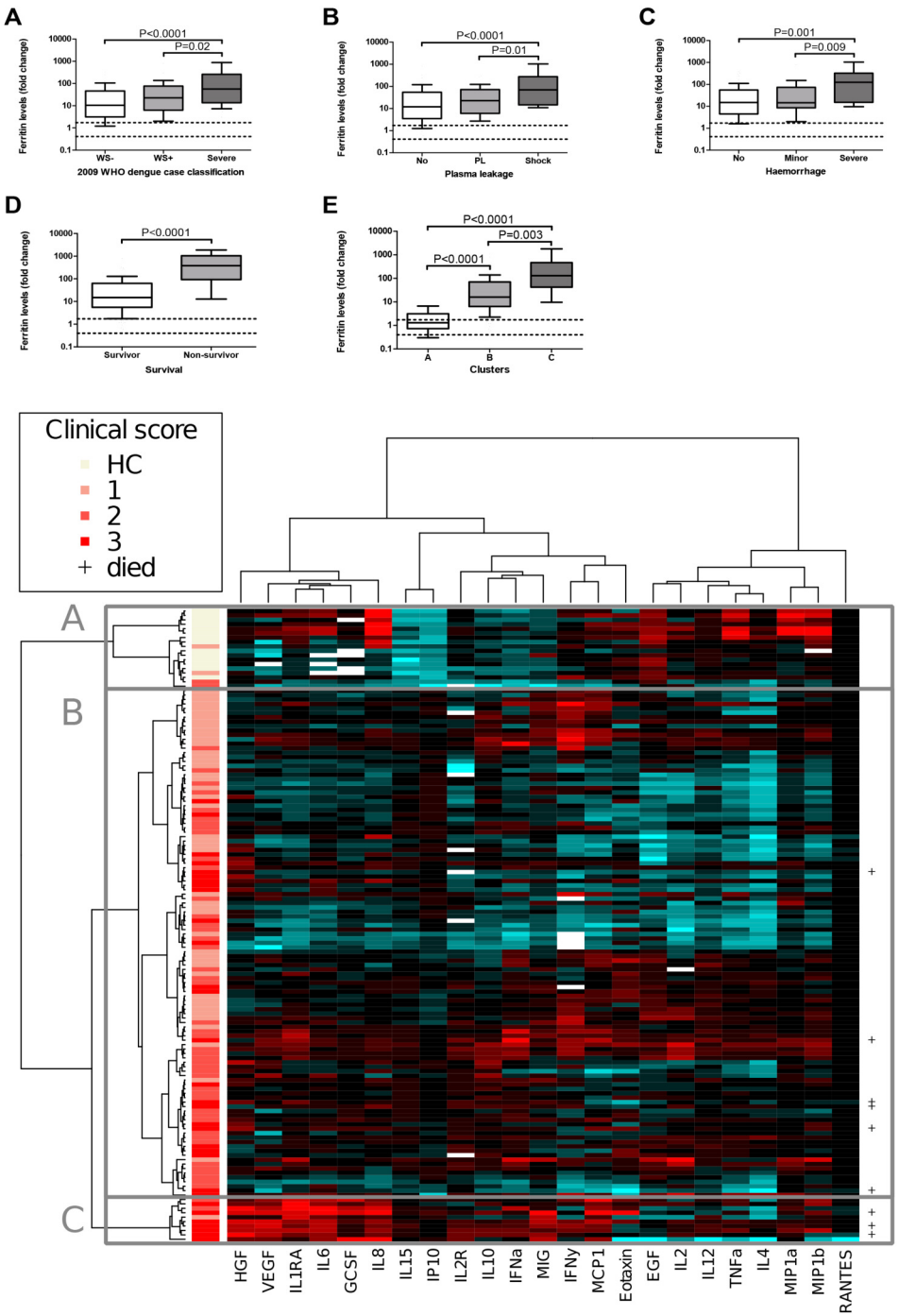

Figure 5 | Ferritin fold change is significantly associated with clinical disease severity and a pro-inflammatory cytokine profile

The ferritin fold change was significantly elevated in patients with clinically severe disease as shown by classifying patients according to the 2009 WHO classification (A), occurrence of plasma leakage and shock (B), occurrence of haemorrhage (C) and survival (D). Moreover, levels were also significantly increased in Cluster C, which contains severely ill dengue patients with a pro-inflammatory cytokine profile (E). Abbreviations: WS- = non-severe dengue without warning signs. WS+ = non-severe dengue with warning signs. PL=plasma leakage.

Area between two dotted horizontal lines: interquartile range from the healthy control group (N=14). Boxplots indicate the interquartile range, the horizontal line inside the box indicates the median. The whiskers reach from the 10th till the 90th percentile. P-value ≤ 0.02 is considered significant.

Heatmap: This heatmap has been published previously [155]. A cluster analysis was performed with 23 cytokines, which resulted in a dendrogram indicated on the left of the heatmap. Every horizontal line indicates one patients. The vertical bar on the left of the heatmap indicates the disease severity of the patient. A: Cluster with mainly healthy controls and four dengue patients. B: Cluster with mild to moderately ill dengue virus infected patients. C: Cluster with severely ill dengue virus infected patients. Abbreviations: 1: non-severe dengue without warning signs. 2: non-severe dengue with warning signs. 3: severe dengue. +: patient died within 14 days after the onset of fever.

Hyperferritinaemia was investigated in association with parameters, indicating the activation of coagulation and fibrinolysis. The APTT and PT showed no significant differences between any of the groups (data not shown).

vWF is released upon endothelial cell activation and plays an important role in the formation of the thrombus. Significantly increased levels were found in patients with hyperferritinaemia compared to OFI at day 2-3 and in both dengue groups compared to OFI at day 4-5 and 6-8 (Figure 4A).

Activation of the coagulation cascade starts with thrombin generation after which it is bound by antithrombin. Thrombin-antithrombin (TAT) complexes are a marker for activation of the coagulation cascade in vivo. Levels were significantly elevated in dengue patients with hyperferritinaemia compared to OFI at day 2-3 and 4-5 and also in patients without hyperferritinaemia compared to OFI at day 4-5 (Figure 4B).

The ability of plasma to generate thrombin in vitro can be investigated by the calibrated automated thrombogram measuring peak thrombin levels. Interestingly, while the levels of TAT were significantly increased, the peak thrombin levels were significantly decreased in patients with hyperferritinaemia compared to OFI at day 2-3 and 4-5 (Figure 4C).

Thrombin generation will lead to fibrin formation and eventually fibrinolysis, resulting in the formation of plasminogen-α2-antiplasmin (PAP) complexes. PAP showed increased levels in patients with hyperferritinaemia compared to patients with OFI at each time point (Figure 4D).

Plasminogen activator inhibitor-1 (PAI-1) can counteract the fibrinolytic system. Levels of PAI-1 were significantly elevated in patients with hyperferritinaemia compared to OFI at day 4-5 (Figure 4E). Activation of the coagulation and fibrinolytic systems eventually result in the production of D-dimers (Figure 4F). Levels of D-dimers were significantly increased in patients with hyperferritinaemia compared to patients with no hyperferritinaemia and OFI at day 2-3 and levels were significantly elevated in patients with hyperferritinaemia compared to OFI at day 4-5 and in patients without hyperferritinaemia compared to OFI at day 6-8.

The coagulation and fibrinolytic systems are highly activated in dengue patients and dengue patients with hyperferritinaemia in particular. The strongest activation was shown at day 2-3 and 4-5 after onset of fever with increased levels of vWF, TAT, PAP and D-dimer.

Ferritin levels in a Brazilian cohort are associated with disease severity and immune activation

To confirm our findings concerning ferritin levels in the cohort from Aruba, we studied ferritin in a previously published dengue cohort obtained during the 2010 DENV outbreak in Brazil. This cohort consisted of 50 WS-, 49 WS+ and 33 severe dengue patients (More clinical details about this cohort are described in the previous publication and information about the sample size in supplementary table 1 [155]).
In this cohort the ferritin fold change was calculated with the same formula as described for the cohort of Aruba, because the autologous control group in Aruba was much larger (N=45) than the healthy control group in Brazil (N=14). The ferritin fold change was significantly elevated in patients with severe dengue according to the 2009 WHO classification, as well as in patients with shock and severe haemorrhage compared to patients with uncomplicated dengue (Figure 5A, B and C). In non-survivors levels were significantly elevated compared to survivors (Figure 5D). The absolute values of the ferritin fold change were on average higher in the Brazilian than in the Aruba cohort. This could be due to the presence of more severe disease in the cohort from Brazil and the use of a different assay.
Patients were clustered based on the expression of the determined cytokines as has been previously described (Figure 5E and heatmap) [155]. Cluster A contained mainly healthy controls, cluster B mild to moderately ill dengue patients and cluster C contained severely ill dengue patients. Severe dengue ($P= 2.2 \times 10^{-16}$), shock (3.4×10^{-5}), severe haemorrhage (P=0.007) and death (P=0.03) occurred significantly more often in cluster C than the other two clusters. Cluster C showed a pro-inflammatory cytokine profile with increased expression of IL-6, IL-8, IL-10, IL-15, IL-1RA, sIL-2R, HGF, VEGF, G-CSF, MCP-1, IP-10, and MIG. Levels of ferritin were significantly increased in cluster C compared to the other two clusters and levels were also significantly elevated in the 'dengue' clusters B and C compared to healthy control cluster A (Figure 5E). In summary, we can conclude that levels of ferritin were significantly associated with clinical disease severity and a pro-inflammatory cytokine profile.

Discussion

In the cohort from Aruba, increased concentrations of ferritin were significantly associated with a confirmed dengue diagnosis and viraemia. Moreover, hyperferritinaemia in dengue was strongly associated with thrombocytopenia and increased levels of liver enzymes and both activation of the coagulation and the fibrinolytic systems. The findings were confirmed in a cohort from Brazil, in which increased levels of ferritin were associated with severe disease and a pro-inflammatory cytokine profile.

Ferritin is an acute-phase reactant and a significant amount is produced by monocytes, macrophages and hepatic cells. It has been shown that synthesis of ferritin can be induced by cytokines and iron [179,180]. We showed that increased levels of ferritin were associated with a pro-inflammatory cytokine profile. Lipopolysaccharide (LPS) was shown to induce iron retention in human monocytic cells, which may subsequently induce ferritin expression [181]. Interestingly, increased levels of LPS have been reported in patients with dengue and were also associated with a pro-inflammatory cytokine profile, suggesting that cytokines, LPS and ferritin all play a role in immune activation in severe dengue [127,155].
It is well known that infectious diseases in general cause hyperferritinaemia (reviewed in [171]). We showed that even in mild dengue, the occurrence of hyperferritinaemia could serve as a discriminatory marker between dengue and other febrile illnesses. Increased levels of cytokines and LPS have been reported in several infectious diseases and therefore these mechanisms cannot solely explain these extremely high ferritin levels. Macrophages, monocytes and lymphocytes in the peripheral

blood are the major target cells of DENV replication in vivo [41]. Monocytes and macrophages are also important producers of ferritin and therefore direct infection and subsequent viral replication in these cells may activate them and increase the ferritin production. In agreement with this, ferritin levels in the convalescent phase correlated strongly with the viral load in the early phase. Interestingly, a high viral load in the early phase of DENV infection has previously been associated with the development of severe symptoms around the time of defervescence [44].

Hepatocytes can also synthesize ferritin and in our study liver enzymes were significantly elevated in patients with hyperferritinaemia. DENV replicates very well in hepatic cell lines in vitro, but whether DENV replicates well in the liver in vivo is still a matter of debate [41]. However, it is likely that liver cells are also indirectly activated by cytokines and/or activated immune cells to produce high amounts of ferritin. It has been shown that DENV infection in mice resulted in NK and CD8+ T cell infiltration of the liver [182].

HLH is characterized by extensive activation and proliferation of NK and CD8+ T cells. CD8+ T-cells can be infected by DENV in vitro [183]. Moreover, apoptosis of CD8+ T cells plays an important role in immune modulation during DENV infection [47,184]. sIL-2R is a marker of T-cell activation and increased levels of sIL-2R have been detected in dengue patients with severe disease [44,50,139]. In a previous study with patients from the Brazilian cohort, increased levels of sIL-2R were associated with mortality [155]. In this study, using the same cohort, levels of ferritin were also significantly associated with mortality, suggesting that extensive activation of monocytes and macrophages with subsequent T-cell activation may be detrimental for the host during DENV infection.
Certain HLH-criteria, such as hypertriglyceridaemia, hypofibrinogenaemia and cytopenia in at least two cell lineages were not found in this study, most probably because the patients in this cohort only suffered from uncomplicated dengue. Increased triglyceride levels and hypofibrinogenaemia have been reported in patients with dengue shock syndrome and non-survivors [90,91]. Therefore, we cannot exclude that HLH-like disease occurs in dengue patients with severe symptoms.
In our study thrombocytopenia was strongly associated with hyperferritinaemia. Thrombocytopenia is a hallmark of DENV infection and it is hypothesized that it can be caused by binding of platelets to activated endothelial cells [185]. Platelets are most probably bound by vWF multimers, which were increased in patients with hyperferritinaemia in this study. Because the cytopenia was limited to the platelet count in DENV infection, it is not very likely that phagocytosis by highly activated macrophages is the cause of thrombocytopenia as in the case of HLH.
Coagulopathy is one of the criteria of HLH and also described in severe dengue (reviewed in [8]). In our cohort, the coagulation and fibrinolytic systems were highly activated in patients with hyperferritinaemia at day 2-3 and 4-5 after the onset of fever resulting in increased levels of vWF, TAT, PAP and D-dimer. Levels of TAT were increased in patients with hyperferritinaemia, while levels of peak thrombin were decreased. TAT is a marker of thrombin generation in vivo, while peak thrombin is a marker for the potential of plasma to generate thrombin in vitro. From these results we may conclude that coagulation activation, thrombin formation and the consumption of coagulation factors decrease the ex vivo capacity for clotting during DENV infection, which may result in clinical bleeding symptoms. In addition to activation of the coagulation cascade, increased levels of PAP, PAI-1 and D-dimer showed that the fibrinolytic system was also highly activated in patients with hyperferritinaemia.

Based on the collective results presented in the manuscript, hyperferritinaemia can be considered as a clinical marker for DENV infection, which can discriminate between dengue and other febrile illness. Moreover, ferritin can also serve as a marker for highly active disease resulting in extensive immune

Supplementary Table 1 | Sample size

Figure 1		Day 2-3	Day 4-5	Day 6-8	Cross-sectional
2009 WHO Classification	WS- WS+	11 16	15 21	14 22	
Hospitalization	Outpatients Hospitalized OFI	19 8 12	24 12 16	23 13 13	
Figure 2					
Viraemia	Not viraemic Viraemic	7 20	7 26	7 25	
Figure 3					
Hyperferritinaemia	No hyperferritinaemia Hyperferritinaemia OFI	20 7 12	21 18 16	23 16 13	
Figure 4					
Hyperferritinaemia	No hyperferritinaemia Hyperferritinaemia OFI	14 7 11	13 16 15	14 14 12	
Figure 5					
2009 WHO case classification	WS- WS+ Severe				49 49 31
Plasma leakage and shock	No plasma leakage Plasma leakage Shock				71 33 25
Haemorrhage	No haemorrhage Minor haemorrhage Severe haemorrhage				86 29 14
Survival	Non-survivors Survivors				9 120
Clusters	A B C				18 115 10

Abbreviations: WS-: non-severe dengue without warning signs. WS+: non-severe dengue with warning signs. OFI: other febrile illnesses

activation, coagulation disturbances and severe clinical symptoms. Therefore, we suggest that patients with hyperferritinaemia are monitored carefully, as they are at higher odds to develop severe disease.

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7

Time since Onset of Symptoms and Classical Clinical Markers Associate with Transcriptional Changes in Uncomplicated Dengue

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Submitted

Abstract

Background

Dengue virus (DENV) infection causes viral hemorrhagic fever that is characterized by extensive activation of the immune system. The aim of this study was to investigate the kinetics of the transcriptome signature changes during the course of disease and the association of genes in these signatures with clinical parameters.

Methodology/principal findings

Sequential whole blood samples from DENV infected patients in Jakarta were profiled using the Affymetrix U133 plus 2.0 microarray platform. Principal component analysis (PCA), limma, gene set analysis and weighted gene coexpression network analysis (WGCNA) were used for the interpretation of gene expression data. We show that time since onset of disease has a large impact on the blood transcriptome of patients with non-severe dengue. Clinical diagnosis (according to the WHO classification) does not associate with differential gene expression. Network analysis, however, indicated that the clinical markers platelet count, fibrinogen, albumin, IV fluid distributed per day and liver enzymes SGOT and SGPT strongly correlate with particular gene modules. Specific genes in these modules show a direct correlation with the platelet count and the liver enzyme SGOT. Overall, we see a shift in the transcriptome from immunity and inflammation to repair and recovery during the course of DENV infection.

Conclusions/significance

Time since onset of disease associates with the shift in transcriptome signatures from immunity and inflammation to cell cycle and repair mechanisms in patients with non-severe dengue. The strong association of time with blood transcriptome changes hampers both the discovery as well as the potential application of biomarkers in dengue. However, we identified gene expression modules that associate with key clinical parameters of dengue that reflect the systemic activity of disease during the course of infection. The expression level of these gene modules may support earlier detection of disease progression as well as clinical management of dengue.

Time since Onset of Symptoms and Classical Clinical Markers Associate with Transcriptional Changes in Uncomplicated Dengue

Author summary

An acute dengue virus infection usually starts with a febrile disease phase that can progress to severe disease around the time fever abates (defervescence). Here we study dengue patients that were included very early after the onset of disease and carefully monitored in a longitudinal cohort study. Our results show that time after the onset of disease has a major impact on the transcriptome profile of patients with dengue. Furthermore, the expression of gene network modules could be linked to specific clinical parameters of dengue virus infection. This analysis shows that the platelet count and the levels of fibrinogen and albumin are good markers for the activity and timing of the disease, reflecting relevant biological processes in the patient. In contrast, conventional WHO classification systems did not show any association with any of the 25 gene modules identified. The expression level of gene modules specific for certain biological processes in dengue, may support earlier detection of progression to severe disease and may improve clinical management.

Introduction

Dengue virus (DENV) infection is endemic in South-East Asia and has a large impact on society, both in terms of burden of disease as in economic costs [186]. DENV belongs to the Flaviviridae family and consists of at least four serotypes, DENV-1,-2,-3 and -4. DENV infection has been described as a triphasic disease in the 2009 WHO dengue case classification [1]. The disease starts with the febrile phase in which all patients suffer from fever and a flu-like disease with general symptoms, such as fever, myalgia, arthralgia, headaches and retro-orbital pain. After 3-5 days, patients enter the critical phase of disease, characterized by resolution of fever. The majority of patients with non-severe dengue recover in this phase, but some patients develop severe symptoms, such as shock, hemorrhage or organ impairment and they are classified as having severe dengue. Typical for the development of severe disease in the critical phase is a rapid decrease in platelet count with a concomitant increase in hemoconcentration due to plasma leakage. The critical phase usually lasts 24-48 hours after which patients enter the recovery phase.

To investigate the underlying biological processes involved in DENV pathogenesis, several studies have applied transcriptome profiling to cohorts of dengue patients [69,77,79]. Some studies report that the acute (febrile) phase of dengue is characterized by an increased expression of genes involved in immunity and inflammation [73,78]. In this phase, transcripts involved in the innate immune response, in particular interferon induced genes and complement, are highly upregulated [73,78]. Other studies report that the convalescent (critical/recovery) phase is characterized by increased abundance of transcripts involved in cell cycle and cell repair mechanisms [70,79]. In terms of disease severity, it has been shown that interferon induced genes have a lower expression in patients with dengue shock syndrome (DSS) compared to patients with uncomplicated dengue [69,73] in the acute phase of disease. In contrast, genes induced by the activation of neutrophils showed an increased expression level in patients with DSS [76,77]. This suggests that impaired viral clearance due to lower levels of in-

terferon and increased activation of neutrophils could contribute to the development of severe dengue.

In this study, we investigated the biology of dengue pathogenesis over time in a cohort of dengue patients from Jakarta, Indonesia, where dengue is endemic and incidence increases during the rainy season [1]. Using a transcriptomics approach, we studied gene expression patterns, focusing on the association with dengue-specific clinical markers over time. From our data, and from a direct comparison of our data with other studies, we conclude that time since onset of disease has the largest impact on the blood transcriptome of patients with uncomplicated dengue. Non-severe dengue patients with (WS+) and without (WS-) warning signs have identical transcriptome profiles with a gene signature that shifts from immunity and inflammation to repair and recovery mechanisms over time. Furthermore, we show that particular clinical parameters correlate with specific gene modules identified in dengue patients, suggesting that they reflect the underlying biological processes and can be used as markers for disease activity.

Methods

Ethics

This study was approved by the research ethics committee of the Faculty of Medicine, University of Indonesia in Jakarta, Indonesia. Patients were included after written informed consent. If patients were younger than 18 years written informed consent was obtained from the parent and/or legal guardian. Data and samples were anonymized with a study number.

Clinical cohort

Between March and June 2010 all patients ≥ 14 years of age with a fever onset ≤ 48 hours before presentation and a clinical suspicion of dengue were included in community health centers (ëpuskesmasí) in Jakarta, Indonesia. Blood was drawn and a NS1 antigen and IgM/IgG antibody rapid test (SD Dengue Duo, Standard Diagnostics, inc, Korea) was performed. If tested positive, patients were admitted to the Cipto Mangunkusomo Hospital in Jakarta for seven days. Clinical data were recorded daily with a standard case report form. Blood samples were collected on every other day (including the day of admission), both for clinical laboratory tests and for transcriptome profiling. At day 3, 5 and 7 of admission ultrasound examination was performed to investigate whether patients suffered from ascites and/or pleural effusion. Patients were classified according to the 2009 WHO dengue case classification [1]. Briefly, patients with fever and general symptoms were classified as non-severe dengue without warning signs (WS-). Patients with one of the following warning signs were classified as non-severe dengue with warning signs (WS+): abdominal pain, vomiting, minor mucosal bleeding, pleural effusion, ascites and hepatomegaly. Patients with shock, respiratory distress, severe bleeding and/or organ impairment were classified as severe dengue.

Laboratory diagnostics

Serology

All diagnostic assays were performed at the Department of Microbiology, Faculty of Medicine, University of Indonesia, Jakarta. The diagnosis of the rapid test was confirmed with detection of NS1 antigen (Panbio) and/or IgM antibodies (Focus diagnostics) and/or an increase in IgG antibodies (Focus diagnostics) with enzyme-linked immunosorbent assay in acute and convalescent phase samples. IgM and IgG antibodies were determined in all collected sequential samples. To determine whether patients suffered from primary or secondary infection the IgM/IgG ratio was determined.

Molecular diagnostics

Reverse transcriptase PCR (RT-PCR) was performed to determine the infecting serotype according to the method described in Lanciotti et al [187]. RNA was extracted from 140 µl of plasma using QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instruction. RNA isolation and PCR were performed in strict containment to avoid contamination. Negative controls were included in both RNA isolation and in RT-PCR.

A semi-nested RT-PCR was performed for serotyping. The first amplification reaction had a reaction volume of 40 µl, consisting of 4 µl 10x PCR buffer with 1.5 mM $MgCl_2$, 3.2 µl 10mM dNTPs, 0.4 µl Super Script II RTase (Invitrogen), 0.15µl 5 U Platinum Taq-polymerase (Invitrogen), 0.8 µl 10 µM D1 primer, 0.8 µl 10 µM D2 primer, 8 µl RNA. The RT step consisted of 53°C for 30 minutes and denaturation at 95°C for 5 minutes. This was followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 90 seconds. The RT-PCR was concluded with a single cycle extension at 72°C for 7 minutes.

The second PCR amplification had a reaction volume of 25µl, consisting of 2.5µl 10x PCR buffer with 1.5 mM $MgCl_2$, 2.0 µl 10 mM dNTPs, 0.15µl 5 U/µl Platinum Taq-polymerase (Invitrogen), 1µl of each D1, TS1, TS2, TS3, TS4 primer (10 µM), 2µl of the product from the first PCR. PCR 2 was performed with initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 60 seconds and one final single cycle extension at 72°C for 7 minutes.

After amplification, PCR products were analyzed by electrophoresis. Eight µl of PCR product was loaded on 2% agarose gel in TAE buffer and stained with Ethidium Bromide. The product was visualized with UV light.

Blood transcriptome profiling

Blood was collected in Tempus Blood RNA tubes (ABI, Foster city, CA, USA). Total RNA was isolated from whole blood using the Tempus Spin RNA isolation kit (Applied Biosystems, Bleiswijk, The Netherlands). Globin RNA was removed from total RNA preparations using the Globiclear kit (Life Technologies). RNA concentrations and OD 260/280 ratios were measured with the NanoDrop ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, Wilmington,USA). Assessment of RNA quality and purity was performed with the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA (100 ng) was labelled using the MessageAmp Premier RNA Amplication kit (Applied Biosystems) and hybridized to Human Genome U133 plus 2 gene chips (Affymetrix), according to the manufacturerís recommendations. Image analysis was performed using GeneChip Operating Software (Affymetrix). Microarray Suite version 5.0 software (Affymetrix) was used to generate .dat and .cel files for each experiment. The raw data has been deposited in the arrayExpress database under access number xxx.

Data normalization and analysis

Analysis of the arrays was performed using R 2.15/Bioconductor [188,189]. Gene expression was evaluated using alternative probeset definitions based on the ensembl genome probeset annotation after quality control and VSN normalization [190-192]. Differential gene expression and Reactome pathway analysis was performed using Limma and the Roast routine [188,193]. Furthermore, we applied weighted gene correlation network analysis (WGCNA) using the available R libraries [194].

Results

A longitudinal cohort of dengue patients

During the 2010 dengue outbreak in Jakarta, Indonesia, 157 patients were recruited into this study. Of these patients, 52 were admitted to the hospital with a positive dengue IgM and/or NS1 rapid test. All four serotypes were circulating during this outbreak. From the admitted patients, 26 were selected for blood transcriptome analysis based on clinical data and a confirmed laboratory diagnosis for DENV infection (see Table 1 for clinical characteristics). During the hospital admission, seven patients were diagnosed with WS-, eighteen with WS+ and one with severe dengue. The patient with severe dengue displayed signs of severe hemorrhage, including melaena. No patients developed shock, although some patients did receive a large amount of IV fluid during their admission. Fifteen matched healthy controls from the same geographical location and similar socio-economic status were also included in this study.

A maximum of three tempus tubes from each patient was included in this analysis. We included 46 tempus tubes from WS- and 19 from WS+ or severe patients. Of the 65 included tempus tubes, 21 were collected at day 0 of admission and 25 at day 4; these time points were therefore analyzed in detail.

Table 1 | **Clinical characteristics of the cohort**

2009 WHO dengue case classification	WS- (N=7)	WS+/severe (N=19)
Sex (male/female)	3/4	11/8
Age*	20 (17-33)	19 (17-27)
Primary/secondary/unknown	2/4/1	6/13/0
Serotype (DENV-1/-2/-3/-4/not detected)	3/2/1/0/1	2/6/7/3/1
Pleural effusion during admission (US)	0	4 (21%)
Ascites during admission (US)	0	9 (47%)
IV fluid during admission (ml)*	7000 (2000-11000)	11000 (6000-13500)
Positive tourniquet at day of admission	2 (33%)#	10 (53%)
Leukocyte count at day of admission*	6060#	
Thrombocytopenia during admission	3 (50%)#	18 (95%)
Mucosal bleeding	0	6 (32%) (1 gum, 2 epistaxis, 2 gum and epistaxis, 1 melaena)
Vomiting during admission	0	6 (32%)
Abdominal pain during admission	0	12 (63%)

** Values are in median (interquartile range). Abbreviations: WS-: non-severe dengue without warning signs. WS+: non-severe dengue with warning signs. # = 1 missing value in the WS- group in the tourniquet test, platelet and leukocyte count.*

Time after the onset of disease accounts for most transcriptome variance

To obtain a global overview of the dengue transcriptome profiles, we applied principal component analysis (PCA) (Figure 1). The first principle component (PC1) accounts for 47% of the variance in the dataset and concurs with time since admission. The second principle component (PC2, Figure 1) accounts for 17% of the variance in gene expression and segregated the dengue samples from the healthy controls. Principle component analysis did not show any segregation of patients by disease severity. Taken together, PCA demonstrates that time since day of admission has the highest impact on the dengue transcriptome profiles in our cohort.

WS- and WS+ dengue patients have indistinguishable transcriptional profiles

To obtain insight into the transcriptional changes that are associated with disease severity and time after the onset of disease, we performed differential gene expression analysis and gene set

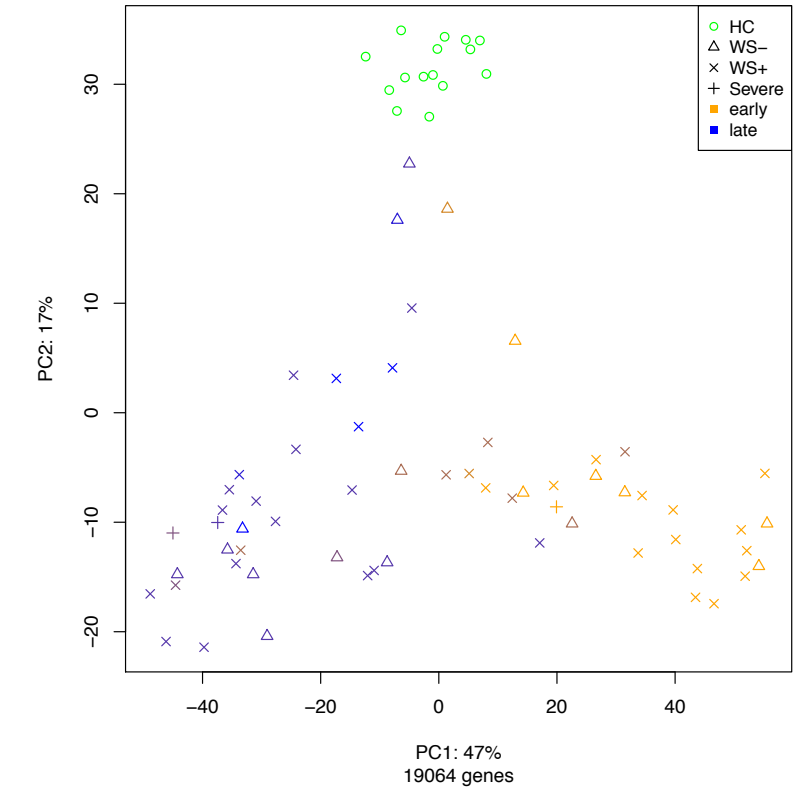


Figure 1 | Principle component analysis of all transcriptome snapshots in this study. Icons and colours indicate type and sampling stage of the samples. All probesets were included for this analysis.

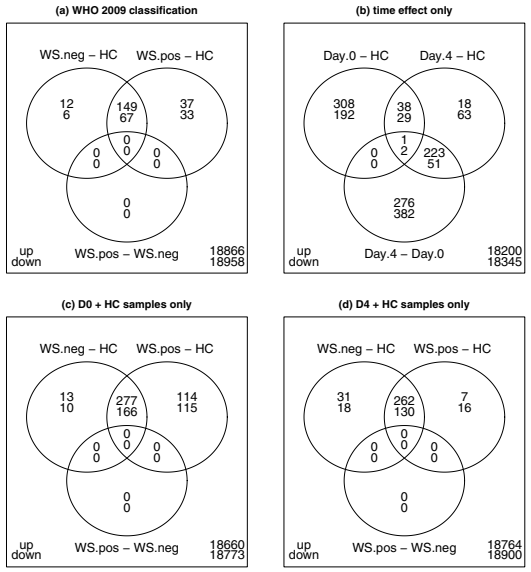


Figure 2 | Differential gene expression analysis. Venn diagrams show the overlap between different sets of differentially expressed genes. FDR <0.05, all differentially expressed genes are at least 2-fold up- or down-regulated.

analysis in dengue patient and control transcriptomes (FDR ≤ 0.05 and fold change ≥ 2). We used the 2009 WHO dengue case classification system to group patients and excluded the single case with severe disease for differential gene expression analysis. The analysis of the samples from all time points together, revealed that 161 genes were up- and 73 genes were downregulated in WS- patients compared to healthy controls (Figure 2a). In WS+ dengue patients, 186 genes were up- and 100 genes were downregulated relative to healthy controls. There is considerable overlap (216 genes) of differentially expressed genes in both dengue groups, suggesting that similar biological processes are ongoing in both WS- and WS+ dengue patients. Indeed, no genes were differentially expressed when comparing WS- to WS+ dengue patients directly. Next, the transcriptome profiles of samples from day 0 and day 4 since admission were compared to identify genes differentially expressed over time, regardless of disease severity (Figure 2b). More genes were differentially expressed in time than between WS- and WS+ disease, confirming the PCA result that time since onset of disease has the largest impact on the transcriptome. To study dengue disease effects independently of time, we restricted our analysis to transcriptome profiles from WS-, WS+ and healthy controls at day 0 and day 4. On day 0, many genes were differentially expressed in each of both dengue groups compared to healthy controls, but no genes were differentially expressed when the severity groups were compared to each other (Figure 2c). At day 4, the number of differentially expressed genes in WS- and WS+ dengue compared to healthy controls was lower than at day 0 (Figure 2d). When severity groups were compared at day 4, again no genes were differentially expressed. Taken together, in our study, WS- and WS+ blood transcriptional profiles cannot be distinguished from each other.

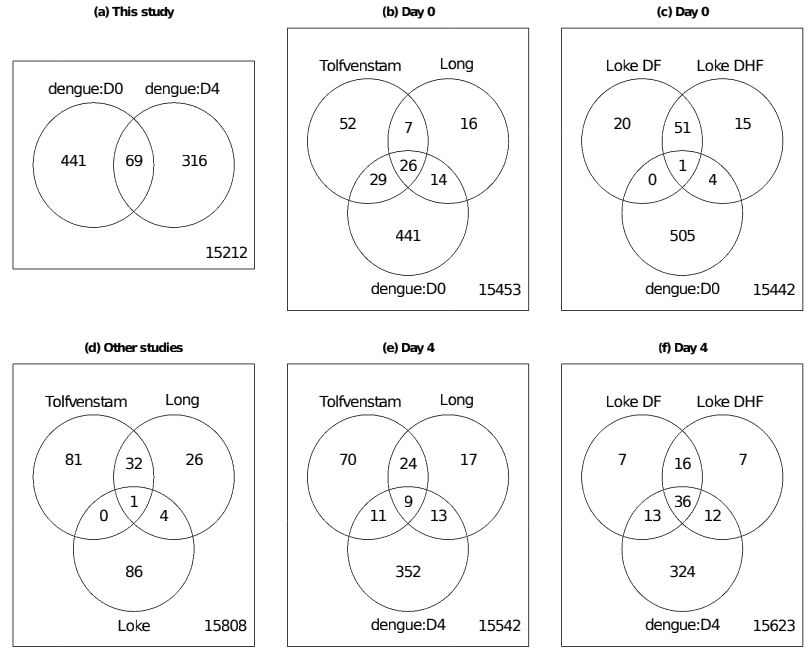


Figure 3 | Overlap of differential gene expression with signatures identified in other studies. (a) indicates the signatures generated in this study, (d) those of the other dengues studies. (b-c) and (e-f) indicate the overlap with the signatures of day 0 and day 4, respectively.

Time after onset of disease explains discordant transcriptional signatures in dengue patients

Over the past few years, several studies have examined the transcriptional profile of dengue infections. Three cross-sectional studies (Tolfvenstam et al., Long et al. and Loke et al.) [73,75,78] were similar in the type of sample used (whole blood) and the included data on time since onset of symptoms, allowing these studies to be compared to results from our cohort (Figure 3a). Tolvenstam et al. and Long et al. have a fairly large overlap in differentially expressed genes (Figure 3d), presumably because both studies included patients early (<72 hours) after onset of disease. The signatures published by Loke et al. have little overlap with those of the other studies (1 and 5 genes only, Loke et al. DF and DHF signatures combined), most likely due to the fact that patients were included at a later time point after the onset of disease (3-6 days after onset). To compare our results to these studies, we compared the early and late general dengue signatures to those of the other studies. 48% of differentially expressed genes in the signature from Tolfvenstam et al. and 63% of Long et al. are also part of our day 0 dengue gene signature (collected <48 hours after onset of disease) (Figure 3b). On the contrary, only 1% of the genes in the DF and 7% of the genes in the DHF signature in Loke et al. were similar to our day 0 signature (Figure 3c). In contrast, our day 4 signature showed the greatest similarity with the signatures in Loke et al. (68% DF, 68% DHF; Figure 3f), but much less so with those from Tolfvenstam et al. and Long et al. (18% and 35%, respectively; Figure 3e). Our results therefore concur with all three studies and confirm that these signatures can occur within one cohort, but at different time points after onset of symptoms. In conclusion, time since the onset of symptoms accounts for most of the transcriptome differences between mRNA profiling studies in dengue patients.

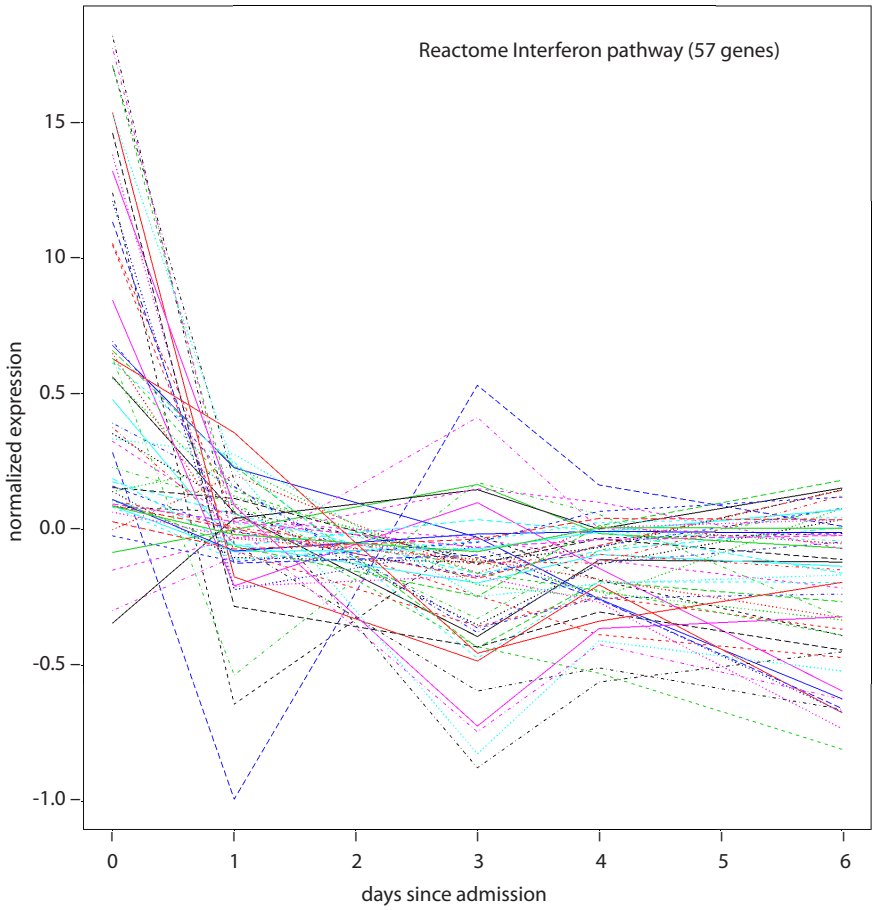
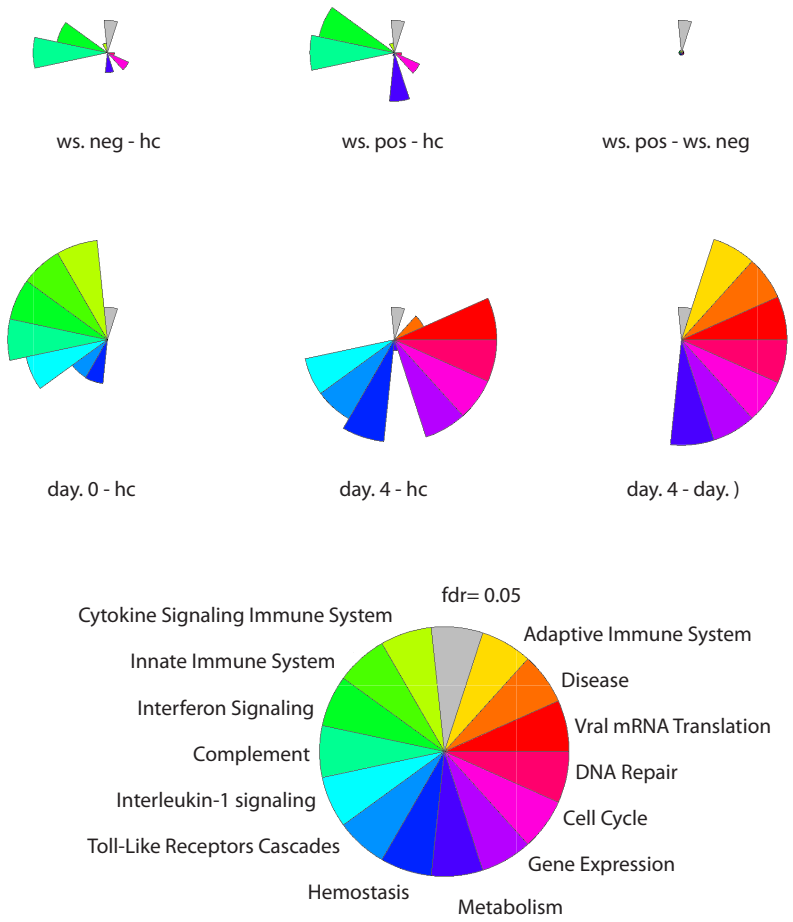


Figure 4 | Gene set analysis of dengue signatures. The interferon pathway (left panel) is upregulated in the early stages of dengue, but not later. Segment plots indicate the significance of enrichment of particular Reactome gene sets and pathways. The longer the segment, the larger the enrichment. All segments longer than the grey segment are significant.

The dengue transcriptome shifts from an immunity and inflammation - to a repair and recovery signature

The type I interferon pathway is known to be differentially expressed in DENV infection [195]. We investigated the interferon response during the course of infection by selecting genes from the interferon pathway (Reactome curated pathway database, 57 genes) and plotting their expression over time (Figure 4). The expression of the majority of interferon genes was highly increased on the first day of admission, but decreased rapidly after that day and continued to be low, consistent with the notion that the interferon pathway is active in the early stages of dengue infection [69].

To functionally annotate the differences in gene expression, we performed gene set analysis using the Reactome curated pathway database [196]. Applying the Roast algorithm [197], we found that when compared with healthy controls, both WS- and WS+ have 'complement' and 'interferon signalling' ranked among the most highly up-regulated pathways (Figure 4). None of the pathways were differentially regulated when comparing the WS- with the WS+ patients.



In addition to severity signatures, we investigated the transcriptional changes related to time after admission in our longitudinal cohort. By comparing day 0 dengue samples to healthy control samples, we observed an up-regulation of pathways related to innate immunity and cytokine signalling (figure 4). When we compared Day 4 to healthy controls, a pronounced shift to cell cycle and DNA repair mechanisms was evident. A direct comparison between day 0 and day 4 samples additionally showed up-regulation of metabolism (Figure 4). Taken together, we see that, initially, innate immunity and interferon is up-regulated, followed by repair mechanisms that mark the beginning of recovery from dengue.

Network analysis shows early immune activation

In addition to conventional differential gene expression analysis, we applied weighted gene correlation network analysis (WGCNA) to identify novel biological processes that are involved in DENV infec-

tion and pathogenesis. WGCNA organizes genes into modules that are subsequently correlated to clinical parameters. Using this approach, we identified 25 gene modules (including one group of unassigned genes) of varying sizes and annotated them using Gene Ontology [198]. All modules were labeled with a functional category that is enriched in this module (Figure 5, left column). Furthermore, the gene expression patterns of the modules were compared with 18 clinical parameters (Figure 5, red and green indicate a positive or negative correlation, respectively). Two modules did not associate with any clinical parameter: Module J-darkmagenta consisted almost exclusively of inactive genes; A-grey is a group of genes that do not cluster – and it is therefore not surprising that these clusters do not correlate with any clinical parameter. Time since onset of disease correlated strongly with a large number of gene modules, confirming that time has a strong effect on the transcriptome of dengue patients. Furthermore, most gene modules involved in immunity showed a negative correlation with time since onset of disease, while the gene modules involved in cellular processes and cell repair mechanisms showed a positive correlation with time. This demonstrates that gene expression in the early phase of disease is dominated by immune response genes, which shifts to genes involved in cell repair mechanisms around the time of defervescence. The modules R-cyan and T-lightyellow had a strong inverse correlation with time. The R-cyan module is enriched for genes involved in the type I interferon response, innate immune response, cytokine production and toll-like receptor 4 signaling pathway; the T-lightyellow module for inflammatory response and lymphocyte and neutrophil activation.

Classical dengue clinical parameters associated with transcriptional patterns in dengue

Next, we investigated the association between the identified gene modules and clinical parameters. Significant associations between gene modules and the clinical parameters platelet count, fibrinogen level, albumin level and volume of IV fluid per day were found. Most modules that displayed a positive correlation with time after admission also did so with the quantity of IV fluid and the liver enzyme SGOT. These same modules displayed a negative association with the platelet count and levels of fibrinogen and albumin. Platelets, albumin and fibrinogen are all part of the blood compartment in which dengue targets monocytic cells to replicate [41]. The pro-inflammatory environment due to DENV replication probably affects the expression of these markers. This may explain the association of these markers with these gene modules.

In contrast to the above-mentioned clinical parameters, the 1997 WHO dengue case classification as well as the 2009 WHO dengue case classification showed no significant association with any of the gene modules, suggesting that these classifications do not reflect the underlying biological processes, or that there are no differences in the underlying biological processes. The non-specific warning signs 'abdominal pain' and 'vomiting' showed no statistical significant associations, which is in line with the generic nature of these symptoms. In contrast, DENV-specific warning signs including 'epistaxis' and 'gum bleeding' did correlate with the gene modules B-antiquewhite4, O-brown4, M-salmon4 and W-sienna3. Enriched GO terms for genes in the O-brown4 module are "cell organelles", such as mitochondrion and cytoplasm. Module W-sienna3 has wound healing and coagulation as enriched GO terms and module M-salmon4 is related to catabolic processes. Ascites, which is a typical sign of plasma leakage, is significantly associated with modules G-palevioletred3 and P-yellowgreen. These two gene modules were not associated with time, suggesting that this is a specific biological pathway. All in all, network analysis showed that the WHO classifications couldn't be related to specific gene modules; however, there are many significant correlations between gene modules and dengue-specific clinical parameters.

Specific genes correlate with the platelet count and the liver enzyme SGOT

In order to identify genes that may serve as a marker of immune activation in the early phase of disease, we focused on modules with a strong negative correlation with time after admission, including

Table 2 | Characteristics of studies profiling whole blood of DENV infected patients

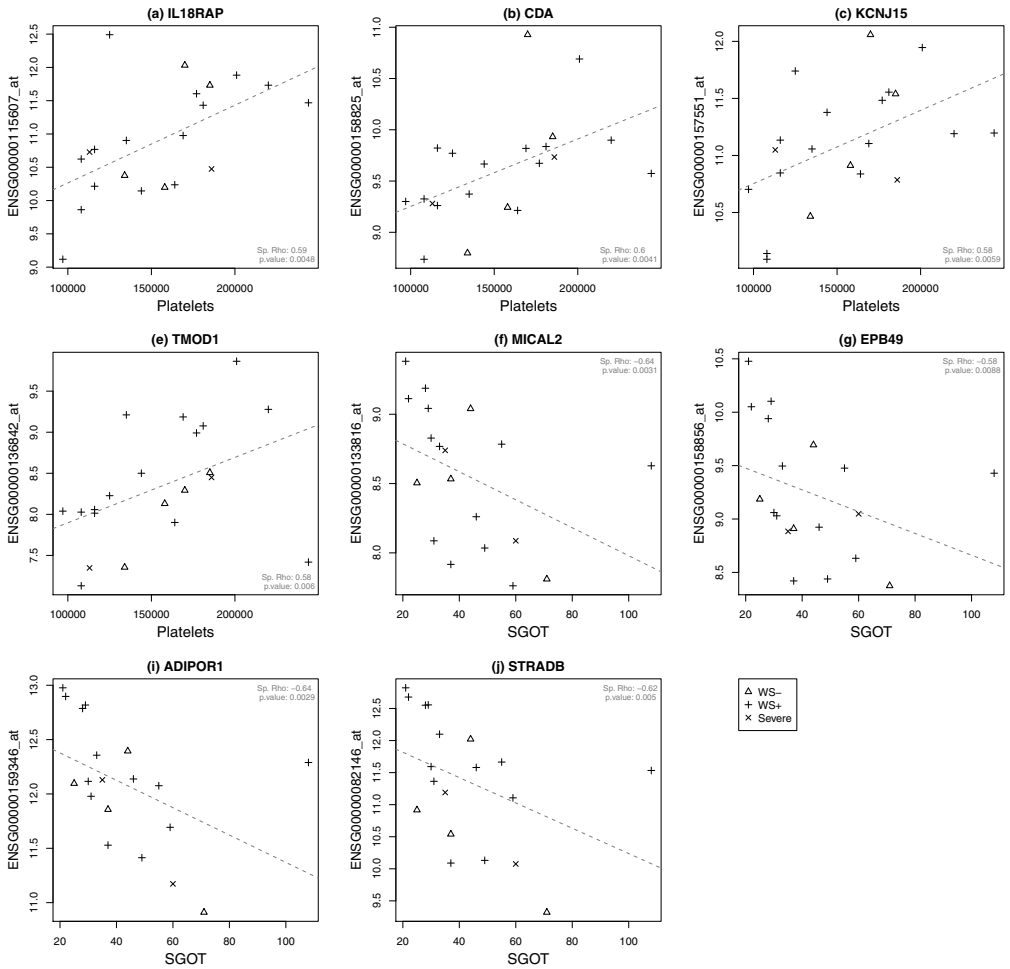
Study	Loke et al.	Long et al.	Tolfvenstam et al.
Journal	PloS Neglected Tropical Diseases	Journal of Virology	BMC Infectious Diseases
Year	2010	2010	2011
Country	Nicaragua	Vietnam	Singapore
Population	Children < 15 years	Adults and children	Adults
Day of illness	Between 3-6 days	<72 hours	<72 hours

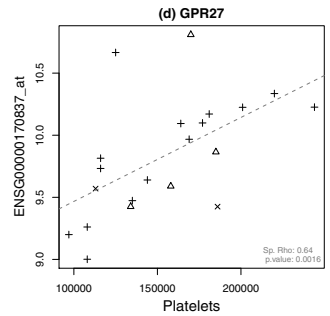
modules M-salmon4, R-cyan, T-lightyellow, U-orange, and X-darkorange. Five genes in the module T-lightyellow showed a significant direct association with the platelet count (Figure 6), including two that play a role in immunological processes. The IL-18 receptor accessory protein (IL-18RAP, Figure 6a) forms the receptor complex with IL-18Rα and is needed for IL-18 signaling. The other gene encoded the protein cytidine deaminase (gene CDA, protein CDD, figure 6b). CDD is highly expressed by activated granulocytes and serves as a negative feedback mechanism of these cells by inhibiting the function of granulocyte-macrophage colony formation in the bone marrow [199]. Two other genes, namely KCNJ15 and G-protein coupled receptor 27 (GPR27) (Figure 6c-d), are both described to play a role in insulin secretion [200,201]. In the module X-darkorange, the gene Tropomodulin 1 (TMOD1) was directly associated with the platelet count (Figure 6e) and the genes Mical2 and dematin (gene EBP49) with SGOT (Figure f-g) all play a role in actin regulation of the cell [202-204]. Seven other genes with an inverse association with SGOT play a role in metabolic processes, including sestrin 3 (SESN3), adiponectin receptor 1 (ADIPOR1) and STE20-related kinase adaptor beta (STRADB) (Figure 6h-j). Sestrin 3 is required for regulation of the blood glucose levels [205] and sestrins can reduce the levels of reactive oxygen and protect cells against cell death [206]. Adiponectin acts through the adiponectin receptor 1, which results in increased fatty acid oxidation in the liver [207]. Adiponectin activates serine/threonine kinase 1 via its receptor (LKB1) [207]. Interestingly, the gene STE20-related kinase adaptor beta was also significantly associated with SGOT and is part of a complex involved in the activation of LKB-1. LKB-1 is important in maintaining cell polarity of hepatocytes, which is essential in the formation and maintenance of the bile canalicular network [208]. In summary, we find genes that correlate with clinical parameters and that can be related to either dengue pathogenesis or tissue physiology, suggesting that these genes may be directly associated with the ongoing biological processes in dengue infection.

Discussion

In this study, we examined DENV infected patients with uncomplicated disease using transcriptome profiling. By using a variety of analysis techniques, we show that time after the onset of disease is the most important determinant of the blood transcriptome profile changes in DENV infected patients. Regardless of the analysis techniques used, we did not observe differences in blood transcriptional profiles between WS- and WS+ patients. Conversely, the clinical parameters platelet count, albumin, fi-

Figure 5 | Dengue coexpression network analysis. Significant correlations between gene modules (y-axis) and clinical parameters (x-axis) are depicted in a red-to-green colour scale. All gene modules are annotated with gene enrichment categories; clinical parameters are grouped by symptom type.





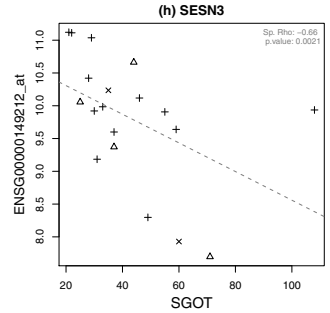


Figure 6 | Correlations between genes (y axis) and clinical parameters (x-axis). Genes are annotated with a gene symbol; those depicted in (a-d) are from module T-lightyellow; (e-j) are from module X-darkorange. Icons depict the class of the sample (see Fig 1). Pearsman Rho correlations and p-values are given in the plot.

brinogen, SGOT, SGPT and volume of IV fluid administered showed a highly significant association with specific gene modules. Gene module expression may serve as a novel marker to monitor the biological processes involved in dengue pathogenesis.

A recurring theme in our results is that time after the onset of disease is the main determinant of transcriptome profile changes in DENV infected patients. This observation is in line with the results reported by Sun et al., although PBMCs were used in that study [79]. Moreover, the comparison of our data to signatures published by Loke et al., Long et al. and Tolfvenstam et al. show that stratifying the expression data by time after onset of disease results in a large overlap with these gene expression profiles. These studies performed transcriptome profiling in unrelated cohorts from populations of patients with diverse genetic backgrounds, geographical locations and age distributions, demonstrating that the impact of time since onset of disease on gene expression is a general and robust feature of DENV infection.

In contrast to the strong signal related to time since onset of disease, there was no detectable transcriptional difference between WS- and WS+ patients. Earlier studies showed that gene expression

patterns from patients with DSS did segregate from those of DF and DHF patients [75,76]. This is the first time that the 2009 WHO classification was used in transcriptome analysis, but our results extend other studies showing that no clear distinction can be made between the transcriptome profile of DF and DHF samples [74-76]. The fact that no differentially expressed genes could be identified in comparisons between WS- and WS+ dengue suggests that the biological processes in these two disease entities are very similar or that the generated blood transcriptome profiles do not accurately reflect disease processes in other parts of the body. The latter is not expected given that DENV infection is a systemic disease that targets monocytic cells in the blood compartment [41]. Our network analysis showed that epistaxis, gum bleeding and ascites were associated with gene modules distinct from those that associate with markers reflecting systemic disease, such as platelet count, fibrinogen, albumin and IV-fluid, suggesting that these markers reflect different biological processes. The network analysis also showed that the parameters platelet count, fibrinogen, albumin and IV fluid reflect the processes of systemic immune activation and subsequent repair mechanisms in the blood. We conclude that distinct dengue-related signatures can be identified, but that these do not concur with the comprehensive WS- and WS+ categories in dengue diagnosis. Furthermore, if WS-/WS+ specific biomarkers could be identified, the strong effect of time upon infection on transcriptome dynamics

would limit the application of such biomarkers in a clinical setting. However, the expression level of the identified gene modules specific for biological processes relevant in dengue disease may support earlier detection of progression to severe disease and improve clinical management of dengue.

The clinical parameter platelet count has frequently been associated with DENV infection [94] and was even one of the four criteria for DHF in the 1997 WHO dengue case classification [17]. It has been shown that children with lower platelet counts in the early phase of disease are more likely to develop DHF later on [5]. Several hypotheses to link DENV infection with platelet depletion have been postulated, such as DENV induced bone marrow suppression [209], complement-induced lysis of platelets through the binding of autoantibodies [99] or the binding of platelets to activated endothelial cells [185]. Our study finds, besides an association between the platelet count and certain gene modules, a strong activation of the innate immunity and complement, which could contribute to all these three mechanisms of platelet depletion. Furthermore, we found that expression of IL-18RAP, which is involved in the induction of IFN-γ production in NK and Th1 cells [210], to be directly associated with the platelet count. Fagundes et al. showed that IL-18 signaling was necessary to inhibit viral replication in DENV infected mice and that IL-18 knock-out mice showed increased virus titers and more severe disease [211].

The occurrence of plasma leakage in dengue patients has been extensively documented. We observe that markers of plasma leakage, including the quantity of IV-fluid supplied and the levels of albumin, were both associated with the same gene modules as platelet count. Plasma leakage tends to correlate inversely with the platelet count [104] and it has been suggested that platelets may directly induce vascular permeability by the release of IL-1β [212]. In a recent study, we showed that dengue shock syndrome and a pro-inflammatory cytokine profile were strongly associated with each other, suggesting that plasma leakage is the result of immune activation during DENV infection [155].

In severe dengue, dysregulation of coagulation is frequently observed. Fibrinogen is consumed after thrombin generation and decreased levels have been detected in severe dengue [91]. The strong association of fibrinogen with gene modules involved in immunity and inflammation suggests that activation of the coagulation cascade is associated with the strong immune response in the acute phase of DENV infection. It has been shown that activation of the coagulation cascade can induce the production of cytokines through NF-κB activation [93], suggesting that crosstalk between coagulation and inflammation may contribute significantly to disease severity.

In our study, gene modules were enriched for metabolic and catabolic processes, kinase activity and organelles, such as mitochondria. Loke et al., also showed that many upregulated genes in the acute phase were involved in metabolic processes and shared between acute DF, DHF and DSS samples [75], suggesting that a highly activated metabolic state is part of the general dengue signature. Acute DENV infection is also characterized by extensive activation of the innate immune response, such as complement and neutrophils [72,78,79]. Complement is suggested to be an important inhibitor of replication of Flaviviruses [213], and was among the most highly overrepresented pathways in our study. Similarly, studies performed in whole blood have shown that neutrophil-related genes are highly expressed in dengue expression signatures that correlated with disease severity [75-77]. In our study, the neutrophil derived protein CDD associated with the platelet count, suggesting increased abundance in the acute phase of disease. This protein could be associated with severe disease, because high levels of CDD have also been shown in patients with meningococcal sepsis [214].

Our results indicate involvement of the liver in dengue pathogenesis. Especially the gene modules involved in inflammation and immunity correlated with the liver enzymes SGOT and SGPT, suggesting that dengue induced inflammation affects the liver. Moreover, ten individual genes showed a significant association with the liver enzyme SGOT. The liver may be affected directly by viral replication or indirectly by cytokines and immune cells. One surprising finding from our studies is differential regulation of several genes that play a role in diabetes. The genes adiponectin receptor 1, sestrin 3, KCNJ15 and G-protein coupled receptor 27 have all been described to play a role in insulin resistance, decreased insulin secretion and impaired blood glucose homeostasis [200,201,215]. It has been shown that inflammation and certain cytokines in particular lead to insulin resistance, which may result in further activation of multiple inflammatory processes [216,217]. In support of this finding, it has been shown that DENV infected patient with diabetes had a higher risk to develop severe disease [218,219]. Altogether, the above suggests involvement of the liver in dengue pathogenesis, in particular related to insulin and blood glucose regulation during DENV infection and pathogenesis.

Future studies should aim to carefully track time since the start of infection, as time is the main source of variance in transcriptomes from dengue patients. Since the transcriptome effects in time are larger than potential transcriptome effects between severity classes, a synchronous longitudinal cohort is an absolute requirement for any biomarker study. Furthermore, individual symptoms and markers appear to better reflect the biological processes underlying dengue pathogenesis. Classification on the basis of gene signatures related to specific symptoms, rather than overall diagnosis, may enable earlier identification of patient subgroups that are at increased risk of developing severe dengue, and to a better understanding of dengue disease pathogenesis.

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Conflict of interest statement

None of the listed authors declare conflict of interest apart from Albert Osterhaus who is a part time employee of Viroclinics BV (for details go to www.erasmusmc.nl). The stated competing interest does not alter the author's adherence to the policies on sharing data and materials.

8

Summarizing Discussion

Summarizing Discussion

A major challenge for medical doctors is to identify DENV infected patients in the early stage of disease, who will develop severe manifestations at a later time point and will need prompt hospital admission and initiation of supportive therapy. Every clinically apparent DENV infection starts with the same non-specific febrile symptoms and the transition to severe disease, if it happens, is usually very sudden. Therefore, clinical dengue research has largely focused on the identification of determinants to predict disease progression and severity.
Fundamental research largely focuses on the question why certain patients develop severe disease, while the majority does not. Disease severity is determined by characteristics of the virus, those of the host or of the environment in which players mutually interact.
The focus of this thesis is on clinical and pathogenesis aspects of DENV infection in an effort to address questions related to the identification of patients at risk of developing severe disease and to contributing mechanisms. In this section results from the respective chapters of this thesis are evaluated and combined to highlight novel insights that this thesis may provide into the pathogenesis of dengue.

Challenges in clinical classification

A clinical classification of dengue accurately reflecting disease severity is necessary for clinical management as well as for clinical research. In clinical practice, consensus about the definition of severe disease is needed to develop universal guidelines for diagnostics and treatment. In clinical research, it is important to use the same definitions of disease severity to be able to compare study results and judge their validity. The challenge is to formulate guidelines that cater for the needs of both basic laboratory research and the clinic. From 1974, the WHO developed several clinical guidelines that enabled classification of dengue cases by severity of disease. Although these guidelines have been updated several times the utility and accuracy of these guidelines have continued to be a matter of debate [6,7,10,15,16]. The most recent WHO dengue case classification was issued in 2009 [1].

In this thesis several classifications were used, e.g. the 1997 and 2009 WHO classification, but also classifications based on signs, symptoms or the physician's judgement of disease severity ('MD classification'). In the first study about microbial translocation (chapter 3), patients were classified according to the 1997 WHO classification and the MD classification. In the 1997 classification patients had to fulfil all four criteria (fever, thrombocytopenia, bleeding tendency and signs of plasma leakage) to be diagnosed with dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [17]. The MD classification was based on the expert physician's judgement about the patient's disease severity and in this classification the 1997 criteria were not stringently applied. As expected, more patients were classified as having DHF or DSS in the MD classification than in the 1997 WHO classification. It has been reported previously [7] that more patients are classified as having severe disease according to the expert physician's judgement than in the 1997 WHO classifi-

cation, because some patients do not fulfil all the stringent criteria of the 1997 WHO classification, although they clinically suffer from severe disease [6,10,14,110].

In chapter 2, a comparison was made between the utility and accuracy of the 1997 and the 2009 WHO clinical case classifications for dengue. We took intensive treatment intervention as an indicator of severe disease with the assumption that the most severely ill patients would be those in need of intensive treatment. The stringency of the 1997 classification became evident in this study, because eight patients diagnosed as having DF presented with signs of shock. In the 2009 classification they were accurately diagnosed as having severe dengue. A consequence of the 2009 classification is that more patients are classified as having severe disease, resulting in more hospital admissions. Moreover, the criteria are less strictly defined leaving room for arbitrary interpretation by the clinician or researcher, which may complicate the comparison of research data from different study settings. Altogether, the conclusion from this study was that the 2009 classification was more specific, albeit at the cost of a lower sensitivity as compared to the 1997 classification.

In the genomics study (chapter 7), gene clusters were linked to clinical parameters of DENV infection, including the 1997 and 2009 WHO classification. Interestingly, no significant associations between these two classifications and clusters of genes were found. This suggests that these classifications might not primarily reflect underlying biological processes adequately. This was in contrast to other markers, such as the platelet count and levels of fibrinogen and albumin, which showed very strong associations with certain gene modules.

In the majority of studies presented in this thesis the 2009 WHO classification was used. Disease manifestations were analysed separately, depending on the research question. For example, in chapter 3 we hypothesized that microbial translocation was caused by the same mechanism as plasma leakage and for that reason we designed a plasma leakage severity score, which indeed showed a strong association with the levels of LPS. In the genomics study (chapter 7) it was shown that symptoms of epistaxis, gum bleeding and ascites were associated with gene modules that differed from markers reflecting systemic disease, such as platelet count, fibrinogen, albumin and IV-fluid administration. This suggests that DENV infection is a syndrome with multiple clinical manifestations, rather than being just a single disease entity. Therefore, the weakness of a general classification for pathogenesis studies is that symptoms with different underlying biological mechanisms are combined. For the purpose of clinical practice, this is no problem as long as the therapeutic intervention strategy is similar for the respective disease manifestations.

In chapter 4,5, 6 and 7 we applied an unsupervised analysis approach in addition to the supervised clinical classifications. In the supervised analyses, patients were classified into certain groups based on clinical signs and symptoms. Examples are the 1997 and 2009 WHO classification. In the unsupervised analyses, patients were clustered based on the expression of biological markers and subsequently disease severity and clinical symptoms were linked to the different clusters. These two approaches work synergistically in providing evidence to support a hypothesis.
An example is presented in chapter 4, in which cytokine expression in DENV infected patients was investigated by clinical classification and by clustering of patients based on the expression of the predetermined cytokines. The outcomes of both analyses were rather similar, although cluster analysis showed more cytokines being associated with severity of disease than with the clinical classifications. Cluster analysis is not subject to classification bias, which may occur because patients with similar disease severity may have different clinical presentations or because MDs differ

in their judgement of disease severity. However, absolute values of cytokines cannot be linked to the clusters, because these levels determine the formation of the clusters. Therefore, the strength of these studies comes from the combination of both clinical classification and cluster analysis. Another example is the endothelial permeability study (chapter 5). When patients were classified according to the occurrence of plasma leakage (supervised) the markers Ang-2, sVEGFR-2, MMP-2 and endothelin-1 were significantly associated with plasma leakage. Cluster analysis (unsupervised) revealed that Ang-2 and sVEGFR-2 were differentially expressed in the cluster with the highest incidence of plasma leakage, suggesting that these two markers were the most important for the occurrence of plasma leakage.

As the 2009 classification, which accurately classifies patients according to disease severity, apparently does not reflect the underlying biological processes, the use of disease severity classification remains a challenge in pathogenesis studies. In order to investigate certain specific pathogenic pathways in humans it is useful to classify patients according to predefined clinical symptoms and laboratory markers. Moreover, unsupervised analyses can circumvent the classification bias and can add significantly to the support of a hypothesis.

Mechanisms of Immune activation

One of the most intriguing aspects of a DENV infection is that severe disease usually starts at a time point that the virus is no longer detectable in the circulation. As in the case of sepsis, it is generally believed that severe dengue is caused by extensive immune activation resulting in a cytokine storm [29]. Pathogenesis research has a strong focus on possible causes of immune activation, in particular antibody-dependent enhancement and original antigenic sin. In this thesis, evidence is provided for additional mechanisms that contribute to immune activation.

Microbial translocation

In two cohorts we detected elevated levels of lipopolysaccharide (LPS) in DENV infected patients, suggestive for the occurrence of microbial translocation (chapter 3 and 4). This was confirmed in

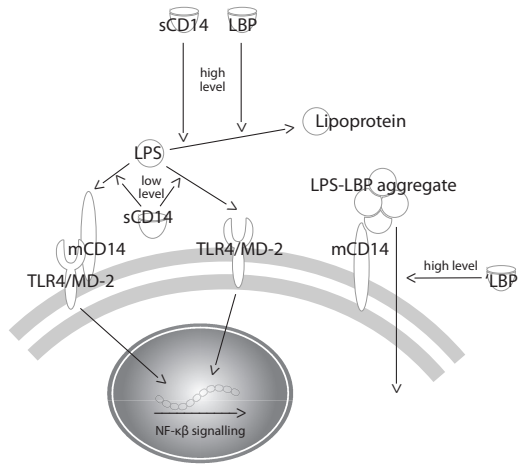

Figure 1 | The LPS pathway. When LPS is present at low levels, LBP and sCD14 facilitate signalling of LPS through the TLR4/MD-2 receptor complex. When present at high levels, LBP and sCD14 shuttle LPS away from the circulation.

the same cohorts by showing increased levels of LPS-binding protein (LBP) and soluble CD14 (sCD14), similar to what is observed in sepsis patients [115,130]. In both studies elevated levels of LPS were associated with severe dengue, especially with regard to plasma leakage and shock. The cohorts differed from each other in several ways: age of the population (children vs. children and adults), geographical location (Indonesia vs. Brazil) and type of samples used (plasma vs. serum). Therefore, we conclude that microbial translocation is a phenomenon that probably generally occurs in DENV infection. Although microbial translocation is known to occur in many systemic infectious diseases, such as HIV/AIDS and Leishmaniasis, this was the first documentation that it occurred during DENV infection [112,133].

LPS is part of a pathogen associated molecular pattern (PAMP), which acts through the TLR4/MD-2-receptor complex resulting in NF-κB signalling and activation of the innate immune system (Figure 1). The immune response to LPS is modulated by LBP and sCD14. After its translocation to the circulation, LPS is transferred to membrane-bound CD14 (mCD14), which facilitates signalling through the TLR4/MD-2 receptor complex. sCD14 is released by activated monocytes/macrophages and plays an important role in modulation of the immune response to LPS. If LPS is present at low levels, sCD14 facilitates the transfer of LPS to the TLR4/MD-2 receptor complex. At high levels of LPS, sCD14 supports its uptake by lipoproteins to shuttle it away from the circulation [117].
LBP is synthesized by the liver upon stimulation with LPS and also plays a dual role in the systemic response to microbial translocation. LBP can accelerate the transfer of LPS monomers from LPS aggregates in the blood to mCD14 resulting in TLR4-signalling. However, if LPS is present at high levels, LBP promotes the transfer of LPS to lipoproteins and facilitates the uptake of LPS-LBP aggregates by the cell [117].
Because of the strong pro-inflammatory properties of LPS, we hypothesized that microbial translocation in DENV infection is associated with immune activation. In the study carried out in Brazil (chapter 4) we could indeed link significantly increased levels of LPS, LBP and sCD14 to a cluster of patients with a pro-inflammatory cytokine profile. Similarly in patients with HIV infection and visceral leishmaniasis it has been shown that microbial translocation contributes to severe disease through excessive immune activation [112,133].
There are several ways in which microbial translocation may contribute to immune activation in patients with DENV infection. First, LPS stimulation can induce the production of several cytokines and growth factors, such as IL-6, IL-8, TNF-α, IL-1β, VEGF, HGF [134,135]. In the study carried out in Brazil high levels of IL-6, IL-8, VEGF and HGF were found in the pro-inflammatory cluster with the increased levels of LPS. Moreover, Bosisio et al. have showed that priming of mononuclear cells with IFN-γ increased the expression of the TLR4 receptor and subsequent LPS-induced cytokine production [136]. This would suggest that DENV induced IFN-γ production could enhance the pro-inflammatory LPS signalling pathway. In addition, Chen et al. [137,138] showed that LPS could prolong DENV infection of monocytes and macrophages. Altogether, the data suggest that microbial translocation can contribute to viraemia and cytokine production in DENV infected patients, which effects are even enhanced by IFN-γ induced TLR4 receptor expression. In the study carried out in Brazil we found an association between microbial translocation and extensive immune activation, suggesting that microbial translocation plays an important role in the induction and perpetuation of the postulated cytokine storm. However, from this study we could not draw any conclusion about the chronologic order of, or the causal relationship between these two events.

In both LPS studies we found an association between microbial translocation and plasma leakage and we hypothesized that both events have a similar underlying cause.

In case of plasma leakage, it is hypothesized that the integrity of the vascular barrier is compromised by either active viral replication or by stimulation of vasoactive agents released by DENV infected cells. A similar mechanism may compromise the intestinal barrier. In HIV infected individuals, microbial translocation was associated with active viral replication in peripheral blood monocytes [112]. A substantial part of these monocytes reside in the gut-associated lymphoid tissue (GALT), which is highly affected by the local pro-inflammatory environment resulting in impaired mucosal integrity [121]. It is not known whether DENV replication takes place in the intestinal tissue or in monocytes and macrophages in the GALT. However, it is likely that intestinal macrophages are also target cells of DENV infection and therefore we hypothesize that DENV replication in intestinal macrophages may lead to a pro-inflammatory environment by which the epithelial cells of the intestine are disrupted. Further studies are needed to further elucidate this mechanism.

Hyperferritinaemia

In the Aruba cohort (chapter 6) we detected increased levels of ferritin in DENV infected patients. It is well known that infectious diseases in general may cause hyperferritinaemia (levels ≥ 500 μg/L) (reviewed in [171]), but in this study levels of ferritin were even significantly increased in mild dengue patients compared to patients with other febrile illnesses. The question was why hyperferritinaemia occurred more often in patients with dengue than in patients with other infectious and inflammatory conditions. The major target cells of DENV replication in vivo (macrophages and monocytes) [41] are also important producers of ferritin. We hypothesized that direct infection and subsequent viral replication activates these cells and increases ferritin production. In agreement with this notion, ferritin levels in the convalescent phase correlated strongly with viral load in the early phase. A high viral load in the early phase of DENV infection has previously been associated with the development of severe symptoms around the time of defervescence [44]. In the cohort study carried out in Brazil, which was also used in the LPS study, increased levels of ferritin were also associated with the cluster of patients with a pro-inflammatory cytokine profile. It has been shown that synthesis of ferritin can be induced by cytokines and iron [179,180]. Moreover, LPS induces iron retention in human monocytic cells, which may subsequently induce ferritin release [181]. Altogether, ferritin expression during DENV infection could be induced by direct viral replication and/or by cytokines released upon DENV infection and/or by LPS.

Coagulation disturbances

The study carried out in Aruba (chapter 6) showed that the coagulation and fibrinolytic systems were highly activated in patients with hyperferritinaemia as illustrated by increased levels of von Willebrand Factor (vWF), thrombin-antithrombin complexes (TAT), plasmin-α2-antiplasmin (PAP) complexes, plasminogen activator inhibitor 1 (PAI-1) and D-dimers. Increased levels of the in vivo marker of thrombin generation TAT and decreased levels of the in vitro marker peak thrombin, suggest that coagulation activation, thrombin formation and the consumption of coagulation factors decrease the ex vivo capacity for clotting during DENV infection. This may be associated with clinical bleeding symptoms as is often observed in DENV infected patients. Hyperferritinaemia is a marker of immune activation. The disturbance of the coagulation system in patients with hyperferritinaemia highlights the cross-talk between the immune system and the coagulation cascade. This cross-talk has also been shown in DENV infected endothelial cells [93].

Biphasic time course of immune activation

In the genomics study carried out in Jakarta (chapter 7) different analysis techniques all pointed towards the finding that time after onset of disease was the main determinant of gene expression patterns in DENV infected patients. The transcriptome signature shifted from immunity and inflammation in the early phase of disease to cell repair mechanisms around the time of defervescence. Several studies have indicated that the early gene signature in DENV infected patients is characterized by over-expression of genes related to inflammation and immunity [69,73,77-79]. The comparison of our data to signatures published by three other studies showed that stratifying the expression data by time after onset of disease resulted in similarities in the top gene lists [73,75,78]. All these studies performed transcriptome profiling in unrelated cohorts from populations with diverse genetic backgrounds, geographical locations and age distributions, which suggests that extensive immune activation in the early phase is a general feature of a DENV infection.

Cross-talk between different players

In a review by Martina et al. it was proposed that several mechanisms during DENV infection could eventually result in coagulation disturbances as well as endothelial cell dysfunction [29]. All the studies combined in this thesis provided more insight in the players that are activated in the human body and contribute to disease severity in DENV infection, which is discussed below.

Soluble markers

As also shown by the studies in this thesis, DENV infection is characterized by extensive immune activation, which may eventually result in a cytokine storm (reviewed in [29,49]). The classical mediators in a cytokine storm are IL-6, IL-8, IL-10, TNF-α and IL-1β. The study carried out in Brazil (chapter 4) contributed to the knowledge about the cytokine storm by identifying other cytokines (IL-1RA, sIL-2R), chemokines (MCP-1, IP-10, MIG, RANTES) and growth factors (HGF, EGF, G-CSF, VEGF), which were also part of the pro-inflammatory state leading to severe disease during DENV infection. In our study the factors IL-6, IL-8, sIL-2R, RANTES, HGF and EGF were strongly linked with severe disease, because they showed a strong association in both the clinical classifications and the cluster analysis.

Innate immune system

Immune responses usually start with activation of the innate immune system and, not surprisingly, we also found extensive activation of the innate immune system in our studies. In the study carried out in Brazil (chapter 4) increased levels of IL-15, IP-10 and G-CSF were detected in the pro-inflammatory cluster. IL-15 and IP-10 [140] play an important role in the NK cell response, whereas G-CSF [61] stimulates neutrophil development and differentiation, which suggests that extensive activation of the innate immune system may contribute to the postulated cytokine storm associated with disease severity.

Several genomics studies, including ours (chapter 7), indicate that acute DENV infection is characterized by extensive activation of the innate immune response [72,78,79]. Especially neutrophil-related genes are highly expressed in the dengue signature and even correlated with disease severity [75-77]. In agreement with this observation, our genomics study showed activation of neutrophils by a significant correlation between the neutrophil derived protein cytidine deaminase (CDD) and the platelet count, suggesting increased abundance of CDD in the acute phase of disease. High levels of CDD were also detected in patients with other infectious diseases, such as

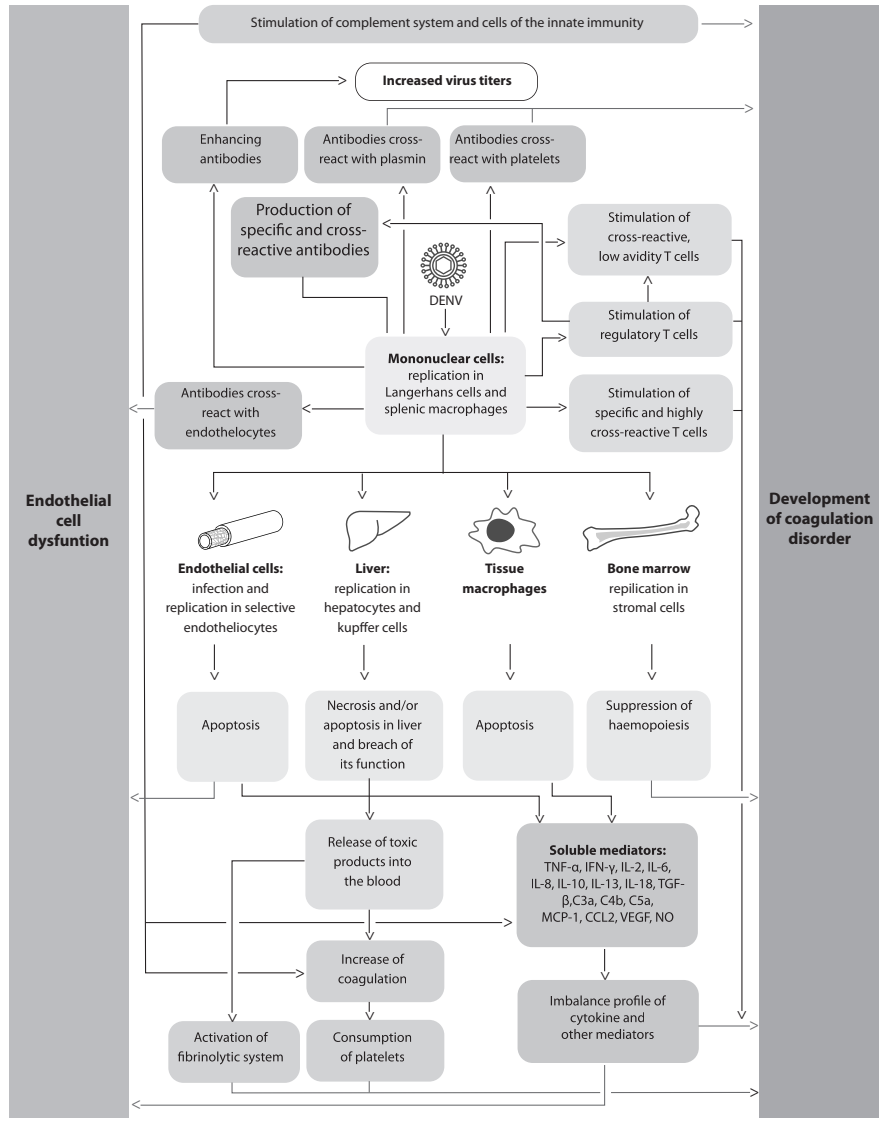

Figure 2 | Players in the pathogenesis of DENV infection. Source: Martina et al. Clinical Microbiology reviews 2010 [29].

meningococcal sepsis [214]. Another finding in the genomics study was that the complement pathway belonged to the most overrepresented ones. Complement plays an important role in clearance of a pathogen and is proposed to inhibit viral replication during flavivirus infections [213].

T cell responses

T cells play an important role in the pathogenesis of DENV infections. First of all, T-cells can be infected by DENV in vitro and in vivo [41,183]. Moreover, T cells are important players in the adaptive immune response to DENV infection, in which the balance between activation and clearance plays a crucial role with implications for the overall pathogenesis.

T-cells are important in the restriction of viral replication. The study carried out in Brazil (chapter 4) showed evidence of a strong Th1 response in the early phase of disease with peak levels of IFN-α [53,139], IL-12 [55-57], MCP-1 [58-60], MIG and MIP-1β [58]. All these Th1 cytokines correlated significantly with the viral load, suggesting that they were associated with a host response aiming at reducing the viral load. The genomics study (chapter 7) also showed increased abundance of the expression of interferon related genes in the early phase of disease. Moreover, the gene IL-18RAP was directly associated with the platelet count, suggesting an increased abundance in the early phase. It has been shown that IL-18 is required to induce IFN-γ production in NK and Th1 cells [210]. Another study showed that both IL-12 and IL-18 were necessary to inhibit viral replication and prevent severe disease in DENV infected mice [211]. The results of the abovementioned studies suggest that a Th1 response is important to restrict viral replication and subsequent disease progression.

If T cell activation is not properly regulated it can be detrimental for the patient. A disease characterized by extensive activation and proliferation of NK and CD8+ T cells is haemophagocytic lymphohistiocytosis (HLH), which is associated with a high morbidity and mortality. A hallmark of this disease is hyperferritinaemia, which was also reported in DENV infected patients in this thesis (chapter 6). Moreover, in the study carried out in Brazil increased ferritin levels were even associated with mortality. HLH develops, because activated NK and CD8+ T cells are not cleared from the circulation. Also for dengue it has been shown that apoptosis of CD8+ T cells is important to prevent the induction of an exaggerated immune response [47,184]. In the study carried out in Brazil (chapter 4) the T cell activation marker sIL-2R was associated with mortality and a pro-inflammatory cytokine profile, suggesting that an exaggerated T cell response contributes highly to pathogenesis in severe DENV infections.

Endothelial cells

Endothelial cell activation most likely plays a crucial role in the pathogenesis of DENV infection, because symptoms like plasma leakage and haemorrhage indicate that these cells are highly affected. Endothelial cells are permissive for DENV infection in vitro, but no clear evidence exists that endothelial cells are indeed infected in vivo [41,220]. It has been shown that DENV infection of endothelial cells in vitro resulted in the production of cytokines, chemokines and complement [80]. With the endothelial permeability study (chapter 5) we provided evidence that endothelial cells play a role in the development of plasma leakage, because four markers, all produced by endothelial cells, were significantly associated with its occurrence. The study carried out in Brazil (chapter 4) also provided evidence that endothelial cells play a role in the pathogenesis, because the endothelial marker VEGF was strongly upregulated in the cluster with the pro-inflammatory cytokine profile. The study carried out in Aruba showed that levels of vWF were significantly increased in patients with hyperferritinaemia. vWF is released upon activation of endothelial cells and therefore this finding suggests that immune activation induces endothelial cell activation, which induces activation of the coagulation cascade.

Liver involvement

Increased levels of liver enzymes indicate that the liver is affected in DENV infected patients. Whether this is the result of direct viral infection or indirect activation by cytokines and immune cells is still a matter of debate [41]. In mice it was shown that DENV infection induced NK and CD8+ T cell infiltration of the liver, which resulted in increased expression of liver enzymes [182].

We have found several signs of liver involvement in our studies. Hepatocytes can synthesize ferritin and in the study in Aruba (chapter 6) liver enzymes were significantly elevated in patients with hyperferritinaemia, suggesting that the liver was involved. Moreover, the genomics study (chapter 7) indicated an important role for the liver in dengue pathogenesis. Especially the gene modules involved in inflammation and immunity correlated with elevated levels of the liver enzymes SGOT and SGPT. Moreover, the genes adiponectin receptor 1, sestrin 3, KCNJ15 and G-protein coupled receptor 27 showed a significant inverse association with the liver enzyme SGOT and have all been described to play a role in insulin resistance, decreased insulin secretion and impaired blood glucose homeostasis [200,201,215]. It has been shown that inflammation and certain cytokines in particular lead to insulin resistance, which may result in further activation of all kind of inflammatory processes [216]. Not surprisingly, it has been shown that DENV infected patient with diabetes had a higher risk to develop severe disease [218,219]. Altogether, this suggests that insulin resistance induced by the liver during DENV infection may also play a role in its pathogenesis.

Clinical markers in the pathogenesis of dengue virus infection

Research in the clinical field of DENV infection has focused on the identification of markers, reflecting disease severity or predicting progression to severe disease. In this thesis we identified several markers, which could be useful in clinical management. Moreover, these markers provide information about the underlying biological processes.

Lipopolysaccharide

As shown in chapter 3 and 4, levels of LPS were increased in DENV infected patients and correlated with disease severity and a pro-inflammatory cytokine profile. Because LPS is a potent immune stimulator, LPS could serve as a marker for immune activation. One drawback is that the LPS assay is quite challenging to perform, because proteins in serum and plasma interfere with the assay [116].

Ferritin

As shown in chapter 6, ferritin may be considered a clinical marker for DENV infection, which can discriminate between dengue and other febrile illness. Moreover, ferritin can also serve as a marker for highly active disease resulting in extensive immune activation, coagulation disturbances and severe clinical symptoms. Therefore, we suggest that patients with hyperferritinaemia should be monitored more closely, as apparently they are at risk to develop severe disease.

Endothelin-1

The study carried out in Brazil (chapter 5) was the first to report increased levels of endothelin-1 in DENV infected patients with plasma leakage. Endothelin-1 is known as a potent inducer of vaso-

constriction [221]. Increased levels have been detected in patients with septic shock and were associated with disease severity and a lower cardiac index due to suppression of the cardiac function [153]. A low cardiac index has also been reported in patients with DSS [159]. Endothelin-1 induced vasoconstriction causes an increase in vascular resistance and a reduction in blood flow, which may eventually result in impaired organ perfusion. In our study, patients with shock were not included, so we can only speculate that endothelin-1 plays a role in the circulatory collapse in DSS.

MMP-2

MMP-2 is produced by microvascular endothelial cells upon DENV infection and increased levels have been detected in patients with plasma leakage in the Brazilian cohort (chapter 5) [160]. MMPs can lyse the subendothelial basement membrane of endothelial cells and may therefore contribute to the development of plasma leakage. Interestingly, in the study carried out in Brazil, MMP-2 was significantly associated with plasma leakage, while MMP-9 was not. MMP-2 is mainly produced by endothelial cells and MMP-9 by macrophages, dendritic cells and endothelial cells and therefore this result suggests that endothelial cells indeed play an important role in the development of plasma leakage.

Angiopoietin-2

In the study carried out in Brazil (chapter 5) Ang-2 was both in the clinical classification and in the cluster analysis, strongly associated with the occurrence of plasma leakage. This is in agreement with a previous study [88]. Ang-2 is synthesized by endothelial cells and quickly released in the circulation upon activation [165]. In vitro, DENV infection of endothelial cells also resulted in an increase of Ang-2 expression [164]. Moreover, adding recombinant Ang-2 to an endothelial cell culture resulted in an increase in vascular permeability due to downregulation of proteins in the adherent and tight junctions between the cells [164]. Ang-1/Tie-2 signalling keeps the endothelium in a quiescent state, while Ang-2 antagonizes this signalling cascade [149]. The strong association of Ang-2 with plasma leakage in our study suggests that Ang-2 is one of the contributors to endothelial cell activation during DENV infection.

Soluble VEGFR-2

sVEGFR-2 levels also showed a strong association with plasma leakage in both supervised and unsupervised analyses (chapter 5). In line with a previous study, levels were significantly decreased in patients with plasma leakage [85]. In vitro, DENV infection of endothelial cells resulted in decreased levels of sVEGFR-2 in the supernatant [85]. VEGF induces vascular permeability by signalling through sVEGFR-2. This signalling results in endocytosis of VE-cadherin in the endothelial cell and perturbation of the junctions between adjacent cells [167]. Both Ang-2 and sVEGFR-2 cause vascular permeability in vitro by disrupting the junctional proteins of endothelial cells and therefore we hypothesize that this is one of the mechanisms causing plasma leakage. Moreover, both markers showed a strong correlation with the occurrence of plasma leakage, which relation was not influenced by time after onset of disease, suggesting they could serve as surrogate markers for plasma leakage in patients with acute DENV infection.

sIL-2R

An exaggerated T cell response can be detrimental for the host. sIL-2R is a T cell activation marker and the study carried out in Brazil showed that sIL-2R was associated with mortality and a pro-inflammatory cytokine profile [141]. This observation was in line with other studies in which sIL-2R has been associated with severe dengue [44,50,139].

Platelet count

From the early days onward, DENV infection has always been associated with the occurrence of thrombocytopenia [94]. It was even one of the four criteria for DHF in the 1997 WHO dengue case classification [17].

There are several hypotheses about how DENV infection leads to platelet depletion, which include cytokine induced bone marrow suppression [209], complement-induced lysis [99] or binding of platelets to activated endothelial cells [104]. In the genomics study (chapter 7) evidence was provided for a strong expression of the immune response and complement in the acute phase of the disease, which may lead to bone marrow suppression and/or complement-induced lysis. The study carried out in Aruba (chapter 6) showed increased expression of vWF in patients with hyperferritinaemia, which results in binding of platelets to endothelial cells to form a haemostatic plug. In the study carried out in Brazil (chapter 5), patients with plasma leakage had significantly lower levels of platelets than those without. Moreover, the platelet count showed a strong correlation with the two major endothelial permeability markers Ang-2 and sVEGFR-2. The platelet count has been shown to correlate inversely with the occurrence of plasma leakage [104]. Besides the low number, it is hypothesized that the function of platelets is also impaired in DENV infected patients [104]. A recent study showed that platelets of DENV-infected patients displayed increased expression of IL-1β, which correlated with the occurrence of plasma leakage [222]. Under normal circumstances, platelets play a crucial role in stabilizing the vascular barrier and therefore, the presence of thrombocytopenia and thrombocytopathy, may contribute to disruption of the vascular barrier (reviewed in reference [106]).

The network analysis in the genomics study (chapter 7) showed that many gene modules were strongly associated with the platelet count. It was concluded that the platelet count was a good marker for systemic disease activity. Altogether, it may be concluded that thrombocytopenia reflects immune activation during DENV infection and we hypothesize that the depletion of platelets during DENV infection plays an important role in the development of plasma leakage.

Albumin

The network analysis (chapter 7) in the genomics study showed that albumin was also a marker of systemic immune activation. Albumin is strongly negatively charged, which prevents leakage from the circulation under normal conditions. However, decreased levels of albumin have been detected in patients with DSS, suggesting that selective restriction by the endothelial barrier is impaired during DENV infection [223]. The genomics study indicates that extensive immune activation may be responsible for that impairment.

Fibrinogen

Fibrinogen is an acute phase reactant and is consumed upon thrombin generation. Decreased levels of fibrinogen have been detected in patients with severe dengue [91]. The genomics study (chapter 7) showed a relation between fibrinogen and immune activation, which suggests that activation of the coagulation cascade is associated with the strong immune response in the acute phase of DENV infection. It has been shown that activation of the coagulation cascade can induce the production of cytokines through NF-κB activation [93], suggesting that cross-talk between coagulation and inflammation contributes significantly to disease severity.

Conclusions

In this thesis two major questions have been addressed: how can we identify patients at risk to develop severe disease and which pathogenetic mechanisms contribute to disease severity.
Markers reflecting general immune activation are ferritin, platelet count, albumin and fibrinogen. Besides being a marker of immune activation, ferritin could also discriminate very accurately between DENV infection and other febrile illnesses. Ang-2 and sVEGFR-2 are strong markers for the occurrence of plasma leakage, even in mild infections. We identified microbial translocation as a novel mechanism in immune activation in DENV infected patients. The release of the potent immune stimulatory molecule LPS can contribute significantly to extensive immune activation, although further studies are warranted to investigate whether there is a causal relationship between microbial translocation and immune activation. The studies in this thesis indicated that the innate immune system, monocytes, macrophages and hepatocytes were highly activated upon DENV infection. They also suggested that extensive immune activation leads to T cell pathology and coagulation disturbances. Altogether, it may be concluded that extensive immune activation due to interplay between different cells and mediators, causes severe disease in patients with acute DENV infection.

Future perspectives

The globally increasing incidence of DENV infection has become a major public health issue and besides vector control, only an effective vaccine would eventually be able to significantly reduce the burden of disease. Because the chances of developing severe disease are increased upon a secondary DENV infection it is important to develop a vaccine, which will induce long-lasting protection directed against all four serotypes. Up till now vaccines have failed to induce neutralizing antibodies against all four serotypes [224,225]. Moreover, it has been claimed that a new serotype of DENV was recently discovered, which may compromise the efficacy of the current vaccines [226]. Therefore, novel vaccine strategies are needed to develop an effective vaccine against DENV infection. Ideally, a vaccine would target an antigen that is conserved among all DENVs. This will be challenging, due to so far poorly understood problems associated with antibody-dependent enhancement [227]. Whether similar problems may also be encountered upon vaccination with e.g. live attenuated vaccines that induce cellular immunity, could be a matter of debate [228,229]. Furthermore, even in the much more active research fields of influenza, malaria and AIDS, the search for safe and effective broadly protective vaccines is ongoing for several decades without major breakthroughs so far [230-232]. It will undoubtedly take several more years before an appropriate safe and effective DENV vaccine will reach the people at risk. Therefore, at least for the near future, it is important to focus on proper identification of patients prone to develop severe disease and the development of therapeutic agents to properly prevent and treat DSS. In the search for prognostic markers it is important to distinguish systemic immune activation markers from markers, specific for certain symptoms, such as haemorrhage and plasma leakage. This issue should be addressed in a new dengue severity classification. Currently, therapeutic options for DSS are limited and consist of symptomatic treatment and supportive care. Pathogenesis research is needed to identify pathways, which could be targeted by therapeutics. In this thesis ET-1 was associated with plasma leakage in DENV infection. ET-1 could serve as a therapeutic target, because it has been shown that medication-induced reduction of ET-1 levels resulted in improvement of cardiac output, peripheral resistance and blood flow in a rat model of sepsis [233]. However, in the sepsis field many promising therapies have failed clinically and therefore miracles may probably not be expected from this strategy [234].
The pathogenesis of DENV infection is still not fully unravelled, while a good understanding is important to develop effective preventive and therapeutic strategies. In this thesis several mechanisms leading to immune activation were identified, which is a significant contribution to the current knowledge about the pathogenesis of DENV infection. However, more research is needed to determine their specific role and impact on disease severity.

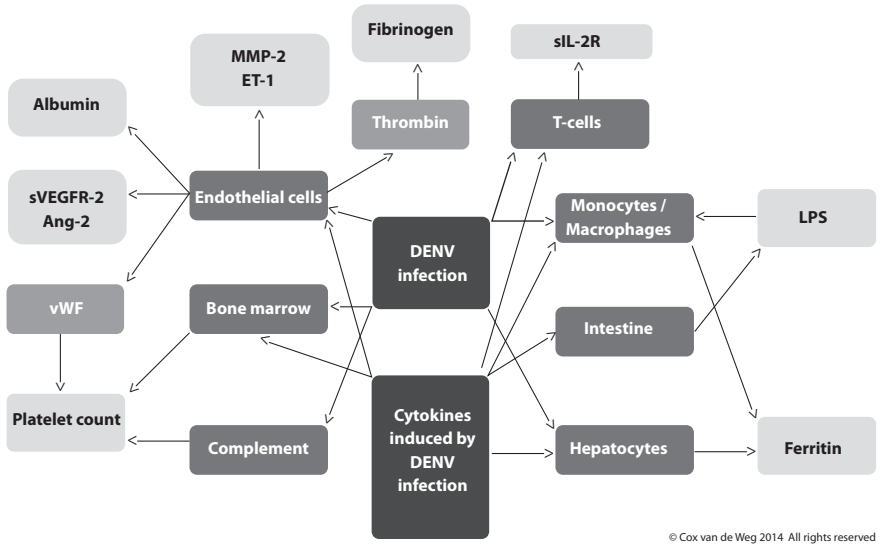

Figure 3 | Summary of the association between clinical parameters and organs/cells affected by DENV infection. It has been proven that DENV replicates in monocytes/macrophages and T cells and there is evidence that it can also replicate in hepatocytes, endothelial cells and bone marrow and that DENV induces complement activation. It has been shown that DENV induced cytokines can activate monocytes/macrophages, T cells, hepatocytes and endothelial cells and that they can induce bone marrow suppression. We hypothesize in this thesis that these cytokines also induce intestinal permeability, resulting in microbial translocation. In the light grey boxes are markers that derive from the depicted organs and/or cells and are indicative for the level of immune activation in case of LPS, ferritin, fibrinogen, platelet count, albumin and sIL-2R and for the occurrence of plasma leakage in case of MMP-2, ET-1, sVEGFR-2 and Ang-2. Thrombin and vWF are intermediate markers.

9

Nederlandse Samenvatting

Nederlandse Samenvatting

Denguevirusinfecties komen wereldwijd voor in tropische en subtropische gebieden. Het denguevirus behoort tot een familie virussen, die wordt aangeduid met de naam Flaviviridae. Het denguevirus wordt overgedragen door zogenoemde tijgermuggen. De denguevirussen verschillen onderling in genetische structuur en kunnen op basis daarvan worden ingedeeld in vier verschillende groepen ook wel serotypes genoemd, namelijk DENV-1, -2, -3 en -4. Een recente studie heeft berekend dat er naar schatting jaarlijks 390 miljoen mensen besmet raken met denguevirus [2]. Echter, enkel een kwart van deze geïnfecteerde groep ontwikkelt klinische symptomen. Alle symptomatische denguevirusinfecties beginnen met koorts, malaise, spierpijn, gewrichtspijn, hoofdpijn en pijn achter de ogen. Vanwege de gewrichtspijn wordt denguevirusinfectie ook wel knokkelkoorts genoemd. Verder tast een denguevirusinfectie het endotheel van de bloedvatwand aan, waardoor er lekkage van vocht uit het bloedvat naar de omliggende weefsels en kleine en grotere bloedingen kunnen ontstaan. De vaatlekkage is meestal beperkt tot de thorax- en de buikholte met respectievelijk pleuravocht en ascites tot gevolg. De verhoogde bloedingsneiging uit zich vaak in kleine puntbloedinkjes in de huid (petechiën) en bloedingen van de slijmvliezen. Bij het merendeel van de patiënten verdwijnt de koorts na 3-5 dagen en begint het herstel. Een kleine groep patiënten ontwikkelt daarentegen ernstige symptomen op dit tijdspunt en komen in de kritieke fase. Ernstige symptomen zijn shock, ernstige bloedingen en orgaanfalen.

Het is nog steeds niet duidelijk hoe een denguevirusinfectie bij een bepaalde groep mensen kan leiden tot ernstige ziekte, terwijl een infectie bij het merendeel van de mensen subklinisch of ongecompliceerd verloopt. Vanwege deze onzekerheid zijn klinische onderzoekers naarstig op zoek naar markers, die het beloop van een infectie met denguevirus in een vroeg stadium kunnen voorspellen. In dit proefschrift worden beide aspecten belicht.

Het uitgangspunt van elk klinisch onderzoek is een goede classificatie van patiënten. In klinische studies met patiënten, die geïnfecteerd zijn met denguevirus, worden meestal de classificaties van de wereldgezondheidsorganisatie (WHO) gebruikt. Deze classificatie is sinds 1974 in ontwikkeling. De meest recente stamt uit 2009 als vervanging voor de klinische classificatie van 1997. De classificatie van 1997 hanteerde strikte criteria om de patiënten in te delen naar ernst van ziekte. Patiënten met een ongecompliceerde denguevirusinfectie werden ingedeeld als 'dengue fever (DF)' [17]. Patiënten, die voldeden aan de volgende vier criteria, koorts, een trombocytopenie (bloedplaatjes < 100.000 cellen/mm3), tekenen van vaatlekkage en een verhoogde bloedingsneiging, werden ingedeeld als 'Dengue hemorrhagic fever (DHF)'. Als een patiënt zich presenteerde met een ernstige bloeding, maar geen tekenen van vaatlekkage vertoonde, dan werd hij/zij dus officieel niet in de DHF groep ingedeeld, terwijl er wel sprake kon zijn van een levensbedreigende ziekte. De 2009 WHO classificatie hanteert minder strikte criteria [1]. Patiënten worden ingedeeld in een groep met niet-ernstige of ernstige ziekte. In de groep met ernstige ziekte lijden patiënten aan shock, ernstige bloedingen of orgaanfalen. Een vergelijking van de classificaties van 1997 en 2009 wordt in hoofdstuk 2 gepresenteerd. Hierbij werd de klinische therapie als een uitkomstmaat gebruikt in de veronderstelling dat ernstig zieke patiënten een intensievere therapie nodig hebben dan minder ernstig zieke patiënten. Uit deze studie bleek dat met de 2009 classificatie inderdaad patiënten met ernstige ziekte accuraat werden inge-

deeld in de ernstige dengue groep, terwijl bij toepassing van de 1997 classificatie een aantal ernstig zieke patiënten niet in de DHF groep terecht kwam.

Een van de centrale onderzoeksvragen is hoe het komt dat sommige patiënten ernstige ziekte ontwikkelen na infectie met een denguevirus en andere niet. Het is fascinerend dat ernstige ziekte tijdens een denguevirusinfectie meestal ontstaat op een moment dat het virus niet meer in het bloed te detecteren is. Dit is een aanwijzing dat ernstige symptomen waarschijnlijk worden veroorzaakt door een heftige afweerreactie van het immuunsysteem tegen het virus en niet direct door het virus zelf. Een van de belangrijkste theorieën, die dit fenomeen probeert te verklaren, is 'antibody-dependent enhancement' [125]. Een denguevirus infecteert in eerste instantie met name bepaalde afweercellen in het bloed, namelijk de monocyten en macrofagen [41]. De theorie van 'antibody-dependent enhancement' veronderstelt dat antistoffen, die aangemaakt zijn tijdens een eerdere denguevirusinfectie met een ander serotype, het infecterende virus wel binden, maar niet volledig kunnen neutraliseren. Door de binding van de antistoffen wordt de opname van het virus in monocyten en macrofagen echter wel verhoogd. Omdat deze antistoffen het virus niet neutraliseren, kan het virus vervolgens in deze cellen repliceren met een hoger aantal virusdeeltjes in de bloedbaan tot gevolg. Studies laten zien dat een groot aantal virusdeeltjes in de vroege fase van een infectie, ernstigere symptomen in een latere fase tot gevolg hebben [44,50].

In dit proefschrift beschreven wij voor het eerst het fenomeen microbiële translocatie in patiënten geïnfecteerd met denguevirus. De darmen van mensen zitten vol met bacteriën. Een deel van deze bacteriën heeft een stofje genaamd lipolysaccharide (LPS) in de celwand zitten. In dit proefschrift was de hypothese dat een denguevirusinfectie een verhoogde doorlaatbaarheid van de mucosale barrière in de darm zou kunnen veroorzaken, waardoor er LPS in de bloedbaan terecht zou kunnen komen. In het onderzoek beschreven in hoofdstuk 3 werd inderdaad gevonden dat er significant verhoogde LPS waarden detecteerbaar waren in het bloed van dengue patiënten in Indonesië. Ook In een volgende studie met een cohort in Brazilië konden we deze bevindingen bevestigen. LPS kan bepaalde delen van het immuunsysteem activeren, wat leidt tot de productie van allerlei eiwitten, zogenaamde cytokinen, die op hun beurt ook weer bijdragen aan activatie van het immuunsysteem en inflammatie. In het cohort in Brazilië waren verhoogde LPS-waarden significant geassocieerd met een cluster van patiënten met een verhoogde expressie van deze cytokinen en eveneens klinisch ernstige ziekte. Door deze resultaten kon geconcludeerd worden dat er een associatie was tussen microbiële translocatie en immuunactivatie tijdens een denguevirusinfectie.

Een ander eiwit, dat werd bestudeerd in relatie met denguevirusinfecties, was ferritine. Ferritine wordt geproduceerd door immuuncellen (monocyten en macrofagen) en levercellen (hepatocyten). Deze cellen worden geactiveerd om ferritine te gaan produceren door het immuunsysteem of door een infectieus pathogeen. In een cohort in Aruba vonden wij sterk verhoogde ferritinewaarden in patiënten met ongecompliceerde dengue. Uit deze studie bleek ook dat patiënten met dengue veel hogere ferritinewaarden hadden dan patiënten met andere koortsende ziekten. Hieruit kon geconcludeerd worden dat ferritine gebruikt zou kunnen worden als marker om patiënten met dengue van patiënten met andere koortsende ziekten te kunnen onderscheiden. Bovendien werd het hebben van een verhoogde ferritinewaarde ook geassocieerd met aantasting van de lever en activatie van de stolling. Bovendien bleek uit het cohort in Brazilië dat, net als LPS, ook ferritine in verband gebracht kon worden met patiënten, die een verhoogde expressie hadden van cytokinen en daarnaast klinisch ernstige ziekte. Uit deze studie kon geconcludeerd worden dat ferritine een goede marker is voor immuunactivatie, die kan leiden tot stollings- en leverfunctiestoornissen en klinisch ernstige ziekte.

Het feit dat klinische dengue leidt tot vaatlekkage en bloedingen is een belangrijke aanwijzing dat de endotheelcellen van de bloedvatwand zijn aangedaan tijdens een infectie. In een cohort in Brazilië bepaalden we de waarden van negen eiwitten, die worden geproduceerd door endotheelcellen. De expressie van deze eiwitten werd geassocieerd met het optreden van klinische symptomen van vaatlekkage. Voor het eerst werden verhoogde waarden van het eiwit endotheline-1 gedetecteerd in patiënten met vaatlekkage ten opzichte van patiënten zonder vaatlekkage. Daarnaast bleek uit verschillende analyses elke keer weer dat de eiwitten angiopoietine-2 en soluble VEGFR-2 significant geassocieerd waren met vaatlekkage in dengue patiënten. Omdat deze twee markers een hele sterke associatie hebben met het optreden van klinische symptomen van vaatlekkage, kunnen ze gezien worden als surrogaatmarkers voor het optreden van dit fenomeen tijdens denguevirusinfecties.

In een cohort van dengue patiënten in Jakarta, Indonesië, bestudeerden we de genexpressie van patiënten tijdens een infectie. We includeerden patiënten binnen 48 uur na het ontstaan van symptomen en volgden hen voor een week, zodat we het hele beloop van de immuunrespons goed konden bestuderen. Deze studie liet zien dat een denguevirusinfectie gekarakteriseerd wordt door een enorme activatie van de immuunrespons in de vroege fase van de ziekte, gevolgd door activatie van cellulaire reparatiemechanismen in een latere fase. Daarnaast bleek de tijd na het ontstaan van ziekte de grootste impact te hebben op de genexpressie in patiënten met een ongecompliceerde denguevirusinfectie. De enorme invloed van tijd op de genexpressie bleek ook door het vergelijken van de resultaten van onze studie met die van drie andere studies. Stratificatie van de data naar tijd na het ontstaan van ziekte, leidde tot grote overlap in de genexpressie. Omdat deze studies verschilden in geografische locatie, leeftijdsgroep en genetische achtergrond, kunnen we concluderen dat genexpressie in de tijd een algemeen verschijnsel is van een denguevirusinfectie. Daarnaast werd ook onderzocht hoe bepaalde klinische parameters geassocieerd waren met clusters genen met dezelfde biologische functie. Uit deze analyse bleek dat bepaalde klinische parameters, zoals de concentratie albumine, fibrinogeen of bloedplaatjes, sterk geassocieerd waren met bepaalde clusters genen. Het interessante was dat de meer algemene WHO classificaties, die uit een combinatie van verschillende klinische parameters bestaan, niet geassocieerd waren met de clusters genen. Hieruit kan de conclusie worden getrokken dat deze twee classificaties weliswaar wel de ernst van ziekte, maar niet de onderliggende biologische processen reflecteren.

In dit proefschrift hebben we enerzijds mechanismen bestudeerd die leiden tot immuunactivatie in dengue patiënten, zoals microbiële translocatie. Anderzijds zijn ook verschillende klinische markers onderzocht, die een indicatie geven voor de mate van immuunactivatie. Een denguevirusinfectie veroorzaakt een hevige systemische immuunrespons, die kan leiden tot klinische symptomen, zoals vaatlekkage en bloedingen. Markers voor systemische immuunactivatie zijn de concentratie van bloedplaatjes, ferritine, albumine en fibrinogeen. Als markers voor vaatlekkage werden angiopoietine-2 en sVEGFR-2 gevonden. De onderzochte markers kunnen worden gebruikt als leidraad voor klinisch handelen en om tot een betere classificatie te komen voor klinisch onderzoek naar de pathogenese van denguevirusinfecties.

10

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Reference List

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About the Author

About the Author

Curriculum vitae

The author of this thesis, Cornelia Anna Maria (Cox) van de Weg, was born on the 1st of April 1983 in Dordrecht, The Netherlands. In this city she attended the gymnasium at the Insula College, from which she graduated 'with honours' in 2001. After high school she studied business administration at the Erasmus University in Rotterdam for a year, before starting medical school at the same university in 2002. Tropical infectious diseases became her interest in the final years of these studies, which resulted in her conducting a graduation research at the Hospital del Niño in Lima, Peru, about acute diarrheal disease and the use of antibiotics in children. Next to that, this interest brought her to the internal medicine department of the Academical Hospital in Paramaribo, Suriname, the Macha Mission Hospital in Zambia as well as the department of infectious diseases at the Erasmus Medical Center for clinical rotations. After graduating from medical school in 2009 she worked as a resident at the intensive care unit of the Albert Schweitzer hospital in Dordrecht for 6 months. In 2009, she not only started her work as a PhD student at the department of virology, but also continued her studies with the postgraduate master 'Infection and Immunity' both at the Erasmus Medical Center. She graduated from the master in 2011. Her PhD research about the pathogenesis of dengue virus infection continued and was conducted in collaboration with researchers from the University of São Paolo, Brazil, the Dr. Oduber Hospitaal in Aruba and the University of Indonesia in Jakarta. Currently, Cox is working at the St. Elisabeth hospital in Tilburg, where she is specializing in internal medicine/infectious diseases.

List of publications

- Evaluation of the 2009 WHO dengue case classification in an Indonesian pediatric cohort.
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- Review: Viral infections and mechanisms of thrombosis and bleeding.
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Journal of Clinical Virology. 2014. Accepted for publication

– Hyperferritinaemia in dengue virus infected patients is associated with immune activation and coagulation disturbances.
Van de Weg CA*, Huits RM*, Pannuti CS, Brouns RM, van den Berg RW, van den Ham HJ, Martina BE, Osterhaus AD, Netea MG, Meijers JC, van Gorp EC, Kallas EG.
Submitted

– Time since onset of symptoms and classical clinical markers associate with transcriptional changes in uncomplicated dengue.
Van de Weg CA*, van den Ham HJ*, Bijl MA, Anfasa F, Zaaraoui-Boutahar F, Dewi BE, Nainggolan L, van IJcken WF, Osterhaus AD, Martine BE, van Gorp EC, Andeweg AC. Submitted
* These authors contributed equally to the study

PhD Portfolio

Name PhD student
Cornelia (Cox) van de Weg

Research group
Department of Viroscience

Research school
Post-graduate Molecular Medicine

PhD period
2009-2014

Promotors
Prof. dr. A.D.M.E. Osterhaus
Prof. dr. E.C.M. van Gorp

Education

2002-2009
– Medical school at Erasmus Medical Center, Rotterdam, The Netherlands
2009-2011
– Master of Science Infection and Immunity. Postgraduate school Molecular Medicine at the Erasmus Medical Center, Rotterdam, The Netherlands.

In-depth courses

2010
– Classical Methods for Data-analysis (NIHES)
– Study Design (NIHES)
– Ensembl (MolMed)
– Basic data analysis on gene expression arrays (MolMed)
2011
– Basiscursus Reizigersadvisering en immunisatie voor artsen (NSPOH)
– The Basic Introduction Course on SPSS (MolMed)
– Research Management for PhD-students (MolMed)
– Photoshop and Illustrator CS5 (MolMed)
2012
– Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers (Erasmus MC)

Presentations

2011
- 15th Molecular Medicine day, Rotterdam (poster),
- European Society of Clinical Virology, Madeira (poster)

2012
- 16th Molecular Medicine day, Rotterdam (poster)
- 15th International Congress on Infectious Diseases, Bangkok (poster)

Attended meetings

2009
Seminar on dengue, University of Jakarta

2010
Indonesian PICU-NICU update
14th Molecular Medicine day, Rotterdam

2011
49th Annual Meeting of the Infectious Diseases Society of America, Boston

Supervision and teaching activities

2010
Mentor graduation research medical students in Jakarta

2011
Mentor graduation research medical student in Curacao

2011-2012
Co-supervision master student

2011-2012
Mentor graduation research medical students in Aruba

2012
Coach 'viruskenner'

2012-2013
Guest lecturer for the master Infection and Immunity

Miscellaneous

Reviewer Dengue Bulletin
Reviewer Journal of Medical Virology
Reviewer Journal of Pediatric Infectious Diseases
Reviewer the Journal of Infection in Developing Countries

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Dankwoord

Dankwoord

Wat voelt het eigenlijk goed om na ruim vier jaar de boekjes van anderen door je handen te laten gaan, nu eindelijk de laatste hand te kunnen leggen aan het eigen boekje. En er is geen mooiere finale dan het dankwoord om even te kunnen reflecteren wie allemaal een steentje hebben bijgedragen aan het tot stand komen van dit werk, hetzij inhoudelijk, hetzij aan de sociale context.

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Mijn medische carrière is begonnen op de intensive care van het Albert Schweitzer ziekenhuis in Dordrecht. Daarom wil ik mijn toenmalige bazen, dr. Ponssen, So, Hendriks, Te Velde en Krist, en de verpleging hartelijk danken voor deze fantastische start van mijn loopbaan. Deze tijd heeft echt een onuitwisbare indruk op me gemaakt en ik had me geen betere eerste baan kunnen bedenken.

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Florien, gisteren voelde ik me toch weer een beetje opgelucht toen ik hoorde dat je veilig uit de Central African Republic was thuisgekomen. Ik heb enorm veel respect en bewondering voor de bevlogenheid waarmee je je werk bij artsen zonder grenzen doet, maar ik mis het wel enorm om een rondje om de kralingse plas met je te wandelen als je op missie bent. Het is zo fijn hoe jij gelukkig kan worden van kleine dingen en daarnaast ben je ook een fantastische en relaxte reispartner. Ik weet niet hoeveel rondjes we inmiddels hebben gelopen om die plas, maar wat mij betreft kunnen er niet genoeg volgen.
Fre, jij bent een van mijn trouwste en meest loyale vriendinnetjes en ik weet dat ik altijd een beroep op je kan doen. De manier waarop jij je werk als geriater doet is voor mij echt een voorbeeld. Het is eigenlijk best bijzonder dat wij in heel veel opzichten precies tegenovergesteld zijn, maar dat is ook juist weer de kracht van onze vriendschap. Ook met jou kan ik heerlijk op de bank of een terrasje hangen en uren bijkletsen. Ik ben heel blij dat jij en Florien mijn paranimfen zijn op dit bijzondere moment.

En dan komen we inderdaad aan bij de familie. Manon, naast mijn schoonzusje ben je ook meteen een goed vriendinnetje en had ik het me niet beter kunnen wensen. Ook mijn schoonouders Ton en Marion wil ik bedanken voor hun steun tijdens mijn promotietraject. Ik voel me altijd welkom in Zuid-Limburg en het is ook altijd gezellig om jullie in Rotterdam te ontvangen.
Opa Voordouw is waarschijnlijk een van de weinige leken, die mijn artikelen ook daadwerkelijk leest en begrijpt. U letterlijk citerend 'bent u wat kwistig geweest met uw genen' en vandaar dat ik nu de vijfde binnen de familie Voordouw ben, die de titel van PhD mag ontvangen. Opa, hartelijk dank voor uw gulheid (en die van oma) wat betreft jullie genen en uw interesse voor mijn leven. Daarnaast ben ik er trots op dat ik een van de weinige 30-ers ben met een opa op facebook.
Ook opa Van de Weg, hoewel nooit heel bewust gekend, heeft invloed gehad op de keuzes in mijn carrière. Zijn 'legacy' als arts en verzetsleider is een dagelijkse bron van inspiratie in mijn werk als arts.
Tante Jeanne, de logeerpartijtjes bij u en oom Ben waren een groot feest en iets waar ik nog met veel plezier aan terugdenk. En nog steeds is het heerlijk om met U op de Veluwe even een 'ruifburger' te gaan eten. Heel veel dank voor alle liefde en gezelligheid.
Onze vaste oppas tijdens mijn kinderjaren was tante Magda Thoeng. Nog steeds heb ik hele warme herinneringen aan deze tijd met alle verhalen over het verre Indonesië. U creëerde een hele fijne en veilige basis tijdens onze kindertijd en daar ben ik u heel dankbaar voor.

Lieve Geert, als broer en zus waren wij al van jongs af aan enorm aan elkaar gewaagd met heel veel ruzietjes tot gevolg. Gelukkig zijn we daar wel overheen gegroeid en ben ik nu een hele dag met jou door de stad aan het dwalen om een leuke locatie voor mijn promotieborrel te zoeken. We delen een passie voor lekker eten en de andere goede dingen van het leven en ik vind het heel knap hoe je het altijd voor elkaar krijgt om je dromen waar te maken. Lieve Christianne, ook op jou ben ik heel trots, want tegen beter weten in, is het je toch gelukt om een carrière op te bouwen in de kunstsector. In dat opzicht ben je net als de rest super eigenwijs en heb je ook een goede portie doorzettingsvermogen. Je bent eigenlijk wel de liefste van ons vieren en ik kan altijd op je rekenen als ik hulp nodig heb en dan zal je ook nooit half werk verrichten. Lieve Goof, qua doen en laten lijken wij eigenlijk wel het meeste op elkaar. Jij bent mijn jongste broertje en daarom vind ik het heerlijk om je grote zus te zijn

en je te helpen met kleding of cadeautjes shoppen. Maar ook kleine broertjes worden groot en zeker de laatste tijd wordt het steeds duidelijker dat ook de benjamin van de familie een serieus leven begint op te bouwen. Ik ben echt super trots op wat je inmiddels al in een hele korte periode hebt bereikt. Ook Merle, bedankt dat je Goof zo gelukkig maakt.

Lieve mama, bedankt voor al je liefde en je steun in alles wat ik doe. Je staat altijd voor ons klaar en bent altijd bereid om te helpen. Als we in Dordrecht op bezoek komen dan worden we gigantisch verwend en gaan nooit met lege handen naar huis. Daarnaast plannen we regelmatig een avondje uit om lekker bij te kletsen. Cees, jij maakt het leven van mijn moeder compleet en dat is een woord fantastisch.

Lieve papa, ik kan wel stellen dat ik hier zonder jou niet gestaan zou hebben. Jij hebt ons altijd meegegeven dat we alles kunnen bereiken, mits we er maar voor gaan. Ik ben alle uitdagingen aangegaan, omdat ik wist dat je er bij falen altijd zou staan om me op te vangen. Jij bent de beste mentor, die ik me ooit had kunnen wensen.

Lieve Pascal, jij bent een van de meest succesvolle projecten uit mijn promotieperiode. Niemand, inclusief mijzelf, had vier jaar geleden gedacht dat ik me nu regelmatig met manlief en twee katten op de bank zou vinden. In vier jaar tijd hebben we al heel veel mooie reizen gemaakt en bijzondere dingen beleefd, waarvan de marathon in New York wel echt een hoogtepunt was. En ook in moeilijke periodes heb ik heel veel steun aan je gehad. We houden elkaar scherp en vullen elkaar aan en eigenlijk zeg ik het veel te weinig, maar ik hou gewoon heel veel van jou!

colofon

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