Arboviruses:
markers of disease severity

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The research described in this thesis was performed at the Department of Viroscience, Erasmus University Medical Center (Erasmus MC), Rotterdam, The Netherlands within the framework of the Erasmus MC Postgraduate School of Molecular Medicine; Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia; and Medical Laboratory Services, Curaçao, Dutch Caribbean.

The studies described in this thesis were financially supported by the Ministry of Research, Technology and Higher Education, Republic of Indonesia, internal grants of Faculty of Medicine Universitas Indonesia, the Erasmus MC Foundation and the European Union program ZIKAlliance (contract no. 734548)

The financial support for printing of this thesis by the I&I fund and Cirion Foundation is gratefully acknowledged.

Cover and design : Labib Ilmi and Fatih Anfasa
Print : Proeschriftmaken (www.proeschriftmaken.nl)
ISBN : 978-94-6380-828-6

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Arboviruses: markers van de ernst van de ziekte

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. R.C.M.E. Engels

and in accordance with the decision of the Doctorate Board.

The public defence shall be held on

Monday, 8 June 2020 at 15.30 hours

by

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I heartily dedicate this thesis to my family, especially to my mother, who has provided unconditional love and faith;

to my wife, Salma Oktaria, who fully support me and always believe in me throughout my journey;

to Safaa and Fathi, the apple of my eye

So verily, with every hardship comes ease

(QS 94:6)

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In: Singh SK, editor. Human Emerging and Re-Emerging Infections: Viral & Parasitic Infections, Volume I. New Jersey: John Wiley& Sons; 2015 p.125-44

Rueshandra Roosenhoff, Fatih Anfasa, Byron E. E. Martina
The pathogenesis of chronic chikungunya: evolving concepts.
Fut Virol. 2016;11:61-77

Arboviruses

Arthropod-borne viruses (arboviruses) are viruses that are transmitted through the bites of mosquitoes and/or ticks, in which they also replicate. There are numerous arboviruses, members of different viral families and genera, which are capable of causing human disease. During the past two decades, the flaviviruses Zika (ZIKV) and dengue (DENV), and the alphavirus chikungunya (CHIKV) have (re-)emerged as increasingly important arboviruses of medical importance. All these viruses are transmitted by the *Aedes (Ae) aegypti* and/or *Ae. Albopictus* mosquitoes (1). Several factors contribute to the resurgence of these viruses, such as the increasing global spread of the mosquito vectors, rise in international trade and travel to arboviruses endemic countries, climatic changes, virus evolution, and urbanization with poor living conditions (2). Figure 1 depicts the countries and areas affected by ZIKV, DENV, and CHIKV, which reflect the distribution of the *Aedes* mosquitoes that transmit these three viruses.

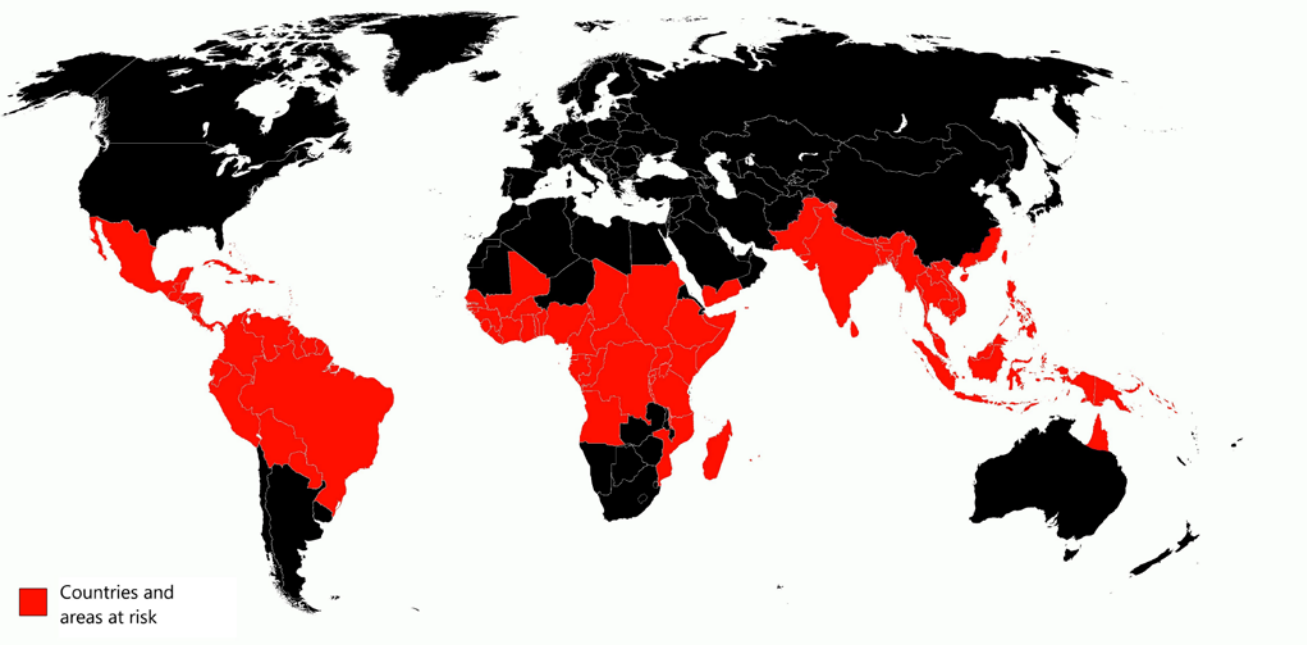

Figure 1. Distribution of Zika, dengue, and chikungunya in areas and countries around the world. The countries and endemic areas are depicted in red. The information is based on reports from WHO and CDC.

To date, there are no antiviral drugs specific for treatment of Zika, dengue and chikungunya infections. Furthermore, there are no licensed vaccines for Zika and chikungunya. Currently, there is only one licensed dengue vaccine available, a recombinant live, attenuated, tetravalent dengue vaccine (CYD-TDV), which varies in performance depending on age and serostatus of the patient (3). Moreover, this vaccine seems to enhance the severity of subsequent dengue infection in seronegative individuals and is only approved for people aged 9-45 years (4). Thus,

an alternative vaccine is warranted for dengue. In addition, efforts are also directed at development of virus targeted or host targeted antiviral drugs. The lack of complete understanding of the disease pathogenesis for all three viruses is a major contributor to the lack of vaccines and therapeutic agents to combat these diseases.

Severe disease manifestations due to Zika, dengue, and chikungunya infections are likely to be a complex interplay between the viral and host factors which determine the disease outcome. Intrinsic properties of a virus, defined in the term virulence, could influence the tropism of the virus, replication in human target cells and the magnitude of the host response (5). On the other side, host factors have been suggested to play an important role in the pathogenesis of infections. Nevertheless, the role of viral and host factors in arbovirus infection has not been completely elucidated. Additionally, there is a lack of reliable (bio)marker of disease severity for important arboviral infections. Hence, studying new potential biomarkers are very important to guide health care workers in managing Zika, dengue, and chikungunya infections.

Epidemiology and disease

Zika

ZIKV is an emerging arbovirus that recently gained a lot of attention as a global public health priority. The virus was first isolated from a rhesus monkey in the Zika forest of Uganda in 1947. Although known to infect humans, ZIKV was not considered to be a significant human pathogen for 60 years, until large scale outbreaks started in 2007 in the Pacific Islands, and different modes of transmission and new severe clinical manifestations and complications were recognized during the 2015-2016 outbreak in South America (6). Similar to most flaviviruses, ZIKV infection is usually asymptomatic or induces a relatively mild febrile disease. However, during the recent outbreaks, severe clinical symptoms and complications were reported, including severe birth defects (7), fetal demise (8), Guillain-Barré syndrome (9), and coagulation disorders (10).

Dengue

Dengue is the most common arboviral disease in humans and the disease is now endemic in more than 100 tropical and subtropical countries. The disease represents a major public health problem, with significant impact on social, economic and political systems. Approximately 2.5 billion people live in dengue endemic areas, with an approximated 100 - 400 million infections annually (11, 12). Around 500,000 patients are admitted to the hospital with severe disease each year. The majority of severe cases are reported in Southeast Asia in young children and result in hospitalization and death in up to 5% of cases. The incidence of DENV infection have

increased more than 30-fold since the 1960s with continuing geographic expansion to new countries, involving both urban and rural settings (11).

Chikungunya

Chikungunya is caused by an emerging arbovirus that caused large outbreaks in recent years. The virus was first isolated from patients suffering from fever and crippling joint pain in 1953 during an outbreak in Tanzania. Locals referred to the disease as "chikungunya", which is a word from the Makonde language that means "to walk bent over", indicating the posture that infected patients suffering from joint pains acquired (13, 14). Starting in 2004, chikungunya went from an endemic viral disease limited to Asia and Africa that caused periodic outbreaks to an important global pathogen. Besides causing millions of cases in the Indian Ocean region it has emerged in new areas, including Europe, the Middle East and the Pacific region (15-18). The attack rate during an epidemic is high, with asymptomatic infections only seen in about 15% of confirmed cases (19, 20).

The pathogens

Flaviviruses: Zika and dengue

ZIKV and DENV are a member of the family *Flaviviridae* and genus Flavivirus, which includes several important arboviruses such as yellow fever virus (YFV), Japanese encephalitis virus (JEV) and West Nile virus (WNV). Flaviviruses are spherical in shape with a diameter of 50 nm, with a positive-sense single stranded RNA. The genome size is approximately 11 kb in length and forms a single open reading frame (ORF) encoding three structural proteins ((capsid (C) protein, a membrane-associated (M) protein and the envelope glycoprotein (E)) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The E protein is the major structural protein exposed on the surface of the viral particle that induces protective immune responses of the host by prompting the production of neutralizing antibodies. This protein is divided into three structural domains, namely envelope domain (ED)I, EDII, and EDIII, held by a helical stem region attached to virus membrane by trans-membrane anchor (21, 22). EDI contains the central region and EDII is involved in virus-mediated membrane fusion. EDIII is an Ig-like domain involved in binding of the virus with cell receptors and contains epitopes recognized by neutralizing antibodies, which makes it to be an important target for the humoral immune response during infection (23).

Two main lineages of ZIKV have been described (African and Asian), which differ by approximately 10% at the nucleotide level (6). ZIKV strains that caused the large outbreaks in the Americas descended from the Asian lineage (24). Before the outbreak of Zika in the Americas, it was unclear whether the different genotypes differed in virulence. ZIKV shares

sequence similarity with other flaviviruses and is most closely related to another African flavivirus named Spondweni virus (approximately 68% E protein amino acid similarity). ZIKV has been classified together with the Spondweni virus into the Spondweni serocomplex, which is closely related to the Japanese encephalitis virus (JEV) serocomplex group (25). Based on the syndrome it causes, Zika could also be classified as a viral neurologic disease.

DENVs are divided into four antigenically distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) and together form the dengue serocomplex group. The classification into serotypes is also supported phylogenetically. DENV serotypes can be further classified into different genotypes by nucleotide sequence comparisons. These serotype and genotype differences have been associated with differences in virulence (26, 27). Based on the syndrome it causes, dengue is also classified as a viral hemorrhagic fever.

Alphavirus: chikungunya

CHIKV is an alphavirus belonging to the family *Togaviridae* and genus alphavirus (28). CHIKV is categorized within the Semliki Forest virus (SFV) serocomplex group. Three CHIKV genotypes are recognized, which historically have spread in the distinct geographical regions after which they are named: West African (WA) genotype, East Central South African (ECSA) genotype, and Asian genotype (29). CHIKV contains a single-stranded positive sense RNA genome of approximately 11 kb that encodes four non-structural proteins (nsP1-4) and five structural proteins (C-E3-E2-6K-E1), including the capsid and two envelope glycoproteins, E1 and E2 (28, 30-32). E1 and E2 are surface glycoproteins and carry the major viral epitopes. The E1 and E2 proteins associate as a heterodimer before being incorporated onto the surface of mature virion as trimeric spikes and participate in the attachment and the entry of CHIKV into target cells (33). Each glycoprotein is further divided into three domains (E1A, E1B, E1C and E1A, E2B, E2C). The most potent neutralizing antibodies target domains A and B of the E2 protein, with those targeting domain B often displaying broad neutralization of multiple strains of CHIKV and other alphaviruses (34-37). Based on the syndrome it causes, CHIKV is also classified as an arthritogenic virus.

Clinical manifestations

Zika

The incubation time of ZIKV is about 3 to 7 days. Infection is asymptomatic in the majority of cases (80%) (38). Symptomatic patients usually develop fever (≥ 38.5°C) accompanied by conjunctivitis, arthralgia, myalgia, headache, fatigue, and rash. Clinical symptoms are usually mild and last for 3-7 days without complications. However, during the recent outbreaks, severe symptoms and complications were also reported, including hemorrhagic manifestations (8, 39-

43), meningitis and encephalitis (44), Guillain-Barré syndrome (9), severe birth defects (7) and even death (8, 41, 42), suggesting a possible tropism of the virus for neurons and endothelial cells. The risk of congenital neurologic defects in fetuses born from mothers infected with ZIKV infection has ranged from 6 to 42% in several reports (7, 45, 46). It has been shown that the risk of microcephaly is higher in the fetus of mothers who are exposed to ZIKV during the first trimester of pregnancy (46, 47). Hemorrhagic manifestations that have been described included subcutaneous bleedings, mucosal bleedings, intracranial hemorrhages, hematospermia, and hematuria (8, 39-43). Interestingly, a case report also demonstrated that ZIKV infection induced coagulation disorders (10). A peculiar feature of ZIKV infection during the recent epidemics is an apparent broadening of cellular tropism and persistence in brain, placenta, and testis which has resulted in apparently new clinical manifestations. It still needs to be determined whether this reflects a fundamental change in ZIKV virulence or whether this is now recognized due to the large number of diagnosed infections.

Dengue

The incubation period following a mosquito bite ranges from 3 to 5 days.The majority of DENV infections are inapparent, producing little clinical illness. Clinically apparent DENV infection is associated with a wide variety of symptoms. Classical dengue fever symptoms are observed more frequently in adults. Clinical signs start suddenly and follow three different phases; an acute febrile phase (AP), a critical phase (CP), which usually ensues at time of defervescence (time when the fever is resolved), and a recovery phase (RP). The AP is usually characterized by fever (≥ 38.5°C) accompanied by headache, fatigue, myalgia, arthralgia, retro-orbital pain, nausea, vomiting, abdominal pain, and sometimes transient rash. Mild hemorrhagic manifestations such as petechiae, gingival bleeding, and spontaneous bruising may also be seen at this stage of the disease. In a minority of patients, mostly in children and young adults, a vascular leakage syndrome may develop around the time of defervescence mainly in the thorax and abdominal cavities. It is not fully understood what drives plasma leakage in the absence of- or at low levels of - viremia during the CP of dengue. Shock may develop as a result of extensive plasma leakage. Without proper treatment, a state of profound shock may set in, in which the blood pressure and pulse become undetectable which eventually may lead to death (48). Major hemorrhagic manifestations such as skin and/or mucosal bleeding or gastro-intestinal bleeding are commonly observed during shock (49). Moderate -to- severe thrombocytopenia is frequent and disturbances of the coagulation pathway is mainly found during this disease phase (50). Those who do not develop serious complications as a result of hyperpermeability of EC are classified as non-severe dengue. It is important to realize that some patients advance to the critical phase without experiencing defervescence. In these patients, the laboratory results

should be used to define the critical phase and guide the diagnosis of plasma leakage and severe dengue. The increased vascular permeability usually lasts for 24-48 hours and rapid improvement in patient condition is seen if supportive treatment is started promptly. The patient then goes into the recovery phase, in which gradual re-absorption of extravascular compartment fluid usually continues for 48-72 hours (11).

Chikungunya

The incubation period of chikungunya is about 2 to 7 days (51). Infection with CHIKV results in development of acute symptoms in the majority of infected individuals, such as fever (temperature is usually ≥ 38.5°C), headache, rigors, petechial or maculopapular rashes, asthenia, edema of the extremities, myalgia and arthralgia (20, 28, 32, 52). Edematous and incapacitating arthralgia with sometimes arthritis are characteristic of the disease, with small joints of the hand, wrist, and ankles being most often affected. Nevertheless, the larger joints of the shoulder and knee may also be involved. Although chikungunya affects all age groups and both males and females, rheumatologic manifestations are less common in children. These clinical manifestations have a major impact on the quality of life of patients and may result in a significant economic burden for the society. The recovery phase starts 1-2 weeks after onset of disease, but, in approximately 30-50% of patients arthralgia/myalgia may persists for weeks, months or even years (53, 54). The underlying mechanism by which CHIKV induces chronic arthralgia is still unclear and requires further investigations.

Pathogenesis of arbovirus infections

The pathogenesis of many arbovirus infections is still not completely elucidated. Severe disease is likely to be caused by a multi-factorial process where complex interactions between viral and host factors determine the disease outcome. Several host factors have been proposed to play an important role in the pathogenesis of these virus infections. The immune response to the virus, pre-immunity to other similar viruses, hemostatic system, host genetics and epigenetics, age, gender, presence of comorbidies and the microbiome have all been shown to contribute to the disease outcome. In addition, the environment of the host may also affect the type of response to a virus infection. On the other hand, intrinsic properties of the virus embedded in the term virulence, could influence the magnitude of the immune and hemostatic responses. Therefore, both viral virulence and host response may be involved in the cell death and/or dysfunction pathways thereby contributing to disease severity.

Coagulation disorders: focus on secondary hemostasis

The release of certain cytokines, such as IL-6, IL-8, and TNF-α, can lead to activation of the coagulation cascade by the tissue factor (TF) pathway (55, 56). TF is an important protein that

induces activation of secondary hemostasis (57). In turn, increased production of coagulation protein may activate protease activated receptors (PARs) to further increase proinflammatory cytokine production and upregulation of adhesion molecule which leads to leukocyte migration to the infection site. PARs are membrane receptors sensitive to coagulation proteases. These receptors are expressed in various cell types, including endothelial cells (ECs), lymphoid cells, platelets, fibroblasts, and others. In addition to its procoagulant role, TF exerts proinflammatory activity by activating PARs to coagulation proteases, such as factor VIIa, factor Xa, and thrombin (56). Cytokines that are being produced bind to specific receptors and, together with coagulation proteins, perpetuate the inflammatory response, which triggers increased activation of activated monocytes, ECs, and platelets. The result is the convergence of signals leading to exacerbated TF expression to sustain coagulation (58). Thus, processes of inflammation and coagulation are closely related, and coagulation may affect inflammation, which then modulates coagulation. This bidirectional interaction has been demonstrated for DENV infection (59, 60). In addition to cytokines, apoptotic cells could also activate the coagulation system (61-63). Although the complete mechanism of how apoptotic cells induce activation of the coagulation system is still not fully understood, it was shown that both adherent and detached apoptotic HUVECs become procoagulant by increased expression of phosphatidylserine (PS) and the loss of anticoagulant components such as thrombomodulin (TM) and tissue factor pathway inhibitor (TFPI) (61). Another possible explanation is that apoptotic ECs produce extracellular vesicles contributing to a procoagulant phenotype of the cells (62). These vesicles contain negatively charged phospholipid and TF that promote coagulation cascade activation (64, 65).

Zika

Two receptors, AXL and TIM1, were suggested as the candidates for ZIKV entry into host cells (66). The target cells for ZIKV in humans are suggested to be keratinocytes, dendritic cells (DCs), dermal fibroblasts, monocytes, macrophages, progenitor cells of the cerebral cortex, cells of ocular tissue, cells of the reproductive tract, cells of renal tissue, trophoblast, and endothelial cells (ECs) (67, 68). The innate immune response plays an important role in controlling ZIKV infection. *In vitro* studies using both primary human cells and human-derived cell lines have been performed to address the interferon (IFN) response to ZIKV infection. Dependent on the cell type used, ZIKV infection induced the production of type I (α and β), type II (γ) and type III (λ) IFN and also the activation of several IFN-stimulated genes (ISGs) (69-72). Furthermore, different mouse models lacking components of the IFN-α/β pathway showed increased susceptibility and succumbed to infection (73). A human study also reported that acute infection induced high expression of toll-like receptor (TLR)-3, IFN-α, IFN-β, and IFN-γ

compared to healthy controls (HCs) (74). Nevertheless, ZIKV has been shown to antagonize type-I IFN-mediated phosphorylation of STAT1 and STAT2 (75). Moreover, it was shown that ZIKV NS5 protein mediates degradation of human STAT2 (76). ZIKV-infected patients have shown elevated levels of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-9, IL-10, IL-13, IL-17, TNF-α, and IFN-γ) and chemokines (CXCL-10, CXCL-12, CCL-2, and CCL-3) during the acute phase (77, 78), whereas, serum levels of IL-1β, IL-6, IL-8, IL-10, IL-13, TNF-α, and IFN-γ were increased in the convalescent phase (77). Patients with neurological complications had elevated levels of IL-18, TNF-α, IFN-γ, and CXCL-10 and reduced expression of IL-10 when compared to patients without neurological manifestations(78). Elevated levels of IL-22, TNF-α, CCL-2, and CCL-10 were identified in ZIKV-infected pregnant woman who gave birth to babies with congenital abnormalities of the central nervous system (CNS) (78). Nevertheless, larger longitudinal studies with sequential samples are needed to confirm these findings, especially in patients with certain disease manifestations, since the aforementioned studies are limited in the number of study participants. ZIKV infects human embryonic cortical neural progenitor cells and induces cell death (79). The virus seems to mainly target neuronal progenitor cells in the developing brain but differentiated neurons in the adult brain are relatively resistant to the virus (80, 81). Early infection is associated with growth attenuation and an increase in cell death. Similar observations were observed in cortical neurospheres (82). Mouse and non-human primate models supported a causal role for ZIKV in neurological complications and adverse pregnancy outcomes observed in humans (38). Interestingly, although both ZIKV lineages could infect fetal brains in pregnant immunodeficient mice (83, 84), it still remains unclear why African ZIKV strains have not been associated so far with microcephaly or other adverse pregnancy outcomes in humans.

A recent study suggests that ZIKV infection also induced coagulation disorders in a patient (10). In addition, a cohort study found that 9% of babies from ZIKV-infected mothers were small for their gestational age and the researchers speculated that this condition may have occurred as a consequence of poor placental perfusion or fetal growth restriction (7). This observation led to the hypothesis that coagulation disorders of the umbilical cord could be one of the explanations for abnormal fetal growth due to reduced vascularization, which has been shown before for cytomegalovirus (CMV) infection (85). CMV infects ECs and leads to thrombosis as a result of endothelial injury (86, 87). ECs play an important role in the regulation of both pro- and anti-coagulation and fibrinolysis through expression and production of several mediators, including TF. Several reports demonstrated that ZIKV also infects ECs in vitro (66, 88, 89). Interestingly, human umbilical vein endothelial cells (HUVECs) were shown to be more susceptible to ZIKV infection compared to human ECs derived from other vasculatures (88). In addition, a recent

study revealed that ZIKV NS1 protein induces endothelial barrier dysfunction in vitro in a vasculature-specific manner. The researchers found that ZIKV NS1 binds mainly on the surface of HUVECs and brain ECs and lead to increased vascular leakage in these cells (90). An *in vivo* study also indicated that pregnant *Ifnar*$^{-/-}$ mice infected with ZIKV developed vascular damage in the placenta (91). Moreover, ZIKV-infected pregnant rhesus macaques developed segmental thrombosis in the umbilical cord (92). Collectively, the above data indicate that coagulation disorders occur during ZIKV infection. Nevertheless, more studies are required to confirm the effects of ZIKV infection on the hemostasis system.

In addition to the innate immune response, ZIKV infection also elicits protective adaptive immunity against infection. The E and PrM proteins along with the secreted protein NS1 represent the important targets for ZIKV-specific antibodies (93, 94). Several studies have shown that ZIKV-specific antibodies are crucial for viral control in mouse models. A vaccine study comprising of ZIKV E and PrM proteins protected mice from viral challenge. This protection was mediated by antibodies against the E protein as was shown by passive transfer experiments (95). The same vaccine was also found to be protective in a study with non-human primates (96). Human monoclonal antibodies (MABs) capable of neutralizing ZIKV both *in vitro* and *in vivo* have also been generated. These include MABs directed to the quaternary E dimer epitope, which showed cross-reactivity with DENV (97, 98). Antibodies recognizing EDIII of E protein, which reduce morbidity and mortality in mice infected with ZIKV, have also been identified (99). Furthermore, two antibodies, which recognize epitopes spanning multiple domains of the E protein, were able to protect mice in a post-exposure experiment (100). Nevertheless, the contribution of antibody functions to the control of viremia and clinical manifestations post-infection still require further studies.

Cellular immune response also contributes against ZIKV infection. CD4+ cells that proliferate in response to stimulation with E and NS1 proteins have been identified in patients who had experienced a recent infection (93). A mouse study also showed that in the absence of CD4+ cells, mice had more severe neurological sequela and increased viral titers in the CNS. Moreover, the transfer of CD4+ cells from ZIKV immune mice protected type I interferon deficient animal from a lethal challenge (101). CD8+ cells also play a role in controlling infection. A study showed that protective effect of induced, activated CD8+ cells was observed in 75% of mice that were injected with IFNAR blocking antibodies prior to injection of ZIKV. The same study involving similarly treated CD8α$^{-/-}$ mice resulted in 100% lethality (102).

Dengue

The DENV envelope binds to receptors on host cells, which may include heparan sulfate or the lectin DC-SIGN, and the E protein is important for virus infectivity. The target cells for DENV in

humans are suggested to be dendritic cells (DCs), monocytes, macrophages, dermal fibroblasts, and hepatocytes (103, 104). ECs are also believed to be a target of infection, although strong *in vivo* evidence in humans is lacking. In the majority of cases, DENV infection triggers an immune response in infected individuals, although non-responders have been described after vaccination with live-attenuated virus, with percentages ranging from 15% to 22% (105). Severe dengue is often associated with low viremia or is seen when virus is undetectable in blood, suggesting that other elements and/or the host factors are the major contributor to the complications of the disease. In general, both the immune system and the hemostatic response have been implicated in development of severe disease. It has been hypothesized that a cytokine storm as a result of DENV infection results in altered hemostasis state leading to hemorrhage. Furthermore, the cytokine storm can induce a state of hyperpermeability of EC, resulting in uncontrolled plasma leakage, leading to hypovolemic shock. It is assumed that infection with one serotype results in life-long protection to the infecting serotype (homotypic immunity) and provides short term protection to the other serotypes (heterotypic immunity) (106). There is evidence that homotypic immunity is primarily exerted by neutralizing antibodies. The short-lived, cross-reactive antibodies of heterotypic immunity are protective when the levels are above a certain treshold. However, at subneutralizing levels, individuals become susceptible to infection with the other DENV serotypes. Several cohort studies have identified secondary infection as a risk factor to develop severe dengue (107, 108). The proposed explanation for this obeservation is that non-neutralizing, cross-reactive antibodies, produced during a primary infection bind the heterologous virus and increase the ability to infect Fc-receptor bearing cells. This phenomenon is called antibody-dependent enhancement (ADE) of infection (109). In this respect, cells infected through ADE are believed to have increased production of virus by inhibition of type I IFN and secretion of proinflammatory cytokines (110). Cells of the monocytic lineage are believed to be the main target of DENV replication when infected through antibodies (109).

Similarly, cellular immune responses have been suggested to play a role in clearing virus infection, but also to contribute to the development of severe dengue. It has been shown that following a primary infection with DENV, the majority of the memory CD8+ T cells are directed against epitopes in the NS3 protein and are cross-reactive with other DENV serotypes (111). A secondary DENV infection shows predominant expansion of T cells with high avidity against the serotype that was encountered during primary infection and low avidity against the current infecting serotype (112). These cross-reactive, low avidity T cells produce high levels of pro-inflammatory cytokines, but express low density of CD107a, a marker of T-cell degranulation (113). This indicates that the high levels of cytokines produced during secondary infection do

not result in an anti-viral effect and in combination with the low cytotoxic potential of the low-avidity T-cells, the cellular immune response fails to achieve early viral control. In addition, the excessive pro-inflammatory response may account for the severe dengue phenotypes observed in some patients.

It is known that serum levels of vasoactive mediators are elevated in patients with severe dengue. These mediators are released by peripheral blood mononuclear cells (PBMCs), ECs and/or the liver upon DENV-infection or stimulation. Cytokines are the most import class of mediators known to be associated with severe dengue. Cytokines can enhance (pro-inflammatory) or inhibit (anti-inflammatory) inflammation in response to virus infections. Under "normal" conditions, the pro-inflammatory response is timely counterbalanced by the anti-inflammatory response. If this compensatory system is destabilized, a "cytokine storm" (or hypercytokinemia) can result, producing a systemic disease characterized by DIC and multiple organ dysfunction. Levels of cytokines produced during infection are determined by the amount of cells that secrete them as well as the secretion rates and concentration per cell. Cytokine storms have also been associated with other infectious diseases such as bacterial sepsis (114), avian influenza infection (115), and HIV infection (116). Mediators associated with severe dengue include IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-18, TNF-α, INF-γ, transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP-1), macrophage migration inhibitory factor (MIF), thrombopoetin, soluble vascular cell adhesion molecule 1 (VCAM-1), soluble intracellular adhesion molecule 1 (ICAM-1), von Willebrand factor antigen, thrombomodulin, E-selectin, tissue factor (TF), plasminogen activator inhibitor 1 (PAI-1), granulocyte colony-stimulating factor (GCSF), hepatocyte growth factor (HGF), platelet activating factor (PAF), matrix metalloproteinase-9 (MMP-9), high mobility group box 1 (HMGB1) and tissue plasminogen activator (TAF) (117). Although differences exist between studies with regards to the group of mediators associated with severe disease, in general, individuals with secondary infections have higher levels of some of these mediators. It is assumed that these soluble mediators have a synergistic interaction that influence the disease process and outcome. However, it is not clear how groups of cytokines interact to induce the pathologic cytokine storm. Several mediators, such as TNF-α, histamine, bradykinin, and thrombin show the capacity to enhance the permeability of ECs *in vitro* and *in vivo* as a result of DENV infection or indirectly, although further studies are needed to confirm these findings. Individually or collectively, these mediators may contribute to the development of plasma leakage in severe dengue. An interesting question is what drives plasma leakage and aberrant production of cytokines in low/absence of viremia. It has been hypothesized that microbial

translocation (MT) during DENV infection contributes to excessive immune activation and plasma leakage that are observed in severe dengue. Two cross-sectional studies provide evidence that microbial translocation was associated with disease severity and excesive immune activation (118, 119). Since dengue is an acute infection, with three different disease phases, it is important to determine whether MT play a role throughout different disease phases. A study using a prospective cohort design would be important to answer this question

Chikungunya

CHIKV infection induces an immune response that should contribute to viral elimination during the acute phase without complications. Acute CHIKV infection initially triggers the activation of the innate type I IFN response (32, 120, 121). IFN-α and IFN-β produced by infected cells binds to IFN-α/β receptors (IFNAR) and induce the expression of antiviral IFN stimulating genes (ISGs) via the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway (28, 32). High levels of IFN-α have been detected during the acute phase in plasma of patients infected with CHIKV (122, 123). The IFN-α concentration positively correlates with viral load in plasma (123). Increased expression of IFN-α has also been detected in CHIKV infected mice and non-human primates (19, 124). Mouse studies have shown that IFN response is required to control CHIKV infection and mice that lack IFN receptors are more susceptible to severe infection compared to wild-type mice (124-127). Despite induction of type 1 IFN response in humans, the virus is not completely cleared from some patients (54). This indicates that the IFN response that is induced in humans is not as efficient to completely clear viral replication compared to that of mice. It is known that there are differences between the immune system of mice and humans (128). For instance, the cellular immune fraction of mice blood consist mainly of lymphocytes, whereas the most abundant cell in human blood are neutrophils (128). Therefore, this species-specificity could be responsible for the different functions of IFN response in mice and human.

CHIKV infection stimulates cells from the innate immune system, resulting in early production of pro-inflammatory cytokines, such as IFN-γ, IL-1 RA, IL-6, IL-8, IL-12, TNF-α, MCP-1 /CCL2, MMP2, IP-10 and CXCL10. Which cells are directly responsible for production of these cytokines is not known, but natural killer (NK) cells and macrophages are the most likely candidates (31, 32, 53, 120, 121). The contribution of the innate immune response to CHIKV clearance and pathogenesis is not completely understood. It has been shown that CHIKV infection promotes the activation and expansion of NK cells in synovial tissue (54, 129). However, the role of NK cells on viral clearance or pathogenesis remains unknown. Acute CHIKV infection is characterized by a vigorous infiltration of macrophages in the target organs, attracted by the chemokine MCP-1/CCL2 (19, 120, 121, 130). The role of macrophages is dual as they may play a

role in the clearance of infection, but on the other hand they are susceptible to CHIKV infection and therefore involved in pathogenesis (19, 32, 120, 121, 130). One study illustrated that depletion of macrophages in mice increased viremia in blood, suggesting that macrophages are involved in CHIKV clearance (126). However, the same group showed that depletion of macrophages also alleviates the mice from arthritic symptoms (126). Inhibition of infiltration of macrophages in joint tissue also led to less clinical manifestation in mice (131). This suggests that similar to Ross River virus (RRV), CHIKV infection of macrophages also accounts for disease progression (132). In addition, as part of the innate immune system, monocytes and macrophages play an important role in iron metabolism. One of the important functions of these immune cells is to function as the regulator of ferritin. Ferritin is a protein that plays an important role in iron regulation and a significant amount is located and produced by monocytes and macrophages (133). Previously, our group has shown that ferritin could be used as a predictive marker of severe dengue infection (134). Both CHIKV and DENV have tropism to monocytes and macrophages and these cells have been shown to play an important role in the pathogenesis of both viral infections (103, 135). Thus, it would be interesting to determine whether ferritin could be used as a predictive marker for disease severity or complications of chikungunya disease.

In addition to the innate immune response, CHIKV infection also elicits a protective adaptive immunity against re-infection. Studies have detected anti-CHIKV antibodies in the sera of infected patients (54, 136-138). CHIKV specific immunoglobulin M and G (IgM, IgG) can be detected respectively 3 - 7 and 4 - 10 days after onset of fever. Several studies have illustrated that these antibodies can neutralize the viral activity and control virus dissemination into the host (138, 139). Although neutralizing antibodies (NABs) have been shown to be important for viral clearance, it is uncertain whether the qualitative and quantitative nature of antibodies, such as neutralization capacity and avidity, play a role in development/protection of chronic disease. Several studies showed that maturation of antibody avidity after natural infection or vaccination may be important for protection against infection and/or disease. Nonetheless, correlation of antibody avidity with protection against infection seemed to be virus-dependent (140, 141). It remains to be determined whether antibody avidity plays a role in persistence or clearance of CHIKV infection. CHIKV specific T cells peak around day 5 post onset of fever (19, 31, 51, 121) and some studies suggested that they have no role in controlling the viral load (54, 142) while others demonstrated that they contribute to the suppression of viremia (143). One animal study implied that $CD4^+$ T cells mediate joint swelling and inflammation (142). Since there is limited evidence of the involvement of T cells in CHIKV infection, more studies are needed to investigate if T cells contribute in the clearance of infection or disease pathogenesis.

The mechanism, by which CHIKV causes persistent myalgia/arthralgia remains unknown. It was shown that CHIKV infected cells undergo apoptotic cell death resulting in damage to infected tissues or stimulation of nerve ends that might be responsible for the myalgic/arthralgic symptoms (144). CHIKV induces a robust acute immune response that can control the viral infection in the majority of cases. However, despite the immune reaction and viral clearance from blood, some patients can still experience chronic clinical symptoms (51, 53, 143). Persistent arthritic symptoms could arise due to viral persistence in affected joints, since alphaviruses are known to induce chronic infection (143, 145-147). Hoarau *et al.* isolated viral antigen and RNA from synovial tissue of a patients suffering from chronic symptoms 18 months post CHIKV infection (54). Additionally, a study with non-human primates also provided evidence of CHIKV persistence, by detecting CHIKV RNA 3 months post infection (19). Consequently, the question arises whether the presence of CHIKV RNA and antigen is the results of active viral replication or delayed antigen clearance. Poo *et al.*(143) revealed that CHIKV RNA has a long half-life of 10 - 11 days and there was still stimulation of the IFN response 60 days post infection. In addition, they also demonstrated that negative strand CHIKV RNA and structural CHIKV proteins were still detectable in feet of C57BL/6 mice 30 - 100 days post infection. Collectively, these data suggest that chronic disease is most likely caused by persistent active CHIKV replication and not delayed antigen clearance (143). How CHIKV can persist in the face of a robust immune response is not understood. In addition, there is no reliable biomarker that clinicians could use to determine whether a patient will develop chronic disease or not.

Aim and outline of this thesis

This thesis primarily aims to study the role of viral and host factors and their association with disease severity in ZIKV, DENV, and CHIKV infections through *in vitro* and *in vivo* studies. The second aim is to explore the potential use of biomarkers that are associated with severe disease manifestations. Figure 2 summarizes the viral and host factors and the disease manifestations that are being investigated in this thesis. **Chapter 1** provides an introduction to the field of re-emerging arboviruses and the host factors that are related to ZIKV, DENV, and CHIKV infections. In **chapter 2**, we used human neural progenitor cells (hNPCs) and several cell lines to study why congenital microcephaly is mainly associated with Asian lineage ZIKV strains. We observed that Asian ZIKV strains infect and induce cell death in hNPCs less efficiently than African ZIKV strains. The observed phenotypic characteristics of Asian ZIKV strains might contribute to their ability to cause chronic infection in tissues of the CNS. **Chapter 3** provides evidence that ZIKV infection of human umbilical vein endothelial cells (HUVECs) induces coagulation disorders. We found increased tissue factor (TF) production and apoptosis in HUVECs infected with two ZIKV

strains. In **chapter 4** we use a prospective cohort study of dengue patients to further investigate the previous cross-sectional studies which suggested that microbial translocation contributes to plasma leakage and altered inflammatory conditions. We found evidence that microbial translocation markers were mainly increased in the critical phase of the disease and are associated with immune activation and plasma leakage. In **chapter 5** we describe the CHIKV outbreak on the Island of Curaçao in 2014-2015. Moreover, we identified ferritin as a potential biomarker to predict chronic disease. **Chapter 6** describes the antibody response in patients with acute and chronic CHIKV infection**.** We found that higher avidity of antibodies was observed in acute patients compared to patients with chronic disease. In the summarizing discussion (**Chapter 7)**, the findings that we observed in our studies together with the other host factors that contribute to the severe disease outcomes of ZIKV, DENV, and CHIKV infections are addressed with suggested direction for future studies.

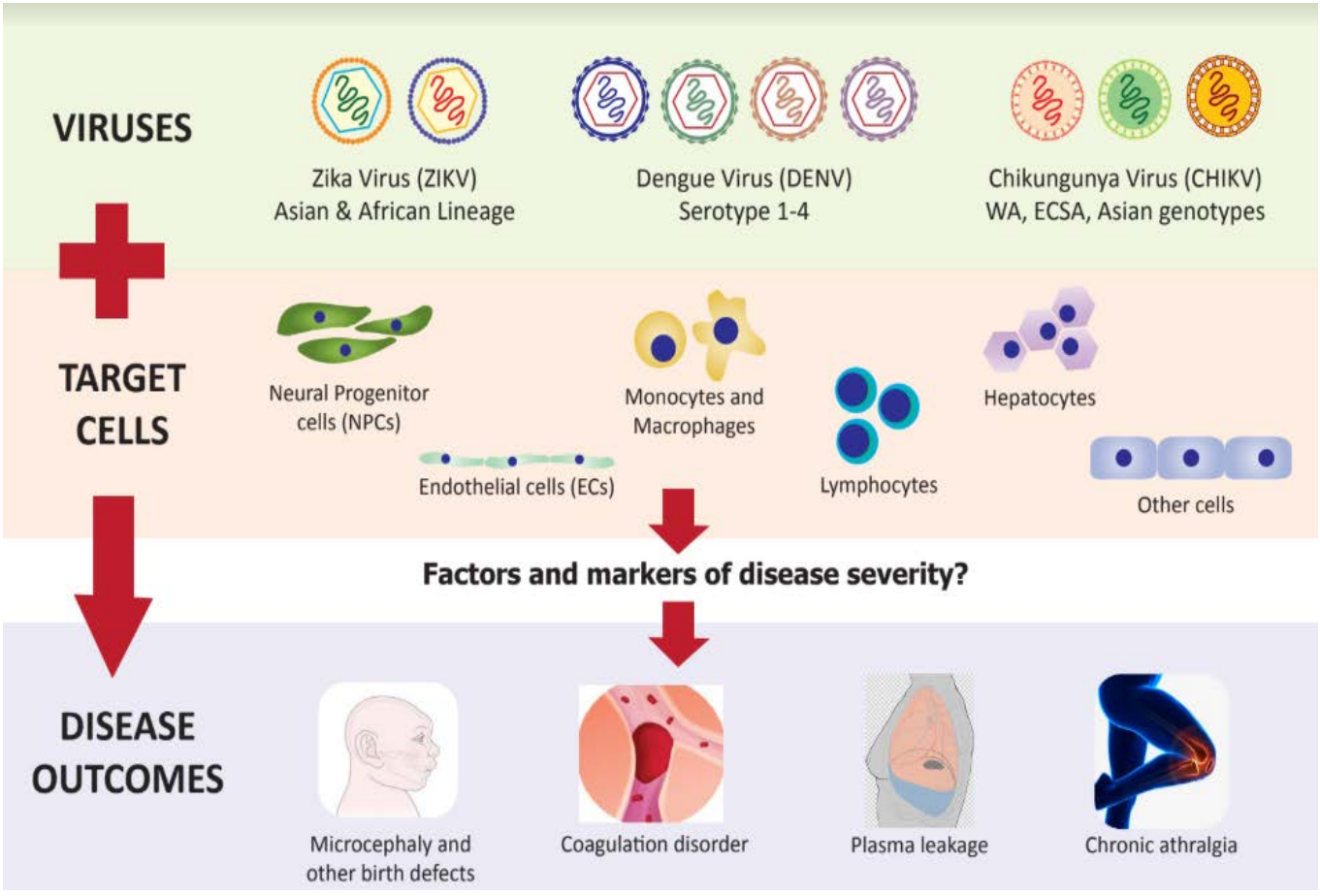

Figure 2. Interaction between arboviruses and host factors

Arboviruses infect target cells of the host that lead to dysfunction and/or cell death. Severe disease and complications are likely to be a multi-factorial process where complex interactions

between host and viral factors determine disease outcome in humans. Virus factors, such as genetic differences, are likely to determine the magnitude of the host response. In contrast, host factors that contribute to certain disease manifestations and complications are still not fully elucidated. Moreover, markers that can be used to predict severe disease or certain complications are still warranted for these arbovirus infections.

Chapter 2

Phenotypic differences between Asian and African lineage Zika viruses in human neural progenitor cells

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MSphere. 2017;2:e00292

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Abstract

Recent Zika virus (ZIKV), infections have been associated with a range of neurological complications, in particular congenital microcephaly. Human neural progenitor cells (hNPC) are thought to play an important role in the pathogenesis of microcephaly and experimental infection of these cells resulted in the induction of cell death. However, there are differences in infection efficiency and induction of cell death between studies, which might be related to intrinsic differences between African and Asian lineage ZIKV strains. Therefore, we determined the replication kinetics, including infection efficiency, burst time, burst size and ability to induce cell death, of two Asian and two African ZIKV strains. African ZIKV strains replicated to higher titers in Vero, human glioblastoma (U87MG), human neuroblastoma (SK-N-SH) cells, and hNPC compared to Asian ZIKV strains. Furthermore, infection with Asian ZIKV strains did not result in significant cell death early after infection, whereas infection with African lineage ZIKV strains resulted in high percentages of cell death in hNPCs. The differences between African and Asian lineage ZIKV strains highlight the importance of including relevant ZIKV strains to study the pathogenesis of congenital microcephaly, and caution against extrapolation of experimental data obtained using historical African ZIKV strains to the current outbreak Finally, the fact that Asian ZIKV strains infect only a minority of cells with a relatively low burst size together with the lack of early cell death induction might contribute to its ability to cause chronic infections within the CNS.

Introduction

Since the emergence of Zika virus (ZIKV) in 2015 in South America, infections have caused a wide spectrum of neurological diseases, such as Guillain-Barr*é* syndrome, myelitis, meningoencephalitis and in particular congenital microcephaly (148). Even though ZIKV was first detected in 1947 in a rhesus monkey, and has caused repeated outbreaks since 2007, not much was known about the pathogenesis of disease caused by ZIKV before the 2015 outbreak. Since then, several studies have shown that ZIKV can infect a variety of neuronal cells, but more insight into the pathogenesis of ZIKV induced central nervous system (CNS) diseases is needed (149).

An important question that remains is whether the emergence of ZIKV in South America and the associated clinical findings are the result of genetic and phenotypic changes of the emerging ZIKV strain, or whether this can be attributed to the introduction of ZIKV in a large naïve population (150). Phylogenetically, two distinct lineages of ZIKV exist, the African lineage and the Asian lineage (151). The current outbreak strain belongs to the Asian lineage and sequence analysis revealed that the virus has changed significantly over the last 50 years, both in nucleotide sequences and amino acid (aa) composition (151, 152). The prototype ancestral ZIKV strain MR766 of the African lineage has been used in many initial studies (79, 153-155), but recent *in vitro* and *in vivo* studies have shown some differences between African and Asian ZIKV strains (73, 82, 156-158). Whether there are also phenotypic differences between Asian ZIKV strains, caused by amino acid substitutions acquired just before the outbreak in South America, is currently unknown (152).

ZIKV infection has been shown to replicate and induce cell death in neuronal cells of fetal mice (152, 159), as well as in human neural progenitor cells and brain organoids (79, 82, 153, 156), a mechanism thought to play an important role in the pathogenesis of ZIKV induced microcephaly. Recent studies have shown that an African ZIKV strain might be able to infect human neural stem cells (hNSCs) and astrocytes more efficiently than Asian ZIKV strains (156). However, a comprehensive study on the replication kinetics and the ability to cause cell death of different African and Asian ZIKV strains is currently lacking (149).

To be able to detect phenotypic differences between Asian and African ZIKV strain, or between recent Asian ZIKV strains, it is important to characterize and understand the *in vitro* replication kinetics—including the infection efficiency, burst size and ability to cause cell death— of these viruses. Therefore, we determined the replication kinetics of two Asian ZIKV strains (isolated in 2013 and 2016) and two African ZIKV strains (isolated in 1947 and 1952) on Induced Pluripotent Stem cell derived human neural progenitor cells (hNPC) and several human neural cell lines.

Results

Four ZIKV strains were included in this study (Figure 1A). Two African ZIKV, Zika virus MR766 ($ZIKV^{AF\text{-}MR766}$) and Uganda 976 ($ZIKV^{AF\text{-}976}$), were isolated in 1947 and 1961 respectively and passaged on mouse brain tissue and vero cells. The two Asian ZIKV strains included were H/PF/2013 ($ZIKV^{AS\text{-}FP13}$) and ZIKVNL00013 ($ZIKV^{AS\text{-}Sur16}$), which were isolated in 2013 and 2016 respectively and passaged 4 times on Vero cells. There are over 50 amino acid differences between the Africa and Asian ZIKV strains which have been described before (152). The aa differences between the Asian ZIKV strains were located in NS1 (R67S; position 863), NS2B (S41T; position 1417) and NS5 (M60V; position 2634) proteins (Fig 1B). Of these aa differences, the mutation on position 2634 is only observed in viruses isolated from the recent outbreak (151, 152, 160). The aa difference at position 1417 of $ZIKV^{AS\text{-}Sur16}$ was not present in the original clinical isolate but was acquired during passaging on Vero cells (161).

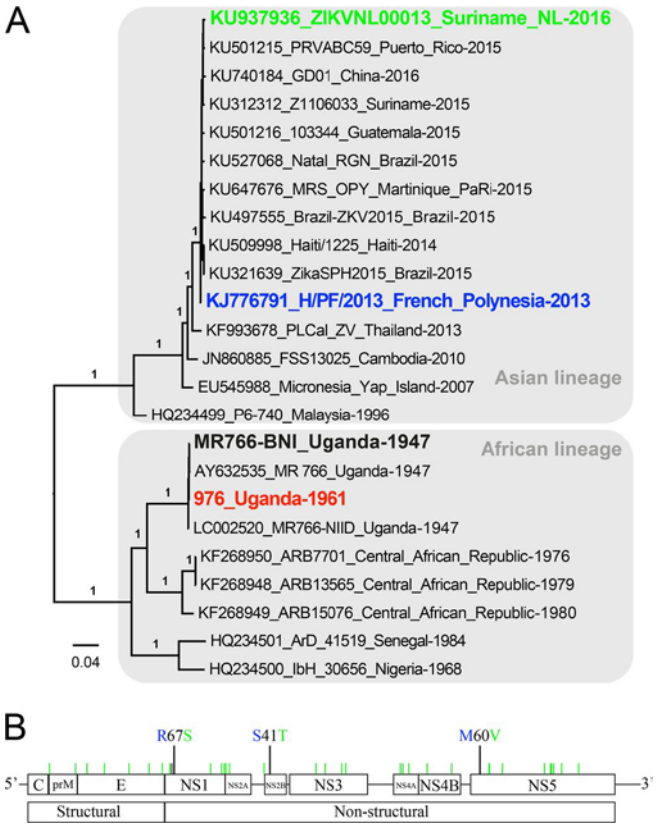

Fig. 1. Phylogenetic analysis of ZIKV strains used in this study and genomic organization and mutations between the Asian lineage ZIKV strains.

(A) Nucleotide sequences of representative Zika virus genomes were analyzed, and a phylogenetic tree was constructed using the PhyML method. Values at branches show the result of the approximate likelihood ratio; values of <0.70 are not shown. **(B)** Genome organization and mutations between Asian lineage H/PF/2013 ($ZIKV^{AS\text{-}FP13}$) and ZIKVNL00013 ($ZIKV^{AS\text{-}Sur16}$) ZIKV strains.

Growth curves of Asian and African ZIKV strains on neuronal cells

Growth curves were determined for ZIKV$^{AS\text{-}FP13}$, ZIKV$^{AS\text{-}Sur16}$, ZIKV$^{AF\text{-}MR766}$ and ZIKV$^{AF\text{-}976}$ by *in vitro* infections using low multiplicity of infection (MOI 0.1 and 0.01) on SK-N-SK cells (human neuroblastoma cells), U87-MG cells (human glioblastoma cells), Vero cells and hNPCs. Growth curves showed that all cells supported replication of all four ZIKV strain included, but virus titers were significantly lower for both Asian strains compared to ZIKV$^{AF\text{-}MR766}$ strains(Fig 2A and B) on Vero, SH-N-SH, U87-MG and hNPCs. On hNPCs ZIKV$^{AF\text{-}MR766}$ grew to significantly higher titers than ZIKV$^{AF\text{-}976}$ (Fig 2A and B). There we no differences in the growth curves between ZIKV$^{AS\text{-}FP13}$ and ZIKV$^{AS\text{-}Sur16}$.

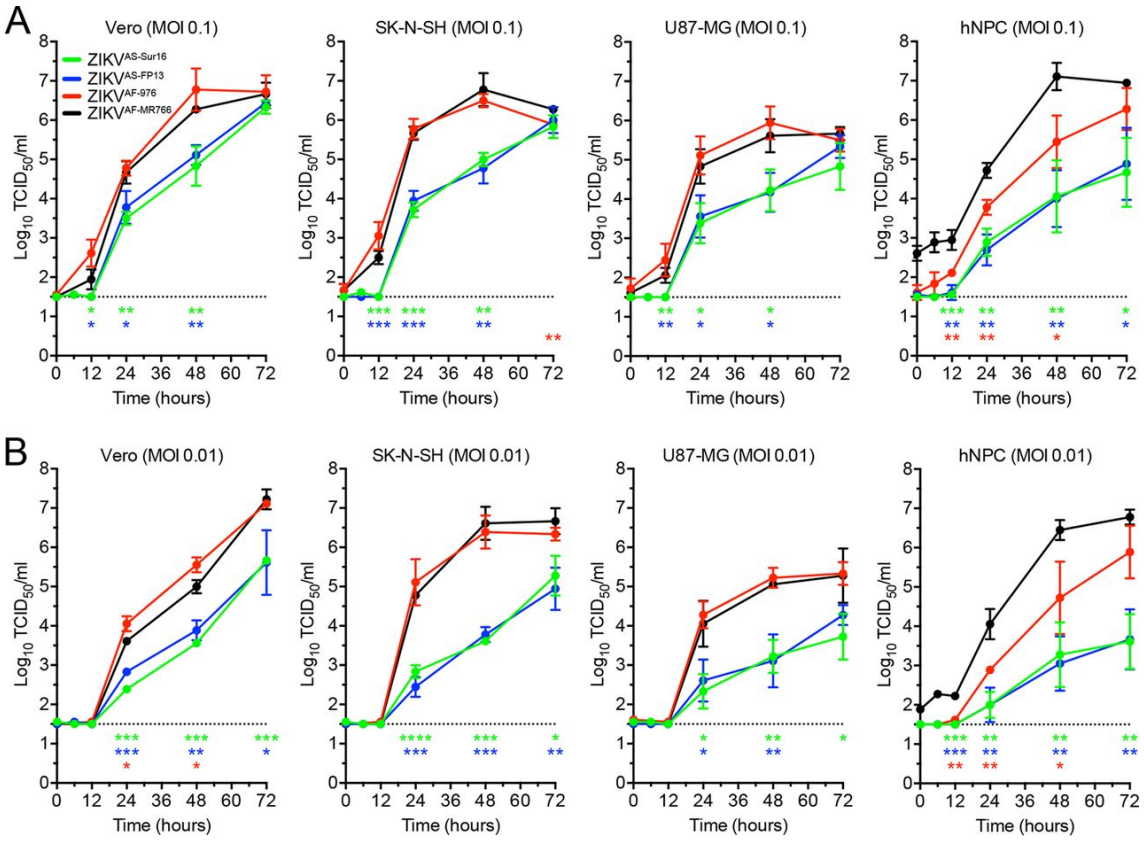

Fig. 2. Growth curves of ZIKV strains on Vero, SK-N-SH, and U87-MG cells and hNPCs. (**A and B**) Growth curves of Asian lineage strains H/PF/2013 (ZIKV$^{AS\text{-}FP13}$ [blue lines]) and ZIKVNL00013 (ZIKV$^{AS\text{-}Sur16}$ [green lines]) and African lineage MR766 (ZIKV$^{AF\text{-}MR766}$ [black lines]) and 976 Uganda (ZIKV$^{AF\text{-}976}$ [red lines]) on Vero, human neuroblastoma (SK-N-SH), and human glioblastoma (U87-MG) cells and human neuronal progenitor cells (hNPCs) at MOI of 0.1 **(A)** and 0.01 **(B)**. Data are presented as means with standard deviations from at least 3 independent experiments. Statistical significance was calculated using the Student *t* test in comparison with ZIKV$^{AF\text{-}MR766}$. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. $TCID_{50}$, 50% tissue culture infectious dose.

One step growth curves of Asian and African ZIKV strains on neuronal cells

One step growth curves (OSGC) were assessed *in vitro* by using a high MOI (MOI 10). Data from OSGC experiments on the different cell lines were used to calculate the percentage of infection and burst size (progeny virus produced per cell). OSGC showed that baseline virus titers were higher for the African ZIKV strains than for the Asian ZIKV strains, and both African ZIKV strains grew to higher titers on all cells (fig 3A). In all cells African ZIKV strains infected more cells than the Asian ZIKV strains (Fig 3B and D). The number of virus particles produces did not differ significantly between the different ZIKV strains. However, there was a trend that the burst size was higher in SK-N-SH cells (~100-400) compared to Vero cells (~20-40), U87-MG (~50-150) and hNPC (~40-75) (Fig 3C).

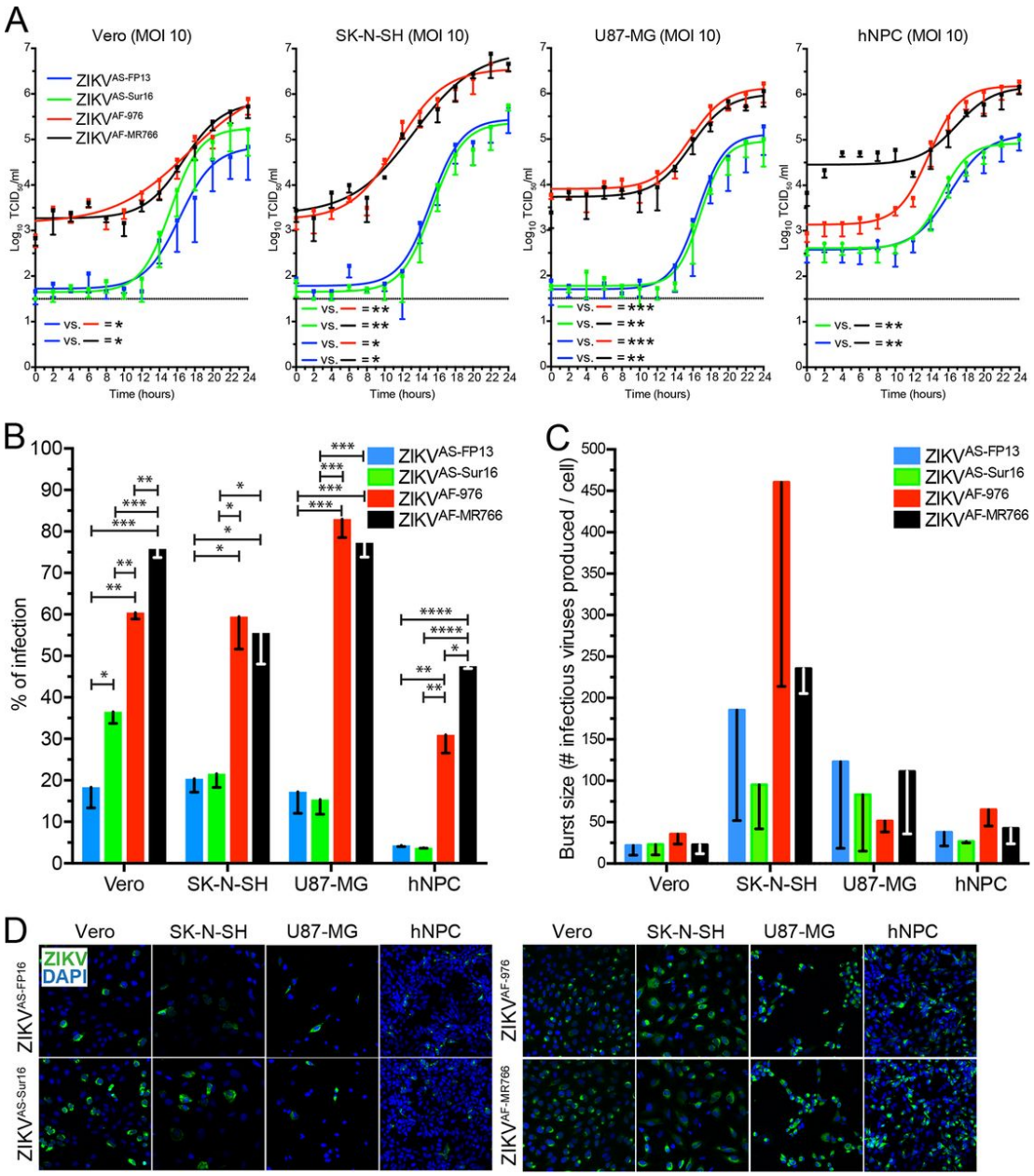

Fig. 3. One-step growth curve (OSGC) kinetics of Asian and African lineage ZIKV strains. **(A)** OSGCs of Asian lineage strains H/PF/2013 ($ZIKV^{AS\text{-}FP13}$ [blue lines]) and ZIKVNL00013 ($ZIKV^{AS\text{-}Sur16}$ [green lines]) and African lineage MR766 ($ZIKV^{AF\text{-}MR766}$ [black lines]) and 976 Uganda ($ZIKV^{AF\text{-}976}$ [red lines]) on Vero, human neuroblastoma (SK-N-SH), and human glioblastoma (U87-MG) cells and human neuronal progenitor cells (hNPCs). **(B)** Percentage of ZIKV infection determined by immunofluorescent microscopy of two Asian and two African ZIKV strains. **(C)** Number of infectious viruses produced per cell (burst size) for each virus in the 4 different cell lines. **(D)** Representative immunofluorescent images of ZIKV-infected cells stained for ZIKV antigen (green). Magnification, ×200. For panels A and B, data are presented as means with standard deviations and nonlinear curve fit for at least 3 independent experiments. For panel C, data are presented as means with standard errors of the means from at least 3 independent experiments. Statistical significance was calculated using a one-way ANOVA with Tukey's multiple comparisons test for panel A. For panels B and C, the Student *t* test was used. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. $TCID_{50}$, 50% tissue culture infectious dose.

Induction of cell death by Asian and African ZIKV strains

The ability of $ZIKV^{AS\text{-}FP13}$, $ZIKV^{AS\text{-}Sur16}$ and $ZIKV^{AF\text{-}MR766}$ to cause cell death in hNPC at 24, 48 and 72 hpi was determined after infection with an MOI of 3. Cells were stained for either ZIKV antigen or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, DNA fragmentation) and measured by FACS. Uninfected cells and beta-propiolactone (BPL) inactivated $ZIKV^{AF\text{-}MR766}$ were included as controls. In addition, cells were fixed at 48 hpi, for immunofluorescent double staining for ZIKV antigen and TUNEL. A maximum of 14% TUNEL positivity was observed in BPL control and negative control cells 72 hpi.

Infection with $ZIKV^{AS\text{-}FP13}$ and $ZIKV^{AS\text{-}Sur16}$ resulted in approximately 20% infection at 72 hpi, and up to 9% of cells were TUNEL positive, the latter comparable to control and BPL treated cells. Immunofluorescent staining revealed that very few TUNEL positive cells were ZIKV infected (Fig. 4A and 4B). In contrast, infection with $ZIKV^{AF\text{-}MR766}$ resulted in 46% infection at 72 hpi and the percentage of TUNEL positive cells increased to 30% at 72hpi (Fig. 4A). Immunofluorescent staining revealed that in $ZIKV^{AF\text{-}MR766}$ infected cells, the majority of TUNEL positive cells were also infected, indicating that $ZIKV^{AF\text{-}MR766}$ is able to induce cell death early after infection in hNPC (Fig 4B).

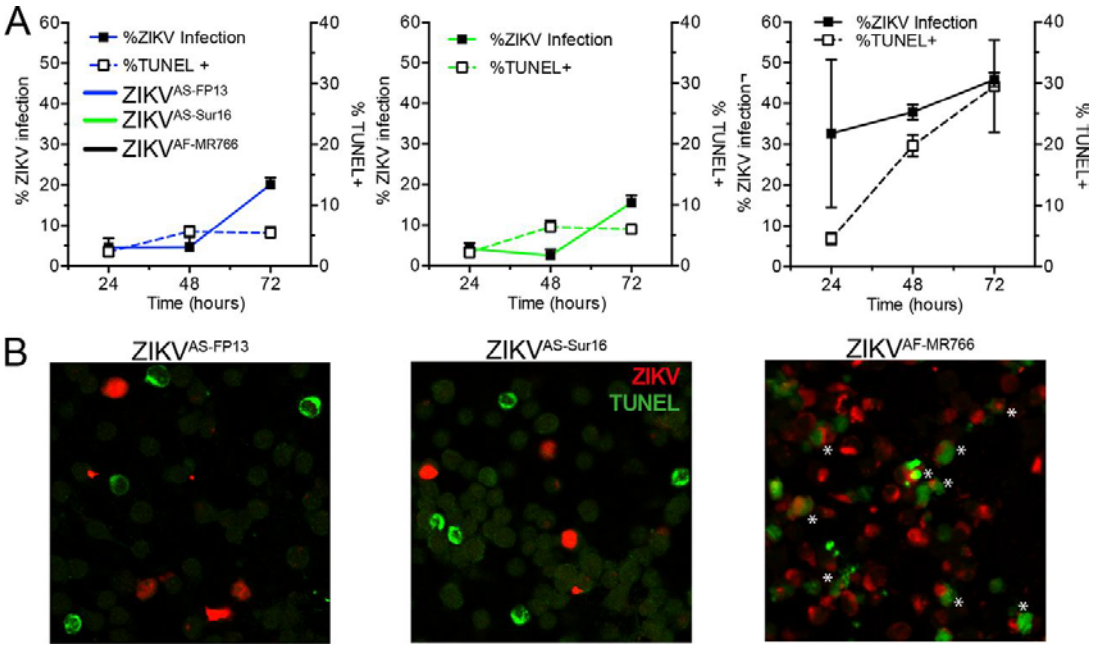

Fig. 4. Ability to cause cell death of African and Asian lineage ZIKV strains in human neural progenitor cells. (A) Percentage of human neural progenitor cells infected with African lineage ZIKV strain ZIKV$^{AF\text{-}MR766}$ (black lines) and Asian lineage ZIKV strains H/PF/2013 (ZIKV$^{AS\text{-}FP13}$ [blue lines]) and ZIKVNL00013 (ZIKV$^{AS\text{-}Sur16}$ [green lines]) and percentage of TUNEL-positive cells measured over 72 h. The left *y* axis represents the percentage of cells infected with ZIKV, and the right *y* axis represents the percentage of TUNEL-positive cells. Data are presented as means with standard errors of the means from at least 3 independent experiments. **(B)** Representative immunofluorescent images of human neural progenitor cells infected with different ZIKV strains 48 h postinfection and double stained for ZIKV antigen (red) and TUNEL (green). Asterisks indicate double-positive cells. Magnification, ×200.

Conclusion

This study on the *in vitro* replication of different ZIKV strains, shows that African ZIKV strains replicated more efficiently in Vero, human glioblastoma, human neuroblastoma cell and hNPCs, than Asian ZIKV strains. In hNPCs, which are consider which are considered important target cell type for the development of congenital microcephaly, African ZIKV strains induced cell death early after infection, which was not observed after infection with Asian ZIKV strains.

Overall, there were few phenotypic differences between ZIKV$^{AS\text{-}FP13}$ and ZIKV$^{AS\text{-}Sur16}$. This suggests that the mutations between these viruses, including position 2634 unique for ZIKV isolated from this outbreak, does not lead to large phenotypic changes, at least, not in these cell lines. The fact that Vero and SK-N-SH cells permit efficient replication of Asian ZIKV strains, supports the usage of these cells for virus isolation from clinical samples (18).

The replication kinetics and ablity to cause cell death in hNPC differed largely between African and Asian ZIKV strains. Asian ZIKV strains infect and replicate less efficiently in than the African ZIKV strains. This 'reduced' replication is not an intrinsic feature of Asian ZIKV strains, since they replicate to high titers in Vero and SK-N-SH cells. One possible explanation for the

increased ability of African ZIKV strains to infect hNPCs in this study could be that these strains have adapted to neural cells due to their passage history in mouse brain tissues (18), and that the 4 aa deletion in the E protein of these viruses contributes to the observed phenotype. However, similar results—high percentage of infection and induction of cell death in hNPC—have been observed with a low passage 1989 African ZIKV strain (ArB41644) (12). Upon sequencing, we did not find any deletion in the E protein (accession number KY576904) of this low passage African-lineage ZIKV strain. Therefore, these studies together suggest that Asian ZIKV strains infect hNPC less efficiently than African ZIKV strains regardless of the passage history of the ZIKV strains. Both Asian-lineage ZIKV strains do not seem to induce cell death early after infection, whereas $ZIKV^{AF\text{-}MR766}$ does. This fits with previous observations, where more apoptotic nuclei were observed after infection with an African ZIKV strain than with an Asian ZIKV strain (156), which suggests that there are intrinsic differences between Asian and African ZIKV strains in their ability to cause cell death in hNPC.

The observed phenotypic characteristics of Asian-lineage ZIKV strains might contribute to its ability to cause chronic infection in tissues of the CNS (161-165). First, Asian ZIKV strains infect relatively few hNPCs. Second, Asian ZIKV strains release less than 50 infectious virus particles per infected hNPC, which is relatively low compared to other viruses, such as influenza and SIV (166, 167). A low burst size has previously also been associated with prolonged virus replication within the CNS for Japanese encephalitis virus, another flavivirus (168). Finally, Asian ZIKV strains do not seem to induce cell death early after infection in neural progenitor cells, which might result in chronic infection and replication within the CNS (163, 164). This fits with a recent animal study using *Stat2*$^{-/-}$ mice demonstrated that African ZIKV strains induce short episodes of severe neurological symptoms followed by lethality while Asian ZIKV strains manifest prolonged signs of neuronal malfunctions. Limited mortality was also only observed in one Asian ZIKV strain (83).

Taken together, we here show that African and Asian ZIKV strains differ in their ability to infect and replicate in different neuronal cells, as well as their ability to cause cell death early after infection. These differences might contribute to the ability of Asian ZIKV strains to cause a wide spectrum of neurological diseases.

Materials and methods

Cells

Human induced pluripotent stem cell (IPSC)-derived neural progenitor cells (NPCs) (ax0015, Axol, Cambridge, UK) were cultured in neural maintenance basal medium with supplements (Ax0031, Axol) according to manufacturer's specification. Human IPSC-derived NPCs were

grown on 20 µg/ml laminin (L2020, Sigma-Aldrich) coated plates. Human neuroblastoma SK-N-SH and human glioblastoma U87-MG were purchased from Sigma-Aldrich and grown in EMEM with EBSS (Lonza, Breda, the Netherlands) containing 10% heat-inactivated fetal bovine serum (HI-FBS, Lonza), 100 U penicillin (Gibco Life Sciences, USA), 100 µg/ml streptomycin (Gibco), 2 mM L-Glutamine (Lonza), 1% Non-essential amino acids (Lonza), 1 mM sodium pyruvate (Gibco), 1.5 mg/ml sodium bicarbonate (Lonza). Both SK-N-SH and U87-MG cells were used below passage 25. Vero cells (ATCC, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% HI-FBS, 100 µg/ml streptomycin, 100 U penicillin, 2 mM L-glutamine, 1% sodium bicarbonate and 1% HEPES buffer (all from Gibco). Human NPCs are primary cells while the other are immortalized cell lines. All cells used in this study were tested negative for *Mycoplasma* sp.

Viruses

Zika virus strain Uganda 976 ($ZIKV^{AF\text{-}976}$) was provided by Dr. Misa Korva (University of Ljubljana; EVAg number: 007V-EVAg1585). Zika virus MR766 ($ZIKV^{AF\text{-}MR766}$) was provided by Dr. Stephan Günther (Bernhard-Nocht-Istitute for Tropical Medicine). This strain has three nucleotides difference (C6258T, G6273T, and G10671A) with the reference MR766 strain (GenBank accession number: KU955594) . Zika virus strain H/PF 2013 ($ZIKV^{AS\text{-}FP13}$) was obtained from UMR 190-Unite des Virus Emergents (EVAg number: 001V-EVA1545). Zika virus Suriname ZIKVNL00013 ($ZIKV^{AS\text{-}Sur16}$) was isolated from a patient in the Netherlands (EVAg number: 011V-01621) (161). All virus stocks used in this study were grown in Vero cells. The following passage numbers were used: passage (P)6 for $ZIKV^{AF\text{-}976}$; unknown for $ZIKV^{AF\text{-}MR766}$, and passage 4 for $ZIKV^{AS\text{-}FP13}$ and $ZIKV^{AS\text{-}Sur16}$. Virus titers were determined in Vero cells 5 days after infection by means of cytopathic effect (CPE) and the 50% tissue culture infective dose ($TCID_{50}$) was calculated using the Spearman-Kärber method (169). All virus stocks were stored at -80°C until further use. A summary of isolation history of all ZIKV strains used in this study and related informations is provided in Table 1.

Next generation sequencing

For genomic characterization of the virus strains, RNA was isolated from 140 µL of the virus stocks with the QIAmp Viral Mini RNA kit (Qiagen, Germany). Subsequently, the product was eluted in 40 µL bidest. Viral metagenomic libraries were constructed with 454 pyrosequencing as previously described (170) and the libraries were sequenced using 454 GS-Junior machine (Roche, USA) according to the manufacturer's instructions.

Phylogenetic analysis

Nearly full length ZIKV genomes of 4 isolates ($ZIKV^{AF\text{-}976}$, $ZIKV^{AF\text{-}MR766}$, $ZIKV^{AS\text{-}FP13}$, and $ZIKV^{AS\text{-}Sur16}$) and other reference sequences were obtained from Genbank database. The sequences

were aligned using ClustalW, and a phylogenetic tree was constructed by using the PhyML method in Seaview 4 (http://pbil.univ-lyon1.fr/software/seaview) with the approximate likelihood ratio test based on a Shimodaira–Hasegawa-like procedure which used general time reversible as substitution model. Nearest neighbor interchange, subtree pruning, and regrafting-based tree search algorithms were used to estimate tree topologies (171). The obtained tree was visualized by using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree).

Replication kinetics of Zika virus strains

Replication kinetics of ZIKV strains $ZIKV^{AS\text{-}976}$, $ZIKV^{AS\text{-}MR766}$, $ZIKV^{AS\text{-}FP13}$ and $ZIKV^{AS\text{-}Sur16}$ were studied *in vitro* by means of one-step growth curve (OSGC) experiments with a multiplicity of infection (MOI) of 10 and focal experiments (growth curves) with a MOI of 0.1 and 0.01. Human neural progenitor cells, SK-N-SH, U87-MG and Vero cells were seeded into 96-well plates (2 x 10^4 cells) (Greiner, USA). After 24 hours, monolayers were inoculated with the different ZIKV strains, or Vero cell culture medium as a control at a MOI of 10, 0.1, or 0.01 for 1 hour at 37°C in 5% CO_2. After 1 hour of virus absorption, the inoculum was removed and cells were washed 3 times and replenished with fresh medium that contains 2% FCS and cultured for 24 or 72 hours at 37°C for the OSGC and growth curve, respectively. For the OSGC, supernatant was collected every 2 hours post infection (hpi) for 24 hours and cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature, washed with PBS and permeabilized and stored in 70% ethanol for immunofluorescent staining. For the growth curves, supernatant was collected at time points 0, 1, 12, 24, 48, and 72 hpi and stored at -80 until use. All growth curves were performed 3 times, and each growth curve included duplo measurements from which the average was used for future analysis

Determination of virus titers

Virus titers ($TCID_{50}$) in the supernatant were determined by end point titrations on Vero cells. Ten-fold serial dilutions were made and inoculated onto a monolayer of Vero cells. Cytopathic effect (CPE) was determined at 5 days post infection (dpi) and virus titers were calculated using the Spearman-Kärber method (169). An initial 1:10 dilution of supernatant resulted in a detection limit of $10^{1.5}$ $TCID_{50}$/mL.

Immunofluorescence microscopy

Infected cells from the OSGC around BT_{50} were fixed with 4% PFA for 20 minutes at room temperature, washed, and permeabilized with 70% ethanol. Subsequently, cells were washed twice in PBS and incubated for 1 hour in the dark and at room temperature with anti-Flavivirus group antigen (MAB10216, clone D1-4G2-4-15 Millipore, Germany, 1:200 dilution) or mouse IgG2a isotype control (MAB003, R&D Systems, 1:50 dilution) in PBS containing 0.1% BSA.

Afterwards, the cells were washed three times with PBS/0.1% BSA and incubated for 1 hour with goat anti-mouse IgG2a conjugated with Alexa Fluor 488 (Life technologies, the Netherlands, 1:250 dilution) in PBS/0.1% BSA at room temperature and in the dark. After 1 hour, cells were washed three times and mounted with ProLong® Diamond Antifade Mountant with DAPI (Life technologies, USA). Zika-virus-infected cells were identified by use of a Zeiss LSM 700 confocal laser scanning microscope fitted on an Axio Observer Z1 inverted microscope (Zeiss). All images were processed using Zen 2010 software (Zeiss). Per sample, 5 high power fields were photographed and scored blindly by three individuals to determine the percentage of infected and non-infected cells.

Calculation of percentage of infection and burst size

Percentage of infection and burst size were calculated from the OSGC experiment. The burst size is defined as the number of progeny virus particles produced per infected cell and was calculated as followed. The time at which half the number of progeny virus were released into the supernatant (EC_{50} for dose-response curve), which was calculated by using a non-linear regression analysis (sigmoidal dose-response, variable slope) in GraphPad Prism 6.0 using the infectious virus titer data from the OSGC measured over 24 hours (2 hour increments) was determined. At this time point infected cells were fixed and stained for ZIKV (as described above) and number of infected cells was calculated by counting virus infected/uninfected cells in 5 randomly chosen panels, in duplo, by 3 blinded assessors. The average number of infected cells from 5 panels was taken and corrected for the surface area of a single 96-well flat bottom plate. Next, the infectious virus titer over 24 hours was calculated by subtracting time point 0 from 24 which then was divided by the number of infected cell resulting in the number of progeny virus particles produced per infected cell.

TUNEL assay

Human neural progenitor cells (hNPCs) were cultured in a 24-well plate and inoculated with $ZIKV^{AS\text{-}MR766}$, $ZIKV^{AS\text{-}FP13}$ or $ZIKV^{AS\text{-}Sur16}$ at a MOI of 3. In addition, $ZIKV^{AS\text{-}MR766}$ was inactivated using beta-propiolactone (BPL) (Sigma Aldrich, USA, 1:4000 v/v) at 4°C for 48-72 hours. Subsequently, BPL was inactivated for 24 hours at 37°C. Both inactivated $ZIKV^{AS\text{-}MR766}$ and Vero cell culture supernatant served as negative controls. Viruses and controls were allowed to absorb for 1 hour, after which hNPCs were washed three times in hNPC medium. Subsequently the medium was replenished with fresh medium and cultured at 37°C for 24, 48, or 72 hours. The number of dead cells were measured using *In Situ* Cell Death Detection Kit, Fluorescein (Sigma Aldrich, USA). Briefly, the cells were first fixed with 4% PFA and permeabilized with a 1:1 dilution of 1% triton X-100 and 70% ethanol. Non-infected cells were treated with 180 IU/ml DNAse (Roche Diagnostics GmbH, Mannheim, Germany) for 15 minutes at room

temperature to serve as a positive control. In Situ Cell Death Detection Kit, Fluorescein was used according to the manufacturer's instructions. Cells stained only with labelling solution were used as negative control as suggested by the manufacturer. The number of TUNEL positive cells was measured using BD FACS Canto™ II (BD Biosciences, USA). Data were analysed using FlowJo 10 software (Ashland, Oregon, USA). All experiments were performed three times and each experiment included duplo measurements from which the average was calculated and used for further analysis

Flowcytometry assay

Cells were infected the same way as described for the TUNEL assay. At time points 24, 48 and 72, cells were collected, fixed and permeabilized using BD Cytofix/Cytoperm™ solution (BD Biosciences, USA) according to the manufacturer's instructions. Cells were blocked using 10% normal goat serum (NGS, Dako, Denmark) for 10 minutes on ice. Subsequently, Zika virus was detected using mouse monoclonal antibody against anti-flavivirus group antigen (MAB10216, clone D1-4G2-4-15, Millipore, Germany) 1:200 dilution or mouse IgG2a isotype control (MAB003, Dako, Denmark) 1:50 dilution in BD perm/wash containing 2% NGS and incubated for 1 hour on ice and in the dark. Cells were washed twice and goat anti-mouse IgG2a conjugated with Alexa Fluor 488 (Life technologies, the Netherlands) 1:250 dilution was incubated for 1 hour in the dark and on ice. After incubation of the secondary antibody, cells were washed twice and resuspended in FACS buffer. The percentage of infected cells were measured using BD FACS Canto™ II (BD Biosciences, USA). Data were analysed using FlowJo 10 software (Ashland, Oregon, USA).

Statistical analysis

The statistical analyses were performed using GraphPad Prism 6.0h software (La Jolla, CA, USA) for Mac. Student's t-test or Mann-Whitney U test were used for the comparison between two groups. P values ≤ 0.05 were considered significant.

Accession numbers

Sequences of E protein African ZIKV strain (ArB41644): KY576904

Funding information

DvR and this study was supported by a fellowship from the Erasmus MC Foundation. FA was supported by the Directorate of Higher Education (DIKTI) PhD grant of the Ministry of Research, Technology and Higher Education of the Republic of Indonesia. Part of this work was further supported by the European Union program ZIKAlliance (contractnumber 734548). Work at UMR1058 was supported by Reacting and La Région Languedoc Roussillon.

Acknowledgements

We acknowledge Claudia Schapendonk for excellent technical assistance; and Thijs Kuiken and Barry Rockx for critical reading of the manuscript.

Chapter 3

Zika virus infection induces elevation of tissue factor production and apoptosis on human umbilical vein endothelial cells

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Front Microbiol. 2019;10:817

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ABSTRACT

Zika virus (ZIKV) infection is typically characterized by a mild disease presenting with fever, maculopapular rash, headache, fatigue, myalgia and arthralgia. A recent animal study found that ZIKV-infected pregnant *Ifnar*$^{-/-}$mice developed vascular damage in the placenta and reduced amount of fetal capillaries. Moreover, ZIKV infection causes segmental thrombosis in the umbilical cord of pregnant rhesus macaques. Furthermore, several case reports suggest that ZIKV infection cause coagulation disorders. These results suggest that ZIKV could cause an alteration in the host hemostatic response, however, the mechanism has not been investigated thus far. This paper aims to determine whether ZIKV infection on HUVECs induces apoptosis and elevation of TF that leads to activation of secondary hemostasis. We infected HUVECs with two ZIKV strains and performed virus titration, immunostaining, and flow cytometry to confirm and quantify infection. We measured tissue factor (TF) concentrations with flow cytometry and performed thrombin generation test (TGT) as a functional assay to assess secondary hemostasis. Furthermore, we determined the amount of cell death using flow cytometry. We also performed enzyme-linked immunosorbent assay (ELISA) to determine interleukin (IL)-6 and IL-8 production and conducted blocking experiments to associate these cytokines with TF expression. Both ZIKV strains infected and replicated to high titers in HUVECs. We found that infection induced elevation of TF expressions. We also showed that increased TF expression led to shortened TGT time. Moreover, the data revealed that infection induced apoptosis. In addition, there was a significant increase of IL-6 and IL-8 production in infected cells. Here we provide *in vitro* evidence that infection of HUVECs with ZIKV induces apoptosis and elevation of TF expression that leads to activation of secondary hemostasis.

INTRODUCTION

Zika virus (ZIKV) is a re-emerging arbovirus that belongs to the genus Flavivirus of the family *Flaviviridae*. ZIKV was first isolated from a rhesus macaque in 1947 in Uganda and has not been recognized as an important viral pathogen until the recent outbreaks in the Americas. Infection is asymptomatic in the majority of cases (80%) and symptomatic patients develop a febrile illness similar to many other infectious diseases (38). Clinical symptoms are usually mild and last for 3-7 days without further complications. However, during the recent outbreaks, severe symptoms and complications were reported, including Guillain-Barré syndrome,(9) severe birth defects,(7) fetal death (8, 41, 42) and coagulation disorders (10). The mechanism of these complications, especially coagulation disorders, are still not fully understood.

Under physiological conditions, hemostasis is orchestrated by the coagulation and the fibrinolytic systems. Endothelial cells (ECs) play an important role in regulating the activities of pro-and anti-coagulation and fibrinolysis through expression and production of several important mediators, including tissue factor (TF), tissue factor pathway inhibitor (TFPI), tissue plasminogen activator (tPA), plasminogen activator inhibitor type-1 (PAI-1) and thrombomodulin (57). TF is an important factor that initiates the activation of secondary hemostasis. Several factors have been shown to activate and down-regulate this protein (172, 173). For instance, interleukin (IL)-6 and 8 are pro-inflammatory cytokines that regulate TF expression on several cells such as human umbilical vein endothelial cells (HUVECs) and monocytes (174, 175). In addition, apoptotic cells could also activate the coagulation system by increasing the surface TF expression (176). It has been shown that several viruses activate the coagulation system especially through TF (177). For instance, treatment of Ebola virus (EBOV) infection with a recombinant inhibitor of factor VIIa/TF was shown to result in prolonged survival, which was associated with reduced activation of coagulation and fibrinolysis (178). Dengue virus (DENV), another flavivirus, has been shown to also cause coagulation disorders and ECs have been shown to play a central role in these pathological conditions (179-181).

Recently, it was found in a cohort study that 9% of infants from ZIKV-infected pregnant woman were small for their gestational age and the authors speculated that this condition occurred as a consequence of fetal growth restriction or poor placental perfusion (7). This study led to the hypothesis that coagulation disorder of the umbilical cord could be one of the explanations for abnormal fetal growth due to reduced perfusion which has been shown for cytomegalovirus (CMV) infection (85, 182). CMV is known to have vascular EC tropism, which causes cell damage and can lead to thrombotic vasculopathy (87). Recent publications demonstrated that ZIKV also infects ECs *in vitro* (66, 88, 89). HUVECs were shown to be more susceptible to ZIKV infection compared to human ECs derived from aorta, coronary artery and saphenous vein (88).

Interestingly, a recent report revealed that ZIKV NS1 protein triggers endothelial barrier dysfunction *in vitro* in a tissue-specific manner. The authors found that ZIKV NS1 bind mainly on the surface of HUVECs and brain ECs and cause increased vascular leakage in these primary cells (90). *In vivo* evidence also revealed that ZIKV-infected pregnant *Ifnar*$^{-/-}$mice developed vascular damage in the placenta and reduced amount of fetal capillaries (91). Furthermore, pregnant rhesus macaques infected with ZIKV developed segmental thrombosis in the umbilical cord (92). Altogether, these data suggest that hemostatic alterations occur during ZIKV infection. However, there is limited evidence of hemostasis disorder in ZIKV infection. Here, we provide *in vitro* evidence that ZIKV infection of HUVECs induce apoptosis and increased TF production which trigger the activation of secondary hemostasis.

MATERIALS AND METHODS

Cells

HUVECs were harvested from patients as previously described (183). Ethical permission to use the leftover materials from mothers who gave birth at Sophia Children Hospital was obtained from the Erasmus MC medical ethics committee. Only cells up to passage four and from one randomly selected donor were used in this study. The identity of HUVECs was confirmed by flow cytometry using Von Willebrand Factor (vWF) staining. HUVECs were grown in human endothelial-SFM medium (Invitrogen, Life Sciences, USA) containing 20% heat-inactivated fetal bovine serum (HI-FBS, Lonza, the Netherlands), 100 U penicillin (Gibco Life Sciences, USA), 100 µg/ml streptomycin (Gibco Life Sciences, USA), 20 ng/ml fibroblast growth factor (Peprotech, USA) and 10 ng/ml endothelial growth factor (Peprotech, USA). Vero cells (ATCC CCL-81, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% HI-FBS (Lonza, the Netherlands), 100 U penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1% HEPES buffer, and 1% sodium bicarbonate (all from Gibco Life Sciences, USA). All cells were grown at 37°C and 5% CO_2. The cells were tested negative for mycoplasma by PCR.

Virus strains

Two ZIKV strains were used in this study; ZIKV strain Uganda 976 ($ZIKV^{AF}$) was kindly provided by Dr. Misa Korva (University of Ljubljana) (European Virus Archive number 007-EVAg1585) and Zika Suriname ZIKVNL00013 ($ZIKV^{AS}$) was isolated from a female patient in the Netherlands who travelled to Suriname (161). Virus stocks used in this study were prepared on Vero cells. ZIKV Uganda 976 with total passage number (P)6 and $ZIKV^{AS}$ P4 were used in the experiments. Viral titers were determined on Vero cells by calculating the 50% Tissue Culture Infective Dose ($TCID_{50}$) using the Spearman-Kärber formula (184) after determining the presence of cytopathic effects (CPE) after five days of incubation. As a control, infectious virus

was inactivated using beta-propiolactone (BPL) (Sigma Aldrich, USA, 1:4000 v/v) at 4°C for 24 hours. Subsequently, BPL was inactivated by incubating for one hour at 37°C. All virus stocks were stored at -80°C until use. A summary of isolation history of the ZIKV strains used in this study with related information is provided in Table 1. A phylogenetic tree of the virus strains used in this study was recently published (185).

Table 1. Source host, isolation and passage history of the ZIKV strains used in the study

Lineage	Strain	Source host	Year of isolation	Location	Passage history	Genbank Accession number	EVAg number
Asian	ZIKVNL 00013	Human	2016	Suriname	4x Vero	KU937936	011V-01621
African	Uganda 976	Monkey	1961	Uganda	2x SMB, 3x Vero E6, 1x Vero	NA	007V-EVAg1585

Abbreviations: SMB= suckling mouse brain; Vero= African green monkey kidney cells; Vero E6= African green monkey kidney clone E6 cells; EVAg= European Virus Archive goes Global; NA= Not available.

Infection experiments

HUVECs were seeded into 96-well- (2 x 10^4 cells per well) (Greiner, USA) or 24-well- (2 x 10^5 cells per well) (Corning, USA) plates depending on the experiment. After 24 hours, confluent monolayers were infected at a multiplicity of infection (MOI) of 0.1, 1 or 5, with infectious, BPL-inactivated virus or a mock (vero cell culture medium) for 1 hour at 37°C in 5% CO_2. Subsequently, the supernatant was discarded and cells were washed three times with RPMI 1640 medium (Gibco, Life Sciences, USA). New medium with 10% heat-inactivated fetal bovine serum (HI-FBS) was added and cells were cultured for 48 hours. All of the experiments were performed according to the local biosecurity safety procedures at the Department of Viroscience, Erasmus Medical Center, which is a WHO collaborating center for hemorrhagic fever viruses.

Determination of virus titer

Virus titers ($TCID_{50}$) in the supernatant were determined by log10 titration of the medium on Vero cells. Presence of CPE was determined at 5 days post-infection (dpi) and the $TCID_{50}$ was calculated using the Spearman-Kärber method (184).

Determination of Percentage of Infection

Cells were cultured on 96-well plates and infected with MOI of 0.1 and 1. Infected cells were harvested, then fixed and permeabilized with BD Cytofix/Cytoperm™ solution (BD Biosciences, USA). To minimize background, cells were incubated with 5% normal goat serum (DAKO, Denmark) in BD Perm/Wash solution (BD Biosciences, USA). ZIKV was detected using mouse monoclonal antibody anti-flavivirus group antigen, MAB10216, clone D1-4G2-4-15 (Millipore, Germany) 1:200 dilution and goat anti-mouse IgG conjugated with APC (Life technologies, the

Netherlands) 1:250 dilution. Non-infected cells and ZIKV-infected cells stained with mouse isotype IgG2a antibody (DAKO, Denmark) were used as negative and isotype controls. The percentage of infected cells was measured using a BD FACS Canto II machine (BD Biosciences, USA). Infected cells were defined as ZIKV Envelope + cells (ENV+) while negative cells were defined as ZIKV ENV - cells (ENV-)

Immunofluorescence staining

Cells were grown on 96-well plates and infected with MOI of 0.1 and 1. Cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with a mixture of 0.1% Triton X-100 and ethanol 70% (1:1 dilution). Subsequently, cells were incubated for 1 hour with mouse monoclonal antibody anti-flavivirus group antigen, MAB10216, clone D1-4G2-4-15 (Millipore, Germany) 1:200 dilution at room temperature. Next, cells were stained for 1 hour with goat anti-mouse IgG conjugated with APC (Life technologies, the Netherlands) 1:250 dilution at room temperature and mounted in ProLong® Diamond Antifade Mountant with DAPI (Life technologies, USA). Non-infected cells and ZIKV-infected cells stained with mouse isotype IgG2a antibody (Dako, Denmark) were used as negative and isotype controls. Representative images of infected cells were generated on a Zeiss LSM 700 confocal laser scanning microscope fitted on an Axio Observer Z1 inverted microscope (Zeiss). All images acquired were processed using Zen 2010 software (Zeiss).

Determination of apoptotic cells

Cells were grown on 96-well plate and infected at an MOI of 5. The number of apoptotic cells was measured using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit, In Situ Cell Death Detection Kit, Fluorescein (Sigma Aldrich, USA). The cells were fixed with 4% PFA and permeabilized with triton X-100 1% and ethanol 70% (1:1 vol/vol). Uninfected cells were treated with 90 IU/ml DNAse (Roche, Germany) for 15 minutes to serve as a positive control (PC). TUNEL assay was performed based on the manufacturer's instructions. Non-infected cells and ZIKV-infected cells stained only with labeling solution were used as negative controls as suggested by the manufacturer. The number of TUNEL positive cells was measured using a BD FACS Canto II machine (BD Biosciences, USA). Data were analyzed using the FlowJo 10 software (FlowJo, USA).

Thrombin generation time test

Cells were grown on 96-well plate and infected with MOI of 0.1 and 5. Thrombin generation time (TGT) test was performed as previously described (183). Briefly, supernatant of cultured HUVECs on 96-well plate was discarded. Cells were washed three times with RPMI 1640 medium (Lonza, the Netherlands) and 80 µL of pooled citrate plasma from healthy donors was added to the monolayer together with 60 µL HEPES buffer [25 mM Hepes, 137 mM NaCl, 0.1%

Albumin]. As a standard, a serial dilution of recombinant TF (Innovin, Germany) was performed in the absence of cells. 60 µL HEPES calcium [25 mM Hepes, 137 mM NaCl, 0.1% Albumin,38 mM CaCl(2)] was then added and the plates were measured directly at optical density (OD) 450 nm using a Tecan Infinite 200 Pro ELISA reader (Tecan Group Ltd., Switzerland) in a kinetic cycle measuring every 45 seconds for 1 hour. Thrombin generation time was defined as the time at half the maximal OD. TF levels were also determined from the ELISA values as previously described (183). BPL-inactivated virus control was not used due to its interference with the assay.

Quantification of Tissue Factor Expression on HUVECs

HUVECs were analyzed for surface TF expression by flow cytometry. Briefly, HUVECs were grown on 96-well plate and infected at an MOI of 5. Infected cells were harvested, then stained with rabbit polyclonal anti-TF conjugated with Alexa Fluor 647 (Bioss Inc, USA) at a 1:200 dilution or isotype control in fluorescence activated cell sorter (FACS) buffer for 45 minutes on ice and in the dark. Subsequently, the cells were fixed and permeabilized with BD Cytofix/Cytoperm™ solution (BD Biosciences, USA). Cells were incubated with 5% normal goat serum (DAKO, Denmark) in BD Perm/Wash solution (BD Biosciences, USA) for 10 minutes to minimize background. ZIKV was detected using mouse monoclonal antibody anti-flavivirus group antigen, MAB10216, clone D1-4G2-4-15 (Millipore, Germany) at a 1:400 dilution for 45 minutes on ice and in the dark. Subsequently, cells were incubated with goat anti-mouse IgG conjugated with Alexa Fluor 488 (Life technologies, the Netherlands) at a 1:250 dilution for 45 minutes on ice and in the dark. Non-infected cells and ZIKV-infected cells stained with mouse isotype IgG2a antibody (DAKO, Denmark) were used as negative and isotype controls. The percentage of TF and infected cells were measured using a BD FACS Canto II machine (BD Biosciences, USA). Data were analyzed using the FlowJo 10 software (FlowJo, USA).

IL-6 and IL-8 ELISA

Cells were grown on 96-well plate and infected at an MOI of 5. Supernatant of infected cells was collected and followed by centrifugation for 10 minutes at 930 g. IL-6 and IL-8 levels from the supernatant were measured using IL-6 (Quantikine R&D, USA) and IL-8 (Quantikine R&D, USA) ELISA kits according to the manufacturer's instructions.

IL-6 and IL-8 blocking experiments

Cells were grown on 96-well plate and infected at an MOI of 5. Following normal infection experiment as the above protocol, anti-IL-6 (R&D, USA) or IL-8 antibody (R&D, USA) was added to the wells according to our ELISA results and the manufacturer's recommendation. TF expression was subsequently determined with flow cytometry at 24 and 48 hpi as the above protocol. To confirm that the blocking experiments work, we incubated HUVECs with IL-6 and

IL-8 concentrations that we observed in our study and determined the downstream activation pathway of these cytokines. Briefly, HUVECs were grown on 96-well plate and was cultured for 24 or 48 h. Dilution of anti-IL-6 or -IL-8 antibody was mixed with either IL-6 or IL-8 protein (R&D, USA) based on the concentrations that we observed in our study for 30 minutes at 37°C before it was added to the cells. The cells were then cultured for 4 h. Next, RNA was isolated from HUVECs with High Pure RNA Isolation kit (Roche, Germany). Subsequently, cDNA synthesis was performed with Superscript IV (Thermo Fisher Scientific, USA). Expression of nuclear factor kappa B subunit 1 (NFKB1), tissue inhibitors of metalloproteinases-1 (TIMP1) and hypoxia-inducible factor 1-alpha (HIF1A) genes were determined with real-time PCR using the Taqman Universal PCR Mastemix II according to the manufacturer's recommendation (Applied Biosystems, USA). Gene expression was corrected for the housekeeping gene glyceraldehyde 3-phospate dehydrogenase (GAPDH) as described previously (186). All the primer-probe assays were obtained from Applied Biosystems, USA.

Statistical analysis

The statistical analyses were performed using GraphPad Prism 5.01 software for Windows. Student's t-test or Mann Whitney U test was used for the comparison of mean and median respectively between two groups. For comparison between multiple groups, one-way analysis of variance (ANOVA) test with Tukey's multiple comparison or Kruskal-Wallis test with Dunn's multiple comparison was used. P values ≤ 0.05 were considered significant.

RESULTS

ZIKV infects and replicates in primary human umbilical vein endothelial cells

To confirm that ZIKV infects and replicate in ECs, we determined the replication kinetics of two strains of ZIKV, one of African lineage and one of Asian lineage, using low MOIs (0.1 and 1). In accordance with the previous study,(88) we found that HUVECs supported infection for both $ZIKV^{AF}$ and $ZIKV^{AS}$. Both virus strains replicated to a titer of 10^7 $TCID_{50}$/ml without noteworthy differences in the replication kinetics (Figure 1A). These results were supported by flow cytometry and IFA staining. The flow cytometry data showed that at both MOI of 0.1 and 1 the number of infected cells increased on average 5-10% (MOI 0.1) and 27-35% (MOI 1) at 24 hpi to ± 25% (MOI 0.1) and ± 40% (MOI 1) at 48 hpi (Figure 1B). The data shown in Figure 1C are representative data of the flow cytometry analyses to determine the percentage of infection. Flow cytometry results were also confirmed by IFA intracellular staining of ZIKV infected cells at low MOIs (Figure 1D). Taken together, these data confirm that ZIKV could infect and replicate efficiently in HUVECs.

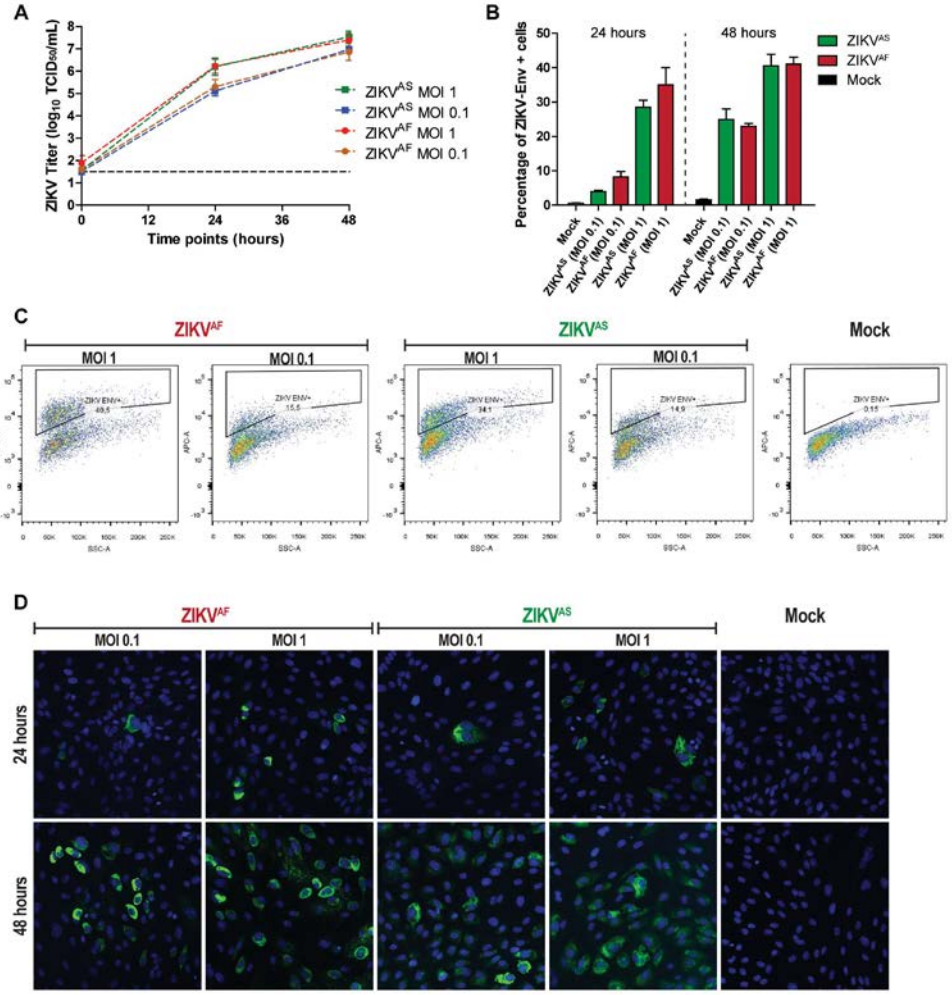

Figure 1. Zika virus (ZIKV) infects and replicates efficiently in human umbilical vein endothelial cells (HUVECs). HUVECs were infected with two ZIKV strains at two multiplicity of infections (MOIs; 1 and 0.1) and samples were collected at the designated times. **A**, Infectious titers of supernatants collected at 0, 24, 48 h post infection (hpi). Experiments were done in triplicate and data are representative of three independent experiments. Bars represent standard error of the mean (SEM) **B**, HUVECs were infected at two MOIs (0.1 and 1) and stained for the presence of ZIKV envelope by flow cytometry at 24 and 48 hpi. Experiments were done in triplicate and data are representative of two independent experiments. The results are displayed as mean and bars represent standard error of the mean (SEM). **C**, Representative flow cytometry analyses plot to determine the percentage of infected cells at 48 hpi. **D**, HUVECs were infected with two ZIKV strains and then imaged for the presence of viral envelope protein at 24 and 48 hpi. 200x magnification.

Increased TF concentrations and shortened thrombin generation time after ZIKV infection of HUVECs

To test whether ZIKV infection induces activation of secondary hemostasis, we incubated HUVECs with both ZIKV strains at a low MOI (0.1) and high MOI (5) or virus-free cultured medium from Vero cells. Two ZIKV lineages were used to assess whether activation of secondary hemostasis is lineage-specific or not. We measured TGT as a functional assay to assess secondary hemostasis. Thrombin generation was quantified directly on infected cells by

incubating plasma on cells and initiating coagulation by the addition of calcium ions. We observed that ZIKV-infected HUVECs triggered plasma clotting faster compared than mock control (Figure 2B). The significant increase was mainly seen at 48 hpi for both strains ($p<0.0001$ MOI 5 $ZIKV^{AF}$, $p=0.0007$ MOI 1 $ZIKV^{AF}$; $p=0.0002$ $ZIKV^{AS}$). We further quantified the mean TF concentrations after ZIKV infection, calculated from TGT standard curve as described previously (183). TF concentrations were significantly increased for $ZIKV^{AF}$ at both MOIs at 48 hpi ($p= 0.0007$ MOI 0.01; $p<0.0001$ MOI 5) and only at MOI 5 for $ZIKV^{AS}$ ($p=0.0002$) (Figure 2D). Since we only observed significant differences mainly with MOI of 5 and based on the *in vitro* studies of DENV (187) and puumala virus (183), which showed that a high MOI is needed to observed permeability and/or hemostatic disorders *in vitro* (HUVECs models), we decided to continue with this MOI for the rest of the experiments. We noticed that increased TF expression were detected since 24 hpi (Figure 2E) and was higher at 48 hpi (Figure 2F). Both ZIKV strains significantly increased TF concentrations compared to BPL at 24 hpi ($ZIKV^{AF}$: $p=0.0022$; $ZIKV^{AS}$: $p=0.002$) and 48 ($ZIKV^{AF}$: $p=0.002$; $ZIKV^{AS}$: $p=0.0004$).

To determine whether increase TF levels occurred in infected or bystander cells, we performed double staining assays against TF and ZIKV envelope. We measured TF on both ENV+ and ENV- cells, but increased TF mainly occurred in the bystander (ENV-) evidenced by the fact that <26% of all the positive TF cells were double positive cells at 48 hpi ($ZIKV^{AF}$: 25.5%; $ZIKV^{AS}$: 7.5%) (Figure 2H). Collectively, our data indicate that ZIKV infection on HUVECs induces increased TF concentration mostly through bystander effect that allows activation of secondary hemostasis.

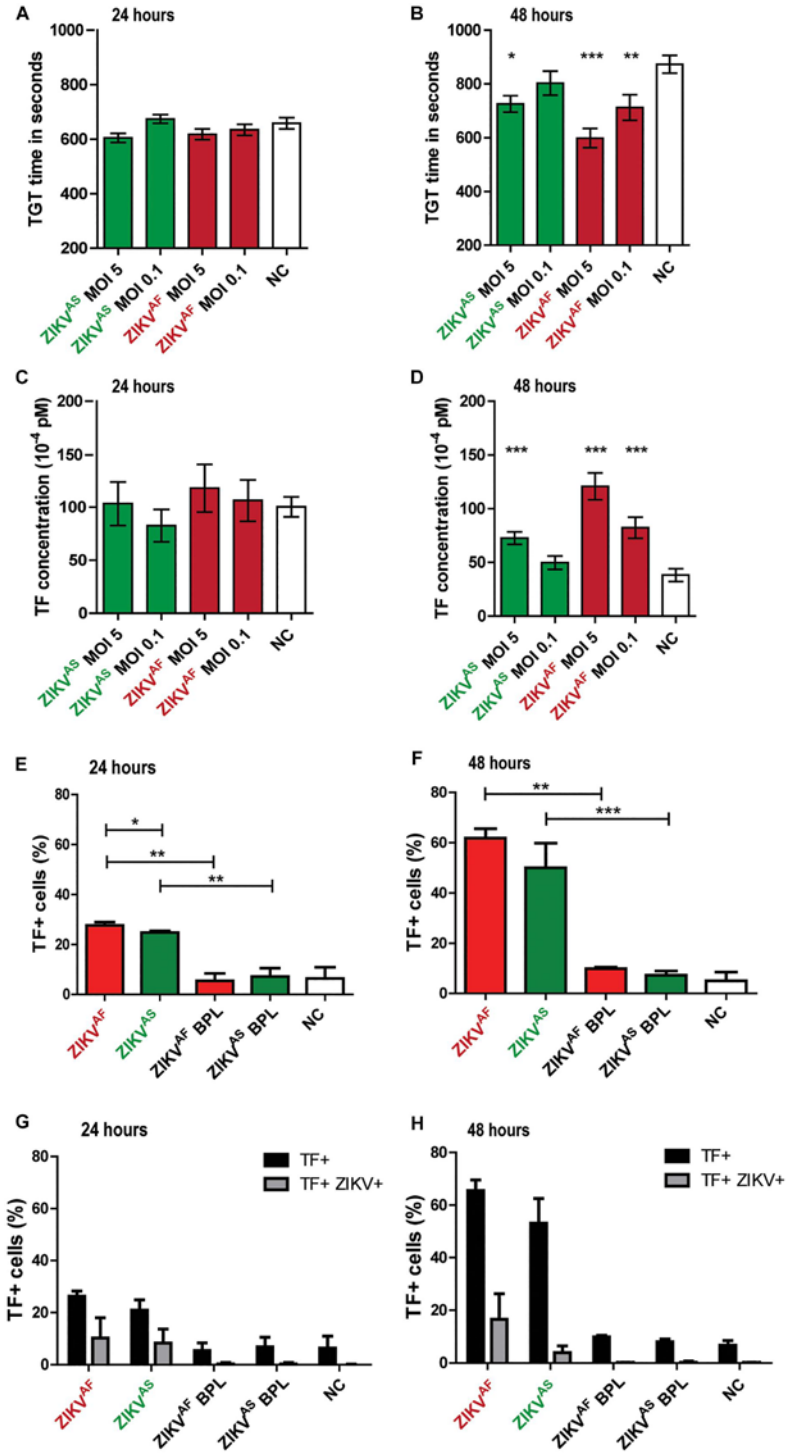

Figure 2. Zika virus (ZIKV) infection of human umbilical vein endothelial cells (HUVECs) leads to increased tissue factor (TF) expression and activation of secondary hemostasis. HUVECs were infected with an MOI of 0.1 or 5 with both ZIKV strains and thrombin generation time (TGT) test was performed as a functional assay to assess secondary hemostasis at 24 and 48 h post infection (**A&B**). TGT was significantly shortened for ECs infected with ZIKV^{AF} (MOI 0.1 and 5) and ZIKV^{AS} (MOI 5) at 48 hpi (**B**). **C&D,** TF concentration was calculated from a standard curve to determine whether there is an increase of TF expression at 24 and 48 hpi based on previous study (183). TF expression increased significantly at 48 hpi for both strains (ZIKV^{AF}: MOI 0.1 and 5; ZIKV^{AS}: MOI 5). Experiments were done in eight replicates and data are representative of three independent experiments. **E&F**, TF expression was also confirmed with

flow cytometry. Increased TF expression was observed for both virus strains against BPL and NC at 24 and 48 hpi. Approximately 61 % of cells expressed TF at 48 hpi for $ZIKV^{AF}$. **G&H,** Double staining experiments were performed to determine whether increased TF expression occurred in infected or bystander cells. Black bars represent the total amount of TF+ cells while gray bars represent the percentage of double positive cells for both ZIKV and TF. Less than 40% of total TF+ cells were double positive for both ZIKV and TF at 24 hpi (**G**). Meanwhile, < 25% of total TF+ cells were double positive for both ZIKV and TF at 48 hpi (**H**). Experiments were done in triplicate and data are representative of two independent experiments. All the results are displayed as mean and bars represent standard error of the mean (SEM). Statistical analyses were performed against mock. Statistical significance is shown: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

ZIKV infection of HUVECs induces apoptosis mainly through infection

Previous studies indicated that cell apoptosis could activate the coagulation system (63, 176). In addition, several studies showed that ZIKV infection of several primary cells induced cell death (71, 185). To determine whether ZIKV infection induced cell death of HUVECs, we assessed cell viability using a TUNEL assay. We found that infection with both strains led to significant morphological changes and increased cell death at 48 hpi (Figure 3A). No significant differences in cell death were observed at 24 hpi between the groups (Figure 3B). Cells treated with DNAse showed approximately 90% of TUNEL+ cells at both 24 and 48 hpi. $ZIKV^{AF}$ induced significantly more cell death ($p=0.005$) than $ZIKV^{AS}$ at 48 hpi (65% vs 55%) (Figure 3C). To determine whether cell death occurred in infected or bystander cells, we performed double staining experiments against ZIKV envelope and TUNEL positive cells. We found that cell death mainly occurred due to infection, especially with $ZIKV^{AF}$ (Figure 3D&E). Approximately 60% of the total death cells were also positive for $ZIKV^{AF}$ while the number was 40% for $ZIKV^{AS}$. Collectively, our data indicate that ZIKV infection of HUVECs leads to increased cell death mainly in the infected cells.

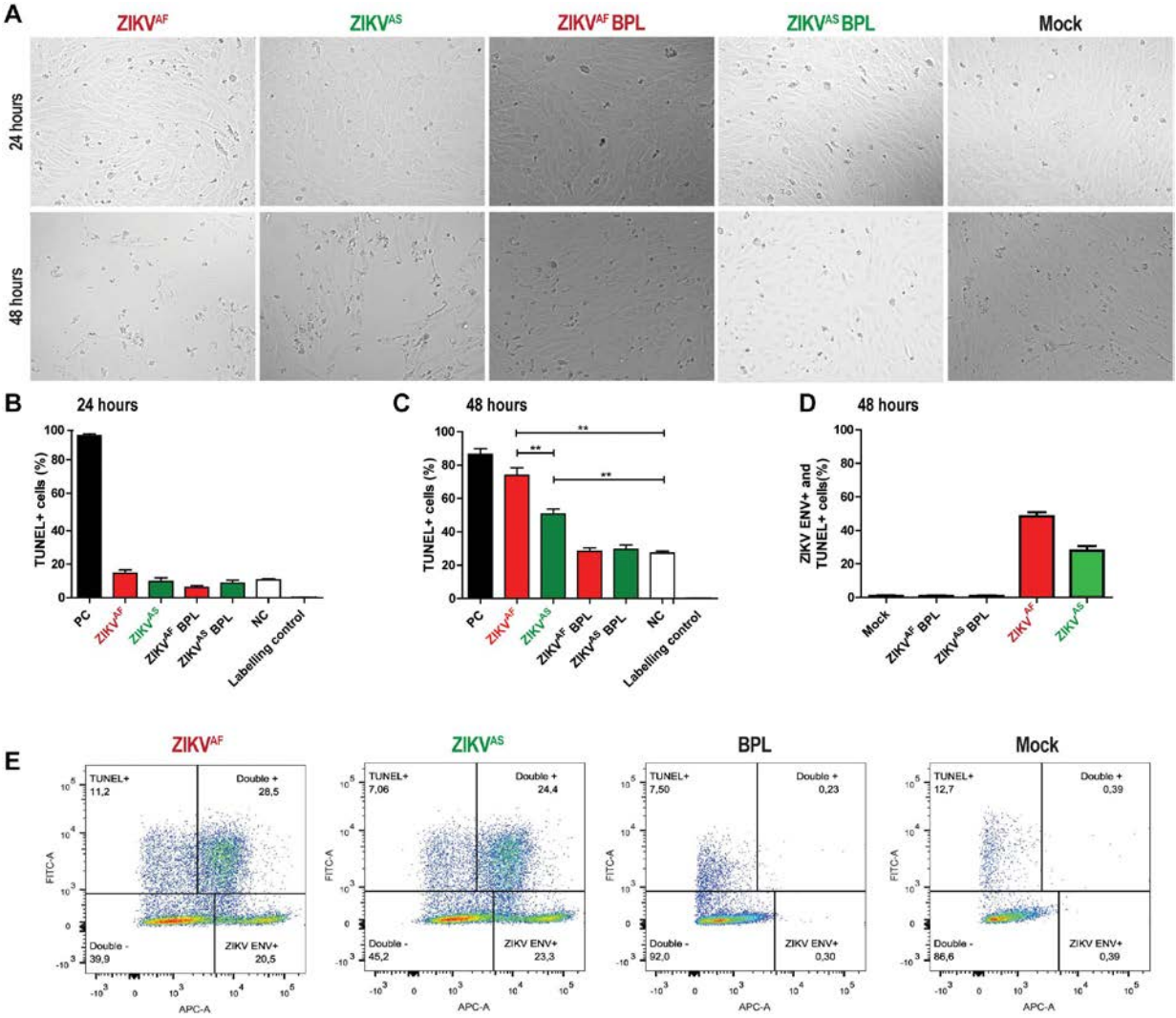

Figure 3. Zika virus (ZIKV) infection on human umbilical vein endothelial cells (HUVECs) induces apoptosis mainly through infection. HUVECs were infected with a MOI of 5 with both ZIKV strains. **A**, Cytophatic effect (CPE) was observed at 48 h post infection (hpi) for both ZIKV strains with light microscopy at 200x magnification. More CPE was observed with ZIKV^{AF}. **B&C**, To measure cell death, cells were stained for DNA fragmentation with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit at 24 and 48 h post infection (hpi). Both ZIKV strains induced cell death at 48 hpi. ZIKV^{AF} induced more cell death compared to ZIKV^{AS} ($p<0.01$). Experiments were done in triplicate and data are representative of two independent experiments. **D**, To determine whether cell death was caused by direct infection or bystander effect, we performed double staining directed against ZIKV envelope protein and TUNEL with flow cytometry. HUVECs were infected at an MOI of 5 and samples were collected at 48 hpi. Majority of the TUNEL+ cells were also positive for ZIKV ENV. **E**, Representative flow cytometry analyses plot to determine the ZIKV ENV+ and TUNEL+ cells at 48 hpi. Experiments were done in triplicate and data are representative of two independent experiments. All the results are displayed as mean and bars represent standard error of the mean (SEM). Statistical significance is shown: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

ZIKV infection on HUVECs triggers production of pro-inflammatory cytokines

Previous studies suggested that IL-6 and IL-8 can also increase TF expression on HUVECs and monocytes (174, 175). To assess whether ZIKV infection led to increased production of IL-6 and IL-8, the culture supernatant of infected HUVECs was collected at 0, 24, and 48 hpi. We found that IL-6 and IL-8 concentrations were significantly increased in the supernatant of ZIKV infected-cells compared to the controls. $ZIKV^{AF}$ induced a higher production of IL-6 and IL-8 than $ZIKV^{AS}$, BPL-inactivated virus and mock control at 48 hpi (Figure 4A). IL-6 and IL-8 levels increased significantly compared to BPL-inactivated virus (IL-6: $p=0.0018$; IL-8: $p<0.0001$) and negative control (IL-6: $p=0.0001$; IL-8: $p<0.0001$) two days after infection. $ZIKV^{AS}$ induced increased levels of IL-6 at 24 ($p<0.0001$) and 48 ($p<0.0001$) hpi compared to mock control (Figure 4A&B). Interestingly, $ZIKV^{AS}$ BPL control also induced elevated levels of IL-6 production.

To determine whether increase IL-6 and IL-8 production was linked to increased TF productions, we further performed blocking experiments against both cytokines and measured TF levels at 24 and 48 hpi. We first investigated whether the concentrations of anti-IL-6 and -IL-8 that we used could neutralize IL-6 and IL-8 by determining the expression levels of several genes of the downstream activation pathway of both cytokines. We found that the gene expression levels of NFKB1 and TIMP1 were significantly reduced in IL-6 with anti-IL-6 treatment (AB), comparable to negative control (NC; medium only), compared to cells with IL-6 only (PC) at both 24 and 48 hpi (Figure 4C). Similarly, NFKB1 and HIF1A expression levels were reduced in the presence of anti-IL-8 (Figure 4D). These results suggest that both monoclonal antibodies could neutralize the respective cytokines. Next, blocking experiments were conducted and TF expression was measured. The results revealed modest reduction of TF (Figure 4E&F), which suggest that IL-6 and IL-8 did not contribute significantly to the increased TF expression on HUVECs.

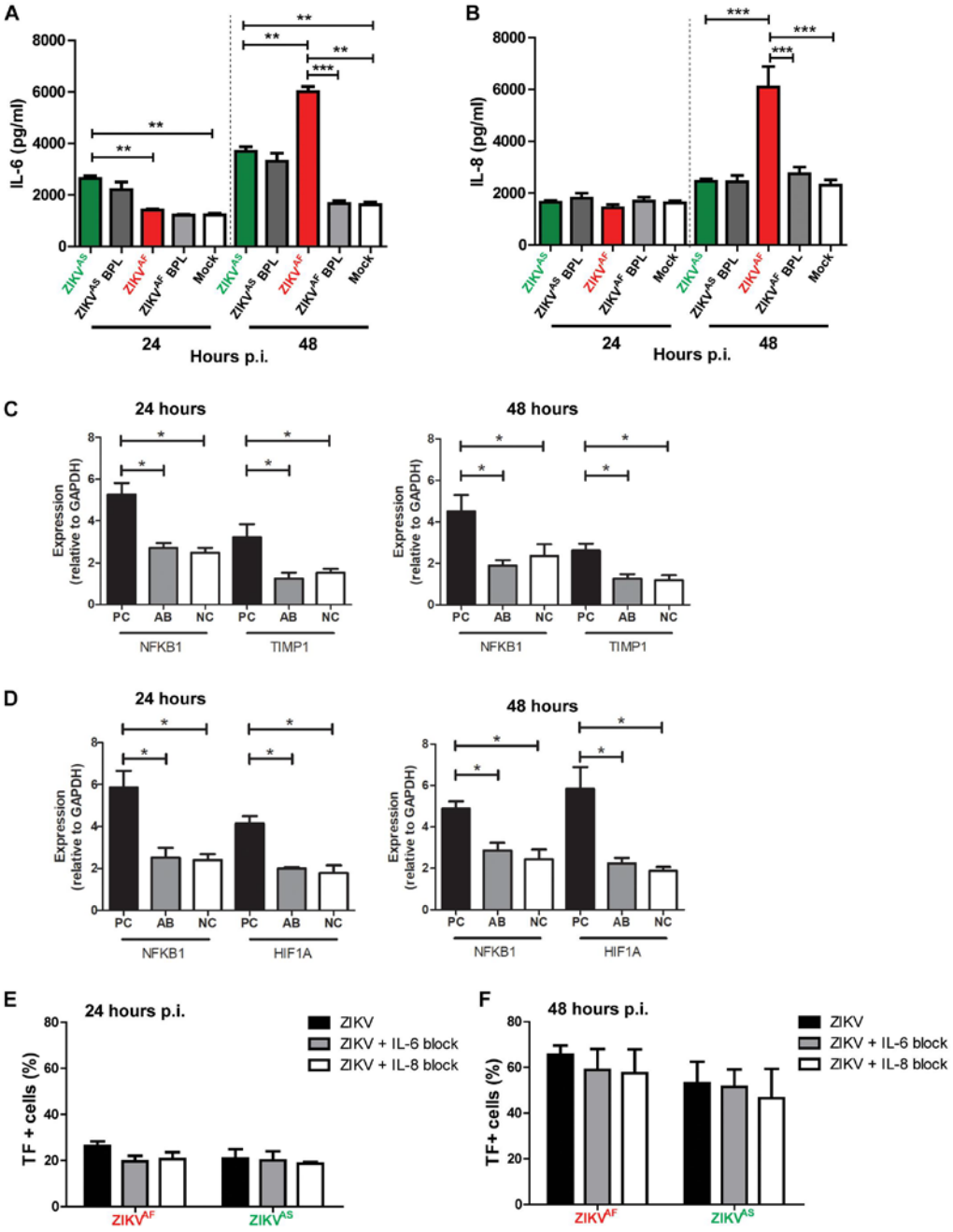

Figure 4. Zika virus (ZIKV)-infected human umbilical vein endothelial cells (HUVECs) produce pro-inflammatory cytokines. HUVECs were infected with a MOI of 5 with both ZIKV strains and supernatants were collected at the designated times. **A&B,** Significantly, increased levels of IL-6 and IL-8 were detected at 48 h post infection (hpi) for ZIKV^{AF}. **C&D,** To investigate whether the concentrations of anti-IL-6 and -IL-8 that we used were sufficient to neutralize IL-6 and IL-8 concentrations at 24 and 48 hpi, we determined the gene expression levels of NFKB1, TIMP-1, and HIF1A, which are the downstream activation pathways of IL-6 (NFKB1 and TIMP1) and IL-8 (NFKB1 and HIF1A). The gene expression levels of NFKB1 and TIMP1 were significantly lower in IL-6 with anti-IL-6 treatment (AB) compared to positive controls (PC; IL-6 protein only) and similar to negative controls (NC) **(C)**. The gene expression levels of NFKB1 and HIF1A were significantly reduced in IL-8 with anti-IL-8 treatment (AB) compared to PC (IL-8 protein only) and equal to NC **(D)**. **E&F,** Modest TF expression reduction was observed after IL-6 and IL-8 blocking experiments at 24 and 48 hpi. The results are based on three (figure A&B) and two (figure C, D, E &F) independent experiments. All experiments were performed in

triplicate except for figure C&D that was performed in duplicate. The results are displayed as mean and bars represent standard error of the mean (SEM). Statistical significance is shown: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

DISCUSSION

The present study addresses the effect of ZIKV infection of ECs *in vitro* on TF expression and apoptosis. We observed that infection with both an African and an Asian strain of ZIKV resulted in increased TF expression on HUVECs, which led to a shortened thrombin generation time. We showed that increased TF expression were mainly induced in ENV-positive cells, which indicate an indirect effect of infection on TF expression. It is important to note that very early stage of infection may lead to increased TF expression where the levels of ENV expression is still under the limit of detection.

TF is a major activator of the coagulation cascade during virus infections (188). A previous study with EBOV indicated that the over-expression of TF in primate monocytes/macrophages plays an important role in triggering the pro-coagulant state and consequently hemorrhagic complications (178). The role of TF in hemorrhagic fevers has also been described in dengue in that infection of ECs with DENV resulted in increased TF expression via the phosphorylation of p38 and ERK1/2 MAPKs (60). Excess TF production during infection could thus lead to increased clotting and eventually consumptive coagulopathy or even disseminated intravascular coagulation (DIC). Our results support the recent observations in humans and animal models, which suggest that hemostasis alterations occur during ZIKV infection (7, 8, 10, 39-43, 91, 92).

Liu et al. (88) showed recently that ZIKV could infect ECs from different vascular beds. Interestingly, the authors found that HUVECs were the most susceptible to ZIKV infection compared to human ECs derived from aortic and coronary artery, as well as the saphenous vein. Moreover, a recent study found that ZIKV NS1 specifically induced vascular leakage in HUVECs and brain ECs from several primary ECs that were tested (90). EC heterogeneity has been described between different organs, both on molecular and functional levels (189). Therefore, infection of ECs from different vascular beds with certain pathogens may lead to different pathogenic outcomes. This hypothesis has been supported for other hemorrhagic viruses such as DENV and puumala virus (183, 190). In contrast to our results, *In vitro* studies using human brain microvascular endothelial cells (hBMECs) and human retinal microvascular endothelial cells observed limited or no cytopathology during ZIKV infection (191, 192). Moreover, Mladinich et al. found that ZIKV infected and persisted in hBMECs (192). Whether vascular heterogeneity play a role in the pathogenesis of ZIKV-induced brain disorders and hemostasis alterations warrants further study.

It is known that pro-inflammatory cytokines play an important role in activation and downregulation of the coagulation system. We found that ZIKV infection of HUVECs leads to increased levels of IL-6 and IL-8 production. Our data is in line with a study that showed increased IL-6 production of ZIKV-infected retinal endothelial cells (191). We observed elevated levels of IL-6 also in HUVEC incubated with BPL-inactivated $ZIKV^{AS}$ suggesting that binding of viral proteins to EC alone may stimulate production of IL-6. This is in line with the study of Roach et al. that used a heat-inactivated Asian strain (PRVABC59) of ZIKV and reported increased levels of IL-6 production compared to mock control (191). We further blocked IL-6 and IL-8 during infection to determine whether these cytokines contributed to increased TF expression using concentrations that were showed to neutralized these cytokines (193). Our results indicated a modest TF expression reduction when both IL-6 or IL-8 were blocked, indicating limited effect of these cytokines neutralization on TF expression.

Several reports revealed that ZIKV infection of primary cells induced apoptosis (79, 185). In the present study, we observed that both ZIKV strains induced significant cell death. It is noteworthy that approximately 20% of dead cells were found in the mock control after 48 hours of culture, which might be explained by the donor since we observed differences among various donors (data not showed). It has been shown that apoptotic cells are procoagulant (61-63). Although the mechanism of how apoptotic cells activate coagulation system remains to be determined, it was shown that both adherent and detached apoptotic HUVECs become procoagulant by increased expression of phosphatidylserine (PS) and the loss of anticoagulant components such as TM and TFPI (61). Another possibility is that apoptotic ECs produce extracellular vesicles contributing to a procoagulant phenotype of the cells (62).

Our study has several limitations. First, we did not determine the whole spectrum of the hemostasis pathway. Second, we did not address the other factors that could trigger TF expression on HUVECs. Therefore, further studies to understand the mechanism of hemostasis activation during ZIKV infection is needed. However, our study provides evidence of increased TF expression, mostly due to bystander effect, that leads to activation of secondary hemostasis.

ACKNOWLEDGEMENTS

The authors would like to thank Cornelia A. M. van de Weg for the HUVECs isolation, Brigitta M. Laksono for her help with the FACS analyses, and Debby Schipper for the IFA staining.

FUNDING

FA was supported by a PhD scholarship grant from the Directorate of Higher Education (DIKTI) of the Ministry of Research, Technology and Higher Education of the Republic of Indonesia. Part

of this study was supported by the European Union program ZIKAlliance (contract no. 734548). The funder had no role in study design, data collection, and interpretation, or the decision to publish the work.

Chapter 4

Plasma leakage is associated with microbial translocation, production of inflammatory mediators and endothelial cell activation in a cohort of dengue patients

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Submitted

ABSTRACT

Introduction

The critical phase (CP) of dengue is characterized by plasma leakage (PL) and extensive immune activation. Most reports showed that virus titers are often low or undetectable at the time patients reach the CP. It is still unclear what drives PL and the aberrant immune response seen in this disease phase. Previous cross-sectional studies demonstrated that microbial translocation (MT) is associated with immune activation and disease severity. This study aim was to determine the kinetics of MT, immune mediators, and endothelial cell (EC) activation markers in relation to the development of PL in a prospective cohort of dengue patients.

Methods

We investigated levels of lipopolysaccharide (LPS), lipopolysaccharide binding protein (LBP), inflammatory mediators and EC activation markers in serial samples of patients that represent the three different phases of dengue. A non-supervised cluster analysis was applied to assess whether patient groups with similar phenotype could be identified by a biomarker profile.

Results

Our results indicate that levels of LPS, LBP, MCP-1, FGF, HGF, VCAM-1, TNF-α, angiopoietin-2, and endocan were significantly increased in the CP of PL patients compared to patients without PL. We also found positive correlations between MT markers and inflammatory and EC activation markers. Cluster analysis indicated that PL patients grouped together and display the highest concentrations of most biomarkers.

Conclusion

Our study provides evidence that MT, production of inflammatory mediators, and endothelial activation are associated with vascular leakage. This longitudinal study also identified for the first time that MT predominantly occurs in the CP of dengue.

INTRODUCTION

Dengue virus (DENV) is an important mosquito-borne virus causing at least 390 million infections annually (12). Clinical disease is divided into three phases: febrile, critical, and recovery (11, 194) The disease starts with a febrile phase that usually lasts 2-7 days in which patients experience fever and other flu-like symptoms. Subsequently, some patients may progress to the critical phase (CP), which is characterized by vascular hyperpermeability resulting in plasma leakage (PL) predominantly in the thorax and abdominal cavities. The onset of this phase usually coincides with defervescence and lasts for 24-48 hours (11, 194). It is hypothesized that the increased permeability observed during the CP is the consequence of aberrant immune activation (118, 195). Several hemorrhagic viruses showed that high viremia levels correlate with excessive immune activation (cytokine storm) and disease severity (196, 197). However, viremia measured in severe dengue, when extensive plasma leakage and hemorrhage are evident, is often low or undetected (198). It is therefore unclear what drives the cytokine storm seen in dengue in the absence of extensive virus replication.

Previously, our group has shown an association between severe dengue and lipopolysaccharide (LPS) levels using cross-sectional studies (118, 119). DENV infects and replicates in monocytes and macrophages, of which many can be found in the gut-associated lymphoid tissue (GALT). Infection of these immune cells may lead to a local pro-inflammatory environment in the intestines that eventually disrupt the gut barrier, resulting in microbial translocation as evidenced by increased serum levels of LPS (118, 119). Given that LPS is known to be a potent immune stimulator(199, 200) and could disrupt endothelial cell (EC) integrity,(201, 202) increased systemic LPS levels may contribute to the cytokine storm and altered vascular permeability in DENV infection. It is hypothesized that if LPS contributes to PL, then elevated levels of LPS should be observed in patients in the CP. Thus, in this study, we investigated the kinetics of systemic LPS presence, immune activation, and EC markers and its association with plasma leakage in a prospective cohort of dengue patients sampled throughout the different stages of disease.

METHODS

Study design

We performed a prospective observational study at a community health center (“Puskesmas”) and Cipto Mangunkusumo Hospital in Jakarta, Indonesia. Patients who experienced an acute fever (axillary temperature >37.5°C) ≤48 hours and two or more additional symptoms (headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations) visiting the Puskesmas from December 2010-May 2011 and June-September 2014 were tested with a NS1

antigen and IgM/IgG antibody rapid tests (SD Dengue Duo, Standard Diagnostics, Republic of Korea). If testing positive for NS1 and/or IgM, patients were admitted to Cipto Mangunkusumo Hospital for further examinations. Subjects were excluded if they were pregnant or breastfeeding or had comorbidities, such as liver problems, enteric disease and severe kidney problems. Clinical data were recorded with a standardized case report form and serum samples were collected on a daily basis. In addition to NS1 and IgM tests, DENV infection was confirmed with conventional reverse–transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time (qRT)-PCR assays. Presence of ascites and/or pleural effusion were assessed by performing serial ultrasound examination on days 3, 5 and 7 after the onset of fever. Patients were classified according to the occurrence of PL (none and positive) and samples that represented the three different phases of dengue were selected from each patient. The subjects were classified as having plasma leakage (PL+) based on the presence of pleural effusions and/or ascites detected by ultrasonography. Acute febrile phase (AP) was defined as the earliest sample that was obtained from each patient ((day 1/2 after onset of illness (AOI)). CP was defined as the sample that was collected when fever resolved and the patients developed PL as detected by an ultrasound examination. CP sample from patients without plasma leakage (PL-) was collected when fever resolved. Recovery phase (RP) was defined as the sample that was collected on day 7 AOI or before the patients were discharged from the hospital. Healthy controls (HCs) were matched for age and sex and were recruited in the same geographical area as the study participants.

Serology

IgM and IgG antibodies (Focus Diagnostics, USA) ELISA test were performed according to the manufacturer's instructions. IgM and IgG antibodies were measured in the serial samples of each patient. Patients with a positive IgG in the sample from day 3/4 AOI were considered as secondary DENV infection. Patients with a negative IgG and positive IgM were considered as primary DENV infection.

Molecular diagnostics

RNA was extracted from plasma using High pure viral RNA kit (Roche, Switzerland) according to the manufacturer's recommendations. DENV serotyping was conducted using a RT-PCR method as described previously (203). Viral load was determined using LightMix dengue virus EC kit (TIB Biomol, Germany) as described before (204).

Other laboratory parameters

Albumin, aspartate transaminase (AST) and alanine transaminase (ALT) concentrations were determined using Cobas 501 machine (Roche, Switzerland). The levels of hematocrit and platelet were analyzed using an automatic hematology analyzer (Sysmex, Japan).

Markers of microbial translocation

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 (119). Detailed LAL method can be found in the **Supplemental Methods** section. All reagents and the machine for the LPS assay were obtained from Associates of Cape Cod, USA. LBP levels were determined using a commercial ELISA assay (Hycult Biotech, USA). The assays were performed according to the manufacturers' recommendations and the samples were measured in duplicate.

Markers of immune activation

The levels of MCP-1, FGF-basic, HGF, IL-8, ICAM-1, VCAM-1, TGF-β1, TNF-α and VEGF were measured using a multiplex immunoassay kit with antibody-conjugated magnetic beads (R&D systems, USA). See **Supplemental Methods** section for more details. The samples were analyzed using the Luminex 200 dual laser detection system (Luminex, USA).

Markers of endothelial cell activation

Levels of Angiopoietin-2 (Ang-2; Quantikine, R&D systems, USA), endothelin-1 (ET-1; Quantikine, R&D systems, USA) and endocan (Lunginnov, France) were measured using commercially available ELISA kits. The assays were performed according to the manufacturers' instructions.

Cluster analysis

Marker measurements were processed and visualized in R (R Core Team (2018), [https://www.R-project.org/]). The heatmap was created using pheatmap (Raivo Kolde, 2019. [R package version 1.0.12], https://CRAN.R-project.org/package=pheatmap). See **Supplemental Methods** for analysis details.

Statistical analysis

GraphPad Prism 5.0 for Windows (GraphPad Software, USA) was used for the statistical analyses. . Statistical significance between two independent groups was determined with unpaired t-test or Mann-Whitney U test, depending on the distribution of the data. Differences of paired data were determined using either a paired t-test or Wilcoxon signed rank test when distribution differed from normal P values for the biomarkers were corrected for multiple testing by controlling the false discovery rate (FDR) according to the Benjamini-Hochberg method.(205) Correlation between continuous variables were determined with the Spearman correlation test. P values ≤.05 were considered significant.

Ethics statement

The study protocol was approved by the ethics committee of the Faculty of Medicine, Universitas Indonesia. Written informed consent was obtained from all the study participants or parent or legal guardian if a patient was younger than 18 years.

RESULTS

Characteristics of the study participants

Eighty-eight patients with laboratory confirmed acute DENV infection were enrolled during the study period. Sixty-four patients were included for further analyses based on the availability of serial serum samples (**Figure 1**). Viral RNA could not be detected in seven patients, but they were included because these patients were tested positive for both NS1 antigen and IgM ELISA tests. There were 31 PL- patients (48.4%) and 33 PL+ patients (51.6%). Routine biochemistry analyses revealed significantly higher levels of AST in the CP (P=.0004) of PL+ patients compared to PL- patients. Furthermore, PL+ patients had significantly lower levels of platelets and albumin in the AP (platelets: P<.0001; albumin: P<.0001) and CP (platelets: P=.0016; albumin: P<.0001) compared to the PL- group. **Table 1** describes the clinical characteristics and laboratory parameters of each study group.

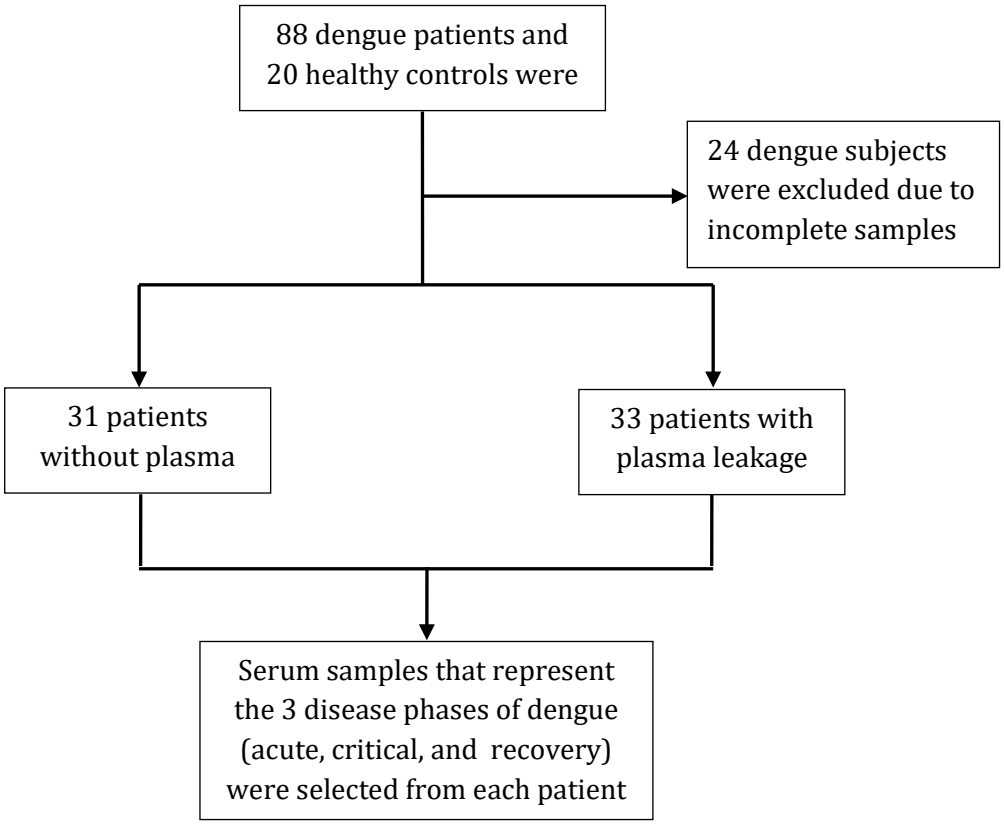

Figure 1. Study layout

Table 1. Clinical characteristics and laboratory parameters of each study group

Variable	PL- (n=31)	PL+ (n=33)	HC (n=20)	Statistics *
Sex, no. male/female	17/14	17/16	11/9	
Age, y, median (IQR)	21 (17-28)	23 (16.5-29.5)	22 (17.5-28.5)	
Day of fever, median (IQR)	3 (2-4)	4 (4-5)	NA	
2011 WHO dengue classification, no. DF/DHF/DSS	31/0/0	0/33/0	NA	
History of infection, no. primary/secondary/unknown	15/13/3	8/23/2	NA	
Hematocrit levels, %, median (IQR)				
AP	38.9 (36.8-41.4)	39.5 (37.85-41.2)	NA	P=.586
CP	39.8 (37.1-44.7)	40.8 (36.25-43.95)	NA	P=.799
RP	37.7 (36.6- 41)	36.8 (33.3-40.55)	NA	P=.190
Platelet count x 1,000 cells/mm^3, median (IQR)				
AP	159 (118-182)	134 (108-183)	NA	P=.285
CP	112 (67-144)	36 (24.5-78.5)	NA	P<.0001
RP	105 (87-166)	77 (57.5-113.5)	NA	P=.0016
Albumin levels, mg/dl, mean (±SD)				
AP	4.33 (0.305)	4.43 (0.302)	NA	P=0.201#
CP	3.97 (0.355)	3.45 (0.486)	NA	P<.0001#
RP	3.98 (0.299)	3.6 (0.284)	NA	P<.0001#
AST levels, U/L, median (IQR)				
AP	34 (22-46)	38 (28.5-60.5)	NA	P=.154
CP	50 (36-69)	83 (55.5-130.5)	NA	P=.0004
RP	91 (66-156)	98 (71-174.5)	NA	P=.301
ALT levels, U/L, median (IQR)				
AP	22 (15-33)	23 (14.5-42)	NA	p=.559
CP	36 (23-57)	44 (33.5-69.5)	NA	p=.061
RP	81 (54-174)	86 (52.5-183)	NA	p=.877
Positive ultrasound results, no.				
Ascites	ND	24	NA	
Pleural effusion	ND	21	NA	

Abbreviations: DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; PL+, plasma leakage positive; PL-, plasma leakage negative; HC, healthy control; AP: acute febrile phase; CP: critical phase; RP: recovery phase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IQR, interquartile range; SD, standard deviation; NA, not applicable; ND, not detected; *between PL+ and PL- groups, Mann Whitney U test was used unless otherwise stated; #, unpaired t test.

Dengue serotype and viral load

Virus serotype data were available in 57 of 64 patients. Thirteen patients (22.8%) were infected with DENV-1, 22 patients (38.6%) with DENV-2, 19 patients (33.3%) with DENV-3, and 3 patients (5.3%) with DENV-4. Serial viral load information was available in 43/57 patients (75.4%). From these patients, 24/43 (55.8%) were in the PL+ group and 19/43 (44.2%) were in the PL- group. Most patients were tested negative for viral RNA from the fifth day of fever onwards (**Supplemental Table 1**). Higher viral load levels were observed throughout the study period in the PL+ patients compared to the PL- patients although the difference was not statistically significant (**Figure 2**).

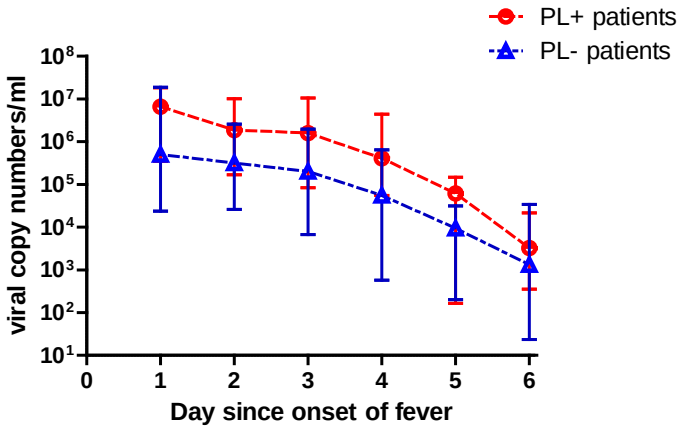

Figure 2. Serial viral load levels in dengue patients with and without plasma leakage.
Serial viral load levels were determined for each group of patients. The horizontal lines represent the median values with interquartile range. Abbreviations: PL-, plasma leakage negative; PL+, plasma leakage positive.

Kinetics of microbial translocation markers

To investigate the kinetics of MT markers, we determined LPS and LPB levels in serum samples collected at the three different phases of disease. Significantly elevated LPS levels were found in the CP of the PL+ group compared to the PL- group (P=.024) (**Figure 3A**). Furthermore, significantly increased levels of LPS were detected in the PL+ group during the CP compared to AP (P=.013) and RP (P=.025). Since LBP production by the liver will mainly be triggered by circulating LPS, LBP represents a specific marker for LPS circulation. In line with this, LBP levels were significantly elevated in the PL+ patients compared to the PL- patients in the CP (P<.0001) **(Figure 3B**). Furthermore, coherent with the LPS data, significantly increased levels of LBP were detected in the PL+ group during the CP compared to AP(P=.0005) and RP (P<.0001).

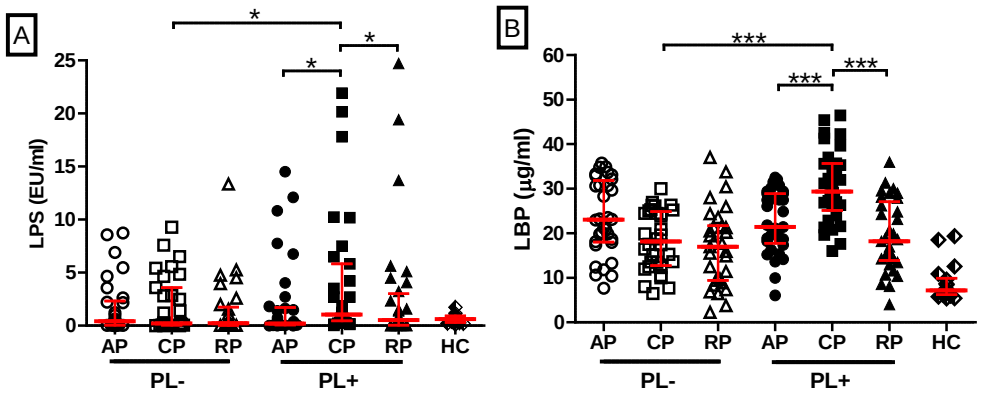

Figure 3. Serum LPS and LBP concentrations in dengue patients with and without plasma leakage. (A) LPS levels are significantly elevated in PL+ patients compared to PL- patients in the CP(P=0.024). Furthermore, LPS levels are significantly increased in the CP compared to AP (P=0.013) and

RP (P=0.025) in the PL+ patients group **(B)** Levels of LBP are significantly elevated in PL+ patients compared to PL- patients in the CP (P<0.0001). Moreover, LBP levels in the CP are significantly higher compared to AP (P=0.0005) and RP (P<0.001) in the PL+ patients group. Data are presented as median with interquartile range. P values were determined by Wilcoxon matched pairs test for paired data and Mann-Whitney U test for non-paired data. *Represents a p value < 0.05; **p value < 0.01; ***p value < 0.001. Abbreviations: PL-, plasma leakage negative; PL+, plasma leakage positive; AP, acute febrile phase; CP, critical phase; RP, recovery phase; HC, healthy control.

Kinetics of cytokines and chemokines serum concentrations

To investigate whether increased production of cytokines and chemokines occurred in PL+ patients, the levels of nine inflammatory mediators were determined. We observed significantly elevated levels of MCP-1 (P=.0017), TNF-α (P=.0014), and FGF (P=.0006) in the PL+ group compared to the PL- group in the CP (**Figure 4**). Moreover, there were significantly increased levels of HGF in the PL+ group both during the CP (P<.0001) and RP (P=.0002) compared to the PL- group.

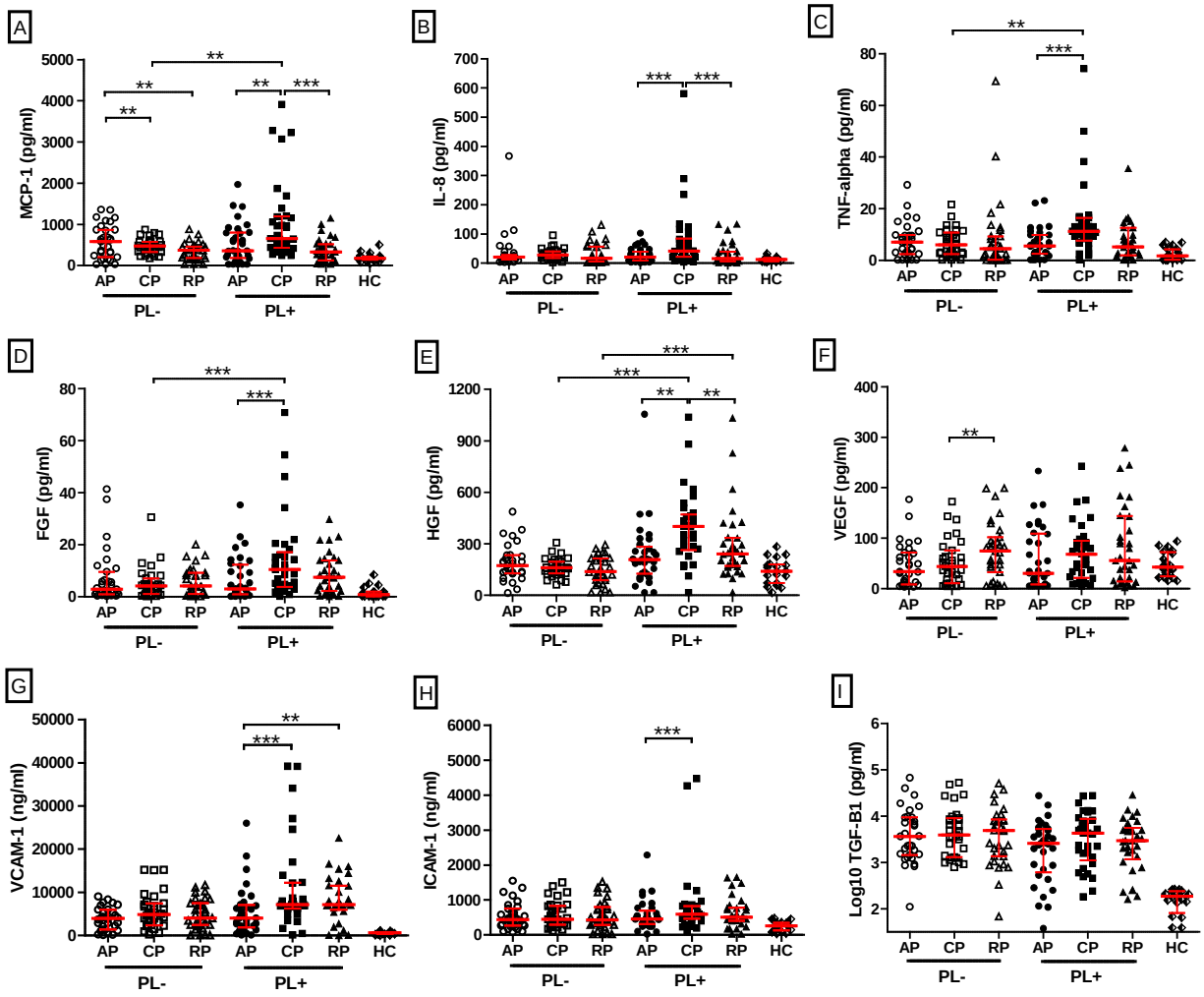

Figure 4. The association of cytokines and chemokines with plasma leakage.
The concentrations of 9 inflammatory mediators were determined in the cohort of patients. (**A**) MCP1: levels in PL+ patients were significantly elevated compared to PL- patients in the CP (P=.0017). Levels

were also significantly increased in the CP of PL+ patients compared to AP (P=.0032) and RP (P<.0001). Concentrations were also significantly higher in the RP of PL- patients compared to AP (P=0.003) and CP (P=.0013). (**B**) IL-8: levels were significantly increased in the CP of PL+ patients compared to AP (P=.0006) and RP (P=.0006). (**C**) TNF-α: Concentrations in PL+ patients were significantly elevated compared to PL- patients in the CP (P=.0014). Levels were also significantly increased in the CP of PL+ patients compared to AP (P=.0002). (**D**) FGF: Levels in PL+ patients were significantly elevated compared to PL- patients in the CP (P=.0006). Concentrations were also significantly increased in the CP of PL+ patients compared to AP (P=.0003). (**E**) HGF: levels in PL+ patients were significantly elevated compared to PL- patients in the CP (P<.0001) and RP (P=.0002). Furthermore, levels were also significantly increased in the CP of PL+ patients compared to AP (P=.0013). (**F**) VEGF: Levels were elevated in the RP of PL- patients compared to CP (P=.0032). (**G**) VCAM-1: Levels were significantly decreased in the AP of PL+ patients compared to CP (P<.0001) and RP (P=.0032). (**G**) ICAM-1: Levels were significantly increased in the CP of PL+ patients compared to AP (P=.0001) (**H**) TGF-β1: Concentrations were elevated in all the samples compared to HCs but there were no significant differences observed between PL+ and PL- or within the groups. Data are presente25d as median with interquartile range. P values were determined by Wilcoxon matched pairs test for paired data and Mann-Whitney U test for non-paired data. **Represents a p value <.0056; ***p value <.001. Abbreviations: PL-, plasma leakage negative; PL+, plasma leakage positive; AP: acute febrile phase; CP: critical phase; RP: recovery phase; HC, healthy control; MCP-1, monocyte chemoattractant protein-1; IL-8, interleukin 8; TNF-α, tumor necrosis factor alpha; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; TGF-β1, transforming growth factor beta 1.

Kinetics of EC activation markers

To evaluate the association between EC activation and PL, the levels of several EC markers were determined. We found that angiopoietin-2 levels were significantly elevated in the PL+ group compared to the PL- group while in the AP (P=.0081), CP (P<.0001) and RP (P=.016) (**Figure 5**). ET-1 levels were significantly higher in the RP of PL+ group compared to AP (P=.0001) and CP (P=.0011). Endocan levels were significantly increased in the PL+ group compared to the PL- group both in the CP (p< .0001) and RP (P=.015). Furthermore, levels were significantly higher in the CP of PL+ patients compared to AP (P<.0001) and CP (P<.0001).

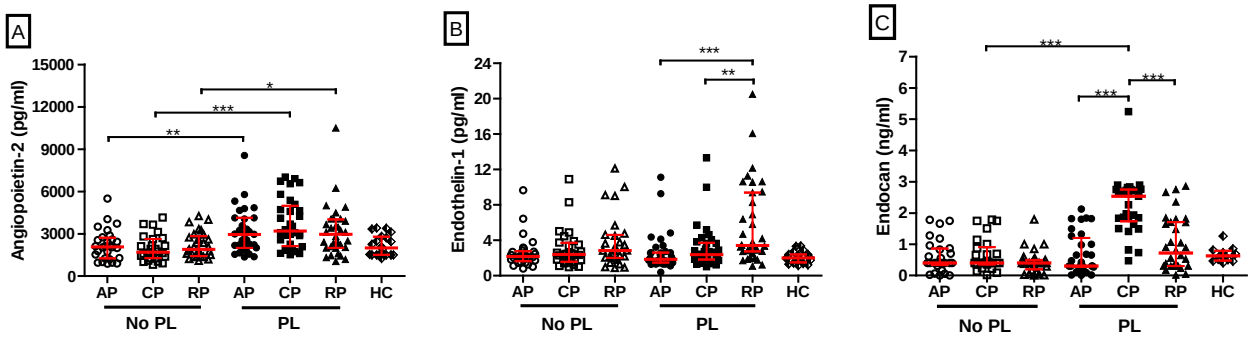

Figure 5. Association of endothelial cell activation markers with plasma leakage.
(**A**) Angiopoietin-2: levels in PL+ patients were significantly elevated compared to PL- patients in the AP (P=.0081), CP (P<.0001) and RP (P=.016). (**B**) Endothelin-1: Levels were significantly increased in the RP of PL+ patients compared to AP (P=.0001) and CP (P=.0011). (**C**) Endocan levels in PL+ patients were significantly elevated compared to PL- patients in the CP (P<.0001). Moreover, levels were significantly higher in the CP of PL+ patients compared to AP (P<.0001) and CP (P<.0001). Data are presented as median with interquartile range. P values were determined by Wilcoxon matched pairs test for paired

data and Mann-Whitney U test for non-paired data. *Represents a p value <.05; **p value <.01; ***p value <.001. Abbreviations: PL-, plasma leakage negative; PL+, plasma leakage positive; AP, acute febrile phase; CP, critical phase; RP, recovery phase; HC, healthy control.

Correlation of MT with immune mediators and EC markers

To investigate whether MT was associated with the activation of EC and cytokines production in the CP, we determined the correlation between these molecules (**Supplemental Table 2**). We found a correlation between LPS and levels of MCP-1 (P=.038; *r*=.259), TNF-α (P=.049; *r*=.345), VEGF (P=.045; *r*=.351), and endocan (P=.018; *r*=.296). Furthermore, we found a positive correlation between LBP and levels of MCP-1 (P=.001; *r*=.402), TNF-α (P=.045; *r*=.252), HGF (P<.0001; *r*=.59), VCAM-1 (P=.01; *r*=.323), angiopoietin-2 (P=.0092; *r*=.323), and endocan (P=<.0001; *r*=.46).

Cluster analysis

Given the observed differences in the marker levels during the CP, we performed unsupervised cluster analysis on all the biomarkers measured for the samples collected during this phase and the result is depicted in a heatmap (**Figure 6**). Samples from HC tend to cluster together in cluster A ($P=1.1x10^{-09}$). PL- patients mainly cluster together in cluster B ($P=3.1x10^{-09}$), while PL+ samples are significantly associated with cluster C ($P=5.3x10^{-14}$). Cluster C displays the highest concentrations of most markers, as indicated by the predominantly red colors in this cluster. Conversely, HCs (cluster A) tend to have low values for most markers and cluster B was a mix of high and low measurements. In addition, we did not identify noteworthy clustering for both the acute and recovery phases (data not shown).

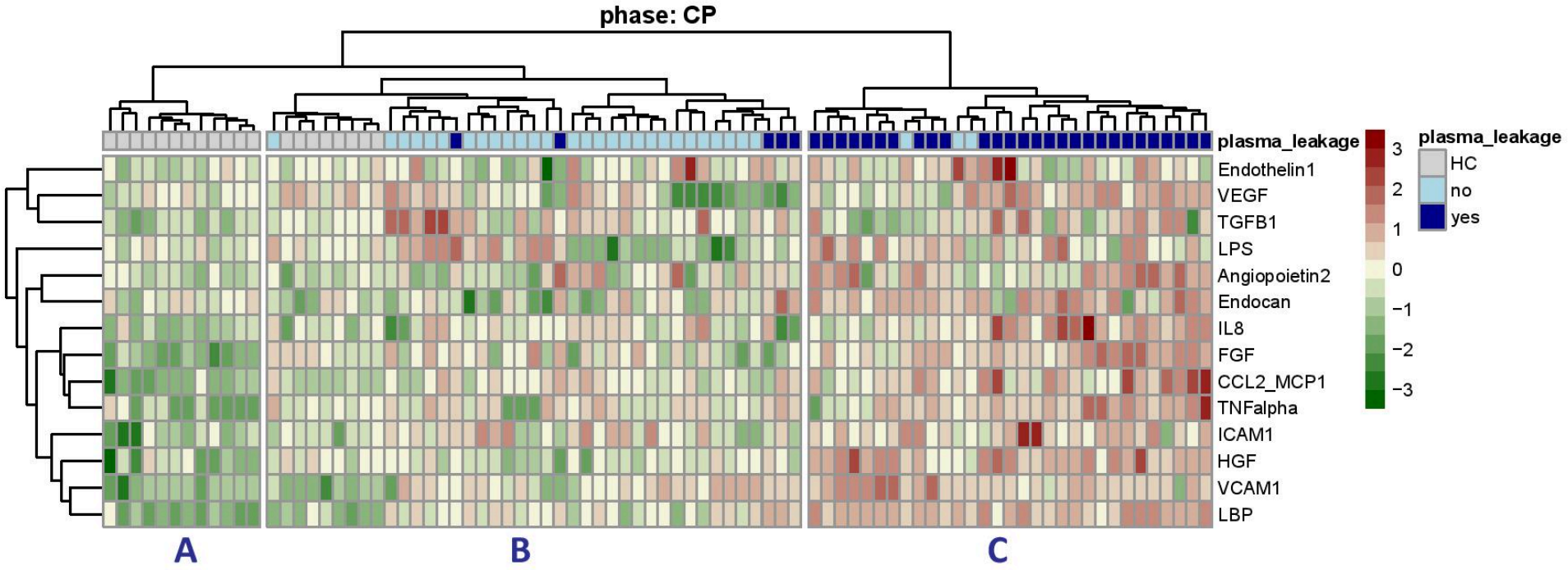

Figure 6. Heatmap of cluster analysis.
A cluster analysis was performed with 14 markers, which resulted in a dendrogram indicated on the top of the heatmap. Every vertical line indicates one patient. The legend indicates the distance from the mean in standard deviations. Red and green are high and low expression, respectively, relative to the mean value of the cytokine or marker. The vertical bar on the right of the heatmap indicates the severity of disease based on the occurrence of plasma leakage. The samples are annotated with either HC for healthy control (grey), or 'yes' /'no' signifying if plasma leakage is present or not (dark/light blue, respectively). A: Cluster with all healthy controls. B: Cluster with mostly PL- patients. C: Cluster with mostly PL+ patients. Abbreviations: CP, critical phase; HC, healthy control.

DISCUSSION

In this study, we investigated a cohort of dengue patients to assess the association of vascular leakage with markers of bacterial translocation, endothelial activation, and inflammatory cascade. To our knowledge, this is the first longitudinal study, which showed an association between elevated LPS and LBP levels and vascular leakage in dengue infection. The rationale for this study was based on earlier observations: it has been shown that LPS disrupt ECs integrity in vitro.(201, 202, 206) LPS induces activation of EC and elicited production of important inflammatory mediators such as IL-6, IL-8, TNF-α, MCP-1 and VEGF, which could disrupt EC permeability.(117, 199, 200, 207) Several animal studies also showed that LPS administration altered vascular permeability.(202, 208) Interestingly, it was shown that in the presence of LPS, DENV-infected monocytes secreted significantly more cytokines than compared to DENV infection alone.(209) LPS interacts with LBP, which is synthesized by hepatocytes upon stimulation with LPS, and serves as an indirect marker of LPS circulation.(210) In view of the above, we decided to do a combined analysis of markers for bacterial translocation, immune activation, and EC activation markers in a prospective cohort of dengue patients with different disease courses.

We confirm earlier observations of elevated levels of MCP-1, FGF, HGF, VCAM-1, TNF-α, angiopoietin-2, and endocan in the PL+ patients compared to the PL- group in the CP.(118, 195, 211) However, our study did not find an association between PL with VEGF, IL-8, ICAM-1, TGF-β1, and ET-1 levels that have previously been reported.(212) One possibility is that the markers for which we did not find an effect are specific for dengue shock syndrome, as our study did not include shock patients.(118, 212, 213)

The consistent grouping of patients to the clinical classes by the use of all markers were also shown when we performed an unsupervised cluster analysis. We identified three different clusters and observed that most PL patients clustered together in cluster C, which displays the highest concentrations of most markers. This analysis further supports that there was an association between PL and the biomarkers investigated in this study.[4] We also showed associations between serum MT markers and inflammatory and EC markers, implying that translocation likely contribute to immune activation in the CP of dengue.

An important question is how these observations fit in the pathogenesis pathway of severe dengue. It is known that increased permeability in dengue is mainly detected in thorax and abdominal cavities, which suggest a localized response to infection. Nevertheless, evidence of *in vivo* local replication in the aforementioned cavities is limited. It has been hypothesized that EC heterogeneity may contribute to the specific altered permeability locations in dengue. This hypothesis is supported by a study which showed that DENV infection of two types of human ECs resulted in both common and specific activation pathways.(190) Moreover, a recent publication using different primary human ECs revealed that DENV NS1 specifically induces altered permeability in vasculature-specific manner.(90) Interestingly, a report also showed diverse inflammatory responses between different types of EC against LPS.(214) Hence, it is important to investigate whether specific activation pathways for ECs in the thorax and abdominal cavities to both DENV and LPS occur.

A previous report of HIV-infected patients indicated that there was a positive correlation between LPS levels and viral load.(215) We found that the PL+ patients had higher viral loads compared to the PL- patients throughout the study duration as observed previously.(216) Our data suggest that viremia was not associated with the amount of MT and immune mediators produced in the CP since higher viral loads were detected in the AP. However, continued post-viremic local viral replication in the intestine or liver may induces bacterial translocation in to the circulation.(217) Previously, we hypothesized[4] that monocytes/macrophages in GALT are infection targets for DENV, supported by observations of enteric involvement in AG-129 mice infected with DENV.(218-220) Moreover, these groups also found altered permeability in the bowel of the animals challenged with different DENV strains.(218-220) Whether local replication in the target organs, specifically intestine and liver, contribute to MT and/or immune activation in dengue warrants further studies.

This study thus confirms the relevance of LPS and LPS-mediated activation markers for risk classification and adds a new potential marker of PL for dengue. We also identified endocan, a soluble proteoglycan that is produced specifically by the vascular ECs of lung, kidney and tumor endothelium, as a potential marker of PL. The levels of this protein were only significantly elevated in the CP of PL+ patients group. This marker was shown as a good marker of endothelial dysfunction and multi-organ failure in

sepsis patients.(221, 222) Moreover, endocan elicited the vascular inflammatory response and disrupted the vascular barrier integrity in both ECs and mouse models. Of interest, endocan production was induced by LPS stimulation in both models.(223) These data suggest that endocan may play a role in the altered permeability of DENV infection.

Although the prospective design of our study is a strong point, our study has some limitations. First, we did not measure biologically active LPS: it is known that not all bacteria produce biologically active LPS, and not all types of LPS can be detected with the LAL assay.(224) Second, the assay that we used only represents gram-negative bacteria translocation. Although gram-negative bacteria represent the majority of the gut microbiota, it is important to further characterize the gut microbial flora that translocate to the circulation. A key question of course is how to translate this information into intervention strategies. Our observations suggest that treatment of severe dengue could benefit from assessing knowledge from the treatment of LPS-mediated inflammatory conditions.

In summary, our cohort study provides evidence that translocation of microbial products, endothelial activation, and production of inflammatory mediators in the CP of dengue are associated with vascular leakage. This longitudinal study also observed for the first time that MT mainly occurs during the CP. Translocation of gut microbiota may play an important role in the production of inflammatory mediators and vascular leakage in dengue. Identifying the factor(s) responsible for disruption of the intestinal mucosal barrier in DENV infection and consequently MT, as well as translation of these findings into treatment options represents an important focus of future studies.

ACKNOWLEDGEMENTS

We thank all the medical and nursing staffs at the Puskemas Kayu Putih and Cipto Mangunkusumo Hospital in Jakarta, Indonesia for patient recruitment and management. We also thank the staffs at the Department of Microbiology, Integrated Laboratory (Lab Terpadu) and INA-REPROMED cluster of Indonesian Medical Education and Research Institute, Faculty of Medicine Universitas Indonesia for their help and contribution in this study. We also thank David van de Vijver and Ahmad Fuady for their help with the statistical analyses and Barry Rockx and Thomas Langerak for their inputs to the manuscript.

FINANCIAL SUPPORT

F.A. was supported by a Directore of Higher Education (DIKTI) PhD grant from the Ministry of Research, Technology and Higher Education of the Republic of Indonesia.

SUPPLEMENTAL DOCUMENTS

METHODS

Measurement of LPS

Samples were diluted 1:20 with LAL reagent water in a depyrogenated glassware (Pyrotube D) to prevent contamination and were heat inactivated at 56°C for 30 minutes. Thereafter, 200 µl of the diluted sample was transferred to a small depyrogenated glassware (Pyrotube K) and 50 µl of LAL diluted in (1-3)-β-D-Glucan inhibiting buffer was added. Subsequently, the samples were measured in a Pyros Kinetik Flex Machine. Commercial *Escherichia coli* endotoxin was used to prepare the standard curve.

Levels of cytokines and chemokines

Cytokines were measured using a multiplex immunoassay kit with antibody-conjugated magnetic beads (R&D systems, USA). The following cytokines were measured: MCP-1, FGF-basic, HGF, IL-8, ICAM-1, VCAM-1, TGF- β1, TNF-α and VEGF. Serum samples were diluted and processed according to the manufacturer's instructions. Standard curves of known concentrations of recombinant human cytokines were used to convert fluorescence units into concentration units (pg/mL). Values below the lower limit of quantification were replaced by the lowest limit of quantification. The samples were analyzed using the Varioskan LUX multimode microplate reader (Thermo Scientific USA). Data generated were processed using the xPONENT software (Luminex, USA).

Cluster analysis

Marker measurements were process and visualized in R (R Core Team (2018), [https://www.R-project.org/]). All measurements were log10 transformed. Two zero values in the measurements were set to half the minimal value of that cytokine. Measurements were subsequently normalized (to a mean=0, standard deviation=1) by cytokine, and were clustered using correlation distance followed by Ward's method. The heatmap was created using pheatmap (Raivo Kolde, 2019. [R package version 1.0.12], https://CRAN.R-project.org/package=pheatmap). The heatmap clusters were sorted by plasma leakage annotation (see side bar of figure 5). Associations per cluster were computed by making a 2-by-2 table of cluster and sample type and computing significance using Fisher's test.

RESULTS

Supplemental Table 1. Number of viral load positive patients on each group in the study period

Viral load results per day	**PL- (n=19)**	**PL+ (n=24)**
Viral load results, positive no./total no. of patient tested (%)		
Day 1	5/5 (100%)	4/4 (100%)
Day 2	18/19 (94.7%)	24/24 (100%)
Day 3	16/19 (84.2%)	24/24 (100%)
Day 4	15/19 (78.9%)	22/24 (91.7%)
Day 5	7/19 (36.8%)	12/24 (50%)
Day 6	4/19 (21%)	4/24 (16.7%)

Abbreviations: PL-, plasma leakage negative; PL+, plasma leakage positive.

Supplemental Table 2. Correlation between LPS and LBP with immune mediators and endothelial activation biomarkers in the critical phase

Markers	Spearman's R	P value
LPS		
MCP-1	.259	.038*
FGF-basic	.189	.135
HGF	.047	.794
IL-8	.123	.333
ICAM-1	-.025	.839
VCAM-1	.034	.792
TGF- β1	-.036	.777
TNF-α	.345	.049*
VEGF	.351	.045*
Endocan	.296	.046*
Angiopoietin-2	.015	.906
Endothelin-1	.112	.392
LBP		
MCP-1	.402	.001*
FGF-basic	.154	.22
HGF	.59	<.0001*
IL-8	.17	.179
ICAM-1	.18	.15
VCAM-1	.323	.01*
TGF- β1	-.05	.67
TNF-α	.252	.045*
VEGF	.156	.21
Endocan	.46	<.0001*
Angiopoietin-2	.323	.0092*
Endothelin-1	.07	.55

Note: *, statistically significant. Abbreviations: LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; MCP-1, monocyte chemoattractant protein-1; IL-8, interleukin 8; TNF-α, tumor necrosis factor alpha; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; TGF-β1, transforming growth factor beta 1.

Chapter 5

Hyperferritinemia is a potential marker of chronic chikungunya: a retrospective study on the Island of Curaçao during the 2014 –2015 outbreak

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J Clin Virol. 2017;86:31-38

*Authors contributed equally

ABSTRACT

Background

Recently Chikungunya virus (CHIKV) outbreaks have been reported in the Carribean. There is no data regarding the outbreak in Curaçao. In addition, to date there is no biomarker that could be used to predict chronic infection.

Objectives

To characterize the first CHIKV outbreak in Curaçao and to identify potential biomarkers for chronic infection.

Study design

A serological test and quantitative polymerase chain reaction (qPCR) were used on samples collected in Curaçao to confirm infection. Subsequently, six samples with high viral load were selected for phylogenetic analysis. Furthermore, we investigated the association of macrophage-related biomarkers during CHIKV infection with chronic arthralgia/arthritis.

Results

116 patients in Curacao were diagnosed with CHIKV infection based on ELISA and 77% were tested positive for CHIKV by qPCR. Phylogenetic analysis showed that an Asian genotype was the cause of the outbreak. Elevated levels of ferritin and CRP were significantly associated with viraemia. In addition, elevated ferritin levels were significantly associated with chronic arthralgia.

Conclusions

The results showed that the presence of an Asian genotype of CHIKV in Curaçao for the first time. Moreover, we found an association between ferritin levels with chronic arthralgia.

1. Background

Chikungunya virus (CHIKV) is an arthropod-borne alphavirus that cause dengue-like illness (30). Three lineages of CHIKV have been identified to date, namely West African, Asian, and East-Central-South-Africa (ECSA) genotypes. In the past, CHIKV outbreaks have only been reported occasionally in Africa and Asia. An Asian lineage was introduced into the island of St Martin in 2013, and quickly spread throughout the Caribbean, South and Central America (225). Between 20% and 50% of CHIKV-infected patients may develop chronic arthralgia. To date there is no marker that could be used to predict chronic CHIKV infection. It is difficult to predict who will develop chronic disease and therefore finding biomarkers associated with disease is an important step in understanding pathogenesis and aiding the therapeutic decision-making to control the inflammation process early enough before any chronic arthritis occurs.

2. Objectives

Our aim was to describe the first CHIKV outbreak on the Island of Curaçao, validate the commercially available diagnostic kits to diagnose CHIKV infection, and identify markers associated with chronic infection.

3. Study design

3.1. Validation of serology for diagnostics of the clinical samples

Samples were obtained from the unit of clinical virology at the Department of Viroscience, Erasmus Medical Center. Fifty-two serum pairs were selected from 26 travelers. To study cross-reactivity of the serological assays, we selected sera from the same serum bank of patients with other viral infections; 12 patients diagnosed with an acute cytomegalovirus (CMV) infection, 17 patients with an acute Epstein-Barr virus (EBV) infection, three patients with an acute Ross River virus (RRV) infection and 20 patients with an acute DENV infection.

3.2. Clinical samples from Curaçao

Serum samples were collected during the 2014-2015 outbreak at the Medical Laboratory Services (MLS) headquarter and aliquoted for RNA isolation and serology. A selection of patients from the MLS database were selected for the retrospective study. Only patients for which paired serum samples were available were selected. Two groups were selected: 1) patients with laboratory confirmed CHIKV infection (based on ELISA) and 2) patients with other febrile illness (OFI). Febrile patients with a negative CHIKV ELISA in the acute and convalescent phase samples were diagnosed as OFI.

3.3. Chikungunya virus serology

CHIKV specific antibodies in sera from patients included in the cohort from Curaçao were determined using an IgM/IgG enzyme-linked immunosorbent assay (ELISA) kit (IBL, Germany). In addition, the IgM/IgG ELISA and the indirect immunofluorescence assay (IFA) for IgM and IgG from Euroimmuun (Germany) were used for comparison. All the assays were performed according to the manufacturer's protocol. The sero-status of all samples for the validation study was confirmed in an in-house developed micro-neutralization assay as previously described (226).

3.4. Virus quantification by real time-PCR

The detection and quantification of CHIKV RNA in patient serum was determined by qRT-PCR as previously described (227, 228) Viral RNA load was determined using a standard curve. CHIKV envelope protein 1 (E1) RNA run-off transcripts were used as a standard curve and were prepared as previously described for West Nile virus (229)

3.5. Sequence and phylogenetic analyses of CHIKV genes

PCR reactions targeting a part of the E1, E2, and non-structural protein 2 (nsP2) were performed from samples of six patients with positive qRT-PCR results. Primers used for PCR and sequencing were derived from previous publications (230, 231). The phylogenetic trees were constructed using 522 nucleotides of the E1 gene, 429 nucleotides of the E2 gene and 483 nucleotides of the nsP2 gene. Sequences were aligned using MUSCLE software (v3.7) and the phylogenetic trees were reconstructed using the maximum likelihood implemented in the PhyML software (v3.0). Both softwares were used from the website, http://phylogeny.lirmm.fr/ as previously described (232).

3.6. Levels of C-reactive protein, ferritin, anti-cyclic citrullinated peptide antibody, rheumatoid factor and neopterin in serum

Serum C-reactive Protein (CRP), ferritin, anti-cyclic citrullinated peptide (anti CCP) antibody and rheumatoid factor (RF) concentrations were determined using a multi-channel Roche Cobas 6000 analyzer under standardized conditions. The threshold/normal values for the biomarkers were CRP (1 mg/dl), ferritin (man 30–400 ng/ml; woman 13–150 ng/ml), anti CCP (17 U/ml), and RF (20 IU/ml). A group of 80 patients were selected based on the ferritin serum levels for determination of serum neopterin, anti CCP and RF levels. Neopterin levels were measured using commercially available ELISA kit (IBL, Germany). The assay was performed according to the manufacturer's instruction.

3.7. Patient questionnaire

All the patients that were selected to determine neopterin levels were contacted and a questionnaire containing the following three questions was completed: 1) development of fever,

2) pain in the muscle(s) or joint(s) and 3) appearance of rash at the time when they visited the general practitioner. Subsequently the patients were asked for how long after their visit to the general practitioner the clinical symptoms persisted.

3.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.01 software (Graphpad Software, USA). For comparison between continuous variables, Student's *t*-test or Mann Whitney test was performed depending on the distribution of the data. Differences in the proportion of patients were analysed by Chi-square test or Fischer's exact test depending on the normality of the data. P values ≤ 0.05 were considered to be statistically significant.

4. Results

4.1. Evaluation of diagnostic methods for detection of CHIKV antibodies

First, a virus neutralization test (VNT) was performed on 52 paired sera from 26 patients with suspicion of CHIKV infection. In 14 out of 26 patients (53.8%), neutralizing antibodies were detected in the acute samples of the paired sera. In order to check if we possibly missed early cases of infection by carrying out only neutralization assay, we additionally performed a CHIKV specific RT-PCR on all the acute samples of the paired sera. We detected CHIKV RNA in four patients that did not have neutralizing antibodies and thereby we confirmed the diagnosis of CHIKV on the acute samples.

The commercially available ELISAs and IFA were compared with the results of the VNT. The specificity of all the tests was above 90% for IgM as well as IgG, indicating that the number of false-positive results is low. The sensitivity of all the tests was lower for both IgM and IgG, varying from 84% to 92% (Table 1). Subsequently, we analysed the cross-reactivity against RRV, EBV, CMV, and DENV of the three commercial tests. All three assays showed IgM cross-reactivity with RRV (33%) and different results were obtained with sera from acute EBV infections (11.8–47.1%). In addition, the Euroimmuun ELISA showed a large amount of cross-reactivity with acute CMV infection (41,7%), whereas the other two assays did not show cross-reaction (Table 2). In addition, the reproducibility of the IFA is largely depending on the experience of the technician performing the test (data not shown).

Table 1. Performance of commercial serological methods versus virus neutralization test (VNT)

		Euroimmuun IFA				Euroimmuun ELISA				Novatec ELISA			
		IgG		IgM		IgG		IgM		IgG		IgM	
		pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
VNT	pos	22	3	23	2	21	4	23	2	22	3	23	2
	neg	0	27	1	26	1	26	0	27	2	25	1	26
	Sensitivity (%)	88		92		84		92		88		92	
	Specificity (%)	100		96.3		96.3		100		92.6		96.3	

Data are depicted as number unless otherwise stated. Abbreviations: VNT: virus neutralization test; IFA: indirect immunofluorescence assay; ELISA: enzyme-linked immunosorbent assay; pos: positive; neg: negative.

Table 2. Cross reactivity of commercial serological assays for CHIKV

	VNT	Euroimmuun IFA		Euroimmuun ELISA		Novatec ELISA		Total no of patients (n)
		IgG	IgM	IgG	IgM	IgG	IgM	
Acute CMV	0	0	0	0	41.7	0	0	12
Acute EBV	0	0	11.8	0	47.1	5.9	23.5	17
Acute RRV	0	67	33	0	33	0	33	3
Acute DENV	0	0	0	0	10	15	5	20

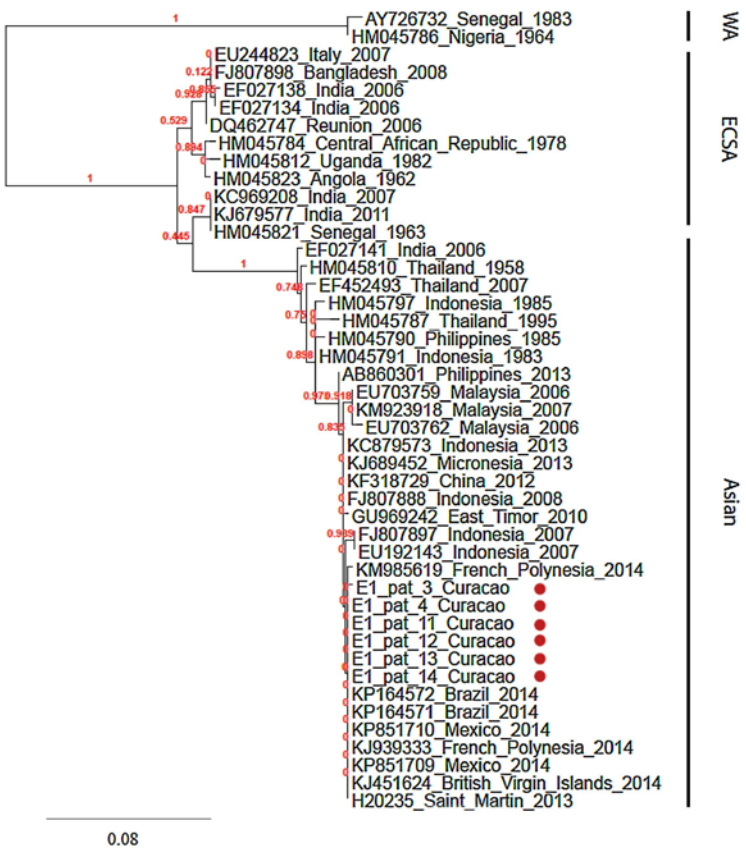
Data are depicted as percentage (%) unless otherwise stated. Abbreviations: VNT: virus neutralization test; IFA: indirect immunofluorescence assay; ELISA: enzyme-linked immunosorbent assay; CMV: cytomegalovirus; EBV: Epstein-Barr virus; RR: Ross River virus; DENV: dengue virus.

4.2. CHIKV diagnostic results of the 2014-2015 outbreak in Curaçao

In total 141 patients were included in the study. 116 patients were diagnosed with CHIKV infection based on the ELISA results. Viral RNA was detected in 77% of the samples and the highest viral RNA load that was found in this study was 10^8 viral copies/ml blood. In contrast, CHIKV RNA was not detected in any of the samples of the OFI group (n=25).

4.3. Asian genotype in the outbreak in Curaçao

The sequences from the Curaçao viruses were obtained from the E1, E2 and nsP2 proteins (Figure 1 A-C). Sequences were deposited in GenBank under accession numbers KU727168-KU727181. Phylogenetic analyses of these partial sequences showed clustering of the Curaçao strains with the Asian Genotype. Closest relation of the E1 Asian genotype isolates was seen with available genomes from the Americas outbreak (from Brazil, Mexico and St Martin). Phylogenetic analyses of the other proteins also showed clustering of the isolates within the Asian genotype.


AY726732_Senegal_1983
HM045786_Nigeria_1964
EU244823_Italy_2007
FJ807898_Bangladesh_2008
EF027138_India_2006
EF027134_India_2006
DQ462747_Reunion_2006
HM045784_Central_African_Republic_1978
HM045812_Uganda_1982
HM045823_Angola_1962
KC969208_India_2007
KJ679577_India_2011
HM045821_Senegal_1963
EF027141_India_2006
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EF452493_Thailand_2007
HM045797_Indonesia_1985
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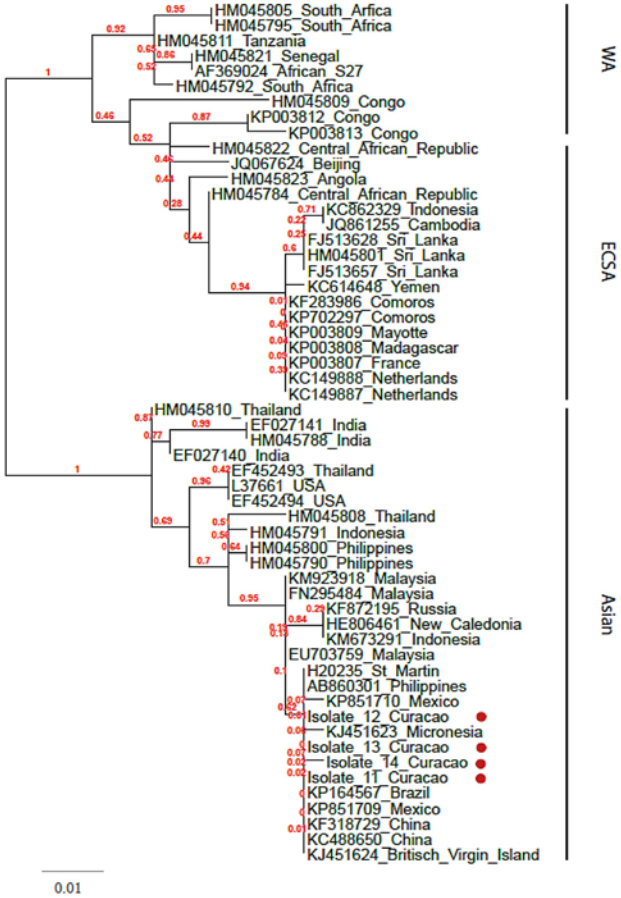
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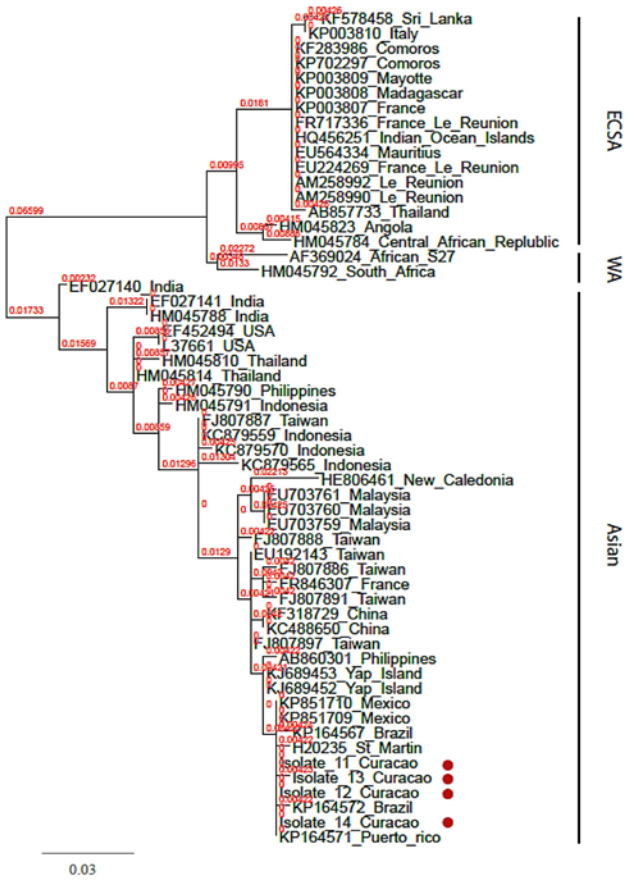
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KP164572_Brazil
Isolate_14_Curacao
KP164571_Puerto_rico
ECSA
WA
Asian
0.03

Fig. 1. Phylogenetic analyses of the CHIKV isolates.
The sequences obtained in this study were marked with red dot. **Panel A** (left) shows phylogenetic analysis of CHIKV isolates based on the E1 gene. **Panel B** (middle) shows phylogenetic analysis of CHIKV isolates based on the E2 gene. **Panel C** (right) shows phylogenetic analysis of CHIKV isolates based on the nsP2 gene. The CHIKV isolates from Curacao cluster with the strains isolated in the Americas and other strains belonging to the Asian genotype. Abbreviations: WA = West Africa; ECSA = East Central South Africa.

4.4. Biomarkers associated with viraemia and chronic arthralgia

For an analysis of biomarkers in relation to viral load, we first divided the included patients in viraemic (qRT-PCR positive) and non-viraemic (qRT-PCR negative) groups. As a negative control, we used serum samples from the OFI patients. We further divided the viraemic group in high viral load (VL) and low VL sub-groups based on arbitrary copy numbers (≥ 10^4 copies were considered high and < 10^4 copies low).

We found a significant increase in ferritin serum level in viraemic patients compared to non-viraemic patients (p=0.0026) and patients with OFI (p=0.0042) (Figure 2A). In addition, the proportion of patients with prolonged chronic arthralgia (≥3 months) was significantly higher in the group with high ferritin levels (p<0.001) compared to normal ferritin levels (table 3). CRP levels were significantly elevated in viraemic group compared to non-viraemic (p<0.001) and OFI group (p<0.001) (Figure 2C). Furthermore, we found significant differences in serum CRP levels when high VL was compared with low VL (p=0.0012) and OFI groups (p<0.001) (Figure 2D).

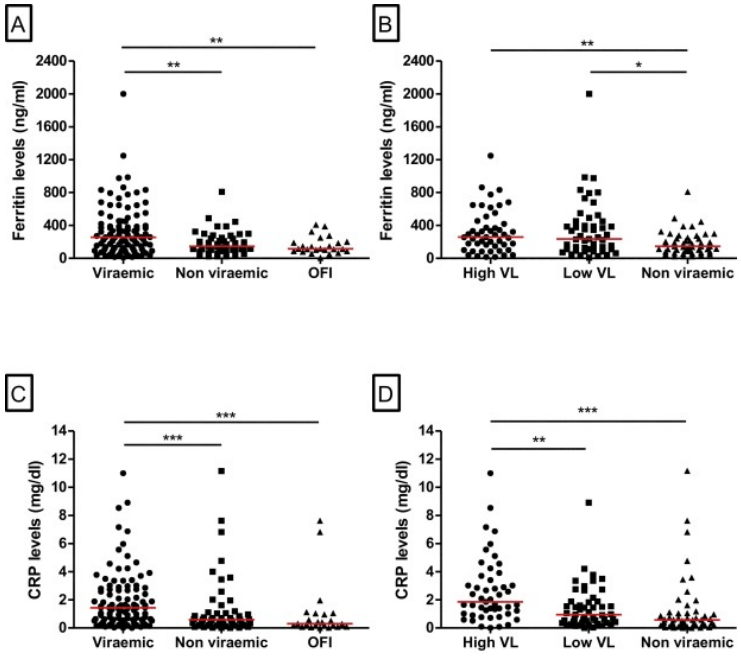

Fig. 2. Ferritin and C-reactive protein (CRP) serum levels in patients infected with CHIKV.
Samples selected in the Curacao 2014–2015 cohort were divided in qPCR positive (viraemic) and qPCR negative (non-viraemic) groups. (A) Ferritin serum levels were significantly elevated

in viraemic patients compared to non-viraemic (p = 0.0026) and OFI group (p = 0.0042) (B) No significant difference in ferritin levels was observed between viraemic samples when divided into high VL and low VL (p = 0.929), indicating that the presence of viraemia rather than viral load determined the serum ferritin levels. (C) Serum CRP levels were significantly increased in viraemic patientscompared to non-viraemic (p < 0.001) and OFI group (p < 0.001). CRP levels of patients classified in high VL group was significantly elevated compared to low VL (p = 0.0012) and non viraemic group (p < 0.001). Horizontal red lines indicate the median of the groups. Abbreviations: OFI: other febrile ilnessess; VL: viral load. *p < 0.05; ** p < 0.01; *** p < 0.001.

Table 3. Comparison of clinical manifestations between high ferritin and normal ferritin levels patients.

	High ferritin levels (n=47)	Normal ferritin levels (n=25)	P
Fever	26 (55.3%)	12 (25%)	0.55
Rash	15 (31.9%)	8 (25%)	0.99
Joint(s)/muscle(s) pain	42 (89.4%)	7 (28%)	<0.001
< 3 months	3 (6.4%)	1 (4%)	0.67
≥ 3 months	39 (83%)	6 (24%)	<0.001

Data are no (%) of patients within the group, unless otherwise indicated. Patients with elevated ferritin levels seem to have more chronic arthralgia (p<0.001) which last more than 3 months (p<0.001) compared to patients with normal ferritin levels.

To determine the neopterin levels, patients with normal or elevated ferritin levels were selected from the cohort. In total 80 CHIKV-infected patients were selected for neopterin ELISA. The group of patients with elevated ferritin levels had significantly higher levels of neopterin (p=0.03) compared to patients with normal ferritin levels (Figure 3).

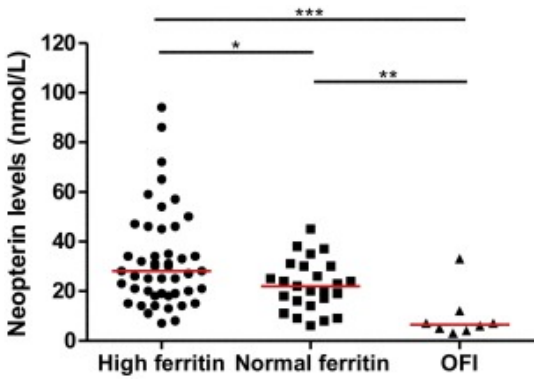

Fig. 3. Correlation of serum neopterin levels with serum ferritin levels.
Serum neopterin levels in patients with elevated ferritin levels were significantly higher compared to the group with normal ferritin levels (p < 0.03). Horizontal red lines indicate the median of the groups. Abbreviations: OFI: other febrile ilnessess. *p < 0.05; ** p < 0.01; *** p < 0.001.

4.5. Biomarkers associated with rheumatoid diseases

To confirm whether the patients that experienced chronic arthralgia/arthritis did not have rheumatoid diseases, we measured anti-CCP and RF serum levels. None of the CHIKV patients showed elevated serum levels for both anti-CCP and RF (data not shown). These results suggest that the chronic disease was caused by CHIKV infection.

5. Discussion

This study reports the first outbreak of CHIKV in Curaçao, which was caused by an Asian genotype. The first CHIKV outbreak in the Caribbean was reported in late 2013 on the Island of St. Martin and shortly thereafter more cases were reported on the other Caribbean Islands (225). The first suspected cases were detected approximately six months later in Curaçao. Afterwards the virus quickly spread throughout Curaçao. The majority of the patients described classic CHIKV clinical symptoms (Table 3) (51).

Our study has several limitations. First, it is a retrospective study. Consequently, owing to the risk of recall bias we could not assess the whole spectrum of clinical symptoms. In addition, the cohort of patients was relatively small, but sufficient enough to justify the conclusions.

The evaluation of the commercial assays was carried out to ensure that the kit that was used in Curaçao is accurate and robust to diagnose CHIKV infection. Our study showed that all the tests evaluated had good sensitivity and specificity. The assays displayed high specificity, which is important in a population with high positivity rates. Sensitivity was lower for the three ELISA kits, which suggest an additional value of either PCR testing in the acute phase or testing follow up (convalescent) sample (233). It is also important to always interpret the results of both IgM and IgG together, which can increase the reliability of the diagnosis. IFA was also tested in this study as several studies have shown that IFA tests have good sensitivity and specificity (234, 235). However, the necessity to have a well-equipped and adapted lab with experienced staff, and high costs limit the use of this assay in daily clinical settings (236). Selection of the commercial kit should be made based on the sensitivity and specificity of the assay and also no/low cross-reactivity with other viruses. This is especially important for IgM, which is an important parameter to diagnose acute infection. Our study showed that all assays showed some degree of IgM cross-reactivity against CMV (Euroimmuun ELISA), EBV (all kits), and DENV (Novatec and Euroimmuun ELISA). Cross-reactivity of the commercially available ELISAs with other alphaviruses was also shown previously (236). These results imply that careful anamneses and physical examinations of patients in addition to other diagnostic tools are important to obtain an accurate diagnosis in selected cases.

Phylogenetic analyses of the CHIKV genes were in line with other reports coming from the Americas. The fact that the CHIKV strain circulating is closely related to the strains currently circulating in the Americas suggests that the Curaçao outbreak was a result of an introduction of the virus from one of the neighboring Islands. Recently a study reported the emergence of ECSA strain in Brazil from a patient that returned from Angola (237). It has been shown that the A226V mutation in the viral E1 protein can increase viral transmission in *A. albopictus,* which was associated with the large CHIKV epidemic in the Indian Ocean (238, 239). Given the presence of *A. albopictus* in many latin American countries and the extensive travelling between the islands in the region, there is a realistic risk for introduction of the Indian Ocean sub-lineage in the future.

We found significantly elevated serum levels of CRP in CHIKV patients with viraemia compared to non-viraemic and OFI groups. C-reactive protein is an acute-phase protein that is synthesized by the liver. Several studies showed that monocytes/macrophages and hepatocytes are the target cell of CHIKV infection (19, 125, 135). It has been shown that CHIKV interactions with monocytes induced a robust and rapid innate immune responses with the production of cytokines and chemokines (135). It is possible that hepatocytes are indirectly activated by pro-inflammatory mediators and/or activated immune cells to synthesize high amounts of CRP. Additionally, direct infection of hepatocytes could also initiate the production of CRP. Our study is in line with previous studies that also found increased CRP levels in patients infected with DENV, another arbovirus, which also infects macrophages and hepatocytes (240, 241).

We also found elevated serum levels of ferritin and neopterin, which are markers of activated marcophages. Studies have shown that synthesis of ferritin can be triggered by cytokines and iron (242, 243). In this study, increased concentrations of ferritin were significantly associated with viraemia. Macrophage and hepatocyte are important producers of the secreted form of ferritin and therefore direct infection and subsequent viral replication in these cells may activate them and increase the production of ferritin. We also found that a significant number of patients with elevated ferritin levels in the acute serum sample developed chronic arthralgia. Hyperferritinaemia has been shown to be associated with other arboviral infections and autoimmune disorders like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (134, 244, 245). We did not find positive rheumatoid markers (anti-CCP and RF) in the patients of our cohort, which suggest that elevated ferritin levels and chronic arthralgia/arthritis were not caused by rheumatoid diseases. In this study, we also found an association between elevated ferritin levels with production of neopterin, which is a specific marker of activated macrophages. Elevated serum levels of both molecules were also shown to be associated with the severity of DENV infection (119, 134). The results presented in the manuscript support the

hypothesis that macrophages might play a role in the development of chronic CHIKV infection. In addition, our results suggest that ferritin could serve as a potential prognostic marker for development of chronic CHIKV infection.

6. Conclusions

This study is the first report of CHIKV emergence in Curaçao. We identified the etiological strain associated with the CHIKV outbreaks in Curaçao, which belongs to the Asian genotype. We found an association between hyperferritinaemia with chronic chikungunya. Further studies are needed to evaluate whether ferritin could serve as a potential prognostic marker for chronic chikungunya.

Ethical approval

Ethical approval of this study was obtained from the Medical Ethics Committee in Curaçao.

Funding

FA was supported by the Directorate of Higher Education (DIKTI) PhD grant of the Ministry of Research, Technology and Higher Education of the Republic of Indonesia. LP was supported by Medical Laboratory Services, Curaçao. The funders had no role in study design, data collection and analysis, preparation of the manuscript, and decision to publish.

Acknowledgements

The authors would like to thank the physicians and the patients in Curaçao for their help and support in this study. We thank Henk-Jan van den Ham for his help with the statistical analyses. We also thank Janienne Klaasse for her excellent technical assistance on the validation study experiments.

Chapter 6

Characterization of antibody response in patients with acute and chronic chikungunya virus disease

Fatih Anfasa, Stephanie M. Lim, Susan Fekken, Robert Wever, Albert D. M. E. Osterhaus, Byron E. E. Martina

J Clin Virol. 2019;117:68-72

ABSTRACT

Background

Chikungunya virus (CHIKV) is a re-emerging arbovirus capable of causing chronic arthralgia, which can last for months to years. Although neutralizing antibodies have been shown to be important for viral clearance, is it not clear whether the quantitative and qualitative nature of antibodies play a role in progression to chronic disease.

Objectives

To characterize and compare the antibody responses in acute and chronic patients in a prospective observational CHIKV study in Curaçao during the 2014-2015 outbreak.

Study design

We performed virus neutralization tests and ELISA on plasma samples collected from a prospective observational chikungunya study in Curaçao to compare the complement-dependent and -independent neutralization capacity, as well as the antibody avidity index of acute and chronic patients.

Results

We found that there was no significant difference in the virus neutralization titers between patients with acute and chronic chikungunya infection. Furthermore, we found that complement increased the neutralization capacity when large amounts of virus was used. Moreover, we found that patients with acute chikungunya disease had a significantly higher antibody avidity index compared to those with chronic disease.

Conclusions

This study suggests that virus neutralization titers in late convalescent sera do not play a role in chronic chikungunya. However, the median antibody avidity was lower in these patients and may therefore suggest a role for antibody avidity in the development of chronic disease.

1. Background

Chikungunya virus (CHIKV) belongs to the genus alphavirus of the *Togaviridae* family. Following acute infection, 20-50% of patients develop chronic symptoms lasting between weeks to years (246, 247). Both innate and adaptive immunity have been proposed to play a role in the development of chronic disease, but the complete mechanisms are still unclear (248). Antibodies to CHIKV have been shown to be important for viral clearance and mediate protection against re-infection (249, 250). Although neutralizing antibodies (NABs) have been shown to be important for CHIKV clearance, it is unclear whether the qualitative and quantitative nature of antibodies, such as avidity and neutralization capacity, play a role in protection/development of chronic disease. For instance, studies showed that maturation of antibody avidity after vaccination/natural infection may be important for protection against infection and/or disease. However, correlation of antibody avidity with protection seemed to be virus-dependent (140, 141). Currently, it is unclear whether antibody avidity contributes to clearance or persistence of CHIKV infection.

In many systems, neutralizing antibodies (NABs) correlates with protection. Two types of neutralization assays have been described: complement-dependent and -independent. Most studies evaluate the levels of complement-independent neutralization. However, several studies revealed that complement inhibits infection of herpesvirus (251), West Nile virus (WNV) (252), and Sindbis virus (SINV) (253-255). While complement activation was associated with protection against SINV (253-255), mice deficient in complement factor 3 developed less severe disease compared to wild-type mice infected with Ross River virus (256). Whether complement plays a role in CHIKV infection still remains to be elucidated.

2. Objectives

To characterize the antibody response in plasma of patients with acute and chronic CHIKV disease.

3. Study design

3.1. Patients

This study was a prospective observational study conducted in Curaçao, which is a continuation from a previous report (257). The selection of patients was based on the availability of clinical symptoms and plasma sample during follow-up. Patients that still experienced chronic symptoms after 3 months (arthralgia and/or myalgia) were defined as “chronic disease” while patients with no symptoms were defined as having had an “acute disease”. IgM/IgG ELISA (IBL,

Germany) and qRT-PCR were also performed on the follow-up samples as previously described (227, 257).

3.2. Cells and viruses

Vero E6 cells were grown and maintained at 37°C with 5% CO_2 as described before.(257) The virus strains used in this study consisted of the African prototype S27 strain passage (P)6 and the CHIKV-IND/NL10 (Asian) strain P5. Both strains were grown on Vero E6 cells and virus titers were determined as the 50% tissue culture infective dose ($TCID_{50}$), calculated using the Kärber method (184, 226).

3.3. Virus neutralization assay

The serostatus of all samples was confirmed using a virus neutralization test (VNT) (226). Briefly, plasma of patients was heat-inactivated (HI) at 56°C for 30 minutes and diluted (1:20-1:10,240) in quadruplicate in 96-wells plates. Subsequently, 100 $TCID_{50}$ of CHIKV-S27 or CHIKV-IND/NL10 was added to each well. To assess the potency of the NABs, the same plasma dilutions were incubated with 10,000 $TCID_{50}$ of CHIKV-S27 or CHIKV/NL10. Neutralization experiments with 100 $TCID_{50}$ of CHIKV were defined as "standard" while experiments with 10,000 $TCID_{50}$ were defined as "potency". After one hour of incubation at 37°C, 2×10^4 Vero E6 cells were added per well and plates were incubated for 5 days at 37°C. The neutralizing titers (VNT_{50}) were determined microscopically and defined as the plasma dilution that reduced CPE by 50% (184).

3.4. IgG antibodies neutralizing capacity

In order to assess the contribution of IgM to the neutralizing capacity, denaturation of IgM was performed using β-mercaptoethanol (258) (Sigma-Aldrich, USA). Briefly, plasma of patients was HI and diluted (1:20 to 1:10,240) in quadruplicate on 96-well plates. Subsequently, the diluted samples were treated for 2 hours at 37°C with β-mercaptoethanol at a final concentration of 250 µM, followed by addition of 100 or 10,000 $TCID_{50}$ of CHIKV-S27 to the designated wells and incubation for 1 hour. Neutralization assays were then performed as described above.

3.5. Avidity assay

In order to assess the avidity of the antibodies directed against CHIKV E1 and E2, avidity ELISA was performed. To this end, 0.1 µg of E1 antigen (MyBioSource, USA) or 50 ng of E2 antigen (Immune Technology, USA) was coated overnight at 4°C on high-binding ELISA plates (Corning, USA). Subsequently, the plates were washed with PBS+0.05% Tween-20 (PBST) and blocked with PBS+2% BSA (Sigma-Aldrich) for 1 hour at 37°C. Plasma (HI) was diluted (1:50 to 1:6400) in six replicates and 1.5 M of NH_4SCN^- (Sigma-Aldrich) or PBS was added (each in triplicate) and incubated for 5 minutes at room temperature. Plates were washed with PBST followed by incubation with rabbit-anti-human antibody (1:6000; Dako, The Netherlands). After one hour of

incubation, TMB (Invitrogen, USA) was added and the reaction was stopped after 5 minutes by addition of 0.5 M of H_2SO_4 (Sigma-Aldrich). Absorbance was measured at 450 nm using an ELISA reader (Tecan, USA). The avidity index (AI) was expressed as the EC_{50} of the OD values obtained using non-linear regression fitting. The AI ratio was calculated as the $EC_{50\ (NH4SCN)}$ / $EC_{50\ (PBS)}$ X 100%.

3.6. Complement-mediated virus neutralization assay

To determine the role of complement-mediated virus neutralization in CHIKV infection, guinea-pig complement was added to the neutralization protocol. To this end, HI-plasma was diluted (1:20 to 1:10,240) in quadruplicate on 96-well plates and 100 or 10,000 $TCID_{50}$ of CHIKV-S27 was added to the wells and incubated for 1 hour at 37°C. Subsequently, 10% guinea-pig complement (MP Biomedicals, USA) was added to the plasma dilutions (1:1 vol/vol) and incubated for 1 hour at 37°C. Neutralization experiments were then performed as described above.

3.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, USA). Mann-Whitney U test was used for comparison between continuous variables. The p-value was adjusted for multiple testing using Bonferroni correction. P-values of ≤0.00625 were considered to be statistically significant.

4. Results

4.1. Cohort description

Fifty-two patients were diagnosed with chronic disease and 38 patients with acute disease. The median follow-up time for the sample collection was 12 months (range 3-14 months) for both groups. All follow-up samples were negative for CHIKV RNA and positive for anti-CHIKV IgGs. Three patients showed measurable levels of anti-CHIKV IgM in the follow-up samples, which were taken 12 months (2 patients) and 6 months (1 patient) post-infection. Two patients belonged to the acute group while one patient belonged to the chronic group.

4.2. CHIKV NABs induced by an Asian genotype cross-neutralize an ECSA genotype

To determine whether NABs in patients infected with an Asian genotype cross-neutralized an East Central South African (ECSA) genotype, we performed VNT with CHIKV strains representing both genotypes. There was no apparent difference in the neutralizing titers against both genotypes (Figure 1A). In addition to performing a standard VNT, we also used 100-fold more virus to test the potency of the NABs, since it has been shown that potent NABs could block infection at concentrations that result in low occupancy of accessible sites on the virion while weak NABs recognize fewer sites on the virion and require almost complete occupancy to

inhibit viral infection (259). Based on this, we presumed that potent NABs could also neutralize when challenged with a higher amount of virus. We found that NABs elicited by an Asian strain cross-neutralized the ECSA strain at similar levels. However, no significant differences in neutralization titers were found between acute and chronic patients for either the standard (Figure 1B) or potency VNT (Figure 1C).

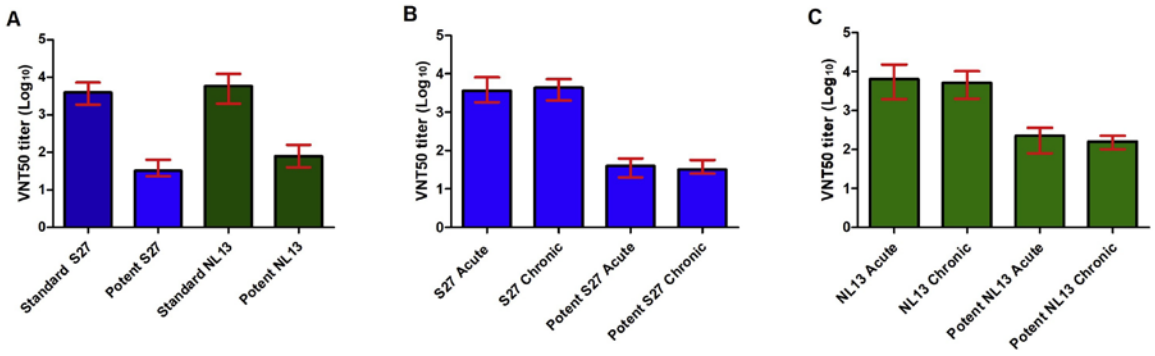

Fig. 1. Neutralization capacity of plasma of patients against ECSA and Asian CHIKV.
(A) Plasma collected from patients during the Asian CHIKV outbreak had similar neutralization titers against CHIKV-S27 (ECSA) and CHIKV/NL10 (Asian) strains in both standard and potency VNTs (virus concentration of 100 and 10,000 $TCID_{50}$, respectively). Neutralization titers of patients with acute and chronic disease were compared for both CHIKV-S27 **(B)** and CHIKV/NL10 strains **(C)**. No significant differences were observed between acute and chronic patients for both virus strains. Data are presented as median with inter-quartile range.

4.3. No differences in IgG neutralization titers between patients with acute and chronic disease

It is known that natural IgM antibodies as well as virus-specific IgM, which can be detected for months post-infection, can contribute to neutralization activity (138, 246, 260). To measure the IgG neutralization activity only, IgM denaturation was performed using β-mercaptoethanol. We first determined the optimal concentration of β-mercaptoethanol, defined as the concentration that was not toxic to cells, did not affect virus infectivity and was able to abolish neutralization activity of IgM antibodies. Plasma samples of two patients in the acute phase (5 days after the onset of symptoms with positive IgM, but no detectable IgG antibodies) were used for validation. The neutralization activity was lost at a concentration of ≥250 μM (data not shown). When the samples were tested in the presence of β-mercaptoethanol, only minor differences in the neutralizing titers were found between the standard and potency groups (Figure 2A). Furthermore, there was no significant difference between acute and chronic groups (Figure 2B).

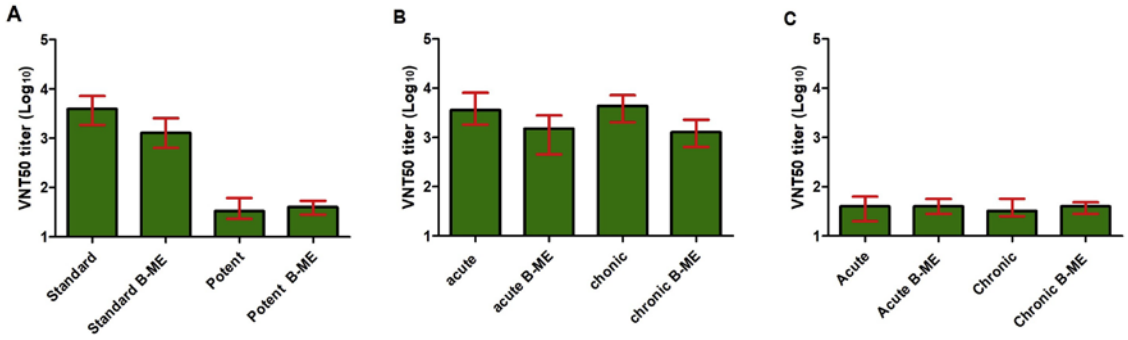

Fig. 2. IgG neutralization titers in patients with acute and chronic CHIKV infections.
(A) IgG neutralization titers of plasma samples were compared in the presence and absence of denaturation agent (B-ME). No significant differences were observed for both standard (100 $TCID_{50}$) and potency (10,000 $TCID_{50}$) virus challenge groups. Neutralization titers of patients with acute and chronic disease were compared in both the standard **(B)** and potency **(C)** groups. No major differences were observed for both groups. Data are presented as median with inter-quartile range. B-ME: β-mercaptoethanol.

4.4. Complement increased the neutralization capacity when high amounts of virus was used

Normally, neutralization is measured in the absence of complement. To investigate the involvement of complement in the neutralization of CHIKV, we incorporated complement in the assay. First, we determined whether complement itself neutralize CHIKV. We found that the complement concentration that we used did not neutralize CHIKV (data not shown). Next, we found that complement did not increase neutralization when 100 $TCID_{50}$ of virus was used. In contrast, the addition of complement significantly increased the neutralization capacity ($p<0.0001$) in the potency group (Figure 3A). This synergistic effect of complement on neutralization was measured in both acute ($p<0.0001$) and chronic ($p<0.0001$) patients (Figure 3C). These results imply that complement-mediated neutralization of CHIKV may play a role in the presence of a high virus load. Nonetheless, no major differences were seen between acute and chronic patients in either the standard or potency complement-mediated neutralization assay using a high viral load.

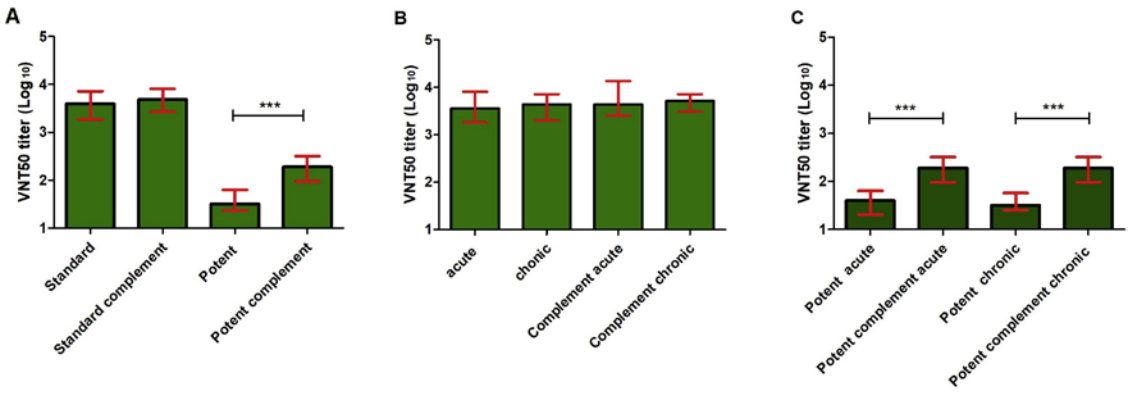

Fig. 3. Complement-mediated enhancement of CHIKV neutralization.

(A) Neutralization titers were compared between plasma samples in the presence and absence of complement. Significant differences were observed for the potency (10,000 $TCID_{50}$ virus) group. Neutralizing titers of patients with acute and chronic disease were compared in both the standard **(B)** and potency **(C)** groups. Increased neutralization was observed for samples that were incubated with 10,000 $TCID_{50}$ of virus (potency) group in comparison to the standard group (100 $TCID_{50}$). Data are presented as median with inter-quartile range. The Mann-Whitney U test was used for group comparisons. *** $p < 0.001$.

4.5. Patients with acute disease had higher antibody avidity compared to chronic disease

To determine whether avidity of the antibodies differed between acute and chronic patients, we compared the antibody AI between these groups. We found a significant difference in the AI of both the anti-E1 ($p<0.0001$) and anti-E2 ($p=0.0003$) antibody response between these groups, which indicates that acute patients had higher antibody avidity against E1 and E2 glycoproteins compared to chronic patients (Figure 4). Despite this difference, the median AI of anti-E2 antibodies was still relatively high in the chronic group (EC_{50} = 81.6%), compared to anti-E1 (EC_{50} = 52.8%).

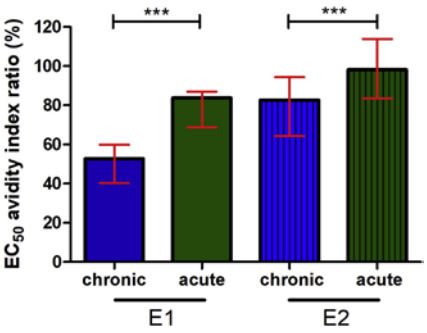

Fig. 4. E1 and E2 protein antibody avidity index in CHIKV patients with acute and chronic infections.
Antibody avidity index ratios for both E1 and E2 protein were significantly higher in patients with acute disease compared to chronic disease (E1: p=<0.0001; E2: p = 0.0003). Data are presented as median with inter-quartile range. The Mann-Whitney U test was used to compare the groups. *** $p < 0.0001$.

5. Discussion

This manuscript describes several properties of the antibody response in a cohort of CHIKV patients. Firstly, our data revealed that NABs produced against one genotype of CHIKV are cross-reactive against another genotype. This result is in agreement with the other studies showing complete cross-neutralization (261, 262). The high percentage of amino acid similarity among all CHIKV strains (29) together with minimal differences in vaccine-NAB responses observed among genotypes (263) indicate that CHIKV NABs to one genotype are highly effective against the other genotypes.

Next, we characterized the neutralizing IgG antibodies. Our results showed that NABs were detected in all the patients, but no apparent differences in VNT_{50} between acute and chronic patients were detected. Consistently, previous study showed that the neutralizing titers of IgG3, the dominant IgG subclass found in the study, were equal for both groups of patients at 2-3 months post-infection (136). This study also found that early induction of anti-CHIKV IgG3 antibodies was associated with protection against chronic arthralgia. As the aforementioned study only looked at shorter time post-infection, we decided to use a longer follow-up time in our study since it has been shown that antibody kinetics can differ over time (141). Lastly, our data is coherent with the data of Kam et al., (136) suggesting that acute and chronic patients developed similar levels of VN titers. As such, quantitative aspects of antibodies may therefore not play a role in the development of chronic disease. If antibodies measured in late convalescent sera are not associated with chronic disease, it is plausible that differences in antibody kinetics early in infection may be important instead.

Serum and cellular factors that intermingle with antibodies have the potential to modulate neutralization activity (264). Our data revealed that complement increased neutralization significantly in the presence of high virus concentration. This effect was equal between the acute and chronic patients, indicating that the neutralization capacity is similar between both groups. Complement is one of the factors that has been shown to play a role in neutralizing several viruses (251, 252, 254). Although there are several possibilities to explain the results in our study, it is likely that in face of high viral titers fewer antibodies per virus are available, making complement activation necessary to reduce viral dissemination and consequently levels of viremia.

Another parameter that could influence the protective capacity of antibodies is avidity. Several studies showed that avidity correlates with protection against infection (140, 265). We found a higher AI against both E1 and E2 glycoproteins in patients with acute disease, which indicates a good immune response maturation. Antibodies produced in the early phase of infection usually show a low avidity for the antigen, which then increases continuously over time following contact with an immunogen. This hypothesis is supported by studies on several viruses, such as measles virus (MV) and CMV (141, 266). Our results are different from another CHIKV study, which reported that antibody avidity was comparable in both arthralgia-positive and -negative patients at 36 months post-infection (247). As the quality and quantity of antibodies can differ over time (141), this discrepancy might be explained by different time intervals used between the studies. Moreover, differences in the protocols may also account for this variance as we used NH_4SCN^- as a chaotropic agent, and several antibody dilutions, while Schilte et al. (247) used urea, and only one antibody dilution.

Interestingly, the AI_{50} values for the antibodies against E1 for the acute group were higher (>80%) compared to the chronic group (<60%). Nevertheless, the AI for the antibody response against the E2 protein in the chronic group was still relatively high (>80%). Therefore, it is unclear whether certain thresholds are needed for protection against chronic disease, or whether a highly avid response against certain epitopes/proteins is more important than others. Although higher levels of avidity were correlated with protection (140, 267), the criteria for defining protective levels of AI is not consistent and could also be virus-specific (141, 268). In our study found similar VNT_{50} was measured in the potency test between both groups, indicating that the differences in avidity were not translated into differences in neutralization potency.

Several reports showed that formalin-inactivated measles vaccine (FIMV) produced low avidity antibodies that bind, but do not neutralize MV, leading to formation of complexes of MV antigen, antibody, and complement in the tissue of macaques (269, 270). In contrast, higher-avidity antibodies were more protective by mediating complement-dependent opsonophagocytosis of both 6B and 23F pneumococci compared to lower-avidity antibodies (271). Whether interaction of antibody avidity and complement contributes to CHIKV neutralization warrants further studies.

Our study has several limitations. We could not assess the whole spectrum of the clinical symptoms, and there was a variation in the follow-up times. Moreover, we did not have sequential samples that would allow measurement of antibody kinetics over time. Although several studies showed that guinea pig complement is comparable to human complement (272, 273), we cannot rule out that the results that we observed are species specific.

6. Conclusions

Our results indicate that VNT_{50} was not different between patients with acute and chronic chikungunya. However, complement increased the neutralization capacity against CHIKV when high amount of virus was used. Finally, the average AI against E1 and E2 glycoproteins was higher in the acute compared to chronic group.

Ethical approval

Ethical clearance for this study was obtained from the Medical Ethics Committee of Curaçao (ref. no. 2014-003). Written informed consent was obtained from enrolled patients.

Acknowledgements

The authors would like to thank the physicians, the lab technicians and the patients in Curaçao for their help and support in this study.

Funding

FA was supported by the Directorate of Higher Education (DIKTI) PhD grant of the Ministry of Research, Technology and Higher Education of the Republic of Indonesia. The funders had no role in study design, data collection and analysis, preparation of the manuscript, and decision to publish.

Chapter 7

Summarizing discussion

Markers associated with disease severity in ZIKV, DENV, and CHIKV infections

For decades, diseases caused by arboviruses were considered to have only limited contribution to global morbidity and mortality. Consequently, low priority was given to arbovirus research and control measures. However, over the past five decades, ZIKV, DENV, and CHIKV have (re-)emerged as important epidemic arboviruses (1). To date, there is no antiviral agent available for physicians to use against Zika, dengue, and chikungunya. Moreover, currently, there is only one licensed dengue vaccine on the market while there is no vaccine available for the other viruses. It is believed that the lack of complete understanding of the disease pathogenesis for all three viruses is a major contributor to the lack of therapeutic agents and vaccines to combat these diseases. Severe disease manifestations and complications are multi-factorial processe in which complex interactions between the host, viral, and environmental factors determine the outcome. Besides an in-depth knowledge of disease pathogenesis for most arboviruses, biomarker(s) for these viruses are also lacking. The usefulness of biomarkers to infectious diseases lies in their ability to provide early detection, establish specific diagnosis, determine accurate prognosis, guide treatment, and monitor disease progression (274). Moreover, biomarkers of severe disease allow targeted studies to identify key mediators of pathogenesis.

The studies in this thesis were performed to study viral and host factors that are associated with disease severity in ZIKV, DENV, and CHIKV infections. Additionally, we aimed to find an association between certain biomarkers and severe disease manifestations and complications.

Zika

ZIKV has been shown to have high affinity for neuronal cells, particularly targeting neural precursors (79). Human neural progenitor cells (hNPCs) are an important target and they are believed to play an important role in the pathogenesis of microcephaly. In **chapter 2** we show that Asian ZIKV strains infect and induce cell death in HNPCs less efficiently compared to African ZIKV strains. Moreover, we found that Asian ZIKV strains infect only minority of cells with a relatively low burst size. Interestingly, a low burst size has previously been shown to be associated with extended virus replication within the CNS for Japanese encephalitis virus (JEV), another flavivirus (168). Hence, the phenotypic characteristics of Asian ZIKV strains may contribute to their ability to cause chronic infections, frequently observed in congenital microcephaly cases. Our study suggests that phenotypic differences between ZIKV strains may, at least partially, contribute to the ability of Asian ZIKV strains to cause chronic infections within the CNS. Additionally, human fetal astrocytes (HFA) can be infected persistently with Asian ZIKV resulting in continuous viral shedding for at least one month (275). A study using Stat2$^{-/-}$ mice also revealed that infection with African ZIKV strains led to short episodes of

severe neurological symptoms followed by mortality, while Asian ZIKV strains showed prolonged signs of neuronal dysfunctions (83). Collectively, our data and those of others suggest that HNPCs and HFAs could be a viral reservoir in the fetal brain leading to chronic infection. To date, no data are available on the ability of the African-lineage strains to cause prolonged infection in humans. Additionally, although a study found that ZIKV has been circulating in West Africa for the past two decades, no information of congenital disorders or severe complications have been reported so far (276). Therefore, epidemiological studies to confirm whether infection with African lineage ZIKV strains is associated with severe disease manifestations and complications in humans, are important to be performed.

Recent studies demonstrated that ZIKV also infects ECs in vitro (66, 88, 89). Interestingly, a study showed that human umbilical vein endothelial cells (HUVECs) were more susceptible to ZIKV infection compared to human ECs derived from other vasculatures (88). In **chapter 3** we show that ZIKV strains infected and replicated to high titers in HUVECs. We also found that infection caused elevated TF expression, which led to shortened thrombin-generation time (TGT). Our study provides evidence that ZIKV infection induces activation of the secondary hemostasis pathway. Interestingly, several case reports revealed that ZIKV-infected patients also developed bleeding disorders and thrombocytopenia (8, 39, 41-43). One possible explanation of these discrepancies relates to EC heterogeneity. EC heterogeneity has been shown between different vascular beds, both on molecular and functional level (189). Of interest, a recent study using different primary human ECs revealed that ZIKV NS1 protein induces endothelial barrier dysfunction in a vasculature-specific manner (90). This study showed that ZIKV NS1 induces hyper-permeability only in brain and umbilical vein ECs. These results, together with our data, suggest that it would be interesting to determine whether ECs derived from different vasculatures give specific hemostasis response upon ZIKV infection. Moreover, it would be important to determine the effect of infection to the whole spectrum of the hemostasis pathway. For human studies, accurate diagnosis of ZIKV infection is important since the clinical presentation of ZIKV infection is greatly similar to dengue, caused by a virus that commonly causes bleeding manifestations in humans. Furthermore, ZIKV cross-reacts serologically with other flaviviruses (277). Due to the similar clinical manifestations and diagnostic difficulties, problems with misdiagnosis and underdiagnosis of ZIKV infection in DENV endemic areas are notorious. Finally, it should be emphasized that data on coagulation disorders in ZIKV-infected patients and the role of hemostatic system in the pathogenesis of ZIKV infection require further investigations.

Dengue

An unresolved question in dengue is what drives aberrant production of cytokines and plasma leakage in the absence of or at low levels of viremia during the critical phase (CP). Other viral infections, such as ebola and influenza virus infections, indicated that viremia levels positively correlates with excessive cytokines and/or chemokines production and disease severity (278, 279). Two possible explanations have been proposed to explain this phenomenon. The first hypothesis is that there is local replication of DENV in several organs that is not reflected in the blood (117). The second hypothesis is that a mediator, such as LPS, contributes to excessive immune activation and plasma leakage that are observed in severe dengue (119). The latter hypothesis was supported by two cross-sectional studies of our group, which demonstrated that microbial translocation (MT) was associated with disease severity and excesive immune activation in patients with DENV infection (118, 119). Since dengue presents with three disease phases, it is important to determine the association of MT with the disease stages. Therefore, it is essential to use a longitudinal cohort which include all three disease phases. In **chapter 4,** we performed a prospective cohort study and demonstrated that LPS and LBP (MT markers) were mainly elevated in the CP of the disease. Moreover, we found an association between translocation, immune activation and plasma leakage. Plasma leakage in dengue patients is mainly detected in two compartments, thorax and abdominal cavities, which indicates a localized response to infection. It has been hypothesized that EC heterogeneity may contribute to the specific altered permeability locations in dengue since evidence of *in vivo* local replication in the aforementioned compartments is limited. This hypothesis is supported by a report by Peyrefitte, *et al*. which showed that DENV infection of two primary human ECs resulted in both common and specific activation pathways (190). In addition, a recent study using several primary human ECs indicate that DENV NS1 protein induced endothelial barrier dysfunction in a tissue-specific manner (90). Interestingly, a report also revealed diverse inflammatory responses between different types of ECs against LPS (214). Thus, it would be interesting to elucidate whether there is a specific activation pathway for ECs in the thorax and abdominal cavities to both DENV and LPS. Taken together, our study provides further evidence that MT plays an important role in the development of plasma leakage and immune activation in the CP of DENV infection. Our data suggest that therapeutic agents targeting MT may be an intervention strategy against severe dengue infection. Nevertheless, further studies are needed to confirm the role of MT in dengue pathogenesis. One limitation of our study is that there are limitations to the assays used to determine translocation. Although we used LPS and LBP assays as convergent evidence, the LAL assay could have been influenced by several factors that limits its ability to detect a different form of LPS (224). Furthermore, the assay that we used is only

representative for gram negative bacteria translocation. Therefore, it is important to further characterize the gut microbial flora that translocates to the circulation during DENV infection. In addition, elimination experiments to confirm the contribution of MT to plasma leakage and excessive immune activation are also warranted.

Our study also identified a new biomarker, endocan, which was significantly elevated in plasma leakage patients in the CP. Endocan is a soluble proteoglycan that is produced mainly by the vascular ECs of lung, kidney, and tumor endothelium (222). A previous report suggested that endocan is a marker of endothelial dysfunction and multi-organ failure in patients with sepsis (221). Moreover, it has been shown that endocan elicited the vascular inflammatory response and disrupted the vascular barrier integrity in ECs and a mice model. Interestingly, endocan production was induced by LPS stimulation in both models (223). These data suggest that endocan may contribute to the development of plasma leakage in DENV infection. More research is needed to determine its specific role in dengue pathogenesis. We also found higher levels of angiopoietin-2 in the plasma leakage group compared to the plasma leakage negative throughout the study duration. Our results are in line with other reports which showed higher levels of angiopoietin-2 in severe dengue and plasma leakage patients (211, 280). Angiopoietin-2 is produced by ECs and rapidly secreted in the circulation upon activation which makes this protein a specific marker for EC activation (281). Our study provides additional evidence that angiopoietin-2 could be used as a predictive marker of plasma leakage as has been shown before (211). Further investigations are needed to confirm whether these biomarkers could be use for different patient populations. Furthermore, the role of these markers in dengue pathogenesis warrant further studies.

Chikungunya

To date, there is no reliable biomarker that physicians could use to determine whether a Chikungunya patient will develop chronic disease or not. Previously, our group has shown that ferritin could be used as a marker to predict severe disease in dengue-infected patients (134). Ferritin is a protein that plays an important role in iron regulation and a significant amount is located and produced by monocytes and macrophages (133). Both CHIKV and DENV have tropism to monocytes and macrophages and these cells have been shown to play an important role in the pathogenesis of both viral infections (103, 135). Therefore, we hypothesized that ferritin could serve as a marker for chronic chikungunya disease. In **chapter 5**, we show that high ferritin levels were significantly associated with chronic disease. Our study provides evidence that ferritin could be used as a marker for chronic disease in chikungunya-infected patients. Macrophages are an important source of ferritin and infection and subsequent viral

replication in these cells may activate them and induce expression and secretion of ferritin into the circulation. This hypothesis was supported by increased neopterin levels, a specific marker for macrophage activation, which we also observed in our study. Nevertheless, we cannot rule out the contribution of damaged cells as the source of the ferritin in the circulation (282). Interestingly, in addition to sequester extracellular and cytosolic iron and store this iron within its core, ferritin also plays a role to prevent formation of harmful reactive oxygen species (ROS) (283). A recent study demonstrated that increased intracellular ROS was found in chikungunya patients with chronic polyarthralgia (284). Both ferritin and ROS have been shown as major factors that contribute to the initiation of ferroptosis, a recently identified cell death mechanism (285). Thus, it will be interesting to determine whether ferroptosis contributes to the pathogenesis of chronic chikungunya. Since ferritin is part of the iron regulation system, the possible role of other iron regulation proteins in chikungunya patients warrants further studies. Furthermore, ferritin is composed of 24 ferritin light (FTL) and ferritin heavy (FTH) subunits that vary in different cells. Therefore, determining the source of ferritin could provide more insight on the pathological processes that occur during CHIKV infection. Taken together, further studies in different cohorts and populations are warranted to determine the contribution of ferritin in chikungunya pathogenesis and to elucidate whether ferritin could serve as a prognostic marker for chronic chikungunya.

CHIKV infection elicits adaptive immunity against infection. Several studies have detected anti-CHIKV antibodies in the sera of infected patients (54, 136-138). Moreover, several reports have demonstrated that these antibodies can neutralize CHIKV and control its dissemination into host cells (138, 139). Even though neutralizing antibodies (NABs) have been shown to be important for viral clearance, it is unknown whether the qualitative and quantitative nature of antibodies, such as neutralization capacity and avidity, play a role in the development or/and protection of chronic chikungunya disease. In **chapter 6** we characterized and compared the antibody responses in acute and chronic patients in a cohort study in Curaçao. We observed that there was no significant difference in the virus neutralization titers between patients with acute and chronic chikungunya disease. Nevertheless, we found that patients with acute disease had a significantly higher antibody avidity index compared to patients with chronic disease. Several studies demonstrated that maturation of antibody avidity after natural infection or vaccination may be important for protection against viral infections. This correlation of antibody avidity with protection against infection seemed to be virus-dependent as has been shown for other viruses like respiratory syncytial virus, measles virus, and mumpsvirus (140, 141). It is unclear whether certain avidity thresholds are needed for protection against chronic chikungunya disease. Nevertheless, it is important to determine, which epitope(s) lead to high avidity

antibody production since it has been shown that different individual epitopes can give rise to antibodies of different avidity within the same individual (286). In addition, it would be interesting to determine whether antibody glycosylation plays a role in protection. Antibody glycosylation dertermines the functional potential of the antibody by delineating the structure of the antibody Fc region and defining which Fc receptors it can bind to in order to recruit effector immune cells. Because glycosylation changes the affinity of antibodies for FC receptors, it has been suggested that glycosylation plays an important role for the immune system to direct a broad range of biological functions (287). Interactions with different combinations of Fc receptors lead to distinct innate and adaptive immune cell responses, including, antibody-dependent cellular cytotoxicity (ADCC), complement-dependent toxicity, and anti-inflammatory activity (288). These effector functions have been suggested to play a role in protection against HIV and Marburg virus(289, 290). Modification of the glycosylation sites of a virus epitope may also influence the antibody response. A previous report showed that changing glycosylation sites in Sindbis virus, another alphavirus, affects antibody reactivity (291). Thus, further studies are needed to elucidate whether and to what extent antibodies are important for protection against the development of chronic chikungunya disease.

Conclusion and future perspectives

Severe disease manifestations and complications due to ZIKV, DENV, and CHIKV infections are the results of a complex interaction between viral and host factors. The work in this thesis describes viral and host factors that are associated with disease severity for these virus infections.

The phenotypic differences between ZIKV Asian and African strains which we observed in our study could be one of the possible explanations of the severe neurological complications detected in humans in the recent epidemics. Thus, our study indicates that it is important to use relevant viral strains to study the pathogenesis of ZIKV infection and develop relevant intervention strategies. Moreover, it is still important to investigate whether the African strains are able to cause the same disease manifestations in humans that are observed in the current epidemics. Good epidemiological and clinical studies in countries with circulating African strains are still warranted. We also showed that ZIKV infection induces apoptosis and increase TF expression, a marker of secondary hemostasis on HUVECs. These phenomena could lead to the damage of placental blood vessel which may reduce the nutrition and oxygen supplies that are needed for the fetus development as was shown in a mouse model (91). Interestingly, a recent publication showed that ZIKV NS1 triggers vascular endothelial dysfunction only in brain

ECs and HUVECs (90). Therefore, whether EC heterogeneity contributes to different or specific hemostasis pathway that lead to certain disease phenotypes requires further studies.

We provide further evidence that significantly increased MT markers are mainly found in the CP of dengue and is associated with severe disease. Hence, therapeutic modalities targeting MT pathway may be an intervention strategy against severe dengue. This approach is promising since patients in endemic countries usually seek medical care when the viraemia is low or no longer detected in the blood. Thus, focusing on therapies that target host factors would be an interesting approach to be added to are limited therapeutic intervention arsenal. In addition, further studies on the microbial compositions that are associated with disease severity as were shown for HIV and HCV infections are needed (292, 293). In addition to MT markers, we also found an interesting marker which is significantly elevated mainly in the CP of PL patients which may be a surrogate marker of plasma leakage in daily clinical practice. Endocan previously was shown as a marker of endothelial dysfunction and multi-organ failure in septic patients (221). Furthermore, stimulation of endocan production with LPS elicited the vascular inflammatory response and disrupted the vascular barrier integrity in ECs and in mouse model (223). Thus, it is interesting to further study the role of this marker in DENV infection.

We provide evidence that elevated ferritin levels are significantly associated with chronic chikungunya disease. Ferritin is an important component of iron metabolism and is composed of different light and heavy subunits that vary in different cells. Therefore, it is important to investigate the iron hemostasis pathway and the source of ferritin in chikungunya infection. Interestingly, increased ferritin levels were detected in the synovial fluid of rheumatoid arthritis patients, a complication that is observed in CHIKV infection (294). Ferritin contributes to oxidative stress process that lead to tissue damage (295). Thus, the role of ferritin in chronic arthralgia of chikungunya infection requires further investigations.

Neutralizing antibody titer solely seems insufficient to protect against the development of chronic chikungunya disease although patients with acute disease had a significantly higher antibody avidity index compared to patients with chronic disease. It was shown that the timely induction of high titers of a neutralizing IgG isotype seems is important to prevent complications of CHIKV infection (136). Thus, understanding the antibody profile kinetics may be crucial to better understand the association of neutralizing antibodies and chronic chikungunya disease which may be important for vaccine design and other related intervention strategies. Therefore, prospective longitudinal studies that allow timely collection of samples are needed.

Overall, we identified several potential biomarkers that are associated with severe disease manifestations of the three arbovirus infections addressed in this thesis. Our results will need to

be replicated and validated in a larger and and more divers study population. Moreover, a better understanding of the role of biomarkers in disease pathogenesis is warranted, and should be subject of further studies.

Chapter 8

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Chapter 9

English Summary/

Nederlandse Sammenvating/

Ringkasan Bahasa

English

Arboviruses are a group of viruses that are transmitted through the bites of mosquitoes and/or ticks. During the past decades, ZIKV, DENV, and CHIKV have (re-)emerged as arboviruses of increasing medical importance. Severe clinical manifestations and complications due to ZIKV, DENV, and CHIKV infections are the results of a complex interaction between viral and host factors. There are limited markers of disease severity available for these virus infections. This thesis describes studies of viral and host markers that are associated with disease severity for ZIKV, DENV, and CHIKV infections.

We observed phenotypic differences between ZIKV Asian and African strains, which could be one of the possible reasons of the severe neurological complications detected in humans in the recent epidemics. Our study illustrates the importance of using appropriate viral strains to study the pathogenesis of ZIKV infection and develop novel intervention strategies. We also demonstrated that ZIKV infection induces apoptosis and increase TF expression on endothelial cell (EC). This might be one of the reasons of vasculopathy observed in several animal models and humans.

We provide evidence that microbial translocation (MT), immune mediators, and EC activation markers were mainly elevated in the critical phase (CP) and are associated with vascular leakage in a cohort of dengue patients. We also identified associations between serum MT markers and inflammatory and EC markers. This suggests that translocation likely contribute to immune activation in the CP of dengue. Thus, therapeutic modalities targeting MT pathway may be an intervention strategy against severe dengue. In addition, we also identified a protein, endocan, which has the potential to be used as a surrogate marker of vascular leakage in clinical practice.

We found that elevated ferritin levels are significantly associated with viremia and chronic chikungunya disease. It is known that macrophages are one of the most important sources of ferritin during inflammatory disease. Moreover, macrophages are one of the primary target cells for CHIKV. Our study suggests that ferritin may serve as a potential prognostic marker for development of chronic chikungunya disease. We also found that neutralizing antibody titer solely seems insufficient to protect against the development of chronic chikungunya. Nevertheless, we observed that patients with acute disease had a significantly higher antibody avidity index compared to patients with chronic disease. Thus, better understanding of the antibody profile may be important to better comprehend the association of neutralizing antibodies and chronic chikungunya disease, which may be important for vaccine design and other related therapeutic strategies.

Collectively, we found several potential markers that are associated with severe disease manifestations of the three arboviruses addressed in this thesis. A better understanding of the role of these biomarkers in disease pathogenesis is warranted to instigate the development of novel intervention strategies against novel arboviruses.

Nederlands

Arbovirussen zijn een groep virussen die worden overgedragen via de beten van muggen en/of teken. In de afgelopen decennia zijn ZIKV, DENV en CHIKV (opnieuw) opgedoken als arbovirussen van toenemend medisch belang. Ernstige klinische verschijnselen en complicaties ten gevolge van ZIKV-, DENV- en CHIKV-infecties zijn het resultaat van een complexe interactie tussen virale- en gastheerfactoren. Er zijn een beperkt aantal biomarkers beschikbaar die een indicatie geven over de ernst van de ziekte voor deze virusinfecties. Dit proefschrift beschrijft studies naar virale en gastheer biomarkers, die geassocieerd zijn met de ernst van de ziekte voor ZIKV-, DENV- en CHIKV-infecties.

We hebben fenotypische verschillen waargenomen tussen de Aziatische en Afrikaanse stammen van ZIKV, wat een van de mogelijke redenen zou kunnen zijn voor de ernstige neurologische complicaties die tijdens de recente epidemieën bij de mens zijn ontdekt. Onze studie illustreert het belang van het gebruik van geschikte virale stammen om de pathogenese van ZIKV-infectie te bestuderen en nieuwe interventiestrategieën te ontwikkelen. We hebben ook aangetoond dat een ZIKV-infectie celdood induceert en verhoogde TF expressie op endotheliale cellen (EC). Dit zou één van de redenen kunnen zijn, waarom vasculopathie in verschillende diermodellen en bij de mens wordt waargenomen.

We laten zien dat microbiële translocatie (MT), immuunmediatoren en EC activeringsmarkers vooral in de kritische fase (CP) verhoogd zijn en geassocieerd zijn met vasculaire lekkage in een cohort van dengue-patiënten. We hebben ook associaties tussen serum MT-markers en inflammatoire en EC-markers geidentificeerd. Dit suggereert dat translocatie waarschijnlijk bijdraagt tot immuunactivatie in de CP van dengue. Therapeutische modaliteiten gericht op de MT mechanismen kunnen dus een interventiestrategie zijn tegen ernstige vormen van dengue. Daarnaast hebben we ook een eiwit, endocan, geïdentificeerd dat de potentie heeft om gebruikt te worden als een surrogaat marker van vasculaire lekkage in de klinische praktijk.

We hebben gevonden dat verhoogde ferritine niveaus significant geassocieerd zijn met viremie en chronische chikungunya ziekte. Het is bekend dat macrofagen een van de belangrijkste bronnen van ferritine zijn tijdens de ontstekingsziekte. Bovendien zijn macrofagen een van de primaire doelwitcellen voor CHIKV. Onze studie suggereert dat ferritine kan dienen als een potentiële prognostische marker voor de ontwikkeling van de chronische ziekte chikungunya. We vonden ook dat alleen een neutraliserende antilichaamtiter onvoldoende lijkt te beschermen tegen de ontwikkeling van chronische chikungunya. Desalniettemin hebben we vastgesteld dat patiënten met een acute ziekte een significant hogere antilichaam aviditeitsindex hadden in vergelijking met patiënten met een chronische ziekte. Daarom kan een

beter begrip van het antilichaamprofiel belangrijk zijn om de associatie van neutraliserende antilichamen en de chronische chikungunya ziekte beter te begrijpen, wat vervolgens belangrijk kan zijn voor het ontwerpen van vaccins en andere gerelateerde therapeutische strategieën.
Gezamenlijk vonden we verschillende potentiële biomarkers die geassocieerd zijn met ernstige ziekteverschijnselen van de drie arbovirussen die in dit proefschrift aan bod komen. Een beter begrip van de rol van deze biomarkers in de pathogenese van de ziekte is gerechtvaardigd om de ontwikkeling van nieuwe interventiestrategieën tegen nieuwe arbovirussen op gang te brengen.

Indonesia

Arbovirus adalah sekelompok virus yang ditularkan melalui gigitan nyamuk atau kutu. Selama beberapa dekade terakhir, ZIKV, DENV, dan CHIKV telah muncul kembali sebagai arbovirus yang penting secara medis. Manifestasi klinis yang berat dan komplikasi akibat infeksi ZIKV, DENV, dan CHIKV adalah hasil dari interaksi yang kompleks antara faktor virus dan pejamu. Penanda keparahan penyakit yang tersedia untuk infeksi virus-virus ini terbatas. Tesis ini menjelaskan studi tentang penanda virus dan pejamu yang dikaitkan dengan tingkat keparahan penyakit untuk infeksi ZIKV, DENV, dan CHIKV.

Kami menemukan perbedaan fenotipe antara strain ZIKV Asia dan Afrika, yang mungkin menjadi salah satu alasan penyebab komplikasi neurologis berat yang ditemukan di manusia pada epidemi baru-baru ini. Penelitian kami menggambarkan pentingnya menggunakan jenis virus yang tepat untuk mempelajari patogenesis infeksi ZIKV dan mengembangkan strategi intervensi baru. Kami juga menunjukkan bahwa infeksi ZIKV menginduksi apoptosis dan meningkatkan ekspresi TF pada sel endotel (EC). Ini mungkin salah satu alasan vaskulopati ditemukan pada beberapa model hewan dan manusia.

Kami memberikan bukti bahwa translokasi mikroba (TM), mediator imun, dan penanda aktivasi EC terutama meningkat pada fase kritis dan berhubungan dengan kebocoran plasma pada pasien dengue. Kami juga mengidentifikasi hubungan antara penanda TM serum dan penanda inflamasi dan EC. Ini menunjukkan bahwa translokasi kemungkinan berkontribusi pada aktivasi sistem imun pada fase kritis demam berdarah. Dengan demikian, modalitas terapi yang menargetkan jalur TM dapat menjadi strategi intervensi terhadap demam berdarah. Selain itu, kami juga mengidentifikasi protein baru, endocan, yang memiliki potensi untuk digunakan sebagai penanda pengganti kebocoran plasma dalam praktik klinis.

Kami menemukan bahwa peningkatan kadar feritin secara signifikan berhubungan dengan viremia dan penyakit chikungunya kronis. Makrofag merupakan salah satu sumber terpenting ferritin untuk penyakit inflamasi. Selain itu, makrofag juga merupakan salah satu sel target utama untuk CHIKV. Studi kami menunjukkan bahwa ferritin dapat berfungsi sebagai penanda prognostik potensial untuk terjadinya penyakit chikungunya kronis. Kami juga menemukan bahwa titer antibodi neutralisasi saja tampaknya tidak adekuat untuk melindungi terhadap terjadinya chikungunya kronis. Meskipun demikian, kami menemukan bahwa pasien dengan penyakit akut memiliki indeks aviditas antibodi yang secara signifikan lebih tinggi dibandingkan pasien dengan penyakit kronis. Oleh karena itu, pemahaman yang lebih baik tentang profil antibodi mungkin penting untuk dapat memahami hubungan antibodi

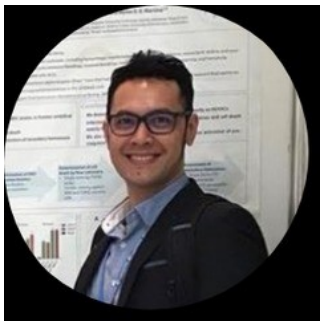
neutralisasi dan penyakit chikungunya kronis. Hal ini penting dalam mendesain vaksin dan strategi terapeutik terkait lainnya.

Secara kolektif, kami menemukan beberapa penanda potensial yang berhubungan dengan manifestasi keparahan penyakit dari tiga arbovirus yang dibahas dalam tesis ini. Pemahaman yang lebih baik tentang peran biomarker ini dalam patogenesis penyakit diperlukan untuk memicu pengembangan strategi intervensi baru terhadap infeksi arbovirus.

Chapter 10

About the Author/ Curriculum Vitae, Ph.D. Portfolio and Publications

Curriculum Vitae



Fatih Anfasa was born in Jakarta, Indonesia, on January 31, 1984. He was raised by his parents, Faik Tadjoedin and Fifin Safina Hafsah, and grew up together with his brother (Ahmad Fenoza) and sister (Fildza Amelia). He studied medicine at the Faculty of Medicine, Universitas Indonesia (FMUI) finishing it in 2008 with honors. Afterwards, he was working as a clinical research assistance for a large multicenter study of an anticoagulant drug for atrial fibrillation patients at the Cardiology Division, Department of Internal Medicine, FMUI – Sanofi Pasteur while also working for a primary health clinic in Jakarta, Indonesia. Subsequently, he continued doing residency at the Department of Internal Medicine FMUI – Cipto Mangunkusumo National Referral Hospital in 2009. He postponed his residency in 2010 and did a Master in Infection and Immunity at Erasmus University Medical Center (Erasmus MC), Rotterdam, the Netherlands with a full scholarship. He graduated as a Master of Science in 2012. He obtained an opportunity to do his PhD study under the supervision of Prof. Marion P. G. Koopmans, Prof. Albert D. M. E. Osterhaus and Dr. Byron E. E. Martina, at the Department of Viroscience, Erasmus MC with a PhD scholarship grant from the Ministry of Research, Technology and Higher Education, Republic of Indonesia. He has been starting with his residency again at the Department of Internal Medicine, FMUI since 2019. He is married to Salma Oktaria and is a proud father of Safaa Nafisa Anfasa and Fathi Ahmad Narendra Anfasa.

Ph.D. Portfolio

Name : Fatih Anfasa
Department : Department of Viroscience, Erasmus MC
Research school : Postgraduate school molecular medicine (PGS Molmed)
Ph.D. period : 2013-2019
Promotors : Prof. dr. Marion P.G. Koopmans
Prof. dr. Albert D.M.E. Osterhaus
Co-promotor : Dr. Byron E.E. Martina

Education

2002 – 2006 : Bachelor of Medicine, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia
2006 – 2008 : Medical Doctor (with honors), Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia
2009 – 2010 : Residency program, Department of Internal Medicine, Faculty of Medicine Universitas Indonesia - Cipto Mangunkusumo National Referral Hospital, Jakarta, Indonesia
2010 – 2012 : Master of science, Erasmus University Medical Center, Rotterdam, the Netherlands
2013 – 2019 : PhD program, Erasmus University Medical Center, Rotterdam, the Netherlands
2019 – now **:** Residency program, Department of Internal Medicine, Faculty of Medicine Universitas Indonesia - Cipto Mangunkusumo National Referral Hospital, Jakarta, Indonesia

Awards

1. I&I fund from research master infection and immunity, Postgraduate School Molecular Medicine, Erasmus Universty Medical Center (2020)
2. PhD scholarship grant from Directorate of Higher Education, Ministry of Education and Culture, Republic of Indonesia (2013)
3. Excellent Research Master Student Award from Royal Netherlands Academy of Arts and Sciences (KNAW) (2012)
4. Full scholarship for research master program in infection and immunity, Postgraduate School Molecular Medicine, Eramus University Medical Center (2010)
5. Cum laude graduate award for medical doctor education from Faculty of Medicine, Universitas of Indonesia (2008)
6. Cum laude graduate award for medical doctor education from Rector of University of Indonesia (2008)
7. Best oral presentation (2nd position), Annual Meeting of Internal Medicine 2008, Jakarta, Indonesia (2008)

Presentations

1. 23rd Molecular Medicine Day 2019
2. 22nd Molecular Medicine Day 2018
3. 10th European Congress of Tropical Medicine and International Health, Antwerp, Belgium 2017
4. 2nd International Meeting on Arboviruses and Their Vectors, Glasgow, Scotland 2017
5. Dutch Arboviral Network Meeting 2017 2017
6. 21st Molecular Medicine Day 2017
7. 1st ICE on IMERI, Jakarta, Indonesia 2016
8. Lab meetings, Departement of Viroscience, Erasmus MC 2013-2019

Courses and conferences

1. Grant proposal writing 2019
2. Research integrity course 2019
3. Dutch Annual Virology Symposium 2018
4. Advance course in immunology 2017
5. Dutch Annual Virology Symposium 2017
6. Course in Virology 2016
7. 19th Molecular Medicine Day 2015
8. Article 9 - certification to design animal experiment project 2014

Teachings

1. 2019: Preparation classes for Erasmus MC & Delft University students that will perform an exchange program to university hospitals in Indonesia (Minor Global Health)
2. 2018: Preparation classes for Erasmus MC students that will perform an exchange program to university hospitals in Indonesia (Minor Global Health)
3. 2015: Supervising a pre-PhD program internship at Erasmus MC (6 months)
4. 2014: Supervising a master student internship from Vrij University Medical Center (6 months)

Miscellaneous

1. Editor of the Journal of Indonesian Medical Association
2. Guest associate editor for Frontiers Microbiology
3. Reviewer:
    a. Frontiers Microbiology
    b. Emerging Microbes and Infection
    c. Vector Borne Zoonotic Diseases
    d. Tropical Medicine International Health

List of publications

Goeijenbier M**, Anfasa F**, Meijers JCM, van Gorp ECM Martina BEE. Vascular bed-specific endothelial cell response and viral hemorrhagic fever. (*manuscript in preparation*)

Anfasa F, Koraka P, Roose J, van Amerongen G, Osterhaus ADME, Martina BEE. Microbial translocation as a measure of dengue virulence in the mouse and non-human primate models. (*manuscript in preparation*)

Nainggolan L, Dewi BE, **Anfasa F**, Martina BEE, van Gorp ECM. A scoring system to predict plasma leakage in dengue patients. (*manuscript in preparation*)

Goeijenbier M, Schuller M, Martina B, **Anfasa F**, Ahmed A, Hartskeerl R, Wagenaar J, Osterhaus A, Planchon S, Sergeant K, Renaut J, van Gorp ECM, Nally J. Comparative proteomic in rodent-borne hemorrhagic fever. (*manuscript in preparation*)

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*These authors contributed equally

Chapter 11

Acknowledgements


SAFAA
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ANGKAT SUMPAH DOKTER
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Jakarta, 01 November 2008
HIMMI

ACKNOWLEDGEMENTS

This PhD journey has been an amazing chapter of my life. During this journey, I met many new friends, learned new values and wisdoms, extended my professional network, and found opportunities to expand my professional carrer. There were many difficult times throughout the way, but I am blessed with families, friends and colleagues that help me to reach the finish line. Now that I am reaching the end of this journey, I would like to express my appreciation to a number of wonderful people for being part of it and making this thesis possible.

I would like to begin by thanking my promotors, Prof. Marion Koopmans and Prof. Ab Osterhaus. Dear Marion, thank you for being my supervisor for the last two years of my PhD and for allowing me to work with Barry while waiting for my defense day. Your passion and enthusiasm on one health always inspire me and you have stimulated me to foresee research from different perspectives. Dear Ab, thank you for accepting me to do my research master at Erasmus MC and to do my PhD at the Viroscience Department. Your passion and enthusiasm on science always inspire me. I also learned how important it is to communicate science to the public, something that I learned first hand when we went to Indonesia and took some shots for a public documentary video. You have never failed to inspire others through your lectures, and many of my colleagues in Jakarta are no exception.

Dr. Byron Martina, thank you for being my supervisor and also for always believing that I can finish this journey. It was difficult at the beginning with your "unique schedule" and "do everything in the lab yourself" motto since I am a medical doctor that has minimal lab experiences. Nevertheless, I can only be thankful at the end of the day for all the valuable knowledge and experience both in and outside the lab that will surely help me in the future. I always enjoy our discussion time, especially with your broad knowledge and good insight on diverse topics. Hopefully, we can continue our collaboration with some projects already on plan.

I would like to thank Prof. Eric van Gorp, Prof. Andre van der Ven, and Dr. Jan Nouwen, for their time and effort to review my thesis.

I am also deeply grateful to all of the co-authors and collaborators for the cooperation resulting the publications in this thesis.

Dr. Barry Rockx, thank you for being such a supportive work group leader. Not only you focus on science, you often put attention to my personal matters. I am deeply thankful for your attention and support to attend my first international conference. I am also grateful that I could work with you for the hanta viruses project. Hopefully, we can collaborate in the future for a research project.

Dr. Debby van Riel, I am grateful that I had the chance to work with you. You are smart, attentive and above all, a wonderful person. I always remember how you and Lonneke helped me to finish my work in the lab after you heard Salma was starting to experience contraction. I admire your spirit and positive mindset even during difficult times. I wish you nothing but the best.

Dr. Jan Nouwen and Dr. Frank van Vliet, thank you for giving me the opportunity to pursue my master study in the Infection and Immunity Master Program. It was a great experience that helped to improve my scientific knowledge and career. Jan, I hope we can continue our collaboration in the future, either through Minor Global Health or other projects. Frank, thank you for your "magic skills" to solve problems when nobody else can. You've been really helpful throughout my time at Erasmus MC and I am deeply grateful for that.

I would like to express my sincere gratitude to Prof. Jon Laman for being such a wonderful mentor during my master study. I always admire your humility and how you organize yourself. I am grateful for your advice on many things. I am indebted for your encouragement that helped me to obtain the award from KNAW.

Dr. Raoul Tan, thank you for your attention and help throughout my PhD study. We often discuss on how we can improve the collaboration between Indonesian medical schools with Erasmus MC. Because of this, we even had the chance to meet the members of the Indonesian House of Parliement. I hope that all your efforts will become fruitful in the near future.

Dr. Penelope Koraka, I am thankful for your help throughout my study time in Rotterdam. You always show that science can be combined with fashion. I esteem your positive mindset and spirit even during difficult times. You are a wondeful person and a loving mother. I wish you nothing but the best.

Dr. Chantal Reusken, thank you for your attention and support throughout my PhD study. You always ask whether I have a starbucks tumbler from a country/city in the middle of your busy trip abroad to add to my collection. You are a wondeful person and I am deeply thankful for your support and attention to me.

Dr. Kirtsy Short and Dr. Keng Yi Chew, thank you for being my sister and brother during your time in the Netherlands. Both of you are such a nice person and I always enjoyed everytime that we spent, both inside and outside the lab. Kirsty, I admire you as a scientist and I am amaze that you can still do "modelling" in the middle of your busy research schedule. Hopefully, we can further collaborate in the future, starting from the COVID-19 multicenter study.

To my paranymphs, Bri and Kim, two of my precious friends that I found in the Netherlands. Dear Bri, thank you very much for everything. Not only for being my paranypmh but also for being a little sister, a good friend and colleague and Safaa's favorite Tante. Thank you for all the good and the bad times. I hope you can be a master on how to draw animals with illustrator :) Kimmy, thank you for being my paranymph, a coffee budy, a good friend to talk about everything and a friend in need during good and difficult times. I am grateful to have you as a friend and I wish all the best with your future career in Amsterdam. I hope you can still enjoy going to "IKEA". :) I will surely keep in touch with both of you.

To the secretaries, Simone, Loubna and Maria, I am really grateful for your help and support throughout the years. Especially during the last months of my PhD study. Without the three of you, getting my PhD would be much more difficult.

I also would like to express my appreciation to the past and current members of the Exotic Viruses Group. Cox, thank you for introducing me on how to work in the lab. Steffie, terima kasih atas segala bantuan dan perhatiannya selama ini. Semoga kamu semakin sukses ke depannya dan bisa lancar bicara Bahasa Indonesia. Zeli, thank you for your help and warm smile everytime I asked for help. Jeroen, thank you for your guidance and support when I was lost in the lab. Petra, it's difficult at the beginning but I am grateful that we can know each other better. Thank you for "the Delft tour" and for all the delicious food. I hope you can pursue your personal passion in science. Marco, amigo, both of us had difficult lab days at the beginning, but we learned how to do it better in the end. It's always fun to work with you! Tarique, thank you for all the discussion time that we had both in and outside the lab. Do, thank you so much for your help with the lab works, the numberless illustrator sessions and the advice to improve my papers. Noreen, I am happy to see how you evolved from day one and know that you do really well and finally started your own PhD research. I wish nothing but the best for you. Melanie, you

are such a warm person and I always enjoy talking with you. Jeer, thank you for the wonderful times that we had in the lab. Delia, it's always nice talking with you about many things. Danny, your Post Malone make over was awesome so it's a pitty that you never play a 1 hour-long song of him in the lab. I believe you will be fine with your PhD. Eleanor and Imke, I wish nothing but the best for your PhD journey.

I also would like to thank these special colleagues at the Department of Viroscience. Jasmine, thank you for being a good listener and a good coffee budy. I wish you a good carreer in a tropical country and I hope that you will have less health issues in the future. Felicity, terima kasih banyak atas bantuan dan diskusinya selama ini. Tetap semangat dan tolong kabari jika sedang berkunjung ke Indonesia. Jurre, the other Zika boy, it was tiring but I had fun when we did the Zika paper. I am relieved that around 1,000 plates that we used are not gone for nothing. Oanh, thank you for all the serious and non-serious discussions that we had. David van de Vijver, thank you for keep reminding me that "Kolmogorov-Smirnov" is not always important when you need to analyze your data. Nisreen, you are a hardworking and a smart person. I wish you a bright future career. Lineke, you are a nice person and I always enjoy talking with you, especially with all the stroopwafels that you always provided at Ee 1714. Nele, you are an example that science is always on the run, literally. Thomas, you are a bright and a hard-working researcher. But I will always remember you as the titration master. Wesley, thank you for inviting me to do the vaccination study and best of luck with your residency. Monique, you are the ultimate "GPS" when I am lost in the lab. Thank you for your kindness and answers to all my questions. Corine, thank you for always trying to provide all the serum samples when I need it. Erwin, thank you for always answering all of my questions about protein microarray. Syriam, your politeness is just amazing. Big Stefan, I always enjoyed the time we ate Bri's snacks together and discussed many things. Pascal L., it was nice to know that you are a neighboor of mine (from a different building) and to meet you on the streets around our apartments. Claudia, it's always nice to have a conversation with you. I hope that you won't have any problem with the earbuds anymore. Miranda, you are a wonderful person to talk with and I am gonna miss the chocolates that you like to share. Rory, you are a brilliant scientist and a martial art expert. A good combo to make sure that everyone listens to you. Gijs, thank you for the charger and the bright smile everytime I ask you something. Eric, thank you for allowing me to visit the clinics and also for helping me with my thesis. Thijs, I am always fascinated with your passion on birds and your sudden appearance with a small piece of chocolate for everyone at the right time. Guus, I enjoyed listening to your awesome PhD story and how you did it with Ab.

To all my office roommates, Ee 1671, Ee 1714, and Ee 1742, especially Marco, Lennert, David van de Vijver, Brooke, Jeer, Carola, Dineke, Byron, Miss P, Steffie, Jeroen, Petra, Chantal, Zeli, Lineke, Jurre, Gijs, Noreen, Nele, Brenda, Bas, Claudia, Miranda, Shirley, Fatiha and Barry. Thank you for all of the wonderful time and delicious snacks that we shared throughout the years.

I am also indebted to many people in the Department of Viroscience (past and present) for their encouragement, kindness and fruitful discussion: Rik, Ron, Arno, Rob, Bart, Georges, Suzan, Bernadette, Mr. Sander, Mathilde, Reina, Bas, Stalin, Arwen, Caroline, Johanna, Brenda, Werner, Zsofi, My Phan, Benjamin, Lennert, Gadissa, small but muscular David, Monique V. Anouk, Carola, Chantal Burghoorn-Maas, Georgina, Marco vdB, Rachel, little Stefan, Dennis, Peter, Patrick, Stella, Nella, Sarah, Soerani, Robert, Mart, Elmoubasher, Stephanie P., Rachel, Cynthia, Diana, Anoushka, Tamana, Laurine, Frederique, Laura, Matthijs, Katy, Eurydice, Zuwena, Victor, Erik, Justin, Minoushka, Varsha, Marwah, Yingying, Max, Shirley, Shanty, Anja, Adinda, Miruna, Diana, Muriel, Sascha, Nadia, Daphne, Alwin, Thao, Anna, Ray, Choi, Stijn, Fiona, Liz, Yvette, Tien + Evelyn and Fleur.

I would also like to acknowledge the colleagues from other departments at Erasmus MC that have been helpful throughout my time in Rotterdam, especially to Dr. Juliette Severin, Dr. Wim

Dik, Dr. Tom Cupedo, Dr. Joost Hegmans, Dr. Ton Langerak, Prof. Rudi Hendriks, Dr. Alex Kleijan, Dr. Maarten Brandt, Dr. Femke de Vrij, Mark van der Kroeg and Dr. Sonja Leonhardt.

To the brothers from "Mascid": Dr. Q. Pan (Abdullah), Mustofa, Muhammad, Ferdows, Fatih, Zouhair and Farhat, thank you for the friendship. Special thanks to Ihsan, Mesut and Adil for the brotherhood and the warm discussions that we had.

Special thanks for the special people that have been really supportive and have been like my second family in the Netherlands: Om Tinus, Tante Ade, Tante Tris, Om Rene, Tante Hermina, Om Ajo, Pak Deden and Pak Hamdi. Thank you for making the Netherlands my second home.

Word cannot express how grateful I am for having good Indonesian friends during my time in the Netherlands. To my band of brothers and sisters in the Netherlands: Dr. Yulia Rosa Saharman, I am grateful for your attention and help on many things. You are like the older sister that I never had and your shopping skill is just amazing. Mbak Rina La Distia Nora, thank you for all the discussions and help throughout these years, and setting a good example that perseverance does pay off. Kang Opik, thank you for being the comedian of the group. You showed me that eating less is forbidden, especially when we are at Tante Ade's apartment. Teh Dowty, thank you for your infectious laugh and kindness. Adit, thank you for your help in taking care of Safaa and Fathi when needed. You also showed that fashion is important even when you are busy doing science. Fuad and Diah, I am grateful for all the things that you have done to me and my family. I wish nothing but all the best for both of you, Birru and Bumi. Raya, I hope that you can get your dream job anywhere else without any income tax again. Tirza, thanks for the many scientific and non-scientif discussions that we had. I always admire your positive mindset. Yanti, thanks for the wonderful times that we shared together. Brian, I admire your broad knowledge and I always enjoyed the time that we talked.

I would also like to thank Pak Zulfa, Mas Dony, Bu Dewi, Kang Aul, Lia, Kang Dudy, Teh Nia, Kang Yopi, Teh Dewi, Pak Harry, Bang Boy, Kak Sasha, Danny, Sasti, Iqbal, Yelvi, Ivan, Niken, Anggi, Yana, Afifa, Nanda, Kimon, Nadia, Hasya, Yoga, Mas Adi, Mas Andi, Adil, Helmi, Hakim, Happy, Astari, Laura, Boy, Bang Utama, Mbak Ririn, Mbak Leli, Devina, Emma, Mbak Maeyta, Pak Wayan, Evy, Novika, Titis, Mikhta, Rara, Yos, Billi, Yudha, Segal, Wisnu, Ario, Mita, Akmal, Tasya, Mas Akram, Sofi, Marion, Ingrid, Ngan, Sandra, Bas, Sandrine, Irene and Stella for the wonderful times and gatherings that we had together in the Netherlands.

To ILUNI UI chapter Netherlands, Mas Edi, Mbak Wulan, Dr. Dicky, Mbak Maria, Pak Jun, Bang Nazar, Hendy, Dito, Maria and many others, thank you for all of your supports.

I would like to extend my appreciation to the Indonesian Embassy in the Netherlands, especially to Prof. Hari, Pak Din, Bu Linda, Pak Lucky and Bu Monica.

Dr. Heriawan, thank you for convincing me to study abroad and broaden my knowledge when I was still unsure about it. I was reluctant at the beginning due to the sudden process, but you convinced me that it is for a greater good. In the end, I am grateful that I followed your advice. I am also thankful for your attention and support throughout my study time in the Netherlands. Your kindness will never be forgotten.

Prof. Pradana, I am grateful for your attention and support during the transition period between my master and the beginning of my PhD. I always enjoyed our discussion time when I met you to inform the progress of my PhD study. I knew that I would get difficult questions from you and it helped me to think better as a scientist.

Dr. Leonard Nainggolan, Bu Jane and Mikha, thank you very much for your attention and support throughout my time in the Netherlands. I am deeply thankful for your attention to my family and personal matters. I always enjoyed everytime the three of you came to visit me in Rotterdam. Dr. Leo, your help and support made it possible for me to finish my PhD, which I am deeply grateful for.

Dr. Beti Ernawati Dewi, thank you very much for your support for the translocation study and all the experiments that need to be done, both at IMERI and the Department of Microbiology Faculty of Medicine Universitas Indonesia (FMUI). Your attention and guidance made it possible for me to finish my PhD.

The former and current Deans of FMUI, Prof. Ratna Sitompul and Prof. Ari Fahrial Syam, I am deeply grateful for your advice and support throughout my study period.

The former and current President Directors of Cipto Mangunkusumo Hospital, Dr Heriawan and Dr. Lies Dina Liastuti, thank you for all of your guidance and support during my PhD study.

The former and current Heads of Department of Internal Medicine, FMUI, Dr. Heriawan, Prof. Imam Subekti and Prof. Dadang Makmun, I would like to extend my sincere gratitude for your support throughout my PhD journey.

The former and current Heads of the Internal Medicine Residency Program at FMUI, Prof. Aru W. Sudoyo, Dr. Aida Lydia and Dr. Kuntjoro Harimurti, I am profoundly grateful for your help and understanding so I could finish my PhD study.

Dr. Em Yunir, my residency academic advisor, I am grateful for your help and provision in my residency training. I also would like to express my gratitude for your support and understanding when I was applying again for the internal medicine residency program.

To my teachers and senior colleagues from FMUI: Prof. Jose Rizal Batubara, Prof. Kusmarinah Bramono, Prof. Endang Basuki, Prof. Rita Sitorus, Prof. Idrus Alwi, Prof. Siti Setiati, Prof. Suhendro, Prof. Rini Sekartini, Dr. Nurjati C. Siregar, Dr. Widayat Djoko Santoso, Dr. Sally Aman Nasution, Dr. Indah Suci Widyahening, Dr. Dyah Purnamasari, Dr. Evy Yunihastuti, Dr. Prasetyanugraheni Kreshanti, Dr. Dicky L. Tahapary and the other staff members of the Department of Internal Medicine, thank you for all of your supports and guidance throughout my study period.

I would like to thank the staff and technicians at the Integrated Lab of FMUI, especially to Dr. Heri Wibowo, Mbak Astrid, Bu Sri, Bu Neneng and Mas Dani for all their help and kindness. My gratitude also goes to the staff of Infectious Disease and Immunology Cluster, Indonesian Medical Research Institute: Dr. Erni Juwita Nelwan, Dr. Atie, Eleanour Louna Urfa, Windi and Mila and the staff of the Department of Microbiology, FMUI: Dr. Fera Ibrahim, Dr. Anis Karuniawati and Dr. Andi Yasmon, for their help when I was working at their department.

To my “Indonesian Sahabat”, Henry, Sandi, Troy, Jaja, Dila, Nate and Diena, thank you for the friendship and all of your support throughout these years.

Saya ingin mengucapkan penghargaan dan terima kasih sebesar-besarnya kepada keluarga besar Chasinah, M. Bakran, Tadjoedin dan Effendi atas dukungan dan kebersamaannya selama ini. Saya dapat meraih gelar ini berkat dukungan dan doa dari banyak anggota keluarga. Saya juga ingin mengucapkan terima kasih yang sebesar-besarnya khususnya kepada alm. Prof. Sujudi, Ibu Faika Sujudi, dr. Heyder Tadjoedin, Tante Farida Baharis, Dr. Hilman Tadjoedin, dan

dr. Feriadi Suwarna atas doa dan perhatiannya selama ini kepada saya dan keluarga. Semoga Allah SWT memberikan balasan atas kebaikan dan perhatian yang diberikan.

Terima kasih kepada Papa Edi dan Mama Tin atas dukungan, perhatian dan doanya sehingga Fasa dapat mencapai gelar ini. Terima kasih pula kepada kakak ipar saya, khususnya kepada Uni Teti, Mas Teguh, Mas Enggi, Mbak Poppy, dan Bunda Laura. Semoga Allah SWT melimpahkan segala rahmat dan perlindunganNya kepada Opa, Oma, dan kakak-kakak semua.

Kepada Mbah Chasinah, terima kasih atas kasih sayang dan doa yang Mbah selalu berikan. Papa, terima kasih atas dukungan, kesabaran, semangat, perhatian serta doa Papa untuk Fasa selama ini. Mama, thank you for always being there for me. Fasa bisa menyelesaikan fase ini berkat perhatian, kasih sayang serta doa tidak terhitung yang terus mama panjatkan. Siapa sangka doa mama agar anaknya bisa mendapatkan kesempatan belajar di luar negeri dikabulkan Allah SWT di negara yang tidak disangka-sangka. Semoga ilmu yang Fasa dapatkan bisa membuat mama bangga dan bahagia. Feno, thank you for being the rock of our family while I am away. I am proud to have you as my brother. Fildza, I always enjoy talking with you about everything. You are a wonderful person and I am grateful to have you as my sister. Labib, thank you for being a reliable little brother and also for your help with the cover and layout of my thesis. Semoga Allah SWT memberikan rahmat dan perlindunganNya kepada kita sekeluarga.

Dear Safaa and Fathi, my life would be meaningless without the two of you. Both of you are “the apple of my eye“. I know that both of you do not yet understand the struggles and the blessings that your father and mother had during our PhD time. But I hope that we can share this story to you one day and showed that chasing your “impossible” dream is possible if you put your passion and hard work to it. Love both of you till the day that I die.

Last but not least, my dearest wife, Salma Oktaria. It’s been a long journey and we face many good and difficult times together. It was really hard when I left you first to study abroad and we got to taste how it fells to do a long-distance relationship. Thank you for all the love and support throughout these years, and for always believing in me throughout my journey. I am blessed to have you as my other half. Hopefully, you can finish your PhD soon and we can continue our next journey together. I love you more than before because of Allah.

Above all, I thank Allah SWT for giving me the strength, endurance and motivation to finish this chapter of my life.

Lastly, I sincerely apologize for not being able to mention here the names of many others who helped me during my study in the Netherlands. But I would like them to know that I am thankful for all the help and support that they have provided.
Now I can officialy say “**I am RotterDONE**”.